

GENETICALLY ENGINEERED MICROORGANISMS FOR THE PRODUCTION OF CHORISMATE-DERIVED PRODUCTS

CROSS REFERENCE TO RELATED APPLICATIONS

0001 This application claims the benefit of U.S. Provisional Patent Application No. 62/167,101 filed May 27, 2015, the entirety of which is incorporated herein by reference.

FIELD OF THE INVENTION

0002 The present invention relates to genetically engineered microorganisms and methods for the production of chorismate-derived products by microbial fermentation, particularly by microbial fermentation of a gaseous substrate.

BACKGROUND OF THE INVENTION

0003 The current generation of biologically-produced commodity chemicals that use either food or non-food crops to produce sugar or cellulose-based feedstocks have drawbacks relating to land use, food security, supply volatility, and environmental issues.

0004 It has long been recognized that catalytic processes may be used to convert gases containing carbon monoxide (CO) and/or carbon dioxide (CO₂) and hydrogen (H₂) into a variety of fuels and chemicals. However, microorganisms may also be used to biologically convert such gases into fuels and chemicals. Biological processes have several advantages over catalytic processes, including higher specificity, higher yields, lower energy costs, and greater catalyst resistance to poisoning.

0005 CO is a major free energy-rich byproduct of the incomplete combustion of organic materials such as coal or oil and oil-derived products. For example, the steel industry in Australia is reported to produce and release into the atmosphere over 500,000 tonnes of CO annually.

0006 The ability of microorganisms to grow on CO as a sole carbon source was first discovered in 1903. This was later determined to be a property of microorganisms that use the acetyl coenzyme A (acetyl-CoA) biochemical pathway of autotrophic growth, also known as the Wood-Ljungdahl pathway. A large number of anaerobic microorganisms including carboxydophilic, photosynthetic, methanogenic, and acetogenic microorganisms have been

shown to metabolize CO to various end products, namely CO₂, H₂, methane, n-butanol, acetate, and ethanol.

0007 The aromatic compound para-hydroxybenzoic acid (pHBA) is a major monomer used in liquid crystal polymers and also used as a precursor for the production of parahydroxybenzoates or parahydroxybenzoic esters, commonly referred to as parabens. Liquid crystal polymers include Kevlar and Vectran, which have multiple uses. Parabens and their salts are used in a range of industries including the cosmetic, pharmaceutical and food industries. They are effective preservatives and can be used for their bactericidal and fungicidal properties in cosmetic and food formulations.

0008 Accordingly, there remains a need for additional microorganisms and methods for producing pHBA and other high-value chorismate-derived products.

SUMMARY OF THE INVENTION

0009 The invention provides a genetically engineered microorganism capable of producing chorismate-derived products. In particular, the invention provides a genetically engineered microorganism capable of producing at least one chorismate-derived product, wherein the bacterium comprises at least one of (a) an exogenous chorismate pyruvate lyase (EC 4.1.3.40), (b) an exogenous isochorismate synthase (EC 5.4.4.2), (c) an exogenous isochorismate pyruvate lyase (EC 4.2.99.21), and (d) a prephenate synthase (EC 5.4.99.5) comprising a disruptive mutation. In particular embodiments, the genetically engineered microorganism is a C1-fixing bacterium, such as a *Clostridium* bacterium, capable of producing at least one chorismate-derived product by fermentation of a C1-containing gaseous substrate.

0010 For example, the chorismate pyruvate lyase may be *ubiC*, the isochorismate synthase may be *pchA*, the isochorismate pyruvate lyase may be *pchB*, and the prephenate synthase may be *pheA*. The disruptive mutation in prephenate synthase may reduce or eliminate the expression or activity of the prephenate synthase. Such a disruptive mutation may yield a bacterium that produces a reduced amount of prephenate or prephenate-derived products compared to a parental bacterium and/or a bacterium that produces substantially no tyrosine or phenylalanine.

0011 The microorganism of the invention may comprise at least one nucleic acid encoding at least one of (a) the exogenous chorismate pyruvate lyase, (b) the exogenous isochorismate synthase, (c) the exogenous isochorismate pyruvate lyase, and (d) the prephenate synthase

comprising a disruptive mutation. In certain embodiments, the nucleic acid is codon optimized for expression in *Clostridium*.

0012 The chorismate-derived product may be any product produced directly or indirectly from chorismate. In particular, the chorismate-derived product may comprise a 6-membered carbon ring, for example, a benzene or cyclohexane ring, substituted with a carboxyl group or carboxylate anion and further substituted with one or more OH groups and/or one or more NH₂ groups. Chorismate-derived products include, but are not limited to, para-hydroxybenzoic acid, salicylate, 2-aminobenzoate, dihydroxybenzoate, and 4-hydroxycyclohexane carboxylic acid.

0013 In one embodiment, the microorganism of the invention expresses a chorismate pyruvate lyase of *ubiC* and produces a chorismate-derived product of para-hydroxybenzoic acid. In one embodiment the microorganism of the invention further expresses feedback-insensitive DAHP synthase.

0014 In one embodiment, the microorganism of the invention expresses an isochorismate synthase of *pchA* and an isochorismate pyruvate lyase of *pchB* and produces a chorismate-derived product of salicylate. In one embodiment the microorganism of the invention further expresses feedback-insensitive DAHP synthase.

0015 In one embodiment, the microorganism of the invention comprises a prephenate synthase comprising a disruptive mutation and produces a one or more of chorismate-derived product of 2-aminobenzoate, 2,3-dihydroxybenzoate, 3,4-dihydroxybenzoate and 4-hydroxycyclohexane carboxylic acid.

0016 In one embodiment, the microorganism of the invention produces at least one chorismate-derived product not produced by a parental microorganism or a greater amount of at least one chorismate-derived product than a parental microorganism.

0017 In one embodiment, the bacterium of the invention is derived from a C1-fixing parental bacterium. In a preferred embodiment, the bacterium of the invention is derived from a parental bacterium selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei*. In a particularly preferred embodiment, the bacterium of the invention is derived from a parental bacterium of *Clostridium autoethanogenum* deposited under DSMZ accession number DSM23693.

0018 The invention further provides a method of producing a fermentation product, comprising fermenting the microorganism of the invention in the presence of a C1-containing

gaseous substrate. Generally, the fermentation product is a chorismate-derived product. In a preferred embodiment, the gaseous substrate comprises at least one C1 carbon source.

BRIEF DESCRIPTION OF THE DRAWINGS

0019 Fig. 1 is a diagram showing production of chorismate via a native shikimate pathway in *Clostridia*.

0020 Fig. 2 is a diagram showing the pathway for production of pHBA in a genetically engineered *Clostridium* bacterium.

0021 Fig. 3 is a diagram showing the pathway for production of salicylate in a genetically engineered *Clostridium* bacterium.

0022 Fig. 4 is a diagram showing the pathway for production of aromatic products in a genetically engineered *Clostridium* bacterium comprising a disruptive mutation in a nucleic acid encoding pheA.

0023 Fig. 5 is a graph of a standard curve showing quantitation of authentic pHBA standards.

0024 Fig. 6a is a graph showing the total ion count of authentic standards (i) authentic standard of pHBA (trimethylsilylated) prepared in supernatant from *C. autoethanogenum* LZ1561 culture medium, (ii) authentic standard of pHBA (trimethylsilylated) prepared in water, and (iii) mass spectrum of trimethylsilylated pHBA.

0025 Fig. 6b is a graph showing selected ion monitoring of fermentation samples and standards: (i) *C. autoethanogenum* LZ1561 without pARO_01 plasmid, (ii) and (iii) samples from *C. autoethanogenum* LZ1561 bearing pARO_01 plasmid, (iv) authentic standard of pHBA, and (v) total ion count comparison between NIST database entry for pHBA and pHBA peak from LZ1561/pARO_01.

0026 Fig. 7 is a diagram of a pARO_01 plasmid. The chorismate pyruvate lyase (ubiC) and feedback-insensitive DAHP synthase (aroG*) are under control of the Wood-Ljungdahl promoter (Pwl). Other shuttle vector features are also shown.

0027 Fig. 8 is a graph showing biomass accumulation in test strains. Biomass was estimated by measuring the absorbance of culture samples at 600 nm at different time points. Data points represent the mean of n=3 replicate cultures $\pm$ 1 standard deviation. LZ1561 refers to untransformed *C. autoethanogenum* LZ1561. pARO_01(1) and pARO_01(2) are

biological replicates of *C. autoethanogenum* LZ1561 transformed with the pARO_01 plasmid.

0028 Figs. 9a and 9b are graphs showing p-hydroxybenzoate accumulation in test strains. Fig. 9a shows quantification of pHBA detected in each sample at 24, 96, 144, and 192 hour time points. Three replicate cultures were sampled for the negative control strain (*C. autoethanogenum* LZ1561) and the two biological replicates of *C. autoethanogenum* LZ1561 carrying pARO_01. Fig. 9b shows mean of n=3 technical replicates $\pm$ 1 SD.

0029 Fig. 10 is a graph showing production of new aromatic compounds in a genetically engineered *Clostridium* bacterium comprising a disruptive mutation in a nucleic acid encoding pheA. The Δ pheA strain produces 4-hydroxy cyclohexane carboxylic acid, 2-aminobenzoic acid, and 3,4-dihydroxybenzoic acid, while the control strain (LZ1561) does not.

0030 Fig. 11a is a graph showing biomass growth of the salicylate production strain with and without induction of the salicylate biosynthetic pathway.

0031 Fig. 11b is a graph showing the difference in accumulation of salicylate in liquid cultures of the test strain with and without induction of the salicylate biosynthetic pathway.

0032 Figure 12 is a graph showing concentration of 4-hydroxy cyclohexane carboxylic acid, 2-aminobenzoic acid, and 3,4-dihydroxybenzoic acid produced by fermentation of an engineered *Clostridium* bacterium comprising a disruptive mutation in a nucleic acid encoding pheA.

0033 Figure 13 is a table identifying exemplary sources of chorismate pyruvate lyase (EC 4.1.3.40).

0034 Figure 14 is a table identifying exemplary sources of isochorismate synthase (EC 5.4.4.2).

0035 Figure 15 is table of identifying exemplary sources of isochorismate pyruvate lyase (EC 4.2.99.21).

DETAILED DESCRIPTION OF THE INVENTION

0036 *Clostridia* natively produce chorismate, which serves as a precursor to the aromatic amino acids tryptophan, tyrosine, and phenylalanine, from phosphoenolpyruvate and

erythrose-4-phosphate via the shikimate pathway (Fig. 1). This pathway is described in detail in Bentley, *Crit Rev Biochem Mol Biol*, 25.5: 307-384, 1990. The invention provides a genetically engineered bacterium capable of producing at least one chorismate-derived product by fermentation of a gaseous substrate.

0037 The inventors have demonstrated that chorismate-derived products can be sustainably produced and recovered from a C1-carbon source. The invention provides a method of producing at least one chorismate-derived product using a C1- containing gaseous substrate as the main carbon and energy source. In this way, the present invention has a number of advantages over processes that rely on sugar- or cellulose-based substrates. For example, sugar- or cellulose-based substrates are typically also useful for food (e.g. sugar cane) and their intensive land use has negative environmental consequences. Further, the invention provides an alternative method for the production of chorismate-derived products, optionally via the use of waste gases (e.g. CO from industrial processes). Thus, the invention provides a source of revenue from waste gases and, furthermore, captures the carbon in those waste gases to reduce the carbon emissions that would occur if the gases were flared to the atmosphere.

0038 Heterotrophic microorganisms such as *E. coli* and *S. cerevisiae* produce relatively high levels of ATP through glycolysis. In contrast, microorganisms which use C1-carbon sources (e.g., CO or CO₂) have poor ATP availability. For example, analysis of the reaction kinetics in a typical carboxydophilic microorganism *C. autoethanogenum* gives a predicted ATP yield when producing pHBA, a chorismate-derived product) of -0.4 ATP per mol of CO fixed. As such, it would not be expected that any pHBA would be produced due to the energy constraints. Similarly it would not be expected that other chorismate-derived products would be produced by a carboxydophilic microorganism due to the metabolic burden of producing such compounds under autotrophic conditions. The inventors have surprisingly shown however that a number of chorismate-derived products can be produced from a gaseous substrate. Further, said products can be produced from industrial waste gases which provide practical, economic, and environmental benefits over other substrates.

0039 In particular, the invention provides genetically engineered microorganisms capable of producing at least one chorismate-derived product by introducing at least one of (a) a nucleic acid encoding an exogenous chorismate pyruvate lyase, (b) a nucleic acid encoding an exogenous isochorismate synthase (a.k.a., isochorismate mutase), (c) a nucleic acid

encoding an exogenous isochorismate pyruvate lyase, and (d) a nucleic acid encoding a prephenate synthase comprising a disruptive mutation. In a preferred embodiment, the genetically engineered microorganism is a C1-fixing bacterium capable of producing at least one chorismate-derived product by fermentation of a gaseous substrate. In preferred embodiments the C1-fixing bacterium is a *Clostridium* bacterium.

0040 A “chorismate-derived product” or “product derived from chorismate” or similar terms encompass products produced directly or indirectly from chorismate (or chorismic acid). Chorismate-derived products typically comprise a 6-membered carbon ring, for example, a benzene or cyclohexane ring, substituted with a carboxyl group or carboxylate anion and further substituted with one or more OH groups and/or one or more NH₂ groups. Specifically, chorismate-derived products include, but are not limited to, para-hydroxybenzoic acid, salicylate, 2-aminobenzoate, 2,3-dihydroxybenzoate, 3,4-dihydroxybenzoate, and 4-hydroxycyclohexane carboxylic acid.

0041 The microorganism of the invention may comprise an exogenous chorismate pyruvate lyase enzyme (EC 4.1.3.40) that catalyzes the conversion of chorismate to para-hydroxybenzoic acid and pyruvate in the first committed step of ubiquinone biosynthesis. The enzyme may be derived from any microorganism having such an enzyme. The enzyme may be a UbiC enzyme. The UbiC enzyme may be derived from *Escherichia coli*, *Klebsiella oxytoca*, *Citrobacter freundii*, or any other microorganism having a UbiC enzyme. In one embodiment, the UbiC enzyme is derived from *Escherichia coli* and comprises SEQ ID NO: 1 or a functionally equivalent variant thereof.

0042 Similarly, the microorganism of the invention may comprise a nucleic acid encoding an exogenous chorismate pyruvate lyase. The nucleic acid may be a chorismate pyruvate lyase gene derived from any microorganism having such a gene. The chorismate pyruvate lyase gene may be a *ubiC* gene. The *ubiC* gene may be derived from *Escherichia coli*, *Klebsiella oxytoca*, *Citrobacter freundii*, or any other microorganism having a *ubiC* gene. In one embodiment, the *ubiC* gene is derived from *Escherichia coli* and comprises SEQ ID NO: 2 or a codon-optimized or functionally equivalent variant thereof.

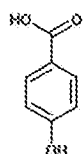
0043 The UbiC enzyme or *ubiC* gene may also be modified (e.g., mutated) to enhance solubility, stability, or other gene/enzyme properties. Such modifications may result in increased product titers. Example 4 describes an experimental protocol to engineer a UbiC enzyme to decrease product inhibition through retention of para-hydroxybenzoic acid. One

particular modification involves engineering the *ubiC* gene to express a UbiC enzyme with two surface-active serines instead of cysteines. The serine residues result in less protein aggregation and, in turn, improved solubility. Accordingly, in a particular embodiment, the UbiC enzyme comprises a mutation to replace at least one surface-active cysteine with a serine.

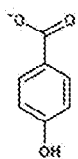
0044 In alternative embodiments, the chorismate pyruvate lyase (EC 4.1.3.40) may be or may be derived, for example, from any of the sources identified in Figure 13.

0045 Introduction of an exogenous chorismate pyruvate lyase (e.g., *ubiC*) or a nucleic acid encoding an exogenous chorismate pyruvate lyase (e.g., *ubiC*) results in production of para-hydroxybenzoic acid, a chorismate-derived product, by the microorganism of the invention. The production of para-hydroxybenzoic acid is illustrated in Fig. 2. C1 fixing bacteria including the species *Acetobacterium woodii*, *Alkalibaculum bacchii*, *Blautia producta*, *Butyribacterium methylotrophicum*, *Clostridium aceticum*, *Clostridium autoethanogenum*, *Clostridium carboxidivorans*, *Clostridium coskatii*, *Clostridium drakei*, *Clostridium formicoaceticum*, *Clostridium ljungdahlii*, *Clostridium magnum*, *Clostridium ragsdalei*, *Clostridium scatologenes*, *Eubacterium limosum*, *Moorella thermautotrophica*, *Moorella thermoacetica*, *Oxobacter pfennigii*, *Sporomusa ovata*, *Sporomusa silvacetica*, *Sporomusa sphaeroides*, and *Thermoanaerobacter kivui*, do not natively produce para-hydroxybenzoic acid. In fact, since ubiquinone is generally only produced in aerobically respiring microorganisms, chorismate pyruvate lyase is not typically found in carboxidotrophic microorganisms. Although it may be expected that the diversion of chorismate to produce pHBA instead of amino acids would have detrimental effects on the growth or survival of the microorganism, the inventors have shown that the microorganism is not affected to a degree that significantly compromises survival and growth under standard conditions.

0046 Para-hydroxybenzoic acid may also be referred to, for example, as pHBA, 4-hydroxybenzoic acid, p-hydroxybenzoic acid, or para-hydroxybenzoate. References to any of these terms, as used herein, encompass both the acid and anion forms of the molecule.



para-hydroxybenzoic acid



para-hydroxybenzoate

0047 The microorganism of the invention may comprise an exogenous isochorismate synthase enzyme, also referred to as isochorismate mutase, (EC 5.4.4.2) that catalyzes the conversion of chorismate to isochorismate. The enzyme may be derived from any microorganism having such an enzyme. The enzyme may be a PchA enzyme. The PchA enzyme may be derived from *Pseudomonas aeruginosa* or any other microorganism having a PchA enzyme. In one embodiment, the PchA enzyme is derived from *Pseudomonas aeruginosa* and comprises SEQ ID NO: 3 or a functionally equivalent variant thereof.

0048 Similarly, the microorganism of the invention may comprise a nucleic acid encoding an exogenous isochorismate synthase. The nucleic acid may be an isochorismate synthase gene derived from any microorganism having such a gene. The isochorismate synthase gene may be a *pchA* gene. The *pchA* gene may be derived from *Pseudomonas aeruginosa* or any other microorganism having a *pchA* gene. In one embodiment, the *pchA* gene is derived from *Pseudomonas aeruginosa* and comprises SEQ ID NO: 4 or a codon-optimized or functionally equivalent variant thereof.

0049 In alternative embodiments, the isochorismate synthase (EC 5.4.4.2) may be or may be derived, for example, from any of the sources identified in Figure 14.

0050 The microorganism of the invention may comprise an exogenous isochorismate pyruvate lyase enzyme (EC 4.2.99.21) that catalyzes the conversion of isochorismate to salicylate and pyruvate. The enzyme may be derived from any microorganism having such an enzyme. The enzyme may be a PchB enzyme. The PchB enzyme may be derived from *Pseudomonas aeruginosa* or any other microorganism having a PchB enzyme. In one embodiment, the PchB enzyme is derived from *Pseudomonas aeruginosa* and comprises SEQ ID NO: 5 or a functionally equivalent variant thereof.

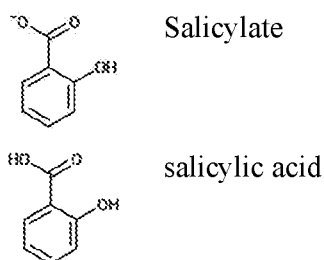
0051 Similarly, the microorganism of the invention may comprise a nucleic acid encoding an exogenous isochorismate pyruvate lyase. The nucleic acid may be an isochorismate pyruvate lyase gene derived from any microorganism having such a gene. The isochorismate pyruvate lyase gene may be a *pchB* gene. The *pchB* gene may be derived from *Pseudomonas*

aeruginosa or any other microorganism having a *pchB* gene. In one embodiment, the *pchB* gene is derived from *Pseudomonas aeruginosa* and comprises SEQ ID NO: 6 or a codon-optimized or functionally equivalent variant thereof.

0052 In alternative embodiments, the isochorismate pyruvate lyase (EC 4.2.99.21) may be or may be derived, for example, from any of the sources identified in Figure 15.

0053 Introduction of (1) an exogenous isochorismate synthase (e.g., *pchA*) and (2) an exogenous isochorismate pyruvate lyase (e.g., *pchB*) results in production of salicylate, a chorismate-derived product, by the microorganism of the invention. The production of salicylate is illustrated in Fig. 3, whereby chorismate is converted to isochorismate by isochorismate synthase and then further converted to salicylate and pyruvate by isochorismate pyruvate lyase. C1 fixing bacteria including the species *Acetobacterium woodii*, *Alkalibaculum bacchii*, *Blautia producta*, *Butyribacterium methylotrophicum*, *Clostridium aceticum*, *Clostridium autoethanogenum*, *Clostridium carboxidivorans*, *Clostridium coskatii*, *Clostridium drakei*, *Clostridium formicoaceticum*, *Clostridium ljungdahlii*, *Clostridium magnum*, *Clostridium ragsdalei*, *Clostridium scatologenes*, *Eubacterium limosum*, *Moorella thermautotrophica*, *Moorella thermoacetica*, *Oxobacter pfennigii*, *Sporomusa ovata*, *Sporomusa silvacetica*, *Sporomusa sphaeroides*, and *Thermoanaerobacter kivui*, do not natively produce salicylate.

0054 Salicylate may also be referred to, for example, as 2-hydroxybenzoate, salicylic acid, or 2-hydroxybenzoic acid. References to any of these terms, as used herein, encompass both the acid and anion forms of the molecule.



(d) Prephenate synthase comprising a disruptive mutation

0055 The microorganism of the invention may comprise a prephenate synthase enzyme (EC 5.4.99.5) comprising a disruptive mutation. Prephenate synthase typically catalyzes the conversion of chorismate to prephenate (i.e., a chorismate $\leftrightarrow$ prephenate mutase reaction).

Accordingly, a prephenate synthase enzyme comprising a disruptive mutation is unable or less able to catalyze the conversion of chorismate to prephenate. The prephenate synthase comprising a disruptive mutation may be *pheA* comprising a disruptive mutation. The prephenate synthase may also be referred to as chorismate mutase.

0056 In some embodiments, the *pheA* may be a bifunctional enzyme that carries out both prephenate synthase (i.e., chorismate mutase) (EC 5.4.99.5) and prephenate dehydratase (EC 4.2.1.51) reactions. In microorganisms where these two reactions are carried out by separate enzymes, knocking out EC 5.4.99.5 activity will result in significantly decreased or eliminated production of prephenate or compounds downstream of prephenate, while knocking out EC 4.2.1.51 activity alone would not achieve the same phenotype, since prephenate may still be produced. In one embodiment, the *pheA* is derived from *Clostridium autoethanogenum* and comprises SEQ ID NO: 11 or a functionally equivalent variant thereof

0057 Similarly, the microorganism of the invention may comprise a nucleic acid encoding a prephenate synthase comprising a disruptive mutation. The nucleic acid may be a *pheA* gene comprising a disruptive mutation. In one embodiment, the disruptive mutation is a knockout mutation of a *pheA* gene. In one embodiment, the *pheA* gene is derived from *Clostridium autoethanogenum* and comprises SEQ ID NO: 10 or a codon-optimized or functionally equivalent variant thereof.

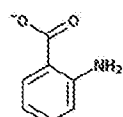
0058 Disrupting prephenate synthase results in reduced or eliminated production of phenylalanine and tyrosine. Surprisingly, disrupting prephenate synthase also results in the production of additional products that are not typically produced or that are produced only at very low levels.

0059 In particular, the introduction of a disruptive mutation to prephenate synthase (e.g., *pheA*) or a nucleic acid encoding prephenate synthase (e.g., *pheA*) results in production of one or more of 2-aminobenzoate, dihydroxybenzoate, and 4-hydroxycyclohexane carboxylic acid, all chorismate-derived products, by the microorganism of the invention. The production pathways of these products is illustrated in Fig. 4. Many microorganisms, including species of *Clostridia* such as *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei*, do not natively produce these products or only produce very low levels of these products.

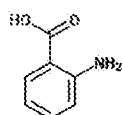
0060 Exemplary sources for pheA are provided. However, it should be appreciated that other suitable sources for pheA may be available. The prephenate dehydratase may be or may be derived, for example, from any of the following sources, the sequences of which are publically available:

Description	Microorganism	Genbank accession
bifunctional chorismate mutase/ prephenate dehydratase	<i>Acetobacterium woodii</i>	AFA49374.1
prephenate dehydratase	<i>Blautia producta</i>	WP_033143345.1
prephenate dehydratase	<i>Clostridium acetivum</i>	WP_044823168.1
prephenate dehydratase	<i>Clostridium autoethanogenum</i>	AGY75132.1
bifunctional chorismate mutase/ prephenate dehydratase	<i>Clostridium carboxidivorans</i>	WP_007060905.1
bifunctional chorismate mutase/ prephenate dehydratase	<i>Clostridium coskatii</i>	WP_063600678.1
bifunctional chorismate mutase/ prephenate dehydratase	<i>Clostridium drakei</i>	WP_032076381.1
bifunctional chorismate mutase/ prephenate dehydratase	<i>Clostridium ljungdahlii</i>	WP_063554005.1
prephenate dehydratase	<i>Clostridium magnum</i>	KZL89370.1
bifunctional chorismate mutase/ prephenate dehydratase	<i>Clostridium scatologenes</i>	WP_029159263.1
chorismate mutase	<i>Eubacterium limosum</i>	WP_058695931.1
chorismate mutase	<i>Oxobacter pfennigii</i>	WP_054874911.1
prephenate dehydratase	<i>Sporomusa ovata</i>	EQB25731.1
prephenate dehydratase	<i>Thermoanaerobacter kivui</i>	WP_049685038.1

0061 2-aminobenzoate may also be referred to, for example, as 2-aminobenzoic acid, o-aminobenzoic acid, anthranilic acid, anthranilate, or vitamin L1. References to any of these terms, as used herein, encompass both the acid and anion forms of the molecule.



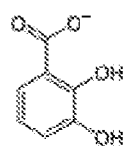
2-aminobenzoate



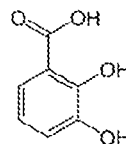
2-aminobenzoic acid

0062 Dihydroxybenzoate may be referred to, for example, as 2,3-dihydroxybenzoate, 2,3-dihydroxybenzoic acid, 3,4-dihydroxybenzoate, 3,4-dihydroxybenzoic acid or Protocatechuic

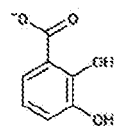
acid. References to any of these terms, as used herein, encompass both the acid and anion forms of the molecule.



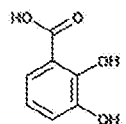
2,3-dihydroxybenzoate



2,3-dihydroxybenzoic acid

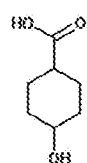


3,4-dihydroxybenzoate

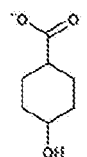


3,4-dihydroxybenzoic acid, Protocatechuic acid

0063 4-hydroxycyclohexane carboxylic acid may also be referred to, for example, as *cis*-4-hydroxycyclohexane carboxylic acid or 4-hydroxycyclohexane-1-carboxylate. References to any of these terms, as used herein, encompass both the acid and anion forms of the molecule.



4-hydroxycyclohexane carboxylic acid



4-hydroxycyclohexane carboxylate

0064 In another embodiment, the microorganism of the invention further comprises a nucleic acid encoding a feedback-insensitive DAHP synthase DAHP synthase catalyses the first committed step in the shikimate pathway (Fig. 1) in which erythrose-4-phosphate and phosphoenolpyruvate are converted to 3-deoxy-D-arabinoheptosonate-7-phosphate. The inventors believe that this step in the pathway is subject to feedback inhibition by aromatic amino acids (tryptophan, phenylalanine, tyrosine) as described for *E. coli* (Hu et al. J. Basic Microbiol. 2003, 43:399-406). Accordingly, the inventors have, based on this prior art,

developed a feedback-insensitive DAHP synthase, which is believed to reduce the risk of flux to chorismate-derived products being reduced by this feedback inhibition. Nucleic acids encoding appropriate DAHP synthases are known to those of skill in the art. However, by way of example, the nucleic acid encoding a DAHP synthase may be derived from *Escherichia coli*, *Clostridium beijerinckii*, or *Saccharomyces cerevisiae*. In one embodiment, the DAHP synthase may be feedback-insensitive DAHP synthase from *Escherichia coli*, having the nucleic acid sequence of SEQ ID NO: 7 and the amino acid sequence of SEQ ID NO: 8. The feedback-insensitive DAHP synthase may be introduced on the same vector as a gene encoding one of the aforementioned enzymes or on a different vector. The feedback-insensitive DAHP synthase may have its own promoter or may follow the promoter for one of the aforementioned enzymes in a bicistronic arrangement, wherein a single promoter drives the transcription of a single mRNA that encodes both the enzyme and the feedback-insensitive DAHP synthase.

0065 In one embodiment, the microorganism of the invention comprises an exogenous chorismate pyruvate lyase enzyme (EC 4.1.3.40), and an exogenous feedback-insensitive DAHP synthase. In particular embodiments the microorganism comprises an exogenous UbiC enzyme, and an exogenous feedback-insensitive DAHP synthase. In a specific embodiment, the invention comprises exogenous ubiC gene having the nucleic acid sequence of SEQ ID NO: 1, and an exogenous feedback-insensitive DAHP synthase having the nucleic acid sequence of SEQ ID NO: 7. In one embodiment, the microorganism comprising both an exogenous chorismate pyruvate lyase enzyme and an exogenous feedback-insensitive DAHP synthase demonstrates greater production of para-hydroxybenzoic acid compared to a microorganism without a feedback-insensitive DAHP synthase.

0066 Similarly, the microorganism of the invention may comprise a nucleic acid encoding both an exogenous chorismate pyruvate lyase and feedback-insensitive DAHP synthase.

0067 In one embodiment, the microorganism of the invention comprises (i) an exogenous isochorismate mutase, (EC 5.4.4.2), (ii) an isochorismate pyruvate lyase enzyme (EC 4.2.99.21), and (iii) an exogenous feedback-insensitive DAHP synthase. In particular embodiments the microorganism comprises an exogenous PchA enzyme, an exogenous PchB enzyme, and an exogenous feedback-insensitive DAHP synthase. In one embodiment, the microorganism comprising an exogenous feedback-insensitive DAHP synthase demonstrates

greater production of salicylic acid compared to a microorganism without a feedback-insensitive DAHP synthase.

0068 Similarly, the microorganism of the invention may comprise a nucleic acid encoding both an exogenous chorismate pyruvate lyase and feedback-insensitive DAHP synthase.

0069 In another embodiment, the microorganism of the invention does not comprise a feedback-insensitive DAHP synthase and instead merely comprises an endogenous DAHP synthase. Where production or natural concentration of aromatic amino acids is expected to be low enough so as to not induce feedback inhibition, it is not necessary to introduce a feedback-insensitive DAHP synthase.

0070 The microorganism of the invention may produce chorismate-derived products at any concentration or in any amount. In one embodiment, the microorganism of the invention produces chorismate-derived products at a concentration of at least about 5 mg/L, 10 mg/L, 15 mg/L, 20 mg/L, 30 mg/L, 50 mg/L, 75 mg/L, 100 mg/L, 200 mg/L, 500 mg/L, 750 mg/L, 1g/L, 1.5g/L or 2 g/L. In one embodiment, the microorganism of the invention produces at least one chorismate-derived product at a concentration of at least 10 mg/L, 50mg/L, 100mg/L, 500mg/L, 800mg/L, or 1g/L

0071 Furthermore, the microorganism of the invention may be engineered to produce products at a certain selectivity or at a minimum selectivity. In one embodiment, a target chorismate-derived product accounts for at least about 5%, 10%, 15%, 20%, 30%, 50%, or 75% of all fermentation products produced by the microorganism of the invention. In one embodiment, the target chorismate-derived product accounts for at least 10% of all fermentation products produced by the microorganism of the invention, such that the microorganism of the invention has a selectivity for the target chorismate-derived product of at least 10%. In another embodiment, the target chorismate-derived product accounts for at least 30% of all fermentation products produced by the microorganism of the invention, such that the microorganism of the invention has a selectivity for the target chorismate-derived product of at least 30%.

0072 The invention further provides a method of producing a fermentation product, specifically a chorismate-derived product, comprising fermenting the microorganism of the invention in the presence of a gaseous substrate.

0073 The invention also provides chorismate-derived products produced by fermenting a microorganism of the invention in the presence of a gaseous substrate.

Definitions and Background

0074 The term “genetic modification” or “genetic engineering” broadly refers to manipulation of the genome or nucleic acids of a microorganism. Methods of genetic modification include, for example, heterologous gene expression, gene or promoter insertion or deletion, nucleic acid mutation, altered gene expression or inactivation, enzyme engineering, directed evolution, knowledge-based design, random mutagenesis methods, gene shuffling, and codon optimization.

0075 “Recombinant” indicates that a nucleic acid, protein, or microorganism is the product of genetic modification, engineering, or recombination. Generally, the term “recombinant” refers to a nucleic acid, protein, or microorganism that contains or is encoded by genetic material derived from multiple sources, such as two or more different strains or species of microorganisms. As used herein, the term “recombinant” may also be used to describe a microorganism that comprises a mutated nucleic acid or protein, including a mutated form of an endogenous nucleic acid or protein.

0076 “Endogenous” refers to a nucleic acid or protein that is present or expressed in the wild-type or parental microorganism from which the microorganism of the invention is derived. For example, an endogenous gene is a gene that is natively present in the wild-type or parental microorganism from which the microorganism of the invention is derived. In one embodiment, the expression of an endogenous gene may be controlled by an exogenous regulatory element, such as an exogenous promoter.

0077 “Exogenous” refers to a nucleic acid or protein that is not present in the wild-type or parental microorganism from which the microorganism of the invention is derived. In one embodiment, an exogenous gene or enzyme may be derived from a heterologous (i.e., different) strain or species and introduced to or expressed in the microorganism of the invention. In another embodiment, an exogenous gene or enzyme may be artificially or recombinantly created and introduced to or expressed in the microorganism of the invention. Exogenous nucleic acids may be adapted to integrate into the genome of the microorganism of the invention or to remain in an extra-chromosomal state in the microorganism of the invention, for example, in a plasmid.

0078 “Enzyme activity” refers broadly to enzymatic activity, including, but not limited, to the activity of an enzyme, the amount of an enzyme, or the availability of an enzyme to catalyze a reaction. Accordingly, “increasing” enzyme activity includes increasing the activity of an enzyme, increasing the amount of an enzyme, or increasing the availability of an enzyme to catalyze a reaction.

0079 “Mutated” refers to a nucleic acid or protein that has been modified in the microorganism of the invention compared to the wild-type or parental microorganism from which the microorganism of the invention is derived. In one embodiment, the mutation may be a deletion, insertion, or substitution in a gene encoding an enzyme. In another embodiment, the mutation may be a deletion, insertion, or substitution of one or more amino acids in an enzyme.

0080 In particular, a “disruptive mutation” is a mutation that reduces or eliminates (i.e., “disrupts”) the expression or activity of a gene or enzyme. The disruptive mutation may partially inactivate, fully inactivate, or delete the gene or enzyme. The disruptive mutation may be a knockout (KO) mutation. The disruptive mutation may be any mutation that reduces, prevents, or blocks the biosynthesis of a product produced by an enzyme. The disruptive mutation may include, for example, a mutation in a gene encoding an enzyme, a mutation in a genetic regulatory element involved in the expression of a gene encoding an enzyme, the introduction of a nucleic acid which produces a protein that reduces or inhibits the activity of an enzyme, or the introduction of a nucleic acid (e.g., antisense RNA, siRNA, CRISPR) or protein which inhibits the expression of an enzyme. The disruptive mutation may be introduced using any method known in the art.

0081 “Codon optimization” refers to the mutation of a nucleic acid, such as a gene, for optimized or improved translation of the nucleic acid in a particular strain or species. Codon optimization may result in faster translation rates or higher translation accuracy. In a preferred embodiment, the genes of the invention are codon optimized for expression in *Clostridium*, particularly *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. In a further preferred embodiment, the genes of the invention are codon optimized for expression in *Clostridium autoethanogenum* LZ1561, which is deposited under DSMZ accession number DSM23693.

0082 “Overexpressed” refers to an increase in expression of a nucleic acid or protein in the microorganism of the invention compared to the wild-type or parental microorganism from

which the microorganism of the invention is derived. Overexpression may be achieved by any means known in the art, including modifying gene copy number, gene transcription rate, gene translation rate, or enzyme degradation rate.

0083 The term “variants” includes nucleic acids and proteins whose sequence varies from the sequence of a reference nucleic acid and protein, such as a sequence of a reference nucleic acid and protein disclosed in the prior art or exemplified herein. The invention may be practiced using variant nucleic acids or proteins that perform substantially the same function as the reference nucleic acid or protein. For example, a variant protein may perform substantially the same function or catalyze substantially the same reaction as a reference protein. A variant gene may encode the same or substantially the same protein as a reference gene. A variant promoter may have substantially the same ability to promote the expression of one or more genes as a reference promoter.

0084 Such nucleic acids or proteins may be referred to herein as “functionally equivalent variants.” By way of example, functionally equivalent variants of a nucleic acid may include allelic variants, fragments of a gene, mutated genes, polymorphisms, and the like.

Homologous genes from other microorganisms are also examples of functionally equivalent variants. These include homologous genes in species such as *Clostridium acetobutylicum*, *Clostridium beijerinckii*, or *Clostridium ljungdahlii*, the details of which are publicly available on websites such as Genbank or NCBI. Functionally equivalent variants also include nucleic acids whose sequence varies as a result of codon optimization for a particular microorganism. A functionally equivalent variant of a nucleic acid will preferably have at least approximately 70%, approximately 80%, approximately 85%, approximately 90%, approximately 95%, approximately 98%, or greater nucleic acid sequence identity (percent homology) with the referenced nucleic acid. A functionally equivalent variant of a protein will preferably have at least approximately 70%, approximately 80%, approximately 85%, approximately 90%, approximately 95%, approximately 98%, or greater amino acid identity (percent homology) with the referenced protein. The functional equivalence of a variant nucleic acid or protein may be evaluated using any method known in the art.

0085 Nucleic acids may be delivered to a microorganism of the invention using any method known in the art. For example, nucleic acids may be delivered as naked nucleic acids or may be formulated with one or more agents, such as liposomes. The nucleic acids may be DNA, RNA, cDNA, or combinations thereof, as is appropriate. Restriction inhibitors may be used

in certain embodiments. Additional vectors may include plasmids, viruses, bacteriophages, cosmids, and artificial chromosomes. In a preferred embodiment, nucleic acids are delivered to the microorganism of the invention using a plasmid. By way of example, transformation (including transduction or transfection) may be achieved by electroporation, ultrasonication, polyethylene glycol-mediated transformation, chemical or natural competence, protoplast transformation, prophage induction, or conjugation. In certain embodiments having active restriction enzyme systems, it may be necessary to methylate a nucleic acid before introduction of the nucleic acid into a microorganism.

0086 Furthermore, nucleic acids may be designed to comprise a regulatory element, such as a promoter, to increase or otherwise control expression of a particular nucleic acid. The promoter may be a constitutive promoter or an inducible promoter. Ideally, the promoter is a Wood-Ljungdahl pathway promoter, a ferredoxin promoter, a pyruvate:ferredoxin oxidoreductase promoter, an Rnf complex operon promoter, an ATP synthase operon promoter, or a phosphotransacetylase/acetate kinase operon promoter.

0087 A “microorganism” is a microscopic organism, especially a bacterium, archaea, virus, or fungus. The microorganism of the invention is typically a bacterium. As used herein, recitation of “microorganism” should be taken to encompass “bacterium.”

0088 A “parental microorganism” is a microorganism used to generate a microorganism of the invention. The parental microorganism may be a naturally-occurring microorganism (i.e., a wild-type microorganism) or a microorganism that has been previously modified (i.e., a mutant or recombinant microorganism). The microorganism of the invention may be modified to express or overexpress one or more enzymes that were not expressed or overexpressed in the parental microorganism. Similarly, the microorganism of the invention may be modified to contain one or more genes that were not contained by the parental microorganism. In one embodiment, the parental microorganism is *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. In a preferred embodiment, the parental microorganism is *Clostridium autoethanogenum* LZ1561, which is deposited under DSMZ accession DSM23693.

0089 The term “derived from” indicates that a nucleic acid, protein, or microorganism is modified or adapted from a different (e.g., a parental or wild-type) nucleic acid, protein, or microorganism, so as to produce a new nucleic acid, protein, or microorganism. Such modifications or adaptations typically include insertion, deletion, mutation, or substitution of

nucleic acids or genes. Generally, the microorganism of the invention is derived from a parental microorganism. In one embodiment, the microorganism of the invention is derived from *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. In a preferred embodiment, the microorganism of the invention is derived from *Clostridium autoethanogenum* LZ1561, which is deposited under DSMZ accession DSM23693.

0090 The microorganism of the invention may be further classified based on functional characteristics. For example, the microorganism of the invention may be or may be derived from a C1-fixing microorganism, an anaerobe, an acetogen, an ethanologen, a carboxydrotroph, and/or a methanogen. Table 1 provides a representative list of microorganisms and identifies their functional characteristics.

Table 1

	C1-fixing	Anaerobe	Acetogen	Ethanologen	Autotroph	Carboxydrotroph	Methanotroph
<i>Acetobacterium woodii</i>	+	+	+	+/- ¹	-	-	-
<i>Alkalibaculum bacchii</i>	+	+	+	+	+	+	-
<i>Blautia producta</i>	+	+	+	-	+	+	-
<i>Butyrivacterium methylotrophicum</i>	+	+	+	+	+	+	-
<i>Clostridium aceticum</i>	+	+	+	-	+	+	-
<i>Clostridium autoethanogenum</i>	+	+	+	+	+	+	-
<i>Clostridium carboxidivorans</i>	+	+	+	+	+	+	-
<i>Clostridium coskatii</i>	+	+	+	+	+	+	-
<i>Clostridium drakei</i>	+	+	+	-	+	+	-
<i>Clostridium formicoaceticum</i>	+	+	+	-	+	+	-
<i>Clostridium ljungdahlii</i>	+	+	+	+	+	+	-
<i>Clostridium magnum</i>	+	+	+	-	+	+/- ²	-
<i>Clostridium ragsdalei</i>	+	+	+	+	+	+	-
<i>Clostridium scatologenes</i>	+	+	+	-	+	+	-
<i>Eubacterium limosum</i>	+	+	+	-	+	+	-
<i>Moorella thermoautotrophica</i>	+	+	+	+	+	+	-
<i>Moorella thermoacetica</i> (formerly <i>Clostridium thermoaceticum</i>)	+	+	+	- ³	+	+	-
<i>Oxobacter pfennigii</i>	+	+	+	-	+	+	-
<i>Sporomusa ovata</i>	+	+	+	-	+	+/- ⁴	-
<i>Sporomusa silvacetica</i>	+	+	+	-	+	+/- ⁵	-
<i>Sporomusa sphaeroides</i>	+	+	+	-	+	+/- ⁶	-
<i>Thermoanaerobacter kivui</i>	+	+	+	-	+	-	-

¹ *Acetobacterium woodii* can produce ethanol from fructose, but not from gas.

² It has not been investigated whether *Clostridium magnum* can grow on CO.

- ³ One strain of *Moorella thermoacetica*, *Moorella* sp. HUC22-1, has been reported to produce ethanol from gas.
- ⁴ It has not been investigated whether *Sporomusa ovata* can grow on CO.
- ⁵ It has not been investigated whether *Sporomusa silvacetica* can grow on CO.
- ⁶ It has not been investigated whether *Sporomusa sphaeroides* can grow on CO.

0091 “C1” refers to a one-carbon molecule, for example, CO, CO₂, CH₄, or CH₃OH. “C1-oxygenate” refers to a one-carbon molecule that also comprises at least one oxygen atom, for example, CO, CO₂, or CH₃OH. “C1-carbon source” refers a one carbon-molecule that serves as a partial or sole carbon source for the microorganism of the invention. For example, a C1-carbon source may comprise one or more of CO, CO₂, CH₄, CH₃OH, or CH₂O₂. Preferably, the C1-carbon source comprises one or both of CO and CO₂. A “C1-fixing microorganism” is a microorganism that has the ability to produce one or more products from a C1-carbon source. Typically, the microorganism of the invention is a C1-fixing bacterium. In a preferred embodiment, the microorganism of the invention is derived from a C1-fixing microorganism identified in Table 1.

0092 An “anaerobe” is a microorganism that does not require oxygen for growth. An anaerobe may react negatively or even die if oxygen is present. Typically, the microorganism of the invention is an anaerobe. In a preferred embodiment, the microorganism of the invention is derived from an anaerobe identified in Table 1.

0093 An “acetogen” is a microorganism that produces or is capable of producing acetate (or acetic acid) as a product of anaerobic respiration. Typically, acetogens are obligately anaerobic bacteria that use the Wood-Ljungdahl pathway as their main mechanism for energy conservation and for synthesis of acetyl-CoA and acetyl-CoA-derived products, such as acetate (Ragsdale, *Biochim Biophys Acta*, 1784: 1873-1898, 2008). Acetogens use the acetyl-CoA pathway as a (1) mechanism for the reductive synthesis of acetyl-CoA from CO₂, (2) terminal electron-accepting, energy conserving process, (3) mechanism for the fixation (assimilation) of CO₂ in the synthesis of cell carbon (Drake, *Acetogenic Prokaryotes*, In: *The Prokaryotes*, 3rd edition, p. 354, New York, NY, 2006). All naturally occurring acetogens are C1-fixing, anaerobic, autotrophic, and non-methanotrophic. Typically, the microorganism of the invention is an acetogen. In a preferred embodiment, the microorganism of the invention is derived from an acetogen identified in Table 1.

0094 An “ethanologen” is a microorganism that produces or is capable of producing ethanol. Typically, the microorganism of the invention is an ethanologen. In a preferred

embodiment, the microorganism of the invention is derived from an ethanologen identified in Table 1.

0095 An “autotroph” is a microorganism capable of growing in the absence of organic carbon. Instead, autotrophs use inorganic carbon sources, such as CO and/or CO₂. Typically, the microorganism of the invention is an autotroph. In a preferred embodiment, the microorganism of the invention is derived from an autotroph identified in Table 1.

0096 A “carboxydotroph” is a microorganism capable of utilizing CO as a sole source of carbon. Typically, the microorganism of the invention is a carboxydotroph. In a preferred embodiment, the microorganism of the invention is derived from a carboxydotroph identified in Table 1.

0097 A “methanotroph” is a microorganism capable of utilizing methane as a sole source of carbon and energy. In certain embodiments, the microorganism of the invention is derived from a methanotroph.

0098 More broadly, the microorganism of the invention may be derived from any genus or species identified in Table 1. In a preferred embodiment, the microorganism of the invention is a *Clostridium* bacterium.

0099 In a preferred embodiment, the microorganism of the invention is derived from the cluster of *Clostridia* comprising the species *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei*. These species were first reported and characterized by Abrini, *Arch Microbiol*, 161: 345-351, 1994 (*Clostridium autoethanogenum*), Tanner, *Int J System Bacteriol*, 43: 232-236, 1993 (*Clostridium ljungdahlii*), and Huhnke, WO 2008/028055 (*Clostridium ragsdalei*).

0100 These three species have many similarities. In particular, these species are all C1-fixing, anaerobic, acetogenic, ethanogenic, and carboxydotrophic members of the genus *Clostridium*. These species have similar genotypes and phenotypes and modes of energy conservation and fermentative metabolism. Moreover, these species are clustered in clostridial rRNA homology group I with 16S rRNA DNA that is more than 99% identical, have a DNA G + C content of about 22-30 mol%, are gram-positive, have similar morphology and size (logarithmic growing cells between 0.5-0.7 x 3-5 µm), are mesophilic (grow optimally at 30-37 °C), have similar pH ranges of about 4-7.5 (with an optimal pH of about 5.5-6), lack cytochromes, and conserve energy via an Rnf complex. Also, reduction of

carboxylic acids into their corresponding alcohols has been shown in these species (Perez, *Biotechnol Bioeng*, 110:1066-1077, 2012). Importantly, these species also all show strong autotrophic growth on CO-containing gases, produce ethanol and acetate (or acetic acid) as main fermentation products, and produce small amounts of 2,3-butanediol and lactic acid under certain conditions.

0101 However, these three species also have a number of differences. These species were isolated from different sources: *Clostridium autoethanogenum* from rabbit gut, *Clostridium ljungdahlii* from chicken yard waste, and *Clostridium ragsdalei* from freshwater sediment. These species differ in utilization of various sugars (e.g., rhamnose, arabinose), acids (e.g., gluconate, citrate), amino acids (e.g., arginine, histidine), and other substrates (e.g., betaine, butanol). Moreover, these species differ in auxotrophy to certain vitamins (e.g., thiamine, biotin). These species have differences in nucleic and amino acid sequences of Wood-Ljungdahl pathway genes and proteins, although the general organization and number of these genes and proteins has been found to be the same in all species (Köpke, *Curr Opin Biotechnol*, 22: 320-325, 2011).

0102 Thus, in summary, many of the characteristics of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei* are not specific to that species, but are rather general characteristics for this cluster of C1-fixing, anaerobic, acetogenic, ethanologenic, and carboxydophilic members of the genus *Clostridium*. However, since these species are, in fact, distinct, the genetic modification or manipulation of one of these species may not have an identical effect in another of these species. For instance, differences in growth, performance, or product production may be observed.

0103 The microorganism of the invention may also be derived from an isolate or mutant of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. Isolates and mutants of *Clostridium autoethanogenum* include JA1-1 (DSM10061) (Abrini, *Arch Microbiol*, 161: 345-351, 1994), LBS1560 (DSM19630) (WO 2009/064200), and LZ1561 (DSM23693). Isolates and mutants of *Clostridium ljungdahlii* include ATCC 49587 (Tanner, *Int J Syst Bacteriol*, 43: 232-236, 1993), PETCT (DSM13528, ATCC 55383), ERI-2 (ATCC 55380) (US 5,593,886), C-01 (ATCC 55988) (US 6,368,819), O-52 (ATCC 55989) (US 6,368,819), and OTA-1 (Tirado-Acevedo, Production of bioethanol from synthesis gas using *Clostridium ljungdahlii*, PhD thesis, North Carolina State University, 2010). Isolates

and mutants of *Clostridium ragsdalei* include PI 1 (ATCC BAA-622, ATCC PTA-7826) (WO 2008/028055).

0104 “Substrate” refers to a carbon and/or energy source for the microorganism of the invention. Typically, the substrate is gaseous and comprises a C1-carbon source, for example, CO, CO₂, and/or CH₄. Preferably, the substrate comprises a C1-carbon source of CO or CO + CO₂. The substrate may further comprise other non-carbon components, such as H₂, N₂, or electrons.

0105 The substrate generally comprises at least some amount of CO, such as about 1, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 mol% CO. The substrate may comprise a range of CO, such as about 20-80, 30-70, or 40-60 mol% CO. Preferably, the substrate comprises about 40-70 mol% CO (e.g., steel mill or blast furnace gas), about 20-30 mol% CO (e.g., basic oxygen furnace gas), or about 15-45 mol% CO (e.g., syngas). In some embodiments, the substrate may comprise a relatively low amount of CO, such as about 1-10 or 1-20 mol% CO. The microorganism of the invention typically converts at least a portion of the CO in the substrate to a product.

0106 The substrate may comprise some amount of H₂. For example, the substrate may comprise about 1, 2, 5, 10, 15, 20, or 30 mol% H₂. In some embodiments, the substrate may comprise a relatively high amount of H₂, such as about 60, 70, 80, or 90 mol% H₂. In further embodiments, the substrate comprises substantially no H₂.

0107 The substrate may comprise some amount of CO₂. For example, the substrate may comprise about 1-80 or 1-30 mol% CO₂. In some embodiments, the substrate may comprise less than about 20, 15, 10, or 5 mol% CO₂. In another embodiment, the substrate comprises substantially no CO₂.

0108 Although the substrate is typically gaseous, the substrate may also be provided in alternative forms. For example, the substrate may be dissolved in a liquid saturated with a CO-containing gas using a microbubble dispersion generator. By way of further example, the substrate may be adsorbed onto a solid support.

0109 The substrate and/or C1-carbon source may be a waste gas obtained as a byproduct of an industrial process or from some other source, such as from automobile exhaust fumes or biomass gasification. In certain embodiments, the industrial process is selected from the group consisting of ferrous metal products manufacturing, such as a steel mill manufacturing,

non-ferrous products manufacturing, petroleum refining processes, coal gasification, electric power production, carbon black production, ammonia production, methanol production, and coke manufacturing. In these embodiments, the substrate and/or C1-carbon source may be captured from the industrial process before it is emitted into the atmosphere, using any convenient method.

0110 The substrate and/or C1-carbon source may be syngas, such as syngas obtained by gasification of coal or refinery residues, gasification of biomass, or reforming of natural gas. The composition of the substrate may have a significant impact on the efficiency and/or cost of the reaction. For example, the presence of oxygen (O₂) may reduce the efficiency of an anaerobic fermentation process. Depending on the composition of the substrate, it may be desirable to treat, scrub, or filter the substrate to remove any undesired impurities, such as toxins, undesired components, or dust particles, and/or increase the concentration of desirable components.

0111 The microorganism of the invention may be cultured to produce one or more products. For instance, *Clostridium autoethanogenum* produces or can be engineered to produce ethanol (WO 2007/117157), acetate (WO 2007/117157), butanol (WO 2008/115080 and WO 2012/053905), butyrate (WO 2008/115080), 2,3-butanediol (WO 2009/151342), lactate (WO 2011/112103), butene (WO 2012/024522), butadiene (WO 2012/024522), methyl ethyl ketone (2-butanone) (WO 2012/024522 and WO 2013/185123), ethylene (WO 2012/026833), acetone (WO 2012/115527), isopropanol (WO 2012/115527), lipids (WO 2013/036147), 3-hydroxypropionate (3-HP) (WO 2013/180581), isoprene (WO 2013/180584), fatty acids (WO 2013/191567), 2-butanol (WO 2013/185123), 1,2-propanediol (WO 2014/0369152), and 1-propanol (WO 2014/0369152). In addition to one or more target products, the microorganism of the invention may also produce ethanol, acetate, and/or 2,3-butanediol.

0112 “Selectivity” refers to the ratio of the production of a target product to the production of all fermentation products produced by a microorganism. The microorganism of the invention may be engineered to produce products at a certain selectivity or at a minimum selectivity. In one embodiment, a target product account for at least about 5%, 10%, 15%, 20%, 30%, 50%, or 75% of all fermentation products produced by the microorganism of the invention. In one embodiment, the target product accounts for at least 10% of all fermentation products produced by the microorganism of the invention, such that the

microorganism of the invention has a selectivity for the target product of at least 10%. In another embodiment, the target product accounts for at least 30% of all fermentation products produced by the microorganism of the invention, such that the microorganism of the invention has a selectivity for the target product of at least 30%.

0113 Typically, the culture is performed in a bioreactor. The term “bioreactor” includes a culture/fermentation device consisting of one or more vessels, towers, or piping arrangements, such as a continuous stirred tank reactor (CSTR), immobilized cell reactor (ICR), trickle bed reactor (TBR), bubble column, gas lift fermenter, static mixer, or other vessel or other device suitable for gas-liquid contact. In some embodiments, the bioreactor may comprise a first growth reactor and a second culture/fermentation reactor. The substrate may be provided to one or both of these reactors. As used herein, the terms “culture” and “fermentation” are used interchangeably. These terms encompass both the growth phase and product biosynthesis phase of the culture/fermentation process.

0114 The culture is generally maintained in an aqueous culture medium that contains nutrients, vitamins, and/or minerals sufficient to permit growth of the microorganism. Preferably the aqueous culture medium is an anaerobic microbial growth medium, such as a minimal anaerobic microbial growth medium. Suitable media are well known in the art.

0115 The culture/fermentation should desirably be carried out under appropriate conditions for production of the target product. Reaction conditions to consider include pressure (or partial pressure), temperature, gas flow rate, liquid flow rate, media pH, media redox potential, agitation rate (if using a continuous stirred tank reactor), inoculum level, maximum gas substrate concentrations to ensure that gas in the liquid phase does not become limiting, and maximum product concentrations to avoid product inhibition. In particular, the rate of introduction of the substrate may be controlled to ensure that the concentration of gas in the liquid phase does not become limiting, since products may be consumed by the culture under gas-limited conditions.

0116 Operating a bioreactor at elevated pressures allows for an increased rate of gas mass transfer from the gas phase to the liquid phase. Accordingly, it is generally preferable to perform the culture/fermentation at pressures higher than atmospheric pressure. Also, since a given gas conversion rate is, in part, a function of the substrate retention time and retention time dictates the required volume of a bioreactor, the use of pressurized systems can greatly reduce the volume of the bioreactor required and, consequently, the capital cost of the

culture/fermentation equipment. This, in turn, means that the retention time, defined as the liquid volume in the bioreactor divided by the input gas flow rate, can be reduced when bioreactors are maintained at elevated pressure rather than atmospheric pressure. The optimum reaction conditions will depend partly on the particular microorganism used. However, in general, it is preferable to operate the fermentation at a pressure higher than atmospheric pressure. Also, since a given gas conversion rate is in part a function of substrate retention time and achieving a desired retention time in turn dictates the required volume of a bioreactor, the use of pressurized systems can greatly reduce the volume of the bioreactor required, and consequently the capital cost of the fermentation equipment.

0117 Target products may be separated or purified from a fermentation broth using any method or combination of methods known in the art, including, for example, fractional distillation, evaporation, pervaporation, gas stripping, phase separation, and extractive fermentation, including for example, liquid-liquid extraction. In certain embodiments, target products are recovered from the fermentation broth by continuously removing a portion of the broth from the bioreactor, separating microbial cells from the broth (conveniently by filtration), and recovering one or more target products from the broth. Alcohols and/or acetone may be recovered, for example, by distillation. Acids may be recovered, for example, by adsorption on activated charcoal. Separated microbial cells are preferably returned to the bioreactor. The cell-free permeate remaining after target products have been removed is also preferably returned to the bioreactor. Additional nutrients (such as B vitamins) may be added to the cell-free permeate to replenish the medium before it is returned to the bioreactor.

EXAMPLES

0118 The following examples further illustrate the invention but, of course, should not be construed to limit its scope in any way.

Example 1

0119 This example describes general methods for culturing *C. autoethanogenum* and *C. ljungdahlii*.

0120 *C. autoethanogenum* DSM10061 and DSM23693 (a derivative of DSM10061) and *C. ljungdahlii* DSM13528 were sourced from DSMZ (The German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7 B, 38124 Braunschweig, Germany).

0121 Strains were grown at 37 °C in PETC medium at pH 5.6 using standard anaerobic techniques (Hungate, *Methods Microbiol*, 3B: 117-132, 1969; Wolfe, *Adv Microbiol Physiol*, 6: 107-146, 1971). Fructose (heterotrophic growth) or 30 psi CO-containing steel mill gas (collected from New Zealand Steel site in Glenbrook, NZ; composition: 44% CO, 32% N₂, 22% CO₂, 2% H₂) in the headspace (autotrophic growth) was used as substrate. For solid media, 1.2 % bacto agar (BD, Franklin Lakes, NJ 07417, USA) was added.

PETC medium component	Amount per 1.0 L of PETC medium
NH ₄ Cl	1 g
KCl	0.1 g
MgSO ₄ · 7H ₂ O	0.2 g
NaCl	0.8 g
KH ₂ PO ₄	0.1 g
CaCl ₂	0.02 g
Trace metal solution (see below)	10 ml
Wolfe's vitamin solution (see below)	10 ml
Yeast extract (optional)	1 g
Resazurin (2 g/L stock)	0.5 ml
NaHCO ₃	2 g
Reducing agent solution (see below)	0.006-0.008 % (v/v)
Fructose (for heterotrophic growth)	5 g

Trace metal solution component	Amount per 1.0 L of trace metal solution
Nitrilotriacetic acid	2 g
MnSO ₄ · H ₂ O	1 g
Fe(SO ₄) ₂ (NH ₄) ₂ · 6H ₂ O	0.8 g
CoCl ₂ · 6H ₂ O	0.2 g
ZnSO ₄ · 7H ₂ O	0.2 mg
CuCl ₂ · 2H ₂ O	0.02 g
NaMoO ₄ · 2H ₂ O	0.02 g
Na ₂ SeO ₃	0.02 g
NiCl ₂ · 6H ₂ O	0.02 g
Na ₂ WO ₄ · 2H ₂ O	0.02 g

Wolfe's vitamin solution component	Amount per 1.0 L of Wolfe's vitamin solution
Biotin	2 mg
Folic acid	2 mg
Pyridoxine hydrochloride	10 mg
Thiamine HCl	5 mg
Riboflavin	5 mg
Nicotinic acid	5 mg
Calcium D-(+)-pantothenate	5 mg
Vitamin B12	0.1 mg

P-aminobenzoic acid	5 mg
Thioctic acid	5 mg

Reducing agent solution component	Amount per 100 mL of reducing agent solution
NaOH	0.9 g
Cysteine-HCl	4 g
Na ₂ S	4 g

Example 2

0122 This example demonstrates the construction of a strain comprising a p-hydroxybenzoate expression plasmid.

0123 The nucleotide sequence for chorismate pyruvate lyase (ubiC) (SEQ ID NO: 1) was optimized (SEQ ID NO: 2) according to the *C. autoethanogenum* codon-usage table by GeneArt and cloned into the pMTL8315 expression vector (Fig. 7) under control of the Wood-Ljungdahl pathway promoter (US 20110256600). The coding sequence for a feedback-insensitive mutant 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase (aroG*) (SEQ ID NO: 8) was also included, following ubiC in a bicistronic format (Fig. 7). The plasmid pARO_01 (SEQ ID NO: 9) was transformed into *C. autoethanogenum* LZ1561 (DSM23693) via conjugation with *E. coli* strain CA434 as donor. Donor strains were grown overnight in LB media supplemented with 25 µg/mL chloramphenicol and 100 µg/mL spectinomycin. Cells from 1.5 mL of culture were harvested by centrifugation and washed in phosphate buffered saline (PBS). Inside an anaerobic workstation, the donor cell pellet was resuspended in 200 µL of exponentially growing recipient LZ1561. The conjugation mixture was spotted on PETC-MES agar medium and incubated at 37 °C. After 24 hours the cells were scraped from the conjugation plate and spread on PETC-MES agar medium supplemented with 7.5 µg thiamphenicol/mL (Sigma) and 10 µg trimethoprim/mL (Sigma). Three plasmid-bearing colonies (i.e. biological triplicates) isolates were grown in PETC-MES liquid medium containing 7.5 µg thiamphenicol/mL and with a gas blend that simulates steel mill off gas as the carbon source (50 % CO, 10 % H₂, 30 % CO₂, 10 % N₂, subsequently referred to as “mill gas” in this application).

0124 Liquid cultures were grown in 10 mL PETC-MES medium in serum bottles containing thiamphenicol and mill gas at 22 psi. Samples were taken daily to measure biomass (Fig. 8) and pHBA (Fig. 9a and Fig. 9b).

0125 To measure pHBA, samples (100 μ L) were spiked with 10 μ L 0.1N NaOH, frozen, and then freeze dried. The samples were then derivatised with 100 μ L BSTFA + TCMS (99:1) and pyridine 100 μ L. The samples were then incubated at 60 °C for 30 min to form trimethyl silyl derivatives of the carboxylic acid functional group. Details of GC-MS method are: Inj. Vol. 1 μ L; Inj. T 250 °C; split ratio 10:1. Initial T 50 °C (hold 5 min); final T 220 °C (20°C/min); const. flow 1 mL/min (He carrier gas); column Zebron ZB-5MS 30m x 0.25mm x 0.25 μ m. Varian Ion Trap 4000 operated in full scan mode 40-400 m/z. Tune PFTBA

0126 In Fig. 9a, LZ1561 (the control strain) has three technical replicates (i.e., grown and sampled three times). Two biological replicates of LZ1561 with pARO_01, were also prepared, each with three technical replicates. “Technical replicate” refers to growing and sampling each strain in separate experiments, while “biological replicate” refers to reproducing the strain from scratch. In this way, the biological replicates account for background biological variation in the microorganism, while technical replicates account for variation due to technical aspects including culture, sampling, and analysis methods. Fig. 9a shows that pHBA was produced repeatedly in separate instances. Fig. 8 and Fig. 9b give an overall representation of growth and pHBA productivity.

Example 3

0127 This example demonstrates the production of p-hydroxybenzoate via gas fermentation.

0128 *C. autoethanogenum* harbouring plasmid pARO_01 (SEQ ID NO: 9) were grown on mill gas as described in Example 1. GC-MS analysis, performed as in Example 1, of the culture determined that pHBA was produced by the bacterium expressing chorismate pyruvate lyase. The linear range for analysis of pHBA using this method spanned 0-12.5 mg/mL (Fig. 5).

0129 pHBA was validated by comparison to retention time and characteristic fragment ions of an authentic pHBA standard and predicted characteristic ions from the NIST mass spectrometry database (Fig. 6).

0130 pHBA production was observed in all cultures expressing the chorismate-pyruvate lyase encoded on the pMTL8315 expression vector. The peak titre of pHBA observed in any one culture was 17 mg pHBA/L after eight days (Fig. 9b). No pHBA was observed in the control sample without the expression vector.

0131 Detectable levels of pHBA were produced by the genetically engineered bacterium and present in the culture.

Example 4

0132 This example demonstrates an experimental protocol for increasing the production of pHBA through enzyme engineering.

0133 UbiC is subject to product inhibition through retention of pHBA. The nucleic acid sequence encoding ubiC may be modified such that amino acids involved in product retention by the enzyme are mutated and release of product is enhanced. To do this, the amino acids involved in pHBA binding are identified by analysis of existing structures with bound product. Product inhibition is then minimised by mutating the amino acids involved in pHBA binding and retention. To identify enzymes with the greatest catalytic efficiency for pHBA yield, a targeted library of ubiC mutants can be produced where different combinations of pHBA-binding amino acids are altered, and these mutant enzymes can be analysed with an enzyme assay. Improved mutants are then expressed in *C. autoethanogenum* LZ1561 to validate the strains with most improved pHBA productivity.

Example 5

0134 This example demonstrates the construction of a strain comprising a salicylate expression plasmid.

0135 The nucleotide sequences for *pchA* (SEQ ID NO: 4) and *pchB* (SEQ ID NO: 6) were codon optimized and cloned into the expression vector under control of a tetracycline-inducible promoter. The plasmid is transformed into *C. autoethanogenum* LZ1561 (DSM23693) via conjugation with *E. coli* strain CA434 as donor. Donor strains were grown overnight in LB media supplemented with 25 µg/mL chloramphenicol and 100 µg/mL spectinomycin. Cells from 1.5 mL culture were harvested by centrifugation and washed in phosphate buffered saline (PBS). Inside an anaerobic workstation, the donor cell pellet was resuspended in 200 µL of exponentially growing recipient *C. autoethanogenum*. The conjugation mixture was spotted on PETC-MES agar medium and incubated at 37 °C. After 24 hours the cells were scraped from the conjugation plate and spread on PETC-MES agar medium supplemented with 7.5 µg thiamphenicol/mL (Sigma) and 10 µg trimethoprim/mL (Sigma). Three plasmid-bearing colonies (i.e. biological triplicates) isolates were grown in

PETC-MES liquid medium containing 7.5 µg thiamphenicol/mL and with mill gas as the carbon source.

0136 Liquid cultures were grown in 10 mL PETC-MES medium in serum bottles containing thiamphenicol and mill gas at 22 psi.

0137 Biomass was monitored spectrophotometrically. At OD₆₀₀ nm = 0.3 expression of the salicylate biosynthetic pathway was induced by addition of 40 ng anhydrotetracycline/mL. Duplicate cultures (technical replicates of the three biological triplicates) were grown without the addition of anhydrotetracycline such that the salicylate biosynthetic pathway remained uninduced. Samples were taken daily

0138 Salicylate concentrations were measured using gas chromatography mass spectrometry analysis (GCMS), employing a Thermo Scientific ISQ LT GCMS equipped an Agilent CP-SIL 5CB-MS (50 m × 0.25 µm × 0.25 µm) column and autosampler. Samples were prepared by diluting 300 µL of sample with 600 µL of acetonitrile and 50 µL 0.1N NaOH. The samples were vortexed then centrifuged for 3 minutes at 14,000 rpm; 800 µL of the supernatant was transferred to a glass vial and the sample was dried in a Thermo SpeedVac®. Once dry, the samples were then suspended in a solution of 100 µL of pyridine containing 22 mg/ml methoxyamine HCl then heated in a sealed glass vial for 60 minutes at 60°C. After which, 300 µL N,O-Bis(trifluoroacetamido) (BSTFA) was added then heated in a sealed glass vial for 60 minutes at 60°C. Samples were transferred to an autosampler for analysis using a 1.5 µL injection, a split ratio of 20 to 1, and an inlet temperature of 250°C. Chromatography was performed with an oven program of 80 °C (no hold) to a ramp of 3 °C/min to 140°C to a ramp of 20 °C/min to 230°C with a 4-min final hold. The column flow rate was 38 cm²/min, with helium as the carrier gas. The MS ion source was kept at 280 °C. Quantitation, was performed using 267 m/z for a quantification ion with 135 and 45 m/z used as qualifier ions.

0139 . Figure 11a shows a comparison of biomass growth in the induced and un-induced samples. Figure 11b shows that salicylate was produced repeatedly.

Example 6

0140 This example demonstrates knockout of pheA for enhanced production of chorismate-derived products.

0141 pheA (e.g. from *C. autoethanogenum*, CAETHG_0905 (CP006763.1:973789..974925)) is a gene that encodes the enzyme prephenate synthase. Prephenate synthase catalyses the conversion of chorismate to prephenate, which is a precursor to the aromatic amino acids phenylalanine and tyrosine. pheA function was knocked out by disrupting the gene using the ClosTron method (Heap et al., J Microbiol Methods. 2010, 80(1):49-55). The ClosTron plasmid pMTL007C-E2 was generated by DNA2.0 and transformed into *C. autoethanogenum* LZ1561 (DSM23693) via conjugation with *E. coli* strain CA434 as donor. Donor strains were grown overnight in LB media supplemented with 25 µg/mL chloramphenicol. Cells from 1.5 mL culture were harvested by centrifugation and washed in phosphate buffered saline (PBS). Inside an anaerobic workstation, the donor cell pellet was resuspended in 200 µL of exponentially growing recipient *C. autoethanogenum* LZ1561. The conjugation mixture was spotted on PETC agar media and incubated at 37 °C. After 24 hours the cells were scraped and resuspended in 500 µL PBS and spread on PETC agar media supplemented with 7.5 µg/mL thiamphenicol (Sigma) and 10 µg/mL trimethoprim (Sigma). Plasmid-bearing isolates were grown in PETC-MES liquid medium containing 7.5 µg thiamphenicol/mL and with mill gas as the carbon source.

0142 Colonies were streaked on PETC solid media containing the antibiotic clarithromycin (5 µg/mL). This step selected for integration of the intron retargeting sequence into the genome. Integration of the intron sequence into the target site results in an 1800 base pair insertion in the genome, which was screened for with colony PCR. The PCR product of the positive ClosTron mutants were purified and sequenced to confirm the insertion site.

0143 Liquid cultures were grown in 10 mL PETC-MES medium in serum bottles containing clarithromycin and mill gas at 22 psi. Glycerol stock was prepared from this serum bottle

0144 Bioreactor experiments were carried out in a 2 L BioFlo 115 water jacket system (New Brunswick Scientific Corp., Edison, NJ) with a working volume of 1.5 L. The CSTR system was equipped with two six-bladed Rushton impellers and baffles enhance the mixing of fermentation broth and the gas to liquid mass transfer. A pH and an oxidation-reduction potential (ORP) electrode (Broadley-James Corporation) were inserted through the headplate and their readings were recorded at 5 min intervals. pH was maintained at 5.0 by automated addition of a 5 M solution of ammonium hydroxide.

0145 The inoculum was prepared from a glycerol stock. One mL of glycerol stock was transferred into 50 mL of PETC media with 22 psi mill gas as carbon source. The culture was incubated at 37 °C for on a shaker two to three days until a visible growth was observed. The culture was then used to inoculate 200 mL of fresh media in 1L-Schott bottle and mill gas was added to a pressure of 22 psi. The Schott bottle was incubated for another 24 to 36 hours before being transferring to the fermenters.

0146 The agitation was set at 200 rpm and the gas flow was set at 35 mL/min/L. After one day, the stirring rate was increased by 25 rpm at 4 hours intervals to the maximum value of 900 rpm. The gas flow was increased by 25 mL/min/L at 4 hours intervals to the maximum flow rate that the target CO uptake can be achieved. The Na₂S was added over the course of the fermentation with an initial pump rate of 0.3 mL/h and later increased in 0.2 mL/h increments when the H₂S concentration in the headspace dropped below 200~ppm. The CO and H₂ consumption and CO₂ production along with the H₂S concentration were measured hourly using gas chromatography (GC). Liquid samples were taken from the fermenter at regular intervals over the course of the fermentation to determine cell mass and metabolite concentrations using HPLC.

0147 After starting up in batch mode, the fermenter was turned to continuous when the OD reached a value of 2. The media and nutrient inflow rates were controlled by one or more precision peristaltic pumps (Masterflex L/S digital drive pumps) while the fermenter volume was held constant by using a level probe that triggers a pump to remove fermentation broth from the CSTR. The dilution rate was set in one step to 0.5 day⁻¹ and further increased to 1 day⁻¹ then to 1.7 day⁻¹ at 24 hour intervals.

0148 An additional equipment was added to the fermentation was a hollow fibre membrane (GE Healthcare) with a pore size of 0.2 µm and a surface area of 1,200 cm². The membrane was used to increase the cell concentration in the fermentation. The fermentation broth was pumped at high speed through the membrane and returned back to the fermenter while a stream of cell-free filtrate was pumped to the filtrate tank at a slower rate than the media pump rate. This allowed the retention time of the bacteria cell in the fermenter to increase.

0149 As shown in Fig. 10, three new compounds were identified using GC-MS. These compounds were cis-4-hydroxycyclohexane carboxylic acid, 3,4-dihydroxybenzoic acid, and 2-aminobenzoic acid. These compounds were only detected in this pheA::CT culture and were not detected in the parental strain (LZ1561) culture.

0150 3,4 dihydroxy benzoic acid, 2-aminobenzoic acid and cis-4-hydroxycyclohexanecarboxylic acid concentrations were measured using gas chromatography (GC) analysis, employing an Agilent 6890N GC equipped a Agilent CP-SIL 5CB-MS (50 m × 0.25 µm × 0.25 µm) column, autosampler and a flame ionization detector (FID). Samples were prepared by diluting 400 µL of sample with 400 µL of acetonitrile, followed by a 3 minute centrifugation at 14,000 rpm; the supernatant was transferred to a glass vial and the sample was dried in a Thermo SpeedVac®. Once dry, the samples were then suspended in a solution of 400 µL of N,O-Bistrifluoroacetamide (BSTFA) and pyridine (3:1 ratio) and heated in a sealed glass vial for 60 minutes at 60°C. Samples were transferred to an autosampler for analysis using a 1 µL injection, a split ratio of 30 to 1, and an inlet temperature of 250°C. Chromatography was performed with an oven program of 70 °C (no hold) to a ramp of 3 °C/min to 110°C to a ramp of 15 °C/min to 230°C, followed by a final ramp of 40°C/min to 310°C with a 3-min hold. The column flow rate was 1.8 ml/min, with helium as the carrier gas. The FID was kept at 320 °C, with hydrogen at 40 ml/min, air at 400 ml/min, and helium at 20 ml/min as the makeup gas.

0151 Figure 12 shows the concentration of cis-4-hydroxycyclohexane carboxylic acid, 3,4-dihydroxybenzoic acid, and 2-aminobenzoic acid over the course of the fermentation run. As shown in Figure 12, compound cis-4-hydroxycyclohexanecarboxylic acid increased to a concentration of about 0.9g/L on day 6 of the fermentation. 2-aminobenzoic acid accumulated to a concentration of about 0.45g/L on day 8-9 of the fermentation. 3,4-dihydroxybenzoic acid was produced in smaller amounts, peaking at a concentration of around 0.3g/L between days 6-8. A total accumulation of cis-4-hydroxycyclohexanecarboxylic acid, 2-aminobenzoic acid and 3,4-dihydroxybenzoic acid of >1.3 g/L was observed on day 6.

0152 Little is known in literature about the production of cis-4-hydroxycyclohexanecarboxylic acid. There is only one report that cis-4-hydroxycyclohexanecarboxylic acid was detected in a child's urine sample using GC-MS. It was hypothesized that the compound was a by-product of enteric bacterial metabolism (Kronick, *Clinica Chimica Acta*, 132: 205-208, 1983). It seems likely that this compound is a direct product of chorismate or prephanate as the reaction mechanism may be explained by a cleavage of the pyruvate molecule followed by a reduction requiring a further 2.5 H₂ molecules that may be provided through NAD(P)H.

0153 2-Aminobenzoic acid is a known intermediate in the chorismate to tryptophan pathway. Anthranilate synthase catalyses the amination followed by the aromatization of chorismate to obtain the aromatic backbone of the tryptophan molecule. It is known that the gene expression of anthranilate synthase is highly regulated and subjected to feedback inhibition by the end product tryptophan (Dosselaere, *Crit Rev Microbiol*, 27: 75-131, 2001). 2-Aminobenzoic acid was only secreted into the fermentation broth when growth ceased indicating that it is an overflow product that was no longer reacted away when growth had stopped.

0154 All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein. The reference to any prior art in this specification is not, and should not be taken as, an acknowledgement that that prior art forms part of the common general knowledge in the field of endeavour in any country.

0155 The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

0156 Preferred embodiments of this invention are described herein. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such

variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

CLAIMS

1. A genetically engineered C1-fixing bacterium capable of producing at least one chorismate-derived product, wherein the bacterium comprises at least one of:
 - a. an exogenous chorismate pyruvate lyase (EC 4.1.3.40),
 - b. an exogenous isochorismate synthase (EC 5.4.4.2),
 - c. an exogenous isochorismate pyruvate lyase (EC 4.2.99.21), and
 - d. a prephenate synthase (EC 5.4.99.5) comprising a disruptive mutation, wherein the disruptive mutation inactivates, deletes, or knocks out the prephenate synthase.
2. The bacterium of claim 1, wherein the bacterium is a *Clostridium* bacterium capable of producing at least one chorismate-derived product by fermentation of a gaseous substrate.
3. The bacterium of claim 1, wherein the chorismate pyruvate lyase is ubiC.
4. The bacterium of claim 1, wherein the isochorismate synthase is pchA.
5. The bacterium of claim 1, wherein the isochorismate pyruvate lyase is pchB.
6. The bacterium of claim 1, wherein the prephenate synthase is pheA.
7. The bacterium of claim 1, wherein the disruptive mutation reduces or eliminates the expression or activity of the prephenate synthase.
8. The bacterium of claim 7, wherein the bacterium produces a reduced amount of prephenate or prephenate-derived products compared to a parental bacterium.
9. The bacterium of claim 7, wherein the bacterium produces substantially no tyrosine or phenylalanine.
10. The bacterium of claim 1, wherein the bacterium comprises at least one nucleic acid encoding at least one of:
 - a. the exogenous chorismate pyruvate lyase,
 - b. the exogenous isochorismate synthase,
 - c. the exogenous isochorismate pyruvate lyase, and
 - d. the prephenate synthase comprising a disruptive mutation.
11. The bacterium of claim 10, wherein the nucleic acid is codon optimized for expression in *Clostridium*.

12. The bacterium of claim 1, wherein the chorismate-derived product comprises a 6-membered carbon ring substituted with a carboxyl group or carboxylate anion and further substituted with one or more OH groups and/or one or more NH₂ groups.
13. The bacterium of claim 1, wherein the chorismate-derived product is selected from the group consisting of para-hydroxybenzoic acid, salicylate, 2-aminobenzoate, dihydroxybenzoate, 4-hydroxycyclohexane carboxylic acid, and salts and ions thereof.
14. The bacterium of claim 1, wherein the bacterium expresses a chorismate pyruvate lyase of ubiC and a feedback-insensitive DAHP synthase and produces a chorismate-derived product of para-hydroxybenzoic acid.
15. The bacterium of claim 1, wherein the bacterium expresses an isochorismate synthase of pchA and an isochorismate pyruvate lyase of pchB and produces a chorismate-derived product of salicylate.
16. The bacterium of claim 15, wherein the bacterium further expresses a feedback-insensitive DAHP synthase.
17. The bacterium of claim 1, wherein the bacterium comprises a prephenate synthase comprising a disruptive mutation and produces a chorismate-derived product of 2-aminobenzoate, 2,3-dihydroxybenzoate, or 4-hydroxycyclohexane carboxylic acid.
18. The bacterium of claim 1, wherein the bacterium produces at least one chorismate-derived product not produced by a parental bacterium.
19. The bacterium of claim 1, wherein the bacterium produces a greater amount of at least one chorismate-derived product than a parental bacterium.
20. The bacterium of claim 1, wherein the bacterium is derived from a parental bacterium selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei*.
21. The bacterium of claim 20, wherein the *Clostridium autoethanogenum* is *Clostridium autoethanogenum* DSM23693.
22. The bacterium of claim 2, wherein the gaseous substrate comprises at least one of CO, CO₂, and H₂.

23. A method of producing a fermentation product, comprising fermenting the bacterium of claim 1 in the presence of a gaseous substrate to produce a fermentation product.
24. The method of claim 23, wherein the gaseous substrate comprises at least one of CO, CO₂, and H₂.