MICROORGANISMS FOR THE PRODUCTION OF 1,4-BUTANEDIOL, 4-HYDROXYBUTANAL, 4-HYDROXYBUTYRYL-COA, PUTRESCINE AND RELATED COMPOUNDS, AND METHODS RELATED THERETO

This application claims the benefit of priority of U.S. Provisional Application No. 61/251,287, filed October 13, 2009, the entire contents of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

This invention relates generally to *in silico* design of organisms and engineering of organisms, more particularly to organisms having 1,4-butanediol, 4-hydroxybutyryl-CoA, 4-hydroxybutanal or putrescine biosynthesis capability.

The compound 4-hydroxybutanoic acid (4-hydroxybutanoate, 4-hydroxybutyrate, 4-HB) is a 4-carbon carboxylic acid that has industrial potential as a building block for various commodity and specialty chemicals. In particular, 4-HB has the potential to serve as a new entry point into the 1,4-butanediol family of chemicals, which includes solvents, resins, polymer precursors, and specialty chemicals. 1,4-Butanediol (BDO) is a polymer intermediate and industrial solvent with a global market of about 3 billion lb/year. BDO is currently produced from petrochemical precursors, primarily acetylene, maleic anhydride, and propylene oxide.

For example, acetylene is reacted with 2 molecules of formaldehyde in the Reppe synthesis reaction (Kroschwitz and Grant, *Encyclopedia of Chem. Tech.*, John Wiley and Sons, Inc., New York (1999)), followed by catalytic hydrogenation to form 1,4-butanediol. It has been estimated that 90% of the acetylene produced in the U.S. is consumed for butanediol production. Alternatively, it can be formed by esterification and catalytic hydrogenation of maleic anhydride, which is derived from butane. Downstream, butanediol can be further transformed; for example, by oxidation to γ-butyrolactone, which can be further converted to pyrrolidone and N-methyl-pyrrolidone, or hydrogenolysis to tetrahydrofuran. These compounds have varied uses as polymer intermediates, solvents, and additives, and have a combined market of nearly 2 billion lb/year.

It is desirable to develop a method for production of these chemicals by alternative means that not only substitute renewable for petroleum-based feedstocks, and also use less energy- and capital-intensive processes. The Department of Energy has proposed 1,4-diacids, and particularly succinic acid, as key biologically-produced intermediates for the manufacture of the

butanediol family of products (DOE Report, "Top Value-Added Chemicals from Biomass", 2004). However, succinic acid is costly to isolate and purify and requires high temperatures and pressures for catalytic reduction to butanediol.

Thus, there exists a need for alternative means for effectively producing commercial quantities of 1,4-butanediol and its chemical precursors. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF INVENTION

The invention provides non-naturally occurring microbial organisms containing a 1,4-butanediol (BDO), 4-hydroxybutanal (4-HBal), 4-hydroxybutyryl-CoA (4-HBCoA) and/or putrescine pathway comprising at least one exogenous nucleic acid encoding a BDO, 4-HBal and/or putrescine pathway enzyme expressed in a sufficient amount to produce BDO, 4-HBal, 4-HBCoA and/or putrescine. The microbial organisms can be further optimized for expression of BDO, 4-HBal, 4-HBCoA and/or putrescine. The invention additionally provides methods of using such microbial organisms to produce BDO, 4-HBal, 4-HBCoA and/or putrescine.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram showing biochemical pathways to 4-hydroxybutyurate (4-HB) and to 1,4-butanediol production. The first 5 steps are endogenous to *E. coli*, while the remainder can be expressed heterologously. Enzymes catalyzing the biosynthetic reactions are: (1) succinyl-CoA synthetase; (2) CoA-independent succinic semialdehyde dehydrogenase; (3) α-ketoglutarate dehydrogenase; (4) glutamate:succinate semialdehyde transaminase; (5) glutamate decarboxylase; (6) CoA-dependent succinic semialdehyde dehydrogenase; (7) 4-hydroxybutanoate dehydrogenase; (8) α-ketoglutarate decarboxylase; (9) 4-hydroxybutyryl CoA:acetyl-CoA transferase; (10) butyrate kinase; (11) phosphotransbutyrylase; (12) aldehyde dehydrogenase; (13) alcohol dehydrogenase.

Figure 2 is a schematic diagram showing homoserine biosynthesis in *E. coli*.

Figure 3 shows the production of 4-HB in glucose minimal medium using *E. coli* strains harboring plasmids expressing various combinations of 4-HB pathway genes. (a) 4-HB concentration in culture broth; (b) succinate concentration in culture broth; (c) culture OD, measured at 600 nm. Clusters of bars represent the 24 hour, 48 hour, and 72 hour (if measured) timepoints. The codes along the x-axis indicate the strain/plasmid combination used. The first

index refers to the host strain: 1, MG1655 lacI^Q; 2, MG1655 ΔgabD lacI^Q; 3, MG1655 ΔgabD ΔaldA lacI^Q. The second index refers to the plasmid combination used: 1, pZE13-0004-0035 and pZA33-0036; 2, pZE13-0004-0035 and pZA33-0010n; 3, pZE13-0004-0008 and pZA33-0036; 4, pZE13-0004-0008 and pZA33-0010n; 5, Control vectors pZE13 and pZA33.

Figure 4 shows the production of 4-HB from glucose in *E. coli* strains expressing α-ketoglutarate decarboxylase from *Mycobacterium tuberculosis.* Strains 1-3 contain pZE13-0032 and pZA33-0036. Strain 4 expresses only the empty vectors pZE13 and pZA33. Host strains are as follows: 1 and 4, MG1655 lacI^Q; 2, MG1655 ΔgabD lacI^Q; 3, MG1655 ΔgabD ΔaldA lacI^Q. The bars refer to concentration at 24 and 48 hours.

Figure 5 shows the production of BDO from 10 mM 4-HB in recombinant *E. coli* strains. Numbered positions correspond to experiments with MG1655 lacI^Q containing pZA33-0024, expressing cat2 from *P. gingivalis*, and the following genes expressed on pZE13: 1, none (control); 2, 0002; 3, 0003; 4, 0003n; 5, 0011; 6, 0013; 7, 0023; 8, 0025; 9, 0008n; 10, 0035. Gene numbers are defined in Table 6. For each position, the bars refer to aerobic, microaerobic, and anaerobic conditions, respectively. Microaerobic conditions were created by sealing the culture tubes but not evacuating them.

Figure 6 shows the mass spectrum of 4-HB and BDO produced by MG1655 lacI^Q pZE13-0004-0035-0002 pZA33-0034-0036 grown in M9 minimal medium supplemented with 4 g/L unlabeled glucose (a, c, e, and g) uniformly labeled ^{13}C-glucose (b, d, f, and h). (a) and (b), mass 116 characteristic fragment of derivatized BDO, containing 2 carbon atoms; (c) and (d), mass 177 characteristic fragment of derivatized BDO, containing 1 carbon atom; (e) and (f), mass 117 characteristic fragment of derivatized 4-HB, containing 2 carbon atoms; (g) and (h), mass 233 characteristic fragment of derivatized 4-HB, containing 4 carbon atoms.

Figure 7 is a schematic process flow diagram of bioprocesses for the production of γ-butyrolactone. Panel (a) illustrates fed-batch fermentation with batch separation and panel (b) illustrates fed-batch fermentation with continuous separation.

Figures 8A and 8B show exemplary 1,4-butanediol (BDO) pathways. Figure 8A shows BDO pathways from succinyl-CoA. Figure 8B shows BDO pathways from alpha-ketoglutarate.

Figures 9A-9C show exemplary BDO pathways. Figure 9A and 9B show pathways from 4-aminobutyrate. Figure 9C shows a pathway from acetoactyl-CoA to 4-aminobutyrate.

Figure 10 shows exemplary BDO pathways from alpha-ketoglutarate.

Figure 11 shows exemplary BDO pathways from glutamate.

Figure 12 shows exemplary BDO pathways from acetoacetyl-CoA.

Figure 13 shows exemplary BDO pathways from homoserine.

Figures 14 shows the nucleotide and amino acid sequences of *E. coli* succinyl-CoA synthetase. Figure 14A shows the nucleotide sequence (SEQ ID NO:) of the *E. coli sucCD* operon. Figures 14B (SEQ ID NO:) and 14C (SEQ ID NO:) show the amino acid sequences of the succinyl-CoA synthetase subunits encoded by the *sucCD* operon.

Figure 15 shows the nucleotide and amino acid sequences of *Mycobacterium bovis* alpha-ketoglutarate decarboxylase. Figure 15A shows the nucleotide sequence (SEQ ID NO:) of *Mycobacterium bovis sucA* gene. Figure 15B shows the amino acid sequence (SEQ ID NO:) of *M. bovis* alpha-ketoglutarate decarboxylase.

Figure 16 shows biosynthesis in *E. coli* of 4-hydroxybutyrate from glucose in minimal medium via alpha-ketoglutarate under anaerobic (microaerobic) conditions. The host strain is ECKh-401. The experiments are labeled based on the upstream pathway genes present on the plasmid pZA33 as follows: 1) *4hbd-sucA*; 2) *sucCD-sucD-4hbd*; 3) *sucCD-sucD-4hbd-sucA*.

Figure 17 shows biosynthesis in *E. coli* of 4-hydroxybutyrate from glucose in minimal medium via succinate and alpha-ketoglutarate. The host strain is wild-type MG1655. The experiments are labeled based on the genes present on the plasmids pZE13 and pZA33 as follows: 1) empty control vectors 2) empty pZE13, pZA33-4hbd; 3) pZE13-sucA, pZA33-4hbd.

Figure 18 A shows the nucleotide sequence (SEQ ID NO:) of CoA-dependent succinate semialdehyde dehydrogenase (*sucD*) from *Porphyromonas gingivalis,* and Figure 18B shows the encoded amino acid sequence (SEQ ID NO:).

Figure 19A shows the nucleotide sequence (SEQ ID NO:) of 4-hydroxybutyrate dehydrogenase (*4hbd*) from *Porphymonas gingivalis*, and Figure 19B shows the encoded amino acid seqence (SEQ ID NO:).

Figure 20A shows the nucleotide sequence (SEQ ID NO:) of 4-hydroxybutyrate CoA transferase (*cat2*) from *Porphyromonas gingivalis*, and Figure 20B shows the encoded amino acid sequence (SEQ ID NO:).

Figure 21A shows the nucleotide sequence (SEQ ID NO:) of phosphotransbutyrylase (*ptb*) from *Clostridium acetobutylicum*, and Figure 21B shows the encoded amino acid sequence (SEQ ID NO:).

Figure 22A shows the nucleotide sequence (SEQ ID NO:) of butyrate kinase (*buk1*) from *Clostridium acetobutylicum*, and Figure 22B shows the encoded amino acid sequence (SEQ ID NO:).

Figure 23 shows alternative nucleotide sequences for *C. acetobutylicum* 020 (phosphtransbutyrylase) with altered codons for more prevalent *E. coli* codons relative to the *C. acetobutylicum* native sequence. Figures 23A-23D (020A-020D, SEQ ID NOS: , respectively) contain sequences with increasing numbers of rare *E. coli* codons replaced by more prevalent codons (A<B<C<D).

Figure 24 shows alternative nucleotide sequences for *C. acetobuytlicum* 021 (butyrate kinase) with altered codons for more prevalent *E. coli* codons relative to the *C. acetobutylicum* native sequence. Figures 24A-24D (021A-021B, SEQ ID NOS: , respectively) contain sequences with increasing numbers of rare *E. coli* codons replaced by more prevalent codons (A<B<C<D).

Figure 25 shows improved expression of butyrate kinase (BK) and phosphotransbutyrylase (PTB) with optimized codons for expression in *E. coli*. Figure 25A shows sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) stained for proteins with Coomassie blue; lane 1, control vector with no insert; lane 2, expression of *C. acetobutylicum* native sequences in *E. coli*; lane 3, expression of 020B-021B codon optimized PTB-BK; lane 4, expression of 020C-021C codon optimized PTB-BK. The positions of BK and PTB are shown. Figure 25B shows the BK and PTB activities of native *C. acetobutylicum* sequence (2021n) compared to codon optimized 020B-021B (2021B) and 020C-021C (2021C).

Figure 26 shows production of BDO and gamma-butyrylactone (GBL) in various strains expressing BDO producing enzymes: Cat2 (034); 2021n; 2021B; 2021C.

Figure 27A shows the nucleotide sequence (SEQ ID NO:) of the native *Clostridium biejerinckii ald* gene (025n), and Figure 27B shows the encoded amino acid sequence (SEQ ID NO:).

Figures 28A-28D show alternative gene sequences for the *Clostridium beijerinckii ald* gene (025A-025D, SEQ ID NOS: , respectively), in which increasing numbers of rare codons are replaced by more prevalent codons (A<B<C<D).

Figure 29 shows expression of native *C. beijerinckii ald* gene and codon optimized variants; no ins (control with no insert), 025n, 025A, 025B, 025C, 025D.

Figure 30 shows BDO or BDO and ethanol production in various strains. Figure 30 shows BDO production in strains containing the native *C. beijerinckii ald* gene (025n) or variants with optimized codons for expression in *E. coli* (025A-025D). Figure 30B shows production of ethanol and BDO in strains expressing the *C. acetobutylicum* AdhE2 enzyme (002C) compared to the codon optimized variant 025B. The third set shows expression of *P. gingivalis sucD* (035). In all cases, *P. gingivalis* Cat2 (034) is also expressed.

Figure 31A shows the nucleotide sequence (SEQ ID NO:) of the *adh1* gene from *Geobacillus thermoglucosidasius*, and Figure 31B shows the encoded amino acid sequence (SEQ ID NO:).

Figure 32A shows the expression of the *Geobacillus thermoglucosidasius adh1* gene in *E. coli*. Either whole cell lysates or supernatants were analyzed by SDS-PAGE and stained with Coomassie blue for plasmid with no insert, plasmid with 083 (*Geotrichum capitatum* N-benzyl-3-pyrrolidinol dehydrogenase) and plasmid with 084 (*Geobacillus thermoglucosidasius adh1*) inserts. Figure 32B shows the activity of 084 with butyraldehyde (diamonds) or 4-hydroxybutyraldehyde (squares) as substrates.

Figure 33 shows the production of BDO in various strains: plasmid with no insert; 025B, 025B-026n; 025B-026A; 025B-026B; 025B-026C; 025B-050; 025B-052; 025B-053; 025B-055; 025B-057; 025B-058; 025B-071; 025B-083; 025B-084; PTSlacO-025B; PTSlacO-025B-026n.

Figure 34 shows a plasmid map for the vector pRE118-V2.

Figure 35 shows the sequence of the ECKh-138 region encompassing the *aceF* and *lpdA* genes. The *K. pneumonia lpdA* gene is underlined, and the codon changed in the Glu354Lys mutant shaded.

Figure 36 shows the protein sequence comparison of the native *E. coli lpdA* and the mutant *K. pneumonia lpdA*.

Figure 37 shows 4-hydroxybutyrate (left bars) and BDO (right bars) production in the strains AB3, MG1655 ΔldhA and ECKh-138. All strains expressed *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd* on the medium copy plasmid pZA33, and *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2 on the high copy plasmid pZE13.

Figure 38 shows the nucleotide sequence of the 5' end of the *aceE* gene fused to the pflB-p6 promoter and ribosome binding site (RBS). The 5' italicized sequence shows the start of the *aroP* gene, which is transcribed in the opposite direction from the *pdh* operon. The 3' italicized sequence shows the start of the *aceE* gene. In upper case: pflB RBS. Underlined: FNR binding site. In bold: pflB-p6 promoter sequence.

Figure 39 shows the nucleotide sequence (SEQ ID NO:) in the *aceF-lpdA* region in the strain ECKh-456.

Figure 40 shows the production of 4-hydroxybutyrate, BDO and pyruvate (left to right bars, respectively) for each of strains ECKh-439, ECKh-455 and ECKh-456.

Figure 41A shows a schematic of the recombination sites for deletion of the *mdh* gene. Figure 41B shows the sequence of the PCR product of the amplification of chloramphenicol resistance gene (CAT) flanked by FRT sites and homology regions from the *mdh* gene from the plasmid pKD3.

Figure 42 shows the sequence of the *arcA* deleted region in strain ECKh-401.

Figure 43 shows the sequence of the region encompassing a mutated *gltA* gene of strain ECKh-422.

Figure 44 shows the citrate synthase activity of wild type *gltA* gene product and the R163L mutant. The assay was performed in the absence (diamonds) or presence of 0.4 mM NADH (squares).

Figure 45 shows the 4-hydroxybutyrate (left bars) and BDO (right bars) production in strains ECKh-401 and ECKh-422, both expressing genes for the complete BDO pathway on plasmids.

Figure 46 shows central metabolic fluxes and associated 95% confidence intervals from metabolic labeling experiments. Values are molar fluxes normalized to a glucose uptake rate of 1 mmol/hr. The result indicates that carbon flux is routed through citrate synthase in the

oxidative direction and that most of the carbon enters the BDO pathway rather than completing the TCA cycle.

Figure 47 shows extracellular product formation for strains ECKh-138 and ECKh-422, both expressing the entire BDO pathway on plasmids. The products measured were acetate (Ace), pyruvate (Pyr), 4-hydroxybutyrate (4HB), 1,4-butanediol (BDO), ethanol (EtOH), and other products, which include gamma-butyrolactone (GBL), succinate, and lactate.

Figure 48 shows the sequence of the region following replacement of PEP carboxylase (ppc) by *H. influenzae* phosphoenolpyruvate carboxykinase (pepck). The pepck coding region is underlined.

Figure 49 shows growth of evolved pepCK strains grown in minimal medium containing 50 mM $NaHCO_3$.

Figure 50 shows product formation in strain ECKh-453 expressing *P. gingivalis* Cat2 and *C. beijerinckii* Ald on the plasmid pZS*13. The products measured were 1,4-butanediol (BDO), pyruvate, 4-hydroxybutyrate (4HB), acetate, γ-butyrolactone (GBL) and ethanol.

Figure 51 shows BDO production of two strains, ECKh-453 and ECKh-432. Both contain the plasmid pZS*13 expressing *P. gingivalis* Cat2 and *C. beijerinckii* Ald. The cultures were grown under microaerobic conditions, with the vessels punctured with 27 or 18 gauge needles, as indicated.

Figure 52 shows the nucleotide sequence of the genomic DNA of strain ECKh-426 in the region of insertion of a polycistronic DNA fragment containing a promoter, *sucCD* gene, *sucD* gene, *4hbd* gene and a terminator sequence.

Figure 53 shows the nucleotide sequence of the chromosomal region of strain ECKh-432 in the region of insertion of a polycistronic sequence containing a promoter, *sucA* gene, *C. kluyveri* *4hbd* gene and a terminator sequence.

Figure 54 shows BDO synthesis from glucose in minimal medium in the ECKh-432 strain having upstream BDO pathway encoding genes intregrated into the chromosome and containing a plasmid harboring downstream BDO pathway genes.

Figure 55 shows a PCR product containing the non-phosphotransferase (non-PTS) sucrose utilization genes flanked by regions of homology to the *rrnC* region.

Figure 56 shows a schematic diagram of the integrations site in the *rrnC* operon.

Figure 57 shows average product concentration, normalized to culture OD600, after 48 hours of growth of strain ECKh-432 grown on glucose and strain ECKh-463 grown on sucrose. Both contain the plasmid pZS*13 expressing *P. gingivalis* Cat2 and *C. beijerinckii* Ald. The data is for 6 replicate cultures of each strain. The products measured were 1,4-butanediol (BDO), 4-hydroxybutyrate (4HB), γ-butyrolactone (GBL), pyruvate (PYR) and acetate (ACE) (left to right bars, respectively).

Figure 58 shows exemplary pathways to 1,4-butanediol from succcinyl-CoA and alpha-ketoglutarate. Abbreviations: A) Succinyl-CoA reductase (aldehyde forming), B) Alpha-ketoglutarate decarboxylase, C) 4-Hydroxybutyrate dehydrogenase, D) 4-Hydroxybutyrate reductase, E) 1,4-Butanediol dehydrogenase.

Figure 59A shows the nucleotide sequence (SEQ ID NO:) of carboxylic acid reductase from *Nocardia iowensis* (GNM_720), and Figure 59B shows the encoded amino acid sequence (SEQ ID NO:).

Figure 60A shows the nucleotide sequence (SEQ ID NO:) of phosphpantetheine transferase, which was codon optimized, and Figure 60B shows the encoded amino acid sequence.

Figure 61 shows a plasmid map of plasmid pZS*-13S-720 721opt.

Figures 62A and 62B show pathways to 1,4-butanediol from succinate, succcinyl-CoA, and alpha-ketoglutarate. Abbreviations: A) Succinyl-CoA reductase (aldehyde forming), B) Alpha-ketoglutarate decarboxylase, C) 4-Hydroxybutyrate dehydrogenase, D) 4-Hydroxybutyrate reductase, E) 1,4-Butanediol dehydrogenase, F) Succinate reductase, G) Succinyl-CoA transferase, H) Succinyl-CoA hydrolase, I) Succinyl-CoA synthetase (or Succinyl-CoA ligase), J) Glutamate dehydrogenase, K) Glutamate transaminase, L) Glutamate decarboxylase, M) 4-aminobutyrate dehydrogenase, N) 4-aminobutyrate transaminase, O) 4-Hydroxybutyrate kinase, P) Phosphotrans-4-hydroxybutyrylase, Q) 4-Hydroxybutyryl-CoA reductase (aldehyde forming), R) 4-hydroxybutyryl-phosphate reductase, S) Succinyl-CoA reductase (alcohol forming), T) 4-Hydroxybutyryl-CoA transferase, U) 4-Hydroxybutyryl-CoA hydrolase, V) 4-Hydroxybutyryl-CoA synthetase (or 4-Hydroxybutyryl-CoA ligase), W) 4-Hydroxybutyryl-CoA reductase (alcohol forming), X) Alpha-ketoglutarate reductase, Y) 5-Hydroxy-2-oxopentanoate dehydrogenase, Z) 5-Hydroxy-2-oxopentanoate decarboxylase, AA) 5-hydroxy-2-oxopentanoate dehydrogenase (decarboxylation).

Figure 63 shows pathways to putrescine from succinate, succcinyl-CoA, and alpha-ketoglutarate. Abbreviations: A) Succinyl-CoA reductase (aldehyde forming), B) Alpha-ketoglutarate decarboxylase, C) 4-Aminobutyrate reductase, D) Putrescine dehydrogenase , E) Putrescine transaminase, F) Succinate reductase, G) Succinyl-CoA transferase, H) Succinyl-CoA hydrolase, I) Succinyl-CoA synthetase (or Succinyl-CoA ligase), J) Glutamate dehydrogenase, K) Glutamate transaminase, L) Glutamate decarboxylase, M) 4-Aminobutyrate dehydrogenase, N) 4-Aminobutyrate transaminase, O) Alpha-ketoglutarate reductase, P) 5-Amino-2-oxopentanoate dehydrogenase, Q) 5-Amino-2-oxopentanoate transaminase, R) 5-Amino-2-oxopentanoate decarboxylase, S) Ornithine dehydrogenase, T) Ornithine transaminase, U) Ornithine decarboxylase.

Figure 64A shows the nucleotide sequence (SEQ ID NO:) of carboxylic acid reductase from *Mycobacterium smegmatis* mc(2)155 (designated 890), and Figure 64B shows the encoded amino acid sequence (SEQ ID NO:).

Figure 65A shows the nucleotide sequence (SEQ ID NO:) of carboxylic acid reductase from *Mycobacterium avium* subspecies *paratuberculosis* K-10 (designated 891), and Figure 65B shows the encoded amino acid sequence (SEQ ID NO:).

Figure 66A shows the nucleotide sequence (SEQ ID NO:) of carboxylic acid reductase from *Mycobacterium marinum M* (designated 892), and Figure 66B shows the encoded amino acid sequence (SEQ ID NO:).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to the design and production of cells and organisms having biosynthetic production capabilities for 4-hydroxybutanoic acid (4-HB), γ-butyrolactone, 1,4-butanediol (BDO), 4-hydroxybutanal (4-HBal), 4-hydroxybutyryl-CoA (4-HBCoA) and/or putrescine. The invention, in particular, relates to the design of microbial organisms capable of producing BDO, 4-HBal, 4-HBCoA and/or putrescine by introducing one or more nucleic acids encoding a BDO, 4-HBal, 4-HBCoA and/or putrescine pathway enzyme.

In one embodiment, the invention utilizes *in silico* stoichiometric models of *Escherichia coli* metabolism that identify metabolic designs for biosynthetic production of 4-hydroxybutanoic acid (4-HB), 1,4-butanediol (BDO), 4-HBal, 4-HBCoA and/or putrescine. The results described herein indicate that metabolic pathways can be designed and recombinantly engineered to achieve the biosynthesis of 4-HBal, 4-HBCoA or 4-HB and downstream products such as 1,4-

butanediol or putrescine in *Escherichia coli* and other cells or organisms. Biosynthetic production of 4-HB, 4-HBal, 4-HBCoA, BDO and/or putrescine, for example, for the *in silico* designs can be confirmed by construction of strains having the designed metabolic genotype. These metabolically engineered cells or organisms also can be subjected to adaptive evolution to further augment 4-HB, 4-HBal, 4-HBCoA, BDO and/or putrescine biosynthesis, including under conditions approaching theoretical maximum growth.

In certain embodiments, the 4-HB, 4-HBal, 4-HBCoA, BDO and/or putrescine biosynthesis characteristics of the designed strains make them genetically stable and particularly useful in continuous bioprocesses. Separate strain design strategies were identified with incorporation of different non-native or heterologous reaction capabilities into *E. coli* or other host organisms leading to 4-HB and 1,4-butanediol producing metabolic pathways from either CoA-independent succinic semialdehyde dehydrogenase, succinyl-CoA synthetase and CoA-dependent succinic semialdehyde dehydrogenase, or glutamate:succinic semialdehyde transaminase. *In silico* metabolic designs were identified that resulted in the biosynthesis of 4-HB in both *E.coli* and yeast species from each of these metabolic pathways. The 1,4-butanediol intermediate γ-butyrolactone can be generated in culture by spontaneous cyclization under conditions at pH<7.5, particularly under acidic conditions, such as below pH 5.5, for example, pH<7, pH<6.5, pH<6, and particularly at pH<5.5 or lower.

Strains identified via the computational component of the platform can be put into actual production by genetically engineering any of the predicted metabolic alterations which lead to the biosynthetic production of 4-HB, 1,4-butanediol or other intermediate and/or downstream products. In yet a further embodiment, strains exhibiting biosynthetic production of these compounds can be further subjected to adaptive evolution to further augment product biosynthesis. The levels of product biosynthesis yield following adaptive evolution also can be predicted by the computational component of the system.

In other specific embodiments, microbial organisms were constructed to express a 4-HB biosynthetic pathway encoding the enzymatic steps from succinate to 4-HB and to 4-HB-CoA. Co-expression of succinate coenzyme A transferase, CoA-dependent succinic semialdehyde dehydrogenase, NAD-dependent 4-hydroxybutyrate dehydrogenase and 4-hydroxybutyrate coenzyme A transferase in a host microbial organism resulted in significant production of 4-HB compared to host microbial organisms lacking a 4-HB biosynthetic pathway. In a further specific embodiment, 4-HB-producing microbial organisms were generated that utilized α-

ketoglutarate as a substrate by introducing nucleic acids encoding α-ketoglutarate decarboxylase and NAD-dependent 4-hydroxybutyrate dehydrogenase.

In another specific embodiment, microbial organisms containing a 1,4-butanediol (BDO) biosynthetic pathway were constructed that biosynthesized BDO when cultured in the presence of 4-HB. The BDO biosynthetic pathway consisted of a nucleic acid encoding either a multifunctional aldehyde/alcohol dehydrogenase or nucleic acids encoding an aldehyde dehydrogenawse and an alcohol dehydrogenase. To support growth on 4-HB substrates, these BDO-producing microbial organisms also expressed 4-hydroxybutyrate CoA transferase or 4-butyrate kinase in conjunction with phosphotranshydroxybutyrlase. In yet a further specific embodiment, microbial organisms were generated that synthesized BDO through exogenous expression of nucleic acids encoding a functional 4-HB biosynthetic pathway and a functional BDO biosynthetic pathway. The 4-HB biosynthetic pathway consisted of succinate coenzyme A transferase, CoA-dependent succinic semialdehyde dehydrogenase, NAD-dependent 4-hydroxybutyrate dehydrogenase and 4-hydroxybutyrate coenzyme A transferase. The BDO pathway consisted of a multifunctional aldehyde/alcohol dehydrogenase. Further described herein are additional pathways for production of BDO (see Figures 8-13).

In a further embodiment, described herein is the cloning and expression of a carboxylic acid reductase enzyme that functions in a 4-hydroxybutanal, 4-hydroxybutyryl-CoA or 1,4-butanediol metabolic pathway. Advantages of employing a carboxylic acid reductase as opposed to an acyl-CoA reductase to form 4-hydroxybutyraldehyde (4-hydroxybutanal) include lower ethanol and GBL byproduct formation accompanying the production of BDO. Also disclosed herein is the application of carboxylic acid reductase as part of additional numerous pathways to produce 1,4-butanediol and putrescine from the tricarboxylic acid (TCA) cycle metabolites, for example, succinate, succinyl-CoA, and/or alpha-ketoglutarate.

As used herein, the term "non-naturally occurring" when used in reference to a microbial organism or microorganism of the invention is intended to mean that the microbial organism has at least one genetic alteration not normally found in a naturally occurring strain of the referenced species, including wild-type strains of the referenced species. Genetic alterations include, for example, modifications introducing expressible nucleic acids encoding metabolic polypeptides, other nucleic acid additions, nucleic acid deletions and/or other functional disruption of the microbial organism's genetic material. Such modifications include, for example, coding regions and functional fragments thereof, for heterologous, homologous or both heterologous and

homologous polypeptides for the referenced species. Additional modifications include, for example, non-coding regulatory regions in which the modifications alter expression of a gene or operon. Exemplary metabolic polypeptides include enzymes or proteins within a biosynthetic pathway for a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine family of compounds.

A metabolic modification refers to a biochemical reaction that is altered from its naturally occurring state. Therefore, non-naturally occurring microorganisms can have genetic modifications to nucleic acids encoding metabolic polypeptides or, functional fragments thereof. Exemplary metabolic modifications are disclosed herein.

As used herein, the term "isolated" when used in reference to a microbial organism is intended to mean an organism that is substantially free of at least one component as the referenced microbial organism is found in nature. The term includes a microbial organism that is removed from some or all components as it is found in its natural environment. The term also includes a microbial organism that is removed from some or all components as the microbial organism is found in non-naturally occurring environments. Therefore, an isolated microbial organism is partly or completely separated from other substances as it is found in nature or as it is grown, stored or subsisted in non-naturally occurring environments. Specific examples of isolated microbial organisms include partially pure microbes, substantially pure microbes and microbes cultured in a medium that is non-naturally occurring.

As used herein, the terms "microbial," "microbial organism" or "microorganism" is intended to mean any organism that exists as a microscopic cell that is included within the domains of archaea, bacteria or eukarya. Therefore, the term is intended to encompass prokaryotic or eukaryotic cells or organisms having a microscopic size and includes bacteria, archaea and eubacteria of all species as well as eukaryotic microorganisms such as yeast and fungi. The term also includes cell cultures of any species that can be cultured for the production of a biochemical.

As used herein, the term "4-hydroxybutanoic acid" is intended to mean a 4-hydroxy derivative of butyric acid having the chemical formula $C_4H_8O_3$ and a molecular mass of 104.11 g/mol (126.09 g/mol for its sodium salt). The chemical compound 4-hydroxybutanoic acid also is known in the art as 4-HB, 4-hydroxybutyrate, gamma-hydroxybutyric acid or GHB. The term as it is used herein is intended to include any of the compound's various salt forms and include, for example, 4-hydroxybutanoate and 4-hydroxybutyrate. Specific examples of salt forms for 4-HB include sodium 4-HB and potassium 4-HB. Therefore, the terms 4-hydroxybutanoic acid, 4-HB,

4-hydroxybutyrate, 4-hydroxybutanoate, gamma-hydroxybutyric acid and GHB as well as other art recognized names are used synonymously herein.

As used herein, the term "monomeric" when used in reference to 4-HB is intended to mean 4-HB in a non-polymeric or underivatized form. Specific examples of polymeric 4-HB include poly-4-hydroxybutanoic acid and copolymers of, for example, 4-HB and 3-HB. A specific example of a derivatized form of 4-HB is 4-HB-CoA. Other polymeric 4-HB forms and other derivatized forms of 4-HB also are known in the art.

As used herein, the term "γ-butyrolactone" is intended to mean a lactone having the chemical formula $C_4H_6O_2$ and a molecular mass of 86.089 g/mol. The chemical compound γ-butyrolactone also is know in the art as GBL, butyrolactone, 1,4-lactone, 4-butyrolactone, 4-hydroxybutyric acid lactone, and gamma-hydroxybutyric acid lactone. The term as it is used herein is intended to include any of the compound's various salt forms.

As used herein, the term "1,4-butanediol" is intended to mean an alcohol derivative of the alkane butane, carrying two hydroxyl groups which has the chemical formula $C_4H_{10}O_2$ and a molecular mass of 90.12 g/mol. The chemical compound 1,4-butanediol also is known in the art as BDO and is a chemical intermediate or precursor for a family of compounds referred to herein as BDO family of compounds.

As used herein, the term "4-hydroxybutanal" is intended to mean an aledehyde having the chemical formula $C_4H_8O_2$ and a molecular mass of 88.10512 g/mol. The chemical compound 4-hydroxybutanal (4-HBal) is also known in the art as 4-hydroxybutyraldehyde.

As used herein, the term "putrescine" is intended to mean a diamine having the chemical formula $C_4H_{12}N_2$ and a molecular mass of 88.15148 g/mol. The chemical compound putrescine is also known in the art as 1,4-butanediamine, 1,4-diaminobutane, butylenediamine, tetramethylenediamine, tetramethyldiamine, and 1,4-butylenediamine.

As used herein, the term "tetrahydrofuran" is intended to mean a heterocyclic organic compound corresponding to the fully hydrogenated analog of the aromatic compound furan which has the chemical formula C_4H_8O and a molecular mass of 72.11 g/mol. The chemical compound tetrahydrofuran also is known in the art as THF, tetrahydrofuran, 1,4-epoxybutane, butylene oxide, cyclotetramethylene oxide, oxacyclopentane, diethylene oxide, oxolane, furanidine,

hydrofuran, tetra-methylene oxide. The term as it is used herein is intended to include any of the compound's various salt forms.

As used herein, the term "CoA" or "coenzyme A" is intended to mean an organic cofactor or prosthetic group (nonprotein portion of an enzyme) whose presence is required for the activity of many enzymes (the apoenzyme) to form an active enzyme system. Coenzyme A functions in certain condensing enzymes, acts in acetyl or other acyl group transfer and in fatty acid synthesis and oxidation, pyruvate oxidation and in other acetylation.

As used herein, the term "substantially anaerobic" when used in reference to a culture or growth condition is intended to mean that the amount of oxygen is less than about 10% of saturation for dissolved oxygen in liquid media. The term also is intended to include sealed chambers of liquid or solid medium maintained with an atmosphere of less than about 1% oxygen.

The non-naturally occurring microbal organisms of the invention can contain stable genetic alterations, which refers to microorganisms that can be cultured for greater than five generations without loss of the alteration. Generally, stable genetic alterations include modifications that persist greater than 10 generations, particularly stable modifications will persist more than about 25 generations, and more particularly, stable genetic modifications will be greater than 50 generations, including indefinitely.

Those skilled in the art will understand that the genetic alterations, including metabolic modifications exemplified herein are described with reference to a suitable source or host organism such as *E. coli*, yeast, or other organisms disclosed herein and their corresponding metabolic reactions or a suitable source organism for desired genetic material such as genes encoding enzymes for their corresponding metabolic reactions for a desired metabolic pathway. However, given the complete genome sequencing of a wide variety of organisms and the high level of skill in the area of genomics, those skilled in the art will readily be able to apply the teachings and guidance provided herein to essentially all other organisms. For example, the *E. coli* metabolic alterations exemplified herein can readily be applied to other species by incorporating the same or analogous encoding nucleic acid from species other than the referenced species. Such genetic alterations include, for example, genetic alterations of species homologs, in general, and in particular, orthologs, paralogs or nonorthologous gene displacements.

An ortholog is a gene or genes that are related by vertical descent and are responsible for substantially the same or identical functions in different organisms. For example, mouse epoxide hydrolase and human epoxide hydrolase can be considered orthologs for the biological function of hydrolysis of epoxides. Genes are related by vertical descent when, for example, they share sequence similarity of sufficient amount to indicate they are homologous, or related by evolution from a common ancestor. Genes can also be considered orthologs if they share three-dimensional structure but not necessarily sequence similarity, of a sufficient amount to indicate that they have evolved from a common ancestor to the extent that the primary sequence similarity is not identifiable. Genes that are orthologous can encode proteins with sequence similarity of about 25% to 100% amino acid sequence identity. Genes encoding proteins sharing an amino acid similarity less that 25% can also be considered to have arisen by vertical descent if their three-dimensional structure also shows similarities. Members of the serine protease family of enzymes, including tissue plasminogen activator and elastase, are considered to have arisen by vertical descent from a common ancestor.

Orthologs include genes or their encoded gene products that through, for example, evolution, have diverged in structure or overall activity. For example, where one species encodes a gene product exhibiting two functions and where such functions have been separated into distinct genes in a second species, the three genes and their corresponding products are considered to be orthologs. For the production, including growth-coupled production, of a biochemical product, those skilled in the art will understand that the orthologous gene harboring the metabolic activity to be introduced or disrupted is to be chosen for construction of the non-naturally occurring microorganism. An example of orthologs exhibiting separable activities is where distinct activities have been separated into distinct gene products between two or more species or within a single species. A specific example is the separation of elastase proteolysis and plasminogen proteolysis, two types of serine protease activity, into distinct molecules as plasminogen activator and elastase. A second example is the separation of mycoplasma 5'-3' exonuclease and *Drosophila* DNA polymerase III activity. The DNA polymerase from the first species can be considered an ortholog to either or both of the exonuclease or the polymerase from the second species and vice versa.

In contrast, paralogs are homologs related by, for example, duplication followed by evolutionary divergence and have similar or common, but not identical functions. Paralogs can originate or derive from, for example, the same species or from a different species. For example, microsomal epoxide hydrolase (epoxide hydrolase I) and soluble epoxide hydrolase (epoxide

hydrolase II) can be considered paralogs because they represent two distinct enzymes, co-evolved from a common ancestor, that catalyze distinct reactions and have distinct functions in the same species. Paralogs are proteins from the same species with significant sequence similarity to each other suggesting that they are homologous, or related through co-evolution from a common ancestor. Groups of paralogous protein families include HipA homologs, luciferase genes, peptidases, and others.

A nonorthologous gene displacement is a nonorthologous gene from one species that can substitute for a referenced gene function in a different species. Substitution includes, for example, being able to perform substantially the same or a similar function in the species of origin compared to the referenced function in the different species. Although generally, a nonorthologous gene displacement will be identifiable as structurally related to a known gene encoding the referenced function, less structurally related but functionally similar genes and their corresponding gene products nevertheless will still fall within the meaning of the term as it is used herein. Functional similarity requires, for example, at least some structural similarity in the active site or binding region of a nonorthologous gene product compared to a gene encoding the function sought to be substituted. Therefore, a nonorthologous gene includes, for example, a paralog or an unrelated gene.

Therefore, in identifying and constructing the non-naturally occurring microbial organisms of the invention having 4-HB, GBL, 4-HBal, 4-HBCoA, BDO and/or putrescine biosynthetic capability, those skilled in the art will understand with applying the teaching and guidance provided herein to a particular species that the identification of metabolic modifications can include identification and inclusion or inactivation of orthologs. To the extent that paralogs and/or nonorthologous gene displacements are present in the referenced microorganism that encode an enzyme catalyzing a similar or substantially similar metabolic reaction, those skilled in the art also can utilize these evolutionally related genes.

Orthologs, paralogs and nonorthologous gene displacements can be determined by methods well known to those skilled in the art. For example, inspection of nucleic acid or amino acid sequences for two polypeptides will reveal sequence identity and similarities between the compared sequences. Based on such similarities, one skilled in the art can determine if the similarity is sufficiently high to indicate the proteins are related through evolution from a common ancestor. Algorithms well known to those skilled in the art, such as Align, BLAST, Clustal W and others compare and determine a raw sequence similarity or identity, and also

determine the presence or significance of gaps in the sequence which can be assigned a weight or score. Such algorithms also are known in the art and are similarly applicable for determining nucleotide sequence similarity or identity. Parameters for sufficient similarity to determine relatedness are computed based on well known methods for calculating statistical similarity, or the chance of finding a similar match in a random polypeptide, and the significance of the match determined. A computer comparison of two or more sequences can, if desired, also be optimized visually by those skilled in the art. Related gene products or proteins can be expected to have a high similarity, for example, 25% to 100% sequence identity. Proteins that are unrelated can have an identity which is essentially the same as would be expected to occur by chance, if a database of sufficient size is scanned (about 5%). Sequences between 5% and 24% may or may not represent sufficient homology to conclude that the compared sequences are related. Additional statistical analysis to determine the significance of such matches given the size of the data set can be carried out to determine the relevance of these sequences.

Exemplary parameters for determining relatedness of two or more sequences using the BLAST algorithm, for example, can be as set forth below. Briefly, amino acid sequence alignments can be performed using BLASTP version 2.0.8 (Jan-05-1999) and the following parameters: Matrix: 0 BLOSUM62; gap open: 11; gap extension: 1; x_dropoff: 50; expect: 10.0; wordsize: 3; filter: on. Nucleic acid sequence alignments can be performed using BLASTN version 2.0.6 (Sept-16-1998) and the following parameters: Match: 1; mismatch: -2; gap open: 5; gap extension: 2; x_dropoff: 50; expect: 10.0; wordsize: 11; filter: off. Those skilled in the art will know what modifications can be made to the above parameters to either increase or decrease the stringency of the comparison, for example, and determine the relatedness of two or more sequences.

Disclosed herein are non-naturally occurring microbial biocatalyst or microbial organisms including a microbial organism having a 4-hydroxybutanoic acid (4-HB) biosynthetic pathway that includes at least one exogenous nucleic acid encoding 4-hydroxybutanoate dehydrogenase, CoA-independent succinic semialdehyde dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, glutamate:succinic semialdehyde transaminase, alpha-ketoglutarate decarboxylase, or glutamate decarboxylase, wherein the exogenous nucleic acid is expressed in sufficient amounts to produce monomeric 4-hydroxybutanoic acid (4-HB). 4-hydroxybutanoate dehydrogenase is also referred to as 4-hydroxybutyrate dehydrogenase or 4-HB dehydrogenase. Succinyl-CoA synthetase is also referred to as succinyl-CoA synthase or succinyl-CoA ligase.

Also disclosed herein is a non-naturally occurring microbial biocatalyst or microbial organism including a microbial organism having a 4-hydroxybutanoic acid (4-HB) biosynthetic pathway having at least one exogenous nucleic acid encoding 4-hydroxybutanoate dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, or α-ketoglutarate decarboxylase, wherein the exogenous nucleic acid is expressed in sufficient amounts to produce monomeric 4-hydroxybutanoic acid (4-HB).

The non-naturally occurring microbial biocatalysts or microbial organisms can include microbial organisms that employ combinations of metabolic reactions for biosynthetically producing the compounds of the invention. The biosynthesized compounds can be produced intracellularly and/or secreted into the culture medium. Exemplary compounds produced by the non-naturally occurring microorganisms include, for example, 4-hydroxybutanoic acid, 1,4-butanediol and γ-butyrolactone.

In one embodiment, a non-naturally occurring microbial organism is engineered to produce 4-HB. This compound is one useful entry point into the 1,4-butanediol family of compounds. The biochemical reactions for formation of 4-HB from succinate, from succinate through succinyl-CoA or from α-ketoglutarate are shown in steps 1-8 of Figure 1.

It is understood that any combination of appropriate enzymes of a BDO, 4-HBal, 4-HBCoA and/or putrescine pathway can be used so long as conversion from a starting component to the BDO, 4-HBal, 4-HBCoA and/or putrescine product is achieved. Thus, it is understood that any of the metabolic pathways disclosed herein can be utilized and that it is well understood to those skilled in the art how to select appropriate enzymes to achieve a desired pathway, as disclosed herein.

In another embodiment, disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a 1,4-butanediol (BDO) pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, the BDO pathway comprising 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, 4-aminobutyrate-CoA ligase, 4-aminobutyryl-CoA oxidoreductase (deaminating), 4-aminobutyryl-CoA transaminase, or 4-hydroxybutyryl-CoA dehydrogenase (see Example VII Table 17). The BDO pathway further can comprise 4-hydroxybutyryl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA reductase, or 1,4-butanediol dehydrogenase.

It is understood by those skilled in the art that various combinations of the pathways can be utilized, as disclosed herein. For example, in a non-naturally occurring microbial organism, the nucleic acids can encode 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, or 4-aminobutyrate-CoA ligase; 4-aminobutyryl-CoA oxidoreductase (deaminating) or 4-aminobutyryl-CoA transaminase; and 4-hydroxybutyryl-CoA dehydrogenase. Other exemplary combinations are specifically describe below and further can be found in Figures 8-13. For example, the BDO pathway can further comprise 4-hydroxybutyryl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA reductase, or 1,4-butanediol dehydrogenase.

Additionally disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a BDO pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, the BDO pathway comprising 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, 4-aminobutyrate-CoA ligase, 4-aminobutyryl-CoA reductase (alcohol forming), 4-aminobutyryl-CoA reductase, 4-aminobutan-1-ol dehydrogenase, 4-aminobutan-1-ol oxidoreductase (deaminating) or 4-aminobutan-1-ol transaminase (see Example VII and Table 18), and can further comprise 1,4-butanediol dehydrogenase. For example, the exogenous nucleic acids can encode 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, or 4-aminobutyrate-CoA ligase; 4-aminobutyryl-CoA reductase (alcohol forming); and 4-aminobutan-1-ol oxidoreductase (deaminating) or 4-aminobutan-1-ol transaminase. In addition, the nucleic acids can encode. 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, or 4-aminobutyrate-CoA ligase; 4-aminobutyryl-CoA reductase; 4-aminobutan-1-ol dehydrogenase; and 4-aminobutan-1-ol oxidoreductase (deaminating) or 4-aminobutan-1-ol transaminase.

Also disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a BDO pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, the BDO pathway comprising 4-aminobutyrate kinase, 4-aminobutyraldehyde dehydrogenase (phosphorylating), 4-aminobutan-1-ol dehydrogenase, 4-aminobutan-1-ol oxidoreductase (deaminating), 4-aminobutan-1-ol transaminase, [(4-aminobutanolyl)oxy]phosphonic acid oxidoreductase (deaminating), [(4-aminobutanolyl)oxy]phosphonic acid transaminase, 4-hydroxybutyryl-phosphate dehydrogenase, or 4-hydroxybutyraldehyde dehydrogenase (phosphorylating) (see Example VII and Table 19). For example, the exogenous nucleic acids can encode 4-aminobutyrate kinase; 4-aminobutyraldehyde dehydrogenase (phosphorylating); 4-aminobutan-1-ol dehydrogenase; and 4-aminobutan-1-ol oxidoreductase (deaminating) or 4-aminobutan-1-ol

transaminase. Alternatively, the exogenous nucleic acids can encode 4-aminobutyrate kinase; [(4-aminobutanolyl)oxy]phosphonic acid oxidoreductase (deaminating) or [(4-aminobutanolyl)oxy]phosphonic acid transaminase; 4-hydroxybutyryl-phosphate dehydrogenase; and 4-hydroxybutyraldehyde dehydrogenase (phosphorylating).

Additionally disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a BDO pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, the BDO pathway comprising alpha-ketoglutarate 5-kinase, 2,5-dioxopentanoic semialdehyde dehydrogenase (phosphorylating), 2,5-dioxopentanoic acid reductase, alpha-ketoglutarate CoA transferase, alpha-ketoglutaryl-CoA hydrolase, alpha-ketoglutaryl-CoA ligase, alpha-ketoglutaryl-CoA reductase, 5-hydroxy-2-oxopentanoic acid dehydrogenase, alpha-ketoglutaryl-CoA reductase (alcohol forming), 5-hydroxy-2-oxopentanoic acid decarboxylase, or 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation) (see Example VIII and Table 20). The BDO pathway can further comprise 4-hydroxybutyryl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA reductase, or 1,4-butanediol dehydrogenase. For example, the exogenous nucleic acids can encode alpha-ketoglutarate 5-kinase; 2,5-dioxopentanoic semialdehyde dehydrogenase (phosphorylating); 2,5-dioxopentanoic acid reductase; and 5-hydroxy-2-oxopentanoic acid decarboxylase. Alternatively, the exogenous nucleic acids can encode alpha-ketoglutarate 5-kinase; 2,5-dioxopentanoic semialdehyde dehydrogenase (phosphorylating); 2,5-dioxopentanoic acid reductase; and 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation). Alternatively, the exogenous nucleic acids can encode alpha-ketoglutarate CoA transferase, alpha-ketoglutaryl-CoA hydrolase, or alpha-ketoglutaryl-CoA ligase; alpha-ketoglutaryl-CoA reductase, 5-hydroxy-2-oxopentanoic acid dehydrogenase; and 5-hydroxy-2-oxopentanoic acid decarboxylase. In another embodiment, the exogenous nucleic acids can encode alpha-ketoglutarate CoA transferase, alpha-ketoglutaryl-CoA hydrolase, or alpha-ketoglutaryl-CoA ligase; alpha-ketoglutaryl-CoA reductase, 5-hydroxy-2-oxopentanoic acid dehydrogenase, and 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation). Alternatively, the exogenous nucleic acids can encode alpha-ketoglutarate CoA transferase, alpha-ketoglutaryl-CoA hydrolase, or alpha-ketoglutaryl-CoA ligase; alpha-ketoglutaryl-CoA reductase (alcohol forming); and 5-hydroxy-2-oxopentanoic acid decarboxylase. In yet another embodiment, the exogenous nucleic acids can encode alpha-ketoglutarate CoA transferase, alpha-ketoglutaryl-CoA hydrolase, or alpha-ketoglutaryl-CoA ligase; alpha-ketoglutaryl-CoA reductase (alcohol forming); and 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation).

Further disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a BDO pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, the BDO pathway comprising glutamate CoA transferase, glutamyl-CoA hydrolase, glutamyl-CoA ligase, glutamate 5-kinase, glutamate-5-semialdehyde dehydrogenase (phosphorylating), glutamyl-CoA reductase, glutamate-5-semialdehyde reductase, glutamyl-CoA reductase (alcohol forming), 2-amino-5-hydroxypentanoic acid oxidoreductase (deaminating), 2-amino-5-hydroxypentanoic acid transaminase, 5-hydroxy-2-oxopentanoic acid decarboxylase, 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation) (see Example IX and Table 21). For example, the exogenous nucleic acids can encode glutamate CoA transferase, glutamyl-CoA hydrolase, or glutamyl-CoA ligase; glutamyl-CoA reductase; glutamate-5-semialdehyde reductase; 2-amino-5-hydroxypentanoic acid oxidoreductase (deaminating) or 2-amino-5-hydroxypentanoic acid transaminase; and 5-hydroxy-2-oxopentanoic acid decarboxylase or 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation). Alternatively, the exogenous nucleic acids can encode glutamate 5-kinase; glutamate-5-semialdehyde dehydrogenase (phosphorylating); glutamate-5-semialdehyde reductase; 2-amino-5-hydroxypentanoic acid oxidoreductase (deaminating) or 2-amino-5-hydroxypentanoic acid transaminase; and 5-hydroxy-2-oxopentanoic acid decarboxylase or 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation). In still another embodiment, the exogenous nucleic acids can encode glutamate CoA transferase, glutamyl-CoA hydrolase, or glutamyl-CoA ligase; glutamyl-CoA reductase (alcohol forming); 2-amino-5-hydroxypentanoic acid oxidoreductase (deaminating) or 2-amino-5-hydroxypentanoic acid transaminase; and 5-hydroxy-2-oxopentanoic acid decarboxylase or 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation). In yet another embodiment, the exogenous nucleic acids can encode glutamate 5-kinase; glutamate-5-semialdehyde dehydrogenase (phosphorylating); 2-amino-5-hydroxypentanoic acid oxidoreductase (deaminating) or 2-amino-5-hydroxypentanoic acid transaminase; and 5-hydroxy-2-oxopentanoic acid decarboxylase or 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation).

Also disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a BDO pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, the BDO pathway comprising 3-hydroxybutyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydratase, vinylacetyl-CoA Δ-isomerase, or 4-hydroxybutyryl-CoA dehydratase (see Example X and Table 22). For example, the exogenous nucleic acids can encode 3-hydroxybutyryl-CoA

dehydrogenase; 3-hydroxybutyryl-CoA dehydratase; vinylacetyl-CoA Δ-isomerase; and 4-hydroxybutyryl-CoA dehydratase.

Further disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a BDO pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, the BDO pathway comprising homoserine deaminase, homoserine CoA transferase, homoserine-CoA hydrolase, homoserine-CoA ligase, homoserine-CoA deaminase, 4-hydroxybut-2-enoyl-CoA transferase, 4-hydroxybut-2-enoyl-CoA hydrolase, 4-hydroxybut-2-enoyl-CoA ligase, 4-hydroxybut-2-enoate reductase, 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, or 4-hydroxybut-2-enoyl-CoA reductase (see Example XI and Table 23). For example, the exogenous nucleic acids can encode homoserine deaminase; 4-hydroxybut-2-enoyl-CoA transferase, 4-hydroxybut-2-enoyl-CoA hydrolase, 4-hydroxybut-2-enoyl-CoA ligase; 4-hydroxybut-2-enoyl-CoA reductase. Alternatively, the exogenous nucleic acids can encode homoserine CoA transferase, homoserine-CoA hydrolase, or homoserine-CoA ligase; homoserine-CoA deaminase; and 4-hydroxybut-2-enoyl-CoA reductase. In a further embodiment, the exogenous nucleic acids can encode homoserine deaminase; 4-hydroxybut-2-enoate reductase; and 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyryl-CoA hydrolase, or 4-hydroxybutyryl-CoA ligase. Alternatively, the exogenous nucleic acids can encode homoserine CoA transferase, homoserine-CoA hydrolase, or homoserine-CoA ligase; homoserine-CoA deaminase; and 4-hydroxybut-2-enoyl-CoA reductase.

Further disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a BDO pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BOD, the BDO pathway comprising succinyl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, 4-hydroxybutanal dehydrogenase (phosphorylating) (see Table 15). Such a BDO pathway can further comprise succinyl-CoA reductase, 4-hydroxybutyrate dehydrogenase, 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyrate kinase, phosphotrans-4-hydroxybutyrylase, 4-hydroxybutyryl-CoA reductase, 4-hydroxybutyryl-CoA reductase (alcohol forming), or 1,4-butanediol dehydrogenase.

Additionally disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a BDO pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, the BDO

pathway comprising glutamate dehydrogenase, 4-aminobutyrate oxidoreductase (deaminating), 4-aminobutyrate transaminase, glutamate decarboxylase, 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, 4-hydroxybutanal dehydrogenase (phosphorylating)(see Table 16). Such a BDO pathway can further comprise alpha-ketoglutarate decarboxylase, 4-hydroxybutyrate dehydrogenase, 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyrate kinase, phosphotrans-4-hydroxybutyrylase, 4-hydroxybutyryl-CoA reductase, 4-hydroxybutyryl-CoA reductase (alcohol forming), or 1,4-butanediol dehydrogenase.

The pathways described above are merely exemplary. One skilled in the art can readily select appropriate pathways from those disclosed herein to obtain a suitable BDO pathway or other metabolic pathway, as desired.

The invention provides genetically modified organisms that allow improved production of a desired product such as BDO by increasing the product or decreasing undesirable byproducts. As disclosed herein, the invention provides a non-naturally occurring microbial organism, comprising a microbial organism having a 1,4-butanediol (BDO) pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO. In one embodiment, the microbial organism is genetically modified to express exogenous succinyl-CoA synthetase (see Example XII). For example, the succinyl-CoA synthetase can be encoded by an *Escherichia coli sucCD* genes.

In another embodiment, the microbial organism is genetically modified to express exogenous alpha-ketoglutarate decarboxylase (see Example XIII). For example, the alpha-ketoglutarate decarboxylase can be encoded by the *Mycobacterium bovis sucA* gene. In still another embodiment, the microbial organism is genetically modified to express exogenous succinate semialdehyde dehydrogenase and 4-hydroxybutyrate dehydrogenase and optionally 4-hydroxybutyryl-CoA/acetyl-CoA transferase (see Example XIII). For example, the succinate semialdehyde dehydrogenase (CoA-dependent), 4-hydroxybutyrate dehydrogenase and 4-hydroxybutyryl-CoA/acetyl-CoA transferase can be encoded by *Porphyromonas gingivalis* W83 genes. In an additional embodiment, the microbial organism is genetically modified to express exogenous butyrate kinase and phosphotransbutyrylase (see Example XIII). For example, the butyrate kinase and phosphotransbutyrylase can be encoded by *Clostridium acetobutilicum buk1* and *ptb* genes.

In yet another embodiment, the microbial organism is genetically modified to express exogenous 4-hydroxybutyryl-CoA reductase (see Example XIII). For example, the 4-hydroxybutyryl-CoA

reductase can be encoded by *Clostridium beijerinckii ald* gene. Additionally, in an embodiment of the invention, the microbial organism is genetically modified to express exogenous 4-hydroxybutanal reductase (see Example XIII). For example, the 4-hydroxybutanal reductase can be encoded by *Geobacillus thermoglucosidasius adh1* gene. In another embodiment, the microbial organism is genetically modified to express exogenous pyruvate dehydrogenase subunits (see Example XIV). For example, the exogenous pyruvate dehydrogenase can be NADH insensitive. The pyruvate dehydrogenase subunit can be encoded by the *Klebsiella pneumonia lpdA* gene. In a particular embodiment, the pyruvate dehydrogenase subunit genes of the microbial organism can be under the control of a pyruvate formate lyase promoter.

In still another embodiment, the microbial organism is genetically modified to disrupt a gene encoding an aerobic respiratory control regulatory system (see Example XV). For example, the disruption can be of the *arcA* gene. Such an organism can further comprise disruption of a gene encoding malate dehydrogenase. In a further embodiment, the microbial organism is genetically modified to express an exogenous NADH insensitive citrate synthase(see Example XV). For example, the NADH insensitive citrate synthase can be encoded by *gltA*, such as an R163L mutant of *gltA*. In still another embodiment, the microbial organism is genetically modified to express exogenous phosphoenolpyruvate carboxykinase (see Example XVI). For example, the phosphoenolpyruvate carboxykinase can be encoded by an *Haemophilus influenza* phosphoenolpyruvate carboxykinase gene.

It is understood that any of a number of genetic modifications, as disclosed herein, can be used alone or in various combinations of one or more of the genetic modifications disclosed herein to increase the production of BDO in a BDO producing microbial organism. In a particular embodiment, the microbial organism can be genetically modified to incorporate any and up to all of the genetic modifications that lead to increased production of BDO. In a particular embodiment, the microbial organism containing a BDO pathway can be genetically modified to express exogenous succinyl-CoA synthetase; to express exogenous alpha-ketoglutarate decarboxylase; to express exogenous succinate semialdehyde dehydrogenase and 4-hydroxybutyrate dehydrogenase and optionally 4-hydroxybutyryl-CoA/acetyl-CoA transferase; to express exogenous butyrate kinase and phosphotransbutyrylase; to express exogenous 4-hydroxybutyryl-CoA reductase; and to express exogenous 4-hydroxybutanal reductase; to express exogenous pyruvate dehydrogenase; to disrupt a gene encoding an aerobic respiratory control regulatory system; to express an exogenous NADH insensitive citrate synthase; and to express exogenous phosphoenolpyruvate carboxykinase. Such strains for improved production

are described in Examples XII-XIX. It is thus understood that, in addition to the modifications described above, such strains can additionally include other modifications disclosed herein. Such modifications include, but are not limited to, deletion of endogenous lactate dehydrogenase (*ldhA*), alcohol dehydrogenase (*adhE*), and/or pyruvate formate lyase (*pflB*)(see Examples XII-XIX and Table 28).

Additionally provided is a microbial organism in which one or more genes encoding the exogenously expressed enzymes are integrated into the fimD locus of the host organism (see Example XVII). For example, one or more genes encoding a BDO pathway enzyme can be integrated into the fimD locus for increased production of BDO. Further provided is a microbial organism expressing a non-phosphotransferase sucrose uptake system that increases production of BDO.

Although the genetically modified microbial organisms disclosed herein are exemplified with microbial organisms containing particular BDO pathway enzymes, it is understood that such modifications can be incorporated into any microbial organism having a BDO, 4-HBal, 4-HBCoA and/or putrescine pathway suitable for enhanced production in the presence of the genetic modifications. The microbial organisms of the invention can thus have any of the BDO, 4-HBal, 4-HBCoA and/or putrescine pathways disclosed herein. For example, the BDO pathway can comprise 4-hydroxybutanoate dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, 4-hydroxybutyrate:CoA transferase, 4-butyrate kinase, phosphotransbutyrylase, alpha-ketoglutarate decarboxylase, aldehyde dehydrogenase, alcohol dehydrogenase or an aldehyde/alcohol dehydrogenase (see Figure 1). Alternatively, the BDO pathway can comprise 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, 4-aminobutyrate-CoA ligase, 4-aminobutyryl-CoA oxidoreductase (deaminating), 4-aminobutyryl-CoA transaminase, or 4-hydroxybutyryl-CoA dehydrogenase (see Table 17). Such a BDO pathway can further comprise 4-hydroxybutyryl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA reductase, or 1,4-butanediol dehydrogenase

Additionally, the BDO pathway can comprise 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, 4-aminobutyrate-CoA ligase, 4-aminobutyryl-CoA reductase (alcohol forming), 4-aminobutyryl-CoA reductase, 4-aminobutan-1-ol dehydrogenase, 4-aminobutan-1-ol oxidoreductase (deaminating) or 4-aminobutan-1-ol transaminase (see Table 18). Also, the BDO pathway can comprise 4-aminobutyrate kinase, 4-aminobutyraldehyde

dehydrogenase (phosphorylating), 4-aminobutan-1-ol dehydrogenase, 4-aminobutan-1-ol oxidoreductase (deaminating), 4-aminobutan-1-ol transaminase, [(4-aminobutanolyl)oxy]phosphonic acid oxidoreductase (deaminating), [(4-aminobutanolyl)oxy]phosphonic acid transaminase, 4-hydroxybutyryl-phosphate dehydrogenase, or 4-hydroxybutyraldehyde dehydrogenase (phosphorylating) (see Table 19). Such a pathway can further comprise 1,4-butanediol dehydrogenase.

The BDO pathway can also comprise alpha-ketoglutarate 5-kinase, 2,5-dioxopentanoic semialdehyde dehydrogenase (phosphorylating), 2,5-dioxopentanoic acid reductase, alpha-ketoglutarate CoA transferase, alpha-ketoglutaryl-CoA hydrolase, alpha-ketoglutaryl-CoA ligase, alpha-ketoglutaryl-CoA reductase, 5-hydroxy-2-oxopentanoic acid dehydrogenase, alpha-ketoglutaryl-CoA reductase (alcohol forming), 5-hydroxy-2-oxopentanoic acid decarboxylase, or 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation)(see Table 20). Such a BDO pathway can further comprise 4-hydroxybutyryl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA reductase, or 1,4-butanediol dehydrogenase. Additionally, the BDO pathway can comprise glutamate CoA transferase, glutamyl-CoA hydrolase, glutamyl-CoA ligase, glutamate 5-kinase, glutamate-5-semialdehyde dehydrogenase (phosphorylating), glutamyl-CoA reductase, glutamate-5-semialdehyde reductase, glutamyl-CoA reductase (alcohol forming), 2-amino-5-hydroxypentanoic acid oxidoreductase (deaminating), 2-amino-5-hydroxypentanoic acid transaminase, 5-hydroxy-2-oxopentanoic acid decarboxylase, 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation)(see Table 21). Such a BDO pathway can further comprise 4-hydroxybutyryl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA reductase, or 1,4-butanediol dehydrogenase.

Additionally, the BDO pathway can comprise 3-hydroxybutyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydratase, vinylacetyl-CoA Δ-isomerase, or 4-hydroxybutyryl-CoA dehydratase (see Table 22). Also, the BDO pathway can comprise homoserine deaminase, homoserine CoA transferase, homoserine-CoA hydrolase, homoserine-CoA ligase, homoserine-CoA deaminase, 4-hydroxybut-2-enoyl-CoA transferase, 4-hydroxybut-2-enoyl-CoA hydrolase, 4-hydroxybut-2-enoyl-CoA ligase, 4-hydroxybut-2-enoate reductase, 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, or 4-hydroxybut-2-enoyl-CoA reductase (see Table 23). Such a BDO pathway can further comprise 4-hydroxybutyryl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA reductase, or 1,4-butanediol dehydrogenase.

The BDO pathway can additionally comprise succinyl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, or 4-hydroxybutanal dehydrogenase (phosphorylating) (see Table 15). Such a pathway can further comprise succinyl-CoA reductase, 4-hydroxybutyrate dehydrogenase, 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyrate kinase, phosphotrans-4-hydroxybutyrylase, 4-hydroxybutyryl-CoA reductase, 4-hydroxybutyryl-CoA reductase (alcohol forming), or 1,4-butanediol dehydrogenase. Also, the BDO pathway can comprise glutamate dehydrogenase, 4-aminobutyrate oxidoreductase (deaminating), 4-aminobutyrate transaminase, glutamate decarboxylase, 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, or 4-hydroxybutanal dehydrogenase (phosphorylating)(see Table 16). Such a BDO pathway can further comprise alpha-ketoglutarate decarboxylase, 4-hydroxybutyrate dehydrogenase, 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyrate kinase, phosphotrans-4-hydroxybutyrylase, 4-hydroxybutyryl-CoA reductase, 4-hydroxybutyryl-CoA reductase (alcohol forming), or 1,4-butanediol dehydrogenase.

The invention additionally provides a non-naturally occurring microbial organism, comprising a 4-hydroxybutanal pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutanal pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutanal, the 4-hydroxybutanal pathway comprising succinyl-CoA reductase (aldehyde forming); 4-hydroxybutyrate dehydrogenase; and 4-hydroxybutyrate reductase (see Figure 58, steps A-C-D). The invention also provides a non-naturally occurring microbial organism, comprising a 4-hydroxybutanal pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutanal pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutanal, the 4-hydroxybutanal pathway comprising alpha-ketoglutarate decarboxylase; 4-hydroxybutyrate dehydrogenase; and 4-hydroxybutyrate reductase (Figure 58, steps B-C-D).

The invention further provides a non-naturally occurring microbial organism, comprising a 4-hydroxybutanal pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutanal pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutanal, the 4-hydroxybutanal pathway comprising succinate reductase; 4-hydroxybutyrate dehydrogenase, and 4-hydroxybutyrate reductase (see Figure 62, steps F-C-D). In yet another embodiment, the invention provides a non-naturally occurring microbial organism, comprising a 4-hydroxybutanal pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutanal pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutanal, the 4-hydroxybutanal pathway comprising alpha-ketoglutarate decarboxylase, or glutamate dehydrogenase or glutamate transaminase and glutamate decarboxylase and 4-aminobutyrate

dehydrogenase or 4-aminobutyrate transaminase; 4-hydroxybutyrate dehydrogenase; and 4-hydroxybutyrate reductase (see Figure 62, steps B or ((J or K)-L-(M or N))-C-D).

The invention also provides a non-naturally occurring microbial organism, comprising a 4-hydroxybutanal pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutanal pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutanal, the 4-hydroxybutanal pathway comprising alpha-ketoglutarate reductase; 5-hydroxy-2-oxopentanoate dehydrogenase; and 5-hydroxy-2-oxopentanoate decarboxylase (see Figure 62, steps X-Y-Z). In yet another embodiment, the invention provides a non-naturally occurring microbial organism, comprising a 4-hydroxybutyryl-CoA pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutyryl-CoA pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutyryl-CoA, the 4-hydroxybutyryl-CoA pathway comprising alpha-ketoglutarate reductase; 5-hydroxy-2-oxopentanoate dehydrogenase; and 5-hydroxy-2-oxopentanoate dehydrogenase (decarboxylation) (see Figure 62, steps X-Y-AA).

The invention additionally provides a non-naturally occurring microbial organism, comprising a putrescine pathway comprising at least one exogenous nucleic acid encoding a putrescine pathway enzyme expressed in a sufficient amount to produce putrescine, the putrescine pathway comprising succinate reductase; 4-aminobutyrate dehydrogenase or 4-aminobutyrate transaminase; 4-aminobutyrate reductase; and putrescine dehydrogenase or putrescine transaminase (see Figure 63, steps F-M/N-C-D/E). In still another embodiment, the invention provides a non-naturally occurring microbial organism, comprising a putrescine pathway comprising at least one exogenous nucleic acid encoding a putrescine pathway enzyme expressed in a sufficient amount to produce putrescine, the putrescine pathway comprising alpha-ketoglutarate decarboxylase; 4-aminobutyrate dehydrogenase or 4-aminobutyrate transaminase; 4-aminobutyrate reductase; and putrescine dehydrogenase or putrescine transaminase (see Figure 63, steps B-M/N-C-D/E). The invention additionally provides a non-naturally occurring microbial organism, comprising a putrescine pathway comprising at least one exogenous nucleic acid encoding a putrescine pathway enzyme expressed in a sufficient amount to produce putrescine, the putrescine pathway comprising glutamate dehydrogenase or glutamate transaminase; glutamate decarboxylase; 4-aminobutyrate reductase; and putrescine dehydrogenase or putrescine transaminase (see Figure 63, steps J/K-L-C-D/E).

The invention provides in another embodiment a non-naturally occurring microbial organism, comprising a putrescine pathway comprising at least one exogenous nucleic acid encoding a

putrescine pathway enzyme expressed in a sufficient amount to produce putrescine, the putrescine pathway comprising alpha-ketoglutarate reductase; 5-amino-2-oxopentanoate dehydrogenase or 5-amino-2-oxopentanoate transaminase; 5-amino-2-oxopentanoate decarboxylase; and putrescine dehydrogenase or putrescine transaminase (see Figure 63, steps O-P/Q-R-D/E). Also provided is a non-naturally occurring microbial organism, comprising a putrescine pathway comprising at least one exogenous nucleic acid encoding a putrescine pathway enzyme expressed in a sufficient amount to produce putrescine, the putrescine pathway comprising alpha-ketoglutarate reductase; 5-amino-2-oxopentanoate dehydrogenase or 5-amino-2-oxopentanoate transaminase; ornithine dehydrogenase or ornithine transaminase; and ornithine decarboxylase (see Figure 63, steps O-P/Q-S/T-U).

In an additional embodiment, the invention provides a non-naturally occurring microbial organism having a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway, wherein the non-naturally occurring microbial organism comprises at least one exogenous nucleic acid encoding an enzyme or protein that converts a substrate of any of the pathways disclosed herein (see, for example, the Examples and Figures 1, 8-13, 58, 62 and 63). In an exmemplary embodiment for producing BDO, the microbial organism can convert a substrate to a product selected from the group consisting of succinate to succinyl-CoA; succinyl-CoA to succinic semialdehyde; succinic semialdehyde to 4-hydroxybutrate; 4-hydroxybutyrate to 4-hydroxybutyryl-phosphate; 4-hydroxybutyryl-phosphate to 4-hydroxtbutyryl-CoA; 4-hydroxybutyryl-CoA to 4-hydroxybutanal; and 4-hydroxybutanal to 1,4-butanediol. In a pathway for producing 4-HBal, a microbial organism can convert, for example, succinate to succinic semialdehyde; succinic semialdehyde to 4-hydroxybutyrate; and 4-hydroxybutyrate to 4-hydroxybutanal. Such an organism can additionally include activity to convert 4-hydroxybutanal to 1,4-butanediol in order to produce BDO. Yet another pathway for producing 4-HBal can be, for example, alpha-ketoglutarate to succinic semialdehyde; succinic semialdehyde to 4-hydroxybutyrate; and 4-hydroxybutyrate to 4-hydroxybutanal. An alternative pathway for producing 4-HBal can be, for example, alpha-ketoglutarate to 2,5-dioxopentanoic acid; 2,5-dioxopentanoic acid to 5-hydroxy-2-oxopentanooic acid; and 5-hydroxy-2-oxopentanoic acid to 4-hydroxybutanal. An exemplary 4-hydroxybutyryl-CoA pathway can be, for example, alpha-ketoglutarate to 2,5-dioxopentanoic acid; 2,5-dioxopentanoic acid to 5-hydroxy-2-oxopentanoic acid; and 5-hydroxy-2-oxopentanoic acid to 4-hydroxybutyryl-CoA. An exemplary putrescine pathway can be, for example, succinate to succinyl-CoA; succinyl-CoA to succinic semialdehyde; succinic semialdehyde to 4-aminobutyrate; 4-aminobutyrate to 4-aminobutanal; and 4-aminobutanal to putrescine. An alternative putrescine pathway can be, for example, succinate to succinic semialdehyde; succinic

semialdehyde to 4-aminobutyrate; 4-aminobutyrate to 4-aminobutanal; and 4-aminobutanal to putrescine. One skilled in the art will understand that these are merely exemplary and that any of the substrate-product pairs disclosed herein suitable to produce a desired product and for which an appropriate activity is available for the conversion can be readily determined by one skilled in the art based on the teachings herein. Thus, the invention provides a non-naturally occurring microbial organism containing at least one exogenous nucleic acid encoding an enzyme or protein, where the enzyme or protein converts the substrates and products of a pathway (see Figures 1, 8-13, 58, 62 and 63).

While generally described herein as a microbial organism that contains a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway, it is understood that the invention additionally provides a non-naturally occurring microbial organism comprising at least one exogenous nucleic acid encoding a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway enzyme or protein expressed in a sufficient amount to produce an intermediate of a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway. For example, as disclosed herein, 4-HB, 4-HBal, 4-HBCoA, BDO and putrescine pathways are exemplified in Figures 1, 8-13, 58, 62 and 63. Therefore, in addition to a microbial organism containing, for example, a BDO pathway that produces BDO, the invention additionally provides a non-naturally occurring microbial organism comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme, where the microbial organism produces a BDO pathway intermediate as a product rather than an intermediate of the pathway. In one exemplary embodiment as shown in Figure 62, for example, the invention provides a microbial organism that produces succinyl-CoA, succinic semialdehyde, 4-hydroxybutyrate, 4-hydroxybutyryl-phosphate, 4-hydroxybutyryl-CoA, or 4-hydroxybutanal as a product rather than an intermediate. Another exemplary embodiment includes, for example, a microbial organism that produces alpha-ketoglutarate, 2,5-dioxopentanoic acid, 5-hydroxy-2-oxopentanoic acid, or 4-hydroxybutanal as a product rather than an intermediate. An exemplary embodiment in a putrescine pathway includes, for example, a microbial organism that produces glutamate, 4-aminobutyrate, or 4-aminobutanal as a product rather than an intermediate. An alternative embodiment in a putrescine pathway can be, for example, a microbial organism that produces 2,5-dioxopentanoate, 5-amino-2-oxopentanoate, or ornithine as a product rather than an intermediate.

It is understood that any of the pathways disclosed herein, as described in the Examples and exemplified in the Figures, including the pathways of Figures 1, 8-13, 58, 62 and 63, can be utilized to generate a non-naturally occurring microbial organism that produces any pathway

intermediate or product, as desired. As disclosed herein, such a microbial organism that produces an intermediate can be used in combination with another microbial organism expressing downstream pathway enzymes to produce a desired product. However, it is understood that a non-naturally occurring microbial organism that produces a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway intermediate can be utilized to produce the intermediate as a desired product.

The invention is described herein with general reference to the metabolic reaction, reactant or product thereof, or with specific reference to one or more nucleic acids or genes encoding an enzyme associated with or catalyzing the referenced metabolic reaction, reactant or product. Unless otherwise expressly stated herein, those skilled in the art will understand that reference to a reaction also constitutes reference to the reactants and products of the reaction. Similarly, unless otherwise expressly stated herein, reference to a reactant or product also references the reaction and that reference to any of these metabolic constitutes also references the gene or genes encoding the enzymes that catalyze the referenced reaction, reactant or product. Likewise, given the well known fields of metabolic biochemistry, enzymology and genomics, reference herein to a gene or encoding nucleic acid also constitutes a reference to the corresponding encoded enzyme and the reaction it catalyzes as well as the reactants and products of the reaction.

The production of 4-HB via biosynthetic modes using the microbial organisms of the invention is particularly useful because it can produce monomeric 4-HB. The non-naturally occurring microbial organisms of the invention and their biosynthesis of 4-HB and BDO family compounds also is particularly useful because the 4-HB product can be (1) secreted; (2) can be devoid of any derivatizations such as Coenzyme A; (3) avoids thermodynamic changes during biosynthesis; (4) allows direct biosynthesis of BDO, and (5) allows for the spontaneous chemical conversion of 4-HB to γ-butyrolactone (GBL) in acidic pH medium. This latter characteristic also is particularly useful for efficient chemical synthesis or biosynthesis of BDO family compounds such as 1,4-butanediol and/or tetrahydrofuran (THF), for example.

Microbial organisms generally lack the capacity to synthesize 4-HB and therefore any of the compounds disclosed herein to be within the 1,4-butanediol family of compounds or known by those in the art to be within the 1,4-butanediol family of compounds. Moreover, organisms having all of the requisite metabolic enzymatic capabilities are not known to produce 4-HB from the enzymes described and biochemical pathways exemplified herein. Rather, with the possible

exception of a few anaerobic microorganisms described further below, the microorganisms having the enzymatic capability to use 4-HB as a substrate to produce, for example, succinate. In contrast, the non-naturally occurring microbial organisms of the invention can generate 4-HB, 4-HBal, 4-HBCoA, BDO and/or putrescine as a product. As described above, the biosynthesis of 4-HB in its monomeric form is not only particularly useful in chemical synthesis of BDO family of compounds, it also allows for the further biosynthesis of BDO family compounds and avoids altogether chemical synthesis procedures.

The non-naturally occurring microbial organisms of the invention that can produce 4-HB, 4-HBal, 4-HBCoA, BDO and/or putrescine are produced by ensuring that a host microbial organism includes functional capabilities for the complete biochemical synthesis of at least one 4-HB, 4-HBal, 4-HBCoA, BDO and/or putrscine biosynthetic pathway of the invention. Ensuring at least one requisite 4-HB, 4-HBal, 4-HBCoA or BDO biosynthetic pathway confers 4-HB biosynthesis capability onto the host microbial organism.

Several 4-HB biosynthetic pathways are exemplified herein and shown for purposes of illustration in Figure 1. Additional 4-HB and BDO pathways are described in Figures 8-13. One 4-HB biosynthetic pathway includes the biosynthesis of 4-HB from succinate (the succinate pathway). The enzymes participating in this 4-HB pathway include CoA-independent succinic semialdehyde dehydrogenase and 4-hydroxybutanoate dehydrogenase. In this pathway, CoA-independent succinic semialdehyde dehydrogenase catalyzes the reverse reaction to the arrow shown in Figure 1. Another 4-HB biosynthetic pathway includes the biosynthesis from succinate through succinyl-CoA (the succinyl-CoA pathway). The enzymes participating in this 4-HB pathway include succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase and 4-hydroxybutanoate dehydrogenase. Three other 4-HB biosynthetic pathways include the biosynthesis of 4-HB from α-ketoglutarate (the α-ketoglutarate pathways). Hence, a third 4-HB biosynthetic pathway is the biosynthesis of succinic semialdehyde through glutamate:succinic semialdehyde transaminase, glutamate decarboxylase and 4-hydroxybutanoate dehydrogenase. A fourth 4-HB biosynthetic pathway also includes the biosynthesis of 4-HB from α-ketoglutarate, but utilizes α-ketoglutarate decarboxylase to catalyze succinic semialdehyde synthesis. 4-hydroxybutanoate dehydrogenase catalyzes the conversion of succinic semialdehyde to 4-HB. A fifth 4-HB biosynthetic pathway includes the biosynthesis from α-ketoglutarate through succinyl-CoA and utilizes α-ketoglutarate dehydrogenase to produce succinyl-CoA, which funnels into the succinyl-CoA pathway described above. Each of these 4-HB biosynthetic pathways, their substrates, reactants and products are described further

below in the Examples. As described herein, 4-HB can further be biosynthetically converted to BDO by inclusion of appropriate enzymes to produce BDO (see Example). Thus, it is understood that a 4-HB pathway can be used with enzymes for converting 4-HB to BDO to generate a BDO pathway.

The non-naturally occurring microbial organisms of the invention can be produced by introducing expressible nucleic acids encoding one or more of the enzymes participating in one or more 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine biosynthetic pathways. Depending on the host microbial organism chosen for biosynthesis, nucleic acids for some or all of a particular 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine biosynthetic pathway can be expressed. For example, if a chosen host is deficient in one or more enzymes in a desired biosynthetic pathway, for example, the succinate to 4-HB pathway, then expressible nucleic acids for the deficient enzyme(s), for example, both CoA-independent succinic semialdehyde dehydrogenase and 4-hydroxybutanoate dehydrogenase in this example, are introduced into the host for subsequent exogenous expression. Alternatively, if the chosen host exhibits endogenous expression of some pathway enzymes, but is deficient in others, then an encoding nucleic acid is needed for the deficient enzyme(s) to achieve 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine biosynthesis. For example, if the chosen host exhibites endogenous CoA-independent succinic semialdehyde dehydrogenase, but is deficient in 4-hydroxybutanoate dehydrogenase, then an encoding nucleic acid is needed for this enzyme to achieve 4-HB biosynthesis. Thus, a non-naturally occurring microbial organism of the invention can be produced by introducing exogenous enzyme or protein activities to obtain a desired biosynthetic pathway or a desired biosynthetic pathway can be obtained by introducing one or more exogenous enzyme or protein activities that, together with one or more endogenous enzymes or proteins, produces a desired product such as 4-HB, 4-HBal, 4-HBCoA, BDO and/or putrescine.

In like fashion, where 4-HB biosynthesis is selected to occur through the succinate to succinyl-CoA pathway (the succinyl-CoA pathway), encoding nucleic acids for host deficiencies in the enzymes succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase and/or 4-hydroxybutanoate dehydrogenase are to be exogenously expressed in the recipient host. Selection of 4-HB biosynthesis through the α-ketoglutarate to succinic semialdehyde pathway (the α-ketoglutarate pathway) can utilize exogenous expression for host deficiencies in one or more of the enzymes for glutamate:succinic semialdehyde transaminase, glutamate decarboxylase and/or 4-hydroxybutanoate dehydrogenase, or α-ketoglutarate decarboxylase and

4-hydroxybutanoate dehydrogenase. One skilled in the art can readily determine pathway enzymes for production of 4-HB or BDO, as disclosed herein.

Depending on the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine biosynthetic pathway constituents of a selected host microbial organism, the non-naturally occurring microbial organisms of the invention will include at least one exogenously expressed 4-HB, 4-HB, 4-HBCoA, BDO or putrescine pathway-encoding nucleic acid and up to all encoding nucleic acids for one or more 4-HB or BDO biosynthetic pathways. For example, 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine biosynthesis can be established in a host deficient in a pathway enzyme or protein through exogenous expression of the corresponding encoding nucleic acid. In a host deficient in all enzymes or proteins of a 4-HB, 4-HB, 4-HBCoA, BDO or putrescine pathway, exogenous expression of all enzyme or proteins in the pathway can be included, although it is understood that all enzymes or proteins of a pathway can be expressed even if the host contains at least one of the pathway enzymes or proteins. If desired, exogenous expression of all enzymes or proteins in a pathway for production of 4-HB, 4-HB, 4-HBCoA, BDO or putrescine can be included. For example, 4-HB biosynthesis can be established from all five pathways in a host deficient in 4-hydroxybutanoate dehydrogenase through exogenous expression of a 4-hydroxybutanoate dehydrogenase encoding nucleic acid. In contrast, 4-HB biosynthesis can be established from all five pathways in a host deficient in all eight enzymes through exogenous expression of all eight of CoA-independent succinic semialdehyde dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, glutamate:succinic semialdehyde transaminase, glutamate decarboxylase, α-ketoglutarate decarboxylase, α-ketoglutarate dehydrogenase and 4-hydroxybutanoate dehydrogenase.

Given the teachings and guidance provided herein, those skilled in the art will understand that the number of encoding nucleic acids to introduce in an expressible form will, at least, parallel the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway deficiencies of the selected host microbial organism. Therefore, a non-naturally occurring microbial organism of the invention can have one, two, three, four, five, six, seven, eight or up to all nucleic acids encoding the enzymes disclosed herein constituting one or more 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine biosynthetic pathways. In some embodiments, the non-naturally occurring microbial organisms also can include other genetic modifications that facilitate or optimize 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine biosynthesis or that confer other useful functions onto the host microbial organism. One such other functionality can include, for example, augmentation of the

synthesis of one or more of the 4-HB pathway precursors such as succinate, succinyl-CoA, α-ketoglutarate, 4-aminobutyrate, glutamate, acetoacetyl-CoA, and/or homoserine.

Generally, a host microbial organism is selected such that it produces the precursor of a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway, either as a naturally produced molecule or as an engineered product that either provides *de novo* production of a desired precursor or increased production of a precursor naturally produced by the host microbial organism. For example, succinyl-CoA, α-ketoglutarate, 4-aminobutyrate, glutamate, acetoacetyl-CoA, and homoserine are produced naturally in a host organism such as *E. coli*. A host organism can be engineered to increase production of a precursor, as disclosed herein. In addition, a microbial organism that has been engineered to produce a desired precursor can be used as a host organism and further engineered to express enzymes or proteins of a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway.

In some embodiments, a non-naturally occurring microbial organism of the invention is generated from a host that contains the enzymatic capability to synthesize 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine. In this specific embodiment it can be useful to increase the synthesis or accumulation of a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway product to, for example, drive 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway reactions toward 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine production. Increased synthesis or accumulation can be accomplished by, for example, overexpression of nucleic acids encoding one or more of the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway enzymes disclosed herein. Over expression of the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway enzyme or enzymes can occur, for example, through exogenous expression of the endogenous gene or genes, or through exogenous expression of the heterologous gene or genes. Therefore, naturally occurring organisms can be readily generated to be non-naturally 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine producing microbial organisms of the invention through overexpression of one, two, three, four, five, six and so forth up to all nucleic acids encoding 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine biosynthetic pathway enzymes. In addition, a non-naturally occurring organism can be generated by mutagenesis of an endogenous gene that results in an increase in activity of an enzyme in the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine biosynthetic pathway.

In particularly useful embodiments, exogenous expression of the encoding nucleic acids is employed. Exogenous expression confers the ability to custom tailor the expression and/or regulatory elements to the host and application to achieve a desired expression level that is

controlled by the user. However, endogenous expression also can be utilized in other embodiments such as by removing a negative regulatory effector or induction of the gene's promoter when linked to an inducible promoter or other regulatory element. Thus, an endogenous gene having a naturally occurring inducible promoter can be up-regulated by providing the appropriate inducing agent, or the regulatory region of an endogenous gene can be engineered to incorporate an inducible regulatory element, thereby allowing the regulation of increased expression of an endogenous gene at a desired time. Similarly, an inducible promoter can be included as a regulatory element for an exogenous gene introduced into a non-naturally occurring microbial organism (see Examples).

"Exogenous" as it is used herein is intended to mean that the referenced molecule or the referenced activity is introduced into the host microbial organism. The molecule can be introduced, for example, by introduction of an encoding nucleic acid into the host genetic material such as by integration into a host chromosome or as non-chromosomal genetic material such as a plasmid. Therefore, the term as it is used in reference to expression of an encoding nucleic acid refers to introduction of the encoding nucleic acid in an expressible form into the microbial organism. When used in reference to a biosynthetic activity, the term refers to an activity that is introduced into the host reference organism. The source can be, for example, a homologous or heterologous encoding nucleic acid that expresses the referenced activity following introduction into the host microbial organism. Therefore, the term "endogenous" refers to a referenced molecule or activity that is present in the host. Similarly, the term when used in reference to expression of an encoding nucleic acid refers to expression of an encoding nucleic acid contained within the microbial organism. The term "heterologous" refers to a molecule or activity derived from a source other than the referenced species whereas "homologous" refers to a molecule or activity derived from the host microbial organism. Accordingly, exogenous expression of an encoding nucleic acid of the invention can utilize either or both a heterologous or homologous encoding nucleic acid.

It is understood that when more than one exogenous nucleic acid is included in a microbial organism that the the more than one exogenous nucleic acids refers to the referenced encoding nucleic acid or biosynthetic activity, as discussed above. It is further understood, as disclosed herein, that such more than one exogenous nucleic acids can be introduced into the host microbial organism on separate nucleic acid molecules, on polycistronic nucleic acid molecules, or a combination thereof, and still be considered as more than one exogenous nucleic acid. For example, as disclosed herein a microbial organism can be engineered to express two or more

exogenous nucleic acids encoding a desired pathway enzyme or protein. In the case where two exogenous nucleic acids encoding a desired activity are introduced into a host microbial organism, it is understood that the two exogenous nucleic acids can be introduced as a single nucleic acid, for example, on a single plasmid, on separate plasmids, can be integrated into the host chromosome at a single site or multiple sites, and still be considered as two exogenous nucleic acids. Similarly, it is understood that more than two exogenous nucleic acids can be introduced into a host organism in any desired combination, for example, on a single plasmid, on separate plasmids, can be integrated into the host chromosome at a single site or multiple sites, and still be considered as two or more exogenous nucleic acids, for example three exogenous nucleic acids. Thus, the number of referenced exogenous nucleic acids or biosynthetic activities refers to the number of encoding nucleic acids or the number of biosynthetic activities, not the number of separate nucleic acids introduced into the host organism.

Sources of encoding nucleic acids for a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway enzyme can include, for example, any species where the encoded gene product is capable of catalyzing the referenced reaction. Such species include both prokaryotic and eukaryotic organisms including, but not limited to, bacteria, including archaea and eubacteria, and eukaryotes, including yeast, plant, insect, animal, and mammal, including human. Exemplary species for such sources include, for example, *Escherichia coli*, *Saccharomyces cerevisiae*, *Saccharomyces kluyveri*, *Clostridium kluyveri*, *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharoperbutylacetonicum*, *Clostridium perfringens*, *Clostridium difficile*, *Clostridium botulinum*, *Clostridium tyrobutyricum*, *Clostridium tetanomorphum*, *Clostridium tetani*, *Clostridium propionicum*, *Clostridium aminobutyricum*, *Clostridium subterminale*, *Clostridium sticklandii*, *Ralstonia eutropha*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, *Porphyromonas gingivalis*, *Arabidopsis thaliana*, *Thermus thermophilus*, *Pseudomonas* species, including *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas stutzeri*, *Pseudomonas fluorescens*, *Homo sapiens*, *Oryctolagus cuniculus*, *Rhodobacter spaeroides*, *Thermoanaerobacter brockii*, *Metallosphaera sedula*, *Leuconostoc mesenteroides*, *Chloroflexus aurantiacus*, *Roseiflexus castenholzii*, *Erythrobacter*, *Simmondsia chinensis*, *Acinetobacter* species, *including Acinetobacter calcoaceticus* and *Acinetobacter baylyi*, *Porphyromonas gingivalis*, *Sulfolobus tokodaii*, *Sulfolobus solfataricus*, *Sulfolobus acidocaldarius*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus brevis*, *Bacillus pumilus*, *Rattus norvegicus*, *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Euglena gracilis*, *Treponema denticola*, *Moorella thermoacetica*, *Thermotoga maritima*, *Halobacterium*

salinarum, Geobacillus stearothermophilus, Aeropyrum pernix, Sus scrofa, Caenorhabditis elegans, Corynebacterium glutamicum, Acidaminococcus fermentans, Lactococcus lactis, Lactobacillus plantarum, Streptococcus thermophilus, Enterobacter aerogenes, Candida, Aspergillus terreus, Pedicoccus pentosaceus, Zymomonas mobilus, Acetobacter pasteurians, Kluyveromyces lactis, Eubacterium barkeri, Bacteroides capillosus, Anaerotruncus colihominis, Natranaerobius thermophilusm, Campylobacter jejuni, Haemophilus influenzae, Serratia marcescens, Citrobacter amalonaticus, Myxococcus xanthus, Fusobacterium nuleatum, Penicillium chrysogenum, marine gamma proteobacterium, butyrate-producing bacterium, *Nocardia iowensis, Nocardia farcinica, Streptomyces griseus, Schizosaccharomyces pombe, Geobacillus thermoglucosidasius, Salmonella typhimurium, Vibrio cholera, Heliobacter pylori, Nicotiana tabacum, Oryza sativa, Haloferax mediterranei, Agrobacterium tumefaciens, Achromobacter denitrificans, Fusobacterium nucleatum, Streptomyces clavuligenus, Acinetobacter baumanii, Mus musculus, Lachancea kluyveri, Trichomonas vaginalis, Trypanosoma brucei, Pseudomonas stutzeri, Bradyrhizobium japonicum, Mesorhizobium loti, Bos taurus, Nicotiana glutinosa, Vibrio vulnificus, Selenomonas ruminantium, Vibrio parahaemolyticus, Archaeoglobus fulgidus, Haloarcula marismortui, Pyrobaculum aerophilum, Mycobacterium smegmatis MC2 155, Mycobacterium avium subsp. paratuberculosis K-10, Mycobacterium marinum M, Tsukamurella paurometabola* DSM 20162, *Cyanobium* PCC7001, *Dictyostelium discoideum AX4*, and others disclosed herein (see Examples). For example, microbial organisms having 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine biosynthetic production are exemplified herein with reference to *E. coli* and yeast hosts. However, with the complete genome sequence available for now more than 550 species (with more than half of these available on public databases such as the NCBI), including 395 microorganism genomes and a variety of yeast, fungi, plant, and mammalian genomes, the identification of genes encoding the requisite 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine biosynthetic activity for one or more genes in related or distant species, including for example, homologues, orthologs, paralogs and nonorthologous gene displacements of known genes, and the interchange of genetic alterations between organisms is routine and well known in the art. Accordingly, the metabolic alterations allowing biosynthesis of 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine and other compounds of the invention described herein with reference to a particular organism such as *E. coli* or yeast can be readily applied to other microorganisms, including prokaryotic and eukaryotic organisms alike. Given the teachings and guidance provided herein, those skilled in the art will know that a metabolic alteration exemplified in one organism can be applied equally to other organisms.

In some instances, such as when an alternative 4-HB, 4-HBal, BDO or putrescine biosynthetic pathway exists in an unrelated species, 4-HB, 4-HBal, BDO or putrescine biosynthesis can be conferred onto the host species by, for example, exogenous expression of a paralog or paralogs from the unrelated species that catalyzes a similar, yet non-identical metabolic reaction to replace the referenced reaction. Because certain differences among metabolic networks exist between different organisms, those skilled in the art will understand that the actual gene usage between different organisms may differ. However, given the teachings and guidance provided herein, those skilled in the art also will understand that the teachings and methods of the invention can be applied to all microbial organisms using the cognate metabolic alterations to those exemplified herein to construct a microbial organism in a species of interest that will synthesize 4-HB, such as monomeric 4-HB, 4-HBal, BDO or putrescine.

Host microbial organisms can be selected from, and the non-naturally occurring microbial organisms generated in, for example, bacteria, yeast, fungus or any of a variety of other microorganisms applicable to fermentation processes. Exemplary bacteria include species selected from *Escherichia coli*, *Klebsiella oxytoca*, *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, *Mannheimia succiniciproducens, Rhizobium etli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Gluconobacter oxydans*, *Zymomonas mobilis*, *Lactococcus lactis*, *Lactobacillus plantarum*, *Streptomyces coelicolor*, *Clostridium acetobutylicum*, *Pseudomonas fluorescens*, and *Pseudomonas putida*. Exemplary yeasts or fungi include species selected from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Aspergillus terreus*, *Aspergillus niger, Pichia pastoris, Rhizopus arrhizus, Rhizobus oryzae*, and the like. *E. coli* is a particularly useful host organisms since it is a well characterized microbial organism suitable for genetic engineering. Other particularly useful host organisms include yeast such as *Saccharomyces cerevisiae*. It is understood that any suitable microbial host organism can be used to introduce metabolic and/or genetic modifications to produce a desired product.

Methods for constructing and testing the expression levels of a non-naturally occurring 4-HB-, 4-HBal-, 4-HBCoA-, BDO-, or putrescine-producing host can be performed, for example, by recombinant and detection methods well known in the art. Such methods can be found described in, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual, Third Ed.*, Cold Spring Harbor Laboratory, New York (2001); Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, MD (1999). 4-HB and GBL can be separated by, for example, HPLC using a Spherisorb 5 ODS1 column and a mobile phase of

70% 10 mM phosphate buffer (pH=7) and 30% methanol, and detected using a UV detector at 215 nm (Hennessy et al. 2004, J. Forensic Sci. 46(6):1-9). BDO is detected by gas chromatography or by HPLC and refractive index detector using an Aminex HPX-87H column and a mobile phase of 0.5 mM sulfuric acid (Gonzalez-Pajuelo et al., *Met. Eng.* 7:329-336 (2005)).

Exogenous nucleic acid sequences involved in a pathway for production of 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine can be introduced stably or transiently into a host cell using techniques well known in the art including, but not limited to, conjugation, electroporation, chemical transformation, transduction, transfection, and ultrasound transformation. For exogenous expression in *E. co*li or other prokaryotic cells, some nucleic acid sequences in the genes or cDNAs of eukaryotic nucleic acids can encode targeting signals such as an N-terminal mitochondrial or other targeting signal, which can be removed before transformation into prokaryotic host cells, if desired. For example, removal of a mitochondrial leader sequence led to increased expression in *E. coli* (Hoffmeister et al., *J. Biol. Chem.* 280:4329-4338 (2005)). For exogenous expression in yeast or other eukaryotic cells, genes can be expressed in the cytosol without the addition of leader sequence, or can be targeted to mitochondrion or other organelles, or targeted for secretion, by the addition of a suitable targeting sequence such as a mitochondrial targeting or secretion signal suitable for the host cells. Thus, it is understood that appropriate modifications to a nucleic acid sequence to remove or include a targeting sequence can be incorporated into an exogenous nucleic acid sequence to impart desirable properties. Furthermore, genes can be subjected to codon optimization with techniques well known in the art to achieve optimized expression of the proteins.

An expression vector or vectors can be constructed to harbor one or more 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine biosynthetic pathway and/or one or more biosynthetic encoding nucleic acids as exemplified herein operably linked to expression control sequences functional in the host organism. Expression vectors applicable for use in the microbial host organisms of the invention include, for example, plasmids, phage vectors, viral vectors, episomes and artificial chromosomes, including vectors and selection sequences or markers operable for stable integration into a host chromosome. Additionally, the expression vectors can include one or more selectable marker genes and appropriate expression control sequences. Selectable marker genes also can be included that, for example, provide resistance to antibiotics or toxins, complement auxotrophic deficiencies, or supply critical nutrients not in the culture media. Expression control sequences can include constitutive and inducible promoters, transcription

enhancers, transcription terminators, and the like which are well known in the art. When two or more exogenous encoding nucleic acids are to be co-expressed, both nucleic acids can be inserted, for example, into a single expression vector or in separate expression vectors. For single vector expression, the encoding nucleic acids can be operationally linked to one common expression control sequence or linked to different expression control sequences, such as one inducible promoter and one constitutive promoter. The transformation of exogenous nucleic acid sequences involved in a metabolic or synthetic pathway can be confirmed using methods well known in the art. Such methods include, for example, nucleic acid analysis such as Northern blots or polymerase chain reaction (PCR) amplification of mRNA, or immunoblotting for expression of gene products, or other suitable analytical methods to test the expression of an introduced nucleic acid sequence or its corresponding gene product. It is understood by those skilled in the art that the exogenous nucleic acid is expressed in a sufficient amount to produce the desired product, and it is further understood that expression levels can be optimized to obtain sufficient expression using methods well known in the art and as disclosed herein.

The non-naturally occurring microbial organisms of the invention are constructed using methods well known in the art as exemplified herein to exogenously express at least one nucleic acid encoding a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway enzyme in sufficient amounts to produce 4-HB, such as monomeric 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine. It is understood that the microbial organisms of the invention are cultured under conditions sufficient to produce 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine. Exemplary levels of expression for 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine enzymes in each pathway are described further below in the Examples. Following the teachings and guidance provided herein, the non-naturally occurring microbial organisms of the invention can achieve biosynthesis of 4-HB, such as monomeric 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine resulting in intracellular concentrations between about 0.1-200 mM or more, for example, 0.1-25 mM or more. Generally, the intracellular concentration of 4-HB, such as monomeric 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine is between about 3-150 mM or more, particularly about 5-125 mM or more, and more particularly between about 8-100 mM, for example, about 3-20mM, particularly between about 5-15 mM and more particularly between about 8-12 mM, including about 10 mM, 20 mM, 50 mM, 80 mM or more. Intracellular concentrations between and above each of these exemplary ranges also can be achieved from the non-naturally occurring microbial organisms of the invention. In particular embodiments, the microbial organisms of the invention, particularly strains such as those disclosed herein (see Examples XII-XIX and Table 28), can provide improved production of a desired product such as 4-HB, 4-HBal, 4-HBCoA,

BDO or putrescine by increasing the production of 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine and/or decreasing undesirable byproducts. Such production levels include, but are not limited to, those disclosed herein and including from about 1 gram to about 25 grams per liter, for example about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or even higher amounts of product per liter.

In addition to the culturing and fermentation conditions disclosed herein, growth condition for achieving biosynthesis of BDO, 4-HB, 4-HBCoA, 4-HBal and/or putrescine can include the addition of an osmoprotectant to the culturing conditions. In certain embodiments, the non-naturally occurring microbial organisms of the invention can be sustained, cultured or fermented as described herein in the presence of an osmoprotectant. Briefly, an osmoprotectant refers to a compound that acts as an osmolyte and helps a microbial organism as described herein survive osmotic stress. Osmoprotectants include, but are not limited to, betaines, amino acids, and the sugar trehalose. Non-limiting examples of such are glycine betaine, praline betaine, dimethylthetin, dimethylslfoniopropionate, 3-dimethylsulfonio-2-methylproprionate, pipecolic acid, dimethylsulfonioacetate, choline, L-carnitine and ectoine. In one aspect, the osmoprotectant is glycine betaine. It is understood to one of ordinary skill in the art that the amount and type of osmoprotectant suitable for protecting a microbial organism described herein from osmotic stress will depend on the microbial organism used. The amount of osmoprotectant in the culturing conditions can be, for example, no more than about 0.1 mM, no more than about 0.5 mM, no more than about 1.0 mM, no more than about 1.5 mM, no more than about 2.0 mM, no more than about 2.5 mM, no more than about 3.0 mM, no more than about 5.0 mM, no more than about 7.0 mM, no more than about 10mM, no more than about 50mM, no more than about 100mM or no more than about 500mM.

In some embodiments, culture conditions include anaerobic or substantially anaerobic growth or maintenance conditions. Exemplary anaerobic conditions have been described previously and are well known in the art. Exemplary anaerobic conditions for fermentation processes are described herein and are described, for example, in U.S. publication 2009/0047719, filed August 10, 2007. Any of these conditions can be employed with the non-naturally occurring microbial organisms as well as other anaerobic conditions well known in the art. Under such anaerobic conditions or substantially anaerobic, the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine producers can synthesize 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine at intracellular concentrations of 5-10 mM or more as well as all other concentrations exemplified herein. It is understood that, even though the above description refers to intracellular concentrations, 4-HB,

4-HBal, 4-HBCoA, BDO or putrescine producing microbial organisms can produce 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine intracellularly and/or secrete the product into the culture medium.

The culture conditions can include, for example, liquid culture procedures as well as fermentation and other large scale culture procedures. As described herein, particularly useful yields of the biosynthetic products of the invention can be obtained under anaerobic or substantially anaerobic culture conditions.

As described herein, one exemplary growth condition for achieving biosynthesis of 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine includes anaerobic culture or fermentation conditions. In certain embodiments, the non-naturally occurring microbial organisms of the invention can be sustained, cultured or fermented under anaerobic or substantially anaerobic conditions. Briefly, anaerobic conditions refers to an environment devoid of oxygen. Substantially anaerobic conditions include, for example, a culture, batch fermentation or continuous fermentation such that the dissolved oxygen concentration in the medium remains between 0 and 10% of saturation. Substantially anaerobic conditions also includes growing or resting cells in liquid medium or on solid agar inside a sealed chamber maintained with an atmosphere of less than 1% oxygen. The percent of oxygen can be maintained by, for example, sparging the culture with an N_2/CO_2 mixture or other suitable non-oxygen gas or gases.

The invention also provides a non-naturally occurring microbial biocatalyst including a microbial organism having 4-hydroxybutanoic acid (4-HB) and 1,4-butanediol (BDO) biosynthetic pathways that include at least one exogenous nucleic acid encoding 4-hydroxybutanoate dehydrogenase, CoA-independent succinic semialdehyde dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, 4-hydroxybutyrate:CoA transferase, glutamate:succinic semialdehyde transaminase, glutamate decarboxylase, CoA-independent aldehyde dehydrogenase, CoA-dependent aldehyde dehydrogenase or alcohol dehydrogenase, wherein the exogenous nucleic acid is expressed in sufficient amounts to produce 1,4-butanediol (BDO). 4-Hydroxybutyrate:CoA transferase also is known as 4-hydroxybutyryl CoA:acetyl-CoA transferase. Additional 4-HB or BDO pathway enzymes are also disclosed herein (see Examples and Figures 8-13).

The invention further provides non-naturally occurring microbial biocatalyst including a microbial organism having 4-hydroxybutanoic acid (4-HB) and 1,4-butanediol (BDO) biosynthetic pathways, the pathways include at least one exogenous nucleic acid encoding 4-

hydroxybutanoate dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, 4-hydroxybutyrate:CoA transferase, 4-butyrate kinase, phosphotransbutyrylase, α-ketoglutarate decarboxylase, aldehyde dehydrogenase, alcohol dehydrogenase or an aldehyde/alcohol dehydrogenase, wherein the exogenous nucleic acid is expressed in sufficient amounts to produce 1,4-butanediol (BDO).

Non-naturally occurring microbial organisms also can be generated which biosynthesize BDO. As with the 4-HB producing microbial organisms of the invention, the BDO producing microbial organisms also can produce intracellularly or secret the BDO into the culture medium. Following the teachings and guidance provided previously for the construction of microbial organisms that synthesize 4-HB, additional BDO pathways can be incorporated into the 4-HB producing microbial organisms to generate organisms that also synthesize BDO and other BDO family compounds. The chemical synthesis of BDO and its downstream products are known. The non-naturally occurring microbial organisms of the invention capable of BDO biosynthesis circumvent these chemical synthesis using 4-HB as an entry point as illustrated in Figure 1. As described further below, the 4-HB producers also can be used to chemically convert 4-HB to GBL and then to BDO or THF, for example. Alternatively, the 4-HB producers can be further modified to include biosynthetic capabilities for conversion of 4-HB and/or GBL to BDO.

The additional BDO pathways to introduce into 4-HB producers include, for example, the exogenous expression in a host deficient background or the overexpression of one or more of the enzymes exemplified in Figure 1 as steps 9-13. One such pathway includes, for example, the enzyme activies necessary to carryout the reactions shown as steps 9, 12 and 13 in Figure 1, where the aldehyde and alcohol dehydrogenases can be separate enzymes or a multifunctional enzyme having both aldehyde and alcohol dehydrogenase activity. Another such pathway includes, for example, the enzyme activities necessary to carry out the reactions shown as steps 10, 11, 12 and 13 in Figure 1, also where the aldehyde and alcohol dehydrogenases can be separate enzymes or a multifunctional enzyme having both aldehyde and alcohol dehydrogenase activity. Accordingly, the additional BDO pathways to introduce into 4-HB producers include, for example, the exogenous expression in a host deficient background or the overexpression of one or more of a 4-hydroxybutyrate:CoA transferase, butyrate kinase, phosphotransbutyrylase, CoA-independent aldehyde dehydrogenase, CoA-dependent aldehyde dehydrogenase or an alcohol dehydrogenase. In the absence of endogenous acyl-CoA synthetase capable of modifying 4-HB, the non-naturally occurring BDO producing microbial organisms can further include an exogenous acyl-CoA synthetase selective for 4-HB, or the combination of multiple

enzymes that have as a net reaction conversion of 4-HB into 4-HB-CoA. As exemplified further below in the Examples, butyrate kinase and phosphotransbutyrylase exhibit BDO pathway activity and catalyze the conversions illustrated in Figure 1 with a 4-HB substrate. Therefore, these enzymes also can be referred to herein as 4-hydroxybutyrate kinase and phosphotranshydroxybutyrylase respectively.

Exemplary alcohol and aldehyde dehydrogenases that can be used for these *in vivo* conversions from 4-HB to BDO are listed below in Table 1.

Table 1. Alcohol and Aldehyde Dehydrogenases for Conversion of 4-HB to BDO.

ALCOHOL DEHYDROGENASES

ec:1.1.1.1	alcohol dehydrogenase
ec:1.1.1.2	alcohol dehydrogenase (NADP+)
ec:1.1.1.4	(R,R)-butanediol dehydrogenase
ec:1.1.1.5	acetoin dehydrogenase
ec:1.1.1.6	glycerol dehydrogenase
ec:1.1.1.7	propanediol-phosphate dehydrogenase
ec:1.1.1.8	glycerol-3-phosphate dehydrogenase (NAD+)
ec:1.1.1.11	D-arabinitol 4-dehydrogenase
ec:1.1.1.12	L-arabinitol 4-dehydrogenase
ec:1.1.1.13	L-arabinitol 2-dehydrogenase
ec:1.1.1.14	L-iditol 2-dehydrogenase
ec:1.1.1.15	D-iditol 2-dehydrogenase
ec:1.1.1.16	galactitol 2-dehydrogenase
ec:1.1.1.17	mannitol-1-phosphate 5-dehydrogenase
ec:1.1.1.18	inositol 2-dehydrogenase
ec:1.1.1.21	aldehyde reductase
ec:1.1.1.23	histidinol dehydrogenase
ec:1.1.1.26	glyoxylate reductase
ec:1.1.1.27	L-lactate dehydrogenase
ec:1.1.1.28	D-lactate dehydrogenase
ec:1.1.1.29	glycerate dehydrogenase
ec:1.1.1.30	3-hydroxybutyrate dehydrogenase
ec:1.1.1.31	3-hydroxyisobutyrate dehydrogenase
ec:1.1.1.35	3-hydroxyacyl-CoA dehydrogenase
ec:1.1.1.36	acetoacetyl-CoA reductase
ec:1.1.1.37	malate dehydrogenase
ec:1.1.1.38	malate dehydrogenase (oxaloacetate-decarboxylating)
ec:1.1.1.39	malate dehydrogenase (decarboxylating)
ec:1.1.1.40	malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+)
ec:1.1.1.41	isocitrate dehydrogenase (NAD+)
ec:1.1.1.42	isocitrate dehydrogenase (NADP+)
ec:1.1.1.54	allyl-alcohol dehydrogenase
ec:1.1.1.55	lactaldehyde reductase (NADPH)
ec:1.1.1.56	ribitol 2-dehydrogenase
ec:1.1.1.59	3-hydroxypropionate dehydrogenase
ec:1.1.1.60	2-hydroxy-3-oxopropionate reductase
ec:1.1.1.61	4-hydroxybutyrate dehydrogenase
ec:1.1.1.66	omega-hydroxydecanoate dehydrogenase
ec:1.1.1.67	mannitol 2-dehydrogenase
ec:1.1.1.71	alcohol dehydrogenase [NAD(P)+]
ec:1.1.1.72	glycerol dehydrogenase (NADP+)
ec:1.1.1.73	octanol dehydrogenase
ec:1.1.1.75	(R)-aminopropanol dehydrogenase
ec:1.1.1.76	(S,S)-butanediol dehydrogenase
ec:1.1.1.77	lactaldehyde reductase
ec:1.1.1.78	methylglyoxal reductase (NADH-dependent)
ec:1.1.1.79	glyoxylate reductase (NADP+)
ec:1.1.1.80	isopropanol dehydrogenase (NADP+)
ec:1.1.1.81	hydroxypyruvate reductase
ec:1.1.1.82	malate dehydrogenase (NADP+)
ec:1.1.1.83	D-malate dehydrogenase (decarboxylating)
ec:1.1.1.84	dimethylmalate dehydrogenase
ec:1.1.1.85	3-isopropylmalate dehydrogenase
ec:1.1.1.86	ketol-acid reductoisomerase
ec:1.1.1.87	homoisocitrate dehydrogenase
ec:1.1.1.88	hydroxymethylglutaryl-CoA reductase
ec:1.1.1.90	aryl-alcohol dehydrogenase
ec:1.1.1.91	aryl-alcohol dehydrogenase (NADP+)
ec:1.1.1.92	oxaloglycolate reductase (decarboxylating)
ec:1.1.1.94	glycerol-3-phosphate dehydrogenase [NAD(P)+]
ec:1.1.1.95	phosphoglycerate dehydrogenase
ec:1.1.1.97	3-hydroxybenzyl-alcohol dehydrogenase
ec:1.1.1.101	acylglycerone-phosphate reductase
ec:1.1.1.103	L-threonine 3-dehydrogenase
ec:1.1.1.104	4-oxoproline reductase
ec:1.1.1.105	retinol dehydrogenase
ec:1.1.1.110	indolelactate dehydrogenase
ec:1.1.1.112	indanol dehydrogenase
ec:1.1.1.113	L-xylose 1-dehydrogenase
ec:1.1.1.129	L-threonate 3-dehydrogenase
ec:1.1.1.137	ribitol-5-phosphate 2-dehydrogenase
ec:1.1.1.138	mannitol 2-dehydrogenase (NADP+)
ec:1.1.1.140	sorbitol-6-phosphate 2-dehydrogenase
ec:1.1.1.142	D-pinitol dehydrogenase
ec:1.1.1.143	sequoyitol dehydrogenase
ec:1.1.1.144	perillyl-alcohol dehydrogenase
ec:1.1.1.156	glycerol 2-dehydrogenase (NADP+)
ec:1.1.1.157	3-hydroxybutyryl-CoA dehydrogenase
ec:1.1.1.163	cyclopentanol dehydrogenase
ec:1.1.1.164	hexadecanol dehydrogenase
ec:1.1.1.165	2-alkyn-1-ol dehydrogenase
ec:1.1.1.166	hydroxycyclohexanecarboxylate dehydrogenase
ec:1.1.1.167	hydroxymalonate dehydrogenase
ec:1.1.1.174	cyclohexane-1,2-diol dehydrogenase
ec:1.1.1.177	glycerol-3-phosphate 1-dehydrogenase (NADP+)
ec:1.1.1.178	3-hydroxy-2-methylbutyryl-CoA dehydrogenase
ec:1.1.1.185	L-glycol dehydrogenase
ec:1.1.1.190	indole-3-acetaldehyde reductase (NADH)
ec:1.1.1.191	indole-3-acetaldehyde reductase (NADPH)
ec:1.1.1.192	long-chain-alcohol dehydrogenase
ec:1.1.1.194	coniferyl-alcohol dehydrogenase
ec:1.1.1.195	cinnamyl-alcohol dehydrogenase
ec:1.1.1.198	(+)-borneol dehydrogenase
ec:1.1.1.202	1,3-propanediol dehydrogenase
ec:1.1.1.207	(-)-menthol dehydrogenase

ec:1.1.1.208 (+)-neomenthol dehydrogenase
ec:1.1.1.216 farnesol dehydrogenase
ec:1.1.1.217 benzyl-2-methyl-hydroxybutyrate dehydrogenase
ec:1.1.1.222 (R)-4-hydroxyphenyllactate dehydrogenase
ec:1.1.1.223 isopiperitenol dehydrogenase
ec:1.1.1.226 4-hydroxycyclohexanecarboxylate dehydrogenase
ec:1.1.1.229 diethyl 2-methyl-3-oxosuccinate reductase
ec:1.1.1.237 hydroxyphenylpyruvate reductase
ec:1.1.1.244 methanol dehydrogenase
ec:1.1.1.245 cyclohexanol dehydrogenase
ec:1.1.1.250 D-arabinitol 2-dehydrogenase
ec:1.1.1.251 galactitol 1-phosphate 5-dehydrogenase
ec:1.1.1.255 mannitol dehydrogenase
ec:1.1.1.256 fluoren-9-ol dehydrogenase
ec:1.1.1.257 4-(hydroxymethyl)benzenesulfonate dehydrogenase
ec:1.1.1.258 6-hydroxyhexanoate dehydrogenase
ec:1.1.1.259 3-hydroxypimeloyl-CoA dehydrogenase
ec:1.1.1.261 glycerol-1-phosphate dehydrogenase [NAD(P)+]
ec:1.1.1.265 3-methylbutanal reductase
ec:1.1.1.283 methylglyoxal reductase (NADPH-dependent)
ec:1.1.1.286 isocitrate-homoisocitrate dehydrogenase
ec:1.1.1.287 D-arabinitol dehydrogenase (NADP+)
butanol dehydrogenase

ALDEHYDE DEHYDROGENASES

ec:1.2.1.2 formate dehydrogenase
ec:1.2.1.3 aldehyde dehydrogenase (NAD+)
ec:1.2.1.4 aldehyde dehydrogenase (NADP+)
ec:1.2.1.5 aldehyde dehydrogenase [NAD(P)+]
ec:1.2.1.7 benzaldehyde dehydrogenase (NADP+)
ec:1.2.1.8 betaine-aldehyde dehydrogenase
ec:1.2.1.9 glyceraldehyde-3-phosphate dehydrogenase (NADP+)
ec:1.2.1.10 acetaldehyde dehydrogenase (acetylating)
ec:1.2.1.11 aspartate-semialdehyde dehydrogenase
ec:1.2.1.12 glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)
ec:1.2.1.13 glyceraldehyde-3-phosphate dehydrogenase (NADP+) (phosphorylating)
ec:1.2.1.15 malonate-semialdehyde dehydrogenase
ec:1.2.1.16 succinate-semialdehyde dehydrogenase [NAD(P)+]
ec:1.2.1.17 glyoxylate dehydrogenase (acylating)
ec:1.2.1.18 malonate-semialdehyde dehydrogenase (acetylating)
ec:1.2.1.19 aminobutyraldehyde dehydrogenase
ec:1.2.1.20 glutarate-semialdehyde dehydrogenase
ec:1.2.1.21 glycolaldehyde dehydrogenase
ec:1.2.1.22 lactaldehyde dehydrogenase
ec:1.2.1.23 2-oxoaldehyde dehydrogenase (NAD+)
ec:1.2.1.24 succinate-semialdehyde dehydrogenase
ec:1.2.1.25 2-oxoisovalerate dehydrogenase (acylating)
ec:1.2.1.26 2,5-dioxovalerate dehydrogenase
ec:1.2.1.27 methylmalonate-semialdehyde dehydrogenase (acylating)
ec:1.2.1.28 benzaldehyde dehydrogenase (NAD+)
ec:1.2.1.29 aryl-aldehyde dehydrogenase
ec:1.2.1.30 aryl-aldehyde dehydrogenase (NADP+)
ec:1.2.1.31 L-aminoadipate-semialdehyde dehydrogenase
ec:1.2.1.32 aminomuconate-semialdehyde dehydrogenase
ec:1.2.1.36 retinal dehydrogenase
ec:1.2.1.39 phenylacetaldehyde dehydrogenase
ec:1.2.1.41 glutamate-5-semialdehyde dehydrogenase
ec:1.2.1.42 hexadecanal dehydrogenase (acylating)
ec:1.2.1.43 formate dehydrogenase (NADP+)
ec:1.2.1.45 4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase
ec:1.2.1.46 formaldehyde dehydrogenase
ec:1.2.1.47 4-trimethylammoniobutyraldehyde dehydrogenase
ec:1.2.1.48 long-chain-aldehyde dehydrogenase
ec:1.2.1.49 2-oxoaldehyde dehydrogenase (NADP+)
ec:1.2.1.51 pyruvate dehydrogenase (NADP+)
ec:1.2.1.52 oxoglutarate dehydrogenase (NADP+)
ec:1.2.1.53 4-hydroxyphenylacetaldehyde dehydrogenase
ec:1.2.1.57 butanal dehydrogenase
ec:1.2.1.58 phenylglyoxylate dehydrogenase (acylating)
ec:1.2.1.59 glyceraldehyde-3-phosphate dehydrogenase (NAD(P)+) (phosphorylating)
ec:1.2.1.62 4-formylbenzenesulfonate dehydrogenase
ec:1.2.1.63 6-oxohexanoate dehydrogenase
ec:1.2.1.64 4-hydroxybenzaldehyde dehydrogenase
ec:1.2.1.65 salicylaldehyde dehydrogenase
ec:1.2.1.66 mycothiol-dependent formaldehyde dehydrogenase
ec:1.2.1.67 vanillin dehydrogenase
ec:1.2.1.68 coniferyl-aldehyde dehydrogenase
ec:1.2.1.69 fluoroacetaldehyde dehydrogenase
ec:1.2.1.71 succinylglutamate-semialdehyde dehydrogenase

Other exmplary enzymes and pathways are disclosed herein (see Examples). Furthermore, it is understood that enzymes can be utilized for carry out reactions for which the substrate is not the natural substrate. While the activity for the non-natural substrate may be lower than the natural substrate, it is understood that such enzymes can be utilized, either as naturally occurring or modified using the directed evolution or adaptive evolution, as disclosed herein (see also Examples).

BDO production through any of the pathways disclosed herein are based, in part, on the identification of the appropriate enzymes for conversion of precursors to BDO. A number of specific enzymes for several of the reaction steps have been identified. For those transformations where enzymes specific to the reaction precursors have not been identified, enzyme candidates have been identified that are best suited for catalyzing the reaction steps. Enzymes have been shown to operate on a broad range of substrates, as discussed below. In addition, advances in the field of protein engineering also make it feasible to alter enzymes to act efficiently on substrates, even if not a natural substrate. Described below are several examples of broad-specificity enzymes from diverse classes suitable for a BDO pathway as well as methods that have been used for evolving enzymes to act on non-natural substrates.

A key class of enzymes in BDO pathways is the oxidoreductases that interconvert ketones or aldehydes to alcohols (1.1.1). Numerous exemplary enzymes in this class can operate on a wide range of substrates. An alcohol dehydrogenase (1.1.1.1) purified from the soil bacterium *Brevibacterium* sp KU 1309 (Hirano et al., *J. Biosc. Bioeng.* 100:318-322 (2005)) was shown to operate on a plethora of aliphatic as well as aromatic alcohols with high activities. Table 2 shows the activity of the enzyme and its K_m on different alcohols. The enzyme is reversible and has very high activity on several aldehydes also (Table 3).

Table 2. Relative activities of an alcohol dehydrogenase from *Brevibacterium* sp KU to oxidize various alcohols.

Substrate	Relative Activity (0%)	K_m (mM)
2-Phenylethanol	100*	0.025
(*S*)-2-Phenylpropanol	156	0.157
(*R*)-2-Phenylpropanol	63	0.020
Bynzyl alcohol	199	0.012
3-Phenylpropanol	135	0.033
Ethanol	76	
1-Butanol	111	
1-Octanol	101	

1-Dodecanol	68	
1-Phenylethanol	46	
2-Propanol	54	

*The activity of 2-phenylethanol, corresponding to 19.2 U/mg, was taken as 100%.

Table 3. Relative activities of an alcohol dehydrogenase from *Brevibacterium* sp KU 1309 to reduce various carbonyl compounds.

Substrate	Relative Activity (%)	K_m (mM)
Phenylacetaldehyde	100	0.261
2-Phenylpropionaldehyde	188	0.864
1-Octylaldehyde	87	
Acetophenone	0	

Lactate dehydrogenase (1.1.1.27) from *Ralstonia eutropha* is another enzyme that has been demonstrated to have high activities on several 2-oxoacids such as 2-oxobutyrate, 2-oxopentanoate and 2-oxoglutarate (a C5 compound analogous to 2-oxoadipate) (Steinbuchel and Schlegel, *Eur. J. Biochem.* 130:329-334 (1983)). Column 2 in Table 4 demonstrates the activities of *ldhA* from *R. eutropha* (formerly *A. eutrophus)* on different substrates (Steinbuchel and Schlegel, *supra*, 1983).

Table 4. The *in vitro* activity of *R. eutropha ldhA* (Steinbuchel and Schlegel, *supra*, 1983) on different substrates and compared with that on pyruvate.

Substrate		Activity (%) of	
	L(+)-lactate dehydrogenase from *A. eutrophus*	**L(+)-lactate dehydrogenase from rabbit muscle**	**D(-)-lactate dehydrogenase from *L. leichmanii***
Glyoxylate	8.7	23.9	5.0
Pyruvate	100.0	100.0	100.0
2-Oxobutyrate	107.0	18.6	1.1
2-Oxovalerate	125.0	0.7	0.0
3-Methyl-2-oxobutyrate	28.5	0.0	0.0
3-Methyl-2-oxovalerate	5.3	0.0	0.0
4-Methyl-2-oxopentanoate	39.0	1.4	1.1
Oxaloacetate	0.0	33.1	23.1
2-Oxoglutarate	79.6	0.0	0.0
3-Fluoropyruvate	33.6	74.3	40.0

Oxidoreductases that can convert 2-oxoacids to their acyl-CoA counterparts (1.2.1) have been shown to accept multiple substrates as well. For example, branched-chain 2-keto-acid

dehydrogenase complex (BCKAD), also known as 2-oxoisovalerate dehydrogenase (1.2.1.25), participates in branched-chain amino acid degradation pathways, converting 2-keto acids derivatives of valine, leucine and isoleucine to their acyl-CoA derivatives and CO_2. In some organisms including *Rattus norvegicus* (Paxton et al., *Biochem. J.* 234:295-303 (1986)) and *Saccharomyces cerevisiae* (Sinclair et al., *Biochem. Mol Biol. Int.* 32:911-922 (1993), this complex has been shown to have a broad substrate range that includes linear oxo-acids such as 2-oxobutanoate and alpha-ketoglutarate, in addition to the branched-chain amino acid precursors.

Members of yet another class of enzymes, namely aminotransferases (2.6.1), have been reported to act on multiple substrates. Aspartate aminotransferase (*aspAT*) from *Pyrococcus fursious* has been identified, expressed in *E. coli* and the recombinant protein characterized to demonstrate that the enzyme has the highest activities towards aspartate and alpha-ketoglutarate but lower, yet significant activities towards alanine, glutamate and the aromatic amino acids (Ward et al., *Archaea* 133-141 (2002)). In another instance, an aminotransferase indentified from *Leishmania mexicana* and expressed in *E. coli* (Vernal et al., *FEMS Microbiol. Lett.* 229:217-222 (2003)) was reported to have a broad substrate specificity towards tyrosine (activity considered 100% on tyrosine), phenylalanine (90%), tryptophan (85%), aspartate (30%), leucine (25%) and methionine (25%), respectively (Vernal et al., *Mol. Biochem. Parasitol* 96:83-92 (1998)). Similar broad specificity has been reported for a tyrosine aminotransferase from *Trypanosoma cruzi*, even though both of these enzymes have a sequence homology of only 6%. The latter enzyme can accept leucine, methionine as well as tyrosine, phenylalanine, tryptophan and alanine as efficient amino donors (Nowicki et al., *Biochim. Biophys. Acta* 1546: 268-281 (2001)).

CoA transferases (2.8.3) have been demonstrated to have the ability to act on more than one substrate. Specifically, a CoA transferase was purified from *Clostridium acetobutylicum* and was reported to have the highest activities on acetate, propionate, and butyrate. It also had significant activities with valerate, isobutyrate, and crotonate (Wiesenborn et al., *Appl. Environ. Microbiol.* 55:323-329 (1989)). In another study, the *E. coli* enzyme acyl-CoA:acetate-CoA transferase, also known as acetate-CoA transferase (EC 2.8.3.8), has been shown to transfer the CoA moiety to acetate from a variety of branched and linear acyl-CoA substrates, including isobutyrate (Matthies and Schink, *App. Environm. Microbiol.* 58:1435-1439 (1992)), valerate (Vanderwinkel et al., *Biochem. Biophys. Res Commun.* 33:902-908 (1968b)) and butanoate (Vanderwinkel et al., *Biochem. Biophys. Res Commun.* 33:902-908(1968a).

Other enzyme classes additionally support broad substrate specificity for enzymes. Some isomerases (5.3.3) have also been proven to operate on multiple substrates. For example, L-rhamnose isomerase from *Pseudomonas stutzeri* catalyzes the isomerization between various aldoalses and ketoses (Yoshida et al., *J. Mol. Biol.* 365:1505-1516 (2007)). These include isomerization between L-rhamnose and L-rhamnulose, L-mannose and L-fructose, L-xylose and L-xylulose, D-ribose and D-ribulose, and D-allose and D-psicose.

In yet another class of enzymes, the phosphotransferases (2.7.1), the homoserine kinase (2.7.1.39) from *E. coli* that converts L-homoserine to L-homoserine phosphate, was found to phosphorylate numerous homoserine analogs. In these substrates, the carboxyl functional group at the R-position had been replaced by an ester or by a hydroxymethyl group (Huo and Viola, *Biochemistry* 35:16180-16185 (1996)). Table 5 demonstrates the broad substrate specificity of this kinase.

Table 5. The substrate specificity of homoserine kinase.

Substrate	k_{cat}	% k_{cat}	K_m (mM)	k_{cat}/K_m
L-homoserine	18.3 ± 0.1	100	0.14 ± 0.04	184 ± 17
D-homoserine	8.3 ± 1.1	32	31.8 ± 7.2	0.26 ± 0.03
L-aspartate β-semialdehyde	2.1 ± 0.1	8.2	0.28 ± 0.02	7.5 ± 0.3
L-2-amino-1,4-butanediol	2.0 ± 0.5	7.9	11.6 ± 6.5	0.17 ± 0.06
L-2-amino-5-hydroxyvalerate	2.5 ± 0.4	9.9	1.1 ± 0.5	2.3 ± 0.3
L-homoserine methyl ester	14.7 ± 2.6	80	4.9 ± 2.0	3.0 ± 0.6
L-homoserine ethyl ester	13.6 ± 0.8	74	1.9 ± 0.5	7.2 ± 1.7
L-homoserine isopropyl ester	13.6 ± 1.4	74	1.2 ± 0.5	11.3 ± 1.1
L-homoserine *n*-propyl ester	14.0 ± 0.4	76	3.5 ± 0.4	4.0 ± 1.2
L-homoserine isobutyl ester	16.4 ± 0.8	84	6.9 ± 1.1	2.4 ± 0.3
L-homserine *n*-butyl ester	29.1 ± 1.2	160	5.8 ± 0.8	5.0 ± 0.5

Another class of enzymes useful in BDO pathways is the acid-thiol ligases (6.2.1). Like enzymes in other classes, certain enzymes in this class have been determined to have broad substrate specificity. For example, acyl CoA ligase from *Pseudomonas putida* has been demonstrated to work on several aliphatic substrates including acetic, propionic, butyric, valeric,

hexanoic, heptanoic, and octanoic acids and on aromatic compounds such as phenylacetic and phenoxyacetic acids (Fernandez-Valverde et al., *Appl. Environ. Microbiol.* 59:1149-1154 (1993)). A related enzyme, malonyl CoA synthetase (6.3.4.9) from *Rhizobium trifolii* could convert several diacids, namely, ethyl-, propyl-, allyl-, isopropyl-, dimethyl-, cyclopropyl-, cyclopropylmethylene-, cyclobutyl-, and benzyl-malonate into their corresponding monothioesters (Pohl et al., J. *Am. Chem. Soc.* 123:5822-5823 (2001)). Similarly, decarboxylases (4.1.1) have also been found with broad substrate ranges. Pyruvate decarboxylase (PDC), also termed keto-acid decarboxylase, is a key enzyme in alcoholic fermentation, catalyzing the decarboxylation of pyruvate to acetaldehyde. The enzyme isolated from *Saccharomyces cerevisiae* has a broad substrate range for aliphatic 2-keto acids including 2-ketobutyrate, 2-ketovalerate, and 2-phenylpyruvate (Li and Jordan, *Biochemistry* 38:10004-10012 (1999)). Similarly, benzoylformate decarboxylase has a broad substrate range and has been the target of enzyme engineering studies. The enzyme from *Pseudomonas putida* has been extensively studied and crystal structures of this enzyme are available (Polovnikova et al., *Biochemistry* 42:1820-1830 (2003); Hasson et al., *Biochemistry* 37:9918-9930 (1998)). Branched chain alpha-ketoacid decarboxylase (BCKA) has been shown to act on a variety of compounds varying in chain length from 3 to 6 carbons (Oku and Kaneda, *J. Biol. Chem.* 263:18386-18396 (1998); Smit et al., *Appl. Environ. Microbiol.* 71:303-311 (2005b)). The enzyme in *Lactococcus lactis* has been characterized on a variety of branched and linear substrates including 2-oxobutanoate, 2-oxohexanoate, 2-oxopentanoate, 3-methyl-2-oxobutanoate, 4-methyl-2-oxobutanoate and isocaproate (Smit et al., *Appl. Environ. Microbiol.* 71:303-311 (2005a).

Interestingly, enzymes known to have one dominant activity have also been reported to catalyze a very different function. For example, the cofactor-dependent phosphoglycerate mutase (5.4.2.1) from *Bacillus stearothermophilus* and *Bacillus subtilis* is known to function as a phosphatase as well (Rigden et al., *Protein Sci.* 10:1835-1846 (2001)). The enzyme from *B. stearothermophilus* is known to have activity on several substrates, including 3-phosphoglycerate, alpha-napthylphosphate, p-nitrophenylphosphate, AMP, fructose-6-phosphate, ribose-5-phosphate and CMP.

In contrast to these examples where the enzymes naturally have broad substrate specificities, numerous enzymes have been modified using directed evolution to broaden their specificity towards their non-natural substrates. Alternatively, the substrate preference of an enzyme has also been changed using directed evolution. Therefore, it is feasible to engineer a given enzyme

for efficient function on a natural, for example, improved efficiency, or a non-natural substrate, for example, increased efficiency. For example, it has been reported that the enantioselectivity of a lipase from *Pseudomonas aeruginosa* was improved significantly (Reetz et al., *Agnew. Chem. Int. Ed Engl.* 36:2830-2832 (1997)). This enzyme hydrolyzed p-nitrophenyl 2-methyldecanoate with only 2% enantiomeric excess (ee) in favor of the *(S)*-acid. However, after four successive rounds of error-prone mutagenesis and screening, a variant was produced that catalyzed the requisite reaction with 81% ee (Reetz et al., *Agnew. Chem. Int. Ed Engl.* 36:2830-2832 (1997)).

Directed evolution methods have been used to modify an enzyme to function on an array of non-natural substrates. The substrate specificity of the lipase in *P. aeruginosa* was broadened by randomization of amino acid residues near the active site. This allowed for the acceptance of alpha-substituted carboxylic acid esters by this enzyme (Reetz et al., *Agnew. Chem. Int. Ed Engl.* 44:4192-4196 (2005)). In another successful modification of an enzyme, DNA shuffling was employed to create an *Escherichia coli* aminotransferase that accepted β-branched substrates, which were poorly accepted by the wild-type enzyme (Yano et al., *Proc. Nat. Acad. Sci. U.S.A.* 95:5511-5515 (1998)). Specifically, at the end of four rounds of shuffling, the activity of aspartate aminotransferase for valine and 2-oxovaline increased by up to five orders of magnitude, while decreasing the activity towards the natural substrate, aspartate, by up to 30-fold. Recently, an algorithm was used to design a retro-aldolase that could be used to catalyze the carbon-carbon bond cleavage in a non-natural and non-biological substrate, 4-hydroxy-4-(6-methoxy-2-naphthyl)-2-butanone (Jiang et al., *Science* 319:1387-1391 (2008)). These algorithms used different combinations of four different catalytic motifs to design new enzyme, and 20 of the selected designs for experimental characterization had four-fold improved rates over the uncatalyzed reaction (Jiang et al., *Science* 319:1387-1391 (2008)). Thus, not only are these engineering approaches capable of expanding the array of substrates on which an enzyme can act, but they allow the design and construction of very efficient enzymes. For example, a method of DNA shuffling (random chimeragenesis on transient templates or RACHITT) was reported to lead to an engineered monooxygenase that had an improved rate of desulfurization on complex substrates as well as 20-fold faster conversion of a non-natural substrate (Coco et al., *Nat. Biotechnol.* 19:354-359 (2001)). Similarly, the specific activity of a sluggish mutant triosephosphate isomerase enzyme was improved up to 19-fold from 1.3 fold (Hermes et al., *Proc. Nat. Acad. Sci. U.S.A.* 87:696-700 1990)). This enhancement in specific activity was accomplished by using random mutagenesis over the whole length of the protein and the improvement could be traced back to mutations in six amino acid residues.

The effectiveness of protein engineering approaches to alter the substrate specificity of an enzyme for a desired substrate has also been demonstrated in several studies. Isopropylmalate dehydrogenase from *Thermus thermophilus* was modified by changing residues close to the active site so that it could now act on malate and D-lactate as substrates (Fujita et al., *Biosci. Biotechnol. Biochem.* 65:2695-2700 (2001)). In this study as well as in others, it was pointed out that one or a few residues could be modified to alter the substrate specificity. For example, the dihydroflavonol 4-reductase for which a single amino acid was changed in the presumed substrate-binding region could preferentially reduce dihydrokaempferol (Johnson et al., *Plant. J.* 25:325-333 (2001)). The substrate specificity of a very specific isocitrate dehydrogenase from *Escherichia coli* was changed form isocitrate to isopropylmalate by changing one residue in the active site (Doyle et al., *Biochemistry* 40:4234-4241 (2001)). Similarly, the cofactor specificity of a NAD^+-dependent 1,5-hydroxyprostaglandin dehydrogenase was altered to $NADP^+$ by changing a few residues near the N-terminal end (Cho et al., *Arch. Biochem. Biophys.* 419:139-146 (2003)). Sequence analysis and molecular modeling analysis were used to identify the key residues for modification, which were further studied by site-directed mutagenesis.

Numerous examples exist spanning diverse classes of enzymes where the function of enzyme was changed to favor one non-natural substrate over the natural substrate of the enzyme. A fucosidase was evolved from a galactosidase in *E. co*li by DNA shuffling and screening (Zhang et al., *Proc. Natl Acad. Sci. U.S.A.* 94:4504-4509 (1997)). Similarly, aspartate aminotransferase from *E. coli* was converted into a tyrosine aminotransferase using homology modeling and site-directed mutagenesis (Onuffer and Kirsch, *Protein Sci.*, 4:1750-1757 (1995)). Site-directed mutagenesis of two residues in the active site of benzoylformate decarboxylase from *P. putida* reportedly altered the affinity (K_m) towards natural and non-natural substrates (Siegert et al., *Protein Eng Des Sel* 18:345-357 (2005)). Cytochrome c peroxidase (CCP) from *Saccharomyces cerevisiae* was subjected to directed molecular evolution to generate mutants with increased activity against the classical peroxidase substrate guaiacol, thus changing the substrate specificity of CCP from the protein cytochrome c to a small organic molecule. After three rounds of DNA shuffling and screening, mutants were isolated which possessed a 300-fold increased activity against guaiacol and up to 1000-fold increased specificity for this substrate relative to that for the natural substrate (Iffland et al., *Biochemistry* 39:10790-10798 (2000)).

In some cases, enzymes with different substrate preferences than either of the parent enzymes have been obtained. For example, biphenyl-dioxygenase-mediated degradation of polychlorinated biphenyls was improved by shuffling genes from two bacteria, *Pseudomonas*

pseudoalcaligens and *Burkholderia cepacia* (Kumamaru et al., *Nat. Biotechnol.* 16:663-666 (1998)). The resulting chimeric biphenyl oxygenases showed different substrate preferences than both the parental enzymes and enhanced the degradation activity towards related biphenyl compounds and single aromatic ring hydrocarbons such as toluene and benzene which were originally poor substrates for the enzyme.

In addition to changing enzyme specificity, it is also possible to enhance the activities on substrates for which the enzymes naturally have low activities. One study demonstrated that amino acid racemase from *P. putida* that had broad substrate specificity (on lysine, arginine, alanine, serine, methionine, cysteine, leucine and histidine among others) but low activity towards tryptophan could be improved significantly by random mutagenesis (Kino et al., *Appl. Microbiol. Biotechnol.* 73:1299-1305 (2007)). Similarly, the active site of the bovine BCKAD was engineered to favor alternate substrate acetyl-CoA (Meng and Chuang, *Biochemistry* 33:12879-12885 (1994)). An interesting aspect of these approaches is that even if random methods have been applied to generate these mutated enzymes with efficacious activities, the exact mutations or structural changes that confer the improvement in activity can be identified. For example, in the aforementioned study, the mutations that facilitated improved activity on tryptophan was traced back to two different positions.

Directed evolution has also been used to express proteins that are difficult to express. For example, by subjecting horseradish peroxidase to random mutagenesis and gene recombination, mutants were identified that had more than 14-fold higher activity than the wild type (Lin et al., *Biotechnol. Prog.* 15:467-471 (1999)).

Another example of directed evolution shows the extensive modifications to which an enzyme can be subjected to achieve a range of desired functions. The enzyme lactate dehydrogenase from *Bacillus stearothermophilus* was subjected to site-directed mutagenesis, and three amino acid substitutions were made at sites that were believed to determine the specificity towards different hydroxyacids (Clarke et al., *Biochem. Biophys. Res. Commun.* 148:15-23 (1987)). After these mutations, the specificity for oxaloacetate over pyruvate was increased to 500 in contrast to the wild type enzyme that had a catalytic specificity for pyruvate over oxaloacetate of 1000. This enzyme was further engineered using site-directed mutagenesis to have activity towards branched-chain substituted pyruvates (Wilks et al., *Biochemistry* 29:8587-8591 (1990)). Specifically, the enzyme had a 55-fold improvement in K_{cat} for alpha-ketoisocaproate. Three structural modifications were made in the same enzyme to change its substrate specificity from

lactate to malate. The enzyme was highly active and specific towards malate (Wilks et al., *Science* 242:1541-1544 (1988)). The same enzyme from *B. stearothermophilus* was subsequently engineered to have high catalytic activity towards alpha-keto acids with positively charged side chains, such as those containing ammonium groups (Hogan et al., *Biochemistry* 34:4225-4230 (1995)). Mutants with acidic amino acids introduced at position 102 of the enzyme favored binding of such side chain ammonium groups. The results obtained proved that the mutants showed up to 25-fold improvements in k_{cat}/K_m values for omega-amino-alpha-keto acid substrates. Interestingly, this enzyme was also structurally modified to function as a phenyllactate dehydrogenase instead of a lactate dehydrogenase (Wilks et al., *Biochemistry* 31:7802-7806 1992). Restriction sites were introduced into the gene for the enzyme which allowede a region of the gene to be excised. This region coded for a mobile surface loop of the polypeptide (residues 98-110) which normally seals the active site from bulk solvent and is a major determinant of substrate specificity. The variable length and sequence loops were inserted so that hydroxyacid dehydrogenases with altered substrate specificities were generated. With one longer loop construction, activity with pyruvate was reduced one-million-fold but activity with phenylpyruvate was largely unaltered. A switch in specificity (k_{cat}/K_m) of 390,000-fold was achieved. The 1700:1 selectivity of this enzyme for phenylpyruvate over pyruvate is that required in a phenyllactate dehydrogenase. The studies described above indicate that various approaches of enzyme engineering can be used to obtain enzymes for the BDO pathways as disclosed herein.

As disclosed herein, biosynthetic pathways to 1,4-butanediol from a number of central metabolic intermediates are can be utilized, including acetyl-CoA, succinyl-CoA, alpha-ketoglutarate, glutamate, 4-aminobutyrate, and homoserine. Acetyl-CoA, succinyl-CoA and alpha-ketoglutarate are common intermediates of the tricarboxylic acid (TCA) cycle, a series of reactions that is present in its entirety in nearly all living cells that utilize oxygen for cellular respiration and is present in truncated forms in a number of anaerobic organisms. Glutamate is an amino acid that is derived from alpha-ketoglutarate via glutamate dehydrogenase or any of a number of transamination reactions (see Figure 8B). 4-aminobutyrate can be formed by the decarboxylation of glutamate (see Figure 8B) or from acetoacetyl-CoA via the pathway disclosed in Figure 9C. Acetoacetyl-CoA is derived from the condensation of two acetyl-CoA molecules by way of the enzyme, acetyl-coenzyme A acetyltransferase, or equivalently, acetoacetyl-coenzyme A thiolase. Homoserine is an intermediate in threonine and methionine metabolism, formed from oxaloacetate via aspartate. The conversion of oxaloacetate to homoserine requires one NADH, two NADPH, and one ATP.

Pathways other than those exemplified above also can be employed to generate the biosynthesis of BDO in non-naturally occurring microbial organisms. In one embodiment, biosynthesis can be achieved using a L-homoserine to BDO pathway (see Figure 13). This pathway has a molar yield of 0.90 mol/mol glucose, which appears restricted by the availability of reducing equivalents. A second pathway synthesizes BDO from acetoacetyl-CoA and is capable of achieving the maximum theoretical yield of 1.091 mol/mol glucose (see Figure 9). Implementation of either pathway can be achieved by introduction of two exogenous enzymes into a host organism such as *E. coli*, and both pathways can additionally complement BDO production via succinyl-CoA. Pathway enzymes, thermodynamics, theoretical yields and overall feasibility are described further below.

A homoserine pathway also can be engineered to generate BDO-producing microbial organisms. Homoserine is an intermediate in threonine and methionine metabolism, formed from oxaloacetate via aspartate. The conversion of oxaloacetate to homoserine requires one NADH, two NADPH, and one ATP (Figure 2). Once formed, homoserine feeds into biosynthetic pathways for both threonine and methionine. In most organisms, high levels of threonine or methionine feedback to repress the homoserine biosynthesis pathway (Caspi et al., *Nucleic Acids Res.* 34:D511-D516 (1990)).

The transformation of homoserine to 4-hydroxybutyrate (4-HB) can be accomplished in two enzymatic steps as described herein. The first step of this pathway is deamination of homoserine by a putative ammonia lyase. In step 2, the product alkene, 4-hydroxybut-2-enoate is reduced to 4-HB by a putative reductase at the cost of one NADH. 4-HB can then be converted to BDO.

Enzymes available for catalyzing the above transformations are disclosed herein. For example, the ammonia lyase in step 1 of the pathway closely resembles the chemistry of aspartate ammonia-lyase (aspartase). Aspartase is a widespread enzyme in microorganisms, and has been characterized extensively (Viola, R.E., *Mol. Biol.* 74:295-341 (2008)). The crystal structure of the *E. coli* aspartase has been solved (Shi et al., *Biochemistry* 36:9136-9144 (1997)), so it is therefore possible to directly engineer mutations in the enzyme's active site that would alter its substrate specificity to include homoserine. The oxidoreductase in step 2 has chemistry similar to several well-characterized enzymes including fumarate reductase in the *E. coli* TCA cycle. Since the thermodynamics of this reaction are highly favorable, an endogenous reductase with broad substrate specificity will likely be able to reduce 4-hydroxybut-2-enoate. The yield of this pathway under anaerobic conditions is 0.9 mol BDO per mol glucose.

The succinyl-CoA pathway was found to have a higher yield due to the fact that it is more energetically efficient. The conversion of one oxaloacetate molecule to BDO via the homoserine pathway will require the expenditure of 2 ATP equivalents. Because the conversion of glucose to two oxaloacetate molecules can generate a maximum of 3 ATP molecules assuming PEP carboxykinase to be reversible, the overall conversion of glucose to BDO via homoserine has a negative energetic yield. As expected, if it is assumed that energy can be generated via respiration, the maximum yield of the homoserine pathway increases to 1.05 mol/mol glucose which is 96% of the succinyl-CoA pathway yield. The succinyl-CoA pathway can channel some of the carbon flux through pyruvate dehydrogenase and the oxidative branch of the TCA cycle to generate both reducing equivalents and succinyl-CoA without an energetic expenditure. Thus, it does not encounter the same energetic difficulties as the homoserine pathway because not all of the flux is channeled through oxaloacetate to succinyl-CoA to BDO. Overall, the homoserine pathway demonstrates a high-yielding route to BDO.

An acetoacetate pathway also can be engineered to generate BDO-producing microbial organisms. Acetoacetate can be formed from acetyl-CoA by enzymes involved in fatty acid metabolism, including acetyl-CoA acetyltransferase and acetoacetyl-CoA transferase. Biosynthetic routes through acetoacetate are also particularly useful in microbial organisms that can metabolize single carbon compounds such as carbon monoxide, carbon dioxide or methanol to form acetyl-CoA.

A three step route from acetoacetyl-CoA to 4-aminobutyrate (see Figure 9C) can be used to synthesize BDO through acetoacetyl-CoA. 4-Aminobutyrate can be converted to succinic semialdehyde as shown in Figure 8B. Succinic semialdehyde, which is one reduction step removed from succinyl-CoA or one decarboxylation step removed from α-ketoglutarate, can be converted to BDO following three reductions steps (Figure 1). Briefly, step 1 of this pathway involves the conversion of acetoacetyl-CoA to acetoacetate by, for example, the *E. coli* acetoacetyl-CoA transferase encoded by the *atoA* and *atoD* genes (Hanai et al., *Appl. Environ. Microbiol.* 73: 7814–7818 (2007)). Step 2 of the acetoacetyl-CoA biopathway entails conversion of acetoacetate to 3-aminobutanoate by an ω-aminotransferase. The ω-amino acid:pyruvate aminotransferase (ω-APT) from *Alcaligens denitrificans* was overexpressed in *E. coli* and shown to have a high activity toward 3-aminobutanoate *in vitro* (Yun et al., *Appl. Environ. Microbiol.* 70:2529-2534 (2004)).

In step 2, a putative aminomutase shifts the amine group from the 3- to the 4- position of the carbon backbone. An aminomutase performing this function on 3-aminobutanoate has not been characterized, but an enzyme from *Clostridium sticklandii* has a very similar mechanism. The enzyme, D-lysine-5,6-aminomutase, is involved in lysine biosynthesis.

The synthetic route to BDO from acetoacetyl-CoA passes through 4-aminobutanoate, a metabolite in *E. coli* that is normally formed from decarboxylation of glutamate. Once formed, 4-aminobutanoate can be converted to succinic semialdehyde by 4-aminobutanoate transaminase (2.6.1.19), an enzyme which has been biochemically characterized.

One consideration for selecting candidate enzymes in this pathway is the stereoselectivity of the enzymes involved in steps 2 and 3. The ω-ABT in *Alcaligens denitrificans* is specific to the L-stereoisomer of 3-aminobutanoate, while D-lysine-5,6-aminomutase likely requires the D-stereoisomer. If enzymes with complementary stereoselectivity are not initially found or engineered, a third enzyme can be added to the pathway with racemase activity that can convert L-3-aminobutanoate to D-3-aminobutanoate. While amino acid racemases are widespread, whether these enzymes can function on ω-amino acids is not known.

The maximum theoretical molar yield of this pathway under anaerobic conditions is 1.091 mol/mol glucose. In order to generate flux from acetoacetyl-CoA to BDO it was necessary to assume that acetyl-CoA:acetoacetyl-CoA transferase is reversible. The function of this enzyme in *E. coli* is to metabolize short-chain fatty acids by first converting them into thioesters.

While the operation of acetyl-CoA:acetoacetyl-CoA transferase in the acetate-consuming direction has not been demonstrated experimentally in *E. coli*, studies on similar enzymes in other organisms support the assumption that this reaction is reversible. The enzyme butyryl-CoA:acetate:CoA transferase in gut microbes *Roseburia* sp. and *F. prasnitzii* operates in the acetate utilizing direction to produce butyrate (Duncan et al., *Appl. Environ. Microbiol* 68:5186-5190 (2002)). Another very similar enzyme, acetyl:succinate CoA-transferase in *Trypanosoma brucei*, also operates in the acetate utilizing direction. This reaction has a $\Delta_{rxn}G$ close to equilibrium, so high concentrations of acetate can likely drive the reaction in the direction of interest. At the maximum theoretical BDO production rate of 1.09 mol/mol glucose simulations predict that *E. coli* can generate 1.098 mol ATP per mol glucose with no fermentation byproducts. This ATP yield should be sufficient for cell growth, maintenance, and production. The acetoacetatyl-CoA biopathway is a high-yielding route to BDO from acetyl-CoA.

Therefore, in addition to any of the various modifications exemplified previously for establishing 4-HB biosynthesis in a selected host, the BDO producing microbial organisms can include any of the previous combinations and permutations of 4-HB pathway metabolic modifications as well as any combination of expression for CoA-independent aldehyde dehydrogenase, CoA-dependent aldehyde dehydrogenase or an alcohol dehydrogenase or other enzymes disclosed herein to generate biosynthetic pathways for GBL and/or BDO. Therefore, the BDO producers of the invention can have exogenous expression of, for example, one, two, three, four, five, six, seven, eight, nine, or up to all enzymes corresponding to any of the 4-HB pathway and/or any of the BDO pathway enzymes disclosed herein.

Design and construction of the genetically modified microbial organisms is carried out using methods well known in the art to achieve sufficient amounts of expression to produce BDO. In particular, the non-naturally occurring microbial organisms of the invention can achieve biosynthesis of BDO resulting in intracellular concentrations between about 0.1-200 mM or more, such as about 0.1-25 mM or more, as discussed above. For example, the intracellular concentration of BDO is between about 3-20mM, particularly between about 5-15 mM and more particularly between about 8-12 mM, including about 10 mM or more. Intracellular concentrations between and above each of these exemplary ranges also can be achieved from the non-naturally occurring microbial organisms of the invention. As with the 4-HB producers, the BDO producers also can be sustained, cultured or fermented under anaerobic conditions.

The invention further provides a method for the production of 4-HB. The method includes culturing a non-naturally occurring microbial organism having a 4-hydroxybutanoic acid (4-HB) biosynthetic pathway comprising at least one exogenous nucleic acid encoding 4-hydroxybutanoate dehydrogenase, CoA-independent succinic semialdehyde dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, glutamate:succinic semialdehyde transaminase, α-ketoglutarate decarboxylase, or glutamate decarboxylase under substantially anaerobic conditions for a sufficient period of time to produce monomeric 4-hydroxybutanoic acid (4-HB). The method can additionally include chemical conversion of 4-HB to GBL and to BDO or THF, for example.

Additionally provided is a method for the production of 4-HB. The method includes culturing a non-naturally occurring microbial organism having a 4-hydroxybutanoic acid (4-HB) biosynthetic pathway including at least one exogenous nucleic acid encoding 4-hydroxybutanoate dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic

semialdehyde dehydrogenase or α-ketoglutarate decarboxylase under substantially anaerobic conditions for a sufficient period of time to produce monomeric 4-hydroxybutanoic acid (4-HB). The 4-HB product can be secreted into the culture medium.

Further provided is a method for the production of BDO. The method includes culturing a non-naturally occurring microbial biocatalyst or microbial organism, comprising a microbial organism having 4-hydroxybutanoic acid (4-HB) and 1,4-butanediol (BDO) biosynthetic pathways, the pathways including at least one exogenous nucleic acid encoding 4-hydroxybutanoate dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, 4-hydroxybutyrate:CoA transferase, 4-hydroxybutyrate kinase, phosphotranshydroxybutyrylase, α-ketoglutarate decarboxylase, aldehyde dehydrogenase, alcohol dehydrogenase or an aldehyde/alcohol dehydrogenase for a sufficient period of time to produce 1,4-butanediol (BDO). The BDO product can be secreted into the culture medium.

Additionally provided are methods for producing BDO by culturing a non-naturally occurring microbial organism having a BDO pathway of the invention. The BDO pathway can comprise at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, 4-aminobutyrate-CoA ligase, 4-aminobutyryl-CoA oxidoreductase (deaminating), 4-aminobutyryl-CoA transaminase, or 4-hydroxybutyryl-CoA dehydrogenase (see Example VII and Table 17).

Alternatively, the BDO pathway can compre at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, 4-aminobutyrate-CoA ligase, 4-aminobutyryl-CoA reductase (alcohol forming), 4-aminobutyryl-CoA reductase, 4-aminobutan-1-ol dehydrogenase, 4-aminobutan-1-ol oxidoreductase (deaminating) or 4-aminobutan-1-ol transaminase (see Example VII and Table 18).

In addition, the invention provides a method for producing BDO, comprising culturing a non-naturally occurring microbial organism having a BDO pathway, the pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising 4-aminobutyrate kinase, 4-aminobutyraldehyde dehydrogenase

(phosphorylating), 4-aminobutan-1-ol dehydrogenase, 4-aminobutan-1-ol oxidoreductase (deaminating), 4-aminobutan-1-ol transaminase, [(4-aminobutanolyl)oxy]phosphonic acid oxidoreductase (deaminating), [(4-aminobutanolyl)oxy]phosphonic acid transaminase, 4-hydroxybutyryl-phosphate dehydrogenase, or 4-hydroxybutyraldehyde dehydrogenase (phosphorylating) (see Example VII and Table 19).

The invention further provides a method for producing BDO, comprising culturing a non-naturally occurring microbial organism having a BDO pathway, the pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising alpha-ketoglutarate 5-kinase, 2,5-dioxopentanoic semialdehyde dehydrogenase (phosphorylating), 2,5-dioxopentanoic acid reductase, alpha-ketoglutarate CoA transferase, alpha-ketoglutaryl-CoA hydrolase, alpha-ketoglutaryl-CoA ligase, alpha-ketoglutaryl-CoA reductase, 5-hydroxy-2-oxopentanoic acid dehydrogenase, alpha-ketoglutaryl-CoA reductase (alcohol forming), 5-hydroxy-2-oxopentanoic acid decarboxylase, or 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation)(see Example VIII and Table 20).

The invention additionally provides a method for producing BDO, comprising culturing a non-naturally occurring microbial organism having a BDO pathway, the pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising glutamate CoA transferase, glutamyl-CoA hydrolase, glutamyl-CoA ligase, glutamate 5-kinase, glutamate-5-semialdehyde dehydrogenase (phosphorylating), glutamyl-CoA reductase, glutamate-5-semialdehyde reductase, glutamyl-CoA reductase (alcohol forming), 2-amino-5-hydroxypentanoic acid oxidoreductase (deaminating), 2-amino-5-hydroxypentanoic acid transaminase, 5-hydroxy-2-oxopentanoic acid decarboxylase, 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation)(see Example IX and Table 21).

The invention additionally includes a method for producing BDO, comprising culturing a non-naturally occurring microbial organism having a BDO pathway, the pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising 3-hydroxybutyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydratase, vinylacetyl-CoA Δ-isomerase, or 4-hydroxybutyryl-CoA dehydratase (see Example X and Table 22).

Also provided is a method for producing BDO, comprising culturing a non-naturally occurring microbial organism having a BDO pathway, the pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising homoserine deaminase, homoserine CoA transferase, homoserine-CoA hydrolase, homoserine-CoA ligase, homoserine-CoA deaminase, 4-hydroxybut-2-enoyl-CoA transferase, 4-hydroxybut-2-enoyl-CoA hydrolase, 4-hydroxybut-2-enoyl-CoA ligase, 4-hydroxybut-2-enoate reductase, 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, or 4-hydroxybut-2-enoyl-CoA reductase (see Example XI and Table 23).

The invention additionally provides a method for producing BDO, comprising culturing a non-naturally occurring microbial organism having a BDO pathway, the pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising succinyl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, 4-hydroxybutanal dehydrogenase (phosphorylating). Such a BDO pathway can further comprise succinyl-CoA reductase, 4-hydroxybutyrate dehydrogenase, 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyrate kinase, phosphotrans-4-hydroxybutyrylase, 4-hydroxybutyryl-CoA reductase, 4-hydroxybutyryl-CoA reductase (alcohol forming), or 1,4-butanediol dehydrogenase.

Also provided is a method for producing BDO, comprising culturing a non-naturally occurring microbial organism having a BDO pathway, the pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising glutamate dehydrogenase, 4-aminobutyrate oxidoreductase (deaminating), 4-aminobutyrate transaminase, glutamate decarboxylase, 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, 4-hydroxybutanal dehydrogenase (phosphorylating).

The invention additionally provides methods of producing a desired product using the genetically modified organisms disclosed herein that allow improved production of a desired product such as BDO by increasing the product or decreasing undesirable byproducts. Thus, the invention provides a method for producing 1,4-butanediol (BDO), comprising culturing the non-naturally occurring microbial organisms disclosed herein under conditions and for a sufficient

period of time to produce BDO. In one embodiment, the invention provides a method of producing BDO using a non-naturally occurring microbial organism, comprising a microbial organism having a 1,4-butanediol (BDO) pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO. In one embodiment, the microbial organism is genetically modified to express exogenous succinyl-CoA synthetase (see Example XII). For example, the succinyl-CoA synthetase can be encoded by an *Escherichia coli sucCD* genes.

In another embodiment, the microbial organism is genetically modified to express exogenous alpha-ketoglutarate decarboxylase (see Example XIII). For example, the alpha-ketoglutarate decarboxylase can be encoded by the *Mycobacterium bovis sucA* gene. In still another embodiment, the microbial organism is genetically modified to express exogenous succinate semialdehyde dehydrogenase and 4-hydroxybutyrate dehydrogenase and optionally 4-hydroxybutyryl-CoA/acetyl-CoA transferase (see Example XIII). For example, the succinate semialdehyde dehydrogenase (CoA-dependent), 4-hydroxybutyrate dehydrogenase and 4-hydroxybutyryl-CoA/acetyl-CoA transferase can be encoded by *Porphyromonas gingivalis* W83 genes. In an additional embodiment, the microbial organism is genetically modified to express exogenous butyrate kinase and phosphotransbutyrylase (see Example XIII). For example, the butyrate kinase and phosphotransbutyrylase can be encoded by *Clostridium acetobutilicum buk1* and *ptb* genes.

In yet another embodiment, the microbial organism is genetically modified to express exogenous 4-hydroxybutyryl-CoA reductase (see Example XIII). For example, the 4-hydroxybutyryl-CoA reductase can be encoded by *Clostridium beijerinckii ald* gene. Additionally, in an embodiment of the invention, the microbial organism is genetically modified to express exogenous 4-hydroxybutanal reductase (see Example XIII). For example, the 4-hydroxybutanal reductase can be encoded by *Geobacillus thermoglucosidasius adh1* gene. In another embodiment, the microbial organism is genetically modified to express exogenous pyruvate dehydrogenase subunits (see Example XIV). For example, the exogenous pyruvate dehydrogenase can be NADH insensitive. The pyruvate dehydrogenase subunit can be encoded by the *Klebsiella pneumonia lpdA* gene. In a particular embodiment, the pyruvate dehydrogenase subunit genes of the microbial organism can be under the control of a pyruvate formate lyase promoter.

In still another embodiment, the microbial organism is genetically modified to disrupt a gene encoding an aerobic respiratory control regulatory system (see Example XV). For example, the

disruption can be of the *arcA* gene. Such an organism can further comprise disruption of a gene encoding malate dehydrogenase. In a further embodiment, the microbial organism is genetically modified to express an exogenous NADH insensitive citrate synthase(see Example XV). For example, the NADH insensitive citrate synthase can be encoded by *gltA*, such as an R163L mutant of *gltA*. In still another embodiment, the microbial organism is genetically modified to express exogenous phosphoenolpyruvate carboxykinase (see Example XVI). For example, the phosphoenolpyruvate carboxykinase can be encoded by an *Haemophilus influenza* phosphoenolpyruvate carboxykinase gene. It is understood that strains exemplified herein for improved production of BDO can similarly be used, with appropriate modifications, to produce other desired products, for example, 4-hydroxybutyrate or other desired products disclosed herein.

The invention additionally provides a method for producing 4-hydroxybutanal by culturing a non-naturally occurring microbial organism, comprising a 4-hydroxybutanal pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutanal pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutanal, the 4-hydroxybutanal pathway comprising succinyl-CoA reductase (aldehyde forming); 4-hydroxybutyrate dehydrogenase; and 4-hydroxybutyrate reductase (see Figure 58, steps A-C-D). The invention also provides a method for producing 4-hydroxybutanal by culturing a non-naturally occurring microbial organism, comprising a 4-hydroxybutanal pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutanal pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutanal, the 4-hydroxybutanal pathway comprising alpha-ketoglutarate decarboxylase; 4-hydroxybutyrate dehydrogenase; and 4-hydroxybutyrate reductase (Figure 58, steps B-C-D).

The invention further provides a method for producing 4-hydroxybutanal by culturing a non-naturally occurring microbial organism, comprising a 4-hydroxybutanal pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutanal pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutanal, the 4-hydroxybutanal pathway comprising succinate reductase; 4-hydroxybutyrate dehydrogenase, and 4-hydroxybutyrate reductase (see Figure 62, steps F-C-D). In yet another embodiment, the invention provides a method for producing 4-hydroxybutanal by culturing a non-naturally occurring microbial organism, comprising a 4-hydroxybutanal pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutanal pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutanal, the 4-hydroxybutanal pathway comprising alpha-ketoglutarate decarboxylase, or glutamate dehydrogenase or glutamate transaminase and glutamate decarboxylase and 4-

aminobutyrate dehydrogenase or 4-aminobutyrate transaminase; 4-hydroxybutyrate dehydrogenase; and 4-hydroxybutyrate reductase (see Figure 62, steps B or ((J or K)-L-(M or N))-C-D).

The invention also provides a method for producing 4-hydroxybutanal by culturing a non-naturally occurring microbial organism, comprising a 4-hydroxybutanal pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutanal pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutanal, the 4-hydroxybutanal pathway comprising alpha-ketoglutarate reductase; 5-hydroxy-2-oxopentanoate dehydrogenase; and 5-hydroxy-2-oxopentanoate decarboxylase (see Figure 62, steps X-Y-Z). The invention furthur provides a method for producing 4-hydroxybutyryl-CoA by culturing a non-naturally occurring microbial organism, comprising a 4-hydroxybutyryl-CoA pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutyryl-CoA pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutyryl-CoA, the 4-hydroxybutyryl-CoA pathway comprising alpha-ketoglutarate reductase; 5-hydroxy-2-oxopentanoate dehydrogenase; and 5-hydroxy-2-oxopentanoate dehydrogenase (decarboxylation) (see Figure 62, steps X-Y-AA).

The invention additionally provides a method for producing putrescine by culturing a non-naturally occurring microbial organism, comprising a putrescine pathway comprising at least one exogenous nucleic acid encoding a putrescine pathway enzyme expressed in a sufficient amount to produce putrescine, the putrescine pathway comprising succinate reductase; 4-aminobutyrate dehydrogenase or 4-aminobutyrate transaminase; 4-aminobutyrate reductase; and putrescine dehydrogenase or putrescine transaminase (see Figure 63, steps F-M/N-C-D/E). In still another embodiment, the invention provides a method for producing putrescine by culturing a non-naturally occurring microbial organism, comprising a putrescine pathway comprising at least one exogenous nucleic acid encoding a putrescine pathway enzyme expressed in a sufficient amount to produce putrescine, the putrescine pathway comprising alpha-ketoglutarate decarboxylase; 4-aminobutyrate dehydrogenase or 4-aminobutyrate transaminase; 4-aminobutyrate reductase; and putrescine dehydrogenase or putrescine transaminase (see Figure 63, steps B-M/N-C-D/E). The invention additionally provides a method for producing putrescine by culturing a non-naturally occurring microbial organism, comprising a putrescine pathway comprising at least one exogenous nucleic acid encoding a putrescine pathway enzyme expressed in a sufficient amount to produce putrescine, the putrescine pathway comprising glutamate dehydrogenase or glutamate transaminase; glutamate decarboxylase; 4-aminobutyrate

reductase; and putrescine dehydrogenase or putrescine transaminase (see Figure 63, steps J/K-L-C-D/E).

The invention provides in another embodiment a method for producing putrescine by culturing a non-naturally occurring microbial organism, comprising a putrescine pathway comprising at least one exogenous nucleic acid encoding a putrescine pathway enzyme expressed in a sufficient amount to produce putrescine, the putrescine pathway comprising alpha-ketoglutarate reductase; 5-amino-2-oxopentanoate dehydrogenase or 5-amino-2-oxopentanoate transaminase; 5-amino-2-oxopentanoate decarboxylase; and putrescine dehydrogenase or putrescine transaminase (see Figure 63, steps O-P/Q-R-D/E). Also provided is a method for producing putrescine by culturing a non-naturally occurring microbial organism, comprising a putrescine pathway comprising at least one exogenous nucleic acid encoding a putrescine pathway enzyme expressed in a sufficient amount to produce putrescine, the putrescine pathway comprising alpha-ketoglutarate reductase; 5-amino-2-oxopentanoate dehydrogenase or 5-amino-2-oxopentanoate transaminase; ornithine dehydrogenase or ornithine transaminase; and ornithine decarboxylase (see Figure 63, steps O-P/Q-S/T-U). It is understood that a microbial organism comprising any of the pathways disclosed herein can be used to produce a a desired product or intermediate, including 4-HB, 4-HBal, BDO or putrescine.

It is understood that, in methods of the invention, any of the one or more exogenous nucleic acids can be introduced into a microbial organism to produce a non-naturally occurring microbial organism of the invention. The nucleic acids can be introduced so as to confer, for example, a 4-HB, BDO, THF or GBL biosynthetic pathway onto the microbial organism. Alternatively, encoding nucleic acids can be introduced to produce an intermediate microbial organism having the biosynthetic capability to catalyze some of the required reactions to confer 4-HB, BDO, THF or GBL biosynthetic capability. For example, a non-naturally occurring microbial organism having a 4-HB biosynthetic pathway can comprise at least two exogenous nucleic acids encoding desired enzymes, such as the combination of 4-hydroxybutanoate dehydrogenase and α-ketoglutarate decarboxylase; 4-hydroxybutanoate dehydrogenase and CoA-independent succinic semialdehyde dehydrogenase; 4-hydroxybutanoate dehydrogenase and CoA-dependent succinic semialdehyde dehydrogenase; CoA-dependent succinic semialdehyde dehydrogenase and succinyl-CoA synthetase; succinyl-CoA synthetase and glutamate decarboxylase, and the like. Thus, it is understood that any combination of two or more enzymes of a biosynthetic pathway can be included in a non-naturally occurring microbial organism of the invention. Similarly, it is understood that any combination of three or more

enzymes of a biosynthetic pathway can be included in a non-naturally occurring microbial organism of the invention, for example, 4-hydroxybutanoate dehydrogenase, α-ketoglutarate decarboxylase and CoA-dependent succinic semialdehyde dehydrogenase; CoA-independent succinic semialdehyde dehydrogenase and succinyl-CoA synthetase; 4-hydroxybutanoate dehydrogenase, CoA-dependent succinic semialdehyde dehydrogenase and glutamate:succinic semialdehyde transaminase, and so forth, as desired, so long as the combination of enzymes of the desired biosynthetic pathway results in production of the corresponding desired product.

Similarly, for example, with respect to any one or more exogenous nucleic acids introduced to confer BDO production, a non-naturally occurring microbial organism having a BDO biosynthetic pathway can comprise at least two exogenous nucleic acids encoding desired enzymes, such as the combination of 4-hydroxybutanoate dehydrogenase and α-ketoglutarate decarboxylase; 4-hydroxybutanoate dehydrogenase and 4-hydroxybutyryl CoA:acetyl-CoA transferase; 4-hydroxybutanoate dehydrogenase and butyrate kinase; 4-hydroxybutanoate dehydrogenase and phosphotransbutyrylase; 4-hydroxybutyryl CoA:acetyl-CoA transferase and aldehyde dehydrogenase; 4-hydroxybutyryl CoA:acetyl-CoA transferase and alcohol dehydrogenase; 4-hydroxybutyryl CoA:acetyl-CoA transferase and an aldehyde/alcohol dehydrogenase, 4-aminobutyrate-CoA transferase and 4-aminobutyryl-CoA transaminase; 4-aminobutyrate kinase and 4-aminobutan-1-ol oxidoreductase (deaminating), and the like. Thus, it is understood that any combination of two or more enzymes of a biosynthetic pathway can be included in a non-naturally occurring microbial organism of the invention. Similarly, it is understood that any combination of three or more enzymes of a biosynthetic pathway can be included in a non-naturally occurring microbial organism of the invention, for example, 4-hydroxybutanoate dehydrogenase, α-ketoglutarate decarboxylase and 4-hydroxybutyryl CoA:acetyl-CoA transferase; 4-hydroxybutanoate dehydrogenase, butyrate kinase and phosphotransbutyrylase; 4-hydroxybutanoate dehydrogenase, 4-hydroxybutyryl CoA:acetyl-CoA transferase and aldehyde dehydrogenase; 4-hydroxybutyryl CoA:acetyl-CoA transferase, aldehyde dehydrogenase and alcohol dehydrogenase; butyrate kinase, phosphotransbutyrylase and an aldehyde/alcohol dehydrogenase; 4-aminobutyryl-CoA hydrolase, 4-aminobutyryl-CoA reductase and 4-amino butan-1-ol transaminase; 3-hydroxybutyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydratase and 4-hydroxybutyryl-CoA dehydratase, and the like. Similarly, any combination of four, five or more enzymes of a biosynthetic pathway as disclosed herein can be included in a non-naturally occurring microbial organism of the invention, as desired, so long as the combination of enzymes of the desired biosynthetic pathway results in production of the corresponding desired product.

Any of the non-naturally occurring microbial organisms described herein can be cultured to produce and/or secrete the biosynthetic products of the invention. For example, the 4-HB producers can be cultured for the biosynthetic production of 4-HB. The 4-HB can be isolated or be treated as described below to generate GBL, THF and/or BDO. Similarly, the BDO producers can be cultured for the biosynthetic production of BDO. The BDO can be isolated or subjected to further treatments for the chemical synthesis of BDO family compounds, as disclosed herein.

The growth medium can include, for example, any carbohydrate source which can supply a source of carbon to the non-naturally occurring microorganism. Such sources include, for example, sugars such as glucose, sucrose, xylose, arabinose, galactose, mannose, fructose and starch. Other sources of carbohydrate include, for example, renewable feedstocks and biomass. Exemplary types of biomasses that can be used as feedstocks in the methods of the invention include cellulosic biomass, hemicellulosic biomass and lignin feedstocks or portions of feedstocks. Such biomass feedstocks contain, for example, carbohydrate substrates useful as carbon sources such as glucose, sucrose, xylose, arabinose, galactose, mannose, fructose and starch. Given the teachings and guidance provided herein, those skilled in the art will understand that renewable feedstocks and biomass other than those exemplified above also can be used for culturing the microbial organisms of the invention for the production of 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine and other compounds of the invention.

Accordingly, given the teachings and guidance provided herein, those skilled in the art will understand that a non-naturally occurring microbial organism can be produced that secretes the biosynthesized compounds of the invention when grown on a carbon source such as a carbohydrate. Such compounds include, for example, 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine and any of the intermediates metabolites in the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathways and/or the combined 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathways. All that is required is to engineer in one or more of the enzyme activities shown in Figure1 to achieve biosynthesis of the desired compound or intermediate including, for example, inclusion of some or all of the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine biosynthetic pathways. Accordingly, the invention provides a non-naturally occurring microbial organism that secretes 4-HB when grown on a carbohydrate, secretes BDO when grown on a carbohydrate and/or secretes any of the intermediate metabolites shown in Figures 1, 8-13, 58, 62 or 63 when grown on a carbohydrate. A BDO producing microbial organisms of the invention can initiate synthesis from, for example, succinate, succinyl-CoA, α-ketogluterate, succinic semialdehyde,

4-HB, 4-hydroxybutyrylphosphate, 4-hydroxybutyryl-CoA (4-HB-CoA) and/or 4-hydroxybutyraldehyde.

In some embodiments, culture conditions include anaerobic or substantially anaerobic growth or maintenance conditions. Exemplary anaerobic conditions have been described previously and are well known in the art. Exemplary anaerobic conditions for fermentation processes are described below in the Examples. Any of these conditions can be employed with the non-naturally occurring microbial organisms as well as other anaerobic conditions well known in the art. Under such anaerobic conditions, the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine producers can synthesize monomeric 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine, respectively, at intracellular concentrations of 5-10 mM or more as well as all other concentrations exemplified previously.

A number of downstream compounds also can be generated for the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine producing non-naturally occurring microbial organisms of the invention. With respect to the 4-HB producing microbial organisms of the invention, monomeric 4-HB and GBL exist in equilibrium in the culture medium. The conversion of 4-HB to GBL can be efficiently accomplished by, for example, culturing the microbial organisms in acid pH medium. A pH less than or equal to 7.5, in particular at or below pH 5.5, spontaneously converts 4-HB to GBL.

The resultant GBL can be separated from 4-HB and other components in the culture using a variety of methods well known in the art. Such separation methods include, for example, the extraction procedures exemplified in the Examples as well as methods which include continuous liquid-liquid extraction, pervaporation, membrane filtration, membrane separation, reverse osmosis, electrodialysis, distillation, crystallization, centrifugation, extractive filtration, ion exchange chromatography, size exclusion chromatography, adsorption chromatography, and ultrafiltration. All of the above methods are well known in the art. Separated GBL can be further purified by, for example, distillation.

Another down stream compound that can be produced from the 4-HB producing non-naturally occurring microbial organisms of the invention includes, for example, BDO. This compound can be synthesized by, for example, chemical hydrogenation of GBL. Chemical hydrogenation reactions are well known in the art. One exemplary procedure includes the chemical reduction of 4-HB and/or GBL or a mixture of these two components deriving from the culture using a

heterogeneous or homogeneous hydrogenation catalyst together with hydrogen, or a hydride-based reducing agent used stoichiometrically or catalytically, to produce 1,4-butanediol.

Other procedures well known in the art are equally applicable for the above chemical reaction and include, for example, WO No. 82/03854 (Bradley, et al.), which describes the hydrogenolysis of gamma-butyrolactone in the vapor phase over a copper oxide and zinc oxide catalyst. British Pat. No. 1,230,276, which describes the hydrogenation of gamma-butyrolactone using a copper oxide-chromium oxide catalyst. The hydrogenation is carried out in the liquid phase. Batch reactions also are exemplified having high total reactor pressures. Reactant and product partial pressures in the reactors are well above the respective dew points. British Pat. No. 1,314,126, which describes the hydrogenation of gamma-butyrolactone in the liquid phase over a nickel-cobalt-thorium oxide catalyst. Batch reactions are exemplified as having high total pressures and component partial pressures well above respective component dew points. British Pat. No. 1,344,557, which describes the hydrogenation of gamma-butyrolactone in the liquid phase over a copper oxide-chromium oxide catalyst. A vapor phase or vapor-containing mixed phase is indicated as suitable in some instances. A continuous flow tubular reactor is exemplified using high total reactor pressures. British Pat. No. 1,512,751, which describes the hydrogenation of gamma-butyrolactone to 1,4-butanediol in the liquid phase over a copper oxide-chromium oxide catalyst. Batch reactions are exemplified with high total reactor pressures and, where determinable, reactant and product partial pressures well above the respective dew points. U.S. Pat. No. 4,301,077, which describes the hydrogenation to 1,4-butanediol of gamma-butyrolactone over a Ru-Ni-Co-Zn catalyst. The reaction can be conducted in the liquid or gas phase or in a mixed liquid-gas phase. Exemplified are continuous flow liquid phase reactions at high total reactor pressures and relatively low reactor productivities. U.S. Pat. No. 4,048,196, which describes the production of 1,4-butanediol by the liquid phase hydrogenation of gamma-butyrolactone over a copper oxide-zinc oxide catalyst. Further exemplified is a continuous flow tubular reactor operating at high total reactor pressures and high reactant and product partial pressures. And U.S. Patent No. 4,652,685, which describes the hydrogenation of lactones to glycols.

A further downstream compound that can be produced form the 4-HB producing microbial organisms of the invention includes, for example, THF. This compound can be synthesized by, for example, chemical hydrogenation of GBL. One exemplary procedure well known in the art applicable for the conversion of GBL to THF includes, for example, chemical reduction of 4-HB and/or GBL or a mixture of these two components deriving from the culture using a

heterogeneous or homogeneous hydrogenation catalyst together with hydrogen, or a hydride-based reducing agent used stoichiometrically or catalytically, to produce tetrahydrofuran. Other procedures well know in the art are equally applicable for the above chemical reaction and include, for example, U.S. Patent No. 6,686,310, which describes high surface area sol-gel route prepared hydrogenation catalysts. Processes for the reduction of maleic acid to tetrahydrofuran (THF) and 1,4-butanediol (BDO) and for the reduction of gamma butyrolactone to tetrahydrofuran and 1,4-butanediol also are described.

The culture conditions can include, for example, liquid culture procedures as well as fermentation and other large scale culture procedures. As described further below in the Examples, particularly useful yields of the biosynthetic products of the invention can be obtained under anaerobic or substantially anaerobic culture conditions.

Suitable purification and/or assays to test for the production of 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine can be performed using well known methods. Suitable replicates such as triplicate cultures can be grown for each engineered strain to be tested. For example, product and byproduct formation in the engineered production host can be monitored. The final product and intermediates, and other organic compounds, can be analyzed by methods such as HPLC (High Performance Liquid Chromatography), GC-MS (Gas Chromatography-Mass Spectroscopy) and LC-MS (Liquid Chromatography-Mass Spectroscopy) or other suitable analytical methods using routine procedures well known in the art. The release of product in the fermentation broth can also be tested with the culture supernatant. Byproducts and residual glucose can be quantified by HPLC using, for example, a refractive index detector for glucose and alcohols, and a UV detector for organic acids (Lin et al., Biotechnol. Bioeng. 90:775-779 (2005)), or other suitable assay and detection methods well known in the art. The individual enzyme or protein activities from the exogenous DNA sequences can also be assayed using methods well known in the art.

The 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine product can be separated from other components in the culture using a variety of methods well known in the art. Such separation methods include, for example, extraction procedures as well as methods that include continuous liquid-liquid extraction, pervaporation, membrane filtration, membrane separation, reverse osmosis, electrodialysis, distillation, crystallization, centrifugation, extractive filtration, ion exchange chromatography, size exclusion chromatography, adsorption chromatography, and ultrafiltration. All of the above methods are well known in the art.

The invention further provides a method of manufacturing 4-HB. The method includes fermenting a non-naturally occurring microbial organism having a 4-hydroxybutanoic acid (4-HB) biosynthetic pathway comprising at least one exogenous nucleic acid encoding 4-hydroxybutanoate dehydrogenase, CoA-independent succinic semialdehyde dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, glutamate:succinic semialdehyde transaminase, a-ketoglutarate decarboxylase, or glutamate decarboxylase under substantially anaerobic conditions for a sufficient period of time to produce monomeric 4-hydroxybutanoic acid (4-HB), the process comprising fed-batch fermentation and batch separation; fed-batch fermentation and continuous separation, or continuous fermentation and continuous separation.

The culture and chemical hydrogenations described above also can be scaled up and grown continuously for manufacturing of 4-HB, 4-HBal, 4-HBCoA, GBL, BDO and/or THF or putrescine. Exemplary growth procedures include, for example, fed-batch fermentation and batch separation; fed-batch fermentation and continuous separation, or continuous fermentation and continuous separation. All of these processes are well known in the art. Employing the 4-HB producers allows for simultaneous 4-HB biosynthesis and chemical conversion to GBL, BDO and/or THF by employing the above hydrogenation procedures simultaneous with continuous cultures methods such as fermentation. Other hydrogenation procedures also are well known in the art and can be equally applied to the methods of the invention.

Fermentation procedures are particularly useful for the biosynthetic production of commercial quantities of 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine. Generally, and as with non-continuous culture procedures, the continuous and/or near-continuous production of 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine will include culturing a non-naturally occurring 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine producing organism of the invention in sufficient nutrients and medium to sustain and/or nearly sustain growth in an exponential phase. Continuous culture under such conditions can be include, for example, 1 day, 2, 3, 4, 5, 6 or 7 days or more. Additionally, continuous culture can include 1 week, 2, 3, 4 or 5 or more weeks and up to several months. Alternatively, organisms of the invention can be cultured for hours, if suitable for a particular application. It is to be understood that the continuous and/or near-continuous culture conditions also can include all time intervals in between these exemplary periods. It is further understood that the time of culturing the microbial organism of the invention is for a sufficient period of time to produce a sufficient amount of product for a desired purpose.

Fermentation procedures are well known in the art. Briefly, fermentation for the biosynthetic production of 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine or other 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine derived products, including intermediates, of the invention can be utilized in, for example, fed-batch fermentation and batch separation; fed-batch fermentation and continuous separation, or continuous fermentation and continuous separation. Examples of batch and continuous fermentation procedures well known in the art are exemplified further below in the Examples.

In addition to the above fermentation procedures using the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine producers of the invention for continuous production of substantial quantities of 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine, including monomeric 4-HB, respectively, the 4-HB producers also can be, for example, simultaneously subjected to chemical synthesis procedures to convert the product to other compounds or the product as described previously for the chemical conversion of monomeric 4-HB to, for example, GBL, BDO and/or THF. The BDO producers can similarly be, for example, simultaneously subjected to chemical synthesis procedures as described previously for the chemical conversion of BDO to, for example, THF, GBL, pyrrolidones and/or other BDO family compounds. In addition, the products of the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine producers can be separated from the fermentation culture and sequentially subjected to chemical conversion to convert the product to other compounds, if desired, as disclosed herein.

Briefly, hydrogenation of GBL in the fermentation broth can be performed as described by Frost et al., *Biotechnology Progress* 18: 201-211 (2002). Another procedure for hydrogenation during fermentation include, for example, the methods described in, for example, U.S. Patent No. 5,478,952. This method is further exemplified in the Examples below.

Therefore, the invention additionally provides a method of manufacturing γ-butyrolactone (GBL), tetrahydrofuran (THF) or 1,4-butanediol (BDO). The method includes fermenting a non-naturally occurring microbial organism having 4-hydroxybutanoic acid (4-HB) and/or 1,4-butanediol (BDO) biosynthetic pathways, the pathways comprise at least one exogenous nucleic acid encoding 4-hydroxybutanoate dehydrogenase, CoA-independent succinic semialdehyde dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, 4-hydroxybutyrate:CoA transferase, glutamate:succinic semialdehyde transaminase, α-ketoglutarate decarboxylase, glutamate decarboxylase, 4-hydroxybutanoate kinase, phosphotransbutyrylase, CoA-independent 1,4-butanediol semialdehyde dehydrogenase,

CoA-dependent 1,4-butanediol semialdehyde dehydrogenase, CoA-independent 1,4-butanediol alcohol dehydrogenase or CoA-dependent 1,4-butanediol alcohol dehydrogenase, under substantially anaerobic conditions for a sufficient period of time to produce 1,4-butanediol (BDO), GBL or THF, the fermenting comprising fed-batch fermentation and batch separation; fed-batch fermentation and continuous separation, or continuous fermentation and continuous separation.

In addition to the biosynthesis of 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine and other products of the invention as described herein, the non-naturally occurring microbial organisms and methods of the invention also can be utilized in various combinations with each other and with other microbial organisms and methods well known in the art to achieve product biosynthesis by other routes. For example, one alternative to produce BDO other than use of the 4-HB producers and chemical steps or other than use of the BDO producer directly is through addition of another microbial organism capable of converting 4-HB or a 4-HB product exemplified herein to BDO.

One such procedure includes, for example, the fermentation of a 4-HB producing microbial organism of the invention to produce 4-HB, as described above and below. The 4-HB can then be used as a substrate for a second microbial organism that converts 4-HB to, for example, BDO, GBL and/or THF. The 4-HB can be added directly to another culture of the second organism or the original culture of 4-HB producers can be depleted of these microbial organisms by, for example, cell separation, and then subsequent addition of the second organism to the fermentation broth can utilized to produce the final product without intermediate purification steps. One exemplary second organism having the capacity to biochemically utilize 4-HB as a substrate for conversion to BDO, for example, is *Clostridium acetobutylicum* (see, for example, Jewell et al., *Current Microbiology*, 13:215-19 (1986)).

Thus, such a procedure includes, for example, the fermentation of a microbial organism that produces a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway intermediate. The 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway intermediate can then be used as a substrate for a second microbial organism that converts the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway intermediate to 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine. The 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway intermediate can be added directly to another culture of the second organism or the original culture of the 4-HB, 4-HBal, 4-HBCoA BDO or putrescine pathway intermediate producers can be depleted of these microbial organisms by, for example,

cell separation, and then subsequent addition of the second organism to the fermentation broth can be utilized to produce the final product without intermediate purification steps.

In other embodiments, the non-naturally occurring microbial organisms and methods of the invention can be assembled in a wide variety of subpathways to achieve biosynthesis of, for example, 4-HB and/or BDO as described. In these embodiments, biosynthetic pathways for a desired product of the invention can be segregated into different microbial organisms and the different microbial organisms can be co-cultured to produce the final product. In such a biosynthetic scheme, the product of one microbial organism is the substrate for a second microbial organism until the final product is synthesized. For example, the biosynthesis of BDO can be accomplished as described previously by constructing a microbial organism that contains biosynthetic pathways for conversion of one pathway intermediate to another pathway intermediate or the product, for example, a substrate such as endogenous succinate through 4-HB to the final product BDO. Alternatively, BDO also can be biosynthetically produced from microbial organisms through co-culture or co-fermentation using two organisms in the same vessel. A first microbial organism being a 4-HB producer with genes to produce 4-HB from succinic acid, and a second microbial organism being a BDO producer with genes to convert 4-HB to BDO. For example, the biosynthesis of 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine can be accomplished by constructing a microbial organism that contains biosynthetic pathways for conversion of one pathway intermediate to another pathway intermediate or the product. Alternatively, 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine also can be biosynthetically produced from microbial organisms through co-culture or co-fermentation using two organisms in the same vessel, where the first microbial organism produces a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine intermediate and the second microbial organism converts the intermediate to 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine.

Given the teachings and guidance provided herein, those skilled in the art will understand that a wide variety of combinations and permutations exist for the non-naturally occurring microbial organisms and methods of the invention together with other microbial organisms, with the co-culture of other non-naturally occurring microbial organisms having subpathways and with combinations of other chemical and/or biochemical procedures well known in the art to produce 4-HB, BDO, GBL and THF products of the invention.

It is understood that, in methods of the invention, any of the one or more exogenous nucleic acids can be introduced into a microbial organism to produce a non-naturally occurring

microbial organism of the invention. The nucleic acids can be introduced so as to confer, for example, a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine biosynthetic pathway onto the microbial organism. Alternatively, encoding nucleic acids can be introduced to produce an intermediate microbial organism having the biosynthetic capability to catalyze some of the required reactions to confer 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine biosynthetic capability. For example, a non-naturally occurring microbial organism having a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine biosynthetic pathway can comprise at least two exogenous nucleic acids encoding desired enzymes or proteins, such as the combination of enzymes as disclosed herein (see Examples and Figures 1, 8-13, 58, 62 and 63), and the like. Thus, it is understood that any combination of two or more enzymes or proteins of a biosynthetic pathway can be included in a non-naturally occurring microbial organism of the invention. Similarly, it is understood that any combination of three or more enzymes or proteins of a biosynthetic pathway can be included in a non-naturally occurring microbial organism of the invention, for example,], and so forth, as desired and disclosed herein, so long as the combination of enzymes and/or proteins of the desired biosynthetic pathway results in production of the corresponding desired product. Similarly, any combination of four or more enzymes or proteins of a biosynthetic pathway as disclosed herein can be included in a non-naturally occurring microbial organism of the invention, as desired, so long as the combination of enzymes and/or proteins of the desired biosynthetic pathway results in production of the corresponding desired product.

To generate better producers, metabolic modeling can be utilized to optimize growth conditions. Modeling can also be used to design gene knockouts that additionally optimize utilization of the pathway (see, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/0168654 and US 2004/0009466, and U.S. Patent No. 7,127,379). Modeling analysis allows reliable predictions of the effects on cell growth of shifting the metabolism towards more efficient production of 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine.

One computational method for identifying and designing metabolic alterations favoring biosynthesis of a desired product is the OptKnock computational framework (Burgard et al., *Biotechnol. Bioeng.* 84:647-657 (2003)). OptKnock is a metabolic modeling and simulation program that suggests gene deletion or disruption strategies that result in genetically stable microorganisms which overproduce the target product. Specifically, the framework examines the complete metabolic and/or biochemical network of a microorganism in order to suggest genetic manipulations that force the desired biochemical to become an obligatory byproduct of

cell growth. By coupling biochemical production with cell growth through strategically placed gene deletions or other functional gene disruption, the growth selection pressures imposed on the engineered strains after long periods of time in a bioreactor lead to improvements in performance as a result of the compulsory growth-coupled biochemical production. Lastly, when gene deletions are constructed there is a negligible possibility of the designed strains reverting to their wild-type states because the genes selected by OptKnock are to be completely removed from the genome. Therefore, this computational methodology can be used to either identify alternative pathways that lead to biosynthesis of a desired product or used in connection with the non-naturally occurring microbial organisms for further optimization of biosynthesis of a desired product.

Briefly, OptKnock is a term used herein to refer to a computational method and system for modeling cellular metabolism. The OptKnock program relates to a framework of models and methods that incorporate particular constraints into flux balance analysis (FBA) models. These constraints include, for example, qualitative kinetic information, qualitative regulatory information, and/or DNA microarray experimental data. OptKnock also computes solutions to various metabolic problems by, for example, tightening the flux boundaries derived through flux balance models and subsequently probing the performance limits of metabolic networks in the presence of gene additions or deletions. OptKnock computational framework allows the construction of model formulations that allow an effective query of the performance limits of metabolic networks and provides methods for solving the resulting mixed-integer linear programming problems. The metabolic modeling and simulation methods referred to herein as OptKnock are described in, for example, U.S. publication 2002/0168654, filed January 10, 2002, in International Patent No. PCT/US02/00660, filed January 10, 2002, and U.S. publication 2009/0047719, filed August 10, 2007.

Another computational method for identifying and designing metabolic alterations favoring biosynthetic production of a product is a metabolic modeling and simulation system termed SimPheny®. This computational method and system is described in, for example, U.S. publication 2003/0233218, filed June 14, 2002, and in International Patent Application No. PCT/US03/18838, filed June 13, 2003. SimPheny® is a computational system that can be used to produce a network model *in silico* and to simulate the flux of mass, energy or charge through the chemical reactions of a biological system to define a solution space that contains any and all possible functionalities of the chemical reactions in the system, thereby determining a range of allowed activities for the biological system. This approach is referred to as constraints-based

modeling because the solution space is defined by constraints such as the known stoichiometry of the included reactions as well as reaction thermodynamic and capacity constraints associated with maximum fluxes through reactions. The space defined by these constraints can be interrogated to determine the phenotypic capabilities and behavior of the biological system or of its biochemical components.

These computational approaches are consistent with biological realities because biological systems are flexible and can reach the same result in many different ways. Biological systems are designed through evolutionary mechanisms that have been restricted by fundamental constraints that all livi ng systems must face. Therefore, constraints-based modeling strategy embraces these general realities. Further, the ability to continuously impose further restrictions on a network model via the tightening of constraints results in a reduction in the size of the solution space, thereby enhancing the precision with which physiological performance or phenotype can be predicted.

Given the teachings and guidance provided herein, those skilled in the art will be able to apply various computational frameworks for metabolic modeling and simulation to design and implement biosynthesis of a desired compound in host microbial organisms. Such metabolic modeling and simulation methods include, for example, the computational systems exemplified above as SimPheny® and OptKnock. For illustration of the invention, some methods are described herein with reference to the OptKnock computation framework for modeling and simulation. Those skilled in the art will know how to apply the identification, design and implementation of the metabolic alterations using OptKnock to any of such other metabolic modeling and simulation computational frameworks and methods well known in the art.

The methods described above will provide one set of metabolic reactions to disrupt. Elimination of each reaction within the set or metabolic modification can result in a desired product as an obligatory product during the growth phase of the organism. Because the reactions are known, a solution to the bilevel OptKnock problem also will provide the associated gene or genes encoding one or more enzymes that catalyze each reaction within the set of reactions. Identification of a set of reactions and their corresponding genes encoding the enzymes participating in each reaction is generally an automated process, accomplished through correlation of the reactions with a reaction database having a relationship between enzymes and encoding genes.

Once identified, the set of reactions that are to be disrupted in order to achieve production of a desired product are implemented in the target cell or organism by functional disruption of at least one gene encoding each metabolic reaction within the set. One particularly useful means to achieve functional disruption of the reaction set is by deletion of each encoding gene. However, in some instances, it can be beneficial to disrupt the reaction by other genetic aberrations including, for example, mutation, deletion of regulatory regions such as promoters or cis binding sites for regulatory factors, or by truncation of the coding sequence at any of a number of locations. These latter aberrations, resulting in less than total deletion of the gene set can be useful, for example, when rapid assessments of the coupling of a product are desired or when genetic reversion is less likely to occur.

To identify additional productive solutions to the above described bilevel OptKnock problem which lead to further sets of reactions to disrupt or metabolic modifications that can result in the biosynthesis, including growth-coupled biosynthesis of a desired product, an optimization method, termed integer cuts, can be implemented. This method proceeds by iteratively solving the OptKnock problem exemplified above with the incorporation of an additional constraint referred to as an integer cut at each iteration. Integer cut constraints effectively prevent the solution procedure from choosing the exact same set of reactions identified in any previous iteration that obligatorily couples product biosynthesis to growth. For example, if a previously identified growth-coupled metabolic modification specifies reactions 1, 2, and 3 for disruption, then the following constraint prevents the same reactions from being simultaneously considered in subsequent solutions. The integer cut method is well known in the art and can be found described in, for example, Burgard et al., *Biotechnol. Prog.* 17:791-797 (2001). As with all methods described herein with reference to their use in combination with the OptKnock computational framework for metabolic modeling and simulation, the integer cut method of reducing redundancy in iterative computational analysis also can be applied with other computational frameworks well known in the art including, for example, SimPheny®.

The methods exemplified herein allow the construction of cells and organisms that biosynthetically produce a desired product, including the obligatory coupling of production of a target biochemical product to growth of the cell or organism engineered to harbor the identified genetic alterations. Therefore, the computational methods described herein allow the identification and implementation of metabolic modifications that are identified by an *in silico* method selected from OptKnock or SimPheny®. The set of metabolic modifications can include, for example, addition of one or more biosynthetic pathway enzymes and/or functional

disruption of one or more metabolic reactions including, for example, disruption by gene deletion.

As discussed above, the OptKnock methodology was developed on the premise that mutant microbial networks can be evolved towards their computationally predicted maximum-growth phenotypes when subjected to long periods of growth selection. In other words, the approach leverages an organism's ability to self-optimize under selective pressures. The OptKnock framework allows for the exhaustive enumeration of gene deletion combinations that force a coupling between biochemical production and cell growth based on network stoichiometry. The identification of optimal gene/reaction knockouts requires the solution of a bilevel optimization problem that chooses the set of active reactions such that an optimal growth solution for the resulting network overproduces the biochemical of interest (Burgard et al., *Biotechnol. Bioeng.* 84:647-657 (2003)).

An *in silico* stoichiometric model of *E. coli* metabolism can be employed to identify essential genes for metabolic pathways as exemplified previously and described in, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/0168654 and US 2004/0009466, and in U.S. Patent No. 7,127,379. As disclosed herein, the OptKnock mathematical framework can be applied to pinpoint gene deletions leading to the growth-coupled production of a desired product. Further, the solution of the bilevel OptKnock problem provides only one set of deletions. To enumerate all meaningful solutions, that is, all sets of knockouts leading to growth-coupled production formation, an optimization technique, termed integer cuts, can be implemented. This entails iteratively solving the OptKnock problem with the incorporation of an additional constraint referred to as an integer cut at each iteration, as discussed above.

The methods exemplified above and further illustrated in the Examples below allow the construction of cells and organisms that biosynthetically produce, including obligatory couple production of a target biochemical product to growth of the cell or organism engineered to harbor the identified genetic alterations. In this regard, metabolic alterations have been identified that result in the biosynthesis of 4-HB and 1,4-butanediol. Microorganism strains constructed with the identified metabolic alterations produce elevated levels of 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine compared to unmodified microbial organisms. These strains can be beneficially used for the commercial production of 4-HB, BDO, THF, GBL, 4-HBal, 4-HBCoA

or putrescine, for example, in continuous fermentation process without being subjected to the negative selective pressures.

Therefore, the computational methods described herein allow the identification and implementation of metabolic modifications that are identified by an *in silico* method selected from OptKnock or SimPheny®. The set of metabolic modifications can include, for example, addition of one or more biosynthetic pathway enzymes and/.or functional disruption of one or more metabolic reactions including, for example, disruption by gene deletion.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also included within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

Any of the non-naturally occurring microbial organisms described herein can be cultured to produce and/or secrete the biosynthetic products of the invention. For example, the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine producers can be cultured for the biosynthetic production of 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine.

For the production of 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine, the recombinant strains are cultured in a medium with carbon source and other essential nutrients. It is highly desirable to maintain anaerobic conditions in the fermenter to reduce the cost of the overall process. Such conditions can be obtained, for example, by first sparging the medium with nitrogen and then sealing the flasks with a septum and crimp-cap. For strains where growth is not observed anaerobically, microaerobic conditions can be applied by perforating the septum with a small hole for limited aeration. Exemplary anaerobic conditions have been described previously and are well-known in the art. Exemplary aerobic and anaerobic conditions are described, for example, in U.S. publication 2009/0047719, filed August 10, 2007. Fermentations can be performed in a batch, fed-batch or continuous manner, as disclosed herein.

If desired, the pH of the medium can be maintained at a desired pH, in particular neutral pH, such as a pH of around 7 by addition of a base, such as NaOH or other bases, or acid, as needed to maintain the culture medium at a desirable pH. The growth rate can be determined by measuring optical density using a spectrophotometer (600 nm), and the glucose uptake rate by monitoring carbon source depletion over time.

In addition to renewable feedstocks such as those exemplified above, the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine producing microbial organisms of the invention also can be modified for growth on syngas as its source of carbon. In this specific embodiment, one or more proteins or enzymes are expressed in the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine producing organisms to provide a metabolic pathway for utilization of syngas or other gaseous carbon source.

Synthesis gas, also known as syngas or producer gas, is the major product of gasification of coal and of carbonaceous materials such as biomass materials, including agricultural crops and residues. Syngas is a mixture primarily of H_2 and CO and can be obtained from the gasification of any organic feedstock, including but not limited to coal, coal oil, natural gas, biomass, and waste organic matter. Gasification is generally carried out under a high fuel to oxygen ratio. Although largely H_2 and CO, syngas can also include CO_2 and other gases in smaller quantities. Thus, synthesis gas provides a cost effective source of gaseous carbon such as CO and, additionally, CO_2.

The Wood-Ljungdahl pathway catalyzes the conversion of CO and H_2 to acetyl-CoA and other products such as acetate. Organisms capable of utilizing CO and syngas also generally have the capability of utilizing CO_2 and CO_2/H_2 mixtures through the same basic set of enzymes and transformations encompassed by the Wood-Ljungdahl pathway. H_2-dependent conversion of CO_2 to acetate by microorganisms was recognized long before it was revealed that CO also could be used by the same organisms and that the same pathways were involved. Many acetogens have been shown to grow in the presence of CO_2 and produce compounds such as acetate as long as hydrogen is present to supply the necessary reducing equivalents (see for example, Drake, *Acetogenesis*, pp. 3-60 Chapman and Hall, New York, (1994)). This can be summarized by the following equation:

$$2\ CO_2 + 4\ H_2 + n\ ADP + n\ Pi \rightarrow CH_3COOH + 2\ H_2O + n\ ATP$$

Hence, non-naturally occurring microorganisms possessing the Wood-Ljungdahl pathway can utilize CO_2 and H_2 mixtures as well for the production of acetyl-CoA and other desired products.

The Wood-Ljungdahl pathway is well known in the art and consists of 12 reactions which can be separated into two branches: (1) methyl branch and (2) carbonyl branch. The methyl branch

converts syngas to methyl-tetrahydrofolate (methyl-THF) whereas the carbonyl branch converts methyl-THF to acetyl-CoA. The reactions in the methyl branch are catalyzed in order by the following enzymes or proteins: ferredoxin oxidoreductase, formate dehydrogenase, formyltetrahydrofolate synthetase, methenyltetrahydrofolate cyclodehydratase, methylenetetrahydrofolate dehydrogenase and methylenetetrahydrofolate reductase. The reactions in the carbonyl branch are catalyzed in order by the following enzymes or proteins: methyltetrahydrofolate:corrinoid protein methyltransferase (for example, AcsE), corrinoid iron-sulfur protein, nickel-protein assembly protein (for example, AcsF), ferredoxin, acetyl-CoA synthase, carbon monoxide dehydrogenase and nickel-protein assembly protein (for example, CooC). Following the teachings and guidance provided herein for introducing a sufficient number of encoding nucleic acids to generate a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway, those skilled in the art will understand that the same engineering design also can be performed with respect to introducing at least the nucleic acids encoding the Wood-Ljungdahl enzymes or proteins absent in the host organism. Therefore, introduction of one or more encoding nucleic acids into the microbial organisms of the invention such that the modified organism contains the complete Wood-Ljungdahl pathway will confer syngas utilization ability.

Additionally, the reductive (reverse) tricarboxylic acid cycle is and/or hydrogenase activities can also be used for the conversion of CO, CO2 and/or H2 to acetyl-CoA and other products such as acetate. Organisms capable of fixing carbon via the reductive TCA pathway can utilize one or more of the following enzymes: ATP citrate-lyase, citrate lyase, aconitase, isocitrate dehydrogenase, alpha-ketoglutarate:ferredoxin oxidoreductase, succinyl-CoA synthetase, succinyl-CoA transferase, fumarate reductase, fumarase, malate dehydrogenase, NAD(P)H:ferredoxin oxidoreductase, carbon monoxide dehydrogenase, and hydrogenase. Specifically, the reducing equivalents extracted from CO and/or H2 by carbon monoxide dehydrogenase and hydrogenase are utilized to fix CO2 via the reductive TCA cycle into acetyl-CoA or acetate. Acetate can be converted to acetyl-CoA by enzymes such as acetyl-CoA transferase, acetate kinase/phosphotransacetylase, and acetyl-CoA synthetase. Acetyl-CoA can be converted to the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine precursors, glyceraldehyde-3-phosphate, phosphoenolpyruvate, and pyruvate, by pyruvate:ferredoxin oxidoreductase and the enzymes of gluconeogenesis. Following the teachings and guidance provided herein for introducing a sufficient number of encoding nucleic acids to generate a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway, those skilled in the art will understand that the same engineering design also can be performed with respect to introducing at least the nucleic acids encoding the reductive TCA pathway enzymes or proteins absent in the host organism.

Therefore, introduction of one or more encoding nucleic acids into the microbial organisms of the invention such that the modified organism contains the complete reductive TCA pathway will confer syngas utilization ability.

Accordingly, given the teachings and guidance provided herein, those skilled in the art will understand that a non-naturally occurring microbial organism can be produced that secretes the biosynthesized compounds of the invention when grown on a carbon source such as a carbohydrate. Such compounds include, for example, 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine and any of the intermediate metabolites in the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway. All that is required is to engineer in one or more of the required enzyme or protein activities to achieve biosynthesis of the desired compound or intermediate including, for example, inclusion of some or all of the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine biosynthetic pathways. Accordingly, the invention provides a non-naturally occurring microbial organism that produces and/or secretes 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine when grown on a carbohydrate or other carbon source and produces and/or secretes any of the intermediate metabolites shown in the 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway when grown on a carbohydrate or other carbon source. The 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine producing microbial organisms of the invention can initiate synthesis from an intermediate in a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway, as disclosed herein.

To generate better producers, metabolic modeling can be utilized to optimize growth conditions. Modeling can also be used to design gene knockouts that additionally optimize utilization of the pathway (see, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/0168654 and US 2004/0009466, and U.S. Patent No. 7,127,379). Modeling analysis allows reliable predictions of the effects on cell growth of shifting the metabolism towards more efficient production of 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine.

One computational method for identifying and designing metabolic alterations favoring biosynthesis of a desired product is the OptKnock computational framework (Burgard et al., *Biotechnol. Bioeng.* 84:647-657 (2003)). OptKnock is a metabolic modeling and simulation program that suggests gene deletion strategies that result in genetically stable microorganisms which overproduce the target product. Specifically, the framework examines the complete metabolic and/or biochemical network of a microorganism in order to suggest genetic manipulations that force the desired biochemical to become an obligatory byproduct of cell

growth. By coupling biochemical production with cell growth through strategically placed gene deletions or other functional gene disruption, the growth selection pressures imposed on the engineered strains after long periods of time in a bioreactor lead to improvements in performance as a result of the compulsory growth-coupled biochemical production. Lastly, when gene deletions are constructed there is a negligible possibility of the designed strains reverting to their wild-type states because the genes selected by OptKnock are to be completely removed from the genome. Therefore, this computational methodology can be used to either identify alternative pathways that lead to biosynthesis of a desired product or used in connection with the non-naturally occurring microbial organisms for further optimization of biosynthesis of a desired product.

Briefly, OptKnock is a term used herein to refer to a computational method and system for modeling cellular metabolism. The OptKnock program relates to a framework of models and methods that incorporate particular constraints into flux balance analysis (FBA) models. These constraints include, for example, qualitative kinetic information, qualitative regulatory information, and/or DNA microarray experimental data. OptKnock also computes solutions to various metabolic problems by, for example, tightening the flux boundaries derived through flux balance models and subsequently probing the performance limits of metabolic networks in the presence of gene additions or deletions. OptKnock computational framework allows the construction of model formulations that allow an effective query of the performance limits of metabolic networks and provides methods for solving the resulting mixed-integer linear programming problems. The metabolic modeling and simulation methods referred to herein as OptKnock are described in, for example, U.S. publication 2002/0168654, filed January 10, 2002, in International Patent No. PCT/US02/00660, filed January 10, 2002, and U.S. publication 2009/0047719, filed August 10, 2007.

Another computational method for identifying and designing metabolic alterations favoring biosynthetic production of a product is a metabolic modeling and simulation system termed SimPheny®. This computational method and system is described in, for example, U.S. publication 2003/0233218, filed June 14, 2002, and in International Patent Application No. PCT/US03/18838, filed June 13, 2003. SimPheny® is a computational system that can be used to produce a network model *in silico* and to simulate the flux of mass, energy or charge through the chemical reactions of a biological system to define a solution space that contains any and all possible functionalities of the chemical reactions in the system, thereby determining a range of allowed activities for the biological system. This approach is referred to as constraints-based

modeling because the solution space is defined by constraints such as the known stoichiometry of the included reactions as well as reaction thermodynamic and capacity constraints associated with maximum fluxes through reactions. The space defined by these constraints can be interrogated to determine the phenotypic capabilities and behavior of the biological system or of its biochemical components.

These computational approaches are consistent with biological realities because biological systems are flexible and can reach the same result in many different ways. Biological systems are designed through evolutionary mechanisms that have been restricted by fundamental constraints that all living systems must face. Therefore, constraints-based modeling strategy embraces these general realities. Further, the ability to continuously impose further restrictions on a network model via the tightening of constraints results in a reduction in the size of the solution space, thereby enhancing the precision with which physiological performance or phenotype can be predicted.

Given the teachings and guidance provided herein, those skilled in the art will be able to apply various computational frameworks for metabolic modeling and simulation to design and implement biosynthesis of a desired compound in host microbial organisms. Such metabolic modeling and simulation methods include, for example, the computational systems exemplified above as SimPheny® and OptKnock. For illustration of the invention, some methods are described herein with reference to the OptKnock computation framework for modeling and simulation. Those skilled in the art will know how to apply the identification, design and implementation of the metabolic alterations using OptKnock to any of such other metabolic modeling and simulation computational frameworks and methods well known in the art.

The methods described above will provide one set of metabolic reactions to disrupt. Elimination of each reaction within the set or metabolic modification can result in a desired product as an obligatory product during the growth phase of the organism. Because the reactions are known, a solution to the bilevel OptKnock problem also will provide the associated gene or genes encoding one or more enzymes that catalyze each reaction within the set of reactions. Identification of a set of reactions and their corresponding genes encoding the enzymes participating in each reaction is generally an automated process, accomplished through correlation of the reactions with a reaction database having a relationship between enzymes and encoding genes.

Once identified, the set of reactions that are to be disrupted in order to achieve production of a desired product are implemented in the target cell or organism by functional disruption of at least one gene encoding each metabolic reaction within the set. One particularly useful means to achieve functional disruption of the reaction set is by deletion of each encoding gene. However, in some instances, it can be beneficial to disrupt the reaction by other genetic aberrations including, for example, mutation, deletion of regulatory regions such as promoters or cis binding sites for regulatory factors, or by truncation of the coding sequence at any of a number of locations. These latter aberrations, resulting in less than total deletion of the gene set can be useful, for example, when rapid assessments of the coupling of a product are desired or when genetic reversion is less likely to occur.

To identify additional productive solutions to the above described bilevel OptKnock problem which lead to further sets of reactions to disrupt or metabolic modifications that can result in the biosynthesis, including growth-coupled biosynthesis of a desired product, an optimization method, termed integer cuts, can be implemented. This method proceeds by iteratively solving the OptKnock problem exemplified above with the incorporation of an additional constraint referred to as an integer cut at each iteration. Integer cut constraints effectively prevent the solution procedure from choosing the exact same set of reactions identified in any previous iteration that obligatorily couples product biosynthesis to growth. For example, if a previously identified growth-coupled metabolic modification specifies reactions 1, 2, and 3 for disruption, then the following constraint prevents the same reactions from being simultaneously considered in subsequent solutions. The integer cut method is well known in the art and can be found described in, for example, Burgard et al., *Biotechnol. Prog.* 17:791-797 (2001). As with all methods described herein with reference to their use in combination with the OptKnock computational framework for metabolic modeling and simulation, the integer cut method of reducing redundancy in iterative computational analysis also can be applied with other computational frameworks well known in the art including, for example, SimPheny®.

The methods exemplified herein allow the construction of cells and organisms that biosynthetically produce a desired product, including the obligatory coupling of production of a target biochemical product to growth of the cell or organism engineered to harbor the identified genetic alterations. Therefore, the computational methods described herein allow the identification and implementation of metabolic modifications that are identified by an *in silico* method selected from OptKnock or SimPheny®. The set of metabolic modifications can include, for example, addition of one or more biosynthetic pathway enzymes and/or functional

disruption of one or more metabolic reactions including, for example, disruption by gene deletion.

As discussed above, the OptKnock methodology was developed on the premise that mutant microbial networks can be evolved towards their computationally predicted maximum-growth phenotypes when subjected to long periods of growth selection. In other words, the approach leverages an organism's ability to self-optimize under selective pressures. The OptKnock framework allows for the exhaustive enumeration of gene deletion combinations that force a coupling between biochemical production and cell growth based on network stoichiometry. The identification of optimal gene/reaction knockouts requires the solution of a bilevel optimization problem that chooses the set of active reactions such that an optimal growth solution for the resulting network overproduces the biochemical of interest (Burgard et al., *Biotechnol. Bioeng.* 84:647-657 (2003)).

An *in silico* stoichiometric model of *E. coli* metabolism can be employed to identify essential genes for metabolic pathways as exemplified previously and described in, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/0168654 and US 2004/0009466, and in U.S. Patent No. 7,127,379. As disclosed herein, the OptKnock mathematical framework can be applied to pinpoint gene deletions leading to the growth-coupled production of a desired product. Further, the solution of the bilevel OptKnock problem provides only one set of deletions. To enumerate all meaningful solutions, that is, all sets of knockouts leading to growth-coupled production formation, an optimization technique, termed integer cuts, can be implemented. This entails iteratively solving the OptKnock problem with the incorporation of an additional constraint referred to as an integer cut at each iteration, as discussed above.

As disclosed herein, a nucleic acid encoding a desired activity of a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway can be introduced into a host organism. In some cases, it can be desirable to modify an activity of a 4-HB, 4-HBal, 4-HBCoA BDO or putrescine pathway enzyme or protein to increase production of 4-HB, 4-HBal, 4-HBCoA BDO or putrescine. For example, known mutations that increase the activity of a protein or enzyme can be introduced into an encoding nucleic acid molecule. Additionally, optimization methods can be applied to increase the activity of an enzyme or protein and/or decrease an inhibitory activity, for example, decrease the activity of a negative regulator.

One such optimization method is directed evolution. Directed evolution is a powerful approach that involves the introduction of mutations targeted to a specific gene in order to improve and/or alter the properties of an enzyme. Improved and/or altered enzymes can be identified through the development and implementation of sensitive high-throughput screening assays that allow the automated screening of many enzyme variants (for example, $>10^4$). Iterative rounds of mutagenesis and screening typically are performed to afford an enzyme with optimized properties. Computational algorithms that can help to identify areas of the gene for mutagenesis also have been developed and can significantly reduce the number of enzyme variants that need to be generated and screened. Numerous directed evolution technologies have been developed (for reviews, see Hibbert et al., *Biomol.Eng* 22:11-19 (2005); Huisman and Lalonde, *In* Biocatalysis in the pharmaceutical and biotechnology industries pgs. 717-742 (2007), Patel (ed.), CRC Press; Otten and Quax. *Biomol.Eng* 22:1-9 (2005).; and Sen et al., *Appl Biochem.Biotechnol* 143:212-223 (2007)) to be effective at creating diverse variant libraries, and these methods have been successfully applied to the improvement of a wide range of properties across many enzyme classes. Enzyme characteristics that have been improved and/or altered by directed evolution technologies include, for example: selectivity/specificity, for conversion of non-natural substrates; temperature stability, for robust high temperature processing; pH stability, for bioprocessing under lower or higher pH conditions; substrate or product tolerance, so that high product titers can be achieved; binding (K_m), including broadening substrate binding to include non-natural substrates; inhibition (K_i), to remove inhibition by products, substrates, or key intermediates; activity (kcat), to increases enzymatic reaction rates to achieve desired flux; expression levels, to increase protein yields and overall pathway flux; oxygen stability, for operation of air sensitive enzymes under aerobic conditions; and anaerobic activity, for operation of an aerobic enzyme in the absence of oxygen.

A number of exemplary methods have been developed for the mutagenesis and diversification of genes to target desired properties of specific enzymes. Such methods are well known to those skilled in the art. Any of these can be used to alter and/or optimize the activity of a 4-HB, 4-HBal, 4-HBCoA, BDO or putrescine pathway enzyme or protein. Such methods include, but are not limited to EpPCR, which introduces random point mutations by reducing the fidelity of DNA polymerase in PCR reactions (Pritchard et al., *J Theor.Biol.* 234:497-509 (2005)); Error-prone Rolling Circle Amplification (epRCA), which is similar to epPCR except a whole circular plasmid is used as the template and random 6-mers with exonuclease resistant thiophosphate linkages on the last 2 nucleotides are used to amplify the plasmid followed by transformation into cells in which the plasmid is re-circularized at tandem repeats (Fujii et al., *Nucleic Acids*

Res. 32:e145 (2004); and Fujii et al., *Nat. Protoc.* 1:2493-2497 (2006)); DNA or Family Shuffling, which typically involves digestion of two or more variant genes with nucleases such as Dnase I or EndoV to generate a pool of random fragments that are reassembled by cycles of annealing and extension in the presence of DNA polymerase to create a library of chimeric genes (Stemmer, *Proc Natl Acad Sci USA* 91:10747-10751 (1994); and Stemmer, *Nature* 370:389-391 (1994)); Staggered Extension (StEP), which entails template priming followed by repeated cycles of 2 step PCR with denaturation and very short duration of annealing/extension (as short as 5 sec) (Zhao et al., *Nat. Biotechnol.* 16:258-261 (1998)); Random Priming Recombination (RPR), in which random sequence primers are used to generate many short DNA fragments complementary to different segments of the template (Shao et al., *Nucleic Acids Res* 26:681-683 (1998)).

Additional methods include Heteroduplex Recombination, in which linearized plasmid DNA is used to form heteroduplexes that are repaired by mismatch repair (Volkov et al, *Nucleic Acids Res.* 27:e18 (1999); and Volkov et al., *Methods Enzymol.* 328:456-463 (2000)); Random Chimeragenesis on Transient Templates (RACHITT), which employs Dnase I fragmentation and size fractionation of single stranded DNA (ssDNA) (Coco et al., *Nat. Biotechnol.* 19:354-359 (2001)); Recombined Extension on Truncated templates (RETT), which entails template switching of unidirectionally growing strands from primers in the presence of unidirectional ssDNA fragments used as a pool of templates (Lee et al., *J. Molec. Catalysis* 26:119-129 (2003)); Degenerate Oligonucleotide Gene Shuffling (DOGS), in which degenerate primers are used to control recombination between molecules; (Bergquist and Gibbs, *Methods Mol.Biol* 352:191-204 (2007); Bergquist et al., *Biomol.Eng* 22:63-72 (2005); Gibbs et al., *Gene* 271:13-20 (2001)); Incremental Truncation for the Creation of Hybrid Enzymes (ITCHY), which creates a combinatorial library with 1 base pair deletions of a gene or gene fragment of interest (Ostermeier et al., *Proc. Natl. Acad. Sci. USA* 96:3562-3567 (1999); and Ostermeier et al., *Nat. Biotechnol.* 17:1205-1209 (1999)); Thio-Incremental Truncation for the Creation of Hybrid Enzymes (THIO-ITCHY), which is similar to ITCHY except that phosphothioate dNTPs are used to generate truncations (Lutz et al., *Nucleic Acids Res* 29:E16 (2001)); SCRATCHY, which combines two methods for recombining genes, ITCHY and DNA shuffling (Lutz et al., *Proc. Natl. Acad. Sci. USA* 98:11248-11253 (2001)); Random Drift Mutagenesis (RNDM), in which mutations made via epPCR are followed by screening/selection for those retaining usable activity (Bergquist et al., *Biomol. Eng.* 22:63-72 (2005)); Sequence Saturation Mutagenesis (SeSaM), a random mutagenesis method that generates a pool of random length fragments using random incorporation of a phosphothioate nucleotide and cleavage, which is used as a template

to extend in the presence of "universal" bases such as inosine, and replication of an inosine-containing complement gives random base incorporation and, consequently, mutagenesis (Wong et al., *Biotechnol. J.* 3:74-82 (2008); Wong et al., *Nucleic Acids Res.* 32:e26 (2004); and Wong et al., *Anal. Biochem.* 341:187-189 (2005)); Synthetic Shuffling, which uses overlapping oligonucleotides designed to encode "all genetic diversity in targets" and allows a very high diversity for the shuffled progeny (Ness et al., *Nat. Biotechnol.* 20:1251-1255 (2002)); Nucleotide Exchange and Excision Technology NexT, which exploits a combination of dUTP incorporation followed by treatment with uracil DNA glycosylase and then piperidine to perform endpoint DNA fragmentation (Muller et al., *Nucleic Acids Res.* 33:e117 (2005)).

Further methods include Sequence Homology-Independent Protein Recombination (SHIPREC), in which a linker is used to facilitate fusion between two distantly related or unrelated genes, and a range of chimeras is generated between the two genes, resulting in libraries of single-crossover hybrids (Sieber et al., *Nat. Biotechnol.* 19:456-460 (2001)); Gene Site Saturation Mutagenesis™ (GSSM™), in which the starting materials include a supercoiled double stranded DNA (dsDNA) plasmid containing an insert and two primers which are degenerate at the desired site of mutations (Kretz et al., *Methods Enzymol.* 388:3-11 (2004)); Combinatorial Cassette Mutagenesis (CCM), which involves the use of short oligonucleotide cassettes to replace limited regions with a large number of possible amino acid sequence alterations (Reidhaar-Olson et al. *Methods Enzymol.* 208:564-586 (1991); and Reidhaar-Olson et al. *Science* 241:53-57 (1988)); Combinatorial Multiple Cassette Mutagenesis (CMCM), which is essentially similar to CCM and uses epPCR at high mutation rate to identify hot spots and hot regions and then extension by CMCM to cover a defined region of protein sequence space (Reetz et al., *Angew. Chem. Int. Ed Engl.* 40:3589-3591 (2001)); the Mutator Strains technique, in which conditional *ts* mutator plasmids, utilizing the *mutD5* gene, which encodes a mutant subunit of DNA polymerase III, to allow increases of 20 to 4000-X in random and natural mutation frequency during selection and block accumulation of deleterious mutations when selection is not required (Selifonova et al., *Appl. Environ. Microbiol.* 67:3645-3649 (2001)); Low et al., *J. Mol. Biol.* 260:359-3680 (1996)).

Additional exemplary methods include Look-Through Mutagenesis (LTM), which is a multidimensional mutagenesis method that assesses and optimizes combinatorial mutations of selected amino acids (Rajpal et al., *Proc. Natl. Acad. Sci. USA* 102:8466-8471 (2005)); Gene Reassembly, which is a DNA shuffling method that can be applied to multiple genes at one time or to create a large library of chimeras (multiple mutations) of a single gene (Tunable

GeneReassembly™ (TGR™) Technology supplied by Verenium Corporation), *in Silico* Protein Design Automation (PDA), which is an optimization algorithm that anchors the structurally defined protein backbone possessing a particular fold, and searches sequence space for amino acid substitutions that can stabilize the fold and overall protein energetics, and generally works most effectively on proteins with known three-dimensional structures (Hayes et al., *Proc. Natl. Acad. Sci. USA* 99:15926-15931 (2002)); and Iterative Saturation Mutagenesis (ISM), which involves using knowledge of structure/function to choose a likely site for enzyme improvement, performing saturation mutagenesis at chosen site using a mutagenesis method such as Stratagene QuikChange (Stratagene; San Diego CA), screening/selecting for desired properties, and, using improved clone(s), starting over at another site and continue repeating until a desired activity is achieved (Reetz et al., *Nat. Protoc.* 2:891-903 (2007); and Reetz et al., *Angew. Chem. Int. Ed Engl.* 45:7745-7751 (2006)).

Any of the aforementioned methods for mutagenesis can be used alone or in any combination. Additionally, any one or combination of the directed evolution methods can be used in conjunction with adaptive evolution techniques, as described herein.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also provided within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

EXAMPLE I
Biosynthesis of 4-Hydroxybutanoic Acid

This example describes exemplary biochemical pathways for 4-HB production.

Previous reports of 4-HB synthesis in microbes have focused on this compound as an intermediate in production of the biodegradable plastic poly-hydroxyalkanoate (PHA) (U.S. Patent No. 6,117,658). The use of 4-HB/3-HB copolymers over poly-3-hydroxybutyrate polymer (PHB) can result in plastic that is less brittle (Saito and Doi, *Intl. J. Biol. Macromol.*16:99-104 (1994)). The production of monomeric 4-HB described herein is a fundamentally distinct process for several reasons: (1) the product is secreted, as opposed to PHA which is produced intracellularly and remains in the cell; (2) for organisms that produce hydroxybutanoate polymers, free 4-HB is not produced, but rather the Coenzyme A derivative is used by the polyhydroxyalkanoate synthase; (3) in the case of the polymer, formation of the granular product changes thermodynamics; and (4) extracellular pH is not an issue for

production of the polymer, whereas it will affect whether 4-HB is present in the free acid or conjugate base state, and also the equilibrium between 4-HB and GBL.

4-HB can be produced in two enzymatic reduction steps from succinate, a central metabolite of the TCA cycle, with succinic semialdehyde as the intermediate (Figure 1). The first of these enzymes, succinic semialdehyde dehydrogenase, is native to many organisms including E. coli, in which both NADH- and NADPH-dependent enzymes have been found (Donnelly and Cooper, *Eur. J. Biochem.* 113:555-561 (1981); Donnelly and Cooper, *J. Bacteriol.* 145:1425-1427 (1981); Marek and Henson, *J. Bacteriol.*170:991-994 (1988)). There is also evidence supporting succinic semialdehyde dehydrogenase activity in S. cerevisiae (Ramos et al., *Eur. J. Biochem.* 149:401-404 (1985)), and a putative gene has been identified by sequence homology. However, most reports indicate that this enzyme proceeds in the direction of succinate synthesis, as shown in Figure 1 (Donnelly and Cooper, *supra*; Lutke-Eversloh and Steinbuchel, *FEMS Microbiol. Lett.* 181:63-71 (1999)), participating in the degradation pathway of 4-HB and gamma-aminobutyrate. Succinic semialdehyde also is natively produced by certain microbial organisms such as *E. coli* through the TCA cycle intermediate α-ketogluterate via the action of two enzymes: glutamate:succinic semialdehyde transaminase and glutamate decarboxylase. An alternative pathway, used by the obligate anaerobe *Clostridium kluyveri* to degrade succinate, activates succinate to succinyl-CoA, then converts succinyl-CoA to succinic semialdehyde using an alternative succinic semialdehyde dehydrogenase which is known to function in this direction (Sohling and Gottschalk, *Eur. J. Biochem.* 212:121-127 (1993)). However, this route has the energetic cost of ATP required to convert succinate to succinyl-CoA.

The second enzyme of the pathway, 4-hydroxybutanoate dehydrogenase, is not native to *E. coli* or yeast but is found in various bacteria such as *C. kluyveri* and *Ralstonia eutropha* (Lutke-Eversloh and Steinbuchel, *supra*; Sohling and Gottschalk, *J. Bacteriol.* 178:871-880 (1996); Valentin et al., *Eur. J. Biochem.* 227:43-60 (1995); Wolff and Kenealy, *Protein Expr. Purif.* 6:206-212 (1995)). These enzymes are known to be NADH-dependent, though NADPH-dependent forms also exist. An additional pathway to 4-HB from alpha-ketoglutarate was demonstrated in *E. coli* resulting in the accumulation of poly(4-hydroxybutyric acid) (Song et al., *Wei Sheng Wu Xue.Bao.* 45:382-386 (2005)). The recombinant strain required the overexpression of three heterologous genes, PHA synthase (*R. eutropha*), 4-hydroxybutyrate dehydrogenase (*R. eutropha*) and 4-hydroxybutyrate:CoA transferase (*C. kluyveri*), along with two native *E. coli* genes: glutamate:succinic semialdehyde transaminase and glutamate decarboxylase. Steps 4 and 5 in Figure 1 can alternatively be carried out by an alpha-

ketoglutarate decarboxylase such as the one identified in *Euglena gracilis* (Shigeoka et al., *Biochem. J.* 282(Pt2):319-323 (1992); Shigeoka and Nakano, *Arch. Biochem. Biophys.* 288:22-28 (1991); Shigeoka and Nakano, *Biochem J.* 292(Pt 2):463-467 (1993)). However, this enzyme has not previously been applied to impact the production of 4-HB or related polymers in any organism.

The microbial production capabilities of 4-hydroxybutyrate were explored in two microbes, *Escherichia coli* and *Saccharomyces cerevisiae*, using *in silico* metabolic models of each organism. Potential pathways to 4-HB proceed via a succinate, succinyl-CoA, or alpha-ketoglutarate intermediate as shown in Figure 1.

A first step in the 4-HB production pathway from succinate involves the conversion of succinate to succinic semialdehyde via an NADH- or NADPH-dependant succinic semialdehyde dehydrogenase. In *E. coli*, *gabD* is an NADP-dependant succinic semialdehyde dehydrogenase and is part of a gene cluster involved in 4-aminobutyrate uptake and degradation (Niegemann et al.,. *Arch. Microbiol.* 160:454-460 (1993); Schneider et al., *J. Bacteriol.* 184:6976-6986 (2002)). *sad* is believed to encode the enzyme for NAD-dependant succinic semialdehyde dehydrogenase activity (Marek and Henson, *supra*). *S. cerevisiae* contains only the NADPH-dependant succinic semialdehyde dehydrogenase, putatively assigned to UGA2 , which localizes to the cytosol (Huh et al., *Nature* 425:686-691 (2003)). The maximum yield calculations assuming the succinate pathway to 4-HB in both *E. coli* and *S. cerevisiae* require only the assumption that a non-native 4-HB dehydrogenase has been added to their metabolic networks.

The pathway from succinyl-CoA to 4-hydroxybutyrate was described in U.S. Patent No. 6,117,658 as part of a process for making polyhydroxyalkanoates comprising 4-hydroxybutyrate monomer units. *Clostridium kluyveri* is one example organism known to possess CoA-dependant succinic semialdehyde dehydrogenase activity (Sohling and Gottschalk, *supra*; Sohling and Gottschalk, *supra*). In this study, it is assumed that this enzyme, from *C. kluyveri* or another organism, is expressed in *E. coli* or *S. cerevisiae* along with a non-native or heterologous 4-HB dehydrogenase to complete the pathway from succinyl-CoA to 4-HB. The pathway from alpha-ketoglutarate to 4-HB was demonstrated in *E. coli* resulting in the accumulation of poly(4-hydroxybutyric acid) to 30% of dry cell weight (Song et al., *supra*). As *E. coli* and *S. cerevisiae* natively or endogenously possess both glutamate:succinic semialdehyde transaminase and glutamate decarboxylase (Coleman et al., *J. Biol. Chem.* 276:244-250 (2001)),

the pathway from AKG to 4-HB can be completed in both organisms by assuming only that a non-native 4-HB dehydrogenase is present.

EXAMPLE II
Biosynthesis of 1,4-Butanediol from Succinate and Alpha-ketoglutarate

This example illustrates the construction and biosynthetic production of 4-HB and BDO from microbial organisms. Pathways for 4-HB and BDO are disclosed herein.

There are several alternative enzymes that can be utilized in the pathway described above. The native or endogenous enzyme for conversion of succinate to succinyl-CoA (Step 1 in Figure 1) can be replaced by a CoA transferase such as that encoded by the *cat1* gene *C. kluyveri* (Sohling and Gottschalk, *Eur.J Biochem.* 212:121-127 (1993)), which functions in a similar manner to Step 9. However, the production of acetate by this enzyme may not be optimal, as it might be secreted rather than being converted back to acetyl-CoA. In this respect, it also can be beneficial to eliminate acetate formation in Step 9. As one alternative to this CoA transferase, a mechanism can be employed in which the 4-HB is first phosphorylated by ATP and then converted to the CoA derivative, similar to the acetate kinase/phosphotransacetylase pathway in *E. coli* for the conversion of acetate to acetyl-CoA. The net cost of this route is one ATP, which is the same as is required to regenerate acetyl-CoA from acetate. The enzymes phosphotransbutyrylase (ptb) and butyrate kinase (bk) are known to carry out these steps on the non-hydroxylated molecules for butyrate production in *C. acetobutylicum* (Cary et al., *Appl Environ Microbiol* 56:1576-1583 (1990); Valentine, R. C. and R. S. Wolfe, *J Biol Chem.* 235:1948-1952 (1960)). These enzymes are reversible, allowing synthesis to proceed in the direction of 4-HB.

BDO also can be produced via α-ketoglutarate in addition to or instead of through succinate. A described previously, and exemplified further below, one pathway to accomplish product biosynthesis is with the production of succinic semialdehyde via α-ketoglutarate using the endogenous enzymes (Figure 1, Steps 4-5). An alternative is to use an α-ketoglutarate decarboxylase that can perform this conversion in one step (Figure 1, Step 8; Tian et al., *Proc Natl Acad Sci U S.A* 102:10670-10675 (2005)).

For the construction of different strains of BDO-producing microbial organisms, a list of applicable genes was assembled for corroboration. Briefly, one or more genes within the 4-HB and/or BDO biosynthetic pathways were identified for each step of the complete BDO-producing pathway shown in Figure 1, using available literature resources, the NCBI genetic

database, and homology searches. The genes cloned and assessed in this study are presented below in in Table 6, along with the appropriate references and URL citations to the polypeptide sequence. As discussed further below, some genes were synthesized for codon optimization while others were cloned via PCR from the genomic DNA of the native or wild-type organism. For some genes both approaches were used, and in this case the native genes are indicated by an "n" suffix to the gene identification number when used in an experiment. Note that only the DNA sequences differ; the proteins are identical.

Table 6. Genes expressed in host BDO-producting microbial organisms.

Gene ID number	Reaction number (Fig. 1)	Gene name	Source organism	Enzyme name	Link to protein sequence	Reference
0001	9	Cat2	*Clostridium kluyveri* DSM 555	4-hydroxybutyrate coenzyme A transferase	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=1228100	1
0002	12/13	adhE	*Clostridium acetobutylicum* ATCC 824	Aldehyde/ alcohol dehydrogenase	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=15004739	2
0003	12/13	adhE2	*Clostridium acetobutylicum* ATCC 824	Aldehyde/ alcohol dehydrogenase	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NP_149325.1	2
0004	1	Cat1	*Clostridium kluyveri* DSM 555	Succinate coenzyme A transferase	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=1228100	1
0008	6	sucD	*Clostridium kluyveri* DSM 555	Succinic semialdehyde dehydrogenase (CoA-dependent)	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=1228100	1
0009	7	4-HBd	*Ralstonia eutropha* H16	4-hydroxybutyrate dehydrogenase (NAD-dependent)	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=YP_726053.1	2
0010	7	4-HBd	*Clostridium kluyveri* DSM 555	4-hydroxybutyrate dehydrogenase (NAD-dependent)	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=1228100	1
0011	12/13	adhE	*E. coli*	Aldehyde/ alcohol dehydrogenase	www.shigen.nig.ac.jp/ecoli/pec/genes.List.DetailAction.do?fromListFlag=true&featureType=1&orfId=1219	
0012	12/13	yqhD	*E. coli*	Aldehyde/ alcohol dehydrogenase	www.shigen.nig.ac.jp/ecoli/pec/genes.List.DetailAction.do	
0013	13	bdhB	*Clostridium acetobutylicum* ATCC 824	Butanol dehydrogenase II	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NP_349891.1	2
0020	11	ptb	*Clostridium acetobutylicum* ATCC 824	Phospho-transbutyrylase	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&id=15896327	2
0021	10	buk1	*Clostridium acetobutylicum* ATCC 824	Butyrate kinase I	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&id=20137334	2
0022	10	buk2	*Clostridium acetobutylicum* ATCC 824	Butyrate kinase II	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&id=20137415	2

0023	13	adhEm	isolated from metalibrary of anaerobic sewage digester microbial consortia	Alcohol dehydrogenase		(37)d}
0024	13	adhE	*Clostridium thermocellum*	Alcohol dehydrogenase	www.genome.jp/dbget-bin/www_bget?cth:Cthe_0423	
0025	13	ald	*Clostridium beijerinckii*	Coenzyme A-acylating aldehyde dehydrogenase	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&id=49036681	(31)d}
0026	13	bdhA	*Clostridium acetobutylicum* ATCC 824	Butanol dehydrogenase	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NP_349892.1	2
0027	12	bld	*Clostridium saccharoperbutylacetonicum*	Butyraldehyde dehydrogenase	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&id=31075383	4
0028	13	bdh	*Clostridium saccharoperbutylacetonicum*	Butanol dehydrogenase	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&id=124221917	4
0029	12/13	adhE	*Clostridium tetani*	Aldehyde/ alcohol dehydrogenase	www.genome.jp/dbget-bin/www_bget?ctc:CTC01366	
0030	12/13	adhE	*Clostridium perfringens*	Aldehyde/ alcohol dehydrogenase	www.genome.jp/dbget-bin/www_bget?cpe:CPE2531	
0031	12/13	adhE	*Clostridium difficile*	Aldehyde/ alcohol dehydrogenase	www.genome.jp/dbget-bin/www_bget?cdf:CD2966	
0032	8	sucA	*Mycobacterium bovis* BCG, Pasteur	α-ketoglutarate decarboxylase	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=YP_977400.1	5
0033	9	cat2	*Clostridium aminobutyricum*	4-hydroxybutyrate coenzyme A transferase	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=6249316	
0034	9	cat2	*Porphyromonas gingivalis* W83	4-hydroxybutyrate coenzyme A transferase	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=34541558	
0035	6	sucD	*Porphyromonas gingivalis* W83	Succinic semialdehyde dehydrogenase (CoA-dependent)	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NP_904963.1	
0036	7	4-HBd	*Porphyromonas gingivalis* W83	NAD-dependent 4-hydroxybutyrate dehydrogenase	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NP_904964.1	
0037	7	gbd	Uncultured bacterium	4-hydroxybutyrate dehydrogenase	www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=5916168	6
0038	1	sucCD	*E. coli*	Succinyl-CoA synthetase	www.shigen.nig.ac.jp/ecoli/pec/genes.List.DetailAction.do	

[1]Sohling and Gottschalk, *Eur. J. Biochem.* 212:121-127 (1993); Sohling and Gottschalk, *J. Bacteriol.* 178:871-880 (1996)
[2]Nolling et al., J., *J. Bacteriol.* 183:4823-4838 (2001)
[3]Pohlmann et al., *Nat. Biotechnol.* 24:1257-1262 (2006)
[4]Kosaka et al., *Biosci. Biotechnol. Biochem.* 71:58-68 (2007)
[5]Brosch et al., *Proc. Natl. Acad. Sci. U.S.A.* 104:5596-5601 (2007)
[6]Henne et al., *Appl. Environ. Microbiol.* 65:3901-3907 (1999)

Expression Vector Construction for BDO pathway. Vector backbones and some strains were obtained from Dr. Rolf Lutz of Expressys (www.expressys.de/). The vectors and strains are based on the pZ Expression System developed by Dr. Rolf Lutz and Prof. Hermann Bujard (Lutz, R. and H. Bujard, *Nucleic Acids Res* 25:1203-1210 (1997)). Vectors obtained were pZE13luc, pZA33luc, pZS*13luc and pZE22luc and contained the luciferase gene as a stuffer fragment. To replace the luciferase stuffer fragment with a lacZ-alpha fragment flanked by appropriate restriction enzyme sites, the luciferase stuffer fragment was first removed from each vector by digestion with EcoRI and XbaI. The lacZ-alpha fragment was PCR amplified from pUC19 with the following primers:

lacZalpha-RI

5'GACGAATTCGCTAGCAAGAGGAGAAGTCGACATGTCCAATTCACTGGCCGTCG TTTTAC3'

lacZalpha 3'BB

5'-GACCCTAGGAAGCTTTCTAGAGTCGACCTATGCGGCATCAGAGCAGA-3'.

This generated a fragment with a 5' end of EcoRI site, NheI site, a Ribosomal Binding Site, a SalI site and the start codon. On the 3' end of the fragment contained the stop codon, XbaI, HindIII, and AvrII sites. The PCR product was digested with EcoRI and AvrII and ligated into the base vectors digested with EcoRI and XbaI (XbaI and AvrII have compatible ends and generate a non-site). Because NheI and XbaI restriction enzyme sites generate compatible ends that can be ligated together (but generate a NheI/XbaI non-site that is not digested by either enzyme), the genes cloned into the vectors could be "Biobricked" together (http://openwetware.org/wiki/Synthetic_Biology:BioBricks). Briefly, this method allows joining an unlimited number of genes into the vector using the same 2 restriction sites (as long as the sites do not appear internal to the genes), because the sites between the genes are destroyed after each addition.

All vectors have the pZ designation followed by letters and numbers indication the origin of replication, antibiotic resistance marker and promoter/regulatory unit. The origin of replication is the second letter and is denoted by E for ColE1, A for p15A and S for pSC101 –based origins. The first number represents the antibiotic resistance marker (1 for Ampicillin, 2 for Kanamycin, 3 for Chloramphenicol, 4 for Spectinomycin and 5 for Tetracycline). The final number defines the promoter that regulated the gene of interest (1 for $P_{LtetO-1}$, 2 for $P_{LlacO-1}$, 3 for $P_{A1lacO-1}$, and 4 for $P_{lac/ara-1}$). The MCS and the gene of interest follows immediately after. For the work

discussed here we employed two base vectors, pZA33 and pZE13, modified for the biobricks insertions as discussed above. Once the gene(s) of interest have been cloned into them, resulting plasmids are indicated using the four digit gene codes given in Table 6; e.g., pZA33-XXXX-YYYY-...

Host Strain Construction. The parent strain in all studies described here is *E. coli* K-12 strain MG1655. Markerless deletion strains in adhE, gabD, and aldA were constructed under service contract by a third party using the redET method (Datsenko, K. A. and B. L. Wanner, *Proc Natl Acad Sci U S.A* 97:6640-6645 (2000)). Subsequent strains were constructed via bacteriophage P1 mediated transduction (Miller, J. Experiments in Molecular Genetics, Cold Spring Harbor Laboratories, New York (1973)). Strain C600Z1 (laci^q, PN25-tetR, Sp^R, lacY1, leuB6,mcrB+, supE44, thi-1, thr-1, tonA21) was obtained from Expressys and was used as a source of a lacI^q allele for P1 transduction. Bacteriophage P1vir was grown on the C600Z1 *E. coli* strain, which has the spectinomycin resistance gene linked to the lacI^q. The P1 lysate grown on C600Z1 was used to infect MG1655 with selection for spectinomycin resistance. The spectinomycin resistant colonies were then screened for the linked lacI^q by determining the ability of the transductants to repress expression of a gene linked to a $P_{A1lacO-1}$ promoter. The resulting strain was designated MG1655 lacI^q. A similar procedure was used to introduce lacI^Q into the deletion strains.

Production of 4-HB From Succinate. For construction of a 4-HB producer from succinate, genes encoding steps from succinate to 4-HB and 4-HB-CoA (1, 6, 7, and 9 in Figure 1) were assembled onto the pZA33 and pZE13 vectors as described below. Various combinations of genes were assessed, as well as constructs bearing incomplete pathways as controls (Tables 7 and 8). The plasmids were then transformed into host strains containing lacI^Q, which allow inducible expression by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). Both wild-type and hosts with deletions in genes encoding the native succinic semialdehyde dehydrogenase (step 2 in Figure 1) were tested.

Activity of the heterologous enzymes were first tested in in vitro assays, using strain MG1655 lacI^Q as the host for the plasmid constructs containing the pathway genes. Cells were grown aerobically in LB media (Difco) containing the appropriate antibiotics for each construct, and induced by addition of IPTG at 1 mM when the optical density (OD600) reached approximately 0.5. Cells were harvested after 6 hours, and enzyme assays conducted as discussed below.

***In Vitro* Enzyme Assays.** To obtain crude extracts for activity assays, cells were harvested by centrifugation at 4,500 rpm (Beckman-Coulter, Allegera X-15R) for 10 min. The pellets were

resuspended in 0.3 mL BugBuster (Novagen) reagent with benzonase and lysozyme, and lysis proceeded for 15 minutes at room temperature with gentle shaking. Cell-free lysate was obtained by centrifugation at 14,000 rpm (Eppendorf centrifuge 5402) for 30 min at 4°C. Cell protein in the sample was determined using the method of Bradford et al., *Anal. Biochem.* 72:248-254 (1976), and specific enzyme assays conducted as described below. Activities are reported in Units/mg protein, where a unit of activity is defined as the amount of enzyme required to convert 1 µmol of substrate in 1 min. at room temperature. In general, reported values are averages of at least 3 replicate assays.

Succinyl-CoA transferase (Cat1) activity was determined by monitoring the formation of acetyl-CoA from succinyl-CoA and acetate, following a previously described procedure Sohling and Gottschalk, *J. Bacteriol.* 178:871-880 (1996). Succinyl-CoA synthetase (SucCD) activity was determined by following the formation of succinyl-CoA from succinate and CoA in the presence of ATP. The experiment followed a procedure described by Cha and Parks, *J. Biol. Chem.* 239:1961-1967 (1964). CoA-dependent succinate semialdehyde dehydrogenase (SucD) activity was determined by following the conversion of NAD to NADH at 340 nm in the presence of succinate semialdehyde and CoA (Sohling and Gottschalk, *Eur. J. Biochem.* 212:121-127 (1993)). 4-HB dehydrogenase (4-HBd) enzyme activity was determined by monitoring the oxidation of NADH to NAD at 340 nm in the presence of succinate semialdehyde. The experiment followed a published procedure Gerhardt et al. *Arch. Microbiol.* 174:189-199 (2000). 4-HB CoA transferase (Cat2) activity was determined using a modified procedure from Scherf and Buckel, *Appl. Environ. Microbiol.* 57:2699-2702 (1991). The formation of 4-HB-CoA or butyryl-CoA formation from acetyl-CoA and 4-HB or butyrate was determined using HPLC.

Alcohol (ADH) and aldehyde (ALD) dehydrogenase was assayed in the reductive direction using a procedure adapted from several literature sources (Durre et al., *FEMS Microbiol. Rev.* 17:251-262 (1995); Palosaari and Rogers, *J. Bacteriol.* 170:2971-2976 (1988) and Welch et al., *Arch. Biochem. Biophys.* 273:309-318 (1989). The oxidation of NADH is followed by reading absorbance at 340 nM every four seconds for a total of 240 seconds at room temperature. The reductive assays were performed in 100 mM MOPS (adjusted to pH 7.5 with KOH), 0.4 mM NADH, and from 1 to 50 µl of cell extract. The reaction is started by adding the following reagents: 100 µl of 100 mM acetaldehyde or butyraldehyde for ADH, or 100 µl of 1 mM acetyl-CoA or butyryl-CoA for ALD. The Spectrophotometer is quickly blanked and then the kinetic read is started. The resulting slope of the reduction in absorbance at 340 nM per minute, along

with the molar extinction coefficient of NAD(P)H at 340 nM (6000) and the protein concentration of the extract, can be used to determine the specific activity.

The enzyme activity of PTB is measured in the direction of butyryl-CoA to butyryl-phosphate as described in Cary et al. *J. Bacteriol.* 170:4613-4618 (1988). It provides inorganic phosphate for the conversion, and follows the increase in free CoA with the reagent 5,5'-dithiobis-(2-nitrobenzoic acid), or DTNB. DTNB rapidly reacts with thiol groups such as free CoA to release the yellow-colored 2-nitro-5-mercaptobenzoic acid (TNB), which absorbs at 412 nm with a molar extinction coefficient of 14,140 M cm^{-1}. The assay buffer contained 150 mM potassium phosphate at pH 7.4, 0.1 mM DTNB, and 0.2 mM butyryl-CoA, and the reaction was started by addition of 2 to 50 µL cell extract. The enzyme activity of BK is measured in the direction of butyrate to butyryl-phosphate formation at the expense of ATP. The procedure is similar to the assay for acetate kinase previously described Rose et al., *J. Biol. Chem.* 211:737-756 (1954). However we have found another acetate kinase enzyme assay protocol provided by Sigma to be more useful and sensitive. This assay links conversion of ATP to ADP by acetate kinase to the linked conversion of ADP and phosphoenol pyruvate (PEP) to ATP and pyruvate by pyruvate kinase, followed by the conversion of pyruvate and NADH to lactate and NAD+ by lactate dehydrogenase. Substituting butyrate for acetate is the only major modification to allow the assay to follow BK enzyme activity. The assay mixture contained 80 mM triethanolamine buffer at pH 7.6, 200 mM sodium butyrate, 10 mM MgCl2, 0.1 mM NADH, 6.6 mM ATP, 1.8 mM phosphoenolpyruvate. Pyruvate kinase, lactate dehydrogenase, and myokinase were added according to the manufacturer's instructions. The reaction was started by adding 2 to 50 µL cell extract, and the reaction was monitored based on the decrease in absorbance at 340 nm indicating NADH oxidation.

Analysis of CoA Derivatives by HPLC. An HPLC based assay was developed to monitor enzymatic reactions involving coenzyme A (CoA) transfer. The developed method allowed enzyme activity characterization by quantitative determination of CoA, acetyl CoA (AcCoA), butyryl CoA (BuCoA) and 4-hydroxybutyrate CoA (4-HBCoA) present in in-vitro reaction mixtures. Sensitivity down to low µM was achieved, as well as excellent resolution of all the CoA derivatives of interest.

Chemical and sample preparation was performed as follows. Briefly, CoA, AcCoA, BuCoA and all other chemicals, were obtained from Sigma-Aldrich. The solvents, methanol and acetonitrile, were of HPLC grade. Standard calibration curves exhibited excellent linearity in the 0.01-

1mg/mL concentration range. Enzymatic reaction mixtures contained 100mM Tris HCl buffer (pH 7), aliquots were taken at different time points, quenched with formic acid (0.04% final concentration) and directly analyzed by HPLC.

HPLC analysis was performed using an Agilent 1100 HPLC system equipped with a binary pump, degasser, thermostated autosampler and column compartment, and diode array detector (DAD), was used for the analysis. A reversed phase column, Kromasil 100 5um C18, 4.6x150mm (Peeke Scientific), was employed. 25mM potassium phosphate (pH 7) and methanol or acetonitrile, were used as aqueous and organic solvents at 1mL/min flow rate. Two methods were developed: a short one with a faster gradient for the analysis of well-resolved CoA, AcCoA and BuCoA, and a longer method for distinguishing between closely eluting AcCoA and 4-HBCoA. Short method employed acetonitrile gradient (0min – 5%, 6min – 30%, 6.5min – 5%, 10min – 5%) and resulted in the retention times 2.7, 4.1 and 5.5min for CoA, AcCoA and BuCoA, respectively. In the long method methanol was used with the following linear gradient: 0min – 5%, 20 min – 35%, 20.5min – 5%, 25min – 5%. The retention times for CoA, AcCoA, 4-HBCoA and BuCoA were 5.8, 8.4, 9.2 and 16.0 min, respectively. The injection volume was 5µL, column temperature 30°C, and UV absorbance was monitored at 260nm.

The results demonstrated activity of each of the four pathway steps (Table 7), though activity is clearly dependent on the gene source, position of the gene in the vector, and the context of other genes with which it is expressed. For example, gene 0035 encodes a succinic semialdehyde dehydrogenase that is more active than that encoded by 0008, and 0036 and 0010n are more active 4-HB dehydrogenase genes than 0009. There also seems to be better 4-HB dehydrogenase activity when there is another gene preceding it on the same operon.

Table 7. *In vitro* enzyme activities in cell extracts from MG1655 $lacI^Q$ containing the plasmids expressing genes in the 4-HB-CoA pathway. Activities are reported in Units/mg protein, where a unit of activity is defined as the amount of enzyme required to convert 1 µmol of substrate in 1 min. at room temperature.

Sample #	pZE13 (a)	pZA33 (b)	OD600	Cell Prot (c)	Cat1	SucD	4HBd	Cat2
1	cat1 (0004)		2.71	6.43	1.232	0.00		
2	cat1 (0004)-sucD (0035)		2.03	5.00	0.761	2.57		
3	cat1 (0004)-sucD (0008)		1.04	3.01	0.783	0.01		
4	sucD (0035)		2.31	6.94		2.32		
5	sucD (0008)		1.10	4.16		0.05		
6		4hbd (0009)	2.81	7.94	0.003		0.25	
7		4hbd (0036)	2.63	7.84			3.31	
8		4hbd (0010n)	2.00	5.08			2.57	
9	cat1 (0004)-sucD (0035)	4hbd (0009)	2.07	5.04	0.600	1.85	0.01	
10	cat1 (0004)-sucD (0035)	4hbd (0036)	2.08	5.40	0.694	1.73	0.41	
11	cat1 (0004)-sucD (0035)	4hbd (0010n)	2.44	4.73	0.679	2.28	0.37	
12	cat1 (0004)-sucD (0008)	4hbd (0009)	1.08	3.99	0.572	-0.01	0.02	
13	cat1 (0004)-sucD (0008)	4hbd (0036)	0.77	2.60	0.898	-0.01	0.04	
14	cat1 (0004)-sucD (0008)	4hbd (0010n)	0.63	2.47	0.776	0.00	0.00	
15		cat2 (0034)	2.56	7.86				1.283
16		cat2(0034)-4hbd(0036)	3.13	8.04			24.86	0.993
17		cat2(0034)-4hbd(0010n)	2.38	7.03			7.45	0.675
18		4hbd(0036)-cat2(0034)	2.69	8.26			2.15	7.490
19		4hbd(0010n)-cat2(0034)	2.44	6.59			0.59	4.101

Genes expressed from Plac on pZE13, a high-copy plasmid with colE1 origin and ampicillin resistance. Gene identification numbers are as given in Table 6
Genes expressed from Plac on pZA33, a medium-copy plasmid with pACYC origin and chloramphenicol resistance.
(c) Cell protein given as mg protein per mL extract.

Recombinant strains containing genes in the 4-HB pathway were then evaluated for the ability to produce 4-HB in vivo from central metabolic intermediates. Cells were grown anaerobically in LB medium to OD600 of approximately 0.4, then induced with 1 mM IPTG. One hour later, sodium succinate was added to 10 mM, and samples taken for analysis following an additional 24 and 48 hours. 4-HB in the culture broth was analyzed by GC-MS as described below. The results indicate that the recombinant strain can produce over 2 mM 4-HB after 24 hours, compared to essentially zero in the control strain (Table 8).

Table 8. Production of 4-HB from succinate in *E. coli* strains harboring plasmids expressing various combinations of 4-HB pathway genes.

				24 Hours			48 Hours		
Sample #	Host Strain	pZE13	pZA33	OD600	4HB, μM	4HB norm. (a)	OD600	4HB, μM	4HB norm. (a)
1	MG1655 laclq	cat1 (0004)-sucD (0035)	4hbd (0009)	0.47	487	1036	1.04	1780	1711
2	MG1655 laclq	cat1 (0004)-sucD (0035)	4hbd (0027)	0.41	111	270	0.99	214	217
3	MG1655 laclq	cat1 (0004)-sucD (0035)	4hbd (0036)	0.47	863	1835	0.48	2152	4484
4	MG1655 laclq	cat1 (0004)-sucD (0035)	4hbd (0010n)	0.46	956	2078	0.49	2221	4533
5	MG1655 laclq	cat1 (0004)-sucD (0008)	4hbd (0009)	0.38	493	1296	0.37	1338	3616
6	MG1655 laclq	cat1 (0004)-sucD (0008)	4hbd (0027)	0.32	26	81	0.27	87	323
7	MG1655 laclq	cat1 (0004)-sucD (0008)	4hbd (0036)	0.24	506	2108	0.31	1448	4672
8	MG1655 laclq	cat1 (0004)-sucD (0008)	4hbd (0010n)	0.24	78	324	0.56	233	416
9	MG1655 laclq gabD	cat1 (0004)-sucD (0035)	4hbd (0009)	0.53	656	1237	1.03	1643	1595
10	MG1655 laclq gabD	cat1 (0004)-sucD (0035)	4hbd (0027)	0.44	92	209	0.98	214	218
11	MG1655 laclq gabD	cat1 (0004)-sucD (0035)	4hbd (0036)	0.51	1072	2102	0.97	2358	2431
12	MG1655 laclq gabD	cat1 (0004)-sucD (0035)	4hbd (0010n)	0.51	981	1924	0.97	2121	2186
13	MG1655 laclq gabD	cat1 (0004)-sucD (0008)	4hbd (0009)	0.35	407	1162	0.77	1178	1530
14	MG1655 laclq gabD	cat1 (0004)-sucD (0008)	4hbd (0027)	0.51	19	36	1.07	50	47
15	MG1655 laclq gabD	cat1 (0004)-sucD (0008)	4hbd (0036)	0.35	584	1669	0.78	1350	1731
16	MG1655 laclq gabD	cat1 (0004)-sucD (0008)	4hbd (0010n)	0.32	74	232	0.82	232	283
17	MG1655 laclq	vector only	vector only	0.8	1	2	1.44	3	2
18	MG1655 laclq gabD	vector only	vector only	0.89	1	2	1.41	7	5

(a) Normalized 4-HB concentration, μM/OD600 units

An alternate to using a CoA transferase (cat1) to produce succinyl-CoA from succinate is to use the native *E. coli* sucCD genes, encoding succinyl-CoA synthetase. This gene cluster was cloned onto pZE13 along with candidate genes for the remaining steps to 4-HB to create pZE13-0038-0035-0036.

Production of 4-HB from Glucose. Although the above experiments demonstrate a functional pathway to 4-HB from a central metabolic intermediate (succinate), an industrial process would require the production of chemicals from low-cost carbohydrate feedstocks such as glucose or sucrose. Thus, the next set of experiments was aimed to determine whether endogenous succinate produced by the cells during growth on glucose could fuel the 4-HB pathway. Cells were grown anaerobically in M9 minimal medium (6.78 g/L Na_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 20 g/L glucose, 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS) to improve the buffering capacity, 10 μg/mL thiamine, and the appropriate antibiotics. 0.25 mM IPTG was added when OD600 reached approximately 0.2, and samples taken for 4-HB analysis every 24 hours following induction. In all cases 4-HB plateaued after 24 hours, with a maximum of about 1 mM in the best strains (Figure 3a), while the succinate concentration continued to rise (Figure 3b). This indicates that the supply of succinate to the pathway is likely not limiting, and that the bottleneck may be in the activity of the enzymes themselves or in NADH availability. 0035 and 0036 are clearly the best gene candidates for CoA-dependent succinic semialdehyde dehydrogenase and 4-HB dehydrogenase, respectively. The elimination of one or both of the

genes encoding known (gabD) or putative (aldA) native succinic semialdehyde dehydrogenases had little effect on performance. Finally, it should be noted that the cells grew to a much lower OD in the 4-HB-producing strains than in the controls (Figure 3c).

An alternate pathway for the production of 4-HB from glucose is via a-ketoglutarate. We explored the use of an α-ketoglutarate decarboxylase from *Mycobacterium tuberculosis* Tian et al., *Proc. Natl. Acad. Sci. USA* 102:10670-10675 (2005) to produce succinic semialdehyde directly from α-ketoglutarate (step 8 in Figure 1). To demonstrate that this gene (0032) was functional *in vivo*, we expressed it on pZE13 in the same host as 4-HB dehydrogenase (gene 0036) on pZA33. This strain was capable of producing over 1.0 mM 4-HB within 24 hours following induction with 1 mM IPTG (Figure 4). Since this strain does not express a CoA-dependent succinic semialdehyde dehydrogenase, the possibility of succinic semialdehyde production via succinyl-CoA is eliminated. It is also possible that the native genes responsible for producing succinic semialdehyde could function in this pathway (steps 4 and 5 in Figure 1); however, the amount of 4-HB produced when the pZE13-0032 plasmid was left out of the host is the negligible.

Production of BDO from 4-HB. The production of BDO from 4-HB required two reduction steps, catalyzed by dehydrogenases. Alcohol and aldehyde dehydrogenases (ADH and ALD, respectively) are NAD+/H and/or NADP+/H-dependent enzymes that together can reduce a carboxylic acid group on a molecule to an alcohol group, or in reverse, can perform the oxidation of an alcohol to a carboxylic acid. This biotransformation has been demonstrated in wild-type *Clostridium acetobutylicum* (Jewell et al., *Current Microbiology*, 13:215-19 (1986)), but neither the enzymes responsible nor the genes responsible were identified. In addition, it is not known whether activation to 4-HB-CoA is first required (step 9 in Figure 1), or if the aldehyde dehydrogenase (step 12) can act directly on 4-HB. We developed a list of candidate enzymes from *C. acetobutylicum* and related organisms based on known activity with the non-hydroxylated analogues to 4-HB and pathway intermediates, or by similarity to these characterized genes (Table 6). Since some of the candidates are multifunctional dehydrogenases, they could potentially catalyze both the NAD(P)H-dependent reduction of the acid (or CoA-derivative) to the aldehyde, and of the aldehyde to the alcohol. Before beginning work with these genes in *E. coli*, we first validated the result referenced above using *C. acetobutylicum* ATCC 824. Cells were grown in Schaedler broth (Accumedia, Lansing, MI) supplemented with 10 mM 4-HB, in an anaerobic atmosphere of 10% CO_2, 10% H_2, and 80% N_2 at 30°C. Periodic culture samples were taken, centrifuged, and the broth analyzed for BDO by

GC-MS as described below. BDO concentrations of 0.1 mM, 0.9 mM, and 1.5 mM were detected after 1 day, 2 days, and 7 days incubation, respectively. No BDO was detected in culture grown without 4-HB addition. To demonstrate that the BDO produced was derived from glucose, we grew the best BDO producing strain MG1655 lacI^Q pZE13-0004-0035-0002 pZA33-0034-0036 in M9 minimal medium supplemented with 4 g/L uniformly labeled ^{13}C-glucose. Cells were induced at OD of 0.67 with 1 mM IPTG, and a sample taken after 24 hours. Analysis of the culture supernatant was performed by mass spectrometry.

Gene candidates for the 4-HB to BDO conversion pathway were next tested for activity when expressed in the *E. coli* host MG1655 lacI^Q. Recombinant strains containing each gene candidate expressed on pZA33 were grown in the presence of 0.25 mM IPTG for four hours at 37°C to fully induce expression of the enzyme. Four hours after induction, cells were harvested and assayed for ADH and ALD activity as described above. Since 4-HB-CoA and 4-hydroxybutyraldehyde are not available commercially, assays were performed using the non-hydroxylated substrates (Table 9). The ratio in activity between 4-carbon and 2-carbon substrates for *C. acetobutylicum adhE2* (0002) and *E. coli adhE* (0011) were similar to those previously reported in the literature a Atsumi et al., *Biochim. Biophys. Acta.* 1207:1-11 (1994).

Table 9. *In vitro* enzyme activities in cell extracts from MG1655 lacI^Q containing pZA33 expressing gene candidates for aldehyde and alcohol dehydrogenases. Activities are expressed in μmol min^{-1} mg cell protein^{-1}. N.D., not determined.

		Aldehyde dehydrogenase		Alcohol dehydrogenase	
Gene	Substrate	Butyryl-CoA	Acetyl-CoA	Butyraldehyde	Acetaldehyde
0002		0.0076	0.0046	0.0264	0.0247
0003n		0.0060	0.0072	0.0080	0.0075
0011		0.0069	0.0095	0.0265	0.0093
0013		N.D.	N.D.	0.0130	0.0142
0023		0.0089	0.0137	0.0178	0.0235
0025		0	0.0001	N.D.	N.D.
0026		0	0.0005	0.0024	0.0008

For the BDO production experiments, cat2 from *Porphyromonas gingivalis* W83 (gene 0034) was included on pZA33 for the conversion of 4-HB to 4-HB-CoA, while the candidate dehydrogenase genes were expressed on pZE13. The host strain was MG1655 lacI^Q. *Along* with the alcohol and aldehyde dehydrogenase candidates, we also tested the ability of CoA-dependent succinic semialdehyde dehydrogenases (sucD) to function in this step, due to the similarity of the substrates. Cells were grown to an OD of about 0.5 in LB medium supplemented with 10 mM 4-HB, induced with 1 mM IPTG, and culture broth samples taken after 24 hours and

analyzed for BDO as described below. The best BDO production occurred using adhE2 from *C. acetobutylicum*, sucD from *C. kluyveri*, or sucD from *P. gingivalis* (Figure 5). Interestingly, the absolute amount of BDO produced was higher under aerobic conditions; however, this is primarily due to the lower cell density achieved in anaerobic cultures. When normalized to cell OD, the BDO production per unit biomass is higher in anaerobic conditions (Table 10).

Table 10. Absolute and normalized BDO concentrations from cultures of cells expressing *adhE2* from *C. acetobutylicum*, *sucD* from *C. kluyveri*, or sucD from *P. gingivalis* (data from experiments 2, 9, and 10 in Figure 3), as well as the negative control (experiment 1).

Gene expressed	Conditions	BDO (μM)	OD (600nm)	BDO/OD
none	Aerobic	0	13.4	0
none	Microaerobic	0.5	6.7	0.09
none	Anaerobic	2.2	1.26	1.75
0002	Aerobic	138.3	9.12	15.2
0002	Microaerobic	48.2	5.52	8.73
0002	Anaerobic	54.7	1.35	40.5
0008n	Aerobic	255.8	5.37	47.6
0008n	Microaerobic	127.9	3.05	41.9
0008n	Anaerobic	60.8	0.62	98.1
0035	Aerobic	21.3	14.0	1.52
0035	Microaerobic	13.1	4.14	3.16
0035	Anaerobic	21.3	1.06	20.1

As discussed above, it may be advantageous to use a route for converting 4-HB to 4-HB-CoA that does not generate acetate as a byproduct. To this aim, we tested the use of phosphotransbutyrylase (ptb) and butyrate kinase (bk) from *C. acetobutylicum* to carry out this conversion via steps 10 and 11 in Figure 1. The native ptb/bk operon from *C. acetobutylicum* (genes 0020 and 0021) was cloned and expressed in pZA33. Extracts from cells containing the resulting construct were taken and assayed for the two enzyme activities as described herein. The specific activity of BK was approximately 65 U/mg, while the specific activity of PTB was approximately 5 U/mg. One unit (U) of activity is defined as conversion of 1 μM substrate in 1 minute at room temperature. Finally, the construct was tested for participation in the conversion of 4-HB to BDO. Host strains were transformed with the pZA33-0020-0021 construct described and pZE13-0002, and compared to use of cat2 in BDO production using the aerobic procedure used above in Figure 5. The BK/PTB strain produced 1 mM BDO, compared to 2 mM when

using cat2 (Table 11). Interestingly, the results were dependent on whether the host strain contained a deletion in the native adhE gene.

Table 11. Absolute and normalized BDO concentrations from cultures of cells expressing adhE2 from *C. acetobutylicum* in pZE13 along with either cat2 from *P. gingivalis* (0034) or the PTB/BK genes from *C. acetobutylicum* on pZA33. Host strains were either MG1655 lacI^Q or MG1655 ΔadhE lacI^Q.

Genes	Host Strain	BDO (μM)	OD (600nm)	BDO/OD
0034	MG1655 lacI^Q	0.827	19.9	0.042
0020+0021	MG1655 lacI^Q	0.007	9.8	0.0007
0034	MG1655 ΔadhE lacI^Q	2.084	12.5	0.166
0020+0021	MG1655 ΔadhE lacI^Q	0.975	18.8	0.052

Production of BDO from Glucose. The final step of pathway corroboration is to express both the 4-HB and BDO segments of the pathway in *E. coli* and demonstrate production of BDO in glucose minimal medium. New plasmids were constructed so that all the required genes fit on two plamids. In general, cat1, adhE, and sucD genes were expressed from pZE13, and cat2 and 4-HBd were expressed from pZA33. Various combinations of gene source and gene order were tested in the MG1655 lacI^Q background. Cells were grown anaerobically in M9 minimal medium (6.78 g/L Na_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 20 g/L glucose, 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS) to improve the buffering capacity, 10 μg/mL thiamine, and the appropriate antibiotics. 0.25 mM IPTG was added approximately 15 hours following inoculation, and culture supernatant samples taken for BDO, 4-HB, and succinate analysis 24 and 48 hours following induction. The production of BDO appeared to show a dependency on gene order (Table 12). The highest BDO production, over 0.5 mM, was obtained with cat2 expressed first, followed by 4-HBd on pZA33, and cat1 followed by *P. gingivalis* sucD on pZE13. The addition of *C. acetobutylicum* adhE2 in the last position on pZE13 resulted in slight improvement. 4-HB and succinate were also produced at higher concentrations.

Table 12. Production of BDO, 4-HB, and succinate in recombinant *E. coli* strains expressing combinations of BDO pathway genes, grown in minimal medium supplemented with 20 g/L glucose. Concentrations are given in mM.

				24 Hours				48 Hours			
Sample	pZE13	pZA33	Induction OD	OD600nm	Su	4HB	BDO	OD600nm	Su	4HB	BDO
1	cat1(0004)-sucD(0035)	4hbd (0036)-cat2(0034)	0.92	1.29	5.44	1.37	0.240	1.24	6.42	1.49	0.280
2	cat1(0004)-sucD(0008N)	4hbd (0036)-cat2(0034)	0.36	1.11	6.90	1.24	0.011	1.06	7.63	1.33	0.011
3	adhE(0002)-cat1(0004)-sucD(0035)	4hbd (0036)-cat2(0034)	0.20	0.44	0.34	1.84	0.050	0.60	1.93	2.67	0.119
4	cat1(0004)-sucD(0035)-adhE(0002)	4hbd (0036)-cat2(0034)	1.31	1.90	9.02	0.73	0.073	1.95	9.73	0.82	0.077
5	adhE(0002)-cat1(0004)-sucD(0008N)	4hbd (0036)-cat2(0034)	0.17	0.45	1.04	1.04	0.008	0.94	7.13	1.02	0.017
6	cat1(0004)-sucD(0008N)-adhE(0002)	4hbd (0036)-cat2(0034)	1.30	1.77	10.47	0.25	0.004	1.80	11.49	0.28	0.003
7	cat1(0004)-sucD(0035)	cat2(0034)-4hbd(0036)	1.09	1.29	5.63	2.15	0.461	1.38	6.66	2.30	0.520
8	cat1(0004)-sucD(0008N)	cat2(0034)-4hbd(0036)	1.81	2.01	11.28	0.02	0.000	2.24	11.13	0.02	0.000
9	adhE(0002)-cat1(0004)-sucD(0035)	cat2(0034)-4hbd(0036)	0.24	1.99	2.02	2.32	0.106	0.89	4.85	2.41	0.186
10	cat1(0004)-sucD(0035)-adhE(0002)	cat2(0034)-4hbd(0036)	0.98	1.17	5.30	2.08	0.569	1.33	6.15	2.14	0.640
11	adhE(0002)-cat1(0004)-sucD(0008N)	cat2(0034)-4hbd(0036)	0.20	0.53	1.38	2.30	0.019	0.91	8.10	1.49	0.034
12	cat1(0004)-sucD(0008N)-adhE(0002)	cat2(0034)-4hbd(0036)	2.14	2.73	12.07	0.16	0.000	3.10	11.79	0.17	0.002
13	vector only	vector only	2.11	2.62	9.03	0.01	0.000	3.00	12.05	0.01	0.000

Analysis of BDO, 4-HB and succinate by GCMS. BDO, 4-HB and succinate in fermentation and cell culture samples were derivatized by silylation and quantitatively analyzed by GCMS using methods adapted from literature reports ((Simonov et al., *J. Anal Chem.*59:965-971 (2004)). The developed method demonstrated good sensitivity down to 1μM, linearity up to at least 25mM, as well as excellent selectivity and reproducibility.

Sample preparation was performed as follows: 100μL filtered (0.2μm or 0.45μm syringe filters) samples, e.g. fermentation broth, cell culture or standard solutions, were dried down in a Speed Vac Concentrator (Savant SVC-100H) for approximately 1 hour at ambient temperature, followed by the addition of 20μL 10mM cyclohexanol solution, as an internal standard, in dimethylformamide. The mixtures were vortexed and sonicated in a water bath (Branson 3510) for 15 min to ensure homogeneity. 100 μL silylation derivatization reagent, N,O-bis(trimethylsilyl)triflouro-acetimide (BSTFA) with 1% trimethylchlorosilane, was added, and the mixture was incubated at 70ºC for 30 min. The derivatized samples were centrifuged for 5 min, and the clear solutions were directly injected into GCMS. All the chemicals and reagents were from Sigma-Aldrich, with the exception of BDO which was purchased from J.T.Baker.

GCMS was performed on an Agilent gas chromatograph 6890N, interfaced to a mass-selective detector (MSD) 5973N operated in electron impact ionization (EI) mode has been used for the analysis. A DB-5MS capillary column (J&W Scientific, Agilent Technologies), 30m x 0.25mm i.d. x 0.25 μm film thickness, was used. The GC was operated in a split injection mode introducing 1μL of sample at 20:1 split ratio. The injection port temperature was 250ºC. Helium was used as a carrier gas, and the flow rate was maintained at 1.0 mL/min. A temperature gradient program was optimized to ensure good resolution of the analytes of interest and minimum matrix interference. The oven was initially held at 80ºC for 1min, then ramped to

120ºC at 2ºC/min, followed by fast ramping to 320ºC at 100ºC/min and final hold for 6min at 320ºC. The MS interface transfer line was maintained at 280ºC. The data were acquired using 'lowmass' MS tune settings and 30-400 m/z mass-range scan. The total analysis time was 29 min including 3 min solvent delay. The retention times corresponded to 5.2, 10.5, 14.0 and 18.2 min for BSTFA-derivatized cyclohexanol, BDO, 4-HB and succinate, respectively. For quantitative analysis, the following specific mass fragments were selected (extracted ion chromatograms): m/z 157 for internal standard cyclohexanol, 116 for BDO, and 147 for both 4-HB and succinate. Standard calibration curves were constructed using analyte solutions in the corresponding cell culture or fermentation medium to match sample matrix as close as possible. GCMS data were processed using Environmental Data Analysis ChemStation software (Agilent Technologies).

The results indicated that most of the 4-HB and BDO produced were labeled with ^{13}C (Figure 6, right-hand sides). Mass spectra from a parallel culture grown in unlabeled glucose are shown for comparison (Figure 6, left-hand sides). Note that the peaks seen are for fragments of the derivatized molecule containing different numbers of carbon atoms from the metabolite. The derivatization reagent also contributes some carbon and silicon atoms that naturally-occurring label distribution, so the results are not strictly quantitative.

Production of BDO from 4-HB using alternate pathways. The various alternate pathways were also tested for BDO production. This includes use of the native *E. coli* SucCD enzyme to convert succinate to succinyl-CoA (Table 13, rows 2-3), use of α-ketoglutarate decarboxylase in the α-ketoglutarate pathway (Table 13, row 4), and use of PTB/BK as an alternate means to generate the CoA-derivative of 4HB (Table 13, row 1). Strains were constructed containing plasmids expressing the genes indicated in Table 13, which encompass these variants. The results show that in all cases, production of 4-HB and BDO occurred (Table 13).

Table 13. Production of BDO, 4-HB, and succinate in recombinant *E. coli* strains genes for different BDO pathway variants, grown anaerobically in minimal medium supplemented with 20 g/L glucose, and harvested 24 hours after induction with 0.1 mM IPTG. Concentrations are given in mM.

Genes on pZE13	Genes on pZA33	Succinate	4-HB	BDO
0002+0004+0035	0020n-0021n-0036	0.336	2.91	0.230
0038+0035	0034-0036	0.814	2.81	0.126
0038+0035	0036-0034	0.741	2.57	0.114
0035+0032	0034-0036	5.01	0.538	0.154

EXAMPLE III
Biosynthesis of 4-Hydroxybutanoic Acid, γ-Butyrolactone and 1,4-Butanediol

This Example describes the biosynthetic production of 4-hydroxybutanoic acid, γ-butyrolactone and 1,4-butanediol using fermentation and other bioprocesses.

Methods for the integration of the 4-HB fermentation step into a complete process for the production of purified GBL, 1,4-butanediol (BDO) and tetrahydrofuran (THF) are described below. Since 4-HB and GBL are in equilibrium, the fermentation broth will contain both compounds. At low pH this equilibrium is shifted to favor GBL. Therefore, the fermentation can operate at pH 7.5 or less, generally pH 5.5 or less. After removal of biomass, the product stream enters into a separation step in which GBL is removed and the remaining stream enriched in 4-HB is recycled. Finally, GBL is distilled to remove any impurities. The process operates in one of three ways: 1) fed-batch fermentation and batch separation; 2) fed-batch fermentation and continuous separation; 3) continuous fermentation and continuous separation. The first two of these modes are shown schematically in Figure 7. The integrated fermentation procedures described below also are used for the BDO producing cells of the invention for biosynthesis of BDO and subsequent BDO family products.

Fermentation protocol to produce 4-HB/GBL (batch): The production organism is grown in a 10L bioreactor sparged with an N_2/CO_2 mixture, using 5 L broth containing 5 g/L potassium phosphate, 2.5 g/L ammonium chloride, 0.5 g/L magnesium sulfate, and 30 g/L corn steep liquor, and an initial glucose concentration of 20 g/L. As the cells grow and utilize the glucose, additional 70% glucose is fed into the bioreactor at a rate approximately balancing glucose consumption. The temperature of the bioreactor is maintained at 30 degrees C. Growth continues for approximately 24 hours, until 4-HB reaches a concentration of between 20-200 g/L, with the cell density being between 5 and 10 g/L. The pH is not controlled, and will typically decrease to pH 3-6 by the end of the run. Upon completion of the cultivation period, the fermenter contents are passed through a cell separation unit (e.g., centrifuge) to remove cells and cell debris, and the fermentation broth is transferred to a product separations unit. Isolation of 4-HB and/or GBL would take place by standard separations procedures employed in the art to separate organic products from dilute aqueous solutions, such as liquid-liquid extraction using a water immiscible organic solvent (e.g., toluene) to provide an organic solution of 4-HB/GBL. The resulting solution is then subjected to standard distillation methods to remove and recycle the organic solvent and to provide GBL (boiling point 204-205°C) which is isolated as a purified liquid.

Fermentation protocol to produce 4-HB/GBL (fully continuous): The production organism is first grown up in batch mode using the apparatus and medium composition described above, except that the initial glucose concentration is 30-50 g/L. When glucose is exhausted, feed medium of the same composition is supplied continuously at a rate between 0.5 L/hr and 1 L/hr, and liquid is withdrawn at the same rate. The 4-HB concentration in the bioreactor remains constant at 30-40 g/L, and the cell density remains constant between 3-5 g/L. Temperature is maintained at 30 degrees C, and the pH is maintained at 4.5 using concentrated NaOH and HCl, as required. The bioreactor is operated continuously for one month, with samples taken every day to assure consistency of 4-HB concentration. In continuous mode, fermenter contents are constantly removed as new feed medium is supplied. The exit stream, containing cells, medium, and products 4-HB and/or GBL, is then subjected to a continuous product separations procedure, with or without removing cells and cell debris, and would take place by standard continuous separations methods employed in the art to separate organic products from dilute aqueous solutions, such as continuous liquid-liquid extraction using a water immiscible organic solvent (e.g., toluene) to provide an organic solution of 4-HB/GBL. The resulting solution is subsequently subjected to standard continuous distillation methods to remove and recycle the organic solvent and to provide GBL (boiling point 204-205°C) which is isolated as a purified liquid.

GBL Reduction Protocol: Once GBL is isolated and purified as described above, it will then be subjected to reduction protocols such as those well known in the art (references cited) to produce 1,4-butanediol or tetrahydrofuran (THF) or a mixture thereof. Heterogeneous or homogeneous hydrogenation catalysts combined with GBL under hydrogen pressure are well known to provide the products 1,4-butanediol or tetrahydrofuran (THF) or a mixture thereof. It is important to note that the 4-HB/GBL product mixture that is separated from the fermentation broth, as described above, may be subjected directly, prior to GBL isolation and purification, to these same reduction protocols to provide the products 1,4-butanediol or tetrahydrofuran or a mixture thereof. The resulting products, 1,4-butanediol and THF are then isolated and purified by procedures well known in the art.

Fermentation and hydrogenation protocol to produce BDO or THF directly (batch): Cells are grown in a 10L bioreactor sparged with an N_2/CO_2 mixture, using 5 L broth containing 5 g/L potassium phosphate, 2.5 g/L ammonium chloride, 0.5 g/L magnesium sulfate, and 30 g/L corn steep liquor, and an initial glucose concentration of 20 g/L. As the cells grow and utilize the glucose, additional 70% glucose is fed into the bioreactor at a rate approximately balancing

glucose consumption. The temperature of the bioreactor is maintained at 30 degrees C. Growth continues for approximately 24 hours, until 4-HB reaches a concentration of between 20-200 g/L, with the cell density being between 5 and 10 g/L. The pH is not controlled, and will typically decrease to pH 3-6 by the end of the run. Upon completion of the cultivation period, the fermenter contents are passed through a cell separation unit (e.g., centrifuge) to remove cells and cell debris, and the fermentation broth is transferred to a reduction unit (e.g., hydrogenation vessel), where the mixture 4-HB/GBL is directly reduced to either 1,4-butanediol or THF or a mixture thereof. Following completion of the reduction procedure, the reactor contents are transferred to a product separations unit. Isolation of 1,4-butanediol and/or THF would take place by standard separations procedures employed in the art to separate organic products from dilute aqueous solutions, such as liquid-liquid extraction using a water immiscible organic solvent (e.g., toluene) to provide an organic solution of 1,4-butanediol and/or THF. The resulting solution is then subjected to standard distillation methods to remove and recycle the organic solvent and to provide 1,4-butanediol and/or THF which are isolated as a purified liquids.

Fermentation and hydrogenation protocol to produce BDO or THF directly (fully continuous): The cells are first grown up in batch mode using the apparatus and medium composition described above, except that the initial glucose concentration is 30-50 g/L. When glucose is exhausted, feed medium of the same composition is supplied continuously at a rate between 0.5 L/hr and 1 L/hr, and liquid is withdrawn at the same rate. The 4-HB concentration in the bioreactor remains constant at 30-40 g/L, and the cell density remains constant between 3-5 g/L. Temperature is maintained at 30 degrees C, and the pH is maintained at 4.5 using concentrated NaOH and HCl, as required. The bioreactor is operated continuously for one month, with samples taken every day to assure consistency of 4-HB concentration. In continuous mode, fermenter contents are constantly removed as new feed medium is supplied. The exit stream, containing cells, medium, and products 4-HB and/or GBL, is then passed through a cell separation unit (e.g., centrifuge) to remove cells and cell debris, and the fermentation broth is transferred to a continuous reduction unit (e.g., hydrogenation vessel), where the mixture 4-HB/GBL is directly reduced to either 1,4-butanediol or THF or a mixture thereof. Following completion of the reduction procedure, the reactor contents are transferred to a continuous product separations unit. Isolation of 1,4-butanediol and/or THF would take place by standard continuous separations procedures employed in the art to separate organic products from dilute aqueous solutions, such as liquid-liquid extraction using a water immiscible organic solvent (e.g., toluene) to provide an organic solution of 1,4-butanediol and/or THF. The

resulting solution is then subjected to standard continuous distillation methods to remove and recycle the organic solvent and to provide 1,4-butanediol and/or THF which are isolated as a purified liquids.

Fermentation protocol to produce BDO directly (batch): The production organism is grown in a 10L bioreactor sparged with an N_2/CO_2 mixture, using 5 L broth containing 5 g/L potassium phosphate, 2.5 g/L ammonium chloride, 0.5 g/L magnesium sulfate, and 30 g/L corn steep liquor, and an initial glucose concentration of 20 g/L. As the cells grow and utilize the glucose, additional 70% glucose is fed into the bioreactor at a rate approximately balancing glucose consumption. The temperature of the bioreactor is maintained at 30 degrees C. Growth continues for approximately 24 hours, until BDO reaches a concentration of between 20-200 g/L, with the cell density generally being between 5 and 10 g/L. Upon completion of the cultivation period, the fermenter contents are passed through a cell separation unit (e.g., centrifuge) to remove cells and cell debris, and the fermentation broth is transferred to a product separations unit. Isolation of BDO would take place by standard separations procedures employed in the art to separate organic products from dilute aqueous solutions, such as liquid-liquid extraction using a water immiscible organic solvent (e.g., toluene) to provide an organic solution of BDO. The resulting solution is then subjected to standard distillation methods to remove and recycle the organic solvent and to provide BDO (boiling point 228-229°C) which is isolated as a purified liquid.

Fermentation protocol to produce BDO directly (fully continuous): The production organism is first grown up in batch mode using the apparatus and medium composition described above, except that the initial glucose concentration is 30-50 g/L. When glucose is exhausted, feed medium of the same composition is supplied continuously at a rate between 0.5 L/hr and 1 L/hr, and liquid is withdrawn at the same rate. The BDO concentration in the bioreactor remains constant at 30-40 g/L, and the cell density remains constant between 3-5 g/L. Temperature is maintained at 30 degrees C, and the pH is maintained at 4.5 using concentrated NaOH and HCl, as required. The bioreactor is operated continuously for one month, with samples taken every day to assure consistency of BDO concentration. In continuous mode, fermenter contents are constantly removed as new feed medium is supplied. The exit stream, containing cells, medium, and the product BDO, is then subjected to a continuous product separations procedure, with or without removing cells and cell debris, and would take place by standard continuous separations methods employed in the art to separate organic products from dilute aqueous solutions, such as continuous liquid-liquid extraction using a water immiscible

organic solvent (e.g., toluene) to provide an organic solution of BDO. The resulting solution is subsequently subjected to standard continuous distillation methods to remove and recycle the organic solvent and to provide BDO (boiling point 228-229°C) which is isolated as a purified liquid (mpt 20°C).

EXAMPLE IV
Exemplary BDO Pathways

This example describes exemplary enzymes and corresponding genes for 1,4-butandiol (BDO) synthetic pathways.

Exemplary BDO synthetic pathways are shown in Figures 8-13. The pathways depicted in Figures 8-13 are from common central metabolic intermediates to 1,4-butanediol. All transformations depicted in Figures 8-13 fall into the 18 general categories of transformations shown in Table 14. Below is described a number of biochemically characterized candidate genes in each category. Specifically listed are genes that can be applied to catalyze the appropriate transformations in Figures 9-13 when cloned and expressed in a host organism. The top three exemplary genes for each of the key steps in Figures 9-13 are provided in Tables 15-23 (see below). Exemplary genes were provided for the pathways depicted in Figure 8 are described herein.

Table 14. Enzyme types required to convert common central metabolic intermediates into 1,4-butanediol. The first three digits of each label correspond to the first three Enzyme Commission number digits which denote the general type of transformation independent of substrate specificity.

Label	Function
1.1.1.a	Oxidoreductase (ketone to hydroxyl or aldehyde to alcohol)
1.1.1.c	Oxidoreductase (2 step, acyl-CoA to alcohol)
1.2.1.b	Oxidoreductase (acyl-CoA to aldehyde)
1.2.1.c	Oxidoreductase (2-oxo acid to acyl-CoA, decarboxylation)
1.2.1.d	Oxidoreductase (phosphorylating/dephosphorylating)
1.3.1.a	Oxidoreductase operating on CH-CH donors
1.4.1.a	Oxidoreductase operating on amino acids
2.3.1.a	Acyltransferase (transferring phosphate group)
2.6.1.a	Aminotransferase
2.7.2.a	Phosphotransferase, carboxyl group acceptor
2.8.3.a	Coenzyme-A transferase
3.1.2.a	Thiolester hydrolase (CoA specific)
4.1.1.a	Carboxy-lyase

4.2.1.a	Hydro-lyase
4.3.1.a	Ammonia-lyase
5.3.3.a	Isomerase
5.4.3.a	Aminomutase
6.2.1.a	Acid-thiol ligase

1.1.1.a - Oxidoreductase (aldehyde to alcohol or ketone to hydroxyl)

Aldehyde to alcohol. Exemplary genes encoding enzymes that catalyze the conversion of an aldehyde to alcohol, that is, alcohol dehydrogenase or equivalently aldehyde reductase, include *alrA* encoding a medium-chain alcohol dehydrogenase for C2-C14 (Tani et al. *Appl.Environ.Microbiol.* 66:5231-5235 (2000)), ADH2 from *Saccharomyces cerevisiae* (Atsumi et al. *Nature* 451:86-89 (2008)), *yqhD* from *E. coli* which has preference for molecules longer than C(3) (Sulzenbacher et al. *Journal of Molecular Biology* 342:489-502 (2004)), and *bdh* I and *bdh* II from *C. acetobutylicum* which converts butyryaldehyde into butanol (Walter et al. *Journal of Bacteriology* 174:7149-7158 (1992)). The protein sequences for each of these exemplary gene products, if available, can be found using the following GenBank accession numbers:

Gene	Accession No.	GI No.	Organism
alrA	BAB12273.1	9967138	*Acinetobacter* sp. Strain M-1
ADH2	NP_014032.1	6323961	*Saccharymyces cerevisiae*
yqhD	NP_417484.1	16130909	*Escherichia coli*
bdh I	NP_349892.1	15896543	*Clostridium acetobutylicum*
bdh II	NP_349891.1	15896542	*Clostridium acetobutylicum*

Enzymes exhibiting 4-hydroxybutyrate dehydrogenase activity (EC 1.1.1.61) also fall into this category. Such enzymes have been characterized in *Ralstonia eutropha* (Bravo et al. *J.Forensic Sci.* 49:379-387 (2004), *Clostridium kluyveri* (Wolff et al. *Protein Expr.Purif.* 6:206-212 (1995)) and *Arabidopsis thaliana* (Breitkreuz et al. *J.Biol.Chem.* 278:41552-41556 (2003)).

Gene	Accession No.	GI No.	Organism
4hbd	YP_726053.1	113867564	*Ralstonia eutropha* H16
4hbd	L21902.1	146348486	*Clostridium kluyveri* DSM 555
4hbd	Q94B07	75249805	*Arabidopsis thaliana*

Another exemplary enzyme is 3-hydroxyisobutyrate dehydrogenase which catalyzes the reversible oxidation of 3-hydroxyisobutyrate to methylmalonate semialdehyde. This enzyme participates in valine, leucine and isoleucine degradation and has been identified in bacteria,

eukaryotes, and mammals. The enzyme encoded by *P84067* from *Thermus thermophilus* HB8 has been structurally characterized (Lokanath et al. *J Mol Biol* 352:905-17 (2005)). The reversibility of the human 3-hydroxyisobutyrate dehydrogenase was demonstrated using isotopically-labeled substrate (Manning et al. *Biochem J* 231:481-484 (1985)). Additional genes encoding this enzyme include *3hidh* in *Homo sapiens* (Hawes et al. *Methods Enzymol.* 324:218-228 (2000)) and *Oryctolagus cuniculus* (Chowdhury et al. *Biosci.Biotechnol Biochem.* 60:2043-2047 (1996); Hawes et al. *Methods Enzymol.* 324:218-228 (2000)), *mmsb* in *Pseudomonas aeruginosa*, and *dhat* in *Pseudomonas putida* (Aberhart et al. *J Chem.Soc. [Perkin 1]* 6:1404-1406 (1979); Chowdhury et al. *Biosci.Biotechnol Biochem.* 67:438-441 (2003); Chowdhury et al. *Biosci.Biotechnol Biochem.* 60:2043-2047 (1996)).

Gene	Accession No.	GI No.	Organism
P84067	P84067	75345323	*Thermus thermophilus*
mmsb	P28811.1	127211	*Pseudomonas aeruginosa*
dhat	Q59477.1	2842618	*Pseudomonas putida*
3hidh	P31937.2	12643395	*Homo sapiens*
3hidh	P32185.1	416872	*Oryctolagus cuniculus*

Several 3-hydroxyisobutyrate dehydrogenase enzymes have also been shown to convert malonic semialdehyde to 3-hydroxyproprionic acid (3-HP). Three gene candidates exhibiting this activity are *mmsB* from *Pseudomonas aeruginosa* PAO1(62), *mmsB* from *Pseudomonas putida* KT2440 (Liao et al., US Publication 2005/0221466) and *mmsB* from *Pseudomonas putida E23* (Chowdhury et al., *Biosci.Biotechnol.Biochem.* 60:2043-2047 (1996)). An enzyme with 3-hydroxybutyrate dehydrogenase activity in *Alcaligenes faecalis* M3A has also been identified (Gokam et al., US Patent No. 7,393,676; Liao et al., US Publication No. 2005/0221466). Additional gene candidates from other organisms including *Rhodobacter spaeroides* can be inferred by sequence similarity.

Gene	Accession No.	GI No.	Organism
mmsB	AAA25892.1	151363	*Pseudomonas aeruginosa*
mmsB	NP_252259.1	15598765	*Pseudomonas aeruginosa* PAO1
mmsB	NP_746775.1	26991350	*Pseudomonas putida* KT2440
mmsB	JC7926	60729613	*Pseudomonas putida* E23
orfB1	AAL26884	16588720	*Rhodobacter spaeroides*

The conversion of malonic semialdehyde to 3-HP can also be accomplished by two other enzymes: NADH-dependent 3-hydroxypropionate dehydrogenase and NADPH-dependent

malonate semialdehyde reductase. An NADH-dependent 3-hydroxypropionate dehydrogenase is thought to participate in beta-alanine biosynthesis pathways from propionate in bacteria and plants (Rathinasabapathi, B. *Journal of Plant Pathology* 159:671-674 (2002); Stadtman, E. R. *J.Am.Chem.Soc.* 77:5765-5766 (1955)). This enzyme has not been associated with a gene in any organism to date. NADPH-dependent malonate semialdehyde reductase catalyzes the reverse reaction in autotrophic CO_2-fixing bacteria. Although the enzyme activity has been detected *in Metallosphaera sedula*, the identity of the gene is not known (Alber et al. *J.Bacteriol.* 188:8551-8559 (2006)).

Ketone to hydroxyl. There exist several exemplary alcohol dehydrogenases that convert a ketone to a hydroxyl functional group. Two such enzymes from *E. coli* are encoded by malate dehydrogenase (*mdh*) and lactate dehydrogenase (*ldhA*). In addition, lactate dehydrogenase from *Ralstonia eutropha* has been shown to demonstrate high activities on substrates of various chain lengths such as lactate, 2-oxobutyrate, 2-oxopentanoate and 2-oxoglutarate (Steinbuchel and. Schlegel, *Eur.J.Biochem.* 130:329-334 (1983)). Conversion of alpha-ketoadipate into alpha-hydroxyadipate can be catalyzed by 2-ketoadipate reductase, an enzyme reported to be found in rat and in human placenta (Suda et al. Arch.Biochem.Biophys. 176:610-620 (1976); Suda et al. *Biochem.Biophys.Res.Commun.* 77:586-591 (1977)). An additional candidate for this step is the mitochondrial 3-hydroxybutyrate dehydrogenase (*bdh*) from the human heart which has been cloned and characterized (Marks et al. *J.Biol.Chem.* 267:15459-15463 (1992)). This enzyme is a dehydrogenase that operates on a 3-hydroxyacid. Another exemplary alcohol dehydrogenase converts acetone to isopropanol as was shown in *C. beijerinckii* (Ismaiel et al. *J.Bacteriol.* 175:5097-5105 (1993)) and *T. brockii* (Lamed et al. *Biochem. J.* 195:183-190 (1981); Peretz and Burstein *Biochemistry* 28:6549-6555 (1989)).

Gene	Accession No.	GI No.	Organism
mdh	AAC76268.1	1789632	*Escherichia coli*
ldhA	NP_415898.1	16129341	*Escherichia coli*
ldh	YP_725182.1	113866693	*Ralstonia eutropha*
bdh	AAA58352.1	177198	*Homo sapiens*
adh	AAA23199.2	60592974	*Clostridium beijerinckii* NRRL B593
adh	P14941.1	113443	*Thermoanaerobacter brockii* HTD4

Exemplary 3-hydroxyacyl dehydrogenases which convert acetoacetyl-CoA to 3-hydroxybutyryl-CoA include *hbd* from *C. acetobutylicum* (Boynton et al. *Journal of Bacteriology* 178:3015-3024 (1996)), *hbd* from *C. beijerinckii* (Colby et al. *Appl Environ.Microbiol* 58:3297-3302

(1992)), and a number of similar enzymes from *Metallosphaera sedula* (Berg et al. Archaea. *Science* 318:1782-1786 (2007)).

Gene	Accession No.	GI No.	Organism
hbd	NP_349314.1	15895965	*Clostridium acetobutylicum*
hbd	AAM14586.1	20162442	*Clostridium beijerinckii*
Msed_1423	YP_001191505	146304189	*Metallosphaera sedula*
Msed_0399	YP_001190500	146303184	*Metallosphaera sedula*
Msed_0389	YP_001190490	146303174	*Metallosphaera sedula*
Msed_1993	YP_001192057	146304741	*Metallosphaera sedula*

1.1.1.c - Oxidoredutase (2 step, acyl-CoA to alcohol)

Exemplary 2-step oxidoreductases that convert an acyl-CoA to alcohol include those that transform substrates such as acetyl-CoA to ethanol (for example, *adhE* from *E. coli* (Kessler et al. *FEBS.Lett.* 281:59-63 (1991)) and butyryl-CoA to butanol (for example, *adhE2* from *C. acetobutylicum* (Fontaine et al. *J.Bacteriol.* 184:821-830 (2002)). In addition to reducing acetyl-CoA to ethanol, the enzyme encoded by *adhE* in *Leuconostoc mesenteroides* has been shown to oxide the branched chain compound isobutyraldehyde to isobutyryl-CoA (Kazahaya et al. *J.Gen.Appl.Microbiol.* 18:43-55 (1972); Koo et al. Biotechnol Lett. 27:505-510 (2005)).

Gene	Accession No.	GI No.	Organism
adhE	NP_415757.1	16129202	*Escherichia coli*
adhE2	AAK09379.1	12958626	*Clostridium acetobutylicum*
adhE	AAV66076.1	55818563	*Leuconostoc mesenteroides*

Another exemplary enzyme can convert malonyl-CoA to 3-HP. An NADPH-dependent enzyme with this activity has characterized in *Chloroflexus aurantiacus* where it participates in the 3-hydroxypropionate cycle (Hugler et al., *J.Bacteriol.* 184:2404-2410 (2002); Strauss and Fuchs, *Eur.J.Biochem.* 215:633-643 (1993)). This enzyme, with a mass of 300 kDa, is highly substrate-specific and shows little sequence similarity to other known oxidoreductases (Hugler et al., *J.Bacteriol.* 184:2404-2410 (2002)). No enzymes in other organisms have been shown to catalyze this specific reaction; however there is bioinformatic evidence that other organisms may have similar pathways (Klatt et al., *Environ.Microbiol.* 9:2067-2078 (2007)). Enzyme candidates in other organisms including *Roseiflexus castenholzii*, *Erythrobacter sp. NAP1* and marine gamma proteobacterium HTCC2080 can be inferred by sequence similarity.

Gene	Accession No.	GI No.	Organism
mcr	AAS20429.1	42561982	*Chloroflexus aurantiacus*
Rcas_2929	YP_001433009.1	156742880	*Roseiflexus castenholzii*
NAP1_02720	ZP_01039179.1	85708113	*Erythrobacter sp. NAP1*
MGP2080_00535	ZP_01626393.1	119504313	marine gamma proteobacterium HTCC2080

Longer chain acyl-CoA molecules can be reduced by enzymes such as the jojoba (*Simmondsia chinensis*) *FAR* which encodes an alcohol-forming fatty acyl-CoA reductase. Its overexpression in *E. coli* resulted in FAR activity and the accumulation of fatty alcohol (Metz et al. *Plant Physiology* 122:635-644) 2000)).

Gene	Accession No.	GI No.	Organism
FAR	AAD38039.1	5020215	*Simmondsia chinensis*

1.2.1.b - Oxidoreductase (acyl-CoA to aldehyde)

Several acyl-CoA dehydrogenases are capable of reducing an acyl-CoA to its corresponding aldehyde. Exemplary genes that encode such enzymes include the *Acinetobacter calcoaceticus acr1* encoding a fatty acyl-CoA reductase (Reiser and Somerville, *J. Bacteriology* 179:2969-2975 (1997)), the *Acinetobacter sp. M-1* fatty acyl-CoA reductase (Ishige et al. *Appl.Environ.Microbiol.* 68:1192-1195 (2002)), and a CoA- and NADP- dependent succinate semialdehyde dehydrogenase encoded by the *sucD* gene in *Clostridium kluyveri* (Sohling and Gottschalk *J Bacteriol* 178:871-80 (1996); Sohling and Gottschalk *J Bacteriol.* 178:871-880 (1996)). *SucD* of *P. gingivalis* is another succinate semialdehyde dehydrogenase (Takahashi et al. *J.Bacteriol.* 182:4704-4710 (2000)). The enzyme acylating acetaldehyde dehydrogenase in *Pseudomonas sp*, encoded by *bphG*, is yet another as it has been demonstrated to oxidize and acylate acetaldehyde, propionaldehyde, butyraldehyde, isobutyraldehyde and formaldehyde (Powlowski et al. *J Bacteriol.* 175:377-385 (1993)).

Gene	Accession No.	GI No.	Organism
acr1	YP_047869.1	50086359	*Acinetobacter calcoaceticus*
acr1	AAC45217	1684886	*Acinetobacter baylyi*
acr1	BAB85476.1	18857901	*Acinetobacter* sp. Strain M-1
sucD	P38947.1	730847	*Clostridium kluyveri*
sucD	NP_904963.1	34540484	*Porphyromonas gingivalis*
bphG	BAA03892.1	425213	*Pseudomonas sp*

An additional enzyme type that converts an acyl-CoA to its corresponding aldehyde is malonyl-CoA reductase which transforms malonyl-CoA to malonic semialdehyde. Malonyl-CoA reductase is a key enzyme in autotrophic carbon fixation via the 3-hydroxypropionate cycle in thermoacidophilic archael bacteria (Berg et al. *Science* 318:1782-1786 (2007); Thauer, R. K. *Science* 318:1732-1733 (2007)). The enzyme utilizes NADPH as a cofactor and has been characterized in *Metallosphaera* and *Sulfolobus spp* (Alber et al. *J.Bacteriol.* 188:8551-8559 (2006); Hugler et al. *J.Bacteriol.* 184:2404-2410 (2002)). The enzyme is encoded by *Msed_0709* in *Metallosphaera sedula* (Alber et al. *J.Bacteriol.* 188:8551-8559 (2006); Berg et al. *Science* 318:1782-1786 (2007)). A gene encoding a malonyl-CoA reductase from *Sulfolobus tokodaii* was cloned and heterologously expressed in *E. coli* (Alber et al. *J.Bacteriol.* 188:8551-8559 (2006)). Although the aldehyde dehydrogenase functionality of these enzymes is similar to the bifunctional dehydrogenase from *Chloroflexus aurantiacus*, there is little sequence similarity. Both malonyl-CoA reductase enzyme candidates have high sequence similarity to aspartate-semialdehyde dehydrogenase, an enzyme catalyzing the reduction and concurrent dephosphorylation of aspartyl-4-phosphate to aspartate semialdehyde. Additional gene candidates can be found by sequence homology to proteins in other organisms including *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius*.

Gene	Accession No.	GI No.	Organism
Msed_0709	YP_001190808.1	146303492	*Metallosphaera sedula*
mcr	NP_378167.1	15922498	*Sulfolobus tokodaii*
asd-2	NP_343563.1	15898958	*Sulfolobus solfataricus*
Saci_2370	YP_256941.1	70608071	*Sulfolobus acidocaldarius*

1.2.1.c - Oxidoreductase (2-oxo acid to acyl-CoA, decarboxylation)

Enzymes in this family include 1) branched-chain 2-keto-acid dehydrogenase, 2) alpha-ketoglutarate dehydrogenase, and 3) the pyruvate dehydrogenase multienzyme complex (PDHC). These enzymes are multi-enzyme complexes that catalyze a series of partial reactions which result in acylating oxidative decarboxylation of 2-keto-acids. Each of the 2-keto-acid dehydrogenase complexes occupies key positions in intermediary metabolism, and enzyme activity is typically tightly regulated (Fries et al. *Biochemistry* 42:6996-7002 (2003)). The enzymes share a complex but common structure composed of multiple copies of three catalytic components: alpha-ketoacid decarboxylase (E1), dihydrolipoamide acyltransferase (E2) and dihydrolipoamide dehydrogenase (E3). The E3 component is shared among all 2-keto-acid dehydrogenase complexes in an organism, while the E1 and E2 components are encoded by

different genes. The enzyme components are present in numerous copies in the complex and utilize multiple cofactors to catalyze a directed sequence of reactions via substrate channeling. The overall size of these dehydrogenase complexes is very large, with molecular masses between 4 and 10 million Da (that is, larger than a ribosome).

Activity of enzymes in the 2-keto-acid dehydrogenase family is normally low or limited under anaerobic conditions in *E. coli*. Increased production of NADH (or NADPH) could lead to a redox-imbalance, and NADH itself serves as an inhibitor to enzyme function. Engineering efforts have increased the anaerobic activity of the *E. coli* pyruvate dehydrogenase complex (Kim et al. *Appl.Environ.Microbiol.* 73:1766-1771 (2007); Kim et al. *J.Bacteriol.* 190:3851-3858) 2008); Zhou et al. *Biotechnol.Lett.* 30:335-342 (2008)). For example, the inhibitory effect of NADH can be overcome by engineering an H322Y mutation in the E3 component (Kim et al. *J.Bacteriol.* 190:3851-3858 (2008)). Structural studies of individual components and how they work together in complex provide insight into the catalytic mechanisms and architecture of enzymes in this family (Aevarsson et al. *Nat.Struct.Biol.* 6:785-792 (1999); Zhou et al. *Proc.Natl.Acad.Sci.U.S.A.* 98:14802-14807 (2001)). The substrate specificity of the dehydrogenase complexes varies in different organisms, but generally branched-chain keto-acid dehydrogenases have the broadest substrate range.

Alpha-ketoglutarate dehydrogenase (AKGD) converts alpha-ketoglutarate to succinyl-CoA and is the primary site of control of metabolic flux through the TCA cycle (Hansford, R. G. *Curr.Top.Bioenerg.* 10:217-278 (1980)). Encoded by genes *sucA*, *sucB* and *lpd* in *E. coli*, AKGD gene expression is downregulated under anaerobic conditions and during growth on glucose (Park et al. *Mol.Microbiol.* 15:473-482 (1995)). Although the substrate range of AKGD is narrow, structural studies of the catalytic core of the E2 component pinpoint specific residues responsible for substrate specificity (Knapp et al. *J.Mol.Biol.* 280:655-668 (1998)). The *Bacillus subtilis* AKGD, encoded by *odhAB* (E1 and E2) and *pdhD* (E3, shared domain), is regulated at the transcriptional level and is dependent on the carbon source and growth phase of the organism (Resnekov et al. *Mol.Gen.Genet.* 234:285-296 (1992)). In yeast, the *LPD1* gene encoding the E3 component is regulated at the transcriptional level by glucose (Roy and Dawes *J.Gen.Microbiol.* 133:925-933 (1987)). The E1 component, encoded by *KGD1*, is also regulated by glucose and activated by the products of HAP2 and HAP3 (Repetto and Tzagoloff *Mol.Cell Biol.* 9:2695-2705 (1989)). The AKGD enzyme complex, inhibited by products NADH and succinyl-CoA, is well-studied in mammalian systems, as impaired function of has been linked to

several neurological diseases (Tretter and dam-Vizi *Philos.Trans.R.Soc.Lond B Biol.Sci.* 360:2335-2345 (2005)).

Gene	Accession No.	GI No.	Organism
sucA	NP_415254.1	16128701	*Escherichia coli str. K12 substr. MG1655*
sucB	NP_415255.1	16128702	*Escherichia coli str. K12 substr. MG1655*
lpd	NP_414658.1	16128109	*Escherichia coli str. K12 substr. MG1655*
odhA	P23129.2	51704265	*Bacillus subtilis*
odhB	P16263.1	129041	*Bacillus subtilis*
pdhD	P21880.1	118672	*Bacillus subtilis*
KGD1	NP_012141.1	6322066	*Saccharomyces cerevisiae*
KGD2	NP_010432.1	6320352	*Saccharomyces cerevisiae*
LPD1	NP_116635.1	14318501	*Saccharomyces cerevisiae*

Branched-chain 2-keto-acid dehydrogenase complex (BCKAD), also known as 2-oxoisovalerate dehydrogenase, participates in branched-chain amino acid degradation pathways, converting 2-keto acids derivatives of valine, leucine and isoleucine to their acyl-CoA derivatives and CO_2. The complex has been studied in many organisms including *Bacillus subtilis* (Wang et al. *Eur.J.Biochem.* 213:1091-1099 (1993)), *Rattus norvegicus* (Namba et al. *J.Biol.Chem.* 244:4437-4447 (1969)) and *Pseudomonas putida* (Sokatch *J.Bacteriol.* 148:647-652 (1981)). In *Bacillus subtilis* the enzyme is encoded by genes *pdhD* (E3 component), *bfmBB* (E2 component), *bfmBAA* and *bfmBAB* (E1 component) (Wang et al. *Eur.J.Biochem.* 213:1091-1099 (1993)). In mammals, the complex is regulated by phosphorylation by specific phosphatases and protein kinases. The complex has been studied in rat hepatocites (Chicco et al. *J.Biol.Chem.* 269:19427-19434 (1994)) and is encoded by genes *Bckdha* (E1 alpha), *Bckdhb* (E1 beta), *Dbt* (E2), and *Dld* (E3). The E1 and E3 components of the *Pseudomonas putida* BCKAD complex have been crystallized (Aevarsson et al. *Nat.Struct.Biol.* 6:785-792 (1999); Mattevi *Science* 255:1544-1550 (1992)) and the enzyme complex has been studied (Sokatch et al. *J.Bacteriol.* 148:647-652 (1981)). Transcription of the *P. putida* BCKAD genes is activated by the gene product of *bkdR* (Hester et al. *Eur.J.Biochem.* 233:828-836 (1995)). In some organisms including *Rattus norvegicus* (Paxton et al. *Biochem.J.* 234:295-303 (1986)) and *Saccharomyces cerevisiae* (Sinclair et al. *Biochem.Mol.Biol.Int.* 31:911-922 (1993)), this complex has been shown to have a broad substrate range that includes linear oxo-acids such as 2-oxobutanoate and alpha-ketoglutarate, in addition to the branched-chain amino acid precursors. The active site of the bovine BCKAD was engineered to favor alternate substrate acetyl-CoA (Meng and Chuang, *Biochemistry* 33:12879-12885 (1994)).

Gene	Accession No.	GI No.	Organism
bfmBB	NP_390283.1	16079459	*Bacillus subtilis*
bfmBAA	NP_390285.1	16079461	*Bacillus subtilis*
bfmBAB	NP_390284.1	16079460	*Bacillus subtilis*
pdhD	P21880.1	118672	*Bacillus subtilis*
lpdV	P09063.1	118677	*Pseudomonas putida*
bkdB	P09062.1	129044	*Pseudomonas putida*
bkdA1	NP_746515.1	26991090	*Pseudomonas putida*
bkdA2	NP_746516.1	26991091	*Pseudomonas putida*
Bckdha	NP_036914.1	77736548	*Rattus norvegicus*
Bckdhb	NP_062140.1	158749538	*Rattus norvegicus*
Dbt	NP_445764.1	158749632	*Rattus norvegicus*
Dld	NP_955417.1	40786469	*Rattus norvegicus*

The pyruvate dehydrogenase complex, catalyzing the conversion of pyruvate to acetyl-CoA, has also been extensively studied. In the *E. coli* enzyme, specific residues in the E1 component are responsible for substrate specificity (Bisswanger, H. *J Biol Chem.* 256:815-822 (1981); Bremer, J. *Eur.J Biochem.* 8:535-540 (1969); Gong et al. *J Biol Chem.* 275:13645-13653 (2000)). As mentioned previously, enzyme engineering efforts have improved the *E. coli* PDH enzyme activity under anaerobic conditions (Kim et al. *Appl.Environ.Microbiol.* 73:1766-1771 (2007); Kim *J.Bacteriol.* 190:3851-3858 (2008); Zhou et al. *Biotechnol.Lett.* 30:335-342 (2008)). In contrast to the *E. coli* PDH, the *B. subtilis* complex is active and required for growth under anaerobic conditions (Nakano *J.Bacteriol.* 179:6749-6755 (1997)). The *Klebsiella pneumoniae* PDH, characterized during growth on glycerol, is also active under anaerobic conditions (Menzel et al. *J.Biotechnol.* 56:135-142 (1997)). Crystal structures of the enzyme complex from bovine kidney (Zhou et al. *Proc.Natl.Acad.Sci.U.S.A.* 98:14802-14807 (2001)) and the E2 catalytic domain from *Azotobacter vinelandii* are available (Mattevi et al. *Science* 255:1544-1550 (1992)). Some mammalian PDH enzymes complexes can react on alternate substrates such as 2-oxobutanoate, although comparative kinetics of *Rattus norvegicus* PDH and BCKAD indicate that BCKAD has higher activity on 2-oxobutanoate as a substrate (Paxton et al. *Biochem.J.* 234:295-303 (1986)).

Gene	Accession No.	GI No.	Organism
aceE	NP_414656.1	16128107	*Escherichia coli str. K12 substr. MG1655*
aceF	NP_414657.1	16128108	*Escherichia coli str. K12 substr. MG1655*
lpd	NP_414658.1	16128109	*Escherichia coli str. K12 substr. MG1655*
pdhA	P21881.1	3123238	*Bacillus subtilis*
pdhB	P21882.1	129068	*Bacillus subtilis*
pdhC	P21883.2	129054	*Bacillus subtilis*

pdhD	P21880.1	118672	*Bacillus subtilis*
aceE	YP_001333808.1	152968699	*Klebsiella pneumonia MGH78578*
aceF	YP_001333809.1	152968700	*Klebsiella pneumonia MGH78578*
lpdA	YP_001333810.1	152968701	*Klebsiella pneumonia MGH78578*
Pdha1	NP_001004072.2	124430510	*Rattus norvegicus*
Pdha2	NP_446446.1	16758900	*Rattus norvegicus*
Dlat	NP_112287.1	78365255	*Rattus norvegicus*
Dld	NP_955417.1	40786469	*Rattus norvegicus*

As an alternative to the large multienzyme 2-keto-acid dehydrogenase complexes described above, some anaerobic organisms utilize enzymes in the 2-ketoacid oxidoreductase family (OFOR) to catalyze acylating oxidative decarboxylation of 2-keto-acids. Unlike the dehydrogenase complexes, these enzymes contain iron-sulfur clusters, utilize different cofactors, and use ferredoxin or flavodixin as electron acceptors in lieu of NAD(P)H. While most enzymes in this family are specific to pyruvate as a substrate (POR) some 2-keto-acid:ferredoxin oxidoreductases have been shown to accept a broad range of 2-ketoacids as substrates including alpha-ketoglutarate and 2-oxobutanoate (Fukuda and Wakagi *Biochim.Biophys.Acta* 1597:74-80 (2002); Zhang et al. *J.Biochem.* 120:587-599 (1996)). One such enzyme is the OFOR from the thermoacidophilic archaeon *Sulfolobus tokodaii 7*, which contains an alpha and beta subunit encoded by gene *ST2300* (Fukuda and Wakagi *Biochim.Biophys.Acta* 1597:74-80 (2002); Zhang et al. *J.Biochem.* 120:587-599 (1996)). A plasmid-based expression system has been developed for efficiently expressing this protein in *E. coli* (Fukuda et al. *Eur.J.Biochem.* 268:5639-5646 (2001)) and residues involved in substrate specificity were determined (Fukuda and Wakagi *Biochim.Biophys.Acta* 1597:74-80 (2002)). Two OFORs from *Aeropyrum pernix str. K1* have also been recently cloned into *E. coli*, characterized, and found to react with a broad range of 2-oxoacids (Nishizawa et al. *FEBS Lett.* 579:2319-2322 (2005)). The gene sequences of these OFOR candidates are available, although they do not have GenBank identifiers assigned to date. There is bioinformatic evidence that similar enzymes are present in all archaea, some anaerobic bacteria and amitochondrial eukarya (Fukuda and Wakagi *Biochim.Biophys.Acta* 1597:74-80 (2005)). This class of enzyme is also interesting from an energetic standpoint, as reduced ferredoxin could be used to generate NADH by ferredoxin-NAD reductase (Petitdemange et al. *Biochim.Biophys.Acta* 421:334-337 (1976)). Also, since most of the enzymes are designed to operate under anaerobic conditions, less enzyme engineering may be required relative to enzymes in the 2-keto-acid dehydrogenase complex family for activity in an anaerobic environment.

Gene	Accession No.	GI No.	Organism
ST2300	NP_378302.1	15922633	*Sulfolobus tokodaii 7*

1.2.1.d - Oxidoreductase (phosphorylating/dephosphorylating)

Exemplary enzymes in this class include glyceraldehyde 3-phosphate dehydrogenase which converts glyceraldehyde-3-phosphate into D-glycerate 1,3-bisphosphate (for example, *E. coli gapA* (Branlant and Branlant *Eur.J.Biochem.* 150:61-66(1985)), aspartate-semialdehyde dehydrogenase which converts L-aspartate-4-semialdehyde into L-4-aspartyl-phosphate (for example, *E. coli asd* (Biellmann et al. *Eur.J.Biochem.* 104:53-58 (1980)), N-acetyl-gamma-glutamyl-phosphate reductase which converts N-acetyl-L-glutamate-5-semialdehyde into N-acetyl-L-glutamyl-5-phosphate (for example, *E. coli argC* (Parsot et al. Gene 68:275-283 (1988)), and glutamate-5-semialdehyde dehydrogenase which converts L-glutamate-5-semialdehyde into L-glutamyl-5-phospate (for example, *E. coli proA* (Smith et al. *J.Bacteriol.* 157:545-551 (1984)).

Gene	Accession No.	GI No.	Organism
gapA	P0A9B2.2	71159358	*Escherichia coli*
asd	NP_417891.1	16131307	*Escherichia coli*
argC	NP_418393.1	16131796	*Escherichia coli*
proA	NP_414778.1	16128229	*Escherichia coli*

1.3.1.a - Oxidoreductase operating on CH-CH donors

An exemplary enoyl-CoA reductase is the gene product of *bcd* from *C. acetobutylicum* (Atsumi et al. *Metab Eng* (2007); Boynton et al. *Journal of Bacteriology* 178:3015-3024 (1996), which naturally catalyzes the reduction of crotonyl-CoA to butyryl-CoA. Activity of this enzyme can be enhanced by expressing *bcd* in conjunction with expression of the *C. acetobutylicum etfAB* genes, which encode an electron transfer flavoprotein. An additional candidate for the enoyl-CoA reductase step is the mitochondrial enoyl-CoA reductase from *E. gracilis* (Hoffmeister et al. *Journal of Biological Chemistry* 280:4329-4338 (2005)). A construct derived from this sequence following the removal of its mitochondrial targeting leader sequence was cloned in *E. coli* resulting in an active enzyme (Hoffmeister et al., supra, (2005)). This approach is well known to those skilled in the art of expressing eukarytotic genes, particularly those with leader sequences that may target the gene product to a specific intracellular compartment, in prokaryotic organisms. A close homolog of this gene, TDE0597, from the prokaryote

Treponema denticola represents a third enoyl-CoA reductase which has been cloned and expressed in *E. coli* (Tucci and Martin *FEBS Letters* 581:1561-1566 (2007)).

Gene	Accession No.	GI No.	Organism
bcd	NP_349317.1	15895968	*Clostridium acetobutylicum*
etfA	NP_349315.1	15895966	*Clostridium acetobutylicum*
etfB	NP_349316.1	15895967	*Clostridium acetobutylicum*
TER	Q5EU90.1	62287512	*Euglena gracilis*
TDE0597	NP_971211.1	42526113	*Treponema denticola*

Exemplary 2-enoate reductase (EC 1.3.1.31) enzymes are known to catalyze the NADH-dependent reduction of a wide variety of α, β-unsaturated carboxylic acids and aldehydes (Rohdich et al. *J.Biol.Chem.* 276:5779-5787 (2001)). 2-Enoate reductase is encoded by *enr* in several species of *Clostridia* (Giesel and Simon *Arch Microbiol.* 135(1): p. 51-57 (2001) including *C. tyrobutyricum*, and *C. thermoaceticum* (now called *Moorella thermoaceticum*) (Rohdich et al., supra, (2001)). In the recently published genome sequence of *C. kluyveri*, 9 coding sequences for enoate reductases have been reported, out of which one has been characterized (Seedorf et al. *Proc Natl Acad Sci U. S. A.* 105(6):2128-33 (2008)). The *enr* genes from both *C. tyrobutyricum* and *C. thermoaceticum* have been cloned and sequenced and show 59% identity to each other. The former gene is also found to have approximately 75% similarity to the characterized gene in *C. kluyveri* (Giesel and Simon *Arch Microbiol* 135(1):51-57 (1983)). It has been reported based on these sequence results that *enr* is very similar to the dienoyl CoA reductase in *E. coli* (*fadH*) (163 Rohdich et al., supra (2001)). The *C. thermoaceticum enr* gene has also been expressed in an enzymatically active form in *E. coli* (163 Rohdich et al., supra (2001)).

Gene	Accession No.	GI No.	Organism
fadH	NP_417552.1	16130976	*Escherichia coli*
enr	ACA54153.1	169405742	*Clostridium botulinum A3 str*
enr	CAA71086.1	2765041	*Clostridium tyrobutyricum*
enr	CAA76083.1	3402834	*Clostridium kluyveri*
enr	YP_430895.1	83590886	*Moorella thermoacetica*

1.4.1.a - Oxidoreductase operating on amino acids

Most oxidoreductases operating on amino acids catalyze the oxidative deamination of alpha-amino acids with NAD+ or NADP+ as acceptor. Exemplary oxidoreductases operating on amino acids include glutamate dehydrogenase (deaminating), encoded by *gdhA*, leucine

dehydrogenase (deaminating), encoded by *ldh*, and aspartate dehydrogenase (deaminating), encoded by *nadX*. The *gdhA* gene product from *Escherichia coli* (Korber et al. *J.Mol.Biol.* 234:1270-1273 (1993); McPherson and Wootton *Nucleic.Acids Res.* 11:5257-5266 (1983)), *gdh* from *Thermotoga maritima* (Kort et al. *Extremophiles* 1:52-60 (1997); Lebbink, et al. *J.Mol.Biol.* 280:287-296 (1998)); Lebbink et al. *J.Mol.Biol.* 289:357-369 (1999)), and *gdhA1* from *Halobacterium salinarum* (Ingoldsby et al. *Gene* 349:237-244 (2005)) catalyze the reversible interconversion of glutamate to 2-oxoglutarate and ammonia, while favoring NADP(H), NAD(H), or both, respectively. The *ldh* gene of *Bacillus cereus* encodes the LeuDH protein that has a wide of range of substrates including leucine, isoleucine, valine, and 2-aminobutanoate (Ansorge and Kula *Biotechnol Bioeng.* 68:557-562 (2000); Stoyan et al. *J.Biotechnol* 54:77-80 (1997)). The *nadX* gene from *Thermotoga maritime* encoding for the aspartate dehydrogenase is involved in the biosynthesis of NAD (Yang et al. *J.Biol.Chem.* 278:8804-8808 (2003)).

Gene	Accession No.	GI No.	Organism
gdhA	P00370	118547	*Escherichia coli*
gdh	P96110.4	6226595	*Thermotoga maritima*
gdhA1	NP_279651.1	15789827	*Halobacterium salinarum*
ldh	P0A393	61222614	*Bacillus cereus*
nadX	NP_229443.1	15644391	*Thermotoga maritima*

The lysine 6-dehydrogenase (deaminating), encoded by *lysDH* gene, catalyze the oxidative deamination of the ε-amino group of L-lysine to form 2-aminoadipate-6-semialdehyde, which in turn nonenzymatically cyclizes to form Δ1-piperideine-6-carboxylate (Misono and Nagasaki *J.Bacteriol.* 150:398-401 (1982)). The *lysDH* gene from *Geobacillus stearothermophilus* encodes a thermophilic NAD-dependent lysine 6-dehydrogenase (Heydari et al. *Appl Environ.Microbiol* 70:937-942 (2004)). In addition, the *lysDH* gene from *Aeropyrum pernix K1* is identified through homology from genome projects.

Gene	Accession No.	GI No.	Organism
lysDH	AB052732	13429872	*Geobacillus stearothermophilus*
lysDH	NP_147035.1	14602185	*Aeropyrum pernix K1*
ldh	P0A393	61222614	*Bacillus cereus*

2.3.1.a - Acyltransferase (transferring phosphate group)

Exemplary phosphate transferring acyltransferases include phosphotransacetylase, encoded by *pta*, and phosphotransbutyrylase, encoded by *ptb*. The *pta* gene from *E. coli* encodes an enzyme

that can convert acetyl-CoA into acetyl-phosphate, and vice versa (Suzuki, T. *Biochim.Biophys.Acta* 191:559-569 (1969)). This enzyme can also utilize propionyl-CoA instead of acetyl-CoA forming propionate in the process (Hesslinger et al. *Mol.Microbiol* 27:477-492 (1998)). Similarly, the *ptb* gene from *C. acetobutylicum* encodes an enzyme that can convert butyryl-CoA into butyryl-phosphate (Walter et al. *Gene* 134(1): p. 107-11 (1993)); Huang et al. *J Mol Microbiol Biotechnol* 2(1): p. 33-38 (2000). Additional *ptb* genes can be found in butyrate-producing bacterium L2-50 (Louis et al. *J.Bacteriol.* 186:2099-2106 (2004)) and *Bacillus megaterium* (Vazquez et al. *Curr.Microbiol* 42:345-349 (2001)).

Gene	Accession No.	GI No.	Organism
pta	NP_416800.1	16130232	*Escherichia coli*
ptb	NP_349676	15896327	*Clostridium acetobutylicum*
ptb	AAR19757.1	38425288	butyrate-producing bacterium L2-50
ptb	CAC07932.1	10046659	*Bacillus megaterium*

2.6.1.a - Aminotransferase

Aspartate aminotransferase transfers an amino group from aspartate to alpha-ketoglutarate, forming glutamate and oxaloacetate. This conversion is catalyzed by, for example, the gene products of *aspC* from *Escherichia coli* (Yagi et al. *FEBS Lett.* 100:81-84 (1979); Yagi et al. *Methods Enzymol.* 113:83-89 (1985)), AAT2 from *Saccharomyces cerevisiae* (Yagi et al. *J Biochem.* 92:35-43 (1982)) and *ASP5* from *Arabidopsis thaliana* (48, 108, 225 48. de la et al. *Plant J* 46:414-425 (2006); Kwok and Hanson *J Exp.Bot.* 55:595-604 (2004); Wilkie and Warren *Protein Expr.Purif.* 12:381-389 (1998)). Valine aminotransferase catalyzes the conversion of valine and pyruvate to 2-ketoisovalerate and alanine. The *E. coli* gene, *avtA*, encodes one such enzyme (Whalen and Berg *J.Bacteriol.* 150:739-746 (1982)). This gene product also catalyzes the amination of α-ketobutyrate to generate α-aminobutyrate, although the amine donor in this reaction has not been identified (Whalen and Berg *J.Bacteriol.* 158:571-574 (1984)). The gene product of the *E. coli serC* catalyzes two reactions, phosphoserine aminotransferase and phosphohydroxythreonine aminotransferase (Lam and Winkler *J.Bacteriol.* 172:6518-6528 (1990)), and activity on non-phosphorylated substrates could not be detected (Drewke et al. *FEBS.Lett.* 390:179-182 (1996)).

Gene	Accession No.	GI No.	Organism
aspC	NP_415448.1	16128895	*Escherichia coli*
AAT2	P23542.3	1703040	*Saccharomyces cerevisiae*
ASP5	P46248.2	20532373	*Arabidopsis thaliana*

avtA	YP_026231.1	49176374	*Escherichia coli*
serC	NP_415427.1	16128874	*Escherichia coli*

Cargill has developed a beta-alanine/alpha-ketoglutarate aminotransferase for producing 3-HP from beta-alanine via malonyl-semialdehyde (PCT/US2007/076252 (Jessen et al)). The gene product of SkPYD4 in *Saccharomyces kluyveri* was also shown to preferentially use beta-alanine as the amino group donor (Andersen et al. *FEBS.J.* 274:1804-1817 (2007)). SkUGA1 encodes a homologue of *Saccharomyces cerevisiae* GABA aminotransferase, UGA1 (Ramos et al. *Eur.J.Biochem.* 149:401-404 (1985)), whereas SkPYD4 encodes an enzyme involved in both β-alanine and GABA transamination (Andersen et al. *FEBS.J.* 274:1804-1817 (2007)). 3-Amino-2-methylpropionate transaminase catalyzes the transformation from methylmalonate semialdehyde to 3-amino-2-methylpropionate. The enzyme has been characterized in *Rattus norvegicus* and *Sus scrofa* and is encoded by *Abat* (Kakimoto et al. *Biochim.Biophys.Acta* 156:374-380 (1968); Tamaki et al. *Methods Enzymol.* 324:376-389 (2000)). Enzyme candidates in other organisms with high sequence homology to 3-amino-2-methylpropionate transaminase include *Gta-1* in *C. elegans* and *gabT* in *Bacillus subtilus*. Additionally, one of the native GABA aminotransferases in *E. coli*, encoded by gene *gabT*, has been shown to have broad substrate specificity (Liu et al. *Biochemistry* 43:10896-10905 (2004); Schulz et al. *Appl Environ Microbiol* 56:1-6 (1990)). The gene product of *puuE* catalyzes the other 4-aminobutyrate transaminase in *E. coli* (Kurihara et al. *J.Biol.Chem.* 280:4602-4608 (2005)).

Gene	**Accession No.**	**GI No.**	**Organism**
SkyPYD4	ABF58893.1	98626772	*Saccharomyces kluyveri*
SkUGA1	ABF58894.1	98626792	*Saccharomyces kluyveri*
UGA1	NP_011533.1	6321456	*Saccharomyces cerevisiae*
Abat	P50554.3	122065191	*Rattus norvegicus*
Abat	P80147.2	120968	*Sus scrofa*
Gta-1	Q21217.1	6016091	*Caenorhabditis elegans*
gabT	P94427.1	6016090	*Bacillus subtilus*
gabT	P22256.1	120779	*Escherichia coli K12*
puuE	NP_415818.1	16129263	*Escherichia coli K12*

The X-ray crystal structures of *E. coli* 4-aminobutyrate transaminase unbound and bound to the inhibitor were reported (Liu et al. *Biochemistry* 43:10896-10905 (2004)). The substrates binding and substrate specificities were studied and suggested. The roles of active site residues were studied by site-directed mutagenesis and X-ray crystallography (Liu et al. *Biochemistry* 44:2982-2992 (2005)). Based on the structural information, attempt was made to engineer *E.*

coli 4-aminobutyrate transaminase with novel enzymatic activity. These studies provide a base for evolving transaminase activity for BDO pathways.

2.7.2.a - Phosphotransferase, carboxyl group acceptor

Exemplary kinases include the *E. coli* acetate kinase, encoded by *ackA* (Skarstedt and Silverstein *J.Biol.Chem.* 251:6775-6783 (1976)), the *C. acetobutylicum* butyrate kinases, encoded by *buk1* and *buk2* (Walter et al. *Gene* 134(1):107-111 (1993) (Huang et al. *J Mol Microbiol Biotechnol* 2(1):33-38 (2000)], and the *E. coli* gamma-glutamyl kinase, encoded by *proB* (Smith et al. *J.Bacteriol.* 157:545-551 (1984)). These enzymes phosphorylate acetate, butyrate, and glutamate, respectively. The *ackA* gene product from *E. coli* also phosphorylates propionate (Hesslinger et al. *Mol.Microbiol* 27:477-492 (1998)).

Gene	Accession No.	GI No.	Organism
ackA	NP_416799.1	16130231	*Escherichia coli*
buk1	NP_349675	15896326	*Clostridium acetobutylicum*
buk2	Q97II1	20137415	*Clostridium acetobutylicum*
proB	NP_414777.1	16128228	*Escherichia coli*

2.8.3.a - Coenzyme-A transferase

In the CoA-transferase family, *E. coli* enzyme acyl-CoA:acetate-CoA transferase, also known as acetate-CoA transferase (EC 2.8.3.8), has been shown to transfer the CoA moiety to acetate from a variety of branched and linear acyl-CoA substrates, including isobutyrate (Matthies and Schink *Appl Environ Microbiol* 58:1435-1439 (1992)), valerate (Vanderwinkel et al. *Biochem.Biophys.Res Commun.* 33:902-908 (1968)) and butanoate (Vanderwinkel, supra (1968)). This enzyme is encoded by *atoA* (alpha subunit) and *atoD* (beta subunit) in *E. coli* sp. K12 (Korolev et al. *Acta Crystallogr.D Biol Crystallogr.* 58:2116-2121 (2002); Vanderwinkel, supra (1968)) and *actA* and *cg0592* in *Corynebacterium glutamicum ATCC 13032* (Duncan et al. *Appl Environ Microbiol* 68:5186-5190 (2002)). Additional genes found by sequence homology include *atoD* and *atoA* in *Escherichia coli UT189.*

Gene	Accession No.	GI No.	Organism
atoA	P76459.1	2492994	*Escherichia coli K12*
atoD	P76458.1	2492990	*Escherichia coli K12*
actA	YP_226809.1	62391407	*Corynebacterium glutamicum ATCC 13032*
cg0592	YP_224801.1	62389399	*Corynebacterium glutamicum ATCC 13032*
atoA	ABE07971.1	91073090	*Escherichia coli UT189*
atoD	ABE07970.1	91073089	*Escherichia coli UT189*

Similar transformations are catalyzed by the gene products of *cat1*, *cat2*, and *cat3* of *Clostridium kluyveri* which have been shown to exhibit succinyl-CoA, 4-hydroxybutyryl-CoA, and butyryl-CoA acetyltransferase activity, respectively (Seedorf et al. *Proc Natl Acad Sci U.S.A.* 105(6):2128-2133 (2008); Sohling and Gottschalk *J Bacteriol* 178(3):871-880 (1996)].

Gene	Accession No.	GI No.	Organism
cat1	P38946.1	729048	*Clostridium kluyveri*
cat2	P38942.2	1705614	*Clostridium kluyveri*
cat3	EDK35586.1	146349050	*Clostridium kluyveri*

The glutaconate-CoA-transferase (EC 2.8.3.12) enzyme from anaerobic bacterium *Acidaminococcus fermentans* reacts with diacid glutaconyl-CoA and 3-butenoyl-CoA (Mack and Buckel *FEBS Lett.* 405:209-212 (1997)). The genes encoding this enzyme are *gctA* and *gctB*. This enzyme has reduced but detectable activity with other CoA derivatives including glutaryl-CoA, 2-hydroxyglutaryl-CoA, adipyl-CoA and acrylyl-CoA (Buckel et al. *Eur.J.Biochem.* 118:315-321 (1981)). The enzyme has been cloned and expressed in *E. coli* (Mac et al. *Eur.J.Biochem.* 226:41-51 (1994)).

Gene	Accession No.	GI No.	Organism
gctA	CAA57199.1	559392	*Acidaminococcus fermentans*
gctB	CAA57200.1	559393	*Acidaminococcus fermentans*

3.1.2.a - Thiolester hydrolase (CoA specific)

In the CoA hydrolase family, the enzyme 3-hydroxyisobutyryl-CoA hydrolase is specific for 3-HIBCoA and has been described to efficiently catalyze the desired transformation during valine degradation (Shimomura et al. *J Biol Chem* 269:14248-14253 (1994)). Genes encoding this enzyme include hibch of *Rattus norvegicus* (Shimomura et al., supra (1994); Shimomura et al. *Methods Enzymol.* 324:229-240 (2000) and Homo sapiens (Shimomura et al., supra, 2000). Candidate genes by sequence homology include hibch of *Saccharomyces cerevisiae* and *BC_2292* of *Bacillus cereus*.

Gene	Accession No.	GI No.	Organism
hibch	Q5XIE6.2	146324906	*Rattus norvegicus*
hibch	Q6NVY1.2	146324905	*Homo sapiens*
hibch	P28817.2	2506374	*Saccharomyces cerevisiae*
BC_2292	Q81DR3	81434808	*Bacillus cereus*

The conversion of adipyl-CoA to adipate can be carried out by an acyl-CoA hydrolase or equivalently a thioesterase. The top *E. coli* gene candidate is *tesB* (Naggert et al. *J Biol Chem.* 266(17):11044-11050 (1991)] which shows high similarity to the human *acot8* which is a dicarboxylic acid acetyltransferase with activity on adipyl-CoA (Westin et al. *J Biol Chem* 280(46): 38125-38132 (2005). This activity has also been characterized in the rat liver (Deana, *Biochem Int.* 26(4): p. 767-773 (1992)).

Gene	Accession No.	GI No.	Organism
tesB	NP_414986	16128437	*Escherichia coli*
acot8	CAA15502	3191970	*Homo sapiens*
acot8	NP_570112	51036669	*Rattus norvegicus*

Other potential *E. coli* thiolester hydrolases include the gene products of *tesA* (Bonner and Bloch, *J Biol Chem.* 247(10):3123-3133 (1972)), *ybgC* (Kuznetsova et al., *FEMS Microbiol Rev.* 29(2):263-279 (2005); Zhuang et al., *FEBS Lett.* 516(1-3):161-163 (2002)) *paaI* (Song et al., *J Biol Chem.* 281(16):11028-11038 (2006)), and *ybdB* (Leduc et al., *J Bacteriol.* 189(19):7112-7126 (2007)).

Gene	Accession No.	GI No.	Organism
tesA	NP_415027	16128478	*Escherichia coli*
ybgC	NP_415264	16128711	*Escherichia coli*
paaI	NP_415914	16129357	*Escherichia coli*
ybdB	NP_415129	16128580	*Escherichia coli*

Several eukaryotic acetyl-CoA hydrolases (EC 3.1.2.1) have broad substrate specificity. The enzyme from *Rattus norvegicus* brain (Robinson et al. *Biochem.Biophys. Res.Commun.* 71:959-965 (1976)) can react with butyryl-CoA, hexanoyl-CoA and malonyl-CoA.

Gene	Accession No.	GI No.	Organism
acot12	NP_570103.1	18543355	*Rattus norvegicus*

4.1.1.a - Carboxy-lyase

An exemplary carboxy-lyase is acetolactate decarboxylase which participates in citrate catabolism and branched-chain amino acid biosynthesis, converting 2-acetolactate to acetoin. In *Lactococcus lactis* the enzyme is composed of six subunits, encoded by gene *aldB*, and is activated by valine, leucine and isoleucine (Goupil et al. *Appl.Environ.Microbiol.* 62:2636-2640 (1996); Goupil-Feuillerat et al. *J.Bacteriol.* 182:5399-5408 (2000)). This enzyme has been overexpressed and characterized in *E. coli* (Phalip et al. *FEBS Lett.* 351:95-99 (1994)). In other

organisms the enzyme is a dimer, encoded by *aldC* in *Streptococcus thermophilus* (Monnet et al. *Lett.Appl.Microbiol.* 36:399-405 (2003)), *aldB* in *Bacillus brevis* (Diderichsen et al. *J.Bacteriol.* 172:4315-4321 (1990); Najmudin et al. *Acta Crystallogr.D.Biol.Crystallogr.* 59:1073-1075 (2003)) and *budA* from *Enterobacter aerogenes* (Diderichsen et al. *J.Bacteriol.* 172:4315-4321 (1990)). The enzyme from *Bacillus brevis* was cloned and overexpressed in *Bacillus subtilis* and characterized crystallographically (Najmudin et al. *Acta Crystallogr.D.Biol.Crystallogr.* 59:1073-1075 (2003)). Additionally, the enzyme from *Leuconostoc lactis* has been purified and characterized but the gene has not been isolated (O'Sullivan et al. *FEMS Microbiol.Lett.* 194:245-249 (2001)).

Gene	Accession No.	GI No.	Organism
aldB	NP_267384.1	15673210	*Lactococcus lactis*
aldC	Q8L208	75401480	*Streptococcus thermophilus*
aldB	P23616.1	113592	*Bacillus brevis*
budA	P05361.1	113593	*Enterobacter aerogenes*

Aconitate decarboxylase catalyzes the final step in itaconate biosynthesis in a strain of *Candida* and also in the filamentous fungus *Aspergillus terreus* (Bonnarme et al. *J Bacteriol.* 177:3573-3578 (1995); Willke and Vorlop *Appl Microbiol Biotechnol* 56:289-295 (2001)). Although itaconate is a compound of biotechnological interest, the aconitate decarboxylase gene or protein sequence has not been reported to date.

4-oxalocronate decarboxylase has been isolated from numerous organisms and characterized. Genes encoding this enzyme include *dmpH* and *dmpE* in *Pseudomonas sp. (strain 600)* (Shingler et al. *J Bacteriol.* 174:711-724 (1992)), *xylII* and *xylIII* from *Pseudomonas putida* (Kato and Asano *Arch.Microbiol* 168:457-463 (1997); Lian and Whitman *J.Am.Chem.Soc.* 116:10403-10411 (1994); Stanley et al. *Biochemistry* 39:3514 (2000)) and *Reut_B5691* and *Reut_B5692* from *Ralstonia eutropha JMP134* (Hughes et al. *J Bacteriol.* 158:79-83 (1984)). The genes encoding the enzyme from *Pseudomonas sp. (strain 600)* have been cloned and expressed in *E. coli* (Shingler et al. *J Bacteriol.* 174:711-724 (1992)).

Gene	Accession No.	GI No.	Organism
dmpH	CAA43228.1	45685	*Pseudomonas sp. CF600*
dmpE	CAA43225.1	45682	*Pseudomonas sp. CF600*
xylII	YP_709328.1	111116444	*Pseudomonas putida*
xylIII	YP_709353.1	111116469	*Pseudomonas putida*
Reut_B5691	YP_299880.1	73539513	*Ralstonia eutropha JMP134*
Reut_B5692	YP_299881.1	73539514	*Ralstonia eutropha JMP134*

An additional class of decarboxylases has been characterized that catalyze the conversion of cinnamate (phenylacrylate) and substituted cinnamate derivatives to the corresponding styrene derivatives. These enzymes are common in a variety of organisms and specific genes encoding these enzymes that have been cloned and expressed in *E. coli* are: *pad 1* from *Saccharomyces cerevisae* (Clausen et al. *Gene* 142:107-112 (1994)), *pdc* from *Lactobacillus plantarum* (Barthelmebs et al. *Appl Environ Microbiol* 67:1063-1069 (2001); Qi et al. *Metab Eng* 9:268-276 (2007); Rodriguez et al. *J.Agric.Food Chem.* 56:3068-3072 (2008)), *pofK* (*pad*) from *Klebsiella oxytoca* (Hashidoko et al. *Biosci.Biotech.Biochem.* 58:217-218 (1994); Uchiyama et al. *Biosci.Biotechnol.Biochem.* 72:116-123 (2008)), *Pedicoccus pentosaceus* (Barthelmebs et al. *Appl Environ Microbiol* 67:1063-1069 (2001)), and *padC* from *Bacillus subtilis* and *Bacillus pumilus* (Lingen et al. *Protein Eng* 15:585-593 (2002)). A ferulic acid decarboxylase from *Pseudomonas fluorescens* also has been purified and characterized (Huang et al. *J.Bacteriol.* 176:5912-5918 (1994)). Importantly, this class of enzymes have been shown to be stable and do not require either exogenous or internally bound co-factors, thus making these enzymes ideally suitable for biotransformations (Sariaslani, *Annu.Rev.Microbiol.* 61:51-69 (2007)).

Gene	Accession No.	GI No.	Organism
pad1	AB368798 BAG32372.1	188496948 188496949	*Saccharomyces cerevisae*
pdc	U63827 AAC45282.1	1762615, 1762616	*Lactobacillus plantarum*
pofK (pad)	AB330293, BAF65031.1	149941607, 149941608	*Klebsiella oxytoca*
padC	AF017117 AAC46254.1	2394281, 2394282	*Bacillus subtilis*
pad	AJ276891 CAC16794.1	11322456, 11322458	*Pedicoccus pentosaceus*
pad	AJ278683 CAC18719.1	11691809, 11691810	*Bacillus pumilus*

Additional decarboxylase enzymes can form succinic semialdehyde from alpha-ketoglutarate. These include the alpha-ketoglutarate decarboxylase enzymes from *Euglena gracilis* (Shigeoka et al. *Biochem.J.* 282(Pt 2):319-323 (1992); Shigeoka and Nakano *Arch.Biochem.Biophys.* 288:22-28 (1991); Shigeoka and Nakano *Biochem.J.* 292 (Pt 2):463-467 (1993)), whose corresponding gene sequence has yet to be determined, and from *Mycobacterium tuberculosis* (Tian et al. *Proc Natl Acad Sci U.S.A.* 102:10670-10675 (2005)). In addition, glutamate decarboxylase enzymes can convert glutamate into 4-aminobutyrate such as the products of the *E. coli gadA* and *gad*B genes (De Biase et al. *Protein.Expr.Purif.* 8:430-438 (1993)).

Gene	Accession No.	GI No.	Organism
kgd	O50463.4	160395583	*Mycobacterium tuberculosis*
gadA	NP_417974	16131389	*Escherichia coli*
gadB	NP_416010	16129452	*Escherichia coli*

Keto-acid decarboxylases

Pyruvate decarboxylase (PDC, EC 4.1.1.1), also termed keto-acid decarboxylase, is a key enzyme in alcoholic fermentation, catalyzing the decarboxylation of pyruvate to acetaldehyde. This enzyme has a broad substrate range for aliphatic 2-keto acids including 2-ketobutyrate, 2-ketovalerate, 3-hydroxypyruvate and 2-phenylpyruvate (Berg et al. *Science* 318:1782-1786 (2007)). The PDC from *Zymomonas mobilus*, encoded by *pdc*, has been a subject of directed engineering studies that altered the affinity for different substrates (Siegert et al. *Protein Eng Des Sel* 18:345-357 (2005)). The PDC from *Saccharomyces cerevisiae* has also been extensively studied, engineered for altered activity, and functionally expressed in *E. coli* (Killenberg-Jabs et al. *Eur.J.Biochem.* 268:1698-1704 (2001); Li and Jordan *Biochemistry* 38:10004-10012 (1999); ter Schure et al. *Appl.Environ.Microbiol.* 64:1303-1307 (1998)). The crystal structure of this enzyme is available (Killenberg-Jabs *Eur.J.Biochem.* 268:1698-1704 (2001)). Other well-characterized PDC candidates include the enzymes from *Acetobacter pasteurians* (Chandra et al. *Arch.Microbiol.* 176:443-451 (2001)) and *Kluyveromyces lactis* (Krieger et al. *Eur.J.Biochem.* 269:3256-3263 (2002)).

Gene	Accession No.	GI No.	Organism
pdc	P06672.1	118391	*Zymomonas mobilus*
pdc1	P06169	30923172	*Saccharomyces cerevisiae*
pdc	Q8L388	75401616	*Acetobacter pasteurians*
pdc1	Q12629	52788279	*Kluyveromyces lactis*

Like PDC, benzoylformate decarboxylase (EC 4.1.1.7) has a broad substrate range and has been the target of enzyme engineering studies. The enzyme from *Pseudomonas putida* has been extensively studied and crystal structures of this enzyme are available (Hasson et al. *Biochemistry* 37:9918-9930 (1998); Polovnikova et al. *Biochemistry* 42:1820-1830 (2003)). Site-directed mutagenesis of two residues in the active site of the Pseudomonas putida enzyme altered the affinity (Km) of naturally and non-naturally occuring substrates (Siegert *Protein Eng Des Sel* 18:345-357 (2005)). The properties of this enzyme have been further modified by directed engineering (Lingen et al. *Protein Eng* 15:585-593 (2002)); Lingen *Chembiochem* 4:721-726 (2003)). The enzyme from *Pseudomonas aeruginosa*, encoded by *mdlC*, has also

been characterized experimentally (Barrowman et al. *FEMS Microbiology Letters* 34:57-60 (1986)). Additional gene candidates from *Pseudomonas stutzeri*, *Pseudomonas fluorescens* and other organisms can be inferred by sequence homology or identified using a growth selection system developed in *Pseudomonas putida* (Henning et al. *Appl.Environ.Microbiol.* 72:7510-7517 (2006)).

Gene	Accession No.	GI No.	Organism
mdlC	P20906.2	3915757	*Pseudomonas putida*
mdlC	Q9HUR2.1	81539678	*Pseudomonas aeruginosa*
dpgB	ABN80423.1	126202187	*Pseudomonas stutzeri*
ilvB-1	YP_260581.1	70730840	*Pseudomonas fluorescens*

4.2.1.a - Hydro-lyase

The 2-(hydroxymethyl)glutarate dehydratase of *Eubacterium barkeri* is an exemplary hydro-lyase. This enzyme has been studied in the context of nicotinate catabolism and is encoded by *hmd* (Alhapel et al. *Proc Natl Acad Sci U S A* 103:12341-12346 (2006)). Similar enzymes with high sequence homology are found in *Bacteroides capillosus*, *Anaerotruncus colihominis*, and *Natranaerobius thermophilius*.

Gene	Accession No.	GI No.	Organism
hmd	ABC88407.1	86278275	*Eubacterium barkeri*
BACCAP_02294	ZP_02036683.1	154498305	*Bacteroides capillosus* ATCC 29799
ANACOL_02527	ZP_02443222.1	167771169	*Anaerotruncus colihominis* DSM 17241
NtherDRAFT_2368	ZP_02852366.1	169192667	*Natranaerobius thermophilus* JW/NM-WN-LF

A second exemplary hydro-lyase is fumarate hydratase, an enzyme catalyzing the dehydration of malate to fumarate. A wealth of structural information is available for this enzyme and researchers have successfully engineered the enzyme to alter activity, inhibition and localization (Weaver, T. *Acta Crystallogr.D Biol Crystallogr.* 61:1395-1401 (2005)). Additional fumarate hydratases include those encoded by *fumC* from *Escherichia coli* (Estevez et al. *Protein Sci.* 11:1552-1557 (2002); Hong and Lee *Biotechnol.Bioprocess Eng.* 9:252-255 (2004); Rose and Weaver *Proc Natl Acad Sci U S.A* 101:3393-3397 (2004)), *Campylobacter jejuni* (Smith et al. *Int.J Biochem.Cell Biol* 31:961-975 (1999)) and *Thermus thermophilus* (Mizobata et al. *Arch.Biochem.Biophys.* 355:49-55 (1998)), and *fumH* from *Rattus norvegicus* (Kobayashi et al. *J Biochem.* 89:1923-1931(1981)). Similar enzymes with high sequence homology include *fum1* from *Arabidopsis thaliana* and *fumC* from *Corynebacterium glutamicum*.

Gene	Accession No.	GI No.	Organism
fumC	P05042.1	120601	*Escherichia coli K12*
fumC	O69294.1	9789756	*Campylobacter jejuni*
fumC	P84127	75427690	*Thermus thermophilus*
fumH	P14408.1	120605	*Rattus norvegicus*
fum1	P93033.2	39931311	*Arabidopsis thaliana*
fumC	Q8NRN8.1	39931596	*Corynebacterium glutamicum*

Citramalate hydrolyase, also called 2-methylmalate dehydratase, converts 2-methylmalate to mesaconate. 2-Methylmalate dehydratase activity was detected in *Clostridium tetanomorphum*, *Morganella morganii*, *Citrobacter amalonaticus* in the context of the glutamate degradation VI pathway (Kato and Asano *Arch.Microbiol* 168:457-463 (1997)); however the genes encoding this enzyme have not been sequenced to date.

The gene product of crt from *C. acetobutylicum* catalyzes the dehydration of 3-hydroxybutyryl-CoA to crotonyl-CoA (Atsumi et al. *Metab Eng.*; 29 (2007)); Boynton et al. *Journal of Bacteriology* 178:3015-3024 (1996)). The enoyl-CoA hydratases, *phaA* and *phaB*, of *P. putida* are believed to carry out the hydroxylation of double bonds during phenylacetate catabolism; (Olivera et al. *Proc Natl Acad Sci U S A* 95(11):6419-6424 (1998)). The *paaA* and *paaB* from *P. fluorescens* catalyze analogous transformations (14 Olivera et al., supra, 1998). Lastly, a number of *Escherichia coli* genes have been shown to demonstrate enoyl-CoA hydratase functionality including *maoC* (Park and Lee *J Bacteriol* 185(18):5391-5397 (2003)), *paaF* (Park and Lee *Biotechnol Bioeng.* 86(6):681-686 (2004a)); Park and Lee *Appl Biochem Biotechnol.* 113-116: 335-346 (2004b)); Ismail et al. *Eur J Biochem* 270(14):p. 3047-3054 (2003), and *paaG* (Park and Lee, supra, 2004; Park and Lee supra, 2004b; Ismail et al., supra, 2003).

Gene	Accession No.	GI No.	Organism
maoC	NP_415905.1	16129348	*Escherichia coli*
paaF	NP_415911.1	16129354	*Escherichia coli*
paaG	NP_415912.1	16129355	*Escherichia coli*
crt	NP_349318.1	15895969	*Clostridium acetobutylicum*
paaA	NP_745427.1	26990002	*Pseudomonas putida*
paaB	NP_745426.1	26990001	*Pseudomonas putida*

phaA	ABF82233.1	106636093	*Pseudomonas fluorescens*
phaB	ABF82234.1	106636094	*Pseudomonas fluorescens*

The *E. coli* genes *fadA* and *fadB* encode a multienzyme complex that exhibits ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, and enoyl-CoA hydratase activities (Yang et al. *Biochemistry* 30(27): p. 6788-6795 (1991); Yang et al. *J Biol Chem* 265(18): p. 10424-10429 (1990); Yang et al. *J Biol Chem* 266(24): p. 16255 (1991); Nakahigashi and Inokuchi *Nucleic Acids Res* 18(16): p. 4937 (1990)). The *fadI* and *fadJ* genes encode similar functions and are naturally expressed only anaerobically (Campbell et al. *Mol Microbiol* 47(3): p. 793-805 (2003). A method for producing poly[(R)-3-hydroxybutyrate] in *E. coli* that involves activating *fadB* (by knocking out a negative regulator, *fadR*) and co-expressing a non-native ketothiolase (*phaA* from *Ralstonia eutropha*) has been described previously (Sato et al. *J Biosci Bioeng* 103(1): 38-44 (2007)). This work clearly demonstrates that a β-oxidation enzyme, in particular the gene product of *fadB* which encodes both 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities, can function as part of a pathway to produce longer chain molecules from acetyl-CoA precursors.

Gene	Accession No.	GI No.	Organism
fadA	YP_026272.1	49176430	*Escherichia coli*
fadB	NP_418288.1	16131692	*Escherichia coli*
fadI	NP_416844.1	16130275	*Escherichia coli*
fadJ	NP_416843.1	16130274	*Escherichia coli*
fadR	NP_415705.1	16129150	*Escherichia coli*

4.3.1.a - Ammonia-lyase

Aspartase (EC 4.3.1.1), catalyzing the deamination of aspartate to fumarate, is a widespread enzyme in microorganisms, and has been characterized extensively (Viola, R. E. *Adv.Enzymol.Relat Areas Mol.Biol* 74:295-341 (2000)). The crystal structure of the *E. coli* aspartase, encoded by *aspA*, has been solved (Shi et al. *Biochemistry* 36:9136-9144 (1997)). The *E. coli* enzyme has also been shown to react with alternate substrates aspartatephenylmethylester, asparagine, benzyl-aspartate and malate (Ma et al. *Ann N.Y.Acad Sci* 672:60-65 (1992)). In a separate study, directed evolution was been employed on this enzyme to alter substrate specificity (Asano et al. *Biomol.Eng* 22:95-101 (2005)). Enzymes with aspartase functionality have also been characterized in *Haemophilus influenzae* (Sjostrom et al.

Biochim.Biophys.Acta 1324:182-190 (1997)), *Pseudomonas fluorescens* (Takagi et al. *J.Biochem.* 96:545-552 (1984)), *Bacillus subtilus* (Sjostrom et al. *Biochim.Biophys.Acta* 1324:182-190 (1997)) and *Serratia marcescens* (Takagi and Kisumi *J Bacteriol.* 161:1-6 (1985)).

Gene	Accession No.	GI No.	Organism
aspA	NP_418562	90111690	*Escherichia coli K12 subsp. MG1655*
aspA	P44324.1	1168534	*Haemophilus influenzae*
aspA	P07346.1	114273	*Pseudomonas fluorescens*
ansB	P26899.1	114271	*Bacillus subtilus*
aspA	P33109.1	416661	*Serratia marcescens*

3-methylaspartase (EC 4.3.1.2), also known as beta-methylaspartase or 3-methylaspartate ammonia-lyase, catalyzes the deamination of threo-3-methylasparatate to mesaconate. The 3-methylaspartase from *Clostridium tetanomorphum* has been cloned, functionally expressed in *E. coli*, and crystallized (Asuncion et al. *Acta Crystallogr.D Biol Crystallogr.* 57:731-733 (2001); Asuncion et al. *J Biol Chem.* 277:8306-8311 (2002); Botting et al. *Biochemistry* 27:2953-2955 (1988); Goda et al. *Biochemistry* 31:10747-10756 (1992). In *Citrobacter amalonaticus*, this enzyme is encoded by *BAA28709* (Kato and Asano *Arch.Microbiol* 168:457-463 (1997)). 3-Methylaspartase has also been crystallized from *E. coli YG1002* (Asano and Kato *FEMS Microbiol Lett.* 118:255-258 (1994)) although the protein sequence is not listed in public databases such as GenBank. Sequence homology can be used to identify additional candidate genes, including *CTC_02563* in *C. tetani* and *ECs0761* in *Escherichia coli O157:H7*.

Gene	Accession No.	GI No.	Organism
MAL	AAB24070.1	259429	*Clostridium tetanomorphum*
BAA28709	BAA28709.1	3184397	*Citrobacter amalonaticus*
CTC_02563	NP_783085.1	28212141	*Clostridium tetani*
ECs0761	BAB34184.1	13360220	*Escherichia coli O157:H7 str. Sakai*

Ammonia-lyase enzyme candidates that form enoyl-CoA products include beta-alanyl-CoA ammonia-lyase (EC 4.3.1.6), which deaminates beta-alanyl-CoA, and 3-aminobutyryl-CoA ammonia-lyase (EC 4.3.1.14). Two beta-alanyl-CoA ammonia lyases have been identified and characterized in *Clostridium propionicum* (Herrmann et al. *FEBS J.* 272:813-821 (2005)). No other beta-alanyl-CoA ammonia lyases have been studied to date, but gene candidates can be identified by sequence similarity. One such candidate is MXAN_4385 in *Myxococcus xanthus*.

Gene	Accession No.	GI No.	Organism
ac12	CAG29275.1	47496504	*Clostridium propionicum*
acl1	CAG29274.1	47496502	*Clostridium propionicum*
MXAN_4385	YP_632558.1	108756898	*Myxococcus xanthus*

5.3.3.a - Isomerase

The 4-hydroxybutyryl-CoA dehydratases from both *Clostridium aminobutyrium* and *C. kluyveri* catalyze the reversible conversion of 4-hydroxybutyryl-CoA to crotonyl-CoA and posses an intrinsic vinylacetyl-CoA Δ-isomerase activity (Scherf and Buckel *Eur.J Biochem.* 215:421-429 (1993); Scherf et al. *Arch.Microbiol* 161:239-245 (1994)). Both native enzymes were purified and characterized, including the N-terminal amino acid sequences (Scherf and Buckel, supra, 1993; Scherf et al., supra, 1994). The *abfD* genes from *C. aminobutyrium* and *C. kluyveri* match exactly with these N-terminal amino acid sequences, thus are encoding the 4-hydroxybutyryl-CoA dehydratases/vinylacetyl-CoA Δ-isomerase. In addition, the *abfD* gene from *Porphyromonas gingivalis ATCC 33277* is identified through homology from genome projects.

Gene	Accession No.	GI No.	Organism
abfD	YP_001396399.1	153955634	*Clostridium kluyveri DSM 555*
abfD	P55792	84028213	*Clostridium aminobutyricum*
abfD	YP_001928843	188994591	*Porphyromonas gingivalis ATCC 33277*

5.4.3.a - Aminomutase

Lysine 2,3-aminomutase (EC 5.4.3.2) is an exemplary aminomutase that converts lysine to (3S)-3,6-diaminohexanoate, shifting an amine group from the 2- to the 3- position. The enzyme is found in bacteria that ferment lysine to acetate and butyrate, including as *Fusobacterium nuleatum* (*kamA*) (Barker et al. *J.Bacteriol.* 152:201-207 (1982)) and *Clostridium subterminale* (*kamA*) (Chirpich et al. *J.Biol.Chem.* 245:1778-1789 (1970)). The enzyme from *Clostridium subterminale* has been crystallized (Lepore et al. *Proc.Natl.Acad.Sci.U.S.A* 102:13819-13824 (2005)). An enzyme encoding this function is also encoded by *yodO* in *Bacillus subtilus* (Chen et al. *Biochem.J.* 348 Pt 3:539-549 (2000)). The enzyme utilizes pyridoxal 5'-phosphate as a cofactor, requires activation by S-Adenosylmethoionine, and is stereoselective, reacting with the only with L-lysine. The enzyme has not been shown to react with alternate substrates.

Gene	Accession No.	GI No.	Organism
yodO	O34676.1	4033499	*Bacillus subtilus*
kamA	Q9XBQ8.1	75423266	*Clostridium subterminale*
kamA	Q8RHX4	81485301	*Fusobacterium nuleatum subsp. nuleatum*

A second aminomutase, beta-lysine 5,6-aminomutase (EC 5.4.3.3), catalyzes the next step of lysine fermentation to acetate and butyrate, which transforms (3S)-3,6-diaminohexanoate to (3S,5S)-3,5-diaminohexanoate, shifting a terminal amine group from the 6- to the 5- position. This enzyme also catalyzes the conversion of lysine to 2,5-diaminohexanoate and is also called lysine-5,6-aminomutase (EC 5.4.3.4). The enzyme has been crystallized in *Clostridium sticklandii* (*kamD, kamE*) (Berkovitch et al. *Proc.Natl.Acad.Sci.U.S.A* 101:15870-15875 (2004)). The enzyme from *Porphyromonas gingivalis* has also been characterized (Tang et al. *Biochemistry* 41:8767-8776 (2002)).

Gene	Accession No.	GI No.	Organism
kamD	AAC79717.1	3928904	*Clostridium sticklandii*
kamE	AAC79718.1	3928905	*Clostridium sticklandii*
kamD	NC_002950.2	34539880, 34540809	*Porphyromonas gingivalis W83*
kamE	NC_002950.2	34539880, 34540810	*Porphyromonas gingivalis W83*

Ornithine 4,5-aminomutase (EC 5.4.3.5) converts D-ornithine to 2,4-diaminopentanoate, also shifting a terminal amine to the adjacent carbon. The enzyme from *Clostridium sticklandii* is encoded by two genes, *oraE* and *oraS*, and has been cloned, sequenced and expressed in *E. coli* (Chen et al. *J.Biol.Chem.* 276:44744-44750 (2001)). This enzyme has not been characterized in other organisms to date.

Gene	Accession No.	GI No.	Organism
oraE	AAK72502	17223685	*Clostridium sticklandii*
oraS	AAK72501	17223684	*Clostridium sticklandii*

Tyrosine 2,3-aminomutase (EC 5.4.3.6) participates in tyrosine biosynthesis, reversibly converting tyrosine to 3-amino-3-(4-hdyroxyphenyl)propanoate by shifting an amine from the 2- to the 3- position. In *Streptomyces globisporus* the enzyme has also been shown to react with tyrosine derivatives (Christenson et al. *Biochemistry* 42:12708-12718 (2003)). Sequence information is not available.

Leucine 2,3-aminomutase (EC 5.4.3.7) converts L-leucine to beta-leucine during leucine degradation and biosynthesis. An assay for leucine 2,3-aminomutase detected activity in many

organisms (Poston, J. M. *Methods Enzymol.* 166:130-135 (1988)) but genes encoding the enzyme have not been identified to date.

Cargill has developed a novel 2,3-aminomutase enzyme to convert L-alanine to β-alanine, thus creating a pathway from pyruvate to 3-HP in four biochemical steps (Liao et al., U.S. Publication No. 2005-0221466).

6.2.1.a - Acid-thiol ligase

An exemplary acid-thiol ligase is the gene products of *sucCD* of *E. coli* which together catalyze the formation of succinyl-CoA from succinate with the concaminant consumption of one ATP, a reaction which is reversible *in vivo* (Buck et al. *Biochemistry* 24(22): p. 6245-6252 (1985)). Additional exemplary CoA-ligases include the rat dicarboxylate-CoA ligase for which the sequence is yet uncharacterized (Vamecq et al. *Biochem J.* 230(3): p. 683-693 (1985)), either of the two characterized phenylacetate-CoA ligases from *P. chrysogenum* (Lamas-Maceiras et al. *Biochem J* 395(1):147-155 (2006); Wang et al. *Biochem Biophys Res Commun*, 360(2):453-458 (2007)), the phenylacetate-CoA ligase from *Pseudomonas putida* (Martinez-Blanco et al. *J Biol Chem.* 265(12):7084-7090 (1990)), and the 6-carboxyhexanoate-CoA ligase from *Bacillus subtilis* (Bower et al. *J Bacteriol* 178(14):4122-4130 (1996)).

Gene	Accession No.	GI No.	Organism
sucC	NP_415256.1	16128703	*Escherichia coli*
sucD	AAC73823.1	1786949	*Escherichia coli*
phl	CAJ15517.1	77019264	*Penicillium chrysogenum*
phlB	ABS19624.1	152002983	*Penicillium chrysogenum*
paaF	AAC24333.2	22711873	*Pseudomonas putida*
bioW	NP_390902.2	50812281	*Bacillus subtilis*

EXAMPLE V
Exemplary BDO Pathway from Succinyl-CoA

This example describes exemplary BDO pathways from succinyl-CoA.

BDO pathways from succinyl-CoA are described herein and have been described previously (see U.S. application serial No. 12/049,256, filed March 14, 2008, and PCT application serial No. US08/57168, filed March 14, 2008, each of which is incorporated herein by reference). Additional pathways are shown in Figure 8A. Enzymes of such exemplary BDO pathways are listed in Table 15, along with exemplary genes encoding these enzymes.

Briefly, succinyl-CoA can be converted to succinic semialdehyde by succinyl-CoA reductase (or succinate semialdehyde dehydrogenase) (EC 1.2.1.b). Succinate semialdehyde can be converted to 4-hydroxybutyrate by 4-hydroxybutyrate dehydrogenase (EC 1.1.1.a), as previously described. Alternatively, succinyl-CoA can be converted to 4-hydroxybutyrate by succinyl-CoA reductase (alcohol forming) (EC 1.1.1.c). 4-Hydroxybutyrate can be converted to 4-hydroxybutyryl-CoA by 4-hydroxybutyryl-CoA transferase (EC 2.8.3.a), as previously described, or by 4-hydroxybutyryl-CoA hydrolase (EC 3.1.2.a) or 4-hydroxybutyryl-CoA ligase (or 4-hydroxybutyryl-CoA synthetase) (EC 6.2.1.a). Alternatively, 4-hydroxybutyrate can be converted to 4-hydroxybutyryl-phosphate by 4-hydroxybutyrate kinase (EC 2.7.2.a), as previously described. 4-Hydroxybutyryl-phosphate can be converted to 4-hydroxybutyryl-CoA by phosphotrans-4-hydroxybutyrylase (EC 2.3.1.a), as previously described. Alternatively, 4-hydroxybutyryl-phosphate can be converted to 4-hydroxybutanal by 4-hydroxybutanal dehydrogenase (phosphorylating) (EC 1.2.1.d). 4-Hydroxybutyryl-CoA can be converted to 4-hydroxybutanal by 4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase) (EC 1.2.1.b). Alternatively, 4-hydroxybutyryl-CoA can be converted to 1,4-butanediol by 4-hydroxybutyryl-CoA reductase (alcohol forming) (EC 1.1.1.c). 4-Hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a), as previously described.

TABLE 15. BDO pathway from succinyl-CoA.

Figure	EC class	Desired substrate	Desired product	Enzyme name	Gene name	GenBank ID (if available)	Organism	Known Substrates
8A	1.2.1.b	succinyl-CoA	succinic semialdehyde	succinyl-CoA reductase (or succinate semialdehyde dehydrogenase)	*sucD*	P38947.1	*Clostridium kluyveri*	succinyl-CoA
					sucD	NP_904963.1	*Porphyromonas gingivalis*	succinyl-CoA
					Msed_0709	YP_001190808.1	*Metallosphaera sedula*	malonyl-CoA
8A	1.1.1.a	succinate semialdehyde	4-hydroxybutyrate	4-hydroxybutyrate dehydrogenase	*4hbd*	YP_726053.1	*Ralstonia eutropha H16*	4-hydroxybutyrate
					4hbd	L21902.1	*Clostridium kluyveri DSM 555*	4-hydroxybutyrate
					4hbd	Q94B07	*Arabidopsis thaliana*	4-hydroxybutyrate
8A	1.1.1.c	succinyl-CoA	4-hydroxybutyrate	succinyl-CoA reductase (alcohol forming)	*adhE2*	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
					mcr	AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
					FAR	AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA

8A	2.8.3.a	4-hydroxybutyrate	4-hydroxybutyryl-CoA	4-hydroxybutyryl-CoA transferase	*cat1, cat2, cat3*	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
					gctA, gctB	CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
					atoA, atoD	P76459.1, P76458.1	*Escherichia coli*	butanoate
8A	3.1.2.a	4-hydroxybutyrate	4-hydroxybutyryl-CoA	4-hydroxybutyryl-CoA hydrolase	*tesB*	NP_414986	*Escherichia coli*	adipyl-CoA
					acot12	NP_570103.1	*Rattus norvegicus*	butyryl-CoA
					hibch	Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA
8A	6.2.1.a	4-hydroxybutyrate	4-hydroxybutyryl-CoA	4-hydroxybutyryl-CoA ligase (or 4-hydroxybutyryl-CoA synthetase)	*sucCD*	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
					phl	CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
					bioW	NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate

8A	2.7.2.a	4-hydroxybutyrate	4-hydroxybutyryl-phosphate	4-hydroxybutyrate kinase	*ackA*	NP_416799.1	*Escherichia coli*	acetate, propionate
					buk1	NP_349675	*Clostridium acetobutylicum*	butyrate
					buk2	Q97II1	*Clostridium acetobutylicum*	butyrate
8A	2.3.1.a	4-hydroxybutyryl-phosphate	4-hydroxybutyryl-CoA	phosphotrans-4-hydroxybutyrylase	*ptb*	NP_349676	*Clostridium acetobutylicum*	butyryl-phosphate
					ptb	AAR19757.1	*butyrate-producing bacterium L2-50*	butyryl-phosphate
					ptb	CAC07932.1	*Bacillus megaterium*	butyryl-phosphate
8A	1.2.1.d	4-hydroxybutyryl-phosphate	4-hydroxybutanal	4-hydroxybutanal dehydrogenase (phosphorylating)	*asd*	NP_417891.1	*Escherichia coli*	L-4-aspartyl-phosphate
					proA	NP_414778.1	*Escherichia coli*	L-glutamyl-5-phospate
					gapA	P0A9B2.2	*Escherichia coli*	Glyceraldehyde-3-phosphate

8A	1.2.1.b	4-hydroxybutyryl-CoA	4-hydroxybutanal	4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase)	*sucD*	P38947.1	*Clostridium kluyveri*	succinyl-CoA
					sucD	NP_904963.1	*Porphyromonas gingivalis*	succinyl-CoA
					Msed_0709	YP_001190808.1	*Metallosphaera sedula*	malonyl-CoA
8A	1.1.1.c	4-hydroxybutyryl-CoA	1,4-butanediol	4-hydroxybutyryl-CoA reductase (alcohol forming)	*adhE2*	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
					mcr	AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
					FAR	AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA
8A	1.1.1.a	4-hydroxybutanal	1,4-butanediol	1,4-butanediol dehydrogenase	*ADH2*	NP_014032.1	*Saccharymyces cerevisiae*	general
					yqhD	NP_417484.1	*Escherichia coli*	>C3
					4hbd	L21902.1	*Clostridium kluyveri DSM 555*	Succinate semialdehyde

EXAMPLE VI
Additional Exemplary BDO Pathways from Alpha-ketoglutarate

This example describes exemplary BDO pathways from alpha-ketoglutarate.

BDO pathways from succinyl-CoA are described herein and have been described previously (see U.S. application serial No. 12/049,256, filed March 14, 2008, and PCT application serial No. US08/57168, filed March 14, 2008, each of which is incorporated herein by reference). Additional pathways are shown in Figure 8B. Enzymes of such exemplary BDO pathways are listed in Table 16, along with exemplary genes encoding these enzymes.

Briefly, alpha-ketoglutarate can be converted to succinic semialdehyde by alpha-ketoglutarate decarboxylase (EC 4.1.1.a), as previously described. Alternatively, alpha-ketoglutarate can be converted to glutamate by glutamate dehydrogenase (EC 1.4.1.a). 4-Aminobutyrate can be converted to succinic semialdehyde by 4-aminobutyrate oxidoreductase (deaminating) (EC 1.4.1.a) or 4-aminobutyrate transaminase (EC 2.6.1.a). Glutamate can be converted to 4-aminobutyrate by glutamate decarboxylase (EC 4.1.1.a). Succinate semialdehyde can be converted to 4-hydroxybutyrate by 4-hydroxybutyrate dehydrogenase (EC 1.1.1.a), as previously described. 4-Hydroxybutyrate can be converted to 4-hydroxybutyryl-CoA by 4-hydroxybutyryl-CoA transferase (EC 2.8.3.a), as previously described, or by 4-hydroxybutyryl-CoA hydrolase (EC 3.1.2.a), or 4-hydroxybutyryl-CoA ligase (or 4-hydroxybutyryl-CoA synthetase) (EC 6.2.1.a). 4-Hydroxybutyrate can be converted to 4-hydroxybutyryl-phosphate by 4-hydroxybutyrate kinase (EC 2.7.2.a). 4-Hydroxybutyryl-phosphate can be converted to 4-hydroxybutyryl-CoA by phosphotrans-4-hydroxybutyrylase (EC 2.3.1.a), as previously described. Alternatively, 4-hydroxybutyryl-phosphate can be converted to 4-hydroxybutanal by 4-hydroxybutanal dehydrogenase (phosphorylating) (EC 1.2.1.d). 4-Hydroxybutyryl-CoA can be converted to 4-hydroxybutanal by 4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase) (EC 1.2.1.b), as previously described. 4-Hydroxybutyryl-CoA can be converted to 1,4-butanediol by 4-hydroxybutyryl-CoA reductase (alcohol forming) (EC 1.1.1.c). 4-Hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a), as previously described.

TABLE 16. BDO pathway from alpha-ketoglutarate.

Figure	EC class	Desired substrate	Desired product	Enzyme name	Gene name	GenBank ID (if available)	Organism	Known Substrates
8B	4.1.1.a	alpha-ketoglutarate	succinic semialdehyde	alpha-ketoglutarate decarboxylase	*kgd*	O50463.4	*Mycobacterium tuberculosis*	alpha-ketoglutarate
					gadA	NP_417974	*Escherichia coli*	glutamate
					gadB	NP_416010	*Escherichia coli*	glutamate
8B	1.4.1.a	alpha-ketoglutarate	glutamate	glutamate dehydrogenase	*gdhA*	P00370	*Escherichia coli*	glutamate
					gdh	P96110.4	*Thermotoga maritima*	glutamate
					gdhA1	NP_279651.1	*Halobacterium salinarum*	glutamate
8B	1.4.1.a	4-aminobutyrate	succinic semialdehyde	4-aminobutyrate oxidoreductase (deaminating)	*lysDH*	AB052732	*Geobacillus stearothermophilus*	lysine
					lysDH	NP_147035.1	*Aeropyrum pernix K1*	lysine
					ldh	P0A393	*Bacillus cereus*	leucine, isoleucine, valine, 2-aminobutanoate

8B	2.6.1.a	4-aminobutyrate	succinic semialdehyde	4-aminobutyrate transaminase	*gabT*	P22256.1	*Escherichia coli*	4-aminobutyryate
					puuE	NP_415818.1	*Escherichia coli*	4-aminobutyryate
					UGA1	NP_011533.1	*Saccharomyces cerevisiae*	4-aminobutyryate
8B	4.1.1.a	glutamate	4-aminobutyrate	glutamate decarboxylase	*gadA*	NP_417974	*Escherichia coli*	glutamate
					gadB	NP_416010	*Escherichia coli*	glutamate
					kgd	O50463.4	*Mycobacterium tuberculosis*	alpha-ketoglutarate
8B	1.1.1.a	succinate semialdehyde	4-hydroxybutyrate	4-hydroxybutyrate dehydrogenase	*4hbd*	YP_726053.1	*Ralstonia eutropha H16*	4-hydroxybutyrate
					4hbd	L21902.1	*Clostridium kluyveri DSM 555*	4-hydroxybutyrate
					4hbd	Q94B07	*Arabidopsis thaliana*	4-hydroxybutyrate
8B	2.8.3.a	4-hydroxybutyrate	4-hydroxybutyryl-CoA	4-hydroxybutyryl-CoA transferase	*cat1, cat2, cat3*	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
					gctA, gctB	CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
					atoA, atoD	P76459.1, P76458.1	*Escherichia coli*	butanoate

8B	3.1.2.a	4-hydroxybutyrate	4-hydroxybutyryl-CoA	4-hydroxybutyryl-CoA hydrolase	*tesB*	NP_414986	*Escherichia coli*	adipyl-CoA
					acot12	NP_570103.1	*Rattus norvegicus*	butyryl-CoA
					hibch	Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA
8B	6.2.1.a	4-hydroxybutyrate	4-hydroxybutyryl-CoA	4-hydroxybutyryl-CoA ligase (or 4-hydroxybutyryl-CoA synthetase)	*sucCD*	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
					phl	CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
					bioW	NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate
8B	2.7.2.a	4-hydroxybutyrate	4-hydroxybutyryl-phosphate	4-hydroxybutyrate kinase	*ackA*	NP_416799.1	*Escherichia coli*	acetate, propionate
					buk1	NP_349675	*Clostridium acetobutylicum*	butyrate
					buk2	Q97II1	*Clostridium acetobutylicum*	butyrate

8B	2.3.1.a	4-hydroxybutyryl-phosphate	4-hydroxybutyryl-CoA	phosphotrans-4-hydroxybutyrylase	*ptb*	NP_349676	*Clostridium acetobutylicum*	butyryl-phosphate
					ptb	AAR19757.1	*butyrate-producing bacterium L2-50*	butyryl-phosphate
					ptb	CAC07932.1	*Bacillus megaterium*	butyryl-phosphate
8B	1.2.1.d	4-hydroxybutyryl-phosphate	4-hydroxybutanal	4-hydroxybutanal dehydrogenase (phosphorylating)	*asd*	NP_417891.1	*Escherichia coli*	L-4-aspartyl-phosphate
					proA	NP_414778.1	*Escherichia coli*	L-glutamyl-5-phospate
					gapA	P0A9B2.2	*Escherichia coli*	Glyceraldehyde-3-phosphate
8B	1.2.1.b	4-hydroxybutyryl-CoA	4-hydroxybutanal	4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase)	*sucD*	P38947.1	*Clostridium kluyveri*	succinyl-CoA
					sucD	NP_904963.1	*Porphyromonas gingivalis*	succinyl-CoA
					Msed_0709	YP_001190808.1	*Metallosphaera sedula*	malonyl-CoA
8B	1.1.1.c	4-hydroxybutyryl-CoA	1,4-butanediol	4-hydroxybutyryl-CoA reductase (alcohol forming)	*adhE2*	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
					mcr	AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
					FAR	AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA

8B	1.1.1.a	4-hydroxybutanal	1,4-butanediol	1,4-butanediol dehydrogenase	*ADH2*	NP_014032.1	*Saccharymyces cerevisiae*	general
					yqhD	NP_417484.1	*Escherichia coli*	>C3
					4hbd	L21902.1	*Clostridium kluyveri DSM 555*	Succinate semialdehyde

EXAMPLE VII
BDO Pathways from 4-Aminobutyrate

This example describes exemplary BDO pathwayd from 4-aminobutyrate.

Figure 9A depicts exemplary BDO pathways in which 4-aminobutyrate is converted to BDO. Enzymes of such an exemplary BDO pathway are listed in Table 17, along with exemplary genes encoding these enzymes.

Briefly, 4-aminobutyrate can be converted to 4-aminobutyryl-CoA by 4-aminobutyrate CoA transferase (EC 2.8.3.a), 4-aminobutyryl-CoA hydrolase (EC 3.1.2.a), or 4-aminobutyrate-CoA ligase (or 4-aminobutyryl-CoA synthetase) (EC 6.2.1.a). 4-aminobutyryl-CoA can be converted to 4-oxobutyryl-CoA by 4-aminobutyryl-CoA oxidoreductase (deaminating) (EC 1.4.1.a) or 4-aminobutyryl-CoA transaminase (EC 2.6.1.a). 4-oxobutyryl-CoA can be converted to 4-hydroxybutyryl-CoA by 4-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.a). 4-hydroxybutyryl-CoA can be converted to 1,4-butanediol by 4-hydroxybutyryl-CoA reductase (alcohol forming) (EC 1.1.1.c). Alternatively, 4-hydroxybutyryl-CoA can be converted to 4-hydroxybutanal by 4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase) (EC 1.2.1.b). 4-hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a).

TABLE 17. BDO pathway from 4-aminobutyrate.

Figure	EC class	Desired substrate	Desired product	Enzyme name	Gene name	GenBank ID (if available)	Organism	Known Substrates
9A	2.8.3.a	4-aminobutyrate	4-aminobutyryl-CoA	4-aminobutyrate CoA transferase	*cat1, cat2, cat3*	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
					gctA, gctB	CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
					atoA, atoD	P76459.1, P76458.1	*Escherichia coli*	butanoate
9A	3.1.2.a	4-aminobutyrate	4-aminobutyryl-CoA	4-aminobutyryl-CoA hydrolase	*tesB*	NP_414986	*Escherichia coli*	adipyl-CoA
					acot12	NP_570103.1	*Rattus norvegicus*	butyryl-CoA
					hibch	Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA
9A	6.2.1.a	4-aminobutyrate	4-aminobutyryl-CoA	4-aminobutyrate-CoA ligase (or 4-aminobutyryl-CoA synthetase)	*sucCD*	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
					phl	CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
					bioW	NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate

Table 17 continued

9A	1.4.1.a	4-aminobutyryl-CoA	4-oxobutyryl-CoA	4-aminobutyryl-CoA oxidoreductase (deaminating)	*lysDH*	AB052732	*Geobacillus stearothermophilus*	lysine
					lysDH	NP_147035.1	*Aeropyrum pernix K1*	lysine
					ldh	P0A393	*Bacillus cereus*	leucine, isoleucine, valine, 2-aminobutanoate
9A	2.6.1.a	4-aminobutyryl-CoA	4-oxobutyryl-CoA	4-aminobutyryl-CoA transaminase	*gabT*	P22256.1	*Escherichia coli*	4-aminobutyryate
					abat	P50554.3	*Rattus norvegicus*	3-amino-2-methylpropionate
					SkyPYD4	ABF58893.1	*Saccharomyces kluyveri*	beta-alanine
9A	1.1.1.a	4-oxobutyryl-CoA	4-hydroxybutyryl-CoA	4-hydroxybutyryl-CoA dehydrogenase	*ADH2*	NP_014032.1	*Saccharymyces cerevisiae*	general
					yqhD	NP_417484.1	*Escherichia coli*	>C3
					4hbd	L21902.1	*Clostridium kluyveri DSM 555*	Succinate semialdehyde

Table 17 continued

8	1.1.1.c	4-hydroxybutyryl-CoA	1,4-butanediol	4-hydroxybutyryl-CoA reductase (alcohol forming)	*adhE2*	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
					mcr	AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
					FAR	AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA
8	1.2.1.b	4-hydroxybutyryl-CoA	4-hydroxybutanal	4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase)	*sucD*	P38947.1	*Clostridium kluyveri*	Succinyl-CoA
					sucD	NP_904963.1	*Porphyromonas gingivalis*	Succinyl-CoA
					Msed_0709	YP_001190808.1	*Metallosphaera sedula*	Malonyl-CoA
8	1.1.1.a	4-hydroxybutanal	1,4-butanediol	1,4-butanediol dehydrogenase	*ADH2*	NP_014032.1	*Saccharymyces cerevisiae*	general
					yqhD	NP_417484.1	*Escherichia coli*	>C3
					4hbd	L21902.1	*Clostridium kluyveri DSM 555*	Succinate semialdehyde

Enzymes for another exemplary BDO pathway converting 4-aminobutyrate to BDO is shown in Figure 9A. Enzymes of such an exemplary BDO pathway are listed in Table 18, along with exemplary genes encoding these enzymes.

Briefly, 4-aminobutyrate can be converted to 4-aminobutyryl-CoA by 4-aminobutyrate CoA transferase (EC 2.8.3.a), 4-aminobutyryl-CoA hydrolase (EC 3.1.2.a) or 4-aminobutyrate-CoA ligase (or 4-aminobutyryl-CoA synthetase) (EC 6.2.1.a). 4-aminobutyryl-CoA can be converted to 4-aminobutan-1-ol by 4-aminobutyryl-CoA reductase (alcohol forming) (EC 1.1.1.c). Alternatively, 4-aminobutyryl-CoA can be converted to 4-aminobutanal by 4-aminobutyryl-CoA reductase (or 4-aminobutanal dehydrogenase) (EC 1.2.1.b), and 4-aminobutanal converted to 4-aminobutan-1-ol by 4-aminobutan-1-ol dehydrogenase (EC 1.1.1.a). 4-aminobutan-1-ol can be converted to 4-hydroxybutanal by 4-aminobutan-1-ol oxidoreductase (deaminating) (EC 1.4.1.a) or 4-aminobutan-1-ol transaminase (EC 2.6.1.a). 4-hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a).

TABLE 18. BDO pathway from 4-aminobutyrate.

Figure	EC class	Desired substrate	Desired product	Enzyme name	Gene name	GenBank ID (if available)	Organism	Known Substrate
9A	2.8.3.a	4-aminobutyrate	4-aminobutyryl-CoA	4-aminobutyrate CoA transferase	*cat1, cat2, cat3*	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
					gctA, gctB	CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
					atoA, atoD	P76459.1, P76458.1	*Escherichia coli*	butanoate
9A	3.1.2.a	4-aminobutyrate	4-aminobutyryl-CoA	4-aminobutyryl-CoA hydrolase	*tesB*	NP_414986	*Escherichia coli*	adipyl-CoA
					acot12	NP_570103.1	*Rattus norvegicus*	butyryl-CoA
					hibch	Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA
9A	6.2.1.a	4-aminobutyrate	4-aminobutyryl-CoA	4-aminobutyrate-CoA ligase (or 4-aminobutyryl-CoA synthetase)	*sucCD*	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
					phl	CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
					bioW	NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate

Table 18 continued

9A	1.1.1.c	4-aminobutyryl-CoA	4-aminobutan-1-ol	4-aminobutyryl-CoA reductase (alcohol forming)	*adhE2*	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
					mcr	AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
					FAR	AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA
9A	1.2.1.b	4-aminobutyryl-CoA	4-aminobutanal	4-aminobutyryl-CoA reductase (or 4-aminobutanal dehydrogenase)	*sucD*	P38947.1	*Clostridium kluyveri*	Succinyl-CoA
					sucD	NP_904963.1	*Porphyromonas gingivalis*	Succinyl-CoA
					Msed_0709	YP_001190808.1	*Metallosphaera sedula*	Malonyl-CoA
9A	1.1.1.a	4-aminobutanal	4-aminobutan-1-ol	4-aminobutan-1-ol dehydrogenase	*ADH2*	NP_014032.1	*Saccharymyces cerevisiae*	general
					yqhD	NP_417484.1	*Escherichia coli*	>C3
					4hbd	L21902.1	*Clostridium kluyveri DSM 555*	Succinate semialdehyde

Table 18 continued

9A	1.4.1.a	4-aminobutan-1-ol	4-hydroxybutanal	4-aminobutan-1-ol oxidoreductase (deaminating)	*lysDH*	AB052732	*Geobacillus stearothermophilus*	lysine
					lysDH	NP_147035.1	*Aeropyrum pernix K1*	lysine
					ldh	P0A393	*Bacillus cereus*	leucine, isoleucine, valine, 2-aminobutanoate
9A	2.6.1.a	4-aminobutan-1-ol	4-hydroxybutanal	4-aminobutan-1-ol transaminase	*gabT*	P22256.1	*Escherichia coli*	4-aminobutyryate
					abat	P50554.3	*Rattus norvegicus*	3-amino-2-methylpropionate
					SkyPYD4	ABF58893.1	*Saccharomyces kluyveri*	beta-alanine
9A	1.1.1.a	4-hydroxybutanal	1,4-butanediol	1,4-butanediol dehydrogenase	*ADH2*	NP_014032.1	*Saccharymyces cerevisiae*	general
					yqhD	NP_417484.1	*Escherichia coli*	>C3
					4hbd	L21902.1	*Clostridium kluyveri DSM 555*	Succinate semialdehyde

Figure 9B depicts exemplary BDO pathway in which 4-aminobutyrate is converted to BDO. Enzymes of such an exemplary BDO pathway are listed in Table 19, along with exemplary genes encoding these enzymes.

Briefly, 4-aminobutyrate can be converted to [(4-aminobutanolyl)oxy] phosphonic acid by 4-aminobutyrate kinase (EC 2.7.2.a). [(4-aminobutanolyl)oxy] phosphonic acid can be converted to 4-aminobutanal by 4-aminobutyraldehyde dehydrogenase (phosphorylating) (EC 1.2.1.d). 4-aminobutanal can be converted to 4-aminobutan-1-ol by 4-aminobutan-1-ol dehydrogenase (EC 1.1.1.a). 4-aminobutan-1-ol can be converted to 4-hydroxybutanal by 4-aminobutan-1-ol oxidoreductase (deaminating) (EC 1.4.1.a) or 4-aminobutan-1-ol transaminase (EC 2.6.1.a). Alternatively, [(4-aminobutanolyl)oxy] phosphonic acid can be converted to [(4-oxobutanolyl)oxy] phosphonic acid by [(4-aminobutanolyl)oxy]phosphonic acid oxidoreductase (deaminating) (EC 1.4.1.a) or [(4-aminobutanolyl)oxy]phosphonic acid transaminase (EC 2.6.1.a). [(4-oxobutanolyl)oxy] phosphonic acid can be converted to 4-hydroxybutyryl-phosphate by 4-hydroxybutyryl-phosphate dehydrogenase (EC 1.1.1.a). 4-hydroxybutyryl-phosphate can be converted to 4-hydroxybutanal by 4-hydroxybutyraldehyde dehydrogenase (phosphorylating) (EC 1.2.1.d). 4-hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a).

TABLE 19. BDO pathway from 4-aminobutyrate.

Figure	EC class	Desired substrate	Desired product	Enzyme name	Gene name	GenBank ID (if available)	Organism	Known Substrate
9B	2.7.2.a	4-aminobutyrate	[(4-aminobutanoyl)oxy] phosphonic acid	4-aminobutyrate kinase	*ackA*	NP_416799.1	*Escherichia coli*	acetate, propionate
					buk1	NP_349675	*Clostridium acetobutylicum*	butyrate
					proB	NP_414777.1	*Escherichia coli*	glutamate
9B	1.2.1.d	[(4-aminobutanolyl)oxy] phosphonic acid	4-aminobutanal	4-aminobutyraldehyde dehydrogenase (phosphorylating)	*asd*	NP_417891.1	*Escherichia coli*	L-4-aspartyl-phosphate
					proA	NP_414778.1	*Escherichia coli*	L-glutamyl-5-phospate
					gapA	P0A9B2.2	*Escherichia coli*	Glyceraldehyde-3-phosphate
9B	1.1.1.a	4-aminobutanal	4-aminobutan-1-ol	4-aminobutan-1-ol dehydrogenase	*ADH2*	NP_014032.1	*Saccharymyces cerevisiae*	general
					yqhD	NP_417484.1	*Escherichia coli*	>C3
					4hbd	L21902.1	*Clostridium kluyveri DSM 555*	Succinate semialdehyde

Table 19 continued

9B	1.4.1.a	4-aminobutan-1-ol	4-hydroxybutanal	4-aminobutan-1-ol oxidoreductase (deaminating)	*lysDH*	AB052732	*Geobacillus stearothermophilus*	lysine
					lysDH	NP_147035.1	*Aeropyrum pernix K1*	lysine
					ldh	P0A393	*Bacillus cereus*	leucine, isoleucine, valine, 2-aminobutanoate
9B	2.6.1.a	4-aminobutan-1-ol	4-hydroxybutanal	4-aminobutan-1-ol transaminase	*gabT*	P22256.1	*Escherichia coli*	4-aminobutyryate
					abat	P50554.3	*Rattus norvegicus*	3-amino-2-methylpropionate
					SkyPYD4	ABF58893.1	*Saccharomyces kluyveri*	beta-alanine
9B	1.4.1.a	[(4-aminobutanolyl)oxy] phosphonic acid	[(4-oxobutanolyl)oxy] phosphonic acid	[(4-aminobutanolyl)oxy]phosphonic acid oxidoreductase (deaminating)	*lysDH*	AB052732	*Geobacillus stearothermophilus*	lysine
					lysDH	NP_147035.1	*Aeropyrum pernix K1*	lysine
					ldh	P0A393	*Bacillus cereus*	leucine, isoleucine, valine, 2-aminobutanoate

Table 19 continued

9B	2.6.1.a	[(4-aminobutanolyl)oxy] phosphonic acid	[(4-oxobutanolyl) oxy] phosphonic acid	[(4-aminobutanolyl)oxy]phosphonic acid transaminase	*gabT*	P22256.1	*Escherichia coli*	4-aminobutyryate
					SkyPYD4	ABF58893.1	*Saccharomyces kluyveri*	beta-alanine
					serC	NP_415427.1	*Escherichia coli*	phosphoserine, phosphohydroxythreonine
9B	1.1.1.a	[(4-oxobutanolyl)oxy] phosphonic acid	4-hydroxybutyryl-phosphate	4-hydroxybutyryl-phosphate dehydrogenase	*ADH2*	NP_014032.1	*Saccharymyces cerevisiae*	general
					yqhD	NP_417484.1	*Escherichia coli*	>C3
					4hbd	L21902.1	*Clostridium kluyveri DSM 555*	Succinate semialdehyde
9B	1.2.1.d	4-hydroxybutyryl-phosphate	4-hydroxybutanal	4-hydroxybutyraldehyde dehydrogenase (phosphorylating)	*asd*	NP_417891.1	*Escherichia coli*	L-4-aspartyl-phosphate
					proA	NP_414778.1	*Escherichia coli*	L-glutamyl-5-phospate
					gapA	P0A9B2.2	*Escherichia coli*	Glyceraldehyde-3-phosphate

Table 19 continued

9B	1.1.1.a	4-hydroxybutanal	1,4-butanediol	1,4-butanediol dehydrogenase	*ADH2*	NP_014032.1	*Saccharymyces cerevisiae*	general
					yqhD	NP_417484.1	*Escherichia coli*	>C3
					4hbd	L21902.1	*Clostridium kluyveri DSM 555*	Succinate semialdehyde

Figure 9C shows an exemplary pathway through acetoacetate.

EXAMPLE VIII
Exemplary BDO Pathways from Alpha-ketoglutarate

This example describes exemplary BDO pathways from alpha-ketoglutarate.

Figure 10 depicts exemplary BDO pathways in which alpha-ketoglutarate is converted to BDO. Enzymes of such an exemplary BDO pathway are listed in Table 20, along with exemplary genes encoding these enzymes.

Briefly, alpha-ketoglutarate can be converted to alpha-ketoglutaryl-phosphate by alpha-ketoglutarate 5-kinase (EC 2.7.2.a). Alpha-ketoglutaryl-phosphate can be converted to 2,5-dioxopentanoic acid by 2,5-dioxopentanoic semialdehyde dehydrogenase (phosphorylating) (EC 1.2.1.d). 2,5-dioxopentanoic acid can be converted to 5-hydroxy-2-oxopentanoic acid by 2,5-dioxopentanoic acid reductase (EC 1.1.1.a). Alternatively, alpha-ketoglutarate can be converted to alpha-ketoglutaryl-CoA by alpha-ketoglutarate CoA transferase (EC 2.8.3.a), alpha-ketoglutaryl-CoA hydrolase (EC 3.1.2.a) or alpha-ketoglutaryl-CoA ligase (or alpha-ketoglutaryl-CoA synthetase) (EC 6.2.1.a). Alpha-ketoglutaryl-CoA can be converted to 2,5-dioxopentanoic acid by alpha-ketoglutaryl-CoA reductase (or 2,5-dioxopentanoic acid dehydrogenase) (EC 1.2.1.b). 2,5-Dioxopentanoic acid can be converted to 5-hydroxy-2-oxopentanoic acid by 5-hydroxy-2-oxopentanoic acid dehydrogenase. Alternatively, alpha-ketoglutaryl-CoA can be converted to 5-hydroxy-2-oxopentanoic acid by alpha-ketoglutaryl-CoA reductase (alcohol forming) (EC 1.1.1.c). 5-hydroxy-2-oxopentanoic acid can be converted to 4-hydroxybutanal by 5-hydroxy-2-oxopentanoic acid decarboxylase (EC 4.1.1.a). 4-hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a). 5-hydroxy-2-oxopentanoic acid can be converted to 4-hydroxybutyryl-CoA by 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation) (EC 1.2.1.c).

TABLE 20. BDO pathway from alpha-ketoglutarate.

Figure	EC class	Desired substrate	Desired product	Enzyme name	Gene name	GenBank ID (if available)	Organism	Known Substrate
10	2.7.2.a	alpha-ketoglutarate	alpha-ketoglutaryl-phosphate	alpha-ketoglutarate 5-kinase	*ackA*	NP_416799.1	*Escherichia coli*	acetate, propionate
					buk1	NP_349675	*Clostridium acetobutylicum*	butyrate
					proB	NP_414777.1	*Escherichia coli*	glutamate
10	1.2.1.d	alpha-ketoglutaryl-phosphate	2,5-dioxopentanoic acid	2,5-dioxopentanoic semialdehyde dehydrogenase (phosphorylating)	*proA*	NP_414778.1	*Escherichia coli*	L-glutamyl-5-phospate
					asd	NP_417891.1	*Escherichia coli*	L-4-aspartyl-phosphate
					gapA	P0A9B2.2	*Escherichia coli*	Glyceraldehyde-3-phosphate
10	1.1.1.a	2,5-dioxopentanoic acid	5-hydroxy-2-oxopentanoic acid	2,5-dioxopentanoic acid reductase	*ADH2*	NP_014032.1	*Saccharymyces cerevisiae*	general
					yqhD	NP_417484.1	*Escherichia coli*	>C3
					4hbd	L21902.1	*Clostridium kluyveri DSM 555*	Succinate semialdehyde

Table 20 continued

10	2.8.3.a	alpha-ketoglutarate	alpha-ketoglutaryl-CoA	alpha-ketoglutarate CoA transferase	*cat1, cat2, cat3*	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
					gctA, gctB	CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
					atoA, atoD	P76459.1, P76458.1	*Escherichia coli*	butanoate
10	3.1.2.a	alpha-ketoglutarate	alpha-ketoglutaryl-CoA	alpha-ketoglutaryl-CoA hydrolase	*tesB*	NP_414986	*Escherichia coli*	adipyl-CoA
					acot12	NP_570103.1	*Rattus norvegicus*	butyryl-CoA
					hibch	Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA
10	6.2.1.a	alpha-ketoglutarate	alpha-ketoglutaryl-CoA	alpha-ketoglutaryl-CoA ligase (or alpha-ketoglutaryl-CoA synthetase)	*sucCD*	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
					phl	CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
					bioW	NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate

Table 20 continued

10	1.2.1.b	alpha-ketoglutaryl-CoA	2,5-dioxopentanoic acid	alpha-ketoglutaryl-CoA reductase (or 2,5-dioxopentanoic acid dehydrogenase)	*sucD*	P38947.1	*Clostridium kluyveri*	Succinyl-CoA
					Msed_0709	YP_001190808.1	*Metallosphaera sedula*	Malonyl-CoA
					bphG	BAA03892.1	*Pseudomonas sp*	Acetaldehyde, Propionaldehyde, Butyraldehyde, Isobutyraldehyde and Formaldehyde
10	1.1.1.a	2,5-dioxopentanoic acid	5-hydroxy-2-oxopentanoic acid	5-hydroxy-2-oxopentanoic acid dehydrogenase	*ADH2* *yqhD* *4hbd*	NP_014032.1 NP_417484.1 L21902.1	*Saccharymyces cerevisiae* *Escherichia coli* *Clostridium kluyveri DSM 555*	general >C3 Succinate semialdehyde
10	1.1.1.c	alpha-ketoglutaryl-CoA	5-hydroxy-2-oxopentanoic acid	alpha-ketoglutaryl-CoA reductase (alcohol forming)	*adhE2*	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
					mcr	AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
					FAR	AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA

10	4.1.1.a	5-hydroxy-2-oxopentanoic acid	4-hydroxybutanal	5-hydroxy-2-oxopentanoic acid decarboxylase	*pdc*	P06672.1	*Zymomonas mobilus*	2-oxopentanoic acid
					mdlC	P20906.2	*Pseudomonas putida*	2-oxopentanoic acid
					pdc1	P06169	*Saccharomyces cerevisiae*	pyruvate
10	1.1.1.a	4-hydroxybutana l	1,4-butanediol	1,4-butanediol dehydrogenase	*ADH2*	NP_014032.1	*Saccharymyces cerevisiae*	general
					yqhD	NP_417484.1	*Escherichia coli*	>C3
					4hbd	L21902.1	*Clostridium kluyveri DSM 555*	Succinate semialdehyde
10	1.2.1.c	5-hydroxy-2-oxopentanoic acid	4-hydroxybutyryl -CoA	5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylatio n)	*sucA, sucB, lpd*	NP_415254.1, NP_415255.1, NP_414658.1	*Escherichia coli*	Alpha-ketoglutarate
					bfmBB, bfmBAA, bfmBAB, bfmBAB, pdhD	NP_390283.1, NP_390285.1, NP_390284.1, P21880.1	*Bacillus subtilis*	2-keto acids derivatives of valine, leucine and isoleucine
					Bckdha, Bckdhb, Dbt, Dld	NP_036914.1, NP_062140.1, NP_445764.1, NP_955417.1	*Rattus norvegicus*	2-keto acids derivatives of valine, leucine and isoleucine

EXAMPLE IX
Exemplary BDO Pathways from Glutamate

This example describes exemplary BDO pathways from glutamate.

Figure 11 depicts exemplary BDO pathways in which glutamate is converted to BDO. Enzymes of such an exemplary BDO pathway are listed in Table 21, along with exemplary genes encoding these enzymes.

Briefly, glutamate can be converted to glutamyl-CoA by glutamate CoA transferase (EC 2.8.3.a), glutamyl-CoA hydrolase (EC 3.1.2.a) or glutamyl-CoA ligase (or glutamyl-CoA synthetase) (EC 6.2.1.a). Alternatively, glutamate can be converted to glutamate-5-phosphate by glutamate 5-kinase (EC 2.7.2.a). Glutamate-5-phosphate can be converted to glutamate-5-semialdehyde by glutamate-5-semialdehyde dehydrogenase (phosphorylating) (EC 1.2.1.d). Glutamyl-CoA can be converted to glutamate-5-semialdehyde by glutamyl-CoA reductase (or glutamate-5-semialdehyde dehydrogenase) (EC 1.2.1.b). Glutamate-5-semialdehyde can be converted to 2-amino-5-hydroxypentanoic acid by glutamate-5-semialdehyde reductase (EC 1.1.1.a). Alternatively, glutamyl-CoA can be converted to 2-amino-5-hydroxypentanoic acid by glutamyl-CoA reductase (alcohol forming) (EC 1.1.1.c). 2-Amino-5-hydroxypentanoic acid can be converted to 5-hydroxy-2-oxopentanoic acid by 2-amino-5-hydroxypentanoic acid oxidoreductase (deaminating) (EC 1.4.1.a) or 2-amino-5-hydroxypentanoic acid transaminase (EC 2.6.1.a). 5-Hydroxy-2-oxopentanoic acid can be converted to 4-hydroxybutanal by 5-hydroxy-2-oxopentanoic acid decarboxylase (EC 4.1.1.a). 4-Hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a). Alternatively, 5-hydroxy-2-oxopentanoic acid can be converted to 4-hydroxybutyryl-CoA by 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation) (EC 1.2.1.c).

TABLE 21. BDO pathway from glutamate.

Figure	EC class	Desired substrate	Desired product	Enzyme name	Gene name	GenBank ID (if available)	Organism	Known Substrate
11	2.8.3.a	glutamate	glutamyl-CoA	glutamate CoA transferase	*cat1, cat2, cat3*	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
					gctA, gctB	CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
					atoA, atoD	P76459.1, P76458.1	*Escherichia coli*	butanoate
11	3.1.2.a	glutamate	glutamyl-CoA	glutamyl-CoA hydrolase	*tesB*	NP_414986	*Escherichia coli*	adipyl-CoA
					acot12	NP_570103.1	*Rattus norvegicus*	butyryl-CoA
					hibch	Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA
11	6.2.1.a	glutamate	glutamyl-CoA	glutamyl-CoA ligase (or glutamyl-CoA synthetase)	*sucCD*	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
					phl	CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
					bioW	NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate
11	2.7.2.a	glutamate	glutamate-5-phosphate	glutamate 5-kinase	*ackA*	NP_416799.1	*Escherichia coli*	acetate, propionate
					buk1	NP_349675	*Clostridium acetobutylicum*	butyrate
					proB	NP_414777.1	*Escherichia coli*	glutamate

Table 21 continued

11	1.2.1.d	glutamate-5-phosphate	glutamate-5-semialdehyde	glutamate-5-semialdehyde dehydrogenase (phosphorylating)	*proA*	NP_414778.1	*Escherichia coli*	L-glutamyl-5-phospate
					asd	NP_417891.1	*Escherichia coli*	L-4-aspartyl-phosphate
					gapA	P0A9B2.2	*Escherichia coli*	Glyceraldehyde-3-phosphate
11	1.2.1.b	glutamyl-CoA	glutamate-5-semialdehyde	glutamyl-CoA reductase (or glutamate-5-semialdehyde dehydrogenase)	*sucD*	P38947.1	*Clostridium kluyveri*	Succinyl-CoA
					Msed_0709	YP_001190808.1	*Metallosphaera sedula*	Malonyl-CoA
					bphG	BAA03892.1	*Pseudomonas sp*	Acetaldehyde, Propionaldehyde, Butyraldehyde, Isobutyraldehyde and Formaldehyde
11	1.1.1.a	glutamate-5-semialdehyde	2-amino-5-hydroxypentanoic acid	glutamate-5-semialdehyde reductase	*ADH2*	NP_014032.1	*Saccharymyces cerevisiae*	general
					yqhD	NP_417484.1	*Escherichia coli*	>C3
					4hbd	L21902.1	*Clostridium kluyveri DSM 555*	Succinate semialdehyde

Table 21 continued

11	1.1.1.c	glutamyl-CoA	2-amino-5-hydroxypentanoic acid	glutamyl-CoA reductase (alcohol forming)	*adhE2*	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
					mcr	AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
					FAR	AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA
11	1.4.1.a	2-amino-5-hydroxypentanoic acid	5-hydroxy-2-oxopentanoic acid	2-amino-5-hydroxypentanoic acid oxidoreductase (deaminating)	*gdhA*	P00370	*Escherichia coli*	glutamate
					ldh	P0A393	*Bacillus cereus*	leucine, isoleucine, valine, 2-aminobutanoate
					nadX	NP_229443.1	*Thermotoga maritima*	aspartate
11	2.6.1.a	2-amino-5-hydroxypentanoic acid	5-hydroxy-2-oxopentanoic acid	2-amino-5-hydroxypentanoic acid transaminase	*aspC*	NP_415448.1	*Escherichia coli*	aspartate
					AAT2	P23542.3	*Saccharomyces cerevisiae*	aspartate
					avtA	YP_026231.1	*Escherichia coli*	valine, alpha-aminobutyrate

Table 21 continued

11	4.1.1.a	5-hydroxy-2-oxopentanoic acid	4-hydroxybutanal	5-hydroxy-2-oxopentanoic acid decarboxylase	*pdc*	P06672.1	*Zymomonas mobilus*	2-oxopentanoic acid
					mdlC	P20906.2	*Pseudomonas putida*	2-oxopentanoic acid
					pdc1	P06169	*Saccharomyces cerevisiae*	pyruvate
11	1.1.1.a	4-hydroxybutanal	1,4-butanediol	1,4-butanediol dehydrogenase	*ADH2*	NP_014032.1	*Saccharymyces cerevisiae*	general
					yqhD	NP_417484.1	*Escherichia coli*	>C3
					4hbd	L21902.1	*Clostridium kluyveri DSM 555*	Succinate semialdehyde
11	1.2.1.c	5-hydroxy-2-oxopentanoic acid	4-hydroxybutyryl-CoA	5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation)	*sucA, sucB, lpd*	NP_415254.1, NP_415255.1, NP_414658.1	*Escherichia coli*	Alpha-ketoglutarate
					bfmBB, bfmBAA, bfmBAB, bfmBAB, pdhD	NP_390283.1, NP_390285.1, NP_390284.1, P21880.1	*Bacillus subtilis*	2-keto acids derivatives of valine, leucine and isoleucine
					Bckdha, Bckdhb, Dbt, Dld	NP_036914.1, NP_062140.1, NP_445764.1, NP_955417.1	*Rattus norvegicus*	2-keto acids derivatives of valine, leucine and isoleucine

EXAMPLE X
Exemplary BDO from Acetoacetyl-CoA

This example describes an exemplary BDO pathway from acetoacetyl-CoA.

Figure 12 depicts exemplary BDO pathways in which acetoacetyl-CoA is converted to BDO. Enzymes of such an exemplary BDO pathway are listed in Table 22, along with exemplary genes encoding these enzymes.

Briefly, acetoacetyl-CoA can be converted to 3-hydroxybutyryl-CoA by 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.a). 3-Hydroxybutyryl-CoA can be converted to crotonoyl-CoA by 3-hydroxybutyryl-CoA dehydratase (EC 4.2.1.a). Crotonoyl-CoA can be converted to vinylacetyl-CoA by vinylacetyl-CoA Δ-isomerase (EC 5.3.3.3). Vinylacetyl-CoA can be converted to 4-hydroxybutyryl-CoA by 4-hydroxybutyryl-CoA dehydratase (EC 4.2.1.a). 4-Hydroxybutyryl-CoA can be converted to 1,4-butanediol by 4-hydroxybutyryl-CoA reductase (alcohol forming) (EC 1.1.1.c). Alternatively, 4-hydroxybutyryl-CoA can be converted to 4-hydroxybutanal by 4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase) (EC 1.2.1.b). 4-Hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a).

TABLE 22. BDO pathway from acetoacetyl-CoA.

Figure	EC class	Desired substrate	Desired product	Enzyme name	Gene name	GenBank ID (if available)	Organism	Known Substrate
12	1.1.1.a	acetoacetyl-CoA	3-hydroxybutyryl-CoA	3-hydroxybutyryl-CoA dehydrogenase	*hbd*	NP_349314.1	*Clostridium acetobutylicum*	3-hydroxybutyryl-CoA
					hbd	AAM14586.1	*Clostridium beijerinckii*	3-hydroxybutyryl-CoA
					Msed_1423	YP_001191505	*Metallosphaera sedula*	presumed 3-hydroxybutyryl-CoA
12	4.2.1.a	3-hydroxybutyryl-CoA	crotonoyl-CoA	3-hydroxybutyryl-CoA dehydratase	*crt*	NP_349318.1	*Clostridium acetobutylicum*	3-hydroxybutyryl-CoA
					maoC	NP_415905.1	*Escherichia coli*	3-hydroxybutyryl-CoA
					paaF	NP_415911.1	*Escherichia coli*	3-hydroxyadipyl-CoA
12	5.3.3.3	crotonoyl-CoA	vinylacetyl-CoA	vinylacetyl-CoA Δ-isomerase	*abfD*	YP_001396399.1	*Clostridium kluyveri DSM 555*	4-hydroxybutyryl-CoA
					abfD	P55792	*Clostridium aminobutyricum*	4-hydroxybutyryl-CoA
					abfD	YP_001928843	*Porphyromonas gingivalis ATCC 33277*	4-hydroxybutyryl-CoA
12	4.2.1.a	vinylacetyl-CoA	4-hydroxybutyryl-CoA	4-hydroxybutyryl-CoA dehydratase	*abfD*	YP_001396399.1	*Clostridium kluyveri DSM 555*	4-hydroxybutyryl-CoA

Table 22 continued

					abfD	P55792	*Clostridium aminobutyricum*	4-hydroxybutyryl-CoA
					abfD	YP_001928843	*Porphyromonas gingivalis ATCC 33277*	4-hydroxybutyryl-CoA
12	1.1.1.c	4-hydroxybutyryl-CoA	1,4-butanediol	4-hydroxybutyryl-CoA reductase (alcohol forming)	*adhE2*	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
					mcr	AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
					FAR	AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA
12	1.2.1.b	4-hydroxybutyryl-CoA	4-hydroxybutanal	4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase)	*sucD*	P38947.1	*Clostridium kluyveri*	Succinyl-CoA
					sucD	NP_904963.1	*Porphyromonas gingivalis*	Succinyl-CoA
					Msed_0709	YP_001190808.1	*Metallosphaera sedula*	Malonyl-CoA
12	1.1.1.a	4-hydroxybutanal	1,4-butanediol	1,4-butanediol dehydrogenase	*ADH2*	NP_014032.1	*Saccharymyces cerevisiae*	general
					yqhD	NP_417484.1	*Escherichia coli*	>C3
					4hbd	L21902.1	*Clostridium kluyveri DSM 555*	Succinate semialdehyde

EXAMPLE XI
Exemplary BDO Pathway from Homoserine

This example describes an exemplary BDO pathway from homoserine.

Figure 13 depicts exemplary BDO pathways in which homoserine is converted to BDO. Enzymes of such an exemplary BDO pathway are listed in Table 23, along with exemplary genes encoding these enzymes.

Briefly, homoserine can be converted to 4-hydroxybut-2-enoate by homoserine deaminase (EC 4.3.1.a). Alternatively, homoserine can be converted to homoserine-CoA by homoserine CoA transferase (EC 2.8.3.a), homoserine-CoA hydrolase (EC 3.1.2.a) or homoserine-CoA ligase (or homoserine-CoA synthetase) (EC 6.2.1.a). Homoserine-CoA can be converted to 4-hydroxybut-2-enoyl-CoA by homoserine-CoA deaminase (EC 4.3.1.a). 4-Hydroxybut-2-enoate can be converted to 4-hydroxybut-2-enoyl-CoA by 4-hydroxybut-2-enoyl-CoA transferase (EC 2.8.3.a), 4-hydroxybut-2-enoyl-CoA hydrolase (EC 3.1.2.a), or 4-hydroxybut-2-enoyl-CoA ligase (or 4-hydroxybut-2-enoyl-CoA synthetase) (EC 6.2.1.a). Alternatively, 4-hydroxybut-2-enoate can be converted to 4-hydroxybutyrate by 4-hydroxybut-2-enoate reductase (EC 1.3.1.a). 4-Hydroxybutyrate can be converted to 4-hydroxybutyryl-coA by 4-hydroxybutyryl-CoA transferase (EC 2.8.3.a), 4-hydroxybutyryl-CoA hydrolase (EC 3.1.2.a), or 4-hydroxybutyryl-CoA ligase (or 4-hydroxybutyryl-CoA synthetase) (EC 6.2.1.a). 4-Hydroxybut-2-enoyl-CoA can be converted to 4-hydroxybutyryl-CoA by 4-hydroxybut-2-enoyl-CoA reductase (EC 1.3.1.a). 4-Hydroxybutyryl-CoA can be converted to 1,4-butanediol by 4-hydroxybutyryl-CoA reductase (alcohol forming) (EC 1.1.1.c). Alternatively, 4-hydroxybutyryl-CoA can be converted to 4-hydroxybutanal by 4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase) (EC 1.2.1.b). 4-Hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a).

TABLE 23. BDO pathway from homoserine.

Figure	EC class	Desired substrate	Desired product	Enzyme name	Gene name	GenBank ID (if available)	Organism	Known Substrate
13	4.3.1.a	homoserine	4-hydroxybut-2-enoate	homoserine deaminase	*aspA*	NP_418562	*Escherichia coli*	aspartate
					aspA	P44324.1	*Haemophilus influenzae*	aspartate
					aspA	P07346	*Pseudomonas fluorescens*	aspartate
13	2.8.3.a	homoserine	homoserine-CoA	homoserine CoA transferase	*cat1, cat2, cat3*	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
					gctA, gctB	CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
					atoA, atoD	P76459.1, P76458.1	*Escherichia coli*	butanoate
13	3.1.2.a	homoserine	homoserine-CoA	homoserine-CoA hydrolase	*tesB*	NP_414986	*Escherichia coli*	adipyl-CoA
					acot12	NP_570103.1	*Rattus norvegicus*	butyryl-CoA
					hibch	Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA

Table 23 continued

13	6.2.1.a	homoserine	homoserine-CoA	homoserine-CoA ligase (or homoserine-CoA synthetase)	*sucCD*	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
					phl	CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
					bioW	NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate
13	4.3.1.a	homoserine-CoA	4-hydroxybut-2-enoyl-CoA	homoserine-CoA deaminase	*acl1*	CAG29274.1	*Clostridium propionicum*	beta-alanyl-CoA
					acl2	CAG29275.1	*Clostridium propionicum*	beta-alanyl-CoA
					MXAN_4385	YP_632558.1	*Myxococcus xanthus*	beta-alanyl-CoA
13	2.8.3.a	4-hydroxybut-2-enoate	4-hydroxybut-2-enoyl-CoA	4-hydroxybut-2-enoyl-CoA transferase	*cat1, cat2, cat3*	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
					gctA, gctB	CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
					atoA, atoD	P76459.1, P76458.1	*Escherichia coli*	butanoate

Table 23 continued

13	3.1.2.a	4-hydroxybut-2-enoate	4-hydroxybut-2-enoyl-CoA	4-hydroxybut-2-enoyl-CoA hydrolase	*tesB*	NP_414986	*Escherichia coli*	adipyl-CoA
					acot12	NP_570103.1	*Rattus norvegicus*	butyryl-CoA
					hibch	Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA
13	6.2.1.a	4-hydroxybut-2-enoate	4-hydroxybut-2-enoyl-CoA	4-hydroxybut-2-enoyl-CoA ligase (or 4-hydroxybut-2-enoyl-CoA synthetase)	*sucCD*	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
					phl	CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
					bioW	NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate
13	1.3.1.a	4-hydroxybut-2-enoate	4-hydroxybutyrate	4-hydroxybut-2-enoate reductase	*enr*	CAA71086.1	*Clostridium tyrobutyricum*	
					enr	CAA76083.1	*Clostridium kluyveri*	
					enr	YP_430895.1	*Moorella thermoacetica*	

Table 23 continued

13	2.8.3.a	4-hydroxybutyrate	4-hydroxybutyryl-coA	4-hydroxybutyryl-CoA transferase	*cat1, cat2, cat3*	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
					gctA, gctB	CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
					atoA, atoD	P76459.1, P76458.1	*Escherichia coli*	butanoate
13	3.1.2.a	4-hydroxybutyrate	4-hydroxybutyryl-coA	4-hydroxybutyryl-CoA hydrolase	*tesB*	NP_414986	*Escherichia coli*	adipyl-CoA
					acot12	NP_570103.1	*Rattus norvegicus*	butyryl-CoA
					hibch	Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA
13	6.2.1.a	4-hydroxybutyrate	4-hydroxybutyryl-coA	4-hydroxybutyryl-CoA ligase (or 4-hydroxybutyryl-CoA synthetase)	*sucCD*	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
					phl	CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
					bioW	NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate

Table 23 continued

13	1.3.1.a	4-hydroxybut-2-enoyl-CoA	4-hydroxybutyryl-CoA	4-hydroxybut-2-enoyl-CoA reductase	*bcd, etfA, etfB*	NP_349317.1, NP_349315.1, NP_349316.1	*Clostridium acetobutylicum*	
					TER	Q5EU90.1	*Euglena gracilis*	
					TDE0597	NP_971211.1	*Treponema denticola*	
8	1.1.1.c	4-hydroxybutyryl-CoA	1,4-butanediol	4-hydroxybutyryl-CoA reductase (alcohol forming)	*adhE2*	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
					mcr	AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
					FAR	AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA
8	1.2.1.b	4-hydroxybutyryl-CoA	4-hydroxybutanal	4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase)	*sucD*	P38947.1	*Clostridium kluyveri*	Succinyl-CoA
					sucD	NP_904963.1	*Porphyromonas gingivalis*	Succinyl-CoA
					Msed_0709	YP_001190808.1	*Metallosphaera sedula*	Malonyl-CoA
8	1.1.1.a	4-hydroxybutanal	1,4-butanediol	1,4-butanediol dehydrogenase	*ADH2*	NP_014032.1	*Saccharymyces cerevisiae*	general
					yqhD	NP_417484.1	*Escherichia coli*	>C3
					4hbd	L21902.1	*Clostridium kluyveri DSM 555*	Succinate semialdehyde

EXAMPLE XII
BDO Producing Strains Expressing Succinyl-CoA Synthetase

This example desribes increased production of BDO in BDO producing strains expressing succinyl-CoA synthetase.

As discussed above, succinate can be a precursor for production of BDO by conversion to succinyl-CoA (see also WO2008/115840, WO 2009/023493, U.S. publication 2009/0047719, U.S. publication 2009/0075351). Therefore, the host strain was genetically modified to overexpress the *E. coli sucCD* genes, which encode succinyl-CoA synthetase. The nucleotide sequence of the *E. coli sucCD* operon is shown in Figure 14A, and the amino acid sequences for the encoded succinyl-CoA synthetase subunits are shown in Figures 14B and 14C. Briefly, the *E. coli sucCD* genes were cloned by PCR from *E. coli* chromosomal DNA and introduced into multicopy plasmids pZS*13, pZA13, and pZE33 behind the PA1lacO-1 promoter (Lutz and Bujard, *Nucleic Acids Res.* 25:1203-1210 (1997)) using standard molecular biology procedures.

The *E. coli sucCD* genes, which encode the succinyl-CoA synthetase, were overexpressed. The results showed that introducing into the strains *sucCD* to express succinyl-CoA synthetase improved BDO production in various strains compared to either native levels of expression or expression of *cat1*, which is a succinyl-CoA/acetyl-CoA transferase. Thus, BDO production was improved by overexpressing the native *E. coli sucCD* genes encoding succinyl-CoA synthetase.

EXAMPLE XIII
Expression of Heterologous Genes Encoding BDO Pathway Enzymes

This example describes the expression of various non-native pathway enzymes to provide improved production of BDO.

Alpha-ketoglutarate decarboxylase. The *Mycobacterium bovis sucA* gene encoding alpha-ketoglutarate decarboxylase was expressed in host strains. Overexpression of *M. bovis sucA* improved BDO production (see also WO2008/115840, WO 2009/023493, U.S. publication 2009/0047719, U.S. publication 2009/0075351). The nucleotide and amino acid sequences of *M. bovis sucA* and the encoded alpha-ketoglutarate decarboxylase are shown in Figure 15.

To construct the *M. bovis sucA* expressing strains, fragments of the *sucA* gene encoding the alpha-ketoglutarate decarboxylase were amplified from the genomic DNA of *Mycobacterium bovis* BCG (ATCC 19015; American Type Culture Collection, Manassas VA) using primers

shown below. The full-length gene was assembled by ligation reaction of the four amplified DNA fragments, and cloned into expression vectors pZS*13 and pZE23 behind the $P_{A1lacO-1}$ promoter (Lutz and Bujard, *Nucleic Acids Res.* 25:1203-1210 (1997)). The nucleotide sequence of the assembled gene was verified by DNA sequencing.

Primers for fragment 1:

5'-ATGTACCGCAAGTTCCGC-3' (SEQ ID NO:)

5'-CAATTTGCCGATGCCCAG-3' (SEQ ID NO:)

Primers for fragment 2:

5'-GCTGACCACTGAAGACTTTG-3' (SEQ ID NO:)

5'-GATCAGGGCTTCGGTGTAG-3' (SEQ ID NO:)

Primers for fragment 3:

5'-TTGGTGCGGGCCAAGCAGGATCTGCTC-3' (SEQ ID NO:)

5'-TCAGCCGAACGCCTCGTCGAGGATCTCCTG-3' (SEQ ID NO:)

Primers for fragment 4:

5'-TGGCCAACATAAGTTCACCATTCGGGCAAAAC-3' (SEQ ID NO:)

5'-TCTCTTCAACCAGCCATTCGTTTTGCCCG-3' (SEQ ID NO:)

Functional expression of the alpha-ketoglutarate decarboxylase was demonstrated using both *in vitro* and *in vivo* assays. The SucA enzyme activity was measured by following a previously reported method (Tian et al., *Proc. Natl. Acad. Sci. USA* 102:10670-10675 (2005)). The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, 0.2 mM thiamine pyrophosphate, 1 mM $MgCl_2$, 0.8 mM ferricyanide, 1 mM alpha-ketoglutarate and cell crude lysate. The enzyme activity was monitored by the reduction of ferricyanide at 430 nm. The *in vivo* function of the SucA enzyme was verified using *E. coli* whole-cell culture. Single colonies of *E. coli* MG1655 $lacI^q$ transformed with plasmids encoding the SucA enzyme and the 4-hydroxybutyrate dehydrogenase (4Hbd) was inoculated into 5 mL of LB medium containing appropriate antibiotics. The cells were cultured at 37°C overnight aerobically. A 200 uL of this overnight culture was introduced into 8 mL of M9 minimal medium (6.78 g/L Na_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 20 g/L glucose, 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS) to improve the buffering capacity, 10 μg/mL thiamine, and the appropriate antibiotics. Microaerobic conditions were

established by initially flushing capped anaerobic bottles with nitrogen for 5 minutes, then piercing the septum with a 23G needle following inoculation. The needle was kept in the bottle during growth to allow a small amount of air to enter the bottles. The protein expression was induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the culture reached mid-log growth phase. As controls, *E. coli* MG1655 lacI^q strains transformed with only the plasmid encoding the 4-hydroxybutyrate dehydrogenase and only the empty vectors were cultured under the same condition (see Table 23). The accumulation of 4-hydroxybutyrate (4HB) in the culture medium was monitored using LCMS method. Only the *E. coli* strain expressing the *Mycobacterium* alpha-ketoglutarate decarboxylase produced significant amount of 4HB (see Figure 16).

Table 24. Three strains containing various plasmid controls and encoding *sucA* and 4-hydroxybutyrate dehydrogenase.

	Host	pZE13	pZA33
1	MG1655 laclq	vector	vector
2	MG1655 laclq	vector	4hbd
3	MG1655 laclq	sucA	4hbd

A separate experiment demonstrated that the alpha-ketoglutarate decarboxylase pathway functions independently of the reductive TCA cycle. *E. coli* strain ECKh-401 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA) was used as the host strain (see Table 25). All the three constructs contained the gene encoding 4HB dehydrogenase (4Hbd). Construct 1 also contained the gene encoding the alpha-ketoglutarate decarboxylase (*sucA*). Construct 2 contained the genes encoding the succinyl-CoA synthetase (*sucCD*) and the CoA-dependent succinate semialdehyde dehydrogenase (*sucD*), which are required for the synthesis of 4HB via the reductive TCA cycle. Construct 3 contains all the genes from 1 and 2. The three *E. coli* strains were cultured under the same conditions as described above except the second culture was under the micro-aerobic condition. By expressing the SucA enzyme, construct 3 produced more 4HB than construct 2, which relies on the reductive TCA cycle for 4HB synthesis (see Figure 17).

Further support for the contribution of alpha-ketoglutarate decarboxylase to production of 4HB and BDO was provided by flux analysis experiments. Cultures of ECKh-432, which contains both *sucCD-sucD* and *sucA* on the chromosome, were grown in M9 minimal medium containing a mixture of 1-13C-glucose (60%) and U-13C-glucose (40%). The biomass was harvested, the protein isolated and hydrolyzed to amino acids, and the label distribution of the amino acids

analyzed by gas chromatography-mass spectrometry (GCMS) as described previously (Fischer and Sauer, *Eur. J. Biochem.* 270:880-891 (2003)). In addition, the label distribution of the secreted 4HB and BDO was analyzed by GCMS as described in WO2008115840 A2. This data was used to calculate the intracellular flux distribution using established methods (Suthers et al., *Metab. Eng.* 9:387-405 (2007)). The results indicated that between 56% and 84% of the alpha-ketoglutarate was channeled through alpha-ketoglutarate decarboxylase into the BDO pathway. The remainder was oxidized by alpha-ketoglutarate dehydrogenase, which then entered BDO via the succinyl-CoA route.

These results demonstrate 4-hydroxybutyrate producing strains that contain the *sucA* gene from *Mycobacterium bovis* BCG expressed on a plasmid. When the plasmid encoding this gene is not present, 4-hydroxybutyrate production is negligible when *sucD* (CoA-dependant succinate semialdehyde dehydrogenase) is not expressed. The *M. bovis* gene is a close homolog of the *Mycobacterium tuberculosis* gene whose enzyme product has been previously characterized (Tian et al., *supra*, 2005).

Succinate semialdehyde dehydrogenase (CoA-dependent), 4-hydroxybutyrate dehydrogenase, and 4-hydroxybutyryl-CoA/acetyl-CoA transferase. The genes from *Porphyromonas gingivalis* W83 can be effective components of the pathway for 1,4-butanediol production (see also WO2008/115840, WO 2009/023493, U.S. publication 2009/0047719, U.S. publication 2009/0075351). The nucleotide sequence of CoA-dependent succinate semialdehyde dehydrogenase (*sucD*) from *Porphyromonas gingivalis* is shown in Figure 18A, and the encoded amino acid sequence is shown in Figure 18B. The nucleotide sequence of 4-hydroxybutyrate dehydrogenase (*4hbd*) from *Porphymonas gingivalis* is shown in Figure 19A, and the encoded amino acid seqence is shown in Figure 19B. The nucleotide sequence of 4-hydroxybutyrate CoA transferase (*cat2*) from *Porphyromonas gingivalis* is shown in Figure 20A, and the encoded amino acid sequence is shown in Figure 20B.

Briefly, the genes from *Porphyromonas gingivalis* W83 encoding succinate semialdehyde dehydrogenase (CoA-dependent) and 4-hydroxybutyrate dehydrogenase, and in some cases additionally 4-hydroxybutyryl-CoA/acetyl-CoA, were cloned by PCR from *P. gingivalis* chromosomal DNA and introduced into multicopy plasmids pZS*13, pZA13, and pZE33 behind the PA1lacO-1 promoter (Lutz and Bujard, *Nucleic Acids Res.* 25:1203-1210 (1997)) using standard molecular biology procedures. These plasmids were then introduced into host strains.

The *Porphyromonas gingivalis* W83 genes were introduced into production strains as described above. Some strains included only succinate semialdehyde dehydrogenase (CoA-dependant) and 4-hydroxybutyrate dehydrogenase without 4-hydroxybutyryl-CoA/acetyl-CoA transferase.

Butyrate kinase and phosphotransbutyrylase. Butyrate kinase (BK) and phosphotransbutyrylase (PTB) enzymes can be utlized to produce 4-hydroxybutyryl-CoA (see also WO2008/115840, WO 2009/023493, U.S. publication 2009/0047719, U.S. publication 2009/0075351). In particular, the *Clostridium acetobutylicum* genes, *buk1* and *ptb*, can be utilized as part of a functional BDO pathway.

Initial experiments involved the cloning and expression of the native *C. acetobutylicum* PTB (020) and BK (021) genes in *E. coli*. Where required, the start codon and stop codon for each gene were modified to "ATG" and "TAA," respectively, for more optimal expression in *E. coli*. The *C. acetobutylicum* gene sequences (020N and 021N) and their corresponding translated peptide sequences are shown in Figures 21 and 22.

The PTB and BK genes exist in *C. acetobutylicum* as an operon, with the PTB (020) gene expressed first. The two genes are connected by the sequence "atta aagttaagtg gaggaatgtt aac" (SEQ ID NO:) that includes a re-initiation ribosomal binding site for the downstream BK (021) gene. The two genes in this context were fused to lac-controlled promoters in expression vectors for expression in *E. coli* (Lutz and Bujard, *Nucleic Acids Res.* 25:1203-1210 (1997)).

Expression of the two proteins from these vector constructs was found to be low in comparison with other exogenously expressed genes due to the high incidence of codons in the C. acetobutylicum genes that occur only rarely in *E. coli*. Therefore new 020 and 021 genes were predicted that changed rare codons for alternates that are more highly represented in *E. coli* gene sequences. This method of codon optimization followed algorithms described previously (Sivaraman et al., *Nucleic Acids Res.* 36:e16(2008)). This method predicts codon replacements in context with their frequency of occurrence when flanked by certain codons on either side. Alternative gene sequences for 020 (Figure 23) and 021 (Figure 24) were determined in which increasing numbers of rare codons were replaced by more prevalent codons (A<B<C<D) based on their incidence in the neighboring codon context. No changes in actual peptide sequence compared to the native 020 and 021 peptide sequences were introduced in these predicted sequences.

The improvement in expression of the BK and PTB proteins resulting from codon optimization is shown in Figure 25A. Expression of the native gene sequences is shown in lane 2, while expression of the 020B-021B and 020C-021C is shown in lanes 3 and 4, respectively. Higher levels of protein expression in the codon-optimized operons 020B-021B (2021B) and 020C-021C (2021C) also resulted in increased activity compared to the native operon (2021n) in equivalently-expressed *E. coli* crude extracts (Figure 25B).

The codon optimized operons were expressed on a plasmid in strain ECKh-432 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd* fimD:: *M. bovis sucA, C. kluyveri 4hbd*) along with the *C. acetobutylicum* aldehyde dehydrogenase to provide a complete BDO pathway. Cells were cultured in M9 minimal medium containing 20 g/L glucose, using a 23G needle to maintain microaerobic conditions as described above. The resulting conversion of glucose to the final product BDO was measured. Also measured was the accumulation of gamma-butyrylactone (GBL), which is a spontaneously rearranged molecule derived from 4Hb-CoA, the immediate product of the PTB-BK enzyme pair. Figure 26 shows that expression of the native 2021n operon resulted in comparable BDO levels to an alternative enzyme function, Cat2 (034), that is capable of converting 4HB and free CoA to 4HB-CoA. GBL levels of 034 were significantly higher than 2021n, suggesting that the former enzyme has more activity than PTB-BK expressed from the native genes. However levels of both BDO and GBL were higher than either 034 or 2021n when the codon-optimized variants 2021B and 2021C were expressed, indicating that codon optimization of the genes for PTB and BK significantly increases their contributions to BDO synthesis in *E. coli*.

These results demonstrate that butyrate kinase (BK) and phosphotransbutyrylase (PTB) enzymes can be employed to convert 4-hydroxybutyrate to 4-hydroxybutyryl-CoA. This eliminates the need for a transferase enzyme such as 4-hydoxybutyryl-CoA/Acetyl-CoA transferase, which would generate one mole of acetate per mol of 4-hydroxybutyryl-CoA produced. The enzymes from *Clostridium acetobutylicum* are present in a number of engineered strains for BDO production.

4-hydroxybutyryl-CoA reductase. The *Clostridium beijerinckii ald* gene can be utilized as part of a functional BDO pathway (see also WO2008/115840, WO 2009/023493, U.S. publication 2009/0047719, U.S. publication 2009/0075351). The *Clostridium beijerinckii ald* can also be

utilized to lower ethanol production in BDO producing strains. Additionally, a specific codon-optimized *ald* variant (GNM0025B) was found to improve BDO production.

The native *C. beijerinckii ald* gene (025n) and the predicted protein sequence of the enzyme are shown in Figure 27. As was seen for the *Clostridium acetobutylicum* PTB and BK genes, expression of the native *C. beijerinckii ald* gene was very low in *E. coli*. Therefore, four codon-optimized variants for this gene were predicted. Figures 28A-28D show alternative gene sequences for 025, in which increasing numbers of rare codons are replaced by more prevalent codons (A<B<C<D) based on their incidence in the neighboring codon context (25A, P=0.05; 25B, P=0.1; 25C, P=0.15; 25D, P=1). No changes in actual peptide sequence compared to the native 025 peptide sequence were introduced in these predictions. Codon optimization significantly increased expression of the *C. beijerinckii ald* (see Figure 29), which resulted in significantly higher conversion of glucose to BDO in cells expressing the entire BDO pathway (Figure 30A).

The native and codon-optimized genes were expressed on a plasmid along with *P. gingivalis* Cat2, in the host strain ECKh-432 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L ΔackA fimD:: *E. coli sucCD*, *P. gingivalis sucD, P. gingivalis 4hbd* fimD:: *M. bovis sucA, C. kluyveri 4hbd*), thus containing a complete BDO pathway. Cells were cultured microaerobically in M9 minimal medium containing 20 g/L glucose as described above. The relative production of BDO and ethanol by the *C. beijerinckii* Ald enzyme (expressed from codon-optimized variant gene 025B) was compared with the *C. acetobutylicum* AdhE2 enzyme (see Figure 30B). The *C. acetobutylicum* AdhE2 enzyme (002C) produced nearly 4 times more ethanol than BDO. In comparison, the *C. beijerinckii* Ald (025B) (in conjunction with an endogenous ADH activity) produced equivalent amounts of BDO, yet the ratio of BDO to ethanol production was reversed for this enzyme compared to 002C. This suggests that the *C. beijerinckii* Ald is more specific for 4HB-CoA over acetyl-coA than the *C. acetobutylicum* AdhE2, and therefore the former is the preferred enzyme for inclusion in the BDO pathway.

The *Clostridium beijerinckii ald* gene (Toth et al., *Appl. Environ. Microbiol.* 65:4973-4980 (1999)) was tested as a candidate for catalyzing the conversion of 4-hydroxybutyryl-CoA to 4-hydroxybutanal. Over fifty aldehyde dehydrogenases were screened for their ability to catalyze the conversion of 4-hydroxybutyryl-CoA to 4-hydroxybutyraldehyde. The *C. beijerinckii ald* gene was chosen for implementation into BDO-producing strains due to the preference of this enzyme for 4-hydroxybutyryl-CoA as a substrate as opposed to acetyl-CoA. This is important

because most other enzymes with aldehyde dehydrogenase functionality (for example, *adhE2* from *C. acetobutylicum* (Fontaine et al., *J Bacteriol.* 184:821-830 (2002)) preferentially convert acetyl-CoA to acetaldehyde, which in turn is converted to ethanol. Utilization of the *C. beijerinckii* gene lowers the amount of ethanol produced as a byproduct in BDO-producing organisms. Also, a codon-optimized version of this gene expresses very well in *E. coli* (Sivaraman et al., *Nucleic Acids Res.* 36:e16 (2008)).

4-hydroxybutanal reductase. 4-hydroxybutanal reductase activity of *adh1* from *Geobacillus thermoglucosidasius* (M10EXG) was utilized. This led to improved BDO production by increasing 4-hydroxybutanal reductase activity over endogenous levels.

Multiple alcohol dehydrogenases were screened for their ability to catalyze the reduction of 4-hydroxybutanal to BDO. Most alcohol dehydrogenases with high activity on butyraldehyde exhibited far lower activity on 4-hydroxybutyraldehyde. One notable exception is the *adh1* gene from *Geobacillus thermoglucosidasius* M10EXG (Jeon et al., *J. Biotechnol.* 135:127-133 (2008)) (GNM0084), which exhibits high activity on both 4-hydroxybutanal and butanal.

The native gene sequence and encoded protein sequence if the *adh1* gene from *Geobacillus thermoglucosidasius* are shown in Figure 31. The *G. thermoglucosidasius ald1* gene was expressed in *E. coli*.

The Adh1 enzyme (084) expressed very well from its native gene in *E. coli* (see Figure 32A). In ADH enzyme assays, the *E. coli* expressed enzyme showed very high reductive activity when butyraldehyde or 4HB-aldehyde were used as the substrates (see Figure 32B). The Km values determined for these substrates were 1.2 mM and 4.0 mM, respectively. These activity values showed that the Adh1 enzyme was the most active on reduction of 4HB-aldehyde of all the candidates tested.

The 084 enzyme was tested for its ability to boost BDO production when coupled with the *C. beijerinckii ald*. The 084 gene was inserted behind the *C. beijerinckii ald* variant 025B gene to create a synthetic operon that results in coupled expression of both genes. Similar constructs linked 025B with other ADH candidate genes, and the effect of including each ADH with 025B on BDO production was tested. The host strain used was ECKh-459 (ΔadhE ldhA ΔpflB ΔlpdA::fnr-pflB6-K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli sucCD*, *P. gingivalis sucD, P. gingivalis 4hbd* fimD:: *M. bovis sucA, C. kluyveri 4hbd* fimD:: *C. acetobutylicum buk1, C. acetobutylicum ptb*), which contains the remainder of the BDO pathway on the chromosome.

The 084 ADH expressed in conjunction with 025B showed the highest amount of BDO (right arrow in Figure 33) when compared with 025B only (left arrow in Figure 33) and in conjunction with endogenous ADH functions. It also produced more BDO than did other ADH enzymes when paired with 025B, indicated as follows: 026A-C, codon-optimized variants of *Clostridium acetobutylicum* butanol dehydrogenase; 050, *Zymomonas mobilis* alcohol dehydrogenase I; 052, *Citrobacter freundii* 1,3-propanediol dehydrogenase; 053, *Lactobacillus brevis* 1,3-propanediol dehydrogenase; 057, *Bacteroides fragilis* lactaldehyde reductase; 058, *E. coli* 1,3-propanediol dehydrogenase; 071, *Bacillus subtilis* 168 alpha-ketoglutarate semialdehyde dehydrogenase. The constructs labeled "PT5lacO" are those in which the genes are driven by the PT5lacO promoter. In all other cases, the PA1lacO-1 promoter was used. This shows that inclusion of the 084 ADH in the BDO pathway increased BDO production.

EXAMPLE XIV
BDO Producing Strains Expressing Pyruvate Dehydrogenase

This example describes the utilization of pyruvate dehydrogenase (PDH) to enhance BDO production. Heterologous expression of the *Klebsiella pneumonia lpdA* gene was used to enhance BDO production.

Computationally, the NADH-generating conversion of pyruvate to acetyl-CoA is required to reach the maximum theoretical yield of 1,4-butanediol (see also WO2008/115840, WO 2009/023493, U.S. publication 2009/0047719, U.S. publication 2009/0075351; WO 2008/018930; Kim et al., *Appl. Environ. Microbiol.* 73:1766-1771 (2007); Kim et al., *J. Bacteriol.* 190:3851-3858 (2008); Menzel et al., *J. Biotechnol.* 56:135-142 (1997)). Lack of PDH activity was shown to reduce the maximum anaerobic theoretical yield of BDO by 11% if phosphoenolpyruvate carboxykinase (PEPCK) activity cannot be attained and by 3% if PEPCK activity can be attained. More importantly, however, absence of PDH activity in the OptKnock strain #439, described in WO 2009/023493 and U.S. publication 2009/0047719, which has the knockout of ADHEr, ASPT, LDH_D, MDH and PFLi, would reduce the maximum anaerobic yield of BDO by 54% or by 43% if PEPCK activity is absent or present, respectively. In the presence of an external electron acceptor, lack of PDH activity would reduce the maximum yield of the knockout strain by 10% or by 3% assuming that PEPCK activity is absent or present, respectively.

PDH is one of the most complicated enzymes of central metabolism and is comprised of 24 copies of pyruvate decarboxylase (E1) and 12 molecules of dihydrolipoyl dehydrogenase (E3),

which bind to the outside of the dihydrolipoyl transacetylase (E2) core. PDH is inhibited by high NADH/NAD, ATP/ADP, and Acetyl-CoA/CoA ratios. The enzyme naturally exhibits very low activity under oxygen-limited or anaerobic conditions in organisms such as *E. coli* due in large part to the NADH sensitivity of E3, encoded by *lpdA*. To this end, an NADH-insensitive version of the *lpdA* gene from *Klebsiella pneumonia* was cloned and expressed to increase the activity of PDH under conditions where the NADH/NAD ratio is expected to be high.

Replacement of the native *lpdA*. The pyruvate dehydrogenase operon of *Klebsiella pneumoniae* is between 78 and 95% identical at the nucleotide level to the equivalent operon of *E. coli*. It was shown previously that *K. pneumoniae* has the ability to grow anaerobically in presence of glycerol (Menzel et al., *J. Biotechnol.* 56:135-142 (1997); Menzel et al., *Biotechnol. Bioeng.* 60:617-626 (1998)). It has also been shown that two mutations in the *lpdA* gene of the operon of *E. coli* would increase its ability to grow anaerobically (Kim et al.. *Appl. Environ. Microbiol.* 73:1766-1771 (2007); Kim et al., *J. Bacteriol.* 190:3851-3858 (2008)). The *lpdA* gene of *K. pneumonia* was amplified by PCR using genomic DNA (ATCC700721D) as template and the primers KP-lpdA-Bam (5'-acacgcggatccaacgtcccgg-3')(SEQ ID NO:) and KP-lpdA-Nhe (5'-agcggctccgctagccgcttatg-3')(SEQ ID NO:). The resulting fragment was cloned into the vector pCR-BluntII-TOPO (Invitrogen; Carlsbad CA), leading to plasmid pCR-KP-lpdA.

The chromosomal gene replacement was performed using a non-replicative plasmid and the *sacB* gene from *Bacillus subtilis* as a means of counterselection (Gay et al., *J. Bacteriol.* 153:1424-1431 (1983)). The vector used is pRE118 (ATCC87693) deleted of the oriT and IS sequences, which is 3.6 kb in size and carrrying the kanamycin resistance gene. The sequence was confirmed, and the vector was called pRE118-V2 (see Figure 34).

The *E. coli* fragments flanking the *lpdA* gene were amplified by PCR using the combination of primers: EC-aceF-Pst (5'-aagccgttgctgcagctcttgagc-3')(SEQ ID NO:) + EC-aceF-Bam2 (5'-atctccggcggtcggatccgtcg-3')(SEQ ID NO:) and EC-yacH-Nhe (5'-aaagcggctagccacgccgc-3')(SEQ ID NO:) + EC-yacH-Kpn (5'-attacacgaggtacccaacg-3')(SEQ ID NO:). A BamHI-XbaI fragment containing the *lpdA* gene of *K. pneumonia* was isolated from plasmid pCR-KP-lpdA and was then ligated to the above *E. coli* fragments digested with PstI +BamHI and NheI-KpnI respectively, and the pRE118-V2 plasmid digested with KpnI and PstI. The resulting plasmid (called pRE118-M2.1 lpdA yac) was subjected to Site Directed Mutagenesis (SDM) using the combination of primers KP-lpdA-HisTyr-F (5'- atgctggcgtacaaaggtgtcc-3')(SEQ ID NO:) and (5'-ggacacctttgtacgccagcat-3')(SEQ ID NO:) for the mutation of the His 322 residue to a Tyr

residue or primers KP-lpdA-GluLys-F (5'-atcgcctacactaaaccagaagtgg-3')(SEQ ID NO:) and KP-lpdA-GluLys-R (5'-ccacttctggtttagtgtaggcgat-3')(SEQ ID NO:) for the mutation of the residue Glu 354 to Lys residue. PCR was performed with the Polymerase Pfu Turbo (Stratagene; San Diego CA). The sequence of the entire fragment as well as the presence of only the desired mutations was verified. The resulting plasmid was introduced into electro competent cells of *E. coli* ΔadhE::Frt-ΔldhA::Frt by transformation. The first integration event in the chromosome was selected on LB agar plates containing Kanamycin (25 or 50 mg/L). Correct insertions were verified by PCR using 2 primers, one located outside the region of insertion and one in the kanamycin gene (5'-aggcagttccataggatggc-3')(SEQ ID NO:). Clones with the correct insertion were selected for resolution. They were sub-cultured twice in plain liquid LB at the desired temperature and serial dilutions were plated on LB-no salt-sucrose 10% plates. Clones that grew on sucrose containing plates were screened for the loss of the kanamycin resistance gene on LB-low salt agar medium and the *lpdA* gene replacement was verified by PCR and sequencing of the encompassing region. Sequence of the insertion region was verified, and is as described below. One clone (named 4-4-P1) with mutation Glu354Lys was selected. This clone was then transduced with P1 lysate of *E. coli* ΔPflB::Frt leading to strain ECKh-138 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322).

The sequence of the ECKh-138 region encompassing the *aceF* and *lpdA* genes is shown in Figure 35. The *K. pneumonia lpdA* gene is underlined, and the codon changed in the Glu354Lys mutant shaded. The protein sequence comparison of the native *E. coli lpdA* and the mutant *K. pneumonia lpdA* is shown in Figure 36.

To evaluate the benefit of using *K. pneumoniae lpdA* in a BDO production strain, the host strains AB3 and ECKh-138 were transformed with plasmids expressing the entire BDO pathway from strong, inducible promoters. Specifically, *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd* were expressed on the medium copy plasmid pZA33, and *P. gingivalis Cat2* and *C. acetobutylicum AdhE2* were expressed on the high copy plasmid pZE13. These plasmids have been described in the literature (Lutz and H. Bujard, Nucleic Acids Res 25:1203-1210 (1997)), and their use for BDO pathway expression is described in Example XIII and WO2008/115840.

Cells were grown anaerobically at 37°C in M9 minimal medium (6.78 g/L Na_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 20 g/L glucose, 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS) to improve the buffering capacity, 10 μg/mL thiamine, and the appropriate antibiotics. Microaerobic conditions were

established by initially flushing capped anaerobic bottles with nitrogen for 5 minutes, then piercing the septum with a 23G needle following inoculation. The needle was kept in the bottle during growth to allow a small amount of air to enter the bottles. 0.25 mM IPTG was added when OD600 reached approximately 0.2 to induce the pathway genes, and samples taken for analysis every 24 hours following induction. The culture supernatants were analyzed for BDO, 4HB, and other by-products as described in Example II and in WO2008/115840. BDO and 4HB production in ECKh-138 was significantly higher after 48 hours than in AB3 or the host used in previous work, MG1655 ΔldhA (Figure 37).

PDH promoter replacement. It was previously shown that the replacement of the pdhR repressor by a transcriptional fusion containing the Fnr binding site, one of the *pflB* promoters, and its ribosome binding site (RBS), thus leading to expression of the aceEF-lpd operon by an anaerobic promoter, should increase pdh activity anaerobically (Zhou et al., *Biotechnol. Lett.* 30:335-342 (2008)). A fusion containing the Fnr binding site, the pflB-p6 promoter and an RBS binding site were constructed by overlapping PCR. Two fragments were amplified, one using the primers aceE-upstream-RC (5'- tgacatgtaacacctaccttctgtgcctgtgccagtggttgctgtgatatagaag-3')(SEQ ID NO:) and pflBp6-Up-Nde (5'- ataataatacatatgaaccatgcgagttacgggcctataagccaggcg-3')(SEQ ID NO:) and the other using primers aceE-EcoRV-EC (5'-agtttttcgatatctgcatcagacaccggcacattgaaacgg-3')(SEQ ID NO:) and aceE-upstream (5'-ctggcacaggcacagaaggtaggtgttacatgtcagaacgtttacacaatgacgtggatc-3')(SEQ ID NO:). The tw fragments were assembled by overlapping PCR, and the final DNA fragment was digested with the restriction enzymes NdeI and BamHI. This fragment was subsequently introduced upstream of the aceE gene of the *E. coli* operon using pRE118-V2 as described above. The replacement was done in strains ECKh-138 and ECKh-422. The nucleotide sequence encompassing the 5' region of the *aceE* gene was verified and is shown in Figure 37. Figure 37 shows the nucleotide sequence of 5' end of the *aceE* gene fused to the pflB-p6 promoter and ribosome binding site (RBS). The 5' italicized sequence shows the start of the *aroP* gene, which is transcribed in the opposite direction from the *pdh* operon. The 3' italicized sequence shows the start of the *aceE* gene. In upper case: pflB RBS. Underlined: FNR binding site. In bold: pflB-p6 promoter sequence.

lpdA promoter replacement. The promoter region containing the fnr binding site, the pflB-p6 promoter and the RBS of the *pflB* gene was amplified by PCR using chromosomal DNA template and primers aceF-pflBp6-fwd (5'- agacaaatcggttgccgtttgttaagccaggcgagatatgatctatatc-3')(SEQ ID NO:) and lpdA-RBS-B-rev (5'-

gagttttgatttcagtactcatcatgtaacacctaccttcttgctgtgatatag-3')(SEQ ID NO:). Plasmid 2-4a was amplified by PCR using primers B-RBS-lpdA fwd (5'-ctatatcacagcaagaaggtaggtgttacatgatgagtactgaaatcaaaactc-3')(SEQ ID NO:) and pflBp6-aceF-rev (5'- gatatagatcatatctcgcctggcttaacaaacggcaaccgatttgtct-3')(SEQ ID NO:). The two resulting fragments were assembled using the BPS cloning kit (BPS Bioscience; San Diego CA). The resulting construct was sequenced verified and introduced into strain ECKh-439 using the pRE118-V2 method described above. The nucleotide sequence encompassing the *aceF-lpdA* region in the resulting strain ECKh-456 is shown in Figure 39.

The host strain ECKh-439 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L ackA fimD:: *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd* fimD:: *M. bovis sucA, C. kluyveri 4hbd*), the construction of which is described below, and the *pdhR* and *lpdA* promoter replacement derivatives ECKh-455 and ECKh-456, were tested for BDO production. The strains were transformed with pZS*13 containing *P. gingivalis* Cat2 and *C. beijerinckii* Ald to provide a complete BDO pathway. Cells were cultured in M9 minimal medium supplemented with 20 g/L glucose as described above. 48 hours after induction with 0.2 mM IPTG, the concentrations of BDO, 4HB, and pyruvate were as shown in Figure 40. The promoter replacement strains produce slightly more BDO than the isogenic parent.

These results demonstrated that expression of pyruvate dehydrogenase increased production of BDO in BDO producing strains.

EXAMPLE XV
BDO Producing Strains Expressing Citrate Synthase and Aconitase

This example describes increasing activity of citrate synthase and aconitase to increase production of BDO. An R163L mutation into *gltA* was found to improve BDO production. Additionally, an *arcA* knockout was used to improve BDO production.

Computationally, it was determined that flux through citrate synthase (CS) and aconitase (ACONT) is required to reach the maximum theoretical yield of 1,4-butanediol (see also WO2008/115840, WO 2009/023493, U.S. publication 2009/0047719, U.S. publication 2009/0075351). Lack of CS or ACONT activity would reduce the maximum theoretical yield by 14% under anaerobic conditions. In the presence of an external electron acceptor, the maximum yield is reduced by 9% or by 6% without flux through CS or ACONT assuming the absence or presence of PEPCK activity, respectively. As with pyruvate dehydrogenase (PDH), the importance of CS and ACONT is greatly amplified in the knockout strain background in

which ADHEr, ASPT, LDH_D, MDH and PFLi are knocked out (design #439)(see WO 2009/023493 and U.S. publication 2009/0047719, which is incorporated herein by reference).

The minimal OptKnock strain design described in WO 2009/023493 and U.S. publication 2009/0047719 had one additional deletion beyond ECKh-138, the *mdh* gene, encoding malate dehydrogenase. Deletion of this gene is intended to prevent flux to succinate via the reductive TCA cycle. The *mdh* deletion was performed using the λ red homologeous recombination method (Datsenko and Wanner, *Proc. Natl. Acad. Sci. USA* 97:6640-6645 (2000)). The following oligonucleotides were used to PCR amplify the chloramphenicol resistance gene (CAT) flanked by FRT sites from pKD3:

S-mdh-Kan 5' - **TAT TGT GCA TAC AGA TGA ATT TTT ATG CAA ACA GTC AGC CCT GAA GAA GG**G TGT AGG CTG GAG CTG CTT C - 3' (SEQ ID NO:)

AS-mdh-Kan 5' - **CAA AAA ACC GGA GTC TGT GCT CCG GTT TTT TAT TAT CCG CTA ATC AAT TAC** ATA TGA ATA TCC TCC TTA G - 3' (SEQ ID NO").

Underlined regions indicate homology to pKD3 plasmid and bold sequence refers to sequence homology upstream and downstream of the *mdh* ORF. After purification, the PCR product was electroporated into ECKh-138 electrocompetent cells that had been transformed with pRedET (tet) and prepared according to the manufacturer's instructions (www.genebridges.com/gb/pdf/K001%20Q%20E%20BAC%20Modification%20Kit-version2.6-2007-screen.pdf). The PCR product was designed so that it integrated into the ECKh-138 genome at a region upstream of the *mdh* gene, as shown in Figure 41.

Recombinants were selected for chloramphenicol resistance and streak purified. Loss of the *mdh* gene and insertion of CAT was verified by diagnostic PCR. To remove the CAT gene, a temperature sensitive plasmid pCP20 containing a FLP recombinase (Datsenko and Wanner, *Proc. Natl. Acad. Sci. USA* 97:6640-6645 (2000)) was transformed into the cell at 30°C and selected for ampicillin resistance (AMP). Transformants were grown nonselectively at 42°C overnight to thermally induce FLP synthesis and to cause lose of the plasmid. The culture was then streak purified, and individual colonies were tested for loss of all antibiotic resistances. The majority lost the FRT-flanked resistance gene and the FLP helper plasmid simultaneously. There was also a "FRT" scar leftover. The resulting strain was named ECKh-172.

CS and ACONT are not highly active or highly expressed under anaerobic conditions. To this end, the *arcA* gene, which encodes for a global regulator of the TCA cycle, was deleted. ArcA

works during microaerobic conditions to induce the expression of gene products that allow the activity of central metabolism enzymes that are sensitive to low oxygen levels, *aceE, pflB* and *adhE*. It was shown that microaerobically, a deletion in *arcA/arcB* increases the specific activities of *ldh, icd, gltA, mdh*, and *gdh* genes (Salmon et al., *J. Biol. Chem.* 280:15084-15096 (2005); Shalel-Levanon et al., *Biotechnol. Bioeng.* 92(2):147-159 (2005). The upstream and downstream regions of the *arcA* gene of *E. coli* MG1655 were amplified by PCR using primers ArcA-up-EcoRI (5'- ataataatagaattcgtttgctacctaaattgccaactaaatcgaaacagg -3')(SEQ ID NO:) with ArcA-up-KpnI (5'-tattattatggtaccaatatcatgcagcaaacggtgcaacattgccg -3')(SEQ ID NO:) and ArcA-down-EcoRI (5'-tgatctggaagaattcatcggctttaccaccgtcaaaaaaaacggcg -3')(SEQ ID NO:) with ArcA-down-PstI (5'-ataaaaccctgcagcggaaacgaagttttatccatttttggttacctg -3')(SEQ ID NO:), respectively. These fragments were subsequently digested with the restriction enzymes EcoRI and KpnI (upstream fragment) and EcoRI and PstI (downstream). They were then ligated into the pRE118-V2 plasmid digested with PstI and KpnI, leading to plasmid pRE118-ΔarcA. The sequence of plasmid pRE118-ΔarcA was verified. pRE118-ΔarcA was introduced into electro-competent cells of *E. coli* strain ECKh-172 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh). After integration and resolution on LB-no salt-sucrose plates as described above, the deletion of the *arcA* gene in the chromosome of the resulting strain ECKh-401 was verified by sequencing and is shown in Figure 42.

The gltA gene of E. coli encodes for a citrate synthase. It was previously shown that this gene is inhibited allosterically by NADH, and the amino acids involved in this inhibition have been identified (Pereira et al., *J. Biol. Chem.* 269(1):412-417 (1994); Stokell et al., *J. Biol. Chem.* 278(37):35435-35443 (2003)). The *gltA* gene of *E. coli* MG1655 was amplified by PCR using primers gltA-up (5'- ggaagagaggctggtacccagaagccacagcagga-3')(SEQ ID NO:) and gltA-PstI (5'-gtaatcactgcgtaagcgccatgccccggcgttaattc -3')(SEQ ID NO:). The amplified fragment was cloned into pRE118-V2 after digestion with KpnI and PstI. The resulting plasmid was called pRE118-gltA. This plasmid was then subjected to site directed mutagensis (SDM) using primers R163L-f (5'- attgccgcgttcctcctgctgtcga-3')(SEQ ID NO:) and R163L-r (5'-cgacagcaggaggaacgcggcaat -3')(SEQ ID NO:) to change the residue Arg 163 to a Lys residue. The sequence of the entire fragment was verified by sequencing. A variation of the λ red homologeous recombination method (Datsenko and Wanner, *Proc. Natl. Acad. Sci. USA* 97:6640-6645 (2000)) was used to replace the native *gltA* gene with the R163L mutant allele without leaving a Frt scar. The general recombination procedure is the same as used to make the *mdh* deletion described above. First, the strain ECKh-172 was made streptomycin resistant by

introducing an rpsL null mutation using the λ red homologeous recombination method. Next, a recombination was done to replace the entire wild-type *gltA* coding region in this strain with a cassette comprised of a kanamycin resistance gene (kanR) and a wild-type copy of the *E. coli rpsL* gene. When introduced into an *E. coli* strain harboring an *rpsL* null mutation, the cassette causes the cells to change from resistance to the drug streptomycin to streptomycin sensitivity. DNA fragments were then introduced that included each of the mutant versions of the *gltA* gene along with appropriate homologous ends, and resulting colony growth was tested in the presence of streptomycin. This selected for strains in which the kanR/rpsL cassette had been replaced by the mutant *gltA* gene. Insertion of the mutant gene in the correct locus was confirmed by PCR and DNA sequencing analyses. The resulting strain was called ECKh-422, and has the genotype ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L. The region encompassing the mutated *gltA* gene of strain ECKh-422 was verified by sequencing, as shown in Figure 43.

Crude extracts of the strains ECKh-401 and the gltAR163L mutant ECKh-422 were then evaluated for citrate synthase activity. Cells were harvested by centrifugation at 4,500 rpm (Beckman-Coulter, Allegera X-15R; Fullerton CA) for 10 min. The pellets were resuspended in 0.3 mL BugBuster (Novagen/EMD; San Diego CA) reagent with benzonase and lysozyme, and lysis proceeded for 15 minutes at room temperature with gentle shaking. Cell-free lysate was obtained by centrifugation at 14,000 rpm (Eppendorf centrifuge 5402; Hamburg Germany) for 30 min at 4°C. Cell protein in the sample was determined using the method of Bradford (Bradford, *Anal. Biochem.* 72:248-254 (1976)).

Citrate synthase activity was determined by following the formation of free coenzyme A (HS-CoA), which is released from the reaction of acetyl-CoA with oxaloacetate. The free thiol group of HS-CoA reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to form 5-thio-2-nitrobenzoic acid (TNB). The concentration of TNB is then monitored spectrophotometrically by measuring the absorbance at 410 nm (maximum at 412 nm). The assay mixture contained 100 mM Tris/HCl buffer (pH 7.5), 20 mM acetyl-CoA, 10 mM DTNB, and 20 mM oxaloacetate. For the evaluation of NADH inhibition, 0.4 mM NADH was also added to the reaction. The assay was started by adding 5 microliters of the cell extract, and the rate of reaction was measured by following the absorbance change over time. A unit of specific activity is defined as the μmol of product converted per minute per mg protein.

Figure 44 shows the citrate synthase activity of wild type *gltA* gene product and the R163L mutant. The assay was performed in the absence or presence of 0.4 mM NADH.

Strains ECKh-401 and ECKh-422 were transformed with plasmids expressing the entire BDO pathway. *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd*, and *M. bovis sucA* were expressed on the low copy plasmid pZS*13, and *P. gingivalis* Cat2 and *C. acetobutylicum* AdhE2 were expressed on the medium copy plasmid pZE23. Cultures of these strains were grown microaerobically in M9 minimal medium supplemented with 20 g/L glucose and the appropriate antibiotics as described above. The 4HB and BDO concentrations at 48 hours post-induction averaged from duplicate cultures are shown in Figure 45. Both are higher in ECKh-422 than in ECKh-401, demonstrating that the enhanced citrate synthase activity due to the *gltA* mutation results in increased flux to the BDO pathway.

The host strain modifications described in this section were intended to redirect carbon flux through the oxidative TCA cycle, which is consistent with the OptKnock strain design described in WO 2009/023493 and U.S. publication 2009/0047719. To demonstrate that flux was indeed routed through this pathway, ^{13}C flux analysis was performed using the strain ECKh-432, which is a version of ECKh-422 in which the upstream pathway is integrated into the chromosome (as described in Example XVII). To complete the BDO pathway, *P. gingivalis* Cat2 and *C. beijerinckii* Ald were expressed from pZS*13. Four parallel cultures were grown in M9 minimal medium (6.78 g/L Na_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) containing 4 g/L total glucose of four different labeling ratios ($^{1\text{-}13}C$, only the first carbon atom in the glucose molecule is labeled with ^{13}C; uniform-^{13}C, all carbon atoms are ^{13}C):

1. 80 mol% unlabeled, 20 mol% uniform-^{13}C
2. 10 mol% unlabeled, 90 mol% uniform-^{13}C
3. 90 mol% $^{1\text{-}13}C$, 10 mol% uniform-^{13}C
4. 40 mol% $^{1\text{-}13}C$, 60 mol% uniform-^{13}C

Parallel unlabeled cultures were grown in duplicate, from which frequent samples were taken to evaluate growth rate, glucose uptake rate, and product formation rates. In late exponential phase, the labeled cultures were harvested, the protein isolated and hydrolyzed to amino acids, and the label distribution of the amino acids analyzed by gas chromatography- mass spectrometry (GCMS) as described previously (Fischer and Sauer, *Eur. J. Biochem.* 270:880-891 (2003)). In addition, the label distribution of the secreted 4HB and BDO in the broth from the labeled cultures was analyzed by GCMS as described in WO2008115840. This data was collectively used to calculate the intracellular flux distribution using established methods

(Suthers et al., *Metab. Eng.* 9:387-405 (2007)). The resulting central metabolic fluxes and associated 95% confidence intervals are shown in Figure 46. Values are molar fluxes normalized to a glucose uptake rate of 1 mmol/hr. The result indicates that carbon flux is routed through citrate synthase in the oxidative direction, and that most of the carbon enters the BDO pathway rather than completing the TCA cycle. Furthermore, it confirms there is essentially no flux between malate and oxaloacetate due to the *mdh* deletion in this strain.

The advantage of using a knockout strain such as strains designed using OptKnock for BDO production (see WO 2009/023493 and U.S. publication 2009/0047719) can be observed by comparing typical fermentation profiles of ECKh-422 with that of the original strain ECKh-138, in which BDO is produced from succinate via the reductive TCA cycle (see Figure 47). Fermentations were performed with 1L initial culture volume in 2L Biostat B+ bioreactors (Sartorius; Cedex France) using M9 minimal medium supplemented with 20 g/L glucose. The temperature was controlled at 37°C, and the pH was controlled at 7.0 using 2 M NH_4OH or Na_2CO_3. Cells were grown aerobically to an OD600 of approximately 10, at which time the cultures were induced with 0.2 mM IPTG. One hour following induction, the air flow rate was reduced to 0.02 standard liters per minute for microaerobic conditions. The agitation rate was set at 700 rpm. Concentrated glucose was fed to maintain glucose concentration in the vessel between 0.5 and 10 g/L. Both strains were transformed with plasmids bearing the entire BDO pathway, as in the examples above. In ECKh-138, acetate, pyruvate, and 4HB dominate the fermentation, while with ECKh-422 BDO is the major product.

EXAMPLE XVI
BDO Strains Expression Phosphoenolpyruvate Carboxykinase

This example describes the utilization of phosphoenolpyruvate carboxykinase (PEPCK) to enhance BDO production. The *Haemophilus influenza* PEPCK gene was used for heterologous expression.

Computationally, it was demonstrated that the ATP-generating conversion of oxaloacetate to phosphoenolpyruvate is required to reach the maximum theoretical yield of 1,4-butanediol (see also WO2008/115840, WO 2009/023493, U.S. publication 2009/0047719, U.S. publication 2009/0075351). Lack of PEPCK activity was shown to reduce the maximum theoretical yield of BDO by 12% assuming anaerobic conditions and by 3% assuming an external electron acceptor such as nitrate or oxygen is present.

In organisms such as *E. coli*, PEPCK operates in the gluconeogenic and ATP-consuming direction from oxaloacetate towards phosphoenolpyruvate. It has been hypothesized that kinetic limitations of PEPCK of *E. coli* prevent it from effectively catalyzing the formation of oxaloacetate from PEP. PEP carboxylase (PPC), which does not generate ATP but is required for efficient growth, is naturally utilized by *E. coli* to form oxaloacetate from phosphoenolpyruvate. Therefore, three non native PEPCK enzymes (Table 26) were tested for their ability to complement growth of a PPC mutant strain of *E. coli* in glucose minimal media.

Table 26. Sources of phosphoenolpyruvate carboxykinase sequences.

PEPCK Source Strain	Accession Number, *GenBank* Reference Sequence
Haemophilus influenza	NC_000907.1
Actinobacillus succinogenes	YP_001343536.1
Mannheimia succiniciproducens	YP_089485.1

Growth complementation studies involved plasmid based expression of the candidate genes in Δppc mutant *E.coli* JW3978 obtained from the Keio collection (Baba et al., Molecular Systems Biology 2:2006.0008 (2006)). The genes were cloned behind the PA1lacO-1 promoter in the expression vectors pZA23 (medium copy) and pZE13 (high copy). These plasmids have been described previously (Lutz and Bujard, *Nucleic Acids Res.* 25:1203-1210 (1997)), and their use in expression BDO pathway genes has been described previously in WO2008115840.

Pre-cultures were grown aerobically in M9 minimal media with 4g/L glucose. All pre-cultures were supplemented with aspartate (2mM) to provide the Δppc mutants with a source for generating TCA cycle intermediates independent of PEPCK expression. M9 minimal media was also used in the test conditions with 4g/L glucose, but no aspartate was added and IPTG was added to 0.5 mM. Table 27 shows the results of the growth complementation studies.

Table 27. Complementation of Δppc mutants with PEPCK from *H. influenzae, A. succinogenes and M. succinoproducens* when expressed from vectors pZA23 or pZE13.

PEPCK Source Strain	Vector	Time (h)	OD_{600}
H.influenzae	pZA23BB	40	0.950
Δppc Control	pZA23BB	40	0.038
A.succinogenes	pZA23BB	40	0.055
M. succinoproducens	pZA23BB	40	0.214
A.succinogenes	pZE13BB	40	0.041
M. succinoproducens	pZE13BB	40	0.024
Δppc Control	pZE13BB	40	0.042

Haemophilus influenza PEPCK was found to complement growth in Δppc mutant *E. coli* best among the genes that were tested in the plasmid based screening. This gene was then integrated into the PPC locus of wild-type *E. coli* (MG1655) using the SacB counter selection method with pRE118-V2 discussed above (Gay et al., *J. Bacteriol.* 153:1424-1431 (1983)). PEPCK was integrated retaining the *E. coli* native PPC promoter, but utilizing the non-native PEPCK terminator. The sequence of this region following replacement of ppc by *H. influenzae* pepck is shown in Figure 48. The pepck coding region is underlined.

Techniques for adaptive evolution were applied to improve the growth rate of the *E. coli* mutant (Δppc::H. inf pepCK). M9 minimal media with 4 g/L glucose and 50mM sodium bicarbonate was used to culture and evolve this strain in an anaerobic environment. The high sodium bicarbonate concentration was used to drive the equilibrium of the PEPCK reaction toward oxaloacetate formation. To maintain exponential growth, the culture was diluted 2-fold whenever an OD600 of 0.5 was achieved. After about 100 generations over 3 weeks of adaptive evolution, anaerobic growth rates improved from about 8h to that of wild type, about 2h. Following evolution, individual colonies were isolated, and growth in anaerobic bottles was compared to that of the initial mutant and wild-type strain (see Figure 49). M9 medium with 4 g/L glucose and 50 mM sodium bicarbonate was used.

The ppc/pepck gene replacement procedure described above was then repeated, this time using the BDO-producing strains ECKh-432 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L ΔackA fimD:: *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd* fimD:: *M. bovis sucA, C. kluyveri 4hbd*) and ECKh-439 as the hosts. These strains contain the TCA cycle enhancements discussed above as well as the upstream pathway integrated in the chromosome. ECKh-439 is a derivative of ECKh-432 that has the *ackA* gene deleted, which encodes acetate kinase. This deletion was performed using the sacB counterselection method described above.

The Δppc::*H. inf* pepCK derivative of ECKh-439, called ECKh-453, was run in a fermentation. The downstream BDO pathway was supplied by pZS*13 containing *P. gingivalis* Cat2 and *C. beijerinckii* Ald. This was performed with 1L initial culture volume in 2L Biostat B+ bioreactors (Sartorius) using M9 minimal medium supplemented with 20 g/L glucose and 50 mM $NaHCO_3$. The temperature was controlled at 37°C, and the pH was controlled at 7.0 using 2 M NH_4OH or Na_2CO_3. Cells were grown aerobically to an OD600 of approximately 2, at which time the cultures were induced with 0.2 mM IPTG. One hour following induction, the air flow rate was reduced to 0.01 standard liters per minute for microaerobic conditions. The

agitation rate was initially set at 700 rpm. The aeration rate was gradually increased throughout the fermentation as the culture density increased. Concentrated glucose solution was fed to maintain glucose concentration in the vessel between 0.5 and 10 g/L. The product profile is shown in Figure 50. The observed phenotype, in which BDO and acetate are produced in approximately a one-to-one molar ratio, is highly similar to that predicted in WO 2009/023493 for design #439 (ADHEr, ASPT, LDH_D, MDH, PFLi). The deletion targeting the ASPT reaction was deemed unnecessary as the natural flux through aspartate ammonia-lyase is low.

A key feature of OptKnock strains is that production of the metabolite of interest is generally coupled to growth, and further, that, production should occur during exponential growth as well as in stationary phase. The growth coupling potential of ECKh-432 and ECKh-453 was evaluated by growth in microaerobic bottles with frequent sampling during the exponential phase. M9 medium containing 4 g/L glucose and either 10 mM $NaHCO_3$ (for ECKh-432) or 50 mM $NaHCO_3$ (for ECKh-453) was used, and 0.2 mM IPTG was included from inoculation. 18G needles were used for microaerobic growth of ECKh-432, while both 18G and 27G needles were tested for ECKh-453. The higher gauge needles result in less aeration. As shown in Figure 51, ECKh-432 does not begin producing BDO until 5 g/L glucose has been consumed, corresponding to the onset of stationary phase. ECKh-453 produces BDO more evenly throughout the experiment. In addition, growth coupling improves as the aeration of the culture is reduced.

EXAMPLE XVII
Integration of BDO Pathway Encoding Genes at Specific Integration Sites

This example describes integration of various BDO pathway genes into the fimD locus to provide more efficient expression and stability.

The entire upstream BDO pathway, leading to 4HB, has been integrated into the *E. coli* chromosome at the fimD locus. The succinate branch of the upstream pathway was integrated into the *E. coli* chromosome using the λ red homologeous recombination method (Datsenko and Wanner, *Proc. Natl. Acad. Sci. USA* 97:6640-6645 (2000)). The recipient *E. coli* strain was ECKh-422 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L). A polycistronic DNA fragment containing a promoter, the *sucCD* gene, the *sucD* gene and the *4hbd* gene and a terminator sequence was inserted into the AflIII site of the pKD3 plasmid. The following primers were used to amplify the operon together with the chloramphenicol marker from the plasmid. The underlined sequences are homologeous to the target insertion site.

5'-GTTTGCACGCTATAGCTGAGGTTGTTGTCTTCCAGCAACGTACCGTATACAA TAGGCGTATCACGAGGCCCTTTC-3' (SEQ ID NO:)
5'-GCTACAGCATGTCACACGATCTCAACGGTCGGATGACCAATCTGGCTGGTAT GGGAATTAGCCATGGTCC-3' (SEQ ID NO:)

Following DpnI treatment and DNA electrophoresis, the purified PCR product was used to transform *E. coli* strain harboring plasmid pKD46. The candidate strain was selected on plates containing chloramphenicol. Genomic DNA of the candidate strain was purified. The insertion sequence was amplified and confirmed by DNA sequencing. The chloramphenicol-resistant marker was removed from chromosome by flipase. The nucleotide sequence of the region after insertion and marker removal is shown in Figure 52.

The alpha-ketoglutarate branch of the upstream pathway was integrated into the chromosome by homologeous recombination. The plasmid used in this modification was derived from vector pRE118-V2, as referenced in Example XIV, which contains a kanamycin-resistant gene, a gene encoding the levansucrase (sacB) and a R6K conditional replication ori. The integration plasmid also contained a polycistronic sequence with a promoter, the *sucA* gene, the *C. kluyveri 4hbd* gene, and a terminator being inserted between two 1.5-kb DNA fragments that are homologeous to the flanking regions of the target insertion site. The resulting plasmid was used to transform *E. coli* strain. The integration candidate was selected on plates containing kanamycin. The correct integration site was verified by PCR. To resolve the antibiotic marker from the chromosome, the cells were selected for growth on medium containing sucrose. The final strain was verified by PCR and DNA sequencing. The nucleotide sequence of the chromosomal region after insertion and marker removal is shown in Figure 53.

The resulting upstream pathway integration strain ECKh-432 was transformed with a plasmid harboring the downstream pathway genes. The construct was able to produce BDO from glucose in minimal medium (see Figure 54).

EXAMPLE XVIII
Use of a Non-Phosphotransferase Sucrose Uptake System to Reduce Pyruvate Byproduct Formation

This example describes the utilization of a non-phosphotransferase (PTS) sucrose uptake system to reduce pyruvate as a byproduct in the conversion of sucrose to BDO.

Strains engineered for the utilization of sucrose via a phosphotransferase (PTS) system produce significant amounts of pyruvate as a byproduct. Therefore, the use of a non-PTS sucrose system can be used to decrease pyruvate formation because the import of sucrose would not be accompanied by the conversion of phosphoenolpyruvate (PEP) to pyruvate. This will increase the PEP pool and the flux to oxaloacetate through PPC or PEPCK.

Insertion of a non-PTS sucrose operon into the *rrnC* region was performed. To generate a PCR product containing the non-PTS sucrose genes flanked by regions of homology to the *rrnC* region, two oligos were used to PCR amplify the *csc* genes from Mach1™ (Invitrogen, Carlsbad, CA). This strain is a descendent of W strain which is an *E. coli* strain known to be able to catabolize sucrose (Orencio-Trejo et al., *Biotechnology Biofuels* 1:8 (2008)). The sequence was derived from *E. coli* W strain KO11 (accession AY314757) (Shukla et al., *Biotechnol. Lett.* 26:689-693 (2004)) and includes genes encoding a sucrose permease (*cscB*), D-fructokinase (*cscK*), sucrose hydrolase (*cscA*), and a LacI-related sucrose-specific repressor (*cscR*). The first 53 amino acids of *cscR* was effectively removed by the placement of the AS primer. The sequences of the oligos were: rrnC 23S del S –CSC 5'-**TGT GAG TGA AAG TCA CCT GCC TTA ATA TCT CAA AAC TCA TCT TCG GGT GA**C GAA ATA TGG CGT GAC TCG ATA C-3' (SEQ ID NO:) and rrnC 23S del AS –CSC 5'-**TCT GTA TCA GGC TGA AAA TCT TCT CTC ATC CGC CAA AAC AGC TTC GGC GT**T AAG ATG CGC GCT CAA GGA C-3' (SEQ ID NO:). Underlined regions indicate homology to the *csc* operon, and bold sequence refers to sequence homology upstream and downstream of the *rrnC* region. The sequence of the entire PCR product is shown in Figure 55.

After purification, the PCR product was electroporated into MG1655 electrocompetent cells which had been transformed with pRedET (tet) and prepared according to manufacturer's instructions (www.genebridges.com/gb/pdf/K001%20Q%20E%20BAC%20Modification%20Kit-version2.6-2007-screen.pdf). The PCR product was designed so that it integrated into genome into the *rrnC* region of the chromosome. It effectively deleted 191 nucleotides upstream of *rrlC* (23S rRNA), all of the *rrlC* rRNA gene and 3 nucleotides downstream of *rrlC* and replaced it with the sucrose operon, as shown in Figure 56.

Transformants were grown on M9 minimal salts medium with 0.4% sucrose and individual colonies tested for presence of the sucrose operon by diagnostic PCR. The entire rrnC::crcAKB region was transferred into the BDO host strain ECKh-432 by P1 transduction (Sambrook et al.,

Molecular Cloning: A Laboratory Manual, Third Ed., Cold Spring Harbor Laboratory, New York (2001), resulting in ECKh-463 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd* fimD:: *M. bovis sucA, C. kluyveri 4hbd* rrnC::cscAKB). Recombinants were selected by growth on sucrose and verified by diagnostic PCR.

ECKh-463 was transformed with pZS*13 containing *P. gingivalis* Cat2 and *C. beijerinckii* Ald to provide a complete BDO pathway. Cells were cultured in M9 minimal medium (6.78 g/L Na_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 10 g/L sucrose. 0.2 mM IPTG was present in the culture from the start. Anaerobic conditions were maintained using a bottle with 23G needle. As a control, ECKh-432 containing the same plasmid was cultured on the same medium, except with 10 g/L glucose instead of sucrose. Figure 57 shows average product concentration, normalized to culture OD600, after 48 hours of growth. The data is for 6 replicate cultures of each strain. This demonstrates that BDO production from ECKh-463 on sucrose is similar to that of the parent strain on sucrose.

EXAMPLE XIX
Summary of BDO Producing Strains

This example describes various BDO producting strains.

Table 28 summarizes various BDO producing strains disclosed above in Examples XII-XVIII.

Table 28. Summary of various BDO production strains.

Strain #	Host Strain #	Host chromosome	Host Description	Plasmid-based
1		ΔldhA	Single deletion derivative of *E. coli* MG1655	*E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2
2	AB3	ΔadhE ΔldhA ΔpflB	Succinate producing strain; derivative of *E. coli* MG1655	*E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2
3	ECKh-138	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322	Improvement of lpdA to increase pyruvate dehydrogenase flux	*E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2
4	ECKh-138	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322		*E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *C.*

				acetobutylicum buk1, *C. acetobutylicum* ptb, *C. acetobutylicum* AdhE2
5	ECKh-401	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA	Deletions in mdh and arcA to direct flux through oxidative TCA cycle	*E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2
6	ECKh-401	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA		*M. bovis* sucA, *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2
7	ECKh-422	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L	Mutation in citrate synthase to improve anaerobic activity	*E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2
8	ECKh-422	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L		*M. bovis* sucA, *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2
9	ECKh-422	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L		*M. bovis* sucA, *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. beijerinckii* Ald
10	ECKh-426	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd	Succinate branch of upstream pathway integrated into ECKh-422	*P. gingivalis* Cat2, *C. beijerinckii* Ald
11	ECKh-432	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd	Succinate and alpha-ketoglutarate upstream pathway branches integrated into ECKh-422	*P. gingivalis* Cat2, *C. beijerinckii* Ald
12	ECKh-432	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd		*C. acetobutylicum* buk1, *C. acetobutylicum* ptb, *C. beijerinckii* Ald
13	ECKh-439	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L ΔackA fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd	Acetate kinase deletion of ECKh-432	*P. gingivalis* Cat2, *C. beijerinckii* Ald
14	ECKh-453	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L ΔackA Δppc::H.i.ppck fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd	Acetate kinase deletion and PPC/PEPCK replacement of ECKh-432	*P. gingivalis* Cat2, *C. beijerinckii* Ald
15	ECKh-456	ΔadhE ΔldhA ΔpflB ΔlpdA::fnr-pflB6-K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli*	Replacement of lpdA promoter with anaerobic promoter in	*P. gingivalis* Cat2, *C. beijerinckii* Ald

		sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd	ECKh-432	
16	ECKh-455	ΔadhE ΔldhA ΔpflB ΔlpdA:: K.p.lpdA322 ΔpdhR:: fnr-pflB6 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd	Replacement of pdhR and aceEF promoter with anaerobic promoter in ECKh-432	*P. gingivalis* Cat2, *C. beijerinckii* Ald
17	ECKh-459	ΔadhE ΔldhA ΔpflB ΔlpdA:: K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd fimD:: *C. acetobutylicum* buk1, *C. acetobutylicum* ptb	Integration of BK/PTB into ECKh-432	*C. beijerinckii* Ald
18	ECKh-459	ΔadhE ΔldhA ΔpflB ΔlpdA:: K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd fimD:: *C. acetobutylicum* buk1, *C. acetobutylicum* ptb		*C. beijerinckii* Ald, *G. thermoglucosidasius* adh1
19	ECKh-463	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd rrnC::cscAKB	Non-PTS sucrose genes inserted into ECKh-432	*P. gingivalis* Cat2, *C. beijerinckii* Ald
20	ECKh-463	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd rrnC::cscAKB		*C. acetobutylicum* buk1, *C. acetobutylicum* ptb, *C. beijerinckii* Ald

The strains summarized in Table 28 are as follows. Strain 1: Single deletion derivative of *E. coli* MG1655, with deletion of endogenous *ldhA*; plasmid expression of *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd, P. gingivalis Cat2, C. acetobutylicum AdhE2*. Strain 2: Host strain AB3, a succinate producing strain, derivative of *E. coli* MG1655, with deletions of endogenous *adhE ldhA pflB*; plasmid expression of *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd, P. gingivalis* Cat2, *C. acetobutylicum* AdhE2.

Strain 3: Host strain ECKh-138, deletion of endogenous *adhE, ldhA, pflB*, deletion of endogenous *lpdA* and chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys mutation at the *lpdA* locus; plasmid expression of *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd, P. gingivalis* Cat2, *C. acetobutylicum* AdhE2; strain provides improvement of lpdA to increase pyruvate dehydrogenase flux. Strain 4: Host strain ECKh-138, deletion of

endogenous *adhE, ldhA, pflB*, and *lpdA*, chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys mutation; plasmid expression *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd, C. acetobutylicum buk1, C. acetobutylicum ptb, C. acetobutylicum* AdhE2.

Strain 5: Host strain ECKh-401, deletion of endogenous *adhE, ldhA, pflB,* deletion of endogenous *lpdA* and chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys mutation at the *lpdA* locus, deletion of endogenous *mdh* and *arcA*; plasmid expression of *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd, P. gingivalis Cat2, C. acetobutylicum* AdhE2; strain has deletions in *mdh* and *arcA* to direct flux through oxidative TCA cycle. Strain 6: host strain ECKh-401, deletion of endogenous *adhE, ldhA, pflB,* deletion of endogenous *lpdA* and chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys mutation at the *lpdA* locus, deletion of endogenous *mdh* and *arcA*; plasmid expression of *M. bovis sucA, E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd, P. gingivalis* Cat2, *C. acetobutylicum* AdhE2.

Strain 7: Host strain ECKh-422, deletion of endogenous *adhE, ldhA, pflB,* deletion of endogenous *lpdA* and chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys mutation at the *lpdA* locus, deletion of endogenous *mdh* and *arcA*, chromosomal replacement of *gltA* with *gltA* Arg163Leu mutant; plasmid expression of *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd, P. gingivalis* Cat2, *C. acetobutylicum* AdhE2; strain has mutation in citrate synthase to improve anaerobic activity. Strain 8: strain ECKh-422, deletion of endogenous *adhE, ldhA, pflB,* deletion of endogenous *lpdA* and chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys mutation at the *lpdA* locus, deletion of endogenous *mdh* and *arcA*, chromosomal replacement of *gltA* with *gltA* Arg163Leu mutant; plasmid expression of *M. bovis sucA, E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd, P. gingivalis* Cat2, *C. acetobutylicum* AdhE2. Strain 9: host strain ECKh-422, deletion of endogenous *adhE, ldhA, pflB,* deletion of endogenous *lpdA* and chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys mutation at the *lpdA* locus, deletion of endogenous *mdh* and *arcA*, chromosomal replacement of *gltA* with *gltA* Arg163Leu mutant; plasmid expression of *M. bovis sucA, E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd, P. gingivalis* Cat2, *C. beijerinckii* Ald.

Strain 10: host strain ECKh-426, deletion of endogenous *adhE, ldhA, pflB,* deletion of endogenous *lpdA* and chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys mutation at the *lpdA* locus, deletion of endogenous *mdh* and *arcA*, chromosomal replacement of *gltA* with *gltA* Arg163Leu mutant, chromosomal insertion at the *fimD* locus of *E. coli sucCD, P.*

gingivalis sucD, P. gingivalis 4hbd; plasmid expression of *P. gingivalis* Cat2, *C. beijerinckii* Ald; strain has succinate branch of upstream pathway integrated into strain ECKh-422 at the *fimD* locus. Strain 11: host strain ECKh-432, deletion of endogenous *adhE, ldhA, pflB,* deletion of endogenous *lpdA* and chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys mutation at the *lpdA* locus, deletion of endogenous *mdh* and *arcA*, chromosomal replacement of *gltA* with *gltA* Arg163Leu mutant, chromosomal insertion at the *fimD* locus of *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd*, chromosomal insertion at the *fimD* locus of *M. bovis sucA, C. kluyveri 4hbd*; plasmid expression of *P. gingivalis* Cat2, *C. beijerinckii* Ald; strain has succinate and alpha-ketoglutarate upstream pathway branches integrated into ECKh-422. Strain 12: host strain ECKh-432, deletion of endogenous *adhE, ldhA, pflB,* deletion of endogenous *lpdA* and chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys mutation at the *lpdA* locus, deletion of endogenous *mdh* and *arcA*, chromosomal replacement of *gltA* with *gltA* Arg163Leu mutant, chromosomal insertion at the *fimD* locus of *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd*, chromosomal insertion at the *fimD* locus of *M. bovis sucA, C. kluyveri 4hbd*; plasmid expression of *C. acetobutylicum buk1, C. acetobutylicum ptb, C. beijerinckii* Ald.

Strain 13: host strain ECKh-439, deletion of endogenous *adhE, ldhA, pflB,* deletion of endogenous *lpdA* and chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys mutation at the *lpdA* locus, deletion of endogenous *mdh* and *arcA*, chromosomal replacement of *gltA* with *gltA* Arg163Leu mutant, deletion of endogenous *ackA*, chromosomal insertion at the *fimD* locus of *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd*, chromosomal insertion at the *fimD* locus of *M. bovis sucA, C. kluyveri 4hbd*; plasmid expression of *P. gingivalis* Cat2, *C. beijerinckii* Ald; strain has acetate kinase deletion in strain ECKh-432. Strain 14: host strain ECKh-453, deletion of endogenous *adhE, ldhA, pflB,* deletion of endogenous *lpdA* and chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys mutation at the *lpdA* locus, deletion of endogenous *mdh* and *arcA*, chromosomal replacement of *gltA* with *gltA* Arg163Leu mutant, deletion of endogenous *ackA*, deletion of endogenous *ppc* and insertion of *Haemophilus influenza ppck* at the ppc locus, chromosomal insertion at the *fimD* locus of *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd*, chromosomal insertion at the *fimD* locus of *M. bovis sucA, C. kluyveri 4hbd*; plasmid expression of *P. gingivalis* Cat2, *C. beijerinckii* Ald; strain has acetate kinase deletion and PPC/PEPCK replacement in strain ECKh-432.

Strain 15: host strain ECKh-456, deletion of endogenous *adhE, ldhA, pflB,* deletion of endogenous *lpdA* and chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys

mutation at the *lpdA* locus, deletion of endogenous *mdh* and *arcA*, chromosomal replacement of *gltA* with *gltA* Arg163Leu mutant, chromosomal insertion at the *fimD* locus of *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd*, chromosomal insertion at the *fimD* locus of *M. bovis sucA, C. kluyveri 4hbd,* replacement of *lpdA* promoter with fnr binding site, pflB-p6 promoter and RBS of *pflB*; plasmid expression of *P. gingivalis* Cat2, *C. beijerinckii* Ald; strain has replacement of *lpdA* promoter with anaerobic promoter in strain ECKh-432. Strain 16: host strain ECKh-455, deletion of endogenous *adhE, ldhA, pflB,* deletion of endogenous *lpdA* and chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys mutation at the *lpdA* locus, deletion of endogenous *mdh* and *arcA*, chromosomal replacement of *gltA* with *gltA* Arg163Leu mutant, chromosomal insertion at the *fimD* locus of *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd*, chromosomal insertion at the *fimD* locus of *M. bovis sucA, C. kluyveri 4hbdI,* replacement of *pdhR* and *aceEF* promoter with fnr binding site, pflB-p6 promoter and RBS of *pflB*; plasmid expression of *P. gingivalis* Cat2, *C. beijerinckii* Ald; strain has replacement of pdhR and aceEF promoter with anaerobic promoter in ECKh-432.

Strain 17: host strain ECKh-459, deletion of endogenous *adhE, ldhA, pflB,* deletion of endogenous *lpdA* and chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys mutation at the *lpdA* locus, deletion of endogenous *mdh* and *arcA*, chromosomal replacement of *gltA* with *gltA* Arg163Leu mutant, chromosomal insertion at the *fimD* locus of *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd*, chromosomal insertion at the *fimD* locus of *M. bovis sucA, C. kluyveri 4hbd*, chromosomal insertion at the *fimD* locus of *C. acetobutylicum buk1, C. acetobutylicum ptb*; plasmid expression of *C. beijerinckii* Ald; strain has integration of BK/PTB into strain ECKh-432. Strain 18: host strain ECKh-459, deletion of endogenous *adhE, ldhA, pflB,* deletion of endogenous *lpdA* and chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys mutation at the *lpdA* locus, deletion of endogenous *mdh* and *arcA*, chromosomal replacement of *gltA* with *gltA* Arg163Leu mutant, chromosomal insertion at the *fimD* locus of *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd*, chromosomal insertion at the *fimD* locus of *M. bovis sucA, C. kluyveri 4hbd*, chromosomal insertion at the *fimD* locus of *C. acetobutylicum buk1, C. acetobutylicum ptb*; plasmid expression of *C. beijerinckii* Ald, *G. thermoglucosidasius adh1*.

Strain 19: host strain ECKh-463, deletion of endogenous *adhE, ldhA, pflB,* deletion of endogenous *lpdA* and chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys mutation at the *lpdA* locus, deletion of endogenous *mdh* and *arcA*, chromosomal replacement of *gltA* with *gltA* Arg163Leu mutant, chromosomal insertion at the *fimD* locus of *E. coli sucCD, P.*

gingivalis sucD, P. gingivalis 4hbd, chromosomal insertion at the *fimD* locus of *M. bovis sucA, C. kluyveri 4hbd*, insertion at the *rrnC* locus of non-PTS sucrose operon genes sucrose permease (*cscB*), D-fructokinase (*cscK*), sucrose hydrolase (*cscA*), and a LacI-related sucrose-specific repressor (*cscR*); plasmid expression of *P. gingivalis* Cat2, *C. beijerinckii* Ald; strain has non-PTS sucrose genes inserted into strain ECKh-432. Strain 20: host strain ECKh-463 deletion of endogenous *adhE, ldhA, pflB,* deletion of endogenous *lpdA* and chromosomal insertion of *Klebsiella pneumoniae lpdA* with a Glu354Lys mutation at the *lpdA* locus, deletion of endogenous *mdh* and *arcA*, chromosomal replacement of *gltA* with *gltA* Arg163Leu mutant, chromosomal insertion at the *fimD* locus of *E. coli sucCD, P. gingivalis sucD, P. gingivalis 4hbd*, chromosomal insertion at the *fimD* locus of *M. bovis sucA, C. kluyveri 4hbd*, insertion at the *rrnC* locus of non-PTS sucrose operon; plasmid expression of *C. acetobutylicum buk1, C. acetobutylicum ptb, C. beijerinckii* Ald.

In addition to the BDO producing strains disclosed herein, including those disclosed in Table 28, it is understood that additional modifications can be incorporated that further increase production of BDO and/or decrease undesirable byproducts. For example, a BDO producing strain, or a strain of Table 28, can incorporate additional knockouts to further increase the production of BDO or decrease an undesirable byproduct. Exemplary knockouts have been described previously (see U.S. publication 2009/0047719). Such knockout strains include, but are not limited to, ADHEr, NADH6; ADHEr, PPCK; ADHEr, SUCD4; ADHEr, ATPS4r; ADHEr, FUM; ADHEr, MDH; ADHEr, PFLi, PPCK; ADHEr, PFLi, SUCD4; ADHEr, ACKr, NADH6; ADHEr, NADH6, PFLi; ADHEr, ASPT, MDH; ADHEr, NADH6, PPCK; ADHEr, PPCK, THD2; ADHEr, ATPS4r, PPCK; ADHEr, MDH, THD2; ADHEr, FUM, PFLi; ADHEr, PPCK, SUCD4; ADHEr, GLCpts, PPCK; ADHEr, GLUDy, MDH; ADHEr, GLUDy, PPCK; ADHEr, FUM, PPCK; ADHEr, MDH, PPCK; ADHEr, FUM, GLUDy; ADHEr, FUM, HEX1; ADHEr, HEX1, PFLi; ADHEr, HEX1, THD2; ADHEr, FRD2, LDH_D, MDH; ADHEr, FRD2, LDH_D, ME2; ADHEr, MDH, PGL, THD2; ADHEr, G6PDHy, MDH, THD2; ADHEr, PFLi, PPCK, THD2; ADHEr, ACKr, AKGD, ATPS4r; ADHEr, GLCpts, PFLi, PPCK; ADHEr, ACKr, ATPS4r, SUCOAS; ADHEr, GLUDy, PFLi, PPCK; ADHEr, ME2, PFLi, SUCD4; ADHEr, GLUDy, PFLi, SUCD4; ADHEr, ATPS4r, LDH_D, SUCD4; ADHEr, FUM, HEX1, PFLi; ADHEr, MDH, NADH6, THD2; ADHEr, ATPS4r, MDH, NADH6; ADHEr, ATPS4r, FUM, NADH6; ADHEr, ASPT, MDH, NADH6; ADHEr, ASPT, MDH, THD2; ADHEr, ATPS4r, GLCpts, SUCD4; ADHEr, ATPS4r, GLUDy, MDH; ADHEr, ATPS4r, MDH, PPCK; ADHEr, ATPS4r, FUM, PPCK; ADHEr, ASPT, GLCpts, MDH; ADHEr, ASPT, GLUDy, MDH; ADHEr, ME2, SUCD4, THD2; ADHEr, FUM, PPCK, THD2; ADHEr, MDH, PPCK,

THD2; ADHEr, GLUDy, MDH, THD2; ADHEr, HEX1, PFLi, THD2; ADHEr, ATPS4r, G6PDHy, MDH; ADHEr, ATPS4r, MDH, PGL; ADHEr, ACKr, FRD2, LDH_D; ADHEr, ACKr, LDH_D, SUCD4; ADHEr, ATPS4r, FUM, GLUDy; ADHEr, ATPS4r, FUM, HEX1; ADHEr, ATPS4r, MDH, THD2; ADHEr, ATPS4r, FRD2, LDH_D; ADHEr, ATPS4r, MDH, PGDH; ADHEr, GLCpts, PPCK, THD2; ADHEr, GLUDy, PPCK, THD2; ADHEr, FUM, HEX1, THD2; ADHEr, ATPS4r, ME2, THD2; ADHEr, FUM, ME2, THD2; ADHEr, GLCpts, GLUDy, PPCK; ADHEr, ME2, PGL, THD2; ADHEr, G6PDHy, ME2, THD2; ADHEr, ATPS4r, FRD2, LDH_D, ME2; ADHEr, ATPS4r, FRD2, LDH_D, MDH; ADHEr, ASPT, LDH_D, MDH, PFLi; ADHEr, ATPS4r, GLCpts, NADH6, PFLi; ADHEr, ATPS4r, MDH, NADH6, PGL; ADHEr, ATPS4r, G6PDHy, MDH, NADH6; ADHEr, ACKr, FUM, GLUDy, LDH_D; ADHEr, ACKr, GLUDy, LDH_D, SUCD4; ADHEr, ATPS4r, G6PDHy, MDH, THD2; ADHEr, ATPS4r, MDH, PGL, THD2; ADHEr, ASPT, G6PDHy, MDH, PYK; ADHEr, ASPT, MDH, PGL, PYK; ADHEr, ASPT, LDH_D, MDH, SUCOAS; ADHEr, ASPT, FUM, LDH_D, MDH; ADHEr, ASPT, LDH_D, MALS, MDH; ADHEr, ASPT, ICL, LDH_D, MDH; ADHEr, FRD2, GLUDy, LDH_D, PPCK; ADHEr, FRD2, LDH_D, PPCK, THD2; ADHEr, ACKr, ATPS4r, LDH_D, SUCD4; ADHEr, ACKr, ACS, PPC, PPCK; ADHEr, GLUDy, LDH_D, PPC, PPCK; ADHEr, LDH_D, PPC, PPCK, THD2; ADHEr, ASPT, ATPS4r, GLCpts, MDH; ADHEr, G6PDHy, MDH, NADH6, THD2; ADHEr, MDH, NADH6, PGL, THD2; ADHEr, ATPS4r, G6PDHy, GLCpts, MDH; ADHEr, ATPS4r, GLCpts, MDH, PGL; ADHEr, ACKr, LDH_D, MDH, SUCD4.

Table 29 shows the reactions of corresponding genes to be knocked out of a host organism such as *E. coli*. The corresponding metabolite corresponding to abbreviations in Table 29 are shown in Table 30.

Table 29. Corresponding genes to be knocked out to prevent a particular reaction from occurring in *E. coli*.

Reaction Abbreviation	Reaction Stoichiometry*	Genes Encoding the Enzyme(s) Catalyzing Each Reaction&
ACKr	[c] : ac + atp <==> actp + adp	(b3115 or b2296 or b1849)
ACS	[c] : ac + atp + coa --> accoa + amp + ppi	b4069
ACt6	ac[p] + h[p] <==> ac[c] + h[c]	Non-gene associated
ADHEr	[c] : etoh + nad <==> acald + h + nadh [c] : acald + coa + nad <==> accoa + h + nadh	(b0356 or b1478 or b1241) (b1241 or b0351)
AKGD	[c] : akg + coa + nad --> co2 + nadh + succoa	(b0116 and b0726

		and b0727)
ASNS2	[c] : asp-L + atp + nh4 --> amp + asn-L + h + ppi	b3744
ASPT	[c] : asp-L --> fum + nh4	b4139
ATPS4r	adp[c] + (4) h[p] + pi[c] <==> atp[c] + (3) h[c] + h2o[c]	(((b3736 and b3737 and b3738) and (b3731 and b3732 and b3733 and b3734 and b3735)) or ((b3736 and b3737 and b3738) and (b3731 and b3732 and b3733 and b3734 and b3735) and b3739))
CBMK2	[c] : atp + co2 + nh4 <==> adp + cbp + (2) h	(b0521 or b0323 or b2874)
EDA	[c] : 2ddg6p --> g3p + pyr	b1850
ENO	[c] : 2pg <==> h2o + pep	b2779
FBA	[c] : fdp <==> dhap + g3p	(b2097 or b2925 or b1773)
FBP	[c] : fdp + h2o --> f6p + pi	(b4232 or b3925)
FDH2	for[p] + (2) h[c] + q8[c] --> co2[c] + h[p] + q8h2[c] for[p] + (2) h[c] + mqn8[c] --> co2[c] + h[p] + mql8[c]	((b3892 and b3893 and b3894) or (b1474 and b1475 and b1476))
FRD2	[c] : fum + mql8 --> mqn8 + succ [c] : 2dmmql8 + fum --> 2dmmq8 + succ	(b4151 and b4152 and b4153 and b4154)
FTHFD	[c] : 10fthf + h2o --> for + h + thf	b1232
FUM	[c] : fum + h2o <==> mal-L	(b1612 or b4122 or b1611)
G5SD	[c] : glu5p + h + nadph --> glu5sa + nadp + pi	b0243
G6PDHy	[c] : g6p + nadp <==> 6pgl + h + nadph	b1852
GLCpts	glc-D[p] + pep[c] --> g6p[c] + pyr[c]	((b2417 and b1101 and b2415 and b2416) or (b1817 and b1818 and b1819 and b2415 and b2416) or (b2417 and b1621 and b2415 and b2416))
GLU5K	[c] : atp + glu-L --> adp + glu5p	b0242
GLUDy	[c] : glu-L + h2o + nadp <==> akg + h + nadph + nh4	b1761
GLYCL	[c] : gly + nad + thf --> co2 + mlthf + nadh + nh4	(b2904 and b2903 and b2905 and b0116)
HEX1	[c] : atp + glc-D --> adp + g6p + h	b2388
ICL	[c] : icit --> glx + succ	b4015
LDH_D	[c] : lac-D + nad <==> h + nadh + pyr	(b2133 or b1380)
MALS	[c] : accoa + glx + h2o --> coa + h + mal-L	(b4014 or b2976)
MDH	[c] : mal-L + nad <==> h + nadh + oaa	b3236

ME2	[c] : mal-L + nadp --> co2 + nadph + pyr	b2463
MTHFC	[c] : h2o + methf <==> 10fthf + h	b0529
NADH12	[c] : h + mqn8 + nadh --> mql8 + nad [c] : h + nadh + q8 --> nad + q8h2 [c] : 2dmmq8 + h + nadh --> 2dmmql8 + nad	b1109
NADH6	(4) h[c] + nadh[c] + q8[c] --> (3) h[p] + nad[c] + q8h2[c] (4) h[c] + mqn8[c] + nadh[c] --> (3) h[p] + mql8[c] + nad[c] 2dmmq8[c] + (4) h[c] + nadh[c] --> 2dmmql8[c] + (3) h[p] + nad[c]	(b2276 and b2277 and b2278 and b2279 and b2280 and b2281 and b2282 and b2283 and b2284 and b2285 and b2286 and b2287 and b2288)
PFK	[c] : atp + f6p --> adp + fdp + h	(b3916 or b1723)
PFLi	[c] : coa + pyr --> accoa + for	(((b0902 and b0903) and b2579) or (b0902 and b0903) or (b0902 and b3114) or (b3951 and b3952))
PGDH	[c] : 6pgc + nadp --> co2 + nadph + ru5p-D	b2029
PGI	[c] : g6p <==> f6p	b4025
PGL	[c] : 6pgl + h2o --> 6pgc + h	b0767
PGM	[c] : 2pg <==> 3pg	(b3612 or b4395 or b0755)
PPC	[c] : co2 + h2o + pep --> h + oaa + pi	b3956
PPCK	[c] : atp + oaa --> adp + co2 + pep	b3403
PRO1z	[c] : fad + pro-L --> 1pyr5c + fadh2 + h	b1014
PYK	[c] : adp + h + pep --> atp + pyr	b1854 or b1676)
PYRt2	h[p] + pyr[p] <==> h[c] + pyr[c]	Non-gene associated
RPE	[c] : ru5p-D <==> xu5p-D	(b4301 or b3386)
SO4t2	so4[e] <==> so4[p]	(b0241 or b0929 or b1377 or b2215)
SUCD4	[c] : q8 + succ --> fum + q8h2	(b0721 and b0722 and b0723 and b0724)
SUCOAS	[c] : atp + coa + succ <==> adp + pi + succoa	(b0728 and b0729)
SULabc	atp[c] + h2o[c] + so4[p] --> adp[c] + h[c] + pi[c] + so4[c]	((b2422 and b2425 and b2424 and b2423) or (b0763 and b0764 and b0765) or (b2422 and b2424 and b2423 and b3917))
TAL	[c] : g3p + s7p <==> e4p + f6p	(b2464 or b0008)
THD2	(2) h[p] + nadh[c] + nadp[c] --> (2) h[c] + nad[c] + nadph[c]	(b1602 and b1603)
THD5	[c] : nad + nadph --> nadh + nadp	(b3962 or (b1602 and b1603))
TPI	[c] : dhap <==> g3p	b3919

Table 30. Metabolite names corresponding to abbreviations used in Table 29.

Metabolite Abbreviation	Metabolite Name
10fthf	10-Formyltetrahydrofolate
1pyr5c	1-Pyrroline-5-carboxylate
2ddg6p	2-Dehydro-3-deoxy-D-gluconate 6-phosphate
2dmmq8	2-Demethylmenaquinone 8
2dmmql8	2-Demethylmenaquinol 8
2pg	D-Glycerate 2-phosphate
3pg	3-Phospho-D-glycerate
6pgc	6-Phospho-D-gluconate
6pgl	6-phospho-D-glucono-1,5-lactone
ac	Acetate
acald	Acetaldehyde
accoa	Acetyl-CoA
actp	Acetyl phosphate
adp	ADP
akg	2-Oxoglutarate
amp	AMP
asn-L	L-Asparagine
asp-L	L-Aspartate
atp	ATP
cbp	Carbamoyl phosphate
co2	CO2
coa	Coenzyme A
dhap	Dihydroxyacetone phosphate
e4p	D-Erythrose 4-phosphate
etoh	Ethanol
f6p	D-Fructose 6-phosphate
fad	Flavin adenine dinucleotide oxidized
fadh2	Flavin adenine dinucleotide reduced
fdp	D-Fructose 1,6-bisphosphate
for	Formate
fum	Fumarate
g3p	Glyceraldehyde 3-phosphate
g6p	D-Glucose 6-phosphate
glc-D	D-Glucose
glu5p	L-Glutamate 5-phosphate
glu5sa	L-Glutamate 5-semialdehyde
glu-L	L-Glutamate
glx	Glyoxylate
gly	Glycine
h	H+
h2o	H2O
icit	Isocitrate
lac-D	D-Lactate
mal-L	L-Malate
methf	5,10-Methenyltetrahydrofolate

mlthf	5,10-Methylenetetrahydrofolate
mql8	Menaquinol 8
mqn8	Menaquinone 8
nad	Nicotinamide adenine dinucleotide
nadh	Nicotinamide adenine dinucleotide - reduced
nadp	Nicotinamide adenine dinucleotide phosphate
nadph	Nicotinamide adenine dinucleotide phosphate - reduced
nh4	Ammonium
oaa	Oxaloacetate
pep	Phosphoenolpyruvate
pi	Phosphate
ppi	Diphosphate
pro-L	L-Proline
pyr	Pyruvate
q8	Ubiquinone-8
q8h2	Ubiquinol-8
ru5p-D	D-Ribulose 5-phosphate
s7p	Sedoheptulose 7-phosphate
so4	Sulfate
succ	Succinate
succoa	Succinyl-CoA
thf	5,6,7,8-Tetrahydrofolate
xu5p-D	D-Xylulose 5-phosphate

EXAMPLE XX
Exemplary Pathways for Producing BDO

This example describes exemplary pathways to produce 4-hydroxybutanal (4-HBal) and/or BDO using a carboxylic acid reductase as a BDO pathway enzyme.

An exemplary pathway for production of BDO includes use of an NAD+ or NADP+ aryl-aldehyde dehydrogenase (E.C.: 1.2.1.29 and 1.2.1.30) to convert 4-hydroxybutyrate to 4-hydroxybutanal and an alcohol dehydrogenase to convert 4-hydroxybutanal to 1,4-butanediol. 4-Hydroxybutyrate can be derived from the tricarboxylic acid cycle intermediates succinyl-CoA and/or alpha-ketoglutarate as shown in Figure 58.

Aryl-Aldehyde Dehydrogenase (or Carboxylic Acid Reductase). An aryl-aldehyde dehydrogenase, or equivalently a carboxylic acid reductase, can be found in *Nocardia iowensis*. Carboxylic acid reductase catalyzes the magnesium, ATP and NADPH-dependent reduction of carboxylic acids to their corresponding aldehydes (Venkitasubramanian et al., *J. Biol. Chem.* 282:478-485 (2007)) and is capable of catalyzing the conversion of 4-hydroxybutyrate to 4-hydroxybutanal. This enzyme, encoded by *car*, was cloned and functionally expressed in *E. coli* (Venkitasubramanian et al., *J. Biol. Chem.* 282:478-485 (2007)). Expression of the *npt* gene

product improved activity of the enzyme via post-transcriptional modification. The *npt* gene encodes a specific phosphopantetheine transferase (PPTase) that converts the inactive apo-enzyme to the active holo-enzyme. The natural substrate of this enzyme is vanillic acid, and the enzyme exhibits broad acceptance of aromatic and aliphatic substrates (Venkitasubramanian et al., in *Biocatalysis in the Pharmaceutical and Biotechnology Industires*, ed. R.N. Patel, Chapter 15, pp. 425-440, CRC Press LLC, Boca Raton, FL. (2006)).

Gene name	GI No.	GenBank Accession No.	Organism
car	40796035	AAR91681.1	*Nocardia iowensis* (sp. NRRL 5646)
npt	114848891	ABI83656.1	*Nocardia iowensis* (sp. NRRL 5646)

Additional *car* and *npt* genes can be identified based on sequence homology.

Gene name	GI No.	GenBank Accession No.	Organism
fadD9	121638475	YP_978699.1	*Mycobacterium bovis* BCG
BCG_2812c	121638674	YP_978898.1	*Mycobacterium bovis* BCG
nfa20150	54023983	YP_118225.1	*Nocardia farcinica* IFM 10152
nfa40540	54026024	YP_120266.1	*Nocardia farcinica* IFM 10152
SGR_6790	182440583	YP_001828302.1	*Streptomyces griseus* subsp. *griseus* NBRC 13350
SGR_665	182434458	YP_001822177.1	*Streptomyces griseus* subsp. *griseus* NBRC 13350
MSMEG_2956	YP_887275.1	YP_887275.1	*Mycobacterium smegmatis MC2 155*
MSMEG_5739	YP_889972.1	118469671	*Mycobacterium smegmatis MC2 155*
MSMEG_2648	YP_886985.1	118471293	*Mycobacterium smegmatis MC2 155*
MAP1040c	NP_959974.1	41407138	*Mycobacterium avium subsp. paratuberculosis K-10*
MAP2899c	NP_961833.1	41408997	*Mycobacterium avium subsp. paratuberculosis K-10*
MMAR_2117	YP_001850422.1	183982131	*Mycobacterium marinum M*
MMAR_2936	YP_001851230.1	183982939	*Mycobacterium marinum M*
MMAR_1916	YP_001850220.1	183981929	*Mycobacterium marinum M*
TpauDRAFT_33060	ZP_04027864.1	227980601	*Tsukamurella paurometabola DSM 20162*
TpauDRAFT_20920	ZP_04026660.1	ZP_04026660.1	*Tsukamurella paurometabola DSM 20162*

CPCC7001_1320	ZP_05045132.1	254431429	*Cyanobium PCC7001*
DDBDRAFT_0187729	XP_636931.1	66806417	*Dictyostelium discoideum AX4*

An additional enzyme candidate found in *Streptomyces griseus* is encoded by the *griC* and *griD* genes. This enzyme is believed to convert 3-amino-4-hydroxybenzoic acid to 3-amino-4-hydroxybenzaldehyde as deletion of either *griC* or *griD* led to accumulation of extracellular 3-acetylamino-4-hydroxybenzoic acid, a shunt product of 3-amino-4-hydroxybenzoic acid metabolism (Suzuki, et al., *J. Antibiot.* 60(6):380-387 (2007)). Co-expression of *griC* and *griD* with SGR_665, an enzyme similar in sequence to the *Nocardia iowensis npt*, can be beneficial.

Gene name	GI No.	GenBank Accession No.	Organism
griC	182438036	YP_001825755.1	*Streptomyces griseus* subsp. *griseus* NBRC 13350
griD	182438037	YP_001825756.1	*Streptomyces griseus* subsp. *griseus* NBRC 13350

An enzyme with similar characteristics, alpha-aminoadipate reductase (AAR, EC 1.2.1.31), participates in lysine biosynthesis pathways in some fungal species. This enzyme naturally reduces alpha-aminoadipate to alpha-aminoadipate semialdehyde. The carboxyl group is first activated through the ATP-dependent formation of an adenylate that is then reduced by NAD(P)H to yield the aldehyde and AMP. Like CAR, this enzyme utilizes magnesium and requires activation by a PPTase. Enzyme candidates for AAR and its corresponding PPTase are found in *Saccharomyces cerevisiae* (Morris et al., *Gene* 98:141-145 (1991)), *Candida albicans* (Guo et al., *Mol. Genet. Genomics* 269:271-279 (2003)), and *Schizosaccharomyces pombe* (Ford et al., *Curr. Genet.* 28:131-137 (1995)). The AAR from *S. pombe* exhibited significant activity when expressed in *E. coli* (Guo et al., *Yeast* 21:1279-1288 (2004)). The AAR from *Penicillium chrysogenum* accepts S-carboxymethyl-L-cysteine as an alternate substrate, but did not react with adipate, L-glutamate or diaminopimelate (Hijarrubia et al., *J. Biol. Chem.* 278:8250-8256 (2003)). The gene encoding the *P. chrysogenum* PPTase has not been identified to date.

Gene name	GI No.	GenBank Accession No.	Organism
LYS2	171867	AAA34747.1	*Saccharomyces cerevisiae*
LYS5	1708896	P50113.1	*Saccharomyces cerevisiae*
LYS2	2853226	AAC02241.1	*Candida albicans*
LYS5	28136195	AAO26020.1	*Candida albicans*
Lys1p	13124791	P40976.3	*Schizosaccharomyces pombe*

Lys7p	1723561	Q10474.1	*Schizosaccharomyces pombe*
Lys2	3282044	CAA74300.1	*Penicillium chrysogenum*

There are several advantages of using carboxylic acid reductase for BDO p.roduction. There are at least two advantages of forming 4-hydroxybutanal from 4-hydroxybutyrate via a carboxylic acid reductase compared to forming 4-hydroxybutanal from an activated version of 4-hydroxybutyrate (for example, 4-hydroxybutyryl-CoA, 4-hydroxybutyryl-Pi) via an acyl-CoA or acyl-phosphate reductase. First, the formation of gamma-butyrolactone (GBL) as a byproduct is greatly reduced. It is believed that the activated versions of 4-hydroxybutyrate cyclize to GBL more readily than unactivated 4-hydroxybutyrate. The use of carboxylic acid reductase eliminates the need to pass through a free activated 4-hydroxybutyrate intermediate, thus reducing the formation of GBL as a byproduct accompanying BDO production. Second, the formation of ethanol as a byproduct is greatly reduced. Ethanol is often formed in varying amounts when an aldehyde- or an alcohol-forming 4-hydroxybutyryl-CoA reductase is used to convert 4-hydroxybutyryl-CoA to 4-hydroxybutanal or 1,4-butanediol, respectively. This is because most, if not all, aldehyde- or alcohol-forming 4-hydroxybutyryl-CoA reductases can accept acetyl-CoA as a substrate in addition to 4-hydroxybutyryl-CoA. Aldehyde-forming enzymes, for example, often catalyze the conversion of acetyl-CoA to acetaldehyde, which is subsequently reduced to ethanol by native or non-native alcohol dehydrogenases. Alcohol-forming 4-hydroxybutyryl-CoA reductases that accept acetyl-CoA as a substrate will convert acetyl-CoA directly to ethanol. It appears that carboxylic acid reductase enzymes have far less activity on acetyl-CoA than aldehyde- or alcohol-forming acyl-CoA reductase enzymes, and thus their application for BDO production results in minimal ethanol byproduct formation (see below).

EXAMPLE XXI
Biosynthesis of 1,4-Butanediol Using A Carboxylic Acid Reductase Enzyme

This example describes the generation of a microbial organism that produces 1,4-butanediol using a carboxylic acid reductase enzyme.

Escherichia coli is used as a target organism to engineer the pathway for 1,4-butanediol synthesis described in Figure 58. *E. coli* provides a good host for generating a non-naturally occurring microorganism capable of producing 1,4-butanediol. *E. coli* is amenable to genetic manipulation and is known to be capable of producing various products, like ethanol, acetic

acid, formic acid, lactic acid, and succinic acid, effectively under various oxygenation conditions.

Integration of 4-Hydroxybutyrate Pathway Genes into Chromosome: Construction of ECKh-432. The carboxylic acid reductase enzyme was expressed in a strain of *E. coli* designated ECKh-432 whose construction is described in Example XVII. This strain contained the components of the BDO pathway, leading to 4HB, integrated into the chromosome of *E. coli* at the *fimD* locus.

As described in Example XVII, the succinate branch of the upstream pathway was integrated into the *E. coli* chromosome using the λ red homologeous recombination method (Datsenko and Wanner, *Proc. Natl. Acad. Sci. USA* 97:6640-6645 (2000)). A polycistronic DNA fragment containing a promoter, the *sucCD* gene of *Escherichia coli* encoding succinyl-CoA ligase, the *sucD* gene of *Porphyromonas gingivalis* encoding succinyl-CoA reductase (aldehyde forming) (step A of Figure 58), the *4hbd* gene of *Porphyromonas gingivalis* encoding 4-hydroxybutyrate dehydrogenase (step C of Figure 58), and a terminator sequence was inserted into the *Afl*III site of the pKD3 plasmid.

As described in Example XVII, the alpha-ketoglutarate branch of the upstream pathway was integrated into the chromosome by homologeous recombination. The plasmid used in this modification was pRE118-V2 (pRE118 (ATCC87693) deleted of the *oriT* and IS sequences), which contains a kanamycin-resistant gene, a gene encoding the levansucrase (sacB) and a R6K conditional replication ori. The integration plasmid also contained a polycistronic sequence with a promoter, the *sucA* gene from *Mycobacterium bovis* encoding alpha-ketoglutarate decarboxylase (step B of Figure 58), the *Clostridium kluyveri 4hbd* gene encoding 4-hydroxybutyrate dehydrogenase (step C of Figure 58), and a terminator being inserted between two 1.5-kb DNA fragments that are homologous to the flanking regions of the target insertion site. The resulting plasmid was used to transform *E. coli* strain. The integration candidate was selected on plates containing kanamycin. The correct integration site was verified by PCR. To resolve the antibiotic marker from the chromosome, the cells were selected for growth on medium containing sucrose. The final strain was verified by PCR and DNA sequencing.

The recipient *E. coli* strain was ECKh-422 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA *glt*AR163L) whose construction is described in Example XV. ECKh-422 contains a mutation *glt*AR163L leading to NADH-insensitivity of citrate synthase encoded by *gltA*. It

further contains an NADH-insensitive version of the *lpdA* gene from *Klebsiella pneumonia* integrated into the chromosome as described below.

Replacement of the native *lpdA* was replaced with a NADH-insensitive *lpdA* from *Klebsiella pneumonie*, as described in Example XIV. The resulting vector was designated pRE118-V2 (see Figure 34).]

Cloning and Expression of Carboxylic Acid Reductase and PPTase. To generate an *E. coli* strain engineered to produce 1,4-butanediol, nucleic acids encoding a carboxylic acid reductase and phosphopantetheine transferase are expressed in *E. coli* using well known molecular biology techniques (see, for example, Sambrook, supra, 2001; Ausubel supra, 1999). In particular, the *car* (AAR91681.1) and *npt* (ABI83656.1) genes were cloned into the pZS*13 vector (Expressys, Ruelzheim, Germany) under the PA1/lacO promoter. The *car* gene (GNM_720) was cloned by PCR from *Nocardia* genomic DNA. Its nucleic acid and protein sequences are shown in Figures 59A and 59B, respectively.

A codon-optimized version of the *npt* gene (GNM_721) was synthesized by GeneArt (Regensburg, Germany). Its nucleic acid and protein sequences are shown in Figures 60A and 60B, respectively. The resulting vector from cloning GNM_720 and GNM_721 into pZS*13 is shown in Figure 61.

The plasmid was transformed into ECKh-432 to express the proteins and enzymes required for 1,4-butanediol production. Alternate versions of the plasmid containing only GNM_720 and only GNM_721 were also constructed.

Demonstration of 1,4-BDO Production using Carboxylic Acid Reductase. Functional expression of the 1,4-butanediol pathway was demonstrated using *E. coli* whole-cell culture. A single colony of *E. coli* ECKh-432 transformed with the pZS*13 plasmid containing both GNM_720 and GNM_721 was inoculated into 5 mL of LB medium containing appropriate antibiotics. Similarly, single colonies of *E. coli* ECKh-432 transformed with the pZS*13 plasmids containing either GNM_720 or GNM_721 were inoculated into additional 5 mL aliquots of LB medium containing appropriate antibiotics. Ten mL micro-aerobic cultures were started by inoculating fresh minimal *in vivo* conversion medium (see below) containing the appropriate antibiotics with 1% of the first cultures.

Recipe of the minimal *in vivo* conversion medium (for 1000 mL) is as follows:

	final concentration
1M MOPS/KOH buffer	40 mM
Glucose (40%)	1%
10XM9 salts solution	1X
MgSO4 (1 M)	1 mM
trace minerals (x1000)	1X
1M NaHCO3	10 mM

Microaerobic conditions were established by initially flushing capped anaerobic bottles with nitrogen for 5 minutes, then piercing the septum with an 18G needle following inoculation. The needle was kept in the bottle during growth to allow a small amount of air to enter the bottles. Protein expression was induced with 0.2 mM IPTG when the culture reached mid-log growth phase. This is considered: time = 0 hr. The culture supernatants were analyzed for BDO, 4HB, and other by-products as described above and in WO2008115840 (see Table 31).

Table 31. Production of BDO, 4-HB and other products in various strains.

				mM						
Strain	pZS*13S	OD600	OD600	PA	SA	LA	4HB	BDO	GBL	$ETOH_{Enz}$
ECKh-432	720	0.420	2.221	6.36	0.00	0.10	7.71	3.03	0.07	>LLOQ
ECKh-432	721	0.323	2.574	1.69	0.00	0.00	12.60	0.00	0.00	>LLOQ
ECKh-432	720/721	0.378	2.469	1.70	0.00	0.01	4.23	9.16	0.24	1.52

PA = pyruvate, SA = succinate, LA = lactate, 4HB = 4-hydroxybutyrate, BDO = 1,4-butanediol, GBL = gamma-butyrolactone, Etoh = ethanol, LLOQ = lower limit of quantification

These results demonstrate that the carboxylic acid reductase gene, GNM_720, is required for BDO formation in ECKh-432 and its effectiveness is increased when co-expressed with the PPTase, GNM_721. GBL and ethanol were produced in far smaller quantities than BDO in the strains expressing GNM_720 by itself or in combination with GNM_721.

Additional Pathways to BDO Employing Carboxylic Acid Reductase. It is expected that carboxylic acid reductase can function as a component of many pathways to 1,4-butanediol from the TCA cycle metabolites: succinate, succinyl-CoA, and alpha-ketoglutarate. Several of these pathways are disclosed in Figure 62. All routes can lead to theoretical BDO yields greater than or equal to 1 mol/mol assuming glucose as the carbon source. Similar high theoretical yields can be obtained from additional substrates including sucrose, xylose, arabinose, synthesis gas, among many others. It is expected that the expression of carboxylic acid reductase alone or in combination with PPTase (that is, to catalyze steps F and D of Figure 62) is sufficient for 1,4-

butanediol production from succinate provided that sufficient endogenous alcohol dehydrogenase activity is present to catalyze steps C and E of Figure 62. Candidate enzymes for steps A through Z of Figure 62 are described in section XXIII.

EXAMPLE XXII
Pathways to Putrescine that Employ Carboxylic Acid Reductase

This example describes exemplary putrescine pathways utilizing carboxylic acid reductase.

Putrescine, also known as 1,4-diaminobutane or butanediamine, is an organic chemical compound of the formula $NH_2(CH_2)_4NH_2$. It can be reacted with adipic acid to yield the polyamide Nylon-4,6, which is marketed by DSM (Heerlen, Netherlands) under the trade name Stanyl™. Putrescine is naturally produced, for example, by the natural breakdown of amino acids in living and dead organisms. *E. coli* has been engineered to produce putrescine by overexpressing the native ornithine biosynthetic machinery as well as an ornithine decarboxylase (Qian, et al., *Biotechnol. Bioeng.* 104(4):651-662 (2009)).

Figure 63 describes a number of additional biosynthetic pathways leading to the production of putrescine from succinate, succinyl-CoA, or alpha-ketoglutarate and employing a carboxylic acid reductase. Note that none of these pathways require formation of an activated version of 4-aminobutyrate such as 4-aminobutyryl-CoA, which can be reduced by an acyl-CoA reductase to 4-aminobutanal but also can readily cyclize to its lactam, 2-pyrrolidinone (Ohsugi, et al., *J. Biol. Chem.* 256:7642-7651 (1981)). All routes can lead to theoretical putrescine yields greater than or equal to 1 mol/mol assuming glucose as the carbon source. Similar high theoretical yields can be obtained from additional substrates including sucrose, xylose, arabinose, synthesis gas, among many others. Candidate enzymes for steps A through U of Figure 63 are described in Example XXIII.

EXAMPLE XXIII
Exemplary Enzymes for Production of C4 Compounds

This example describes exemplary enzymes for production of C4 compounds such as 1,4-butanediol, 4-hydroxybutanal and putrescine.

Enzyme classes. All transformations depicted in Figures 58, 62 and 63 fall into the general categories of transformations shown in Table 32. This example describes a number of biochemically characterized genes in each category. Specifically listed are genes that can be applied to catalyze the appropriate transformations in Figures 58, 62 and 63 when cloned and

expressed. The first three digits of each label correspond to the first three Enzyme Commission number digits which denote the general type of transformation independent of substrate specificity.

Table 32. Classes of Enzyme Transformations Depicted in Figures 58, 62 and 63.

LABEL	FUNCTION
1.1.1.a	Oxidoreductase (oxo to alcohol)
1.1.1.c	Oxidoreductase (2 step, acyl-CoA to alcohol)
1.2.1.b	Oxidoreductase (acyl-CoA to aldehyde)
1.2.1.c	Oxidoreductase (2-oxo acid to acyl-CoA, decarboxylation)
1.2.1.d	Oxidoreductase (phosphonate reductase)
1.2.1.e	Acid reductase
1.4.1.a	Oxidoreductase (aminating)
2.3.1.a	Acyltransferase (transferring phosphate group to CoA)
2.6.1.a	Aminotransferase
2.7.2.a	Phosphotransferase (carboxy acceptor)
2.8.3.a	CoA transferase
3.1.2.a	CoA hydrolase
4.1.1.a	Carboxy-lyase
6.2.1.a	CoA synthetase

1.1.1.a Oxidoreductase (oxo to alcohol)

Aldehyde to alcohol. Three transformations described in Figures 58, 62 and 63 involve the conversion of an aldehyde to alcohol. These are 4-hydroxybutyrate dehydrogenase (step C, Figures 58 and 62), 1,4-butanediol dehydrogenase (step E, Figures 58 and 62), and 5-hydroxy-2-pentanoic acid (step Y, Figure 62). Exemplary genes encoding enzymes that catalyze the conversion of an aldehyde to alcohol, that is, alcohol dehydrogenase or equivalently aldehyde reductase, include *alrA* encoding a medium-chain alcohol dehydrogenase for C2-C14 (Tani et al. *Appl. Environ. Microbiol.* 66:5231-5235 (2000)), ADH2 from *Saccharomyces cerevisiae* (Atsumi et al. *Nature* 451:86-89 (2008)), *yqhD* from *E. coli,* which has preference for molecules longer than C(3) (Sulzenbacher et al. *J. Mol. Biol.* 342:489-502 (2004)), and *bdh* I and *bdh* II from *C. acetobutylicum,* which converts butyryaldehyde into butanol (Walter et al. *J. Bacteriol.* 174:7149-7158 (1992)). The protein sequences for each of exemplary gene products can be found using the following GenBank accession numbers:

Gene	Accession No.	GI No.	Organism
alrA	BAB12273.1	9967138	*Acinetobacter* sp. Strain M-1
ADH2	NP_014032.1	6323961	*Saccharymyces cerevisiae*
yqhD	NP_417484.1	16130909	*Escherichia coli*
bdh I	NP_349892.1	15896543	*Clostridium acetobutylicum*
bdh II	NP_349891.1	15896542	*Clostridium acetobutylicum*

Enzymes exhibiting 4-hydroxybutyrate dehydrogenase activity (EC 1.1.1.61) also fall into this category. Such enzymes have been characterized in *Ralstonia eutropha* (Bravo et al. *J. Forensic Sci.* 49:379-387 (2004)), *Clostridium kluyveri* (Wolff et al., *Protein Expr. Purif.* 6:206-212 (1995)) and *Arabidopsis thaliana* (Breitkreuz et al. *J. Biol. Chem.* 278:41552-41556 (2003)).

Gene	Accession No.	GI No.	Organism
4hbd	YP_726053.1	113867564	*Ralstonia eutropha* H16
4hbd	EDK35022.1	146348486	*Clostridium kluyveri* DSM 555
4hbd	Q94B07	75249805	*Arabidopsis thaliana*

The *adh1* gene from *Geobacillus thermoglucosidasius* M10EXG (Jeon et al., *J. Biotechnol.* 135:127-133 (2008)) was shown to exhibit high activity on both 4-hydroxybutanal and butanal (see above). Thus this enzyme exhibits 1,4-butanediol dehydrogenase activity.

Gene	Accession No.	GI No.	Organism
adh1	AAR91477.1	40795502	*Geobacillus thermoglucosidasius M10EXG*

Another exemplary enzyme is 3-hydroxyisobutyrate dehydrogenase, which catalyzes the reversible oxidation of 3-hydroxyisobutyrate to methylmalonate semialdehyde. This enzyme participates in valine, leucine and isoleucine degradation and has been identified in bacteria, eukaryotes, and mammals. The enzyme encoded by *P84067* from *Thermus thermophilus* HB8 has been structurally characterized (Lokanath et al. *J. Mol. Biol.* 352:905-17 (2005)). The reversibility of the human 3-hydroxyisobutyrate dehydrogenase was demonstrated using isotopically-labeled substrate (Manning et al., *Biochem. J.* 231:481-484 (1985)). Additional genes encoding this enzyme include *3hidh* in *Homo sapiens* (Hawes et al., *Methods Enzymol.* 324:218-228 (2000)) and *Oryctolagus cuniculus* (Chowdhury et al., *Biosci. Biotechnol. Biochem.* 60:2043-2047 (1996); Hawes et al. *Methods Enzymol.* 324:218-228 (2000)), *mmsb* in *Pseudomonas aeruginosa*, and *dhat* in *Pseudomonas putida* (Aberhart et al., *J. Chem. Soc. [Perkin 1]* 6:1404-1406 (1979); Chowdhury et al., *Biosci. Biotechnol Biochem.* 67:438-441 (2003); Chowdhury et al., *Biosci. Biotechnol. Biochem.* 60:2043-2047 (1996)).

Gene	Accession No.	GI No.	Organism
P84067	P84067	75345323	*Thermus thermophilus*
mmsb	P28811.1	127211	*Pseudomonas aeruginosa*
dhat	Q59477.1	2842618	*Pseudomonas putida*
3hidh	P31937.2	12643395	*Homo sapiens*
3hidh	P32185.1	416872	*Oryctolagus cuniculus*

Several 3-hydroxyisobutyrate dehydrogenase enzymes have also been shown to convert malonic semialdehyde to 3-hydroxyproprionic acid (3-HP). Three gene candidates exhibiting this activity are *mmsB* from *Pseudomonas aeruginosa* PAO1(62), *mmsB* from *Pseudomonas putida* KT2440 (Liao et al., US Publication 2005/0221466) and *mmsB* from *Pseudomonas putida E23* (Chowdhury et al., *Biosci. Biotechnol. Biochem.* 60:2043-2047 (1996)). An enzyme with 3-hydroxybutyrate dehydrogenase activity in *Alcaligenes faecalis* M3A has also been identified (Gokam et al., US Patent No. 7,393,676; Liao et al., US Publication No. 2005/0221466). Additional gene candidates from other organisms including *Rhodobacter spaeroides* can be inferred by sequence similarity.

Gene	**Accession No.**	**GI No.**	**Organism**
mmsB	AAA25892.1	151363	*Pseudomonas aeruginosa*
mmsB	NP_252259.1	15598765	*Pseudomonas aeruginosa* PAO1
mmsB	NP_746775.1	26991350	*Pseudomonas putida* KT2440
mmsB	JC7926	60729613	*Pseudomonas putida* E23
orfB1	AAL26884	16588720	*Rhodobacter spaeroides*

The conversion of malonic semialdehyde to 3-HP can also be accomplished by two other enzymes, NADH-dependent 3-hydroxypropionate dehydrogenase and NADPH-dependent malonate semialdehyde reductase. An NADH-dependent 3-hydroxypropionate dehydrogenase is thought to participate in beta-alanine biosynthesis pathways from propionate in bacteria and plants (Rathinasabapathi, *J. Plant Pathol.* 159:671-674 (2002); Stadtman, *J. Am. Chem. Soc.* 77:5765-5766 (1955)). This enzyme has not been associated with a gene in any organism to date. NADPH-dependent malonate semialdehyde reductase catalyzes the reverse reaction in autotrophic CO_2-fixing bacteria. Although the enzyme activity has been detected *in Metallosphaera sedula*, the identity of the gene is not known (Alber et al. *J. Bacteriol.* 188:8551-8559 (2006)).

1.1.1.c Oxidoreductase (2 step, acyl-CoA to alcohol).

Steps S and W of Figure 62 depict bifunctional reductase enzymes that can form 4-hydroxybutyrate and 1,4-butanediol, respectively. Exemplary 2-step oxidoreductases that

convert an acyl-CoA to alcohol include those that transform substrates such as acetyl-CoA to ethanol (for example, *adhE* from *E. coli* (Kessler et al., FEBS. Lett. 281:59-63 (1991)) and butyryl-CoA to butanol (for example, *adhE2* from *C. acetobutylicum* (Fontaine et al., *J.Bacteriol.* 184:821-830 (2002)). The *C. acetobutylicum adhE2* gene was shown to convert 4-hydroxybutyryl-CoA to 1,4-butanediol (see above). In addition to reducing acetyl-CoA to ethanol, the enzyme encoded by *adhE* in *Leuconostoc mesenteroides* has been shown to oxidize the branched chain compound isobutyraldehyde to isobutyryl-CoA (Kazahaya et al. J., *Gen. Appl. Microbiol.* 18:43-55 (1972); Koo et al., *Biotechnol. Lett.* 27:505-510 (2005)).

Gene	Accession No.	GI No.	Organism
adhE	NP_415757.1	16129202	*Escherichia coli*
adhE2	AAK09379.1	12958626	*Clostridium acetobutylicum*
adhE	AAV66076.1	55818563	*Leuconostoc mesenteroides*

Another exemplary enzyme can convert malonyl-CoA to 3-HP. An NADPH-dependent enzyme with this activity has characterized in *Chloroflexus aurantiacus,* where it participates in the 3-hydroxypropionate cycle (Hugler et al., *J. Bacteriol.* 184:2404-2410 (2002); Strauss and Fuchs, *Eur. J. Biochem.* 215:633-643 (1993)). This enzyme, with a mass of 300 kDa, is highly substrate-specific and shows little sequence similarity to other known oxidoreductases (Hugler et al., *J. Bacteriol.* 184:2404-2410 (2002)). No enzymes in other organisms have been shown to catalyze this specific reaction; however there is bioinformatic evidence that other organisms may have similar pathways (Klatt et al., *Environ. Microbiol.* 9:2067-2078 (2007)). Enzyme candidates in other organisms including *Roseiflexus castenholzii*, *Erythrobacter sp. NAP1* and marine gamma proteobacterium HTCC2080 can be inferred by sequence similarity.

Gene	Accession No.	GI No.	Organism
mcr	AAS20429.1	42561982	*Chloroflexus aurantiacus*
Rcas_2929	YP_001433009.1	156742880	*Roseiflexus castenholzii*
NAP1_02720	ZP_01039179.1	85708113	*Erythrobacter sp. NAP1*
MGP2080_00535	ZP_01626393.1	119504313	marine gamma proteobacterium HTCC2080

Longer chain acyl-CoA molecules can be reduced by enzymes such as the jojoba (*Simmondsia chinensis*) *FAR,* which encodes an alcohol-forming fatty acyl-CoA reductase. Its overexpression in *E.* coli resulted in FAR activity and the accumulation of fatty alcohol (Metz et al., *Plant Physiol.* 122:635-644 2000)).

Gene	Accession No.	GI No.	Organism
FAR	AAD38039.1	5020215	*Simmondsia chinensis*

1.2.1.b Oxidoreductase (acyl-CoA to aldehyde).

Step A of Figures 58, 62 and 63 involves the conversion of succinyl-CoA to succinate semialdehyde by an aldehyde forming succinyl-CoA reductase. Step Q of Figure 62 depicts the conversion of 4-hydroxybutyryl-CoA to 4-hydroxybutanal by an aldehyde-forming 4-hydroxybutyryl-CoA reductase. Several acyl-CoA dehydrogenases are capable of reducing an acyl-CoA to its corresponding aldehyde. Exemplary genes that encode such enzymes include the *Acinetobacter calcoaceticus acr1* encoding a fatty acyl-CoA reductase (Reiser and Somerville, *J. Bacteriol.* 179:2969-2975 (1997)), the *Acinetobacter sp. M-1* fatty acyl-CoA reductase (Ishige et al., *Appl. Environ. Microbiol.* 68:1192-1195 (2002)), and a CoA- and NADP- dependent succinate semialdehyde dehydrogenase encoded by the *sucD* gene in *Clostridium kluyveri* (Sohling and Gottschalk, *J. Bacteriol.* 178:871-80 (1996); Sohling and Gottschalk, *J. Bacteriol.* 178:871-880 (1996)). *SucD* of *P. gingivalis* is another aldehyde-forming succinyl-CoA reductase (Takahashi et al., *J.Bacteriol.* 182:4704-4710 (2000)). The enzyme acylating acetaldehyde dehydrogenase in *Pseudomonas sp*, encoded by *bphG*, is yet another as it has been demonstrated to oxidize and acylate acetaldehyde, propionaldehyde, butyraldehyde, isobutyraldehyde and formaldehyde (Powlowski et al., *J. Bacteriol.* 175:377-385 (1993)). In addition to reducing acetyl-CoA to ethanol, the enzyme encoded by *adhE* in *Leuconostoc mesenteroides* has been shown to oxidize the branched chain compound isobutyraldehyde to isobutyryl-CoA (Koo et al., *Biotechnol. Lett.* 27:505-510 (2005)). Butyraldehyde dehydrogenase catalyzes a similar reaction, conversion of butyryl-CoA to butyraldehyde, in solventogenic organisms such as *Clostridium saccharoperbutylacetonicum* (Kosaka et al., *Biosci. Biotechnol. Biochem.* 71:58-68 (2007)).

Gene	Accession No.	GI No.	Organism
acr1	YP_047869.1	50086359	*Acinetobacter calcoaceticus*
acr1	AAC45217	1684886	*Acinetobacter baylyi*
acr1	BAB85476.1	18857901	*Acinetobacter* sp. Strain M-1
sucD	P38947.1	730847	*Clostridium kluyveri*
sucD	NP_904963.1	34540484	*Porphyromonas gingivalis*

bphG	BAA03892.1	425213	*Pseudomonas sp*
adhE	AAV66076.1	55818563	*Leuconostoc mesenteroides*
bld	AAP42563.1	31075383	*Clostridium saccharoperbutylacetonicum*

An additional enzyme type that converts an acyl-CoA to its corresponding aldehyde is malonyl-CoA reductase, which transforms malonyl-CoA to malonic semialdehyde. Malonyl-CoA reductase is a key enzyme in autotrophic carbon fixation via the 3-hydroxypropionate cycle in thermoacidophilic archael bacteria (Berg et al., *Science* 318:1782-1786 (2007); Thauer, *Science* 318:1732-1733 (2007)). The enzyme utilizes NADPH as a cofactor and has been characterized in *Metallosphaera* and *Sulfolobus spp* (Alber et al., *J. Bacteriol.* 188:8551-8559 (2006); Hugler et al., *J. Bacteriol.* 184:2404-2410 (2002)). The enzyme is encoded by *Msed_0709* in *Metallosphaera sedula* (Alber et al., *J. Bacteriol.* 188:8551-8559 (2006); Berg et al., *Science* 318:1782-1786 (2007)). A gene encoding a malonyl-CoA reductase from *Sulfolobus tokodaii* was cloned and heterologously expressed in *E. coli* (Alber et al., *J. Bacteriol.* 188:8551-8559 (2006)). Although the aldehyde dehydrogenase functionality of these enzymes is similar to the bifunctional dehydrogenase from *Chloroflexus aurantiacus*, there is little sequence similarity. Both malonyl-CoA reductase enzyme candidates have high sequence similarity to aspartate-semialdehyde dehydrogenase, an enzyme catalyzing the reduction and concurrent dephosphorylation of aspartyl-4-phosphate to aspartate semialdehyde. Additional gene candidates can be found by sequence homology to proteins in other organisms including *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius*. Yet another candidate for CoA-acylating aldehyde dehydrogenase is the *ald* gene from *Clostridium beijerinckii* (Toth et al., *Appl. Environ.Microbiol.* 65:4973-4980 (1999)). This enzyme has been reported to reduce acetyl-CoA and butyryl-CoA to their corresponding aldehydes. This gene is very similar to *eutE* that encodes acetaldehyde dehydrogenase of *Salmonella typhimurium* and *E. coli* (Toth et al., *Appl. Environ. Microbiol.* 65:4973-4980 (1999)).

Gene	**Accession No.**	**GI No.**	**Organism**
Msed_0709	YP_001190808.1	146303492	*Metallosphaera sedula*
mcr	NP_378167.1	15922498	*Sulfolobus tokodaii*
asd-2	NP_343563.1	15898958	*Sulfolobus solfataricus*
Saci_2370	YP_256941.1	70608071	*Sulfolobus acidocaldarius*

Ald	AAT66436	49473535	*Clostridium beijerinckii*
eutE	AAA80209	687645	*Salmonella typhimurium*
eutE	P77445	2498347	*Escherichia coli*

1.2.1.c Oxidoreductase (2-oxo acid to acyl-CoA, decarboxylation).

Step AA in Figure 62 depicts the conversion of 5-hydroxy-2-oxopentanoic acid to 4-hydroxybutyryl-CoA. Candidate enzymes for this transformation include 1) branched-chain 2-keto-acid dehydrogenase, 2) alpha-ketoglutarate dehydrogenase, and 3) the pyruvate dehydrogenase multienzyme complex (PDHC). These enzymes are multi-enzyme complexes that catalyze a series of partial reactions which result in acylating oxidative decarboxylation of 2-keto-acids. Each of the 2-keto-acid dehydrogenase complexes occupies key positions in intermediary metabolism, and enzyme activity is typically tightly regulated (Fries et al. *Biochemistry* 42:6996-7002 (2003)). The enzymes share a complex but common structure composed of multiple copies of three catalytic components: alpha-ketoacid decarboxylase (E1), dihydrolipoamide acyltransferase (E2) and dihydrolipoamide dehydrogenase (E3). The E3 component is shared among all 2-keto-acid dehydrogenase complexes in an organism, while the E1 and E2 components are encoded by different genes. The enzyme components are present in numerous copies in the complex and utilize multiple cofactors to catalyze a directed sequence of reactions via substrate channeling. The overall size of these dehydrogenase complexes is very large, with molecular masses between 4 and 10 million Da (that is, larger than a ribosome).

Activity of enzymes in the 2-keto-acid dehydrogenase family is normally low or limited under anaerobic conditions in *E. coli*. Increased production of NADH (or NADPH) could lead to a redox-imbalance, and NADH itself serves as an inhibitor to enzyme function. Engineering efforts have increased the anaerobic activity of the *E. coli* pyruvate dehydrogenase complex (Kim et al. *Appl.Environ.Microbiol.* 73:1766-1771 (2007); Kim et al. *J.Bacteriol.* 190:3851-3858) 2008); Zhou et al. *Biotechnol.Lett.* 30:335-342 (2008)). For example, the inhibitory effect of NADH can be overcome by engineering an H322Y mutation in the E3 component (Kim et al. *J.Bacteriol.* 190:3851-3858 (2008)). Structural studies of individual components and how they work together in complex provide insight into the catalytic mechanisms and architecture of enzymes in this family (Aevarsson et al. *Nat.Struct.Biol.* 6:785-792 (1999); Zhou et al. *Proc.Natl.Acad.Sci.U.S.A.* 98:14802-14807 (2001)). The substrate specificity of the

dehydrogenase complexes varies in different organisms, but generally branched-chain keto-acid dehydrogenases have the broadest substrate range.

Alpha-ketoglutarate dehydrogenase (AKGD) converts alpha-ketoglutarate to succinyl-CoA and is the primary site of control of metabolic flux through the TCA cycle (Hansford, R. G. *Curr.Top.Bioenerg.* 10:217-278 (1980)). Encoded by genes *sucA*, *sucB* and *lpd* in *E. coli*, AKGD gene expression is downregulated under anaerobic conditions and during growth on glucose (Park et al. *Mol.Microbiol.* 15:473-482 (1995)). Although the substrate range of AKGD is narrow, structural studies of the catalytic core of the E2 component pinpoint specific residues responsible for substrate specificity (Knapp et al. *J.Mol.Biol.* 280:655-668 (1998)). The *Bacillus subtilis* AKGD, encoded by *odhAB* (E1 and E2) and *pdhD* (E3, shared domain), is regulated at the transcriptional level and is dependent on the carbon source and growth phase of the organism (Resnekov et al. *Mol.Gen.Genet.* 234:285-296 (1992)). In yeast, the *LPD1* gene encoding the E3 component is regulated at the transcriptional level by glucose (Roy and Dawes *J.Gen.Microbiol.* 133:925-933 (1987)). The E1 component, encoded by *KGD1*, is also regulated by glucose and activated by the products of HAP2 and HAP3 (Repetto and Tzagoloff *Mol.Cell Biol.* 9:2695-2705 (1989)). The AKGD enzyme complex, inhibited by products NADH and succinyl-CoA, is well-studied in mammalian systems, as impaired function of has been linked to several neurological diseases (Tretter and dam-Vizi *Philos.Trans.R.Soc.Lond B Biol.Sci.* 360:2335-2345 (2005)).

Gene	Accession No.	GI No.	Organism
sucA	NP_415254.1	16128701	*Escherichia coli str. K12 substr. MG1655*
sucB	NP_415255.1	16128702	*Escherichia coli str. K12 substr. MG1655*
lpd	NP_414658.1	16128109	*Escherichia coli str. K12 substr. MG1655*
odhA	P23129.2	51704265	*Bacillus subtilis*
odhB	P16263.1	129041	*Bacillus subtilis*
pdhD	P21880.1	118672	*Bacillus subtilis*
KGD1	NP_012141.1	6322066	*Saccharomyces cerevisiae*
KGD2	NP_010432.1	6320352	*Saccharomyces cerevisiae*
LPD1	NP_116635.1	14318501	*Saccharomyces cerevisiae*

Branched-chain 2-keto-acid dehydrogenase complex (BCKAD), also known as 2-oxoisovalerate dehydrogenase, participates in branched-chain amino acid degradation pathways, converting 2-keto acids derivatives of valine, leucine and isoleucine to their acyl-CoA derivatives and CO_2.

The complex has been studied in many organisms including *Bacillus subtilis* (Wang et al. *Eur.J.Biochem.* 213:1091-1099 (1993)), *Rattus norvegicus* (Namba et al. *J.Biol.Chem.* 244:4437-4447 (1969)) and *Pseudomonas putida* (Sokatch *J.Bacteriol.* 148:647-652 (1981)). In *Bacillus subtilis* the enzyme is encoded by genes *pdhD* (E3 component), *bfmBB* (E2 component), *bfmBAA* and *bfmBAB* (E1 component) (Wang et al. *Eur.J.Biochem.* 213:1091-1099 (1993)). In mammals, the complex is regulated by phosphorylation by specific phosphatases and protein kinases. The complex has been studied in rat hepatocites (Chicco et al. *J.Biol.Chem.* 269:19427-19434 (1994)) and is encoded by genes *Bckdha* (E1 alpha), *Bckdhb* (E1 beta), *Dbt* (E2), and *Dld* (E3). The E1 and E3 components of the *Pseudomonas putida* BCKAD complex have been crystallized (Aevarsson et al. *Nat.Struct.Biol.* 6:785-792 (1999); Mattevi *Science* 255:1544-1550 (1992)) and the enzyme complex has been studied (Sokatch et al. *J.Bacteriol.* 148:647-652 (1981)). Transcription of the *P. putida* BCKAD genes is activated by the gene product of *bkdR* (Hester et al. *Eur.J.Biochem.* 233:828-836 (1995)). In some organisms including *Rattus norvegicus* (Paxton et al. *Biochem.J.* 234:295-303 (1986)) and *Saccharomyces cerevisiae* (Sinclair et al. *Biochem.Mol.Biol.Int.* 31:911-922 (1993)), this complex has been shown to have a broad substrate range that includes linear oxo-acids such as 2-oxobutanoate and alpha-ketoglutarate, in addition to the branched-chain amino acid precursors. The active site of the bovine BCKAD was engineered to favor alternate substrate acetyl-CoA (Meng and Chuang, *Biochemistry* 33:12879-12885 (1994)).

Gene	Accession No.	GI No.	Organism
bfmBB	NP_390283.1	16079459	*Bacillus subtilis*
bfmBAA	NP_390285.1	16079461	*Bacillus subtilis*
bfmBAB	NP_390284.1	16079460	*Bacillus subtilis*
pdhD	P21880.1	118672	*Bacillus subtilis*
lpdV	P09063.1	118677	*Pseudomonas putida*
bkdB	P09062.1	129044	*Pseudomonas putida*
bkdA1	NP_746515.1	26991090	*Pseudomonas putida*
bkdA2	NP_746516.1	26991091	*Pseudomonas putida*
Bckdha	NP_036914.1	77736548	*Rattus norvegicus*
Bckdhb	NP_062140.1	158749538	*Rattus norvegicus*
Dbt	NP_445764.1	158749632	*Rattus norvegicus*
Dld	NP_955417.1	40786469	*Rattus norvegicus*

The pyruvate dehydrogenase complex, catalyzing the conversion of pyruvate to acetyl-CoA, has also been extensively studied. In the *E. coli* enzyme, specific residues in the E1 component are responsible for substrate specificity (Bisswanger, H. *J Biol Chem.* 256:815-822 (1981); Bremer,

J. *Eur.J Biochem.* 8:535-540 (1969); Gong et al. *J Biol Chem.* 275:13645-13653 (2000)). As mentioned previously, enzyme engineering efforts have improved the *E. coli* PDH enzyme activity under anaerobic conditions (Kim et al. *Appl.Environ.Microbiol.* 73:1766-1771 (2007); Kim *J.Bacteriol.* 190:3851-3858 (2008); Zhou et al. *Biotechnol.Lett.* 30:335-342 (2008)). In contrast to the *E. coli* PDH, the *B. subtilis* complex is active and required for growth under anaerobic conditions (Nakano *J.Bacteriol.* 179:6749-6755 (1997)). The *Klebsiella pneumoniae* PDH, characterized during growth on glycerol, is also active under anaerobic conditions (Menzel et al. *J.Biotechnol.* 56:135-142 (1997)). Crystal structures of the enzyme complex from bovine kidney (Zhou et al. *Proc.Natl.Acad.Sci.U.S.A.* 98:14802-14807 (2001)) and the E2 catalytic domain from *Azotobacter vinelandii* are available (Mattevi et al. *Science* 255:1544-1550 (1992)). Some mammalian PDH enzymes complexes can react on alternate substrates such as 2-oxobutanoate, although comparative kinetics of *Rattus norvegicus* PDH and BCKAD indicate that BCKAD has higher activity on 2-oxobutanoate as a substrate (Paxton et al. *Biochem.J.* 234:295-303 (1986)).

Gene	Accession No.	GI No.	Organism
aceE	NP_414656.1	16128107	*Escherichia coli str. K12 substr. MG1655*
aceF	NP_414657.1	16128108	*Escherichia coli str. K12 substr. MG1655*
lpd	NP_414658.1	16128109	*Escherichia coli str. K12 substr. MG1655*
pdhA	P21881.1	3123238	*Bacillus subtilis*
pdhB	P21882.1	129068	*Bacillus subtilis*
pdhC	P21883.2	129054	*Bacillus subtilis*
pdhD	P21880.1	118672	*Bacillus subtilis*
aceE	YP_001333808.1	152968699	*Klebsiella pneumonia MGH78578*
aceF	YP_001333809.1	152968700	*Klebsiella pneumonia MGH78578*
lpdA	YP_001333810.1	152968701	*Klebsiella pneumonia MGH78578*
Pdha1	NP_001004072.2	124430510	*Rattus norvegicus*
Pdha2	NP_446446.1	16758900	*Rattus norvegicus*
Dlat	NP_112287.1	78365255	*Rattus norvegicus*
Dld	NP_955417.1	40786469	*Rattus norvegicus*

As an alternative to the large multienzyme 2-keto-acid dehydrogenase complexes described above, some anaerobic organisms utilize enzymes in the 2-ketoacid oxidoreductase family (OFOR) to catalyze acylating oxidative decarboxylation of 2-keto-acids. Unlike the

dehydrogenase complexes, these enzymes contain iron-sulfur clusters, utilize different cofactors, and use ferredoxin or flavodixin as electron acceptors in lieu of NAD(P)H. While most enzymes in this family are specific to pyruvate as a substrate (POR) some 2-keto-acid:ferredoxin oxidoreductases have been shown to accept a broad range of 2-ketoacids as substrates including alpha-ketoglutarate and 2-oxobutanoate (Fukuda and Wakagi *Biochim.Biophys.Acta* 1597:74-80 (2002); Zhang et al. *J.Biochem.* 120:587-599 (1996)). One such enzyme is the OFOR from the thermoacidophilic archaeon *Sulfolobus tokodaii 7*, which contains an alpha and beta subunit encoded by gene *ST2300* (Fukuda and Wakagi *Biochim.Biophys.Acta* 1597:74-80 (2002); Zhang et al. *J.Biochem.* 120:587-599 (1996)). A plasmid-based expression system has been developed for efficiently expressing this protein in *E. coli* (Fukuda et al. *Eur.J.Biochem.* 268:5639-5646 (2001)) and residues involved in substrate specificity were determined (Fukuda and Wakagi *Biochim.Biophys.Acta* 1597:74-80 (2002)). Two OFORs from *Aeropyrum pernix str. K1* have also been recently cloned into *E. coli*, characterized, and found to react with a broad range of 2-oxoacids (Nishizawa et al. *FEBS Lett.* 579:2319-2322 (2005)). The gene sequences of these OFOR candidates are available, although they do not have GenBank identifiers assigned to date. There is bioinformatic evidence that similar enzymes are present in all archaea, some anaerobic bacteria and amitochondrial eukarya (Fukuda and Wakagi *Biochim.Biophys.Acta* 1597:74-80 (2005)). This class of enzyme is also interesting from an energetic standpoint, as reduced ferredoxin could be used to generate NADH by ferredoxin-NAD reductase (Petitdemange et al. *Biochim.Biophys.Acta* 421:334-337 (1976)). Also, since most of the enzymes are designed to operate under anaerobic conditions, less enzyme engineering may be required relative to enzymes in the 2-keto-acid dehydrogenase complex family for activity in an anaerobic environment.

Gene	Accession No.	GI No.	Organism
ST2300	NP_378302.1	15922633	*Sulfolobus tokodaii 7*

1.2.1.d Oxidoreductase (phosphonate reductase).

The conversion of 4-hydroxybutyryl-phosphate to 4-hydroxybutanal can be catalyzed by an oxidoreductase in the EC class 1.2.1. Aspartate semialdehyde dehydrogenase (ASD, EC 1.2.1.11) catalyzes the NADPH-dependent reduction of 4-aspartyl phosphate to aspartate-4-semialdehyde. ASD participates in amino acid biosynthesis and recently has been studied as an antimicrobial target (Hadfield et al., *Biochemistry* 40:14475-14483 (2001). The *E. coli* ASD structure has been solved (Hadfield et al.,. *J. Mol. Biol.* 289:991-1002 (1999)) and the enzyme has been shown to accept the alternate substrate beta-3-methylaspartyl phosphate (Shames et al.,

J. Biol. Chem. 259:15331-15339 (1984)). The *Haemophilus influenzae* enzyme has been the subject of enzyme engineering studies to alter substrate binding affinities at the active site (Blanco et al., *Acta Crystallogr. D. Biol. Crystallogr.* 60:1388-1395 (2004); Blanco et al., *Acta Crystallogr. D. Biol. Crystallogr.* 60:1808-1815 (2004)). Other ASD candidates are found in *Mycobacterium tuberculosis* (Shafiani et al., *J. Appl. Microbiol.* 98:832-838 (2005), *Methanococcus jannaschii* (Faehnle et al.,. *J. Mol. Biol.* 353:1055-1068 (2005)), and the infectious microorganisms *Vibrio cholera* and *Heliobacter pylori* (Moore et al., *Protein Expr. Purif.* 25:189-194 (2002)). A related enzyme candidate is acetylglutamylphosphate reductase (EC 1.2.1.38), an enzyme that naturally reduces acetylglutamylphosphate to acetylglutamate-5-semialdehyde, found in *S. cerevisiae* (Pauwels et al., *Eur. J. Biochem.* 270:1014-1024 (2003), *B. subtilis* (O'Reilly and Devine, *Microbiology* 140 (Pt 5):1023-1025 (1994)). and other organisms.

Gene	Accession No.	GI No.	Organism
asd	NP_417891.1	16131307	*Escherichia coli*
asd	YP_248335.1	68249223	*Haemophilus influenzae*
asd	AAB49996	1899206	*Mycobacterium tuberculosis*
VC2036	NP_231670	15642038	*Vibrio cholera*
asd	YP_002301787.1	210135348	*Heliobacter pylori*
ARG5,6	NP_010992.1	6320913	*Saccharomyces cerevisiae*
argC	NP_389001.1	16078184	*Bacillus subtilis*

Other exemplary enzymes in this class include glyceraldehyde 3-phosphate dehydrogenase which converts glyceraldehyde-3-phosphate into D-glycerate 1,3-bisphosphate (for example, *E. coli gapA* (Branlant and Branlant,. *Eur. J. Biochem.* 150:61-66 (1985)), N-acetyl-gamma-glutamyl-phosphate reductase which converts N-acetyl-L-glutamate-5-semialdehyde into N-acetyl-L-glutamyl-5-phosphate (for example, *E. coli argC* (Parsot et al., *Gene* 68:275-283 (1988)), and glutamate-5-semialdehyde dehydrogenase, which converts L-glutamate-5-semialdehyde into L-glutamyl-5-phospate (for example, *E. coli proA* (Smith et al., *J. Bacteriol.* 157:545-551 (1984)). Genes encoding glutamate-5-semialdehyde dehydrogenase enzymes from *Salmonella typhimurium* (Mahan and Csonka, *J. Bacteriol.* 156:1249-1262 (1983)) and *Campylobacter jejuni* (Louie and Chan, *Mol. Gen. Genet.* 240:29-35 (1993)) were cloned and expressed in *E. coli.*

Gene	Accession No.	GI No.	Organism
gapA	P0A9B2.2	71159358	*Escherichia coli*
argC	NP_418393.1	16131796	*Escherichia coli*
proA	NP_414778.1	16128229	*Escherichia coli*

proA	NP_459319.1	16763704	*Salmonella typhimurium*
proA	P53000.2	9087222	*Campylobacter jejuni*

1.2.1.e Acid reductase.

Several steps in Figures 58, 62 and 63 depict the conversion of unactivated acids to aldehydes by an acid reductase. These include the conversion of 4-hydroxybutyrate, succinate, alpha-ketoglutarate, and 4-aminobutyrate to 4-hydroxybutanal, succinate semialdehyde, 2,5-dioxopentanoate, and 4-aminobutanal, respectively. One notable carboxylic acid reductase can be found in *Nocardia iowensis* which catalyzes the magnesium, ATP and NADPH-dependent reduction of carboxylic acids to their corresponding aldehydes (Venkitasubramanian et al., *J. Biol. Chem.* 282:478-485 (2007)). This enzyme is encoded by the *car* gene and was cloned and functionally expressed in *E. coli* (Venkitasubramanian et al., *J. Biol. Chem.* 282:478-485 (2007)). Expression of the *npt* gene product improved activity of the enzyme via post-transcriptional modification. The *npt* gene encodes a specific phosphopantetheine transferase (PPTase) that converts the inactive apo-enzyme to the active holo-enzyme. The natural substrate of this enzyme is vanillic acid, and the enzyme exhibits broad acceptance of aromatic and aliphatic substrates (Venkitasubramanian et al., in *Biocatalysis in the Pharmaceutical and Biotechnology Industires*, ed. R.N. Patel, Chapter 15, pp. 425-440, CRC Press LLC, Boca Raton, FL. (2006)).

Gene	Accession No.	GI No.	Organism
car	AAR91681.1	40796035	*Nocardia iowensis* (sp. NRRL 5646)
npt	ABI83656.1	114848891	*Nocardia iowensis* (sp. NRRL 5646)

Additional *car* and *npt* genes can be identified based on sequence homology.

Gene	Accession No.	GI No.	Organism
fadD9	YP_978699.1	121638475	*Mycobacterium bovis* BCG
BCG_2812c	YP_978898.1	121638674	*Mycobacterium bovis* BCG
nfa20150	YP_118225.1	54023983	*Nocardia farcinica* IFM 10152
nfa40540	YP_120266.1	54026024	*Nocardia farcinica* IFM 10152
SGR_6790	YP_001828302.1	182440583	*Streptomyces griseus* subsp. griseus NBRC 13350
SGR_665	YP_001822177.1	182434458	*Streptomyces griseus* subsp. griseus NBRC 13350

An additional enzyme candidate found in *Streptomyces griseus* is encoded by the *griC* and *griD* genes. This enzyme is believed to convert 3-amino-4-hydroxybenzoic acid to 3-amino-4-hydroxybenzaldehyde as deletion of either *griC* or *griD* led to accumulation of extracellular 3-acetylamino-4-hydroxybenzoic acid, a shunt product of 3-amino-4-hydroxybenzoic acid metabolism (Suzuki, et al., *J. Antibiot.* 60(6):380-387 (2007)). Co-expression of *griC* and *griD* with SGR_665, an enzyme similar in sequence to the *Nocardia iowensis npt*, can be beneficial.

Gene	Accession No.	GI No.	Organism
griC	182438036	YP_001825755.1	*Streptomyces griseus* subsp. griseus NBRC 13350
griD	182438037	YP_001825756.1	*Streptomyces griseus* subsp. griseus NBRC 13350
MSMEG_2956	YP_887275.1	YP_887275.1	*Mycobacterium smegmatis MC2 155*
MSMEG_5739	YP_889972.1	118469671	*Mycobacterium smegmatis MC2 155*
MSMEG_2648	YP_886985.1	118471293	*Mycobacterium smegmatis MC2 155*
MAP1040c	NP_959974.1	41407138	*Mycobacterium avium* subsp. *paratuberculosis* K-10
MAP2899c	NP_961833.1	41408997	*Mycobacterium avium* subsp. *paratuberculosis* K-10
MMAR_2117	YP_001850422.1	183982131	*Mycobacterium marinum M*
MMAR_2936	YP_001851230.1	183982939	*Mycobacterium marinum M*
MMAR_1916	YP_001850220.1	183981929	*Mycobacterium marinum M*
TpauDRAFT_33060	ZP_04027864.1	227980601	*Tsukamurella paurometabola DSM 20162*
TpauDRAFT_20920	ZP_04026660.1	227979396	*Tsukamurella paurometabola DSM 20162*
CPCC7001_1320	ZP_05045132.1	254431429	*Cyanobium PCC7001*
DDBDRAFT_0187729	XP_636931.1	66806417	*Dictyostelium discoideum AX4*

An enzyme with similar characteristics, alpha-aminoadipate reductase (AAR, EC 1.2.1.31), participates in lysine biosynthesis pathways in some fungal species. This enzyme naturally reduces alpha-aminoadipate to alpha-aminoadipate semialdehyde. The carboxyl group is first

activated through the ATP-dependent formation of an adenylate that is then reduced by NAD(P)H to yield the aldehyde and AMP. Like CAR, this enzyme utilizes magnesium and requires activation by a PPTase. Enzyme candidates for AAR and its corresponding PPTase are found in *Saccharomyces cerevisiae* (Morris et al., *Gene* 98:141-145 (1991)), *Candida albicans* (Guo et al., *Mol. Genet. Genomics* 269:271-279 (2003)), and *Schizosaccharomyces pombe* (Ford et al., *Curr. Genet.* 28:131-137 (1995)). The AAR from *S. pombe* exhibited significant activity when expressed in *E. coli* (Guo et al., *Yeast* 21:1279-1288 (2004)). The AAR from *Penicillium chrysogenum* accepts S-carboxymethyl-L-cysteine as an alternate substrate, but did not react with adipate, L-glutamate or diaminopimelate (Hijarrubia et al., *J. Biol. Chem.* 278:8250-8256 (2003)). The gene encoding the *P. chrysogenum* PPTase has not been identified to date.

Gene	Accession No.	GI No.	Organism
LYS2	AAA34747.1	171867	*Saccharomyces cerevisiae*
LYS5	P50113.1	1708896	*Saccharomyces cerevisiae*
LYS2	AAC02241.1	2853226	*Candida albicans*
LYS5	AAO26020.1	28136195	*Candida albicans*
Lys1p	P40976.3	13124791	*Schizosaccharomyces pombe*
Lys7p	Q10474.1	1723561	*Schizosaccharomyces pombe*
Lys2	CAA74300.1	3282044	*Penicillium chrysogenum*

1.4.1.a Oxidoreductase (aminating).

Glutamate dehydrogenase (Step J, Figures 62 and 63), 4-aminobutyrate dehydrogenase (Step M, Figures 62 and 63), putrescine dehydrogenase (Step D, Figure 63), 5-amino-2-oxopentanoate dehydrogenase (Step P, Figure 63), and ornithine dehydrogenase (Step S, Figure 63) can be catalyzed by aminating oxidoreductases. Enzymes in this EC class catalyze the oxidative deamination of alpha-amino acids with NAD+ or NADP+ as acceptor, and the reactions are typically reversible. Exemplary oxidoreductases operating on amino acids include glutamate dehydrogenase (deaminating), encoded by *gdhA*, leucine dehydrogenase (deaminating), encoded by *ldh*, and aspartate dehydrogenase (deaminating), encoded by *nadX*. The *gdhA* gene product from *Escherichia coli* (Korber et al., *J. Mol. Biol.* 234:1270-1273 (1993); McPherson and Wootton, *Nucleic Acids Res.* 11:5257-5266 (1983)), *gdh* from *Thermotoga maritima* (Kort et al., *Extremophiles* 1:52-60 (1997); Lebbink, et al. *J. Mol. Biol.* 280:287-296 (1998); Lebbink et al. *J. Mol. Biol.* 289:357-369 (1999)), and *gdhA1* from *Halobacterium salinarum* (Ingoldsby et al., *Gene* 349:237-244 (2005)) catalyze the reversible interconversion of glutamate to 2-oxoglutarate and ammonia, while favoring NADP(H), NAD(H), or both, respectively. The *ldh* gene of *Bacillus cereus* encodes the LeuDH protein that has a wide of range of substrates including

leucine, isoleucine, valine, and 2-aminobutanoate (Ansorge and Kula, *Biotechnol. Bioeng.* 68:557-562 (2000); Stoyan et al. *J. Biotechnol.* 54:77-80 (1997)). The *nadX* gene from *Thermotoga maritime* encoding for the aspartate dehydrogenase is involved in the biosynthesis of NAD (Yang et al., *J. Biol. Chem.* 278:8804-8808 (2003)).

Gene	Accession No.	GI No.	Organism
gdhA	P00370	118547	*Escherichia coli*
gdh	P96110.4	6226595	*Thermotoga maritima*
gdhA1	NP_279651.1	15789827	*Halobacterium salinarum*
ldh	P0A393	61222614	*Bacillus cereus*
nadX	NP_229443.1	15644391	*Thermotoga maritima*

Additional glutamate dehydrogenase gene candidates are found in *Bacillus subtilis* (Khan et al., *Biosci. Biotechnol. Biochem.* 69:1861-1870 (2005)), *Nicotiana tabacum* (Purnell et al., *Planta* 222:167-180 (2005)), *Oryza sativa* (Abiko et al., *Plant Cell Physiol.* 46:1724-1734 (2005)), *Haloferax mediterranei* (Diaz et al., *Extremophiles* 10:105-115 (2006)) and *Halobactreium salinarum* (Hayden et al., *FEMS Microbiol. Lett.* 211:37-41 (2002)). The *Nicotiana tabacum* enzyme is composed of alpha and beta subunits encoded by *gdh1* and *gdh2* (Purnell et al., *Planta* 222:167-180 (2005)). Overexpression of the NADH-dependent glutamate dehydrogenase was found to improve ethanol production in engineered strains of *S. cerevisiae* (Roca et al., *Appl. Environ. Microbiol.* 69:4732-4736 (2003)).

Gene	Accession No.	GI No.	Organism
rocG	NP_391659.1	16080831	*Bacillus subtilis*
gdh1	AAR11534.1	38146335	*Nicotiana tabacum*
gdh2	AAR11535.1	38146337	*Nicotiana tabacum*
GDH	Q852M0	75243660	*Oryza sativa*
GDH	Q977U6	74499858	*Haloferax mediterranei*
GDH	P29051	118549	*Halobactreium salinarum*
GDH2	NP_010066.1	6319986	*Saccharomyces cerevisiae*

An exemplary enzyme for catalyzing the conversion of aldehydes to their corresponding primary amines is lysine 6-dehydrogenase (EC 1.4.1.18), encoded by the *lysDH* genes. The lysine 6-dehydrogenase (deaminating), encoded by *lysDH* gene, catalyze the oxidative deamination of the ε-amino group of L-lysine to form 2-aminoadipate-6-semialdehyde, which in turn nonenzymatically cyclizes to form Δ1-piperideine-6-carboxylate (Misono and Nagasaki, *J. Bacteriol.* 150:398-401 (1982)). The *lysDH* gene from *Geobacillus stearothermophilus* encodes a thermophilic NAD-dependent lysine 6-dehydrogenase (Heydari et al., *Appl. Environ. Microbiol* 70:937-942 (2004)). The *lysDH* gene from *Aeropyrum pernix K1* is identified

through homology from genome projects. Additional enzymes can be found in *Agrobacterium tumefaciens* (Hashimoto et al., *J. Biochem.* 106:76-80 (1989); Misono and Nagasaki, *J. Bacteriol.* 150:398-401 (1982)) and *Achromobacter denitrificans* (Ruldeekulthamrong et al., *BMB. Rep.* 41:790-795 (2008)).

Gene	Accession No.	GI No.	Organism
lysDH	BAB39707	13429872	*Geobacillus stearothermophilus*
lysDH	NP_147035.1	14602185	*Aeropyrum pernix K1*
lysDH	NP_353966	15888285	*Agrobacterium tumefaciens*
lysDH	AAZ94428	74026644	*Achromobacter denitrificans*

An enzyme that converts 3-oxoacids to 3-amino acids is 3,5-diaminohexanoate dehydrogenase (EC 1.4.1.11), an enzyme found in organisms that ferment lysine. The gene encoding this enzyme, *kdd*, was recently identified in *Fusobacterium nucleatum* (Kreimeyer et al., *J. Biol. Chem.* 282:7191-7197 (2007)). The enzyme has been purified and characterized in other organisms (Baker et al., *J. Biol. Chem.* 247:7724-7734 (1972); Baker and and van der Drift, *Biochemistry* 13:292-299 (1974)), but the genes associated with these enzymes are not known. Candidates in *Myxococcus xanthus*, *Porphyromonas gingivalis W83* and other sequenced organisms can be inferred by sequence homology.

Gene	Accession No.	GI No.	Organism
kdd	AAL93966.1	19713113	*Fusobacterium nucleatum*
mxan_4391	ABF87267.1	108462082	*Myxococcus xanthus*
pg_1069	AAQ66183.1	34397119	*Porphyromonas gingivalis*

2.3.1.a Acyltransferase (transferring phosphate group to CoA).

Step P of Figure 62 depicts the transformation of 4-hydroxybutyryl-CoA to 4-hydroxybutyryl-Pi. Exemplary phosphate transferring acyltransferases include phosphotransacetylase, encoded by *pta*, and phosphotransbutyrylase, encoded by *ptb*. The *pta* gene from *E. coli* encodes an enzyme that can convert acetyl-CoA into acetyl-phosphate, and vice versa (Suzuki, *Biochim. Biophys. Acta* 191:559-569 (1969)). This enzyme can also utilize propionyl-CoA instead of acetyl-CoA forming propionate in the process (Hesslinger et al., *Mol. Microbiol.* 27:477-492 (1998)). Similarly, the *ptb* gene from *C. acetobutylicum* encodes an enzyme that can convert butyryl-CoA into butyryl-phosphate (Walter et al., *Gene* 134:107-111 (1993)); Huang et al., *J Mol. Microbiol. Biotechno.l* 2:33-38 (2000). Additional *ptb* genes can be found in butyrate-producing bacterium L2-50 (Louis et al., *J.Bacteriol.* 186:2099-2106 (2004)) and *Bacillus megaterium* (Vazquez et al., *Curr. Microbiol.* 42:345-349 (2001)).

Gene	Accession No.	GI No.	Organism
pta	NP_416800.1	16130232	*Escherichia coli*
ptb	NP_349676	15896327	*Clostridium acetobutylicum*
ptb	AAR19757.1	38425288	butyrate-producing bacterium L2-50
ptb	CAC07932.1	10046659	*Bacillus megaterium*

2.6.1. Aminotransferase.

Aminotransferases reversibly convert an aldehyde or ketone to an amino group. Common amino donor/acceptor combinations include glutamate/alpha-ketoglutarate, alanine/pyruvate, and aspartate/oxaloacetate. Several enzymes have been shown to convert aldehydes to primary amines, and vice versa, such as 4-aminobutyrate, putrescine, and 5-amino-2-oxopentanoate. These enzymes are particularly well suited to carry out the following transformations: Step N in Figures 62 and 63, Steps E and Q in Figure 63. Lysine-6-aminotransferase (EC 2.6.1.36) is one exemplary enzyme capable of forming a primary amine. This enzyme function, converting lysine to alpha-aminoadipate semialdehyde, has been demonstrated in yeast and bacteria. Candidates from *Candida utilis* (Hammer and Bode, *J. Basic Microbiol.* 32:21-27 (1992)), *Flavobacterium lutescens* (Fujii et al., *J. Biochem.* 128:391-397 (2000)) and *Streptomyces clavuligenus* (Romero et al., *J. Ind. Microbiol. Biotechnol.* 18:241-246 (1997)) have been characterized. A recombinant lysine-6-aminotransferase from *S. clavuligenus* was functionally expressed in *E. coli* (Tobin et al., *J. Bacteriol.* 173:6223-6229 (1991)). The *F. lutescens* enzyme is specific to alpha-ketoglutarate as the amino acceptor (Soda and Misono, *Biochemistry* 7:4110-4119 (1968)). Other enzymes which convert aldehydes to terminal amines include the *dat* gene product in *Acinetobacter baumanii* encoding 2,4-diaminobutanoate:2-ketoglutarate 4-transaminase (Ikai and Yamamoto, *J. Bacteriol.* 179:5118-5125 (1997)). In addition to its natural substrate, 2,4-diaminobutyrate, DAT transaminates the terminal amines of lysine, 4-aminobutyrate and ornithine.

Gene	Accession No.	GI No.	Organism
lat	BAB13756.1	10336502	*Flavobacterium lutescens*
lat	AAA26777.1	153343	*Streptomyces clavuligenus*
dat	P56744.1	6685373	*Acinetobacter baumanii*

The conversion of an aldehyde to a terminal amine can also be catalyzed by gamma-aminobutyrate transaminase (GABA transaminase or 4-aminobutyrate transaminase). This enzyme naturally interconverts succinic semialdehyde and glutamate to 4-aminobutyrate and alpha-ketoglutarate and is known to have a broad substrate range (Liu et al., *Biochemistry* 43:10896-10905 2004); Schulz et al., *Appl. Environ. Microbiol.* 56:1-6 (1990)). The two GABA

transaminases in *E. coli* are encoded by *gabT* (Bartsch et al., *J. Bacteriol.* 172:7035-7042 (1990)) and *puuE* (Kurihara et al., *J. Biol. Chem.* 280:4602-4608. (2005)). GABA transaminases in *Mus musculus*, *Pseudomonas fluorescens*, and *Sus scrofa* have been shown to react with a range of alternate substrates including 6-aminocaproic acid (Cooper, *Methods Enzymol.* 113:80-82 (1985); Scott and Jakoby, *J. Biol. Chem.* 234:932-936 (1959)).

Gene	Accession No.	GI No.	Organism
gabT	NP_417148.1	16130576	*Escherichia coli*
puuE	NP_415818.1	16129263	*Escherichia coli*
abat	NP_766549.2	37202121	*Mus musculus*
gabT	YP_257332.1	70733692	*Pseudomonas fluorescens*
abat	NP_999428.1	47523600	*Sus scrofa*

Additional enzyme candidates for interconverting aldehydes and primary amines are putrescine transminases or other diamine aminotransferases. The *E. coli* putrescine aminotransferase is encoded by the *ygjG* gene, and the purified enzyme also was able to transaminate cadaverine and spermidine (Samsonova et al., *BMC Microbiol.* 3:2 (2003)). In addition, activity of this enzyme on 1,7-diaminoheptane and with amino acceptors other than 2-oxoglutarate (for example, pyruvate, 2-oxobutanoate) has been reported (Kim, *J. Biol. Chem.* 239:783-786 (1964); Samsonova et al., *BMC Microbiol.* 3:2 (2003)). A putrescine aminotransferase with higher activity with pyruvate as the amino acceptor than alpha-ketoglutarate is the *spuC* gene of *Pseudomonas aeruginosa* (Lu et al., *J. Bacteriol.* 184:3765-3773 (2002)).

Gene	Accession No.	GI No.	Organism
ygjG	NP_417544	145698310	*Escherichia coli*
spuC	AAG03688	9946143	*Pseudomonas aeruginosa*

Enzymes that transaminate 3-oxoacids include GABA aminotransferase (described above), beta-alanine/alpha-ketoglutarate aminotransferase and 3-amino-2-methylpropionate aminotransferase. Beta-alanine/alpha-ketoglutarate aminotransferase (WO08027742) reacts with beta-alanine to form malonic semialdehyde, a 3-oxoacid. The gene product of *SkPYD4* in *Saccharomyces kluyveri* was shown to preferentially use beta-alanine as the amino group donor (Andersen and Hansen, *Gene* 124:105-109 (1993)). *SkUGA1* encodes a homologue of *Saccharomyces cerevisiae* GABA aminotransferase, *UGA1* (Ramos et al., *Eur. J. Biochem.* 149:401-404 (1985)), whereas *SkPYD4* encodes an enzyme involved in both beta-alanine and GABA transamination (Andersen and Hansen, *Gene* 124:105-109 (1993)). 3-Amino-2-methylpropionate transaminase catalyzes the transformation from methylmalonate semialdehyde to 3-amino-2-methylpropionate. The enzyme has been characterized in *Rattus norvegicus* and

Sus scrofa and is encoded by *Abat* (Kakimoto et al., *Biochim. Biophys. Acta* 156:374-380 (1968); Tamaki et al., *Methods Enzymol.* 324:376-389 (2000)).

Gene	Accession No.	GI No.	Organism
SkyPYD4	ABF58893.1	98626772	*Lachancea kluyveri*
SkUGA1	ABF58894.1	98626792	*Lachancea kluyveri*
UGA1	NP_011533.1	6321456	*Saccharomyces cerevisiae*
Abat	P50554.3	122065191	*Rattus norvegicus*
Abat	P80147.2	120968	*Sus scrofa*

Several aminotransferases transaminate the amino groups of amino acids to form 2-oxoacids. Aspartate aminotransferase is an enzyme that naturally transfers an oxo group from oxaloacetate to glutamate, forming alpha-ketoglutarate and aspartate. Aspartate is similar in structure to OHED and 2-AHD. Aspartate aminotransferase activity is catalyzed by, for example, the gene products of *aspC* from *Escherichia coli* (Yagi et al., *FEBS Lett.* 100:81-84 (1979); Yagi et al., *Methods Enzymol.* 113:83-89 (1985)), *AAT2* from *Saccharomyces cerevisiae* (Yagi et al., *J. Biochem.* 92:35-43 (1982)) and *ASP5* from *Arabidopsis thaliana* (de la Torre et al., *Plant J.* 46:414-425 (2006); Kwok and Hanson. *J. Exp. Bot.* 55:595-604 (2004); Wilkie and Warren, *Protein Expr. Purif.* 12:381-389 (1998)). The enzyme from *Rattus norvegicus* has been shown to transaminate alternate substrates such as 2-aminohexanedioic acid and 2,4-diaminobutyric acid (Recasens et al., *Biochemistry* 19:4583-4589 (1980)). Aminotransferases that work on other amino-acid substrates can also be able to catalyze this transformation. Valine aminotransferase catalyzes the conversion of valine and pyruvate to 2-ketoisovalerate and alanine. The *E. coli* gene, *avtA*, encodes one such enzyme (Whalen and Berg, *J. Bacteriol.* 150:739-746 (1982)). This gene product also catalyzes the transamination of α-ketobutyrate to generate α-aminobutyrate, although the amine donor in this reaction has not been identified (Whalen and Berg, *J. Bacteriol.* 158:571-574 1984)). The gene product of the *E. coli serC* catalyzes two reactions, phosphoserine aminotransferase and phosphohydroxythreonine aminotransferase (Lam and Winkler, *J. Bacteriol.* 172:6518-6528 (1990)), and activity on non-phosphorylated substrates could not be detected (Drewke et al., *FEBS Lett.* 390:179-182 (1996)).

Gene	Accession No.	GI No.	Organism
aspC	NP_415448.1	16128895	*Escherichia coli*
AAT2	P23542.3	1703040	*Saccharomyces cerevisiae*
ASP5	P46248.2	20532373	*Arabidopsis thaliana*
Got2	P00507	112987	*Rattus norvegicus*
avtA	YP_026231.1	49176374	*Escherichia coli*
serC	NP_415427.1	16128874	*Escherichia coli*

Another enzyme candidate is alpha-aminoadipate aminotransferase (EC 2.6.1.39), an enzyme that participates in lysine biosynthesis and degradation in some organisms. This enzyme interconverts 2-aminoadipate and 2-oxoadipate, using alpha-ketoglutarate as the amino acceptor. Gene candidates are found in *Homo sapiens* (Okuno et al., *Enzyme Protein* 47:136-148 (1993)) and *Thermus thermophilus* (Miyazaki et al., *Microbiology* 150:2327-2334 (2004)). The *Thermus thermophilus* enzyme, encoded by *lysN,* is active with several alternate substrates including oxaloacetate, 2-oxoisocaproate, 2-oxoisovalerate, and 2-oxo-3-methylvalerate.

Gene	Accession No.	GI No.	Organism
lysN	BAC76939.1	31096548	*Thermus thermophilus*
AadAT-II	Q8N5Z0.2	46395904	*Homo sapiens*

2.7.2.a Phosphotransferase (carboxy acceptor).

Phosphotransferase enzymes in the EC class 2.7.2 transform carboxylic acids to phosphonic acids with concurrent hydrolysis of one ATP. Step O of Figure 62 involves the conversion of 4-hydroxybutyrate to 4-hydroxybutyryl-phosphate by such an enzyme. Butyrate kinase (EC 2.7.2.7) carries out the reversible conversion of butyryl-phosphate to butyrate during acidogenesis in *C. acetobutylicum* (Cary et al., *Appl. Environ. Microbiol.* 56:1576-1583 (1990)). This enzyme is encoded by either of the two *buk* gene products (Huang et al., *J. Mol. Microbiol. Biotechnol.* 2:33-38 (2000)). Other butyrate kinase enzymes are found in *C. butyricum* and *C. tetanomorphum* (Twarog and Wolfe, *J. Bacteriol.* 86:112-117 (1963)). Related enzyme isobutyrate kinase from *Thermotoga maritima* has also been expressed in *E. coli* and crystallized (Diao et al., *Acta Crystallogr. D. Biol. Crystallogr.* 59:1100-1102 (2003); Diao and Hasson, *J. Bacteriol.* 191:2521-2529 (2009)). Aspartokinase catalyzes the ATP-dependent phosphorylation of aspartate and participates in the synthesis of several amino acids. The aspartokinase III enzyme in *E. coli,* encoded by *lysC,* has a broad substrate range, and the catalytic residues involved in substrate specificity have been elucidated (Keng and Viola, *Arch. Biochem. Biophys.* 335:73-81 (1996)). Two additional kinases in *E. coli* are also good candidates: acetate kinase and gamma-glutamyl kinase. The *E. coli* acetate kinase, encoded by *ackA* (Skarstedt and Silverstein, *J. Biol. Chem.* 251:6775-6783 (1976)), phosphorylates propionate in addition to acetate (Hesslinger et al., *Mol. Microbiol.* 27:477-492 (1998)). The *E. coli* gamma-glutamyl kinase, encoded by *proB* (Smith et al., *J. Bacteriol.* 157:545-551 (1984)), phosphorylates the gamma carbonic acid group of glutamate.

Gene	Accession No.	GI No.	Organism
buk1	NP_349675	15896326	*Clostridium acetobutylicum*
buk2	Q97II1	20137415	*Clostridium acetobutylicum*
buk2	Q9X278.1	6685256	*Thermotoga maritima*
lysC	NP_418448.1	16131850	*Escherichia coli*
ackA	NP_416799.1	16130231	*Escherichia coli*
proB	NP_414777.1	16128228	*Escherichia coli*

Acetylglutamate kinase phosphorylates acetylated glutamate during arginine biosynthesis. This enzyme is not known to accept alternate substrates; however, several residues of the *E. coli* enzyme involved in substrate binding and phosphorylation have been elucidated by site-directed mutagenesis (Marco-Marin et al., *J. Mol. Biol.* 334:459-476 (2003); Ramon-Maiques et al., *Structure* 10:329-342 (2002)). The enzyme is encoded by *argB* in *Bacillus subtilis* and *E. coli* (Parsot et al., *Gene* 68:275-283 (1988)), and *ARG5,6* in *S. cerevisiae* (Pauwels et al., *Eur. J. Biochem.* 270:1014-1024 (2003)). The ARG5,6 gene of *S. cerevisiae* encodes a polyprotein precursor that is matured in the mitochondrial matrix to become acetylglutamate kinase and acetylglutamylphosphate reductase.

Gene	Accession No.	GI No.	Organism
argB	NP_418394.3	145698337	*Escherichia coli*
argB	NP_389003.1	16078186	*Bacillus subtilis*
ARG5,6	NP_010992.1	6320913	*Saccharomyces cerevisiae*

2.8.3.a CoA transferase.

The gene products of *cat1*, *cat2*, and *cat3* of *Clostridium kluyveri* have been shown to exhibit succinyl-CoA (Step G, Figures 62 and 63), 4-hydroxybutyryl-CoA (Step T, Figure 62), and butyryl-CoA acetyltransferase activity, respectively (Seedorf et al., *Proc. Natl. Acad. Sci. USA* 105:2128-2133 (2008); Sohling and Gottschalk, *J Bacteriol* 178:871-880 (1996)). Similar CoA transferase activities are also present in *Trichomonas vaginalis* (van Grinsven et al., *J. Biol. Chem.* 283:1411-1418 (2008)) and *Trypanosoma brucei* (Riviere et al., *J. Biol. Chem.* 279:45337-45346 (2004)).

Gene	Accession No.	GI No.	Organism
cat1	P38946.1	729048	*Clostridium kluyveri*
cat2	P38942.2	1705614	*Clostridium kluyveri*
cat3	EDK35586.1	146349050	*Clostridium kluyveri*
TVAG_395550	XP_001330176	123975034	*Trichomonas vaginalis G3*
Tb11.02.0290	XP_828352	71754875	*Trypanosoma brucei*

An additionally useful enzyme for this type of transformation is acyl-CoA:acetate-CoA transferase, also known as acetate-CoA transferase (EC 2.8.3.8), which has been shown to transfer the CoA moiety to acetate from a variety of branched and linear acyl-CoA substrates, including isobutyrate (Matthies and Schink, *Appl. Environ. Microbiol.* 58:1435-1439 (1992)), valerate (Vanderwinkel et al., *Biochem. Biophys. Res. Commun.* 33:902-908 (1968)) and butanoate (Vanderwinkel, *supra* (1968)). This enzyme is encoded by *atoA* (alpha subunit) and *atoD* (beta subunit) in *E. coli* sp. K12 (Korolev et al., *Acta Crystallogr. D Biol. Crystallogr.* 58:2116-2121 (2002); Vanderwinkel, *supra* (1968)). Similar enzymes exist in *Corynebacterium glutamicum* ATCC 13032 (Duncan et al., *Appl. Environ. Microbiol.* 68:5186-5190 (2002)), *Clostridium acetobutylicum* (Cary et al., *Appl. Environ. Microbiol.* 56:1576-1583 (1990); Wiesenborn et al., *Appl. Environ. Microbiol.* 55:323-329 (1989)), and *Clostridium saccharoperbutylacetonicum* (Kosaka et al., *Biosci. Biotechnol. Biochem.* 71:58-68 (2007)).

Gene	Accession No.	GI No.	Organism
atoA	P76459.1	2492994	*Escherichia coli K12*
atoD	P76458.1	2492990	*Escherichia coli K12*
actA	YP_226809.1	62391407	*Corynebacterium glutamicum*
cg0592	YP_224801.1	62389399	*Corynebacterium glutamicum*
ctfA	NP_149326.1	15004866	*Clostridium acetobutylicum*
ctfB	NP_149327.1	15004867	*Clostridium acetobutylicum*
ctfA	AAP42564.1	31075384	*Clostridium saccharoperbutylacetonicum*
ctfB	AAP42565.1	31075385	*Clostridium saccharoperbutylacetonicum*

The glutaconate-CoA-transferase (EC 2.8.3.12) enzyme from anaerobic bacterium *Acidaminococcus fermentans* reacts with diacid glutaconyl-CoA and 3-butenoyl-CoA (Mack and Buckel, *FEBS Lett.* 405:209-212 (1997)). The genes encoding this enzyme are *gctA* and *gctB*. This enzyme has reduced but detectable activity with other CoA derivatives including glutaryl-CoA, 2-hydroxyglutaryl-CoA, adipyl-CoA and acrylyl-CoA (Buckel et al., *Eur. J. Biochem.* 118:315-321 (1981)). The enzyme has been cloned and expressed in *E. coli* (Mac et al., *Eur.J.Biochem.* 226:41-51 (1994)).

Gene	Accession No.	GI No.	Organism
gctA	CAA57199.1	559392	*Acidaminococcus fermentans*
gctB	CAA57200.1	559393	*Acidaminococcus fermentans*

3.1.2.a CoA hydrolase.

Enzymes in the 3.1.2 family hydrolyze acyl-CoA molecules to their corresponding acids. However, such enzymes can be modified to empart CoA-ligase or synthetase functionality if coupled to an energy source such as a proton pump or direct ATP hydrolysis. Several eukaryotic acetyl-CoA hydrolases (EC 3.1.2.1) have broad substrate specificity. For example, the enzyme from *Rattus norvegicus* brain (Robinson et al., *Biochem. Biophys. Res. Commun.* 71:959-965 (1976)) can react with butyryl-CoA, hexanoyl-CoA and malonyl-CoA. Though its sequence has not been reported, the enzyme from the mitochondrion of the pea leaf also has a broad substrate specificity, with demonstrated activity on acetyl-CoA, propionyl-CoA, butyryl-CoA, palmitoyl-CoA, oleoyl-CoA, succinyl-CoA, and crotonyl-CoA (Zeiher and Randall, *Plant. Physiol.* 94:20-27 (1990)). The acetyl-CoA hydrolase, *ACH1*, from *S. cerevisiae* represents another candidate hydrolase (Buu et al., *J. Biol. Chem.* 278:17203-17209 (2003)).

Gene	Accession No.	GI No.	Organism
acot12	NP_570103.1	18543355	*Rattus norvegicus*
ACH1	NP_009538	6319456	*Saccharomyces cerevisiae*

Another candidate hydrolase is the human dicarboxylic acid thioesterase, *acot8*, which exhibits activity on glutaryl-CoA, adipyl-CoA, suberyl-CoA, sebacyl-CoA, and dodecanedioyl-CoA (Westin et al., *J. Biol. Chem.* 280:38125-38132 (2005)) and the closest *E. coli* homolog, *tesB*, which can also hydrolyze a broad range of CoA thioesters (Naggert et al., *J. Biol. Chem.* 266:11044-11050 (1991)). A similar enzyme has also been characterized in the rat liver (Deana, *Biochem. Int.* 26:767-773 (1992)). Other potential *E. coli* thioester hydrolases include the gene products of *tesA* (Bonner and Bloch, *J. Biol. Chem.* 247:3123-3133 (1972)), *ybgC* (Kuznetsova et al., *FEMS Microbiol. Rev.* 29:263-279 (2005); Zhuang et al., *FEBS Lett.* 516:161-163 (2002)), *paaI* (Song et al., *J. Biol. Chem.* 281:11028-11038 (2006)), and *ybdB* (Leduc et al., *J. Bacteriol.* 189:7112-7126 (2007)).

Gene	Accession No.	GI No.	Organism
acot8	CAA15502	3191970	*Homo sapiens*
tesB	NP_414986	16128437	*Escherichia coli*
acot8	NP_570112	51036669	*Rattus norvegicus*
tesA	NP_415027	16128478	*Escherichia coli*
ybgC	NP_415264	16128711	*Escherichia coli*
paaI	NP_415914	16129357	*Escherichia coli*
ybdB	NP_415129	16128580	*Escherichia coli*

Yet another candidate hydrolase is the glutaconate CoA-transferase from *Acidaminococcus fermentans*. This enzyme was transformed by site-directed mutagenesis into an acyl-CoA hydrolase with activity on glutaryl-CoA, acetyl-CoA and 3-butenoyl-CoA (Mack and Buckel, *FEBS Lett.* 405:209-212 (1997)). This indicates that the enzymes encoding succinyl-CoA:3-ketoacid-CoA transferases and acetoacetyl-CoA:acetyl-CoA transferases can also serve as candidates for this reaction step but would likely require certain mutations to change their function.

Gene	Accession No.	GI No.	Organism
gctA	CAA57199.1	559392	*Acidaminococcus fermentans*
gctB	CAA57200.1	559393	*Acidaminococcus fermentans*

Additional hydrolase enzymes include 3-hydroxyisobutyryl-CoA hydrolase which has been described to efficiently catalyze the conversion of 3-hydroxyisobutyryl-CoA to 3-hydroxyisobutyrate during valine degradation (Shimomura et al., *J. Biol. Chem.* 269:14248-14253 (1994)). Genes encoding this enzyme include *hibch* of *Rattus norvegicus* (Shimomura et al., *supra* (1994); Shimomura et al., *Methods Enzymol.* 324:229-240 (2000)) and *Homo sapiens* (Shimomura et al., *supra* (1994). Candidate genes by sequence homology include *hibch* of *Saccharomyces cerevisiae* and *BC_2292* of *Bacillus cereus*.

Gene	Accession No.	GI No.	Organism
hibch	Q5XIE6.2	146324906	*Rattus norvegicus*
hibch	Q6NVY1.2	146324905	*Homo sapiens*
hibch	P28817.2	2506374	*Saccharomyces cerevisiae*
BC_2292	AP09256	29895975	*Bacillus cereus*

4.1.1.a Carboxy-lyase.

Decarboxylation of Alpha-Keto Acids. Alpha-ketoglutarate decarboxylase (Step B, Figures 58, 62 and 63), 5-hydroxy-2-oxopentanoic acid decarboxylase (Step Z, Figure 62), and 5-amino-2-oxopentanoate decarboxylase (Step R, Figure 63) all involve the decarboxylation of an alpha-ketoacid. The decarboxylation of keto-acids is catalyzed by a variety of enzymes with varied substrate specificities, including pyruvate decarboxylase (EC 4.1.1.1), benzoylformate decarboxylase (EC 4.1.1.7), alpha-ketoglutarate decarboxylase and branched-chain alpha-ketoacid decarboxylase.

Pyruvate decarboxylase (PDC), also termed keto-acid decarboxylase, is a key enzyme in alcoholic fermentation, catalyzing the decarboxylation of pyruvate to acetaldehyde. The enzyme from *Saccharomyces cerevisiae* has a broad substrate range for aliphatic 2-keto acids including

2-ketobutyrate, 2-ketovalerate, 3-hydroxypyruvate and 2-phenylpyruvate (Davie et al., *J. Biol. Chem.* 267:16601-16606 (1992)). This enzyme has been extensively studied, engineered for altered activity, and functionally expressed in *E. coli* (Killenberg-Jabs et al., *Eur. J. Biochem.* 268:1698-1704 (2001); Li and Jordan, *Biochemistry* 38:10004-10012 (1999); ter Schure et al., *Appl. Environ. Microbiol.* 64:1303-1307 (1998)). The PDC from *Zymomonas mobilus,* encoded by *pdc*, also has a broad substrate range and has been a subject of directed engineering studies to alter the affinity for different substrates (Siegert et al., *Protein Eng. Des. Sel.* 18:345-357 (2005)). The crystal structure of this enzyme is available (Killenberg-Jabs et al., *Eur. J. Biochem.* 268:1698-1704 (2001)). Other well-characterized PDC candidates include the enzymes from *Acetobacter pasteurians* (Chandra et al., *Arch. Microbiol.* 176:443-451 (2001)) and *Kluyveromyces lactis* (Krieger et al., *Eur. J. Biochem.* 269:3256-3263 (2002)).

Gene	Accession No.	GI No.	Organism
pdc	P06672.1	118391	*Zymomonas mobilus*
pdc1	P06169	30923172	*Saccharomyces cerevisiae*
pdc	AM21208	20385191	*Acetobacter pasteurians*
pdc1	Q12629	52788279	*Kluyveromyces lactis*

Like PDC, benzoylformate decarboxylase (EC 4.1.1.7) has a broad substrate range and has been the target of enzyme engineering studies. The enzyme from *Pseudomonas putida* has been extensively studied and crystal structures of this enzyme are available (Hasson et al., *Biochemistry* 37:9918-9930 (1998); Polovnikova et al., *Biochemistry* 42:1820-1830 (2003). Site-directed mutagenesis of two residues in the active site of the *Pseudomonas putida* enzyme altered the affinity (Km) of naturally and non-naturally occurring substrates (Siegert et al., *Protein Eng. Des. Sel.* 18:345-357 (2005)). The properties of this enzyme have been further modified by directed engineering (Lingen et al., *Protein Eng*. 15:585-593 (2002); Lingen et al., *Chembiochem.* 4:721-726 (2003)). The enzyme from *Pseudomonas aeruginosa,* encoded by *mdlC,* has also been characterized experimentally (Barrowman et al., *FEMS Microbiol. Lett.* 34:57-60 (1986)). Additional gene candidates from *Pseudomonas stutzeri*, *Pseudomonas fluorescens* and other organisms can be inferred by sequence homology or identified using a growth selection system developed in *Pseudomonas putida* (Henning et al., *Appl. Environ. Microbiol.* 72:7510-7517 (2006)).

Gene	Accession No.	GI No.	Organism
mdlC	P20906.2	3915757	*Pseudomonas putida*
mdlC	Q9HUR2.1	81539678	*Pseudomonas aeruginosa*
dpgB	ABN80423.1	126202187	*Pseudomonas stutzeri*
ilvB-1	YP_260581.1	70730840	*Pseudomonas fluorescens*

A third enzyme capable of decarboxylating 2-oxoacids is alpha-ketoglutarate decarboxylase (KGD). The substrate range of this class of enzymes has not been studied to date. The KDC from *Mycobacterium tuberculosis* (Tian et al., *Proc. Natl. Acad. Sci. USA* 102:10670-10675 (2005)) has been cloned and functionally expressed. However, it is not an ideal candidate for strain engineering because it is large (~130 kD) and GC-rich. KDC enzyme activity has been detected in several species of rhizobia including *Bradyrhizobium japonicum* and *Mesorhizobium loti* (Green et al., *J. Bacteriol.* 182:2838-2844 (2000). Although the KDC-encoding gene(s) have not been isolated in these organisms, the genome sequences are available, and several genes in each genome are annotated as putative KDCs. A KDC from *Euglena gracilis* has also been characterized, but the gene associated with this activity has not been identified to date (Shigeoka and Nakano, *Arch. Biochem. Biophys.* 288:22-28 (1991)). The first twenty amino acids starting from the N-terminus were sequenced (MTYKAPVKDVKFLLDKVFKV; SEQ ID NO:) (Shigeoka and Nakano, *Arch. Biochem. Biophys.* 288:22-28 (1991)). The gene can be identified by testing candidate genes containing this N-terminal sequence for KDC activity.

Gene	Accession No.	GI No.	Organism
kgd	O50463.4	160395583	*Mycobacterium tuberculosis*
kgd	NP_767092.1	27375563	*Bradyrhizobium japonicum*
kgd	NP_105204.1	13473636	*Mesorhizobium loti*

A fourth candidate enzyme for catalyzing this reaction is branched chain alpha-ketoacid decarboxylase (BCKA). This class of enzyme has been shown to act on a variety of compounds varying in chain length from 3 to 6 carbons (Oku and Kaneda, *J. Biol. Chem.* 263:18386-18396 (1988); Smit et al., B. A., J. E. Hylckama Vlieg, W. J. Engels, L. Meijer, J. T. Wouters, and G. Smit. Identification, cloning, and characterization of a Lactococcus lactis branched-chain alpha-keto acid decarboxylase involved in flavor formation. *Appl. Environ. Microbiol.* 71:303-311 (2005)). The enzyme in *Lactococcus lactis* has been characterized on a variety of branched and linear substrates including 2-oxobutanoate, 2-oxohexanoate, 2-oxopentanoate, 3-methyl-2-oxobutanoate, 4-methyl-2-oxobutanoate and isocaproate (Smit et al., *Appl. Environ. Microbiol.* 71:303-311 (2005)). The enzyme has been structurally characterized (Berg et al., *Science*

318:1782-1786 (2007)). Sequence alignments between the *Lactococcus lactis* enzyme and the pyruvate decarboxylase of *Zymomonas mobilus* indicate that the catalytic and substrate recognition residues are nearly identical (Siegert et al., *Protein Eng. Des. Sel.* 18:345-357 (2005)), so this enzyme would be a promising candidate for directed engineering.

Decarboxylation of alpha-*ketoglutarate* by a BCKA was detected in *Bacillus subtilis*; however, this activity was low (5%) relative to activity on other branched-chain substrates (Oku and Kaneda. Biosynthesis of branched-chain fatty acids in Bacillus subtilis. A decarboxylase is essential for branched-chain fatty acid synthetase. *J. Biol. Chem.* 263:18386-18396 (1988)), and the gene encoding this enzyme has not been identified to date. Additional BCKA gene candidates can be identified by homology to the *Lactococcus lactis* protein sequence. Many of the high-scoring BLASTp hits to this enzyme are annotated as indolepyruvate decarboxylases (EC 4.1.1.74). Indolepyruvate decarboxylase (IPDA) is an enzyme that catalyzes the decarboxylation of indolepyruvate to indoleacetaldehyde in plants and plant bacteria.

Gene	Accession No.	GI No.	Organism
kdcA	AAS49166.1	44921617	*Lactococcus lactis*

Recombinant branched chain alpha-keto acid decarboxylase enzymes derived from the E1 subunits of the mitochondrial branched-chain keto acid dehydrogenase complex from *Homo sapiens* and *Bos taurus* have been cloned and functionally expressed in *E. coli* (Davie et al., *J. Biol. Chem.* 267:16601-16606 1992); Wynn et al., *J. Biol. Chem.* 267:1881-1887 (1992); Wynn et al., *J. Biol. Chem.* 267:12400-12403 (1992)). In these studies, the authors found that co-expression of chaperonins GroEL and GroES enhanced the specific activity of the decarboxylase by 500-fold (Wynn et al., *J. Biol. Chem.* 267:12400-12403 (1992)). These enzymes are composed of two alpha and two beta subunits.

Gene	Accession No.	GI No.	Organism
BCKDHB	NP_898871.1	34101272	*Homo sapiens*
BCKDHA	NP_000700.1	11386135	*Homo sapiens*
BCKDHB	P21839	115502434	*Bos taurus*
BCKDHA	P11178	129030	*Bos taurus*

Decarboxylation of Alpha-Keto Acids. Several ornithine decarboxylase (Step U, Figure 63) enzymes also exhibit activity on lysine and other similar compounds. Such enzymes are found in *Nicotiana glutinosa* (Lee and Cho, *Biochem. J.* 360:657-665 (2001)), *Lactobacillus sp. 30a* (Guirard and Snell, *J. Biol. Chem.* 255:5960-5964 (1980)) and *Vibrio vulnificus* (Lee et al., *J.*

Biol. Chem. 282:27115-27125 (2007)). The enzymes from *Lactobacillus sp. 30a* (Momany et al., *J. Mol. Biol.* 252:643-655 (1995)) and *V. vulnificus* have been crystallized. The *V. vulnificus* enzyme efficiently catalyzes lysine decarboxylation, and the residues involved in substrate specificity have been elucidated (Lee et al., *J. Biol. Chem.* 282:27115-27125 (2007)). A similar enzyme has been characterized in *Trichomonas vaginalis,* but the gene encoding this enzyme is not known (Yarlett et al., *Biochem. J.* 293 (Pt 2):487-493 (1993)).

Gene	Accession No.	GI No.	Organism
AF323910.1:1..1299	AAG45222.1	12007488	*Nicotiana glutinosa*
odc1	P43099.2	1169251	*Lactobacillus sp. 30a*
VV2_1235	NP_763142.1	27367615	*Vibrio vulnificus*

Glutamate decarboxylase enzymes (Step L, Figures 62 and 63) are also well-characterized. Exemplary glutamate decarboxylases can be found in *E. coli* (De Biase et al., *Protein Expr. Purif.* 8:430-438 (1996)), *S. cerevisiae* (Coleman et al., *J. Biol. Chem.* 276:244-250 (2001)), and *Homo sapiens* (Bu et al., *Proc. Natl. Acad. Sci. USA* 89:2115-2119 (1992); Bu and Tobin, *Genomics* 21:222-228 (1994)).

Gene	Accession No.	GI No.	Organism
GAD1	NP_000808	58331246	*Homo sapiens*
GAD2	NP_001127838	197276620	*Homo sapiens*
gadA	NP_417974	16131389	*Escherichia coli*
gadB	NP_416010	16129452	*Escherichia coli*
GAD1	NP_013976	6323905	*Saccharomyces cerevisiae*

Lysine decarboxylase (EC 4.1.1.18) catalyzes the decarboxylation of lysine to cadaverine. Two isozymes of this enzyme are encoded in the *E. coli* genome by genes *cadA* and *ldcC*. CadA is involved in acid resistance and is subject to positive regulation by the *cadC* gene product (Lemonnier and Lane, *Microbiology* 144 (Pt 3):751-760 (1998)). CadC accepts hydroxylysine and S-aminoethylcysteine as alternate substrates, and 2-Aminopimelate and 6-ACA act as competitive inhibitors to this enzyme (Sabo et al., *Biochemistry* 13:662-670 (1974)). Directed evolution or other enzyme engineering methods can be utilized to increase the activity for this enzyme to decarboxylate 2-aminopimelate. The constitutively expressed *ldc* gene product is less active than CadA (Lemonnier and Lane, *Microbiology* 144 (Pt 3):751-760 (1998)). A lysine decarboxylase analogous to CadA was recently identified in *Vibrio parahaemolyticus* (Tanaka et al., *J. Appl. Microbiol.* 104:1283-1293 (2008)). The lysine decarboxylase from *Selenomonas ruminantium*, encoded by *ldc,* bears sequence similarity to eukaryotic ornithine decarboxylases, and accepts both L-lysine and L-ornithine as substrates (Takatsuka et al., *Biosci. Biotechnol.*

Biochem. 63:1843-1846 (1999)). Active site residues were identified and engineered to alter the substrate specificity of the enzyme (Takatsuka et al., *J. Bacteriol.* 182:6732-6741 (2000)).

Gene	Accession No.	GI No.	Organism
cadA	AAA23536.1	145458	*Escherichia coli*
ldcC	AAC73297.1	1786384	*Escherichia coli*
ldc	O50657.1	13124043	*Selenomonas ruminantium*
cadA	AB124819.1	44886078	*Vibrio parahaemolyticus*

6.2.1.a CoA synthetase.

CoA synthetase or ligase reactions are required by Step I of Figures 62 and 63, and Step V of Figure 62. Succinate or 4-hydroxybutyrate are the required substrates. Exemplary genes encoding enzymes likely to carry out these transformations include the *sucCD* genes of *E. coli,* which naturally form a succinyl-CoA synthetase complex. This enzyme complex naturally catalyzes the formation of succinyl-CoA from succinate with the concomitant consumption of one ATP, a reaction which is reversible *in vivo* (Buck et al., *Biochem.* 24:6245-6252 (1985)).

Gene	Accession No.	GI No.	Organism
sucC	NP_415256.1	16128703	*Escherichia coli*
sucD	AAC73823.1	1786949	*Escherichia coli*

Additional exemplary CoA-ligases include the rat dicarboxylate-CoA ligase for which the sequence is yet uncharacterized (Vamecq et al., *Biochemical J.* 230:683-693 (1985)), either of the two characterized phenylacetate-CoA ligases from *P. chrysogenum* (Lamas-Maceiras et al., *Biochem. J.* 395:147-155 (2005); Wang et al., *Biochem Biophy Res Commun* 360(2):453-458 (2007)), the phenylacetate-CoA ligase from *Pseudomonas putida* (Martinez-Blanco et al., *J. Biol. Chem.* 265:7084-7090 (1990)), and the 6-carboxyhexanoate-CoA ligase from *Bacilis subtilis* (Boweret al., *J. Bacteriol.* 178(14):4122-4130 (1996)). Additional candidate enzymes are acetoacetyl-CoA synthetases from *Mus musculus* (Hasegawa et al., *Biochim. Biophys. Acta* 1779:414-419 (2008)) and *Homo sapiens* (Ohgami et al., *Biochem. Pharmacol.* 65:989-994 (2003)), which naturally catalyze the ATP-dependant conversion of acetoacetate into acetoacetyl-CoA. 4-Hydroxybutyryl-CoA synthetase activity has been demonstrated in *Metallosphaera sedula* (Berg et al., *Science* 318:1782-1786 (2007)). This function has been tentatively assigned to the Msed_1422 gene.

Gene	Accession No.	GI No.	Organism
phl	CAJ15517.1	77019264	*Penicillium chrysogenum*
phlB	ABS19624.1	152002983	*Penicillium chrysogenum*
paaF	AAC24333.2	22711873	*Pseudomonas putida*
bioW	NP_390902.2	50812281	*Bacillus subtilis*
AACS	NP_084486.1	21313520	*Mus musculus*
AACS	NP_076417.2	31982927	*Homo sapiens*
Msed_1422	YP_001191504	146304188	*Metallosphaera sedula*

ADP-forming acetyl-CoA synthetase (ACD, EC 6.2.1.13) is another candidate enzyme that couples the conversion of acyl-CoA esters to their corresponding acids with the concurrent synthesis of ATP. Several enzymes with broad substrate specificities have been described in the literature. ACD I from *Archaeoglobus fulgidus*, encoded by AF1211, was shown to operate on a variety of linear and branched-chain substrates including acetyl-CoA, propionyl-CoA, butyryl-CoA, acetate, propionate, butyrate, isobutyryate, isovalerate, succinate, fumarate, phenylacetate, indoleacetate (Musfeldt et al., *J. Bacteriol.* 184:636-644 (2002)). The enzyme from *Haloarcula marismortui* (annotated as a succinyl-CoA synthetase) accepts propionate, butyrate, and branched-chain acids (isovalerate and isobutyrate) as substrates, and was shown to operate in the forward and reverse directions (Brasen et al., *Arch. Microbiol.* 182:277-287 (2004)). The ACD encoded by PAE3250 from hyperthermophilic crenarchaeon *Pyrobaculum aerophilum* showed the broadest substrate range of all characterized ACDs, reacting with acetyl-CoA, isobutyryl-CoA (preferred substrate) and phenylacetyl-CoA (Brasen et al., *supra* (2004)). The enzymes from *A. fulgidus, H. marismortui* and *P. aerophilum* have all been cloned, functionally expressed, and characterized in *E. coli* (Musfeldt et al., *supra*;Brasen et al., *supra* (2004)).

Gene	Accession No.	GI No.	Organism
AF1211	NP_070039.1	11498810	*Archaeoglobus fulgidus DSM 4304*
scs	YP_135572.1	55377722	*Haloarcula marismortui ATCC 43049*
PAE3250	NP_560604.1	18313937	*Pyrobaculum aerophilum str. IM2*

EXAMPLE XXIII
Production of BDO Utilizing Carboxylic Acid Reductase

This example describes the generation of a microbial organism that produces 1,4-butanediol using carboxylic acid reductase enzymes.

Escherichia coli is used as a target organism to engineer the pathway for 1,4-butanediol synthesis described in Figure 58. *E. coli* provides a good host for generating a non-naturally

occurring microorganism capable of producing 1,4-butanediol. *E. coli* is amenable to genetic manipulation and is known to be capable of producing various products, like ethanol, acetic acid, formic acid, lactic acid, and succinic acid, effectively under various oxygenation conditions.

Integration of 4-Hydroxybutyrate Pathway Genes into Chromosome: Construction of ECKh-432. The carboxylic acid reductase enzymes were expressed in a strain of *E. coli* designated ECKh-761 which is a descendent of ECKh-432 with additional deletions of the *sad* and *gabD* genes encoding succinate semialdehyde dehydrogenase enzymes. This strain contained the components of the BDO pathway, leading to 4HB, integrated into the chromosome of *E. coli* at the *fimD* locus as described in Example XXI.

Cloning and Expression of Carboxylic Acid Reductase and PPTase. To generate an *E. coli* strain engineered to produce 1,4-butanediol, nucleic acids encoding a carboxylic acid reductase and phosphopantetheine transferase are expressed in *E. coli* using well known molecular biology techniques (see, for example, Sambrook, supra, 2001; Ausubel supra, 1999). In particular, *car* genes from *Nocardia iowensis* (designated 720), *Mycobacterium smegmatis* mc(2)155 (designated 890), *Mycobacterium avium* subspecies *paratuberculosis* K-10 (designated 891) and *Mycobacterium marinum M* (designated 892) were cloned into pZS*13 vectors (Expressys, Ruelzheim, Germany) under control of PA1/lacO promoters. The *npt* (ABI83656.1) gene (i.e., 721) was cloned into the pKJL33S vector, a derivative of the original mini-F plasmid vector PML31 under control of promoters and ribosomal binding sites similar to those used in pZS*13.

The *car* gene (GNM_720) was cloned by PCR from *Nocardia* genomic DNA. Its nucleic acid and protein sequences are shown in Figures 59A and 59B, respectively. A codon-optimized version of the *npt* gene (GNM_721) was synthesized by GeneArt (Regensburg, Germany). Its nucleic acid and protein sequences are shown in Figures 60A and 60B, respectively. The nucleic acid and protein sequences for the *Mycobacterium smegmatis* mc(2)155 (designated 890), *Mycobacterium avium* subspecies *paratuberculosis* K-10 (designated 891) and *Mycobacterium marinum M* (designated 892) genes and enzymes can be found in Figures 64, 65, and 66, respectively. The plasmids were transformed into ECKh-761 to express the proteins and enzymes required for 1,4-butanediol production.

Demonstration of 1,4-BDO Production using Carboxylic Acid Reductase. Functional expression of the 1,4-butanediol pathway was demonstrated using *E. coli* whole-cell culture. Single colonies of *E. coli* ECKh-761 transformed with the pZS*13 and pKJL33S plasmids

containing a *car* gene and GNM_721, respectively, were inoculated into 5 mL of LB medium containing appropriate antibiotics. Similarly, single colonies of *E. coli* ECKh-761 transformed with *car*-containing pZS*13 plasmids and pKJL33S plasmids with no insert were inoculated into additional 5 mL aliquots of LB medium containing appropriate antibiotics. Ten mL micro-aerobic cultures were started by inoculating fresh minimal *in vivo* conversion medium (see below) containing the appropriate antibiotics with 1.5% of the first cultures.

Recipe of the minimal *in vivo* conversion medium (for 1000 mL) is as follows:

	Final concentration
1M MOPS/KOH buffer	100 mM
Glucose (40%)	1%
10XM9 salts solution	1X
MgSO4 (1 M)	1 mM
trace minerals (x1000)	1X
1M NaHCO3	10 mM

Microaerobic conditions were established by initially flushing capped anaerobic bottles with nitrogen for 5 minutes, then piercing the septum with an 18G needle following inoculation. The needle was kept in the bottle during growth to allow a small amount of air to enter the bottles. Protein expression was induced with 0.2 mM IPTG when the culture reached mid-log growth phase. This is considered: time = 0 hr. The culture supernatants were analyzed for BDO, 4HB, and other by-products as described above and in WO2008115840 (see Table 31).

Table 33 shows the production of various products in the strains expressing various carboxylic acid reductases, including production of BDO.

Table 33. Production of various products in strains expressing various carboxylic acid reductases.

					0 h	
		Cm10	Carb100	Carb100		
	Strain	**pKLJ33S**	**pZS*13S**	**pZShc13S**	**OD600**	**OD600**
1	761	034rbs55	no insert		0.54	2.13
5	761	721	720		0.48	1.88
7	761	721	890		0.45	1.63
8	761	721	891		0.48	1.65
9	761	721	892		0.45	1.31
12	761	no insert	720		0.50	1.72
14	761	no insert	890		0.51	1.96
15	761	no insert	891		0.19	2.36
16	761	no insert	892		0.05	1.40

48 h						
48 h, mM						
	PA	Su	La	4HB	BDO	GBL	$EtOH_{Enz}$
1	10.60	0.00	0.20	8.08	2.40	2.97	0.65
5	3.41	0.00	0.02	6.93	8.53	0.24	1.82
7	0.00	0.00	0.00	6.26	12.30	0.47	5.85
8	2.16	0.00	0.00	7.61	9.08	0.46	2.84
9	0.36	0.00	0.00	5.89	7.83	0.15	2.89
12	8.30	0.00	0.13	9.91	1.99	0.14	0.64
14	2.57	0.00	0.01	9.77	3.53	0.14	1.44
15	1.73	0.00	0.00	9.71	2.68	0.10	0.79
16	0.02	0.00	0.00	10.80	1.30	0.07	0.55

48 h, mM/OD						
	PA	Su	La	4HB	BDO	GBL	$EtOH_{Enz}$
1	4.98	0.00	0.09	3.80	1.13	1.40	0.31
5	1.81	0.00	0.01	3.69	4.54	0.13	0.97
7	0.00	0.00	0.00	3.84	7.55	0.29	3.59
8	1.31	0.00	0.00	4.61	5.50	0.28	1.72
9	0.27	0.00	0.00	4.50	5.99	0.12	2.21
12	4.83	0.00	0.07	5.76	1.16	0.08	0.37
14	1.31	0.00	0.01	4.99	1.80	0.07	0.74
15	0.73	0.00	0.00	4.11	1.13	0.04	0.33
16	0.01	0.00	0.00	7.71	0.93	0.05	0.39

PA = pyruvate, SA = succinate, LA = lactate, 4HB = 4-hydroxybutyrate, BDO = 1,4-butanediol, GBL = gamma-butyrolactone, Etoh = ethanol, LLOQ = lower limit of quantification

These results show that various carboxylic acid reductases can function in a BDO pathway to produce BDO.

Throughout this application various publications have been referenced. The disclosures of these publications in their entireties, including GenBank and GI number publications, are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains. Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention.

What is claimed is:

1. A non-naturally occurring microbial organism, comprising a microbial organism having a 4-hydroxybutanal pathway comprising exogenous nucleic acids encoding 4-hydroxybutanal pathway enzymes expressed in a sufficient amount to produce 4-hydroxybutanal, said 4-hydroxybutanal pathway comprising 4-hydroxybutyrate reductase; succinyl-CoA reductase (aldehyde forming); and 4-hydroxybutyrate dehydrogenase.

2. A non-naturally occurring microbial organism, comprising a microbial organism having a 4-hydroxybutanal pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutanal pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutanal, said 4-hydroxybutanal pathway comprising succinyl-CoA reductase (aldehyde forming); 4-hydroxybutyrate dehydrogenase; and 4-hydroxybutyrate reductase.

3. The non-naturally occurring microbial organism of claims 1 or 2, wherein said at least one exogenous nucleic acid is a heterologous nucleic acid.

4. A method for producing 4-hydroxybutanal, comprising culturing the non-naturally occurring microbial organism of any of claims 1-3 under conditions and for a sufficient period of time to produce 4-hydroxybutanal.

5. The method of claim 4, wherein said non-naturally occurring microbial organism is in a substantially anaerobic culture medium.

6. A non-naturally occurring microbial organism, comprising a microbial organism having a 4-hydroxybutanal pathway comprising exogenous nucleic acids encoding a 4-hydroxybutanal pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutanal, said 4-hydroxybutanal pathway comprising 4-hydroxybutyrate reductase; alpha-ketoglutarate decarboxylase; and 4-hydroxybutyrate dehydrogenase.

7. A non-naturally occurring microbial organism, comprising a microbial organism having a 4-hydroxybutanal pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutanal pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutanal, said 4-hydroxybutanal pathway comprising alpha-ketoglutarate decarboxylase; 4-hydroxybutyrate dehydrogenase; and 4-hydroxybutyrate reductase.

8. The non-naturally occurring microbial organism of any of claims 6 or 7, wherein said at least one exogenous nucleic acid is a heterologous nucleic acid.

9. A method for producing 4-hydroxybutanal, comprising culturing the non-naturally occurring microbial organism of any of claims 6-8 under conditions and for a sufficient period of time to produce 4-hydroxybutanal.

10. The method of claim 9, wherein said non-naturally occurring microbial organism is in a substantially anaerobic culture medium.

11. A non-naturally occurring microbial organism, comprising a microbial organism having a 4-hydroxybutanal pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutanal pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutanal, said 4-hydroxybutanal pathway comprising succinate reductase; 4-hydroxybutyrate dehydrogenase, and 4-hydroxybutyrate reductase.

12. The non-naturally occurring microbial organism of claim 11, wherein said microbial organism comprises three exogenous nucleic acids encoding succinate reductase; 4-hydroxybutyrate dehydrogenase, and 4-hydroxybutyrate reductase.

13. The non-naturally occurring microbial organism of claim 11, wherein said at least one exogenous nucleic acid is a heterologous nucleic acid.

14. A method for producing 4-hydroxybutanal, comprising culturing the non-naturally occurring microbial organism of any of claims 11-13 under conditions and for a sufficient period of time to produce 4-hydroxybutanal.

15. The method of claim 14, wherein said non-naturally occurring microbial organism is in a substantially anaerobic culture medium.

16. A non-naturally occurring microbial organism, comprising a microbial organism having a 4-hydroxybutanal pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutanal pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutanal, said 4-hydroxybutanal pathway comprising alpha-ketoglutarate decarboxylase, or glutamate dehydrogenase or glutamate transaminase and glutamate decarboxylase and 4-aminobutyrate dehydrogenase or 4-aminobutyrate transaminase; 4-hydroxybutyrate dehydrogenase; and 4-hydroxybutyrate reductase.

17. The non-naturally occurring microbial organism of claim 16, wherein said microbial organism comprises at least three exogenous nucleic acids encoding alpha-ketoglutarate decarboxylase, or glutamate dehydrogenase or glutamate transaminase and glutamate decarboxylase and 4-aminobutyrate dehydrogenase or 4-aminobutyrate transaminase; 4-hydroxybutyrate dehydrogenase; and 4-hydroxybutyrate reductase.

18. The non-naturally occurring microbial organism of claim 16, wherein said at least one exogenous nucleic acid is a heterologous nucleic acid.

19. A method for producing 4-hydroxybutanal, comprising culturing the non-naturally occurring microbial organism of any of claims 16-18 under conditions and for a sufficient period of time to produce 4-hydroxybutanal.

20. The method of claim 19, wherein said non-naturally occurring microbial organism is in a substantially anaerobic culture medium.

21. A non-naturally occurring microbial organism, comprising a microbial organism having a 4-hydroxybutanal pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutanal pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutanal, said 4-hydroxybutanal pathway comprising alpha-ketoglutarate reductase; 5-hydroxy-2-oxopentanoate dehydrogenase; and 5-hydroxy-2-oxopentanoate decarboxylase.

22. The non-naturally occurring microbial organism of claim 21, wherein said microbial organism comprises three exogenous nucleic acids encoding alpha-ketoglutarate reductase; 5-hydroxy-2-oxopentanoate dehydrogenase; and 5-hydroxy-2-oxopentanoate decarboxylase.

23. The non-naturally occurring microbial organism of claim 21, wherein said at least one exogenous nucleic acid is a heterologous nucleic acid.

24. A method for producing 4-hydroxybutanal, comprising culturing the non-naturally occurring microbial organism of any of claims 21-23 under conditions and for a sufficient period of time to produce 4-hydroxybutanal.

25. The method of claim 24, wherein said non-naturally occurring microbial organism is in a substantially anaerobic culture medium.

26. A non-naturally occurring microbial organism, comprising a microbial organism having a putrescine pathway comprising at least one exogenous nucleic acid encoding a putrescine pathway enzyme expressed in a sufficient amount to produce putrescine, said putrescine pathway comprising succinate reductase; 4-aminobutyrate dehydrogenase or 4-aminobutyrate transaminase; 4-aminobutyrate reductase; and putrescine dehydrogenase or putrescine transaminase.

27. The non-naturally occurring microbial organism of claim 26, wherein said microbial organism comprises four exogenous nucleic acids encoding succinate reductase; 4-

aminobutyrate dehydrogenase or 4-aminobutyrate transaminase; 4-aminobutyrate reductase; and putrescine dehydrogenase or putrescine transaminase.

28. The non-naturally occurring microbial organism of claim 26, wherein said at least one exogenous nucleic acid is a heterologous nucleic acid.

29. A method for producing putrescine, comprising culturing the non-naturally occurring microbial organism of any of claims 26-28 under conditions and for a sufficient period of time to produce putrescine.

30. The method of claim 29, wherein said non-naturally occurring microbial organism is in a substantially anaerobic culture medium.

31. A non-naturally occurring microbial organism, comprising a microbial organism having a putrescine pathway comprising at least one exogenous nucleic acid encoding a putrescine pathway enzyme expressed in a sufficient amount to produce putrescine, said putrescine pathway comprising alpha-ketoglutarate decarboxylase; 4-aminobutyrate dehydrogenase or 4-aminobutyrate transaminase; 4-aminobutyrate reductase; and putrescine dehydrogenase or putrescine transaminase.

32. The non-naturally occurring microbial organism of claim 31, wherein said microbial organism comprises four exogenous nucleic acids encoding alpha-ketoglutarate decarboxylase; 4-aminobutyrate dehydrogenase or 4-aminobutyrate transaminase; 4-aminobutyrate reductase; and putrescine dehydrogenase or putrescine transaminase.

33. The non-naturally occurring microbial organism of claim 31, wherein said at least one exogenous nucleic acid is a heterologous nucleic acid.

34. A method for producing putrescine, comprising culturing the non-naturally occurring microbial organism of any of claims 31-33 under conditions and for a sufficient period of time to produce putrescine.

35. The method of claim 34, wherein said non-naturally occurring microbial organism is in a substantially anaerobic culture medium.

36. A non-naturally occurring microbial organism, comprising a microbial organism having a putrescine pathway comprising at least one exogenous nucleic acid encoding a putrescine pathway enzyme expressed in a sufficient amount to produce putrescine, said putrescine pathway comprising glutamate dehydrogenase or glutamate transaminase; glutamate

decarboxylase; 4-aminobutyrate reductase; and putrescine dehydrogenase or putrescine transaminase.

37. The non-naturally occurring microbial organism of claim 36, wherein said microbial organism comprises four exogenous nucleic acids encoding glutamate dehydrogenase or glutamate transaminase; glutamate decarboxylase; 4-aminobutyrate reductase; and putrescine dehydrogenase or putrescine transaminase.

38. The non-naturally occurring microbial organism of claim 36, wherein said at least one exogenous nucleic acid is a heterologous nucleic acid.

39. A method for producing putrescine, comprising culturing the non-naturally occurring microbial organism of any of claims 36-38 under conditions and for a sufficient period of time to produce putrescine.

40. The method of claim 39, wherein said non-naturally occurring microbial organism is in a substantially anaerobic culture medium.

41. A non-naturally occurring microbial organism, comprising a microbial organism having a putrescine pathway comprising at least one exogenous nucleic acid encoding a putrescine pathway enzyme expressed in a sufficient amount to produce putrescine, said putrescine pathway comprising alpha-ketoglutarate reductase; 5-amino-2-oxopentanoate dehydrogenase or 5-amino-2-oxopentanoate transaminase; 5-amino-2-oxopentanoate decarboxylase; and putrescine dehydrogenase or putrescine transaminase.

42. The non-naturally occurring microbial organism of claim 41, wherein said microbial organism comprises four exogenous nucleic acids encoding alpha-ketoglutarate reductase; 5-amino-2-oxopentanoate dehydrogenase or 5-amino-2-oxopentanoate transaminase; 5-amino-2-oxopentanoate decarboxylase; and putrescine dehydrogenase or putrescine transaminase.

43. The non-naturally occurring microbial organism of claim 41, wherein said at least one exogenous nucleic acid is a heterologous nucleic acid.

44. A method for producing putrescine, comprising culturing the non-naturally occurring microbial organism of any of claims 41-43 under conditions and for a sufficient period of time to produce putrescine.

45. The method of claim 44, wherein said non-naturally occurring microbial organism is in a substantially anaerobic culture medium.

46. A non-naturally occurring microbial organism, comprising a microbial organism having a putrescine pathway comprising at least one exogenous nucleic acid encoding a putrescine pathway enzyme expressed in a sufficient amount to produce putrescine, said putrescine pathway comprising alpha-ketoglutarate reductase; 5-amino-2-oxopentanoate dehydrogenase or 5-amino-2-oxopentanoate transaminase; ornithine dehydrogenase or ornithine transaminase; and ornithine decarboxylase.

47. The non-naturally occurring microbial organism of claim 46, wherein said microbial organism comprises four exogenous nucleic acids encoding alpha-ketoglutarate reductase; 5-amino-2-oxopentanoate dehydrogenase or 5-amino-2-oxopentanoate transaminase; ornithine dehydrogenase or ornithine transaminase; and ornithine decarboxylase.

48. The non-naturally occurring microbial organism of claim 46, wherein said at least one exogenous nucleic acid is a heterologous nucleic acid.

49. A method for producing putrescine, comprising culturing the non-naturally occurring microbial organism of any of claims 46-48 under conditions and for a sufficient period of time to produce putrescine.

50. The method of claim 49, wherein said non-naturally occurring microbial organism is in a substantially anaerobic culture medium.

51. A non-naturally occurring microbial organism, comprising a microbial organism having a 4-hydroxybutyryl-CoA (4-HBCoA) pathway comprising at least one exogenous nucleic acid encoding a 4-hydroxybutyryl-CoA pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutyryl-CoA, said 4-hydroxybutyryl-CoA pathway comprising alpha-ketoglutarate reductase; 5-hydroxy-2-oxopentanoate dehydrogenase; and 5-hydroxy-2-oxopentanoate dehydrogenase (decarboxylation).

52. The non-naturally occurring microbial organism of claim 51, wherein said microbial organism comprises three exogenous nucleic acids encoding alpha-ketoglutarate reductase; 5-hydroxy-2-oxopentanoate dehydrogenase; and 5-hydroxy-2-oxopentanoate dehydrogenase (decarboxylation).

53. The non-naturally occurring microbial organism of claim 51, wherein said at least one exogenous nucleic acid is a heterologous nucleic acid.

54. A method for producing 4-hydroxybutyryl-CoA, comprising culturing the non-naturally occurring microbial organism of any of claims 51-53 under conditions and for a sufficient period of time to produce 4-hydroxybutyryl-CoA.

55. The method of claim 54, wherein said non-naturally occurring microbial organism is in a substantially anaerobic culture medium.