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Canadian Intellectual Property Office

CA 2848253 C 2020/11/03

(11)(21) 2 848 253

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 2012/03/08
(87) Date publication PCT/PCT Publication Date: 2013/03/21
(45) Date de délivrance/Issue Date: 2020/11/03
(85) Entrée phase nationale/National Entry: 2014/03/10
(86) N° demande PCT/PCT Application No.: US 2012/028256
(87) N° publication PCT/PCT Publication No.: 2013/039563
(30) Priorité/Priority: 2011/09/14 (US13/232,927)

(51) Cl.Int./Int.Cl. *C12P 5/02* (2006.01),
C12N 1/21 (2006.01), *C12N 15/63* (2006.01),
C12P 7/64 (2006.01)

(72) Inventeurs/Inventors:
LEE, GRACE J., US;
HALIBURTON, JOHN R., US;
HU, ZHIHAO, US;
SCHIRMER, ANDREAS W., US

(73) Propriétaire/Owner:
REG LIFE SCIENCES, LLC, US

(74) Agent: BERESKIN & PARR LLP/S.E.N.C.R.L.,S.R.L.

(54) Titre : PRODUCTION DE DERIVES D'ACIDE GRAS A CHAINE PAIRE DANS CELLULES MICROBIENNES RECOMBINANTES

(54) Title: PRODUCTION OF ODD CHAIN FATTY ACID DERIVATIVES IN RECOMBINANT MICROBIAL CELLS

(57) Abrégé/Abstract:
Recombinant microbial cells are provided which have been engineered to produce fatty acid derivatives having linear chains containing an odd number of carbon atoms by the fatty acid biosynthetic pathway. Also provided are methods of making odd chain fatty acid derivatives using the recombinant microbial cells, and compositions comprising odd chain fatty acid derivatives produced by such methods.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 March 2013 (21.03.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/039563 A1

(51) International Patent Classification:
C12P 5/02 (2006.01) *C12N 15/63* (2006.01)
C12N 1/21 (2006.01) *C12P 7/64* (2006.01)

(21) International Application Number:
PCT/US2012/028256

(22) International Filing Date:
8 March 2012 (08.03.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
13/232,927 14 September 2011 (14.09.2011) US

(71) Applicant *(for all designated States except US)*: **LS9, INC.** [US/US]; 600 Gateway Boulevard, South San Francisco, California 94080 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **LEE, Grace J.** [US/US]; 600 Gateway Boulevard, South San Francisco, California 94080 (US). **HALIBURTON, John R.** [US/US]; 29 Lapidge, Apt. 1, San Francisco, California 94110 (US). **HU, Zhihao** [CN/US]; 600 Gateway Boulevard, South San Francisco, California 94080 (US). **SCHIRMER, Andreas W.** [DE/US]; 600 Gateway Boulevard, South San Francisco, California 94080 (US).

(74) Agent: **JUDGE, Linda R.**; 600 Gateway Boulevard, South San Francisco, California 94080 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with information concerning one or more priority claims considered void (Rule 26bis.2(d))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2013/039563 A1

(54) **Title**: PRODUCTION OF ODD CHAIN FATTY ACID DERIVATIVES IN RECOMBINANT MICROBIAL CELLS

(57) **Abstract**: Recombinant microbial cells are provided which have been engineered to produce fatty acid derivatives having linear chains containing an odd number of carbon atoms by the fatty acid biosynthetic pathway. Also provided are methods of making odd chain fatty acid derivatives using the recombinant microbial cells, and compositions comprising odd chain fatty acid derivatives produced by such methods.

PRODUCTION OF ODD CHAIN FATTY ACID DERIVATIVES IN RECOMBINANT MICROBIAL CELLS

BACKGROUND

Crude petroleum is a very complex mixture containing a wide range of hydrocarbons. It is converted into a diversity of fuels and chemicals through a variety of chemical processes in refineries. Crude petroleum is a source of transportation fuels as well as a source of raw materials for producing petrochemicals. Petrochemicals are used to make specialty chemicals such as plastics, resins, fibers, elastomers, pharmaceuticals, lubricants, and gels.

The most important transportation fuels -- gasoline, diesel, and jet fuel -- contain distinctively different mixtures of hydrocarbons which are tailored toward optimal engine performance. For example, gasoline comprises straight chain, branched chain, and aromatic hydrocarbons generally ranging from about 4 to 12 carbon atoms, while diesel predominantly comprises straight chain hydrocarbons ranging from about 9 to 23 carbon atoms. Diesel fuel quality is evaluated by parameters such as cetane number, kinematic viscosity, oxidative stability, and cloud point (Knothe G., *Fuel Process Technol.* 86:1059-1070 (2005)). These parameters, among others, are impacted by the hydrocarbon chain length as well as by the degree of branching or saturation of the hydrocarbon.

Microbially-produced fatty acid derivatives can be tailored by genetic manipulation. Metabolic engineering enables microbial strains to produce various mixtures of fatty acid derivatives, which can be optimized, for example, to meet or exceed fuel standards or other commercially relevant product specifications. Microbial strains can be engineered to produce chemicals or precursor molecules that are typically derived from petroleum. In some instances, it is desirable to mimic the product profile of an existing product, for example the product profile of an existing petroleum-derived fuel or chemical product, for efficient drop-in compatibility or substitution. Recombinant cells and methods described herein demonstrate microbial production of fatty acid derivatives with varied ratios of odd: even length chains as a means to precisely control the structure and function of, *e.g.*, hydrocarbon-based fuels and chemicals.

There is a need for cost-effective alternatives to petroleum products that do not require exploration, extraction, transportation over long distances, or substantial refinement, and avoid the types of environmental damage associated with processing of petroleum. For similar reasons, there is a need for alternative sources of chemicals which are typically derived from petroleum. There is also a need for efficient and cost-effective methods for producing high-quality biofuels, fuel alternatives, and chemicals from renewable energy sources.

Recombinant microbial cells engineered to produce fatty acid precursor molecules having desired chain lengths (such as, chains having odd numbers of carbons), and fatty acid derivatives made therefrom, methods using these recombinant microbial cells to produce compositions comprising fatty acid derivatives having desired acyl chain lengths and desired ratios of odd: even length chains, and compositions produced by these methods, address these needs.

SUMMARY

The present invention provides novel recombinant microbial cells which produce odd chain length fatty acid derivatives and cell cultures comprising such novel recombinant microbial cells. The invention also provides methods of making compositions comprising odd chain length fatty acid derivatives comprising culturing recombinant microbial cells of the invention, compositions made by such methods, and other features apparent upon further review.

In a first aspect, the invention provides a recombinant microbial cell comprising a polynucleotide encoding a polypeptide having enzymatic activity effective to increase the production of propionyl-CoA in the cell relative to the production of propionyl-CoA in a parental microbial cell lacking or having a reduced amount of said enzymatic activity, wherein the recombinant microbial cell produces a fatty acid derivative composition comprising odd chain fatty acid derivatives when the cell is cultured in the presence of a carbon source under conditions effective to express the polynucleotide. The recombinant microbial cell comprises: (a) a polynucleotide encoding a polypeptide having enzymatic activity effective to produce an increased amount of propionyl-CoA in the recombinant microbial cell, relative to the amount of propionyl-CoA produced in a parental microbial cell lacking or having a reduced amount of said enzymatic activity, wherein the polypeptide is exogenous to the recombinant microbial cell, or expression of the polynucleotide is modulated in the recombinant microbial cell as compared to the expression of the polynucleotide in the parental microbial cell; (b) a polynucleotide encoding a polypeptide having β-ketoacyl-ACP synthase ("FabH") activity that utilizes propionyl-CoA as a substrate, and (c) a polynucleotide encoding a polypeptide having fatty acid derivative enzyme activity, wherein the recombinant microbial cell produces a fatty acid derivative composition comprising odd chain fatty acid derivatives when the cell is cultured in the presence of a carbon source under conditions effective to express the polynucleotides according to (a), (b), and (c). In some embodiments, expression of at least one

polynucleotide according to (a) is modulated by overexpression of the polynucleotide, such as by operatively linking the polynucleotide to an exogenous promoter.

In some embodiments, at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the fatty acid derivatives in the composition produced by the microbial cell of the first aspect are odd chain fatty acid derivatives. In some embodiments, the recombinant microbial cell produces at least 50 mg/L, at least 75 mg/L, at least 100 mg/L, at least 200 mg/L, at least 500 mg/L, at least 1000 mg/L, at least 2000 mg/L, at least 5000 mg/L, or at least 10000 mg/L odd chain fatty acid derivatives when cultured in a culture medium containing a carbon source under conditions effective to express the polynucleotides according to (a), (b), and (c).

In some embodiments, the polynucleotide encoding a polypeptide having enzymatic activity effective to produce an increased amount of propionyl-CoA in the recombinant microbial cell according to (a) is selected from: (i) one or more polynucleotide encoding a polypeptide having aspartokinase activity, homoserine dehydrogenase activity, homoserine kinase activity, threonine synthase activity, or threonine deaminase activity; (ii) one or more polynucleotide encoding a polypeptide having (R)-citramalate synthase activity , isopropylmalate isomerase activity, or beta-isopropylmalate dehydrogenase activity; and (iii) one or more polynucleotide encoding a polypeptide having methylmalonyl-CoA mutase activity, methylmalonyl-CoA decarboxylase activity, methylmalonyl-CoA carboxyltransferase activity, or methylmalonyl-CoA epimerase activity. In some embodiments, the microbial cell comprises one or more polynucleotide according to (i) and one or more polynucleotide according to (ii). In some embodiments, the microbial cell comprises one or more polynucleotide according to (i) and/or (ii), and one or more polynucleotide according to (iii).

In some embodiments, the polypeptide having β-ketoacyl-ACP synthase activity that utilizes propionyl-CoA as a substrate is exogenous to the recombinant microbial cell. In a more particular embodiment, expression of a polypeptide having β-ketoacyl-ACP synthase activity endogenous to the recombinant microbial cell is attenuated.

The fatty acid derivative enzyme activity may be endogenous ("native") or exogenous. In some embodiments, the fatty acid derivative enzyme activity comprises thioesterase activity, and the fatty acid derivative composition produced by the recombinant microbial cell comprises odd chain fatty acids and even chain fatty acids. In some embodiments, at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the fatty acids in the composition are odd chain fatty acids. In some embodiments, the recombinant microbial cell produces at least 50 mg/L, at least 75 mg/L, at least 100 mg/L, at least 200 mg/L, at least 500 mg/L, at least 1000 mg/L, at least 2000 mg/L, at least 5000 mg/L, or at least 10000 mg/L odd chain fatty acids when cultured in a culture medium containing a carbon source under conditions effective to express the polynucleotides.

In some embodiments of the first aspect, the fatty acid derivative enzyme activity comprises ester synthase activity, and the fatty acid derivative composition produced by the recombinant microbial comprises odd chain fatty esters and even chain fatty esters. In some embodiments, at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the fatty esters in the composition are odd chain fatty esters . In some embodiments, the recombinant microbial cell produces at least 50 mg/L, at least 75 mg/L, at least 100 mg/L, at least 200 mg/L, at least 500 mg/L, at least 1000 mg/L, at least 2000 mg/L, at least 5000 mg/L, or at least 10000 mg/L odd chain fatty esters when cultured in a culture medium containing a carbon source under conditions effective to express the polynucleotides.

In some embodiments of the first aspect, the fatty acid derivative enzyme activity comprises fatty aldehyde biosynthesis activity, and the fatty acid derivative composition produced by the recombinant microbial cell comprises odd chain fatty aldehydes and even chain fatty aldehydes. In some embodiments, at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the fatty aldehydes in the composition are odd chain fatty aldehydes. In some embodiments, the recombinant microbial cell produces at least 50 mg/L, at least 75 mg/L, at least 100 mg/L, at least 200 mg/L, at least 500 mg/L, at least 1000 mg/L, at least 2000 mg/L, at least 5000 mg/L, or at least 10000 mg/L odd chain fatty aldehydes when cultured in a culture medium containing a carbon source under conditions effective to express the polynucleotides.

In some embodiments of the first aspect, the fatty acid derivative enzyme activity comprises fatty alcohol biosynthesis activity, and the fatty acid derivative composition produced by the recombinant microbial cell comprises odd chain fatty alcohols and even chain fatty alcohols. In some embodiments, at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the fatty alcohols in the composition are odd chain fatty alcohols. In some embodiments, the recombinant microbial cell produces at least 50 mg/L, at least 75 mg/L, at least 100 mg/L, at least 200 mg/L, at least 500 mg/L, at least 1000 mg/L, at least 2000 mg/L, at least 5000 mg/L, or at least 10000 mg/L odd chain fatty alcohols when cultured in a culture medium containing a carbon source under conditions effective to express the polynucleotides.

In some embodiments of the first aspect, the fatty acid derivative enzyme activity comprises hydrocarbon biosynthesis activity, and the fatty acid derivative composition produced by the recombinant microbial cell is a hydrocarbon composition, such as an alkane composition, an alkene composition, a terminal olefin composition, an internal olefin composition, or a ketone composition, the hydrocarbon composition comprising odd chain hydrocarbons and even chain hydrocarbons. In some embodiments, at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the hydrocarbons in the composition are even chain hydrocarbons. In some embodiments, the recombinant microbial cell produces at least 50 mg/L,

at least 75 mg/L, at least 100 mg/L, at least 200 mg/L, at least 500 mg/L, at least 1000 mg/L, at least 2000 mg/L, at least 5000 mg/L, or at least 10000 mg/L even chain hydrocarbons when cultured in a culture medium containing a carbon source under conditions effective to express the polynucleotides.

In various embodiments, the carbon source comprises a carbohydrate, such as a sugar, e.g., a monosaccharide, a disaccharide, an oligosaccharide, or a polysaccharide. In some embodiments, the carbon source is obtained from biomass, such as a cellulosic hydrolysate.

In various embodiments, the parental (*e.g.*, host) microbial cell is a filamentous fungi, an algae, a yeast, or a prokaryote such as a bacterium. In various preferred embodiments, the host cell is a bacterial cell. In more preferred embodiments the host cell is an *E. coli* cell or a *Bacillus* cell.

Exemplary pathways for making even chain fatty acid derivatives and odd chain fatty acid derivatives are shown in Figs. 1A and 1B, respectively. Figs. 2 and 3 provide an overview of various approaches to direct metabolic flux through propionyl-CoA to increase odd chain fatty acid derivative production; Fig. 2 showing exemplary pathways through the intermediate α-ketobutyrate, and Fig. 3 showing an exemplary pathway through the intermediate methylmalonyl-CoA.

In one embodiment, the recombinant microbial cell according to the first aspect comprises a polynucleotide encoding a polypeptide having β-ketoacyl-ACP synthase activity that utilizes propionyl-CoA as a substrate, preferably a β-ketoacyl-ACP synthase III activity categorized as EC 2.3.1.180. In one embodiment, the polypeptide having β-ketoacyl-ACP synthase activity is encoded by a *fabH* gene. In one embodiment, the polypeptide having β-ketoacyl-ACP synthase activity is endogenous to the parental microbial cell. In another embodiment, the polypeptide having β-ketoacyl-ACP synthase activity is exogenous to the parental microbial cell. In another embodiment, expression of a polynucleotide encoding a polypeptide having β-ketoacyl-ACP synthase activity is modulated in the recombinant microbial cell. In some instances, expression of the polynucleotide is modulated by operatively linking the polynucleotide to an exogenous promoter, such that the polynucleotide is overexpressed in the recombinant microbial cell. In another embodiment, the polypeptide having β-ketoacyl-ACP synthase activity comprises a sequence selected from SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 146, 147, 148, or 149, or a variant or a fragment thereof having β-ketoacyl-ACP synthase activity that utilizes propionyl-CoA as a substrate and catalyzes the condensation of propionyl-CoA with malonyl-ACP to form an odd chain acyl-ACP *in vitro* or *in vivo*, preferably *in vivo*. In another embodiment, the polypeptide having β-ketoacyl-ACP synthase activity that utilizes propionyl-CoA as a substrate comprises one or more sequence motif selected from SEQ ID NOs:14-19 and catalyzes the condensation of propionyl-CoA with malonyl-ACP to form an odd chain acyl-ACP *in vitro* or *in vivo*, preferably *in vivo*.

In one embodiment, the recombinant microbial cell according to the first aspect comprises an endogenous polynucleotide sequence (such as, an endogenous *fabH* gene) encoding a polypeptide having β-ketoacyl-ACP synthase activity, and expression of such endogenous polynucleotide

sequence in the recombinant microbial cell is attenuated. In some embodiments, expression of the endogenous polynucleotide is attenuated by deletion of all or part of the sequence of the endogenous polynucleotide in the recombinant microbial cell. Such a recombinant microbial cell comprising an attenuated endogenous β-ketoacyl-ACP synthase gene preferably further comprises a polynucleotide sequence encoding an exogenous polypeptide having β-ketoacyl-ACP synthase activity that utilizes propionyl-CoA as a substrate.

In one embodiment, the recombinant microbial cell according to the first aspect comprises a polynucleotide encoding a polypeptide having aspartokinase activity which is categorized as EC 2.7.2.4 (Figure 2, pathway (A)). In some embodiments, the polypeptide having aspartokinase activity is encoded by a *thrA*, a *dapG* or a *hom3* gene. In one embodiment, the polypeptide having aspartokinase activity is endogenous to the parental microbial cell, or is exogenous to the parental microbial cell. In another embodiment, expression of the polynucleotide encoding the polypeptide having aspartokinase activity is modulated in the recombinant microbial cell. In some instances, expression of the polynucleotide is modulated by operatively linking the polynucleotide to an exogenous promoter, such that the polynucleotide is overexpressed in the recombinant microbial cell. In another embodiment, the polypeptide having aspartokinase activity comprises a sequence selected from SEQ ID NOs:20, 21, 22, 23, 24, or a variant or a fragment thereof having aspartokinase activity and which catalyzes the conversion of aspartate to aspartyl phosphate *in vitro* or *in vivo*, preferably *in vivo*.

In one embodiment, the recombinant microbial cell according to the first aspect comprises a polynucleotide encoding a polypeptide having homoserine dehydrogenase activity which is categorized as EC 1.1.1.3. In some embodiments, the polypeptide having homoserine dehydrogenase activity is encoded by a *thrA*, a *hom* or a *hom6* gene. In one embodiment, the polypeptide having homoserine dehydrogenase activity is endogenous to the parental microbial cell, or is exogenous to the parental microbial cell. In another embodiment, expression of the polynucleotide encoding the polypeptide having homoserine dehydrogenase activity is modulated in the recombinant microbial cell. In some instances, expression of the polynucleotide is modulated by operatively linking the polynucleotide to an exogenous promoter, such that the polynucleotide is overexpressed in the recombinant microbial cell. In another embodiment, the polypeptide having homoserine dehydrogenase activity comprises a sequence selected from SEQ ID NOs:20, 21, 25, 26, 27, or a variant or a fragment thereof having homoserine dehydrogenase activity and which catalyzes the conversion of aspartate semialdehyde to homoserine *in vitro* or *in vivo*, preferably *in vivo*.

In a particular embodiment, the recombinant microbial cell according to the first aspect comprises a polynucleotide encoding a polypeptide having both aspartokinase and homoserine dehydrogenase activity. In one embodiment, the polypeptide having aspartokinase and homoserine dehydrogenase activity is endogenous to the parental microbial cell, or is exogenous to the parental

microbial cell. In another embodiment, expression of the polynucleotide encoding the polypeptide having aspartokinase and homoserine dehydrogenase activity is modulated in the recombinant microbial cell. In some instances, expression of the polynucleotide is modulated by operatively linking the polynucleotide to an exogenous promoter, such that the polynucleotide is overexpressed in the recombinant microbial cell. In one embodiment the polypeptide having aspartokinase and homoserine dehydrogenase activity comprises the sequence SEQ ID NO:20 or a variant or a fragment thereof, such as SEQ ID NO:21, which catalyzes the conversion of aspartate to aspartyl phosphate and the conversion of aspartate semialdehyde to homoserine *in vitro* or *in vivo*, preferably *in vivo*.

In one embodiment, the recombinant microbial cell according to the first aspect comprises a polynucleotide encoding a polypeptide having homoserine kinase activity which is categorized as EC 2.7.1.39. In some embodiments, the polypeptide having homoserine kinase activity is encoded by a *thrB* gene or a *thr1* gene. In one embodiment, the polypeptide having homoserine kinase activity is endogenous to the parental microbial cell, or is exogenous to the parental microbial cell. In another embodiment, expression of the polynucleotide encoding the polypeptide having homoserine kinase activity is modulated in the recombinant microbial cell. In some instances, expression of the polynucleotide is modulated by operatively linking the polynucleotide to an exogenous promoter, such that the polynucleotide is overexpressed in the recombinant microbial cell. In another embodiment, the polypeptide having homoserine kinase activity comprises a sequence selected from SEQ ID NOs:28, 29, 30, 31, or a variant or a fragment thereof having homoserine kinase activity and which catalyzes the conversion of homoserine to O-phospho-L-homoserine *in vitro* or *in vivo*, preferably *in vivo*.

In one embodiment, the recombinant microbial cell according to the first aspect comprises a polynucleotide encoding a polypeptide having threonine synthase activity which is categorized as EC 4.2.3.1. In one embodiment, the polypeptide having threonine synthase activity is encoded by a *thrC* gene. In one embodiment, the polypeptide having threonine synthase activity is endogenous to the parental microbial cell, or is exogenous to the parental microbial cell. In another embodiment, expression of the polynucleotide encoding the polypeptide having threonine synthase activity is modulated in the recombinant microbial cell. In some instances, expression of the polynucleotide is modulated by operatively linking the polynucleotide to an exogenous promoter, such that the polynucleotide is overexpressed in the recombinant microbial cell. In another embodiment, the polypeptide having threonine synthase activity comprises a sequence selected from SEQ ID NOs:32, 33, 34, or a variant or a fragment thereof having threonine synthase activity and which catalyzes the conversion of O-phospho-L-homoserine to threonine *in vitro* or *in vivo*, preferably *in vivo*.

In one embodiment, the recombinant microbial cell according to the first aspect comprises a polynucleotide encoding a polypeptide having threonine deaminase activity which is categorized as EC 4.3.1.19. In some embodiments, the polypeptide having threonine deaminase activity is encoded

by a *tdcB* gene or an *ilvA* gene. In one embodiment, the polypeptide having threonine deaminase activity is endogenous to the parental microbial cell, or is exogenous to the parental microbial cell. In another embodiment, expression of the polynucleotide encoding the polypeptide having threonine deaminase activity is modulated in the recombinant microbial cell. In some instances, expression of the polynucleotide is modulated by operatively linking the polynucleotide to an exogenous promoter, such that the polynucleotide is overexpressed in the recombinant microbial cell. In another embodiment, the polypeptide having threonine deaminase activity comprises a sequence selected from SEQ ID NOs:35, 36, 37, 38, 39, or a variant or a fragment thereof having threonine deaminase activity and which catalyzes the conversion of threonine to 2-ketobutyrate *in vitro* or *in vivo*, preferably *in vivo*.

In one embodiment, the recombinant microbial cell according to the first aspect comprises a polynucleotide encoding a polypeptide having (R)-citramalate synthase activity which is categorized as EC 2.3.1.182 (Figure 2, pathway (B)). In one embodiment, the polypeptide having (R)-citramalate synthase activity is encoded by a *cimA* gene. In one embodiment, the polypeptide having (R)-citramalate synthase activity is endogenous to the parental microbial cell, or is exogenous to the parental microbial cell. In another embodiment, expression of the polynucleotide encoding the polypeptide having (R)-citramalate synthase activity is modulated in the recombinant microbial cell. In some instances, expression of the polynucleotide is modulated by operatively linking the polynucleotide to an exogenous promoter, such that the polynucleotide is overexpressed in the recombinant microbial cell. In another embodiment, the polypeptide having (R)-citramalate synthase activity comprises a sequence selected from SEQ ID NOs:40, 41, 42, 43, or a variant or a fragment thereof having (R)-citramalate synthase activity and which catalyzes the reaction of acetyl-CoA and pyruvate to (R)-citramalate *in vitro* or *in vivo*, preferably *in vivo*.

In one embodiment, the recombinant microbial cell according to the first aspect comprises a polynucleotide encoding a polypeptide having isopropylmalate isomerase activity which is categorized as EC 4.2.1.33. In one embodiment, the polypeptide having isopropylmalate isomerase activity comprises a large subunit and a small subunit encoded by *leuCD* genes. In one embodiment, the polypeptide having isopropylmalate isomerase activity is endogenous to the parental microbial cell, or is exogenous to the parental microbial cell. In another embodiment, expression of the polynucleotide encoding the polypeptide having isopropylmalate isomerase activity is modulated in the recombinant microbial cell. In some instances, expression of the polynucleotide is modulated by operatively linking the polynucleotide to an exogenous promoter, such that the polynucleotide is overexpressed in the recombinant microbial cell. In another embodiment, the polypeptide having isopropylmalate isomerase activity comprises a large subunit and a small subunit. In other embodiments, the polypeptide having isopropylmalate isomerase activity comprises a large subunit sequence selected from SEQ ID NOs:44 and 46 and a small subunit sequence selected from SEQ ID

NOs:45 and 47, or variants or fragments thereof having isopropylmalate isomerase activity and which catalyzes the conversion of (R)-citramalate to citraconate and citraconate to beta-methyl-D-malate *in vitro* or *in vivo*, preferably *in vivo*.

In one embodiment, the recombinant microbial cell according to the first aspect comprises a polynucleotide encoding a polypeptide having beta-isopropylmalate dehydrogenase activity which is categorized as EC 1.1.1.85. In some embodiments, the polypeptide having beta-isopropyl malate dehydrogenase activity is encoded by a *leuB* gene or a *leu2* gene. In one embodiment, the polypeptide having beta-isopropylmalate dehydrogenase activity is endogenous to the parental microbial cell, or is exogenous to the parental microbial cell. In another embodiment, expression of the polynucleotide encoding the polypeptide having beta-isopropylmalate dehydrogenase activity is modulated in the recombinant microbial cell. In some instances, expression of the polynucleotide is modulated by operatively linking the polynucleotide to an exogenous promoter, such that the polynucleotide is overexpressed in the recombinant microbial cell. In another embodiment, the polypeptide having beta-isopropyl malate dehydrogenase activity comprises a sequence selected from SEQ ID NOs:48, 49, 50, or a variant or a fragment thereof having beta-isopropylmalate dehydrogenase activity and which catalyzes conversion of beta-methyl-D-malate to 2-ketobutyrate *in vitro* or *in vivo*, preferably *in vivo*.

In one embodiment, the recombinant microbial cell according to the first aspect comprises a polynucleotide encoding a polypeptide having methylmalonyl-CoA mutase activity which is categorized as EC 5.4.99.2 (Figure 3). In some embodiments, the polypeptide having methylmalonyl-CoA mutase activity is encoded by an *scpA* (also known as *sbm*) gene. In one embodiment, the polypeptide having methylmalonyl-CoA mutase activity is endogenous to the parental microbial cell, or is exogenous to the parental microbial cell. In another embodiment, expression of the polynucleotide encoding the polypeptide having methylmalonyl-CoA mutase activity is modulated in the recombinant microbial cell. In some instances, expression of the polynucleotide is modulated by operatively linking the polynucleotide to an exogenous promoter, such that the polynucleotide is overexpressed in the recombinant microbial cell. In another embodiment, the polypeptide having methylmalonyl-CoA mutase activity comprises a sequence selected from SEQ ID NOs:51, 52, 53, 54, 55, 56, 57, 58, or a variant or a fragment thereof having methylmalonyl-CoA mutase activity and which catalyzes conversion of succinyl-CoA to methylmalonyl-CoA *in vitro* or *in vivo*, preferably *in vivo*.

In one embodiment, the recombinant microbial cell according to the first aspect comprises a polynucleotide encoding a polypeptide having methylmalonyl-CoA decarboxylase activity which is categorized as EC 4.1.1.41. In some embodiments, the polypeptide having methylmalonyl-CoA decarboxylase activity is encoded by an *scpB* (also known as *ygfG*) gene. In one embodiment, the polypeptide having methylmalonyl-CoA decarboxylase activity is endogenous to the parental

microbial cell, or is exogenous to the parental microbial cell. In another embodiment, expression of the polynucleotide encoding the polypeptide having methylmalonyl-CoA decarboxylase activity is modulated in the recombinant microbial cell. In some instances, expression of the polynucleotide is modulated by operatively linking the polynucleotide to an exogenous promoter, such that the polynucleotide is overexpressed in the recombinant microbial cell. In another embodiment, the polypeptide having methylmalonyl-CoA decarboxylase activity comprises a sequence selected from SEQ ID NOs:59, 60, 61, or a variant or a fragment thereof having methylmalonyl-CoA decarboxylase activity and which catalyzes conversion of methylmalonyl-CoA to propionyl-CoA *in vitro* or *in vivo*, preferably *in vivo*.

In one embodiment, the recombinant microbial cell according to the first aspect comprises a polynucleotide encoding a polypeptide having methylmalonyl-CoA carboxyltransferase activity which is categorized as EC 2.1.3.1. In one embodiment, the polypeptide having methylmalonyl-CoA carboxyltransferase activity is endogenous to the parental microbial cell, or is exogenous to the parental microbial cell. In another embodiment, expression of the polynucleotide encoding the polypeptide having methylmalonyl-CoA carboxyltransferase activity is modulated in the recombinant microbial cell. In some instances, expression of the polynucleotide is modulated by operatively linking the polynucleotide to an exogenous promoter, such that the polynucleotide is overexpressed in the recombinant microbial cell. In another embodiment, the polypeptide having methylmalonyl-CoA carboxyltransferase activity comprises the sequence SEQ ID NO:62, or a variant or a fragment thereof having methylmalonyl-CoA carboxyltransferase activity and which catalyzes conversion of methylmalonyl-CoA to propionyl-CoA *in vitro* or *in vivo*, preferably *in vivo*.

In one embodiment, the recombinant microbial cell according to the first aspect comprises a polynucleotide encoding a polypeptide having methylmalonyl-CoA epimerase activity which is categorized as EC 5.1.99.1. In one embodiment, the polypeptide having methylmalonyl-CoA epimerase activity is endogenous to the parental microbial cell, or is exogenous to the parental microbial cell. In another embodiment, expression of the polynucleotide encoding the polypeptide having methylmalonyl-CoA epimerase activity is modulated in the recombinant microbial cell. In some instances, expression of the polynucleotide is modulated by operatively linking the polynucleotide to an exogenous promoter, such that the polynucleotide is overexpressed in the recombinant microbial cell. In another embodiment, the polypeptide having methylmalonyl-CoA epimerase activity comprises the sequence SEQ ID NO:63, or a variant or a fragment thereof having methylmalonyl-CoA epimerase activity and which catalyzes conversion of (R)-methylmalonyl-CoA to (S)-methylmalonyl-CoA *in vitro* or *in vivo*, preferably *in vivo*.

In one embodiment, the recombinant microbial cell according to the first aspect comprises an endogenous polynucleotide sequence (such as, an endogenous *scpC* gene (also known as *ygfH*)) encoding a polypeptide having propionyl-CoA::succinyl-CoA transferase activity, and expression of

the endogenous polynucleotide in the recombinant microbial cell is attenuated. In some embodiments, expression of the endogenous polynucleotide is attenuated by deletion of all or part of the sequence of the endogenous polynucleotide in the recombinant microbial cell.

In one embodiment, the recombinant microbial cell according to the first aspect comprises an endogenous polynucleotide sequence (such as, an endogenous *fadE* gene) encoding a polypeptide having acyl-CoA dehydrogenase activity, and expression of the endogenous polynucleotide in the recombinant microbial cell may or may not be attenuated.

In other embodiments, a recombinant microbial cell according to the first aspect comprises a polynucleotide encoding a polypeptide having a fatty acid derivative enzyme activity, wherein the recombinant microbial cell produces a fatty acid derivative composition comprising odd chain fatty acid derivatives when cultured in the presence of a carbon source.

In various embodiments, the fatty acid derivative enzyme activity comprises a thioesterase activity, an ester synthase activity, a fatty aldehyde biosynthesis activity, a fatty alcohol biosynthesis activity, a ketone biosynthesis activity, and/or a hydrocarbon biosynthesis activity. In some embodiments, the recombinant microbial cell comprises polynucleotides encoding two or more polypeptides, each polypeptide having a fatty acid derivative enzyme activity. In more particular embodiments, the recombinant microbial cell expresses or overexpresses one or more polypeptides having fatty acid derivative enzyme activity selected from: (1) a polypeptide having thioesterase activity; (2) a polypeptide having decarboxylase activity; (3) a polypeptide having carboxylic acid reductase activity; (4) a polypeptide having alcohol dehydrogenase activity (EC 1.1.1.1); (5) a polypeptide having aldehyde decarbonylase activity (EC 4.1.99.5); (6) a polypeptide having acyl-CoA reductase activity (EC 1.2.1.50); (7) a polypeptide having acyl-ACP reductase activity; (8) a polypeptide having ester synthase activity (EC 3.1.1.67); (9) a polypeptide having OleA activity; or (10) a polypeptide having OleCD or OleBCD activity; wherein the recombinant microbial cell produces a composition comprising odd chain fatty acids, odd chain fatty esters, odd chain wax esters, odd chain fatty aldehydes, odd chain fatty alcohols, even chain alkanes, even chain alkenes, even chain internal olefins, even chain terminal olefins, or even chain ketones.

In one embodiment, the fatty acid derivative enzyme activity comprises a thioesterase activity, wherein a culture comprising the recombinant microbial cell produces a fatty acid composition comprising odd chain fatty acids when cultured in the presence of a carbon source. In some embodiments, the polypeptide has a thioesterase activity which is categorized as EC 3.1.1.5, EC 3.1.2.- , or EC 3.1.2.14. In some embodiments, the polypeptide having a thioesterase activity is encoded by a *tesA*, a *tesB*, a *fatA*, or a *fatB* gene. In some embodiments, the polypeptide having thioesterase activity is endogenous to the parental microbial cell, or is exogenous to the parental microbial cell. In another embodiment, expression of the polynucleotide encoding the polypeptide having thioesterase activity is modulated in the recombinant microbial cell. In some instances,

expression of the polynucleotide is modulated by operatively linking the polynucleotide to an exogenous promoter, such that the polynucleotide is overexpressed in the recombinant microbial cell. In another embodiment, the polypeptide having thioesterase activity comprises a sequence selected from SEQ ID NO: 64, 65, 66, 67, 68, 69, 70, 71 and 72, or a variant or a fragment thereof having thioesterase activity and which catalyzes the hydrolysis of an odd chain acyl-ACP to an odd chain fatty acid, or catalyzes the alcoholysis of an odd chain acyl-ACP to an odd chain fatty ester, *in vitro* or *in vivo*, preferably *in vivo*. In some embodiments, the recombinant microbial cell according to the first aspect, comprising a polynucleotide encoding a polypeptide having thioesterase activity, when cultured in the presence of a carbon source, produces at least 50 mg/L, at least 75 mg/L, at least 100 mg/L, at least 200 mg/L, at least 500 mg/L, at least 1000 mg/L, or at least 2000 mg/L odd chain fatty acids when cultured in a culture medium containing a carbon source under conditions effective to express the polynucleotides. In some embodiments, the recombinant microbial cell according to the first aspect, comprising a polynucleotide encoding a polypeptide having thioesterase activity, produces a fatty acid composition comprising odd chain fatty acids and even chain fatty acids. In some embodiments, at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the fatty acids in the composition are odd chain fatty acids.

The invention includes a cell culture comprising the recombinant microbial cell according to the first aspect.

In a second aspect, the invention includes a method of producing odd chain fatty acid derivatives (or a fatty acid derivative composition comprising odd chain fatty acid derivatives) in a recombinant microbial cell, the method comprising expressing in the cell a recombinant polypeptide having enzymatic activity effective to increase the production of propionyl-CoA in the cell, and culturing the cell in the presence of a carbon source under conditions effective to express the recombinant polypeptide and produce the odd chain fatty acid derivatives.

In one embodiment, the method of making a fatty acid derivative composition comprising odd chain fatty acid derivatives comprises obtaining a recombinant microbial cell according to the first aspect, culturing the cell in a culture medium containing a carbon source under conditions effective to express the polynucleotides according to (a), (b), and (c) and produce a fatty acid derivative composition comprising odd chain fatty acid derivatives, and optionally recovering the composition from the culture medium.

In some embodiments, the fatty acid derivative composition produced by the method according to the second aspect comprises odd chain fatty acid derivatives and even chain fatty acid derivatives, wherein at least 5%, at least 6%, at least 8%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% by weight of the fatty acid derivatives in the composition are odd chain fatty acid derivatives. In some embodiments, the

fatty acid derivative composition comprises odd chain fatty acid derivatives in an amount (*e.g.*, a titer) of at least 50 mg/L, at least 75 mg/L, at least 100 mg/L, at least 200 mg/L, at least 500 mg/L, at least 1000 mg/L, at least 2000 mg/L, at least 5000 mg/L, at least 10000 mg/L, or at least 20000 mg/L.

In various embodiments of the second aspect, the fatty acid derivative enzyme activity comprises a thioesterase activity, an ester synthase activity, a fatty aldehyde biosynthesis activity, a fatty alcohol biosynthesis activity, a ketone biosynthesis activity, and/or a hydrocarbon biosynthesis activity. In some embodiments, the recombinant microbial cell comprises polynucleotides encoding two or more polypeptides, each polypeptide having a fatty acid derivative enzyme activity. In more particular embodiments, the recombinant microbial cell expresses or overexpresses one or more polypeptides having fatty acid derivative enzyme activity selected from: (1) a polypeptide having thioesterase activity; (2) a polypeptide having decarboxylase activity; (3) a polypeptide having carboxylic acid reductase activity; (4) a polypeptide having alcohol dehydrogenase activity (EC 1.1.1.1); (5) a polypeptide having aldehyde decarbonylase activity (EC 4.1.99.5); (6) a polypeptide having acyl-CoA reductase activity (EC 1.2.1.50); (7) a polypeptide having acyl-ACP reductase activity; (8) a polypeptide having ester synthase activity (EC 3.1.1.67); (9) a polypeptide having OleA activity; or (10) a polypeptide having OleCD or OleBCD activity; wherein the recombinant microbial cell produces a composition comprising one or more of odd chain fatty acids, odd chain fatty esters, odd chain wax esters, odd chain fatty aldehydes, odd chain fatty alcohols, even chain alkanes, even chain alkenes, even chain internal olefins, even chain terminal olefins, and even chain ketones.

The invention includes a fatty acid derivative composition comprising odd chain fatty acid derivatives produced by the method according to the second aspect.

In a third aspect, the invention includes a method of making a recombinant microbial cell which produces a higher titer or higher proportion of odd chain fatty acid derivatives than a parental microbial cell, the method comprising obtaining a parental microbial cell comprising a polynucleotide encoding a polypeptide having fatty acid derivative enzyme activity, and engineering the parental microbial cell to obtain a recombinant microbial cell which produces or is capable of producing a greater amount of propionyl-CoA than the amount of propionyl-CoA produced by the parental microbial cell when cultured under the same conditions, wherein the recombinant microbial cell produces a higher titer or higher proportion of odd chain fatty acid derivatives when cultured in the presence of a carbon source under conditions effective to produce propionyl-CoA and fatty acid derivatives in the recombinant microbial cell, relative to the titer or proportion of odd chain fatty acid derivatives produced by the parental microbial cell cultured under the same conditions.

In a fourth aspect, the invention includes a method of increasing the titer or proportion of odd chain fatty acid derivatives produced by a microbial cell, the method comprising obtaining a parental microbial cell that is capable of producing a fatty acid derivative, and engineering the parental

microbial cell to obtain a recombinant microbial cell which produces or is capable of producing a greater amount of propionyl-CoA than the amount of propionyl-CoA produced by the parental microbial cell when cultured under the same conditions, wherein the recombinant microbial cell produces a higher titer or higher proportion of odd chain fatty acid derivatives when cultured in the presence of a carbon source under conditions effective to produce propionyl-CoA and fatty acid derivatives in the recombinant microbial cell, relative to the titer or proportion of odd chain fatty acid derivatives produced by the parental microbial cell cultured under the same conditions.

In some embodiments according to the third or fourth aspect, the step of engineering the parental microbial cell comprises engineering the cell to express polynucleotides encoding polypeptides selected from (a) one or more polypeptides having aspartokinase activity, homoserine dehydrogenase activity, homoserine kinase activity, threonine synthase activity, and threonine deaminase activity; (b) one or more polypeptides having (R)-citramalate synthase activity, isopropylmalate isomerase activity, and beta-isopropylmalate dehydrogenase activity; and (c) one or more polypeptides having methylmalonyl-CoA mutase activity, methylmalonyl-CoA decarboxylase activity, methylmalonyl-CoA carboxyltransferase activity, and methylmalonyl-CoA epimerase activity; wherein at least one polypeptide according to (a), (b) or (c) is exogenous to the parental microbial cell, or wherein expression of at least one polynucleotide according to (a), (b) or (c) is modulated in the recombinant microbial cell as compared to the expression of the polynucleotide in the parental microbial cell. In some embodiments, expression of at least one polynucleotide is modulated by overexpression of the polynucleotide, such as by operatively linking the polynucleotide to an exogenous promoter. In some embodiments, the engineered cell expresses one or more polypeptide according to (a) and one or more polypeptide according to (b).

In some embodiments according to the third or fourth aspect, the parental microbial cell comprises a polynucleotide encoding a polypeptide having β-ketoacyl-ACP synthase activity that utilizes propionyl-CoA as a substrate. In some embodiments, the recombinant microbial cell is engineered to express an exogenous polynucleotide or to overexpress an endogenous polynucleotide encoding a polypeptide having β-ketoacyl-ACP synthase activity that utilizes propionyl-CoA as a substrate. In some embodiments, the recombinant microbial cell is engineered to express an exogenous polynucleotide encoding a polypeptide having β-ketoacyl-ACP synthase activity that utilizes propionyl-CoA as a substrate, and expression of an endogenous polynucleotide encoding a polypeptide having β-ketoacyl-ACP synthase activity is attenuated. In some embodiments, the polynucleotide encoding a polypeptide having β-ketoacyl-ACP synthase is a modified, mutant or variant form of an endogenous polynucleotide, which has been selected for enhanced affinity or activity for propionyl-CoA as a substrate relative to the unmodified endogenous polynucleotide. Numerous methods for generation of modified, mutant or variant polynucleotides are well known in the art, examples of which are described herein below.

These and other objects and features of the invention will become more fully apparent when the following detailed description is read in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B compare exemplary intermediates and products of fatty acid biosynthetic pathways when supplied with different acyl-CoA "primer" molecules: Fig. 1A shows a reaction pathway utilizing the two-carbon primer acetyl-CoA , which generates the even chain length β-ketoacyl-ACP intermediate acetoacetyl-ACP, leading to even chain (ec)-acyl-ACP intermediates and even chain fatty acid derivatives produced therefrom; and Fig. 1B shows a reaction pathway utilizing the three carbon primer propionyl-CoA, which generates the odd chain length β-ketoacyl-ACP intermediate 3-oxovaleryl-ACP, leading to odd chain (oc)-acyl-ACP intermediates and odd chain fatty acid derivatives produced therefrom.

Fig. 2 depicts exemplary pathways for increased production of propionyl-CoA via the intermediate α-ketobutyrate, by a threonine biosynthetic pathway (pathway (A)) and by a citramalate biosynthetic pathway (pathway (B)) as described herein.

Fig. 3 depicts an exemplary pathway for increased production of propionyl-CoA via a methylmalonyl-CoA biosynthetic pathway (pathway (C)) as described herein.

DETAILED DESCRIPTION

The invention is not limited to the specific compositions and methodology described herein, as these may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention.

Accession Numbers: Sequence Accession numbers throughout this description were obtained from databases provided by the NCBI (National Center for Biotechnology Information) maintained by the National Institutes of Health, U.S.A. (which are identified herein as "NCBI Accession Numbers" or alternatively as "GenBank Accession Numbers") , and from the UniProt Knowledgebase (UniProtKB) and Swiss-Prot databases provided by the Swiss Institute of Bioinformatics (which are identified herein as "UniProtKB Accession Numbers"). Unless otherwise expressly indicated, the sequence identified by an NCBI / GenBank Accession number is version number 1 (that is, the Version Number of the sequence is "AccessionNumber.1"). The NCBI and UniProtKB accession numbers provided herein were current as of August 2, 2011.

Enzyme Classification (EC) Numbers: EC numbers are established by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB), description of which is available on the IUBMB Enzyme Nomenclature website on the World Wide Web. EC numbers classify enzymes according to the reaction catalyzed. EC numbers referenced herein are

derived from the KEGG Ligand database, maintained by the Kyoto Encyclopedia of Genes and Genomics, sponsored in part by the University of Tokyo. Unless otherwise indicated, EC numbers are as provided in the KEGG database as of August 2, 2011.

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 invention belongs. Although any materials and methods similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred compositions and methods are now described.

Definitions

As used herein, the term "fatty acid" refers to a carboxylic acid having the formula R-(C=O)-OH, wherein R represents a carbon chain which can be between about 4 and about 36 carbon atoms in length, more generally between about 4 and about 22 carbon atoms in length. Fatty acids can be saturated or unsaturated. If unsaturated, R can have one or more points of unsaturation, that is, R can be monounsaturated or polyunsaturated. R can be a straight chain (also referred to herein as a "linear chain") or a branched chain. The term "fatty acid" may be used herein to refer to a "fatty acid derivative" which can include one or more different fatty acid derivative, or mixtures of fatty acids derivatives.

An "odd chain fatty acid" (abbreviated "oc-FA") as used herein refers to a fatty acid molecule having a linear carbon chain containing an odd number of carbon atoms, inclusive of the carbonyl carbon. Non-limiting examples of oc-FAs include tridecanoic acid (C13:0), pentadecanoic acid (C15:0), and heptadecanoic acid (C17:0), which are saturated oc-FAs, and heptadecenoic acid (C17:1), which is an unsaturated (*i.e.*, a monounsaturated) oc-FA.

The term "β-ketoacyl-ACP" as used herein refers to the product of the condensation of an acyl-CoA primer molecule with malonyl-ACP catalyzed by an enzyme having beta ketoacyl-ACP synthase activity (*e.g.*, EC 2.3.1.180) as represented by part (D) of the pathways shown in Figs. 1A and 1B. The acyl-CoA primer molecule may have an acyl group containing an even number of carbon atoms, such as acetyl-CoA as represented in Fig. 1A, in which case the resulting β-ketoacyl-ACP intermediate is acetoacetyl-ACP, which is an even chain (ec-)β-ketoacyl-ACP. The acyl-CoA primer molecule may have an acyl group containing an odd number of carbon atoms, such as propionyl-CoA as represented in Fig. 1B, in which case the resulting β-ketoacyl-ACP intermediate is 3-oxovaleryl-ACP, which is an odd chain (oc-)β-ketoacyl-ACP. The β-ketoacyl-ACP intermediate enters the fatty acid synthase (FAS) cycle, represented by part (E) of Figs. 1A and 1B, where it is subjected to a round of elongation (*i.e.*, keto reduction, dehydration, and enoyl reduction), adding two carbon units to the acyl chain, followed by additional elongation cycles, which each involve condensation with another malonyl-ACP molecule, keto reduction, dehydration, and enoyl reduction, such that the acyl chain of the acyl-ACP is elongated by two carbon units per elongation cycle.

An "acyl-ACP" generally refers to the product of one or more rounds of FAS-catalyzed elongation of a β-ketoacyl-ACP intermediate. Acyl-ACP is an acyl thioester formed between the carbonyl carbon of an alkyl chain and the sulfhydryl group of the 4'-phosphopantethionyl moiety of an acyl carrier protein (ACP), and, in the case of a linear carbon chain, typically has the formula CH3-(CH2)n-C(=O)-s-ACP wherein n may be an even number (*e.g.*, an "even chain acyl-ACP" or "ec-acyl-ACP", which is produced, for example, when acetyl-CoA is the primer molecule, see Fig. 1A) or an odd number (*e.g.*, an "odd chain acyl-ACP" or "oc-acyl-ACP", which is produced, for example, when propionyl-CoA is the primer molecule, see Fig. 1B).

Unless otherwise specified, a "fatty acid derivative" (abbreviated "FA derivative") is intended to include any product made at least in part by the fatty acid biosynthetic pathway of the recombinant microbial cell. A fatty acid derivative also includes any product made at least in part by a fatty acid pathway intermediate, such as an acyl-ACP intermediate. The fatty acid biosynthetic pathways described herein can include fatty acid derivative enzymes which can be engineered to produce fatty acid derivatives, and in some instances additional enzymes can be expressed to produce fatty acid derivatives having desired carbon chain characteristics, such as, for example, compositions of fatty acid derivatives having carbon chains containing a desired number of carbon atoms, or compositions of fatty acid derivatives having a desired proportion of derivatives containing odd numbered carbon chains, and the like. Fatty acid derivatives include, but are not limited to, fatty acids, fatty aldehydes, fatty alcohols, fatty esters (such as waxes), hydrocarbons (such as alkanes and alkenes (including terminal olefins and internal olefins)) and ketones.

The term "odd chain fatty acid derivative" (abbreviated "oc-FA derivative") refers to a product of the reaction of an oc-acyl-ACP, as defined above, with one or more fatty acid derivative enzymes. The resulting fatty acid derivative product likewise has a linear carbon chain containing an odd number of carbon atoms, unless the fatty acid derivative is itself the product of decarbonylation or decarboxylation of an oc-FA derivative or an oc-acyl-ACP, in which case the resulting oc-FA derivative has an even number of carbon atoms; for example, when the fatty acid derivative is an ec-alkane or ec-alkene produced by decarbonylation of an oc-fatty aldehyde, an ec-terminal olefin produced by decarboxylation of an oc-fatty acid, an ec-ketone or an ec-internal olefin produced by decarboxylation of an oc-acyl-ACP, and so forth. It is to be understood that such even chain length products of oc-FA derivatives or oc-acyl-ACP precursor molecules, despite having linear chains containing an even number of carbon atoms, are nevertheless considered to fall under the definition of "oc-FA derivatives".

An "endogenous" polypeptide refers to a polypeptide encoded by the genome of the parental microbial cell (also termed "host cell") from which the recombinant cell is engineered (or "derived").

An "exogenous" polypeptide refers to a polypeptide which is not encoded by the genome of the parental microbial cell. A variant (*i.e.*, mutant) polypeptide is an example of an exogenous polypeptide.

In embodiments of the invention wherein a polynucleotide sequence encodes an endogenous polypeptide, in some instances the endogenous polypeptide is overexpressed. As used herein, "overexpress" means to produce or cause to be produced a polynucleotide or a polypeptide in a cell at a greater concentration than is normally produced in the corresponding parental cell (such as, a wild-type cell) under the same conditions. A polynucleotide or a polypeptide can be "overexpressed" in a recombinant microbial cell when the polynucleotide or polypeptide is present in a greater concentration in the recombinant microbial cell as compared to its concentration in a non-recombinant microbial cell of the same species (such as, the parental microbial cell) under the same conditions. Overexpression can be achieved by any suitable means known in the art.

In some embodiments, overexpression of the endogenous polypeptide in the recombinant microbial cell can be achieved by the use of an exogenous regulatory element. The term "exogenous regulatory element" generally refers to a regulatory element (such as, an expression control sequence or a chemical compound) originating outside of the host cell. However, in certain embodiments, the term "exogenous regulatory element" (*e.g.*, "exogenous promoter") can refer to a regulatory element derived from the host cell whose function is replicated or usurped for the purpose of controlling the expression of the endogenous polypeptide in the recombinant cell. For example, if the host cell is an *E. coli* cell, and the polypeptide is an endogenous polypeptide, then expression of the endogenous polypeptide the recombinant cell can be controlled by a promoter derived from another *E. coli* gene. In some embodiments, the exogenous regulatory element that causes an increase in the level of expression and/or activity of an endogenous polypeptide is a chemical compound, such as a small molecule.

In some embodiments, the exogenous regulatory element which controls the expression of a polynucleotide (*e.g.*, an endogenous polynucleotide) encoding an endogenous polypeptide is an expression control sequence which is operably linked to the endogenous polynucleotide by recombinant integration into the genome of the host cell. In certain embodiments, the expression control sequence is integrated into a host cell chromosome by homologous recombination using methods known in the art (*e.g.*, Datsenko *et al., Proc. Natl. Acad. Sci. U.S.A.*, 97(12): 6640-6645 (2000)).

Expression control sequences are known in the art and include, for example, promoters, enhancers, polyadenylation signals, transcription terminators, internal ribosome entry sites (IRES), and the like, that provide for the expression of the polynucleotide sequence in a host cell. Expression control sequences interact specifically with cellular proteins involved in transcription (Maniatis *et al.*, *Science, 236*: 1237-1245 (1987)). Exemplary expression control sequences are described in, for

example, Goeddel, *Gene Expression Technology: Methods in Enzymology*, Vol. 185, Academic Press, San Diego, Calif. (1990).

In the methods of the invention, an expression control sequence is operably linked to a polynucleotide sequence. By "operably linked" is meant that a polynucleotide sequence and expression control sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (*e.g.*, transcriptional activator proteins) are bound to the expression control sequence(s). Operably linked promoters are located upstream of the selected polynucleotide sequence in terms of the direction of transcription and translation. Operably linked enhancers can be located upstream, within, or downstream of the selected polynucleotide. Additional nucleic acid sequences, such as nucleic acid sequences encoding selection markers, purification moieties, targeting proteins, and the like, can be operatively linked to the polynucleotide sequence, such that the additional nucleic acid sequences are expressed together with the polynucleotide sequence.

In some embodiments, the polynucleotide sequence is provided to the recombinant cell by way of a recombinant vector, which comprises a promoter operably linked to the polynucleotide sequence. In certain embodiments, the promoter is a developmentally-regulated, an organelle-specific, a tissue-specific, an inducible, a constitutive, or a cell-specific promoter.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid, *i.e.*, a polynucleotide sequence, to which it has been linked. One type of useful vector is an episome (*i.e.*, a nucleic acid capable of extra-chromosomal replication). Useful vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors." In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids," which refer generally to circular double stranded DNA loops that, in their vector form, are not bound to the chromosome. The terms "plasmid" and "vector" are used interchangeably herein, inasmuch as a plasmid is the most commonly used form of vector. However, also included are such other forms of expression vectors that serve equivalent functions and that become known in the art subsequently hereto.

In some embodiments, the recombinant vector comprises at least one sequence selected from the group consisting of (a) an expression control sequence operatively linked to the polynucleotide sequence; (b) a selection marker operatively linked to the polynucleotide sequence; (c) a marker sequence operatively linked to the polynucleotide sequence; (d) a purification moiety operatively linked to the polynucleotide sequence; (e) a secretion sequence operatively linked to the polynucleotide sequence; and (f) a targeting sequence operatively linked to the polynucleotide sequence.

The expression vectors described herein include a polynucleotide sequence described herein in a form suitable for expression of the polynucleotide sequence in a host cell. It will be appreciated

by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of polypeptide desired, etc. The expression vectors described herein can be introduced into host cells to produce polypeptides, including fusion polypeptides, encoded by the polynucleotide sequences as described herein.

Expression of genes encoding polypeptides in prokaryotes, for example, *E. coli*, is often carried out with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion polypeptides. Fusion vectors add a number of amino acids to a polypeptide encoded therein, usually to the amino- or carboxy-terminus of the recombinant polypeptide. Such fusion vectors typically serve one or more of the following three purposes: (1) to increase expression of the recombinant polypeptide; (2) to increase the solubility of the recombinant polypeptide; and (3) to aid in the purification of the recombinant polypeptide by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant polypeptide. This enables separation of the recombinant polypeptide from the fusion moiety after purification of the fusion polypeptide. Examples of such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin, and enterokinase. Exemplary fusion expression vectors include pGEX (Pharmacia Biotech, Inc., Piscataway, NJ; Smith *et al.*, *Gene, 67*: 31-40 (1988)), pMAL (New England Biolabs, Beverly, MA), and pRITS (Pharmacia Biotech, Inc., Piscataway, N.J.), which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant polypeptide.

Vectors can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in, for example, Sambrook *et al.* (*supra*).

For stable transformation of bacterial cells, it is known that, depending upon the expression vector and transformation technique used, only a small fraction of cells will take up and replicate the expression vector. In order to identify and select these transformants, a gene that encodes a selectable marker (*e.g.*, resistance to an antibiotic) can be introduced into the host cells along with the gene of interest. Selectable markers include those that confer resistance to drugs such as, but not limited to, ampicillin, kanamycin, chloramphenicol, or tetracycline. Nucleic acids encoding a selectable marker can be introduced into a host cell on the same vector as that encoding a polypeptide described herein or can be introduced on a separate vector. Host cells which are stably transformed with the introduced nucleic acid, resulting in recombinant cells, can be identified by growth in the presence of an appropriate selection drug.

Similarly, for stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to an antibiotic) can be introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin, and methotrexate. Nucleic acids encoding a selectable marker can be introduced into a host cell on the same vector as that encoding a polypeptide described herein or can be introduced on a separate vector. Host cells stably transfected with the introduced nucleic acid, resulting in recombinant cells, can be identified by growth in the presence of an appropriate selection drug.

"Gene knockout", as used herein, refers to a procedure by which a gene encoding a target protein is modified or inactivated so to reduce or eliminate the function of the intact protein. Inactivation of the gene may be performed by general methods such as mutagenesis by UV irradiation or treatment with N-methyl-N′-nitro-N-nitrosoguanidine, site-directed mutagenesis, homologous recombination, insertion-deletion mutagenesis, or "Red-driven integration" (Datsenko *et al., Proc. Natl. Acad. Sci. USA*, 97:6640-45, 2000). For example, in one embodiment, a construct is introduced into a parental cell, such that it is possible to select for homologous recombination events in the resulting recombinant cell. One of skill in the art can readily design a knock-out construct including both positive and negative selection genes for efficiently selecting transfected (*i.e.*, recombinant) cells that undergo a homologous recombination event with the construct. The alteration in the parental cell may be obtained, for example, by replacing through a single or double crossover recombination a wild type (*i.e.*, endogenous) DNA sequence by a DNA sequence containing the alteration. For convenient selection of transformants (*i.e.*, recombinant cells), the alteration may, for example, be a DNA sequence encoding an antibiotic resistance marker or a gene complementing a possible auxotrophy of the host cell. Mutations include, but are not limited to, deletion-insertion mutations. An example of such an alteration in a recombinant cell includes a gene disruption, *i.e.*, a perturbation of a gene such that the product that is normally produced from this gene is not produced in a functional form. This could be due to a complete deletion, a deletion and insertion of a selective marker, an insertion of a selective marker, a frameshift mutation, an in-frame deletion, or a point mutation that leads to premature termination. In some instances, the entire mRNA for the gene is absent. In other situations, the amount of mRNA produced varies.

The phrase "increasing the level of expression of an endogenous polypeptide" means to cause the overexpression of a polynucleotide sequence encoding the endogenous polypeptide, or to cause the overexpression of an endogenous polypeptide sequence. The degree of overexpression can be about 1.5-fold or more, about 2-fold or more, about 3-fold or more, about 5-fold or more, about 10-

fold or more, about 20-fold or more, about 50-fold or more, about 100-fold or more, or any range therein.

The phrase "increasing the level of activity of an endogenous polypeptide" means to enhance the biochemical or biological function (*e.g.*, enzymatic activity) of an endogenous polypeptide. The degree of enhanced activity can be about 10% or more, about 20% or more, about 50% or more, about 75% or more, about 100% or more, about 200% or more, about 500% or more, about 1000% or more, or any range therein.

The phrase, "the expression of said polynucleotide sequence is modified relative to the wild type polynucleotide sequence", as used herein means an increase or decrease in the level of expression and/or activity of an endogenous polynucleotide sequence. In some embodiments, an exogenous regulatory element which controls the expression of an endogenous polynucleotide is an expression control sequence which is operably linked to the endogenous polynucleotide by recombinant integration into the genome of the host cell. In some embodiments, the expression control sequence is integrated into a host cell chromosome by homologous recombination using methods known in the art.

As used herein, the phrase "under conditions effective to express said polynucleotide sequence(s)" means any conditions that allow a recombinant cell to produce a desired fatty acid derivative. Suitable conditions include, for example, fermentation conditions. Fermentation conditions can comprise many parameters, such as temperature ranges, levels of aeration, and media composition. Each of these conditions, individually and in combination, allows the host cell to grow. Exemplary culture media include broths or gels. Generally, the medium includes a carbon source that can be metabolized by a recombinant cell directly. Fermentation denotes the use of a carbon source by a production host, such as a recombinant microbial cell of the invention. Fermentation can be aerobic, anaerobic, or variations thereof (such as micro-aerobic). As will be appreciated by those of skill in the art, the conditions under which a recombinant microbial cell can process a carbon source into an oc-acyl-ACP or a desired oc-FA derivative (*e.g.*, an oc-fatty acid, an oc-fatty ester, an oc-fatty aldehyde, an oc-fatty alcohol, an ec-alkane, an ec-alkene or an ec-ketone) will vary in part, based upon the specific microorganism. In some embodiments, the process occurs in an aerobic environment. In some embodiments, the process occurs in an anaerobic environment. In some embodiments, the process occurs in a micro-aerobic environment.

As used herein, the term "carbon source" refers to a substrate or compound suitable to be used as a source of carbon for prokaryotic or simple eukaryotic cell growth. Carbon sources can be in various forms, including, but not limited to polymers, carbohydrates (*e.g.*, sugars, such as monosaccharides, disaccharides, oligosaccharides, and polysaccharides), acids, alcohols, aldehydes, ketones, amino acids, peptides, and gases (*e.g.*, CO and CO_2). Exemplary carbon sources include, but are not limited to: monosaccharides, such as glucose, fructose, mannose, galactose, xylose, and

arabinose; disaccharides, such as sucrose, maltose, cellobiose, and turanose; oligosaccharides, such as fructo-oligosaccharide and galacto-oligosaccharide; polysaccharides, such as starch, cellulose, pectin, and xylan; cellulosic material and variants such as hemicelluloses, methyl cellulose and sodium carboxymethyl cellulose; saturated or unsaturated fatty acids, succinate, lactate, and acetate; alcohols, such as ethanol, methanol, and glycerol, or mixtures thereof. The carbon source can be a product of photosynthesis, such as glucose. In certain preferred embodiments, the carbon source is derived from biomass. In another preferred embodiment, the carbon source comprises sucrose. In another preferred embodiment, the carbon source comprises glucose.

As used herein, the term "biomass" refers to any biological material from which a carbon source is derived. In some embodiments, a biomass is processed into a carbon source, which is suitable for bioconversion. In other embodiments, the biomass does not require further processing into a carbon source. The carbon source can be converted into a biofuel. An exemplary source of biomass is plant matter or vegetation, such as corn, sugar cane, or switchgrass. Another exemplary source of biomass is metabolic waste products, such as animal matter (*e.g.*, cow manure). Further exemplary sources of biomass include algae and other marine plants. Biomass also includes waste products from industry, agriculture, forestry, and households, including, but not limited to, fermentation waste, ensilage, straw, lumber, sewage, garbage, cellulosic urban waste, and food leftovers. The term "biomass" also can refer to sources of carbon, such as carbohydrates (*e.g.*, monosaccharides, disaccharides, or polysaccharides).

To determine if conditions are sufficient to allow production of a product or expression of a polypeptide, a recombinant microbial cell can be cultured, for example, for about 4, 8, 12, 24, 36, 48, 72, or more hours. During and/or after culturing, samples can be obtained and analyzed to determine if the conditions allow production or expression. For example, the recombinant microbial cells in the sample or the medium in which the recombinant microbial cells were grown can be tested for the presence of a desired product. When testing for the presence of a desired product, such as an odd chain fatty acid derivative (*e.g.*, an oc-fatty acid, an oc-fatty ester, an oc-fatty aldehyde, an oc-fatty alcohol, or an ec-hydrocarbon), assays such as, but not limited to, gas chromatography (GC), mass spectroscopy (MS), thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), liquid chromatography (LC), GC coupled with a flame ionization detector (GC-FID), GC-MS, and LC-MS, can be used. When testing for the expression of a polypeptide, techniques such as, but not limited to, Western blotting and dot blotting, may be used.

As used herein, the term "microorganism" means prokaryotic and eukaryotic microbial species from the domains *Archaea*, *Bacteria* and *Eucarya*, the latter including yeast and filamentous fungi, protozoa, algae, and higher *Protista*. The terms "microbes" and "microbial cells" (*i.e.*, cells from microbes) and are used interchangeably with "microorganisms" and refer to cells or small organisms that can only be seen with the aid of a microscope.

In some embodiments, the host cell (*e.g.*, parental cell) is a microbial cell. In some embodiments, the host cell is a microbial cell selected from the genus *Escherichia, Bacillus, Lactobacillus, Pantoea, Zymomonas, Rhodococcus, Pseudomonas, Aspergillus, Trichoderma, Neurospora, Fusarium, Humicola, Rhizomucor, Kluyveromyces, Pichia, Mucor, Myceliophtora, Penicillium, Phanerochaete, Pleurotus, Trametes, Chrysosporium, Saccharomyces, Stenotrophamonas, Schizosaccharomyces, Yarrowia, Streptomyces, Synechococcus, Chlorella, or Prototheca.*

In other embodiments, the host cell is a *Bacillus lentus* cell, a *Bacillus brevis* cell, a *Bacillus stearothermophilus* cell, a *Bacillus lichenoformis* cell, a *Bacillus alkalophilus* cell, a *Bacillus coagulans* cell, a *Bacillus circulans* cell, a *Bacillus pumilis* cell, a *Bacillus thuringiensis* cell, a *Bacillus clausii* cell, a *Bacillus megaterium* cell, a *Bacillus subtilis* cell, or a *Bacillus amyloliquefaciens* cell.

In other embodiments, the host cell is a *Trichoderma koningii* cell, a *Trichoderma viride* cell, a *Trichoderma reesei* cell, a *Trichoderma longibrachiatum* cell, an *Aspergillus awamori* cell, an *Aspergillus fumigates* cell, an *Aspergillus foetidus* cell, an *Aspergillus nidulans* cell, an *Aspergillus niger* cell, an *Aspergillus oryzae* cell, a *Humicola insolens* cell, a *Humicola lanuginose* cell, a *Rhodococcus opacus* cell, a *Rhizomucor miehei* cell, or a *Mucor michei* cell.

In yet other embodiments, the host cell is a *Streptomyces lividans* cell or a *Streptomyces murinus* cell.

In yet other embodiments, the host cell is an *Actinomycetes* cell.

In some embodiments, the host cell is a *Saccharomyces cerevisiae* cell.

In still other embodiments, the host cell is a CHO cell, a COS cell, a VERO cell, a BHK cell, a HeLa cell, a Cvl cell, an MDCK cell, a 293 cell, a 3T3 cell, or a PC12 cell.

In some embodiments, the host cell is a cell from an eukaryotic plant, algae, cyanobacterium, green-sulfur bacterium, green non-sulfur bacterium, purple sulfur bacterium, purple non-sulfur bacterium, extremophile, yeast, fungus, an engineered organism thereof, or a synthetic organism. In some embodiments, the host cell is light-dependent or fixes carbon. In some embodiments, the host cell has autotrophic activity. In some embodiments, the host cell has photoautotrophic activity, such as in the presence of light. In some embodiments, the host cell is heterotrophic or mixotrophic in the absence of light.

In certain embodiments, the host cell is a cell from *Avabidopsis thaliana, Panicum virgatum, Miscanthus giganteus, Zea mays, Botryococcuse braunii, Chlamydomonas reinhardtii, Dunaliela salina, Synechococcus Sp. PCC 7002, Synechococcus Sp. PCC 7942, Synechocystis Sp. PCC 6803, Thermosynechococcus elongates BP-1, Chlorobium tepidum, Chlorojlexus auranticus, Chromatiumm vinosum, Rhodospirillum rubrum, Rhodobacter capsulatus, Rhodopseudomonas palusris, Clostridium ljungdahlii, Clostridiuthermocellum, Penicillium chrysogenum, Pichia pastoris, Saccharomyces*

cerevisiae, Schizosaccharomyces pombe, Pseudomonas jluorescens, Pantoea citrea or *Zymomonas mobilis.* In certain embodiments, the host cell is a cell from *Chlorella fusca, Chlorella protothecoides, Chlorella pyrenoidosa, Chlorella kessleri, Chlorella vulgaris, Chlorella saccharophila, Chlorella sorokiniana, Chlorella ellipsoidea, Prototheca stagnora, Prototheca portoricensis, Prototheca moriformis, Prototheca wickerhamii, or Prototheca zopfii.*

In some embodiments, the host cell is a bacterial cell. In some embodiments, the host cell is a Gram-positive bacterial cell. In some embodiments, the host cell is a Gram-negative bacterial cell.

In certain embodiments, the host cell is an *E. coli* cell. In some embodiments, the *E. coli* cell is a strain B, a strain C, a strain K, or a strain W *E. coli* cell.

In certain embodiments of the invention, the host cell is engineered to express (or overexpress) a transport protein. Transport proteins can export polypeptides and organic compounds (*e.g.*, fatty acids or derivatives thereof) out of a host cell.

As used herein, the term "metabolically engineered" or "metabolic engineering" involves rational pathway design and assembly of polynucleotides corresponding to biosynthetic genes, genes associated with operons, and control elements of such polynucleotides, for the production of a desired metabolite such as, for example, an oc-β-ketoacyl-ACP, an oc-acyl-ACP, or an oc-fatty acid derivative, in a recombinant cell, such as a recombinant microbial cell as described herein. "Metabolic engineering" can further include optimization of metabolic flux by regulation and optimization of transcription, translation, protein stability and protein functionality using genetic engineering and appropriate culture conditions including the reduction of, disruption, or knocking out of, a competing metabolic pathway that competes with an intermediate leading to a desired pathway. A "biosynthetic gene" can be endogenous (native) to the host cell (*i.e.*, a gene which is not modified from the host cell), or, can be exogenous (heterologous) to the host cell either by virtue of being foreign to the host cell, or by being modified by mutagenesis, recombination, and/or association in the recombinant cell with a exogenous (heterologous) expression control sequence . A biosynthetic gene encodes a "biosynthetic polypeptide" or a "biosynthetic enzyme".

The term "biosynthetic pathway", also referred to as "metabolic pathway", refers to a set of biochemical reactions, catalyzed by biosynthetic enzymes, which convert one chemical species into another. As used herein, the term "fatty acid biosynthetic pathway" (or more simply, "fatty acid pathway") refers to a set of biochemical reactions that produces fatty acid derivatives (*e.g.*, fatty acids, fatty esters, fatty aldehydes, fatty alcohols, alkanes, alkenes, ketones, and so forth). The fatty acid pathway includes fatty acid pathway biosynthetic enzymes (*i.e.*, "fatty acid pathway enzymes") that can be engineered, as described herein, to produce fatty acid derivatives, and in some embodiments can be expressed with additional enzymes to produce fatty acid derivatives having desired carbon chain characteristics. For example, an "odd chain fatty acid biosynthetic pathway" (*i.e.*, an "oc-FA pathway") as described herein includes enzymes sufficient to produce oc-fatty acid derivatives.

The term "recombinant microbial cell" refers to a microbial cell (*i.e.*, a microorganism) that has been genetically modified (*i.e.*, "engineered") by the introduction of genetic material into a "parental microbial cell" (*i.e.*, host cell) of choice, thereby modifying or altering the cellular physiology and biochemistry of the parental microbial cell. Through the introduction of genetic material, the recombinant microbial cell acquires a new or improved property compared to that of the parental microbial cell, such as, for example, the ability to produce a new intracellular metabolite, or greater quantities of an existing intracellular metabolite. Recombinant microbial cells provided herein express a plurality of biosynthetic enzymes (*e.g.*, fatty acid pathway enzymes, such as oc-FA pathway enzymes) involved in pathways for the production of, for example, an oc-acyl-ACP intermediate or an oc-fatty acid derivative, from a suitable carbon source. The genetic material introduced into the parental microbial cell may contain gene(s), or parts of genes, encoding one or more of the enzymes involved in a biosynthetic pathway (that is, biosynthetic enzymes) for the production of an oc-fatty acid derivative, and may alternatively or in addition include additional elements for the expression and/or regulation of expression of genes encoding such biosynthetic enzymes, such as promoter sequences. Accordingly, recombinant microbial cells described herein have been genetically engineered to express or overexpress biosynthetic enzymes involved in oc-fatty acid (oc-FA) biosynthetic pathways as described herein.

It is understood that the terms "recombinant microbial cell" and "recombinant microorganism" refer not only to the particular recombinant microbial cell/microorganism, but to the progeny or potential progeny of such a cell.

A recombinant microbial cell can, alternatively or in addition to comprising genetic material introduced into the parental microbial cell, include a reduction, disruption, deletion or a "knocking-out" of a gene or polynucleotide to alter the cellular physiology and biochemistry of the parental microbial cell. Through the reduction, disruption, deletion or knocking-out of a gene or polynucleotide (also known as "attenuation" of the gene or polynucleotide), the recombinant microbial cell acquires a new or improved property (such as, for example, the ability to produce a new or greater quantities of an intracellular metabolite, the ability to improve the flux of a metabolite through a desired pathway, and/or the ability to reduce the production of an undesirable by-product) compared to that of the parental microbial cell.

Engineering Recombinant Microbial Cells to Produce Odd Chain Fatty Acid Derivatives

Many microbial cells normally produce straight chain fatty acids in which the linear aliphatic chains predominantly contain an even number of carbon atoms, and generally produce relatively low amounts of fatty acids having linear aliphatic chains containing an odd number of carbon atoms. The relatively low amounts of linear odd chain fatty acids (oc-FAs) and other linear odd chain fatty acid derivatives (oc-FA derivatives) produced by such microbial cells, such as *E. coli*, can in some instances be attributed to low levels of propionyl-CoA present in such cells. Such cells

predominantly utilize acetyl-CoA as the primer molecule for fatty acid biosynthesis, leading to the majority of fatty acids and other fatty acid derivatives produced in such cells being linear even chain fatty acids (ec-FAs) and other linear even chain fatty acid derivatives (ec-FA derivatives).

The invention is based in part on the discovery that by engineering a microorganism to produce an increased amount of propionyl-CoA compared to that produced by a parental microorganism, the engineered microorganism produces a greater amount (titer) of oc-FA derivatives compared to the amount of oc-FA derivatives produced by the parental microorganism, and/or produces a fatty acid derivative composition having a higher proportion of oc-FA derivatives compared to the proportion of oc-FA derivatives in the fatty acid derivative composition produced by the parental microorganism.

As the ultimate goal is to provide environmentally responsible and cost-effective methods for the production of fatty acid derivatives, including oc-FA derivatives, on an industrial scale starting from a carbon source (such as, for example, carbohydrate or biomass), improvements in yield of microbially produced oc-FA derivative molecules and/or optimization of the composition of microbially produced fatty acid derivative molecules (such as by increasing the proportion of odd chain product relative to even chain product) is desirable. Accordingly, strategies for the overproduction of various pathway intermediates have been examined to increase metabolic flux through pathways leading to odd chain fatty acid production. Pathways that direct metabolic flux from a starting material, such as a sugar, to propionyl-CoA, through an odd chain acyl-ACP (oc-acyl-ACP) intermediate, to an oc-FA derivative product, can be engineered in an industrially useful microorganism.

In one aspect, the invention includes a recombinant microbial cell comprising one or more polynucleotides encoding polypeptides (*e.g.*, enzymes) having enzymatic activities which participate in the biosynthesis of propionyl-CoA, and/or participate in the biosynthesis of an oc-acyl-ACP intermediate, when the recombinant microbial cell is cultured in the presence of a carbon source under conditions effective to expresses the polynucleotides. In some embodiments, the recombinant microbial cell further comprises one or more polynucleotides each encoding a polypeptide having fatty acid derivative enzyme activity, wherein the recombinant microbial cell produces an odd chain fatty acid derivative when cultured in the presence of a carbon source under conditions sufficient to expresses the polynucleotides. The invention also includes methods of making compositions comprising odd chain fatty acid derivatives, comprising culturing a recombinant microbial cell of the invention. The invention also includes methods of increasing the amount of propionyl-CoA produced by a microbial cell, and methods of increasing the amount or proportion of odd chain fatty acid derivatives produced by a microbial cell, and other features apparent upon further review.

The recombinant microbial cell can be a filamentous fungi, an algae, a yeast, or a prokaryote such as a bacterium (*e.g.*, an *E. coli* or a *Bacillus sp*).

In general, odd chain fatty acid derivatives (such as, odd chain fatty acids, odd chain fatty esters (including odd chain fatty acid methyl esters (oc-FAMEs), odd chain fatty acid ethyl esters (oc-FAEEs), and odd chain wax esters), odd chain fatty aldehydes, odd chain fatty alcohols, and, due to decarbonylation or decarboxylation of an odd chain precursor, even chain hydrocarbons such as even chain alkanes, even chain alkenes, even chain terminal olefins, even chain internal olefins, and even chain ketones) can be produced in a recombinant microbial cell of the invention via the odd chain fatty acid biosynthetic pathway ("oc-FA pathway") depicted in Fig. 1B.

To produce an odd chain fatty acid derivative, the recombinant microbial cell utilizes propionyl-CoA as a "primer" for the initiation of the fatty acyl chain elongation process. As shown in Fig. 1B, the fatty acyl elongation process initially involves condensation of the odd chain length primer molecule propionyl-CoA with a malonyl-ACP molecule, catalyzed by an enzyme having β-ketoacyl ACP synthase activity (such as, a β-ketoacyl ACP synthase III enzyme), to form an initial odd chain β-ketoacyl-ACP intermediate (*e.g.*, 3-oxovaleryl-ACP), as depicted in step (D) of Fig. 1B. The odd chain β-ketoacyl-ACP intermediate undergoes keto-reduction, dehydration and enoyl-reduction at the β-carbon via the fatty acid synthase (FAS) complex to form an initial odd chain acyl-ACP intermediate, which undergoes further cycles of condensation with malonyl-ACP, keto-reduction, dehydration, and enoyl-reduction, adding two carbon units per cycle to form acyl-ACP intermediates of increasing odd-numbered carbon chain lengths ("oc-acyl-ACP") as depicted in step (E) of Fig. 1B. The oc-acyl-ACP intermediate reacts with one or more fatty acid derivative enzymes, as depicted in step (F) of Fig. 1B, resulting in an odd chain fatty acid derivative (oc-FA derivative) product. This is in contrast to the process in a cell that produces relatively low levels of propionyl-CoA (such as, for example, a wild-type *E. coli* cell). Such a cell produces predominantly straight-chain fatty acids having an even number of carbon atoms, and low or trace amounts of straight-chain fatty acids having an odd number of carbon atoms. As depicted in Fig. 1A, the even chain length primer molecule acetyl-CoA initially condenses with a malonyl-ACP molecule to form an even chain β-keto acyl-ACP intermediate (*e.g.*, acetoacetyl-ACP), as depicted in step (D) of Fig. 1A, which likewise undergoes FAS-catalyzed cycles of keto-reduction, dehydration, enoyl-reduction and condensation with additional malonyl-ACP molecules, likewise adding two carbon units per cycle, this time to form acyl-ACP intermediates of increasing even-numbered carbon chain lengths ("ec-acyl-ACP") as depicted in step (E) of Fig. 1A. The ec-acyl-ACP intermediate reacts with one or more fatty acid derivative enzymes, as depicted in step (F) of Fig. 1A, resulting in an even chain fatty acid derivative.

The propionyl-CoA "primer" molecule can be supplied to the oc-FA biosynthetic pathway of the recombinant microbial cell of the invention by a number of methods. Methods to increase the production of propionyl-CoA in a microbial cell include, but are not limited to, the following:

Propionyl-CoA can be generated by the native biosynthetic machinery of the parental microbial cell (*e.g.*, by enzymes endogenous to the parental microbial cell). If increasing the amount of propionyl-CoA produced in the parental microbial cell is desired , one or more enzymes endogenous to the parental microbial cell which contribute to the production of propionyl-CoA can be overexpressed in the recombinant microbial cell.

Propionyl-CoA can be generated by engineering the cell to overexpress endogenous enzymes and/or express exogenous enzymes which divert metabolic flux through the intermediate α-ketobutyrate, as shown in Fig. 2. Non-limiting examples of enzymes for use in engineering such pathways are provided in Tables 1 and 2, below.

Propionyl-CoA can be generated by engineering the cell to overexpress endogenous enzymes and/or express exogenous enzymes which divert metabolic flux from succinyl-CoA through the intermediate methylmalonyl-CoA, as shown Fig. 3. Non-limiting examples of enzymes for use in engineering such pathways are provided in Table 3, below.

In an exemplary approach, propionyl-CoA can be generated by engineering the cell to overexpress endogenous enzymes and/or express exogenous enzymes which divert metabolic flux from malonyl-CoA through the intermediates malonate semialdehyde and 3-hydroxypropionate. Non-limiting examples of enzymes for use in engineering such pathways are provided, for example, in United States Patent Application Publication Number US20110201068A1.

In another approach, propionyl-CoA can be generated by engineering the cell to overexpress endogenous enzymes and/or express exogenous enzymes which divert metabolic flux from D-lactate through the intermediates lactoyl-CoA and acryloyl-CoA. Non-limiting examples of enzymes for use in engineering such pathways are provided, for example, in United States Patent Application Publication Number US20110201068A1.

As noted above, initiation of the odd chain elongation process involves condensation of propionyl-CoA with a malonyl-ACP molecule to form an oc-β-ketoacyl-ACP intermediate. This step, as represented by part (D) of Fig. 1B, is catalyzed in the recombinant microbial cell by an enzyme having β-ketoacyl-ACP synthase activity, preferably β-ketoacyl-ACP synthase III activity (*e.g.*, EC 2.3.1.180) which utilizes propionyl-CoA as a substrate. The enzyme can be endogenous to the recombinant microbial cell, or can exogenous to the recombinant microbial cell.

In one embodiment, a polynucleotide encoding a polypeptide endogenous to the parental microbial cell having β-ketoacyl-ACP synthase activity that utilizes propionyl-CoA as a substrate is expressed or is overexpressed in the recombinant microbial cell. In another embodiment, a polynucleotide encoding a polypeptide having β-ketoacyl-ACP synthase activity that utilizes propionyl-CoA as a substrate which is exogenous to the parental microbial cell is expressed in the recombinant microbial cell.

The oc-β-ketoacyl-ACP intermediate generated in step (D) of the oc-FA pathway (Fig. 1B) can undergo elongation by successive cycles of keto-reduction, dehydration and enoyl-reduction at the beta carbon and further condensation with malonyl-ACP molecules catalyzed by a fatty acid synthase (FAS) complex, such as for example a Type II FAS complex, adding 2-carbon units to the lengthening odd-carbon chain of the oc-acyl-ACP intermediate as represented by step (E) of Fig. 1B. In one embodiment, an endogenous FAS complex native to the recombinant microbial cell catalyzes cycles of condensation with malonyl-ACP / keto-reduction / dehydration / enoyl-reduction to produce the oc-acyl-ACP intermediate.

Odd chain fatty acid derivatives (such as oc-fatty acids, oc-fatty esters, oc-fatty aldehydes, oc-fatty alcohols, ec-ketones, and ec-hydrocarbons) can be produced from the oc-acyl-ACP intermediate, as will be described in more detail below. Accordingly, in some embodiments, the recombinant microbial cell further comprises one or more polynucleotide sequences each encoding a polypeptide having fatty acid derivative enzyme activity, such as thioesterase (*e.g.*, TesA), decarboxylase, carboxylic acid reductase (CAR; *e.g.*, CarA, CarB, or FadD9), alcohol dehydrogenase/aldehyde reductase; aldehyde decarbonylase (ADC), fatty alcohol forming acyl-CoA reductase (FAR), acyl ACP reductase (AAR), ester synthase, acyl-CoA reductase (ACR1), OleA, OleCD, or OleBCD, wherein the microbial cell produces a composition comprising an oc-fatty acid, an oc-fatty ester (such as an oc-fatty acid methyl ester, an oc-fatty acid ethyl ester, an oc-wax ester), an oc-fatty aldehyde, an oc-fatty alcohol, an ec-ketone, or an ec-hydrocarbon (such as an ec-alkane, an ec-alkene, an ec-terminal olefin, or an ec-internal olefin), when the recombinant microbial cell is cultured in the presence of a carbon source under conditions effective to expresses the polynucleotides. The invention also includes methods for the production of an oc-fatty acid derivative comprising culturing a recombinant microbial cell of the invention.

Engineering Microbial Cells to Produce Increased Amounts of Propionyl-CoA

In one aspect, the invention includes a method of increasing the amount of odd chain fatty acid derivatives produced by a microbial cell, which comprises engineering a parental microbial cell to produce an increased amount of propionyl-CoA. Engineering the parental microbial cell to produce an increased amount of propionyl-CoA can be accomplished, for example, by engineering the cell to express polynucleotides encoding: (a) polypeptides having aspartokinase activity, homoserine dehydrogenase activity, homoserine kinase activity, threonine synthase activity, and threonine deaminase activity; (b) polypeptides having (R)-citramalate synthase activity , isopropylmalate isomerase activity, and beta-isopropylmalate dehydrogenase activity; or (c) a polypeptide having methylmalonyl-CoA mutase activity and one or more polypeptides having methylmalonyl-CoA decarboxylase activity and methylmalonyl carboxyltransferase activity, and optionally a polypeptide having methylmalonyl epimerase activity; wherein at least one polypeptide is exogenous to the recombinant microbial cell, or expression of at least one polynucleotide is modulated in the

recombinant microbial cell as compared to the expression of the polynucleotide in the parental microbial cell, and wherein the recombinant microbial cell produces a greater amount of propionyl-CoA when cultured in the presence of a carbon source under conditions effective to express the polynucleotides, relative to the amount of propionyl-CoA produced by the parental microbial cell cultured under the same conditions.

In some embodiments, at least one polypeptide encoded by a polynucleotide according to (a) is an exogenous polypeptide (for example, a polypeptide originating from an organism other than the parental microbial cell, or, a variant of a polypeptide native to the parental microbial cell). In some instances, at least one polypeptide encoded by a polynucleotide according to (a) is an endogenous polypeptide (that is, a polypeptide native to the parental microbial cell), and the endogenous polypeptide is overexpressed in the recombinant microbial cell.

In some embodiments, at least one polypeptide encoded by a polynucleotide according to (b) is an exogenous polypeptide. In some instances, at least one polypeptide encoded by a polynucleotide according to (b) is an endogenous polypeptide, and the endogenous polypeptide is overexpressed in the recombinant microbial cell.

In some embodiments, the recombinant microbial cell comprises one or more polynucleotide according to (a) and one or more polynucleotide according to (b). In some instances, at least one polypeptide encoded by a polynucleotide according to (a) or (b) is an exogenous polypeptide. In some instances, at least one polypeptide encoded by a polynucleotide according to (a) or (b) is an endogenous polypeptide, and the endogenous polypeptide is overexpressed in the recombinant microbial cell.

In some embodiments, at least one polypeptide encoded by a polynucleotide according to (c) is an exogenous polypeptide. In some instances, at least one polypeptide encoded by a polynucleotide according to (c) is an endogenous polypeptide, and the endogenous polypeptide is overexpressed in the recombinant microbial cell.

By engineering a parental microbial cell to obtain a recombinant microbial cell that has increased metabolic flux through propionyl-CoA compared to the parental (*e.g.*, non-engineered) microbial cell, the engineered microbial cell produces a greater amount (titer) of oc-FA derivative compared to the amount of oc-FA derivative produced by the parental microbial cell, and/or produces a fatty acid derivative composition having a higher proportion of oc-FA derivative compared to the proportion of oc-FA derivative in the fatty acid derivative composition produced by the parental microbial cell.

Accordingly, in another aspect, the invention includes a method of increasing the amount or proportion of odd chain fatty acid derivatives produced by a microbial cell, the method comprising engineering a parental microbial cell to obtain a recombinant microbial cell which produces a greater amount, or is capable of producing a greater amount, of propionyl-CoA relative to the amount of

propionyl-CoA produced by the parental microbial cell cultured under the same conditions, wherein, when the recombinant microbial cell and the parental microbial cell are each cultured in the presence of a carbon source under identical conditions effective to increase the level of propionyl-CoA in the recombinant microbial cell relative to the parental microbial cell, the culture of the recombinant microbial cell produces a greater amount or a greater proportion of odd chain fatty acid derivatives relative to the amount or proportion of odd chain fatty acid derivatives produced by the parental microbial cell. In some embodiments, the recombinant microbial cell comprises polynucleotides encoding polypeptides according to one or more of pathways (a), (b), and (c), as described in more detail below, wherein at least one encoded polypeptide is exogenous to the recombinant microbial cell, or wherein expression of at least one polynucleotide is modulated in the recombinant microbial cell as compared to the expression of the polynucleotide in the parental microbial cell. In some embodiments, the recombinant microbial cell comprises at least one polynucleotide encoding a polypeptide having fatty acid derivative enzyme activity. In some embodiments, the recombinant microbial cell comprises a polynucleotide encoding a polypeptide having β-ketoacyl-ACP synthase activity that utilizes propionyl-CoA as a substrate.

Exemplary metabolic pathways useful for increasing propionyl-CoA production in a recombinant microbial cell are described below. It is to be understood that these exemplary pathways for increasing propionyl-CoA production in a recombinant cell are not intended to limit the scope of the invention; any suitable metabolic pathway that increases propionyl-CoA production in the cell and/or increases metabolic flux in the cell through the propionyl-CoA intermediate is suitable for use in recombinant microbial cells, compositions, and methods of the invention. Metabolic pathways which increase propionyl-CoA production and/or increase metabolic flux through the propionyl-CoA intermediate are therefore suitable for use in recombinant microbial cells, compositions, and methods of the invention.

Production of Propionyl-CoA via an α-Ketobutyrate Intermediate

Manipulation of various amino acid biosynthetic pathways has been shown to increase the production of those various amino acids in microbial cells (Guillouet S., *et al.*, *Appl. Environ. Microbiol.* 65:3100-3107 (1999); Lee K.H., *et al., Mol. Syst. Biol.* 3:149 (2007)). Amino acid biosynthetic pathways have been used in the production of short chain branched alcohols in *E. coli* (Atsumi S. and Liao J.C., *Appl. Environ. Microbiol.* 74(24): 7802-7808 (2008); Cann A.F. and Liao J.C., *Appl Microbiol Biotechnol.* 81(1):89-98(2008); Zhang K., *et al.*, *Proc. Natl. Acad. Sci. U S A.* 105(52):20653-20658(2008)).

Directing the flux of certain amino acid biosynthetic metabolites to the production of the intermediate α-ketobutyrate (also known as alpha-ketobutyrate, 2-ketobutyrate, 2-ketobutanoate, 2-oxobutyrate and 2-oxobutanoate) results in increased propionyl-CoA production. Accordingly, in one embodiment, the invention includes a recombinant microbial cell comprising polynucleotides

encoding one or more enzymes (*i.e.*, "oc-FA pathway enzymes") which participate in the conversion of a carbon source (for example, a carbohydrate, such as a sugar) to α-ketobutyrate when the recombinant microbial cell is cultured in the presence of the carbon source under conditions sufficient to expresses the polynucleotides. The α-ketobutyrate molecule is an intermediate in the microbial production of propionyl-CoA which serves as a primer in the production of linear odd chain fatty acid derivatives according to the oc-FA pathway (Fig. 1B).

Pyruvate dehydrogenase complex (PDC) catalyzes the oxidative decarboxylation of α-ketobutyrate to produce propionyl-CoA in bacteria (Danchin, A. *et al.*, *Mol. Gen. Genet.* 193: 473 - 478 (1984); Bisswanger, H., *J. Biol .Chem.* 256:815-822 (1981)). The pyruvate dehydrogenase complex is a multienzyme complex that contains three activities: a pyruvate decarboxylase (E1), a dihydrolipoyl transacetylase (E2), and a dihydrolipoyl dehydrogenase (E3). Other suitable ketoacid dehydrogenase complexes exist that use a similar catalytic scheme employing α-ketoacid substrates other than pyruvate. The TCA cycle α-ketoglutarate dehydrogenase complex is an example. In one embodiment, the pyruvate dehydrogenase complex endogenous to the host cell (*i.e.*, the pyruvate dehydrogenase complex native to the parental cell) is utilized to catalyze the conversion of α-ketobutyrate to propionyl-CoA. In other embodiments, genes encoding one or more PDC complex polypeptides having pyruvate decarboxylase, dihydrolipoyl transacetylase, and/or dihydrolipoyl dehydrogenase activity are overexpressed in the recombinant microbial cell. Other enzymes or enzyme complexes which catalyze the conversion of α-ketobutyrate to propionyl-CoA can be expressed or overexpressed in the recombinant microbial cell to further increase metabolic flux from α-ketobutyrate to propionyl-CoA.

Conversion of α-ketobutyrate to propionyl-CoA can also be accomplished by conversion of α-ketobutyrate to propionate and activation of propionate to propionyl-CoA. Conversion of α-ketobutyrate to propionate can be catalyzed by pyruvate oxidase (E.C. 1.2.3.3), such as *E. coli* pyruvate oxidase encoded by the *poxB* gene (Grabau and Cronan, *Nucleic Acids Res.* 14(13): 5449–5460 (1986)). The native *E. coli* PoxB enzyme reacts with α-ketobutyrate and with pyruvate, with a preference for pyruvate; however, Chang and Cronan (*Biochem J.* 352:717-724 (2000)) described PoxB mutant enzymes which retained full activity towards α-ketobutyrate and reduced activity towards pyruvate. Activation of propionate to propionyl-CoA can be catalyzed by an acyl-CoA synthase, such as Acetyl-CoA synthetase (Doi et al., J. Chem Soc. 23: 1696 (1986)). Yeast acetyl-CoA synthetase has been shown to catalyze the activation of propionate to propionyl-CoA (Patel and Walt, *J. Biol. Chem.* 262: 7132 (1987)). Propionate can also be activated to propionyl-CoA by the actions of acetate kinase (*ackA*) and phosphotransacetylase (*pta*).

One or more enzymes endogenous to the parental microbial cell may compete for substrate with enzymes of the engineered oc-FA biosynthetic pathway in the recombinant microbial cell, or may break down or otherwise divert an intermediate (such as, α-ketobutyrate) away from the oc-FA

biosynthetic pathway; genes encoding such undesired endogenous enzymes may be attenuated to increase the production of odd chain fatty acid derivatives by the recombinant microbial cell. For example, in *E. coli*, endogenous acetohydroxyacid synthase (AHAS) complexes, such as AHAS I (*e.g.*, encoded by *ilvBN* genes), AHAS II (*e.g.*, encoded by *ilvGM* genes) and AHAS III (*e.g.*, encoded by *ilvIH* genes), catalyze the conversion of α-ketobutyrate to α-aceto-α-hydroxybutyrate and may thus divert metabolic flux away from propionyl-CoA and reduce oc-FA production. Deleting or otherwise reducing the expression of one or more endogenous AHAS genes may thus direct biosynthesis in the recombinant microbial cell more towards propionyl-CoA and ultimately more towards odd chain fatty acid production. Other endogenous enzymes which may compete with oc-FA biosynthetic pathway enzymes include enzymes with acetohydroxyacid isomeroreductase activity (*e.g.*, encoded by an *ilvC* gene) which catalyzes the conversion of α-aceto-α-hydroxybutyrate to 2,3-dihydroxy-3-methylvalerate, and dihydroxy acid dehydratase activity (*e.g.,* encoded by an *ilvD* gene), which catalyzes the conversion of 2,3-dihydroxy-3-methylvalerate to 2-keto-3-methylvalerate; deleting or otherwise reducing the expression of one or more of these genes may direct biosynthesis in the recombinant microbial cell more towards propionyl-CoA and ultimately more towards odd chain fatty acid production.

Either or both of the following exemplary pathways can be engineered in the recombinant microbial cell to increase metabolic flux through the common α-ketobutyrate intermediate resulting in increased propionyl-CoA production in the cell. These exemplary pathways are shown in Fig. 2 and are described in more detail below.

Pathway A (Threonine Intermediate)

The first pathway leading to the common α-ketobutyrate intermediate, as represented by pathway (A) of Fig. 2, involves production of the intermediate threonine by threonine biosynthetic enzymes, followed by the deamination of threonine to α-ketobutyrate catalyzed by an enzyme with threonine dehydratase activity.

In pathway (A), increasing metabolic flux to threonine can be accomplished by expressing polynucleotides encoding enzymes involved in threonine biosynthesis, including enzymes with aspartate kinase activity (*e.g.*, EC 2.7.2.4; also termed aspartokinase activity), which catalyzes the conversion of aspartate to aspartyl phosphate; aspartate-semialdehyde dehydrogenase activity (*e.g.*, EC 1.2.1.11), which catalyzes the conversion of aspartyl phosphate to aspartate semialdehyde; homoserine dehydrogenase activity (*e.g.*, EC 1.1.1.3), which catalyzes the conversion of aspartate semialdehyde to homoserine; homoserine kinase activity (*e.g.*, EC 2.7.1.39), which catalyzes the conversion of homoserine to O-phospho-L-homoserine; and threonine synthase activity (*e.g.*, EC 4.2.3.1), which catalyzes the conversion of O-phospho-L-homoserine to threonine. Not all of the activities listed above need be engineered in the recombinant microbial cell to increase metabolic flux through the threonine intermediate; in some instances, an activity already present in the parental

microbial cell (for example, a polypeptide having that activity which is produced by a native gene in the parental microbial cell) will be sufficient to catalyze a step listed above. In one embodiment, the recombinant microbial cell is engineered to recombinantly express one or more polynucleotides selected from: a polynucleotide encoding a polypeptide having aspartate kinase activity, wherein the polypeptide catalyzes the conversion of aspartate to aspartyl phosphate; a polynucleotide encoding a polypeptide having aspartate-semialdehyde dehydrogenase activity, wherein the polypeptide catalyzes the conversion of aspartyl phosphate to aspartate semialdehyde; a polynucleotide encoding a polypeptide having homoserine dehydrogenase activity, wherein the polypeptide catalyzes the conversion of aspartate semialdehyde to homoserine; a polynucleotide encoding a polypeptide having homoserine kinase activity, wherein the polypeptide catalyzes the conversion of homoserine to O-phospho-L-homoserine; a polynucleotide encoding a polypeptide having threonine synthase activity, wherein the polypeptide catalyzes the conversion of O-phospho-L-homoserine to threonine; wherein the recombinant microbial cell has increased metabolic flux through the pathway intermediate threonine compared to the parental microbial cell. In some instances, the polypeptide encoded by recombinantly expressed polynucleotide is present in the recombinant microbial cell at a greater concentration compared to its concentration in the parent microbial cell when cultured under the same conditions, *i.e.*, the polypeptide is "overexpressed" in the recombinant cell. For example, the recombinantly expressed polynucleotide can be operatively linked to a promoter which expresses the polynucleotide in the recombinant microbial cell at a greater concentration than is normally expressed in the parental microbial cell when cultured under the same conditions. In one embodiment, an *E. coli thrA* gene is used, which encodes a bifunctional ThrA with aspartate kinase and homoserine dehydrogenase activities. In another embodiment, a mutant *E. coli thrA* gene is used, encoding a variant enzyme with aspartate kinase and homoserine dehydrogenase activities and with reduced feedback inhibition relative to the parent ThrA enzyme (designated ThrA*; Ogawa-Miyata,Y., *et al.*, *Biosci. Biotechnol. Biochem.* 65:1149-1154 (2001); Lee J.-H., *et al.*, *J. Bacteriol.* 185: 5442-5451 (2003)).

Threonine can be deaminated to α-ketobutyrate by an enzyme with threonine deaminase activity (*e.g.*, EC 4.3.1.19; also known as threonine ammonia-lyase activity, and was previously classified as EC 4.2.1.16, threonine dehydratase). In one embodiment, threonine deaminase activity which is already present in (*i.e.*, is endogenous to) the parental microbial cell is sufficient to catalyze the conversion of threonine to α-ketobutyrate. In another embodiment, the recombinant microbial cell is engineered to recombinantly express a polypeptide having threonine deaminase activity, wherein the polypeptide catalyzes the conversion of threonine to α-ketobutyrate. In some embodiments, the polypeptide having threonine deaminase activity is overexpressed in the recombinant microbial cell.

Non-limiting examples of enzymes and polynucleotides encoding such enzymes for use in engineering pathway (A) are provided in Table 1.

Table 1. Non-limiting examples of enzymes and nucleic acid coding sequences for use in pathway A of the oc-FA biosynthetic pathway shown in Fig. 2.

EC Number	Organism	Gene symbol	UniProtKB (SwissProt) Accession Number, or literature reference	NCBI Protein Accession Number	SEQ ID NO
EC 2.7.2.4	aspartate kinase (aspartokinase)				
	E. coli K-12 MG1655	*thrA*	P00561	NP_414543	20
	E. coli (mutant)	*thrA**	Ogawa-Miyata et al, 2001; Lee et al, 2003		21
	B. subtilis 168	*dapG*	Q04795	ZP_03591402	22
	P. putida F1	*Pput1442*	A5W0E0	YP_001266784	23
	S. cerevisiae	*hom3*		NP_010972	24
EC 1.1.1.3	homoserine dehydrogenase				
	E. coli K12 MG1655	*thrA*	P00561	NP_414543	20
	E. coli (mutant)	*thrA**	Ogawa-Miyata et al, 2001; Lee et al, 2003		21
	B. subtilis 168	*hom*	P19582	NP_391106	25
	P. putida F1	*Pput_4251*	A5W8B5	YP_001269559	26
	S. cerevisiae	*hom6*	P31116	NP_012673	27
EC 2.7.1.39	homoserine kinase				
	E. coli K12 MG1655	*thrB*	P00547	NP_414544	28
	B. subtilis 168	*thrB*	P04948	NP_391104	29
	P. putida F1	*Pput_0138*	A5VWQ3	YP_001265497	30
	S. cerevisiae	*thr1*	P17423	NP_011890	31
EC 4.2.3.1	threonine synthase				
	E. coli K12 MG1655	*thrC*	P00934	NP_414545	32
	B. subtilis 168	*thrC*	P04990	NP_391105	33
	C. glutamicum ATCC 13032	*thrC*	P23669	YP_226461	34
EC 4.3.1.19	threonine deaminase (threonine ammonia-lyase; previously termed threonine dehydratase)				
	E. coli K12 MG1655	*tdcB*	P0AGF6	NP_417587	35
	E. coli K12 MG1655	*ilvA*	P04968	NP_418220	36
	B. subtilis 168	*ilvA*	P37946	NP_390060	37
	C. glutamicum ATCC 13032	*ilvA*	Q04513	YP_226365	38
	C. glutamicum ATCC 13032	*tdcB*	Q8NRR7	YP_225271	39

Additional polypeptides can be identified, for example, by searching a relevant database (such as the KEGG database (University of Tokyo), the PROTEIN or the GENE databases (Entrez databases; NCBI), the UNIPROTKB or ENZYME databases (ExPASy; Swiss Institute of Bioinformatics), and the BRENDA database (The Comprehensive Enzyme Information System; Technical University of Braunschweig)), all which are available on the World Wide Web, for polypeptides categorized by the above noted EC numbers. For example, additional aspartokinase polypeptides can be identified by searching for polypeptides categorized under EC 2.7.2.4; additional homoserine dehydrogenase polypeptides can be identified by searching for polypeptides categorized under EC 1.1.1.3; additional homoserine kinase polypeptides can be identified by searching for polypeptides categorized under EC 2.7.1.39; additional threonine synthase polypeptides can be identified by searching for polypeptides categorized under EC 4.2.3.1; and additional threonine deaminase polypeptides can be identified by searching for polypeptides categorized under EC 4.3.1.19.

In some embodiments, a polynucleotide encoding a parent fatty acid pathway polypeptide (such as a polypeptide described in Table 1 or identified by EC number or by homology to an exemplary polypeptide) is modified using methods well known in the art to generate a variant polypeptide having an enzymatic activity noted above (*e.g.*, aspartokinase activity, homoserine dehydrogenase activity, homoserine kinase activity, threonine synthase activity, threonine deaminase activity) and an improved property, compared to that of the parent polypeptide, which is more suited to the microbial cell and/or to the pathway being engineered; such as, for example, increased catalytic activity or improved stability under conditions in which the recombinant microbial cell is cultured; reduced inhibition (*e.g.*, reduced feedback inhibition) by a cellular metabolite or by a culture media component, and the like.

Pathway B (Citramalate Intermediate)

The second pathway leading to the common α-ketobutyrate intermediate, as represented by pathway (B) of Fig. 2, involves the production of the intermediate citramalate (which is also known as 2-methylmalate) via an enzyme with citramalate synthase activity, and the conversion of citramalate to α-ketobutyrate by the action of enzymes with isopropylmalate isomerase and alcohol dehydrogenase activities.

Citramalate synthase activity (*e.g.*, EC 2.3.1.182), which catalyzes the reaction of acetyl-CoA and pyruvate to form (R)-citramalate, can be supplied by expression of a *cimA* gene from a bacterium such as *Methanococcus jannaschi or Leptospira interrogans* (Howell, D.M. *et al.*, *J. Bacteriol.* 181(1):331-3 (1999); Xu, H., *et al.*, *J. Bacteriol.* 186:5400-5409(2004)) which encodes a CimA polypeptide such as CimA from *M. jannaschii* (SEQ ID NO: 40) or *L. interrogans* (SEQ ID NO:42). Alternatively, a modified *cimA* nucleic acid sequence encoding a CimA variant with improved catalytic activity or stability in the recombinant microbial cell and/or reduced feedback inhibition can

be used, such as, for example, a CimA variant described by Atsumi S. and Liao J.C. (*Appl. Environ. Microbiol.* 74(24): 7802-7808 (2008)), preferably the CimA3.7 variant (SEQ ID NO:41) encoded by the *cimA3.7* gene. Alternatively, a *Leptospira interrogans* CimA variant (SEQ ID NO:43) can be used. Isopropylmalate isomerase activity (EC 4.2.1.33; also termed isopropylmalate dehydratase), which catalyzes the conversion of (R)-citramalate first to citraconate and then to beta-methyl-D-malate, can be provided, for example, by expression of a heterodimeric protein encoded by *E. coli* or *B. subtilis leuCD* genes. Alcohol dehydrogenase activity (EC 1.1.1.85; beta-isopropyl malate dehydrogenase), which catalyzes the conversion of beta-methyl-D-malate to 2-ketobutyrate (*i.e.*, α-ketobutyrate) can be provided, for example, by expression of an *E. coli* or *B. subtilis leuB* gene or a yeast *leu2* gene. Non-limiting examples of fatty acid pathway enzymes and polynucleotides encoding such enzymes for use in engineering pathway (B) of the oc-FA pathway are provided in Table 2.

Table 2. Non-limiting examples of enzymes and nucleic acid coding sequences for use in pathway (B) of the oc-FA biosynthetic pathway shown in Fig. 2.

EC number	Organism	Gene symbol	UniProtKB (Swiss-Prot) Protein Accession Number, or literature reference	NCBI Protein Accession Number	SEQ ID NO
EC 2.3.1.182	(R)-citramalate synthase				
	M. jannaschii	*cimA*	Q58787	NP_248395	40
	M.jannaschii (mutant)	*cimA 3.7*	Atsumi and Liao (2008)		41
	Leptospira interrogans	*cimA*	Q8F3Q1	AAN49549	42
	Leptospira interrogans (mutant)	*cimA**	(this disclosure)		43
EC 4.2.1.33	isopropylmalate isomerase (3-isopropylmalate dehydratase)				
	E. coli K12 MG1655	*leuCD*	P0A6A6 (C, Lg subunit); P30126 (D, Sm subunit)	(C) NP_414614 (D) NP_414613	44 45
	B. subtilis 168	*leuCD*	P80858 (C, Lg subunit); P94568 (D, Sm subunit)	(C) NP_390704 (D) NP_390703	46 47
EC 1.1.1.85	beta-isopropylmalate dehydrogenase (3-isopropylmalate dehydrogenase)				
	E. coli K12 MG1655	*leuB*	P30125	NP_414615	48
	B. subtilis	*leuB*	P05645	NP_390705.2	49
	S. cerevisiae	*leu2*	P04173	NP_009911.2	50

Additional polypeptides can be identified, for example, by searching a relevant database (such as the KEGG database (University of Tokyo), the PROTEIN or the GENE databases (Entrez databases; NCBI), the UNIPROTKB or ENZYME databases (ExPASy; Swiss Institute of

Bioinformatics), and the BRENDA database (The Comprehensive Enzyme Information System; Technical University of Braunschweig)), all which are available on the World Wide Web, for polypeptides categorized by the above noted EC numbers. For example, additional (R)-citramalate synthase polypeptides can be identified by searching for polypeptides categorized under EC 2.3.1.182; additional isopropyl malate isomerase polypeptides can be identified by searching for polypeptides categorized under EC 4.2.1.33; and additional beta-isopropyl malate dehydrogenase polypeptides can be identified by searching for polypeptides categorized under EC 1.1.1.85.

In some embodiments, a polynucleotide encoding a parent fatty acid pathway polypeptide (such as a polypeptide described in Table 2 or identified by EC number or by homology to an exemplary polypeptide) is modified using methods well known in the art to generate a variant polypeptide having an enzymatic activity noted above (*e.g.*, (R)-citramalate synthase activity, isopropyl malate isomerase activity, beta-isopropyl malate dehydrogenase activity) and an improved property, compared to that of the parent polypeptide, which is more suited to the microbial cell and/or to the pathway being engineered; such as, for example, increased catalytic activity or improved stability under conditions in which the recombinant microbial cell is cultured; reduced inhibition (*e.g.*, reduced feedback inhibition) by a cellular metabolite or by a culture media component, and the like.

Production of Propionyl-CoA via Methylmalonyl-CoA

Pathway C (Methylmalonyl-CoA Intermediate)

The following exemplary pathway can be engineered in the recombinant microbial cell to increase metabolic flux through a methylmalonyl-CoA intermediate resulting in increased propionyl-CoA production in the cell. This exemplary pathway is shown in Fig. 3 and is described in more detail below.

Directing metabolic flux through methylmalonyl-CoA can result in increased propionyl-CoA production. Accordingly, in one embodiment, the invention includes a recombinant microbial cell comprising polynucleotides encoding which participate in the conversion of a carbon source (for example, a carbohydrate, such as a sugar) to succinyl-CoA and to methylmalonyl-CoA when the recombinant microbial cell is cultured in the presence of the carbon source under conditions sufficient to expresses the polynucleotides. Succinyl-CoA and methylmalonyl-CoA are intermediates in the microbial production of propionyl-CoA, which serves as a primer in the production of linear odd chain fatty acid derivatives according to the oc-FA pathway (Fig. 1B).

The pathway leading to propionyl-CoA as shown in Fig. 3 (also referred to herein as "pathway (C)") involves the conversion of succinyl-CoA to methylmalonyl-CoA via an enzyme having methylmalonyl-CoA mutase activity, and the conversion of methylmalonyl-CoA to propionyl-CoA by the action of an enzyme having methylmalonyl-CoA decarboxylase activity, and/or by the action of an enzyme having methylmalonyl-CoA carboxyltransferase activity. In some instances, depending on the stereoisomer of methylmalonyl-CoA utilized by the particular methylmalonyl-CoA

decarboxylase or methylmalonyl-CoA carboxyltransferase employed, an enzyme having methylmalonyl-CoA epimerase activity may be utilized to interconvert (R)- and (S)-methylmalonyl-CoA.

Succinyl-CoA can be provided to this pathway by the cellular TCA cycle. In some instances, flux from fumarate to succinate can be increased by, for example, overexpressing endogenous *frd* (fumurate reductase) or other gene(s) involved in production of succinate or succinyl-CoA. The conversion of succinyl-CoA to methylmalonyl-CoA can be catalyzed by an enzyme having methylmalonyl-CoA mutase activity (*e.g.*, EC 5.4.99.2). Such activity can be supplied to the recombinant microbial cell by expression of an exogenous *scpA* (also known as *sbm*) gene or by overexpression of an endogenous *scpA* gene. An exemplary *sbm* gene includes that from *E. coli* (Haller, T. *et al.*, *Biochemistry* 39:4622-4629 (2000)) which encodes an Sbm polypeptide (Accession NP_417392, SEQ ID NO: 51) having methylmalonyl-CoA mutase activity. Alternatively, a methylmalonyl-CoA mutase from, for example, *Propionibacterium freundenreichii subsp. shermanii* which comprises an α-subunit or "large subunit" (MutB, Accession YP_003687736) and a β-subunit or "small subunit" (MutA, Accession CAA33089) can be used. Non-limiting examples of polypeptides that catalyze the conversion of succinyl-CoA to methylmalonyl-CoA are provided in Table 3, below.

In one embodiment, conversion of methylmalonyl-CoA to propionyl-CoA can be catalyzed by a polypeptide having methylmalonyl-CoA decarboxylase activity (*e.g.*, EC 4.1.1.41), which catalyzes the decarboxylation of methylmalonyl-CoA to propionyl-CoA. Such activity can be supplied to the recombinant microbial cell by expression of an exogenous *scpB* (also known as *ygfG*) gene or by overexpression of an endogenous *scpB* gene. Exemplary methylmalonyl-CoA decarboxylase polypeptides include, for example, a methylmalonyl-CoA decarboxylase polypeptide encoded by the *E. coli scpB* gene (Haller *et al.*, *supra*), or a methylmalonyl-CoA decarboxylase polypeptide encoded by *Salmonella enterica* or *Yersinia enterocolitica*. In another embodiment, conversion of methylmalonyl-CoA to propionyl-CoA can be catalyzed by a polypeptide having methylmalonyl-CoA carboxyltransferase activity (*e.g.*, EC 2.1.3.1), such as, for example, a methylmalonyl-CoA carboxyltransferase from *P. freundenreichii subsp. shermanii* (*mmdA*, NBCI Accession No. Q8GBW6.3). Depending on the stereoisomer of methylmalonyl-CoA utilized by the methylmalonyl-CoA decarboxylase or by the methylmalonyl-CoA carboxyltransferase, conversion between (R)-methylmalonyl-CoA and (S)-methylmalonyl-CoA may be desired, which can be catalyzed by a polypeptide having methylmalonyl-CoA epimerase activity (*e.g.*, EC 5.1.99.1), such as, for example, a methylmalonyl-CoA epimerase from *Bacillus subtilis* (*yqjC*; Haller *et al.*, Biochemistry 39:4622-4629 (2000)) or *Propionibacterium freundenreichii subsp. shermanii* (NCBI Accession No. YP_003688018).

One or more enzymes endogenous to the parental microbial cell may compete for substrate with enzymes of the engineered oc-FA biosynthetic pathway in the recombinant microbial cell, or may break down or otherwise divert an intermediate away from the oc-FA biosynthetic pathway; genes encoding such undesired endogenous enzymes may be attenuated to increase the production of odd chain fatty acid derivatives by the recombinant microbial cell. For example, in *E. coli*, the endogenous propionyl-CoA:succinyl-CoA transferase (NCBI Accession Number NP_417395), encoded by the *E. coli scpC* (also known as *ygfH*) gene, catalyzes the conversion of propionyl-CoA to succinyl-CoA and may thus divert metabolic flux away from propionyl-CoA and reduce oc-FA production. Deleting or otherwise reducing the expression of the *scpC (ygfH)* gene may thus direct biosynthesis in the recombinant microbial cell more towards propionyl-CoA and ultimately more towards odd chain fatty acid production.

Non-limiting examples of fatty acid pathway enzymes and polynucleotides encoding such enzymes that catalyze the conversion of succinyl-CoA to methylmalonyl-CoA and the conversion of methylmalonyl-CoA to propionyl-CoA for use in engineering pathway (C) of the oc-FA pathway are provided in Table 3.

Table 3. Non-limiting examples of enzymes and nucleic acid coding sequences for use in pathway (C) of the oc-FA biosynthetic pathway shown in Fig. 3.

EC number	Organism	Gene symbol	UniProtKB (Swiss-Prot) Protein Accession Number, or literature reference	NCBI Protein Accession Number	SEQ ID NO
EC 5.4.99.2	Methylmalonyl-CoA mutase				
	E. coli	*scpA (sbm)*	P27253	NP_417392	51
	Salmonella enterica	*SARI_04585*	A9MRG0	YP_001573500	52
	P. freundenreichii subsp. shermanii	*mutA* *mutB*	(sm) P11652 (lg) D7GCN5	(sm) CAA33089 (lg) YP_003687736	53 54
	Bacillus megaterium	*mutA* *mutB*	(sm) D5DS48 (lg) D5DS47	(sm) YP_003564880 (lg) YP_003564879	55 56
	Corynebacterium glutamicum	*mcmA* *mcmB*	(sm) Q8NQA8 (lg) Q8NQA9	(sm) YP_225814 (lg) YP_225813	57 58
EC 4.1.1.41	Methylmalonyl-CoA decarboxylase				
	E. coli	*scpB (ygfG)*	C6UT22	YP_001731797	59
	Salmonella enterica	*SARI_04583*	A9MRF8	YP_001573498	60
	Yersinia enterocolitica	*YE1894*	A1JMG8	YP_001006155	61
EC 2.1.3.1	Methylmalonyl-CoA carboxyltransferase				
	P.freudenreichii subsp. shermanii	*mmdA*	Q8GBW6	Q8GBW6.3	62

EC number	Organism	Gene symbol	UniProtKB (Swiss-Prot) Protein Accession Number, or literature reference	NCBI Protein Accession Number	SEQ ID NO
EC 5.1.99.1	Methylmalonyl-CoA epimerase				
	P. freudenreichii subsp. shermanii	*PFREUD_10590; mmcE*	D7GDH1	YP_003688018	63

Additional polypeptides can be identified, for example, by searching a relevant database (such as the KEGG database (University of Tokyo), the PROTEIN or the GENE databases (Entrez databases; NCBI), the UNIPROTKB or ENZYME databases (ExPASy; Swiss Institute of Bioinformatics), and the BRENDA database (The Comprehensive Enzyme Information System; Technical University of Braunschweig)), all which are available on the World Wide Web, for polypeptides categorized by the above noted EC numbers. For example, additional methylmalonyl-CoA mutase polypeptides can be identified by searching for polypeptides categorized under EC 5.4.99.2, additional methylmalonyl-CoA decarboxylase polypeptides can be identified by searching for polypeptides categorized under EC 4.1.1.41, additional methylmalonyl-CoA carboxyltransferase polypeptides can be identified by searching for polypeptides categorized under EC 2.1.3.1, and additional methylmalonyl-CoA epimerase polypeptides can be identified by searching for polypeptides categorized under EC 5.1.99.1.

In some embodiments, a polynucleotide encoding a parent fatty acid pathway polypeptide (such as a polypeptide described in Table 3 or identified by EC number or by homology to an exemplary polypeptide) is modified using methods well known in the art to generate a variant polypeptide having an enzymatic activity noted above (*e.g.*, methylmalonyl-CoA mutase activity, methylmalonyl-CoA decarboxylase activity, methylmalonyl-CoA epimerase activity, methylmalonyl-CoA carboxyltransferase activity) and an improved property, compared to that of the parent polypeptide, which is more suited to the microbial cell and/or to the pathway being engineered; such as, for example, increased catalytic activity or improved stability under conditions in which the recombinant microbial cell is cultured; reduced inhibition (*e.g.*, reduced feedback inhibition) by a cellular metabolite or by a culture media component, and the like.

Engineering Microbial Cells to Produce Increased Amounts of oc-FA Derivatives

Propionyl-CoA to oc-β-Ketoacyl-ACP

As discussed above, propionyl-CoA serves as a primer for subsequent FAS-catalyzed elongation steps in the production of oc-FA derivatives. The initiation of this process involves condensation of propionyl-CoA with a malonyl-ACP molecule to form the oc-β-ketoacyl-ACP intermediate 3-oxovaleryl-ACP (Fig. 1B). This initiation step, as represented by step (D) of Fig. 1B, is catalyzed in the recombinant microbial cell by an enzyme having β-ketoacyl-ACP synthase activity

(such as, a Type III β-ketoacyl-ACP synthase (*e.g.*, EC 2.3.1.180)) that utilizes propionyl-CoA as a substrate.

The substrate specificity of a β-ketoacyl-ACP synthase from a particular microorganism often reflects the fatty acid composition of that microorganism (Han, L., *et al.*, *J. Bacteriol.* 180:4481-4486 (1998); Qui, X., *et al.*, *Protein Sci.* 14:2087-2094 (2005)). For example, the *E. coli* FabH enzyme utilizes propionyl-CoA and acetyl-CoA with a very strong preference for acetyl-CoA (Choi, K.H., *et al., J. Bacteriology* 182:365-370 (2000); Qui, *et al.*, *supra*) reflecting the high proportion of linear even chain fatty acids produced, while the enzyme from *Streptococcus pneumoniae* utilizes short straight chain acyl-CoA primers of between two and four carbons in length as well as various branched-chain acyl-CoA primers (Khandekar S.S., *et al.*, *J. Biol. Chem.* 276:30024-30030 (2001)) reflecting the variety of linear chain and branched chain fatty acids produced. A polynucleotide sequence encoding a polypeptide having β-ketoacyl-ACP synthase activity that utilizes propionyl-CoA as a substrate can generally be obtained from a microbial cell containing a β-ketoacyl-ACP synthase with a broad acyl-CoA substrate specificity. Sources of broad-specificity β-ketoacyl-ACP synthases may include bacteria that produce a variety of fatty acid structures including branched chain fatty acids, such as, for example, *Bacillus (e.g., B. subtilis), Listeria (e.g., L. monocytogenes), Streptomyces (e.g., S. coelicolor),* and *Propionibacterium (e.g., P. freudenreichii subsp. shermanii*). Particularly preferred β-ketoacyl-ACP synthase enzymes include those with a greater preference for propionyl-CoA vs. acetyl-CoA than that exhibited by the endogenous FabH. For example, when an *E. coli* cell is engineered, preferred β-ketoacyl-ACP synthase enzymes may include, but are not limited to, *B. subtilis* FabH1 (Choi *et al.* 2000, *supra*), *Streptomyces glauscens* FabH (Han, L., *et al.*, *J. Bacteriol.* 180:4481-4486 (1998)), *Streptococcus pneumoniae* FabH (Khandekar S.S., *et al.*, *J. Biol. Chem.* 276:30024-30030 (2001), and *Staphylococcus aureus* FabH (Qui, X. *et al.*, *Protein Sci.* 14:2087-2094 (2005)).

One or more endogenous enzymes may compete for substrate with enzymes of the engineered oc-FA biosynthetic pathway in the recombinant microbial cell, or may break down an oc-FA pathway intermediate or may otherwise divert metabolic flux away from oc-FA production; genes encoding such undesired endogenous enzymes may be attenuated to increase the production of oc-FA derivatives by the recombinant microbial cell. For example, while the endogenous *fabH*-encoded β-ketoacyl-ACP synthase of *E. coli* utilizes propionyl-CoA as a substrate, it has a much greater preference for the two-carbon acetyl-CoA molecule than for the three-carbon propionyl-CoA molecule (Choi *et al.* 2000, *supra*). Cells expressing the *E. coli fabH* gene thus preferentially utilize acetyl-CoA as a primer for fatty acid synthesis and predominantly produce even chain fatty acid molecules *in vivo*. Deleting or otherwise reducing the expression of an endogenous *fabH* gene and expressing an exogenous gene encoding a β-ketoacyl-ACP synthase with greater preference for propionyl-CoA than that exhibited by the endogenous FabH (for example, when engineering *E. coli*,

replacing the endogenous *E. coli* FabH with *B. subtilis* FabH1 or an alternative exogenous FabH with a greater preference for propionyl-CoA than acetyl-CoA relative to that exhibited by *E. coli* FabH) may direct metabolic flux in the recombinant microbial cell more towards an oc-β-ketoacyl-ACP intermediate and ultimately more towards production of oc-FA derivatives.

Non-limiting examples of fatty acid pathway enzymes and polynucleotides encoding such enzymes for use in engineering step D of the oc-FA pathway are provided in Table 4.

Table 4. Non-limiting examples of enzymes and coding sequences for use in step D of the oc-FA biosynthetic pathways shown in Fig. 1B.

EC number	Organism	Gene symbol	UniProtKB (Swiss-Prot) Protein Accession Number, or literature reference	NCBI Protein Accession Number	SEQ ID NO
EC 2.3.1.180	β-ketoacyl-ACP synthase III				
	E. coli	*fabH*	P0A6R0	AAC74175	1
	B. subtilis 168	*fabH1*	O34746	NP_389015	2
	B. subtilis 168	*fabH2*	O07600	NP_388898	3
	Streptomyces coelicolor	*fabH*	Q9K3G9	CAB99151	4
	Streptomyces glaucescens	*fabH*	Q54206	AAA99447	5
	Streptomyces avermitilis MA-4680	*fabH3*	Q82KT2	NP_823466	6
	Listeria monocytogenes	*fabH*	B8DFA8	YP_002349314	7
	L. monocytogenes (mutant)	*fabH2*	(this disclosure)		8
	Staphylococcus aureus MW2	*fabH*	Q8NXE2	NP_645682	9
	Streptococcus pneumoniae	*fabH*	P0A3C5	AAK74580	10
	Streptococcus mutans UA159	*fabH*	Q8DSN2	NP_722071	11
	Lactococcus lactis subsp. lactis	*fabH*	Q9CHG0	NP_266927	12
	Propionibacterium freundenreichii subsp. shermanii	*fabH*	D7GD58	YP_003687907	13
	Stenotrophomonas maltophila	*fabH*	B2FR86	YP_001970902	146
	Alicyclobacillus acidocaldarius	*fabH*	C8WPY3	YP_003183476	147
	Desulfobulbus propionicus	*fabH1*	E8RF72	YP_004195454	148
	Desulfobulbus propionicus	*fabH2*	E8RBR5	YP_004196088	149

Additional β-ketoacyl-ACP synthase polypeptides can be identified, for example, by searching a relevant database (such as the KEGG database (University of Tokyo), the PROTEIN or the GENE databases (Entrez databases; NCBI), the UNIPROTKB or ENZYME databases (ExPASy; Swiss Institute of Bioinformatics), and the BRENDA database (The Comprehensive Enzyme Information System; Technical University of Braunschweig)), all which are available on the World Wide Web, for polypeptides categorized under EC 2.3.1.180.

Additional β-ketoacyl-ACP synthase polypeptides can also be identified by searching a sequence pattern database, such as the Prosite database (ExPASy Proteomics Server, Swiss Institute of Bioinformatics) for a polypeptide comprising one or more of the sequence motifs listed below. This is readily accomplished, for example, by using the ScanProsite tool which is available on the World Wide Web site of the ExPASy Proteomics Server.

In one embodiment, the β-ketoacyl-ACP synthase polypeptide comprises one or more sequence motif selected from:

D-T-[N,S]D-[A,E]-W-I-x(2)-[M,R]-T-G-I-x-[N,E]-R-[R,H] (SEQ ID NO:14)

[S,A]-x-D-x(2)-A-[A,V]-C-[A,S]-G-F-x(3)-[M,L]-x(2)-A (SEQ ID NO:15)

D-R-x-T-[A,I]-[I,V]-x-F-[A,G]-D-G-A-[A,G]-[G,A]-[A,V] (SEQ ID NO:16)

H-Q-A-N-x-R-I-[M,L] (SEQ ID NO:17)

G-N-T-[G,S]-A-A-S-[V,I]-P-x(2)-[I,L]-x(6)-G (SEQ ID NO:18)

[I,V]-x-L-x(2)-F-G-G-G-[L,F]-[T,S]-W-G (SEQ ID NO:19)

wherein the amino acid residues in each of the brackets indicate alternative amino acid residues at the particular position, each x indicates any amino acid residue, and each n in "x(n)" indicates the number of x residues in a contiguous stretch of amino acid residues.

In some embodiments, a polynucleotide encoding a parent fatty acid pathway polypeptide (such as a polypeptide described in Table 4 or identified by EC number or by motif or by homology to an exemplary polypeptide) is modified using methods well known in the art to generate a variant polypeptide having β-ketoacyl-ACP synthase activity, and an improved property, compared to that of the parent polypeptide, which is more suited to the microorganism and/or to the pathway being engineered; such as, for example, increased catalytic activity and/or increased specificity for propionyl-CoA (relative to, *e.g.*, acetyl-CoA); improved catalytic activity or improved stability under conditions in which the recombinant microbial cell is cultured; reduced inhibition (*e.g.*, reduced feedback inhibition) by a cellular metabolite or by a culture media component, and the like.

The invention includes a recombinant microbial cell comprising a polynucleotide encoding a polypeptide, said polypeptide comprising a polypeptide sequence having at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to one of SEQ ID NOs:1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 146, 147, 148, and 149, wherein the polypeptide has β-ketoacyl-ACP synthase activity that utilizes

propionyl-CoA as a substrate. In some instances, the polypeptide sequence comprises one or more sequence motif selected from SEQ ID NOs:14-19. The invention also includes an isolated polypeptide comprising said polypeptide sequence, and an isolated polynucleotide encoding said polypeptide. In one embodiment, the polypeptide comprises a substitution at position W310 or at an equivalent position thereto. In one embodiment, the polypeptide comprises a W310G substitution. In one embodiment, the polypeptide comprises a sequence having at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to SEQ ID NO:7 and comprises the substitution W310G. In some embodiments, the polypeptide exhibits greater specificity for propionyl-CoA than for acetyl-CoA.

As used herein, "a polypeptide having β-ketoacyl-ACP synthase activity that utilizes propionyl-CoA as a substrate" includes any polypeptide having a detectable level of β-ketoacyl-ACP synthase activity when supplied with the substrate propionyl-CoA.

Enzymatic activity and specificity of β-ketoacyl-ACP synthases for substrates, such as propionyl-CoA, can be determined using known methods. For example, Choi *et al.* (*J. Bacteriology* 182(2):365-370 (2000)) described in detail a filtered disc assay suitable for determining β-ketoacyl-ACP synthase ("FabH") activity against acetyl-CoA substrate, which can be modified to assay propionyl-CoA as a substrate. The assay contains 25 μM ACP, 1 mM β-mercaptoethanol, 65 μM malonyl-CoA, 45 μM [1-^{14}C]acetyl-CoA (specificity activity about 45.8 Ci/mol), *E.coli* FadD (0.2 μg), and 0.1 M sodium phosphate buffer (pH 7.0) in a final volume of 40 μL. To assay β-ketoacyl-ACP synthase activity, [1-^{14}C]acetyl-CoA can be substituted with ^{14}C labeled propionyl-CoA. The reaction is initiated by the addition of FabH, and the mixture is incubated at 37°C for 12 minutes. A 35 mL aliquot is then removed and deposited on a Whatman 3 MM filter disc. The discs are then washed with three changes (20 mL/disc for 20 minutes each) of ice-cold trichloroacetic acid. The concentration of the trichloroacetic acid is then reduced from 10 to 5 to 1% in each successive wash. The filters are dried an counted in 3 mL of scintillation cocktail.

Alternatively, FabH activity can be determined using a radioactively labeled malonyl-CoA substrate and gel electrophoresis to separate and quantitate the products (Choi *et al.* 2000, *supra*). The assay mixture contains 25 μM ACP, 1 mM β-mercaptoethanol, 70 μM [2-^{14}C] malonyl-CoA (specific activity, ~ 9 Ci/mol), 45 μM of a CoA-substrate (such as acetyl-CoA or propionyl-CoA), FadD (0.2 μg), 100 μM NADPH, FabG (0.2 μg) and 0.1 M sodium phosphate buffer (pH 7.0) in a final volume of 40 μL. The reaction can be initiated by the addition of FabH. The mixture is incubated at 37°C for 12 minutes and then placed in an ice slurry, gel loading buffer is then added, and the mixture is loaded onto a conformationally sensitive 13% polyacrylamide gel containing 0.5 to 2.0 M urea. Electrophoresis can be performed at 25°C at 32 mA/gel. The gels are then dried, and the bands quantitated by exposure of the gel to a PhosphoImager screen. Specific activity can be

calculated from the slopes of the plot of product formation *vs*. FabH protein concentration in the assay.

oc-β-Ketoacyl-ACP to oc-Acyl-ACP

The oc-β-ketoacyl-ACP intermediate 3-oxovaleryl-ACP generated in step (D) can undergo elongation by successive cycles of condensation with malonyl-ACP / keto-reduction / dehydration / enoyl-reduction, catalyzed by a fatty acid synthase (FAS) complex, such as, for example, a type II fatty acid synthase complex, thereby adding 2-carbon units to the lengthening fatty acid chain of the resulting oc-acyl-ACP, as represented by step (E) of Fig. 1B. In one embodiment, a FAS enzyme complex (such as, for example, a Type II FAS complex) endogenous to the microbial cell is used to catalyze cycles of condensation with malonyl-ACP / keto-reduction / dehydration / enoyl-reduction to produce the oc-acyl-ACP intermediate.

oc-Acyl-ACP to oc-FA Derivative

Odd chain fatty acid derivatives can be produced by a recombinant microbial cell of the invention. The oc-acyl-ACP intermediate is converted to an oc-FA derivative in a reaction catalyzed by one or more enzymes each having fatty acid derivative activity (*i.e.*, fatty acid derivative enzymes), as represented by step (F) of Fig. 1B. A fatty acid derivative enzyme can, for example, convert an oc-acyl-ACP to an initial oc-FA derivative, or, can convert the initial oc-FA derivative to a second oc-FA derivative. In some instances, the initial oc-FA derivative is converted to a second oc-FA derivative by an enzyme having a different fatty acid derivative activity. In some instances, the second oc-FA derivative is further converted to a third oc-FA derivative by another fatty acid derivative enzyme, and so on.

Accordingly, in some embodiments, the recombinant microbial cell further comprises one or more polynucleotides, each polynucleotide encoding a polypeptide having a fatty acid derivative enzyme activity, wherein the recombinant microbial cell produces an oc-FA derivative when cultured in the presence of a carbon source under conditions effective to express the polynucleotides.

In various embodiments, the fatty acid derivative activity comprises thioesterase activity, wherein the recombinant microbial cell produces oc-fatty acids; ester synthase activity, wherein the recombinant microbial cell produces oc-fatty esters; fatty aldehyde biosynthesis activity, wherein the recombinant microbial cell produces oc-fatty aldehydes; fatty alcohol biosynthesis activity, wherein the recombinant microbial cell produces oc-fatty alcohols; ketone biosynthesis activity, wherein the recombinant microbial cell produces ec-ketones; or hydrocarbon biosynthesis activity, wherein the recombinant microbial cell produces ec-hydrocarbons. In some embodiments, the recombinant microbial cell comprises polynucleotides encoding two or more polypeptides, each polypeptide having fatty acid derivative enzyme activity.

In more particular embodiments, the recombinant microbial cell expresses or overexpresses one or more polypeptides having fatty acid derivative enzyme activity as described hereinabove,

wherein the recombinant microbial cell produces an oc-FA composition comprising oc-fatty acids, oc-fatty esters, oc-wax esters, oc-fatty aldehydes, oc-fatty alcohols, ec-ketones, ec-alkanes, ec-alkanes, ec-internal olefins, or ec-terminal olefins.

The following are further examples of fatty acid derivative enzymes, and oc-FA derivatives produced by reactions catalyzed by such enzymes, in accordance with various embodiments of the invention.

oc-Fatty Acid

In one embodiment, the recombinant microbial cell comprises a polynucleotide encoding a thioesterase, and the oc-acyl-ACP intermediate produced by the recombinant microbial cell is hydrolyzed by the thioesterase (*e.g.*, 3.1.1.5, EC 3.1.2.- ; such as, for example, EC 3.1.2.14) resulting in production of an oc-fatty acid. In some embodiments, a composition comprising fatty acids (also referred to herein as a "fatty acid composition") comprising oc-fatty acids is produced by culturing the recombinant cell in the presence of a carbon source under conditions effective to express the polynucleotide. In some embodiments, the fatty acid composition comprises oc-fatty acids and ec-fatty acids. In some embodiments, the composition is recovered from the cell culture.

In some embodiments, the recombinant microbial cell comprises a polynucleotide encoding a polypeptide having thioesterase activity, and one or more additional polynucleotides encoding polypeptides having other fatty acid derivative enzyme activities. In some such instances, the oc-fatty acid produced by the action of the thioesterase is converted by one or more enzymes having different fatty acid derivative enzyme activities to another oc-fatty acid derivative, such as, for example, an oc-fatty ester, oc-fatty aldehyde, oc-fatty alcohol, or ec-hydrocarbon.

In one embodiment, an oc-acyl-ACP intermediate reacts with a thioesterase to form an oc-fatty acid. The oc-fatty acid can be recovered from the cell culture, or can be further converted to another oc-FA derivative, such as an oc-fatty ester, an oc-fatty aldehyde, an oc-fatty alcohol, or an ec-terminal olefin.

The chain length of a fatty acid, or a fatty acid derivative made therefrom, can be selected for by modifying the expression of certain thioesterases. Thioesterase influences the chain length of fatty acids produced as well as that of the derivatives made therefrom. Hence, the recombinant microbial cell can be engineered to express, overexpress, have attenuated expression, or not to express one or more selected thioesterases to increase the production of a preferred fatty acid or fatty acid derivative substrate. For example, C_{10} fatty acids can be produced by expressing a thioesterase that has a preference for producing C_{10} fatty acids and attenuating thioesterases that have a preference for producing fatty acids other than C_{10} fatty acids (*e.g.*, a thioesterase which prefers to produce C_{14} fatty acids). This would result in a relatively homogeneous population of fatty acids that have a carbon chain length of 10. In other instances, C_{14} fatty acids can be produced by attenuating endogenous thioesterases that produce non-C_{14} fatty acids and expressing thioesterases that use C_{14}-ACP. In some

situations, C_{12} fatty acids can be produced by expressing thioesterases that use C_{12}-ACP and attenuating thioesterases that produce non-C_{12} fatty acids. Fatty acid overproduction can be verified using methods known in the art, for example, by use of radioactive precursors, HPLC, or GC-MS subsequent to cell lysis.

Additional non-limiting examples of thioesterases and polynucleotides encoding them for use in the oc-fatty acid pathway are provided in Table 5 and in PCT Publication No. WO 2010/075483.

Table 5. Non-limiting examples of thioesterases and coding sequences thereof for use in the oc-FA pathway shown in Fig. 1B.

EC number	Organism	Gene symbol	UniProtKB (Swiss-Prot) Protein Accession Number, or literature reference	NCBI Protein Accession Number	SEQ ID NO
EC 3.1.1.5, EC 3.1.2.-	Thioesterase				
	E. coli K-12 MG1655	*tesA*	P0ADA1	AAC73596	64
	E. coli (without leader sequence)	*'tesA*	*Cho et al, J. Biol. Chem.*, 270:4216-4219 (1995)		65
	E. coli K-12 MG1655	*tesB*	P0AGG2	AAC73555	66
	Arabidopsis thaliana	*fatA*	Q42561	NP_189147	67
	Arabidopsis thaliana	*fatB*	Q9SJE2	NP_172327	68
	Umbellularia california	*fatB*	Q41635	AAA34215	69
	Cuphea hookeriana	*fatA1*	Q9ZTF7	AAC72883	70
	Cuphea hookeriana	*fatB2*	Q39514	AAC49269	71
	Cuphea hookeriana	*fatB3*	Q9ZTF9	AAC72881	72

oc-Fatty Ester

In one embodiment, the recombinant microbial cell produces an oc-fatty ester, such as, for example, an oc-fatty acid methyl ester or an oc-fatty acid ethyl ester or an oc-wax ester. In some embodiments, an oc-fatty acid produced by the recombinant microbial cell is converted into the oc-fatty ester.

In some embodiments, the recombinant microbial cell comprises a polynucleotide encoding a polypeptide (*i.e.*, an enzyme) having ester synthase activity (also referred to herein as an "ester synthase polypeptide" or an "ester synthase enzyme"), and the oc-fatty ester is produced by a reaction catalyzed by the ester synthase polypeptide expressed or overexpressed in the recombinant microbial

cell. In some embodiments, a composition comprising fatty esters (also referred to herein as a "fatty ester composition") comprising oc-fatty esters is produced by culturing the recombinant cell in the presence of a carbon source under conditions effective to express the polynucleotide. In some embodiments, the fatty ester composition comprises oc-fatty esters and ec-fatty esters . In some embodiments, the composition is recovered from the cell culture.

Ester synthase polypeptides include, for example, an ester synthase polypeptide classified as EC 2.3.1.75, or any other polypeptide which catalyzes the conversion of an acyl-thioester to a fatty ester, including, without limitation, a wax-ester synthase, an acyl-CoA:alcohol transacylase, an acyltransferase, or a fatty acyl-CoA:fatty alcohol acyltransferase. For example, the polynucleotide may encode wax/dgat, a bifunctonal ester synthase/acyl-CoA:diacylglycerol acyltransferase from *Simmondsia chinensis, Acinetobacter sp. Strain* ADP1, *Alcanivorax borkumensis, Pseudomonas aeruginosa, Fundibacter jadensis, Arabidopsis thaliana,* or *Alkaligenes eutrophus.* In a particular embodiment, the ester synthase polypeptide is an *Acinetobacter sp.* diacylglycerol O-acyltransferase (*wax-dgaT*; UniProtKB Q8GGG1, GenBank AAO17391) or *Simmondsia chinensis* wax synthase (UniProtKB Q9XGY6 , GenBank AAD38041). In a particular embodiment, the polynucleotide encoding the ester synthase polypeptide is overexpressed in the recombinant microbial cell. In some embodiments the recombinant microbial cell further comprises a polynucleotide encoding a thioesterase.

In another embodiment, the recombinant microbial cell produces an oc-fatty ester, such as, for example, an oc-fatty acid methyl ester or an oc-fatty acid ethyl ester, wherein the recombinant microbial cell expresses a polynucleotide encoding an ester synthase / acyltransferase polypeptide classified as 2.3.1.20, such as AtfA1 (an acyltransferase derived from *Alcanivorax borkumensis* SK2, UniProtKB Q0VKV8, GenBank YP_694462) or AtfA2 (another acyltransferase derived from *Alcanivorax borkumensis* SK2, UniProtKB Q0VNJ6, GenBank YP_693524). In a particular embodiment, the polynucleotide encoding the ester synthase polypeptide is overexpressed in the recombinant microbial cell. In some embodiments the recombinant microbial cell further comprises a polynucleotide encoding a thioesterase.

In another embodiment, the recombinant microbial cell produces an oc-fatty ester, such as, for example, an oc-fatty acid methyl ester or an oc-fatty acid ethyl ester, wherein the recombinant microbial cell expresses a polynucleotide encoding a ester synthase polypeptide, such as ES9 (a wax ester synthase from *Marinobacter hydrocarbonoclasticus* DSM 8798, UniProtKB A3RE51, GenBank ABO21021, encoded by the *ws2* gene), or ES376 (another wax ester synthase derived from *Marinobacter hydrocarbonoclasticus* DSM 8798, UniProtKB A3RE50, GenBank ABO21020, encoded by the *ws1* gene). In a particular embodiment, the polynucleotide encoding the ester synthase polypeptide is overexpressed in the recombinant microbial cell. In some embodiments the recombinant microbial cell further comprises a polynucleotide encoding a thioesterase.

Additional non-limiting examples of ester synthase polypeptides and polynucleotides encoding them suitable for use in these embodiments include those described in PCT Publication Nos. WO 2007/136762 and WO2008/119082.

oc-Fatty Aldehyde

In one embodiment, the recombinant microbial cell produces an oc-fatty aldehyde. In some embodiments, an oc-fatty acid produced by the recombinant microbial cell is converted into the an oc-fatty aldehyde. In some embodiments, the oc-fatty aldehyde produced by the recombinant microbial cell is then converted into an oc-fatty alcohol or an ec-hydrocarbon.

In some embodiments, the recombinant microbial cell comprises a polynucleotide encoding a polypeptide (*i.e.*, an enzyme) having fatty aldehyde biosynthesis activity (also referred to herein as a "fatty aldehyde biosynthesis polypeptide" or a "fatty aldehyde biosynthesis enzyme"), and the oc-fatty aldehyde is produced by a reaction catalyzed by the fatty aldehyde biosynthesis polypeptide expressed or overexpressed in the recombinant microbial cell. In some embodiments, a composition comprising fatty aldehydes (also referred to herein as a "fatty aldehyde composition") comprising oc-fatty aldehydes is produced by culturing the recombinant cell in the presence of a carbon source under conditions effective to express the polynucleotide. In some embodiments, the fatty aldehyde composition comprises oc-fatty aldehydes and ec-fatty aldehydes. In some embodiments, the composition is recovered from the cell culture.

In some embodiments, the oc-fatty aldehyde is produced by expressing or overexpressing in the recombinant microbial cell a polynucleotide encoding a polypeptide having a fatty aldehyde biosynthesis activity such as carboxylic acid reductase (CAR) activity (encoded, for example, by a *car* gene). Examples of carboxylic acid reductase (CAR) polypeptides and polynucleotides encoding them useful in accordance with this embodiment include, but are not limited to, FadD9 (EC 6.2.1.-, UniProtKB Q50631, GenBank NP_217106), CarA (GenBank ABK75684), CarB (GenBank YP889972) and related polypeptides described in PCT Publication No. WO 2010/042664.

In some embodiments the recombinant microbial cell further comprises a polynucleotide encoding a thioesterase.

In some embodiments, the oc-fatty aldehyde is produced by expressing or overexpressing in the recombinant microbial cell a polynucleotide encoding a fatty aldehyde biosynthesis polypeptide, such as a polypeptide having acyl-ACP reductase (AAR) activity, encoded by, for example, an *aar* gene. Examples of acyl-ACP reductase polypeptides useful in accordance with this embodiment include, but are not limited to, acyl-ACP reductase from *Synechococcus elongatus* PCC 7942 (GenBank YP_400611) and related polypeptides described in PCT Publication No. WO 2010/042664.

In some embodiments, the oc-fatty aldehyde is produced by expressing or overexpressing in the recombinant microbial cell a polynucleotide encoding a fatty aldehyde biosynthesis polypeptide,

such as a polypeptide having acyl-CoA reductase activity (*e.g.*, EC 1.2.1.x), encoded by, for example, an *acr1* gene. Examples of acyl-CoA reductase polypeptides useful in accordance with this embodiment include, but are not limited to, ACR1 from *Acinetobacter sp.* strain ADP1 (GenBank YP_047869) and related polypeptides described in PCT Publication No. WO 2010/042664.

In some embodiments the recombinant microbial cell further comprises polynucleotides encoding a thioesterase and an acyl-CoA synthase.

oc-Fatty Alcohol

In one embodiment, the recombinant microbial cell produces an oc-fatty alcohol. In some embodiments, an oc-fatty aldehyde produced by the recombinant microbial cell is converted to the oc-fatty alcohol. In other embodiments, an oc-fatty acid produced by the recombinant microbial cell is converted to the oc-fatty alcohol

In some embodiments, the recombinant microbial cell comprises a polynucleotide encoding a polypeptide (*i.e.*, an enzyme) having fatty alcohol biosynthesis activity (also referred to herein as a "fatty alcohol biosynthesis polypeptide" or a "fatty alcohol biosynthesis enzyme"), and the oc-fatty alcohol is produced by a reaction catalyzed by the fatty alcohol biosynthesis enzyme expressed or overexpressed in the recombinant microbial cell. In some embodiments, a composition comprising fatty alcohols (also referred to herein as a "fatty alcohol composition") comprising oc-fatty alcohols is produced by culturing the recombinant cell in the presence of a carbon source under conditions effective to express the polynucleotide. In some embodiments, the fatty alcohol composition comprises oc-fatty alcohols and ec-fatty alcohols. In some embodiments, the composition is recovered from the cell culture.

In some embodiments, the oc-fatty alcohol is produced by expressing or overexpressing in the recombinant microbial cell a polynucleotide encoding a polypeptide having fatty alcohol biosynthesis activity such as alcohol dehydrogenase (aldehyde reductase) activity, *e.g.*, EC 1.1.1.1. Examples of alcohol dehydrogenase polypeptides useful in accordance with this embodiment include, but are not limited to, *E. coli* alcohol dehydrogenase YqhD (GenBank AP_003562) and related polypeptides described in PCT Publication Nos. WO 2007/136762 and WO2008/119082.

In some embodiments the recombinant microbial cell further comprises a polynucleotide encoding a fatty aldehyde biosynthesis polypeptide. In some embodiments the recombinant microbial cell further comprises a polynucleotide encoding a thioesterase.

In some embodiments, the oc-fatty alcohol is produced by expressing or overexpressing in the recombinant microbial cell a polynucleotide encoding a fatty alcohol biosynthesis polypeptide, such as a polypeptide having fatty alcohol forming acyl-CoA reductase (FAR) activity, *e.g.*, EC 1.1.1.x. Examples of FAR polypeptides useful in accordance with this embodiment include, but are not limited to, those described in PCT Publication No. WO 2010/062480.

In some embodiments the recombinant microbial cell further comprises polynucleotides encoding a thioesterase and an acyl-CoA synthase.

ec- Hydrocarbon

In one embodiment, the recombinant microbial cell produces an ec-hydrocarbon, such as an ec-alkane or an ec-alkene (*e.g.*, an ec-terminal olefin or an ec-internal olefin) or an ec-ketone. In some embodiments, an oc-acyl-ACP intermediate is converted by decarboxylation, removing a carbon atom to form an ec-internal olefin or an ec-ketone. In some embodiments, an oc-fatty aldehyde produced by the recombinant microbial cell is converted by decarbonylation, removing a carbon atom to form an ec-hydrocarbon. In some embodiments, an oc-fatty acid produced by the recombinant microbial cell is converted by decarboxylation, removing a carbon atom to form an ec-terminal olefin.

In some embodiments, the recombinant microbial cell comprises a polynucleotide encoding a polypeptide (*i.e.*, an enzyme) having hydrocarbon biosynthesis activity (also referred to herein as a "hydrocarbon biosynthesis polypeptide" or a "hydrocarbon biosynthesis enzyme"), and the ec-hydrocarbon is produced by a reaction catalyzed by the hydrocarbon biosynthesis enzyme expressed or overexpressed in the recombinant microbial cell. In some embodiments, a composition comprising hydrocarbons (also referred to herein as a "hydrocarbon composition") comprising ec-hydrocarbons is produced by culturing the recombinant cell in the presence of a carbon source under conditions effective to express the polynucleotide. In some embodiments, the hydrocarbon composition comprises ec-hydrocarbons and oc-hydrocarbons. In some embodiments, the hydrocarbon composition is recovered from the cell culture.

In some embodiments, the ec-hydrocarbon is produced by expressing or overexpressing in the recombinant microbial cell a polynucleotide encoding a polypeptide having hydrocarbon biosynthesis activity such as an aldehyde decarbonylase (ADC) activity (*e.g.*, EC 4.1.99.5), for example, a polynucleotide encoding an aldehyde decarbonylase from *Prochlorococcus marinus* MIT9313 (GenBank NP_895059) or *Nostoc punctiforme* (GenBank Accession No. YP_001865325). Additional examples of aldehyde decarbonylase and related polypeptides useful in accordance with this embodiment include, but are not limited to, those described in PCT Publication Nos. WO 2008/119082 and WO 2009/140695 . In some embodiments the recombinant microbial cell further comprises a polynucleotide encoding a fatty aldehyde biosynthesis polypeptide. In some embodiments the recombinant microbial cell further comprises a polynucleotide encoding an acyl-ACP reductase .

In some embodiments, an ec-terminal olefin is produced by expressing or overexpressing in the recombinant microbial cell a polynucleotide encoding a hydrocarbon biosynthesis polypeptide, such as a polypeptide having decarboxylase activity as described, for example, in PCT Publication No. WO 2009/085278 . In some embodiments the recombinant microbial cell further comprises a polynucleotide encoding a thioesterase.

In some embodiments, an ec-internal olefin is produced by expressing or overexpressing in the recombinant microbial cell a polynucleotide encoding a hydrocarbon biosynthesis polypeptide, such as a polypeptide having OleCD or OleBCD activity as described, for example, in PCT Publication No. WO 2008/147781.

In some embodiments, an ec-ketone is produced by expressing or overexpressing in the recombinant microbial cell a polynucleotide encoding a hydrocarbon biosynthesis polypeptide, such as a polypeptide having OleA activity as described, for example, in PCT Publication No. WO 2008/147781.

Saturation Levels of oc-FA Derivatives

The degree of saturation of oc-acyl-ACPs (which can then be converted into various oc-FA derivatives as described hereinabove) can be controlled by regulating the degree of saturation of fatty acid intermediates. For example, the *sfa*, *gns*, and *fab* families of genes can be expressed, overexpressed, or expressed at reduced levels (*e.g.*, attenuated), to control the amount of saturation of an oc-acyl-ACP.

oc-FA Pathway Polypeptides and Polynucleotides

The disclosure identifies polynucleotides useful in the recombinant microbial cells, methods, and compositions of the invention; however it will be recognized that absolute sequence identity to such polynucleotides is not necessary. For example, changes in a particular polynucleotide sequence can be made and the encoded polypeptide screened for activity. Such changes typically comprise conservative mutations and silent mutations (such as, for example, codon optimization). Modified or mutated (*i.e.*, mutant) polynucleotides and encoded variant polypeptides can be screened for a desired function, such as, an improved function compared to the parent polypeptide, including but not limited to increased catalytic activity, increased stability, or decreased inhibition (*e.g.*, decreased feedback inhibition), using methods known in the art.

The disclosure identifies enzymatic activities involved in various steps (*i.e.*, reactions) of the oc-FA biosynthetic pathways described herein according to Enzyme Classification (EC) number, and provides exemplary polypeptides (*i.e.*, enzymes) categorized by such EC numbers, and exemplary polynucleotides encoding such polypeptides. Such exemplary polypeptides and polynucleotides, which are identified herein by Accession Numbers and/or Sequence Identifier Numbers (SEQ ID NOs), are useful for engineering oc-FA pathways in parental microbial cells to obtain the recombinant microbial cells described herein. It is to be understood, however, that polypeptides and polynucleotides described herein are exemplary and non-limiting. The sequences of homologues of exemplary polypeptides described herein are available to those of skill in the art using databases such as, for example, the Entrez databases provided by the National Center for Biotechnology Information (NCBI) , the ExPasy databases provided by the Swiss Institute of Bioinformatics, the BRENDA database provided by the Technical University of Braunschweig, and the KEGG database provided

by the Bioinformatics Center of Kyoto University and University of Tokyo, all which are available on the World Wide Web.

It is to be further understood that a variety of microbial cells can be modified to contain an oc-FA pathway described herein, resulting in recombinant microbial cells suitable for the production of odd chain fatty acid derivatives. It is also understood that a variety of cells can provide sources of genetic material, including sequences of polynucleotides encoding polypeptides suitable for use in a recombinant microbial cell provided herein.

The disclosure provides numerous examples of polypeptides (*i.e.*, enzymes) having activities suitable for use in the oc-FA biosynthetic pathways described herein. Such polypeptides are collectively referred to herein as "oc-FA pathway polypeptides" (alternatively, "oc-FA pathway enzymes"). Non-limiting examples of oc-FA pathway polypeptides suitable for use in recombinant microbial cells of the invention are provided in the Tables and Description and in the Examples herein.

In some embodiments, the invention includes a recombinant microbial cell comprising a polynucleotide sequence (also referred to herein as an "oc-FA pathway polynucleotide" sequence) which encodes an oc-FA pathway polypeptide.

Additional oc-FA pathway polypeptides and polynucleotides encoding them suitable for use in engineering an oc-FA pathway in a recombinant microbial cell of the invention can be obtained by a number of methods. For example, EC numbers classify enzymes according to the reaction catalyzed. Enzymes that catalyze a reaction in a biosynthetic pathway described herein can be identified by searching the EC number corresponding to that reaction in a database such as, for example: the KEGG database (Kyoto Encyclopedia of Genes and Genomes; Kyoto University and University of Tokyo); the UNIPROTKB database or the ENZYME database (ExPASy Proteomics Server; Swiss Institute of Bioinformatics); the PROTEIN database or the GENE database (Entrez databases; National Center for Biotechnology Information (NCBI)); or the BRENDA database (The Comprehensive Enzyme Information System; Technical University of Braunschweig); all of which are available on the World Wide Web. In one embodiment, an oc-FA pathway polynucleotide encoding an oc-FA pathway polypeptide having an enzymatic activity categorized by an EC number (such as, an EC number listed in the Description or in one of Tables herein), or a fragment or a variant thereof having that activity, is used in engineering the corresponding step of an oc-FA pathway in a recombinant microbial cell.

In some embodiments, an oc-FA pathway polynucleotide sequence encodes a polypeptide which is endogenous to the parental cell of the recombinant cell being engineered. Some such endogenous polypeptides are overexpressed in the recombinant microbial cell. An "endogenous polypeptide", as used herein, refers to a polypeptide which is encoded by the genome of the parental (e.g., wild-type) cell that is being engineered to produce the recombinant microbial cell.

An oc-FA pathway polypeptide, such as for example an endogenous oc-FA pathway polypeptide, can be overexpressed by any suitable means. As used herein, "overexpress" means to express or cause to be expressed a polynucleotide or polypeptide in a cell at a greater concentration than is normally expressed in a corresponding parental (for example, wild-type) cell under the same conditions. For example, a polypeptide is "overexpressed" in a recombinant microbial cell when it is present in a greater concentration in the recombinant cell as compared to its concentration in a non-recombinant host cell of the same species (*e.g.*, the parental cell) when cultured under the same conditions.

In some embodiments, the oc-FA pathway polynucleotide sequence encodes an exogenous or heterologous polypeptide. In other words, the polypeptide encoded by the polynucleotide is exogenous to the parental microbial cell. An "exogenous" (or "heterologous") polypeptide, as used herein, refers to a polypeptide not encoded by the genome of the parental (e.g., wild-type) microbial cell that is being engineered to produce the recombinant microbial cell. Such a polypeptide can also be referred to as a "non-native" polypeptide.

In certain embodiments, an oc-FA pathway polypeptide comprises an amino acid sequence other than that of one of the exemplary polypeptides provided herein; for example, an oc-FA pathway polypeptide can comprise a sequence which is a homologue, a fragment, or a variant of the sequence of the exemplary polypeptide.

The terms "homolog," "homologue," and "homologous" as used herein refer to a polynucleotide or a polypeptide comprising a sequence that is at least 50%, preferably at least 60%, more preferably at least 70% (*e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%) homologous to the corresponding polynucleotide or polypeptide sequence. One of ordinary skill in the art is well aware of methods to determine homology between two or more sequences. Briefly, calculations of "homology" between two sequences can be performed as follows. 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 polynucleotide sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a first sequence that is aligned for comparison purposes is at least about 30%, preferably at least about 40%, more preferably at least about 50%, even more preferably at least about 60%, and even more preferably at least about 70%, at least about 80%, at least about 90%, or about 100% of the length of a second sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions of the first and second sequences 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.

The comparison of sequences and determination of percent homology (*i.e.*, percent identity) between two sequences can be accomplished using a mathematical algorithm, such as BLAST (Altschul *et al.*, *J. Mol. Biol., 215(3)*: 403-410 (1990)). The percent homology between two amino acid sequences also can be determined using the Needleman and Wunsch algorithm that has been incorporated into the GAP program in the GCG software package, using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3,4, 5, or 6 (Needleman and Wunsch, *J. Mol. Biol., 48*: 444-453 (1970)). The percent homology between two nucleotide sequences also can be determined using the GAP program in the GCG software package, using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. One of ordinary skill in the art can perform initial homology calculations and adjust the algorithm parameters accordingly. A preferred set of parameters (and the one that should be used if a practitioner is uncertain about which parameters should be applied to determine if a molecule is within a homology limitation of the claims) are a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5. Additional methods of sequence alignment are known in the biotechnology arts (see, *e.g.*, Rosenberg, *BMC Bioinformatics*, *6*: 278 (2005); Altschul *et al.*, *FEBS J.*, *272(20)*: 5101–5109 (2005)).

An "equivalent position" (for example, an "equivalent amino acid position" or "equivalent nucleic acid position") is defined herein as a position (such as, an amino acid position or nucleic acid position) of a test polypeptide (or test polynucleotide) sequence which aligns with a corresponding position of a reference polypeptide (or reference polynucleotide) sequence, when optimally aligned using an alignment algorithm as described herein. The equivalent amino acid position of the test polypeptide need not have the same numerical position number as the corresponding position of the reference polypeptide; likewise, the equivalent nucleic acid position of the test polynucleotide need not have the same numerical position number as the corresponding position of the reference polynucleotide.

In some embodiments, the oc-FA pathway polypeptide is a variant of a reference (*e.g.*, a parent) polypeptide, such as a variant of an exemplary oc-FA pathway polypeptide described herein. A "variant" (alternatively, "mutant") polypeptide as used herein refers to a polypeptide having an amino acid sequence that differs from that of a parent (*e.g.*, wild-type) polypeptide by at least one amino acid. The variant can comprise one or more conservative amino acid substitutions, and/or can comprise one or more non-conservative substitutions, compared to the parent polypeptide sequence. In some embodiments, the variant polypeptide has 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, or more amino acid substitutions, additions, insertions, or deletions compared to the parent polypeptide

sequence. In some embodiments, the sequence of the variant polypeptide is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the sequence of the parent polypeptide.

In some embodiments, the oc-FA pathway polypeptide is a fragment of a reference (*e.g.*, a parent) polypeptide, such as a fragment of an exemplary oc-FA pathway polypeptide described herein. The term "fragment" refers to a shorter portion of a full-length polypeptide or protein ranging in size from four amino acid residues to the entire amino acid sequence minus one amino acid residue. In certain embodiments of the invention, a fragment refers to the entire amino acid sequence of a domain of a polypeptide or protein (*e.g.*, a substrate binding domain or a catalytic domain).

In some embodiments, a homologue, a variant, or a fragment comprises one or more sequence motif as defined herein. In one embodiment, a homologue, a variant, or a fragment of a β-ketoacyl-ACP synthase polypeptide comprises one or more sequence motif selected from SEQ ID NOs:14-19. Determination that a sequence contains a particular sequence motif can be readily accomplished, for example, using the ScanProsite tool available on the World Wide Web site of the ExPASy Proteomics Server.

It is understood that an oc-FA polypeptide may have conservative or non-essential amino acid substitutions, relative to a parent polypeptide, which does not have a substantial effect on a biological function or property of the oc-FA polypeptide. Whether or not a particular substitution will be tolerated (*i.e.*, will not adversely affect a desired biological function, such as enzymatic activity) can be determined, for example, as described in Bowie *et al.* (*Science, 247*: 1306-1310 (1990)).

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).

Variants can be naturally occurring or created *in vitro*. In particular, variants can be created using genetic engineering techniques, such as site directed mutagenesis, random chemical mutagenesis, exonuclease III deletion procedures, or standard cloning techniques. Alternatively, such variants, fragments, analogs, or derivatives can be created using chemical synthesis or modification procedures.

Methods of making variants are well known in the art. These include procedures in which nucleic acid sequences obtained from natural isolates are modified to generate nucleic acids that encode polypeptides having characteristics that enhance their value in industrial or laboratory

applications (including, but not limited to, increased catalytic activity (turnover number), improved stability, and reduced feedback inhibition). In such procedures, a large number of modified nucleic acid sequences having one or more nucleotide differences with respect to the sequence obtained from the natural isolate are generated and characterized. Typically, these nucleotide differences result in amino acid changes with respect to the polypeptides encoded by the nucleic acids from the natural isolates. For example, variants can be prepared by using random or site-directed mutagenesis.

Variants can also be created by *in vivo* mutagenesis. In some embodiments, random mutations in a nucleic acid sequence are generated by propagating the sequence in a bacterial strain, such as an *E. coli* strain, which carries mutations in one or more of the DNA repair pathways. Such "mutator" strains have a higher random mutation rate than that of a wild-type strain. Propagating a DNA sequence in one of these strains will eventually generate random mutations within the DNA. Mutator strains suitable for use for *in vivo* mutagenesis are described in, for example, International Patent Application Publication No. WO 1991/016427.

Variants can also be generated using cassette mutagenesis. In cassette mutagenesis, a small region of a double-stranded DNA molecule is replaced with a synthetic oligonucleotide "cassette" that differs from the native sequence. The oligonucleotide often contains a completely and/or partially randomized native sequence.

Recursive ensemble mutagenesis can also be used to generate variants. Recursive ensemble mutagenesis is an algorithm for protein engineering (*i.e.*, protein mutagenesis) developed to produce diverse populations of phenotypically related mutants whose members differ in amino acid sequence. This method uses a feedback mechanism to control successive rounds of combinatorial cassette mutagenesis. Recursive ensemble mutagenesis is described in, for example, Arkin *et al.*, *Proc. Natl. Acad. Sci., U.S.A., 89*: 7811-7815 (1992).

In some embodiments, variants are created using exponential ensemble mutagenesis. Exponential ensemble mutagenesis is a process for generating combinatorial libraries with a high percentage of unique and functional mutants, wherein small groups of residues are randomized in parallel to identify, at each altered position, amino acids which lead to functional proteins. Exponential ensemble mutagenesis is described in, for example, Delegrave *et al.*, *Biotech. Res, 11*: 1548-1552 (1993).

Preferred fragments or variants of a parent polypeptide (e.g., fragments or variants of a parent oc-FA pathway polypeptide) retain some or all of a biological function or property (such as, enzymatic activity, thermal stability) of the parent polypeptide. In some embodiments, the fragment or variant retains at least 75% (*e.g.*, at least 80%, at least 90%, or at least 95%) of a biological function or property of the parent polypeptide. In other embodiments, the fragment or variant retains about 100% of a biological function or property of the parent polypeptide.

In some embodiments, the fragment or variant of the parent polypeptide exhibits an increased catalytic activity (as reflected by, for example, a higher turnover number, an altered pH optimum, a decreased K_m for a desired substrate, or an increased k_{cat}/K_m for a desired substrate), relative to that of the parent polypeptide, under conditions in which the recombinant microbial cell is cultured. For example, if the parent polypeptide is endogenous to (that is, is derived from) a thermophilic cell, and if the recombinant microbial cell is generally cultured at a lower temperature than the thermophilic cell, the parent polypeptide may exhibit significantly reduced activity at the lower temperature; in which case, the variant polypeptide preferably exhibits an increased catalytic activity (such as, a higher turnover number), relative to that of the parent polypeptide, at that lower temperature.

In other embodiments, the fragment or variant of the parent polypeptide exhibits improved stability, relative to that of the parent polypeptide, under conditions in which the recombinant microbial cell is cultured. Such stability can include stability towards changes in temperature, ionic strength, pH, or any other differences in growth or media conditions between the recombinant microbial cell and the cell from which the parent polypeptide was derived. For example, if the parent polypeptide is derived from a psychrotrophic cell, and if the recombinant microbial cell is generally cultured at a higher temperature than the psychrotrophic cell, the parent polypeptide may be relatively unstable at the higher temperature; in which case, the variant polypeptide preferably exhibits improved stability relative to that of the parent polypeptide at that higher temperature.

In other embodiments, the fragment or variant of the parent polypeptide exhibits reduced inhibition of catalytic activity (such as, reduced feedback inhibition) by a cellular metabolite or by a culture media component, relative to such inhibition exhibited by the parent polypeptide, under conditions in which the recombinant microbial cell is cultured.

In certain embodiments, an oc-FA pathway polypeptide is a homologue, a fragment, or a variant of a parent polypeptide, wherein the oc-FA pathway polypeptide is effective in carrying out an oc-FA pathway reaction in a recombinant microbial cell. Such an oc-FA pathway polypeptide is suitable for use in a recombinant microbial cell of the invention.

The effectiveness of a test polypeptide (such as, for example, an oc-FA pathway polypeptide described herein, or a homologue, a fragment, or a variant thereof) in carrying out a reaction of an oc-FA pathway can be determined by a number of methods. For example, to determine the effectiveness of a test polypeptide in catalyzing a specific reaction of a biochemical pathway, first a cell is engineered (if necessary) to obtain a parental cell that comprises all the activities needed to catalyze the reactions of the biochemical pathway in question, except for the specific pathway reaction being tested (although, in some instances, the parental cell may express endogenous polypeptide(s) that catalyze the specific pathway reaction being tested; in such instances the endogenous activity will preferably be low enough to readily detect an increase in product owing to the activity of the test polypeptide). A polynucleotide encoding the test polypeptide, operatively linked to a suitable

promoter (*e.g.*, in an expression vector), is then introduced into the parental cell, generating a test cell. The test cell and the parental cell are cultured separately under identical conditions which are sufficient for expression of the pathway polypeptides in the parental and test cell cultures and expression of the test polypeptide in the test cell culture. At various times during and/or after culturing, samples are obtained from the test cell culture and the parental cell culture. The samples are analyzed for the presence of a particular pathway intermediate or product. Presence of the pathway intermediate or product can be determined by methods including, but not limited to, gas chromatography (GC), mass spectroscopy (MS), thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), liquid chromatography (LC), GC coupled with a flame ionization detector (GC-FID), GC-MS, and LC-MS. The presence of an oc-FA pathway intermediate or product in the test cell culture sample(s), and the absence (or a reduced amount) of the oc-FA pathway intermediate or product in the parent cell culture sample(s), indicates that the test polypeptide is effective in carrying out an oc-FA pathway reaction and is suitable for use in a recombinant microbial cell of the invention.

Production of Odd Chain Fatty Acid Derivatives in Recombinant Microbial Cells

In one aspect, the invention includes a method of making an odd chain fatty acid derivative composition, the method comprising culturing a recombinant microbial cell of the invention in a culture medium containing a carbon source under conditions effective to express the recombinant polynucleotide sequences, and optionally isolating the produced odd chain fatty acid derivative composition.

An "odd chain fatty acid derivative composition" (abbreviated "oc-FA derivative composition") is a composition comprising an odd chain fatty acid derivative as defined herein, such as, for example, an odd chain fatty acid, an odd chain fatty ester (*e.g.*, an odd chain fatty methyl ester, an odd chain fatty ethyl ester , an odd chain wax ester), an odd chain fatty aldehyde, an odd chain fatty alcohol, an even chain hydrocarbon (such as an even chain alkane, an even chain alkene, an even chain terminal olefin, an even chain internal olefin), or an even chain ketone. Similarly, an "odd chain fatty acid composition" is a composition comprising odd chain fatty acids, an "odd chain fatty alcohol composition" is a composition comprising odd chain fatty alcohols, an "even chain alkane composition" is a composition comprising even chain alkanes, and so on. It is to be understood that a composition comprising odd chain fatty acid derivatives may also comprise even chain fatty acid derivatives.

In one aspect, the invention includes a method of making a composition comprising an odd chain fatty acid derivative, the method comprising: obtaining a recombinant microbial cell (such as, a culture comprising a recombinant microbial cell) comprising : (a) polynucleotides encoding polypeptides having enzymatic activities effective to produce an increased amount of propionyl-CoA in the recombinant microbial cell, relative to the amount of propionyl-CoA produced in a parental

microbial cell lacking or having a reduced amount of said enzymatic activity, wherein at least one polypeptide is exogenous to the recombinant microbial cell or wherein expression of at least one polynucleotide is modulated in the recombinant microbial cell as compared to the expression of the polynucleotide in the parental microbial cell; (b) a polynucleotide encoding a polypeptide having β-ketoacyl-ACP synthase activity that utilizes propionyl-CoA as a substrate; and (c) one or more polynucleotides encoding a polypeptide having fatty acid derivative enzyme activity, wherein the recombinant microbial cell produces a fatty acid derivative composition comprising odd chain fatty acid derivatives and even chain fatty acid derivatives when cultured in the presence of a carbon source under conditions effective to express the polynucleotides according to (a), (b), and (c); culturing the recombinant microbial cell in a culture medium containing a carbon source under conditions effective to express the polynucleotides according to (a), (b), and (c) and produce a fatty acid derivative composition comprising odd chain fatty acid derivatives and even chain fatty acid derivatives, and optionally recovering the composition from the culture medium.

In some embodiments, at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% by weight of the fatty acid derivatives in the composition are odd chain fatty acid derivatives. In some embodiments, the fatty acid derivative composition comprises odd chain fatty acid derivatives in an amount (e.g., a titer) of at least 10 mg/L, at least 15 mg/L, at least 20 mg/L, at least 25 mg/L, at least 50 mg/L, at least 75 mg/L, at least 100 mg/L, at least 125 mg/L, at least 150 mg/L, at least 175 mg/L, at least 200 mg/L, at least 225 mg/L, at least 250 mg/L, at least 275 mg/L, at least 300 mg/L, at least 325 mg/L, at least 350 mg/L, at least 375 mg/L, at least 400 mg/L, at least 425 mg/L, at least 450 mg/L, at least 475 mg/L, at least 500 mg/L, at least 525 mg/L, at least 550 mg/L, at least 575 mg/L, at least 600 mg/L, at least 625 mg/L, at least 650 mg/L, at least 675 mg/L, at least 700 mg/L, at least 725 mg/L, at least 750 mg/L, at least 775 mg/L, at least 800 mg/L, at least 825 mg/L, at least 850 mg/L, at least 875 mg/L, at least 900 mg/L, at least 925 mg/L, at least 950 mg/L, at least 975 mg/L, at least 1000 mg/L, at least 1050 mg/L, at least 1075 mg/L, at least 1100 mg/L, at least 1125 mg/L, at least 1150 mg/L, at least 1175 mg/L, at least 1200 mg/L, at least 1225 mg/L, at least 1250 mg/L, at least 1275 mg/L, at least 1300 mg/L, at least 1325 mg/L, at least 1350 mg/L, at least 1375 mg/L, at least 1400 mg/L, at least 1425 mg/L, at least 1450 mg/L, at least 1475 mg/L, at least 1500 mg/L, at least 1525 mg/L, at least 1550 mg/L, at least 1575 mg/L, at least 1600 mg/L, at least 1625 mg/L, at least 1650 mg/L, at least 1675 mg/L, at least 1700 mg/L, at least 1725 mg/L, at least 1750 mg/L, at least 1775 mg/L, at least 1800 mg/L, at least 1825 mg/L, at least 1850 mg/L, at least 1875 mg/L, at least 1900 mg/L, at least 1925 mg/L, at least 1950 mg/L, at least 1975 mg/L, at least 2000 mg/L, at least 3000 mg/L, at least 4000 mg/L, at least 5000 mg/L, at least 6000 mg/L, at least 7000 mg/L, at least 8000 mg/L, at least 9000 mg/L, at least 10000 mg/L, at least 20000 mg/L, or a range bounded by any two of the foregoing values.

In various embodiments, the fatty acid derivative enzyme activity comprises a thioesterase activity, an ester synthase activity, a fatty aldehyde biosynthesis activity, a fatty alcohol biosynthesis activity, a ketone biosynthesis activity, and/or a hydrocarbon biosynthesis activity. In some embodiments, the recombinant microbial cell comprises polynucleotides encoding two or more polypeptides, each polypeptide having a fatty acid derivative enzyme activity.

In various embodiments, the recombinant microbial cell produces a composition comprising odd chain fatty acids, odd chain fatty esters, odd chain wax esters, odd chain fatty aldehydes, odd chain fatty alcohols, even chain alkanes, even chain alkenes, even chain internal olefins, even chain terminal olefins, or even chain ketones.

In various embodiments, the recombinant microbial cell comprises polynucleotides encoding polypeptides having enzymatic activities effective to produce an increased amount of propionyl-CoA in the recombinant microbial cell, selected from: (i) polynucleotides encoding polypeptides having aspartokinase activity, homoserine dehydrogenase activity, homoserine kinase activity, threonine synthase activity, and threonine deaminase activity, or (ii) polynucleotides encoding polypeptides having (R)-citramalate synthase activity, isopropylmalate isomerase activity, and beta-isopropyl malate dehydrogenase activity, or (iii) polypeptides having methylmalonyl-CoA mutase activity, methylmalonyl-CoA decarboxylase activity and/or methylmalonyl-CoA carboxyltransferase activity, or (i) and (ii), or (i) and (iii), or (ii) and (iii), or (i), (ii), and (iii), wherein at least one polypeptide is exogenous to the recombinant microbial cell, or wherein expression of at least one polynucleotide is modulated in the recombinant microbial cell as compared to the expression of the polynucleotide in the parental microbial cell.

The fatty acid derivative compositions comprising odd chain fatty acid derivatives produced by the methods of invention may be recovered or isolated from the recombinant microbial cell culture. The term "isolated" as used herein with respect to products, such as fatty acid derivatives, refers to products that are separated from cellular components, cell culture media, or chemical or synthetic precursors. The fatty acid derivatives produced by the methods described herein can be relatively immiscible in the fermentation broth, as well as in the cytoplasm. Therefore, the fatty acid derivatives can collect in an organic phase either intracellularly or extracellularly. The collection of the products in the organic phase can lessen the impact of the fatty acid derivative on cellular function and can allow the recombinant microbial cell to produce more product.

In some embodiments, the fatty acid derivative composition (which comprises odd chain fatty acid derivatives) produced by the methods of invention are purified. As used herein, the term "purify," "purified," or "purification" means the removal or isolation of a molecule from its environment by, for example, isolation or separation. "Substantially purified" molecules are at least about 60% free (*e.g.*, at least about 70% free, at least about 75% free, at least about 85% free, at least about 90% free, at least about 95% free, at least about 97% free, at least about 99% free) from other

components with which they are associated. As used herein, these terms also refer to the removal of contaminants from a sample. For example, the removal of contaminants can result in an increase in the percentage of a fatty acid derivative (such as, a fatty acid or a fatty alcohol or a fatty ester or a hydrocarbon) relative to other components in a sample. For example, when a fatty ester or a fatty alcohol is produced in a recombinant microbial cell , the fatty ester or fatty alcohol can be purified by the removal of recombinant microbial cell proteins. After purification, the percentage of the fatty ester or fatty alcohol in the sample relative to other components is increased.

As used herein, the terms "purify," "purified," and "purification" are relative terms which do not require absolute purity. Thus, for example, when a fatty acid derivative composition is produced in recombinant microbial cells, a purified fatty acid derivative composition is a fatty acid derivative composition that is substantially separated from other cellular components (*e.g.*, nucleic acids, polypeptides, lipids, carbohydrates, or other hydrocarbons).

The fatty acid derivative composition (which comprises odd chain fatty acid derivatives) may be present in the extracellular environment, or it may be isolated from the extracellular environment of the recombinant microbial cell. In certain embodiments, the fatty derivative is secreted from the recombinant microbial cell. In other embodiments, the fatty acid derivative is transported into the extracellular environment. In yet other embodiments, the fatty acid derivative is passively transported into the extracellular environment. The fatty acid derivative can be isolated from a recombinant microbial cell using methods known in the art.

Fatty acid derivatives (including odd chain fatty acid derivatives produced according to the methods of the present invention) can be distinguished from organic compounds derived from petrochemical carbon on the basis of dual carbon-isotopic fingerprinting or ^{14}C dating. Additionally, the specific source of biosourced carbon (*e.g.*, glucose vs. glycerol) can be determined by dual carbon-isotopic fingerprinting (see, *e.g.*, U.S. Patent 7,169,588).

The ability to distinguish fatty acid derivatives produced by recombinant microbial cells from petroleum-based organic compounds is beneficial in tracking these materials in commerce. For example, organic compounds or chemicals comprising both biologically-based and petroleum-based carbon isotope profiles may be distinguished from organic compounds and chemicals made only of petroleum-based materials. Hence, the materials prepared in accordance with the inventive methods may be followed in commerce on the basis of their unique carbon isotope profile.

Fatty acid derivatives produced by recombinant microbial cells can be distinguished from petroleum-based organic compounds by comparing the stable carbon isotope ratio ($^{13}C/^{12}C$) in each fuel. The $^{13}C/^{12}C$ ratio in a given fatty acid derivative thereof produced according to the methods of the invention is a consequence of the $^{13}C/^{12}C$ ratio in atmospheric carbon dioxide at the time the carbon dioxide is fixed. It also reflects the precise metabolic pathway. Regional variations also occur. Petroleum, C_3 plants (the broadleaf), C_4 plants (the grasses), and marine carbonates all show

significant differences in $^{13}C/^{12}C$ and the corresponding $\delta^{13}C$ values. Furthermore, lipid matter of C_3 and C_4 plants analyze differently than materials derived from the carbohydrate components of the same plants as a consequence of the metabolic pathway.

The ^{13}C measurement scale was originally defined by a zero set by Pee Dee Belemnite (PDB) limestone, where values are given in parts per thousand deviations from this material. The "$\delta^{13}C$" values are expressed in parts per thousand (per mil), abbreviated, ‰, and are calculated as follows:

$$\delta^{13}C\ (‰) = [(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}] / (^{13}C/^{12}C)_{standard} \times 1000$$

In some embodiments, a fatty acid derivative produced according to the methods of the invention has a $\delta^{13}C$ of about -30 or greater, about -28 or greater, about -27 or greater, about -20 or greater, about -18 or greater, about -15 or greater, about -13 or greater, or about -10 or greater. Alternatively, or in addition, a fatty acid derivative has a $\delta^{13}C$ of about -4 or less, about -5 or less, about -8 or less, about -10 or less, about -13 or less, about -15 or less, about -18 or less, or about -20 or less. Thus, the fatty acid derivative can have a $\delta^{13}C$ bounded by any two of the above endpoints. For example, a fatty acid derivative can have a $\delta^{13}C$ of about -30 to about -15, about -27 to about -19, about -25 to about -21, about -15 to about -5, about -13 to about -7, or about -13 to about -10. In some embodiments, a fatty acid derivative can have a $\delta^{13}C$ of about -10, -11, -12, or -12.3. In other embodiments, a fatty acid derivative has a $\delta^{13}C$ of about -15.4 or greater. In yet other embodiments, a fatty acid derivative has a $\delta^{13}C$ of about -15.4 to about -10.9, or a $\delta^{13}C$ of about -13.92 to about -13.84.

A fatty acid derivative produced by a recombinant microbial cell can also be distinguished from petroleum-based organic compounds by comparing the amount of ^{14}C in each compound. Because ^{14}C has a nuclear half-life of 5730 years, petroleum based fuels containing "older" carbon can be distinguished from fatty acids or derivatives thereof which contain "newer" carbon (see, *e.g.*, Currie, "Source Apportionment of Atmospheric Particles", *Characterization of Environmental Particles*, J. Buffle and H. P. van Leeuwen, Eds., Vol. I of the IUPAC Environmental Analytical Chemistry Series, Lewis Publishers, Inc., pp. 3-74 (1992)).

As used herein, "fraction of modern carbon" or f_M has the same meaning as defined by National Institute of Standards and Technology (NIST) Standard Reference Materials (SRMs) 4990B and 4990C, known as oxalic acids standards HOxI and HOxII, respectively. The fundamental definition relates to 0.95 times the ^{14}C /^{12}C isotope ratio HOxI (referenced to AD 1950). This is roughly equivalent to decay-corrected pre-Industrial Revolution wood. For the current living biosphere (plant material), f_M is approximately 1.1.

In some embodiments, a fatty acid derivative produced according to the methods of the invention has a $f_M{}^{14}C$ of at least about 1, *e.g.*, at least about 1.003, at least about 1.01, at least about 1.04, at least about 1.111, at least about 1.18, or at least about 1.124. Alternatively, or in addition, the fatty acid derivative has an $f_M{}^{14}C$ of about 1.130 or less, *e.g.*, about 1.124 or less, about 1.18 or less,

about 1.111 or less, or about 1.04 or less. Thus, the fatty acid derivative can have a $f_M{}^{14}C$ bounded by any two of the above endpoints. For example, the fatty acid derivative can have a $f_M{}^{14}C$ of about 1.003 to about 1.124, a $f_M{}^{14}C$ of about 1.04 to about 1.18, or a $f_M{}^{14}C$ of about 1.111 to about 1.124.

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

Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

EXAMPLES

Media Compositions:

Che-9 media: M9 supplemented with extra NH_4Cl (an additional 1 g/L), Bis-Tris buffer (0.2 M), Triton X-100 (0.1% v/v), and trace minerals (27mg/L $FeCl_3.6\ H_2O$, 2mg/L $ZnCl.4H_2O$, 2mg/L $CaCl_2.\ 6H_2O$, 2mg/L $Na_2MoO_4.2H_2O$, 1.9mg/L $CuSO_4.5H_2O$, 0.5mg/L H_3BO_3, 100 mL/L concentrated HCl).

2NBT: Che-9 supplemented with 20 g/L (2% w/v) glucose.

4NBT: Che-9 supplemented with 40 g/L (4% w/v) glucose.

Example 1. Bacterial Strains and Plasmids

E.coli MG1655 *ΔfadE* (Strain "D1")

This example describes the construction of a recombinant microbial cell in which the expression of a fatty acid degradation enzyme is attenuated. The *fadE* gene of *E.coli* (also known as *yafH*), which encodes an acyl coenzyme A dehydrogenase (GenBank Accession No. AAC73325) involved in fatty acid degradation, was deleted from *E. coli* strain MG1655 using the Red system described by Datsenko, K.A. *et al.* (*Proc. Natl. Acad. Sci. USA* 97: 6640-6645 (2000)), with the following modifications.

The following two primers were used to create the deletion of *fadE*:

Del-*fadE*-F 5' AAAAACAGCA ACAATGTGAG CTTTGTTGTAATTAT ATTGTAA ACATATT GATTCCGGGGATCCGTCGACC (SEQ ID NO:82); and

Del-*fadE*-R 5' AAACGGAGCCT TTCGGCTCCGTTATT CATTTACGCGGCTTCAAC TTTCCTG TAGGCTGGAGCTGCTTC (SEQ ID NO:83)

The Del-*fadE*-F and Del-*fadE*-R primers were used to amplify the kanamycin resistance (Km^R) cassette from plasmid pKD13 (Datsenko *et al.*, *supra*) by PCR. The PCR product was then used to transform electrocompetent *E. coli* MG1655 cells containing plasmid pKD46, which expresses Red recombinase (Datsenko *et al.*, *supra*), which had been previously induced with arabinose for 3-4 hours. Following a 3-hour outgrowth in SOC medium at 37°C, the cells were plated on Luria agar plates containing 50 μg/mL of kanamycin. Resistant colonies were identified and isolated after an overnight incubation at 37°C. Disruption of the *fadE* gene was confirmed in some of the colonies by PCR amplification using primers *fadE*-L2 and *fadE*-R1, which were designed to flank the *E.coli fadE* gene.

fadE-L2 5'-CGGGCAGGTGCTATGACCAGGAC (SEQ ID NO:84); and

fadE-R1 5'-CGCGGCGTTGACCGGCAGCCTGG (SEQ ID NO:85)

After the *fadE* deletion was confirmed, a single colony was used to remove the Km^R marker using the pCP20 plasmid (Datsenko *et al.*, *supra*). The resulting MG1655 *E.coli* strain with the *fadE* gene deleted and the Km^R marker removed was designated *E.coli* MG1655 *ΔfadE*, or strain "D1".

E.coli MG1655 *ΔfadE ΔtonA* (Strain "DV2")

This example describes the construction of a recombinant microbial cell in which the expression of a fatty acid degradation enzyme and the expression of an outer membrane protein receptor are attenuated. The *tonA* (also known as *fhuA*) gene of *E.coli* MG1655 , which encodes a ferrichrome outer membrane transporter which also acts as a bacteriophage receptor (GenBank

Accession No. NP_414692) was deleted from strain D1 (described above) using the Red system according to Datsenko *et al.*, *supra*, with the following modifications:

The primers used to create the *tonA* deletion were:

Del-*tonA*-F 5'-ATCATTCTCGTTTACGTTATCATTCACTTTACATCAGAGATATAC CAATGATTCCGGGGATCCGTCGACC (SEQ ID NO:86); and

Del-*tonA*-R 5'-GCACGGAAATCCGTGCCCCAAAAGAGAAATTAGAAACGGAAG GTTGCGG TTGTAGGCTGGAGCTGCTTC (SEQ ID NO:87)

The Del-*tonA*-F and Del-*tonA*-R primers were used to amplify the kanamycin resistance (Km^R) cassette from plasmid pKD13 by PCR. The PCR product obtained in this way was used to transform electrocompetent *E. coli* MG1655 D1 cells containing pKD46 (Datsenko *et al.*, *supra)*, which cells had been previously induced with arabinose for 3-4 hours. Following a 3-hour outgrowth in SOC medium at 37°C, cells were plated on Luria agar plates containing 50 µg/mL of kanamycin. Resistant colonies were identified and isolated after an overnight incubation at 37°C. Disruption of the *tonA* gene was confirmed in some of the colonies by PCR amplification using primers flanking the *E.coli tonA* gene: *tonA*-verF and *tonA*-verR:.

tonA-verF 5'-CAACAGCAACCTGCTCAGCAA (SEQ ID NO:88); and

tonA-verR 5'-AAGCTGGAGCAGCAAAGCGTT (SEQ ID NO:89)

After the *tonA* deletion was confirmed, a single colony was used to remove the Km^R marker using the pCP20 plasmid (Datsenko *et al., supra*). The resulting MG1655 *E.coli* strain having *fadE* and *tonA* gene deletions was designated *E. coli* MG1655 Δ*fadE* Δ*tonA*, or strain "DV2".

E. coli MG1655 *ΔfadE ΔtonA lacI:tesA* (Strain "DV2 'tesA")

This example describes the construction of a recombinant microbial cell comprising a polynucleotide encoding a polypeptide having a fatty acid derivative enzyme activity. The *tesA* polynucleotide sequence encoding *E.coli* acyl-CoA thioesterase I (EC 3.1.1.5, 3.1.2.- ; *e.g.,* GenBank Accession AAC73596; SEQ ID NO:64) was modified to remove the leader sequence, such that the resulting *'tesA* gene product was truncated by 25 amino acids and the amino acid at the original position 26, alanine, was replaced with methionine, which then became the first amino acid of the 'TesA polypeptide sequence (SEQ ID NO:65; Cho *et al.*, *J. Biol. Chem.*, 270:4216-4219 (1995)).

An integration cassette containing the *'tesA* coding sequence operatively linked to the P_{Trc} promoter plus a kanamycin resistance gene was PCR- amplified from plasmid pACYC-P_{Trc}-tesA (Example 1, below) using the primers

lacI-forward: GGCTGGCTGGCATAAATATCTC (SEQ ID NO:90) and lacZ-reverse: GCGTTAAAGTTGTTCTGCTTCATCAGCAGGATATCCTGCACCATCGTCTGGATTTTGAACT TTTGCTTTGCCACGGAAC (SEQ ID NO:91), electroporated into strain DV2 and integrated into the chromosome using Red recombinase expressed from the pKD46 plasmid (Datsenko *et al., supra*).

The transformants were selected on LB plates supplemented with kanamycin. Correct integration was assessed using diagnostic PCR.

pDG2 expression vector

The pDG2 expression vector was the base plasmid for many of the constructs described below. The pCDFDuet-1 vector (Novagen/EMD Biosciences) carries the CloDF13 replicon, *lacI* gene and streptomycin/spectinomycin resistance gene (*aadA*). To construct the pDG2 plasmid, the C-terminal portion of the *plsX* gene, which contains an internal promoter for the downstream *fabH* gene (Podkovyrov and Larson, *Nucl. Acids Res.* (1996) 24 (9): 1747-1752 (1996)) was amplified from *E. coli* MG1655 genomic DNA using primers

5'- TGAATTCCATGGCGCAACTCACTCTTCTTTTAGTCG-3' (SEQ ID NO:92) and

5'- CAGTACCTCGAGTCTTCGTATACATATGCGCT CAGTCAC-3' (SEQ ID NO:93)

These primers introduced *NcoI* and *XhoI* restriction sites near the ends, as well as an internal *NdeI* site.

Both the *plsX* insert (containing the *EcfabH* promoter), and the pCDFDuet-1 vector, were digested with restriction enzymes *NcoI* and *XhoI*. The cut vector was treated with Antarctic phosphatase. The insert was ligated into the vector and transformed into transformation-competent *E. coli* cells. Clones were screened by DNA sequencing. The pDG2 plasmid sequence is provided herein as SEQ ID NO:73.

FabH expression plasmids

The pDG6 plasmid, expressing *B. subtilis* FabH1, was constructed using the pDG2 plasmid. The *fabH1* coding sequence was amplified from *Bacillus subtilis* strain 168 using primers

5'- CCTTGGGGCATATGAAAGCTG-3' (SEQ ID NO:94) and

5'- TTTAGTCATCTCGAGTGCACCTCACCTTT-3' (SEQ ID NO:95). These primers introduced *NdeI* and *XhoI* restriction sites at the ends of the amplification product.

Both the *fabH1* insert and the pDG2 vector were digested with restriction enzymes *NdeI* and *XhoI*. The cut vector was treated with Antarctic phosphatase. The insert was ligated into the vector and transformed into transformation-competent *E. coli* cells. Clones were screened by DNA sequencing. The pDG6 plasmid sequence is provided herein as SEQ ID NO:74, and expresses the *B. subtilis* FabH1 polypeptide (SEQ ID NO:2) under the control of the *EcfabH* promoter.

Other plasmids based on pDG2 were prepared using a similar strategy as employed for the pDG6 plasmid. Plasmid pDG7 comprises a *Bacillus subtilis fabH2* coding sequence which expresses the *B. subtilis* FabH2 polypeptide (SEQ ID NO:3). Plasmid pDG8 comprises a *Streptomyces coelicolor fabH* coding sequence which expresses the *S. coelicolor* FabH polypeptide (SEQ ID NO:4).

pACYC-P_{Trc}-tesA and pACYC-P_{Trc2}-tesA plasmids

Plasmid pACYC-P_{Trc} was constructed by PCR-amplifying the $lacI^q$, P_{Trc} promoter and terminator region from pTrcHis2A (Invitrogen, Carlsbad, CA) using primers

pTrc_F TTTCGCGAGGCCGGCCCCGCCAACACCCGCTGACG (SEQ ID NO:96) and

pTrc_R AAGGACGTCTTAATTAATCAGGAGAGCGTTCACCGACAA (SEQ ID NO:97)

The PCR product was then digested with *AatII* and *NruI* and inserted into plasmid pACYC177 (Rose, R.E., *Nucleic Acids Res.*, 16:356 (1988)) digested with *AatII* and *ScaI*. The nucleotide sequence of the pACYC-P_{Trc} vector is provided herein as SEQ ID NO: 75.

To generate the pACYC-P_{Trc2} plasmid, a single point mutation was introduced in the P_{Trc} promoter of the pACYC-P_{Trc} plasmid to generate the variant promoter P_{Trc2} and the pACYC-P_{Trc2} plasmid. The wild-type P_{Trc} promoter sequence is provided herein as SEQ ID NO:76, and the P_{Trc2} variant promoter is provided herein as SEQ ID NO:77.

The nucleotide sequence encoding *E.coli* acyl-CoA thioesterase I (TesA, EC 3.1.1.5, 3.1.2.- ; *e.g.,* GenBank Accession AAC73596; SEQ ID NO:64) was modified to remove the leader sequence, such that the resulting '*tesA* gene product was truncated by 25 amino acids and the amino acid at the original position 26, alanine, was replaced with methionine, which then became the first amino acid of the 'TesA polypeptide (SEQ ID NO:65; Cho *et al.*, *J. Biol. Chem.*, 270:4216-4219 (1995)). DNA encoding the 'TesA polypeptide was inserted into the *NcoI* and *EcoRI* sites of the pACYC-P_{Trc} vector and the pACYC-P_{Trc2} vector, producing the pACYC-P_{Trc}-'*tesA* and pACYC-P_{Trc2}-'*tesA* plasmids, respectively. Correct insertion of '*tesA* sequence into the plasmids was confirmed by restriction digestion.

pOP80 plasmid

The pOP80 plasmid was constructed by digesting the cloning vector pCL1920 (GenBank AB236930; Lerner C.G. and Inouye M., *Nucleic Acids Res.* 18:4631 (1990)) with the restriction enzymes *AflII* and *SfoI*. Three DNA fragments were produced by this digestion. The 3737 bp fragment was gel-purified using a gel-purification kit (Qiagen, Inc., Valencia, CA). In parallel, a DNA sequence fragment containing the P_{Trc} promoter and *lacI* region from the commercial plasmid pTrcHis2 (Invitrogen, Carlsbad, CA) was amplified by PCR using primers LF302 (5'-atatgacgtcGGCATCCGCTTACAGACA-3', SEQ ID NO:98) and LF303 (5'-aattcttaagTCAGGAGAGCGTTCACCGACAA-3', SEQ ID NO:99) introducing the recognition sites for the *ZraI* and *AflII* enzymes, respectively. After amplification, the PCR products were purified using a PCR-purification kit (Qiagen, Inc. Valencia, CA) and digested with *ZraI* and *AflII* following the recommendations of the supplier (New England BioLabs Inc., Ipswich, MA). After digestion, the PCR product was gel-purified and ligated with the 3737 bp DNA sequence fragment derived from pCL1920 to generate the expression vector pOP80 containing the P_{Trc} promoter.

L. monocytogenes fabH1 and fabH2 plasmids (pTB.079 and pTB.081)

The genomic DNA of *Listeria monocytogenes* Li23 (ATCC 19114D-5) was used as template to amplify the *fabH* gene using the following primers:

TREE044 (fabH_forward) GAGGAATAAACCATGAACGCAGGAATTTTAGGAGTAG (SEQ ID NO:100); primer 61 (fabH_reverse)

CCCAAGCTTCGAATTCTTACTTACCCCAACGAATGATTAGG (SEQ ID NO:101)

The PCR product was then cloned into the NcoI/ EcoRI sites of pDS80 (a pCL1920-based vector carrying the phage lambda P_L promoter; SEQ ID NO:78) and transformed into transformation-competent *E. coli* cells. Individual colonies were picked for sequence verification of cloned inserts. The nucleic acid sequence of wild type *L. monocytogenes fabH* encodes the wild type LmFabH1 protein (SEQ ID NO:7), and the plasmid expressing this sequence was designated pTB.079.

A mutant *L. monocytogenes fabH* gene was discovered containing a T to G change at position 928, resulting in a change in the expressed protein at amino acid position 310 from Tryptophan (W) to Glycine (G) , *i.e.*, a W310G variant. The mutant *L. monocytogenes fabH* gene encoding the FabH W310G variant (SEQ ID NO:8) was designated LmFabH2, and the plasmid expressing this sequence pTB.081.

pOP80-based *fabH* expression plasmids

Each gene was PCR amplified from the indicated template and primers (Table 6). The native sequence versions of each gene were used, except forPffabH in which the *E. coli* codon-optimized sequence was used (SEQ ID NO:150). Genes PffabH(opt), DpfabH1, and DpfabH2 were synthesized by DNA2.0 (Menlo Park, CA). The cloning vector was also PCR amplified with primers PTrc_vector_F and PTrc_vector_R (Table 7), using plasmid OP80 as a template. The different fabH genes were then cloned into the PCR-amplified OP80 vector backbone using InFusion cloning (Clontech, Mountain View CA). The standard protocol, as outlined by the manufacturer, was followed. All constructs were verified by sequencing.

Table 6. FabH genes, primers and templates

Gene	Forward PCR primer	Reverse PCR primer	Template	Construct Name
BsfabH1	BsfabH1_IFF	BsfabH1_IFR	*B. subtilis* genomic DNA	pCL-*BsH1*
BsfabH2	BsfabH2_IFF	BsfabH2_IFR	*B. subtilis* genomic DNA	pCL-*BsH2*
LmfabH1	LmfabH1-2_IFF	LmfabH1_IFR	pTB.079	pCL-*LmH*
LmfabH2	LmfabH1-2_IFF	LmfabH2_IFR	pTB.081	pCL-*LmH2*
SmfabH	SmfabH_IFF	SmfabH_IFR	*S.maltophila* genomic DNA	pCL-*SmH*
PffabH(opt)	PffabHopt_IFF	PffabHopt_IFR	synthetic	pCL-*PfH* (opt)
AafabH	AafabH_IFF	AafabH_IFR	pSN-21	pCL-*AaH*

Gene	Forward PCR primer	Reverse PCR primer	Template	Construct Name
DpfabH1	DpfabH1_IFF	DpfabH1_IFR	synthetic	pCL-*DpH1*
DpfabH2	DpfabH2_IFF	DpfabH2_IFR	synthetic	pCL-*DpH2*

Table 7. FabH primer sequences

Primer	Sequence (5' → 3')	SEQ ID NO
PTrc_vector_F	GAATTCGAAGCTTGGGCCCGAAC	151
PTrc_vector_R	CATGGTTTATTCCTCCTTATTTAATCGATAC	152
BsfabH1_IFF	GAGGAATAAACCATGAAAGCTGGAATACTTGGTGTTGGAC	153
BsfabH1_IFR	CCAAGCTTCGAATTCttaTCGGCCCCAGCGGATTGC	154
BsfabH2_IFF	GAGGAATAAACCATGTCAAAAGCAAAAATTACAGCTATCGGC	155
BsfabH2_IFR	CCAAGCTTCGAATTCttaCATCCCCCATTTAATAAGCAATCCTG	156
LmfabH1-2_IFF	GAGGAATAAACCATGAACGCAGGAATTTTAGGAGTAGG	157
LmfabH1_IFR	CCAAGCTTCGAATTCttaCTTACCCCAACGAATGATTAGGGC	158
LmfabH2_IFR	CCAAGCTTCGAATTCttaCTTACCCCCACGAATGATTAGGG	159
DpfabH1_IFF	GAGGAATAAACCATGaatagagcagttatcttgggaacc	160
DpfabH1_IFR	CCAAGCTTCGAATTCttaccaacgcatgagcagcgaacc	161
DpfabH2_IFF	GAGGAATAAACCATGactttgcgttacacccaggtc	162
DpfabH2_IFR	CCAAGCTTCGAATTCttaccagtcgatgcccagcatg	163
AafabH_IFF	GAGGAATAAACCATGTACAAGGCCGTGATTCGCG	164
AafabH_IFR	CCAAGCTTCGAATTCtcaATACTCCACCATCGCGCC	165
PffabHopt_IFF	GAGGAATAAACCATGATTGATAGCACACCGGAATGG	166
PffabHopt_IFR	CCAAGCTTCGAATTCttaCGGCAGAACAACAACACGACC	167
SmfabH_IFF	GAGGAATAAACCATGAGCAAGCGGATCTATTCGAGG	168
SmfabH_IFR	CCAAGCTTCGAATTCtcaATAGCGCAGCAGGGCCG	169

Example 2. Engineering *E. coli* for Production of Odd Chain Fatty Acids by Pathway (A)

The following example describes the construction of recombinant *E. coli* strains which express exogenous genes and/or overexpress endogenous genes encoding enzymes which serve to increase metabolic flux through the intermediates threonine and α-ketobutyrate to propionyl-CoA by pathway (A) of Fig. 2, leading to the increased production of odd chain acyl-ACPs and odd chain fatty acid derivatives in these recombinant cells.

This example also demonstrates the effect on oc-FA production of attenuating the expression of an endogenous gene and replacing it with an exogenous gene; in this example, expression of the endogenous *E. coli fabH* gene encoding β-ketoacyl-ACP synthase was attenuated by deletion of the gene, and β-ketoacyl-ACP synthase activity was supplied by expression of the exogenous *B. subtilis fabH1* gene.

DV2 P_L-*thrA*BC*

A recombinant *E. coli* strain was constructed in which chromosomal genes involved in threonine biosynthesis were placed under control of a strong chromosomally-integrated lambda P_L promoter, and one of the genes was mutated.

To introduce a single mutation in the native aspartokinase I (*thrA*) gene, the gene was amplified from *E.coli* MG1655 DNA in two parts. The first part was amplified using primers TREE026 and TREE028 while the second part was amplified using TREE029 and TREE030 (Table 6). The primers used to amplify the two components contained overlapping sequences which were then used to "stitch" the individual pieces together. The two PCR products were combined in a single PCR reaction and primers TREE026 and TREE030 to amplify the entire *thrA* gene. Primers TREE028 and TREE029 were designed to create a mutation in the native *thrA* at codon 345, which resulted in an S345F variant of aspartokinase I (SEQ ID NO: 21). This mutation has been shown to eliminate feedback inhibition of the enzyme by threonine in the host strain (Ogawa-Miyata,Y., *et al.*, *Biosci. Biotechnol. Biochem.* 65:1149-1154 (2001); Lee J.-H., *et al.*, *J. Bacteriol.* 185: 5442-5451 (2003)). The modified version of this gene was designated "*thrA**".

The P_L promoter was amplified using primers Km_trc_overF and TREE027 (Table 8) using plasmid pDS80 (a pCL1920-based vector carrying the phage lambda P_L promoter; SEQ ID NO:78) as a template. This fragment was then stitched to a kanamycin resistance cassette flanked by FRT sites, which was amplified from plasmid pKD13 using primers TREE025 and Km_trc_overR (Table 8). The resulting PCR product containing the KmFRT cassette and P_L promoter was stitched to the thrA* PCR product. Primers TREE025 and TREE030 were used to amplify the entire KmFRT-P_L-*thrA** mutagenic cassette. These primers also contain approximately 50 bp of homology to the integration site at the 5' end and the entire *thrA* gene as homology on the 3' end, targeting the cassette to the native *thrA* site in *E. coli*, which is part of an operon comprising the *thrA*, *thrB* and *thrC* genes. This mutagenic cassette was electroporated into the parental strain, *E.coli* DV2 (Example 1) containing the helper plasmid pKD46 expressing Red recombinase (Datsenko *et al.*, *supra*). Clones containing the chromosomal integration were selected in the presence of kanamycin, and verified by diagnostic PCR. The kanamycin marker was then removed by expression of the pCP20 plasmid (Datsenko *et al.*, *supra*). Proper integration and marker removal were verified by PCR and sequencing. The resulting strain , in which the mutant *thrA** gene and the endogenous *thrB* and *thrC* genes were overexpressed by the chromosomally-integrated lambda P_L promoter, was designated DV2 P_L *thrA*BC*.

Table 8 : Primers

Primer	Sequence (5' → 3')	SEQ ID NO
TREE025	CCTGACAGTGCGGGCTTTTTTTTTCGACCAAAGGTAACGAGGTA ACAACCGTGTAGGCTGGAGCTGCTTCG	102
TREE026	GTATATATTAATGTATCGATTAAATAAGGAGGAATAAACCATGC GAGTGTTGAAGTTCGGCG	103
TREE027	CTGATGTACCGCCGAACTTCAACACTCGCATGGTTTATTCCTCCT TATTTAATCGATAC	104
TREE028	GCGCCCGTATTTTCGTGGTGCTGATTAC	105
TREE029	GTAATCAGCACCACGTAAATACGGGCGC	106
TREE030	TCAGACTCCTAACTTCCATGAGAGG	107
Km_trc_ overR	AATATTTGCCAGAACCGTTATGATGTCGGCATTCCGGGGATCCGT CGACC	108
Km_trc_ overF	CTTCGAACTGCAGGTCGACGGATCCCCGGAATGCCGACATCATA ACGGTTCTGGC	109
EG238	GCTGATCATTAACTATCCGCTGGATGACC	110
TREE017	ACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTAAG	111
TREE018	TCACTGCCCGCTTTCC	112
TREE019	ACCGGCAGATCGTATGTAATATGCATGGTTTATTCCTCCTTATTT AATCGATACA	113
TREE020	ATGCATATTACATACGATCTGCC	114
TREE021	GGTCGACGGATCCCCGGAATTAAGCGTCAACGAAACCG	115
TREE022	GAAGCAGCTCCAGCCTACACCAGACGATGGTGCAGGAT	116
TREE023	GCAAAGACCAGACCGTTCATA	117
Kan/Chlor 1	ATTCCGGGGATCCGTCGACC	118
Kan/Chlor 4	TGTAGGCTGGAGCTGCTTCG	119

DV2 P_L *thrA*BC* P_L *tdcB*

The native *E.coli* catabolic threonine deaminase (*tdcB*) gene (also known as threonine ammonia-lyase) was overexpressed by integrating an extra copy of the gene into the *lacZ* locus and placing it under the control of a strong chromosomally-integrated lambda P_L promoter.

Catabolic threonine deaminase catalyzes the degradation of threonine to α-keto-butyrate, the first reaction of the threonine degradation / isoleucine production pathway. The reaction catalyzed likely involves initial elimination of water (hence the earlier classification of this enzyme as a threonine dehydratase), followed by isomerization and hydrolysis of the product with C-N bond breakage. Increased expression of this gene has been shown to dramatically increase levels of isoleucine in heterologous organisms (Guillouet S. *et al.*, *Appl. Environ. Microbiol.* 65:3100-3107 (1999)). Furthermore, threonine deaminase is relatively resistant to isoleucine feedback mechanisms (Guillouet *et al., supra*).

E.coli MG1655 genomic DNA was amplified using primers TREE020 and TREE021 (Table 8) to obtain the native *tdcB* gene. At the same time, primers Kan/Chlor 1 and Kan/Chlor 4 (Table 8) were used to amplify an FRT-Kanamycin resistance cassette to be used for integration selection/screening as previously described. Using *E.coli* MG1655 genomic DNA as template, primers EG238 and TREE018 (Table 8) were used to amplify a region of homology 3' to the lacZ integration site, while primers TREE022 and TREE023 (Table 8) were used to amplify a region of homology 5' to the lacZ site. The plasmid pDS80 (a pCL1920-based vector carrying the phage lambda P_L promoter; SEQ ID NO:78) was used as a template to amplify a fragment containing the P_L promoter by using primers TREE017 and TREE018 (Table 8). Each of these fragments were designed with overlaps for corresponding adjacent piece and were stitched together using SOEing PCR techniques. The resulting P_L *tdcB* mutagenic cassette (approx. 4.3kb) contained approximately 700bp of homology to the integration site at the 5' end and 750bp of homology to the integration site at the 3' end. The P_L *tdcB* mutagenic cassette was electroporated into the host strain, *E.coli* DV2 P_L *thrA*BC* (above) containing the helper plasmid, pKD46 (Datsenko *et al.*, *supra*). Clones containing the chromosomal integration were selected for in the presence of kanamycin, and verified by PCR and sequencing analysis. The kanamycin marker was then removed using the pCP22 plasmid (Datsenko *et al.*, *supra*). The resulting strain was designated DV2 P_L *thrA*BC* P_L *tdcB*. The strain was transformed with the plasmid pACYC-p_{trc2}-'*tesA* (Example 1), which expressed a truncated form of *E. coli tesA*.

The strain was also transformed with plasmid pDG6 (Example 1) expressing the *B. subtilis* FabH1 enzyme. Fermentation experiments were conducted, and the titers of free fatty acids (FFA), odd chain fatty acids (oc-FA), and the fraction of FFA produced as oc-FA were determined, as shown in Example 5 and Table 11. Alternatively, the strain can be transformed with a plasmid expressing a different FabH polypeptide, such as, for example, pDG7 expressing *B. subtilis* FabH2, pDG8 expressing *Streptomyces coelicolor* FabH, pTB.079 expressing *Listeria monocytogenes* FabH, pTB.081 expressing a *Listeria monocytogenes* FabH W310G variant, or a FabH plasmid described in Example 5 and Tables 12A-12C. Fermentation experiments are conducted, and the titers of free fatty acids (FFA), odd chain fatty acids (oc-FA), and the fraction of FFA produced as oc-FA are determined.

DV2 P_L-*thrA*BC* P_{T5}-*BsfabH1*

A recombinant *E. coli* strain was constructed in which the *B. subtilis fabH1* gene was integrated into the chromosome and placed under transcriptional control of the strong constitutive T5 promoter.

First, a PCR product was generated for the chromosomal integration of a loxPcat integration cassette comprising a chloramphenicol resistance gene, a T5 promoter (P_{T5}), and *BsfabH1* coding sequence, at the site of the *fadE* deletion scar of DV2 P_L *thrA*BC*. The individual components of the

integration cassette were first PCR-amplified. The loxP-cat-loxP P_{T5} component was amplified from plasmid p100.38 (SEQ ID NO:79) using primers TREE133 and TREE135 (Table 9). The *BsfabH1* gene was amplified from a plasmid carrying the *BsfabH1* gene using primers TREE134 and TREE136. Primers TREE133 and TREE136 contain the 5' and 3' 50 bp of homology sequence for integration. The primers used to amplify the components contain overlapping sequence which were then used to "stitch" the individual pieces together. The loxP-cat-P_{T5} and *BsfabH1* PCR products were stitched together by combining both pieces in a single PCR reaction and using primers TREE133 and TREE136 to amplify the final loxPcat-P_{T5}-BsfabH1 integration cassette.

Table 9: Primers

Primer Name	Sequence	SEQ ID NO
TREE133	AAAAACAGCAACAATGTGAGCTTTGTTGTAATTATATTGTAAACATAT TGTCCGCTGTTTCTGCATTCTTACgt	120
TREE134	GATGACGACGAACACGCATTaagGAGGTGAATAAGGAGGAATAAcatAT GAAAGCTGGCATTCTTGGTGTTG	121
TREE135	GTAACGTCCAACACCAAGAATGCCAGCTTTCATatgTTATTCCTC CTTATTCACCTCcttAATGCGTGTTCG	122
TREE136	AAACGGAGCCTTTCGGCTCCGTTATTCATTTACGCGGCTTCAAC TTTCCGTTATCGGCCCCAGCGGATTG	123
TREE137	CGCAGTTTGCAAGTGACGGTATATAACCGAAAAGTGACTGAGC GTACatgATTCCGGGGATCCGTCGACC	124
TREE138	GCAAATTGCGTCATGTTTTAATCCTTATCCTAGAAACGAACCAG CGCGGATGTAGGCTGGAGCTGCTTCG	125
TREE139	GCAGCGACAAGTTCCTCAGC	126
TREE140	CCGCAGAAGCTTCAGCAAACG	127
fadE-L2	CGGGCAGGTGCTATGACCAGGAC	128
fadE-R2	GGGCAGGATAAGCTCGGGAGG	129

The loxP-cat-P_{T5}-*BsfabH1* cassette was integrated using the Red recombinase system (Datsenko, *et al.*, *supra*). The loxP-cat-P_{T5}-*BsfabH1* PCR product was used to transform electrocompetent DV2 P_L-*thrA*BC* cells containing plasmid pKD46, which had been previously induced with arabinose for 3 – 4 hours at 30°C. Following a 3 hour 37°C outgrowth in SOC medium, cells were plated on Luria agar plates containing 17µg/mL chloramphenicol and incubated overnight at 37°C. Chloramphenicol-resistant colonies were screened by PCR for proper integration of loxP-cat-P_{T5}-*BsfabH1*. Primers fadE-L2 and fadE-R2 (Table 9) which flank the chromosomal integration site, were used to confirm the integration. Upon verification of integration, the chloramphenicol marker gene was removed by expressing a Cre recombinase which promotes recombination between the two loxP sites that flank the chloramphenicol resistance gene. The plasmid pJW168, which harbors the *cre* recombinase gene, was transformed into strain DV2 P_L-*thrA*BC* loxP-cat-P_{T5}-*BsfabH1* and the marker was removed according to the method described by Palmeros *et al.* (*Gene*

247:255-264 (2000)). The resulting strain DV2 P_L-*thrA*BC* P_{T5}-*BsfabH1* was verified by sequencing.

DV2 P_L-*thrA*BC* P_{T5}-*BsfabH1* ΔEcfabH

A recombinant *E. coli* strain was constructed in which the expression of an endogenous gene (in this instance, the *fabH* gene of *E. coli*) was attenuated by deletion of that gene.

The *fabH* gene of *E.coli* was deleted from DV2 P_L-*thrA*BC* P_{T5}-*BsfabH1* using the Red recombinase system (Datsenko *et al.*, *supra*). Primers TREE137 and TREE138 (Table 9), were used to amplify the kanamycin resistance cassette from plasmid pKD13 by PCR. The PCR product was then used to transform electrocompetent DV2 P_L-*thrA*BC* P_{T5}-*BsfabH1* cells containing plasmid pKD46. Deletion of *EcfabH* and removal of the kanamycin marker were carried out according to the method described by Wanner and Datsenko, *supra*. Primers TREE139 and TREE140 were used to confirm the deletion of *EcfabH*. The final markerless strain was designated DV2 P_L-*thrA*BC* P_{T5}-*BsfabH1* Δ*EcfabH*.

DV2 P_L-*thrA*BC* P_L-*tdcB* P_{T5}-*BsfabH1* ΔEcfabH

A recombinant *E. coli* strain was constructed containing chromosomally-integrated genes overexpressing enzymes of pathway (A) and step (D) of the oc-FA biosynthetic pathway shown in Fig. 2 and Fig. 1B, respectively. The P_L-*tdcB* mutagenic cassette (prepared as described above) was integrated into strain DV2 P_L-*thrA*BC* P_{T5}-*BsfabH1* Δ*EcfabH* to generate the strain DV2 P_L-*thrA*BC* P_L-*tdcB* P_{T5}-*BsfabH1* Δ*EcfabH*. In this strain, the integrated *E. coli thrA*BC* genes and the integrated *E. coli tdcB* gene were both under the control of strong lambda P_L promoters, the integrated *B. subtilis fabH1* gene was under the control of the strong T5 promoter, and the endogenous *E. coli fabH* gene was deleted. Fermentation experiments were conducted, and the results are provided in Table 11.

Example 3. Engineering *E. coli* for Production of Odd Chain Fatty Acids by Pathway (B)

The following example describes the construction of recombinant *E. coli* strains which express exogenous genes and/or overexpress endogenous genes encoding enzymes which serve to increase metabolic flux through the intermediates citramalate and α-ketobutyrate to propionyl-CoA by pathway (B) of Fig. 2, leading to the increased production of odd chain acyl-ACPs and odd chain fatty acid derivatives in these recombinant cells.

DV2 P_{Trc}-*cimA3.7 leuBCD*

To prepare an *E. coli* strain overexpressing endogenous *leuBCD* genes and an exogenous *cimA3.7* gene, a PCR product was generated for the chromosomal integration of a KmFRT cassette, a P_{Trc} promoter, and *cimA3.7* between the endogenous chromosomal *E. coli leuA* and *leuB* genes. This integration disrupted the native *leuABCD* operon, placing *cimA3.7* and *leuBCD* in an operon under control of the strong IPTG-inducible promoter, P_{Trc}.

DNA encoding CimA3.7 was synthesized by Geneart AG (Regensburg, Germany). The DNA was cloned into the *SfiI* site of plasmid pMK-RQ (kanR) (Geneart AG, Regensburg, Germany). Flanking the coding sequence, a 5' *KpnI* restriction site and a 3' *SacI* restriction site were introduced directly upstream of the ATG start codon and immediately downstream of the TAA stop codon respectively. The cimA 3.7 cloning vector was verified by sequencing.

The individual components of the integration cassette were PCR-amplified as follows. The KmFRT component was amplified from plasmid pKD13 using primers TREE146 and Km_trc_overR (Table 10). The P_{Trc} promoter was amplified from pOP80 (Example 1) using primers Km_trc_overF and TREE033.

The *cimA3.7* coding sequence was amplified from the cimA 3.7 cloning vector described above using primers TREE032 and TREE035. To provide the 3' homology sequence for integration, *E. coli* native *leuBC* genes were amplified using *E. coli* genomic DNA and primers TREE034 and TREE104. The forward primer TREE146, which was used to amplify the KmFRT cassette, included the 5' 50bp of homology sequence for integration. Each of the primers used to amplify the components contained overlapping sequence which were used to "stitch" the individual pieces together. First, KmFRT and P_{Trc} were stitched together by combining both pieces in a single PCR reaction and using primers TREE146 and TREE033 to amplify the KmFRT-P_{Trc} product. KmFRT-P_{Trc} was then stitched with *cimA3.7* using primers TREE146 and TREE035 to generate KmFRT-P_{Trc}-*cimA3.7*. The final piece, *leuBC* was stitched to KmFRT-P_{Trc}-*cimA3.7* using primers TREE146 and TREE104 to generate the final integration cassette: KmFRT-P_{Trc}-*cimA3.7 leuBC*.

Table 10: Primers

Primer Name	Primer Sequence (5' → 3')	SEQ ID NO
Km_trc_overF	CTTCGAACTGCAGGTCGACGGATCCCCGGAATGCCGACATCATAACGGTTCTGGC	130
Km_trc_overR	AATATTTGCCAGAACCGTTATGATGTCGGCATTCCGGGGATCCGTCGACC	131
TREE032	GTATATATTAATGTATCGATTAAATAAGGAGGAATAAACCatgatggtaaggatatttgatacaacac	132
TREE033	ctaagtgttgtatcaaatatccttaccatcatGGTTTATTCCTCCTTATTTAATCGATAC	133
TREE034	gatttgttggctatagttagagaagttactggaaaattgTAACAAGGAAACCGTGTGATGTCGAAG	134
TREE035	GTAATTCTTCGACATCACACGGTTTCCTTGTTAcaattttccagtaacttctctaactatag	135
TREE104	GGTAGCGAAGGTTTTGCCCGGC	136
TREE106	GATTGGTGCCCCAGGTGACCTG	137
TREE146	GAGTTGCAACGCAAAGCTCAACACAACGAAAACAACAAGGAAACCGTGTGaGTGTAGGCTGGAGCTGCTTCG	138
TREE151	CTTCCACGGCGTCGGCCTG	139

The KmFRT-P_{Trc}-*cimA3.7 leuBC* cassette was integrated into the *E. coli* genome using the Red recombinase system (Datsenko *et al.*, *supra*). The KmFRT-P_{Trc}-*cimA3.7 leuBC* PCR product was used to transform electrocompetent *E. coli* MG1655 DV2 cells containing plasmid pKD46, which had been previously induced with arabinose for 3 – 4 hours at 30°C. Following a 3-hour 37°C outgrowth in SOC medium, cells were plated on Luria agar plates containing 50μg/mL kanamycin and incubated overnight at 37°C. Kanamycin-resistant colonies were screened by PCR for proper integration of KmFRT-P_{Trc}-*cimA3.7*. Primers TREE151 and TREE106, which flank the chromosomal integration site, were used to confirm the integration. Upon verification of integration, the kanamycin marker gene was removed in accordance with the method described by Datsenko *et al.*, *supra*. Successful integration of P_{Trc}-*cimA3.7* and removal of the kanamycin marker gene in the final strain, DV2 P_{Trc} *cimA3.7 leuBCD*, was verified by sequencing.

The strain was transformed with the plasmid pACYC-p_{trc2}-tesA, which expressed a truncated form of *E. coli tesA*, and, in some instances, pDG6, which expressed *B. subtilis fabH1*. Fermentation experiments were conducted, and the titers of free fatty acids (FFA), odd chain fatty acids (oc-FA), and the fraction of FFA produced as oc-FA, are provided in **Table 11**.

Example 4. Engineering *E. coli* for Production of Odd Chain Fatty Acids by Pathways (A) and (B) Combined

The following example describes the construction of recombinant *E. coli* strains which express exogenous genes and/or overexpress endogenous genes encoding enzymes which serve to increase metabolic flux through the common intermediate α-ketobutyrate to propionyl-CoA by the combined (A) and (B) pathways of Fig. 2, leading to even greater production of oc-acyl-ACPs and odd chain fatty acids in these recombinant cells.

DV2 P_L-*thrA*BC* P_{Trc}-*cimA3.7_leuBCD* P_{T5}-*BsfabH1* Δ*EcfabH* (strain "G1")

To begin combining pathways (A) and (B) of Fig. 2, the P_{Trc}-*cimA3.7_leuBCD* cassette (Example 5) was integrated into strain DV2 P_L-*thrA*BC* P_{T5}-*BsfabH1* Δ*EcfabH* (Example 4) to generate the strain DV2 P_L-*thrA*BC* P_{Trc}-*cimA3.7_leuBCD* P_{T5}-*BsfabH1* Δ*EcfabH*, which was also called strain G1. This strain overexpressed polypeptides having (R)-citramalate synthase activity, isopropylmalate isomerase activity, and beta-isopropyl malate dehydrogenase activity according to pathway (B) of the oc-FA pathway, and overexpressed polypeptides having aspartokinase activity, homoserine dehydrogenase activity, homoserine kinase activity, and threonine synthase activity according to pathway (A) of the oc-FA pathway (Fig. 2).

DV2 P_L-*thrA*BC* P_L-*tdcB* P_{Trc}-*cimA3.7_leuBCD* P_{T5}-*BsfabH1* Δ*EcfabH* (strain "G2")

To create a strain engineered to overexpress polypeptides having activities corresponding to the combined pathways (A) and (B) of the of the oc-FA pathway, the P_L-*tdcB* cassette (Example 4) was integrated into strain G1, to generate strain DV2 P_L-*thrA*BC* P_L-*tdcB* P_{Trc}-*cimA3.7_leuBCD* P_{T5}-

BsfabH1 ΔEcfabH, which was also called strain G2. In this strain, the integrated *E. coli thrA*BC* genes and the integrated *E. coli tdcB* gene (encoding polypeptides having aspartokinase activity, homoserine dehydrogenase activity, homoserine kinase activity, threonine synthase activity, and threonine deaminase activity, corresponding to pathway (A)) were placed under the control of strong lambda P_L promoters, and were overexpressed. The exogenous *cimA3.7* gene and the native *E. coli leuBCD* genes (encoding polypeptides having (R)-citramalate synthase activity , isopropylmalate isomerase activity, and beta-isopropyl malate dehydrogenase activity corresponding pathway (B)), were also integrated into the *E. coli* chromosome under control of the strong IPTG-inducible promoter P_{Trc} and therefore were also overexpressed. The integrated *B. subtilis fabH1* gene, encoding a branched chain beta ketoacyl-ACP synthase corresponding to part (D) of the oc-FA pathway (Fig. 1B), was under the control of the strong T5 promoter. The endogenous *E. coli fabH* gene was deleted from this strain.

Example 5. Evaluation of Odd Chain Fatty Acid Production

The following example demonstrates the production of linear odd-chain fatty acids in *E. coli* strains engineered to express exogenous genes and/or overexpress endogenous genes encoding enzymes which increase metabolic flux through the common α-ketobutyrate intermediate to produce propionyl-CoA, by way of either the threonine-dependent pathway (pathway (A) of Fig. 2) or via the citramalate pathway (pathway (B) of Fig. 2). Propionyl-CoA, which serves as a "primer" molecule for odd-chain fatty acid production, then condenses with malonyl-ACP by the action of β-ketoacyl-ACP synthase III (FabH) to form the odd-chain β-ketoacyl-ACP intermediate which enters the fatty acid synthase cycle to produce odd-chain fatty acids and oc-FA derivatives. Accordingly, this example also demonstrates the effect of exogenous FabH enzymes on odd-chain fatty acid production.

In the first set of experiments, strains were evaluated for free fatty acid (FFA) production by performing a 96 deep-well plate fermentation using the 4N-BT protocol. Single colonies or a scraping from a glycerol stock were used to inoculate 300 µL of LB + antibiotic(s). LB seed cultures were grown for 6 – 8 hours at 37°C with shaking at 250 rpm until turbid. 20 µL of the LB cultures were used to inoculate 400 µL of 2N-BT. These were allowed to grow overnight at 32°C with shaking at 250 rpm. The following morning, 20 µL of 2N-BT culture was transferred to 400 µL of 4N-BT. The 4N-BT cultures were allowed to grow for 6 hours at 32°C with shaking at 250 rpm at which point, cells were induced with 1 mM IPTG. Upon induction, cultures were allowed to grow for an additional 16 – 18 hours before being extracted and analyzed for FFA production. 40 µL of 1M HCl was added to each well, followed by 400µL of butyl acetate spiked with 500 mg/L C24 alkane internal standard. Cells were extracted by vortexing for 15 minutes at 2000 rpm. Extracts were derivatized with an equal volume of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) before being analyzed by GC/MS.

Table 11: Production of Odd Chain Fatty Acids in Recombinant *E. coli* Strains

	Strain	*fabH*	*tesA*	Total FFA titer	oc-FA titer	oc-FA / Total FFA
1	DV2	*Ec*	p	2054	6	< 0.01
2	DV2 *thrA*BC tdcB*	*Ec*	p	1364	246	0.18
3	DV2 *thrA*BC tdcB*	*Ec pBsH1*	p	1460	545	0.37
4	DV2 *thrA*BC tdcB*	*ΔEc IntBsH1*	p	1148	832	0.72
5	DV2 *cimA3.7 leuBCD*	*Ec*	p	1617	73	0.04
6	DV2 *cimA3.7 leuBCD*	*Ec pBsH1*	p	1650	214	0.13
7	"G1": DV2 *thrA*BC cimA3.7 leuBCD*	*ΔEc IntBsH1*	p	1104	286	0.26
8	G1 / Tn7-tesA	*ΔEc IntBsH1*	int	885	267	0.30
9	"G2": DV2 *thrA*BC tdcB cimA3.7 leuBCD*	*ΔEc IntBsH1*	p	617	551	0.89
10	G2 / Tn7-tesA	*ΔEc IntBsH1*	int	923	840	0.91

all titers are in milligrams per liter (mg/L)
FFA = free fatty acid (oc-FA + ec-FA)
oc-FA = odd chain fatty acid; ec-FA = even chain fatty acid
Ec = chromosomal (native) *E. coli fabH* gene
ΔEc = deleted chromosomal *E. coli fabH* gene
pBsH1 = plasmid-expressed *BsfabH1* (pDG6 plasmid)
IntBsH1 = chromosomally integrated *BsfabH1*
p = plasmid-expressed *'tesA* gene (pACYC-p_{Trc2}-tesA)
int = chromosomally integrated *'tesA* gene

The odd chain fatty acids produced in these experiments generally included C13:0, C15:0, C17:0 and C17:1 fatty acids, with C15:0 being the predominant oc-FA produced.

Comparison of strains 1 and 2 in Table 11 demonstrates that microbial cells overexpressing genes involved in the biosynthesis and degradation of threonine, which increased metabolic flux through the pathway intermediate α-ketobutyrate, significantly increased the proportion of odd chain length fatty acids produced by the cells. While the parental DV2 strain produced straight chain fatty acids with only a negligible amount of odd chain length fatty acids, the DV2 strain overexpressing the *thrA*BC* and *tdcB* genes (encoding polypeptides having aspartokinase activity, homoserine dehydrogenase activity, homoserine kinase activity, threonine synthase activity and threonine deaminase activity) produced a significantly greater amount and significantly greater proportion of

odd chain length fatty acids; about 18% (by weight) of the straight chain fatty acids produced were odd chain length fatty acids.

Strains 2 and 3 demonstrate the effect on oc-FA production by including an exogenous β-ketoacyl ACP synthase with high specificity towards propionyl-CoA. Strain 2 contained the native (endogenous) *E. coli fabH* gene. By introducing a plasmid expressing the *B. subtilis fabH1* gene, oc-FA production was markedly increased from about 18% (in Strain 2) to about 37% of the straight chain fatty acids produced (in Strain 3).

A striking effect on oc-FA production was observed when the endogenous *E. coli fabH* gene was deleted and the *B. subtilis fabH1* gene was chromosomally integrated. In Strain 4, the proportion of oc-FA increased to 72% of the straight chain fatty acids produced.

Strains 5 and 6 demonstrate that increasing metabolic flux through α-ketobutyrate by another approach, this time by a pathway involving citramalate biosynthesis and degradation , also increased the proportion of odd chain length fatty acids produced. Engineering the DV2 strain to overexpress the *cimA3.7* and *leuBCD* genes (encoding polypeptides having (R)-citramalate synthase activity , isopropylmalate isomerase activity, and β-isopropylmalate dehydrogenase activity) resulted in about 4% of the straight chain fatty acids produced having odd chain lengths, which increased to about 13% when plasmid-expressed *B. subtilis fabH1* was included.

Strains 7 and 9 show the effect of combining the threonine and citramalate pathways on oc-FA production. In strain G1, in which the *thrA*BC, cimA3.7* and *leuBCD* genes were overexpressed, the endogenous *E. coli fabH* gene was deleted and the *B. subtilis fabH1* gene was chromosomally integrated, about 26% of the straight chain fatty acids produced were odd chain fatty acids. In strain G2, in which the *thrA*BC, tdcB, cimA3.7 and leuBCD* genes were overexpressed, the endogenous *E. coli fabH* gene was deleted and the *B. subtilis fabH1* gene was chromosomally integrated, nearly 90% of the straight chain fatty acids produced were odd chain fatty acids. Strains G1/Tn7-tesA and G /Tn7-tesA (strains 8 and 10, respectively), in which the '*tesA* gene was chromosomally integrated at the Tn7 attachment site, showed amounts and proportions of oc-FA similar to those in strains G1 and G2 (strains 7 and 9, respectively) in which the '*tesA* gene was plasmid-expressed.

In the second set of experiments, the role of propionyl-CoA production and the effect of FabH enzymes on oc-FA production was examined. In these experiments, exogenous *fabH* coding sequences were cloned into the pOP80 expression vector (Example 1), where expression was controlled by the strong P_{Trc} promoter. The *fabH* expression constructs (or, in strains lacking exogenous *fabH*, the pOP80 vector alone) were transformed, along with '*tesA* plasmid pACYC-PTrc2-*tesA*, into the following strains:

- DV2
- DV2 *cimA3.7_leuBCD* (increased propionyl-CoA via the citramalate pathway (B) of Fig. 2)
- DV2 *thrA*BC tdcB* (increased propionyl-CoA via the thr-dependent pathway (A) of Fig. 2)

Single colonies or a scraping from a frozen glycerol stock were used to inoculate 300µL of LB + antibiotic(s). LB seed cultures were grown for 6 - 8 hours at 37°C with shaking at 250rpm until turbid. 20µL of the LB cultures were used to inoculate 400µL of 2N-BT media. These were allowed to grow overnight at 32°C with shaking at 250rpm, at least 14 hours. The following morning, 20µL of 2N-BT culture was transferred to 400µL of 4N-BT. The 4N-BT cultures were allowed to grow for 6 hours at 32°C with shaking at 250rpm at which point, cells were induced with 1mM IPTG. Upon induction, cultures were allowed to grow for an additional 20 - 22 hours before being extracted and analyzed for free fatty acid (FFA) production. 40µL of 1M HCl was added to each well, followed by 400µL of butyl acetate. Cells were extracted by vortexing for 15 minutes at 2000rpm. Extracts were derivatized with an equal volume of N,O - bis(trimethylsilyl) trifluoroacetamide (BSTFA) before being analyzed by GC coupled with a flame ionization detector (GC-FID).

Ratios of odd-chain fatty acids relative to total free fatty acids produced by strains expressing various *fabH* genes are presented in Tables 12A-C below. Odd chain fatty acid ratios produced in the control DV2 strain are presented in Table 12A, while odd chain fatty acid ratios in strains engineered for increased metabolic flux to propionyl-CoA by way of either the citramalate pathway (pathway (B) of Fig. 2) or the threonine-dependent pathway (pathway (A) of Fig. 2) are shown in Tables 12B and 12C, respectively.

Table 12A: Production of Odd Chain Fatty Acids in Recombinant *E. coli* Strains

	fabH	**Strain**	**Total FFA titer**	**oc-FA titer**	**oc-FA / Total FFA**
1 a	*Ec*	DV2	2488	23	< 0.01
2 a	*Ec pCL-BsH1*	DV2	193	7	0.04
3 a	*Ec pCL-BsH2*	DV2	314	8	0.03
4 a	*Ec pCL-LmH*	DV2	571	24	0.04
5 a	*Ec pCL-LmH2*	DV2	2501	29	0.01
6 a	*Ec pCL-PfH(opt)*	DV2	2132	47	0.02
7 a	*Ec pCL-SmH*	DV2	806	31	0.04
8 a	*Ec pCL-AaH*	DV2	569	22	0.04
9 a	*Ec pCL-DpH1*	DV2	323	7	0.02
10 a	*Ec pCL-DpH2*	DV2	2381	20	< 0.01

Table 12B: Production of Odd Chain Fatty Acids in Recombinant *E. coli* Strains with Increased Flux to Propionyl-CoA via the Citramalate Pathway (B) of Fig. 2.

	fabH	Strain	Total FFA titer	oc-FA titer	oc-FA / Total FFA
1 b	*Ec*	DV2 *cimA3.7 leuBCD*	1994	106	0.05
2 b	*Ec pCL-BsH1*	DV2 *cimA3.7 leuBCD*	189	17	0.09
3 b	*Ec pCL-BsH2*	DV2 *cimA3.7 leuBCD*	268	14	0.05
4 b	*Ec pCL-LmH*	DV2 *cimA3.7 leuBCD*	826	90	0.04
5 b	*Ec pCL-LmH2*	DV2 *cimA3.7 leuBCD*	641	89	0.14
6 b	*Ec pCL-PfH(opt)*	DV2 *cimA3.7 leuBCD*	2135	211	0.10
7 b	*Ec pCL-SmH*	DV2 *cimA3.7 leuBCD*	2054	240	0.12
8 b	*Ec pCL-AaH*	DV2 *cimA3.7 leuBCD*	618	93	0.15
9 b	*Ec pCL-DpH1*	DV2 *cimA3.7 leuBCD*	222	18	0.08
10 b	*Ec pCL-DpH2*	DV2 *cimA3.7 leuBCD*	2033	101	0.05

Table 12C: Production of Odd Chain Fatty Acids in Recombinant *E. coli* Strains with Increased Flux to Propionyl-CoA via the Threonine-Dependent Pathway (A) of Fig. 2.

	fabH	Strain	Total FFA titer	oc-FA titer	oc-FA / Total FFA
1 c	*Ec*	DV2 *thrA*BC tdcB*	1871	376	0.20
2 c	*Ec pCL-BsH1*	DV2 *thrA*BC tdcB*	500	132	0.26
3 c	*Ec pCL-BsH2*	DV2 *thrA*BC tdcB*	236	39	0.17
4 c	*Ec pCL-LmH*	DV2 *thrA*BC tdcB*	560	151	0.27
5 c	*Ec pCL-LmH2*	DV2 *thrA*BC tdcB*	1968	622	0.32
6 c	*Ec pCL-PfH(opt)*	DV2 *thrA*BC tdcB*	1708	404	0.23
7 c	*Ec pCL-SmH*	DV2 *thrA*BC tdcB*	471	131	0.28
8 c	*Ec pCL-AaH*	DV2 *thrA*BC tdcB*	528	137	0.26
9 c	*Ec pCL-DpH1*	DV2 *thrA*BC tdcB*	240	45	0.19
10 c	*Ec pCL-DpH2*	DV2 *thrA*BC tdcB*	1614	434	0.27

all titers are in milligrams per liter (mg/L)
all strains also contained plasmid-expressed *'tesA* (pACYC-p_{Trc2}-tesA)
FFA = free fatty acid (oc-FA + ec-FA)
oc-FA = odd chain fatty acid; ec-FA = even chain fatty acid
Ec = chromosomal (native) *E. coli fabH* gene
pCL-BsH1 = pOP80-expressed *Bacillus subtilis fabH1*
pCL-BsH2 = pOP80-expressed *Bacillus subtilis fabH2*
pCL-LmH = pOP80-expressed *Listeria monocytogenes fabH*
pCL-LmH2 = pOP80-expressed *Listeria monocytogenes fabH2*
pCL-PfH(opt) = pOP80-expressed *Propionibacterium freudenreichii fabH(codon-optimized)*
pCL-SmH = pOP80-expressed *Stenotrophomonas maltophila fabH*
pCL-AaH = pOP80-expressed *Alicyclobacillus acidocaldarius fabH*
pCL-DpH1 = pOP80-expressed *Desulfobulbus propionicus fabH1*
pCL-DpH2 = pOP80-expressed *Desulfobulbus propionicus fabH2*

All of the strains depicted in Tables 12A-12C expressed the endogenous *E. coli fabH* gene. Strains 2-10 each contained in addition a plasmid-expressed exogenous *fabH* gene. It was shown in Table 11 (above) that deletion of the endogenous *E. coli fabH* gene and chromosomal integration of the exogenous *B. subtilis fabH1* gene produced a larger amount and greater proportion of oc-FA (Table 11, strain 4) compared to the strain containing endogenous *E. coli fabH* plus plasmid-expressed exogenous *B. subtilis fabH1* (Table 11, strain 3). Nevertheless, the results presented in Tables 12A-12C demonstrate that (a) propionyl-CoA is a necessary precursor for recombinant linear odd chain fatty acid production in bacteria, since all of the *fabH*-expressing strains tested exhibited significant linear oc-fatty acid production in the strains engineered for elevated α-ketobutyrate and propionyl-CoA levels -- DV2 *cimA3.7 leuBCD* (Table 12B) and DV2 *thrA*BC tdcB* (Table 12C) -- but no significant oc-fatty acid production was observed in the DV2 control strains (Table 12A), and (b) recombinant linear oc-fatty acid production occurs in the presence of a variety of heterologous FabH enzymes isolated from organisms whose membranes contain branched chain fatty acids and/or odd chain fatty acids. Such FabH enzymes are capable of utilizing the propionyl-CoA molecule in the priming reaction for fatty acid biosynthesis and confer odd-chain fatty acid biosynthetic capablities to the recombinant microorganism.

In conclusion, this Example demonstrates that a microoganism which normally produces even-chain fatty acids can be engineered to produce odd-chain fatty acids by increasing metabolic flux through propionyl-CoA and expressing a β-ketoacyl synthase (FabH) enzyme that utilizes propionyl-CoA. Example 6 (below) demonstrates an alternative pathway than can be engineered to increase metabolic flux through propionyl-CoA. Recombinant microorganisms engineered to produce odd-chain fatty acids can be further modified to produce odd-chain fatty acid derivatives, such as odd-chain fatty alcohols (Example 7) and even-chain alkanes (Example 8).

Example 6: Engineering *E. coli* for Production of Odd Chain Fatty Acids by Pathway (C)

The following example describes the construction of recombinant *E. coli* strains which express exogenous genes and/or overexpress endogenous genes encoding enzymes which serve to increase metabolic flux through the intermediate methylmalonyl-CoA to produce propionyl-CoA by pathway (C) of Fig. 3, leading to the increased production of odd chain acyl-ACPs and odd chain fatty acid derivatives in these recombinant cells. In particular, this example describes production of odd chain fatty acids in an *E. coli* strain which overexpresses endogenous methylmalonyl-CoA mutase (*scpA/sbm)* and methylmalonyl-CoA decarboxylase (*scpB/ygfG*) genes on a plasmid and the chromosomal propionyl-CoA:succinyl-CoA transferase (*scpC/ygfH*) and *scpB/ygfG* genes are deleted.

E. coli strain DV2, plasmid pDG6 (expressing *B. subtilis* FabH1), and plasmid pACYC-p_{Trc2}-tesA (expressing the truncated 'TesA polypeptide) were prepared as described in Example 1.

Plasmid pACYC-P_{Trc}-*sbm-ygfG*

Plasmid pACYC-P_{Trc}-*sbm-ygfG* is the pACYC-P_{Trc} plasmid (Example 1), which overexpresses *E. coli sbm* encoding methylmalonyl-CoA mutase and *E. coli ygfG* encoding methylmalonyl-CoA decarboxylase. The sequence of pACYC-P_{Trc}-*sbm-ygfG* is provided herein as SEQ ID NO:80

Strain sDF4

Strain sDF4 is *E. coli* strain DV2 from which the chromosomal *scpB* and *scpC* genes were deleted, the native *frd* promoter replaced with the *trc* promoter, and the *'tesA* gene was chromosomally integrated at the Tn7 attachment site.

To integrate the *'tesA* gene, a P_{Trc}-*'tesA* integration cassette was first prepared by amplifying the pACYC-P_{Trc}-*'tesA* plasmid (Example 1) using the following primers:

IFF: 5'- GGGTCAATAGCGGCCGCCAATTCGCGCGCGAAGGCG (SEQ ID NO:140)

IFR: 5'- TGGCGCGCCTCCTAGGGCATTACGCTGACTTGACGGG (SEQ ID NO:141)

The integration cassette was inserted into the *NotI* and *AvrII* restriction sites of pGRG25 (GenBank Accession No. DQ460223) creating the Tn7tes plasmid (SEQ ID NO: 81), in which the *lacIq,* P_{Trc}-*'tesA* cassette is flanked by the left and right Tn7 ends.

To prepare strain sDF4, plasmid Tn7tes was first electroporated into *E. coli* strain DV2 (Example 1) using a protocol described by McKenzie *et al.*, *BMC Microbiology* 6:39 (2006). After electroporation, ampicillin-resistant cells were selected by growth in an LB medium containing 0.1 % glucose and 100 μg/mL carbenicilin at 32°C overnight. This was followed by selection of plasmids comprising the Tn7-transposition fractions, using the growth of cells on an LB plus 0.1% arabinose plates overnight at 32°C. Single colonies were selected and streaked onto new LB medium plates with and without ampicillin, and they were grown overnight at 42°C to cure of Tn7tes plasmid. Thus, the *lacIq,* P_{Trc}-*'tesA* was integrated into the attTn7 site on the *E.coli* chromosome located between the

pstS and *glmS* genes. Integration of these genes was confirmed by PCR and sequencing. The resulting strain was designated DV2 Tn7-*tesA*.

To delete the *scpBC* genes from DV2 Tn7-*tesA*, the following two primers were used : ScpBC-KOfwd 5'- GCTCAGTGAATTTATCCAGACGCAATATTTTGATTAAAGGA ATTTT TATGATTCCG GGGATCCGTCGACC (SEQ ID NO:142); and ScpBC-KOrc 5'- ATTGCTGAAGATCGTGACGGGACGAGTCATTAACCCAGCATCGAGCCGGTTGT AGGCTG GAGCTGCTTC (SEQ ID NO:143)

The ScpBC-KOfwd and ScpBC-KOrc primers were used to amplify the kanamycin resistance (Km^R) cassette from plasmid pKD13 (Datsenko *et al.*, *supra*) by PCR. The PCR product was then used to transform electrocompetent *E. coli* DV2 Tn7-tesA cells containing plasmid pKD46, which expresses Red recombinase (Datsenko *et al.*, *supra*) which had been previously induced with arabinose for 3-4 hours. Following a 3-hour outgrowth in SOC medium at 37°C, the cells were plated on Luria agar plates containing 50 µg/mL of kanamycin. Resistant colonies were identified and isolated after an overnight incubation at 37°C. Disruption of the *scpBC* genes was confirmed by PCR amplification using the following primers designed to flank the chromosomal *scpBC* genes:

ScpBC check -60 fwd 5' - CGGGTTCTGACTTGTAGCG (SEQ ID NO:144)

ScpBC check +60 rc 5'- CCAACTTCGAAGCAATGATTGATG (SEQ ID NO:145)

After the *scpBC* deletion was confirmed, a single colony was picked and used to remove the Km^R marker using the pCP20 plasmid (Datsenko *et al.*, *supra*). The native fumarate reductase (*frd*) promoter was replaced with the PTrc promoter using a modification of the procedure of Datsenko *et al.* (*supra*). The resulting *E. coli* DV2 Δ*scpBC*::FRT, Δ*Pfrd*::FRT-PTrc, attTn7::PTrc-'*tesA* strain was designated "sDF4".

Strains were transformed with plasmids as indicated below and evaluated for fatty acid production using the 96 deep-well plate fermentation procedure described in Example 5; since ScpA is a B-12 dependent enzyme, the 4N-BT culture media was supplemented with cobalamin.

Table 13: Production of Odd Chain Fatty Acids in Recombinant *E. coli* Strains

	Strain	***fabH***	***tesA***	**Total FFA**	**oc-FA**	**oc-FA / total FFA**
1	DV2 pACYC-*PTrc2-'tesA*	*Ec*	p	2054	6	< 0.01
2	sDF4 pACYC-*PTrc-sbm-ygfG*	*Ec*	int	973	39	0.04
3	sDF4 pACYC-*PTrc-sbm-ygfG* pDG6	*Ec pBsH1*	int	863	140	0.16

all titers are in milligrams per liter (mg/L)
FFA = free fatty acid (oc-FA + ec-FA)
oc-FA = odd chain fatty acid; ec-FA = even chain fatty acid
Ec = chromosomal *E. coli fabH* gene ; *pBsH1* = plasmid-expressed *BsfabH1* (pDG6)
p = plasmid-expressed *'tesA* gene (pACYC-p_{Trc2}-tesA);

int = chromosomally integrated '*tesA* gene

Microbial cells overexpressing genes involved in the production of propionyl-CoA via the intermediates succinyl-CoA and methylmalonyl-CoA increased the proportion of odd chain length fatty acids produced by the cells. While the DV2 strain (strain 1 of Table 13) produced only a negligible amount of odd chain length fatty acids, the sDF4 strain overexpressing the endogenous *E. coli sbm* and *ygfG* genes (encoding polypeptides having methylmalonyl-CoA mutase activity and methylmalonyl-CoA decarboxylase activity) produced an increased amount of odd chain length fatty acids.

Strains 2 and 3 of Table 13 demonstrate the effect on oc-FA production by including an exogenous β-ketoacyl ACP synthase with high specificity towards propionyl-CoA. Strain 2 contained the native *E. coli fabH* gene. By introducing a plasmid expressing the *B. subtilis fabH1* gene, oc-FA production further increased from about 4% of the fatty acids produced in Strain 2 to about 16% of the fatty acids produced in Strain 3 .

Example 7: Production of Odd Chain Fatty Alcohols in *E. coli*

The following demonstrates the production of odd chain fatty alcohols by previously-described strains, which, in this example, also expressed a polypeptide having acyl-ACP reductase (AAR) activity. The AAR activity converted the oc-acyl-ACP intermediate to oc-fatty aldehyde, which reacted with endogenous aldehyde reductase to form oc-fatty alcohol.

Strains DV2, DV2 P_L-*thrA*BC* P_L-*tdcB* P_{T5}-*BsfabH1* Δ*EcfabH*, and G1 (prepared as described in Examples 1, 2, and 4, respectively) were transformed either with plasmid pLS9185 or pDS171s. Plasmid pLS9185 expressed a *Synechococcus elongatus* fatty acyl-ACP reductase (AAR; GenBank Accession No. YP_400611). Plasmid pDS171s expressed *S. elongatus* AAR, an acyl carrier protein (ACP) from the cyanobacterium *Nostoc punctiforme* (cACP; GenBank Accession No. YP_001867863) and a phosphopantetheinyl transferase from *Bacillus subtilis* (Sfp; GenBank Accession No. YP_004206313). These strains were evaluated for fatty alcohol production using the 96 deep-well plate fermentation procedure described in Example 5.

Table 14: Production of Odd Chain Fatty Alcohols in Recombinant *E. coli* Strains

	Strain	pLS9185	pDS171s	**Total FAlc titer**	**oc-FAlc titer**	**oc-FAlc / Total FAlc**
1	DV2	x		432	23	0.05
4	DV2 *thrA*BC tdcB ΔEcFabH IntBsFabH1*	x		398	325	0.82
7	"G1": DV2 *thrA*BC cimA3.7 leuBCD ΔEcFabH IntBsFabH1*	x		420	157	0.37

	Strain	pLS9185	pDS171s	Total FAlc titer	oc-FAlc titer	oc-FAlc / Total FAlc
1	DV2		x	847	37	0.04
4	DV2 *thrA*BC tdcB ΔEcFabH IntBsFabH1*		x	906	735	0.81
7	"G1": DV2 *thrA*BC cimA3.7 leuBCD ΔEcFabH IntBsFabH1*		x	775	344	0.44

all titers are in milligrams per liter (mg/L)
FAlc = fatty alcohol (oc-FAlc + ec-FAlc)
oc-FAlc = odd chain fatty alcohol; ec-FAlc = even chain fatty alcohol
ΔEcFabH = deleted chromosomal *E. coli fabH* gene
IntBsH1 = chromosomally integrated *BsfabH1*
pLS9185 = plasmid-expressed AAR
pDS171s = plasmid-expressed AAR, cACP, and Sfp

Compared to the control strain DV2, both strains DV2 *thrA*BC tdcB BsfabH1 ΔEcfabH* and G1 produced significantly higher titers and proportions of odd chain fatty alcohols when transformed with a plasmid expressing AAR, or a plasmid expressing AAR, cACP, and Sfp (Table 14). The proportion of fatty alcohols produced as odd chain fatty alcohols roughly reflects the proportions observed when these strains were evaluated for fatty acid production (Table 11), suggesting that AAR does not show a preference for odd or even chain fatty acyl-ACPs of similar overall chain length.

Example 8: Production of Even Chain Alkanes in *E. coli*

The following example demonstrates the production of even chain alkanes by a strain which expressed a polypeptide having acyl-ACP reductase (AAR) activity and a polypeptide having aldehyde decarbonylase (ADC) activity. The AAR activity converted the oc-acyl-ACP intermediate to oc-fatty aldehyde, and the ADC activity decarbonylated the oc-fatty aldehyde to form even chain (ec-)alkane.

Strains DV2, DV2 *thrA*BC tdcB BsfabH1 ΔEcfabH*, and G1 (prepared as described in Examples 1, 2, and 4, respectively) were transformed with plasmids pLS9185 and pLS9181. Plasmid pLS9185 expressed a *Synechococcus elongatus* fatty acyl-ACP reductase (AAR; GenBank Accession No. YP_400611). Plasmid pLS9181 expressed a *Nostoc punctiforme* aldehyde decarbonylase (ADC; GenBank Accession No. YP_001865325). Strains transformed with both plasmids were analyzed for alkane production using the 96 deep-well plate fermentation procedure described in Example 5 above, but with the added supplementation of 25μM $MnSO_4$ (final concentration) at induction.

Table 15: Production of Even Chain Alkanes in Recombinant *E. coli* Strains

	Strain	AAR	ADC	Total Alk titer	ec-Alk titer	ec-Alk / Total Alk
1	DV2	x	x	432	23	0.05
4	DV2 *thrA*BC tdcB ΔEcFabH IntBsFabH1*	x	x	398	325	0.82
7	"G1": DV2 *thrA*BC cimA3.7 leuBCD ΔEcFabH IntBsFabH1*	x	x	420	157	0.37

all titers are in milligrams per liter (mg/L)
Alk = alkane (oc-Alk + ec-Alk); oc-Alk = odd chain alkane; ec-Alk = even chain alkane
ΔEcFabH = deleted chromosomal *E. coli fabH* gene
IntBsFabH1 = chromosomally integrated *BsfabH1*
AAR = plasmid-expressed *aar* gene (pLS9185)
ADC = plasmid-expressed *adc* gene (pLS9181)

Compared to the control strain DV2, both DV2 *thrA*BC tdcB BsfabH1 ΔEcfabH* and G1 produced significantly higher titers and proportions of even chain alkanes when transformed with plasmids expressing AAR and ADC (Table 15). The proportion of alkanes produced as even chain alkanes roughly reflects the proportions of odd chain products produced when these strains were evaluated for fatty acid production (Table 11) and for fatty alcohol production (Table 14), suggesting that ADC, like AAR, does not show a preference between odd or even chain substrates of comparable overall chain length.

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A recombinant microbial cell comprising:

(a) one or more polynucleotides encoding a polypeptide having enzymatic activity effective to produce an increased amount of propionyl-CoA in the recombinant microbial cell relative to the amount of propionyl-CoA produced in a parental microbial cell lacking or having a reduced amount of said enzymatic activity, wherein said polypeptide is exogenous to the recombinant microbial cell or wherein expression of said polynucleotide is modulated in the recombinant microbial cell as compared to the expression of the polynucleotide in the parental microbial cell, and wherein the one or more polynucleotides are selected from the group consisting of:

(i) a polynucleotide encoding a polypeptide having aspartokinase activity, homoserine dehydrogenase activity, homoserine kinase activity, threonine synthase activity, or threonine deaminase activity;
(ii) a polynucleotide encoding a polypeptide having (R)-citramalate synthase activity, isopropylmalate isomerase activity, or beta-isopropylmalate dehydrogenase activity; and
(iii) a polynucleotide encoding a polypeptide having methylmalonyl-CoA mutase activity, methylmalonyl-CoA decarboxylase activity or methylmalonyl-CoA carboxyltransferase activity,

(b) a polynucleotide encoding a polypeptide having β-ketoacyl-ACP synthase activity that utilizes propionyl-CoA as a substrate, and

(c) a polynucleotide encoding a polypeptide having fatty acid derivative enzyme activity,

wherein the recombinant microbial cell produces a fatty acid derivative composition comprising odd chain fatty acid derivatives when cultured in the presence of a carbon source under conditions effective to express the polynucleotides according to (a), (b), and (c), and

wherein at least 10% of the fatty acid derivatives in the fatty acid derivative composition are odd chain fatty acid derivatives.

2. The recombinant microbial cell of claim 1, wherein at least 20% of the fatty acid derivatives in the fatty acid derivative composition are odd chain fatty acid derivatives.

3. The recombinant microbial cell of claim 1, wherein the cell produces at least 100 mg/L of odd chain fatty acid derivatives.

4. The recombinant microbial cell of claim 1, wherein expression of the at least one polynucleotide according to (a) is modulated by overexpression of the polynucleotide in the recombinant microbial cell.

5. The recombinant microbial cell of claim 1, comprising one or more polynucleotides according to (i) and one or more polynucleotides according to (ii).

6. The recombinant microbial cell of claim 1, wherein the polypeptide having β-ketoacyl-ACP synthase activity that utilizes propionyl-CoA as a substrate is exogenous to the recombinant microbial cell, and expression of a polypeptide having β-ketoacyl-ACP synthase activity endogenous to the recombinant microbial cell is attenuated.

7. The recombinant microbial cell of claim 1, wherein the fatty acid derivative enzyme activity comprises thioesterase activity and the recombinant microbial cell produces a fatty acid composition comprising odd chain fatty acids,

wherein at least 10% of the fatty acids in the composition are odd chain fatty acids.

8. The recombinant microbial cell of claim 1, wherein the fatty acid derivative enzyme activity comprises ester synthase activity and the recombinant microbial cell produces a fatty ester composition comprising odd chain fatty esters,

wherein at least 10% of the fatty esters in the composition are odd chain fatty esters.

9. The recombinant microbial cell of claim 1, wherein the fatty acid derivative enzyme activity comprises fatty aldehyde biosynthesis activity and the recombinant microbial cell produces a fatty aldehyde composition comprising odd chain fatty aldehydes,

wherein at least 10% of the fatty aldehydes in the composition are odd chain fatty aldehydes.

10. The recombinant microbial cell of claim 1, wherein the fatty acid derivative enzyme activity comprises fatty alcohol biosynthesis activity and the recombinant microbial cell produces a fatty alcohol composition comprising odd chain fatty alcohols,

wherein at least 10% of the fatty alcohols in the composition are odd chain fatty alcohols.

11. The recombinant microbial cell of claim 1, wherein the fatty acid derivative enzyme activity comprises hydrocarbon biosynthesis activity and the recombinant microbial cell produces a hydrocarbon composition comprising even chain hydrocarbons,

wherein at least 10% of the hydrocarbons in the composition are even chain hydrocarbons.

12. A cell culture comprising the recombinant microbial cell of claim 1.

13. A method of making a fatty acid derivative composition comprising odd chain fatty acid derivatives, the method comprising:

obtaining the recombinant microbial cell of claim 1,

culturing the recombinant microbial cell in a culture medium containing a carbon source under conditions effective to express the polynucleotides according to (a), (b), and (c) and produce a fatty acid derivative composition comprising odd chain fatty acid derivatives wherein at least 10% of the fatty acid derivatives in the composition are odd chain fatty acid derivatives, and

optionally recovering the odd chain fatty acid derivative composition from the culture medium.

14. The method of claim 13, wherein the recombinant microbial cell expresses one or more polynucleotides encoding a polypeptide having a fatty acid derivative enzyme activity selected from the group consisting of:

(1) a polypeptide having thioesterase activity;

(2) a polypeptide having decarboxylase activity;

(3) a polypeptide having carboxylic acid reductase activity;

(4) a polypeptide having alcohol dehydrogenase activity (EC 1.1.1.1);

(5) a polypeptide having aldehyde decarbonylase activity (EC 4.1.99.5);

(6) a polypeptide having acyl-CoA reductase activity (EC 1.2.1.50);

(7) a polypeptide having acyl-ACP reductase activity;

(8) a polypeptide having ester synthase activity (EC 3.1.1.67);

(9) a polypeptide having OleA activity; and

(10) a polypeptide having OleCD or OleBCD activity;

wherein the recombinant microbial cell produces a composition comprising one or more of odd chain fatty acids, odd chain fatty esters, odd chain fatty aldehydes, odd chain fatty alcohols, even chain alkanes, even chain alkenes, even chain terminal olefins, even chain internal olefins, or even chain ketones.

15. A method of making a recombinant microbial cell which produces a higher titer or higher proportion of odd chain fatty acid derivatives than produced by a parental microbial cell, the method comprising:

transforming the parental microbial cell with

(a) one or more polynucleotides encoding a polypeptide having enzymatic activity effective to produce an increased amount of propionyl-CoA in the recombinant microbial cell compared to the amount of propionyl-CoA produced by the parental microbial cell when cultured under

the same conditions, wherein the one or more polynucleotides are selected from the group consisting of:

(i) a polynucleotide encoding a polypeptide having aspartokinase activity, homoserine dehydrogenase activity, homoserine kinase activity, threonine synthase activity, or threonine deaminase activity;
(ii) a polynucleotide encoding a polypeptide having (R)-citramalate synthase activity, isopropylmalate isomerase activity, or beta-isopropylmalate dehydrogenase activity; and
(iii) a polynucleotide encoding a polypeptide having methylmalonyl-CoA mutase activity, methylmalonyl-CoA decarboxylase activity or methylmalonyl-CoA carboxyltransferase activity,

(b) a polynucleotide encoding a polypeptide having β-ketoacyl-ACP synthase activity that utilizes propionyl-CoA as a substrate, and

(c) a polynucleotide encoding a polypeptide having fatty acid derivative enzyme activity,

to obtain the recombinant microbial cell, wherein the recombinant microbial cell produces a higher titer or higher proportion of odd chain fatty acid derivatives when cultured in the presence of a carbon source under conditions effective to express the polynucleotides, relative to the titer or proportion of odd chain fatty acid derivatives produced by the parental microbial cell cultured under the same conditions.

16. The method of claim 15, wherein expression of an endogenous polynucleotide encoding a polypeptide having β-ketoacyl-ACP synthase activity is attenuated.

17. A method of increasing the titer or the proportion of odd chain fatty acid derivatives produced by a microbial cell, the method comprising:

obtaining a parental microbial cell which produces fatty acid derivatives, and

transforming the parental microbial cell with

(a) one or more polynucleotides encoding a polypeptide having enzymatic activity effective to produce an increased amount of propionyl-CoA in the recombinant microbial cell compared to the amount of of propionyl-CoA produced by the parental microbial cell when cultured under the same conditions, wherein the one or more polynucleotides are selected from the group consisting of:

(i) a polynucleotide encoding a polypeptide having aspartokinase activity, homoserine dehydrogenase activity, homoserine kinase activity, threonine synthase activity, or threonine deaminase activity;

(ii) a polynucleotide encoding a polypeptide having (R)-citramalate synthase activity, isopropylmalate isomerase activity, or beta-isopropylmalate dehydrogenase activity; and

(iii) a polynucleotide encoding a polypeptide having methylmalonyl-CoA mutase activity, methylmalonyl-CoA decarboxylase activity or methylmalonyl-CoA carboxyltransferase activity,

(b) a polynucleotide encoding a polypeptide having β-ketoacyl-ACP synthase activity thatutilizes propionyl-CoA as a substrate, and

(c) a polynucleotide encoding a polypeptide having fatty acid derivative enzyme activity,

to obtain the recombinant microbial cell, wherein the recombinant microbial cell produces a higher titer or higher proportion of odd chain fatty acid derivatives when cultured in the presence of a carbon source under conditions effective to produce propionyl-CoA and fatty acid derivatives in the recombinant microbial cell, relative to the titer or proportion of odd chain fatty acid derivatives produced by the parental microbial cell cultured under the same conditions.

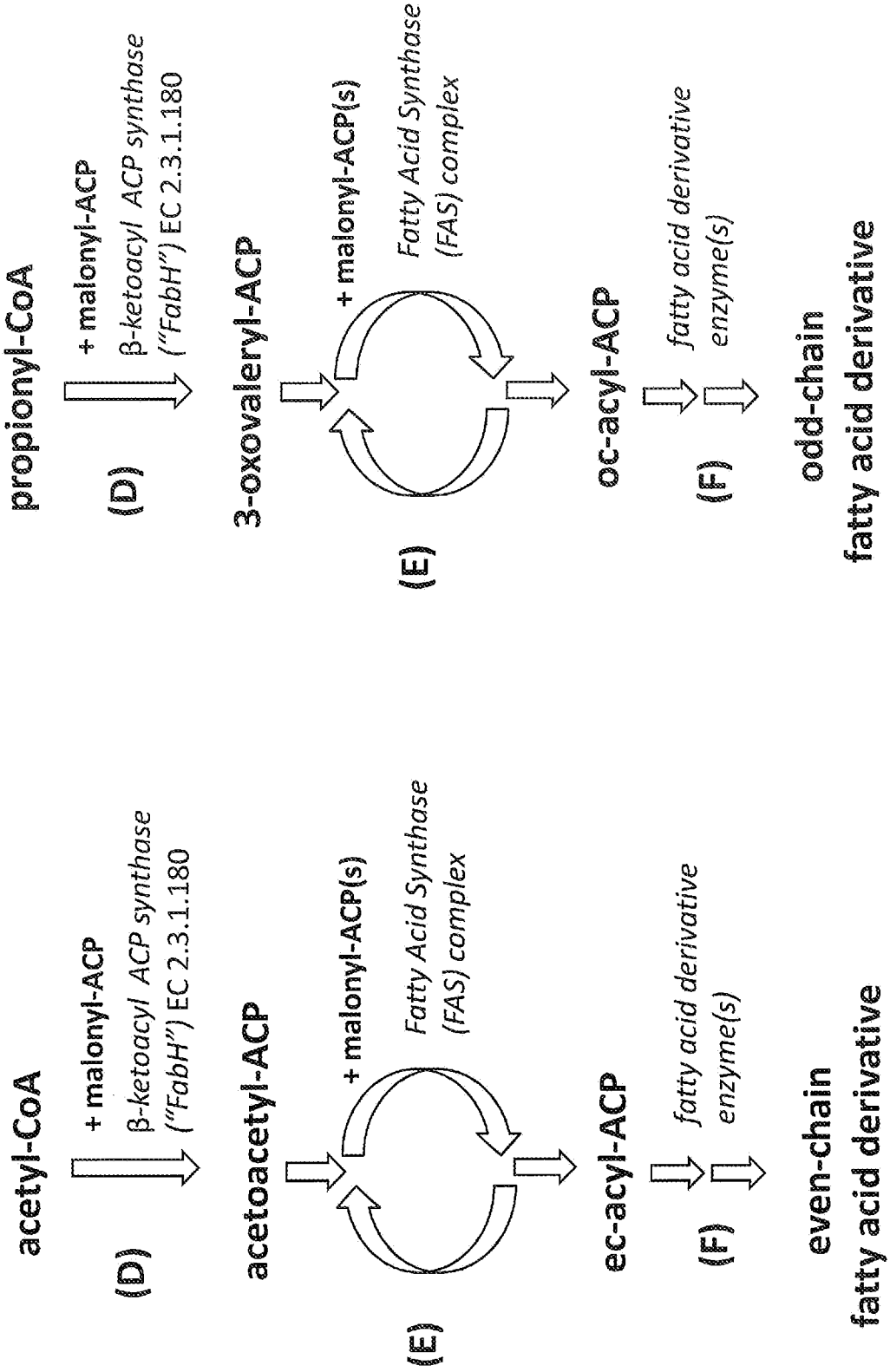
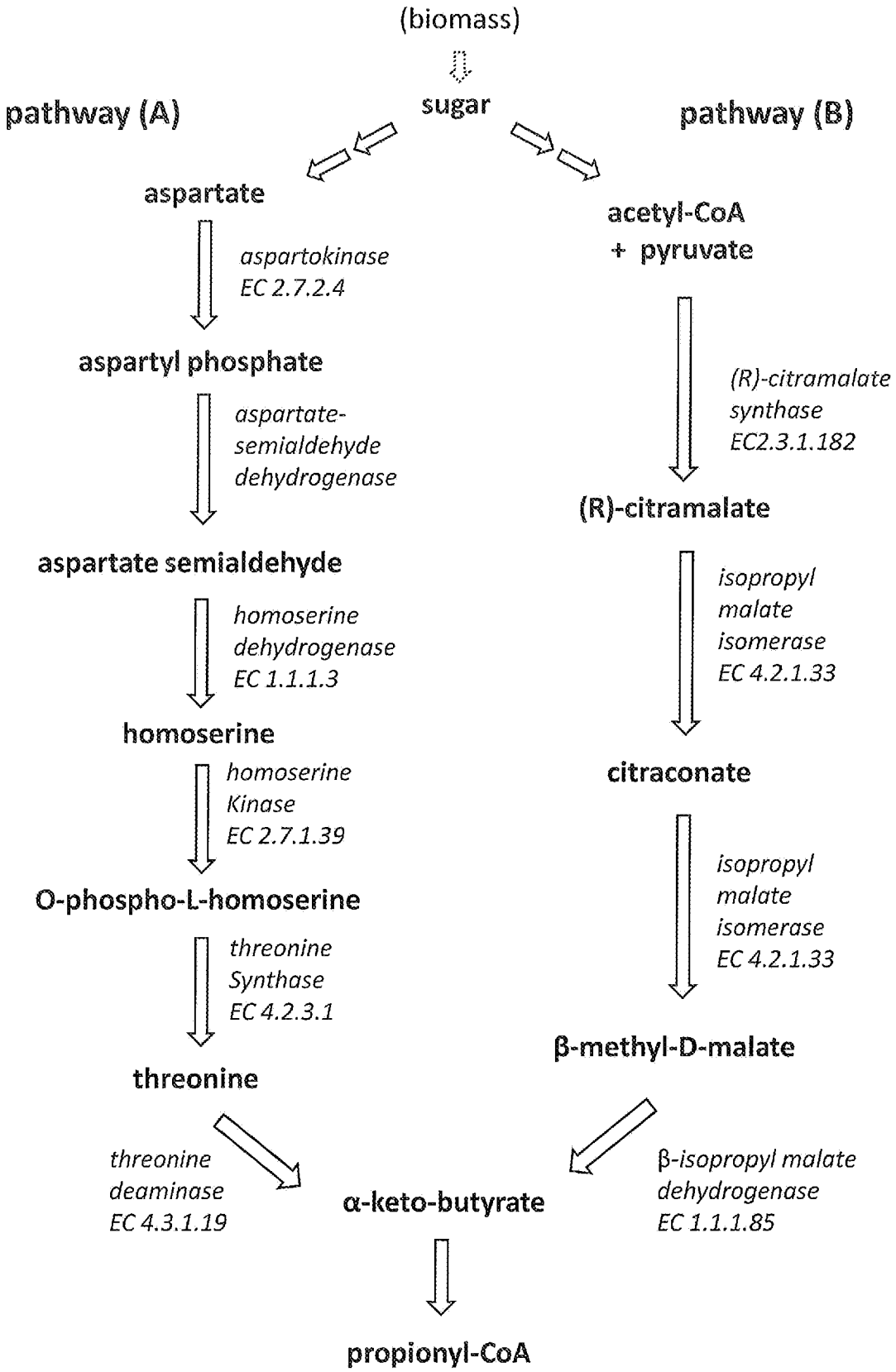

propionyl-CoA
(D)
+ malonyl-ACP
β-ketoacyl ACP synthase
("FabH") EC 2.3.1.180
3-oxovaleryl-ACP
(E)
+ malonyl-ACP(s)
Fatty Acid Synthase
(FAS) complex
oc-acyl-ACP
(F)
fatty acid derivative
enzyme(s)
odd-chain
fatty acid derivative
acetyl-CoA
(D)
+ malonyl-ACP
β-ketoacyl ACP synthase
("FabH") EC 2.3.1.180
acetoacetyl-ACP
(E)
+ malonyl-ACP(s)
Fatty Acid Synthase
(FAS) complex
ec-acyl-ACP
(F)
fatty acid derivative
enzyme(s)
even-chain
fatty acid derivative

Fig. 1A

Fig. 1B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pathway (A)
pathway (B)
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aspartokinase
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aspartyl phosphate
aspartate-
semialdehyde
dehydrogenase
aspartate semialdehyde
homoserine
dehydrogenase
EC 1.1.1.3
homoserine
homoserine
Kinase
EC 2.7.1.39
O-phospho-L-homoserine
threonine
Synthase
EC 4.2.3.1
threonine
threonine
deaminase
EC 4.3.1.19
acetyl-CoA
+ pyruvate
(R)-citramalate
synthase
EC2.3.1.182
(R)-citramalate
isopropyl
malate
isomerase
EC 4.2.1.33
citraconate
isopropyl
malate
isomerase
EC 4.2.1.33
β-methyl-D-malate
β-isopropyl malate
dehydrogenase
EC 1.1.1.85
α-keto-butyrate
propionyl-CoA

Fig. 2

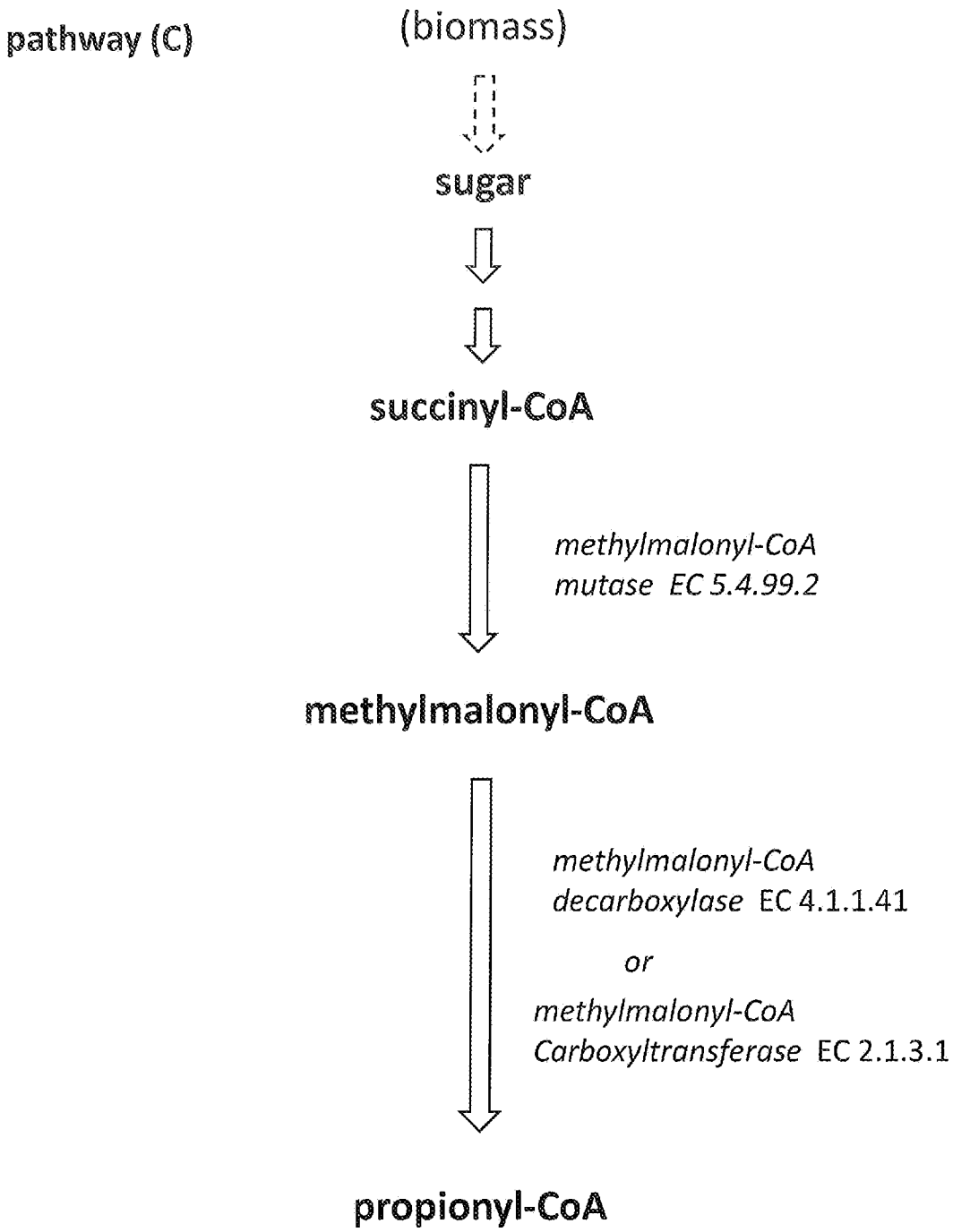
pathway (C)
(biomass)
sugar
succinyl-CoA
methylmalonyl-CoA mutase EC 5.4.99.2
methylmalonyl-CoA
methylmalonyl-CoA decarboxylase EC 4.1.1.41
or
methylmalonyl-CoA Carboxyltransferase EC 2.1.3.1
propionyl-CoA

Fig. 3