

propanediol, etc. Therefore, the fusions can be expressed in cells that naturally have such product pathways, or cells engineered to express the fusion proteins can further be engineered to include target product pathway enzymes that use the increased amounts of intermediate products available when the fusion proteins of the disclosure are expressed.

5 The fusion proteins of the disclosure improve the ability of a microorganism to grow on methanol as a carbon source. The improved growth on methanol using methanol pathway fusions is likely due to several benefits the fusions provide: 1) rapid conversion of formaldehyde, a potentially toxic product of the MeDH reaction, to hexulose-6-phosphate (or alternatively to DHA and G3P) due to the localized presence of fused HPS; 2) increased
10 efficiency of the fusion protein(s) in the methanol pathway reduces the amount of total methanol pathway protein required for efficient methanol utilization; 3) the presence of methanol pathway fusion proteins pushes the reaction in the forward, desired direction with respect to methanol utilization.

 In one aspect, the disclosure provides a fusion protein comprising: (5a) a first region
15 comprising methanol dehydrogenase or an enzymatically active portion thereof, (5b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and (5c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; or (6a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (6b) a second region comprising 3-hexulose-6-phosphate
20 dehydrogenase or an enzymatically active portion thereof; (6c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; and (6d) a fourth region comprising methanol dehydrogenase activator or an enzymatically active portion thereof; wherein the fusion protein optionally comprises one or more linker amino acid

sequence(s) positioned between two or more of the first, second, and third regions of the fusion protein.

5 In another aspect, the disclosure provides a fusion protein comprising: first and second portions of a methanol dehydrogenase, 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof, and 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof, and optionally methanol dehydrogenase activator or an enzymatically active portion thereof, positioned between the first and second portions of a methanol dehydrogenase.

10 In yet another aspect, the disclosure provides a nucleic acid encoding the fusion protein as described herein.

In yet another aspect, the disclosure provides an expression construct comprising the nucleic acid as described herein.

In yet another aspect, the disclosure provides an engineered cell comprising the fusion protein as described herein, the nucleic acid as described herein, or the expression construct as described herein, or an engineered cell that heterologously expresses methanol dehydrogenase, 3-hexulose-6-phosphate dehydrogenase, and 6-phospho-3-hexuloisomerase, wherein heterologous expression includes expression of a fusion protein comprising (1a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, and (1b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; (2a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (2b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and (2c) a third region comprising methanol dehydrogenase activator or an enzymatically active portion thereof; (3a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, and (3b) a second region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; (4a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (4b) a second region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; and (4c) a third region comprising methanol dehydrogenase activator or an enzymatically active portion thereof; (5a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (5b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and (5c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; (6a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (6b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; (6c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; and (6d) a fourth region comprising methanol dehydrogenase activator or an enzymatically active portion thereof; or (7a) first and second portions of a methanol dehydrogenase, and 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof, and/or 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof, and optionally methanol dehydrogenase activator or an enzymatically active portion thereof, positioned between the first and second portions of a methanol dehydrogenase.

In yet another aspect, the disclosure provides an engineered cell comprising (i) a fusion protein comprising a first region comprising methanol dehydrogenase and a second region comprising methanol dehydrogenase activator, or enzymatically active portions thereof, and (ii) and a fusion protein comprising a first region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and a second region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof.

In yet another aspect, the disclosure provides a composition comprising the engineered cell as described herein.

In yet another aspect, the disclosure provides a method of producing a target product or its intermediate comprising culturing the engineered cell as disclosed herein in a culture medium comprising methanol to produce the target product or an intermediate of the target product.

What is claimed is:

1. A fusion protein comprising:

(5a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (5b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and (5c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; or

(6a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (6b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; (6c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; and (6d) a fourth region comprising methanol dehydrogenase activator or an enzymatically active portion thereof;

wherein the fusion protein optionally comprises one or more linker amino acid sequence(s) positioned between two or more of the first, second, and third regions of the fusion protein.

2. A fusion protein comprising:

first and second portions of a methanol dehydrogenase, 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof, and 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof, and optionally methanol dehydrogenase activator or an enzymatically active portion thereof, positioned between the first and second portions of a methanol dehydrogenase.

3. The fusion protein of claim 1 having a structure as follows:

(v) 5a-L¹-5b-L²-5c, 5a-L¹-5c-L²-5b, 5b-L¹-5a-L²-5c, 5b-L¹-5c-L²-5a, 5c-L¹-5a-L²-5b, or 5c-L¹-5b-L²-5a; or

(vi) 6a-L¹-6b-L²-6c-L³-6d, 6a-L¹-6b-L²-6d-L³-6c, 6a-L¹-6c-L²-6b-L³-6d, 6a-L¹-6c-L²-6d-L³-6b, 6a-L¹-6d-L²-6b-L³-6c, 6a-L¹-6d-L²-6c-L³-6b, 6b-L¹-6a-L²-6c-L³-6d, 6b-L¹-6a-L²-6c-L³-6d, 6b-L¹-6c-L²-6a-L³-6d, 6b-L¹-6c-L²-6d-L³-6a, 6b-L¹-6d-L²-6a-L³-6c, 6b-L¹-6d-L²-6c-L³-6a, 6c-L¹-6a-L²-6b-L³-6d, 6c-L¹-6a-L²-6d-L³-6b, 6c-L¹-6b-L²-6a-L³-6d, 6c-L¹-6b-L²-6d-L³-6a, 6c-L¹-6d-L²-6a-L³-6b, 6c-L¹-6d-L²-6b-L³-6a, 6d-L¹-6a-L²-6b-L³-6c, 6d-L¹-6a-L²-6c-L³-6b, 6d-L¹-6b-L²-6a-L³-6c, 6d-L¹-6b-L²-6c-L³-6a, 6d-L¹-6c-L²-6b-L³-6a, and 6d-L¹-6c-L²-6a-L³-6b;

wherein L¹, L², and L³ are independently selected from (-) (a covalent bond) and a linker amino acid sequence.

4. The fusion protein of claim 1, 2 or 3 wherein one or more of the methanol dehydrogenase, methanol dehydrogenase activator, the 3-hexulose-6-phosphate dehydrogenase, the 6-phospho-3-hexuloisomerase, or enzymatically active portions thereof, is from bacteria.
- 5 5. The fusion protein of claim 4 wherein one or more of the methanol dehydrogenase, the methanol dehydrogenase activator, the 3-hexulose-6-phosphate dehydrogenase, the 6-phospho-3-hexuloisomerase, or enzymatically active portions thereof, is from *Bacillus*.
6. The fusion protein of claim 5 wherein one or more of the methanol dehydrogenase, the
10 methanol dehydrogenase activator, the 3-hexulose-6-phosphate dehydrogenase, the 6-phospho-3-hexuloisomerase, or enzymatically active portions thereof, is from *Bacillus methanolicus* MGA3 or *Bacillus methanolicus* PBI.
7. The fusion protein of claim 1, 2 or 3 wherein the methanol dehydrogenase (MeDH) or an
15 enzymatically active portion thereof is from *Bacillus methanolicus* MGA3 MeDH (3EIJ77596.1); *Bacillus methanolicus* C1 MeDH (AAA22593.1); *Bacillus methanolicus* PB1 MeDH (EIJ77618.1); *Bacillus methanolicus* PB1 MeDH (EIJ78790.1); *Bacillus methanolicus* MGA3 MeDH (EIJ80770.1); *Bacillus methanolicus* PB1 MeDH (EIJ78397.1); *Bacillus methanolicus* MGA3 MeDH (EIJ83020.1); *Lysinibacillus fusiformis* MeDH (EFI69743.1);
20 *Bacillus coagulans* 36D1 MeDH (YP_004860127.1); *Lysinibacillus sphaericus* MeDH (YP_001699778.1); *Bacillus azotoformans* LMG 9581 MeDH (ZP_11313277.1); *Burkholderia thailandensis* E264 MeDH (ZP_05587334.1); *Cupriavidus necator* N-1 MeDH (YP_004681552.1); uncultured organism MeDH (AGF87161); *Geobacter bemidjiensis* Bem MeDH (YP_002138168.1); *Carboxydotherrmus hydrogenoformans* Z-2901 MeDH (YP_359772.1); *Actinobacillus succinogenes* 130Z MeDH (YP_001343716.1); *Acinetobacter baumannii* Naval-82 MeDH (ZP_16224338.1); *Clostridium pasteurianum* DSM 525 MeDH (AAC45651.1); *Methanosarcina mazei* Tuc01 MeDH (YP_007491369.1); *Desulfovibrio vulgaris* str. 'Miyazaki F' MeDH (YP_002434746); *Desulfovibrio africanus* str. Walvis Bay MeDH (YP_005052855); *Clostridium perfringens* str. 13 MeDH (NP_561852.1); *Vibrio campbellii* ATCC BAA-1116 MeDH (YP_001447544); *Desulfotomaculum reducens* MI-1 MeDH (YP_001113612.1); *Desulfovibrio vulgaris* str. Hildenborough MeDH (YP_011618); *Photobacterium profundum* 3TCK MeDH (ZP_01220157.1); *Geobacillus* sp. Y4.1MC1 MeDH (YP_003990729.1); *Desulfovibrio fructosovorans* JJ MeDH (ZP_07335453.1); *Shewanella oneidensis* MR-1 MeDH (NP_717107); *Sealdella termitidis* ATCC 33386 MeDH
30 (YP_003310546.1); *Paenibacillus peoriae* KCTC 3763 MeDH (ZP_10241531.1); *Klebsiella*

- pneumoniae* subsp. *pneumoniae* MGH 78578 MeDH (YP_001337153.1); *Escherichia coli* MeDH (YP_026233.1); *Clostridium perfringens* ATCC 13124 MeDH (YP_694908); *Ralstonia eutropha* H16 MeDH (YP_725376.1); *Thermoanaerobacter* sp. X514 MeDH (YP_001663549); human gut metagenome MeDH (EKC54576); or *Geobacillus* 5 *themodenitrificans* NG80-2 MeDH (YP_001126968.1).
8. The fusion protein of claim 1, 2 or 3 wherein the methanol dehydrogenase or an enzymatically active portion thereof is a polypeptide having 50% or greater, 60% or greater, 70% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater, 96% or 10 greater, 97% or greater, 98% or greater, or 99% or greater identity to *Bacillus methanolicus* MGA3MeDH (3EIJ77596.1) or to any other MeDH according to claim 7.
9. The fusion protein of claim 8 comprising one or more amino acid substitutions that differ from a native sequence of the methanol dehydrogenase, 3-hexulose-6-phosphate 15 dehydrogenase, 6-phospho-3-hexuloisomerase, or methanol dehydrogenase activator.
10. The fusion protein of claim 1, 2 or 3 wherein the region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof is from *Bacillus methanolicus* MGA3 HPS (AAR39392.1); *Bacillus methanolicus* PB1 HPS (EIJ81375.1); 20 *Methylobacillus flagellatus* HPS (YP 544362.1); *Methylobacillus flagellatus* HPS (YP 544362.1); *Bacillus subtilis* HPS (NP_388228.1); *Methylophilus methylotrophus* HPS (WP_018986666.1); or *Methylophilus methylotrophus* ATCC 53528 HPS (WP_018985298.1).
11. The fusion protein of claim 1, 2 or 3, wherein 3-hexulose-6-phosphate dehydrogenase or an 25 enzymatically active portion thereof is a polypeptide having 50% or greater, 60% or greater, 70% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater, 96% or greater, 97% or greater, 98% or greater, or 99% or greater identity to *Bacillus methanolicus* MGA3 HPS (AAR39392.1) or to any other HPS according to claim 10.
- 30 12. The fusion protein of claim 1, 2 or 3, wherein 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof is from *Bacillus methanolicus* MGA3 PHI (AAR39393.1); *Bacillus methanolicus* PB1 PHI (EIJ81376.1); *Mycobacterium gastri* PB1 PHI (BAA90545.1); *Methylobacillus flagellatus* KT PHI (YP 545762.1); *Bacillus subtilis* PHI (NP_388227.1); or *Methylophilus methylotrophus* ATCC 53528 HPS (WP_018985297.1).

13. The fusion protein of claim 1, 2 or 3, wherein the region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof is a polypeptide having 50% or greater, 60% or greater, 70% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater, 96% or greater, 97% or greater, 98% or greater, or 99% or greater identity to
5 *Bacillus methanolicus* MGA3 PHI (AAR39393.1) or to any other PHI according to claim 12;.
14. The fusion protein of claim 1, 2 or 3, wherein the region comprising the methanol dehydrogenase activator or an enzymatically active portion thereof is a polypeptide having 50% or greater, 60% or greater, 70% or greater, 80% or greater, 85% or greater, 90% or
10 greater, 95% or greater, 96% or greater, 97% or greater, 98% or greater, or 99% or greater identity to *Bacillus methanolicus* MGA3 ACT (Q8KP10) or a paralog or ortholog of MGA3ACT.
15. The fusion protein of any one of the previous claims having one linker amino acid
15 sequence.
16. The fusion protein of any of claims 1-15 having two linker amino acid sequences, wherein the two linker amino acid sequences are the same or different.
- 20 17. The fusion protein of any one of the previous claims, wherein the linker amino acid sequence comprises one or more amino acids selected from the group consisting of glycine, alanine, serine, and threonine.
18. The fusion protein of any one of the previous claims, wherein the linker amino acid
25 sequence has a length in the range of 1-50 amino acid residues, or 1-20 amino acid residues.
19. The fusion protein of any one of the previous claims, wherein the linker amino acid sequence is $[(\text{GGS})_x(\text{GS})_y(\text{GGS})_z]_n$ where x is 1-6, y is 1-6, z is 0-3, and n is 1-4, preferably where x is 1-3, y is 1-3, z is 1-2, and n is 1-2.
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20. A nucleic acid encoding the fusion protein of any of the previous claims.
21. An expression construct comprising the nucleic acid of claim 20.

22. An engineered cell comprising the fusion protein of any one of claims 1-19, the nucleic acid of claim 20, or the expression construct of claim 21, or

an engineered cell that heterologously expresses methanol dehydrogenase, 3-hexulose-6-phosphate dehydrogenase, and 6-phospho-3-hexuloisomerase, wherein heterologous

5 expression includes expression of a fusion protein comprising

(1a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, and (1b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof;

10 (2a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (2b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and (2c) a third region comprising methanol dehydrogenase activator or an enzymatically active portion thereof;

15 (3a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, and (3b) a second region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof;

20 (4a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (4b) a second region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; and (4c) a third region comprising methanol dehydrogenase activator or an enzymatically active portion thereof;

(5a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (5b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and (5c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof;

25 (6a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (6b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; (6c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; and (6d) a fourth region comprising methanol dehydrogenase activator or an
30 enzymatically active portion thereof; or

(7a) first and second portions of a methanol dehydrogenase, and 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof, and/or 6-phospho-

3-hexuloisomerase or an enzymatically active portion thereof, and optionally methanol dehydrogenase activator or an enzymatically active portion thereof, positioned between the first and second portions of a methanol dehydrogenase.

5 23. The engineered cell of claim 22 which is a microbial cell.

24. The engineered cell of claim 23 which is a bacterial cell.

25. The engineered cell of claim 24 which is *E. coli*.

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26. The engineered cell of any one of claims 22-25 further comprising one or more transgene(s) encoding a protein of a metabolic pathway that promotes production of a target product or intermediate thereof, wherein the metabolic pathway uses a compound resulting from methanol consumption by the cell.

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27. The engineered cell of any one of claims 22-25 wherein expression of the fusion provides an increased amount of reducing equivalents for an increase in a target product and/or for increased fixation of carbon from formaldehyde into a target product.

20 28. The engineered cell of any one of claims 22-25 further comprising a transgene encoding an enzyme to convert the formaldehyde to formate thereby generating reducing equivalents useful to product the target product and/or able to fix carbon of formate into the target product.

25 29. The engineered cell of any one of claims 22-25 capable of producing a greater amount of acetyl CoA in the presence of methanol as compared to a cell that does not express the fusion protein.

30 30. The engineered cell of any one of claims 22-25 comprising a metabolic pathway capable of producing a target product selected from the group consisting of 1,4-butanediol, 1,3-butanediol, butadiene, succinate, adipate, HMDA, or 6-aminocaproic acid (6ACA).

31. An engineered cell comprising (i) a fusion protein comprising a first region comprising methanol dehydrogenase and a second region comprising methanol dehydrogenase activator, or enzymatically active portions thereof, and (ii) and a fusion protein comprising a first region

comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and a second region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof.

- 5 32. The engineered cell of any one of claims 22-31 comprising any one or more enzymes selected from the group consisting of DHA (dihydroxyacetone) synthase, F6P (fructose-6-phosphate) aldolase, DHA kinase, fructose-bisphosphate aldolase, wherein any one or more of the enzymes is optionally expressed from an exogenous nucleic acid.
- 10 33. The engineered cell of any one of claims 22-32 comprising any one or more enzymes selected from the group consisting of succinyl-CoA synthetase; CoA-independent succinic semialdehyde dehydrogenase; α -ketoglutarate dehydrogenase; glutamate:succinate semialdehyde transaminase; glutamate decarboxylase; CoA-dependent succinic semialdehyde dehydrogenase; 4-hydroxybutanoate dehydrogenase; α -ketoglutarate decarboxylase; 4-
- 15 hydroxybutyryl CoA: acetyl-CoA transferase; butyrate kinase; phosphotransbutyrylase; aldehyde dehydrogenase; alcohol dehydrogenase, wherein any one or more of the enzymes is optionally expressed from an exogenous nucleic acid.
34. A composition comprising the engineered cell of any of claims 22-33.
- 20 35. The composition of claim 34 wherein the composition is a cell culture composition, optionally comprising a target product or intermediate thereof.
36. A method of producing a target product or its intermediate comprising culturing the
- 25 engineered cell of any of claims 22-33 in a culture medium comprising methanol to produce the target product or an intermediate of the target product.
37. The method of claim 36 wherein the culture medium comprises methanol and a second carbon source.
- 30 38. The method of claim 36 or 37 further comprising a step of isolating or purifying the target product or an intermediate of the target product.