

RECOMBINANT MICROORGANISMS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Serial No. 63/051,672, filed July 14, 2020, the disclosures of which are incorporated herein by reference.

TECHNICAL FIELD

[0002] Metabolically-modified microorganisms and methods of producing such organisms are provided.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

[0003] Accompanying this filing is a Sequence Listing entitled "Sequence-Listing_ST25.txt", created on July 14, 2021 and having 110,913 bytes of data, machine formatted on IBM-PC, MS-Windows operating system. The sequence listing is hereby incorporated herein by reference in its entirety for all purposes.

BACKGROUND

[0004] Methanol, being electron-rich and derivable from methane or CO₂, is a potentially renewable one-carbon (C1) feedstock for microorganisms. Although the ribulose monophosphate (RuMP) cycle used by methylotrophs to assimilate methanol differs from the typical sugar metabolism by only three enzymes, turning a non-methylotrophic organism to a synthetic methylotroph that grows to a high cell density has been challenging.

SUMMARY

[0004a] In one aspect, the present disclosure provides a synthetic methylotroph (SM) that grows on methanol as a sole carbon source and has a doubling time (tD) of less than 12 hours.

[0004b] In another aspect, the present disclosure provides a method for producing a metabolite, comprising growing a SM as disclosed herein in a medium comprising methanol, wherein the methanol is the only carbon source for the SM microbe, whereby the metabolite is produced.

[0004c] In another aspect, the present disclosure provides a synthetic methylotroph that grows on methanol and has a doubling time (tD) of less than 12 hours and expresses: a polypeptide having methanol dehydrogenase activity, a polypeptide having

hexulose-6-phosphate synthase activity, a polypeptide having hexulose-6-phosphate isomerase activity and comprises increased activity of a polypeptide having phosphoglucoisomerase activity.

[0004d] In another aspect, the present disclosure provides a synthetic methylotroph that assimilates a C1 carbon source, has a double time (tD) of less than 12 hours and comprises a plurality of enzymes selected from the group consisting of Medh, Hps, Phi, Pgi, RpiA, Tkt, Tal and any combination thereof.

[0004e] In another aspect, the present disclosure provides a synthetic methylotroph that has a doubling time (tD) of less than 12 hours and expresses one or more heterologous polynucleotide or over-expression of one or more heterologous polynucleotide encoding a polypeptide having methanol dehydrogenase activity, hexulose-6-phosphate synthase activity, 6-phospho-3-hexulose isomerase activity, glucose phosphate isomerase activity and ribose-phosphate isomerase A activity, with a concomitant reduction or elimination of glyceraldehyde-3-phosphate dehydrogenase activity, reduction or elimination of S-(hydroxymethyl)glutathione dehydrogenase (FrmA) activity, reduction or deletion of phosphocarrier protein HPr (also referred to as Histidine-containing protein, HPr and/or PtsH) activity, and the reduction or elimination of ProQ provides, wherein the microorganism grows on methanol.

[0005] The disclosure provides a synthetic methylotroph (SM) that grows on methanol as the sole carbon source, has a doubling time (tD) of about 12 hours or less. In another example, the SM has a methanol tolerance of ~1.2 M (e.g., from about 50 mM to about 1.2M). In one example, the SM expresses a polypeptide having methanol dehydrogenase activity, a polypeptide having hexulose-6-phosphate synthase activity, a polypeptide having 3-hexulose-6-phosphate isomerase (sometimes referred to as 6-phospho-3-hexuloisomerase) activity and comprises increased activity of a polypeptide having phosphoglucoisomerase activity, wherein the SM can grow on methanol up to ~1.2M (e.g., 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 1M, 1.1M, 1.2M, 1.3M, 1.4M or a value between any two of the

foregoing values). In another or further example, the SM contains a deletion or reduction in the expression or activity of a glyceraldehyde dehydrogenase A polypeptide, S-(hydroxymethyl) glutathione dehydrogenase A polypeptide, phosphofructokinase polypeptide, histidine-containing protein, and/or a proQ polypeptide. In yet another or further example, the SM has an increased in copy number variation of 2 to 85 of a region between *yggE* to *yghO*, *rrsA* to *rrlB*, and/or *ygiG* to *smf*. In still yet another or further example, the SM is obtained by engineering a parental microorganism selected from the group consisting of *Escherichia*, *Bacillus*, *Clostridium*, *Enterobacter*, *Klebsiella*, *Enterobacteria*, *Mannheimia*, *Pseudomonas*, *Acinetobacter*, *Shewanella*, *Ralstonia*, *Geobacter*, *Zymomonas*, *Acetobacter*, *Geobacillus*, *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Corynebacterium*, *Streptomyces*, *Propionibacterium*, *Synechocystis*, *Synechococcus*, *Cyanobacteria*, *Chlorobi*, *Deinococcus* and *Saccharomyces* sp. In a further example, the parental microorganism is *E. coli*. In yet another or further example, the SM further expresses a ribose-5-phosphate isomerase A. In another example, the SM comprises the genetic make up of ATCC deposit accession number.

[0006] In yet another aspect, the present disclosure provides a synthetic methylotroph designated *Escherichia Coli* SM1 having ATCC accession no. PTA-126783. The disclosure further provides progeny and cultures of the microorganism having accession no. PTA-126783.

[0007] The disclosure provides a method for producing a metabolite, comprising growing a SM of any of the foregoing examples in a medium comprising methanol, whereby the metabolite is produced. In a further example, the metabolite is selected from the group consisting of 4-carbon chemicals, diacids, 3-carbon chemicals, higher carboxylic acids, alcohols of higher carboxylic acids, carotenoids, isoprenoids, cannabinoids and polyhydroxyalkanoates.

[0008] The disclosure provides a recombinant microorganism that assimilates a C1 carbon source and comprises a plurality of enzymes selected from the group consisting of Medh, Hps, Phi, Pgi, RpiA, Tkt, Tal and any combination thereof. In one example, the microorganism is obtained by engineering a parental microorganism of

the species *E. coli*. In a further example, the recombinant microorganism comprises a reduction or knockout of a gene selected from the group consisting of *pfkA*, *gapA*, *frmA*, *ptsH*, *proQ* and any combination thereof. In a further example, of any of the foregoing, the recombinant microorganism comprises an increased copy number of a region of the genome.

[0009] The disclosure provides a recombinant microorganism that expresses one or more heterologous polynucleotide or over-expression of one or more heterologous polynucleotide encoding a polypeptide having methanol dehydrogenase activity, hexulose-6-phosphate synthase activity, 6-phospho-3-hexulose isomerase activity, glucose phosphate isomerase activity and ribose-phosphate isomerase A activity, with a concomitant reduction or elimination of glyceraldehyde-3-phosphate dehydrogenase activity, reduction or elimination of S-(hydroxymethyl)glutathione dehydrogenase (FrmA) activity, reduction or deletion of phosphocarrier protein HPr (also referred to as Histidine-containing protein, HPr and/or PtsH) activity, and the reduction or elimination of ProQ provides, wherein the microorganism grows on methanol.

[0010] The disclosure also provides a recombinant microorganism that grows on methanol and comprises the metabolic pathway of Figure 1A.

[0011] The details of one or more examples of the disclosure are set forth in the accompanying drawings and the description below. Other features, objects, and advantages will be apparent from the description and drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] The accompanying drawings, which are incorporated into and constitute a part of this specification, illustrate one or more embodiments of the disclosure and, together with the detailed description, serve to explain the principles and implementations of the invention.

[0013] **Figure 1A-B** presents the build and evolution a synthetic methylotrophic *E. coli* strain. **(A)** Pathway and mutations relevant to synthetic methylotrophic *E. coli* SM1. The cog icons represent rationally designed and engineered gene modifications. Solid boxes indicate up-regulated or high copy number genes, while dashed boxes

WHAT IS CLAIMED IS:

1. A synthetic methylotroph (SM) that grows on methanol as a sole carbon source and has a doubling time (t_D) of less than 12 hours.
2. The SM of claim 1, wherein the SM expresses:
 - a polypeptide having methanol dehydrogenase activity,
 - a polypeptide having hexulose-6-phosphate synthase activity,
 - a polypeptide having 3-hexulose-6-phosphate isomerase activityand
 - comprises increased activity of a polypeptide having phosphoglucoisomerase activity,
 - wherein the SM can grow on methanol as the sole carbon source.
3. The SM of claim 1 or 2, wherein the SM contains deletions or reductions in expression or activity of one or more of a glyceraldehyde dehydrogenase polypeptide, phosphofructokinase polypeptide, S-(hydroxymethyl) glutathione dehydrogenase polypeptide, histidine-containing protein, and/or a ProQ polypeptide.
4. The SM of claim 1, 2 or 3, wherein the SM has increased copy number variation of any region within the SM's genome.
5. The SM of claim 1, 2, 3 or 4, wherein the SM has an increase of one or more copy number variations of 2 to 85 of a region between *yggE* to *yghO*, *rrsA* to *rrlB*, and/or *ygiG* to *smf*, and/or *osmC* to *dosP*.
6. The SM of any one of claims 1-5, wherein the SM is obtained by engineering a parental microorganism selected from the group consisting of *Escherichia*, *Bacillus*, *Clostridium*, *Enterobacter*, *Klebsiella*, *Enterobacteria*, *Mannheimia*, *Pseudomonas*, *Acinetobacter*, *Shewanella*, *Ralstonia*, *Geobacter*, *Zymomonas*, *Acetobacter*, *Geobacillus*, *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Corynebacterium*, *Streptomyces*, *Propionibacterium*, *Synechocystis*, *Synechococcus*, *Cyanobacteria*, *Chlorobi*, *Deinococcus* and *Saccharomyces sp.*
7. The SM of claim 6, wherein the parental microorganism is *E. coli*.

8. The SM of any one of the foregoing claims, wherein the SM further expresses a ribose-5-phosphate isomerase A.
9. The SM of any one of claims 1-7, having the doubling time and product profile of ATCC deposit accession number PTA-126783, when grown on methanol.
10. A synthetic methylotroph designated *Escherichia coli* SM1 having ATCC accession no. PTA-126783.
11. A method for producing a metabolite, comprising growing a SM of any one of claims 1-10 in a medium comprising methanol, wherein the methanol is the only carbon source for the SM microbe, whereby the metabolite is produced.
12. The method of claim 11, wherein the metabolite is selected from the group consisting of 4-carbon chemicals, diacids, 3-carbon chemicals, higher carboxylic acids, alcohols of higher carboxylic acids, carotenoids, cannabinoids, isoprenoids, and polyhydroxyalkanoates.
13. The method of claim 11, wherein the metabolite is selected from the group consisting of succinate, ethanol, and n-butanol.
14. A synthetic methylotroph that grows on methanol and has a doubling time (tD) of less than 12 hours and expresses:
a polypeptide having methanol dehydrogenase activity,
a polypeptide having hexulose-6-phosphate synthase activity,
a polypeptide having hexulose-6-phosphate isomerase activity
and
comprises increased activity of a polypeptide having phosphoglucoisomerase activity.
15. A synthetic methylotroph that assimilates a C1 carbon source, has a double time (tD) of less than 12 hours and comprises a plurality of enzymes selected from the group consisting of Medh, Hps, Phi, Pgi, RpiA, Tkt, Tal and any combination thereof.
16. The synthetic methylotroph of claim 15, wherein the microorganism is *E. coli*.

17. The synthetic methylotroph of claim 15, further comprising a reduction or knockout of a gene selected from the group consisting of *pfkA*, *gapA*, *frmA*, *ptsH*, *proQ* and any combination thereof.

18. The synthetic methylotroph of claim 15, further comprising an amplified region of the genome.

19. A synthetic methylotroph that has a doubling time (tD) of less than 12 hours and expresses one or more heterologous polynucleotide or over-expression of one or more heterologous polynucleotide encoding a polypeptide having methanol dehydrogenase activity, hexulose-6-phosphate synthase activity, 6-phospho-3-hexulose isomerase activity, glucose phosphate isomerase activity and ribose-phosphate isomerase A activity, with a concomitant reduction or elimination of glyceraldehyde-3-phosphate dehydrogenase activity, reduction or elimination of S-(hydroxymethyl)glutathione dehydrogenase (FrmA) activity, reduction or deletion of phosphocarrier protein HPr (also referred to as Histidine-containing protein, HPr and/or PtsH) activity, and the reduction or elimination of ProQ provides, wherein the microorganism grows on methanol.