Abstract:

The disclosure herein provides five distinct pathways to synthesize malonyl-CoA from central metabolites such as phosphoenolpyruvate and pyruvate. The disclosure provides a recombinant microorganism that produces malonyl-CoA at levels greater than a parental organism comprising a pathway selected from the group consisting of: (a) phosphoenolpyruvate to malonate semialdehyde; and (b) pyruvate to malonate semialdehyde; wherein the pathway comprises a malonyl-CoA reductase.
RECOMBINANT PATHWAY AND ORGANISMS FOR MALONYL-CoA SYNTHESIS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 61/870,488, filed August 27, 2013, the disclosure of which is incorporated herein by reference in its entirety.

BACKGROUND

[0002] Malonyl-Coenzyme A (CoA), the starting substrate for n-butanol pathway, is naturally produced by acetyl-CoA carboxylase, which is a highly regulated protein and difficult to engineer.

SUMMARY

[0003] The disclosure provides five distinct pathways to synthesize malonyl-CoA from central metabolites such as phosphoenolpyruvate and pyruvate.

[0004] The disclosure provides both cell-free systems and recombinant microorganism for the production of malonyl-CoA.

[0005] The disclosure provides a recombinant microorganism that produces malonyl-CoA at levels greater than a parental organism comprising a pathway selected from the group consisting of: (a) phosphoenolpyruvate to malonate semialdehyde; and (b) pyruvate to malonate semialdehyde; wherein the pathway comprises a malonyl-CoA reductase. In one embodiment, the recombinant microorganism is engineered to express an acetyl-CoA carboxylase and a malonyl-CoA reductase. In another embodiment, the recombinant microorganism is engineered to express or overexpress one or more enzymes that carries out a metabolic function selected from the group consisting of (i) converting PEP to Glyceraldehyde-3P; (ii) converting Glyceraldehyde-3P to DHAP; (iii) converting DHAP to glycerol-3P; (iv) converting glycerol-3P to glycerol; (v) converting glycerol to 3-HPA; (vi) converting 3HPA to 3HP; (vii) converting 3HP to malonate semialdehyde; and (viii) converting malonate semialdehyde to malonyl-CoA. In another embodiment, the recombinant microorganism is engineered to express or overexpress one or more enzymes that carries out a metabolic function selected from the group consisting of (i) converting PEP to oxaloacetate; (ii) converting oxaloacetate to malonate semialdehyde; and (iii) converting malonate semialdehyde to malonyl-CoA. In yet another embodiment, the recombinant
microorganism is engineered to express or overexpress one or more enzymes that carries out a metabolic function selected from the group consisting of (i) converting PEP to oxaloacetate; (ii) converting oxaloacetate to aspartate, (iii) converting aspartate to beta-alanine; (iv) converting beta-alanine to malonate semialdehyde; and (v) converting malonate semialdehyde to malonyl-coA. In still another embodiment, the recombinant microorganism is engineered to express or overexpress one or more enzymes that carries out a metabolic function selected from the group consisting of (i) optionally converting PEP to pyruvate; (ii) converting pyruvate to alanine; (iii) converting alanine to bet-alanine; (iv) converting beta-alanine to malonate semialdehyde; and (v) converting malonate semialdehyde to malonyl-coA. In another embodiment, the recombinant microorganism is engineered to express or overexpress one or more enzymes that carries out a metabolic function selected from the group consisting of (i) converting PEP to pyruvate; (ii) converting pyruvate to lactate; (iii) converting lactate to lactoyl-CoA; (iv) converting lactoyl-CoA to acrylyl-CoA; (v) converting acrylyl-CoA to 3-hydroxypropionyl-CoA; (vi) 3-hydroxypropionyl-CoA to 3-hydroxypropionate; (vii) converting 3-hydroxypropionate to malonate semialdehyde; and (viii) converting malonate semialdehyde to malonyl-coA. In a further embodiment, the recombinant microorganism is engineered to express or overexpress one or more enzyme selected from the group consisting of trios phosphate isomerase, glycerol-3-phosphate dehydrogenase, glycerol-3-phoshatase, glycerol dehydratase, glycerol dehydratase reactivase, aldehyde dehydrogenase, malonate semialdehyde dehydrogenase and malonyl-CoA reductase. In yet a further embodiment, the recombinant microorganism is engineered to express or overexpress one or more enzymes selected from the group consisting of a phosphoenolpyruvate carboxylase, a oxaloacetate 1-decarboxylase and malonyl-CoA reductase. In yet another further embodiment, the recombinant microorganism is engineered to express or overexpress one or more enzymes selected from the group consisting of a phosphoenolpyruvate carboxylase, an aspartate aminotransferase, an aspartate 1-decarboxylase or a PLP-dependent aspartate 1-decarboxylase, a beta-alanine aminotransferase and a malonyl-CoA reductase. In still
another embodiment, the recombinant microorganism is engineered to express or overexpress one or more enzymes selected from the group consisting of a pyruvate kinase, an alanine aminotransferase, an alanine aminomutase and a malonyl-CoA reductase. In another embodiment, the recombinant microorganism is engineered to express or overexpress one or more enzymes selected from the group consisting of a pyruvate kinase, a lactate dehydrogenase, a lactoyl-CoA transferase, a propionyl-CoA synthase, a lactoyl-CoA dehydratase, a hydroxypropionyl-CoA dehydratase, a hydroxypropionyl-CoA hydrolase, a malonate semialdehyde dehydrogenase and a malonyl-CoA reductase. In yet another embodiment, the recombinant microorganism comprises an enzyme or homologs thereof selected from the group consisting of Tpi, GpsA, GPP, DhaB123/GdrAB, PuuC, Msr and Mcr. In yet another embodiment, the recombinant microorganism comprises enzyme or homologs thereof selected from the group consisting of Ppc, Oad and Mcr. In yet another embodiment, the recombinant microorganism comprises enzyme or homologs thereof selected from the group consisting of Ppc, AspC, PanD or AeADC, SkPYD4 and Mcr. In yet another embodiment, the recombinant microorganism comprises enzyme or homologs thereof selected from the group consisting of Pyk, Aat, Aam and Mcr. In yet another embodiment, the recombinant microorganism comprises enzyme or homologs thereof selected from the group consisting of Pyk, Ldh, Pet (and Pes), Led, Hpd, Hph, Msr and Mcr. In certain embodiments, the aspartate amino transferase can comprise a sequence having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:2 and having aspartate aminotransferase activity; the aspartate 1-decarboxylase can comprise a sequences having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:4 and having aspartate 1-decarboxylase activity; the PLP-dependent aspartate 1-decarboxylase can comprise a sequence having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:6 and having PLP-dependent aspartate 1-decarboxylase; and/or the beta-alanine aminotransferase can comprise a sequence having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:8 and having beta-alanine aminotransferase. In another embodiment, the malonyl-CoA reductase can comprise a sequence having
at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:10 and having malonyl-CoA reductase.

The disclosure also provides an in vitro metabolic pathway for producing malonyl-CoA comprising the enzymes triose phosphate isomerase, glycerol-3-phosphate dehydrogenase, glycerol-3-phosphatase, glycerol dehydratase, glycerol dehydratase reactivase, aldehyde dehydrogenase, malonate semialdehyde dehydrogenase and malonyl-CoA reductase. In one embodiment, the in vitro metabolic pathway comprises an enzyme or homologs thereof selected from the group consisting of Tpi, GpsA, GPP, DhaB123/GdrA, PuuC, Msr and Mcr.

The disclosure also provides an in vitro metabolic pathway for producing malonyl-CoA comprising the enzymes phosphoenolpyruvate carboxylase, oxaloacetate 1-decarboxylase and malonyl-CoA reductase. In one embodiment, the in vitro metabolic pathway comprises an enzyme or homologs thereof selected from the group consisting of Ppc, Oad and Mcr.

The disclosure also provides an in vitro metabolic pathway for producing malonyl-CoA comprising the enzymes phosphoenolpyruvate carboxylase, aspartate aminotransferase, aspartate 1-decarboxylase or PLP-dependent aspartate 1-decarboxylase, beta-alanine aminotransferase and a malonyl-CoA reductase. In one embodiment, the in vitro metabolic pathway comprises an enzyme or homologs thereof selected from the group consisting of Ppc, AspC, PanD or AeADC, SkPYD4 and Mcr.

The disclosure also provides an in vitro metabolic pathway for producing malonyl-CoA comprising the enzymes pyruvate kinase, alanine aminotransferase, alanine aminotransferase and malonyl-CoA reductase. In one embodiment, the in vitro metabolic pathway comprises an enzyme or homologs thereof selected from the group consisting of Pyk, Aat, Aam and Mcr.

The disclosure provides an in vitro metabolic pathway for producing malonyl-CoA comprising the enzymes pyruvate kinase, lactate dehydrogenase, lactoyl-CoA transferase, propionyl-CoA synthase, lactoyl-CoA dehydratase, hydroxypropionyl-CoA dehydratase, hydroxypropionyl-CoA hydratase, malonate semialdehyde dehydrogenase and malonyl-CoA reductase. In one embodiment, the in vitro metabolic
pathway comprises an enzyme or homologs thereof selected from the group consisting of Pyk, Ldh, Pet (and Pes), Led, Hpd, Hph, Msr and Mcr.

The disclosure also provides a method for producing a chemical or malonyl-CoA, the method comprising: (a) providing a recombinant microorganism as described herein; (b) culturing the microorganism (b) of (a) in the presence of a carbon substrate under conditions suitable for the conversion of the substrate to the chemical or malonyl-CoA; and (c) purifying the chemical or malonyl-CoA.

The disclosure also provides a method for producing a chemical or malonyl-CoA, the method comprising: (a) providing an in vitro metabolic pathway as described herein; (b) incubating the enzymes of the in vitro metabolic pathway in (a) in the presence of a carbon substrate under conditions suitable for the conversion of the substrate to the chemical or malonyl-CoA; and (c) purifying the chemical or malonyl-CoA.

The disclosure provides a recombinant microorganism that produces malonyl-CoA at levels greater than a parental organism comprising a pathway selected from the group consisting of: (a) phosphoenolpyruvate to malonate semialdehyde; and (b) pyruvate to malonate semialdehyde; wherein the pathway comprises a malonyl-CoA reductase. In one embodiment the organism comprises expression or elevated expression of an enzyme that converts (i) PEP to Glyceraldehyde-3P; (ii) Glyceraldehyde-3P to DHAP; (iii) DHAP to glycerol-3P; (iv) glycerol-3P to glycerol; (v) glycerol to 3-HPA; (vi) 3HPA to 3HP; (vii) 3HP to malonate semialdehyde; and (viii) malonate semialdehyde to malonyl-coA. In another embodiment, the organism comprises expression or elevated expression of an enzyme that converts (i) PEP to oxaloacetate; (ii) oxaloacetate to malonate semialdehyde; and (iii) malonate semialdehyde to malonyl-coA. In yet another embodiment, the organism comprises expression or elevated expression of an enzyme that
converts (i) PEP to pyruvate; (ii) pyruvate to alanine; (iii) alanine to bet-alanine; (iv) beta-alanine to malonate semialdehyde; and (v) malonate semialdehyde to malonyl-coA. In another embodiment, the organism comprises expression or elevated expression of an enzyme that converts (i) PEP to pyruvate; (ii) pyruvate to lactate; (iii) lactate to lactoyl-CoA; (iv) lactoyl-CoA to acrylyl-CoA; (v) acrylyl-CoA to 3-hydroxypropionyl-CoA; (vi) 3-
hydroxypropionyl-CoA to 3-hydroxypropionate; (vii) 3-
hydroxypropionate to malonate semialdehyde; and (viii) malonate semialdehyde to malonyl-coA. In one embodiment, the microorganism comprises an enzyme or homologs thereof selected from the group consisting of Tpi, GpsA, GPP, DhaB123/GdrAB, PuuC, Msr and Mcr. In one embodiment, the microorganism comprises an or homologs thereof selected from the group consisting of Ppc, Oad and Mcr. In one embodiment, the microorganism comprises an enzyme or homologs thereof selected from the group consisting of Ppc, AspC, PanD or AeADC, SkPYD4 and Mcr. In one embodiment, the microorganism comprises an enzyme or homologs thereof selected from the group consisting of Pyk, Aat, Aam and Mcr. In one embodiment, the microorganism comprises an enzyme or homologs thereof selected from the group consisting of Pyk, Ldh, Pet (and Pes), Led, Hpd, Hph, Msr and Mcr. In yet another embodiment, the microorganism is engineered to express or overexpress (a) an aspartate aminotransferase (EC 2.6.1.1); (b) an aspartate 1-decarboxylase or a PLP-dependent aspartate 1-decarboxylase (EC 4.1.1.11); (c) a beta-alanine aminotransferase (EC 2.6.1.19); and (d) a malonyl-CoA reductase (Mcr, EC 1.2.1.75). In a further embodiment, the aspartate amino transferase comprises a sequence having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:2 and having aspartate aminotransferase activity wherein the aspartate 1-decarboxylase comprises a sequences having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:4 and having aspartate 1-decarboxylase activity wherein the PLP-dependent aspartate 1-decarboxylase comprises a sequence having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:6 and having PLP-dependent aspartate 1-
deboxylase; wherein the beta-alanine aminotransferase comprises a sequence having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to
SEQ ID NO:8 and having beta-alanine aminotransferase. In any of the foregoing embodiments, the malonyl-CoA reductase comprises a sequence having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:10 and having malonyl-CoA reductase.

[0014] The disclosure also provides a method for producing a chemical or malonyl-CoA, the method comprising (a) providing a recombinant microorganism as described above; (b) culturing the microorganism (s) of (a) in the presence of a carbon substrate under conditions suitable for the conversion of the substrate to the chemical or malonyl-CoA; and (c) purifying the chemical or malonyl-CoA.

[0015] The details of one or more embodiments 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**

[0016] 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.

[0017] **Figure 1A-E** is a schematics malonyl-CoA biosynthesis. 
A) Natural malonyl-CoA biosynthesis via acetyl-CoA. Synthetic malonyl-CoA biosynthesis pathway via malonate semialdehyde which can be produced by
B) sequential carboxylation and decarboxylation of PEP, 
C) functionalization of a-keto to a-amino followed by a-amino transfer and regeneration of keto moiety, 
D) reduction of pyruvate to lactate in which net hydroxyl transfer can be achieved by sequential dehydration and hydration followed by re-oxidation of β-hydroxyl to β-keto, 
E) hydration of PEP to 2-phosphoglycerate and reduction to glycerol followed by dehydration of hydroxyl on carbon 2 of glycerol to 3-hydroxypropionaldehyde which can be oxidized to malonate semialdehyde.

[0018] **Figure 2** shows a further detailed schematic of the sequential carboxylation and decarboxylation of PEP to produce malonyl-CoA.
Figure 3 shows a further detailed schematic of the sequential carboxylation and decarboxylation of PEP to produce malonyl-CoA.

Figure 4 shows a further detailed schematic of the isomerization via amino transfer for production of malonyl-CoA.

Figure 5 shows a further detailed schematic of the isomerization via net hydroxyl transfer (dehydration first followed by hydration) in the production of malonyl-CoA.

Figure 6 shows a further detailed schematic of isomerization via net hydroxyl transfer (hydration first followed by dehydration) in the production of malonyl-CoA.

Figure 7 shows precursors to malonate semialdehyde. Dashed lines represent enzymes not found in nature.

Figure 8 shows the thermodynamics of malonyl-CoA biosynthesis pathways. Change in energy was calculated using eQuilibrator with parameters of physiological conditions. Each pathway is named based on their representative metabolite.

Figure 9 shows the aspartate shunt to bypass oxaloacetate 1-decarboxylase. Dash line represents that enzyme not yet identified.

Figure 10A-F shows biosynthesis of malonyl-CoA from β-alanine. A) schematics of the coupled reaction containing SkPYD4 and Mcr. Titration of substrates and cofactors: B) β-alanine, C) α-ketoglutarate, D) NADP+, and E) CoA. Except the substrate being titrated, concentrations of other substrates were: 1 mM of NADP+, 1 mM of CoA, 30 mM β-alanine, and 30 mM α-ketoglutarate. Reaction was catalyzed using 200 nM of SkPYD4, and 200 nM of Mcr.

Figure 11A-E shows one pot biosynthesis of malonyl-CoA from oxaloacetate. A) HPLC chromatogram showing synthesis of malonyl-CoA only upon presence of all enzymes AspC, PanD, SkPYD4, and Mcr. Titration of individual enzymes in aspartate dependent biosynthesis of malonyl-CoA: B) AspC, C) PanD, D) SkPYD4, and E) Mcr. Reaction mixture contained at 1 mM of NADP+, 1 mM of CoA, 20 mM oxaloacetate, 15 mM α-ketoglutarate, 15 mM glutamate, 100 µM of PLP. Except the enzyme being titrated, concentrations of other enzymes were kept constant at 1 µM.
Figure 12A-D shows titration of individual enzymes in malonyl-CoA biosynthesis with PLP-dependent aspartate decarboxylase. Titration of individual enzymes: B) ADC, C) AspC, D) SkPYD4, and E) Mcr. Mcr. Reaction mixture contained at 1 mM of NADP+, 1 mM of CoA, 20 mM oxaloacetate, 15 mM a-ketoglutarate, 15 mM glutamate, and 100 µM of PLP. Except the enzyme being titrated, concentrations of other enzymes were kept constant at 1 µM.

Figure 13 shows a pathway schematic useful for understanding the disclosure. Complete metabolic interconnections between the synthetic malonyl-CoA pathways. Dash lines represents enzymes not identified. Abbreviations: Tpi, triose phosphate isomerase; GpsA, glycerol-3-phosphate dehydrogenase; GPP, glycerol-S-phosphatase; DhaB123, glycerol dehydratase; GdrAB, glycerol dehydratase reactivase; PuuC, aldehyde dehydrogenase; Mr, malonate semialdehyde dehydrogenase; Ppc, phosphoenolpyruvate carboxylase; AspC, aspartate aminotransferase; PanD, aspartate 1-decarboxylase; AeADC, PLP-dependent aspartate 1-decarboxylase; SkPYD4, β-alanine aminotransferase; Oad, oxaloacetate 1-decarboxylase; Mcr, malonyl-CoA reductase; Pyk, pyruvate kinase; Aat; alanine aminotransferase; Aam, alanine aminomutase; Pdh, pyruvate dehydrogenase; AccDA, acetyl-CoA carboxylase; AccC, biotin carboxylase; Ldh, lactate dehydrogenase; Pet, lactoyl-CoA transferase; Pes, propionyl-CoA synthase; Led, lactoyl-CoA dehydratase; Hpd, hydroxypropionyl-CoA dehydratase; Hph, hydroxypropionyl-CoA hydrolase; MatB, malonyl-CoA synthase.

Like reference symbols in the various drawings indicate like elements.

DETAILED DESCRIPTION

As used herein and in the appended claims, the singular forms "a," "and," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a microorganism" includes a plurality of such microorganisms and reference to "the polypeptide" includes reference to one or more polypeptides and equivalents thereof, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs.
Although any methods and reagents similar or equivalent to those described herein can be used in the practice of the disclosed methods and compositions, the exemplary methods and materials are now described.

[0033] Also, the use of "or" means "and/or" unless stated otherwise. Similarly, "comprise," "comprises," "comprising" "include," "includes," and "including" are interchangeable and not intended to be limiting.

[0034] It is to be further understood that where descriptions of various embodiments use the term "comprising," those skilled in the art would understand that in some specific instances, an embodiment can be alternatively described using language "consisting essentially of" or "consisting of."

[0035] All publications mentioned herein are incorporated herein by reference in full for the purpose of describing and disclosing the methodologies, which are described in the publications, which might be used in connection with the description herein. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior disclosure.

[0036] The first century of metabolic research has successfully discovered almost all of the enzymatic steps and mechanisms in metabolism that supports life. The study of metabolism is further enriched by genome sequencing that leads to rapid identification of genes coding for the enzymes. Meanwhile, genetic techniques and engineering principles have enabled modification and transferring foreign pathways to almost any organism to produce compounds for industrial purposes. After optimizing the terminal pathways for a specific product, the production is often limited by central metabolites that are produced from pathways optimized for a cell's benefit rather than for production. As such, redesign of essential primary metabolic pathways for the purpose of production has become a priority.

[0037] Malonyl-CoA is an essential metabolite that serves as the common building block for fatty acids, polyketides, and flavonoids.
Malonyl-CoA is synthesized via carboxylation of acetyl-CoA catalyzed by acetyl-CoA carboxylase (Acc) (Fig. 1A), which typically is a four subunit enzyme complex or a multi-domain enzyme. Biotin-carboxyl carrier protein (BCCP) domain is first biotinylated into active carboxyl-carrier. Biotin carboxylase then carboxylates BCCP-biotin complex while consuming ATP. Malonyl-CoA is subsequently produced upon transfer of the carboxyl group from AccB-biotin complex to acetyl-CoA by acetyl-CoA carboxyltransf erase. Recently, the Acc-dependent malonyl-CoA synthesis has also been used in an engineered pathway for acetoacetyl-CoA synthesis that leads to n-butanol production. Because Acc catalyzes the first step for directing acetyl-CoA flux towards secondary metabolite biosynthesis instead of amino acid biosynthesis or energy production, Acc expression is tightly regulated at all levels including: transcriptional regulation by growth-rate, translational regulation by mRNA binding, and feedback inhibition by product acyl-ACP. As a result, biosynthesis of downstream products of malonyl-CoA is believed to be mainly controlled by Acc. Currently, almost all engineering approaches to increase malonyl-CoA flux are based on overexpression of Acc or increasing acetyl-CoA flux. Although these efforts achieved reasonable success, Acc overexpression is not always effective and often results in conservative beneficial effects.

[0038] Synthetic metabolism enables biosynthesis of various valuable compounds and introduces new functions to cells. Nature uses limited sets of metabolites to construct a diverse space of complex molecules. Carbon chain elongation offers most diversity and complexity in molecules produced. Malonyl-CoA is one of the principle carbon extender for achieving this chemical diversity. Thus, the disclosure provides for design and demonstration of a synthetic malonyl-CoA biosynthesis pathway.

[0039] The disclosure provides methods and compositions for the production of malonyl-CoA using a culture of microorganisms that utilizes oxaloacetate, malonate semialdehyde or malonate semialdehyde-CoA as an intermediate. The disclosure can used photoautotrophic organisms, photoheterotrophic organisms or combinations thereof. Simply, the recombinant organism can be any
organism that produces pyruvate or phosphoenolpyruvate (PEP) as an intermediate.

From a biochemical stand point, malonate, oxaloacetate, malonate semialdehyde-CoA, and malonate semialdehyde are the possible direct precursors to malonyl-CoA (Fig. 7). However, among them malonate semialdehyde is more biologically feasible. Malonate semialdehyde is a naturally occurring metabolite found in 3-hydroxypropionate dependent CO2 fixation pathway, indicating its intracellular stability.

Isomerization of pyruvate to malonate semialdehyde requires migration of the keto group from α-position to β-position. Because keto isomerization is not available, alternative reaction schemes are necessary to achieve this net isomerization. Four general solutions exist for this isomerization: 1) Carboxylation at β-position followed by α-decarboxylation (Fig. 1B), 2) transamination of α-keto into α-amino followed by amino transfer and re-transamination (Fig. 1C), and 3) Reduction of α-keto into α-hydroxyl followed by either dehydration and re-hydration (Fig. ID) or 4) hydration and dehydrogenation (Fig. IE) and re-oxidation of β-hydroxyl.

PEP was chosen as a starting reference point for thermodynamics analysis because it is the common intermediate for all synthetic pathways for malonyl-CoA biosynthesis. The thermodynamics of these pathways were calculated based on physiological conditions and are shown in Fig. 8. These synthetic pathways can be divided into three groups based on their ATP yield. Group 1 pathways has an ATP yield of zero from either PEP or glucose and include Natural Acc dependent pathway (Fig. 1A), oxaloacetate (Fig. IB), and aspartate (Fig. 9) dependent pathways. Group 2 pathway has ATP yield greater than zero and contains only represented by alanine (Fig. 1C) pathway. Group 3 pathways have ATP yield less than one, indicating extra energy cost, and include both the lactate (Fig. ID) and glycerol (Fig. IE) pathways.

In one embodiment, the disclosure shows the in vitro conversion of oxaloacetate to malonyl-CoA in four enzymatic steps. In particular, the rate of malonyl-CoA biosynthesis demonstrated is
comparable to that of in vitro fatty acid biosynthesis, suggesting compatibility of this synthetic pathway for biofuel production.

Decarboxylative Claisen condensation of malonyl-CoA and its derivative malonyl-acyl carrier protein (ACP) with Acyl-ACP is irreversible and provides the driving force necessary for iterative carbon chain elongation. β-Carboxylate of malonyl-CoA serves as a leaving group to facilitate this otherwise unfavorable condensation of two acetyl-CoA. Using pyruvate as a reference, nature's solution for achieving this β-carboxylate is by oxidative decarboxylation of pyruvate to acetyl-CoA and followed by an energy dependent carboxylation to malonyl-CoA (Fig. 1A). From a biochemical standpoint, β-carboxylate of malonyl-CoA can also be achieved by CoA-acylating oxidation of malonate semialdehyde, a naturally occurring metabolite.

Accordingly, the disclosure describes at least four general alternatives (Fig. 1B, C, D, E and Fig. 2, 3, 4, 5 and 6) and other variations thereof (Fig. 13 for complete metabolic interconnections between the pathways) to achieve biosynthesis of malonyl-CoA via malonate semialdehyde, a structural isomer of pyruvate. The four general alternatives are: sequential carboxylation and decarboxylation (Fig. 1B, 2 and 3), one step isomerization by amino transfer (Fig. 1C and 4), and multi-step isomerization by hydroxyl transfer mediated by hydration and dehydration (Fig. 1D, E and 5, 6).

Of these approaches, sequential carboxylation and decarboxylation is the most promising pathway to construct because it is thermodynamically favorable and requires fewer enzymatic steps. In particular, oxaloacetate, a β-keto acid, may favor decarboxylation of the β-carboxylate over the a-carboxylate.

Reversed reaction of malonyl-CoA reductase (Mcr), catalyzing the oxidation of malonyl-CoA to malonate semialdehyde using NADPH, converts malonate semialdehyde to malonyl-CoA. Two types of Mcr exist in nature: unifunctional and bifunctional. The reversibility of the unifunctional Mcr from Sulfolobus tokodaii is uncertain and has not been reported. On the other hand, bifunctional Mcr from Chloroflexus aurantiacus, which catalyzes two step malonyl-CoA reduction to 3-hydroxypropionate, is reversible but with strong
preference for malonyl-CoA reducing direction. Nevertheless, the calculated Gibbs free energy for malonate semialdehyde oxidation to malonyl-CoA under physiological conditions (1 mM concentration with 0.1M ionic strength at pH 7.0) is -7.5 kJ/mol, suggesting its thermodynamic feasibility.

In one embodiment, the recombinant microorganism comprises a pathway that includes at least one recombinant enzyme that converts phosphoenolpyruvate (PEP) to malonyl-CoA, wherein the pathway includes converting: (i) PEP to glyceraldehyde-3P; (ii) glyceraldehyde-3P to dihydroxyacetone phosphate (DHAP); (iii) DHAP to glycerol-3P; (iv) glycerol-3P to glycerol; (v) glycerol to 3-hydroxypropionaldehyde (3-HPA); (vi) 3HPA to 3-hydroxypropionate (3HP); (vii) 3HP to malonate semialdehyde; and (viii) malonate semialdehyde to malonyl-CoA (see, e.g., Figure 6).

In further embodiment, the recombinant microorganism comprises a pathway having enzyme or homologs thereof selected from the group consisting of a triose phosphate isomerase (e.g., Tpi), a glycerol-3-phosphate dehydrogenase (e.g., GpsA), a glycerol-3-phosphatase (e.g., GPP), an aglycerol dehydratase (e.g., DhaB123)/a glycerol dehydratase reactivase (e.g., GdrAB), an aldehyde dehydrogenase (e.g., PuuC), a malonate semialdehyde dehydrogenase (e.g., Msr) and a malonyl-CoA reductase (e.g., Mcr). In yet a further embodiment, at least one of the foregoing enzymes or homologs thereof is recombinantly engineered into the microorganism to produce malonyl-CoA from PEP.

This pathway uses gluconeogenesis. PEP is first hydrated at β-position to 2-phosphoglycerate. Following gluconeogenesis, glycerol is synthesized by reduction of dihydroxyacetone-phosphate using glycerol-3-phosphate dehydrogenase followed by dephosphorylation using glycerol phosphatase. Subsequently, glycerol is dehydrated to 3-hydroxypropionaldehyde using glycerol dehydratase (Gdh). Two types of Gdh exist in nature. Klebsiella Gdh is composed of three subunits and utilizes coenzyme B12 as a prosthetic group. However, Klebsiella Gdh is irreversibly inactivated upon reaction with glycerol. A reactivating factor is necessary for subsequent glycerol dehydration. Glycerol dehydratase reactivating factor (Gdr) utilizes energy from ATP hydrolysis to re activates Gdh, increasing
additional ATP cost and contributing to the net ATP deficiency of this synthetic pathway. *Clostridium* Gdh on the other hand does not require Coenzyme B12 for catalysis. Instead, *Clostridium* Gdh requires an activation enzyme to introduce a glycyl radical required for catalysis. Similar to pyruvate :formate lyase, *Clostridium* Gdh is oxygen sensitive, prohibiting its function in some aerobic processes. Thus, the disclosure can use either a combination of the glycerol dehydratase reactivating factor (GdrAB) and glycerol dehydratase (DhaB123) or an oxygen sensitive Gdh. One of skill depending upon the balance of ATP and oxygen can readily appreciate and select the appropriate enzyme. While all enzymes of this synthetic pathway are available in nature (although not found in a single organism), and overall thermodynamics is more favorable than all other pathways, both the ATP demand and the number of enzymes required are significantly larger than the other pathways described below. Net hydroxyl transfer can be achieved via a hydration pattern different from the lactate pathway described in more detail below.

[0051] In another embodiment, the recombinant microorganism comprises a pathway that includes at least one recombinant enzyme that converts PEP to malonyl-CoA, wherein the pathway includes the steps: (i) PEP to oxaloacetate; (ii) oxaloacetate to malonate semialdehyde; and (iii) malonate semialdehyde to malonyl-coA (see, Figure IB).

[0052] In one embodiment, the recombinant microorganism comprises a pathway having enzyme or homologs thereof selected from the group consisting of a phosphoenolpyruvate carboxylase (e.g., Ppc, or homolog thereof), an oxaloacetate 1-decarboxylase (e.g., Oad, or homolog thereof) and a malonyl-CoA reductase (e.g., Mcr or homolog thereof). In yet a further embodiment, at least one of the foregoing enzymes or homologs thereof is recombinantly engineered into the microorganism to produce malonyl-coA from PEP.

[0053] Carboxylation of PEP at β-position followed by ω-decarboxylation yielding malonate semialdehyde (Fig. IB) is the shortest route, containing only two enzymatic steps, to achieve a to β keto-migration. Notably, this pathway has an overall thermodynamics similar to that of Acc dependent malonyl-CoA.
synthesis, where its ATP yield from both PEP and glucose is zero and all steps have negative AG' (Fig. 3). a-decarboxylation occurs naturally in many biological processes such as pyruvate decarboxylase in ethanol fermentation and a-ketoglutarate decarboxylase in cyanobacteria TCA cycle.

[0054] In another embodiment, the recombinant microorganism includes an aspartate shunt, wherein in this embodiment an oxaloacetate 1-decarboxylase (e.g., Oad, or homolog thereof) is not required, but may be present. In embodiments where the recombinant microorganism comprises an aspartate shunt, the recombinant microorganism comprises a pathway that includes at least one recombinant enzyme that converts PEP to malonyl-CoA, wherein the pathway includes the steps: (i) PEP to oxaloacetate; (ii) oxaloacetate to aspartate, (iii) aspartate to beta-alanine; (iv) beta-alanine to malonate semialdehyde; and (v) malonate semialdehyde to malonyl-coA.

[0055] In a further embodiment, the recombinant microorganism comprises a pathway having enzyme or homologs thereof selected from the group consisting of a phosphoenolpyruvase (e.g., Ppc or homolog thereof), an aspartate aminotransferase (e.g., AspC, or homolog thereof), an aspartate 1-decarboxylase (e.g., PanD, or homolog thereof) and/or a PLP-dependent aspartate 1-decarboxylase (e.g., AeADC, or homolog thereof), a beta-alanine aminotransferase (e.g., SkPYD4, or homolog thereof) and a malonyl-CoA reductase (e.g., Mcr, or homolog thereof). In yet another embodiment, at least one of the foregoing enzymes or homologs thereof is recombinant engineered into the microorganism to produce malonyl-coA from PEP.

[0056] Migration of the a-keto group of pyruvate to the β-position, forming malonate semialdehyde, is a major challenge for designing synthetic malonyl-CoA pathway. Carboxylation of PEP to oxaloacetate introduces a carboxyl group at the γ-position of pyruvate. The original a-keto group of pyruvate therefore becomes β-keto relative to this new carboxyl group (Fig. 1B). Subsequent decarboxylation of the original pyruvate carboxyl group yields malonate semialdehyde. Instead of decarboxylation of oxaloacetate the disclosure uses the decarboxylation of aspartate which is readily available in nature. In this embodiment, the disclosure
describes a shunt utilizing aspartate decarboxylase. The limiting step, cofactor independent aspartate decarboxylase, was further solved by recruiting a PLP-dependent aspartate decarboxylase. Using this system, the steady state kinetics of this synthetic pathway and effects of substrate concentration were analyzed. These results represent the construction of a synthetic malonyl-CoA biosynthesis and present an alternative method for increasing production of malonyl-CoA derived products.

[0057] In the synthetic pathway comprising the aspartate shunt (see, e.g., Figs. 3 and 9), a-keto group of oxaloacetate is transaminated into amino group to facilitate the a-decarboxylation into β-alanine. Subsequent transamination returns keto functionality and forms malonate semialdehyde. Using this aspartate shunt, the disclosure demonstrates that conversion of oxaloacetate into malonate semialdehyde. This synthetic pathway has the same ATP cost as the natural Acc dependent pathway.

[0058] While the direct decarboxylation of oxaloacetate to malonate semialdehyde is described above (see Fig. 1B), decarboxylation of aspartate, the transaminated form of oxaloacetate (Figs. 3 and 9) can also be used. In this pathway, oxaloacetate is transaminated into aspartate by aspartate aminotransferase (AspC) using glutamate as the amino donor. Aspartate then undergoes a-decarboxylation using aspartate 1-decarboxylase into β-alanine, a metabolite in panthothenate biosynthesis. Once β-alanine is formed, amino-group can then be transaminated back into keto functionality using a-ketoglutarate as the amino receptor to produce malonate semialdehyde. In one embodiment, transamination of β-alanine is catalyzed by a β-Alanine aminotransferase (SkPYD4) found in Saccharomyces kluyveri. Effectively, transamination of oxaloacetate to aspartate serves as a shunt to facilitate a-decarboxylation. Aspartate a-decarboxylase (PanD) is an unusual enzyme using pyruvate as a prosthetic group. PanD is translated as an inactive proenzyme, which after self-proteolysis forms two functional subunits and the pyruvyl-group. Decarboxylation of aspartate provides an irreversible trap for carbon flux, which serves as a driving force for this synthetic malonyl-CoA pathway.
In another embodiment, the recombinant microorganism comprises a pathway that includes at least one recombinant enzyme that converts PEP (or pyruvate) to malonyl-CoA, wherein the pathway includes the steps: (ia) PEP to pyruvate and/or (ib) pyruvate to alanine; (ii) alanine to bet-alanine; (iii) beta-alanine to malonate semialdehyde; and (iv) malonate semialdehyde to malonyl-coA.

In a further embodiment, the recombinant microorganism comprises a pathway having enzyme or homologs thereof selected from the group consisting of an optional pyruvate kinase (e.g., Pyk, or homolog thereof), an alanine aminotransferase (e.g., Aat, or homolog thereof), an alanine aminomutase (e.g., Aam, or homolog thereof) and a malonyl-CoA reductase (e.g., Mcr, or homolog thereof). In yet a further embodiment, at least one of the foregoing enzymes or homologs thereof is recombinant engineered into the microorganism to produce malonyl-coA from PEP.

The pathway in the preceding paragraph is the least energy demanding and second shortest pathway for malonyl-CoA biosynthesis is mediated by amino transfer (Figs. 1C and 4). Pyruvate is first transaminated into alanine, effectively converting α-keto to α-amino which is converted into β-amino by an alanine aminomutase. Subsequent transamination of β-alanine returns keto group by using either pyruvate or a-ketoglutarate as an amino receptor. This synthetic pathway has a net positive ATP yield. However, this synthetic pathway uses alanine aminomutase. Enzymatic functional relatives of alanine aminomutase such as lysine 2,3-aminomutase and glutamate 2,3-aminomutase contains iron sulfur clusters for generating radical SAM, which facilitates amino transfer.

In another embodiment, the recombinant microorganism comprises a pathway that includes at least one recombinant enzyme that converts PEP (or pyruvate) to malonyl-CoA, wherein the pathway includes the steps: (ia) PEP to pyruvate and/or (ib) pyruvate to lactate; (ii) lactate to lactoyl-CoA; (iii) lactoyl-CoA to acrylyl-CoA; (iv) acrylyl-CoA to 3-hydroxypropionyl-CoA; (v) 3-hydroxypropionyl-CoA to 3-hydroxypropionate; (vi) 3-hydroxypropionate to malonate semialdehyde; and (vii) malonate semialdehyde to malonyl-coA.
In a further embodiment, the recombinant microorganism comprises a pathway having enzyme or homologs thereof selected from the group consisting of an optional pyruvate kinase (e.g., Pyk, or homolog thereof), a lactate dehydrogenase (e.g., Ldh, or homolog thereof), a lactoyl-CoA transferase (e.g., Pet, or homolog thereof), a propionyl-CoA synthase (e.g., Pes, or homolog thereof), a lactoyl-CoA dehydratase (e.g., Led, or homolog thereof), a hydroxypropionyl-CoA dehydratase (e.g., Hpd, or homolog thereof), a hydroxypropionyl-CoA hydrolase (e.g., Hph, or homolog thereof), a malonate semialdehyde dehydrogenase (e.g., Msr, or homolog thereof) and a malonyl-CoA reductase (e.g., Mcr, or homolog thereof). In yet a further embodiment, at least one of the foregoing enzymes or homologs thereof is recombinant engineered into the microorganism to produce malonyl-CoA from PEP.

In this embodiment, reduction of pyruvate yields lactate in which its α-hydroxyl can be removed by dehydration, resulting in formation of acrylate (Figs. 1D and 5). Hydration at β-position of acrylate followed by oxidation returns keto functionality at the β-position, forming malonate semialdehyde. To facilitate dehydration of α-hydroxyl group, lactate is first activated into lactoyl-CoA using propionyl-CoA as CoA donor. Propionyl-CoA is synthesized from propionate, CoA, and ATP using AMP forming propionyl-CoA synthase. Two ATP are used to convert AMP back into ATP, which increases ATP cost of this pathway and contributes to the net ATP deficiency of this pathway. Lactoyl-CoA dehydrates into acryloyl-CoA which is then rehydrated at the β-position, forming 3-hydroxypropionyl-CoA. 3-hydroxypropionyl-CoA is then hydrolyzed to 3-hydroxypropionate, structural isomer of lactate. Subsequent re-oxidation of β-hydroxyl group of 3-hydroxypropionate yields malonate semialdehyde. Alternatively, 3-hydroxypropionyl-CoA can be converted directly to malonyl-CoA in two reduction steps. The ATP demand of this pathway and the number of enzymes required is larger than the aspartate dependent pathway (described above).

The disclosure provides microorganisms that comprise an artificially engineered pathway to produce malonyl-CoA and may further include additional enzymes to produce fatty acids, and other chemicals from malonyl-CoA. The disclosure demonstrates
recombinant pathways for the production of malonyl-CoA from a suitable carbon source (e.g., via pyruvate or PEP).

[0066] The malonyl-CoA produced by the recombinant microorganism described herein can also be used for CoA-dependent chain elongation. Decarboxylative condensation of malonyl-CoA with acetyl-CoA is irreversible and thus provides driving force for downstream pathways, an important design principle for engineering photosynthetic organisms. CoA dependent chain elongation includes the Clostridium pathway for 1-butanol production and mevalonate pathway for production of diverse downstream isoprenoids-based compounds. Incorporation of the synthetic malonyl-CoA pathway into in vivo systems would enhance production of these diverse malonyl-CoA derived products.

[0067] The term "biosynthetic pathway", also referred to as "metabolic pathway", refers to a set of anabolic or catabolic biochemical reactions for converting (transmuting) one chemical species into another. Gene products belong to the same "metabolic pathway" if they, in parallel or in series, act on the same substrate, produce the same product, or act on or produce a metabolic intermediate (i.e., metabolite) between the same substrate and metabolite end product.

[0068] As used herein, the term "metabolically engineered" or "metabolic engineering" involves rational pathway design and assembly of biosynthetic genes, genes associated with operons, and control elements of such polynucleotides, for the production of a desired metabolite, such as an acetoacetyl-CoA or higher alcohol, in a microorganism. "Metabolically engineered" can further include optimization of metabolic flux by regulation and optimization of transcription, translation, protein stability, reducing agents and protein functionality using genetic engineering and appropriate culture condition including the reduction of, disruption, or knocking out of, a competing metabolic pathway that competes with an intermediate or use of a cofactor or energy source, leading to a desired pathway. A biosynthetic gene can be heterologous to the host microorganism, either by virtue of being foreign to the host, or being modified by mutagenesis, recombination, and/or association with a heterologous expression control sequence in an endogenous
host cell. In one embodiment, where the polynucleotide is xenogenetic to the host organism, the polynucleotide can be codon optimized.

[0069] Microorganisms provided herein are modified to produce metabolites in quantities not available in the parental microorganism. A "metabolite" refers to any substance produced by metabolism or a substance necessary for or taking part in a particular metabolic process. A metabolite can be an organic compound that is a starting material (e.g., glucose or pyruvate), an intermediate (e.g., malonate semialdehyde) in, or an end product (e.g., malonyl-CoA), of metabolism. Metabolites can be used to construct more complex molecules, or they can be broken down into simpler ones. Intermediate metabolites may be synthesized from other metabolites, perhaps used to make more complex substances, or broken down into simpler compounds, often with the release of chemical energy.

[0070] The term "substrate" or "suitable substrate" refers to any substance or compound that is converted or meant to be converted into another compound by the action of an enzyme. The term includes not only a single compound, but also combinations of compounds, such as solutions, mixtures and other materials which contain at least one substrate, or derivatives thereof. Further, the term "substrate" encompasses not only compounds that provide a carbon source suitable for use as a starting material, such as CO2, or any biomass derived sugar, but also intermediate and end product metabolites used in a pathway associated with a metabolically engineered microorganism as described herein.

[0071] Recombinant microorganisms provided herein can express a plurality of target enzymes involved in pathways described above and herein for the production of malonyl-CoA from a suitable carbon substrate and may further include pathways for the synthesis of fatty acids, polyketides and flavonoids.

[0072] Accordingly, metabolically "engineered" or "modified" microorganisms are recombinant microorganisms produced via the introduction of genetic material into a host or parental microorganism of choice thereby modifying or altering the cellular physiology and biochemistry of the microorganism. Through the
introduction of genetic material the parental microorganism acquires new properties, e.g. the ability to produce a new, or greater quantities of, a metabolite. In an illustrative embodiment, the introduction of genetic material into a parental microorganism results in a new or modified ability to produce malonyl-CoA. The genetic material introduced into the parental microorganism contains gene(s) or parts of genes, coding for one or more of the enzymes involved in a biosynthetic pathway for the production of malonyl-CoA or an intermediate or downstream product thereof and may also include additional elements for the expression and/or regulation of expression of these genes, e.g. promoter sequences.

[0073] In general, the recombinant microorganisms comprises at least one recombinant metabolic pathway that comprises a target enzyme and may further include a reduction in activity or reduction in expression of an enzyme in a competitive biosynthetic pathway. The pathway acts to modify a substrate or metabolic intermediate in the production of malonyl-CoA. The target enzyme is encoded by, and expressed from, a polynucleotide derived from a suitable biological source.

[0074] In some embodiments, the polynucleotide comprises a gene derived from a bacterial or yeast source and recombinantly engineered into a microorganism of the disclosure. In one embodiment, the disclosure provides a recombinant microorganism comprising elevated expression of at least one target enzyme as compared to a parental microorganism or encodes an enzyme not found in the parental organism. For example, in one embodiment, a microorganism is engineered to express or overexpress one or more polypeptides that convert pyruvate to malonyl-CoA or PEP to malonyl-CoA. As described herein a plurality of pathways can be engineered to provide for metabolic pathways to produce malonyl-CoA. For example, in a first embodiment, a recombinant microorganism is engineered to express an acetyl-CoA carboxylase. In a second embodiment, a recombinant microorganism can be engineered to expresses or overexpresses one or more enzymes that carries out a metabolic function selected from the group consisting of (i) converting PEP to Glyceraldehyde-3P; (ii) converting Glyceraldehyde-3P to DHAP; (iii) converting DHAP to glycerol-3P; (iv) converting
glycerol-3P to glycerol; (v) converting glycerol to 3-HPA; (vi) converting 3HPA to 3HP; (vii) converting 3HP to malonate semialdehyde; and (viii) converting malonate semialdehyde to malonyl-coA. In a third embodiment, a recombinant microorganism can be engineered to express or overexpress one or more enzymes that carries out a metabolic function selected from the group consisting of (i) converting PEP to oxaloacetate; (ii) converting oxaloacetate to malonate semialdehyde; and (iii) converting malonate semialdehyde to malonyl-coA. In a fourth embodiment, a recombinant microorganism can be engineered to express or overexpress one or more enzymes that carries out a metabolic function selected from the group consisting of (i) converting PEP to oxaloacetate; (ii) converting oxaloacetate to aspartate, (iii) converting aspartate to beta-alanine; (iv) converting beta-alanine to malonate semialdehyde; and (v) converting malonate semialdehyde to malonyl-coA. In a fifth embodiment, a recombinant microorganism can be engineered to express or overexpress one or more enzymes that carries out a metabolic function selected from the group consisting of (i) optionally converting PEP to pyruvate; (ii) converting pyruvate to alanine; (iii) converting alanine to beta-alanine; (iv) converting beta-alanine to malonate semialdehyde; and (v) converting malonate semialdehyde to malonyl-coA. In a sixth embodiment, a recombinant microorganism can be engineered to express or overexpress one or more enzymes that carries out a metabolic function selected from the group consisting of (i) converting PEP to pyruvate; (ii) converting pyruvate to lactate; (iii) converting lactate to lactoyl-CoA; (iv) converting lactoyl-CoA to acrylyl-CoA; (v) converting acrylyl-CoA to 3-hydroxypropionyl-CoA; (vi) 3-hydroxypropionyl-CoA to 3-hydroxypropionate; (vii) converting 3-hydroxypropionate to malonate semialdehyde; and (viii) converting malonate semialdehyde to malonyl-coA.

[0075] For example, in one embodiment, the microorganism comprises a photautotrophic, photoheterotrophic, chemotrophic, or autotrophic organism that is engineered to express or overexpress a trios phosphate isomerase (e.g., Tpi, or homolog thereof), glycerol-3-phosphate dehydrogenase (e.g., GpsA, or homolog thereof), glycerol-3-phosphatase (e.g., GPP, or homolog thereof), glycerol
dehydratase (e.g., DhaB123, or homolog thereof), glycerol dehydratase reactivase (GdrAB, or homolog thereof), aldehyde dehydrogenase (e.g., PuuC, or homolog thereof), malonate semialdehyde dehydrogenase (e.g., Msr, or homolog thereof) and malonyl-CoA reductase (e.g., Mcr, or homolog thereof). In yet a further embodiment, at least one of the foregoing enzymes or homologs thereof is recombinant engineered into the microorganism to produce malonyl-coA from PEP.

[0076] For example, in another embodiment, the recombinant microorganism is engineered to express or overexpress a phosphoenolpyruvate carboxylase (e.g., Ppc, or homolog thereof), an oxaloacetate 1-decarboxylase (e.g., Oad, or homolog thereof) and malonyl-CoA reductase (e.g., Mcr or homolog thereof). In yet a further embodiment, at least one of the foregoing enzymes or homologs thereof is recombinant engineered into the microorganism to produce malonyl-coA from PEP.

[0077] For example, in yet another embodiment, the recombinant microorganism is engineered to express or overexpress a phosphoenolpyruvase (e.g., Ppc or homolog thereof), an aspartate aminotransferase (e.g., AspC, or homolog thereof), an aspartate 1-decarboxylase (e.g., PanD, or homolog thereof) or a PLP-dependent aspartate 1-decarboxylase (e.g., AeADC, or homolog thereof), a beta-alanine aminotransferase (e.g., SkPYD4, or homolog thereof) and a malonyl-CoA reductase (e.g., Mcr, or homolog thereof). In yet a further embodiment, at least one of the foregoing enzymes or homologs thereof is recombinant engineered into the microorganism to produce malonyl-coA from PEP. In another embodiment, the recombinant microorganism is engineered to express or overexpress a pyruvate kinase (e.g., Pyk, or homolog thereof), an alanine aminotransferase (e.g., Aat, or homolog thereof), an alanine aminotransferase (e.g., Aam, or homolog thereof) and a malonyl-CoA reductase (e.g., Mcr, or homolog thereof). In yet a further embodiment, at least one of the foregoing enzymes or homologs thereof is recombinant engineered into the microorganism to produce malonyl-coA from PEP.

[0078] In another embodiment, the recombinant microorganism is engineered to express or overexpress a pyruvate kinase (e.g., Pyk,
or homolog thereof), a lactate dehydrogenase (e.g., Ldh, or homolog thereof), a lactoyl-CoA transferase (e.g., Pet, or homolog thereof), a propionyl-CoA synthase (e.g., Pes, or homolog thereof), a lactoyl-CoA dehydratase (e.g., Led, or homolog thereof), a hydroxypropionyl-CoA dehydratase (e.g., Hpd, or homolog thereof), a hydroxypropionyl-CoA hydrolase (e.g., Hph, or homolog thereof), a malonate semialdehyde dehydrogenase (e.g., Msr, or homolog thereof) and a malonyl-CoA reductase (e.g., Mcr, or homolog thereof). In yet a further embodiment, at least one of the foregoing enzymes or homologs thereof is recombinant engineered into the microorganism to produce malonyl-CoA from PEP.

[0079] Tables A-G provide information regarding the various enzymes and pathways described above and elsewhere herein including substrate specificity, products, and co-factors.

[0080] Table A. Synthetic malonyl-CoA biosynthesis pathways with enzymes available in nature

<table>
<thead>
<tr>
<th>Step</th>
<th>Enzyme</th>
<th>E.C. number</th>
<th>Substrate</th>
<th>Product</th>
<th>AG&lt;sup&gt;0&lt;/sup&gt;</th>
<th>AG&lt;sup&gt;'&lt;/sup&gt;</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phosphoenol pyruvate carboxylase</td>
<td>4.1.1.31</td>
<td>Phosphoenol-pyruvate + CO2</td>
<td>Oxaloacetate + phosphate</td>
<td>-43.2</td>
<td>-43.2</td>
<td>Irreversible</td>
</tr>
<tr>
<td>2</td>
<td>Aspartate aminotransferase</td>
<td>2.6.1.1</td>
<td>Oxaloacetate + glutamate</td>
<td>Aspartate + α-ketoglutarate</td>
<td>1.5</td>
<td>1.5</td>
<td>Reversible</td>
</tr>
<tr>
<td>3</td>
<td>Aspartate 1-decarboxylase</td>
<td>4.1.1.11</td>
<td>Aspartate</td>
<td>β-alanine + CO2</td>
<td>-19.9</td>
<td>-35.8</td>
<td>Irreversible</td>
</tr>
<tr>
<td>4</td>
<td>β-Alanine aminotransferase</td>
<td>2.6.1.19</td>
<td>β-alanine + α-ketoglutarate</td>
<td>Malonate semialdehyde + glutamate</td>
<td>9.8</td>
<td>11</td>
<td>Reversible</td>
</tr>
<tr>
<td>5</td>
<td>Malonyl-CoA reductase</td>
<td>1.2.1.75</td>
<td>Malonate semialdehyde + CoA + NADP+</td>
<td>Malonyl-CoA + NADPH</td>
<td>-14.9</td>
<td>-7.5</td>
<td>Reversible</td>
</tr>
</tbody>
</table>

Glycerol pathway

<table>
<thead>
<tr>
<th>Step</th>
<th>Enzyme</th>
<th>E.C. number</th>
<th>Substrate</th>
<th>Product</th>
<th>AG&lt;sup&gt;0&lt;/sup&gt;</th>
<th>AG&lt;sup&gt;'&lt;/sup&gt;</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Enolase</td>
<td>4.2.1.11</td>
<td>Phosphoenol-pyruvate + H2O</td>
<td>2-phosphoglycerate</td>
<td>2.0</td>
<td>3.3</td>
<td>Reversible</td>
</tr>
<tr>
<td>2</td>
<td>Phosphoglycerate mutase</td>
<td>5.4.2.1</td>
<td>2-phosphoglycerate</td>
<td>3-phosphoglycerate</td>
<td>-5.7</td>
<td>-5.9</td>
<td>Reversible</td>
</tr>
<tr>
<td>3</td>
<td>Phosphoglycerate kinase</td>
<td>2.7.2.3</td>
<td>3-phosphoglycerate + ATP</td>
<td>1.3-bis-phosphoglycerate + ADP + phosphate</td>
<td>7.8</td>
<td>8.1</td>
<td>Reversible</td>
</tr>
<tr>
<td></td>
<td>Reaction</td>
<td>Enzyme Name</td>
<td>EC Number</td>
<td>Reaction Type</td>
<td>ΔG₀° (kJ/mol)</td>
<td>ΔGₚ₀° (kJ/mol)</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>--------------------------------------------------------------------------</td>
<td>------------------------------------</td>
<td>-----------</td>
<td>---------------</td>
<td>---------------</td>
<td>----------------</td>
<td>---</td>
</tr>
<tr>
<td>4</td>
<td>Glycer-                        3-phosphate  de-                         hydrogenase</td>
<td>2.7.2.3</td>
<td></td>
<td>reversible</td>
<td>-7.8</td>
<td>-3.1</td>
<td>Reversible</td>
</tr>
<tr>
<td>5</td>
<td>Triose-                        phosphate isomerase</td>
<td>5.3.1.1</td>
<td></td>
<td>reversible</td>
<td>-7.7</td>
<td>-7.7</td>
<td>Reversible</td>
</tr>
<tr>
<td>6</td>
<td>Glycerol-3-                        phosphate de-                          hydrogenase</td>
<td>1.1.1.8</td>
<td></td>
<td>irreversible</td>
<td>-46.3</td>
<td>-43.3</td>
<td>Irreversible</td>
</tr>
<tr>
<td>7</td>
<td>Glycerol phosphatase</td>
<td>3.1.3.21</td>
<td></td>
<td>irreversible</td>
<td>1.1</td>
<td>-15.3</td>
<td>Irreversible</td>
</tr>
<tr>
<td>8</td>
<td>Glycerol dehydratase</td>
<td>4.2.1.30</td>
<td></td>
<td>irreversible</td>
<td>-36</td>
<td>-36</td>
<td>Irreversible</td>
</tr>
<tr>
<td>9</td>
<td>3-hydroxy-                        propion-phyde de-                          hydrogenase</td>
<td>1.2.1.30</td>
<td></td>
<td>irreversible</td>
<td>-41.5</td>
<td>-45.2</td>
<td>Irreversible</td>
</tr>
<tr>
<td>10</td>
<td>3-hydroxy-                        propionate de-                          hydrogenase</td>
<td>1.1.1.59</td>
<td></td>
<td>reversible</td>
<td>23.7</td>
<td>18.8</td>
<td>Reversible</td>
</tr>
<tr>
<td>11</td>
<td>Malonyl-CoA reductase</td>
<td>1.2.1.75</td>
<td></td>
<td>irreversible</td>
<td>-14.9</td>
<td>-7.5</td>
<td>Reversible</td>
</tr>
</tbody>
</table>

**Lactate pathway**

<table>
<thead>
<tr>
<th></th>
<th>Reaction</th>
<th>Enzyme Name</th>
<th>EC Number</th>
<th>Reaction Type</th>
<th>ΔG₀° (kJ/mol)</th>
<th>ΔGₚ₀° (kJ/mol)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pyruvate kinase</td>
<td>2.7.1.40</td>
<td></td>
<td>irreversible</td>
<td>-30.6</td>
<td>-29.3</td>
<td>Irreversible</td>
</tr>
<tr>
<td>2</td>
<td>Lactate de-                        hydrogenase</td>
<td>1.1.1.27</td>
<td></td>
<td>reversible</td>
<td>-20.2</td>
<td>-17.8</td>
<td>Reversible</td>
</tr>
<tr>
<td>3a</td>
<td>Propionyl-CoA transferase</td>
<td>2.8.3.1</td>
<td></td>
<td>reversible</td>
<td>0</td>
<td>0</td>
<td>Reversible</td>
</tr>
<tr>
<td>3b</td>
<td>Propionate-CoA ligase</td>
<td>6.2.1.17</td>
<td></td>
<td>irreversible</td>
<td>-10.5</td>
<td>-10.4</td>
<td>Reversible</td>
</tr>
<tr>
<td>4</td>
<td>lactoyl-CoA dehydratase</td>
<td>4.2.1.54</td>
<td></td>
<td>reversible</td>
<td>9.9</td>
<td>9.9</td>
<td>Reversible</td>
</tr>
<tr>
<td>5</td>
<td>3-hydroxy-                        propionyl-CoA dehydratase</td>
<td>4.2.1.116</td>
<td></td>
<td>irreversible</td>
<td>-2.4</td>
<td>-2.4</td>
<td>Reversible</td>
</tr>
<tr>
<td>6</td>
<td>3-hydroxy-                        propionyl-CoA</td>
<td>3.1.2.1</td>
<td></td>
<td>irreversible</td>
<td>-25.8</td>
<td>-27.1</td>
<td>Irreversible</td>
</tr>
<tr>
<td>Step</td>
<td>Enzyme</td>
<td>E.C number</td>
<td>Substrate</td>
<td>Product</td>
<td>( \Delta G^\circ )</td>
<td>( \Delta G' )</td>
<td>Reversibility</td>
</tr>
<tr>
<td>------</td>
<td>---------------------------------------</td>
<td>------------</td>
<td>-----------------------------------</td>
<td>------------------------------------------</td>
<td>---------------------</td>
<td>----------------</td>
<td>---------------</td>
</tr>
<tr>
<td>1</td>
<td>Pyruvate kinase</td>
<td>2.7.1.40</td>
<td>Phosphoenol-pyruvate + ADP + phosphate</td>
<td>Pyruvate + ATP</td>
<td>-30.6</td>
<td>-29.3</td>
<td>Irreversible</td>
</tr>
<tr>
<td>2</td>
<td>( \beta )-alanine aminotransferase</td>
<td>2.6.1.18</td>
<td>Pyruvate + ( \beta )-alanine</td>
<td>( \alpha )-alanine + malonate semialdehyde</td>
<td>9.8</td>
<td>9.8</td>
<td>Reversible</td>
</tr>
<tr>
<td>3</td>
<td>Alanine aminotransferase</td>
<td>---</td>
<td>( \alpha )-alanine</td>
<td>( \beta )-alanine</td>
<td>0.4</td>
<td>0.4</td>
<td>Reversible</td>
</tr>
<tr>
<td>4</td>
<td>Malonyl-CoA reductase</td>
<td>1.2.1.75</td>
<td>Malonate semialdehyde + CoA + NADP(^+)</td>
<td>Malonyl-CoA + NADPH</td>
<td>-14.9</td>
<td>-7.5</td>
<td>Reversible</td>
</tr>
</tbody>
</table>

**Oxaloacetate pathway**

<table>
<thead>
<tr>
<th>Step</th>
<th>Enzyme</th>
<th>E.C number</th>
<th>Substrate</th>
<th>Product</th>
<th>( \Delta G^\circ )</th>
<th>( \Delta G' )</th>
<th>Reversibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phosphoenol-pyruvate carboxylase</td>
<td>4.1.1.31</td>
<td>Phosphoenol-pyruvate + CO(_2)</td>
<td>Oxaloacetate + phosphate</td>
<td>-43.2</td>
<td>-43.2</td>
<td>Irreversible</td>
</tr>
<tr>
<td>2</td>
<td>Oxaloacetate</td>
<td>---</td>
<td>oxaloacetate</td>
<td>Malonate semialdehyde</td>
<td>-13.9</td>
<td>-29.7</td>
<td>Irreversible</td>
</tr>
<tr>
<td>3</td>
<td>Malonyl-CoA reductase</td>
<td>1.2.1.75</td>
<td>Malonate semialdehyde + CoA + NADP(^+)</td>
<td>Malonyl-CoA + NADPH</td>
<td>-14.9</td>
<td>-7.5</td>
<td>Reversible</td>
</tr>
</tbody>
</table>

**Table C: Enzymes for oxaloacetate dependent pathway.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Enzyme</th>
<th>E.C number</th>
<th>Substrate</th>
<th>Product</th>
<th>( \Delta G^\circ )</th>
<th>( \Delta G' )</th>
<th>Reversibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phosphoenol-pyruvate carboxylase</td>
<td>4.1.1.31</td>
<td>Phosphoenol-pyruvate + CO(_2)</td>
<td>Oxaloacetate + phosphate</td>
<td>-43.2</td>
<td>-43.2</td>
<td>Irreversible</td>
</tr>
<tr>
<td>2</td>
<td>Oxaloacetate</td>
<td>---</td>
<td>oxaloacetate</td>
<td>Malonate semialdehyde</td>
<td>-13.9</td>
<td>-29.7</td>
<td>Irreversible</td>
</tr>
<tr>
<td>3</td>
<td>Malonyl-CoA reductase</td>
<td>1.2.1.75</td>
<td>Malonate semialdehyde + CoA + NADP(^+)</td>
<td>Malonyl-CoA + NADPH</td>
<td>-14.9</td>
<td>-7.5</td>
<td>Reversible</td>
</tr>
</tbody>
</table>
### Table D: Enzymes for α-alanine dependent pathway.

<table>
<thead>
<tr>
<th>Step</th>
<th>Enzyme</th>
<th>E.C number</th>
<th>Substrate</th>
<th>Product</th>
<th>$\Delta G^{\circ}$</th>
<th>$\Delta G'$</th>
<th>Reversibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pyruvate kinase</td>
<td>2.7.1.40</td>
<td>Phosphoenolpyruvate + ADP + phosphate</td>
<td>Pyruvate + ATP</td>
<td>-30.6</td>
<td>-29.3</td>
<td>Irreversible</td>
</tr>
<tr>
<td>2</td>
<td>β-alanine aminotransferase</td>
<td>2.6.1.18</td>
<td>Pyruvate + β-alanine</td>
<td>α-alanine + malonate semialdehyde</td>
<td>9.8</td>
<td>9.8</td>
<td>Reversible</td>
</tr>
<tr>
<td>3</td>
<td>Alanine aminotransferase</td>
<td>---</td>
<td>α-alanine</td>
<td>β-alanine</td>
<td>0.4</td>
<td>0.4</td>
<td>Reversible</td>
</tr>
<tr>
<td>4</td>
<td>Malonyl-CoA reductase</td>
<td>1.2.1.75</td>
<td>Malonate semialdehyde + CoA + NADP$^+$</td>
<td>Malonyl-CoA + NADPH</td>
<td>-14.9</td>
<td>-7.5</td>
<td>Reversible</td>
</tr>
</tbody>
</table>

### Table E: Enzymes for lactate dependent pathway.

<table>
<thead>
<tr>
<th>Step</th>
<th>Enzyme</th>
<th>E.C number</th>
<th>Substrate</th>
<th>Product</th>
<th>$\Delta G^{\circ}$</th>
<th>$\Delta G'$</th>
<th>Reversibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pyruvate kinase</td>
<td>2.7.1.40</td>
<td>Phosphoenolpyruvate + ADP + phosphate</td>
<td>Pyruvate + ATP</td>
<td>-30.6</td>
<td>-29.3</td>
<td>Irreversible</td>
</tr>
<tr>
<td>2</td>
<td>Lactate dehydrogenase</td>
<td>1.1.1.27</td>
<td>Pyruvate + NADH</td>
<td>Lactate + NAD</td>
<td>-20.2</td>
<td>-17.8</td>
<td>Reversible</td>
</tr>
<tr>
<td>3a</td>
<td>Propionyl-CoA transferase</td>
<td>2.8.3.1</td>
<td>Lactate + Propionyl-CoA</td>
<td>Lactoyl-CoA + Propionate</td>
<td>0</td>
<td>0</td>
<td>Reversible</td>
</tr>
<tr>
<td>3b</td>
<td>Propionate-CoA ligase</td>
<td>6.2.1.17</td>
<td>Propionate + ATP + CoA</td>
<td>Propionyl-CoA + AMP + PPI</td>
<td>-10.5</td>
<td>-10.4</td>
<td>Reversible</td>
</tr>
<tr>
<td>4</td>
<td>Lactoyl-CoA dehydratase</td>
<td>4.2.1.54</td>
<td>Lactoyl-CoA</td>
<td>Acryloyl-CoA + H$_2$O</td>
<td>9.9</td>
<td>9.9</td>
<td>Reversible</td>
</tr>
<tr>
<td>5</td>
<td>3-hydroxypropionyl-CoA dehydratase</td>
<td>4.2.1.116</td>
<td>Acryloyl-CoA + H$_2$O</td>
<td>3-hydroxypropionyl-CoA</td>
<td>-2.4</td>
<td>-2.4</td>
<td>Reversible</td>
</tr>
<tr>
<td>6</td>
<td>3-hydroxypropionyl-CoA hydrolyase</td>
<td>3.1.2.---</td>
<td>3-hydroxypropionyl-CoA + H$_2$O</td>
<td>3-hydroxypropionate + CoA</td>
<td>-25.8</td>
<td>-27.1</td>
<td>Irreversible</td>
</tr>
<tr>
<td>7</td>
<td>3-hydroxypropionate dehydrogenase</td>
<td>1.1.1.59</td>
<td>3-hydroxypropionate + NADP</td>
<td>Malonate semialdehyde + NADPH</td>
<td>23.7</td>
<td>18.8</td>
<td>Reversible</td>
</tr>
<tr>
<td>8</td>
<td>Malonyl-CoA reductase</td>
<td>1.2.1.75</td>
<td>Malonate semialdehyde + CoA + NADP$^+$</td>
<td>Malonyl-CoA + NADPH</td>
<td>-14.9</td>
<td>-7.5</td>
<td>Reversible</td>
</tr>
</tbody>
</table>
### Table F: Enzymes for glycerol dependent pathway.

<table>
<thead>
<tr>
<th>Step</th>
<th>Enzyme</th>
<th>E.C number</th>
<th>Substrate</th>
<th>Product</th>
<th>AG°</th>
<th>AG'</th>
<th>Reversibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Enolase</td>
<td>4.2.1.11</td>
<td>Phosphoenolpyruvate + H₂O</td>
<td>2-phosphoglycerate</td>
<td>2.0</td>
<td>3.3</td>
<td>Reversible</td>
</tr>
<tr>
<td>2</td>
<td>Phosphoglycerate mutase</td>
<td>5.4.2.1</td>
<td>2-phosphoglycerate</td>
<td>3-phosphoglycerate</td>
<td>-5.7</td>
<td>-5.9</td>
<td>Reversible</td>
</tr>
<tr>
<td>3</td>
<td>Phosphoglycerate kinase</td>
<td>2.7.2.3</td>
<td>3-phosphoglycerate + ATP</td>
<td>1,3-bisphosphoglycerate + ADP + phosphate</td>
<td>7.8</td>
<td>8.1</td>
<td>Reversible</td>
</tr>
<tr>
<td>4</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>2.7.2.3</td>
<td>1,3-bisphosphoglycerate + NADH</td>
<td>glyceraldehyde 3-phosphate + NAD + phosphate</td>
<td>-7.8</td>
<td>-3.1</td>
<td>Reversible</td>
</tr>
<tr>
<td>5</td>
<td>Triosephosphate isomerase</td>
<td>5.3.1.1</td>
<td>glyceraldehyde 3-phosphate</td>
<td>Dihydroxyacetone phosphate</td>
<td>-7.7</td>
<td>-7.7</td>
<td>Reversible</td>
</tr>
<tr>
<td>6</td>
<td>Glycerol-3-phosphate dehydrogenase</td>
<td>1.1.1.8</td>
<td>Dihydroxyacetone phosphate + NADH</td>
<td>Glycerol-3-phosphate + NAD</td>
<td>-46.3</td>
<td>-43.3</td>
<td>Irreversible</td>
</tr>
<tr>
<td>7</td>
<td>Glycerol phosphatase</td>
<td>3.1.3.21</td>
<td>Glycerol-3-phosphate + NADH</td>
<td>Glycerol + phosphate</td>
<td>1.1</td>
<td>-15.3</td>
<td>Irreversible</td>
</tr>
<tr>
<td>8</td>
<td>Glycerol dehydratase</td>
<td>4.2.1.30</td>
<td>Glycerol</td>
<td>3-hydroxypropionate dehydrogenase + H₂O</td>
<td>-36</td>
<td>-36</td>
<td>Irreversible</td>
</tr>
<tr>
<td>9</td>
<td>3-hydroxypropion-aldehyde dehydrogenase</td>
<td>1.2.1.-</td>
<td>3-hydroxypropion-aldehyde + NAD</td>
<td>3-hydroxypropionate + NAD</td>
<td>-41.5</td>
<td>-45.2</td>
<td>Irreversible</td>
</tr>
<tr>
<td>10</td>
<td>3-hydroxypropionate dehydrogenase</td>
<td>1.1.1.59</td>
<td>3-hydroxypropionate + NADP</td>
<td>Malonate semialdehyde + NADPH</td>
<td>23.7</td>
<td>18.8</td>
<td>Reversible</td>
</tr>
<tr>
<td>11</td>
<td>Malonyl-CoA reductase</td>
<td>1.2.1.75</td>
<td>Malonate semialdehyde + CoA + NADPH</td>
<td>Malonyl-CoA + NADPH</td>
<td>-14.9</td>
<td>-7.5</td>
<td>Reversible</td>
</tr>
</tbody>
</table>

### Table G: Enzymes for aspartate dependent pathway.

<table>
<thead>
<tr>
<th>Step</th>
<th>Enzyme</th>
<th>E.C number</th>
<th>Substrate</th>
<th>Product</th>
<th>AG°</th>
<th>AG'</th>
<th>Reversibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phosphoenolpyruvate carboxylase</td>
<td>4.1.1.31</td>
<td>Phosphoenolpyruvate + CO₂</td>
<td>Oxaloacetate + phosphate</td>
<td>-43.2</td>
<td>-43.2</td>
<td>Irreversible</td>
</tr>
<tr>
<td>2</td>
<td>Aspartate aminotransferase</td>
<td>2.6.1.1</td>
<td>Oxaloacetate + glutamate</td>
<td>Aspartate + α-ketoglutarate</td>
<td>1.5</td>
<td>1.5</td>
<td>Reversible</td>
</tr>
<tr>
<td>3</td>
<td>Aspartate 1-decarboxylase</td>
<td>4.1.1.11</td>
<td>Aspartate</td>
<td>β-alanine + CO₂</td>
<td>-19.9</td>
<td>-35.8</td>
<td>Irreversible</td>
</tr>
<tr>
<td>4</td>
<td>β-Alanine aminotransferase</td>
<td>2.6.1.19</td>
<td>β-alanine + α-ketoglutarate</td>
<td>Malonate semialdehyde + glutamate</td>
<td>9.8</td>
<td>11</td>
<td>Reversible</td>
</tr>
</tbody>
</table>
[0087] In another or further embodiment, the microorganism comprises a reduction, disruption or knockout of at least one gene encoding an enzyme that competes with a metabolite necessary for the production of a malonyl-CoA or a downstream product of malonyl-CoA metabolism.

[0088] As described above, in one embodiment, a recombinant microorganism of the disclosure comprise expression of a heterologous aspartate aminotransferase or elevated expression of an endogenous aspartate aminotransferase. As used herein, the term "aspartate aminotransferase" (aspartate aminotransferase (AspC, EC 2.6.1.1)) means an enzyme that catalyzes the conversion of oxaloacetate and glutamate to aspartate and alpha-ketoglutarate. In one embodiment, aspartate aminotransferase is derived from E. coli. For example, an aspartate aminotransferase can comprise a nucleotide sequence or codon optimized sequence of SEQ ID NO:1, which encodes the amino acid sequence of SEQ ID NO:2. In another embodiment, an aspartate amino transferase can include polypeptides having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:2 and having aspartate aminotransferase activity.

[0089] As described above, in another or further embodiment, a recombinant microorganism of the disclosure comprise expression of a heterologous aspartate 1-decarboxylase or elevated expression of an endogenous aspartate 1-decarboxylase. As used herein, the term "aspartate 1-decarboxylase" (aspartate 1-decarboxylase (PanD, EC 4.1.1.11)) means an enzyme that catalyzes the conversion of aspartate to beta-alanine and CO2. In one embodiment, aspartate 1-decarboxylase is derived from Corynebacterium glutamicum. For example, an aspartate 1-decarboxylase can comprise a nucleotide sequence or codon optimized sequence of SEQ ID NO:3, which encodes the amino acid sequence of SEQ ID NO:4. In another embodiment, an aspartate 1-decarboxylase can include polypeptides having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:4 and having aspartate 1-decarboxylase activity.
As described above, in another or further embodiment, a recombinant microorganism of the disclosure comprise expression of a heterologous PLP-dependent aspartate 1-decarboxylase or elevated expression of an endogenous PLP-dependent aspartate 1-decarboxylase. As used herein, the term "PLP-dependent aspartate 1-decarboxylase" (PLP-dependent aspartate 1-decarboxylase (ADC, EC 4.1.1.11)) means an enzyme that catalyzes the conversion of aspartate to beta-alanine and CO2. In one embodiment, PLP-dependent aspartate 1-decarboxylase is derived from Aedes aegypti. For example, a PLP-dependent aspartate 1-decarboxylase can comprise a codon optimized nucleotide sequence of SEQ ID NO:5, which encodes the amino acid sequence of SEQ ID NO:6. In another embodiment, a PLP-dependent aspartate 1-decarboxylase can include polypeptides having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:6 and having PLP-dependent aspartate 1-decarboxylase activity.

As described above, in another embodiment or further embodiment, a recombinant microorganism of the disclosure comprise expression of a heterologous beta-alanine aminotransferase or elevated expression of an endogenous beta-alanine aminotransferase. As used herein, the term "beta-alanine aminotransferase" (beta-alanine aminotransferase (SKPYD4, EC 2.6.1.19)) means an enzyme that catalyzes the conversion of beta-alanine and alphaketoglutarate to malonate semialdehyde and glutamate. In one embodiment, beta-alanine aminotransferase is derived from Saccharomyces kluveri. For example, a beta-alanine aminotransferase can comprise a codon optimized nucleotide sequence of SEQ ID NO:7, which encodes the amino acid sequence of SEQ ID NO:8. In another embodiment, a beta-alanine aminotransferase can include polypeptides having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:8 and having beta-alanine aminotransferase activity.

As described above, in another embodiment or further embodiment, a recombinant microorganism of the disclosure comprise expression of a heterologous malonyl-CoA reductase or elevated expression of an endogenous malonyl-CoA reductase. As used herein, the term "malonyl-CoA reductase" (malonyl-CoA reductase (Mcr, EC 1.2.1.75)) means the enzyme that catalyzes the conversion of malonate semialdehyde and CoA and NADP+ to malonyl-CoA and NADPH. In
one embodiment, malonyl-CoA reductase is derived from *Sulfolobus tokodaii*. For example, a malonyl-CoA reductase can comprise a codon optimized nucleotide sequence of SEQ ID NO: 9, which encodes the amino acid sequence of SEQ ID NO: 10. In another embodiment, a malonyl-CoA reductase can include polypeptide having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO: 10 and having malonyl-CoA reductase activity.

[0093] As described above, in another embodiment or further embodiment, a recombinant microorganism of the disclosure comprise expression of a heterologous phosphoenolpyruvate carboxylase or elevated expression of an endogenous phosphoenolpyruvate carboxylase. As used herein, the term "phosphoenolpyruvate carboxylase" (phosphoenolpyruvate carboxylase (Ppc, EC 4.1.1.31)) means the enzyme that catalyzes the conversion of phosphoenolpyruvate and CO2 to oxaloacetate. In one embodiment, phosphoenolpyruvate carboxylase is derived from *E. coli*. For example, a phosphoenolpyruvate carboxylase can comprise a nucleotide sequence or codon optimized sequence of SEQ ID NO: 11, which encodes the amino acid sequence of SEQ ID NO: 12. In another embodiment, a phosphoenolpyruvate carboxylase can include polypeptide having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO: 12 and having phosphoenolpyruvate carboxylase activity.

[0094] An alternative enzyme for converting phosphoenolpyruvate to oxaloacetate is PEP carboxykinase, which simultaneously forms an ATP while carboxylating PEP. In most organisms PEP carboxykinase serves a gluconeogenic function and converts oxaloacetate to PEP at the expense of one ATP. *S. cerevisiae* is one such organism whose native PEP carboxykinase, PCK1, serves a gluconeogenic role (Valdes-Hevia et al., FEBS Lett. 258:313-316 (1989)). *E. coli* is another such organism, as the role of PEP carboxykinase in producing oxaloacetate is believed to be minor when compared to PEP carboxylyase, which does not form ATP, possibly due to the higher *K*<sub>m</sub> for bicarbonate of PEP carboxykinase (Kim et al., Appl. Environ. Microbiol. 70:1238-1241 (2004)). Nevertheless, activity of the native *E. coli* PEP carboxykinase from PEP towards oxaloacetate has been demonstrated in ppc mutants of *E. coli* K-12 (Kwon et al., J. Microbiol. Biotechnol. 16:1448-1452 (2006)). These strains exhibited no growth defects and
had increased succinate production at high NaHCO3 concentrations. Alternately, the activity of the E. coli enzyme in the oxaloacetate-consuming direction can be reduced by introducing an amino acid substitution at the oxaloacetate binding site (pck R65Q) (Cotelesage et al., Int. J. Biochem. Cell Biol. 39:1204-1210 (2007)). Mutant strains of E. coli can adopt Pck as the dominant CO2-fixing enzyme following adaptive evolution (Zhang et al., supra, 2009). In some organisms, particularly rumen bacteria, PEP carboxykinase is quite efficient in producing oxaloacetate from PEP and generating ATP.

Examples of PEP carboxykinase genes that have been cloned into E. coli include those from Mannheimia succiniciproducens (Lee et al., Biotechnol. Bioprocess Eng. 7:95-99 (2002)), Anaerobiospirillum succiniciproducens (Laivenieks et al., Appl. Environ. Microbiol. 63:2273-2280 (1997), and Actinobacillus succinogenes (Kim et al. supra). The PEPCK enzyme from Megathyrsus maximus has a low Km for CO2, a substrate thought to be rate-limiting in the E. coli enzyme (Chen et al., Plant Physiol 128:160-164 (2002); Cotelesage et al., Int. J Biochem. Cell Biol. 39:1204-1210 (2007)). The PEP carboxykinase enzyme of Haemophilus influenza is effective at forming oxaloacetate from PEP.

[0095] As described above, in another embodiment or further embodiment, a recombinant microorganism of the disclosure comprise expression of a heterologous pyruvate kinase or elevated expression of an endogenous pyruvate kinase. As used herein, the term "pyruvate kinase" (pyruvate kinase (Pyk, EC 2.7.1.40)) means the enzyme that catalyzes the conversion of phosphoenolpyruvate and ADP to pyruvate and ATP. In one embodiment, pyruvate kinase is derived from E. coli. For example, a pyruvate kinase can comprise a nucleotide sequence or codon optimized sequence of SEQ ID NO:13, which encodes the amino acid sequence of SEQ ID NO:14. In another embodiment, a pyruvate kinase can include polypeptide having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:14 and having pyruvate kinase activity.

[0096] As described above, in another embodiment or further embodiment, a recombinant microorganism of the disclosure comprise expression of a heterologous beta alanine aminotransferase or elevated expression of an endogenous beta alanine aminotransferase.
As used herein, the term "beta alanine aminotransferase" (beta alanine aminotransferase (Baat, EC 2.6.1.18)) means the enzyme that catalyzes the conversion of pyruvate and beta-alanine to alanine and malonate semialdehyde. In one embodiment, a beta alanine aminotransferase is derived from Streptomyces avermitilis. For example, a beta alanine aminotransferase can comprise a nucleotide sequence or codon optimized sequence of SEQ ID NO: 15, which encodes the amino acid sequence of SEQ ID NO: 16. In another embodiment, a beta alanine aminotransferase can include polypeptide having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO: 16 and having beta alanine aminotransferase activity.

[0097] As described above, in another embodiment or further embodiment, a recombinant microorganism of the disclosure comprise expression of a heterologous alanine aminomutase or elevated expression of an endogenous alanine aminomutase. As used herein, the term "alanine aminomutase" (alanine aminomutase (Aam) means the enzyme that catalyzes the conversion of alanine to beta-alanine.

Various alanine aminomutases are known in the art and include engineered alanine aminomutases (see, e.g., U.S. Patent Publication No. 20120040437A1, which is incorporated herein by reference).

[0098] As described above, in another embodiment or further embodiment, a recombinant microorganism of the disclosure comprise expression of a heterologous lactate dehydrogenase or elevated expression of an endogenous lactate dehydrogenase. As used herein, the term "lactate dehydrogenase" (lactate dehydrogenase (Ldh, EC 1.1.1.27)) means the enzyme that catalyzes the conversion of pyruvate and NADH to lactate. Lactate Dehydrogenase (also referred to as D-lactate dehydrogenase and fermentive dehydrogenase) is encoded in E.coli by ldhA and catalyzes the NADH-dependent conversion of pyruvate to D-lactate. LdhA homologs and variants are known. In fact there are currently 1664 bacterial lactate dehydrogenases available through NCBI. For example, such homologs and variants include, for example, D-lactate dehydrogenase (D-LDH) (Fermentative lactate dehydrogenase) gi|11730102|sp|P52643.1|LDHD_ECOLI (1730102); D-lactate dehydrogenase gi|11049265|gb|IAAB51772.1| (1049265); D-lactate dehydrogenase (Escherichia coli APEC 01) gi|117623655|ref|YP_852568.1|(117623655);
D-lactate dehydrogenase (Escherichia coli CFT073)
gi 6247689 Ref NP_753729.1 (26247689); D-lactate dehydrogenase
(Escherichia coli 0157:H7 EDL933)
gi 15801748 Ref NP_287766.1 (15801748); D-lactate dehydrogenase
(Escherichia coli APEC 01) gi|115512779|gb|ABJO0584.1|(115512779);
D-lactate dehydrogenase (Escherichia coli CFT073)
gi 6108091 Ljb|AA80291.1|AE01 6760_150 (26108091); fermentative D-
lactate dehydrogenase, NAD-dependent (Escherichia coli K12)
gi 16129341 Ref NP_415898.1 (16129341); fermentative D-lactate
dehydrogenase, NAD-dependent (Escherichia coli UTI89)
gi 91210646 Ref NP_540632.1 (91210646); fermentative D-lactate
dehydrogenase, NAD-dependent (Escherichia coli K12)
gi 1787645 Ljb|AAC74462.1 (1787645); fermentative D-lactate
dehydrogenase, NAD-dependent (Escherichia coli W3110)
gi 89108227 Ref NP_002007.1 (89108227); fermentative D-lactate
dehydrogenase, NAD-dependent (Escherichia coli W3110)
gi 1742259 Ljb|EAA14990.1 (1742259); fermentative D-lactate
dehydrogenase, NAD-dependent (Escherichia coli UTI89)
gi 91072220 Ljb|DBE07101.1 (91072220); fermentative D-lactate
dehydrogenase, NAD-dependent (Escherichia coli 0157:H7 EDL933)
gi 2515320 |gb|AAG56380.1|AE005366_6 (12515320); fermentative D-
lactate dehydrogenase (Escherichia coli 0157:H7 str. Sakai)
gi 13361468 Ljb|EAB35425.1 (13361468); COG1052: Lactate
dehydrogenase and related dehydrogenases (Escherichia coli 101-1)
gi 83588593 Ref EP_00927217.1 (83588593); COG1052: Lactate
dehydrogenase and related dehydrogenases (Escherichia coli 53638)
gi 75515985 Ref EP_00738103.1 (75515985); COG1052: Lactate
dehydrogenase and related dehydrogenases (Escherichia coli E22)
gi 5260157 Ref EP_00731425.1 (75260157); COG1052: Lactate
dehydrogenase and related dehydrogenases (Escherichia coli Fl1)
gi 5242656 Ref EP_00726400.1 (75242656); COG1052: Lactate
dehydrogenase and related dehydrogenases (Escherichia coli E110019)
gi 5237491 Ref EP_00721524.1 (75237491); COG1052: Lactate
dehydrogenase and related dehydrogenases (Escherichia coli B7A)
gi 5231601 Ref EP_00717959.1 (75231601); and COG1052: Lactate
dehydrogenase and related dehydrogenases (Escherichia coli B171)
gi 5211308 Ref EP_00711407.1 (75211308), each sequence associated
with the accession number is incorporated herein by reference in its entirety

[0099] As described above, in another embodiment or further embodiment, a recombinant microorganism of the disclosure comprise expression of a heterologous triose phosphate isomerase or elevated expression of an endogenous triose phosphate isomerase. In addition to the foregoing, the terms "triose phosphate isomerase" or "Tpi" refer to proteins that are capable of catalyzing the formation of dihydroxyacetone phosphate from glyceraldehyde-3-phosphate, and which share at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or greater sequence identity, or at least about 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or greater sequence similarity, as calculated by NCBI BLAST, using default parameters, to SEQ ID NO:18. Additional homologs include: 

Rattus norvegicus AAA42278.1 having 45% identity to SEQ ID NO:18; 
Homo sapiens AAH17917.1 having 45% identity to SEQ ID NO:18; 
Bacillus subtilis BEST7613 NP_391272.1 having 40% identity to SEQ ID NO:18; 
Synechococcus elongatus PCC 6301 YP_171000.1 having 40% identity to SEQ ID NO:18; and 
Salmonella enterica subsp. enterica serovar Typhi str. AG3 ZP_06540375.1 having 98% identity to SEQ ID NO:18. The sequences associated with the foregoing accession numbers are incorporated herein by reference.

[00100] As described above, in another embodiment or further embodiment, a recombinant microorganism of the disclosure comprise expression of a heterologous glycerol-3-phosphate dehydrogenase or elevated expression of an endogenous glycerol-3-phosphate dehydrogenase. As used herein, "glycerol-3-phosphate dehydrogenase" or "GpsA" refer to an enzyme that converts DHAP and NADH to glycerol-3-phosphate. In one embodiment, the gpsA gene is of prokaryotic origin. In another embodiment, the gpsA gene is of bacterial origin. Exemplary embodiments of gpsA genes include, but are not limited to, those originating from E. coli (SEQ ID NO: 19). Other homologs can be found in Shigella flexneri, Salmonella typhimurium, Salmonella enterica, Yersinia pestis, Yersinia pseudotuberculosis, Serratia marcescens, Photorhabdus luminescens, Erwinia carotovora or conservative substitutions thereof. For example, a glycerol-3-phosphate dehydrogenase can comprise a
nucleotide sequence or codon optimized sequence of SEQ ID NO: 19, which encodes the amino acid sequence of SEQ ID NO: 20. In another embodiment, a glycerol-3-phosphate dehydrogenase can include polypeptide having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO: 20 and having glycerol-3-phosphate activity.

[00101] As described above, in another embodiment or further embodiment, a recombinant microorganism of the disclosure comprise expression of a heterologous glycerol-3-phosphatase or elevated expression of an endogenous glycerol-3-phosphatase. As used herein, "glycerol-3-phosphatase" or "GPP" refers to an enzyme that converts glycerol-3-phosphate to glycerol. For example, a "glycerol-3-phosphatase" or "sn-glycerol-3-phosphatase" or "α, 1-glycerol phosphatase" or "G3P phosphatase" refer to the polypeptide (s) responsible for an enzyme activity that is capable of catalyzing the conversion of glycerol-3-phosphate to glycerol. For example, a glycerol-3-phosphatase can comprise a nucleotide sequence or codon optimized sequence of SEQ ID NO: 21, which encodes the amino acid sequence of SEQ ID NO: 22. In another embodiment, a glycerol-3-phosphatase can include polypeptide having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO: 22 and having glycerol-3-phosphatase activity.

[00102] As described above, in another embodiment or further embodiment, a recombinant microorganism of the disclosure comprise expression of a heterologous aldehyde dehydrogenase or elevated expression of an endogenous aldehyde dehydrogenase. As used herein, the term "aldehyde dehydrogenase" (aldehyde dehydrogenase \((P\text{U}\text{U}\text{C}, \text{EC} 1.2.1.-)) means the enzyme that catalyzes the conversion of 3-hydroxypropionatealdehyde to 3-hydroxypropionate. In one embodiment, aldehyde dehydrogenase is derived from \(E\text{. coli}\). For example, an aldehyde dehydrogenase can comprise a nucleotide sequence or codon optimized sequence of SEQ ID NO: 23, which encodes the amino acid sequence of SEQ ID NO: 24. In another embodiment, a phosphoenolpyruvate carboxylase can include polypeptide having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO: 24 and having aldehyde dehydrogenase activity.

[00103] In another embodiment, recombinant microorganisms are described that are capable of metabolizing a carbon source for
producing malonyl-CoA at a yield of at least 4% of theoretical, and, in some cases, a yield of over 50% or more (e.g., 60%, 70%, 80% or 90%) of theoretical. As used herein, the term "yield" refers to the molar yield. For example, the yield equals 100% when one mole of glucose is converted to one mole of malonyl-CoA. In particular, the term "yield" is defined as the mole of product obtained per mole of carbon source monomer and may be expressed as percent. Unless otherwise noted, yield is expressed as a percentage of the theoretical yield. "Theoretical yield" is defined as the maximum moles of product that can be generated per a given mole of substrate as dictated by the stoichiometry of the metabolic pathway used to make the product. In one embodiment, the yield is at least 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12 or more. In another example, the yield of a recombinant microorganism can be from 5% to 90% of theoretical.

[00104] In another embodiment, the disclosure provides a culture of recombinant microorganisms of the disclosure comprising a population that is substantially homogenous (e.g., from about 70-100% homogenous). In another embodiment, a culture can comprise a combination of microorganism each having distinct biosynthetic pathways that produced metabolites that can be used by at least one other microorganism in culture leading to the production of malonyl-CoA or a product derived therefrom. In these embodiments, at least one "population" of recombinant microorganism comprises the ability to make more malonyl-CoA compared to the same microorganism that has not been recombinantly engineered.

[00105] The disclosure also provides an in vitro method of producing malonyl-CoA and chemicals and biofuels that use malonyl-CoA as a substrate. In this embodiment, of the disclosure an in vitro metabolic pathway can be obtained in a number of ways. In one embodiment, the enzymes of the any of the pathways described herein (see, e.g., Figures 1, 3, 4, 5, and 6), are purchased and mixed in a suitable buffer and a suitable substrate is added and incubated under conditions suitable for malonyl-CoA production. In some embodiments, the enzyme can be bound to a support or expressed in a phage display or other surface expression system and, for example, fixed in a fluid pathway corresponding to points in the metabolic pathway. In another embodiment, a pathway is partially cloned and
expressed in a recombinant microorganism of the disclosure and then the cells are disrupted and the necessary addition enzymes and substrates are added. In a third method, the full pathways of any of the malonyl-CoA production pathways described herein are cloned into a recombinant microorganism. The microorganism is then cultured to express the polypeptides and the cells are disrupted. The disrupted milieu can then be used directly or the polypeptide for the pathway additionally purified.

[00106] The accession numbers for various genes, homologs and variants (and EC enzyme reference numbers) useful in the generation of recombinant microorganism described herein can be identified using the information in Tables A-G. It is to be understood that homologs and variants described herein are exemplary and non-limiting. Additional homologs, variants and sequences are available to those of skill in the art using various databases including, for example, the National Center for Biotechnology Information (NCBI) access to which is available on the World-Wide-Web.

[00107] The disclosure identifies genes useful in the methods, compositions and organisms of the disclosure; however it will be recognized that absolute identity to such genes is not necessary. For example, changes in a particular gene or polynucleotide comprising a sequence encoding a polypeptide or enzyme can be performed and screened for activity. Typically such changes comprise conservative mutation and silent mutations. Such modified or mutated polynucleotides and polypeptides can be screened for expression of a functional enzyme activity using methods known in the art.

[00108] As will be understood by those of skill in the art, it can be advantageous to modify a coding sequence to enhance its expression in a particular host. The genetic code is redundant with 64 possible codons, but most organisms typically use a subset of these codons. The codons that are utilized most often in a species are called optimal codons, and those not utilized very often are classified as rare or low-usage codons. Codons can be substituted to reflect the preferred codon usage of the host, a process sometimes called "codon optimization" or "controlling for species codon bias."
[00109] Optimized coding sequences containing codons preferred by a particular prokaryotic or eukaryotic host (see also, Murray et al. (1989) Nucl. Acids Res. 17:477-508) can be prepared, for example, to increase the rate of translation or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, as compared with transcripts produced from a non-optimized sequence. Translation stop codons can also be modified to reflect host preference. For example, typical stop codons for S. cerevisiae and mammals are UAA and UGA, respectively. The typical stop codon for monocotyledonous plants is UGA, whereas insects and E. coli commonly use UAA as the stop codon (Dalphin et al. (1996) Nucl. Acids Res. 24: 216-218). Methodology for optimizing a nucleotide sequence for expression in a plant is provided, for example, in U.S. Pat. No. 6,015,891, and the references cited therein.

[00110] Those of skill in the art will recognize that, due to the degenerate nature of the genetic code, a variety of DNA compounds differing in their nucleotide sequences can be used to encode a given enzyme of the disclosure. The native DNA sequence encoding the biosynthetic enzymes described above are referenced herein merely to illustrate an embodiment of the disclosure, and the disclosure includes DNA compounds of any sequence that encode the amino acid sequences of the polypeptides and proteins of the enzymes utilized in the methods of the disclosure. In similar fashion, a polypeptide can typically tolerate one or more amino acid substitutions, deletions, and insertions in its amino acid sequence without loss or significant loss of a desired activity. The disclosure includes such polypeptides with different amino acid sequences than the specific proteins described herein so long as they modified or variant polypeptides have the enzymatic anabolic or catabolic activity of the reference polypeptide. Furthermore, the amino acid sequences encoded by the DNA sequences shown herein merely illustrate embodiments of the disclosure.

[00111] In addition, homologs of enzymes useful for generating metabolites are encompassed by the microorganisms and methods provided herein. The term "homologs" used with respect to an original enzyme or gene of a first family or species refers to distinct enzymes or genes of a second family or species which are
determined by functional, structural or genomic analyses to be an enzyme or gene of the second family or species which corresponds to the original enzyme or gene of the first family or species. Most often, homologs will have functional, structural or genomic similarities. Techniques are known by which homologs of an enzyme or gene can readily be cloned using genetic probes and PCR. Identity of cloned sequences as homolog can be confirmed using functional assays and/or by genomic mapping of the genes.

[00112] A protein has "homology" or is "homologous" to a second protein if the nucleic acid sequence that encodes the protein has a similar sequence to the nucleic acid sequence that encodes the second protein. Alternatively, a protein has homology to a second protein if the two proteins have "similar" amino acid sequences. (Thus, the term "homologous proteins" is defined to mean that the two proteins have similar amino acid sequences).

[00113] As used herein, two proteins (or a region of the proteins) are substantially homologous when the amino acid sequences have at least about 30%, 40%, 50% 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity. To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In one embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, typically at least 40%, more typically at least 50%, even more typically at least 60%, and even more typically at least 70%, 80%, 90%, 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the
sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

[00114] When "homologous" is used in reference to proteins or peptides, it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions. A "conservative amino acid substitution" is one in which an amino acid residue is substituted by another amino acid residue having a side chain (R group) with similar chemical properties (e.g., charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of a protein. In cases where two or more amino acid sequences differ from each other by conservative substitutions, the percent sequence identity or degree of homology may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the art (see, e.g., Pearson et al., 1994, hereby incorporated herein by reference).

[00115] A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). The following six groups each contain amino acids that are conservative substitutions for one another: 1) Serine (S), Threonine (T); 2) Aspartic Acid (D), Glutamic Acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Alanine (A), Valine (V), and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).
Sequence homology for polypeptides, which can also be referred to as percent sequence identity, is typically measured using sequence analysis software. See, e.g., the Sequence Analysis Software Package of the Genetics Computer Group (GCG), University of Wisconsin Biotechnology Center, 910 University Avenue, Madison, Wis. 53705. Protein analysis software matches similar sequences using measure of homology assigned to various substitutions, deletions and other modifications, including conservative amino acid substitutions. For instance, GCG contains programs such as "Gap" and "Bestfit" which can be used with default parameters to determine sequence homology or sequence identity between closely related polypeptides, such as homologous polypeptides from different species of organisms or between a wild type protein and a mutein thereof. See, e.g., GCG Version 6.1.

A typical algorithm used comparing a molecule sequence to a database containing a large number of sequences from different organisms is the computer program BLAST (Altschul, 1990; Gish, 1993; Madden, 1996; Altschul, 1997; Zhang, 1997), especially blastp or tblastn (Altschul, 1997). Typical parameters for BLASTp are: Expectation value: 10 (default); Filter: seg (default); Cost to open a gap: 11 (default); Cost to extend a gap: 1 (default); Max. alignments: 100 (default); Word size: 11 (default); No. of descriptions: 100 (default); Penalty Matrix: BLOWSUM62.

When searching a database containing sequences from a large number of different organisms, it is typical to compare amino acid sequences. Database searching using amino acid sequences can be measured by algorithms other than blastp known in the art. For instance, polypeptide sequences can be compared using FASTA, a program in GCG Version 6.1. FASTA provides alignments and percent sequence identity of the regions of the best overlap between the query and search sequences (Pearson, 1990, hereby incorporated herein by reference). For example, percent sequence identity between amino acid sequences can be determined using FASTA with its default parameters (a word size of 2 and the PAM250 scoring matrix), as provided in GCG Version 6.1, hereby incorporated herein by reference.
It is understood that a range of microorganisms can be modified to include a recombinant metabolic pathway suitable for the production of e.g., malonyl-CoA or products derived therefrom. It is also understood that various microorganisms can act as "sources" for genetic material encoding target enzymes suitable for use in a recombinant microorganism provided herein. The term "microorganism" includes prokaryotic and eukaryotic photosynthetic microbial species. The terms "microbial cells" and "microbes" are used interchangeably with the term microorganism.

"Bacteria", or "eubacteria", refers to a domain of prokaryotic organisms. Bacteria include at least 11 distinct groups as follows: (1) Gram-positive (gram+) bacteria, of which there are two major subdivisions: (1) high G+C group (Actinomycetes, Mycobacteria, Micrococcus, others) (2) low G+C group (Bacillus, Clostridia, Lactobacillus, Staphylococci, Streptococci, Mycoplasmas); (2) Proteobacteria, e.g., Purple photosynthetic + non-photosynthetic Gram-negative bacteria (includes most "common" Gram-negative bacteria); (3) Cyanobacteria, e.g., oxygenic phototrophs; (4) Spirochetes and related species; (5) Planctomycetes; (6) Bacteroides, Flavobacteria; (7) Chlamydia; (8) Green sulfur bacteria; (9) Green non-sulfur bacteria (also anaerobic phototrophs); (10) Radioresistant micrococci and relatives; (11) Thermotoga and Thermosiphon thermophiles.

Gram-negative bacteria include cocci, nonenteric rods, and enteric rods. The genera of Gram-negative bacteria include, for example, Neisseria, Spirillum, Pasteurella, Brucella, Yersinia, Francisella, Haemophilus, Bordetella, Escherichia, Salmonella, Shigella, Klebsiella, Proteus, Vibrio, Pseudomonas, Bacteroides, Acetobacter, Aerobacter, Agrobacterium, Azotobacter, Spirilla, Serratia, Vibrio, Rhizobium, Chlamydia, Rickettsia, Treponema, and Fusobacterium.

Gram positive bacteria include cocci, nonsporulating rods, and sporulating rods. The genera of gram positive bacteria include, for example, Actinomyces, Bacillus, Clostridium, Corynebacterium, Erysipelothrix, Lactobacillus, Listeria, Mycobacterium, Myxococcus, Nocardia, Staphylococcus, Streptococcus, and Streptomycetes.
[00123] Photoautotrophic bacteria are typically Gram-negative rods which obtain their energy from sunlight through the processes of photosynthesis. In this process, sunlight energy is used in the synthesis of carbohydrates, which in recombinant photoautotrophs can be further used as intermediates in the synthesis of biofuels. In other embodiment, the photoautotrophs serve as a source of carbohydrates for use by non-photosynthetic microorganism (e.g., recombinant E.coli) to produce biofuels by a metabolically engineered microorganism. Certain photoautotrophs called anoxygenic photoautotrophs grow only under anaerobic conditions and neither use water as a source of hydrogen nor produce oxygen from photosynthesis. Other photoautotrophic bacteria are oxygenic photoautotrophs. These bacteria are typically cyanobacteria. They use chlorophyll pigments and photosynthesis in photosynthetic processes resembling those in algae and complex plants. During the process, they use water as a source of hydrogen and produce oxygen as a product of photosynthesis.

[00124] Cyanobacteria include various types of bacterial rods and cocci, as well as certain filamentous forms. The cells contain thylakoids, which are cytoplasmic, platelike membranes containing chlorophyll. The organisms produce heterocysts, which are specialized cells believed to function in the fixation of nitrogen compounds.

[00125] The term "recombinant microorganism" and "recombinant host cell" are used interchangeably herein and refer to microorganisms that have been genetically modified to express or over-express endogenous nucleic acid sequences, or to express non-endogenous sequences, such as those included in a vector. The nucleic acid sequence generally encodes a target enzyme involved in a metabolic pathway for producing a desired metabolite as described above. Accordingly, recombinant microorganisms described herein have been genetically engineered to express or over-express target enzymes not previously expressed or over-expressed by a parental microorganism. It is understood that the terms "recombinant microorganism" and "recombinant host cell" refer not only to the particular recombinant microorganism but to the progeny or potential progeny of such a microorganism.
A "parental microorganism" refers to a cell used to generate a recombinant microorganism. The term "parental microorganism" describes a cell that occurs in nature, i.e. a "wild-type" cell that has not been genetically modified. The term "parental microorganism" also describes a cell that has been genetically modified but which does not express or over-express a target enzyme e.g., an enzyme involved in the biosynthetic pathway for the production of a desired metabolite such as malonyl-CoA. For example, a wild-type microorganism can be genetically modified to express or over-express a first target enzyme such as phosphoenolpyruvate carboxylase. This microorganism can act as a parental microorganism in the generation of a microorganism modified to express or over-express a second target enzyme e.g., aspartate aminotransferase. In turn, the microorganism modified to express or over express e.g., aspartate 1-decarboxylase, which can be modified to express or over express a yet another target enzyme e.g., beta alanine aminotransferase. Accordingly, a parental microorganism functions as a reference cell for successive genetic modification events. Each modification event can be accomplished by introducing a nucleic acid molecule into the reference cell. The introduction facilitates the expression or over-expression of a target enzyme. It is understood that the term "facilitates" encompasses the activation of endogenous nucleic acid sequences encoding a target enzyme through genetic modification of e.g., a promoter sequence in a parental microorganism. It is further understood that the term "facilitates" encompasses the introduction of exogenous nucleic acid sequences encoding a target enzyme into a parental microorganism.

In another embodiment a method of producing a recombinant microorganism that converts a suitable carbon substrate to e.g., malonyl-CoA is provided. The method includes transforming a microorganism with one or more recombinant nucleic acid sequences as described above and elsewhere herein (see, e.g., Figures 1-6). Nucleic acid sequences that encode enzymes useful for generating metabolites including homologs, variants, fragments, related fusion proteins, or functional equivalents thereof, are used in recombinant nucleic acid molecules that direct the expression of such polypeptides in appropriate host cells, such as bacterial or yeast
cells (such enzymes are described in Tables A-G). It is understood that the addition of sequences which do not alter the encoded activity of a nucleic acid molecule, such as the addition of a non-functional or non-coding sequence, is a conservative variation of the basic nucleic acid. The "activity" of an enzyme is a measure of its ability to catalyze a reaction resulting in a metabolite, i.e., to "function", and may be expressed as the rate at which the metabolite of the reaction is produced. For example, enzyme activity can be represented as the amount of metabolite produced per unit of time or per unit of enzyme (e.g., concentration or weight), or in terms of affinity or dissociation constants.

[00128] A "protein" or "polypeptide", which terms are used interchangeably herein, comprises one or more chains of chemical building blocks called amino acids that are linked together by chemical bonds called peptide bonds. An "enzyme" means any substance, composed wholly or largely of protein, that catalyzes or promotes, more or less specifically, one or more chemical or biochemical reactions. The term "enzyme" can also refer to a catalytic polynucleotide (e.g., RNA or DNA). A "native" or "wild-type" protein, enzyme, polynucleotide, gene, or cell, means a protein, enzyme, polynucleotide, gene, or cell that occurs in nature.

[00129] It is understood that the nucleic acid sequences described above include "genes" and that the nucleic acid molecules described above include "vectors" or "plasmids." For example, a nucleic acid sequence encoding a keto thiolase can be encoded by an atoB gene or homolog thereof, or a fadA gene or homolog thereof. Accordingly, the term "gene", also called a "structural gene" refers to a nucleic acid sequence that codes for a particular sequence of amino acids, which comprise all or part of one or more proteins or enzymes, and may include regulatory (non-transcribed) DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed. The transcribed region of the gene may include untranslated regions, including introns, 5'-untranslated region (UTR), and 3'-UTR, as well as the coding sequence. The term "nucleic acid" or "recombinant nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and,
where appropriate, ribonucleic acid (RNA). The term "expression" with respect to a gene sequence refers to transcription of the gene and, as appropriate, translation of the resulting mRNA transcript to a protein. Thus, as will be clear from the context, expression of a protein results from transcription and translation of the open reading frame sequence.

[00130] The term "operon" refers two or more genes which are transcribed as a single transcriptional unit from a common promoter. In some embodiments, the genes comprising the operon are contiguous genes. It is understood that transcription of an entire operon can be modified (i.e., increased, decreased, or eliminated) by modifying the common promoter. Alternatively, any gene or combination of genes in an operon can be modified to alter the function or activity of the encoded polypeptide. The modification can result in an increase in the activity of the encoded polypeptide. Further, the modification can impart new activities on the encoded polypeptide. Exemplary new activities include the use of alternative substrates and/or the ability to function in alternative environmental conditions.

[00131] A "vector" is any means by which a nucleic acid can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include viruses, bacteriophage, pro-viruses, plasmids, phagemids, transposons, and artificial chromosomes such as YACs (yeast artificial chromosomes), BACs (bacterial artificial chromosomes), and PLACs (plant artificial chromosomes), and the like, that are "episomes," that is, that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine -conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that are not episomal in nature, or it can be an organism which comprises one or more of the above polynucleotide constructs such as an agrobacterium or a bacterium.

[00132] "Transformation" refers to the process by which a vector is introduced into a host cell. Transformation (or transduction, or transfection), can be achieved by any one of a number of means
including electroporation, microinjection, biolistics (or particle bombardment-mediated delivery), or agrobacterium mediated transformation.

[00133] The disclosure provides nucleic acid molecules in the form of recombinant DNA expression vectors or plasmids, as described in more detail below, that encode one or more target enzymes. Generally, such vectors can either replicate in the cytoplasm of the host microorganism or integrate into the chromosomal DNA of the host microorganism. In either case, the vector can be a stable vector (i.e., the vector remains present over many cell divisions, even if only with selective pressure) or a transient vector (i.e., the vector is gradually lost by host microorganisms with increasing numbers of cell divisions). The disclosure provides DNA molecules in isolated (i.e., not pure, but existing in a preparation in an abundance and/or concentration not found in nature) and purified (i.e., substantially free of contaminating materials or substantially free of materials with which the corresponding DNA would be found in nature) forms.

[00134] Provided herein are methods for the heterologous expression of one or more of the biosynthetic genes involved in malonyl-CoA biosynthesis and recombinant DNA expression vectors useful in the method. Thus, included within the scope of the disclosure are recombinant expression vectors that include such nucleic acids. The term expression vector refers to a nucleic acid that can be introduced into a host microorganism or cell-free transcription and translation system. An expression vector can be maintained permanently or transiently in a microorganism, whether as part of the chromosomal or other DNA in the microorganism or in any cellular compartment, such as a replicating vector in the cytoplasm. An expression vector also comprises a promoter that drives expression of an RNA, which typically is translated into a polypeptide in the microorganism or cell extract. For efficient translation of RNA into protein, the expression vector also typically contains a ribosome-binding site sequence positioned upstream of the start codon of the coding sequence of the gene to be expressed. Other elements, such as enhancers, secretion signal sequences, transcription termination sequences, and one or more
marker genes by which host microorganisms containing the vector can be identified and/or selected, may also be present in an expression vector. Selectable markers, i.e., genes that confer antibiotic resistance or sensitivity, are used and confer a selectable phenotype on transformed cells when the cells are grown in an appropriate selective medium.

[00135] The various components of an expression vector can vary widely, depending on the intended use of the vector and the host cell(s) in which the vector is intended to replicate or drive expression. Expression vector components suitable for the expression of genes and maintenance of vectors in *E. coli*, yeast, Streptomyces, and other commonly used cells are widely known and commercially available. For example, suitable promoters for inclusion in the expression vectors of the disclosure include those that function in eukaryotic or prokaryotic host microorganisms. Promoters can comprise regulatory sequences that allow for regulation of expression relative to the growth of the host microorganism or that cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus. For *E. coli* and certain other bacterial host cells, promoters derived from genes for biosynthetic enzymes, antibiotic-resistance conferring enzymes, and phage proteins can be used and include, for example, the galactose, lactose (lac), maltose, tryptophan (*trp*), beta-lactamase (*bla*), bacteriophage lambda PL, and T5 promoters. In addition, synthetic promoters, such as the tac promoter (U.S. Pat. No. 4,551,433), can also be used. For *E. coli* expression vectors, it is useful to include an *E. coli* origin of replication, such as from pUC, pLP, pi, and pBR.

[00136] Thus, recombinant expression vectors contain at least one expression system, which, in turn, is composed of at least a portion of a biosynthetic gene coding sequences operably linked to a promoter and optionally termination sequences that operate to effect expression of the coding sequence in compatible host cells. The host cells are modified by transformation with the recombinant DNA expression vectors of the disclosure to contain the expression system sequences either as extrachromosomal elements or integrated into the chromosome.
As previously noted, the term "host cell" is used interchangeably with the term "recombinant microorganism" and includes any cell type which is suitable for producing e.g., malonyl-CoA and susceptible to transformation with a nucleic acid construct such as a vector or plasmid.

A nucleic acid of the disclosure can be amplified using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques and those procedures described in the Examples section below. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to nucleotide sequences can be prepared by standard synthetic techniques, e.g., using an automated DNA synthesizer.

It is also understood that an isolated nucleic acid molecule encoding a polypeptide homologous to the enzymes described herein can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence encoding the particular polypeptide, such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced into the nucleic acid sequence by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. In contrast to those positions where it may be desirable to make a non-conservative amino acid substitutions (see above), in some positions it is preferable to make conservative amino acid substitutions. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and
aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

[00140] In another embodiment a method for producing e.g., malonyl-CoA is provided. The method includes culturing a recombinant autotroph, photoautotroph or photoheterotroph microorganism (s) or culture comprising an autotroph, photoautotroph or photoheterotroph and a recombinant non-photosynthetic or photoheterotroph microorganism as provided herein in the presence of a suitable substrate and under conditions suitable for the conversion of the substrate to malonyl-CoA. The malonyl-CoA produced by a microorganism or culture provided herein can be detected by any method known to the skilled artisan. Culture conditions suitable for the growth and maintenance of a recombinant microorganism provided herein are described in the Examples below. The skilled artisan will recognize that such conditions can be modified to accommodate the requirements of each microorganism.


EXAMPLES

Materials and methods

[00142] Chemicals and reagents. All chemicals were purchase from Sigma-Aldrich. KOD and KOD-extreme DNA polymerases were purchased from EMD Biosciences. Phusion DNA polymerase, and ligases were purchased from New England Biolabs (Ipswich, MA). T5-Exonuclease was purchased from Epicentre Biotechnologies. Oligonucleotides were obtained from IDT (San Diego, CA).

[00143] Plasmid constructions. A list of plasmids used in this work is presented in Table 1. All plasmids were constructed by isothermal DNA assembly method. Plasmids were propagated and stored in E. coli strain XL-1 blue. Promoter and enzyme coding regions of all plasmids were verified by DNA sequencing performed by Genewiz (San Diego, CA). Gene mcr was synthesized by Genewiz (South Plainfield, NJ) with codon optimization for E. coli. Genes skpyd4 and adc were synthesized by DNA2.0 (Menlo Park, CA) with codon optimization for E. coli.

[00144] Protein expression and purification. All enzymes used in this study were expressed in BL21 (DE3). Cells were inoculated from an overnight preculture at 100X dilution and grown in 2XYT rich medium containing 50 mg/L spectinomycin. At OD600 of 0.6, cell cultures were induced with 1 mM IPTG and incubated at 30 °C.
overnight. Cells were pelleted by centrifuging at 4300 x g for 20 minutes. Cell pellets were resuspended in His-Binding buffer (either from Zymo or EMD Millipore) and lysed either using TissueLyser II (Qiagen) or Sonicator (Branson Sonifier 250). Soluble proteins were separated from insoluble cell debris by centrifugation at 16,000 x g for 20 minutes. His-tagged enzymes were then purified using either His-Spin Protein Miniprep (Zymo) or Amicon® Pro Affinity Concentration Kit Ni-NTA (EMD Millipore) according to manufacturer's instruction. Purified enzymes were subjected to buffer exchange and stored in solution containing 50 mM Tris-HCl pH 8.5, ImM DTT, and 20% glycerol at -80°C. For AspC, SkPYD4, and ADC, additional 100 µM of PLP was added to storage buffer.

Thermodynamics of each reaction step was calculated using eQuilibrator with either standard condition (AG'°) or physiological relevant condition (AG' at 1 mM substrate and product concentration, pH 7.0, and ionic strength of 0.1 M).

[00146] Kinetic analysis of malonyl-CoA biosynthesis. Since NADPH is produced at 1 to 1 stoichiometric ratio with malonyl-CoA from either β-alanine or aspartate, spectrophotometric assay monitoring the disappearance of NADPH corresponding to absorbance at 340 nm was used. Absorbance at 340 nm was monitored using Bio-TEK PowerWave XS microplate spectrophotometer. For determining the effect of substrate concentration on rate of malonyl-CoA biosynthesis from β-alanine, individual substrate was varied as indicated while keeping the concentration of other substrates constant at 1 mM of NADP+, 1 mM of CoA, 30 mM β-alanine, 30 mM α-ketoglutarate. α-Ketoglutarate was neutralized with NaOH prior to mixing with the enzymatic reaction. The reaction also contained 200 nM SkPYD4, and 200 nM Mcr in 50 mM Tris-HCl pH 8.5. reaction was started by addition of β-alanine.

[00147] For determining the effect of individual enzyme concentration on rate of malonyl-CoA biosynthesis from oxaloacetate, concentration of each enzyme was varied as indicated while keeping the concentration of other enzymes at 1 µM. The reaction mixture contained 20 mM oxaloacetate, 15 mM α-ketoglutarate, 15 mM glutamate, and 80 µM PLP in 50 mM Tris-HCl pH 8.5.
HPLC analysis of malonyl-CoA. Separation of CoA and malonyl-CoA was achieved using HPLC equipped with an autosample and a C18 column (TOSOH, TSKgel ODS-100Z 4.6 x 150 mm, 5 µm). Product elution was carried out using gradient program with 50 mM sodium acetate pH 3.7 as aqueous solvent and acetonitrile as organic solvent with flow rate of 1 mL/min. The gradient program used is summarized in Table 2. Product elution was monitored at 254 nm using diode array detector. Injection volume was 20 µL. Column temperature was kept at 30 °C.

Table 1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotypes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCDFDuets</td>
<td>Spec&lt;sup&gt;R&lt;/sup&gt;; CDF ori; P&lt;sub&gt;T7&lt;/sub&gt;:MCS (His tagged)</td>
<td>Novagen</td>
</tr>
<tr>
<td>pCDF-SkPYD4</td>
<td>Spec&lt;sup&gt;R&lt;/sup&gt;; CDF ori; P&lt;sub&gt;T7&lt;/sub&gt;:Skpyd4 (His tagged)</td>
<td>This work</td>
</tr>
<tr>
<td>pCDF-AspC</td>
<td>Spec&lt;sup&gt;R&lt;/sup&gt;; CDF ori; P&lt;sub&gt;T7&lt;/sub&gt;:aspC (His tagged)</td>
<td>This work</td>
</tr>
<tr>
<td>pCDF-PanD</td>
<td>Spec&lt;sup&gt;R&lt;/sup&gt;; CDF ori; P&lt;sub&gt;T7&lt;/sub&gt;:panD (His tagged)</td>
<td>This work</td>
</tr>
<tr>
<td>pEL230</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;; pUC ori; P&lt;sub&gt;T5&lt;/sub&gt;:ade (His tagged)</td>
<td>This work</td>
</tr>
<tr>
<td>pEL141</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;; CDF ori; P&lt;sub&gt;T5&lt;/sub&gt;:mcr (His tagged)</td>
<td>This work</td>
</tr>
<tr>
<td>pEL147</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;; p15A ori; lac; P&lt;sub&gt;T5&lt;/sub&gt;:His&lt;sub&gt;6&lt;/sub&gt;mcr</td>
<td>This work</td>
</tr>
</tbody>
</table>

Table 2. HPLC parameter for separation of malonyl-CoA

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>18</td>
<td>30</td>
</tr>
<tr>
<td>22</td>
<td>100</td>
</tr>
<tr>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>27</td>
<td>10</td>
</tr>
</tbody>
</table>

In vitro biosynthesis of malonyl-CoA from β-alanine.

From the thermodynamics and feasibility analysis, an in vitro analysis of the aspartate dependent pathway was performed. While the thermodynamics analysis suggests the reversibility of Mcr, Mcr has not been demonstrated to function in the oxidative direction. Since malonate semialdehyde is not commercially available, a coupled reaction using SkPYD4 and Mcr (Fig. 10) was used to convert β-alanine to malonyl-CoA. SkPYD4 and Mcr were individually purified using poly-His- tag. The results are shown in HPLC chromatogram (Fig. 10)
10B). Malonyl-CoA is synthesized upon presence of the components in the reaction, indicating both the reversibility of Mcr and the feasibility of malonyl-CoA biosynthesis from β-alanine.

[00152] To explore the dependence of this synthetic malonyl-CoA pathway on the substrate concentrations of these two steps, each reactant was titrated individually into the system and their effect observed on the rate of malonyl-CoA formation. Rate of malonyl-CoA synthesis reaches maximum at around 20 to 30 mM of β-alanine (Fig. 10C) and α-ketoglutarate (Fig. 10D). Both of these reactants achieve half-maximum rate around 4 to 5 mM which is consistent with the literature $K_m$ value of SkPYD4 for β-alanine and α-ketoglutarate. On the other hand, both NADP+ (Fig. 10E) and CoA (Fig. 10F) saturates rate of malonyl-CoA formation within concentration of 1.5 mM. These results indicated that rate of malonyl-CoA biosynthesis uses a high concentration of β-alanine and α-ketoglutarate. Natural flux to β-alanine from aspartate is not expected to be large because its biosynthesis is mainly for synthesizing CoA, which only minor amounts are required. Therefore, it is likely that formation of β-alanine may be a limiting factor in this pathway.

[00153] **Effect of individual enzyme concentration on rate of malonyl-CoA biosynthesis from oxaloacetate**. To study the overall rate of malonyl-CoA biosynthesis using the aspartate dependent pathway, an in vitro system composed of AspC, PanD, SkPYD4, and Mcr was constructed to convert oxaloacetate to malonyl-CoA (Fig. 9). PanD from Corynebacterium glutamicum was chosen for its more efficient maturation. As shown in HPLC chromatogram (Fig. 11A), malonyl-CoA is synthesized using the four enzymes in the system, indicating the functional constitution of this synthetic pathway. To analyze the concentration effect of each enzyme on the biosynthesis of malonyl-CoA from oxaloacetate, each enzyme was titrated in this four step reaction while keeping the concentration of other enzymes constant at 1 μM. No change in rate of malonyl-CoA formation was observed when the concentration of AspC was increased (Fig. 11B), indicating that AspC bares no bottleneck for this pathway. On the other hand, reaction rate increased with PanD concentration until 40 μM (Fig. 11C). This result indicated that PanD is a limiting step in this synthetic pathway, which is consistent with the expectation as
β-alanine is not naturally produced at large flux. Both SkPYD4 (Fig. 11D) and Mcr (Fig. HE) reaches maximum reaction rate at around 4 uM of enzyme concentration. From these results, we deduced that the optimum ratio of AspC : PanD : SkPYD4 : Mcr for malonyl-CoA biosynthesis is 1:40:4:4.

[00154] **PLP-dependent aspartate a-decarboxylase enhanced rate of malonyl-CoA biosynthesis.** Considering the large concentration of PanD used to reach optimum rate of malonyl-CoA biosynthesis, an alternative candidate enzyme for aspartate decarboxylation was examined. Most amino acid a-decarboxylases such as glutamate decarboxylase and lysine decarboxylase use pyridoxal 5-phosphate (PLP) for catalysis. Turnover number of glutamate decarboxylase (24.85 s⁻¹) and lysine decarboxylase (30 s⁻¹) are both higher than that of PanD (0.65 s⁻¹) in E. coli, indicating more efficient catalysis with PLP. Inspired by this observation, an aspartate decarboxylase using PLP as a cofactor was identified. Aspartate decarboxylase (ADC) from Aedes aegypti is a structural homologue of glutamate decarboxylase using PLP as a cofactor. Accordingly, ADC was used to replace PanD in the pathway.

[00155] Each enzyme was again titrated in the four step reaction with PLP dependent aspartate decarboxylase. Malonyl-CoA synthesis reaches maximum rate at 8 uM of ADC (Fig. 12A), which is significantly less than the 40 uM of PanD required for achieving maximum rate. AspC (Fig. 12B) and SkPYD4 (Fig. 12C) achieved highest rate at 1 uM and 4 uM, respectively, which is identical to the PanD dependent system. Mcr required 8 uM to reach highest rate in this system (Fig. 12D). Together, these results showed that using PLP dependent ADC, the amount of enzyme required to achieve higher rate of malonyl-CoA synthesis was reduced. The optimum ratio of AspC : ADC : SkPYD4 : Mcr is 1:8:4:8, which may be easier to implement in vivo.

[00156] A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.
WHAT IS CLAIMED IS

1. A recombinant microorganism that produces malonyl-CoA at levels greater than a parental organism comprising a pathway selected from the group consisting of:
   (a) phosphoenolpyruvate to malonate semialdehyde; and
   (b) pyruvate to malonate semialdehyde;
wherein the pathway comprises a malonyl-CoA reductase.

2. The recombinant microorganism of claim 1, wherein the recombinant microorganism is engineered to express an acetyl-CoA carboxylase and a malonyl-CoA reductase.

3. The recombinant microorganism of claim 1, wherein the recombinant microorganism is engineered to express or overexpress one or more enzymes that carries out a metabolic function selected from the group consisting of (i) converting PEP to Glyceraldehyde-3P; (ii) converting Glyceraldehyde-3P to DHAP; (iii) converting DHAP to glycerol-3P; (iv) converting glycerol-3P to glycerol; (v) converting glycerol to 3-HPA; (vi) converting 3HPA to 3HP; (vii) converting 3HP to malonate semialdehyde; and (viii) converting malonate semialdehyde to malonyl-coA.

4. The recombinant microorganism of claim 1, wherein the recombinant microorganism is engineered to express or overexpress one or more enzymes that carries out a metabolic function selected from the group consisting of (i) converting PEP to oxaloacetate; (ii) converting oxaloacetate to malonate semialdehyde; and (iii) converting malonate semialdehyde to malonyl-coA.

5. The recombinant microorganism of claim 1, wherein the recombinant microorganism is engineered to express or overexpress one or more enzymes that carries out a metabolic function selected from the group consisting of (i) converting PEP to oxaloacetate; (ii) converting oxaloacetate to aspartate, (iii) converting aspartate to beta-alanine; (iv) converting beta-alanine to malonate semialdehyde; and (v) converting malonate semialdehyde to malonyl-coA.
6. The recombinant microorganism of claim 1, wherein the recombinant microorganism is engineered to express or overexpress one or more enzymes that carries out a metabolic function selected from the group consisting of (i) optionally converting PEP to pyruvate; (ii) converting pyruvate to alanine; (iii) converting alanine to beta-alanine; (iv) converting beta-alanine to malonate semialdehyde; and (v) converting malonate semialdehyde to malonyl-CoA.

7. The recombinant microorganism of claim 1, wherein the recombinant microorganism is engineered to express or overexpress one or more enzymes that carries out a metabolic function selected from the group consisting of (i) converting PEP to pyruvate; (ii) converting pyruvate to lactate; (iii) converting lactate to lactoyl-CoA; (iv) converting lactoyl-CoA to acrylyl-CoA; (v) converting acrylyl-CoA to 3-hydroxypropionyl-CoA; (vi) 3-hydroxypropionyl-CoA to 3-hydroxypropionate; (vii) converting 3-hydroxypropionate to malonate semialdehyde; and (viii) converting malonate semialdehyde to malonyl-CoA.

8. The recombinant microorganism of claim 3, wherein the recombinant microorganism is engineered to express or overexpress one or more enzyme selected from the group consisting of trios phosphate isomerase, glycerol-3-phosphate dehydrogenase, glycerol-3-phosphatase, glycerol dehydratase, glycerol dehydratase reactivase, aldehyde dehydrogenase, malonate semialdehyde dehydrogenase and malonyl-CoA reductase.

9. The recombinant microorganism of claim 4, wherein the recombinant microorganism is engineered to express or overexpress one or more enzymes selected from the group consisting of a phosphoenolpyruvate carboxylase, a oxaloacetate 1-decarboxylase and malonyl-CoA reductase.

10. The recombinant microorganism of claim 5, wherein the recombinant microorganism is engineered to express or overexpress
one or more enzymes selected from the group consisting of a phosphoenolpyruvate carboxylase, an aspartate aminotransferase, an aspartate 1-decarboxylase or a PLP-dependent aspartate 1-decarboxylase, a beta-alanine aminotransferase and a malonyl-CoA reductase.

11. The recombinant microorganism of claim 6, wherein the recombinant microorganism is engineered to express or overexpress one or more enzymes selected from the group consisting of a pyruvate kinase, an alanine aminotransferase, an alanine aminomutase and a malonyl-CoA reductase.

12. The recombinant microorganism of claim 7, wherein the recombinant microorganism is engineered to express or overexpress one or more enzymes selected from the group consisting of a pyruvate kinase, a lactate dehydrogenase, a lactoyl-CoA transferase, a propionyl-CoA synthase, a lactoyl-CoA dehydratase, a hydroxypropionyl-CoA dehydratase, a hydroxypropionyl-CoA hydrolase, a malonate semialdehyde dehydrogenase and a malonyl-CoA reductase.

13. The recombinant microorganism of claim 8, comprising an enzyme or homologs thereof selected from the group consisting of Tpi, GpsA, GPP, DhaB123/GdrAB, PuuC, Msr and Mcr.

14. The recombinant microorganism of claim 9, comprising enzyme or homologs thereof selected from the group consisting of Ppc, Oad and Mcr.

15. The recombinant microorganism of claim 10, comprising enzyme or homologs thereof selected from the group consisting of Ppc, AspC, PanD or AeADC, SkPYD4 and Mcr.

16. The recombinant microorganism of claim 11, comprising enzyme or homologs thereof selected from the group consisting of Pyk, Aat, Aam and Mcr.
17. The recombinant microorganism of claim 12, comprising enzyme or homologs thereof selected from the group consisting of Pyk, Ldh, Pet (and Pes), Led, Hpd, Hph, Msr and Mcr.

18. The recombinant microorganism of claim 10, wherein the aspartate amino transferase comprises a sequence having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:2 and having aspartate aminotransferase activity; wherein the aspartate 1-decarboxylase comprises a sequences having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:4 and having aspartate 1-decarboxylase activity; wherein the PLP-dependent aspartate 1-decarboxylase comprises a sequence having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:6 and having PLP-dependent aspartate 1-decarboxylase; wherein the beta-alanine aminotransferase comprises a sequence having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:8 and having beta-alanine aminotransferase.

19. The recombinant microorganism of any of the foregoing claims wherein the malonyl-CoA reductase comprises a sequence having at least 70%, 80%, 90%, 95%, 98%, or 99% identity to SEQ ID NO:10 and having malonyl-CoA reductase.

20. An in vitro metabolic pathway for producing malonyl-CoA comprising the enzymes trios phosphate isomerase, glycerol-3-phosphate dehydrogenase, glycerol-3-phosphatase, glycerol dehydratase, glycerol dehydratase reactivase, aldehyde dehydrogenase, malonate semialdehyde dehydrogenase and malonyl-CoA reductase.


22. An in vitro metabolic pathway for producing malonyl-CoA comprising the enzymes phosphoenolpyruvate carboxylase, aspartate aminotransferase, aspartate 1-decarboxylase or PLP-dependent
aspartate 1-decarboxylase, beta-alanine aminotransferase and a malonyl-CoA reductase.

23. An in vitro metabolic pathway for producing malonyl-CoA comprising the enzymes pyruvate kinase, alanine aminotransferase, alanine aminomutase and malonyl-CoA reductase.


25. The in vitro metabolic pathway of claim 20, comprising an enzyme or homologs thereof selected from the group consisting of Tpi, GpsA, GPP, DhaB123/GdrAB, PuuC, Msr and Mcr.

26. The in vitro metabolic pathway of claim 21, comprising enzyme or homologs thereof selected from the group consisting of Ppc, Oad and Mcr.

27. The in vitro metabolic pathway of claim 22, comprising enzyme or homologs thereof selected from the group consisting of Ppc, AspC, PanD or AeADC, SkPYD4 and Mcr.

28. The in vitro metabolic pathway of claim 23, comprising enzyme or homologs thereof selected from the group consisting of Pyk, Aat, Aam and Mcr.

29. The in vitro metabolic pathway of claim 24, comprising enzyme or homologs thereof selected from the group consisting of Pyk, Ldh, Pet (and Pes), Led, Hpd, Hph, Msr and Mcr.

30. A method for producing a chemical or malonyl-CoA, the method comprising:

62
a) providing a recombinant microorganism of any of claims 1-19;
b) culturing the microorganism(s) of (a) in the presence of a carbon substrate under conditions suitable for the conversion of the substrate to the chemical or malonyl-CoA; and
c) purifying the chemical or malonyl-CoA.

31. A method for producing a chemical or malonyl-CoA, the method comprising:
   a) providing an in vitro metabolic pathway of any of claims 20-29;
   b) incubating the enzymes of the in vitro metabolic pathway in (a) in the presence of a carbon substrate under conditions suitable for the conversion of the substrate to the chemical or malonyl-CoA; and
c) purifying the chemical or malonyl-CoA.
Figure 1 A-E
FIGURE 5
**Carboxylation – decarboxylation**

PEP $\xrightarrow{\text{O}=	ext{C}:=\text{O}}$ Oxaloacetate $\xrightarrow{\text{O}=	ext{C}:=\text{O}}$ Malonate semi-aldehyde $\xrightarrow{\text{NADPH}_{\text{Mc}}}$ Malonyl-CoA

**Aspartate shunt**

AspC $\xrightarrow{\text{Aspartate}}$ Glutamate $\xrightarrow{\alpha$-Ketoglutarate}$ SkPYD4 $\xrightarrow{\text{Aspartate}}$ PanD $\xrightarrow{\text{O}=	ext{C}:=\text{O}}$ β-Alanine

**FIGURE 9**
INTERNATIONAL SEARCH REPORT

International application No.
PCT/US20 14/052960

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 1/11 (201 4.01 )
CPC - C12N 9/00 (2014.1 2)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12N 1/00, 1/11, 1/19, 1/21; C12P 19/60, 19/62, 29/00 (2014.01)
USPC - 435/69.2, 106, 109, 116, 143, 145, 170, 171

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - C12N 9/000, 9/0004, 9/0008, 9/88 9/93; C12P 7/52, 7/66, 17/18, 19/62, 29/00 (2014.12) (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Orbit, Google. Patents, Google Scholar
Search terms used: "lactyl-CoA transferase" "propionyl-CoA synthase" "acetyl-CoA dehydratase" "hydroxy propionyl-CoA dehydratase" "hydroxy propionyl-CoA hydrolase" malonyl-coa beta-alanine aspartate pathway

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>US 2013/0122541 A1 (LYNCH et al) 16 May 2013 (16.05.2013) entire document 1-17, 19, 21, 22, 26, 27</td>
<td>18, 20, 23-25, 28, 29</td>
</tr>
</tbody>
</table>

Special documents are listed in the continuation of Box C

1. "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
2. "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
3. "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Date of the actual completion of the international search
06 December 2014

Date of mailing of the international search report
17 DEC 2014

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Form PCT/ISA/2 10 (second sheet) (July 2009)
Box No. 1  Nucleotide and/or amino acid sequence(s) (Continuation of item 1c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:
   a. (means)
      □ on paper
      □ in electronic form
   b. (time)
      □ in the international application as filed
      □ together with the international application in electronic form
      □ subsequently to this Authority for the purposes of search

2. □ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:
   SEQ ID NOs:1-1 were searched.
INTERNATIONAL SEARCH REPORT

International application No. 
PCT/US2014/052960

Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. □ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. □ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. □ Claims Nos.: 30, 31
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. □ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. □ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. □ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. □ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest □ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

□ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

□ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)