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Description

Cross-reference to Related Applications

[0001] This application claims the benefit of US Provisional Application No. 60/802,016 filed May 19, 2006, US Provisional Application No. 60/801,995 filed May 19, 2006, US Provisional Application 60/908,547 filed March 28, 2007 and PCT application number PCT/US2007/003736 filed February 13, 2007.

Field

[0002] Genetically engineered microorganisms are provided that produce products from the fatty acid biosynthetic pathway (fatty acid derivatives), as well as methods of their use.

Background

[0003] Developments in technology have been accompanied by an increased reliance on fuel sources and such fuel sources are becoming increasingly limited and difficult to acquire. With the burning of fossil fuels taking place at an unprecedented rate, it has likely that the world's fuel demand will soon outweigh the current fuel supplies.

[0004] As a result, efforts have been directed toward harnessing sources of renewable energy, such as sunlight, water, wind, and biomass. The use of biomasses to produce new sources of fuel which are not derived from petroleum sources, (i.e. biofuel) has emerged as one alternative option. Biofuel (biodiesel) is a biodegradable, clean-burning combustible fuel made of long chain alkanes and esters. Biodiesel can be used in most internal combustion diesel engines in either a pure form, which is referred to as "neat" biodiesel, or as a mix in any concentration with regular petroleum diesel. Current methods of making biodiesel involve transesterification of triacylglycerides (mainly vegetable oil) which leads to a mixture of fatty acid esters and the unwanted side product glycerin, thus, providing a product that is heterogeneous and a waste product that causes economic inefficiencies.

Summary

[0005] Disclosed herein are recombinant microorganisms that are capable of synthesizing products derived from the fatty acid biosynthetic pathway (fatty acid derivatives), and optionally releasing such products into the fermentation broth. Such fatty acid derivatives are useful, *inter alia*, as biofuels and specialty chemicals. These biofuels and specialty chemicals can be used to make additional products, such as nutritional supplements, polymers, paraffin replacements, and personal care products.

[0006] The recombinant microorganisms disclosed herein can be engineered to yield various fatty acid derivatives including, but not limited to, short chain alcohols such as ethanol, propanol isopropanol and butanol, fatty alcohols, fatty acid esters, hydrocarbons and wax esters.

[0007] In one example, the disclosure provides a method for modifying a microorganism so that it produces, and optionally releases, fatty acid derivatives generated from a renewable carbon source. Such microorganisms are genetically engineered, for example, by introducing an exogenous DNA sequence encoding one or more proteins capable of metabolizing a renewable carbon source to produce, and in some examples secrete, a fatty acid derivative. The modified microorganisms can then be used in a fermentation process to produce useful fatty acid derivatives using the renewable carbon source (biomass) as a starting material. In some examples, an existing genetically tractable microorganism is used because of the ease of engineering its pathways for controlling growth, production and reducing or eliminating side reactions that reduce biosynthetic pathway efficiencies. In addition, such modified microorganisms can be used to consume renewable carbon sources in order to generate fuels that can be directly used as biofuels, without the need for special methods for storage, or transportation. In other examples, microorganisms that naturally produce hydrocarbons are engineered to overproduce hydrocarbons by expressing exogenous nucleic acid sequences that increase fatty acid production.

[0008] Provided herein are microorganisms that produce fatty acid derivatives having defined carbon chain length, branching, and saturation levels. In particular examples, the production of homogeneous products decreases the overall cost associated with fermentation and separation. In some examples microorganisms including one or more exogenous nucleic acid sequences encoding at least one thioesterase (EC 3.1.2.14), and at least one wax synthase (EC 2.3.1.75) are provided. In other examples microorganisms are provided that include one or more exogenous nucleic acid sequences encoding at least one thioesterase (EC 3.1.2.14) and at least one alcohol acetyltransferase (2.3.1.84). In yet other examples, microorganisms including one or more exogenous nucleic acid sequences encoding at least one thioesterase (EC 3.1.2.14), at least one acyl-CoA reductase (EC 1.2.1.50) and at least one alcohol dehydrogenase (EC 1.1.1.1) are provided. Microorganisms expressing one or more exogenous nucleic acid sequences encoding at least one thioesterase

(EC 3.1.2.14) and at least one fatty alcohol forming acyl-CoA reductase (1.1.1.*) are also provided. The thioesterase peptides encoded by the exogenous nucleic acid sequences can be chosen to provide homogeneous products.

[0009] In some examples the microorganism that is engineered to produce the fatty acid derivative is *E. coli, Z. mobilis, Rhodococcus opacus, Ralstonia eutropha, Vibrio furnissii, Saccharomyces cerevisiae, Lactococcus lactis, Streptomycetes, Stenotrophomonas maltophila, Pseudomonas* or *Micrococus leuteus* and their relatives.

[0010] In other examples microorganisms that produce hydrocarbons endogenously can be engineered to overproduce hydrocarbons by optimizing the fatty acid biosynthetic pathway as described herein. Exemplary microorganisms that are known to produce hydrocarbons and can be engineered to over-produce hydrocarbons using the teachings provided herein include *Arthrobacter sp., Bacillus sp., Botryococcus braunii, Chromatium sp., Cladosporium resina* (ATCC22711), *Clostridium pasteurianum VKM, Clostridium tenanomorphum, Clostridium acidiurici, Corynebacterium species, cyanobacterial species (Nostoc muscorum, Anacystis (Synechococcus) nidulans, Phormidium luridum, Chlorogloea fritschii, Trichodesmium erythaeum, Oscillatoria williamsii, Microcoleus chthonoplaseis, Coccochloris elabens, Agmenellum quadruplicatum, Plectonema terebrans, M vaginatus,* and *C. scopulorum), Desulfovibrio desulfuricans* (ATCC29577), *Kineococcus radiotolerans* (BAA-149), *Micrococcus luteus* (FD533, ATCC 272, 381, 382, ISU, 540, 4698, 7468, 27141), *Micrococcus sp.* (ATCC 146, 398, 401, 533), *Micrococcus roseus* (ATCC 412, 416, 516), *Micrococcus lysodeikticus, Mycobacterium species, Penicillium sp., Aspergillus sp., Trichoderma virida,Pullularia pullulans, Jeotgalicoccus sp.* (*M. candicans*)(ATCC 8456), *Rhodopseudomonas spheroids Chlorobium sp., Rhodospirillium rubrum* (ATCC11170), *Rhodomicrobium vannielii, Stenotrophomonas maltophilia* (ATCC 13637, 17444, 17445, 17666, 17668, 17673, 17674, 17679, 17677), *Saccharomycodes ludwigii* (ATCC 22711), *Saccharomyces sp. (oviformus, ludwiggi, tropicalis), Vibrio furnissii* M1, *Vibrio marinus* MP-1, *Vibrio ponticus, Serratia marinorubra, Ustilago maydis, Ustilago nuda, Urocystis agropyri, Sphacelotheca reiliana,* and *Tilletia sp. (foetida, caries, controversa).*

[0011] In addition to being engineered to express exogenous nucleic acid sequences that allow for the production of fatty acid derivatives, the microorganism can additionally have one or more endogenous genes functionally deleted or attenuated. For example, *ackA* (EC 2.7.2.1), *ackB* (EC 2.7.2.1), *adhE.* (EC 1.1.1.1, 1.2.1.10), *fabF* (EC 2.3.1.179), *fabR* (accession NP_418398), *fadE* (EC 1.3.99.3, 1.3.99.-), GST (EC 6.3.2.3), *gpsA* (EC 1.1.1.94), *ldhA* (EC 1.1.1.28), *pflB* (EC 2.3.1.54), *plsB* (EC 2.3.1.15), *poxB* (EC 1.2.2.2), *pta* (EC 2.3.1.8), glutathione synthase (EC 6.3.2.3) and combinations thereofcan be attenuated.

[0012] In addition to being engineered to express exogenous nucleic acid sequences that allow for the production of fatty acid derivatives, the microorganism can additionally have one or more additional genes over-expressed. For example, *pdh, panK, aceEF* (encoding the E1p dehydrogenase component and the E2p dihydrolipoamide acyltransferase component of the pyruvate and 2-oxoglutarate dehydrogenase complexes, Accessions: NP_414656, NP_414657, EC: 1.2.4.1. 2.3.1.61, 2.3.1.12), *accABCD /fabH /fabD/fabG/acpP/fabF* (encoding FAS, Accessions: CAD85557, CAD85558, NP_842277, NP_841683, NP_415613, EC: 2.3.1.180, 2.3.1.39, 1.1.1.100, 1.6.5.3, 2.3.1.179), genes encoding fatty-acyl-coA reductases (Accessions: AAC45217, EC 1.2.1.-), *UdhA* or similar genes (encoding pyridine nucleotide transhydrogenase, Accession: CAA46822 , EC:1.6.1.1) and genes encoding fatty-acyl-coA reductases (Accessions: AAC45217, EC 1.2.1.-).

[0013] In some examples, the microorganisms described herein produce at least 1 mg of fatty acid derivative per liter fermentation broth. In other examples the microorganisms produce at least 100 mg/L, 500 mg/L, 1 g/L, 5 g/L, 10 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, 50 g/L, 100 g/L, or 120 g/L of fatty acid derivative per liter fermentation broth. In some examples, the fatty acid derivative is produced and released from the microorganism and in yet other examples the microorganism is lysed prior to separation of the product.

[0014] In some examples, the fatty acid derivative includes a carbon chain that is at least 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, or 34 carbons long. In some examples at least 50%, 60%, 70%, 80%, 85%, 90%, or 95% of the fatty acid derivative product made contains a carbon chain that is 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, or 34 carbons long. In yet other examples, at least 60%, 70%, 80%, 85%, 90%, or 95% of the fatty acid derivative product contain 1, 2, 3, 4, or 5, points of unsaturation

[0015] Also provided are methods of producing fatty acid derivatives. These methods include culturing the microorganisms described herein and separating the product from the fermentation broth.

[0016] These and other examples are described further in the following detailed description.

Brief Description of the Figures

[0017]

Fig. 1 shows the FAS biosynthetic pathway.

Fig. 2 shows biosynthetic pathways that produce waxes. Waxes can be produced in a host cell using alcohols produced within the host cell or they can be produced by adding exogenous alcohols in the medium. A microorganism designed to produce waxes will produce wax synthase enzymes (EC 2.3.1.75) using exogenous nucleic acid sequences as well as thioesterase (EC 3.1.2.14) sequences. Other enzymes that can be also modulated to increase the production of waxes include enzymes involved in fatty acid synthesis (FAS enzymes EC 2.3.1.85), acyl-CoA synthase (EC 2.3.1.86), fatty alcohol forming acyl-CoA reductase (EC 1.1.1.*), acyl-CoA reductase (1.2.1.50) and alcohol dehydrogenase (EC 1.1.1.1).

Fig. 3 shows biosynthetic pathways that produce fatty alcohols. Fatty alcohols having defined carbon chain lengths can be produced by expressing exogenous nucleic acid sequences encoding thioesterases (EC 3.1.2.14), and combinations of acyl-CoA reductases (EC 1.2.1.50), alcohol dehydrogenases (EC 1.1.1.1) and fatty alcohol forming acyl-CoA reductases (FAR, EC 1.1.1*). Other enzymes that can be also modulated to increase the production of fatty alcohols include enzymes involved in fatty acid synthesis (FAS enzymes EC 2.3.1.85), and acyl-CoA synthase (EC 2.3.1.86).

Fig. 4 shows biosynthetic pathways that produce fatty acids esters. Fatty acids esters having defined carbon chain lengths can be produced by exogenously expressing various thioesterases (EC 3.1.2.14), combinations of acyl-CoA reductase (1.2.1.50), alcohol dehydrogenases (EC 1.1.1.1), and fatty alcohol forming Acyl-CoA reductase (FAR, EC 1.1.1*), as well as, acetyl transferase (EC 2.3.1.84). Other enzymes that can be modulated to increase the production of fatty acid esters include enzymes involved in fatty acid synthesis (FAS enzymes EC 2.3.1.85), and acyl-CoA synthase (EC 2.3.1.86).

Fig. 5 shows fatty alcohol production by the strain described in Example 4, co-transformed with pCDFDuet-1-fadD-acr1 and plasmids containing various thioesterase genes. The strains were grown aerobically at 25°C in M9 mineral medium with 0.4% glucose in shake flasks. Saturated C10, C12, C14, C16 and C18 fatty alcohol were identified. Small amounts of C16:1 and C18:1 fatty alcohols were also detected in some samples. Fatty alcohols were extracted from cell pellets using ethyl acetate and derivatized with N-trimethylsilyl (TMS) imidazole to increase detection.

Fig. 6 shows the release of fatty alcohols from the production strain. Approximately 50% of the fatty alcohol produced was released from the cells when they were grown at 37°C.

Figs. 7A-7D show GS-MS spectrum of octyl octanoate (C8C8) produced by a production hosts expressing alcohol acetyl transferase (AATs, EC 2.3.1.84) and production hosts expressing wax synthase (EC 2.3.1.75). **Fig. 7A** shows acetyl acetate extract of strain C41(DE3, ΔfadE/pHZ1.43)/pRSET B+pAS004.114B) wherein the pHZ1.43 plasmid expressed ADP1 (wax synthase). **Fig. 7B** shows acetyl acetate extract of strain C41(DE3, ΔfadE/pHZ1.43)/pRSET B+pAS004.114B) wherein the pHZ1.43 plasmid expressed SAAT. **Fig. 7C** shows acetyl acetate extract of strain C41(DE3, ΔfadE/pHZ1.43)/pRSET B+pAS004.114B) wherein the pHZ1.43 plasmid did not contain ADP1 (wax synthase) or SAAT. **Fig. 7D** shows the mass spectrum and fragmentation pattern of C8C8 produced by C41(DE3, ΔfadE/pHZ1.43)/pRSET B+pAS004.114B) wherein the pHZ1.43 plasmid expressed SAAT).

Fig. 8 shows the distribution of ethyl esters made when the wax synthase from *A. baylyi* ADP1 (WSadp1) was co-expressed with thioesterase gene from *Cuphea hookeriana* in a production host.

Figs. 9A and 9B show chromatograms of GC/MS analysis. **Fig. 9A** shows a chromatogram of the ethyl extract of the culture *of E. coli* LS9001 strain transformed with plasmids pCDFDuet-1-fadD-WSadp1, pETDuet-1-'tesA. Ethanol was fed to fermentations. **Fig. 9B** shows a chromatogram of ethyl hexadecanoate and ethyl oleate used as reference.

Fig. 10 shows a table that identifies various genes that can be over-expressed or attenuated to increase fatty acid derivative production. The table also identifies various genes that can be modulated to alter the structure of the fatty acid derivative product. One of ordinary skill in the art will appreciate that some of the genes that are used to alter the structure of the fatty acid derivative will also increase the production of fatty acid derivatives.

Abbreviations and Terms

[0018] The following explanations of terms and methods are provided to better describe the present disclosure and to guide those of ordinary skill in the art in the practice of the present disclosure. As used herein, "comprising" means "including" and the singular forms "a" or "an" or "the" include plural references unless the context clearly dictates otherwise. For example, reference to "comprising a cell" includes one or a plurality of such cells, and reference to "comprising the thioesterase" includes reference to one or more thioesterase peptides and equivalents thereof known to those of ordinary skill in the art, and so forth. The term "or" refers to a single element of stated alternative elements or a combination of two or more elements, unless the context clearly indicates otherwise. For example, the phrase "thioesterase activity or fatty alcohol-forming acyl-CoA reductase activity" refers to thioesterase activity, fatty alcohol forming acyl-CoA reductase activity, or a combination of both fatty alcohol forming acyl-CoA reductase activity, and thioesterase activity.

[0019] Unless explained otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. The materials, methods, and examples are illustrative only and not intended to be limiting. Other features of the disclosure are apparent from the following detailed description and the claims.

[0020] **Accession Numbers:** The accession numbers throughout this description are derived from the NCBI database

(National Center for Biotechnology Information) maintained by the National Institute of Health, U.S.A. The accession numbers are as provided in the database on March 27, 2007.

[0021] Enzyme Classification Numbers (EC): The EC numbers provided throughout this description are derived from the KEGG Ligand database, maintained by the Kyoto Encyclopedia of Genes and Genomics, sponsored in part by the University of Tokyo. The EC numbers are as provided in the database on March 27, 2007.

[0022] Attenuate: To lessen the impact, activity or strength of something. In one example, the sensitivity of a particular enzyme to feedback inhibition or inhibition caused by a composition that is not a product or a reactant (non-pathway specific feedback) is lessened such that the enzyme activity is not impacted by the presence of a compound. For example, the *fabH* gene and its corresponding amino acid sequence are temperature sensitive and can be altered to decrease the sensitivity to temperature fluctuations. The attenuation of the *fabH* gene can be used when branched amino acids are desired. In another example, an enzyme that has been altered to be less active can be referred to as attenuated.

[0023] A functional deletion of an enzyme can be used to attenuate an enzyme. A functional deletion is a mutation, partial or complete deletion, insertion, or other variation made to a gene sequence or a sequence controlling the transcription of a gene sequence, which reduces or inhibits production of the gene product, or renders the gene product non-functional (*i.e.* the mutation described herein for the *plsB* gene). For example, functional deletion of *fabR* in *E. coli* reduces the repression of the fatty acid biosynthetic pathway and allows *E. coli* to produce more unsaturated fatty acids (UFAs). In some instances a functional deletion is described as a knock-out mutation.

[0024] One of ordinary skill in the art will appreciate that there are many methods of attenuating a enzyme activity. For example, attenuation can be accomplished by introducing amino acid sequence changes via altering the nucleic acid sequence, placing the gene under the control of a less active promoter, expressing interfering RNA, ribozymes or antisense sequences that targeting the gene of interest, or through any other technique known in the art.

[0025] Carbon source: Generally 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, acids, alcohols, aldehydes, ketones, amino acids, peptides, etc. These include, for example, various monosaccharides such as glucose, oligosaccharides, polysaccharides, cellulosic material, xylose, and arabinose, disaccharides, such sucrose, saturated or unsaturated fatty acids, succinate, lactate, acetate, ethanol, etc., or mixtures thereof. The carbon source can additionally be a product of photosynthesis, including, but not limited to glucose.

[0026] cDNA (complementary DNA): A piece of DNA lacking internal, non-coding segments (introns) and regulatory sequences which determine transcription. cDNA can be synthesized by reverse transcription from messenger RNA extracted from cells.

[0027] Deletion: The removal of one or more nucleotides from a nucleic acid molecule or one or more amino acids from a protein, the regions on either side being joined together.

[0028] Detectable: Capable of having an existence or presence ascertained. For example, production of a product from a reactant, for example, the production of C18 fatty acids, is detectable using the method provided in Example 11 below.

[0029] DNA: Deoxyribonucleic acid. DNA is a long chain polymer which includes the genetic material of most living organisms (some viruses have genes including ribonucleic acid, RNA), The repeating units in DNA polymers are four different nucleotides, each of which includes one of the four bases, adenine, guanine, cytosine and thymine bound to a deoxyribose sugar to which a phosphate group is attached. Triplets of nucleotides, referred to as codons, in DNA molecules code for amino acid in a peptide. The term codon is also used for the corresponding (and complementary) sequences of three nucleotides in the mRNA into which the DNA sequence is transcribed.

[0030] Endogenous: As used herein with reference to a nucleic acid molecule and a particular cell or microorganism refers to a nucleic acid sequence or peptide that is in the cell and was not introduced into the cell using recombinant engineering techniques. For example, a gene that was present in the cell when the cell was originally isolated from nature. A gene is still considered endogenous if the control sequences, such as a promoter or enhancer sequences that activate transcription or translation have been altered through recombinant techniques.

[0031] Exogenous: As used herein with reference to a nucleic acid molecule and a particular cell refers to any nucleic acid molecule that does not originate from that particular cell as found in nature. Thus, a non-naturally-occurring nucleic acid molecule is considered to be exogenous to a cell once introduced into the cell. A nucleic acid molecule that is naturally-occurring also can be exogenous to a particular cell. For example, an entire coding sequence isolated from cell X is an exogenous nucleic acid with respect to cell Y once that coding sequence is introduced into cell Y, even if X and Y are the same cell type.

[0032] Expression: The process by which a gene's coded information is converted into the structures and functions of a cell, such as a protein, transfer RNA, or ribosomal RNA. Expressed genes include those that are transcribed into mRNA and then translated into protein and those that are transcribed into RNA but not translated into protein (for example, transfer and ribosomal RNAs).

[0033] Fatty ester: Includes any ester made from a fatty acid. The carbon chains in fatty acids can contain any combination of the modifications described herein. For example, the carbon chain can contain one or more points of unsaturation, one or more points of branching, including cyclic branching, and can be engineered to be short or long. Any alcohol can be used to form fatty acid esters, for example alcohols derived from the fatty acid biosynthetic pathway, alcohols produced by the production host through non-fatty acid biosynthetic pathways, and alcohols that are supplied in the fermentation broth.

[0034] Fatty acid derivative: Includes products made in part from the fatty acid biosynthetic pathway of the host organism. The fatty acid biosynthetic pathway includes fatty acid synthase enzymes which can be engineered as described herein to produce fatty acid derivatives, and in some examples can be expressed with additional enzymes to produce fatty acid derivatives having desired carbon chain characteristics. Exemplary fatty acid derivatives include for example, short and long chain alcohols, hydrocarbons, and fatty acid esters including waxes.

[0035] Fermentation Broth: Includes any medium which supports microorganism life (*i.e.* a microorganism that is actively metabolizing carbon). A fermentation medium usually contains a carbon source. The carbon source can be anything that can be utilized, with or without additional enzymes, by the microorganism for energy.

[0036] Hydrocarbon: includes chemical compounds that containing the elements carbon (C) and hydrogen (H). All hydrocarbons consist of a carbon backbone and atoms of hydrogen attached to that backbone. Sometimes, the term is used as a shortened form of the term "aliphatic hydrocarbon." There are essentially three types of hydrocarbons: (1) aromatic hydrocarbons, which have at least one aromatic ring; (2) saturated hydrocarbons, also known as alkanes, which lack double, triple or aromatic bonds; and (3) unsaturated hydrocarbons, which have one or more double or triple bonds between carbon atoms, are divided into: alkenes, alkynes, and dienes. Liquid geologically-extracted hydrocarbons are referred to as petroleum (literally "rock oil") or mineral oil, while gaseous geologic hydrocarbons are referred to as natural gas. All are significant sources of fuel and raw materials as a feedstock for the production of organic chemicals and are commonly found in the Earth's subsurface using the tools of petroleum geology. Oil reserves in sedimentary rocks are the principal source of hydrocarbons for the energy and chemicals industries. Hydrocarbons are of prime economic importance because they encompass the constituents of the major fossil fuels (coal, petroleum, natural gas, etc.) and biofuels, as well as plastics, waxes, solvents and oils.

[0037] Isolated: An "isolated" biological component (such as a nucleic acid molecule, protein, or cell) has been substantially separated or purified away from other biological components in which the component naturally occurs, such as other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acid molecules and proteins that have been "isolated" include nucleic acid molecules and proteins purified by standard purification methods. The term also embraces nucleic acid molecules and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acid molecules and proteins.

[0038] In one example, isolated refers to a naturally-occurring nucleic acid molecule that is not immediately contiguous with both of the sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally-occurring genome of the organism from which it is derived.

[0039] Microorganism: Includes prokaryotic and eukaryotic microbial species from the Domains Archaea, Bacteria and Eucarya, the latter including yeast and filamentous fungi, protozoa, algae, or higher Protista. The terms "microbial cells" and "microbes" are used interchangeably with the term microorganism.

[0040] Nucleic Acid Molecule: Encompasses both RNA and DNA molecules including, without limitation, cDNA, genomic DNA, and mRNA. Includes synthetic nucleic acid molecules, such as those that are chemically synthesized or recombinantly produced. The nucleic acid molecule can be double-stranded or single-stranded. Where single-stranded, the nucleic acid molecule can be the sense strand or the antisense strand. In addition, nucleic acid molecule can be circular or linear.

[0041] Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame. Configurations of separate genes that are transcribed in tandem as a single messenger RNA are denoted as operons. Thus placing genes in close proximity, for example in a plasmid vector, under the transcriptional regulation of a single promoter, constitutes a synthetic operon.

[0042] ORF (open reading frame): A series of nucleotide triplets (codons) coding for amino acids without any termination codons. These sequences are usually translatable into a peptide.

[0043] Over-expressed: When a gene is caused to be transcribed at an elevated rate compared to the endogenous transcription rate for that gene. In some examples, over-expression additionally includes an elevated rate of translation of the gene compared to the endogenous translation rate for that gene. Methods of testing for over-expression are well known in the art, for example transcribed RNA levels can be assessed using rtPCR and protein levels can be assessed using SDS page gel analysis.

[0044] Purified: The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified fatty acid derivative preparation, such as a wax, or a fatty acid ester preparation, is one in which the product is more concentrated than the product is in its environment within a cell. For example, a purified wax is one that is substantially separated from cellular components (nucleic acids, lipids, carbohydrates, and other peptides) that can accompany it. In another example, a purified wax preparation is one in which the wax is substantially-free from contaminants, such as those that might be present following fermentation.

[0045] In one example, a fatty acid ester is purified when at least about 50% by weight of a sample is composed of the fatty acid ester, for example when at least about 60%, 70%, 80%, 85%, 90%, 92%, 95%, 98%, or 99% or more of a sample is composed of the fatty acid ester. Examples of methods that can be used to purify a waxes, fatty alcohols, and fatty acid esters, include the methods described in Example 11 below.

[0046] **Recombinant:** A recombinant nucleic acid molecule or protein is one that has a sequence that is not naturally occurring, has a sequence that is made by an artificial combination of two otherwise separated segments of sequence, or both. This artificial combination can be achieved, for example, by chemical synthesis or by the artificial manipulation of isolated segments of nucleic acid molecules or proteins, such as genetic engineering techniques. Recombinant is also used to describe nucleic acid molecules that have been artificially manipulated, but contain the same regulatory sequences and coding regions that are found in the organism from which the nucleic acid was isolated. A recombinant cell or microorganism is one that contains an exogenous nucleic acid molecule, such as a recombinant nucleic acid molecule.

[0047] **Release:** The movement of a compound from inside a cell (intracellular) to outside a cell (extracellular). The movement can be active or passive. When release is active it can be facilitated by one or more transporter peptides and in some examples it can consume energy. When release is passive, it can be through diffusion through the membrane and can be facilitated by continually collecting the desired compound from the extracellular environment, thus promoting further diffusion. Release of a compound can also be accomplished by lysing a cell.

[0048] **Surfactants:** Substances capable of reducing the surface tension of a liquid in which they are dissolved. They are typically composed of a water-soluble head and a hydrocarbon chain or tail. The water soluble group is hydrophilic and can be either ionic or nonionic, and the hydrocarbon chain is hydrophobic. Surfactants are used in a variety of products, including detergents and cleaners, and are also used as auxiliaries for textiles, leather and paper, in chemical processes, in cosmetics and pharmaceuticals, in the food industry and in agriculture. In addition, they can be used to aid in the extraction and isolation of crude oils which are found hard to access environments or as water emulsions.

[0049] There are four types of surfactants characterized by varying uses. Anionic surfactants have detergent-like activity and are generally used for cleaning applications. Cationic surfactants contain long chain hydrocarbons and are often used to treat proteins and synthetic polymers or are components of fabric softeners and hair conditioners. Amphoteric surfactants also contain long chain hydrocarbons and are typically used in shampoos. Non-ionic surfactants are generally used in cleaning products.

[0050] **Transformed or recombinant cell:** A cell into which a nucleic acid molecule has been introduced, such as an acyl-CoA synthase encoding nucleic acid molecule, for example by molecular biology techniques. Transformation encompasses all techniques by which a nucleic acid molecule can be introduced into such a cell, including, but not limited to, transfection with viral vectors, conjugation, transformation with plasmid vectors, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration.

[0051] **Under conditions that permit product production:** Any fermentation conditions that allow a microorganism to produce a desired product, such as fatty acids, hydrocarbons, fatty alcohols, waxes, or fatty acid esters. Fermentation conditions usually include temperature ranges, levels of aeration, and media selection, which when combined allow the microorganism to grow. Exemplary mediums include broths or gels. Generally, the medium includes a carbon source such as glucose, fructose, cellulose, or the like that can be metabolized by the microorganism directly, or enzymes can be used in the medium to facilitate metabolizing the carbon source. To determine if culture conditions permit product production, the microorganism can be cultured for 24, 36, or 48 hours and a sample can be obtained and analyzed. For example, the cells in the sample or the medium in which the cells were grown can be tested for the presence of the desired product. When testing for the presence of a product assays, such as those provided in the Examples below, can be used.

[0052] **Vector:** A nucleic acid molecule as introduced into a cell, thereby producing a transformed cell. A vector can include nucleic acid sequences that permit it to replicate in the cell, such as an origin of replication. A vector can also include one or more selectable marker genes and other genetic elements known in the art.

[0053] **Wax:** A variety of fatty acid esters which form solids or pliable substances under an identified set of physical conditions. Fatty acid esters that are termed waxes generally have longer carbon chains than fatty acid esters that are not waxes. For example, a wax generally forms a pliable substance at room temperature.

Detailed Description

I. Production of fatty acid derivatives

[0054] The host organism that exogenous DNA sequences are transformed into can be a modified host organism, such as an organism that has been modified to increase the production of acyl-ACP or acyl-CoA, reduce the catabolism of fatty acid derivatives and intermediates, or to reduce feedback inhibition at specific points in the biosynthetic pathway. In addition to modifying the genes described herein additional cellular resources can be diverted to over produce fatty acids, for example the lactate, succinate and/or acetate pathways can be attenuated, and acetyl-CoA carboxylase (ACC) can be over expressed. The modifications to the production host described herein can be through genomic alterations, extrachromosomal expression systems, or combinations thereof. An overview of the pathway is provided in Figs. 1 and 2.

A. Acetyl-CoA - Malonyl-CoA to Acyl-ACP

[0055] Fatty acid synthase (FAS) is a group of peptides that catalyze the initiation and elongation of acyl chains (Marrakchi et al., Biochemical Society, 30:1050-1055, 2002). The acyl carrier protein (ACP) along with the enzymes in the FAS pathway control the length, degree of saturation and branching of the fatty acids produced. Enzymes that can be included in FAS include AccABCD, FabD, FabH, FabG, FabA, FabZ, FabI, FabK, FabL, FabM, FabB, and FabF. Depending upon the desired product one or more of these genes can be attenuated or over-expressed.

[0056] For example, the fatty acid biosynthetic pathway in the production host suses the precursors acetyl-CoA and malonyl-CoA (Fig. 2). *E. coli* or other host organisms engineered to overproduce these components can serve as the starting point for subsequent genetic engineering steps to provide the specific output product (such as, fatty acid esters, hydrocarbons, fatty alcohols). Several different modifications can be made, either in combination or individually, to the host strain to obtain increased acetyl CoA/malonyl CoA/fatty acid and fatty acid derivative production. For example, to increase acetyl CoA production, a plasmid with *pdh, panK, aceEF,* (encoding the E1p dehydrogenase component and the E2p dihydrolipoamide acyltransferase component of the pyruvate and 2-oxoglutarate dehydrogenase complexes), *fabh /fabD/fabG/acpP/fabF,* and in some examples additional DNA encoding fatty-acyl-CoA reductases and aldehyde decarbonylases, all under the control of a constitutive, or otherwise controllable promoter, can be constructed. Exemplary Genbank accession numbers for these genes are: *pdh* (BAB34380, AAC73227, AAC73226), *panK* (also known as coaA, AAC76952), *aceEF* (AAC73227, AAC73226), *fabH* (AAC74175), *fabD* (AAC74176), *fabG* (AAC74177), *acpP* (AAC74178), *fabF* (AAC74179).

[0057] Additionally, *fadE, gpsA, ldhA, pflb, adhE, pta, poxB, ackA,* and/or *ackB* can be knocked-out, or their expression levels can be reduced, in the engineered microorganism by transformation with conditionally replicative or non-replicative plasmids containing null or deletion mutations of the corresponding genes, or by substituting promoter or enhancer sequences. Exemplary Genbank accession numbers for these genes are; *fadE* (AAC73325), *gspA* (AAC76632), *ldhA* (AAC74462), *pflb* (AAC73989), *adhE* (AAC74323), *pta* (AAC75357), *poxB* (AAC73958), *ackA* (AAC75356), and *ackB* (BAB81430).

[0058] The resulting engineered microorganisms can be grown in a desired environment, for example one with limited glycerol (less than 1% w/v in the culture medium). As such, these microorganisms will have increased acetyl-CoA production levels. Malonyl-CoA overproduction can be effected by engineering the microorganism as described above, with DNA encoding *accABCD* (acetyl CoA carboxylase, for example accession number AAC73296, EC 6.4.1.2) included in the plasmid synthesized *de novo.* Fatty acid overproduction can be achieved by further including DNA encoding lipase (for example Accessions numbers CAA89087, CAA98876) in the plasmid synthesized *de novo.*

[0059] In some examples, acetyl-CoA carboxylase (ACC) is over-expressed to increase the intracellular concentration thereof by at least 2-fold, such as at least 5-fold, or at least 10-fold, for example relative to native expression levels.

[0060] In addition, the *plsB* (for example Accession number AAC77011) D311E mutation can be used to remove limitations on the pool of acyl-CoA.

[0061] In addition, over-expression of an *sfa* gene (suppressor of FabA, for example Accession number AAN79592) can be included in the production host to increase production of monounsaturated fatty acids (Rock et al., J. Bacteriology 178:5382-5387, 1996).

B. Acyl-ACP to Fatty Acid

[0062] To engineer a production host for the production of a homogeneous population of fatty acid derivatives, one or more endogenous genes can be attenuated or functionally deleted and one or more thioesterases can be expressed. For example, C10 fatty acid derivatives can be produced by attenuating thioesterase C18 (for example accession numbers AAC73596 and P0ADA1), which uses C18:1-ACP and expressing thioesterase C10 (for example accession number Q39513), which uses C10-ACP. Thus, resulting in a relatively homogeneous population of fatty acid derivatives that have a carbon chain length of 10. In another example, C14 fatty acid derivatives can be produced by attenuating endogenous thioesterases that produce non-C14 fatty acids and expressing the thioesterase accession number Q39473 (which uses C14-ACP). In yet another example, C12 fatty acid derivatives can be produced by expressing thioesterases that use C12-ACP (for example accession number Q41635) and attenuating thioesterases that produce non-C12 fatty acids. Acetyl CoA, malonyl CoA, and fatty acid overproduction can be verified using methods known in the art, for example by using radioactive precursors, HPLC, and GC-MS subsequent to cell lysis.

Table 1

Thioesterases			
Accession Number	Source Organism	*Gene*	Preferential product produced
AAC73596	*E. coli*	*tesA* without leader sequence	C18:1
Q41635	*Umbellularia california*	*fatB*	C12:0
Q39513;	*Cuphea hookeriana*	*fatB2*	C8:0 - C10:0
AAC49269	*Cuphea hookeriana*	*fatB3*	C14:0 - C16:0
Q39473	*Cinnamonum camphorum*	*fatB*	C14:0
CAA85388	*Arabidopsis thaliana*	*fatB*[M141T]*	C16:1
NP 189147; NP 193041	*Arabidopsis thaliana*	*fatA*	C18:1
CAC39106	*Bradyrhiizobium japonicum*	*fatA*	C18:1
AAC72883	*Cuphea hookeriana*	*fatA*	C18:1
*Mayer *et al., BMC Plant Biology* 7:1-11, 2007.			

C. Fatty Acid to Acyl-CoA

[0063] Production hosts can be engineered using known peptides to produce fatty acids of various lengths. One method of making fatty acids involves increasing the expression of, or expressing more active forms of, one or more acyl-CoA synthase peptides (EC 2.3.1.86).

[0064] As used herein, acyl-CoA synthase includes peptides in enzyme classification number EC 2.3.1.86, as well as any other peptide capable of catalyzing the conversion of a fatty acid to acyl-CoA. Additionally, one of ordinary skill in the art will appreciate that some acyl-CoA synthase peptides will catalyze other reactions as well, for example some acyl-CoA synthase peptides will accept other substrates in addition to fatty acids. Such non-specific acyl-CoA synthase peptides are, therefore, also included. Acyl-CoA synthase peptide sequences are publicly available. Exemplary GenBank Accession Numbers are provided in **Fig. 10.**

D. Acyl-CoA to fatty alcohol

[0065] Production hosts can be engineered using known polypeptides to produce fatty alcohols from acyl-CoA. One method of making fatty alcohols involves increasing the expression of or expressing more active forms of fatty alcohol forming acyl-CoA reductase (FAR, EC 1.1.1.*), or acyl-CoA reductases (EC 1.2.1.50) and alcohol dehydrogenase (EC 1.1.1.1). Hereinafter fatty alcohol forming acyl-CoA reductase (FAR, EC 1.1.1.*), acyl-CoA reductases (EC 1.2.1.50) and alcohol dehydrogenase (EC 1.1.1.1) are collectively referred to as fatty alcohol forming peptides. In some examples all three of the fatty alcohol forming genes can be over expressed in a production host, and in yet other examples one or more of the fatty alcohol forming genes can be over-expressed.

[0066] As used herein, fatty alcohol forming peptides include peptides in enzyme classification numbers EC 1.1.1.*, 1.2.1.50, and 1.1.1.1, as well as any other peptide capable of catalyzing the conversion of acyl-CoA to fatty alcohol. Additionally, one of ordinary skill in the art will appreciate that some fatty alcohol forming peptides will catalyze other reactions as well, for example some acyl-CoA reductase peptides will accept other substrates in addition to fatty acids. Such non-specific peptides are, therefore, also included. Fatty alcohol forming peptides sequences are publicly available. Exemplary GenBank Accession Numbers are provided in **Fig. 10.**

[0067] Fatty alcohols can also be described as hydrocarbon-based surfactants. For surfactant production the microorganism is modified so that it produces a surfactant from a renewable carbon source. Such a microorganism includes a first exogenous DNA sequence encoding a protein capable of converting a fatty acid to a fatty aldehyde and a second exogenous DNA sequence encoding a protein capable of converting a fatty aldehyde to an alcohol. In some examples, the first exogenous DNA sequence encodes a fatty acid reductase. In one embodiment, the second exogenous DNA sequence encodes mammalian microsomal aldehyde reductase or long-chain aldehyde dehydrogenase. In a further example, the first and second exogenous DNA sequences are from a multienzyme complex from *Arthrobacter AK 19,* *Rhodotorula glutinins, Acinobacter sp strain M-1,* or *Candida lipolytica.* In one embodiment, the first and second heterologous DNA sequences are from a multienzyme complex from *Acinobacter sp strain M-1* or *Candida lipolytica.*

[0068] Additional sources of heterologous DNA sequences encoding fatty acid to long chain alcohol converting proteins that can be used in surfactant production include, but are not limited to, *Mortierella alpina* (ATCC 32222), *Crytococcus curvatus,* (also referred to as *Apiotricum curvatum*), *Alcanivorax jadensis* (T9T =DSM 12718 =ATCC 700854), *Acinetobacter sp.* HO1-N, (ATCC 14987) and *Rhodococcus opacus* (PD630 DSMZ 44193).

[0069] In one example, the fatty acid derivative is a saturated or unsaturated surfactant product having a carbon atom content limited to between 6 and 36 carbon atoms. In another example, the surfactant product has a carbon atom content limited to between 24 and 32 carbon atoms.

[0070] Appropriate hosts for producing surfactants can be either eukaryotic or prokaryotic microorganisms. Exemplary hosts include *Arthrobacter AK 19, Rhodotorula glutinins, Acinobacter sp strain M-1, Arabidopsis thalania,* or *Candida lipolytica, Saccharomyces cerevisiae,* and *E. coli* engineered to express acetyl CoA carboxylase. Hosts which demonstrate an innate ability to synthesize high levels of surfactant precursors in the form of lipids and oils, such as *Rhodococcus opacus, Arthrobacter AK 19, Rhodotorula glutinins E. coli* engineered to express acetyl CoA carboxylase, and other oleaginous bacteria, yeast, and fungi can also be used.

E. Fatty Alcohols to Fatty Esters

[0071] Production hosts can be engineered using known polypeptides to produce fatty esters of various lengths. One method of making fatty esters includes increasing the expression of, or expressing more active forms of, one or more alcohol O-acetyltransferase peptides (EC 2.3.1.84). These peptides catalyze the reaction of acetyl-CoA and an alcohol to form CoA and an acetic ester. In some examples the alcohol O-acetyltransferase peptides can be expressed in conjunction with selected thioesterase peptides, FAS peptides and fatty alcohol forming peptides, thus, allowing the carbon chain length, saturation and degree of branching to be controlled. In some. cases the *bkd* operon can be coexpressed to enable branched fatty acid precursors to be produced.

[0072] As used herein, alcohol O-acetyltransferase peptides include peptides in enzyme classification number EC 2.3.1.84, as well as any other peptide capable of catalyzing the conversion of acetyl-CoA and an alcohol to form CoA and an acetic ester. Additionally, one of ordinary skill in the art will appreciate that alcohol *O*-acetyltransferase peptides will catalyze other reactions as well, for example some alcohol *O*-acetyltransferase peptides will accept other substrates in addition to fatty alcohols or acetyl-CoA thiosester i.e such as other alcohols and other acyl-CoA thioesters. Such non-specific or divergent specificty alcohol *O*-acetyltransferase peptides are, therefore, also included. Alcohol *O*-acetyltransferase peptide sequences are publicly available. Exemplary GenBank Accession Numbers are provided in **Fig. 10.** Assays for characterizing the activity of a particular alcohol *O*-acetyltransferase peptides are well known in the art. Engineered *O*-acetyltransferases and O-acyltransferases can be also created that have new activities and specificities for the donor acyl group or acceptor alcohol moiety. Engineered enzymes could be generated through rational and evolutionary approaches well documented in the art.

F. Acyl-CoA to Fatty Esters (biodiesels and waxes)

[0073] Production hosts can be engineered using known peptides to produce fatty acid esters from acyl-CoA and alcohols. In some examples the alcohols are provided in the fermentation media and in other examples the production host can provide the alcohol as described herein. One of ordinary skill in the art will appreciate that structurally, fatty acid esters have an A and a B side. As described herein, the A side of the ester is used to describe the carbon chain contributed by the alcohol, and the B side of the ester is used to describe the carbon chain contributed by the acyl-CoA. Either chain can be saturated or unsaturated, branched or unbranched. The production host can be engineered to produce fatty alcohols or short chain alcohols. The production host can also be engineered to produce specific acyl-CoA molecules. As used herein fatty acid esters are esters derived from a fatty acyl-thioester and an alcohol, wherein the A side and the B side of the ester can vary in length independently. Generally, the A side of the ester is at least 1, 2, 3, 4, 5, 6, 7, or 8 carbons in length, while the B side of the ester is 8, 10, 12, 14, 16, 18, 20, 22, 24, or 26 carbons in length. The A side and the B side can be straight chain or branched, saturated or unsaturated.

[0074] The production of fatty esters, including waxes from acyl-CoA and alcohols can be engineered using known polypeptides. As used herein waxes are long chain fatty acid esters, wherein the A side and the B side of the ester can vary in length independently. Generally, the A side of the ester is at least 8, 10, 12, 14, 16, 18, 20, 22, 24, or 26 carbons in length. Similarly the B side of the ester is at least 8, 10, 12, 14, 16, 18, 20, 22, 24, or 26 carbons in length. The A side and the B side can be mono-, di-, tri- unsaturated. The production of fatty esters, including waxes from acyl-CoA and alcohols can be engineered using known polypeptides. One method of making fatty esters includes increasing the expression of or expressing more active forms of one or more wax synthases (EC 2.3.1.75).

[0075] As used herein, wax synthases includes peptides in enzyme classification number EC 2.3.1.75, as well as any other peptide capable of catalyzing the conversion of an acyl-thioester to fatty esters. Additionally, one of ordinary skill in the art will appreciate that some wax synthase peptides will catalyze other reactions as well, for example some wax synthase peptides will accept short chain acyl-CoAs and short chain alcohols to produce fatty esters. Such non-specific wax synthases are, therefore, also included. Wax synthase peptide sequences are publicly available. Exemplary GenBank Accession Numbers are provided in **Fig. 10.** Methods to identify wax synthase activity are provided in U.S. patent number 7,118,896, which is herein incorporated by reference.

[0076] In particular examples, if the desired product is a fatty ester based biofuel, the microorganism is modified so that it produces a fatty ester generated from a renewable energy source. Such a microorganism includes an exongenous DNA sequence encoding a wax ester synthase that is expressed so as to confer upon said microorganism the ability to synthesize a saturated, unsaturated, or branched fatty ester from a renewable energy source. In some embodiments, the wax ester synthesis proteins include, but are not limited to,: fatty acid elongases, acyl-CoA reductases, acyltransferases or wax synthases, fatty acyl transferases, diacylglycerol acyltransferases, acyl-coA wax alcohol acyltransferases, bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltransferase selected from a multienzyme complex from *Simmondsia chinensis, Acinetobacter* sp. strain ADP1 (formerly *Acinetobacter calcoaceticus* ADP1), *Pseudomonas aeruginosa, Fundibacter jadensis, Arabidopsis thaliana,* or *Alkaligenes eutrophus.* In one embodiment, the fatty acid elongases, acyl-CoA reductases or wax synthases are from a multienzyme complex from *Alkaligenes eutrophus* and other organisms known in the literature to produce wax and fatty acid esters.

[0077] Additional sources of heterologous DNA encoding wax synthesis proteins useful in fatty ester production include, but are not limited to, *Mortierella alpina* (for example ATCC 32222), *Crytococcus curvatus,* (also referred to as *Apiotricum curvatum), Alcanivorax jadensis* (for example T9T =DSM 12718 =ATCC 700854), *Acinetobacter sp.* HO1-N, (for example ATCC 14987) and *Rhodococcus opacus* (for example PD630, DSMZ 44193).

[0078] The methods of described herein permit production of fatty esters of varied length. In one example, the fatty ester product is a saturated or unsaturated fatty ester product having a carbon atom content between 24 and 46 carbon atoms. In one embodiment, the fatty ester product has a carbon atom content between 24 and 32 carbon atoms. In another embodiment the fatty ester product has a carbon content of 14 and 20 carbons. In another embodiment the fatty ester is the methyl ester of C18:1. In another embodiment the fatty acid ester is the ethyl ester of C16:1. In another embodiment the fatty ester is the methyl ester of C16:1. In another embodiment the fatty acid ester is octadecyl ester of octanol.

[0079] Useful hosts for producing fatty esters can be either eukaryotic or prokaryotic microorganisms. In some embodiments such hosts include, but are not limited to, *Saccharomyces cerevisiae, Candida lipolytica, E. coli Arthrobacter AK 19, Rhodotorula glutinins, Acinobacter sp strain M-1, Candida lipolytica* and other oleaginous microorganisms.

[0080] In one example the wax ester synthase from Acinetobacter sp. ADP1 at locus AAO17391 (described in Kalscheuer and Steinbuchel, J. Biol. Chem. 278:8075-8082, 2003). is used. In another example the wax ester synthase from *Simmondsia chinensis,* at locus AAD38041 is used.

[0081] Optionally a wax ester exporter such as a member of the FATP family can be used to facilitate the release of waxes or esters into the extracellular environment. One example of a wax ester exporter that can be used is fatty acid (long chain) transport protein CG7400-PA, isoform A from *Drosophila melanogaster,* at locus NP_524723.

G. Acyl-ACP, Acyl-CoA to Hydrocarbon

[0082] A diversity of microorganisms are known to produce hydrocarbons, such as alkanes, olefins, and isoprenoids. Many of these hydrocarbons are derived from fatty acid biosynthesis. The production of these hydrocarbons can be controlled by controlling the genes associated with fatty acid biosynthesis in the native hosts. For example, hydrocarbon biosynthesis in the algae *Botryococcus braunii* occurs through the decarbonylation of fatty aldehydes. The fatty aldehydes are produced by the reduction of fatty acyl - thioesters by fatty acyl-CoA reductase. Thus, the structure of the final alkanes can be controlled by engineering *B. braunii* to express specific genes, such as thioesterases, which control the chain length of the fatty acids being channeled into alkane biosynthesis. Expressing the enzymes that result in branched chain fatty acid biosynthesis in *B. braunii* will result in the production of branched chain alkanes. Introduction of genes effecting the production of desaturation of fatty acids will result in the production of olefins. Further combinations of these genes can provide further control over the final structure of the hydrocarbons produced. To produce higher levels of the native or engineered hydrocarbons, the genes involved in the biosynthesis of fatty acids and their precursors or the degradation to other products can be expressed, overexpressed, or attenuated. Each of these approaches can be applied to the production of alkanes in *Vibrio furnissi* M1 and its functional homologues, which produces alkanes through the reduction of fatty alcohols (see above for the biosynthesis and engineering of fatty alcohol production). Each of these approaches can also be applied to the production of the olefins produced by many strains of *Micrococcus leuteus, Stenotrophomonas maltophilia, Jeogalicoccus sp.* (ATCC8456), and related microorganisms. These microorganisms produce long chain internal olefins that are derived from the head to head condensation of fatty acid precursors. Controlling the structure and level of the fatty acid precursors using the methods described herein will result in formation of olefins of different chain length, branching, and level of saturation.

[0083] Hydrocarbons can also be produced using evolved oxido/reductases for the reduction of primary alcohols. Primary fatty alcohols are known to be used to produce alkanes in microorganisms such as *Vibrio furnissii* M1 (Myong-Ok, J. Bacteriol., 187:1426-1429, 2005). An NAD(P)H dependent oxido/reductase is the responsible catalyst. Synthetic NAD(P)H dependent oxidoreductases can be produced through the use of evolutionary engineering and be expressed in production hosts to produce fatty acid derivatives. One of ordinary skill in the art will appreciate that the process of "evolving" a fatty alcohol reductase to have the desired activity is well known (Kolkman and Stemmer Nat Biotechnol. 19:423-8, 2001, Ness et al., Adv Protein Chem. 55:261-92, 2000, Minshull and Stemmer Curr Opin Chem Biol. 3:284-90, 1999, Huisman and Gray Curr Opin Biotechnol. Aug;13:352-8, 2002, and see U.S. patent application 2006/0195947). A library of NAD(P)H depenedent oxidoreductases is generated by standard methods, such as error prone PCR, site-specific random mutagenesis, site specific saturation mutagenesis, or site directed specific mutagenesis. Additionally, a library can be created through the "shuffling" of naturally occurring NAD(P)H dependent oxidoreductase encoding sequences. The library is expressed in a suitable host, such as *E. coli.* Individual colonies expressing a different member of the oxido/reductase library is then analyzed for its expression of an oxido/reductase that can catalyze the reduction of a fatty alcohol. For example, each cell can be assayed as a whole cell bioconversion, a cell extract, a permeabilized cell, or a purified enzyme. Fatty alcohol reductases are identified by the monitoring the fatty alcohol dependent oxidation of NAD(P)H spectrophotometrically or fluorometrically. Production of alkanes is monitored by GC/MS, TLC, or other methods. An oxido/reductase identified in this manner is used to produce alkanes, alkenes, and related branched hydrocarbons. This is achieved either *in vitro* or *in vivo.* The later is achieved by expressing the evolved fatty alcohol reductase gene in an organism that produces fatty alcohols, such as those described herein. The fatty alcohols act as substrates for the alcohol reductase which would produce alkanes. Other oxidoreductases can be also engineered to catalyze this reaction, such as those that use molecular hydrogen, glutathione, FADH, or other reductive coenzymes.

II. Genetic Engineering of Production Strain to increase Fatty Acid Derivative Production

[0084] Heterologous DNA sequences involved in a biosynthetic pathway for the production of fatty acid derivatives can be introduced stably or transiently into a host cell using techniques well known in the art for example electroporation, calcium phosphate precipitation, DEAE-dextran mediated transfection, liposome-mediated transfection, conjugation, transduction, and the like. For stable transformation, a DNA sequence can further include a selectable marker, such as, antibiotic resistance, for example resistance to neomycin, tetracycline, chloramphenicol, kanamycin, genes that complement auxotrophic deficiencies, and the like.

[0085] Various embodiments of this disclosure utilize an expression vector that includes a heterologous DNA sequence encoding a protein involved in a metabolic or biosynthetic pathway. Suitable expression vectors include, but are not limited to, viral vectors, such as baculovirus vectors, phage vectors, such as bacteriophage vectors, plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes, viral vectors (*e.g.* viral vectors based on vaccinia virus, poliovirus, adenovirus, adeno-associated virus, SV40, herpes simplex virus, and the like), P1-based artificial chromosomes, yeast plasmids, yeast artificial chromosomes, and any other vectors specific for specific hosts of interest (such as *E. coli, Pseudomonas pisum* and *Saccharomyces cerevisiae*).

[0086] Useful expression vectors can include one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells. The selectable marker gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selectable marker gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli.* In alternative embodiments, the selectable marker gene is one that encodes dihydrofolate reductase or confers neomycin resistance (for use in eukaryotic cell culture), or one that confers tetracycline or ampicillin resistance (for use in a prokaryotic host cell, such as *E. coli*).

[0087] The biosynthetic pathway gene product-encoding DNA sequence in the expression vector is operably linked to an appropriate expression control sequence, (promoters, enhancers, and the like) to direct synthesis of the encoded gene product. Such promoters can be derived from microbial or viral sources, including CMV and SV40. Depending on the host/vector system utilized, any of a number of suitable transcription and translation control elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, *etc.* can be used in the expression vector (see *e.g.,* Bitter et al., Methods in Enzymology, 153:516- 544, 1987).

[0088] Suitable promoters for use in prokaryotic host cells include, but are not limited to, promoters capable of recognizing the T4, T3, Sp6 and T7 polymerases, the P_R and P_L promoters of bacteriophage lambda, the trp, recA, heat shock, and lacZ promoters of *E. coli,* the alpha-amylase and the sigma-specific promoters of *B. subtilis,* the promoters of the bacteriophages of *Bacillus, Streptomyces* promoters, the int promoter of bacteriophage lambda, the bla promoter of the beta-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene. Prokaryotic promoters are reviewed by Glick, J. Ind. Microbiol. 1:277, 1987; Watson et al., MOLECULAR BIOLOGY OF THE GENE, 4th Ed., Benjamin Cummins (1987); and Sambrook *et al.*, *supra.*

[0089] Non-limiting examples of suitable eukaryotic promoters for use within a eukaryotic host are viral in origin and include the promoter of the mouse metallothionein I gene (Hamer et al., J. Mol. Appl. Gen. 1:273, 1982); the TK promoter of Herpes virus (McKnight, Cell 31:355, 1982); the SV40 early promoter (Benoist et al., Nature (London) 290:304, 1981); the Rous sarcoma virus promoter; the cytomegalovirus promoter (Foecking et al., Gene 45:101, 1980); the yeast gal4 gene promoter (Johnston, et al., PNAS (USA) 79:6971, 1982; Silver, et al., PNAS (USA) 81:5951, 1984); and the IgG promoter (Orlandi et al., PNAS (USA) 86:3833, 1989).

[0090] The microbial host cell can be genetically modified with a heterologous DNA sequence encoding a biosynthetic pathway gene product that is operably linked to an inducible promoter. Inducible promoters are well known in the art. Suitable inducible promoters include, but are not limited to promoters that are affected by proteins, metabolites, or chemicals. These include: a bovine leukemia virus promoter, a metallothionein promoter, a dexamethasone-inducible MMTV promoter, a SV40 promoter, a MRP polIII promoter, a tetracycline-inducible CMV promoter (such as the human immediate-early CMV promoter) as well as those from the trp and lac operons.

[0091] In some examples a genetically modified host cell is genetically modified with a heterologous DNA sequence encoding a biosynthetic pathway gene product that is operably linked to a constitutive promoter. Suitable constitutive promoters are known in the art and include, constitutive adenovirus major late promoter, a constitutive MPSV promoter, and a constitutive CMV promoter.

[0092] In some examples a modified host cell is one that is genetically modified with an exongenous DNA sequence encoding a single protein involved in a biosynthesis pathway. In other embodiments, a modified host cell is one that is genetically modified with exongenous DNA sequences encoding two or more proteins involved in a biosynthesis pathway -- for example, the first and second enzymes in a biosynthetic pathway.

[0093] Where the host cell is genetically modified to express two or more proteins involved in a biosynthetic pathway, those DNA sequences can each be contained in a single or in separate expression vectors. When those DNA sequences are contained in a single expression vector, in some embodiments, the nucleotide sequences will be operably linked to a common control element (*e.g.*, a promoter), e.g., the common control element controls expression of all of the biosynthetic pathway protein-encoding DNA sequences in the single expression vector.

[0094] When a modified host cell is genetically modified with heterologous DNA sequences encoding two or more proteins involved in a biosynthesis pathway, one of the DNA sequences can be operably linked to an inducible promoter, and one or more of the DNA sequences can be operably linked to a constitutive promoter.

[0095] In some embodiments, the intracellular concentration (*e.g.*, the concentration of the intermediate in the genetically modified host cell) of the biosynthetic pathway intermediate can be increased to further boost the yield of the final product. The intracellular concentration of the intermediate can be increased in a number of ways, including, but not limited to, increasing the concentration in the culture medium of a substrate for a biosynthetic pathway; increasing the catalytic activity of an enzyme that is active in the biosynthetic pathway; increasing the intracellular amount of a substrate (*e.g.*, a primary substrate) for an enzyme that is active in the biosynthetic pathway; and the like.

[0096] In some examples the fatty acid derivative or intermediate is produced in the cytoplasm of the cell. The cytoplasmic concentration can be increased in a number of ways, including, but not limited to, binding of the fatty acid to coenzyme A to form an acyl-CoA thioester. Additionally, the concentration of acyl-CoAs can be increased by increasing the biosynthesis of CoA in the cell, such as by over-expressing genes associated with pantothenate biosynthesis (*panD*) or knocking out the genes associated with glutathione biosynthesis (glutathione synthase).

III. Carbon chain characteristics

[0097] Using the teachings provided herein a range of products can be produced. These products include hydrocarbons, fatty alcohols, fatty acid esters, and waxes. Some of these products are useful as biofuels and specialty chemicals. These products can be designed and produced in microorganisms. The products can be produced such that they contain branch points, levels of saturation, and carbon chain length, thus, making these products desirable starting materials for use in many applications (**Fig. 10** provides a description of the various enzymes that can be used alone or in combination to make various fatty acid derivatives).

[0098] In other examples, the expression of exongenous FAS genes originating from different species or engineered variants can be introduced into the host cell to result in the biosynthesis of fatty acid metabolites structurally different (in length, branching, degree of unsaturation, etc.) as that of the native host. These heterologous gene products can be also chosen or engineered so that they are unaffected by the natural complex regulatory mechanisms in the host cell and, therefore, function in a manner that is more controllable for the production of the desired commercial product. For example the FAS enzymes from *Bacillus subtilis, Saccharomyces cerevisiae, Streptomyces spp, Ralstonia, Rhodococcus, Corynebacteria, Brevibacteria, Mycobacteria,* oleaginous yeast, and the like can be expressed in the production host.

[0099] One of ordinary skill in the art will appreciate that when a production host is engineered to produce a fatty acid from the fatty acid biosynthetic pathway that contains a specific level of unsaturation, branching, or carbon chain length the resulting engineered fatty acid can be used in the production of the fatty acid derivatives. Hence, fatty acid derivatives generated from the production host can display the characteristics of the engineered fatty acid. For example, a production host can be engineered to make branched, short chain fatty acids, and then using the teachings provided herein relating to fatty alcohol production (i.e. including alcohol forming enzymes such as FAR) the production host produce branched, short chain fatty alcohols. Similarly, a hydrocarbon can be produced by engineering a production host to produce a fatty acid having a defined level of branching, unsaturation, and/or carbon chain length, thus, producing a homogenous hydrocarbon population. Moreover, when an unsaturated alcohol, fatty acid ester, or hydrocarbon is desired the fatty acid biosynthetic pathway can be engineered to produce low levels of saturated fatty acids and an additional desaturase can be expressed to lessen the saturated product production.

A. Saturation

[0100] Production hosts can be engineered to produce unsaturated fatty acids by engineering the production host to over-express *fabB*, or by growing the production host at low temperatures (for example less than 37°C). FabB has preference to cis-δ^3decenoyl-ACP and results in unsaturated fatty acid production in *E. coli.* Over-expression of FabB resulted in the production of a significant percentage of unsaturated fatty acids (de Mendoza et al., J. Biol. Chem., 258:2098-101, 1983). These unsaturated fatty acids can then be used as intermediates in production hosts that are engineered to produce fatty acid derivatives, such as fatty alcohols, esters, waxes, olefins, alkanes, and the like. One of ordinary skill in the art will appreciate that by attenuating *fabA*, or over-expressing *FabB* and expressing specific thioesterases (described below), unsaturated fatty acid derivatives having a desired carbon chain length can be produced. Alternatively, the repressor of fatty acid biosynthesis, FabR (Genbank accession NP_418398), can be deleted, which will also result in increased unsaturated fatty acid production in *E. coli* (Zhang et al., J. Biol. Chem. 277:pp. 15558, 2002.). Further increase in unsaturated fatty acids may be achieved by over-expression of FabM (trans-2, cis-3-decenoyl-ACP isomerase, Genbank accession DAA05501) and controlled expression of FabK (trans-2-enoyl-ACP reductase II, Genbank accession NP_357969) from *Streptococcus pneumoniae* (Marrakchi et al., J. Biol. Chem. 277: 44809, 2002), while deleting *E. coli* Fab I ((trans-2-enoyl-ACP reductase, Genbank accession NP_415804). Additionally, to increase the percentage of unsaturated fatty acid esters, the microorganism can also have *fabB* (encoding β-ketoacyl-ACP synthase I, Accessions: BAA16180, EC:2.3.1.41), *Sfa* (encoding a suppressor of fabA, Accession: AAC44390) and *gnsA* and *gnsB* (both encoding secG null mutant suppressors, a.k.a. cold shock proteins, Accession: ABD18647.1, AAC74076.1) over-expressed.

[0101] In some examples, the endogenous fabF gene can be attenuated, thus, increasing the percentage of palmitoleate (C16:1) produced.

B. Branching including cyclic moieties

[0102] Fatty acid derivatives can be produced that contain branch points, cyclic moieties, and combinations thereof, using the teachings provided herein.

[0103] Microorganisms that naturally produce straight fatty acids (sFAs) can be engineered to produce branched chain fatty acids (brFAs) by expressing one or more exogenous nucleic acid sequences. For example, *E. coli* naturally produces straight fatty acids (sFAs). To engineer *E. coli* to produce brFAs, several genes can be introduced and expressed that provide branched precursors (bkd operon) and allow initiation of fatty acid biosynthesis from branched precursors (fabH). Additionally, the organism can express genes for the elongation of brFAs (e.g. ACP, FabF) and/or deleting the corresponding *E. coli* genes that normally lead to sFAs and would compete with the introduced genes (e.g. FabH, FabF).

[0104] The branched acyl-CoAs 2-methyl-buturyl-CoA, isovaleryl-CoA and isobuturyl-CoA are the precursors of brFA. In most brFA-containing microorganisms they are synthesized in two steps (described in detail below) from branched amino acids (isoleucine, leucine and valine) (Kadena, Microbiol. Rev. 55: pp. 288, 1991). To engineer a microorganism to produce brFAs, or to overproduce brFAs, expression or over-expression of one or more of the enzymes in these two steps can be engineered. For example, in some instances the production host may have an endogenous enzyme that can accomplish one step and therefore, only enzymes involved in the second step need to be expressed recombinantly.

[0105] The first step in forming branched fatty acids is the production of the corresponding α-keto acids by a branched-chain amino acid aminotransferase. *E. coli* has such an enzyme, IlvE (EC 2.6.1.42; Genbank accession YP_026247). In some examples, a heterologous branched-chain amino acid aminotransferase may not be expressed. However, *E. coli* IlvE or any other branched-chain amino acid aminotransferase, e.g. ilvE from *Lactococcus lactis* (Genbank accession AAF34406), ilvE from *Pseudomonas putida* (Genbank accession NP_745648) or ilvE from *Streptomyces coelicolor* (Genbank accession NP_629657) can be over-expressed in a host microorganism, should the aminotransferase reaction turn out to be rate limiting in brFA biosynthesis in the host organism chosen for fatty acid derivative production.

[0106] The second step, the oxidative decarboxylation of the α-ketoacids to the corresponding branched-chain acyl-

CoA, is catalyzed by a branched-chain α-keto acid dehydrogenase complexes (bkd; EC 1.2.4.4.) (Denoya et al. J. Bacteriol. 177:pp. 3504, 1995), which consist of E1α/β (decarboxylase), E2 (dihydrolipoyl transacylase) and E3 (dihydrolipoyl dehydrogenase) subunits and are similar to pyruvate and α-ketoglutarate dehydrogenase complexes. **Table 2** shows potential *bkd* genes from several microorganisms, that can be expressed in a production host to provide branched-chain acyl-CoA precursors. Basically, every microorganism that possesses brFAs and/or grows on branched-chain amino acids can be used as a source to isolate *bkd* genes for expression in production hosts such as, for example, *E. coli.* Furthermore, *E. coli* has the E3 component (as part of its pyruvate dehydrogenase complex; lpd, EC 1.8.1.4, Genbank accession NP_414658), it can therefore, be sufficient to only express the *E1 α/β* and *E2 bkd* genes.

Table 2

Bkd genes from selected microorganisms		
Organism	Gene	Genbank Accession #
Streptomyces coelicolor	*bkdA1* (E1α) *bkdB1* (E1β) *bkdC1* (E2)	NP_ 628006 NP_628005 NP_638004
Streptomyces coelicolor	*bkdA2* (E1α) *bkdB2* (E1β) *bkdC2* (E2)	NP_733618 NP_628019 NP_628018
Streptomyces avermitilis	*bkdA* (E1a) *bkdB* (E1b) *bkdC* (E2)	BAC72074 BAC72075 BAC72076
Streptomyces avermitilis	*bkdF* (E1α) *bkdG* (E1β) *bkdH (E2)*	BAC72088 BAC72089 BAC72090
Bacillus subtilis	*bkdAA* (E1α) *bkdAB* (E1β) *bkdB* (E2)	NP_390288 NP_390288 NP_390288
Pseudomonas putida	*bkdA1* (E1α) *bkdA2* (E1β) *bkdC* (E2)	AAA65614 AAA65615 AAA65617

[0107] In another example, isobutyryl-CoA can be made in a production host, for example in *E. coli* through the coexpression of a crotonyl-CoA reductase (Ccr, EC 1.1.1.9) and isobutyryl-CoA mutase (large subunit IcmA, EC 5.4.99.2; small subunit IcmB, EC 5.4.99.13) (Han and Reynolds J. Bacteriol. 179:pp. 5157, 1997). Crotonyl-CoA is an intermediate in fatty acid biosynthesis in *E. coli* and other microorganisms. Examples for *ccr* and *icm* genes from selected microorganisms are given in **Table 3.**

Table 3

***Ccr* and *icm* genes from selected microorganisms**		
Organism	**Gene**	Genbank Accession #
Streptomyces coelicolor	*ccr* *icmA* *icmB*	NP_630556 NP_629554 NP_630904
Streptomyces cinnamonensis	*ccr* *icmA* *icmB*	AAD53915 AAC08713 AJ246005

[0108] In addition to expression of the *bkd* genes (see above), the initiation of brFA biosynthesis utilizes β-ketoacyl-acyl-carrier-protein synthase III (FabH, EC 2.3.1.41) with specificity for branched chain acyl CoAs (Li et al. J. Bacteriol. 187:pp. 3795, 2005). Examples of such FabHs are listed in **Table 4**. *FabH* genes that are involved in fatty acid biosynthesis of any brFA-containing microorganism can be expressed in a production host. The Bkd and FabH enzymes from production hosts that do not naturally make brFA may not support brFA production and therefore, Bkd and FabH can be expressed recombinantly. Similarly, the endogenous level of Bkd and FabH production may not be sufficient to produce brFA, therefore, they can be over-expressed. Additionally, other components of fatty acid biosynthesis machinery can be expressed such as acyl carrier proteins (ACPs) and β-ketoacyl-acyl-carrier-protein synthase II candidates are acyl carrier proteins (ACPs) and β-ketoacyl-acyl-carrier-protein synthase II (*fabF,* EC 2.3.1.41) (candidates are listed in **Table 4**). In addition to expressing these genes, some genes in the endogenous fatty acid biosynthesis pathway may be attenuated in the production host. For example, in *E. coli* the most likely candidates to interfere with brFA biosynthesis are *fabH* (Genbank accession # NP_415609) and/or *fabF* genes (Genbank accession # NP_415613).

[0109] As mentioned above, through the combination of expressing genes that support brFA synthesis and alcohol synthesis branched chain alcohols can be produced. For example, when an alcohol reductase such as Acr1 from Acinetobacter *baylyi* ADP1 is coexpressed with a *bkd* operon, *E. coli* can synthesize isopentanol, isobutanol or 2-methyl butanol. Similarly, when Acr1 is coexpressed with ccr/icm genes, *E. coli* can synthesize isobutanol.

[0110] In order to convert a production host such as *E. coli* into an organism capable of synthesizing ω-cyclic fatty acids (cyFAs), several genes need to be introduced and expressed that provide the cyclic precursor cyclohexylcarbonyl-CoA (Cropp et al. Nature Biotech. 18:pp. 980, 2000). The genes listed in **Table 4** (*fabH, ACP* and *fabF*) can then be expressed to allow initiation and elongation of ω-cyclic fatty acids. Alternatively, the homologous genes can be isolated from microorganisms that make cyFAs and expressed in *E. coli.*

Table 4

***FabH, ACP* and *fabF* genes from selected microorganisms with brFAs**		
Organism	Gene	Genbank Accession #
Streptomyces coelicolor	*fabH1* *ACP* *fabF*	NP_626634 NP_626635 NP_626636
Streptomyces avermitilis	*fabH3* *fabC3 (ACP)* *fabF*	NP_823466 NP_823467 NP_823468
Bacillus subtilis	*fabH_A* *fabH_B* *ACP* *fabF*	NP_389015 NP_388898 NP_389474 NP_389016
Stenotrophomonas maltophilia	SmalDRAFT_0818 (*FabH*) SmalDRAFT_0821 (*ACP*) SmalDRAFT_0822 (*FabF*)	ZP_01643059 ZP_01643063 ZP_01643064
Legionella pneumophila	*FabH* *ACP* *fabF*	YP_123672 YP_123675 YP_123676

[0111] Expression of the following genes are sufficient to provide cyclohexylcarbonyl-CoA in *E. coli: ansJ, ansK, ansL, chcA* and *ansM* from the ansatrienin gene cluster of *Streptomyces collinus* (Chen et al., Eur. J. Biochem. 261:pp. 1999, 1999) or *plmJ, plmK, plmL, chcA* and *plmM* from the phoslactomycin B gene cluster of *Streptomyces* sp. HK803 (Palaniappan et al., J. Biol. Chem. 278:pp. 35552, 2003) together with the *chcB* gene (Patton et al. Biochem., 39:pp. 7595, 2000) from *S. collinus, S. avermitilis* or *S. coelicolor* (see **Table 5** for Genbank accession numbers).

Table 5

Genes for the synthesis of cyclohexylcarbonyl-CoA		
Organism	Gene	Genbank Accession #
Streptomyces collinus	*ansJK* *ansL* *chcA* *ansL* *chcB*	U72144* AF268489
Streptomyces sp. HK803	*pmlJK* *pmlL* *chcA* *pmlM*	AAQ84158 AAQ84159 AAQ84160 AAQ84161
Streptomyces coelicolor	*chcB/caiD*	NP_ 629292
Streptomyces avermitilis	*chcB/caiD*	NP_ 629292
Only *chcA* is annotated in Genbank entry U72144, *ansJKLM* are according to Chen et al. (Eur. J. Biochem. 261:pp. 1999, 1999)		

[0112] The genes listed in **Table 4** (*fabH, ACP* and *fabF*) are sufficient to allow initiation and elongation of ω-cyclic fatty acids, because they can have broad substrate specificity. In the event that coexpression of any of these genes with the ansJKLM/chcAB or pmlJKLM/chcAB genes from **Table 5** does not yield cyFAs, *fabH, ACP* and/or fabF homologs from microorganisms that make cyFAs can be isolated (e.g. by using degenerate PCR primers or heterologous DNA probes) and coexpressed. **Table 6** lists selected microorganisms that contain ω-cyclic fatty acids.

Table 6

Examples of microorganisms that contain ω-cyclic fatty acids	
Organism	Reference
Curtobacterium pusillum	ATCC19096
Alicyclobacillus acidoterrestris	ATCC49025
Alicyclobacillus acidocaldarius	ATCC27009
Alicyclobacillus cycloheptanicum *	Moore, J. Org. Chem. 62:pp. 2173, 1997.
*uses cycloheptylcarbonyl-CoA and not cyclohexylcarbonyl-CoA as precursor for cyFA biosynthesis	

C. Ester characteristics

[0113] One of ordinary skill in the art will appreciate that an ester includes an A side and a B side. As described herein, the B side is contributed by a fatty acid produced from *de novo* synthesis in the host organism. In some instances where the host is additionally engineered to make alcohols, including fatty alcohols, the A side is also produced by the host organism. In yet other examples the A side can be provided in the medium. As described herein, by selecting the desired thioesterase genes the B side, and when fatty alcohols are being made the A side, can be designed to be have certain carbon chain characteristics. These characteristics include points of unsaturation, branching, and desired carbon chain lengths. Exemplary methods of making long chain fatty acid esters, wherein the A and B side are produced by the production host are provided in Example 6, below. Similarly, Example 5 provides methods of making medium chain fatty acid esters. When both the A and B side are contributed by the production host and they are produced using fatty acid biosynthetic pathway intermediates they will have similar carbon chain characteristics. For example, at least 50%, 60%, 70%, or 80% of the fatty acid esters produced will have A sides and B sides that vary by 6, 4, or 2 carbons in length. The A side and the B side will also display similar branching and saturation levels.

[0114] In addition to producing fatty alcohols for contribution to the A side, the host can produce other short chain alcohols such as ethanol, propanol, isopropanol, isobutanol, and butanol for incorporation on the A side using techniques well known in the art. For example, butanol can be made by the host organism. To create butanol producing cells, the

LS9001 strain (described in Example 1, below) can be further engineered to express atoB (acetyl-CoA acetyltransferase) from *Escherichia coli* K12, β-hydroxybutyryl-CoA dehydrogenase from *Butyrivibrio fibrisolvens,* crotonase from *Clostridium beijerinckii,* butyryl CoA dehydrogenase from *Clostridium beijerinckii,* CoA-acylating aldehyde dehydrogenase (ALDH) from *Cladosporium fulvum,* and adhE encoding an aldehyde-alchol dehydrogenase of *Clostridium acetobutylicum* in the pBAD24 expression vector under the *prpBCDE* promoter system. Similarly, ethanol can be produced in a production host using the methods taught by Kalscheuer et al., Microbiology 152:2529-2536, 2006).

IV. Fermentation

[0115] The production and isolation of fatty acid derivatives can be enhanced by employing specific fermentation techniques. One method for maximizing production while reducing costs is increasing the percentage of the carbon source that is converted to hydrocarbon products. During normal cellular lifecycles carbon is used in cellular functions including producing lipids, saccharides, proteins, organic acids, and nucleic acids. Reducing the amount of carbon necessary for growth-related activities can increase the efficiency of carbon source conversion to output. This can be achieved by first growing microorganisms to a desired density, such as a density achieved at the peak of the log phase of growth. At such a point, replication checkpoint genes can be harnessed to stop the growth of cells. Specifically, quorum sensing mechanisms (reviewed in Camilli and Bassler Science 311:1113,2006; Venturi FEMS Microbio Rev 30:274-291, 2006; and Reading and Sperandio FEMS Microbiol Lett 254:1-11, 2006) can be used to activate genes such as p53, p21, or other checkpoint genes. Genes that can be activated to stop cell replication and growth in *E. coli* include umuDC genes, the over-expression of which stops the progression from stationary phase to exponential growth (Murli et al., J. of Bact. 182:1127, 2000). UmuC is a DNA polymerase that can carry out translesion synthesis over non-coding lesions - the mechanistic basis of most UV and chemical mutagenesis. The *umuDC* gene products are used for the process of translesion synthesis and also serve as a DNA damage checkpoint. UmuDC gene products include UmuC, UmuD, umuD', $UmuD'_2C$, $UmuD'_2$ and $UmuD_2$. Simultaneously, the product producing genes would be activated, thus minimizing the need for replication and maintenance pathways to be used while the fatty acid derivative is being made.

[0116] The percentage of input carbons converted to hydrocarbon products is a cost driver. The more efficient (i.e. the higher the percentage), the less expensive the process. For oxygen-containing carbon sources (i.e. glucose and other carbohydrate based sources), the oxygen must be released in the form of carbon dioxide. For every 2 oxygen atoms released, a carbon atom is also released leading to a maximal theoretical metabolic efficiency of -34% (w/w) (for fatty acid derived products). This figure, however, changes for other hydrocarbon products and carbon sources. Typical efficiencies in the literature are ~<5%. Engineered microorganisms which produce hydrocarbon products can have greater than 1, 3, 5, 10, 15, 20, 25, and 30% efficiency. In one example microorganisms will exhibit an efficiency of about 10% to about 25%. In other examples, such microorganisms will exhibit an efficiency of about 25% to about 30%, and in other examples such microorganisms will exhibit >30% efficiency.

[0117] In some examples where the final product is released from the cell, a continuous process can be employed. In this approach, a reactor with organisms producing fatty acid derivatives can be assembled in multiple ways. In one example, a portion of the media is removed and let to sit. Fatty acid derivatives are separated from the aqueous layer, which will in turn, be returned to the fermentation chamber.

[0118] In one example, the fermentation chamber will enclose a fermentation that is undergoing a continuous reduction. In this instance, a stable reductive environment would be created. The electron balance would be maintained by the release of carbon dioxide (in gaseous form). Efforts to augment the NAD/H and NADP/H balance can also facilitate in stabilizing the electron balance.

[0119] The availability of intracellular NADPH can be also enhanced by engineering the production host to express an NADH:NADPH transhydrogenase. The expression of one or more NADH:NADPH transhydrogenase converts the NADH produced in glycolysis to NADPH which enhances the production of fatty acid derivatives.

[0120] Disclosed herein is a system for continuously producing and exporting fatty acid derivatives out of recombinant host microorganisms via a transport protein. Many transport and efflux proteins serve to excrete a large variety of compounds and can be evolved to be selective for a particular type of fatty acid derivatives. Thus, in some embodiments an exogenous DNA sequence encoding an ABC transporter will be functionally expressed by the recombinant host microorganism, so that the microorganism exports the fatty acid derivative into the culture medium. In one example, the ABC transporter is an ABC transporter from *Caenorhabditis elegans, Arabidopsis thalania, Alkaligenes eutrophus* or *Rhodococcus erythropolis* (locus AAN73268). In another example, the ABC transporter is an ABC transporter chosen from CER5 (locuses At1g51500 or AY734542), AtMRP5, AmiS2 and AtPGP1. In some examples, the ABC transporter is CER5. In yet another example, the CER5 gene is from Arabidopsis (locuses At1g51500, AY734542, At3g21090 and At1g51460).

[0121] The transport protein, for example, can also be an efflux protein selected from: AcrAB, TolC and AcrEF from *E. coli,* or tll1618, tll1619 and tll0139 from *Thermosynechococcus elongatus* BP-1.

[0122] In addition, the transport protein can be, for example, a fatty acid transport protein (FATP) selected from

Drosophila melanogaster, Caenorhabditis elegans, Mycobacterium tuberculosis or *Saccharomyces cerevisiae* or any one of the mammalian FATP's. The FATPs can additionally be resynthesized with the membranous regions reversed in order to invert the direction of substrate flow. Specifically, the sequences of amino acids composing the hydrophilic domains (or membrane domains) of the protein, could be inverted while maintaining the same codons for each particular amino acid. The identification of these regions is well known in the art.

[0123] Production hosts can also be chosen for their endogenous ability to release fatty acid derivatives. The efficiency of product production and release into the fermentation broth can be expressed as a ratio intracellular product to extracellular product. In some examples the ratio can be 5:1, 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4, or 1:5.

[0124] The production host can be additionally engineered to express recombinant cellulosomes, such as those described in PCT application number PCT/US2007/003736, which will allow the production host to use cellulosic material as a carbon source. For example, the production host can be additionally engineered to express invertases (EC 3.2.1.26) so that sucrose can be used as a carbon source.

[0125] Similarly, the production host can be engineered using the teachings described in U.S. Patent Numbers 5,000,000, 5,028,539, 5,424,202, 5,482,846, and 5,602,030 to Ingram et al. so that the production host can assimilate carbon efficiently and use cellulosic materials as carbons sources.

IV. Post production processing

[0126] The fatty acid derivatives produced during fermentation can be separated from the fermentation media. Any technique known for separating fatty acid derivatives from aqueous media can be used. One exemplary separation process provided herein is a two phase (bi-phasic) separation process. This process involves fermenting the genetically engineered production hosts under conditions sufficient to produce a fatty acid derivative, allowing the derivative to collect in an organic phase and separating the organic phase from the aqueous fermentation broth. This method can be practiced in both a batch and continuous fermentation setting.

[0127] Bi-phasic separation uses the relative immisiciblity of fatty acid derivatives to facilitate separation. Immiscible refers to the relative inability of a compound to dissolve in water and is defined by the compounds partition coefficient. The partition coefficient, P, is defined as the equilibrium concentration of compound in an organic phase (in a bi-phasic system the organic phase is usually the phase formed by the fatty acid derivative during the production process, however, in some examples an organic phase can be provided (such as a layer of octane to facilitate product separation) divided by the concentration at equilibrium in an aqueous phase (i.e. fermentation broth). When describing a two phase system the P is usually discussed in terms of logP. A compound with a logP of 10 would partition 10:1 to the organic phase, while a compound of logP of 0.1 would partition 10:1 to the aqueous phase. One or ordinary skill in the art will appreciate that by choosing a fermentation broth and the organic phase such that the fatty acid derivative being produced has a high logP value, the fatty acid derivative will separate into the organic phase, even at very low concentrations in the fermentation vessel.

[0128] The fatty acid derivatives produced by the methods described herein will be relatively immiscible in the fermentation broth, as well as in the cytoplasm. Therefore, the fatty acid derivative will collect in an organic phase either intracellularly or extracellularly. The collection of the products in an organic phase will lessen the impact of the fatty acid derivative on cellular function and will allow the production host to produce more product. Stated another way, the concentration of the fatty acid derivative will not have as significant of an impact on the host cell.

[0129] The fatty alcohols, fatty acid esters, waxes, and hydrocarbons produced as described herein allow for the production of homogeneous compounds wherein at least 60%, 70%, 80%, 90%, or 95% of the fatty alcohols, fatty acid esters, and waxes produced will have carbon chain lengths that vary by less than 4 carbons, or less than 2 carbons. These compounds can also be produced so that they have a relatively uniform degree of saturation, for example at least 60%, 70%, 80%, 90%, or 95% of the fatty alcohols, fatty acid esters, hydrocarbons and waxes will be mono-, di-, or tri-unsaturated. These compounds can be used directly as fuels, personal care additives, nutritional supplements. These compounds can also be used as feedstock for subsequent reactions for example transesterification, hydrogenation, catalytic cracking via either hydrogenation, pyrolisis, or both or epoxidations reactions to make other products.

V. Fuel Compositions

[0130] The fatty acid derivatives described herein can be used as fuel. One of ordinary skill in the art will appreciate that depending upon the intended purpose of the fuel different fatty acid derivatives can be produced and used. For example, for automobile fuel that is intended to be used in cold climates a branched fatty acid derivative may be desirable and using the teachings provided herein, branched hydrocarbons, fatty acid esters, and alcohols can be made. Using the methods described herein fuels comprising relatively homogeneous fatty acid derivatives that have desired fuel qualities can be produced. Such fuels can be characterized by carbon fingerprinting, their lack of impurities when compared to petroleum derived fuels or bio-diesel derived from triglycerides and, moreover, the fatty acid derivative based fuels can be combined with other fuels or fuel additives to produce fuels having desired properties.

A. Carbon fingerprinting

[0131] Biologically produced fatty acid derivatives represent a new feedstock for fuels, such as alcohols, diesel and gasoline. Some biofuels made using fatty acid derivatives have not been produced from renewable sources and as such, are new compositions of matter. These new fuels can be distinguished from fuels derived form petrochemical carbon on the basis of dual carbon-isotopic fingerprinting. Additionally, the specific source of biosourced carbon (e.g. glucose vs. glycerol) can be determined by dual carbon-isotopic fingerprinting (see, US Patent Number 7,169,588, which is herein incorporated by reference).

[0132] This method usefully distinguishes chemically-identical materials, and apportions carbon in products by source (and possibly year) of growth of the biospheric (plant) component. The isotopes, ^{14}C and ^{13}C, bring complementary information to this problem. The radiocarbon dating isotope (^{14}C), with its nuclear half life of 5730 years, clearly allows one to apportion specimen carbon between fossil ("dead") and biospheric ("alive") feedstocks [Currie, L. A. "Source Apportionment of Atmospheric Particles," Characterization of Environmental Particles, J. Buffle and H. P. van Leeuwen, Eds., 1 of Vol. I of the IUPAC Environmental Analytical Chemistry Series (Lewis Publishers, Inc) (1992) 3 74]. The basic assumption in radiocarbon dating is that the constancy of ^{14}C concentration in the atmosphere leads to the constancy of ^{14}C in living organisms. When dealing with an isolated sample, the age of a sample can be deduced approximately by the relationship t=(-5730/0.693)ln(A/A.sub.O) (Equation 5) where t=age, 5730 years is the half-life of radiocarbon, and A and A.sub.O are the specific ^{14}C activity of the sample and of the modem standard, respectively [Hsieh, Y., Soil Sci. Soc. Am J., 56, 460, (1992)]. However, because of atmospheric nuclear testing since 1950 and the burning of fossil fuel since 1850, ^{14}C has acquired a second, geochemical time characteristic. Its concentration in atmospheric CO2--and hence in the living biosphere--approximately doubled at the peak of nuclear testing, in the mid-1960s. It has since been gradually returning to the steady-state cosmogenic (atmospheric) baseline isotope rate ($^{14}C/^{12}C$) of ca. $1.2x10^{12}$, with an approximate relaxation "half-life" of 7-10 years. (This latter half-life must not be taken literally; rather, one must use the detailed atmospheric nuclear input/decay function to trace the variation of atmospheric and biospheric ^{14}C since the onset of the nuclear age.) It is this latter biospheric ^{14}C time characteristic that holds out the promise of annual dating of recent biospheric carbon. ^{14}C can be measured by accelerator mass spectrometry (AMS), with results given in units of "fraction of modern carbon" (f_M). f_M is 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 approx 1.1.

[0133] The stable carbon isotope ratio ($^{13}C/^{12}C$) provides a complementary route to source discrimination and apportionment. The $^{13}C/^{12}C$ ratio in a given biosourced material is a consequence of the $^{13}C/^{12}C$ ratio in atmospheric carbon dioxide at the time the carbon dioxide is fixed and also reflects the precise metabolic pathway. Regional variations also occur. Petroleum, C3 plants (the broadleaf), C.sub.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 C3 and C4 plants analyze differently than materials derived from the carbohydrate components of the same plants as a consequence of the metabolic pathway. Within the precision of measurement, ^{13}C shows large variations due to isotopic fractionation effects, the most significant of which for the instant invention is the photosynthetic mechanism. The major cause of differences in the carbon isotope ratio in plants is closely associated with differences in the pathway of photosynthetic carbon metabolism in the plants, particularly the reaction occurring during the primary carboxylation, *i.e.,* the initial fixation of atmospheric CO_2. Two large classes of vegetation are those that incorporate the "C3" (or Calvin-Benson) photosynthetic cycle and those that incorporate the "C4" (or Hatch-Slack) photosynthetic cycle. C3 plants, such as hardwoods and conifers, are dominant in the temperate climate zones. In C3 plants, the primary CO_2 fixation or carboxylation reaction involves the enzyme ribulose-1,5-diphosphate carboxylase and the first stable product is a 3-carbon compound. C4 plants, on the other hand, include such plants as tropical grasses, corn and sugar cane. In C4 plants, an additional carboxylation reaction involving another enzyme, phosphoenol-pyruvate carboxylase, is the primary carboxylation reaction. The first stable carbon compound is a 4-carbon acid which is subsequently decarboxylated. The CO_2 thus released is refixed by the C3 cycle.

[0134] Both C4 and C3 plants exhibit a range of $^{13}C/^{12}C$ isotopic ratios, but typical values are ca. -10 to -14 per mil (C4) and -21 to -26 per mil (C3) [Weber et al., J. Agric. Food Chem., 45, 2942 (1997)]. Coal and petroleum fall generally in this latter range. 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 in parts per thousand (per mil), abbreviated %, and are calculated as follows:

$$\delta^{13}C = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}}{(^{13}C/^{12}C)_{standard}} \times 100\% \quad \text{(Equation 6)}$$

Since the PDB reference material (RM) has been exhausted, a series of alternative RMs have been developed in cooperation with the IAEA, USGS, NIST, and other selected international isotope laboratories. Notations for the per mil deviations from PDB is $\Delta^{13}C$, Measurements are made on CO_2 by high precision stable ratio mass spectrometry (IRMS) on molecular ions of masses 44, 45 and 46.

[0135] The fatty acid derivatives and the associated biofuels, chemicals, and mixtures may be completely distinguished from their petrochemical derived counterparts on the basis of ^{14}C (fM) and dual carbon-isotopic fingerprinting, indicating new compositions of matter.

[0136] The fatty acid derivatives described herein have utility in the production of biofuels and chemicals. The new fatty acid derivative based product compositions provided by the instant invention additionally may be distinguished on the basis of dual carbon-isotopic fingerprinting from those materials derived solely from petrochemical sources. The ability to distinguish these products is beneficial in tracking these materials in commerce. For example, fuels or chemicals comprising both "new" and "old" carbon isotope profiles may be distinguished from fuels and chemicals made only of "old" materials. Hence, the instant materials may be followed in commerce on the basis of their unique profile and for the purposes of defining competition, and for determining shelf life.

[0137] In some examples a biofuel composition is made that includes a fatty acid derivative having $\delta^{13}C$ of from about -10.9 to about -15.4, wherein the fatty acid derivative accounts for at least about 85% of biosourced material (derived from a renewable resource such as cellulosic materials and sugars) in the composition. In other examples, the biofuel composition includes a fatty acid derivative having the formula

$$X\text{-}(CH(R))_nCH_3$$

wherein X represents CH_3, $-CH_2OR^1$; $-C(O)OR^2$; or $-C(O)NR^3R^4$;
R is, for each n, independently absent, H or lower aliphatic;
n is an integer from 8 to 34, such as from 10 to 24; and
R^1, R^2, R^3 and R^4 independently are selected from H and lower alkyl. Typically, when R is lower aliphatic, R represents a branched, unbranched or cyclic lower alkyl or lower alkenyl moiety. Exemplary R groups include, without limitation, methyl, isopropyl, isobutyl, *sec*-butyl, cyclopentenyl and the like. The fatty acid derivative is additionally characterized as having a $\delta^{13}C$ of from about - 10.9 to about -15.4; and the fatty acid derivative accounts for at least about 85% of biosourced material in the composition. In some examples the fatty acid derivative in the biofuel composition is characterized by having a fraction of modern carbon (f_M ^{14}C) of at least about 1.003, 1.010, or 1.5.

B. Fatty acid derivatives

[0138] The centane number (CN), viscosity, melting point, and heat of combustion for various fatty acid esters have been characterized in for example, Knothe, Fuel Processing Technology 86:1059-1070, 2005, which is herein incorporated by reference. Using the teachings provided herein a production host can be engineered to produce anyone of the fatty acid esters described in the Knothe, Fuel Processing Technology 86:1059-1070, 2005.

[0139] Alcohols (short chain, long chain, branched or unsaturated) can be produced by the production hosts described herein. Such alcohols can be used as fuels directly or they can be used to create an ester, i.e. the A side of an ester as described above. Such ester alone or in combination with the other fatty acid derivatives described herein are useful a fuels.

[0140] Similarly, hydrocarbons produced from the microorganisms described herein can be used as biofuels. Such hydrocarbon based fuels can be designed to contain branch points, defined degrees of saturation, and specific carbon lengths. When used as biofuels alone or in combination with other fatty acid derivatives the hydrocarbons can be additionally combined with additives or other traditional fuels (alcohols, diesel derived from triglycerides, and petroleum based fuels).

C. Impurities

[0141] The fatty acid derivatives described herein are useful for making bio-fuels. These fatty acid derivatives are made directly from fatty acids and not from the chemical processing of triglycerides. Accordingly, fuels comprising the disclosed fatty acid derivatives will contain less of the impurities than are normally associated with bio-fuels derived from triglycerides, such as fuels derived from vegetable oils and fats.

[0142] The crude fatty acid derivative bio-fuels described herein (prior to mixing the fatty acid derivative with other fuels such as traditional fuels) will contain less transesterification catalyst than petrochemical diesel or bio-diesel. For example, the fatty acid derivative can contain less than about 2%, 1.5%, 1.0%, 0.5%, 0.3%, 0.1%. 0.05%, or 0% of a transesterification catalyst or an impurity resulting from a transesterification catalyst. Transesterification catalysts include for example, hydroxide catalysts such as NaOH, KOH, LiOH, and acidic catalysts, such as mineral acid catalysts and Lewis acid catalysts. Catalysts and impurities resulting from transesterification catalysts include, without limitation, tin, lead, mercury, cadmium, zinc, titanium, zirconium, hafnium, boron, aluminum, phosphorus, arsenic, antimony, bismuth, calcium, magnesium, strontium, uranium, potassium, sodium, lithium, and combinations thereof.

[0143] Similarly, the crude fatty acid derivative bio-fuels described herein (prior to mixing the fatty acid derivative with other fuels such as petrochemical diesel or bio-diesel) will contain less glycerol (or glycerin) than bio-fuels made from triglycerides. For example, the fatty acid derivative can contain less than about 2%, 1.5%, 1.0%, .5%, .3%, .1%, .05%, or 0% glycerol.

[0144] The crude biofuel derived from fatty acid derivatives will also contain less free alcohol (i.e. alcohol that is used to create the ester) than bio-diesel made from triglycerides. This is in-part due to the efficiency of utilization of the alcohol by the production host. For example, the fatty acid derivative will contain less than about 2%, 1.5%, 1.0%, .5%, .3%, .1%, .05%, or 0% free alcohol.

[0145] Biofuel derived from the disclosed fatty acid derivatives can be additionally characterized by its low concentration of sulfur compared to petroleum derived diesel. For example, biofuel derived from fatty acid derivatives can have less than about 2%, 1.5%, 1.0%, .5%, .3%, .1%, .05%, or 0% sulfur.

D. Additives

[0146] Fuel additives are used to enhance the performance of a fuel or engine. For example, fuel additives can be used to alter the freezing/gelling point, cloud point, lubricity, viscosity, oxidative stability, ignition quality, octane level, and flash point. In the United States, all fuel additives must be registered with Environmental Protection Agency and companies that sell the fuel additive and the name of the fuel additive are publicly available on the agency website and also by contacting the agency. One of ordinary skill in the art will appreciate that the fatty acid derivatives described herein can be mixed with one or more such additives to impart a desired quality.

[0147] One of ordinary skill in the art will also appreciate that the fatty acid derivatives described herein are can be mixed with other fuels such as bio-diesel derived from triglycerides, various alcohols such as ethanol and butanol, and petroleum derived products such as gasoline. In some examples, a fatty acid derivative, such as C16:1 ethyl ester or C18:1 ethyl ester, is produced which has a low gel point. This low gel point fatty acid derivative is mixed with bio-diesel made from triglycerides to lessen the overall gelling point of the fuel. Similarly, a fatty acid derivative such as C16:1 ethyl ester or C18:1 ethyl ester can be mixed with petroleum derived diesel to provide a mixture that is at least and often greater than 5% biodiesel. In some examples, the mixture includes at least 20% or greater of the fatty acid derivative.

[0148] For example, a biofuel composition can be made that includes at least about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90% or 95% of a fatty acid derivative that includes a carbon chain that is 8:0, 10:0, 12:0, 14:0, 14:1, 16:0, 16:1, 18:0,18:1, 18:2, 18:3, 20:0, 20:1, 20:2, 20:3, 22:0, 22:1 or 22:3. Such biofuel compositions can additionally include at least one additive selected from a cloud point lowering additive that can lower the cloud point to less than about 5°C, or 0°C, a surfactant, or a microemulsion, at least about 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70% or 80%, 85%, 90%, or 95% diesel fuel from triglycerides, petroleum derived gasoline or diesel fuel from petroleum.

EXAMPLES

[0149]

Fig. 1 is a diagram of the FAS pathway showing the enzymes directly involved in the synthesis of acyl-ACP. To increase the production of waxes/fatty acid esters, and fatty alcohols one or more of the enzymes can be over expressed or mutated to reduce feedback inhibition. Additionally, enzymes that metabolize the intermediates to make non-fatty acid based products (side reactions) can be functionally deleted or attenuated to increase the flux of carbon through the fatty acid biosynthetic pathway. Examples 1, 2, and 8 below provide exemplary production hosts that have been modified to increase fatty acid production.

Figs. 2, 3 and 4 show biosynthetic pathways that can be engineered to make fatty alcohols and wax/fatty acid esters, respectively. As illustrated in **Fig. 2** the conversion of each substrate (acetyl-CoA, malonyl-CoA, acyl-ACP, fatty acid, and acyl-CoA) to each product (acetyl-CoA, malonyl-CoA, acyl-ACP, fatty acid, and acyl-CoA) can be accomplished using several different polypeptides that are members of the enzyme classes indicated. The Examples below describe microorganisms that have been engineered or can be engineered to produce specific fatty alcohols and waxes/fatty acid esters and hydrocarbons.

Example 1, Production Host Construction

[0150] An exemplary production host is LS9001. LS9001 was produced by modifying C41(DE3) from Overexpress.com (Saint Beausine, France) to functionally deleting the *fadE* gene (acyl-CoA dehydrogenase).

[0151] Briefly, the *fadE* knock-out strain *of E. coli* was made using primers YafV_NotI and Ivry_OI to amplify about 830 bp upstream of *fadE* and primers Lpcaf_ol and LpcaR_Bam to amplify about 960 bp downstream of *fadE*. Overlap PCR was used to create a construct for in frame deletion of the complete *fadE* gene. The *fadE* deletion construct was cloned into the temperature sensitive plasmid pKOV3, which contained a *SacB* gene for counterselection, and a chromosomal deletion of *fadE* was made according to the method of Link et al., J. Bact. 179:6228-6237, 1997. The resulting strain was not capable of degrading fatty acids and fatty acyl-CoAs (this functional deletion is herein designated as *ΔfadE*)

[0152] Additional modifications that can be included in a production host include introducing a plasmid carrying the four genes which are responsible for acetyl-CoA carboxylase activity in *E. coli* (accA, B, C, and D, Accessions: NP_414727, NP_417721, NP_417722, NP_416819, EC 6.4.1.2). The accABCD genes were cloned in two steps as bicistronic operons into the *Nco*I/*Hind*III and *Nde*I/*Avr*II sites of pACYCDuet-1 (Novagen, Madison, WI) the resulting plasmid was termed pAS004.126.

[0153] Additional modifications that can be included in a production host include the following: over-expression of *aceEF* (encoding the E1p dehydrogase component and the E2p dihydrolipoamide acyltransferase component of the pyruvate and 2-oxoglutarate dehydrogenase complexes); and *fabH/fabD/fabG/acpP/fabF* (encoding FAS) from any organism known in the art to encode such proteins, including for example *E. coli, Nitrosomonas europaea* (ATCC 19718), *Bacillus subtilis, Saccharomyces cerevisiae, Streptomyces spp, Ralstonia, Rhodococcus, Corynebacteria, Brevibacteria, Mycobacteria,* oleaginous yeast, and the like can be expressed in the production host. Similarly, production hosts can be engineered to express *accABCD* (encoding acetyl co-A carboxylase) from *Pisum savitum* instead of, or in addition to, the *E.coli* homologues. However, when the production host is also producing butanol it is less desirable to express the *Pisum savitum homologue.*

[0154] In some exemplary production hosts, genes can be knocked out or attenuated using the method of Link, et al., J. Bacteriol. 179:6228-6237, 1997. For example, genes that can be knocked out or attenuated include *gpsA* (encoding biosynthetic sn-glycerol 3-phosphate dehydrogenase, accession NP_418065, EC: 1.1.1.94); *ldhA* (encoding lactate dehydrogenase, accession NP_415898, EC: 1.1.1.28); *pflb* (encoding formate acetyltransferase 1, accessions: P09373, EC: 2.3.1.54); *adhE* (encoding alcohol dehydrogenase, accessions: CAA47743, EC: 1.1.1.1, 1.2.1.10); *pta* (encoding phosphotransacetylase, accessions: NP_416800, EC: 2.3.1.8); *poxB* (encoding pyruvate oxidase, accessions: NP_415392, EC: 1.2.2.2); *ackA* (encoding acetate kinase, accessions: NP_416799, EC: 2.7.2.1) and combinations thereof.

[0155] Similarly, the PlsB[D311E] mutation can be introduced into LS9001 to attenuate PlsB using the method described above for the *fadE* deletion. Once introduced, this mutation will decrease the amount of carbon being diverted to phospholipid production (see, **Fig. 1**). Briefly, an allele encoding PlsB[D311E] is made by replacing the GAC codon for aspartate 311 with a GAA codon for glutamate. The altered allele is made by gene synthesis and the chromosomal *plsB* wildtype allele is exchanged for the mutant *plsB*[D311E] allele using the method of *Link et al.* (see above).

Example 2, Production host modifications

[0156] The following plasmids were constructed for the expression of various proteins that are used in the synthesis of fatty acid derivatives. The constructs were made using standard molecular biology methods and all the cloned genes were put under the control of IPTG-inducible promoters (T7, tac or lac promoters).

[0157] The *'tesA* gene (thioesterase A gene accession NP_415027 without leader sequence (Cho and Cronan, The J. of Biol Chem., 270:4216-9, 1995, EC: 3.1.1.5, 3.1.2.-) *of E. coli* was cloned into *Nde*I/*Avr*II digested pETDuet-1 (pETDuet-1 described herein is available from Novagen, Madison, WI). Genes encoding for FatB-type plant thioesterases (TEs) from *Umbellularia California, Cuphea hookeriana* and *Cinnamonum camphorum* (accessions: UcFatB1=AAA34215, ChFatB2=AAC49269, ChFatB3=AAC72881, CcFatB=AAC49151 were individually cloned into three different vectors: (i) *Nde*I/*Avr*II digested pETDuet-1, (ii) *Xho*I/*Hind*III digested pBluescript KS+ (Stratagene, La Jolla, CA)(used to create N-terminal lacZ::TE fusion proteins) and (iii) *Xba*I/*Hind*III digested pMAL-c2X (New England Lab, Ipswich, MA) (used to create n-terminal MalE::TE fusions). The *fadD* gene (encoding acyl-CoA synthetase) from *E. coli* was cloned into a *Nco*I/*Hind*III digested pCDFDuet-1 derivative, which contained the *acr1* gene (acyl-CoA reductase) from *Acinetobacter baylyi* ADP1 within its *Nde*I/*Avr*II sites. **Table 7** provides a summary of the plasmids generated to make several exemplary production strains, one of ordinary skill in the art will appreciate that different plasmids and genomic modifications can be used to achieve similar strains.

Table 7

Summary of Plasmids used in Production hosts		
Plasmid	Source Organism Gene Product	Accession No., EC number
pETDuet-1-*tesA*	*E. coli* TesA	Accessions: NP_415027, EC: 3.1.1.5, 3.1.2.-
pETDuet-1-TEuc pBluescript-TEuc pMAL-c2X-TEuc	*Umbellularia California* UcFatB1	Q41635 AAA34215
pETDuet-1-TEch pBluescript-TEch pMAL-c2X-TEch	*Cuphea hookeriana* ChFatB2 ChFatB3	ABB71581 AAC49269 AAC72881
pETDuet-1-TEcc pBluescript-TEcc TEci	*Cinnamonum camphorum* CcFatB	AAC49151
pCDFDuet-1-fadD-acr1	*E. coli*	fadD:Accessions NP_416319, EC 6.2.1.3 acr1:Accessions YP_047869

[0158] The chosen expression plasmids contain compatible replicons and antibiotic resistance markers, so that a four-plasmid expression system can be established. Therefore, LS9001 can be co-transformed with (i) any of the TE-expressing plasmids, (ii) the FadD-expressing plasmid, which also expresses acr1 and (iii) wax synthase expression plasmid. When induced with IPTG, the resulting strain will produce increased concentrations of fatty-alcohols from carbon sources such as glucose. The carbon chain length and degree of saturation of the fatty alcohol produced is dependent on the thioesterase gene that is expressed.

Example 3, Production of fatty alcohol in the recombinant *E. coli* strain

[0159] Fatty alcohols were produced by expressing a thioesterase gene and an acyl-CoA reductase gene (FAR) exogenously in a production host. More specifically, plasmids pCDFDuet-1-fadD-acr1 (acyl-CoA reductase) and pETDuet-1-'tesA (thioesterase) were transformed into *E. coli* strain LS9001 (described in Example 1) and corresponding transformants were selected in LB plate supplemented with 100 mg/L of spectinomycin and 50 mg/L of carbenicillin. Four transformants of LS9001/pCDFDuet-1-fadD-acr1 were independently inoculated into 3 mL of M9 medium supplemented with 50 mg/L of carbenicillin and 100 mg/L of spectinomycin). The samples containing the transformants were grown in at 25°C in a shaker (250 rpm) until they reached 0.5 OD_{600}. 1.5 mL of each sample was transferred into a 250 mL flask containing 30 mL of the medium described above. The resulting culture was grown at 25°C in a shaker until the culture reached between 0.5 -1.0 OD_{600}. IPTG was then added to a final concentration of 1 mM. and growth continued for 40 hours.

[0160] The cells were then spun down at 4000 rpm and the cell pellets were suspended in 1.0 mL of methanol. 3 mL of ethyl acetate was then mixed with the suspended cells. 3 mL of H_2O were then added to the mixture and the mixture was sonicated for 20 minutes. The resulting sample was centrifuged at 4000 rpm for 5 minutes and the organic phase (the upper phase) which contained fatty alcohol and was subjected to GC/MS analysis. Total alcohol (including tetradecanol, hexadecanol, hexadecenol and octadecenol) yield was about 1-10 mg/L. When an *E. coli* strain carrying only empty vectors was cultured in the same way, only 0.2-0.5 mg/L of fatty alcohols were found in the ethyl acetate extract.

Example 4, Production and release of fatty alcohol from production host

[0161] Acr1 (acyl-CoA reductase) was expressed in *E. coli* grown on glucose as the sole carbon and energy source. The *E. coli* produced small amounts of fatty alcohols such as dodecanol (C12:0-OH), tetradecanol (C14:0-OH) and hexadecanol (C16:0-OH). In other samples, *FadD* (acyl-CoA synthetase) was expressed together with *acr1* in *E. coli* and a five-fold increase in fatty alcohol production was observed.

[0162] In other samples, *acr1, fadD, accABCD* (acetyl-CoA Carboxylase) (plasmid carrying accABCD constructed as described in Example 1) were expressed along with various individual thioesterases (TEs) in wildtype *E. coli*C41 (DE3) and an *E. coli* C41(DE3 *ΔfadE*, a strain lacking acyl-CoA dehydrogenase. This resulted in additional increases in fatty alcohol production and modulating the profiles of fatty alcohols (see **Fig. 5**). For example, over-expression *of E. coli 'tesA* (pETDuet-1-'tesA) in this system achieved approximately a 60-fold increase in C12:0-OH, C14:0-OH and C16:0-OH with C14:0-OH being the major fatty alcohol. A very similar result was obtained when the ChFatB3 enzyme (FatB3 from *Cuphea hookeriana* in pMAL-c2X-TEcu) was expressed. When the UcFatB1 enzyme (FatB1 from *Umbellularia californicain* in pMAL-c2X-TEuc) was expressed, fatty alcohol production increased approximately 20-fold and C12:0-OH was the predominant fatty alcohol.

[0163] Expression of ChFatB3 and UcFatB1 also led to the production of significant amounts of the unsaturated fatty alcohols C16:1-OH and C14:1-OH, respectively. The presence of fatty alcohols was also found in the supernatant of samples generated from the expression of *tesA* (**Fig. 6**). At 37°C approximately equal amounts of fatty alcohols were found in the supernatant and in the cell pellet, whereas at 25°C approximately 25% of the fatty alcohols were found in the supernatant

Example 5, Medium Chain fatty acid esters

[0164] Alcohol acetyl transferases (AATs, EC 2.3.1.84), which is responsible for acyl acetate production in various plants, can be used to produce medium chain length waxes, such as octyl octanoate, decyl octanoate, decyl decanoate, and the like. Fatty esters, synthesized from medium chain alcohol (such as C6, C8) and medium chain acyl-CoA (or fatty acids, such as C6 or C8) have a relative low melting point. For example, hexyl hexanoate has a melting point of -55°C and octyl octanoate has a melting point of -18 to -17°C. The low melting points of these compounds makes them good candidates for use as biofuels.

[0165] In this example, a SAAT gene was co-expressed in a production host C41(DE3, ΔfadE) with *fadD* from *E. coli* and *acr1* (alcohol reductase from *A. baylyi* ADP1) and octanoic acid was provided in the fermentation broth. This resulted in the production of octyl octanoate. Similarly, when the wax synthase gene from A. *baylyi* ADP1 was expressed in the production host instead of the SAAT gene octyl octanoate was produced.

[0166] A recombinant SAAT gene was synthesized using DNA 2.0 (Menlo Park, CA 94025). The synthesized DNA was based on the published gene sequence (accession number AF193789) and modified to eliminate the *Nco*I site. The synthesized *SAAT* gene (as a BamHI-HindIII fragment) was cloned in pRSET B (Invitrogen, Calsbad, California), linearized with *Bam*HI and *Hind*III. The resulted plasmid, pHZ1.63A was cotransformed into an *E. coli* production host with pAS004.114B, which carries a *fadD* gene from *E. coli* and *acr1* gene from *A. baylyi* ADP1. The transformants were grown in 3 mL of M9 medium with 2% of glucose. After IPTG induction and the addition of 0.02% of octanoic acid, the culture was continued at 25°C from 40 hours. After that, 3 mL of acetyl acetate was added to the whole culture and mixed several times with mixer. The acetyl acetate phase was analyzed by GC/MS.

[0167] Surprising, in the acetyl acetate extract, there is no acyl acetate found. However, a new compound was found and the compound was octyl octanoate. Whereas the control strain without the SAAT gene [C41(DE3, ΔfadE)/pRSET B+pAS004.114B] did not produce octyl octanoate. Also the strain [C41(DE3, ΔfadE)/pHZ1 .43 B+pAS004.114B], in which the wax synthase gene from *A. baylyi* ADP1 was carried by pHZ1.43 produced octyl octanoate (see Figs. 7B).

[0168] The finding that SAAT activity produces octyl octanoate has not reported before and makes it possible to produce medium chain waxes such as octyl octanoate, octyl decanoate, which have low melting point and are good candidates to be use for biofuel to replace triglyceride based biodiesel.

Example 6, Production of wax ester in E. coli strain LS9001

[0169] Wax esters were produced by engineering an *E. coli* production host to express a fatty alcohol forming acyl-CoA reductase, thioesterase, and a wax synthase. Thus, the production host produced both the A and the B side of the ester and the structure of both sides was influenced by the expression of the thioesterase gene.

[0170] More specifically, wax synthase from *A. baylyi* ADP1 (termed WSadp1, accessions AA017391, EC: 2.3.175) was amplified with the following primers using genomic DNA from *A. baylyi* ADP1 as the template. The primers were (1) WSadp1_NdeI, 5'-TCATATGCGCCCATTACATCCG-3' and (2) WSadp1_Avr, 5'-TCCTAGGAGGGCTAATT-TAGCCCTTTAGTT-3'. The PCR product was digested with *Nde*I and *Avr*II and cloned into pCOALDeut-1 to give pHZ 1.43. The plasmid carrying WSadp1 was then co-transformed into *E. coli* strain LS9001 with both pETDuet-1'tesA and pCDFDuet-1-fadD-acr1 and transformants were selected in LB plates supplemented with 50 mg/L of kanamycin, 50 mg/L of carbenicillin and 100 mg/L of spectinomycin. Three transformants were inoculated in 3 mL of LBKCS (LB broth supplement with 50 mg/L of kanamycin, 50 mg/L of carbenicillin, 100 mg/L of spectinomycin and 10 g/L of glucose) and cultured at 37°C shaker (250 rpm). When the cultures reached 0.5 OD_{600}, 1.5 mL of each culture was transferred into 250 mL flasks containing 50 mL of LBKCS and the flasks were grown in a shaker (250 rpm) at 37°C until the culture reached 0.5-1.0 OD_{600}. IPTG was then added to a final concentration of 1 mM. The induced cultures were grown at 37°C shaker for another 40-48 hours.

[0171] The culture was then placed into 50 mL conical tubes and the cells were spun down at 3500 X g for 10 minutes. The cell pellet was then mixed with 5 mL of ethyl acetate. The ethyl acetate extract was analyzed with GC/MS. The intracellular yield of waxes (including C16C16, C14:1C16, C18:1C18:1,C2C14, C2C16, C2C16:1, C16C16:1 and C2C18:1)was about 10 mg/L. When an *E. coli* strain only carrying empty vectors was cultured in the same way, only 0.2 mg/L of wax was found in the ethyl acetate extract.

Example 7, Production and release of fatty -ethyl ester from production host

[0172] The LS9001 strain was modified by transforming it with the plasmids carrying a wax synthase gene from *A. baylyi* (plasmid pHZ1.43), a thioesterase gene from *Cuphea hookeriana* (plasmid pMAL-c2X-TEcu) and a *fadD* gene from *E. coli* (plasmid pCDFDuet-1-fadD). This recombinant strain was grown at 25°C in 3 mL of M9 medium with 50mg/L of kanamycin, 100 mg/L of carbenicillin and 100 mg/L of spectinomycin. After IPTG induction, the media was adjusted to a final concentration of 1% ethanol and 2% glucose. The culture was allowed to grow for 40 hours after IPTG induction. The cells were separated from the spent medium by centrifugation at 3500 X g for 10 minutes). The cell pellet was re-suspended with 3 mL of M9 medium. The cell suspension and the spent medium were then extracted with 1 volume of ethyl acetate. The resulting ethyl acetate phases from the cells suspension and the supernatant were subjected to GC-MS analysis. The results showed that the C16 ethyl ester was the most prominent ester species (as expected for this thioesterase, see **Table 1**), and that 20% of the fatty acid ester produced was released from the cell (see **Fig. 8**). A control *E. coli* strain C41(DE3, ΔfadE) containing pCOLADuet-1 (empty vector for the wax synthase gene), pMAL-c2X-TEuc (containing *fatB* from *U. california*) and pCDFDuet-1-fadD (*fadD* gene from *E. coli*) failed to produce detectable amounts of fatty ethyl esters. The fatty acid esters were quantified using commercial palmitic acid ethyl ester as the reference. Fatty acid esters were also made using the methods described herein except that methanol, or isopropanol was added to the fermentation broth and the expected fatty acid esters were produced.

Example 8, The influence of various thioesterases on the composition of fatty-ethyl esters produced in recombinant *E. coli* strains.

[0173] The thioesterases FatB3 (C. *hookeriana*), TesA (*E. coli*), and FatB (*U. california*) were expressed simultaneously with wax synthase (*A. baylyi*). A plasmid termed pHZ1.61 was constructed by replacing the *Not*I-*Avr*II fragment (carrying the *acr1* gene) with the *Not*I-*Avr*II fragment from pHZ1.43 so that *fadD* and the *ADP1* wax synthase were in one plasmid and both coding sequences were under the control of separate T7 promoter. The construction of pHZ1.61 made it possible to use a two plasmid system instead of the three plasmid system as described in Example 6. pHZ1.61 was then co-transformed into *E. coli* C41 (DE3, ΔfadE) with one of the various plasmids carrying the different thioesterase genes stated above.

[0174] The total fatty acid ethyl esters (supernatant and intracellular fatty acid ethyl esters) produced by these transformants were evaluated using the technique described herein. The yields and the composition of fatty acid ethyl esters are summarized in **Table 8**.

Table 8

The yields (mg/L) and the composition of fatty acid ethyl esters by recombinant *E. coli* C41(DE3, ΔfadE) /pHZ1.61 and plasmids carrying various thioesterase genes.

Thioesterases	C2C10	C2C12:1	C2C12	C2C14:1	C2C14	C2C16:1	C2C16	C2C18:1	Total
'TesA	0.0	0.0	6.5	0.0	17.5	6.9	21.6	18.1	70.5
ChFatB3	0.0	0.0	0.0	0.0	10.8	12.5	11.7	13.8	48.8
ucFatB	6.4	8.5	25.3	14.7	0.0	4.5	3.7	6.7	69.8
pMAL	0.0	0.0	0.0	0.0	5.6	0.0	12.8	7.6	26.0

Note: 'TesA, pETDuet-1-'*tesA*; chFatB3, pMAL-c2X-TEcu; ucFatB, pMAL-c2X-TEuc; pMAL, pMAL-c2X, the empty vector for thioesterase genes used in the study.

Example 9, Production Host Construction

[0175] The genes that control fatty acid production are conserved between microorganisms. For example, **Table 9** identifies the homologues of many of the genes described herein which are known to be expressed in microorganisms that produce hydrocarbons. To increase fatty acid production and, therefore, hydrocarbon production in microorganisms such as those identified in **Table 9**, heterologous genes, such as those from *E. coli* can be expressed. One of ordinary skill in the art will also appreciate that genes that are endogenous to the micoorganisms provided in **Table 9** can also be over-expressed, or attenuated using the methods described herein. Moreover, genes that are described in **Fig. 10** can be expressed or attenuated in microorganisms that endogenously produce hydrocarbons to allow for the production of specific hydrocarbons with defined carbon chain length, saturation points, and branch points.

[0176] For example, exogenous nucleic acid sequences encoding acetyl-CoA carboxylase are introduced into *K. radiotolerans.* The following genes comprise the acetyl-CoA carboxylase protein product in *K. radiotolerans;* acetyl CoA carboxylase, alpha subunit (*accA*/ZP_00618306), acetyl-CoA carboxylase, biotin carboxyl carrier protein (*accB*/ZP_00618387), acetyl-CoA carboxylase, biotin carboxylase subunit (*accC*/ZP_00618040), and acetyl-CoA carboxylase, beta (carboxyltranferase) subunit (*accD*/ZP_00618306). These genes are cloned into a plasmid such that they make a synthetic acetyl-CoA carboxylase operon (accABCD) under the control of a *K. radiotolerαns* expression system such as the expression system disclosed in Ruyter et al., Appl Environ Microbiol. 62:3662-3667, 1996. Transformation of the plasmid into *K. radiotolerans* will enhance fatty acid production. The hydrocarbon producing strain of *K. radiotolerans* can also be engineered to make branched, unsaturated hydrocarbons having specific carbon chain lengths using the methods disclosed herein.

Table 9

Hydrocarbon production hosts

Organism	Gene Name	Accession No./Seq ID/Loci	EC No.
Desulfovibrio desulfuricans G20	accA	YP_388034	6.4.1.2
Desulfovibrio desulfuricans G22	accC	YP_388573/YP_388033	6.3.4.14, 6.4.1.2
Desulfovibrio desulfuricans G23	accD	YP_388034	6.4.1.2
Desulfovibrio desulfuricans G28	fabH	YP_38S920	2.3.1.180
Desulfovibrio desulfuricans G29	fabD	YP_388786	2.3.1.39
Desulfovibrio desulfuricans G30	fabG	YP_388921	1.1.1.100
Desulfovibrio desulfuricans G31	acpP	YP_388922/YP_389150	3.1.26.3, 1.6.5.3, 1.6.99.3
Desulfovibrio desulfuricans G32	fabF	YP_388923	2.3.1.179
Desulfovibrio desulfuricans G33	gpsA	YP_389667	1.1.1.94
Desulfovibrio desulfuricans G34	ldhA	YP_388173/YP_390177	1.1.1.27, 1.1.1.28
Erwinia (micrococcus) amylovora	accA	942060 - 943016	6.4.1.2
Erwinia (micrococcus) amylovora	accB	3440869 - 3441336	6.4.1.2
Erwinia (micrococcus) amylovora	accC	3441351 - 3442697	6.3.4.14,6.4.1.2
Erwinia (micrococcus) amylovora	accD	2517571 - 2516696	6.4.1.2
Erwinia (micrococcus) amylovora	fadE	1003232 - 1000791	1.3.99.-
Erwinia (micrococcus) amylovora	plsB (D311E)	333843 - 331423	2.3.1.15
Erwinia (micrococcus) amylovora	aceE	840558 - 843218	1.2.4.1

(continued)

Hydrocarbon production hosts

Organism	Gene Name	Accession No./Seq ID/Loci	EC No.
Erwinia (micrococcus) amylovora	aceF	843248 - 844828	2.3.1.12
Erwinia (micrococcus) amylovora	fabH	1579839 - 1580789	2.3.1.180
Erwinia (micrococcus) amylovora	fabD	1580826 - 1581749	2.3.1.39
Erwinia (micrococcus) amylovora	fabG	CAA74944	1.1.1.100
Erwinia (micrococcus) amylovora	acpP	1582658 - 1582891	3.1.26.3, 1.6.5.3, 1.6.99.3
Erwinia (micrococcus) amylovora	fabF	1582983 - 1584221	2.3.1.179
Erwinia (micrococcus) amylovora	gpsA	124800 - 125810	1.1.1.94
Erwinia (micrococcus) amylovora	ldhA	1956806 - 1957789	1.1.1.27, 1.1.1.28
Kineococcus radiotolerans SRS30216	accA	ZP_00618306	6.4.1.2
Kineococcus radiotoferans SRS30216	accB	ZP_00618387	6.4.1.2
Kineococcus radiotolerans SRS30216	accC	ZP_00618040/ZP_00618387	6.3.4.14, 6.4.1.2
Kineococcus radiotolerans SRS30216	accD	ZP_00618306	6.4.1.2
Kineacoccus radiotolerans SRS30216	fadE	ZP_00617773	1.3.99.-
Kineococcus radiotolerans SRS30216	plsB (D311E)	ZP_00617279	2.3.1.15
Kineococcus radiotolerans SRS30216	aceE	ZP_00617600	1.2.4.1
Kineococcus radiolol erans SRS30216	aceF	ZP_00619307	2.3.1.12
Kineococcus radiotolerans SRS30216	fabH	ZP_00618003	2.3.1.180
Kineococcus radiotolerans SRS30216	fabD	ZP_00617602	2.3.1.39
Kineococcus radiotolerans SRS30216	fabG	ZP_00615651	1.1.1.100
Kineococcus radiotolerans SRS30216	acpP	ZP_00617604	3.1.26.3, 1.6.5.3, 1.6.99.3
Kineococcus radiotolerans SRS30216	fabF	ZP_00617605	2.3.1.179
Kineococcus radiotolerans SRS30216	gpsA	ZP_00618825	1.1.1.94
Kineococcus radiotolerans SRS30216	ldhA	ZP_00618879	1.1.1.27, 1.1.1.28
Rhodospirillum rubrum	accA	YP_425310	6.4.1.2
Rhodospirillum rubrum	accB	YP_427521	6.4.1.2
Rhodospirillum rubrum	accC	YP_427522/YP_425144/YP_427028/YP_ 426209/YP_427404	6.3.4.14, 6.4.1.2

(continued)

Hydrocarbon production hosts

Organism	Gene Name	Accession No./Seq ID/Loci	EC No.
Rhodospirillum rubrum	accD	YP_428511	6.4.1.2
Rhodospirillum rubrum	fadE	YP_427035	1.3.99.-
Rhodospirillum rubrum	aceE	YP_427492	1.2.4.1
Rhodospirillum rubrum	aceF	YP_426966	2.3.1.12
Rhodospirillum rubrum	fabH	YP_426754	2.3.1.180
Rhodospirillum rubrum	fabD	YP_425507	2.3.1.39
Rhodospirillum rubrum	fabG	YP_425508/YP_425365	1.1.1.100
Rhodospirillum rubrum	acpP	YP_425509	1.6.5.3, 1.6.99.3
Rhodospirillum rubrum	fabF	YP_425510/YP_425510/YP_425285	2.3.1.179
Rhodospirillum rubrum	gpsA	YP_428652	1.1.1.94
Rhodosplrillum rubrum	ldhA	YP_426902/YP_428871	1.1.1.28
Vibrio furnissii	accA	1,16	6.4.1.2
Vibrio furnissii	accB	2, 17	6.4.1.2
Vibrio furnissii	accC	3, 18	6.3.4.14, 6.4.1.2
Vibrio furnissii	accD	4, 19	6.4.1.2
Vibrio furnissii	fadE	5, 20	1.3.99.-
Vibrio furnissii	plsB (D311E)	6, 21	2.3.1.15
Vibrio furnissii	aceE	7, 22	1.2.4.1
Vibrio furnissii	aceF	8, 23	2.3.1.12
Vibrio furnissii	fabH	9, 24	2.3.1.180
Vibrio furnissii	fabD	10, 25	2.3.1.39
Vibrio furnissii	fabG	11, 26	1.1.1.100 3.1.26.3,
Vibrio furnissii	acpP	12, 27	1.6.5.3, 1.6.99.3
Vibrio furnissii	fabF	13, 28	2.3.1.179
Vibrio furnissii	gpsA	14, 29	1.1.1.94
Vibrio furnissii	ldhA	15, 30	1.1.1.27, 1.1.1.28
Stenotrophomonas maltophilia R551-3	accA	ZP_01643799	6.4.1.2
Stenotrophomonas maltophilia R551-3	accB	ZP_01644036	6.4.1.2
Stenotrophomonas maltophilia R551-3	accC	ZP_01644037	6.3.4.14, 6.4.1.2
Stenotrophomonas maltophilia R551-3	accD	ZP_01644801	6.4.1.2
Stenotrophomonas maltophilia R551-3	fadE	ZP_01645823	1.3.99.-
Stenotrophomonas maltophilia R551-3	plsB (D311E)	ZP_01644152	2.3.1.15
Stenotrophomonas maltophilia R551-3	aceE	ZP_01644724	1.2.4.1
Stenotrophomonas maltophilia R551-3	aceF	ZP_01645795	2.3.1.12
Stenotrophomonas maltophilia R551-3	fabH	ZP_01643247	2.3.1.180
Stenotrophomonas maltophilia R551-3	fabD	ZP_01643535	2.3.1.39

(continued)

Hydrocarbon production hosts

Organism	Gene Name	Accession No./Seq ID/Loci	EC No.
Stenotrophomonas maltophilia R551-3	fabG	ZP_01643062	1.1.1.100
Stenotrophomonas maltophilia R551-3	acpP	ZP_01643063	3.1.26.3, 1.6.5.3, 1.6.99.3
Stenotrophomonas maltophilia R551-3	fabF	ZP_01643064	2.3.1.179
Stenotrophomonas maltophilia R551-3	gpsA	ZP_01643216	1.1.1.94
Stenotrophomonas maltophilia R551-3	ldhA	ZP_01645395	1.1.1.27, 1.1.1.28

For Table 9, Accession Numbers are from GenBank, Release 159.0 as of April 15 2007, EC Numbers are from KEGG, Release 42.0 as of April 2007 (plus daily updates up to and including 05/09/07), results for Erwinia amylovora strain Ea273 are taken from the Sanger sequencing center, completed shotgun sequence as of 5/9/07, positions for Erwinia represent locations on the Sanger psuedo-chromosome, sequences from Vibrio furnisii M1 are from the LS9 VFM1 pseudochromosome, v2 build, as of 9/28/06, and include the entire gene, and may also include flanking sequence

Example 10, Additional Exemplary Production strains

[0177] **Table 10,** below provides additional exemplary production strains. Two example biosynthetic pathways are described for producing fatty acids, fatty alcohols, and wax esters. A genetically engineered host can be produced by cloning the expression of the accABCD genes from E. coli, the 'tesA gene from *E. coli,* and *fadD* gene from *E. coli* into a host cell. Host cells can be selected from *E. coli,* yeast, add to the list. These genes can also be transformed into a host cell that is modified to contain one or more of the genetic manipulations described in Examples 1 and 2, above. As provided in **Table 10**, additional production hosts can be created using the indicated exogenous genes.

Table 10

Combination of genes useful for making genetically engineered production strains								
			Fatty acids		**Fatty alcohols**		**wax/fatty esters**	
Peptide	**Sources of genes**	**Genes**	example 1	example 2	example 1	example 2	example 1	example 2
acetyl-CoA carboxylase	*E. coil*	accABCD	x	x	x	x	x	x
thioesterase	*E. coli*	tesA	x		x		x	x
	Cinnamomum camphore	ccFatB						
	Umbellularia californica	umFatB		x		x		
	Cuphea hookariana	chFatB2						
	Cuphea hookeriana	chFatB3						
	Cuphea hookerian	chFatA						
	Arabidopsis thaliana	AtFatA1						
	Arabidopsis thalian	AtFatB1[M141T]						
acyl-CoA synthase	*E.coli*	fadD	x	x	x	x	x	x
acyl-CoA reductase	*Bombyx mori*	bFAR						
	Acinetobacter baylyi ADP1	acr 1			x		x	
	Simmondsia chinensis	jjFAR				x		x
	Triticum aestivum							
	Mus musculus	mFAR1						
	Mus musculus	mFAR2						
	Acinetpbacter sp M1	acr M1						
	Homo sapiens	hFAR						

(continued)

Combination of genes useful for making genetically engineered production strains								
			Fatty acids		**Fatty alcohols**		**wax/fatty esters**	
Peptide	**Sources of genes**	**Genes**	example 1	example 2	example 1	example 2	example 1	example 2
wax synthase/alcohol acyltransferase	*Fundibacter jedensis* DSM 12178	WST9						
	Acinetobacter sp. HO1-N	WSHN					x	
	Acinetobacter beylyl ADP1	WSadp1						x
	Mus musculus	mWS						
	Homo saplens	hWS						
	Fragaria x ananassa	SAAT						
	Malus x domestica	MpAAT						
	Simmondsia chinensis	JjWS (AAD38041)						
Decarbonylase	*Arabidopsis thaliana*	cer1						
	Oryza sativa	cer1						
Transporter	*Acinetobacter sp. HO1-N*						x	x
	Arabidopsis thaliana	Cer5						

Example 11, Fermentation

[0178] Host microorganisms can be also engineered to express umuC and umuD from *E. coli* in pBAD24 under the *prpBCDE* promoter system through *de novo* synthesis of this gene with the appropriate end-product production genes. For small scale hydrocarbon product production, *E. coli* BL21(DE3) cells harbouring pBAD24 (with ampicillin resistance and the end-product synthesis pathway) as well as pUMVC1 (with kanamycin resistance and the acetyl CoA/malonyl CoA over-expression system) are incubated overnight at at 37°C shaken at >200 rpm 2L flasks in 500 ml LB medium supplemented with 75 μg/mL ampicillin and 50 μg/ml kanamycin until cultures reached an OD_{600} of> 0.8. Upon achieving an OD_{600} of > 0.8, cells are supplemented with 25 mM sodium proprionate (pH 8.0) to activate the engineered gene systems for production as well as to stop cellular proliferation (through activation of umuC and umuD proteins). Induction is performed for 6 hours at 30°C. After incubation, media is examined for product using GC-MS (as described below).

[0179] For large scale product production, the engineered microorganisms are grown in 10 L, 100 L or larger batches, fermented and induced to express desired products based on the specific genes encoded in plasmids as appropriate. *E. coli* BL21(DE3) cells harbouring pBAD24 (with ampicillin resistance and the end-product synthesis pathway) as well as pUMVC1 (with kanamycin resistance and the acetyl-CoA/malonyl-CoA over-expression system) are incubated from a 500 mL seed culture for 10 L fermentations (5 L for 100 L fermentations) in LB media (glycerol free) at 37°C shaken at >200 rpm until cultures reached an OD_{600} of > 0.8 (typically 16 hours) incubated with 50 μg/mL kanamycin and 75 μg/mL ampicillin. Media is treated with continuously supplemented to maintain a 25 mM sodium proprionate (pH 8.0) to activate the engineered in gene systems for production as well as to stop cellular proliferation (through activation of umuC and umuD proteins). Media is continuously supplemented with glucose to maintain a concentration 90g/100 mL. After the first hour of induction, aliquots of no more than 10% of the total cell volume are removed each hour and allowed to sit unaggitated so as to allow the hydrocarbon product to rise to the surface and undergo a spontaneous phase separation. The hydrocarbon component is then collected and the aqueous phase returned to the reaction chamber. The reaction chamber is operated continuously. When the OD_{600} drops below 0.6, the cells are replaced with a new batch grown from a seed culture.

[0180] For wax ester production, subsequent to isolation, the wax esters are washed briefly in 1 M HCl to split the ester bond, and returned to pH 7 with extensive washing with distilled water.

Example 12, Product Characterization

[0181] To characterize and quantify the fatty alcohols and fatty acid esters, gas chromatography (GC) coupled with electron impact mass spectra (MS) detection was used. Fatty alcohol samples were first derivatized with an excess of N-trimethylsilyl (TMS) imidazole to increase detection sensitivity. Fatty acid esters did not required derivatization. Both fatty alcohol-TMS derivatives and fatty acid esters were dissolved in an appropriate volatile solvent, like ethyl acetate. The samples were analyzed on a 30m DP-5 capillary column using the following method. After a 1 μL splitless injection onto the GC/MS column, the oven is held at 100°C for 3 minutes. The temperature was ramped up to 320°C at a rate of 20°C/minute. The oven was held at 320°C for an additional 5 minutes. The flow rate of the carrier gas helium was 1.3 mL/minute. The MS quadrapole scans from 50 to 550 m/z. Retention times and fragmentation patterns of product peaks were compared with authentic references to confirm peak identity.

[0182] For example, hexadeconic acid ethyl ester eluted at 10.18 minutes (Figs. 9A **and** 9B). The parent ion of 284 mass units was readily observed. More abundent were the daughter ions produced during mass fragmentation. This included the most prevalent daughter ion of 80 mass units. The derivatized fatty alcohol hexadecanol-TMS eluted at 10.29 minutes and the parent ion of 313 could be observed. The most prevalent ion was the M-14 ion of 299 mass units.

[0183] Quantification was carried out by injecting various concentrations of the appropriate authentic references using the GC/MS method described above. This information was used to generate a standard curve with response (total integrated ion count) versus concentration.

EQUIVALENTS

[0184] While specific examples of the subject inventions are explicitly disclosed herein, the above specification and examples herein are illustrative and not restrictive. Many variations of the inventions will become apparent to those skilled in the art upon review of this specification including the examples.

SEQUENCE LISTING

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<210> 4
<211> 927
<212> DNA
<213> Vibrio furnisii

<400> 4

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<210> 5
<211> 2445
<212> DNA
<213> Vibrio furnisii

<400> 5

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tttggcccag tgggtatcat cagctgggcg ctgtacttag ccgctatcgc ggtattggca    180
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gccgaactgt tcaaaggcaa accggactgg caacagctga gccatatcaa agcgcccaca 360

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aacgactatc aggtgactca cgaattggcg gatttgcctc cggaagtgtg gcaatacctg 480

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gcgtacgcgc aatcgctggt gctacaaaag ctgacgggcg tatcgggcgt gctctcttcc 600

accgtcggcg tgccgaactc tctcggcccg ggcgaactgc tgcaacatta cggcaccgac 660

gatcagaaag attactacct ccctcgtttg gcggaaggca aagagattcc atgtttcgcg 720

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ttagcagcaa aaaagtcaca acccaagctg gtcgaggtcg cgtga 2445

<210> 6
<211> 2424
<212> DNA
<213> Vibrio furnisii

<400> 6

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gattctatcg ccacattcag cgaaatgctc agcctgcatc aagaagattc ggagttggat 360

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cgtccatatt tgcaagcctt gaatggcccg caaaaagcca aagcggtctt tgccgccgga 480

cgggactgtt tggtgcgctt tagccccgtg gtctcgctgc gttatatggc cgactcgcac 540

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<210> 7
<211> 2661
<212> DNA
<213> Vibrio furnisii

<400> 7

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<210> 8
<211> 1893
<212> DNA
<213> Vibrio furnisii

<400> 8

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<210> 9
<211> 951
<212> DNA
<213> Vibrio furnisii

<400> 9

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<210> 10
<211> 924

<212> DNA
<213> Vibrio furnisii

<400> 10
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ggttacgacc tatgggcatt ggttcagaac ggtcctgttg aagatctcaa ccagactttc 180

cgtacgcaac ctgcactgct ggcgtcttct gtggcgattt ggcgtgtatg gcaagcgctg 240

ggtcttgagc agccagaagt gctggcaggc cacagccttg gtgaatactc tgcactggtt 300

tgtgccggtg tgattgattt taaagccgcg atcaaattgg tcgaactgcg tggtcaactg 360

atgcaagaag cagtacctgc aggaaccggc gcaatgtacg cgatcatcgg tttggatgat 420

gcggcgattg ccaaagcgtg tgaagacgct gcgcaaggcg acgtggtgtc tccggtgaac 480

ttcaactcac caggccaagt ggtcattgcc ggtcagaaag atgcggtaga acgcgcgggc 540

gcactgtgta aagaagcggg cgcgaaacgt gcactgccac tgccggtgtc agtgccttca 600

cactgcgcgc tgatgaaacc tgcagcagaa aaactggctg tggcgctaga agcgcttgag 660

ttcaacgcgc cgcaaatccc agtgattaac aacgtggacg ttgcgacaga aacggatcca 720

gcgaaaatca aagatgcgtt ggttcgtcaa ctacacagcc cagtccgctg gacagaaggc 780

gtggagaaga tggcagcaca aggcattgaa aaactaattg aagttggccc aggcaaagta 840

ctgactggtt tgactaaacg tattgtgaaa acgcttgatg cagcagcagt gaacgacatc 900

gcttcactgg aagccgttaa gtaa 924

<210> 11
<211> 747
<212> DNA
<213> Vibrio furnisii

<400> 11

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gcgttgaatg tgacagatgt agcgtctatc gaatccgtgc tgaaaagcat taacgatgaa 240
ttcggcggtg ttgatattct ggtgaacaac gcgggtatca cgcgtgacaa cctgctgatg 300
cgtatgaaag atgacgagtg gaccgatatt ctggatacca acttgacgtc gatcttccgt 360
ctgtctaaag ctgtacttcg tggcatgatg aaaaaacgcc aaggccgtat cattaatgtc 420
ggttctgttg tcggtacaat gggtaacgcg ggtcaaacaa actacgcagc cgcaaaagcg 480
ggcgtaatcg gctttacgaa gtcaatggca cgtgaagttg catcccgtgg cgtgaccgtg 540
aacacagttg caccaggttt catcgaaacg gatatgacaa aagcgctgaa tgacgaccaa 600
cgtgctgcta cacttgcaca agtgccagca ggtcgtctgg gtgatccacg tgaaatcgca 660
tccgcggttg cattcttggc atctccagaa gcagcgtaca ttaccggtga aactctgcac 720
gttaacggcg gaatgtacat ggtttaa 747

<210> 12
<211> 525
<212> DNA
<213> Vibrio furnisii

<400> 12

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tttgtggttt gaccagcaag gtccccccttg caactttcac tagtttgaat aaactacgga 180
atcatcgcat taggcgaaat ctgtaaagga aaagaaaaaa tgagcaacat cgaagaacgc 240
gtaaagaaaa tcatcgttga acagctaggc gtagacgaag cagaagtgaa aaacgaagct 300
tctttcgttg aagacctagg tgcggattct ctagacactg ttgagcttgt tatggctctg 360
gaagaagaat tcgacactga gattcctgat gaagaagcag agaaaatcac tactgttcaa 420
gctgcgatcg attacgtaaa cagcgctcag taatgtctct ccccaggcgg ccctctggcc 480
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<210> 13
<211> 1251
<212> DNA
<213> Vibrio furnisii

<400> 13

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gaacactttg atacaacaaa tttctcaact cgtttcgcag gtctggtaaa agatttcaac     180
tgcgaagagt acatgtctaa aaaagatgcc cgtaaaatgg atttatttat ccagtacggt     240
attgctgcgg gcatccaagc gctagacgat tctggtctgg tgatcactga agaaaacgcg     300
ccacgcgtcg gtgttgcaat cggctcgggc atcggtggtc ttgatttgat cgaaaaaggt     360
catcaagcgc ttatggagaa aggtccacgt aaagtgagcc cattcttcgt cccttcaacc     420
atcgtgaaca tggttgccgg taacttatct atcatgcgtg gtcttcgtgg tcctaacatc     480
gcgatttcaa ctgcatgtac cacaggttta cataacatcg gccacgcggc gcgtatgatt     540
gcatacggcg atgcggaagc gatggttgct ggtggtagtg aaaaagcgtc taccсctctg     600
ggtatggctg gcttcggtgc cgctaaagcg ctgtctacac gcaacgatga acctgcaaaa     660
gcttctcgcc cttgggacaa agaccgtgac ggttttgttc tgggtgacgg cgcaggcgtg     720
atggttctgg aaggatacga acacgcaaaa gcgcgtggcg cgaaaatcta cgcagaaatc     780
gtaggcttcg gtatgtccgg tgacgcgtac cacatgactt cgccaagcga agatggttca     840

ggtggcgcgc tggctatgga agcggcgatg cgtgatgcag cactagcggg tacacaaatc     900
ggctacgtga acgcgcacgg tacgtcaaca ccagcaggtg acgtagcgga agtgaaaggt     960
atcaaacgtg cacttggcga agacggtgcg aaacaagtac tgatctcttc aaccaaatcg    1020
atgaccggtc acctactggg tgctgcaggc tcggtagaag ccatcattac cgtgatgtct    1080
ctggttgacc aaatcgttcc gccaaccatc aacctggata atccagaaga aggtttgggc    1140
gtggatttgg ttccgcacac agcacgtaaa gtggaaggca tggaatacgc gatgtgtaac    1200
tcgtttggct ttggtggcac aaacggttca ctgatcttca agcgcgtata a             1251
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<210> 14
<211> 1035
<212> DNA
<213> Vibrio furnisii

<400> 14

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atgactgatt cacacacaaa caatgcttac ggtaaagcga tcgccatgac cgtcattggc      60
gcgggttcgt acggcacatc tctggccatt tctttggctc gcaacggcgc caatgttgtc     120
ctgtggggac acgatccggt ccacatggcg cgtttggaag cggaacgtgc taaccacgaa     180
ttcctccctg acatcgattt tccaccgtcg ctgatcattg aatccgattt gcaaaaagcg     240
gtgcaagcga gccgcgatct gctggtggtg gtgccaagcc atgtgtttgc gattgtgctc     300
aacagcctgc aaccttactt gcgagaagat acccgtatct gctgggcaac caaagggttg     360
gaaccggaca caggacgttt gctgcaagat gtggcgcatg acgtgctggg tgaatcccat     420
ccattggcgg tgctgtctgg cccgacgttt gcgaaagagc tggcgatggg tatgcccact     480
gcgatttcag tggcatcgcc tgacgcgcag tttgtcgccg atctgcagga aaagattcac     540
tgcagcaaaa ccttccgtgt ttatgccaac agcgatttca tcggcatgca actggggggc     600
gctgtgaaga acgtgattgc cattggtgcg gggatgtcgg atggcatcgg ctttggtgcc     660
aacgctcgta cggcgctgat tacccgtggt ttggcggaaa tgacccgtct gggcgcggcg     720
ctgggcgcgc agccggaaac cttcatgggc atggcggggc tgggtgattt ggtgctgacg     780
tgtaccgata accaatcgcg caaccgtcgt tttggtttgg ccttgggcca aggcaaagat     840
gtcgatacgg cgcaacaaga tatcggtcaa gtggtggaag ggtatcggaa caccaaagag     900
gtgtggctac tggcgcaacg catgggcgtg gagatgccaa tagttgaaca aatttatcaa     960
gtattgtatc aaggaaagga cgcccgcatg gcagcacaag atttgctggc gcgcgataaa    1020
aaagcagaac gataa                                                     1035
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<210> 15
<211> 855
<212> DNA
<213> Vibrio furnisii

<400> 15

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gcaaaacgca ttggcatgca ggtggttcgc gtacctgcgt attcaccaga agcggtggca   180
gagcacgcgg tcgggttgat gatgtgtctg aaccgccgtt accacaaagc gtatcagcgc   240
acacgtgagg ccaacttctc gttggaaggc ttggtgggct ttaacttcta tggcaaaacc   300
gtgggtgtga ttggttcagg caagattggc attgcagcga tgcgtatcct caaaggcctt   360
ggcatgaaca ttctctgctt tgacccgtat gaaaacccat tggccattga aatcggcgcg   420
aaatacgttc aattgccgga gctgtatgca aacagcgaca tcattacgct gcactgcccg   480
atgaccaaag aaaactacca cctgctggat gagcaagcgt tcgctcaaat gaaggatggg   540
gtgatgatca tcaataccag ccgtggcgaa ttgcttgatt cagtcgcagc cattgaagcg   600
ctcaaacgtg gccgtattgg cgcgctgggc ttagacgtat acgacaacga aaaagatctg   660
ttcttccaag acaagtcgaa cgatgtgatt gtagatgacg tgttccgccg cctgtccgcc   720
tgccataacg tgctgtttac cggccatcag gcgtttttga cagaagatgc cctgcacaat   780
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<210> 16
<211> 177
<212> PRT
<213> Vibrio furnisii

<220>
<221> misc_feature
<222> (2)..(2)
<223> Xaa can be any naturally occurring amino acid

<400> 16

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Phe Xaa Asn Leu Glu Lys Pro Ile Val Glu Leu Glu Ala Lys Ile Gln
1               5                   10                  15

Ala Leu Arg Asp Val Ser Arg His Gly Gly Gly Thr Ser Val Asp Leu
            20                  25                  30

Glu Lys Glu Ile Glu Gln Leu Glu Lys Lys Ser Leu Glu Leu Lys Lys
        35                  40                  45

Lys Ile Phe Gly Asp Leu Gly Ala Trp Gln Val Ala Gln Met Ala Arg
    50                  55                  60

His Pro Gln Arg Pro Tyr Thr Leu Asp Tyr Ile Asn Asn Met Phe Thr
65                  70                  75                  80
```

```
Glu Phe Asp Glu Leu Ala Gly Asp Arg Ala Phe Ala Asp Asp Lys Ala
                85                  90                  95

Ile Val Gly Gly Met Ala Arg Leu Asp Gly Arg Pro Val Met Val Ile
            100                 105                 110

Gly His Gln Lys Gly Arg Glu Thr Arg Glu Lys Val Lys Arg Asn Phe
        115                 120                 125

Gly Met Pro Lys Pro Glu Gly Tyr Arg Lys Ala Leu Arg Leu Met Glu
    130                 135                 140

Met Ala Glu Arg Phe Asn Met Pro Ile Ile Thr Phe Ile Asp Thr Ala
145                 150                 155                 160

Gly Ala Tyr Pro Gly Val Gly Ala Glu Glu Arg Gly Gln Ser Glu Ala
                165                 170                 175

Ile
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<210> 17
<211> 187
<212> PRT
<213> Vibrio furnisii

<400> 17

```
Met Asn Ser Leu Cys Arg Gln Pro Phe Ala Arg Cys Lys Gln Ser Lys
1               5                   10                  15

Pro Lys His Ser Ala Ala Gln Leu Ser Cys Leu Ile His Lys Ile Lys
            20                  25                  30

Glu Lys Glu Thr Met Asp Ile Arg Lys Ile Lys Lys Leu Ile Glu Leu
        35                  40                  45

Val Glu Glu Ser Gly Ile Ala Glu Leu Glu Ile Ser Glu Gly Glu Glu
    50                  55                  60

Ser Val Arg Ile Ser Arg His Gly Val Ala Pro Val Ala Pro Ile Gln
65                  70                  75                  80

Tyr Ala Ala Pro Ala Pro Met Ala Ala Pro Val Ala Ala Pro Ala Ala
                85                  90                  95

Ala Pro Val Ala Glu Ala Pro Ala Ala Ala Lys Thr Pro Ala Gly His
            100                 105                 110
```

Met Val Leu Ser Pro Met Val Gly Thr Phe Tyr Arg Ser Pro Ser Pro
115 120 125

Asp Ala Lys Ser Phe Ile Glu Val Gly Gln Thr Val Lys Ala Gly Asp
130 135 140

Thr Leu Cys Ile Val Glu Ala Met Lys Met Met Asn Gln Ile Glu Ala
145 150 155 160

Asp Lys Ser Gly Val Val Thr Glu Ile Leu Val Glu Asp Gly Gln Ala
165 170 175

Val Glu Phe Asp Gln Pro Leu Val Val Ile Glu
180 185

<210> 18
<211> 447
<212> PRT
<213> Vibrio furnisii

<400> 18

Met Leu Asp Lys Leu Val Ile Ala Asn Arg Gly Glu Ile Ala Leu Arg
1 5 10 15

Ile Leu Arg Ala Cys Lys Glu Leu Gly Ile Lys Thr Val Ala Val His
20 25 30

Ser Thr Ala Asp Arg Asp Leu Lys His Val Leu Leu Ala Asp Glu Thr
35 40 45

Val Cys Ile Gly Pro Ala Lys Gly Ile Asp Ser Tyr Leu Asn Ile Pro
50 55 60

Arg Ile Ile Ser Ala Ala Glu Val Thr Gly Ala Val Ala Ile His Pro
65 70 75 80

Gly Tyr Gly Phe Leu Ser Glu Asn Ala Asp Phe Ala Glu Gln Val Glu
85 90 95

Arg Ser Gly Phe Ile Phe Val Gly Pro Lys Ala Asp Thr Ile Arg Leu
100 105 110

Met Gly Asp Lys Val Ser Ala Ile Thr Ala Met Lys Lys Ala Gly Val
115 120 125

Pro Cys Val Pro Gly Ser Asp Gly Pro Leu Asp Asn Asp Glu Val Lys
130 135 140

```
Asn Arg Ala His Ala Lys Arg Ile Gly Tyr Pro Val Ile Ile Lys Ala
145                 150                 155                 160

Ser Gly Gly Gly Gly Gly Arg Gly Met Arg Val Val Arg Ser Glu Ala
                165                 170                 175

Glu Leu Val Asn Ala Ile Ser Met Thr Arg Ala Glu Ala Lys Ala Ala
            180                 185                 190

Phe Asn Asn Asp Met Val Tyr Met Glu Lys Tyr Leu Glu Asn Pro Arg
        195                 200                 205

His Val Glu Val Gln Val Leu Ala Asp Gly Gln Gly Ser Ala Ile His
    210                 215                 220

Leu Gly Glu Arg Asp Cys Ser Met Gln Arg Arg His Gln Lys Val Val
225                 230                 235                 240

Glu Glu Ala Pro Ala Pro Gly Ile Thr Glu Glu Met Arg Lys Tyr Ile
                245                 250                 255

Gly Glu Arg Cys Thr Arg Ala Cys Ile Glu Ile Gly Tyr Arg Gly Ala
            260                 265                 270

Gly Thr Phe Glu Phe Leu Tyr Glu Asn Gly Glu Phe Tyr Phe Ile Glu
        275                 280                 285

Met Asn Thr Arg Ile Gln Val Glu His Pro Val Thr Glu Met Val Thr
    290                 295                 300

Gly Val Asp Leu Ile Lys Glu Gln Leu Arg Ile Ala Ala Gly Gln Pro
305                 310                 315                 320

Leu Ser Phe Thr Gln Asp Asp Ile Lys Ile Arg Gly His Ala Met Glu
                325                 330                 335

Cys Arg Ile Asn Ala Glu Asp Pro Glu Arg Phe Leu Pro Cys Pro Gly
            340                 345                 350

Lys Ile Thr Arg Phe His Ser Pro Gly Gly Met Gly Val Arg Trp Glu
        355                 360                 365

Ser His Ile Tyr Ser Gly Tyr Thr Val Pro Ala Tyr Tyr Asp Ser Met
    370                 375                 380

Ile Gly Lys Leu Ile Thr Phe Gly Glu Asn Arg Asp Val Ala Ile Ala
```

385 390 395 400

Arg Met Arg Asn Ala Leu Asp Glu Met Ile Val Glu Gly Ile Lys Thr
405 410 415

Asn Ile Pro Leu Gln Gln Val Ile Met Lys Asp Glu Asn Phe Gln His
420 425 430

Gly Gly Thr Asn Ile His Tyr Leu Glu Lys Lys Leu Gly Leu Gln
435 440 445

<210> 19
<211> 308
<212> PRT
<213> Vibrio furnisii

<400> 19

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Met Ser Trp Leu Glu Lys Ile Leu Glu Lys Ser Asn Ile Gly Ser Ser
1               5                   10                  15

Arg Lys Ala Ser Ile Pro Glu Gly Val Trp Thr Lys Cys Thr Ser Cys
            20                  25                  30

Glu Gln Val Leu Tyr Tyr Ala Glu Leu Glu Arg Asn Leu Glu Val Cys
        35                  40                  45

Pro Lys Cys Asn His His Met Arg Met Lys Ala Arg Arg Arg Leu Glu
    50                  55                  60

Thr Phe Leu Asp Glu Ala Asn Arg Tyr Glu Ile Ala Asp Glu Leu Glu
65                  70                  75                  80

Pro Gln Asp Lys Leu Lys Phe Lys Asp Ser Lys Arg Tyr Lys Glu Arg
                85                  90                  95

Leu Ala Thr Ala Gln Lys Ser Ser Gly Glu Lys Asp Ala Leu Ile Val
            100                 105                 110

Met Lys Gly Glu Leu Met Thr Ile Pro Val Val Ala Cys Ala Phe Glu
        115                 120                 125

Phe Ser Phe Met Gly Gly Ser Met Gly Ser Val Val Gly Ala Arg Phe
    130                 135                 140

Val Arg Ala Val Glu Ala Ala Ile Glu Ala Asn Cys Gly Leu Val Cys
145                 150                 155                 160

Phe Ser Ala Ser Gly Gly Ala Arg Met Gln Glu Ala Leu Met Ser Leu
```

165 170 175

Met Gln Met Ala Lys Thr Ser Ala Ala Leu Glu Arg Leu Thr Ala Lys
180 185 190

Gly Leu Pro Phe Ile Ser Val Met Thr Asp Pro Thr Met Gly Gly Val
195 200 205

Ser Ala Ser Leu Ala Met Leu Gly Asp Ile Asn Ile Gly Glu Pro Lys
210 215 220

Ala Leu Ile Gly Phe Ala Gly Arg Arg Val Ile Glu Gln Thr Val Arg
225 230 235 240

Glu Glu Leu Pro Glu Gly Phe Gln Arg Ser Glu Phe Leu Leu Glu His
245 250 255

Gly Ala Ile Asp Met Ile Val Asp Arg Arg Glu Met Arg Gln Arg Val
260 265 270

Ala Gly Leu Leu Ala Lys Met Thr Arg Gln Glu Ser Pro Leu Val Val
275 280 285

Ser Val Asn Asp Ala Pro Asn Glu Ala Ala Tyr Ser Val Pro Glu Ala
290 295 300

Asn Lys Lys Gly
305

<210> 20
<211> 814
<212> PRT
<213> Vibrio furnisii

<400> 20

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Met Asp Ile Leu Leu Ser Ile Leu Gly Phe Val Val Val Leu Ser Gly
1               5                   10                  15

Cys Leu Tyr His Arg Thr Ser Leu Met Thr Ala Leu Ala Ala Leu Thr
            20                  25                  30

Val Thr Met Leu Val Leu Ser Leu Phe Gly Pro Val Gly Ile Ile Ser
        35                  40                  45

Trp Ala Leu Tyr Leu Ala Ala Ile Ala Val Leu Ala Val Pro Ser Ile
    50                  55                  60

Arg Gln Ser Leu Ile Ser Gly Lys Thr Leu Lys Val Phe Lys Lys Val
```

```
65                  70                  75                  80

Leu Pro Ala Met Ser Gln Thr Glu Lys Glu Ala Leu Asp Ala Gly Thr
                85                  90                  95

Val Trp Trp Glu Ala Glu Leu Phe Lys Gly Lys Pro Asp Trp Gln Gln
            100                 105                 110

Leu Ser His Ile Lys Ala Pro Thr Leu Ser Ala Glu Glu Gln Ala Phe
        115                 120                 125

Leu Asp Gly Pro Val Asn Glu Val Cys Ala Met Val Asn Asp Tyr Gln
    130                 135                 140

Val Thr His Glu Leu Ala Asp Leu Pro Pro Glu Val Trp Gln Tyr Leu
145                 150                 155                 160

Lys Asp His Lys Phe Phe Ala Met Ile Ile Lys Lys Gln Tyr Gly Gly
                165                 170                 175

Leu Glu Phe Ser Ala Tyr Ala Gln Ser Leu Val Leu Gln Lys Leu Thr
            180                 185                 190

Gly Val Ser Gly Val Leu Ser Ser Thr Val Gly Val Pro Asn Ser Leu
        195                 200                 205

Gly Pro Gly Glu Leu Leu Gln His Tyr Gly Thr Asp Asp Gln Lys Asp
    210                 215                 220

Tyr Tyr Leu Pro Arg Leu Ala Glu Gly Lys Glu Ile Pro Cys Phe Ala
225                 230                 235                 240

Leu Thr Ser Pro Glu Ala Gly Ser Asp Ala Gly Ser Ile Pro Asp Tyr
                245                 250                 255

Gly Ile Val Cys Lys Asp Glu Trp Glu Gly Lys Glu Val Leu Gly Met
            260                 265                 270

Arg Leu Thr Trp Asn Lys Arg Tyr Ile Thr Leu Ala Pro Val Ala Thr
        275                 280                 285

Val Leu Gly Leu Ala Phe Lys Leu Arg Asp Pro Asp Gly Leu Leu Gly
    290                 295                 300

Asp Gln Lys Glu Ile Gly Ile Thr Cys Ala Leu Ile Pro Thr His Leu
305                 310                 315                 320
```

```
Lys Gly Val Glu Ile Gly Asn Arg His Phe Pro Leu Asn Val Pro Phe
                325                 330                 335

Gln Asn Gly Pro Thr Arg Ala Asn Asp Leu Phe Val Pro Leu Asp Phe
            340                 345                 350

Ile Ile Gly Gly Pro Ser Met Ala Gly Gln Gly Trp Arg Met Leu Val
        355                 360                 365

Glu Cys Leu Ser Val Gly Arg Gly Ile Thr Leu Pro Ser Asn Ser Thr
    370                 375                 380

Gly Gly Ile Lys Ala Ala Ala Met Ala Thr Gly Ala Tyr Ala Arg Ile
385                 390                 395                 400

Arg Arg Gln Phe Lys Gln Pro Ile Gly His Met Glu Gly Ile Glu Glu
                405                 410                 415

Pro Leu Ala Arg Leu Ala Gly Asn Ala Tyr Val Met Asp Ala Ala Ser
            420                 425                 430

Asn Leu Thr Val Ala Gly Ile Asp Ala Gly Glu Lys Pro Ser Val Ile
        435                 440                 445

Ser Ala Ile Val Lys Tyr His Cys Thr His Arg Gly Gln Arg Ser Ile
    450                 455                 460

Ile Asp Ala Met Asp Ile Val Gly Gly Lys Gly Ile Cys Leu Gly Pro
465                 470                 475                 480

Ser Asn Phe Leu Ala Arg Gly Tyr Gln Gly Ser Pro Ile Ala Ile Thr
                485                 490                 495

Val Glu Gly Ala Asn Ile Leu Thr Arg Ser Met Ile Ile Phe Gly Gln
            500                 505                 510

Gly Ala Ile Arg Cys His Pro Tyr Val Leu Lys Glu Met Glu Ala Ala
        515                 520                 525

Tyr Ser Asp Ser Ala Asn Ala Val Glu Gln Phe Asp Ala Ala Leu Ala
    530                 535                 540

Gly His Val Ser Phe Thr Met Ser Asn Leu Val Arg Cys Ile Trp Phe
545                 550                 555                 560

Gly Leu Thr Asp Gly Leu Gly Ser Ala Ala Pro Thr Lys Asp Ala Thr
                565                 570                 575
```

```
Lys Arg Tyr Tyr Gln Gln Leu Asn Arg Tyr Ser Ala Asn Leu Ala Leu
            580                 585                 590

Leu Ala Asp Ile Ser Met Ala Val Leu Gly Gly Ser Leu Lys Arg Lys
        595                 600                 605

Glu Arg Leu Ser Ala Arg Leu Gly Asp Ile Leu Ser Gln Leu Tyr Leu
    610                 615                 620

Ser Ser Ala Thr Leu Lys Arg Phe Glu Asn Asp Gly Arg Pro Ala Glu
625                 630                 635                 640

Asp Leu Ala Leu Val His Trp Gly Leu Gln Asp Ser Leu Lys Gln Thr
                645                 650                 655

Glu Val Ala Ile Asp Glu Phe Leu Ala Asn Phe Pro Asn Lys Val Ile
            660                 665                 670

Gly Lys Ala Leu Arg Val Leu Ile Met Pro Phe Gly Arg Val Arg Lys
        675                 680                 685

Ala Pro Asn Asp Lys Leu Asp Ser Lys Val Ala Gln Ile Ile Gln Thr
    690                 695                 700

Pro Ser Ala Thr Arg Ser Arg Ile Gly Arg His Gln Tyr Leu Glu Pro
705                 710                 715                 720

Thr Ala His Asn Ala Val Gly Lys Ile Glu Leu Ala Leu Asn Val Ile
                725                 730                 735

Leu Gln Ala Glu Pro Val Phe Asp Lys Val Cys Lys Ala Leu Asn Glu
            740                 745                 750

Arg Arg Pro Phe Thr Gln Leu Asp Gln Val Ala Gln Cys Gly Leu Glu
        755                 760                 765

Gln Lys Leu Ile Thr Glu Gln Glu Ala Glu Leu Leu Ile Glu Ala Glu
    770                 775                 780

Gln His Arg Leu Tyr Thr Ile Asn Val Asp Asp Phe Ala Pro Gln Glu
785                 790                 795                 800

Leu Ala Ala Lys Lys Ser Gln Pro Lys Leu Val Glu Val Ala
                805                 810
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<210> 21
<211> 807

<212> PRT
<213> Vibrio furnisii

<400> 21

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Met Ser Ser Gly His Ser Phe Ser Arg Ser Leu Leu Lys Leu Pro Leu
1               5                   10                  15

Ser Val Leu Val Lys Gly Thr Val Ile Pro Ser Asn Pro Ile Asp Asp
            20                  25                  30

Leu Glu Ile Asp Ile Asn Lys Pro Ile Val Tyr Ala Leu Pro Phe Arg
        35                  40                  45

Ser Asn Val Asp Leu Leu Thr Leu Gln Thr His Ala Leu Gln Ala Gly
    50                  55                  60

Leu Pro Asp Pro Leu Glu Pro Leu Thr Ile His Ser His Thr Leu Lys
65                  70                  75                  80

Arg Tyr Val Phe Ile Ser Ser Arg Pro Thr Leu Leu Gln Asp Asp Asn
                85                  90                  95

Gln Val Pro Thr Asp Ser Ile Ala Thr Phe Ser Glu Met Leu Ser Leu
            100                 105                 110

His Gln Glu Asp Ser Glu Leu Asp Val Gln Val Ile Pro Ala Thr Val
        115                 120                 125

Leu Trp Gly Arg Lys Pro Gly Lys Glu Gly Arg Glu Arg Pro Tyr Leu
    130                 135                 140

Gln Ala Leu Asn Gly Pro Gln Lys Ala Lys Ala Val Phe Ala Ala Gly
145                 150                 155                 160

Arg Asp Cys Leu Val Arg Phe Ser Pro Val Val Ser Leu Arg Tyr Met
                165                 170                 175

Ala Asp Ser His Gly Thr Asp Ala Ser Ile Ala His Lys Leu Ala Arg
            180                 185                 190

Val Ala Arg Ile His Phe Ser Arg Gln Lys Leu Ala Ala Ser Gly Pro
        195                 200                 205

Asn Leu Pro Gln Arg His Gln Leu Phe Gln Arg Leu Met Asn Ser Pro
    210                 215                 220

Ala Ile Glu Lys Ala Ile Ala Asp Glu Ala Ala Ala Lys Asn Ile Ser
225                 230                 235                 240
```

```
Leu Glu Lys Ala Arg Lys Glu Ala His Asp Met Leu Asp Glu Ile Ala
                245                 250                 255

Ala Asp Phe Ser Tyr Ser Leu Val Arg Lys Gly Asp Arg Ile Leu Gly
            260                 265                 270

Trp Leu Trp Asn Arg Ile Tyr Gln Gly Leu Asn Ile Asn Asn Ala Ala
        275                 280                 285

Thr Val Arg Arg Leu Ala Gln Asp Gly His Glu Ile Val Tyr Val Pro
    290                 295                 300

Cys His Arg Ser His Met Asp Tyr Leu Leu Leu Ser Tyr Val Leu Tyr
305                 310                 315                 320

His Glu Gly Met Val Pro Pro His Ile Ala Ala Gly Ile Asn Leu Asn
                325                 330                 335

Phe Phe Pro Ala Gly Pro Ile Phe Arg Arg Gly Gly Ala Phe Phe Ile
            340                 345                 350

Arg Arg Ser Phe Lys Gly Asn Lys Leu Tyr Ser Thr Ile Phe Arg Glu
        355                 360                 365

Tyr Leu Ala Glu Leu Phe Ala Lys Gly Tyr Ser Val Glu Tyr Phe Ser
    370                 375                 380

Glu Gly Gly Arg Ser Arg Thr Gly Arg Leu Leu Gln Ala Lys Thr Gly
385                 390                 395                 400

Met Leu Ala Met Thr Ile Gln Ala Met Leu Arg Gly Leu Asn Arg Pro
                405                 410                 415

Val Thr Leu Val Pro Val Tyr Ile Gly Tyr Glu His Val Met Glu Val
            420                 425                 430

Gly Thr Tyr Ala Lys Glu Leu Arg Gly Lys Arg Lys Glu Lys Glu Asn
        435                 440                 445

Ala Ser Leu Val Leu Arg Thr Ile Arg Lys Leu Arg Asn Phe Gly Gln
    450                 455                 460

Gly Tyr Val Asn Phe Gly Glu Pro Ile Pro Leu Asn Gln Phe Leu Asn
465                 470                 475                 480

Glu Gln Val Pro Glu Trp Thr Gln Asp Ile Asp Ala Met Gly Ala Ser
```

485 490 495

Lys Pro Gln Trp Met Thr Pro Val Val Asn Lys Leu Ala Thr Lys Met
500 505 510

Met Thr His Ile Asn Asp Ala Ala Ala Ala Asn Ala Met Thr Leu Cys
515 520 525

Ala Thr Ala Leu Leu Ala Ser Arg Gln Arg Ala Leu Ala Arg Asp Asn
530 535 540

Leu Val Lys Gln Ile Asp Cys Tyr Leu Gln Leu Leu Arg Asn Val Pro
545 550 555 560

Tyr Ser Asn Thr Tyr Thr Val Pro Ser Asp Ser Ala Glu Ser Leu Val
565 570 575

Gln His Ala Glu Ser Leu Asp Lys Phe Val Val Glu Thr Asp Thr Met
580 585 590

Gly Asp Ile Ile Ser Leu Asp Arg Asn Gln Ser Ile Leu Met Thr Tyr
595 600 605

Tyr Arg Asn Asn Ile Ile His Leu Leu Ala Leu Pro Ser Leu Ile Ala
610 615 620

Gln Met Leu Ile Arg Gln Gln Gln Met Pro Val Glu Gln Ile Gln Thr
625 630 635 640

Cys Val Ala Lys Val Tyr Pro Phe Leu Lys Gln Glu Leu Phe Leu Ser
645 650 655

His Asp Glu Thr Gln Leu Asp Glu Val Val Met His Tyr Leu Ala Glu
660 665 670

Leu Gln Arg Gln Gln Leu Val Thr Leu Asp Asp Gly Ile Ala Thr Ile
675 680 685

Asn Gln Ala Gln Thr Gln Val Leu Met Leu Leu Gly Arg Thr Ile Ser
690 695 700

Glu Thr Leu Gln Arg Tyr Ala Ile Thr Leu Asn Leu Leu Val Ala Asn
705 710 715 720

Pro Glu Leu Gly Lys Ser Asp Leu Glu Ser Lys Ser Gln Glu Ile Ala
725 730 735

```
Gln Arg Leu Gly Arg Leu His Gly Ile Asn Ala Pro Glu Phe Phe Asp
            740                 745                 750

Lys Gly Val Phe Ser Ser Met Phe Val Thr Leu Lys Gln Gln Gly Tyr
        755                 760                 765

Leu Asp Ser Asp Gly Asn Cys His Leu Asp Gln Thr Lys His Phe Ser
    770                 775                 780

Arg Met Leu Tyr Thr Met Leu Tyr Pro Glu Val Arg Leu Thr Ile Gln
785                 790                 795                 800

Glu Ser Ile Cys Gln Val Glu
                805
```

<210> 22
<211> 886
<212> PRT
<213> Vibrio furnisii

<400> 22

```
Met Ser Asp Met Lys His Asp Val Asp Ala Leu Glu Thr Gln Glu Trp
1               5                   10                  15

Leu Ala Ala Leu Glu Ser Val Val Arg Glu Glu Gly Val Glu Arg Ala
            20                  25                  30

Gln Tyr Leu Leu Glu Glu Val Leu Glu Lys Ala Arg Leu Asp Gly Val
        35                  40                  45

Asp Met Pro Thr Gly Ile Thr Thr Asn Tyr Ile Asn Thr Ile Pro Ala
    50                  55                  60

Ala Gln Glu Pro Ala Tyr Pro Gly Asp Thr Thr Ile Glu Arg Arg Ile
65                  70                  75                  80

Arg Ser Ile Ile Arg Trp Asn Ala Ile Met Ile Val Leu Arg Ala Ser
                85                  90                  95

Lys Lys Asp Leu Asp Leu Gly Gly His Met Ala Ser Phe Gln Ser Ser
            100                 105                 110

Ala Ala Phe Tyr Glu Thr Cys Phe Asn His Phe Phe Arg Ala Pro Asn
        115                 120                 125

Glu Lys Asp Gly Gly Asp Leu Val Tyr Tyr Gln Gly His Ile Ser Pro
    130                 135                 140
```

```
Gly Ile Tyr Ala Arg Ala Phe Val Glu Gly Arg Leu Thr Glu Glu Gln
145                 150                 155                 160

Leu Asp Asn Phe Arg Gln Glu Val Asp Gly Lys Gly Ile Pro Ser Tyr
                165                 170                 175

Pro His Pro Lys Leu Met Pro Glu Phe Trp Gln Phe Pro Thr Val Ser
            180                 185                 190

Met Gly Leu Gly Pro Ile Ala Ser Ile Tyr Gln Ala Arg Phe Leu Lys
        195                 200                 205

Tyr Leu Glu Gly Arg Gly Met Lys Asp Thr Ala Glu Gln Arg Val Tyr
    210                 215                 220

Ala Phe Leu Gly Asp Gly Glu Met Asp Glu Pro Glu Ser Arg Gly Ala
225                 230                 235                 240

Ile Ser Phe Ala Ala Arg Glu Lys Leu Asp Asn Leu Cys Phe Leu Ile
                245                 250                 255

Asn Cys Asn Leu Gln Arg Leu Asp Gly Pro Val Met Gly Asn Gly Lys
            260                 265                 270

Ile Ile Gln Glu Leu Glu Gly Leu Phe Lys Gly Ala Gly Trp Asn Val
        275                 280                 285

Val Lys Val Ile Trp Gly Asn Asn Trp Asp Ser Leu Leu Ala Lys Asp
    290                 295                 300

Thr Ser Gly Lys Leu Leu Gln Leu Met Asn Glu Thr Ile Asp Gly Asp
305                 310                 315                 320

Tyr Gln Thr Phe Lys Ala Lys Asp Gly Ala Tyr Val Arg Glu His Phe
                325                 330                 335

Phe Gly Lys Tyr Pro Glu Thr Ala Ala Leu Val Ala Asp Met Thr Asp
            340                 345                 350

Asp Glu Val Phe Ala Leu Lys Arg Gly Gly His Glu Ser Ser Lys Leu
        355                 360                 365

Tyr Ala Ala Phe Lys Asn Ala Gln Asp Thr Lys Gly Arg Pro Thr Val
    370                 375                 380

Ile Leu Ala Lys Thr Val Lys Gly Tyr Gly Met Gly Asp Ala Ala Gln
385                 390                 395                 400
```

```
Gly Lys Asn Ile Ala His Gln Val Lys Lys Met Asp Met Thr His Val
                405                 410                 415

Ile Ala Met Arg Asn Arg Leu Gly Leu Gln Asp Ile Ile Ser Asp Glu
            420                 425                 430

Glu Val Asn Asn Leu Pro Tyr Leu Lys Leu Glu Glu Gly Ser Lys Glu
        435                 440                 445

Phe Glu Tyr Leu His Ala Arg Arg Lys Ala Leu His Gly Tyr Thr Pro
    450                 455                 460

Gln Arg Leu Pro Lys Phe Thr Gln Glu Leu Val Ile Pro Glu Leu Glu
465                 470                 475                 480

Glu Phe Lys Pro Leu Leu Glu Glu Gln Lys Arg Glu Ile Ser Ser Thr
                485                 490                 495

Met Ala Tyr Val Arg Ala Leu Asn Ile Leu Leu Lys Asp Lys Asn Ile
            500                 505                 510

Gly Lys Asn Ile Val Pro Ile Ile Ala Asp Glu Ala Arg Thr Phe Gly
        515                 520                 525

Met Glu Gly Leu Phe Arg Gln Ile Gly Ile Tyr Asn Pro His Gly Gln
    530                 535                 540

Thr Tyr Thr Pro Glu Asp Arg Gly Val Val Ser Tyr Tyr Lys Glu Asp
545                 550                 555                 560

Thr Ala Gly Gln Val Leu Gln Glu Gly Ile Asn Glu Leu Gly Ala Met
                565                 570                 575

Ser Ser Trp Val Ala Ala Ala Thr Ser Tyr Ser Thr Asn Asn Leu Pro
            580                 585                 590

Met Ile Pro Phe Tyr Ile Tyr Tyr Ser Met Phe Gly Phe Gln Arg Val
        595                 600                 605

Gly Asp Met Ala Trp Met Ala Gly Asp Gln Gln Ala Arg Gly Phe Leu
    610                 615                 620

Leu Gly Ala Thr Ala Gly Arg Thr Thr Leu Asn Gly Glu Gly Leu Gln
625                 630                 635                 640

His Glu Asp Gly His Ser His Ile Gln Ala Ala Thr Ile Pro Asn Cys
                645                 650                 655
```

```
Ile Ser Tyr Asp Pro Thr Phe Ala Tyr Glu Val Ala Val Ile Met Gln
            660                 665                 670

Asp Gly Ile Arg Arg Met Tyr Gly Asp Gln Glu Asn Val Phe Tyr Tyr
        675                 680                 685

Met Thr Leu Met Asn Glu Asn Tyr Ala His Pro Ala Met Pro Glu Gly
    690                 695                 700

Ala Glu Glu Gly Ile Arg Lys Gly Ile Tyr Lys Leu Glu Thr Leu Ser
705                 710                 715                 720

Gly Ser Lys Gly Lys Val Gln Leu Met Ser Ser Gly Thr Ile Met Asn
                725                 730                 735

Glu Val Arg Lys Ala Ala Val Ile Leu Ser Glu Glu Tyr Gly Ile Ala
            740                 745                 750

Ser Asp Val Tyr Ser Val Thr Ser Phe Asn Glu Leu Ala Arg Asp Gly
        755                 760                 765

Gln Asn Val Glu Arg Tyr Asn Met Leu His Pro Glu Ala Glu Ala Gln
    770                 775                 780

Val Pro Tyr Ile Ala Ser Val Met Gly Thr Glu Pro Ala Ile Ala Ala
785                 790                 795                 800

Thr Asp Tyr Met Lys Asn Tyr Ala Asp Gln Val Arg Ala Phe Ile Pro
                805                 810                 815

Ala Glu Ser Tyr Lys Val Leu Gly Thr Asp Gly Phe Gly Arg Ser Asp
            820                 825                 830

Ser Arg Glu Asn Leu Arg Arg His Phe Glu Val Asn Ala Gly Tyr Val
        835                 840                 845

Val Val Ala Ala Leu Asn Glu Leu Ala Lys Arg Gly Glu Val Glu Lys
    850                 855                 860

Ser Val Val Ala Glu Ala Ile Lys Lys Phe Asp Ile Asp Thr Glu Lys
865                 870                 875                 880

Thr Asn Pro Leu Tyr Ala
                885
```

<210> 23
<211> 630

<212> PRT
<213> Vibrio furnisii

<400> 23

```
Met Ala Ile Glu Ile Tyr Val Pro Asp Ile Gly Ala Asp Glu Val Glu
1               5                   10                  15

Val Thr Glu Ile Leu Val Ser Val Gly Asp Lys Val Glu Glu Glu Gln
            20                  25                  30

Ser Leu Ile Thr Val Glu Gly Asp Lys Ala Ser Met Glu Val Pro Ala
        35                  40                  45

Ser Gln Ala Gly Ile Val Lys Glu Ile Lys Val Val Thr Gly Asp Lys
    50                  55                  60

Val Thr Thr Gly Ser Leu Ile Met Val Phe Glu Ala Glu Gly Ala Ala
65                  70                  75                  80

Ala Ala Ala Pro Ala Pro Ala Ala Glu Ala Ala Pro Val Ala Ala Ala
                85                  90                  95

Pro Ala Ala Val Glu Leu Lys Glu Val Asn Val Pro Asp Ile Gly Gly
            100                 105                 110

Asp Glu Val Glu Val Thr Glu Ile Met Val Ala Val Gly Asp Thr Val
        115                 120                 125

Ser Glu Glu Gln Ser Leu Ile Thr Val Glu Gly Asp Lys Ala Ser Met
    130                 135                 140

Glu Val Pro Ala Pro Phe Ala Gly Thr Val Lys Glu Ile Lys Ile Ala
145                 150                 155                 160

Ser Gly Asp Lys Val Thr Thr Gly Ser Leu Ile Met Val Phe Glu Val
                165                 170                 175

Ala Gly Ser Gly Ala Pro Ala Ala Ala Ala Pro Ala Gln Ala Ala Ala
            180                 185                 190

Pro Ala Ala Ala Pro Ala Val Ala Ala Asp Lys Glu Val Asn Val Pro
        195                 200                 205

Asp Ile Gly Gly Asp Glu Val Glu Val Thr Glu Ile Met Val Ala Val
    210                 215                 220

Gly Asp Met Val Ser Glu Glu Gln Ser Leu Ile Thr Val Glu Gly Asp
```

225 230 235 240

Lys Ala Ser Met Glu Val Pro Ala Pro Phe Ala Gly Lys Val Lys Ala
245 250 255

Ile Lys Val Ala Ala Gly Asp Lys Val Ser Thr Gly Ser Leu Ile Met
260 265 270

Val Phe Glu Val Ala Gly Ala Ala Pro Ala Ala Val Ser Ala Pro Ala
275 280 285

Gln Ala Ala Ala Pro Ala Ala Ala Ala Pro Lys Ala Glu Ala Pro Ala
290 295 300

Ala Ala Ala Pro Ala Ala Ala Thr Gly Asp Phe Gln Glu Asn Asn Glu
305 310 315 320

Tyr Ala His Ala Ser Pro Val Val Arg Arg Leu Ala Arg Glu Phe Gly
325 330 335

Val Asn Leu Ser Lys Val Lys Gly Ser Gly Arg Lys Ser Arg Ile Leu
340 345 350

Lys Glu Asp Val Gln Asn Tyr Val Lys Glu Ala Leu Lys Arg Leu Glu
355 360 365

Ser Gly Ala Ala Ser Ala Ala Ser Gly Lys Gly Asp Gly Ala Ala Leu
370 375 380

Gly Leu Leu Pro Trp Pro Lys Val Asp Phe Ser Lys Phe Gly Asp Thr
385 390 395 400

Glu Ile Gln Pro Leu Ser Arg Ile Lys Lys Ile Ser Gly Ala Asn Leu
405 410 415

His Arg Asn Trp Val Met Ile Pro His Val Thr Gln Trp Asp Asn Ala
420 425 430

Asp Ile Thr Glu Leu Glu Ala Phe Arg Lys Glu Gln Asn Ala Ile Glu
435 440 445

Ala Lys Lys Asp Thr Gly Met Lys Ile Thr Pro Leu Val Phe Ile Met
450 455 460

Lys Ala Ala Ala Lys Ala Leu Glu Ala Phe Pro Ala Phe Asn Ser Ser
465 470 475 480

```
Leu Ser Glu Asp Gly Glu Ser Leu Ile Leu Lys Lys Tyr Val Asn Ile
                485                 490                 495

Gly Ile Ala Val Asp Thr Pro Asn Gly Leu Val Val Pro Val Phe Lys
            500                 505                 510

Asp Val Asn Lys Lys Gly Ile Tyr Glu Leu Ser Glu Glu Leu Ala Val
        515                 520                 525

Val Ser Lys Lys Ala Arg Ala Gly Lys Leu Thr Ala Ser Asp Met Gln
    530                 535                 540

Gly Gly Cys Phe Thr Ile Ser Ser Leu Gly Gly Ile Gly Gly Thr Ala
545                 550                 555                 560

Phe Thr Pro Ile Val Asn Ala Pro Glu Val Gly Ile Leu Gly Val Ser
                565                 570                 575

Lys Ser Glu Met Lys Pro Val Trp Asn Gly Lys Glu Phe Ala Pro Arg
            580                 585                 590

Leu Gln Leu Pro Leu Ser Leu Ser Tyr Asp His Arg Val Ile Asp Gly
        595                 600                 605

Ala Glu Gly Ala Arg Phe Ile Thr Tyr Leu Asn Gly Cys Leu Ser Asp
    610                 615                 620

Ile Arg Arg Leu Val Leu
625                 630
```

<210> 24
<211> 316
<212> PRT
<213> Vibrio furnisii

<400> 24

```
Met Tyr Ser Lys Ile Leu Gly Thr Gly Ser Tyr Leu Pro Ser Gln Val
1               5                   10                  15

Arg Thr Asn Ala Asp Leu Glu Lys Met Val Asp Thr Ser Asp Glu Trp
            20                  25                  30

Ile Val Thr Arg Thr Gly Ile Arg Glu Arg Arg Ile Ala Ala Asp Asn
        35                  40                  45

Glu Thr Val Ala Asp Met Gly Phe Tyr Ala Ala Gln Asn Ala Ile Glu
    50                  55                  60
```

```
Met Ala Gly Ile Asp Lys Asn Asp Ile Asp Leu Ile Ile Leu Ala Thr
65                  70                  75                  80

Thr Ser Ser Ser His Thr Phe Pro Ser Ser Ala Cys Gln Val Gln Ala
                85                  90                  95

Lys Leu Gly Ile Lys Gly Cys Pro Ala Phe Asp Leu Ala Ala Ala Cys
            100                 105                 110

Ser Gly Phe Ile Tyr Gly Leu Ser Val Ala Asp Gln His Ile Lys Ser
        115                 120                 125

Gly Met Cys Lys Asn Val Leu Val Ile Gly Ala Asp Ala Leu Ser Lys
    130                 135                 140

Thr Cys Asp Pro Thr Asp Arg Ser Thr Ile Ile Leu Phe Gly Asp Gly
145                 150                 155                 160

Ala Gly Ala Val Val Val Gly Ala Ser Glu Glu Pro Gly Ile Leu Ser
                165                 170                 175

Thr His Val Tyr Ala Asp Gly Gln Phe Gly Asp Leu Leu Ser Leu Glu
            180                 185                 190

Val Pro Glu Arg Gly Gly Asp Val Asp Lys Trp Leu Tyr Met Ala Gly
        195                 200                 205

Asn Glu Val Phe Lys Val Ala Val Thr Gln Leu Ser Lys Leu Val Lys
    210                 215                 220

Asp Thr Leu Ala Ala Asn Asn Met His Lys Ser Glu Leu Asp Trp Leu
225                 230                 235                 240

Val Pro His Gln Ala Asn Tyr Arg Ile Ile Ser Ala Thr Ala Lys Lys
                245                 250                 255

Leu Ser Met Ser Leu Asp Gln Val Val Ile Thr Leu Asp Arg His Gly
            260                 265                 270

Asn Thr Ser Ala Ala Thr Val Pro Thr Ala Leu Asp Glu Ala Val Arg
        275                 280                 285

Asp Gly Arg Ile Lys Arg Gly Gln Thr Leu Leu Leu Glu Ala Phe Gly
    290                 295                 300

Gly Gly Phe Thr Trp Gly Ser Ala Leu Val Lys Phe
305                 310                 315
```

<210> 25
<211> 307
<212> PRT
<213> Vibrio furnisii

<400> 25

```
Met Ser Lys Phe Ala Ile Val Phe Pro Gly Gln Gly Ser Gln Ala Val
1               5                   10                  15

Gly Met Leu Ala Glu Leu Gly Glu Gln Tyr Asp Val Val Lys Gln Thr
            20                  25                  30

Phe Ala Glu Ala Ser Asp Ala Leu Gly Tyr Asp Leu Trp Ala Leu Val
        35                  40                  45

Gln Asn Gly Pro Val Glu Asp Leu Asn Gln Thr Phe Arg Thr Gln Pro
    50                  55                  60

Ala Leu Leu Ala Ser Ser Val Ala Ile Trp Arg Val Trp Gln Ala Leu
65                  70                  75                  80

Gly Leu Glu Gln Pro Glu Val Leu Ala Gly His Ser Leu Gly Glu Tyr
                85                  90                  95

Ser Ala Leu Val Cys Ala Gly Val Ile Asp Phe Lys Ala Ala Ile Lys
            100                 105                 110

Leu Val Glu Leu Arg Gly Gln Leu Met Gln Glu Ala Val Pro Ala Gly
        115                 120                 125

Thr Gly Ala Met Tyr Ala Ile Ile Gly Leu Asp Asp Ala Ala Ile Ala
    130                 135                 140

Lys Ala Cys Glu Asp Ala Ala Gln Gly Asp Val Val Ser Pro Val Asn
145                 150                 155                 160

Phe Asn Ser Pro Gly Gln Val Val Ile Ala Gly Gln Lys Asp Ala Val
                165                 170                 175

Glu Arg Ala Gly Ala Leu Cys Lys Glu Ala Gly Ala Lys Arg Ala Leu
            180                 185                 190

Pro Leu Pro Val Ser Val Pro Ser His Cys Ala Leu Met Lys Pro Ala
        195                 200                 205

Ala Glu Lys Leu Ala Val Ala Leu Glu Ala Leu Glu Phe Asn Ala Pro
    210                 215                 220
```

Gln Ile Pro Val Ile Asn Asn Val Asp Val Ala Thr Glu Thr Asp Pro
225 230 235 240

Ala Lys Ile Lys Asp Ala Leu Val Arg Gln Leu His Ser Pro Val Arg
245 250 255

Trp Thr Glu Gly Val Glu Lys Met Ala Ala Gln Gly Ile Glu Lys Leu
260 265 270

Ile Glu Val Gly Pro Gly Lys Val Leu Thr Gly Leu Thr Lys Arg Ile
275 280 285

Val Lys Thr Leu Asp Ala Ala Ala Val Asn Asp Ile Ala Ser Leu Glu
290 295 300

Ala Val Lys
305

<210> 26
<211> 248
<212> PRT
<213> Vibrio furnisii

<400> 26

Met Ser Asn Phe Met Asn Leu Glu Gly Lys Ile Val Leu Val Thr Gly
1 5 10 15

Ala Ser Arg Gly Ile Gly Lys Ala Ile Ala Glu Leu Leu Val Glu Arg
20 25 30

Gly Ala Thr Val Ile Gly Thr Ala Thr Ser Glu Ser Gly Ala Asp Ala
35 40 45

Ile Ser Ala Tyr Leu Gly Asp Asn Gly Lys Gly Leu Ala Leu Asn Val
50 55 60

Thr Asp Val Ala Ser Ile Glu Ser Val Leu Lys Ser Ile Asn Asp Glu
65 70 75 80

Phe Gly Gly Val Asp Ile Leu Val Asn Asn Ala Gly Ile Thr Arg Asp
85 90 95

Asn Leu Leu Met Arg Met Lys Asp Asp Glu Trp Thr Asp Ile Leu Asp
100 105 110

Thr Asn Leu Thr Ser Ile Phe Arg Leu Ser Lys Ala Val Leu Arg Gly
115 120 125

```
Met Met Lys Lys Arg Gln Gly Arg Ile Ile Asn Val Gly Ser Val Val
    130                 135                 140

Gly Thr Met Gly Asn Ala Gly Gln Thr Asn Tyr Ala Ala Ala Lys Ala
145                 150                 155                 160

Gly Val Ile Gly Phe Thr Lys Ser Met Ala Arg Glu Val Ala Ser Arg
                165                 170                 175

Gly Val Thr Val Asn Thr Val Ala Pro Gly Phe Ile Glu Thr Asp Met
            180                 185                 190

Thr Lys Ala Leu Asn Asp Asp Gln Arg Ala Ala Thr Leu Ala Gln Val
        195                 200                 205

Pro Ala Gly Arg Leu Gly Asp Pro Arg Glu Ile Ala Ser Ala Val Ala
    210                 215                 220

Phe Leu Ala Ser Pro Glu Ala Ala Tyr Ile Thr Gly Glu Thr Leu His
225                 230                 235                 240

Val Asn Gly Gly Met Tyr Met Val
                245
```

<210> 27
<211> 77
<212> PRT
<213> Vibrio furnisii

<400> 27

```
Met Ser Asn Ile Glu Glu Arg Val Lys Lys Ile Ile Val Glu Gln Leu
1               5                   10                  15

Gly Val Asp Glu Ala Glu Val Lys Asn Glu Ala Ser Phe Val Glu Asp
            20                  25                  30

Leu Gly Ala Asp Ser Leu Asp Thr Val Glu Leu Val Met Ala Leu Glu
        35                  40                  45

Glu Glu Phe Asp Thr Glu Ile Pro Asp Glu Glu Ala Glu Lys Ile Thr
    50                  55                  60

Thr Val Gln Ala Ala Ile Asp Tyr Val Asn Ser Ala Gln
65                  70                  75
```

<210> 28
<211> 416
<212> PRT

<213> Vibrio furnisii

<400> 28

```
Met Ile Val Ser Lys Arg Arg Val Val Val Thr Gly Met Gly Met Leu
1               5                   10                  15

Ser Pro Val Gly Asn Thr Val Glu Ser Ser Trp Lys Ala Leu Leu Ala
            20                  25                  30

Gly Gln Ser Gly Ile Val Asn Ile Glu His Phe Asp Thr Thr Asn Phe
        35                  40                  45

Ser Thr Arg Phe Ala Gly Leu Val Lys Asp Phe Asn Cys Glu Glu Tyr
    50                  55                  60

Met Ser Lys Lys Asp Ala Arg Lys Met Asp Leu Phe Ile Gln Tyr Gly
65                  70                  75                  80

Ile Ala Ala Gly Ile Gln Ala Leu Asp Asp Ser Gly Leu Val Ile Thr
                85                  90                  95

Glu Glu Asn Ala Pro Arg Val Gly Val Ala Ile Gly Ser Gly Ile Gly
            100                 105                 110

Gly Leu Asp Leu Ile Glu Lys Gly His Gln Ala Leu Met Glu Lys Gly
        115                 120                 125

Pro Arg Lys Val Ser Pro Phe Phe Val Pro Ser Thr Ile Val Asn Met
    130                 135                 140

Val Ala Gly Asn Leu Ser Ile Met Arg Gly Leu Arg Gly Pro Asn Ile
145                 150                 155                 160

Ala Ile Ser Thr Ala Cys Thr Thr Gly Leu His Asn Ile Gly His Ala
                165                 170                 175

Ala Arg Met Ile Ala Tyr Gly Asp Ala Glu Ala Met Val Ala Gly Gly
            180                 185                 190

Ser Glu Lys Ala Ser Thr Pro Leu Gly Met Ala Gly Phe Gly Ala Ala
        195                 200                 205

Lys Ala Leu Ser Thr Arg Asn Asp Glu Pro Ala Lys Ala Ser Arg Pro
    210                 215                 220

Trp Asp Lys Asp Arg Asp Gly Phe Val Leu Gly Asp Gly Ala Gly Val
225                 230                 235                 240
```

```
Met Val Leu Glu Gly Tyr Glu His Ala Lys Ala Arg Gly Ala Lys Ile
                245                 250                 255

Tyr Ala Glu Ile Val Gly Phe Gly Met Ser Gly Asp Ala Tyr His Met
            260                 265                 270

Thr Ser Pro Ser Glu Asp Gly Ser Gly Gly Ala Leu Ala Met Glu Ala
        275                 280                 285

Ala Met Arg Asp Ala Ala Leu Ala Gly Thr Gln Ile Gly Tyr Val Asn
    290                 295                 300

Ala His Gly Thr Ser Thr Pro Ala Gly Asp Val Ala Glu Val Lys Gly
305                 310                 315                 320

Ile Lys Arg Ala Leu Gly Glu Asp Gly Ala Lys Gln Val Leu Ile Ser
                325                 330                 335

Ser Thr Lys Ser Met Thr Gly His Leu Leu Gly Ala Ala Gly Ser Val
            340                 345                 350

Glu Ala Ile Ile Thr Val Met Ser Leu Val Asp Gln Ile Val Pro Pro
        355                 360                 365

Thr Ile Asn Leu Asp Asn Pro Glu Glu Gly Leu Gly Val Asp Leu Val
    370                 375                 380

Pro His Thr Ala Arg Lys Val Glu Gly Met Glu Tyr Ala Met Cys Asn
385                 390                 395                 400

Ser Phe Gly Phe Gly Gly Thr Asn Gly Ser Leu Ile Phe Lys Arg Val
                405                 410                 415
```

<210> 29
<211> 344
<212> PRT
<213> Vibrio furnisii

<400> 29

```
Met Thr Asp Ser His Thr Asn Asn Ala Tyr Gly Lys Ala Ile Ala Met
1               5                   10                  15

Thr Val Ile Gly Ala Gly Ser Tyr Gly Thr Ser Leu Ala Ile Ser Leu
            20                  25                  30

Ala Arg Asn Gly Ala Asn Val Val Leu Trp Gly His Asp Pro Val His
        35                  40                  45
```

Met Ala Arg Leu Glu Ala Glu Arg Ala Asn His Glu Phe Leu Pro Asp
50 55 60

Ile Asp Phe Pro Pro Ser Leu Ile Ile Glu Ser Asp Leu Gln Lys Ala
65 70 75 80

Val Gln Ala Ser Arg Asp Leu Leu Val Val Val Pro Ser His Val Phe
85 90 95

Ala Ile Val Leu Asn Ser Leu Gln Pro Tyr Leu Arg Glu Asp Thr Arg
100 105 110

Ile Cys Trp Ala Thr Lys Gly Leu Glu Pro Asp Thr Gly Arg Leu Leu
115 120 125

Gln Asp Val Ala His Asp Val Leu Gly Glu Ser His Pro Leu Ala Val
130 135 140

Leu Ser Gly Pro Thr Phe Ala Lys Glu Leu Ala Met Gly Met Pro Thr
145 150 155 160

Ala Ile Ser Val Ala Ser Pro Asp Ala Gln Phe Val Ala Asp Leu Gln
165 170 175

Glu Lys Ile His Cys Ser Lys Thr Phe Arg Val Tyr Ala Asn Ser Asp
180 185 190

Phe Ile Gly Met Gln Leu Gly Gly Ala Val Lys Asn Val Ile Ala Ile
195 200 205

Gly Ala Gly Met Ser Asp Gly Ile Gly Phe Gly Ala Asn Ala Arg Thr
210 215 220

Ala Leu Ile Thr Arg Gly Leu Ala Glu Met Thr Arg Leu Gly Ala Ala
225 230 235 240

Leu Gly Ala Gln Pro Glu Thr Phe Met Gly Met Ala Gly Leu Gly Asp
245 250 255

Leu Val Leu Thr Cys Thr Asp Asn Gln Ser Arg Asn Arg Arg Phe Gly
260 265 270

Leu Ala Leu Gly Gln Gly Lys Asp Val Asp Thr Ala Gln Gln Asp Ile
275 280 285

Gly Gln Val Val Glu Gly Tyr Arg Asn Thr Lys Glu Val Trp Leu Leu
290 295 300

```
Ala Gln Arg Met Gly Val Glu Met Pro Ile Val Glu Gln Ile Tyr Gln
305                 310                 315                 320

Val Leu Tyr Gln Gly Lys Asp Ala Arg Met Ala Ala Gln Asp Leu Leu
                325                 330                 335

Ala Arg Asp Lys Lys Ala Glu Arg
            340
```

<210> 30
<211> 284
<212> PRT
<213> Vibrio furnisii

<400> 30

```
Val Val Cys Ala Phe Val Asn Asp Asp Leu Ser Ala Thr Val Leu Glu
1               5                   10                  15

Glu Leu Tyr Gln Gly Gly Thr Arg Leu Ile Ala Met Arg Cys Ala Gly
            20                  25                  30

Phe Asp Lys Val Asp Leu Asp Ala Ala Lys Arg Ile Gly Met Gln Val
        35                  40                  45

Val Arg Val Pro Ala Tyr Ser Pro Glu Ala Val Ala Glu His Ala Val
    50                  55                  60

Gly Leu Met Met Cys Leu Asn Arg Arg Tyr His Lys Ala Tyr Gln Arg
65                  70                  75                  80

Thr Arg Glu Ala Asn Phe Ser Leu Glu Gly Leu Val Gly Phe Asn Phe
                85                  90                  95

Tyr Gly Lys Thr Val Gly Val Ile Gly Ser Gly Lys Ile Gly Ile Ala
            100                 105                 110

Ala Met Arg Ile Leu Lys Gly Leu Gly Met Asn Ile Leu Cys Phe Asp
        115                 120                 125

Pro Tyr Glu Asn Pro Leu Ala Ile Glu Ile Gly Ala Lys Tyr Val Gln
    130                 135                 140

Leu Pro Glu Leu Tyr Ala Asn Ser Asp Ile Ile Thr Leu His Cys Pro
145                 150                 155                 160

Met Thr Lys Glu Asn Tyr His Leu Leu Asp Glu Gln Ala Phe Ala Gln
                165                 170                 175
```

```
Met Lys Asp Gly Val Met Ile Ile Asn Thr Ser Arg Gly Glu Leu Leu
            180                 185                 190

Asp Ser Val Ala Ala Ile Glu Ala Leu Lys Arg Gly Arg Ile Gly Ala
        195                 200                 205

Leu Gly Leu Asp Val Tyr Asp Asn Glu Lys Asp Leu Phe Phe Gln Asp
    210                 215                 220

Lys Ser Asn Asp Val Ile Val Asp Asp Val Phe Arg Arg Leu Ser Ala
225                 230                 235                     240

Cys His Asn Val Leu Phe Thr Gly His Gln Ala Phe Leu Thr Glu Asp
                245                 250                 255

Ala Leu His Asn Ile Ala Gln Thr Thr Leu Asn Asn Val Leu Ala Phe
            260                 265                 270

Glu Gln Gly Thr Lys Ser Gly Asn Glu Leu Val Asn
        275                 280
```

Claims

1. A recombinant *E. coli* cell comprising one or more exogenous nucleic acid sequences encoding at least one enzyme from a fatty alcohol biosynthetic pathway for use in the production of a fatty alcohol, wherein the fatty alcohol is released from the cell into the culture medium at a 1:1 ratio of intracellular product to extracellular product, and wherein the one or more exogenous nucleic acid sequences are overexpressed.

2. The recombinant *E. coli* cell of claim 1, wherein the enzyme is selected from the group consisting of an acetyl-CoA carboxylase of E.C. 6.4.1.2, a thioesterase of EC 3.1.2.14, an acyl-CoA synthase of EC 2.3.1.86, a fatty alcohol forming acyl-CoA reductase of EC 1.1.1.*, an acyl-CoA reductase of 1.2.1.50, an alcohol dehydrogenase of EC 1.1.1.1, and a FAS enzyme of EC 2.3.1.85.

3. A culture medium comprising the recombinant *E. coli cell* of claim 1.

4. The culture medium of claim 3, further comprising a fatty alcohol which is produced by the recombinant E. *coli* cell of claim 1.

5. The culture medium of claim 4, wherein the fatty acid derivative comprises from 1 to 5 double bonds.

6. The culture medium of claim 4, wherein the fatty acid derivative comprises a carbon chain length from 8 to 30.

7. The culture medium of claim 4, wherein the fatty acid derivative comprises from 1 to 5 branch points.

8. Use of a recombinant *E. coli* cell comprising one or more exogenous nucleic acid sequences encoding at least one polypeptide from a fatty alcohol biosynthetic pathway for the production of a fatty alcohol, wherein the fatty alcohol is released from the cell into the culture medium at a 1:1 ratio of intracellular product to extracellular product, and wherein the one or more exogenous nucleic acid sequences are overexpressed.

9. A method of producing a fatty alcohol comprising culturing a recombinant *E. coli* cell comprising one or more exogenous nucleic acid sequences encoding at least one polypeptide from a fatty alcohol biosynthetic pathway, wherein the fatty acid derivative is released from the cell into the culture medium at a 1:1 ratio of intracellular product

REFERENCES CITED IN THE DESCRIPTION

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PATENTKRAV

1. Rekombinant *E. coli*-celle, der omfatter én eller flere eksogene nukleinsyresekvenser, der koder for mindst ét enzym fra en biosyntesevej for en fedtalkohol til anvendelse i fremstillingen af en fedtalkohol, hvor fedtalkoholen frigives fra cellen i dyrkningsmediet ved et 1:1 forhold mellem intracellullært produkt og ekstracellulært produkt, og hvor den ene eller flere eksogene nukleinsyresekvenser er overudtrykt.

2. Rekombinant *E. coli*-celle ifølge krav 1, hvor enzymet er udvalgt fra gruppen bestående af en acetyl-CoA-carboxylase af E.C. 6.4.1.2, en thioesterase af EC 3.1.2.14, en acyl-CoA-syntase af EC 2.3.1.86, en fedtalkoholdannende acyl-CoA-reduktase af EC 1.1.1.*, en acyl-CoA-reduktase af 1.2.1.50, en alkoholdehydrogenase af EC 1.1.1.1 og et FAS-enzym af EC 2.3.1.85.

3. Dyrkningsmedium, der omfatter den rekombinante *E. coli*-celle ifølge krav 1.

4. Dyrkningsmedium ifølge krav 3, der endvidere omfatter en fedtalkohol, der er fremstillet ved hjælp af den rekombinante E. *coli*-celle ifølge krav 1.

5. Dyrkningsmedium ifølge krav 4, hvor fedtsyrederivatet omfatter fra 1 til 5 dobbeltbindinger.

6. Dyrkningsmedium ifølge krav 4, hvor fedtsyrederivatet omfatter en carbonkædelængde fra 8 til 30.

7. Dyrkningsmedium ifølge krav 4, hvor fedtsyrederivatet omfatter fra 1 til 5 forgreningspunkter.

8. Anvendelse af en rekombinant *E. coli*-celle, der omfatter én eller flere eksogene nukleinsyresekvenser, der koder for mindst ét polypeptid fra en biosyntesevej for en fedtalkohol til fremstilling af en fedtalkohol, hvor fedtalkoholen frigives fra cellen i dyrkningsmediet ved et 1:1 forhold mellem intracellulært produkt og ekstracellulært produkt, og

hvor den ene eller flere eksogene nukleinsyresekvenser er overudtrykt.

9. Fremgangsmåde til fremstilling af en fedtalkohol, der omfatter dyrkning af en rekombinant *E. coli*-celle omfattende én eller flere eksogene nukleinsyresekvenser, der koder for mindst ét polypeptid fra en biosyntesevej for en fedtalkohol,

hvor fedtsyrederivatet frigives fra cellen ind i dyrkningsmediet ved et 1:1 forhold mellem intracellulært produkt og ekstracellulært produkt, og hvor den ene eller flere eksogene nukleinsyresekvenser er overudtrykt.

10. Anvendelse ifølge krav 8, eller fremgangsmåde ifølge krav 9, hvor enzymet er udvalgt fra gruppen bestående af en acetyl-CoA-carboxylase af E.C. 6.4.1.2, en thioesterase af EC 3.1.2.14, en acyl-CoA-syntase af EC 2.3.1.86, en fedtalkohol, der danner en acyl-CoA-reduktase af EC 1.1.1.*, en acyl-CoA-reduktase af 1.2.1.50, en alkoholdehydrogenase af EC 1.1.1.1 og et FAS-enzym af EC 2.3.1.85.

11. Anvendelse ifølge krav 8, eller fremgangsmåde ifølge krav 9, hvor fedtsyrederivatet omfatter fra 1 til 5 dobbeltbindinger.

12. Anvendelse ifølge krav 8, eller fremgangsmåde ifølge krav 9, hvor fedtsyrederivatet omfatter en carbonkædelængde fra 8 til 30.

13. Anvendelse ifølge krav 8, eller fremgangsmåde ifølge krav 9, hvor fedtsyrederivatet omfatter fra 1 til 5 forgreningspunkter.

14. Beholder, der indeholder den rekombinante *E. coli*-celle ifølge krav 1 eller 2, eller dyrkningsmedium ifølge et hvilket som helst af krav 3 til 7.

15. Fermenteringsbouillon, der omfatter den rekombinante *E. coli*-celle ifølge krav 1 eller 2, eller dyrkningsmedium ifølge et hvilket som helst af krav 3 til 7.

DRAWINGS

FIG. 1

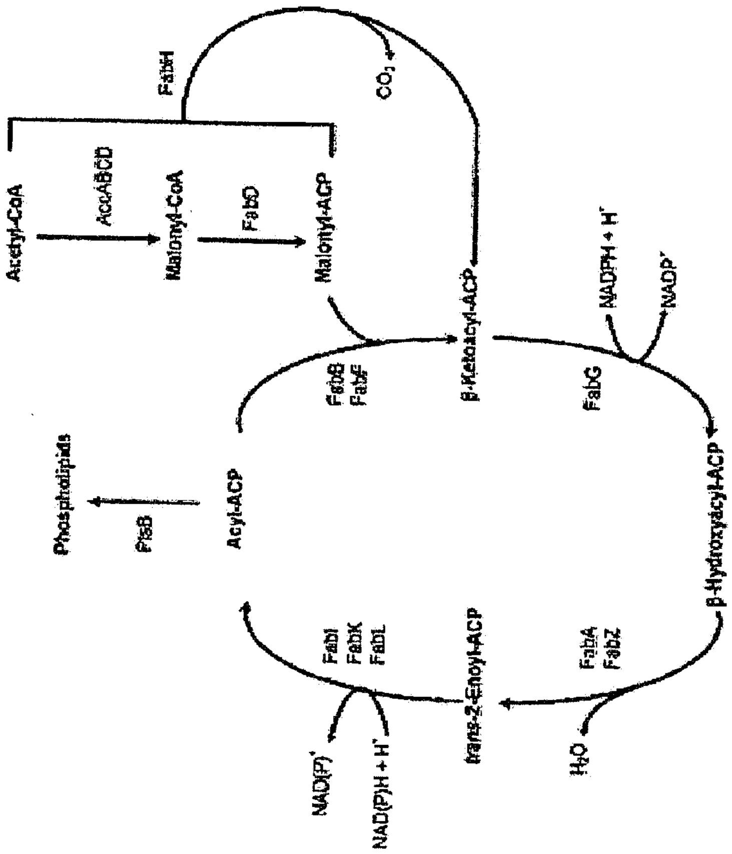
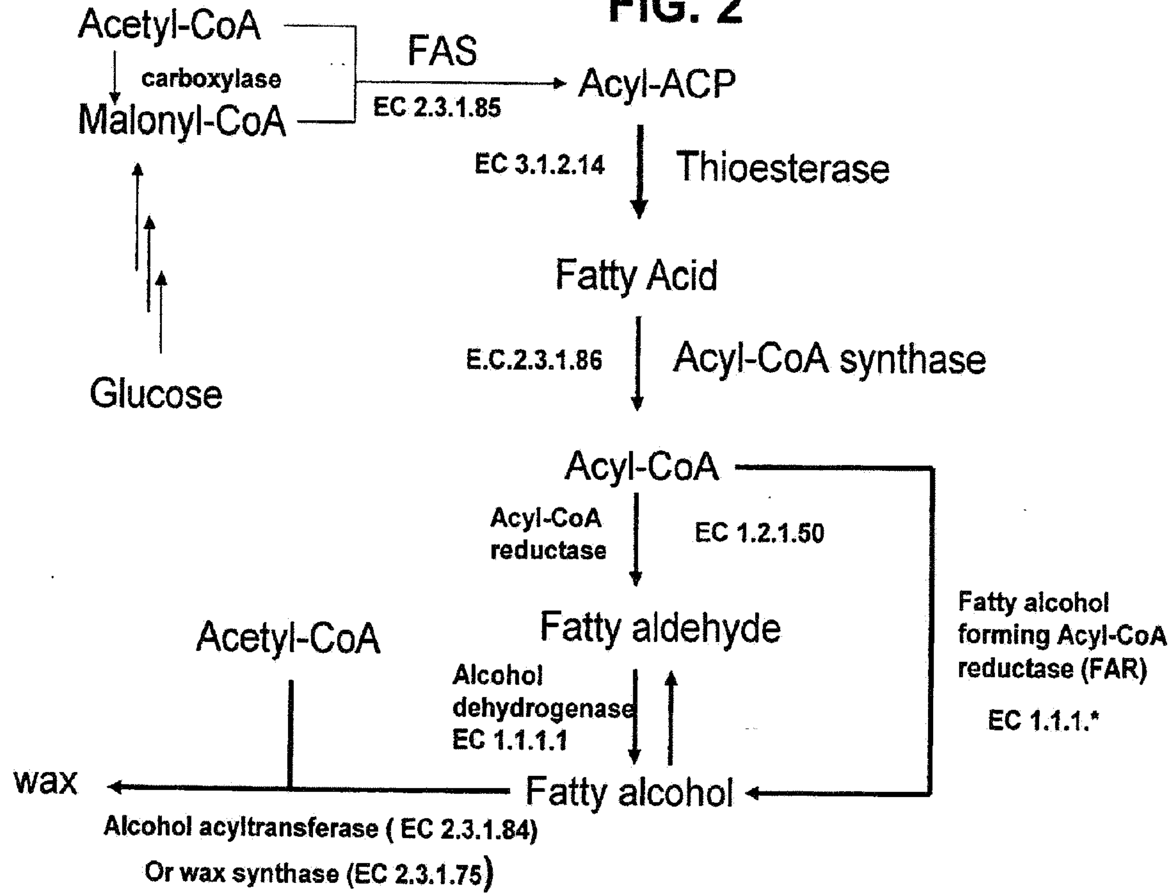
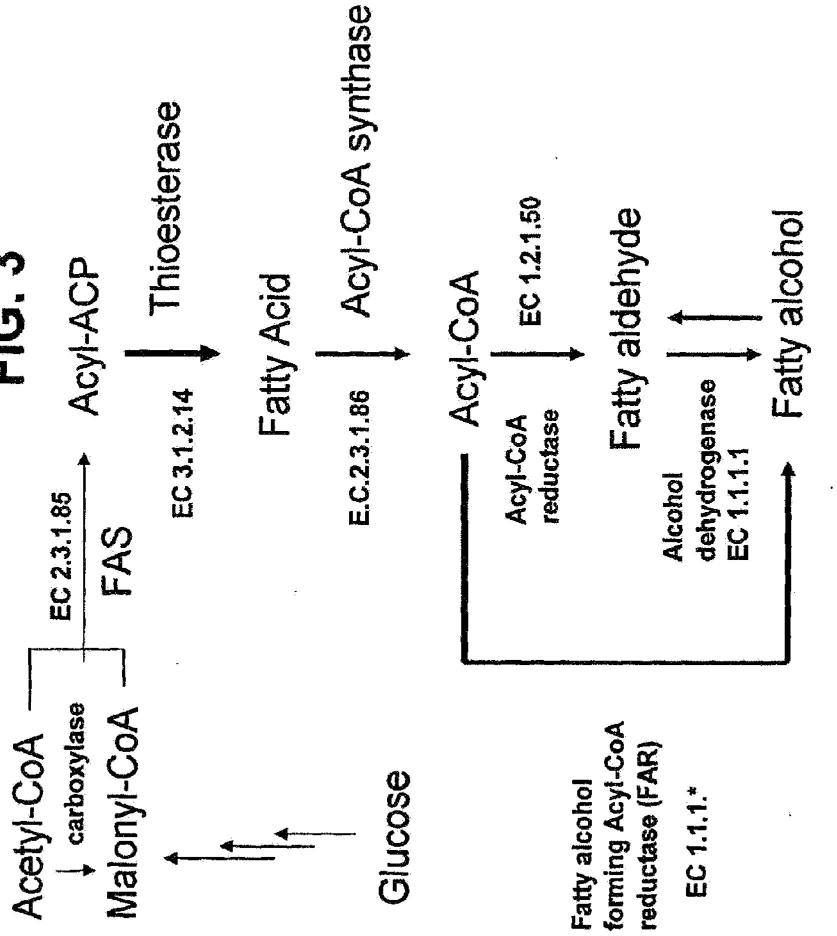


FIG. 2



Fatty alcohol forming acyl-CoA reductase references: Kalscheurer 2006; Metz 2000; Cheng 2004a

FIG. 3


Acetyl-CoA
carboxylase
Malonyl-CoA
Glucose
EC 2.3.1.85
FAS
Acyl-ACP
EC 3.1.2.14
Thioesterase
Fatty Acid
E.C.2.3.1.86
Acyl-CoA synthase
Acyl-CoA
Acyl-CoA
reductase
EC 1.2.1.50
Fatty aldehyde
Alcohol
dehydrogenase
EC 1.1.1.1
Fatty alcohol
Fatty alcohol
forming Acyl-CoA
reductase (FAR)
EC 1.1.1.*

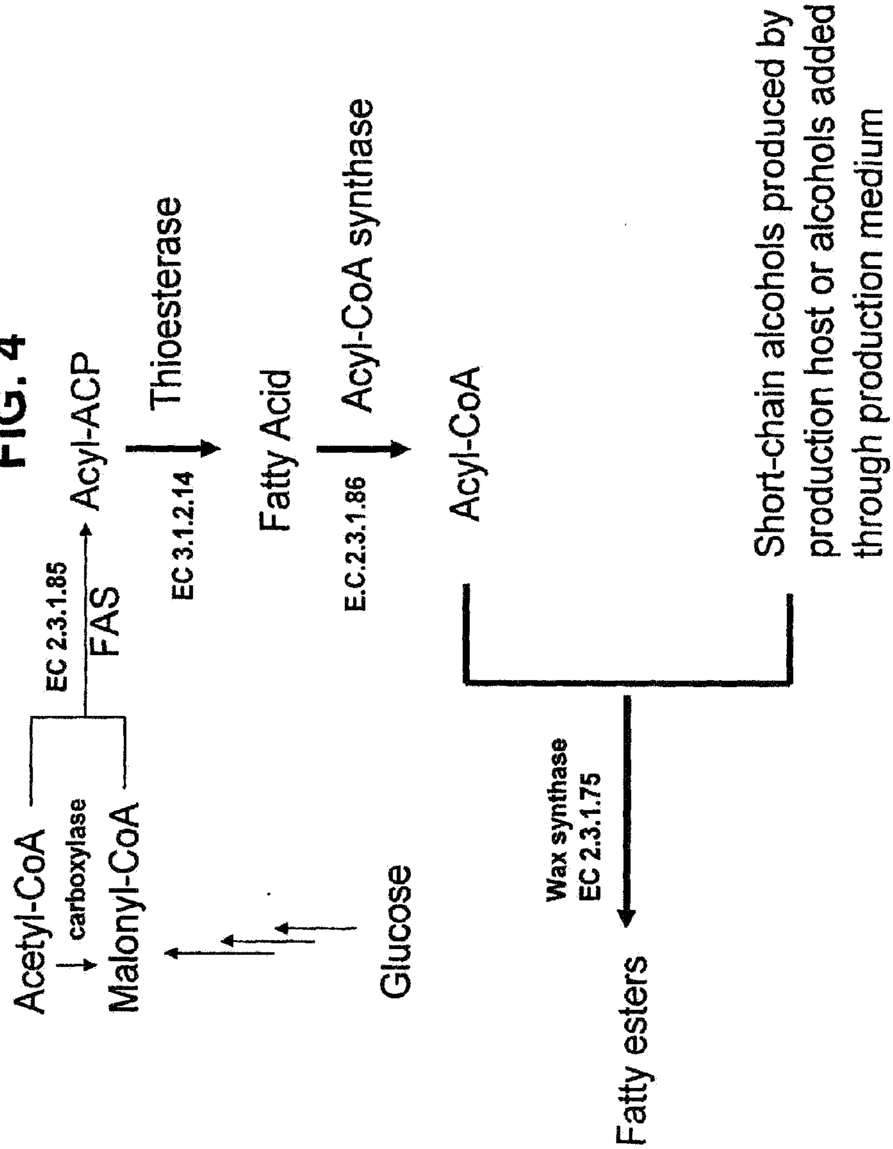

Acetyl-CoA
carboxylase
Malonyl-CoA
Glucose
EC 2.3.1.85
FAS
Acyl-ACP
EC 3.1.2.14
Thioesterase
Fatty Acid
E.C.2.3.1.86
Acyl-CoA synthase
Acyl-CoA
Short-chain alcohols produced by production host or alcohols added through production medium
Wax synthase
EC 2.3.1.75
Fatty esters

FIG. 4

FIG. 5

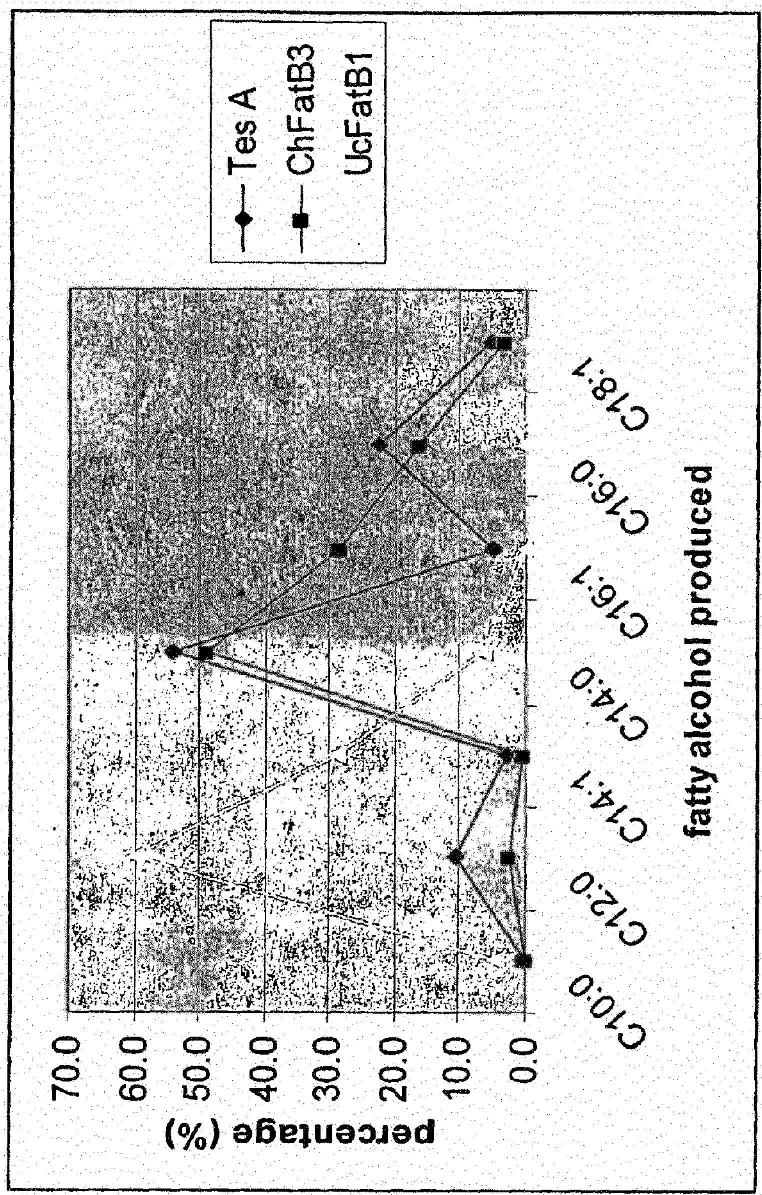
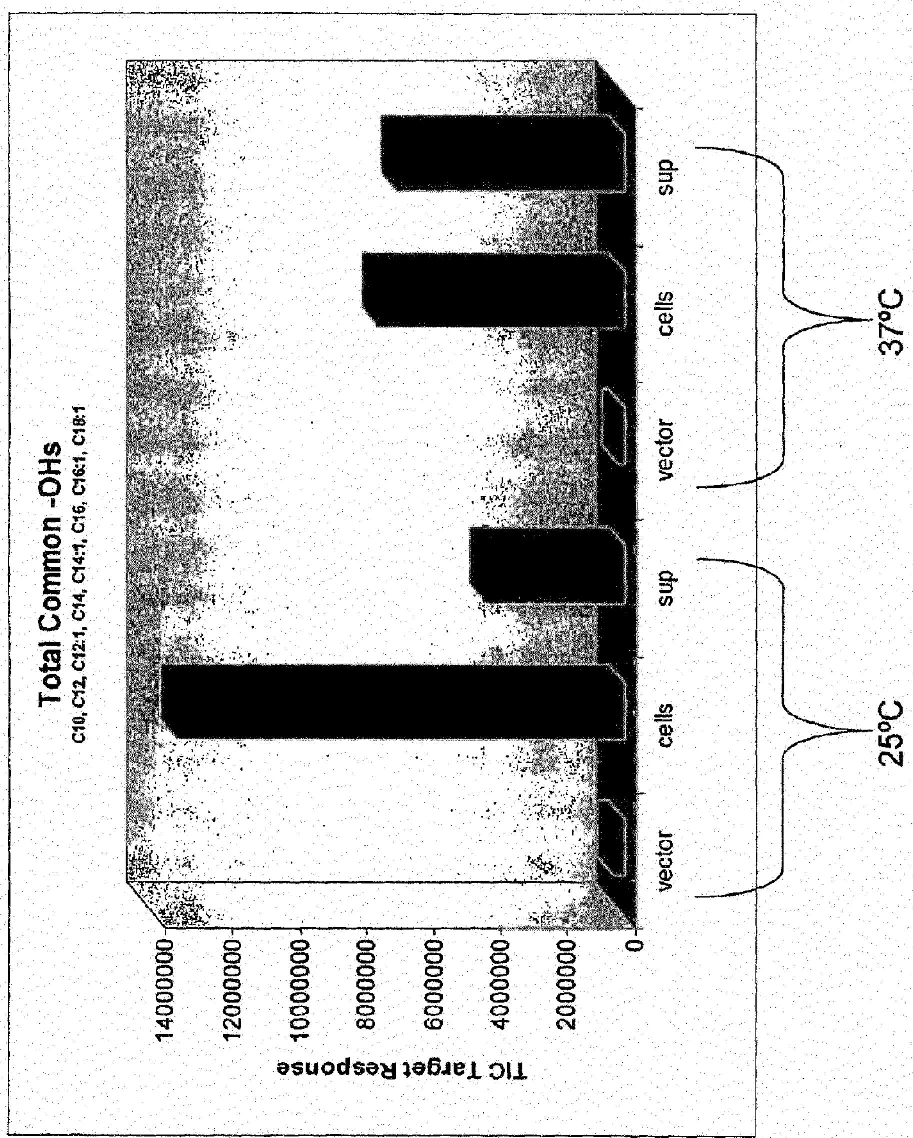

Tes A
ChFatB3
UcFatB1
percentage (%)
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
C10:0
C12:0
C14:1
C14:0
C16:1
C16:0
C18:1
fatty alcohol produced

FIG. 6


Total Common -OHs
C10, C12, C12:1, C14, C14:1, C16, C16:1, C18:1
TIC Target Response
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
vector
cells
sup
vector
cells
sup
25°C
37°C

FIGS. 7A-7D

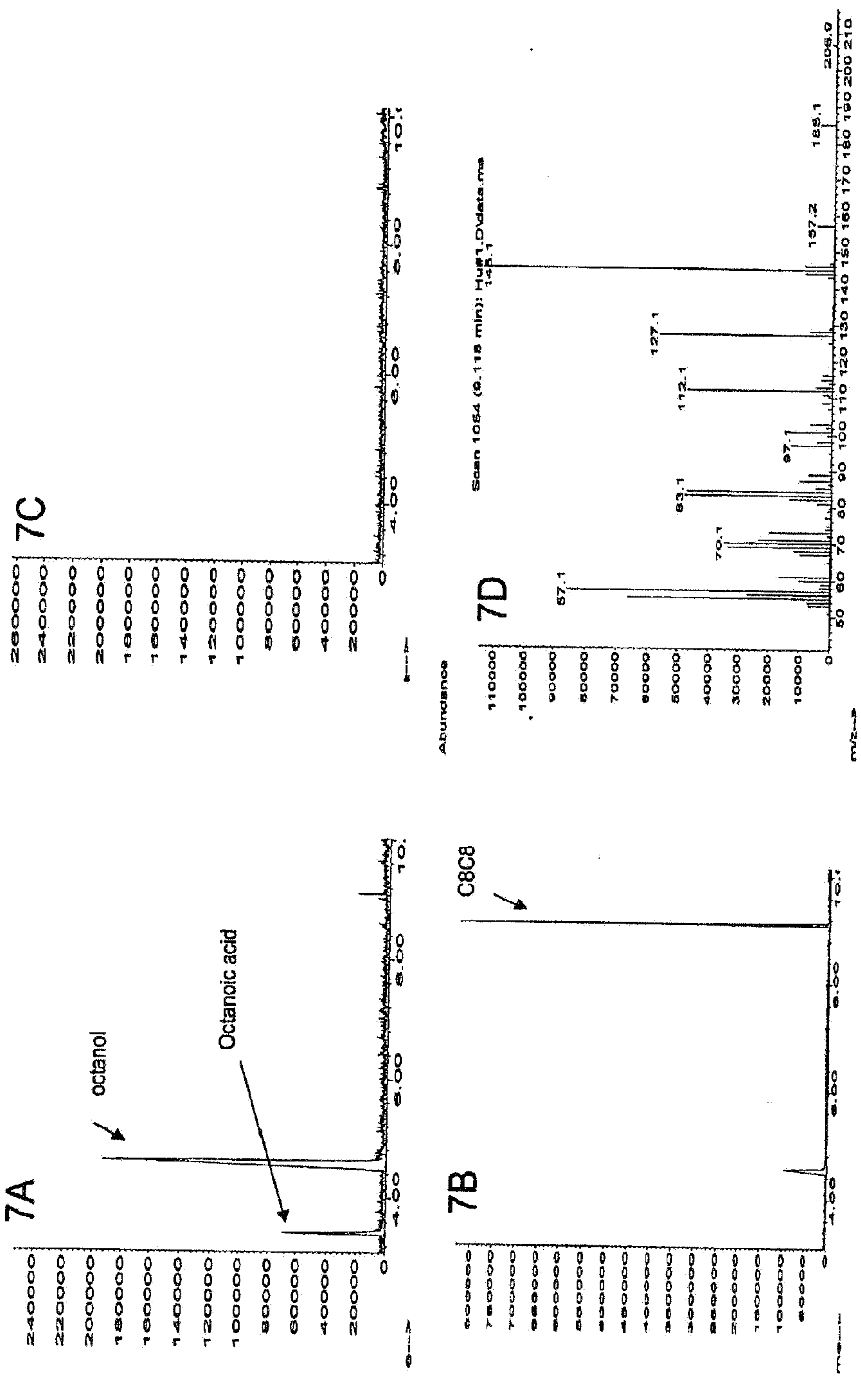


FIG. 8

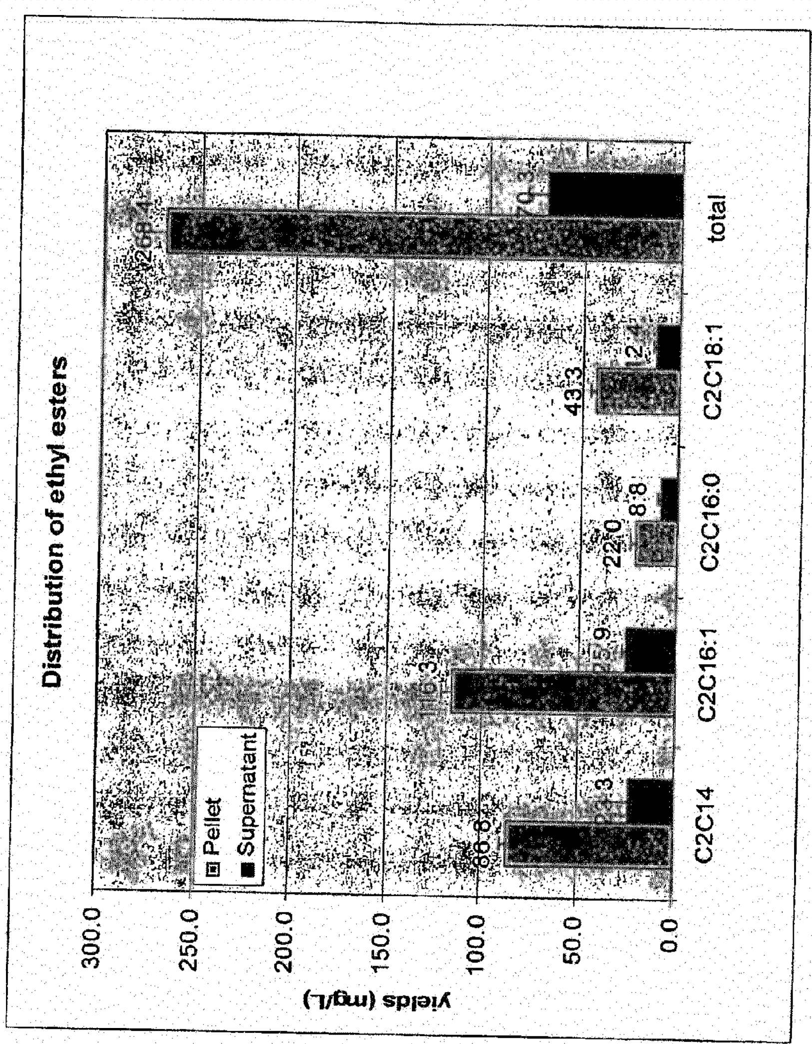

Distribution of ethyl esters
yields (mg/L)
300.0
250.0
200.0
150.0
100.0
50.0
0.0
Pellet
Supernatant
C2C14
C2C16:1
C2C16:0
C2C18:1
total
116.3
22.0
8.8
43.3

FIG. 9A

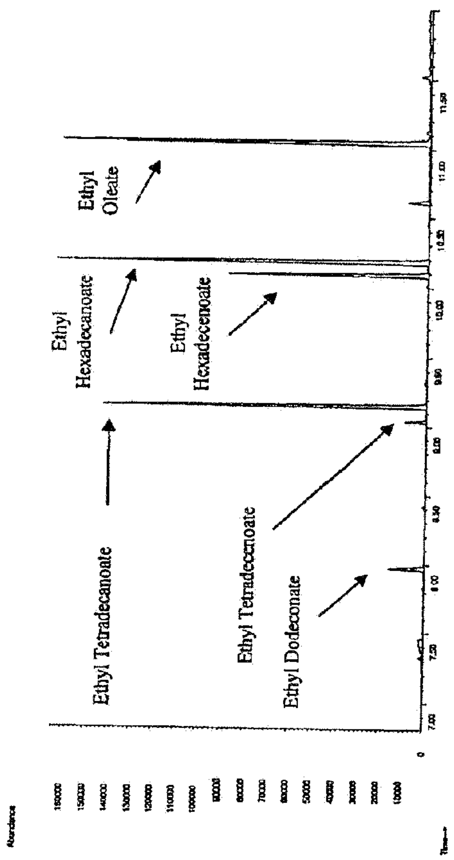

Ethyl Oleate
Ethyl Hexadecanoate
Ethyl Hexadecenoate
Ethyl Tetradecanoate
Ethyl Tetradecenoate
Ethyl Dodeconate

FIG. 9B

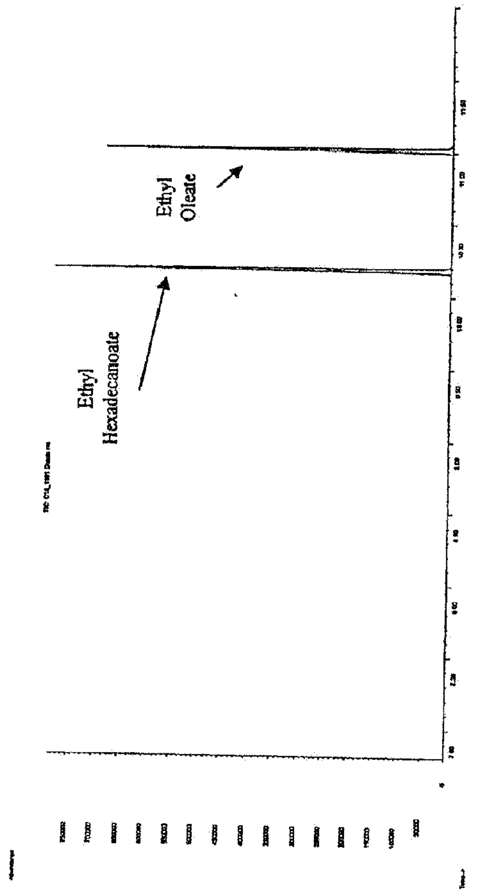

Ethyl Oleate
Ethyl Hexadecanoate

FIG. 10

Accession Numbers are from NCBI, GenBank, Release 159.0 as of April 15 2007
EC Numbers are from KEGG, Release 42.0 as of April 2007 (plus daily updates up to and including the date for this patent)

CATEGORY	GENE	NAME	ACCESSION	EC NUMBER	MODIFICATION	USE	ORGANISM
1. Fatty Acid Production Increase / Product Production Increase							
increase acyl-CoA *reduce catabolism of derivatives and intermediates* *reduce feedback inhibition* *attenuate other pathways that consume fatty acids*							
	accA	Acetyl-CoA carboxylase, subunit	AAC73296, NP_414727	6.4.1.2	Over-express	increase Malonyl-CoA production	*Escherichia coli*
	accB	Acetyl-CoA carboxylase, subunit	NP_417721	6.4.1.2	Over-express	increase Malonyl-CoA production	*Escherichia coli*
	accC	Acetyl-CoA carboxylase, subunit	NP_417722	6.4.1.2	Over-express	increase Malonyl-CoA production	*Escherichia coli*
	accD	Acetyl-CoA carboxylase, subunit	NP_416819	6.4.1.2	Over-express	increase Malonyl-CoA production	*Escherichia coli*
	aceE	pyruvate dehydrogenase, subunit E1	NP_414656, AAC73226	1.2.4.1, 2.3.1.61,2.3.1.12	Over-express	increase Acetyl-CoA production	*Escherichia coli*
	aceF	pyruvate dehydrogenase, subunit E2	NP_414657, AAC73227	1.2.4.1, 2.3.1.61,2.3.1.12	Over-express	increase Acetyl-CoA production	*Escherichia coli*
	ackA	acetate kinase	AAC75356, NP_416799	2.7.2.1	Delete or reduce	increase Acetyl-CoA production	*Escherichia coli*
	ackB	acetate kinase AckB	BAB81430	2.7.2.1	Delete or reduce	increase Acetyl-CoA production	*Escherichia coli*
	acpP	acyl carrier protein	AAC74178	NONE	Over-express	increase Acetyl-CoA production	*Escherichia coli*
	fadD	acyl-CoA synthase	AP_002424	2.3.1.86	Over-express	increase Fatty acid production	*Escherichia coli W3110*

adhE	alcohol dehydrogenase	AAC74323, CAA47743	1.1.1.1, 1.2.1.10	Delete or reduce	increase Acetyl-CoA production	*Escherichia coli W3111*
cer1	Aldehyde decarbonylase	BAA11024	4.1.99.5	Over-express	increase Acetyl-CoA production	*Arabidopsis thaliana*
fabA	beta-hydroxydecanoyl thioester dehydrase	NP_415474	4.2.1.60	express	fatty acyl-CoA production	*E. coli K12*
fabD	[acyl-carrier-protein] S-malonyltransferase	AAC74176	2.3.1.39	Over-express	increase Acetyl-CoA production	*E. coli K12*
fabF	3-oxoacyl-[acyl-carrier-protein] synthase II	AAC74179	2.3.1.179	Delete or OverExpress	increase Acetyl-CoA production	*E. coli K12*
fabG	3-oxoacyl-[acyl-carrier protein] reductase	AAC74177	1.1.1.100	Over-express	increase Acetyl-CoA production	*E. coli K12*
fabH	3-oxoacyl-[acyl-carrier-protein] synthase III	AAC74175	2.3.1.180	Over-express	increase Acetyl-CoA production	*E. coli K12*
fabI	enoyl-[acyl-carrier-protein] reductase, NADH-dependent	NP_415804	1.3.1.9	express	fatty acyl-CoA production	*E. coli K12*
fabR	Transcriptional Repressor	NP_418398	NONE	Delete or reduce	modulate unsaturated fatty acid production	*E. coli K12*
fabZ	(3R)-hydroxymyristol acyl carrier protein dehydratase	NP_414722	4.2.1.-			*E. coli K12*
fadE	acyl-CoA dehydrogenase	AAC73325	1.3.99.3, 1.3.99.-	Delete or reduce	increase Acetyl-CoA production	
acr1	Fatty Acyl-CoA reductase	AAC45217	1.2.1.-	Over-express	for fatty alcohol production	*E. coli K12*
GST	Glutathione synthase	P04425	6.3.2.3	Delete or reduce	increase Acyl-CoA	*E. coli K12*
gpsA	biosynthetic sn-glycerol 3-phosphate dehydrogenase	AAC76632, NP_418065	EC: 1.1.1.94	Delete or reduce	increase Acetyl-CoA production	*E. coli K12*
ldhA	lactate dehydrogenase	AAC74462, NP_415898	EC: 1.1.1.28	Delete or reduce	increase Acetyl-CoA production	*E. coli K12*
Lipase	Triglyceride Lipase	CAA89087, CAA98876	3.1.1.3	express	increase Fatty acid production	*E. coli K12*
	Malonyl-CoA decarboxylase	AAA26500	4.1.1.9, 4.1.1.41	Over-express		*Saccharopolyspora erythraea*
panD	aspartate 1-decarboxylase	BAB96708	4.1.1.11	Over-express	increase Acyl-CoA	*Escherichia coli W3110*
panK a.k.a. coaA	pantothenate kinase	AAC76952	2.7.1.33	Over-express	increase Acetyl-CoA production	

	pdh	Pyruvate dehydrogenase	BAB34380, AAC73227, AAC73226	1.2.4.1	Over-express	increase Acetyl-CoA production	
	pflB	formate acetyltransferase	AAC73989, P09373	EC: 2.3.1.54	Delete or reduce	increase Acetyl-CoA production	
	plsB	acyltransferase	AAC77011	2.3.1.15	D311E mutation	reduce limits on Acyl-CoA pool	*E. coli K12*
	poxB	pyruvate oxidase	AAC73958, NP_415392	1.2.2.2	Delete or reduce	increase Acetyl-CoA production	
	pta	phosphotransacetylase	AAC75357, NP_416800	2.3.1.8	Delete or reduce	increase Acetyl-CoA production	
	udhA	pyridine nucleotide transhydrogenase	CAA46822	1.6.1.1	Over-express	conversion NADH to NADPH or vice versa	
	fadB	fused 3-hydroxybutyryl-CoA epimerase/delta(3)-cis-delta(2)-trans enoyl-CoA Isomerase/enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase	AP_003956	4.2.1.17, 5.1.2.3, 5.3.3.8, 1.1.1.35	Delete or reduce	Block fatty acid degradation	*E. coli*
	fadJ	3-hydroxyacyl-CoA dehydrogenase; K01692 enoyl-CoA hydratase; K01782 3-hydroxybutyryl-CoA epimerase	AAC75401	1.1.1.35, 4.2.1.17, 5.1.2.3	Delete or reduce	Block fatty acid degradation	*E. coli*
	fadA	3-ketoacyl-CoA thiolase	BAE77458	2.3.1.16	Delete or reduce	Block fatty acid degradation	*E. coli*
	fadI	beta-ketoacyl-CoA thiolase	AAC75402	1.5.1.29, 1.16.	Delete or reduce	Block fatty acid degradation	*E. coli*
	YdiO	acyl-coA dehydrogenase	YP_852786	1.3.99.-	Delete or reduce	Block fatty acid degradation	*E. coli*

2. Structure Control

2A. Chain Length Control

2	tesA	thioester	P0ADA1	3.1.2.-	Delete 1 and express	C18 Chain Length	
	tesA without leader sequence	thioesterase	AAC73596, NP_415027	3.1.1.-	express or overexpress	C18:1	*E.coli*
	fatB (umbellularia)	thioesterase	Q41635	3.1.1.-	express or overexpress	C12:0	*Umbellularia californica*
	fatB2 (umbellularia)	thioesterase	AAC49269	3.1.1.-	express or overexpress	C8:0 - C10:0	*Cuphea hookeriana*

fatB3	thioesterase	AAC72881	3.1.1.-	express or overexpress	C14:0 - C16:0	*Cuphea hookeriana*
fatB (cinnamonum)	thioesterase	Q39473	3.1.1.-	express or overexpress	C14:0	*Cinnamomum camphora*
fatB[M141T]*	thioesterase	CAA85388	3.1.1.-	express or overexpress	C16:1	*Arabidopsis thaliana*
fatA1 (Helianthus)	thioesterase	AAL79361	3.1.1.-	express or overexpress	*C18:1*	*Helianthus annuus*
atfata	thioesterase	NP 189147, NP 193041	3.1.1.-	express or overexpress	C18:1	*Arabidopsis thaliana*
fatA	thioesterase	CAC39106	3.1.1.-	express or overexpress	C18:1	*Brassica juncea*
fatA (cuphea)	thioesterase	AAC72883	3.1.1.-	express or overexpress	C18:1	*Cuphea hookeriana*

2B. Branching Control

attenuate FabH						
express FabH from S. glaucescens and knock out endogenouse FabH					increase branched chain fatty acid derivatives	
express FabH from B. subtilis and knock out endogenouse FabH						
bdk - E3 - dihydroplipoyl dehyrodgenase subunit			EC 1.2.4.4			
bkd - E1- alpha/beta subunit			EC 1.2.4.4			

bkd - E2 - dihydrolipoyl transacylase subunit			EC 1.2.4.4			
bkdA1	branched-chain a-ketoacid decarboxylase a-subunit (E1a)	NP_628006	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Streptomyces coelicolor*
bkdB1	branched-chain a-ketoacid decarboxylase b-subunit (E1b)	NP_628005	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Streptomyces coelicolor*
bkdC1	dihydrolipoyl transacetylase (E2)	NP_638004	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Streptomyces coelicolor*
bkdA2	branched-chain a-ketoacid decarboxylase a-subunit (E1a)	NP_733618	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Streptomyces coelicolor*
bkdB2	branched-chain a-ketoacid decarboxylase b-subunit (E1b)	NP_628019	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Streptomyces coelicolor*
bkdC2	dihydrolipoyl transacetylase (E2)	NP_628018	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Streptomyces coelicolor*
bkdA	branched-chain a-ketoacid decarboxylase a-subunit (E1a)	BAC72074	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Streptomyces avermitilis*
bkdB	branched-chain a-ketoacid decarboxylase b-subunit (E1b)	BAC72075	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Streptomyces avermitilis*
bkdC	dihydrolipoyl transacetylase (E2)	BAC72076	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Streptomyces avermitilis*
bkdF	branched-chain a-ketoacid decarboxylase a-subunit (E1a)	BAC72088	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Streptomyces avermitilis*
bkdG	branched-chain a-ketoacid decarboxylase b-subunit (E1b)	BAC72089	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Streptomyces avermitilis*
bkdH	dihydrolipoyl transacetylase (E2)	BAC72090	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Streptomyces avermitilis*
bkdAA	branched-chain a-ketoacid decarboxylase a-subunit (E1a)	NP_390285	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Bacillus subtilis*
bkdAB	branched-chain a-ketoacid decarboxylase b-subunit (E1b)	NP_390284	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Bacillus subtilis*
bkdB	dihydrolipoyl transacetylase (E2)	NP_390283	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Bacillus subtilis*
bkdA1	branched-chain a-ketoacid decarboxylase a-subunit (E1a)	AAA65614	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Pseudomonas putida*
bkdA2	branched-chain a-ketoacid decarboxylase b-subunit (E1b)	AAA65615	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Pseudomonas putida*

bkdC	dihydrolipoyl transacetylase (E2)	AAA65617	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Pseudomonas putida*
lpd	dihydrolipoamide dehydrogenase (E3)	NP_414658	1.8.1.4	express or Over-Express	make branched-chain acyl-CoA precursors	*Escherichia coli*
IlvE	branched-chain amino acid aminotransferase	YP_026247	2.6.1.42	express or Over-Express	make branched a-ketoacids	*Escherichia coli*
IlvE	branched-chain amino acid aminotransferase	AAF34406	2.6.1.42	express or Over-Express	make branched a-ketoacids	*Lactococcus lactis*
IlvE	branched-chain amino acid aminotransferase	NP_745648	2.6.1.43	express or Over-Express	make branched a-ketoacids	*Pseudomonas putida*
IlvE	branched-chain amino acid aminotransferase	NP_629657	2.6.1.42	express or Over-Express	make branched a-ketoacids	*Streptomyces coelicolor*
ccr	crotonyl-CoA reductase	NP_630556	1.1.1.9	express or Over-Express	Converting crotonyl-CoA to butyryl-CoA	*Streptomyces coelicolor*
ccr	crotonyl-CoA reductase	AAD53915	1.1.1.9	express or Over-Express	Converting crotonyl-CoA to butyryl-CoA	*Streptomyces cinnamonensis*
IcmA, isobutyryl-CoA mutase	isobutyryl-CoA mutase, subunit A	NP_629554	5.4.99.2	express or Over-Express	converting butyryl-CoA to isobutyryl-CoA	*Streptomyces coelicolor*
IcmA, isobutyryl-CoA mutase	isobutyryl-CoA mutase, subunit A	AAC08713	5.4.99.2	express or Over-Express	converting butyryl-CoA to isobutyryl-CoA	*Streptomyces cinnamonensis*
IcmB, isobutyryl-CoA mutase	isobutyryl-CoA mutase, subunit B	NP_630904	5.4.99.13	express or Over-Express	converting butyryl-CoA to isobutyryl-CoA	*Streptomyces coelicolor*
IcmB, isobutyryl-CoA mutase	isobutyryl-CoA mutase, subunit B	AJ246005	5.4.99.13	express or Over-Express	converting butyryl-CoA to isobutyryl-CoA	*Streptomyces cinnamonensis*
FabH, ACPs and fabF genes with specificity for branched chain acyl-CoAs						
IlvE		CAC12788	EC2.6.1.42	over express	branched chain amino acid amino transferase	S. carnosus
FabH1	beta-ketoacyl-ACP synthase III	NP_626634	2.3.1.180	express or Over-Express	initiation of branched-chain fatty acid biosynthesis	Streptomyces coelicolor

ACP	acyl-carrier protein	NP_626635	NONE	express or Over-Express	initiation and elongation of branched-chain fatty acid biosynthesis	*Streptomyces coelicolor*
FabF	beta-ketoacyl-ACP synthase II	NP_626636	2.3.1.179	express or Over-Express	elongation of branched-chain fatty acid biosynthesis	*Streptomyces coelicolor*
FabH3	beta-ketoacyl-ACP synthase III	NP_823466	2.3.1.180	express or Over-Express	initiation of branched-chain fatty acid biosynthesis	*Streptomyces avermitilis*
FabC3 (ACP)	acyl-carrier protein	NP_823467	NONE	express or Over-Express	initiation and elongation of branched-chain fatty acid biosynthesis	*Streptomyces avermitilis*
FabF	beta-ketoacyl-ACP synthase II	NP_823468	2.3.1.179	express or Over-Express	elongation of branched-chain fatty acid biosynthesis	*Streptomyces avermitilis*
FabH_A	beta-ketoacyl-ACP synthase III	NP_389015	2.3.1.180	express or Over-Express	initiation of branched-chain fatty acid biosynthesis	*Bacillus subtillis*
FabH_B	beta-ketoacyl-ACP synthase III	NP_388898	2.3.1.180	express or Over-Express	initiation of branched-chain fatty acid biosynthesis	*Bacillus subtillis*
ACP	acyl-carrier protein	NP_389474	NONE	express or Over-Express	initiation and elongation of branched-chain fatty acid biosynthesis	*Bacillus subtillis*
FabF	beta-ketoacyl-ACP synthase II	NP_389016	2.3.1.179	express or Over-Express	elongation of branched-chain fatty acid biosynthesis	*Bacillus subtillis*
SmalDRAFT_0818	beta-ketoacyl-ACP synthase III	ZP_01643059	2.3.1.180	express or Over-Express	initiation of branched-chain fatty acid biosynthesis	*Stenotrophomonas maltophilia*
SmalDRAFT_0821	acyl-carrier protein	ZP_01643063	NONE	express or Over-Express	initiation and elongation of branched-chain fatty acid biosynthesis	*Stenotrophomonas maltophilia*
SmalDRAFT_0822	beta-ketoacyl-ACP synthase II	ZP_01643064	2.3.1.179	express or Over-Express	elongation of branched-chain fatty acid biosynthesis	*Stenotrophomonas maltophilia*
FabH	beta-ketoacyl-ACP synthase III	YP_123672	2.3.1.180	express or Over-Express	initiation of branched-chain fatty acid biosynthesis	*Legionella pneumophila*
ACP	acyl-carrier protein	YP_123675	NONE	express or Over-Express	initiation and elongation of branched-chain fatty acid biosynthesis	*Legionella pneumophila*
FabF	beta-ketoacyl-ACP synthase II	YP_123676	2.3.1.179	express or Over-Express	elongation of branched-chain fatty acid biosynthesis	*Legionella pneumophila*
FabH	beta-ketoacyl-ACP synthase III	NP_415609	2.3.1.180	delete or reduce	initiation of branched-chain fatty acid biosynthesis	*Escherichia coli*

FabF	beta-ketoacyl-ACP synthase II	NP_415613	2.3.1.179	delete or reduce	elongation of branched-chain fatty acid biosynthesis	*Escherichia coli*
To Produce Cyclic Fatty Acids						
AnsJ	dehydratase (putative)	not available	not available	express or Over-Express	cyclohexylcarbonyl-CoA boiosynthesis	*Streptomyces collinus*
AnsK	CoA ligase (putative)	not available	not available	express or Over-Express	cyclohexylcarbonyl-CoA boiosynthesis	*Streptomyces collinus*
AnsL	dehydrogenase (putative)	not available	not available	express or Over-Express	cyclohexylcarbonyl-CoA boiosynthesis	*Streptomyces collinus*
ChcA	enoyl-CoA reductase	U72144	E1.3.1.34	express or Over-Express	cyclohexylcarbonyl-CoA boiosynthesis	*Streptomyces collinus*
AnsM	oxidorecutase (putative)	not available	not available	express or Over-Express	cyclohexylcarbonyl-CoA boiosynthesis	*Streptomyces collinus*
PlmJ	dehydratase (putative)	AAQ84158	not available	express or Over-Express	cyclohexylcarbonyl-CoA boiosynthesis	*Streptomyces sp. HK803*
PlmK	CoA ligase (putative)	AAQ84158	not available	express or Over-Express	cyclohexylcarbonyl-CoA boiosynthesis	*Streptomyces sp. HK803*
PlmL	dehydrogenase (putative)	AAQ84159	not available	express or Over-Express	cyclohexylcarbonyl-CoA boiosynthesis	*Streptomyces sp. HK803*
ChcA	enoyl-CoA reductase	AAQ84160	E1.3.1.34	express or Over-Express	cyclohexylcarbonyl-CoA boiosynthesis	*Streptomyces sp. HK803*
PlmM	oxidorecutase (putative)	AAQ84161	not available	express or Over-Express	cyclohexylcarbonyl-CoA boiosynthesis	*Streptomyces sp. HK803*
ChcB	enoyl-CoA isomerase	AF268489	not available	express or Over-Express	cyclohexylcarbonyl-CoA boiosynthesis	*Streptomyces collinus*
ChcB/CaiD	enoyl-CoA isomerase	NP_629292	4.2.1.-	express or Over-Express	cyclohexylcarbonyl-CoA boiosynthesis	*Streptomyces coelicolor*
ChcB/CaiD	enoyl-CoA isomerase	NP_824296	4.2.1.-	express or Over-Express	cyclohexylcarbonyl-CoA boiosynthesis	*Streptomyces avermitilis*
2C. Saturation Level Control						
Sfa also see FabA in sec. 1	Suppressor of FabA	AAN79592, AAC44390	Can't find	Over-express	increase monounsaturated fatty acids	*E.coli*
GnsA	suppressors of the secG null mutation	ABD18647.1	NONE	express Over-express	produce unsaturated fatty acids increase unsaturated fatty acid esters	*E.coli*

GnsB	suppressors of the secG null mutation	AAC74076.1	NONE	Over-express	increase unsaturated fatty acid esters	*E.coli*
also see section 2A - items with :0 are unsaturated (no double bonds) and with :1 are saturated (1 double bond)						
fabB	3-oxoacyl-[acyl-carrier-protein] synthase I	BAA16180	EC:2.3.1.41	overexpress	modulate unsaturated fatty acid production	*Escherichia coli*
fabK	trans-2-enoyl-ACP reductase II	AAF98273	1.3.1.9	express	modulate unsaturated fatty acid production	*Streptococcus pneumoniae*
fabL	enoyl-(acyl carrier protein) reductase	AAU39821	1.3.1.9	express	modulate unsaturated fatty acid production	*Bacillus licheniformis DSM 13*
fabM	trans-2, cis-3-decenoyl-ACP isomerase	DAA05501	5.3.3.14	Over-express	modulate unsaturated fatty acid production	*Streptococcus mutans*
<u>3. Final Product Output</u>						
<u>3A. Wax Output</u>						
AT3G51970	long-chain-alcohol O-fatty-acyltransferase	NP_190765	2.3.1.75	express	wax production	*Arabidopsis thaliana*
	thioesterase (see chain length control section)		3.1.2.14	express	increase fatty acid production	
	fatty alcohol forming acyl-CoA reductase		1.1.1.*	express	convert acyl-coa to fatty alcohol	
acr1	acyl-CoA reductase (ACR1)	YP_047869	1.2.1.50	express	convert acyl-coa to fatty alcohol	*Acinetobacter sp. ADP1*
yqhD	alcohol dehydrogenase	AP_003562	1.1.1.1	express	increase	*E. coli W3110*
ELO1	Fatty acid elongase	BAD98251	2.3.1.74	express	produce very long chain length fatty acids	*Pichia angusta*
plsC	acyltransferase	AAA16514	2.3.1.-	express		*Saccharomyces cerevisiae*
DAGAT	diacylglycerol acyltransferase	AAF19262	2.3.1.20	express	wax production	*Arabidopsis thaliana*

hWS	acyl-CoA wax alcohol acyltransferase	AAX48018	Can't find	express	wax production	*Homo sapiens*
aft1	bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltransferase	AAO17391	2.3.1.20	express	wax production	*Acinetobacter sp. ADP1*
mWS	wax ester synthase (simmondsia)	AAD38041	2.3.1.75	express	wax production	*Simmondsia chinensis*
<u>3B. Fatty Alcohol Output</u>						
	various thioesterases (refer to Sec. 2A)		3.1.2.14	express	produce	
acr1	acyl-CoA reductase	YP_047869	1.2.1.50	express	produce	*Acinetobacter sp. ADP1*
yqhD	alcohol dehydrogenase	AP_003562	1.1.1.1	express	produce	*Escherichia coli W3110*
BmFAR	FAR (fatty alcohol forming acyl-CoA reductase)	BAC79425	1.1.1.*	express	reduce fatty acyl-CoA to fatty alcohol	*Bombyx mori*
Akr1a4	Mammalian microsomal aldehyde reductase	NP_067448	1.1.1.21	express	produce	*Mus musculus*
GTNG_1865	Long-chain aldehyde dehydrogenase	YP_001125970	1.2.1.48	express	produce	*Geobacillus thermodenitrificans NG80-2*
FadD	acyl-CoA synthetase	NP_416319	EC 6.2.1.3	express	produce more	*E. Coli K12*
To make Butanol						
atoB	acetyl-CoA acetyltransferase	YP_049388	2.3.1.9	express	produce	*Erwinia carotovora*
hbd	Beta-hydroxybutyryl-CoA dehydrogenase	BAD51424	1.1.1.157	express	produce	*Butyrivibrio fibrisolvens*
CPE0095	crotonase	BAB79801	4.2.1.17	express	produce	*Clostridium perfringens*
bcd	butyryl-CoA dehydryogenase	AAM14583	Can't find	express	produce	*Clostridium beijerinckii*
ALDH	CoA-acylating dehydrogenase	AAT66436	Can't find	express	produce	*Clostridium beijerinckii*
AdhE	aldehyde-alcohol dehydrogenase	AAN80172	1.1.1.1 1.2.1.10	express	produce	*Escherichia coli CFT073*

3C. Fatty Acid Ester Output

thioesterase	see chain length control section		3.1.2.14	express	produce	
acr1	acyl-CoA reductase	YP_047869	1.2.1.50	express	produce	*Acinetobacter sp. ADP1*
yqhD	alcohol dehydrogenase	AP_003562	1.1.1.1	express	produce	*E. Coli K12*
AAT	alcohol O-acetyltransferase	AAG13130	2.3.1.84	express	produce	*Fragaria x ananassa*

4. Export

Wax ester exporter (FATP family, Fatty Acid (long chain) Transport Protein)		NP_524723	NONE	express	export wax	*Drosophila melanogaster*
ABC transporter	putative alkane transporter	AAN73268		express	export products	*Rhodococcus erythropolis*
CER5	wax transporter	At1g51500, AY734542, At3g21090, At1g51460	NONE	express	export products	*Arabidopsis thaliana*
AtMRP5	Arabidopsis thaliana multidrug resistance-associated	NP_171908	NONE	express	export products	*Arabidopsis thaliana*
AmiS2	ABC transporter AmiS2	JC5491	Can't Find	express	export products	*Rhodococcus sp.*
AtPGP1	ARABIDOPSIS THALIANA P GLYCOPROTEIN1	NP_181228	NONE	express	export products	*Arabidopsis thaliana*
AcrA	putative multidrug-efflux transport protein acrA	CAF23274	NONE	express	export products	*Candidatus Protochlamydia amoebophila UWE25*
AcrB	probable multidrug-efflux transport protein, acrB	CAF23275	NONE	express	export products	*Candidatus Protochlamydia amoebophila UWE25*
TolC	Outer membrane protein [Cell envelope biogenesis,	ABD59001	NONE	express	export products	*Francisella tularensis subsp. novicida*

AcrE	transmembrane protein affects septum formation and cell membrane permeability	YP_312213	NONE	express	export products	*Shigella sonnei Ss046*
AcrF	Acriflavine resistance protein F	P24181	NONE	express	export products	*Escherichia coli*
tll1618	multidrug efflux transporter	NP_682408.1		express	export products	*Thermosynechococcus elongatus BP-1]*
tll1619	multidrug efflux transporter	NP_682409.1		express	export products	*Thermosynechococcus elongatus BP-1]*
tll0139	multidrug efflux transporter	NP_680930.1		express	export products	*Thermosynechococcus elongatus BP-1]*
5. Fermentation						
replication checkpoint genes					increase output efficiency	
umuD	DNA polymerase V, subunit	YP_310132	3.4.21.-	Over-express	increase output efficiency	*Shigella sonnei Ss046*
umuC	DNA polymerase V, subunit	ABC42261	3.4.21.-	Over-express	increase output efficiency	*Escherichia coli*
NADH:NADPH transhydrogenase (alpha and beta subunits)		P07001, P0AB70	1.6.1.1. 1.6.1.2	express	increase output efficiency	*Shigella flexneri*

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