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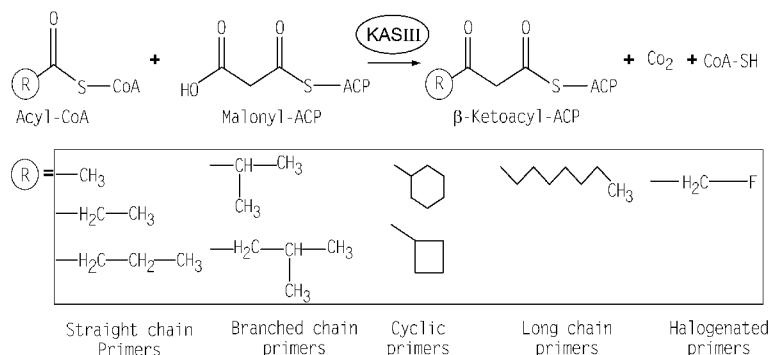


FIG. 1

(57) Abstract: A mutant *Bacillus subtilis*, which does not express a functional KASIIIA and/or KASIIIB, and method of making; a mutant *Rhodospirillum rubrum*, which does not express a functional PhaC1, PhaC2, and/or PhaC3, and method of making; method of characterizing substrate specificity of KASIII; method of making mutant KASIII with altered substrate specificity and/or altered level of activity and nucleic acid, vector, host cell/organism, and mutant KASIII; an *in vitro*, high-throughput spectrophotometric method of assaying KASIII activity; and materials and methods for using KASIII for production of bi-functional fatty acids and the materials so produced.

MATERIALS AND METHODS FOR CHARACTERIZING AND USING KASIII FOR PRODUCTION OF BI-FUNCTIONAL FATTY ACIDS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. provisional patent application no. 61/755,946, which was filed January 23, 2013, and which is incorporated by reference in its entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing, which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on January 22, 2014, is named ISURF 1201 PCT_SEQ_ST25.txt.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0003] The work described herein was supported, at least in part, by The National Science Foundation under contract nos. EEC0813570 and IIP1237247. Therefore, the Government of the United States of America has certain rights in the invention.

TECHNICAL FIELD

[0004] The present disclosure relates to enzymes, mutants thereof, fatty acid synthesis, nucleic acids, host cells and organisms, assays, bi-functional fatty acid compositions, and uses thereof.

BACKGROUND

[0005] The biochemical mechanism of fatty acid biosynthesis is universally similar among all organisms. Generally, fatty acids are synthesized by the repeated iteration of four-reactions, which start with an acyl-primer, which is elongated, two carbons per cycle, using carbon atoms derived from a malonyl moiety. The four sequential reactions that make up this cycle generate 3-ketoacyl-thioester, 3-hydroxyacyl-thioester, and 2-enoyl-thioester derivative intermediates, and finally an acyl-thioester derivative that is two carbons longer than the initial acyl primer. In bacteria, typified

by the *E. coli* system, and higher plant plastids, these reactions are catalyzed by a dissociable, type II fatty acid synthase that is composed of the four enzymes 3-ketoacyl-ACP synthase (KAS), 3-ketoacyl-ACP reductase (encoded by *fabG*), 3-hydroxyacyl-ACP dehydratase (encoded by *fabA*), and enoyl-ACP reductase (encoded by *fabI*) (Rock et al., Biochim. Biophys. Acta 1302: 1-16 (1996)). In contrast, a type I fatty acid synthase, which is composed of four enzyme components that occur as domains on a multifunctional protein(s), occurs in other eukaryotes (Jenni et al., Science 311: 1263-1267 (2006); and Maier et al., Science 311: 1258-1262 (2006)). However, in both type I and type II fatty acid synthase systems acyl derivatives are bound to phosphopantetheine cofactors.

[0006] In the type II fatty acid synthase system, there are three genetically and biochemically distinct KAS isomers, namely KASI (encoded by *fabB*), KASII (encoded by *fabF*), and KASIII (encoded by *fabH*) (Rock et al. (1996), *supra*; Garwin et al., J. Biol. Chem. 255: 11949-11956 (1980)); and Jackowski et al., J. Biol. Chem. 262: 7927-7931 (1987)). Their functions have been studied extensively in *E. coli*. They differ in their specificities for acyl-thioester substrates, having optimum activities for substrates of different acyl-chain lengths and different thioesters. While KASI and KASII catalyze the condensation between acyl-ACP (of longer acyl-chain length) with malonyl-ACP substrates, KASIII specifically utilizes acetyl-CoA as a substrate for the condensing reaction with malonyl-ACP (Tsay et al., J. Biol. Chem. 267: 6807-6814 (1992); and Heath et al., J. Biol. Chem. 271: 1833-1836 (1996)), and thus initiates fatty acid biosynthesis.

[0007] The general mechanism of fatty acid biosynthesis in Gram-positive bacteria, such as *B. subtilis*, is similar to that of *E. coli* (Magnuson et al., Microbiol. Rev. 57: 522-542 (1993)). One major difference is that *B. subtilis* produces large quantities of branched-chain fatty acids (BCFAs) and unsaturated fatty acids as a result of the expression of a unique $\Delta 5$ desaturase (Aguilar et al., J. Bacteriol. 180: 2194-2200 (1998)). The BCFAs and the unsaturated fatty acids together maintain membrane fluidity in response to lower growth temperatures. The BCFAs are branched with methyl groups at the iso- and anteiso positions (i.e., 13-methyltetradecanoic, 12-methyltetradecanoic acid, and 14-methylpentadecanoic acid), and they are biosynthesized by a type II FAS that has the ability to initiate this process by using

branched acyl-CoAs that are derived from the branched chain amino acids, leucine, isoleucine, and valine (Willecke et al., J. Biol. Chem. 246: 5264-5272 (1971)). Thus, the *B. subtilis* FAS enzyme must have the capacity to utilize such branched acyl-CoA substrates. Genomics-based analysis of the *B. subtilis* genome has led to the identification of KASII (Shujman et al., J. Bacteriol. 183: 3032-3040 (2001)) and KASIII homologous genes; however, it appears that this bacterium does not contain a sequence-recognizable KASI homolog. In *B. subtilis* KASII is an essential enzyme, which is encoded by *yjaY*. Two *B. subtilis* KASIII-encoding genes, *bfabHA* (*yjaX*) and *bfabHB* (*yhfB*), have been characterized, and these have the capacity to catalyze the condensation of branched acyl-CoAs with malonyl-ACP (Choi et al., J. Bacteriol. 182: 365-370 (2000); and Smirnova et al., J. Bacteriol. 183: 2335-2342 (2001)). These two genes code for 312- and 325-residue proteins that share 43% sequence identity.

[0008] KASIII has been characterized in several bacterial (Tsay et al., J. Biol. Chem. 267: 6807-6814 (1992); Han et al., J. Bacteriol. 180: 4481-4486 (1998); Qiu et al., J. Biol. Chem. 274: 36465-36471 (1999); Choi et al., J. Bacteriol. 182: 365-370 (2000a); Choi et al., J. Bacteriol. 182: 365-370 (2000b); Choi et al., J. Biol. Chem. 275: 28201-28207 (2000c); Davies et al., Structure 8: 185-195 (2000); Khandekar et al., Biochem. Biophys. Res. Comm. 270: 100-107 (2000); Khandekar et al., J. Biol. Chem. 276: 30024-30030 (2001); Qiu et al., J. Mol. Biol. 307: 341-356 (2001); Revill et al., J. Bacteriol. 183: 3526-3530 (2001); Huynh et al., Acta Crystallogr. Sect. F. Struct. Biol. Cryst. Comm. 65: 460-462 (2009); Wen et al., Protein Expr. Purif. 65: 83-91 (2009); Singh et al., FEMS Microbiol. Lett. 301: 188-192 (2009); Gajiwala et al., FEBS Lett. 583: 2939-2946 (2009); and Pereira et al., Acta Crystallogr. D. Biol. Crystallogr. 68: 1320-1328 (2012)), protozoan (Waters et al., Mol. Biochem. Parasitol. 123: 85-94 (2002); and Prigge et al., Biochem. 42: 1160-1169 (2003)), and plant species (Clough et al., J. Biol. Chem. 267: 20992-20998 (1992); Jaworski et al., Plant Physiol. 90: 41-44 (1989); Tai et al., Plant Physiol. 106: 801-802 (1994); Abbadi et al., Biochem. J. 345 (Pt. 1): 153-160 (2000); Dehesh et al., Plant Physiol. 125: 1103-1114 (2001); Li et al., Tree Physiol. 28: 921-927 (2008); and Gonzalez-Mellado et al., Planta 231: 1277-1289 (2010)). Functionally characterized KASIII enzymes exhibit diverse substrate specificities, utilizing acyl-CoA substrates ranging from short, straight-chain acyl-CoAs (e.g. acetyl-CoA, propionyl-CoA (Choi et al. (2000a), *supra*; Clough et al.

(1992), *supra*; and Abbadi et al. (2000), *supra*), branched-chain acyl-CoAs (e.g., isobutyryl-CoA and ante-isovaleryl-CoA (Han et al. (1998), *supra*; Choi et al. (2000a), *supra*; Khandekar et al. (2001), *supra*; Singh et al. (2009), *supra*; and Qiu et al., Protein Sci. 14: 2087-2094 (2005)) to long-chain acyl-CoAs (e.g., lauroyl-CoA, palmitoyl-CoA (Choi et al. (2000b), *supra*; and Scarsdale et al., J. Biol. Chem. 276: 20516-20522 (2001)).

[0009] By virtue of diverse substrate specificities shown by KASIII enzymes from different organisms, this enzyme is thought to determine the fatty acid profile of the organism, particularly the structure of the omega-end of the fatty acid products (Choi et al. (2000a), *supra*; Gajiwala et al. (2009), *supra*; and Pereira et al. (2012), *supra*). For example, in many Gram-positive bacteria (i.e., *Bacillus subtilis*, *Streptomyces glaucescens*, and *Staphylococcus aureus*), KASIII can utilize both branched-chain and straight-chain substrates, resulting in the production of both branched- and straight-chain fatty acids (Han et al. (1998), *supra*; Choi et al. (2000a), *supra*; Pereira et al. (2012), *supra*; and Qiu et al. (2005), *supra*). In contrast, KASIII from Gram-negative bacteria (e.g., *E. coli*) appears to prefer straight-chain acyl-CoA substrates, which results in the production of straight-chain fatty acids (Choi et al. (2000a), *supra*).

[0010] The active site residues and substrate binding pocket are well conserved among KASIII from different species. Three residues, Cys112, His244 and Asn274, form the catalytic triad in *E. coli* KASIII and carry out two half reactions as a part of the Claisen condensation (Fig. 1) of acyl-CoA and malonyl-ACP (Heath et al., Nat. Prod. Rep. 19: 581-596 (2002)). Qiu et al. (J. Biol. Chem. 274: 36465-36471 (1999)) and Davies et al. (Structure 8: 185-195 (2000)) have proposed different mechanisms for the reaction at the active site. The mechanism proposed by Davies et al., which is supported by crystallographic data, is shown in Fig. 2. The first step is the transfer of the acyl group from the acyl-CoA primer to the enzyme and covalent linkage of the acyl group to the Cys112 residue. As per Davies *et al.*, the thiol group of Cys112 is deprotonated by the dipole effect of the α -helix in which it is located. The resulting nucleophilic thiolate ion on Cys112 attacks acyl-CoA and forms a thioester with the acyl group, with the release of CoA-SH. The second step is the entry of ionized malonyl-ACP into the active site, where it is decarboxylated through the aid of Phe205.

[0011] After decarboxylation, the resulting negative charge on its thioester carbonyl is stabilized by His244 and Asn274. A carbanion is formed on its α -carbon that attacks the acetate bound to Cys112. The tetrahedral transition state is stabilized by an oxyanion hole formed by Cys112 and Gly306, which eventually breaks down to give acetoacetyl-ACP as the product.

[0012] The US imports almost 10 million barrels of petroleum a day (U.S.E.I. Administration Monthly Energy Review (2011), www.eia.gov/energy_in_brief/foreign_oil_dependence.cfm) to create a multi-billion dollar plastics and specialty chemical industry that obtains its monomers from petroleum feedstocks. Currently, only a limited number of bio-based products are available in the market including polylactic acid (PLA), polyhydroxybutyrate (PHB), and polyethylene terephthalate (PET), which is based on 1,3-propanediol, and emerging products based on succinic acid and adipic acid (Frost and Sullivan, Global Bio-Based Plastics Market (2009), www.frost.com/prod/servlet/report-toc.pag?repid=M4A1-01-00-00-00). However, the global marketplace for the bio-plastics "green" market is projected to expand to over a billion dollars (Ceresana Research, Market Study: Bioplastics (2011), www.ceresana.com/en/market-studies/plastics/bioplastics), and with increasing awareness about reduced environmental impacts of bio-based plastics, the market for these products will continue to grow.

[0013] The present disclosure seeks to provide materials and methods for characterizing and using 3-ketoacyl-acyl carrier protein (ACP) synthases III (KASIII) in the production of bi-functional fatty acids. This and other objects and advantages, as well as inventive features, will become apparent from the detailed description provided herein.

SUMMARY

[0014] A mutant *Bacillus subtilis* is provided. The mutant *B. subtilis* does not express a functional 3-ketoacyl-acyl carrier protein (ACP) synthase III (KASIII) selected from the group consisting of KASIIIA and KASIIIB. In an embodiment, the mutant *B. subtilis* does not express a functional KASIIIA and a functional KASIIIB.

[0015] A method of making the mutant *B. subtilis* is also provided. The method comprises introducing into wild-type *B. subtilis* a mutation selected from the group

consisting of a mutation that prevents expression of a functional KASIIIA and a mutation that prevents expression of a functional KASIIIB. In an embodiment, the method comprises introducing into wild-type *B. subtilis* a mutation that prevents expression of a functional KASIIIA and a mutation that prevents expression of a functional KASIIIB.

[0016] Also provided is a mutant *Rhodospirillum rubrum*. The mutant *R. rubrum* does not express a functional polyhydroxyalkanoate (PHA) polymerase selected from the group consisting of PhaC1, PhaC2, and PhaC3. In an embodiment, the mutant *R. rubrum* does not express a functional PhaC1, a functional PhaC2, and a functional PhaC3.

[0017] Thus, also provided is a method of making the mutant *R. rubrum*. The method comprises introducing into wild-type *R. rubrum* a mutation selected from the group consisting of a mutation that prevents expression of a functional PhaC1, a mutation that prevents expression of a functional PhaC2, and a mutation that prevents expression of a functional PhaC3. In an embodiment, the method comprises introducing into wild-type *R. rubrum* a mutation that prevents expression of a functional PhaC1, a mutation that prevents expression of a functional PhaC2, and a mutation that prevents expression of a functional PhaC3.

[0018] Further provided is a method of characterizing substrate specificity of a KASIII. The method comprises expressing the KASIII, which is not expressed in wild-type *B. subtilis*, in an above-described mutant *B. subtilis* and assessing the production of ω -functionalized fatty acids. In an embodiment, the KASIII is derived from an organism, the wild-type of which produces ω -functionalized fatty acids. In another embodiment, the KASIII is derived from an organism, the wild-type of which does not normally produce ω -functionalized fatty acids.

[0019] Still further provided is another method of characterizing substrate specificity of a KASIII enzyme. The method comprises expressing the KASIII, which is not expressed in wild-type *R. rubrum*, in an above-described mutant *R. rubrum* and assessing the production of ω -functionalized fatty acids. In an embodiment, the KASIII is derived from an organism, the wild-type of which produces ω -functionalized fatty acids. In another embodiment, the KASIII is derived from an organism, the wild-type of which does not normally produce ω -functionalized fatty acids.

[0020] Yet still further provided is a method of altering the specificity of a KASIII for at least one of its substrates. The method comprises introducing into the KASIII one or more mutations comprising at least one mutation, which causes the rotamer conformation of a phenylalanine in the KASIII corresponding to Phe304 in KASIII from *E. coli* to change. In one embodiment, the KASIII is from *E. coli*, and the one or more mutations comprises a mutation of Leu220, alone or in further combination with Val215. The mutation of Leu220 can be Leu220Met, and the mutation of Val215 can be Val215Phe. The rotamer conformation of Phe304 can be changed from an active site-distal rotamer conformation, in which the Phe304 is oriented away from the active site, to an active site-proximal rotamer conformation, in which the side chain of Phe304 faces towards the active site. In another embodiment, the KASIII is KASIIIA from *B. subtilis*, and the one or more mutations comprises a mutation of Met213, alone or in further combination with Phe208. The mutation of Met213 can be Met213Leu, and the mutation of Phe208 can be Phe208Val. The rotamer conformation of Phe297 can be changed from an active site-distal rotamer conformation, in which the Phe297 is oriented away from the active site, to an active site-proximal rotamer conformation, in which the side chain of Phe297 faces towards the active site. In yet another embodiment, the KASIII is KASIIIB from *B. subtilis*, and the one or more mutations comprise a mutation of Trp221 and a mutation of Val226. The mutation of Trp221 can be Trp221Val, and the mutation of Val226 can be Val226Leu. The rotamer conformation of Phe310 can be changed from an active site-distal rotamer conformation, in which the Phe310 is oriented away from the active site, to an active site-proximal rotamer conformation, in which the side chain of Phe310 faces towards the active site. In still yet another embodiment, the KASIII is from the KASIII is from *Aeromonas hydrophila*, *Bacteroides vulgatus*, *Brevibacterium linens*, *Capnocytophaga gingivalis*, *Thermus aquaticus*, *Bacillus licheniformis*, *Desulfovibrio vulgaris*, *Bacillus subtilis subsp. S*, *Haliangium ochraceum*, *Alicyclobacillus acidocaldarius*, *Staphylococcus aureus*, *Legionella pneumophila*, or *Myxococcus xanthus*.

[0021] An isolated or purified nucleic acid molecule comprising a nucleotide sequence encoding a mutant KASIII is also provided. The isolated or purified nucleic acid molecule can be a vector. The mutant KASIII comprises one or more mutations comprising at least one mutation, which causes the rotamer conformation of a

phenylalanine in the KASIII corresponding to Phe304 in KASIII from *E. coli* to change. In one embodiment, the KASIII is from *E. coli*, and the one or more mutations comprises a mutation of Leu220, alone or in further combination with Val215. The mutation of Leu220 can be Leu220Met, and the mutation of Val215 can be Val215Phe. The rotamer conformation of Phe304 can be changed from an active site-distal rotamer conformation, in which the Phe304 is oriented away from the active site, to an active site-proximal rotamer conformation, in which the side chain of Phe304 faces towards the active site. In another embodiment, the KASIII is KASIIIA from *B. subtilis*, and the one or more mutations comprises a mutation of Met213, alone or in further combination with Phe208. The mutation of Met213 can be Met213Leu, and the mutation of Phe208 can be Phe208Val. The rotamer conformation of Phe297 can be changed from an active site-proximal rotamer conformation to an active site-distal rotamer conformation. In yet another embodiment, the KASIII is KASIIIB from *B. subtilis*, and the one or more mutations comprise a mutation of Trp221 and a mutation of Val226. The mutation of Trp221 can be Trp221Val, and the mutation of Val226 can be Val226Leu. The rotamer conformation of Phe310 can be changed from an active site-proximal rotamer conformation, in which the side chain of Phe310 faces towards the active site, to an active site-distal rotamer conformation, in which the Phe310 is oriented away from the active site. In still yet another embodiment, the KASIII is from the KASIII is from *Aeromonas hydrophila*, *Bacteroides vulgatus*, *Brevibacterium linens*, *Capnocytophaga gingivalis*, *Thermus aquaticus*, *Bacillus licheniformis*, *Desulfovibrio vulgaris*, *Bacillus subtilis subsp. S*, *Haliangium ochraceum*, *Alicyclobacillus acidocaldarius*, *Staphylococcus aureus*, *Legionella pneumophila*, or *Myxococcus xanthus*.

[0022] A host cell or organism is also provided. The host cell or organism comprises an above-described isolated or purified nucleic acid molecule.

[0023] Further provided is an isolated or purified mutant KASIII. The mutant KASIII comprises one or more mutations comprising at least one mutation, which causes the rotamer conformation of a phenylalanine in the KASIII corresponding to Phe304 in KASIII from *E. coli* to change. In one embodiment, the KASIII is from *E. coli*, and the one or more mutations comprises a mutation of Leu220, alone or in further combination with Val215. The mutation of Leu220 can be Leu220Met, and the

mutation of Val215 can be Val215Phe. The rotamer conformation of Phe304 can be changed from an active site-distal rotamer conformation, in which the Phe304 is oriented away from the active site, to an active site-proximal rotamer conformation, in which the side chain of Phe304 faces towards the active site. In another embodiment, the KASIII is KASIIIA from *B. subtilis*, and the one or more mutations comprises a mutation of Met213, alone or in further combination with Phe208. The mutation of Met213 can be Met213Leu, and the mutation of Phe208 can be Phe208Val. The rotamer conformation of Phe297 can be changed from an active site-proximal rotamer conformation, in which the side chain of Phe297 faces towards the active site, to an active site-distal rotamer conformation, in which Phe297 is oriented away from the active site. In yet another embodiment, the KASIII is KASIIIB from *B. subtilis*, and the one or more mutations comprise a mutation of Trp221 and a mutation of Val226. The mutation of Trp221 can be Trp221Val, and the mutation of Val226 can be Val226Leu. The rotamer conformation of Phe310 can be changed from an active site-proximal rotamer conformation, in which the side chain of Phe310 faces towards the active site, to an active site-distal rotamer conformation, in which Phe310 is oriented away from the active site. In still yet another embodiment, the KASIII is from the KASIII is from *Aeromonas hydrophila*, *Bacteroides vulgatus*, *Brevibacterium linens*, *Capnocytophaga gingivalis*, *Thermus aquaticus*, *Bacillus licheniformis*, *Desulfovibrio vulgaris*, *Bacillus subtilis subsp. S*, *Haliangium ochraceum*, *Alicyclobacillus acidocaldarius*, *Staphylococcus aureus*, *Legionella pneumophila*, or *Myxococcus xanthus*.

[0024] Still further provided is an *in vitro*, high-throughput spectrophotometric method of assaying KASIII activity. The method comprises (i) incubating holo-ACP, malonyl-CoA, dithiothreitol (DTT), acyl-CoA, NADPH, and malonyl-CoA:ACP transacylase (FabD), (ii) adding KASIII and 3-ketoacyl-ACP reductase (FabG), and (iii) measuring the change in absorbance at 340 nm when NADPH is converted to NADP⁺ during reduction of 3-ketoacyl-ACP to 3-hydroxyl-acyl-ACP by FabG. In an embodiment, the acyl-CoA is a straight-chain-CoA, a branched-chain-CoA or a hydroxylated-CoA. Preferably, incubating in (i) is for about two minutes, e.g., two minutes, in the presence of a buffer, such as sodium phosphate buffer, at neutral pH, e.g., pH 7.2.

[0025] Even still further provided is a method of producing bi-functional fatty acids in a host cell or organism. The method comprises introducing into a host cell or organism, which comprises one or more ω -functionalized acyl-CoAs, and expressing therein a nucleic acid molecule comprising a nucleotide sequence encoding a KASIII, which can use one or more of the ω -functionalized acyl-CoAs as a substrate. The one or more ω -functionalized acyl-CoAs can be functionalized at the ω -position with a moiety comprising a hydroxyl group, a benzoyl group, a cyclic group, a branched-chain group, an amino group, or a halo group. The host cell or organism can be a mutant *R. rubrum*, such as a mutant *R. rubrum* described above.

BRIEF DESCRIPTION OF THE FIGURES

[0026] Fig. 1 is a schematic diagram of the Claisen condensation reaction catalyzed by KASIII.

[0027] Fig. 2 is a schematic diagram of the reaction mechanism of *E. coli* FabH proposed by Davies et al.

[0028] Fig. 3a is a graph of time (hours (h)) vs. OD at 600 nm, wherein WT is wild-type, $\Delta bfabHA$ is the deletion mutant for *bfabHA*, and $\Delta bfabHB$ is the deletion mutant for *bfabHB* (i.e., *bfabHB::erm*, in which the *bfabHB* gene has been replaced with a gene conferring resistance to erythromycin (*erm*)) cultured on minimal medium at 37°C. Data represent the average of three determinations $\pm$ standard error.

[0029] Fig. 3b is a graph of time (h) vs. OD at 600 nm for WT, $\Delta bfabHA$, and $\Delta bfabHB$ (*bfabHB::erm*) cultured on minimal medium at 16°C. Data represent the average of three determinations $\pm$ standard error.

[0030] Fig. 4 is a graph of OD at 600 nm vs. β -galactosidase activity (Miller units), which shows the expression of the *bfabHA* and *bfabHB* genes. The strains *bfabHA::pM4* (—■—, —□—), and *bfabHB::pM4B* (—▲—, —△—), which carry *lacZ* reporter fusions for each KASIII-coding paralog were grown on LB medium at either 37 °C (—■—, —▲—) or 16 °C (—□—, —△—). At the indicated optical density, aliquots of the cultures were removed and β -galactosidase activity was determined. Data represent average of three determinations $\pm$ standard error.

[0031] Fig. 5a is a graph of fatty acid vs. mol % for cultures of WT strain 169 (□) and mutant strains $\Delta bfabHA$ (⊗) and $\Delta bfabHB$ (*bfabHB::erm*; ⊗) grown on minimal

medium at 37 °C showing the effect of deleting the *bfabHA* and *bfabHB* genes on fatty acid composition of *B. subtilis*. Data represent average of three determinations $\pm$ standard error.

[0032] Fig. 5b is a graph of anteiso:iso ratio for cultures of WT strain 168 ($\square$) and mutant strains $\Delta bfabHA$ ($\otimes$) and $\Delta bfabHB$ (*bfabHB::erm*; $\boxtimes$) grown on minimal medium at 37 °C. Data represent average of three determinations $\pm$ standard error.

[0033] Fig. 5c is a graph of fatty acid chain length (<16, 16 or >16 carbon atoms) vs. mol% for cultures of WT strain 168 ($\square$) and mutant strains $\Delta bfabHA$ ($\otimes$) and $\Delta bfabHB$ (*bfabHB::erm*; $\boxtimes$) grown on minimal medium at 37 °C. Data represent average of three determinations $\pm$ standard error.

[0034] Fig. 5d is a graph of fatty acid vs. mol % showing the effect of deleting the *bfabHA* and *bfabHB* genes on fatty acid composition of *B. subtilis*. Cultures of WT strain 168 ($\square$) and mutant strains $\Delta bfabHA$ ($\otimes$) and $\Delta bfabHB$ (*bfabHB::erm*; $\boxtimes$) were grown on minimal medium at 16 °C. Data represent average of three determinations $\pm$ standard error.

[0035] Fig. 5e is a graph of anteiso:iso ratio for cultures of WT strain 168 ($\square$) and mutant strains $\Delta bfabHA$ ($\otimes$) and $\Delta bfabHB$ (*bfabHB::erm*; $\boxtimes$) grown on minimal medium at 16 °C. Data represent average of three determinations $\pm$ standard error.

[0036] Fig. 5f is a graph of fatty acid chain length (<16, 16 or >16 carbon atoms) vs. mol % for cultures of WT strain 168 ($\square$) and mutant strains $\Delta bfabHA$ ($\otimes$) and $\Delta bfabHB$ (*bfabHB::erm*; $\boxtimes$) grown on minimal medium at 16 °C. Data represent average of three determinations $\pm$ standard error.

[0037] Fig. 5g is a graph of the proportion of different classes of fatty acids (based on the acyl-CoA primers used in their biosynthesis) vs. mol % at 37 °C, wherein "odd-i" is odd-numbered iso-fatty acids, "even-i" is even-numbered iso-fatty acids, "a" is anteiso-fatty acids, "odd-n" is odd-numbered normal fatty acids, and "even-n" is even-numbered normal fatty acids. Data represent the average of three determinations $\pm$ standard error.

[0038] Fig. 5h is a graph of the proportion of different classes of fatty acids (based on the acyl-CoA primers used in their biosynthesis) vs. mol % at 16 °C, wherein "odd-i" is odd-numbered iso-fatty acids, "even-i" is even-numbered iso-fatty acids, "a" is anteiso-fatty acid, "odd-n" is odd-numbered normal fatty acids, and "even-n" is even-

numbered normal fatty acids. Data represent the average of three determinations $\pm$ standard error.

[0039] Fig. 6a is a graph of fatty acid vs. mol % of WT *B. subtilis* grown without (⌘) or with (⌘) 10 μ M anteiso-C16 fatty acid, compared to the *AbfabHA AbfabHB::erm* double mutant rescued by the inclusion of 10 μ M anteiso-C16 fatty acid in the media (□).

[0040] Fig. 6b is a graph of fatty acids vs. mol% of WT *B. subtilis* grown without (⌘) or with (⌘) 10 μ M anteiso-C16 fatty acid, compared to the *AbfabHA AbfabHB::erm* double mutant rescued by the inclusion of 10 μ M anteiso-C16 fatty acid in the media (□), wherein BCFA is branched chain fatty acids, normal FA is normal fatty acids, and exogenous FA is exogenous fatty acids.

[0041] Fig. 7a is a graph of time (hours (h)) vs. OD at 600 nm with doubling times indicated for *B. subtilis AbfabHA AbfabHB::erm* double mutant strain grown in LB medium supplied with 30 μ M anteiso-C16:0, iso-C16:0, palmitoleic acid or n-C16:0 at 37°C. Data represent average of three determinations $\pm$ standard error. NA = not applicable.

[0042] Fig. 7b is a graph of fatty acids vs. mol % for *B. subtilis AbfabHA AbfabHB::erm* double mutant strain grown in LB medium supplied with 30 μ M anteiso-C16:0, iso-C16:0, palmitoleic acid or n-C16:0 at 37°C.

[0043] Fig. 7c is a graph of type of fatty acids vs. mol % for *B. subtilis AbfabHA AbfabHB::erm* double mutant strain grown in LB medium supplied with 30 μ M anteiso-C16:0, iso-C16:0, palmitoleic acid or n-C16:0 at 37°C, wherein BCFA is branched chain fatty acids, normal FA is normal fatty acids, and exogenous FA is exogenous fatty acids.

[0044] Fig. 7d is a graph of time (hours (h)) vs. OD at 600 nm with doubling times indicated for *B. subtilis AbfabHA AbfabHB::erm* double mutant strain grown in LB medium supplied with 30 μ M anteiso-C16:0, iso-C16:0, palmitoleic acid or n-C16:0 at 30 °C. Data represent average of three determinations $\pm$ standard error. NA = not applicable.

[0045] Fig. 7e is a graph of fatty acids vs. mol % for *B. subtilis AbfabHA AbfabHB::erm* double mutant strain grown in LB medium supplied with 30 μ M anteiso-C16:0, iso-C16:0, palmitoleic acid or n-C16:0 at 30 °C.

[0046] Fig. 7f is a graph of type of fatty acids vs. mol % for *B. subtilis* $\Delta bfabHA \Delta bfabHB::erm$ double mutant strain grown in LB medium supplied with 30 μ M anteiso-C16:0, iso-C16:0, palmitoleic acid or n-C16:0 at 30 °C, wherein BCFA is branched chain fatty acids, normal FA is normal fatty acids, and exogenous FA is exogenous fatty acids.

[0047] Fig. 7g is a graph of time (hours (h)) vs. OD at 600 nm with doubling times indicated for *B. subtilis* $\Delta bfabHA \Delta bfabHB::erm$ double mutant strain grown in LB medium supplied with 30 μ M anteiso-C16:0, iso-C16:0, palmitoleic acid or n-C16:0 at 20 °C. Data represent average of three determinations $\pm$ standard error. NA = not applicable.

[0048] Fig. 7h is a graph of fatty acids vs. mol % for *B. subtilis* $\Delta bfabHA \Delta bfabHB::erm$ double mutant strain grown in LB medium supplied with 30 μ M anteiso-C16:0, iso-C16:0, palmitoleic acid or n-C16:0 at 20 °C.

[0049] Fig. 7i is a graph of type of fatty acids vs. mol % for *B. subtilis* $\Delta bfabHA \Delta bfabHB::erm$ double mutant strain grown in LB medium supplied with 30 μ M anteiso-C16:0, iso-C16:0, palmitoleic acid or n-C16:0 at 20 °C, wherein BCFA is branched chain fatty acids, normal FA is normal fatty acids, and exogenous FA is exogenous fatty acids.

[0050] Fig. 7j is a graph of time (hours (h)) vs. OD at 600 nm with doubling times indicated for *B. subtilis* $\Delta bfabHA \Delta bfabHB::erm$ double mutant strain grown in LB medium supplied with 30 μ M anteiso-C16:0, iso-C16:0, palmitoleic acid or n-C16:0 at 16 °C. Data represent average of three determinations $\pm$ standard error. NA = not applicable.

[0051] Fig. 7k is a graph of fatty acids vs. mol % for *B. subtilis* $\Delta bfabHA \Delta bfabHB::erm$ double mutant strain grown in LB medium supplied with 30 μ M anteiso-C16:0, iso-C16:0, palmitoleic acid or n-C16:0 at 16 °C.

[0052] Fig. 7l is a graph of type of fatty acids vs. mol % for *B. subtilis* $\Delta bfabHA \Delta bfabHB::erm$ double mutant strain grown in LB medium supplied with 30 μ M anteiso-C16:0, iso-C16:0, palmitoleic acid or n-C16:0 at 16 °C, wherein BCFA is branched chain fatty acids, normal FA is normal fatty acids, and exogenous FA is exogenous fatty acids.

[0053] Fig. 8a is a schematic drawing of the crystal structure of *E. coli*'s KASIII (PDB code 3IL9) using Swiss Model in (A) showing the active site, which consists of C112, N274, and H244.

[0054] Fig. 8b is a schematic drawing of the crystal structure of *E. coli*'s KASIII showing the proposed effect of mutations L220M and V215F (modeled using PyMol (B)) on the orientation of Phe 304 and substrate specificity based on the orientation of F304 relative to V215 and L220 in the layer behind it.

[0055] Fig. 9a is a schematic drawing of the predicted crystal structure of *B. subtilis*'s KASIIIA using Swiss Model in (A) showing the active site, which consists of C122, N267, and H237.

[0056] Fig. 9b is a schematic drawing of the predicted crystal structure of *B. subtilis*'s KASIIIA showing the proposed effect of mutations M213L and F208V (modeled using PyMol (B)) on the orientation of F297 and substrate specificity based on the orientation of F297 relative to M213 and F208 in the layer behind it.

[0057] Fig. 10a is a schematic diagram showing the saturation transfer from KASIII enzymes to different ligand binding epitopes of acetyl-CoA and isobutyryl-CoA.

[0058] Fig. 10b is a graph of protons of ligand acetyl-CoA vs. STD amplification factor (STD_{af}) for *E. coli* KASIII.

[0059] Fig. 10c is a graph of protons of ligand isobutyryl-CoA vs. STD amplification factor (STD_{af}) for *E. coli* KASIII.

[0060] Fig. 10d is a graph of protons of ligand acetyl-CoA vs. STD amplification factor (STD_{af}) for *B. subtilis* KASIIIA.

[0061] Fig. 10e is a graph of protons of ligand isobutyryl-CoA vs. STD amplification factor (STD_{af}) for *B. subtilis* KASIIIA.

[0062] Fig. 10f is a graph of protons of ligand acetyl-CoA vs. STD amplification factor (STD_{af}) for *B. subtilis* KASIIIB.

[0063] Fig. 10g is a graph of protons of ligand isobutyryl-CoA vs. STD amplification factor (STD_{af}) for *B. subtilis* KASIIIB.

[0064] Fig. 11a is a graph of protons of ligand acetyl-CoA vs. STD amplification factor (STD_{af}) for *E. coli* containing an F304A mutation.

[0065] Fig. 11b is a graph of protons of ligand isobutyryl-CoA vs. STD amplification factor (STD_{af}) for *E. coli* KASIII containing an F304A mutation.

[0066] Fig. 11c is a graph of protons of ligand acetyl-CoA vs. STD amplification factor (STD_{af}) for *B. subtilis* KASIII containing an F297A mutation (nucleotide sequence [SEQ ID NO:125]; amino acid sequence [SEQ ID NO:126]).

[0067] Fig. 11d is a graph of protons of ligand isobutyryl-CoA vs. STD amplification factor (STD_{af}) for *B. subtilis* KASIII containing an F297A mutation.

[0068] Fig. 12a is a graph of ligand molar excess vs. STD amplification factor for *E. coli* KASIII wild-type enzyme, which shows competition binding data.

[0069] Fig. 12b is a graph of ligand molar excess vs. STD amplification factor for *B. subtilis* KASIIIA wild-type enzyme, which shows competition binding data.

[0070] Fig. 12c is a graph of ligand molar excess vs. STD amplification factor for *B. subtilis* KASIIIB wild-type enzyme, which shows competition binding data.

[0071] Fig. 13a is a gas chromatogram (GC) profile of the fatty acids produced by recombinant *R. rubrum* lacking *phaC* activity but expressing *A. acidocaldarius* KASIII (line 1) and the parallel profile of the fatty acids produced by control *R. rubrum* (line 2) lacking *A. acidocaldarius* KASIII.

[0072] Fig. 13b is the mass spectra of the largest peaks of lines 1 and 2, wherein the peak of line 1 is the silylated form of the methyl-ester of 15-hydroxypalmitate.

[0073] Fig. 14a is a graph of time (minutes) vs. NADPH absorbance (340 nm), which shows the *in vitro* activity of *B. vulgaris* KASIII with strain-chain substrate (acetyl-CoA).

[0074] Fig. 14b is a graph of time (minutes) vs. NADPH absorbance (340 nm), which shows the *in vitro* activity of *B. vulgaris* KASIII with branched-chain substrate (isobutyryl-CoA).

[0075] Fig. 14c is a graph of time (minutes) vs. NADPH absorbance (340 nm), which shows the *in vitro* activity of *B. vulgaris* KAS III with hydroxylated substrate (β -hydroxybutyryl-CoA).

[0076] Fig. 15a is a graph of time (minutes) vs. NADPH absorbance (340 nm), which shows the *in vitro* activity of *L. pneumophila* KASIII with strain-chain substrate (acetyl-CoA).

[0077] Fig. 15b is a graph of time (minutes) vs. NADPH absorbance (340 nm), which shows the *in vitro* activity of *L. pneumophila* KASIII with branched-chain substrate (isobutyryl-CoA).

[0078] Fig. 15c is a graph of time (minutes) vs. NADPH absorbance (340 nm), which shows the *in vitro* activity of *L. pneumophila* KAS III with hydroxylated substrate (β -hydroxybutyryl-CoA).

[0079] Fig. 16a is a graph of substrate vs. thermal shift (ΔT_m) relative to water ($^{\circ}\text{C}$).

[0080] Fig. 16b is a graph of substrate vs. thermal shift (ΔT_m) relative to water ($^{\circ}\text{C}$).

[0081] Fig. 16c is a graph of substrate vs. thermal shift (ΔT_m) relative to water ($^{\circ}\text{C}$).

[0082] Fig. 16d is a graph of substrate vs. thermal shift (ΔT_m) relative to water ($^{\circ}\text{C}$).

[0083] Fig. 17 shows an alignment of conserved residues of KASIIIs from Gram-negative and Gram-positive bacteria, including those KASIIIs analyzed in the Examples (*), nine experimentally confirmed KASIII enzymes, and six uncharacterized KASIII proteins ($\dagger$). Seventeen conserved residues are shown, which are grouped into five structural categories. Branched-chain fatty acid content (%) is provided for each bacterium (Ratledge, C., and Wilkinson, S.G. (1988) *Microbial Lipids*, Academic Press, UK). Phylogenetic sub-family from KASIII tree is also provided for each bacterium (Chen et al., Protein Sci. 20: 1659-1667 (2011)). Residues conserved in KASIIIs from Gram-negative and Gram-positive bacteria are highlighted in black, whereas homologous substitutions are shown in gray, and non-conserved residues are shown in white. Residue numbering corresponds to the residue position in *E. coli* KASIII (GenBank Accession AAG55837.1).

[0084] Fig. 18 shows a classification of KASIII enzymes into five distinct structure-function groups. The enzymes include those analyzed in the Examples (*), those for which structural data are available (highlighted in dark gray), those for which functional data are available (highlighted in light gray), or those for which structural and functional data are available (highlighted in gradation of light to dark gray).

[0085] Fig. 19 shows a comparison of structural and functional features of KASIIIs, including those analyzed in the Examples (*). The enzymes are grouped into five structure-function groups based on phylogenetic analysis (see Examples) and structural and functional features. Residues highlighted in black are similar or identical to the residues occurring in KASIII with broad substrate specificity, such as *S. aureus*

KASIII. Non-functional KASIII proteins are highlighted in gray. N.D. = not determined.

DETAILED DESCRIPTION

[0086] The present disclosure is predicated, at least in part, on the discovery that bi-functional fatty acids, including bi-functional fatty acids that do not occur naturally, can be made by introducing into a host organism, which expresses one or more acyl-CoA starter substrates of interest, an exogenous 3-ketoacyl-acyl carrier protein (ACP) synthase III (KASIII), such as a wild-type KASIII from another organism, a natural variant thereof, or a mutant thereof. KASIII catalyzes a reaction that creates a new carbon-carbon bond that links two precursor molecules together. Precursor molecules (also referred to herein as "substrates" and "starter substrates"; e.g., acetyl-CoA, isobutyryl-CoA, hydroxyl acyl-CoA, or benzoyl-CoA) are extended by two carbon atoms by condensation with malonyl-ACP. The host organism can be modified to express more or less of a given acyl-CoA starter substrate or a different acyl-CoA starter substrate. Additionally or alternatively, the host organism can be modified to reduce, preferably eliminate, fatty acid degradation and/or termination of fatty acid elongation and/or to secrete fatty acids so produced. The materials and methods have application for bio-based chemicals, such as surfactants, lubricants, food oils, polymers, and the like.

[0087] "Bi-functional fatty acid" is used herein to refer to a fatty acid, which comprises a functional group, such as a carboxyl-containing group (-COOH), at the α end and another functional group, which can be the same or different, such as a hydroxyl-containing group (-OH), a benzoyl group, a nitrogen-containing group (-N), such as an amino-containing group (e.g., an amino-carboxylic acid), or a halogen-containing group (-X) at the ω end. The functional group can be a straight chain, a branched chain (e.g., iso or ante-iso), or a cyclic group. While the production of bi-functional fatty acids has been demonstrated in bacteria herein, such production can be adapted to other hosts, such as algae, yeast, plants, and animals.

[0088] Amino acids may be identified herein in accordance with convention. Either a three-letter code or a single-letter code may be used, wherein the 20 naturally occurring amino acids are identified as follows:

Amino acid	Three-Letter Code	Single-Letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

[0089] In view of the above, a mutant *Bacillus subtilis* is provided. The mutant *B. subtilis* does not express a functional KASIII selected from the group consisting of KASIIIA (BsKASIIIA (bfabHA (yjaX) locus); GenBank Accession No. CAB12974.1; nucleotide sequence [SEQ ID NO:63]; amino acid sequence [SEQ ID NO:64]) and KASIIIB (BsKASIIIB (bfabHB (yhfB) locus); GenBank Accession No. CAB12857.1; nucleotide sequence [SEQ ID NO:65]; amino acid sequence [SEQ ID NO:66]). In one

embodiment, the mutant *B. subtilis* does not express a functional KASIIIA and a functional KASIIIB. While wild-type *B. subtilis* normally produces linear, iso-branched, and ante-iso-branched fatty acids, the double deletion mutant lacks KASIII activity and fails to grow unless provided with exogenous fatty acids.

[0090] Accordingly, also provided is a method of making a mutant *B. subtilis*. The method comprises introducing into wild-type *B. subtilis* a mutation selected from the group consisting of a mutation that prevents expression of a functional KASIIIA and a mutation that prevents expression of a functional KASIIIB. The method can comprise introducing into wild-type *B. subtilis* a mutation that prevents expression of a functional KASIIIA and a mutation that prevents expression of a functional KASIIIB. Preferably, the mutation is a gene knock-out. A gene-deletion knock-out mutation can be preferred because it eliminates the possibility of revertants occurring.

[0091] The double deletion mutant can be "rescued" by the expression therein of a functional KASIII gene (such as an exogenous KASIII gene, e.g., a KASIII gene from *Alicyclobacillus acidocaldarius*). Thus, the double deletion mutant can be used to characterize an exogenous KASIII, such as in accordance with the methods exemplified herein. Chemically modified carboxylic acids can be fed to the rescued double deletion mutant to determine whether or not the exogenous KASIII can incorporate these modified carboxylic acids into the ω -end of fatty acids. For example, the KASIII from *A. acidocaldarius* can use 3-hydroxybutyryl-CoA (generated from the 3-hydroxybutyric acid that was provided in the media) to produce hydroxypalmitate (16-carbon chain) and hydroxystearate (18-carbon chain).

[0092] In this regard, the mutant *B. subtilis* can be used to characterize an exogenous KASIII, which is a wild-type KASIII, a naturally occurring variant thereof, or a mutant thereof, e.g., a mutant with altered starter substrate specificity. "Altered starter substrate specificity," as used herein, can mean a change in the relative preference of a KASIII for one substrate over another substrate, the loss of the ability to use a given substrate, or the gain of the ability to use a given substrate. Thus, also provided is a method of characterizing substrate specificity of a KASIII. The method comprises expressing the KASIII, which is not expressed in wild-type *B. subtilis*, in a mutant *B. subtilis*, such as a mutant *B. subtilis*, which lacks a functional KASIIIA and a functional KASIIIB, in the presence of ω -functionalized carboxylic acid starter

substrate and assessing the production of ω -functionalized fatty acids. The KASIII can be derived from an organism, the wild-type of which produces ω -functionalized fatty acids. Alternatively, the KASIII can be derived from an organism, the wild-type of which does not normally produce ω -functionalized fatty acids.

[0093] Also in view of the above, a mutant *Rhodospirillum rubrum* is provided. Wild-type *R. rubrum* generates large quantities of hydroxybutyryl-CoA, which is usually polymerized by polyhydroxyalkanoate (PHA) polymerase (encoded by the *phaC* gene), as a means of storing carbon and energy (Jin et al., J. Bacteriol. 194: 5522-5529 (2012)). *R. rubrum* specifically assembles polyhydroxybutyrate from hydroxybutyryl-CoA, which is produced from acetyl-CoA in a two-step reaction. The first step is the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA (catalyzed by acetoacetyl-CoA thiolase, which is encoded by the *phaA* gene), and the second step is the reduction of acetoacetyl-CoA to form hydroxybutyryl-CoA (catalyzed by acetoacetyl-CoA reductase, which is encoded by the *phaB* gene). The *R. rubrum* genome contains three genes (Rru_A0275, Rru_A2413 and Rru_A1816) encoding PHA polymerases, which are designated *phaC1*, *phaC2*, and *phaC3* (Jin et al., J. Bacteriol. 194: 5522-5529 (2012)). One of them (*phaC1*) is located in the *pha* operon, adjoining the *phaA* and the *phaB* genes. PhaC2 and PhaC3 share highest sequence conservation (50.2% identity), and PhaC1 is equally distinct from PhaC2 and PhaC3 (14.3% and 18.4% sequence identity, respectively). The mutant *R. rubrum* provided herein does not express a functional PHA polymerase selected from the group consisting of PhaC1, PhaC2, and PhaC3. In one embodiment, the mutant *R. rubrum* does not express a functional PhaC1, a functional PhaC2, and a functional PhaC3. The triple mutant fails to accumulate any PHA polymer, with only a slight impact on growth characteristics. The triple mutant still has the capacity to generate hydroxybutyryl-CoA.

[0094] A method of making a mutant *R. rubrum* is also provided. The method comprises introducing into wild-type *R. rubrum* a mutation selected from the group consisting of a mutation that prevents expression of a functional PhaC1, a mutation that prevents expression of a functional PhaC2, and a mutation that prevents expression of a functional PhaC3. The method can comprise introducing into wild-type *R. rubrum* a mutation that prevents expression of a functional PhaC1, a mutation that prevents

expression of a functional PhaC2, and a mutation that prevents expression of a functional PhaC3. Preferably, the mutation is a gene knock-out. A gene knock-out mutation can be preferred because it eliminates the possibility of revertants occurring.

[0095] The mutant *R. rubrum* can be used to characterize an exogenous KASIII, such as in accordance with the methods described herein. The hydroxyacyl-CoA, such as hydroxybutyryl-CoA, present in the mutant *R. rubrum* is available as a starter substrate for the exogenous KASIII. Whether or not the exogenous KASIII incorporates the hydroxylacyl-CoA into fatty acids can be determined. Expression of *A. acidocaldarius* KASIII in the mutant *R. rubrum*, for example, results in the production of 15-hydroxy-palmitate, which does not occur normally in any known system.

[0096] In this regard, the mutant *R. rubrum* can be used to characterize an exogenous KASIII, which is a wild-type KASIII, a naturally occurring variant thereof, or a mutant thereof, e.g., a mutant with altered starter substrate specificity. Thus, also provided is a method of characterizing substrate specificity of a KASIII enzyme. The method comprises expressing the KASIII enzyme, which is not expressed in wild-type *R. rubrum*, in a mutant *R. rubrum*, such as a mutant *R. rubrum*, which lacks functional PhaC1, PhaC2, and PhaC3, in the presence of ω -functionalized acyl-CoA starter substrate and assessing the production of ω -functionalized fatty acids. The KASIII enzyme can be derived from an organism, the wild-type of which produces ω -functionalized fatty acids. Alternatively, the KASIII can be derived from an organism, the wild-type of which does not normally produce ω -functionalized fatty acids.

[0097] The above mutant *B. subtilis* and mutant *R. rubrum* can be generated in accordance with any suitable method known in the art, including, but not limited to, methods described and exemplified herein. Mutations, such as substitutions, insertions, deletions, and/or side chain modifications, can be introduced into the nucleotide and amino acid sequences of the gene of interest using any suitable technique known in the art, including site-directed mutagenesis (Wu, ed., Meth. Enzymol. 217, Academic Press (1993)). The lambda red recombinase method can be used to "knock out" genes (Datsenko et al., PNAS USA 97: 6640-6645 (2000)). A kanamycin disruption cassette flanked by FLP recognition target sites can be used to excise a cassette precisely and create an in-frame deletion (Baba et al., Mol. System. Biol. 2: 2006-2008 (2006)).

Permanent, marker-free, multiple gene disruptions can be created. Non-naturally occurring nucleotides and amino acids also can be used.

An isolated or purified mutant (or chimeric) KASIII derived from a wild-type KASIII, such as a KASIII from family KS1 (ketoacyl synthase 1) from www.enzyme.cbirc.iastate.edu, which website is incorporated by reference herein for its teachings regarding KASIII, is also provided. Examples include a KASIII from a bacterium, such as *E. coli* and *B. subtilis*, as described and exemplified herein. Other examples include *Aeromonas hydrophila* (ATCC Deposit No. 7966; GenBank Accession No. YP_857537.1; nucleotide sequence [SEQ ID NO:67]; amino acid sequence [SEQ ID NO:68]), *Bacteroides vulgatus* (ATCC Deposit No. 8482; GenBank Accession No. ABR38167.1; YP_001297789.1; nucleotide sequence [SEQ ID NO:77]; amino acid sequence [SEQ ID NO:78]), *Brevibacterium linens* (KAS_BvL1: GenBank Accession No. AAGP010000; ZP_05913013.1; nucleotide sequence [SEQ ID NO:85]; amino acid sequence [SEQ ID NO:86]; KAS_BvL2: GenBank Accession No. AAGP010000; ZP_05912949.1; nucleotide sequence [SEQ ID NO:87]; amino acid sequence [SEQ ID NO:88]), *Capnocytophaga gingivalis* (KAS_CG1: ATCC Deposit No. 33624; GenBank Accession No. EEK13720.1; ZP_04058441.1; nucleotide sequence [SEQ ID NO:79]; amino acid sequence [SEQ ID NO:80]; KAS_CG2: ATCC Deposit No. 33624; GenBank Accession No. EEK14223.1; ZP_04057621; nucleotide sequence [SEQ ID NO:81]; amino acid sequence [SEQ ID NO:82]; KAS_CG3: ATCC Deposit No. 33624; GenBank Accession No. EEK14078.1; ZP_04058132.1; nucleotide sequence [SEQ ID NO:83]; amino acid sequence [SEQ ID NO:84]), *Thermus aquaticus* (GenBank Accession No. EED09603.1; ZP_03497263.1; nucleotide sequence [SEQ ID NO:89]; amino acid sequence [SEQ ID NO:90]), *Bacillus licheniformis* (KAS_BaL1: ATCC Deposit No. 14580; GenBank Accession No. AAU22783.1; YP_078421.1; nucleotide sequence [SEQ ID NO:91]; amino acid sequence [SEQ ID NO:92]; KAS_BaL2: ATCC Deposit No. 14580; GenBank Accession No. AAU22657.1; nucleotide sequence [SEQ ID NO:93]; amino acid sequence [SEQ ID NO:94]), *Desulfovibrio vulgaris* (GenBank Accession No. ACL10038.1; YP_002437506.1; nucleotide sequence [SEQ ID NO:107]; amino acid sequence [SEQ ID NO:108]), *Bacillus subtilis subsp. S* (ATCC Deposit No. 6633; GenBank Accession No. EFG91245.1; ZP_06874900.1; nucleotide sequence [SEQ ID NO:101]; amino acid

sequence [SEQ ID NO:102]), *Haliangium ochraceum* (GenBank Accession No. ACY12771.1; YP_003264664.1; nucleotide sequence [SEQ ID NO: 103]; amino acid sequence [SEQ ID NO: 104]), *Alicyclobacillus acidocaldarius* (GenBank Accession No. ACV57087.1; nucleotide sequence [SEQ ID NO: 105]; amino acid sequence [SEQ ID NO: 106]), *Staphylococcus aureus* (GenBank Accession No. BAB57145.1; NP_371507.1; nucleotide sequence [SEQ ID NO:109]; amino acid sequence [SEQ ID NO:110]), *Legionella pneumophila* (KAS_LP1: GenBank Accession No. CAH12499.1; YP_123672.1; nucleotide sequence [SEQ ID NO:69]; amino acid sequence [SEQ ID NO:70]; KAS_LP2: GenBank Accession No. CAH13332.1; YP_124492; nucleotide sequence [SEQ ID NO:71]; amino acid sequence [SEQ ID NO:72]; KAS_LP3: GenBank Accession No. CAH13337.1; YP_124497; nucleotide sequence [SEQ ID NO:73]; amino acid sequence [SEQ ID NO:74]; KAS_LP4: GenBank Accession No. CAH12752.1; YP_123920; nucleotide sequence [SEQ ID NO:75]; amino acid sequence [SEQ ID NO:76]), *Myxococcus xanthus* (KAS_MX1: GenBank Accession No. ABF92307.1; YP_628497.1; nucleotide sequence [SEQ ID NO:95]; amino acid sequence [SEQ ID NO:96]; KAS_MX2: GenBank Accession No. ABF89527.1; YP_635461.1; nucleotide sequence [SEQ ID NO:97]; amino acid sequence [SEQ ID NO:98]; KAS_MX3: GenBank Accession No. ABF92876.1; YP_629114.1; nucleotide sequence [SEQ ID NO:99]; amino acid sequence [SEQ ID NO:100]), and the like. Yet other examples include *Yersinia pestis* (ZP_02318831.1), *Stappia aggregata* (ZP_01545409.1), *Erythrobacter* sp. SD-21 (ZP_01865126.1 and ZP_01040217.1), *Physcomitrella patens* (XP_001754014.1), *Synechococcus* sp. CC9605 (ABB36118.1), *Renibacterium salmoninarum* (ATCC Deposit No. 33209; YP_001626411.1), *Chloroflexus aurantiacus*, and those set forth below in the "KASIII Enzymes" table. Also, see the "Examples" herein. Depending on the particular KASIII (e.g., a KASIII from family KS1 from www.enzyme.cbirc.iastate.edu) that has been mutated, such mutants can have altered substrate specificity and/or altered level of activity. Mutants can be derived from wild-type KASIIIs in accordance with methods known in the art (e.g., site-directed mutagenesis) and described and exemplified herein. Also, the same amino acid sequence can be encoded by nucleotide sequences that vary due to the degeneracy of the genetic code.

KASIII Enzymes

Genus Species	RefSeq
<i>Yersinia pestis</i>	ZP_02318831.1, YP_001678077.1
<i>Yersinia enterocoliticas</i>	YP_001005922.1
<i>Vibrio Cholerae</i> , <i>O1 biovar E1</i>	ZP_04418348.1, NP_231657.1, ZP_04411352.1, ZP_04961806.1
<i>Vibrio splendidus</i>	YP_002416637.1
<i>Vibrio parahaemolyticus</i> <i>RIMD</i>	NP_798435.1, ZP_05904665.1
<i>Vibrio alginolyticus</i>	ZP_06181412.1
<i>Vibrio harveyi</i> , <i>ATCC BAA</i>	ZP_06176396.1, ZP_06176396.1
<i>Aeromonas salmonicida</i>	YP_001141871.1
<i>Aeromonas hydrophila</i>	YP_856773.1
<i>Pasteurella multocida</i>	NP_246853.1
<i>Haemophilus influenzae</i>	ZP_00154722.2, YP_001290328.1, ZP_01789313.1, ZP_01784255.1, ZP_01797068.1, NP_438327.1, ZP_01792978.1, YP_003519776.1, YP_003557294.1
<i>Haemophilus ducreyi</i>	NP_873290.1
<i>Serratia odorifera</i>	ZP_06640853.1, ZP_06189616.1
<i>Serratia proteamaculans</i>	YP_001478135.1
<i>Vibrio harveyi</i>	ZP_06176396.1
<i>Vibrio harveyi</i> , <i>ATCC BAA-1</i>	YP_001446090.1
<i>Vibrio splendidus</i>	YP_002416637.1
<i>Vibrio vulnificus</i>	NP_761811.1
<i>Xanthomonas campestris</i> pv. <i>c. a</i>	YP_244293.1, NP_636392.1, ZP_06490218.1, YP_244293.1, NP_636392.1, ZP_06490218.1, YP_362874.1
<i>Xanthomonas oryzae</i> pv. <i>oryz</i>	ZP_02242169.1, YP_449832.1,
<i>Legionella longbeachae</i>	ZP_06185395.1, YP_003454953.1
<i>Legionella pneumophila</i> str	YP_123672.1, YP_126694.1
<i>Neisseria cinerea</i>	ZP_05982749.1
<i>Neisseria elongata</i>	ZP_06735426.1
<i>Neisseria flavescens</i>	ZP_04757560.1, ZP_03720464.1
<i>Neisseria gonorrhoeae</i>	ZP_04735631.1, YP_209174.1, ZP_05795165.1
<i>Neisseria lactamica</i>	ZP_05986719.1
<i>Neisseria meningitidis</i>	YP_974421.1, YP_002342022.1, NP_274910.1, YP_003082489.1, CBA07454.1, CAX50870.1
<i>Neisseria sicca</i>	ZP_05317661.1
<i>Neisseria subflava</i>	ZP_05985366.2
<i>Francisella tularensis</i> subsp	ABK78999.1, YP_513827.1, YP_001428636.1, ABK78992.1, YP_763623.1
<i>Thiobacillus denitrificans</i>	YP_315309.1
<i>Escherichia coli</i>	YP_002292430.1, 1MZS, YP_001457935.1, YP_001002812.1,
<i>Escherichia coli</i> <i>K-12</i>	NP_669073.1
<i>Shigella dysenteriae</i>	YP_403645.1
<i>Shigella flexneri</i> 2a str	NP_707007.1
<i>Klebsiella pneumoniae</i>	YP_002239290.1, YP_001334752.1
<i>Enterobacter cloacae</i> sub s	YP_003613037.1
<i>Proteus mirabilis</i>	YP_002150616.1, ZP_03842098.1
<i>Providencia stuartii</i>	ZP_02960986.1
<i>Bordetella parapertussis</i>	NP_885474.1
<i>Bordetella pertussis</i>	NP_881071.1

<i>Xanthobacter autotrophicus</i>	P3H77, YP_001419263.1
<i>Rhodomicrobium vannielii</i>	ZP_06349548.1
<i>Rhodopseudomonas palustris</i>	YP_569816.1, YP_486263.1, ZP_06361298.1, NP_948084.1, YP_781743.1, YP_532544.1
<i>Anabaena variabilis</i>	YP_323237.1
<i>Synechocystis</i> sp.	BAA18018.1
<i>Synechococcus</i> spp.	YP_001733788.1, ZP_01084650.1, ZP_01084650.1, ZP_01471198.1, YP_376322.1, ZP_01468767.1, NP_898337.1, ZP_05789459.1, ABB36118.1, YP_382673.2, ZP_01081021.1
<i>Bacteroides ovatus</i>	ZP_06616069, ZP_02063379, ZP_02067060
<i>Bacteroides thetaiotaomicron</i>	NP_81275, NP_809035
<i>Bacteroides fragilis</i>	ZP_05283826, YP_101365, YP_100487, ZP_05282747
<i>Bacteroides vulgatus</i>	YP_001297789
<i>Bacteroides</i> sp.	ZP_06077075
<i>Capnocytophaga ochracea</i>	YP_003140158.1
<i>Capnocytophaga gingivalis</i>	ZP_04058441.1
<i>Algoriphagus</i> sp.	ZP_01718224.1
<i>Salinibacter ruber</i> DSM 13855	YP_444197.1
<i>Cytophaga hutchinsonii</i>	YP_678233.1
<i>Micrococcus luteus</i>	YP_002957006.1, ZP_06503185.1
<i>Peptostreptococcus anaerobius</i>	ZP_06425389.1
<i>Mycobacterium tuberculosis</i>	P2AJ9, 2AHB, PNP_215047.1, 1M1M, ZP_02548981.1, ZP_01977036.1, ZP_04417767.1, ZP_06048029.1, ZP_01679181.1, ZP_01868999.1,
<i>Vibrio Cholerae</i>	ZP_00993243.1, YP_002395289.1
<i>Vibrio splendidus</i>	ZP_00993243.1, YP_002395289.1
<i>Vibrio parahaemolyticus</i> RIMD	ZP_05118776.1, ZP_05905223.1, NP_800481.1, ZP_05909890.1
<i>Vibrioharveyi</i> , ATCC BAA	YP_001447821.1, ZP_06178483.1, ZP_01985466.1
<i>Vibrio vulnificus</i>	NP_936962.1, NP_762318.1
<i>Aeromonas salmonicida</i>	YP_001142999.1
<i>Aeromonas hydrophila</i>	YP_855582.1
<i>Arthrobacter aurescens</i> TCI	YP_948164.1
<i>Brevibacterium linens</i>	ZP_05913013.1
<i>Cellulomonas flavigena</i>	YP_003637243.1
<i>Rothia dentocariosa</i>	ZP_06906913.1
<i>Streptomyces coelicolor</i>	NP_627458.1, NP_630009.1, NP_625558.1
<i>Streptomyces griseus</i>	YP_001828147.1, AAQ08929.1
<i>Streptomyces griseus</i> subsp	YP_001826619.1
<i>Vibrioharveyi</i> ATCC_BAA -1	YP_001447821.1
<i>Vibrio harveyi</i>	ZP_06178483.1, ZP_01985466.1
<i>Vibrio splendidus</i>	ZP_00993243.1, YP_002395289.1
<i>Vibriovulnificus</i>	NP_936962.1, NP_762318.1
<i>Clostridium perfringens</i> E, str, C, CP	ZP_02633999.1, NP_561984.1, ZP_02863431, YP_695770.1, ZP_02640909.1, YP_698458.1
<i>Clostridium botulinum</i> D st, B s, E3, B, Ba4, A s, F s, A3	ZP_04862054, YP_001885344.1, YP_001920474.1, YP_001783240.1, YP_002864614.1, ZP_02618347.1, ZP_02615736.1, YP_001256083.1, YP_001392959.1, YP_001788947.1
<i>Clostridium thermocellum</i>	YP_001037363.1, YP_001036566.1
<i>Clostridium sporogenes</i>	ZP_02993884.1

<i>Geobacillus Y412MC10</i>	YP_002948901.1, ZP_05371368.1, ADI27688.1, ZP_03146734
<i>Staphylococcus aureus</i>	ZP_05601460.1, PNP_645682.1, CBI48861.1
<i>Staphylococcus epidermidis</i>	NP_764232.1, ZP_04818718.1
<i>Staphylococcus warneri</i>	ZP_04677499.1
<i>Staphylococcus capitis</i>	ZP_03612995.1
<i>Staphylococcus haemolyticus</i>	YP_253888.1
<i>Staphylococcus hominis</i>	ZP_04059117.1
<i>Staphylococcus saprophyticus</i>	YP_301888.1
<i>Ruminococcus flavefaciens</i>	ZP_06142884.1
<i>Ruminococcus albus</i>	ZP_06720344.1
<i>Bacillus clausii</i>	YP_176043.1
<i>Bacillus halodurans</i>	NP_243749.1
<i>Bacillus pseudofirmus</i>	YP_003425377.1
<i>Bacillus cellulosilyticus</i>	ZP_06363736.1
<i>Bacillus selenitireducens</i>	ADH99048.1
<i>Bacillus cytotoxicus</i>	YP_001374220.1
<i>Bacillus cereus</i>	ZP_04216549.1, YP_002450207.1, ZP_03111602.1, YP_002365961.1, ZP_04232574.1, ZP_04299488.1, ZP_04226741.1, ZP_04202127.1
<i>Bacillus mycoides</i>	ZP_04155996.1, ZP_04161820.1
<i>Bacillus thuringiensis</i>	ZP_04144529.1, ZP_04119301.1, ZP_04064103.1
<i>Bacillus coagulans</i>	ZP_04430458.1, ZP_01862223.1
<i>Bacillus sp.</i>	ZP_01726102.1
<i>Bacillus coahuilensis</i>	ZP_03225732.1
<i>Bacillus amyloliquefacien</i>	YP_001420728.1
<i>Bacillus pumilus</i>	YP_001486301.1
<i>Bacillus licheniformis</i>	YP_078421.1
<i>Desulfotomaculum acetoxid</i>	YP_003190685.1
<i>Thermus aquaticus</i>	ZP_03497263.1
<i>Thermus thermophilus</i>	YP_004024.1, P1UB7, ZP_05405201.1, YP_001113417.1, ZP_03734375.1
<i>Campylobacter fetus subsp. f</i>	YP_891441.1
<i>Campylobacter jejuni subsp</i>	ZP_06372697.1, ZP_06373244.1, YP_001398621.1, ZP_01067178.1, YP_178392.1, ZP_03222790.1, ZP_01810106.1, PZP_01100387.1, ZP_01069410.1, YP_001481881.1
<i>Fusobacterium gonidiaformans</i>	ZP_05630604.1
<i>Fusobacterium mortiferum</i>	ZP_04568082.1
<i>Fusobacterium varium</i>	ZP_04859066.1
<i>Myxococcus xanthus</i>	YP_635461.1
<i>Stigmatella aurantiaca</i>	ZP_01460905.1
<i>Capnocytophaga ochracea</i>	YP_003140583.1
<i>Capnocytophaga gingivalis</i>	ZP_04057621
<i>Algoriphagus sp.</i>	ZP_01720592.1
<i>Flavobacterium johnsoniae</i>	YP_001192610.1
<i>Cytophaga hutchinsonii</i>	YP_678504.1
<i>Clostridium botulinum</i>	YP_001390078.1, YP_001780359.1, YP_002861584.1, ZP_02612773.1, YP_002803086.1, YP_001253259.1, YP_001786083.1, ZP_02618062.1, YP_001884875.1, ZP_04823833.1, YP_001920032.1
<i>F s, B1, Ba4, A s, A 2, A 3, B s, E1 s, E3</i>	
<i>Clostridium sporogenes</i>	ZP_02994303.1
<i>Bacillus thuringiensis ser</i>	ZP_04143036.1
<i>Myxococcus xanthus</i>	YP_628497.1

<i>Stigmatella aurantiaca</i>	ZP_01461080.1, ZP_01464359.1
<i>Lactobacillus casei</i>	YP_807311.1
<i>Lactobacillus plantarum</i> L, su	NP_785253.1, ZP_04012979.1, YP_003062988.1
<i>Lactobacillus helveticus</i>	YP_001578074.1
<i>Lactobacillus fermentum</i>	YP_001843126.1
<i>Lactobacillus delbrueckii</i> su	YP_812862.1, YP_618929.1
<i>Desulfovibrio vulgaris</i>	YP_002437506.1, YP_010426.1
<i>Desulfovibrio desulfuricans</i>	YP_388920.1
<i>Streptococcus equi</i> subsp	YP_002122793.1
<i>Streptococcus equi</i> subsp	YP_002745087.1
<i>Legionella pneumophila</i> _str	YP_127488.1, YP_124492.1, YP_096240.1
<i>Bacteroides fragilis</i>	YP_098105.1, ZP_05279764, YP_098107.1, ZP_05279766.1, ZP_05283454.1, ZP_05283453.1
<i>Bacteroides</i> sp.	ZP_05284648, ZP_05286338.1, ZP_06076430.1, ZP_05288125.1, ZP_04850303.1, ZP_05284665.1, ZP_06093726.1, ZP_05285443.1
<i>Parabacteroides distasoni</i>	ZP_05544531, YP_001304951.1
<i>Parabacteroides</i> sp.	ZP_05544549, ZP_05544544, ZP_05547823.1, ZP_05547825.1, ZP_05544531.1
<i>Capnocytophaga gingivalis</i>	ZP_04058132.1
<i>Clostridium thermocellum</i>	YP_001036564.1
<i>Geobacillus</i> sp.	YP_003241015.1
<i>Bacillus cereus</i>	ZP_04197006.1, ZP_04294582.1, ZP_04222169.1, ZP_03114282.1, YP_002450917.1, YP_083343.1, ZP_04283649.1, ZP_00239031.1, ZP_04322923.1, ZP_04185735.1
<i>Bacillus mycoides</i>	ZP_04168455.1
<i>Bacillus thuringiensis</i>	YP_894541.1, ZP_04078161.1
<i>Vibrio Cholerae</i>	ZP_01982749.1
<i>Vibrioharveyi</i> ATCC BAA	YP_001443904.1
<i>Pseudomonas fluorescens</i>	YP_002873982.1, YP_258752.1, YP_347256.1, YP_002871305.1, YP_258783.1, YP_002871302.1, YP_002872773.1
<i>Pseudomonas aeruginosa</i> PAO	ZP_03854283.1
<i>Pseudomonas aeruginosa</i> _PA	YP_001670166.1, YP_002505552.1, YP_001349750.1
<i>Pseudomonas aeruginosa</i>	ZP_06091406.1, YP_001349639.1, AAP35715.1, YP_560887.1, ZP_01364358.1, YP_002441910.1, PNP_249690.1, NP_252023.1
<i>Pseudomonas aeruginosa</i> _PAOI	NP_106422.1
<i>Pseudomonas putida</i>	YP_001266829.1, YP_001750557.1
<i>Pseudomonas syringae</i> _pv._t, tom, aver, pha, o, s, syr	ZP_03395719.1, NP_791771.1, ABQ23410.1, YP_275551.1, ZP_05638972.1, ZP_04587683.1, ZP_06499892.1, YP_236537.1, ZP_03395750.1, YP_275581.1, ZP_06460699.1
<i>Vibrio harveyi</i> _ATCC_BAA-1	YP_001443904.1
<i>Xanthomonas campestris</i> _pv._v	YP_363742.1, ZP_06491606.1
<i>Xanthomonas oryzae</i> _pv._oryz	ZP_02243250.1, YP_451479.1, YP_201231.1,
<i>Legionella longbeachae</i>	ZP_06187897.1
<i>Legionella pneumophila</i> _str	YP_127493.1, YP_096244.1, YP_001250982.1, YP_124497.1, NP_866833.1, ZP_04769890.1,
<i>Campylobacter coli</i>	ZP_00367350.1

<i>Campylobacter jejuni</i> subsp	ZP_06372437.1, YP_001397612.1, ZP_01068304.1, ZP_01069191.1, YP_179478.1, YP_001000977.1, ZP_03223081.1, ZP_06374174.1, ZP_01809975.1, ZP_01071227.1
<i>Bacteroides</i> sp.	ZP_05287210.1
<i>Parabacteroides johnsoni</i>	ZP_03477145
<i>Lactobacillus plantarum</i>	YP_003062116.1, BAA93641.1
<i>Staphylococcus epidermidis</i>	NP_863215.1, ZP_04818028.1
<i>Staphylococcus capitis</i>	ZP_03613727.1
<i>Bacillus cereus</i>	ZP_04218294.1, YP_002366551.1, NP_831535.1, ZP_04202709.1, ZP_04316975.1, YP_002445213.1, ZP_04227324.1, ZP_04294488.1, ZP_04196905.1, NP_978230.1, ZP_00236595.1, ZP_04288820.1, ZP_03108471.1, YP_083243.1, ZP_04283554.1, YP_002529555.1, YP_002337903.1
<i>Bacillus cereus</i>	YP_002450821.1, ZP_04222067.1, ZP_04174069.1, ZP_04322830.1, ZP_04185649.1, ZP_04300078.1, YP_002749110.1, YP_245896.1
<i>Bacillus mycoides</i>	ZP_04163379.1, ZP_04157778.1, ZP_04168363.1
<i>Bacillus thuringiensis</i>	ZP_04071417.1, ZP_04101582.1, YP_003664162.1, ZP_04114339.1, ZP_04083919.1, ZP_04125963.1, ZP_00742862.1, YP_036004.1, ZP_04107829.1, ZP_04089938.1, YP_894441.1
<i>Bacillus thuringiensis</i> ser	ZP_04096011.1, ZP_04078067.1
<i>Bacillus subtilis</i> subsp.	ZP_06874900.1
<i>Bacillus licheniformis</i>	YP_078295.1
<i>Bacillus anthracis</i> str	NP_844246.1
<i>Bacillus megaterium</i>	YP_003597285.1, YP_003562589.1, YP_003565870.1
<i>Aeromonas salmonicida</i>	YP_001142803.1
<i>Aeromonas hydrophila</i>	YP_857537.1
<i>Haemophilus influenzae</i>	P3IL3
<i>Pseudomonas putida</i>	YP_001670278.1, NP_746654.1, YP_001266689.1
<i>Pseudomonas syringae</i> pv. <i>t, tom, a, o, pha</i>	ZP_03399527.1, NP_793855.1, ZP_04587307.1, ZP_06482619.1, ZP_06460756.1, ZP_05640738.1, YP_273685.1
<i>Myxococcus xanthus</i>	YP_629114.1
<i>Rhodococcus vanniellii</i>	ZP_06349538.1
<i>Bacteroides</i> sp.	YP_001250373.1
<i>Clostridium botulinum</i> A3, A s, B1, F s, A2	ZP_02617328.1, YP_001787903.1, 001255081.1, 001782200.1, 001391882.1, 002805031.1, 02615189.1
<i>Clostridium sporogenes</i>	ZP_02995301.1
<i>Micrococcus luteus</i>	ZP_06503261.1, YP_002957382.1
<i>Bacillus cereus</i>	ZP_03105077.1, ZP_04223379.1, ZP_04279606.1, YP_084527.1, ZP_04301428.1, ZP_04295615.1, ZP_04220174.1,
<i>Bacillus mycoides</i>	ZP_04155556.1
<i>Bacillus thuringiensis</i> ser	ZP_04085281.1, YP_895664.1, ZP_04109142.1
<i>Bacillus subtilis</i>	yjaXTNP_389015.1
<i>Bacillus subtilis</i>	yhfBTNP_388898.1
<i>Bacillus subtilis</i> subsp.	BAI85702.1, NP_390087.1
<i>Bacillus anthracis</i> str	NP_845551.1
<i>Renibacterium salmoninarum</i>	YP_001626411.1
<i>Yersinia pseudotuberculosis</i>	YP_070488.1, YP_001401081.1
<i>Vibrio alginolyticus</i>	ZP_01258726.1, ZP_06180329.1
<i>Vibrio vulnificus</i>	NP_760725.1
<i>Brevibacterium linens</i>	ZP_05912949.1
<i>Streptomyces griseus</i> subsp. g	AAF81237.1
<i>Pseudomonas fluorescens</i>	YP_263190.1
<i>Pseudomonas aeruginosa</i>	YP_001349751.1

<i>Pseudomonas putida</i>	YP_095658.1
<i>Pseudomonas syringae</i> _pv. _a, s	ZP_06494049.1, ZP_06478421.1
<i>Serratia odorifera</i>	ZP_06190585.1
<i>Serratia proteamaculans</i>	YP_001479098.1
<i>Vibrio vulnificus</i>	NP_760725.1
<i>Xanthomonas campestris</i> _pv. _c, a, pv, ca, v	ZP_06486563.1, AAM41717.1, YP_001903132.1, ZP_06483318.1, YP_364501.1
<i>Xanthomonas oryzae</i> _pv. _oryz,	ZP_02241404.1, ZP_06486563.1,
<i>Legionella longbeachae</i>	ZP_06186504.1
<i>Legionella pneumophila</i> _str	YP_123920.1
<i>Neisseria sicca</i>	ZP_05319573.1
<i>Fusobacterium nucleatum</i> _su	ZP_06870819.1
<i>Myxococcus xanthus</i>	YP_635355.1
<i>Stigmatella aurantiaca</i>	ZP_01461725.1
<i>Escherichia coli</i> _K-12	P3IL9
<i>Escherichia coli</i> _O157:H7, coli_O127:H6	fabHNP_287225.1, YP_002328892.1
<i>Escherichia coli</i>	PIEBL, P1HNI, BAI54746.1, NP_753594.1

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[0098] A KASIII can be mutated, for example, to alter the three-dimensional conformation of the active site, which, in turn can alter substrate specificity and/or level of activity. For example, by increasing the space available for substrate binding, a KASIII may utilize a branched-chain substrate whereas the corresponding wild-type KASIII is only able to utilize a straight-chain substrate. Conversely, by decreasing the space available for substrate binding, a KASIII may utilize a strain-chain substrate whereas the corresponding wild-type KASIII is additionally or only able to utilize a branched-chain substrate.

[0099] By way of example, a KASIII from *E. coli* can be mutated in such a manner as to alter the orientation of F304 in relation to the active site, which consists of C112, N274, and H244 (see Fig. 8a, which is a schematic drawing of the crystal structure of *E. coli*'s KASIII (PDB code 3IL9) using Swiss Model). As shown in Fig. 8b, which is another schematic drawing of the crystal structure of *E. coli*'s KASIII, mutations L220M and V215F (modeled using PyMol (B)) affect the orientation of F304 relative to the active site and substrate specificity based on the orientation of F304 relative to V215 and L220 in the layer behind it. By altering the orientation of F304 in relation to the active site, substrate specificity can be altered, such as by altering the relative

specificity of the KASIII for one substrate over another or by changing the substrate specificity of the KASIII such that it is no longer specific for a given substrate and/or it is now specific for a new substrate. As demonstrated in Example 3 herein, altering the orientation of F304 in KASIII of *E. coli* to resemble the orientation of the corresponding amino acid in KASIII of *B. subtilis* resulted in altered, e.g., broadened, substrate specificity.

[00100] By way of another example, a KASIII from *B. subtilis* can be mutated in such a manner as to alter the orientation of F297 in relation to the active site, which consists of C122, N267, and H237 (see Fig. 9a, which is a schematic drawing of the crystal structure of *E. coli*'s KASIII (PDB code 3IL9) using Swiss Model). As shown in Fig. 9b, which is another schematic drawing of the crystal structure of *B. subtilis*'s KASIII, mutations M213L and F208V (modeled using PyMol (B)) affect the orientation of F297 relative to the active site and substrate specificity based on the orientation of F297 relative to M213 and F208 in the layer behind it. By altering the orientation of F297 in relation to the active site, substrate specificity can be altered, such as by altering the relative specificity of the KASIII for one substrate over another or by changing the substrate specificity of the KASIII such that it is no longer specific for a given substrate and/or it is now specific for a new substrate. As demonstrated in Example 3 herein, altering the orientation of F297 in KASIII of *B. subtilis* to resemble the orientation of the corresponding amino acid in KASIII of *E. coli* resulted in altered, e.g., narrowed, substrate specificity.

[00101] Other KASIII, such as a KASIII from family KS1 (ketoacyl synthase 1) from www.enzyme.cbirc.iastate.edu, which website is incorporated by reference herein for its teachings regarding KASIII), in particular a KASIII from another bacterium, can be similarly mutated. For example, a KASIII from *Aeromonas hydrophila*, *Bacteroides vulgatus*, *Brevibacterium linens*, *Capnocytophaga gingivalis*, *Thermus aquaticus*, *Bacillus licheniformis*, *Desulfovibrio vulgaris*, *Bacillus subtilis* subsp. *S*, *Haliangium ochraceum*, *Alicyclobacillus acidocaldarius*, *Staphylococcus aureus*, *Legionella pneumophila*, or *Myxococcus xanthus* can be mutated.

[00102] Thus, in view of the above, a method of altering the specificity of a KASIII for at least one of its substrates is provided. The method comprises introducing into the KASIII one or more mutations comprising at least one mutation, which causes the

rotamer conformation of a phenylalanine in the KASIII corresponding to Phe304 in KASIII from *E. coli* to change. In one embodiment, the KASIII is from *E. coli* (GenBank Accession No. AAG55837.1; nucleotide sequence [SEQ ID NO:61]; amino acid sequence [SEQ ID NO:62]), and the one or more mutations comprises a mutation of Leu220, alone or in further combination with Val215. The mutation of Leu220 can be Leu220Met, and the mutation of Val215 can be Val215Phe. The rotamer conformation of Phe304 can be changed from the active site-distal rotamer conformation, in which the Phe304 is oriented away from the active site, to the active site-proximal rotamer conformation, in which the side chain of Phe304 faces towards the active site. In another embodiment, the KASIII is KASIIIA from *B. subtilis*, and the one or more mutations comprises a mutation of Met213, alone or in further combination with Phe208. The mutation of Met213 can be Met213Leu, and the mutation of Phe208 can be Phe208Val. The rotamer conformation of Phe297 can be changed from the active site-proximal rotamer conformation, in which the side chain of Phe297 faces towards the active site, to the active site-distal rotamer conformation, in which Phe297 is oriented away from the active site. In yet another embodiment, the KASIII is KASIIIB from *B. subtilis*, and the one or more mutations comprise a mutation of Trp221 and a mutation of Val226. The mutation of Trp221 can be Trp221Val, and the mutation of Val226 can be Val226Leu. The rotamer conformation of Phe310 can be changed from the active site-proximal rotamer conformation, in which the side chain of Phe310 faces towards the active site, to the active site-distal rotamer conformation, in which Phe310 is oriented away from the active site. In still yet another embodiment, the KASIII is from the KASIII is from *Aeromonas hydrophila*, *Bacteroides vulgatus*, *Brevibacterium linens*, *Capnocytophaga gingivalis*, *Thermus aquaticus*, *Bacillus licheniformis*, *Desulfovibrio vulgaris*, *Bacillus subtilis* subsp. *S*, *Haliangium ochraceum*, *Alicyclobacillus acidocaldarius*, *Staphylococcus aureus*, *Legionella pneumophila*, or *Myxococcus xanthus*.

[00103] The structure of KASIII can be found in the PDB database www.rcsb.org/pdb/home/home.do, which is hereby incorporated by reference for its teachings regarding same. The KASIII from *E. coli* has the PDB ID number 1EBL. The KASIII from *S. aureus* has the PDB ID number 1ZOW. The phenylalanine in *S. aureus*, which corresponds to F304 in *E. coli*, has the active site-proximal rotamer

orientation. "Altering the specificity of a KASIII for at least one of its substrates" can mean a change in the relative specificity of a given mutant KASIII for two or more substrates compared to the corresponding wild-type KASIII, gain of specificity for a substrate not utilized by the corresponding wild-type KASIII, or loss of specificity for a substrate utilized by the corresponding wild-type KASIII. Additionally or alternatively, the level of activity of a KASIII can be altered; for example, the level of activity of the mutant KASIII can be increased or decreased compared to the activity level of the corresponding wild-type KASIII. Preferably, activity levels are increased.

[00104] In view of the above, also provided is an isolated or purified nucleic acid molecule comprising a nucleotide sequence encoding a mutant KASIII. The isolated or purified nucleic acid molecule can be a vector. The mutant KASIII comprises one or more mutations comprising at least one mutation, which causes the rotamer conformation of a phenylalanine in the KASIII corresponding to Phe304 in KASIII from *E. coli* to change. In one embodiment, the KASIII is from *E. coli*, and the one or more mutations comprises a mutation of Leu220, alone or in further combination with Val215. The mutation of Leu220 can be Leu220Met, and the mutation of Val215 can be Val215Phe. The rotamer conformation of Phe304 can be changed from the active site-distal rotamer conformation, in which the Phe304 is oriented away from the active site, to the active site-proximal rotamer conformation, in which the side chain of Phe304 faces towards the active site. In another embodiment, the KASIII is KASIIIA from *B. subtilis*, and the one or more mutations comprises a mutation of Met213, alone or in further combination with Phe208. The mutation of Met213 can be Met213Leu, and the mutation of Phe208 can be Phe208Val. The rotamer conformation of Phe297 can be changed from the active site-proximal rotamer conformation, in which the side chain of Phe297 faces towards the active site, to the active site-distal rotamer conformation, in which Phe297 is oriented away from the active site. In yet another embodiment, the KASIII is KASIIIB from *B. subtilis*, and the one or more mutations comprise a mutation of Trp221 and a mutation of Val226. The mutation of Trp221 can be Trp221Val, and the mutation of Val226 can be Val226Leu. The rotamer conformation of Phe310 can be changed from the active site-proximal rotamer conformation, in which the side chain of Phe310 faces towards the active site, to the active site-distal rotamer conformation, in which Phe310 is oriented away from the

active site. In still yet another embodiment, the KASIII is from *Aeromonas hydrophila*, *Bacteroides vulgatus*, *Brevibacterium linens*, *Capnocytophaga gingivalis*, *Thermus aquaticus*, *Bacillus licheniformis*, *Desulfovibrio vulgaris*, *Bacillus subtilis* subsp. *S*, *Haliangium ochraceum*, *Alicyclobacillus acidocaldarius*, *Staphylococcus aureus*, *Legionella pneumophila*, or *Myxococcus xanthus*.

[00105] When a KASIII gene is being mutated, such as to alter starter substrate specificity, mutations to the nucleotide sequence should not place the sequence out of reading frame and should not create complementary regions that could produce secondary mRNA structures. The mutant (or chimeric, such as when domains are swapped between genes) KASIII may have altered substrate specificity, e.g., reacts with an acyl-CoA substrate that differs in chain length, degree of saturation, or presence/absence of a side group (e.g., methyl group), from that which is acted upon by the wild-type (also referred to as "native") KASIII. Alternatively, the mutant or chimeric KASIII may have altered relative substrate specificity between two or more substrates, both of which are acted upon by the wild-type KASIII. Both types of alterations in substrate specificity are encompassed by references to alterations of substrate specificity and substrate specificity-altering mutations herein. Alternatively or additionally to altered substrate specificity, the mutant or chimeric KASIII may have an altered activity level, e.g., level of synthase activity, such as the total amount of fatty acids produced, including increased or decreased activity. Altered substrate specificity and altered activity can be detected by expression of the mutant KASIII in *E. coli*, for example, and assay of enzyme activity.

[00106] A nucleotide sequence encoding all or a part of a KASIII can be chemically synthesized, such as by the phosphoramidite method (Beaucage et al., Tetrahedron Letters 22: 1859-1869 (1981); and Matthes et al., EMBO J. 3: 801-805 (1984)). Alternatively, a nucleotide sequence encoding all or a part of a KASIII can be amplified from the genome or mRNA of an appropriate host using polymerase chain reaction (PCR) methods or amplified from an environmental DNA sample using PCR (see, e.g., metagenomics methods). Polynucleotides can be synthesized, purified, annealed to their complementary strand, ligated, and then, optionally, cloned into suitable vectors.

[00107] The isolated or purified nucleic acid molecule comprising a nucleotide sequence encoding a KASIII, such as a mutant/chimeric KASIII, can be a vector. The vector can contain, and preferably does contain, transcription and translation control regions. A promoter can be constitutive or regulatable, such as inducible. Additional sequences that can be present in the vector include pre-processing sequences, such as transit peptide sequences and plastid transit peptide sequences.

[00108] The KASIIIs and mutant/chimeric KASIIIs identified herein can be used in whole or in part as probes in hybridization assays to identify other KASIIIs that can be used in the methods described herein. The KASIIIs or fragments thereof also can be used as primers to amplify target DNA, such as by polymerase chain reaction (PCR) and other nucleic acid amplification methods. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2001); Ausubel et al., eds., *Short Protocols in Molecular Biology*, 5th ed., John Wiley & Sons (2002).

[00109] The nucleic acid molecule comprising a nucleotide sequence encoding a KASIII or a mutant/chimeric KASIII can be introduced into a host cell or a host organism using any suitable technique as is known in the art. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2001); Ausubel et al., eds., *Short Protocols in Molecular Biology*, 5th ed., John Wiley & Sons (2002). Such methods include microinjection, DNA particle bombardment, electroporation, liposome fusion, *Agrobacterium*-mediated transformation, and methods exemplified herein. Depending on the host cell or the host organism, one method can be preferred over another as readily appreciated by one of ordinary skill in the art. The nucleotide sequence can be codon-optimized for the recipient host cell or organism.

[00110] Also provided is an isolated or purified mutant KASIII. The mutant KASIII comprises one or more mutations comprising at least one mutation, which causes the rotamer conformation of a phenylalanine in the KASIII corresponding to Phe304 in KASIII from *E. coli* to change. In one embodiment, the KASIII is from *E. coli*, and the one or more mutations comprises a mutation of Leu220, alone or in further combination with Val215. The mutation of Leu220 can be Leu220Met, and the mutation of Val215 can be Val215Phe. The rotamer conformation of Phe304 can be

changed from the active site-distal rotamer conformation, in which Phe304 is oriented away from the active site, to the active-site proximal rotamer conformation, in which the side chain of Phe304 faces towards the active site. In another embodiment, the KASIII is KASIIIA from *B. subtilis*, and the one or more mutations comprises a mutation of Met213, alone or in further combination with Phe208. The mutation of Met213 can be Met213Leu, and the mutation of Phe208 can be Phe208Val. The rotamer conformation of Phe297 can be changed from the active site-proximal rotamer conformation, in which the side chain of Phe297 faces towards the active site, to the active site-distal rotamer conformation, in which Phe297 is oriented away from the active site. In yet another embodiment, the KASIII is KASIIIB from *B. subtilis*, and the one or more mutations comprise a mutation of Trp221 and a mutation of Val226. The mutation of Trp221 can be Trp221Val, and the mutation of Val226 can be Val226Leu. The rotamer conformation of Phe310 can be changed from the active site-proximal rotamer conformation, in which the side chain of Phe310 faces towards the active site, to the active site-distal rotamer conformation, in which the Phe310 is oriented away from the active site. In still yet another embodiment, the KASIII is from *Aeromonas hydrophila*, *Bacteroides vulgatus*, *Brevibacterium linens*, *Campylobacter jejuni*, *Thermus aquaticus*, *Bacillus licheniformis*, *Desulfovibrio vulgaris*, *Bacillus subtilis* subsp. *S*, *Haliangium ochraceum*, *Alicyclobacillus acidocaldarius*, *Staphylococcus aureus*, *Legionella pneumophila*, or *Myxococcus xanthus*.

[00111] Once sequenced, polypeptides can be synthesized using methods known in the art, such as, for example, exclusive solid phase synthesis, partial solid phase synthesis, fragment condensation, and classical solution synthesis. See, e.g., Merrifield, J. Am. Chem. Soc. 85: 2149 (1963), and Stewart and Young in *Solid Phase Peptide Syntheses* (2nd Ed., Pierce Chemical Company, 1984). Automated peptide synthesizers are commercially available, as are services that make peptides to order.

[00112] In view of the above, a host cell comprising an above-described isolated or purified nucleic acid molecule is also provided. The host cell or organism can be any suitable host cell or organism. The host cell or organism can be prokaryotic or eukaryotic, unicellular or multicellular, and undifferentiated or differentiated. If large-scale production of short-chain fatty acids is desired, e.g., as a source of a bio-based chemical (such as surfactants, lubricants, food oils, polymers, and the like) bacteria

(see, e.g., U.S. Pat. App. Pub. No. 2012/0164700, which discloses examples of cyanobacteria, and U.S. Pat. App. Pub. No. 2009/0298143, which discloses methods of expression in bacteria, and both of which are hereby incorporated by reference for their teachings regarding same), yeast (see, e.g., U.S. Pat. App. Pub. No. 2011/0294174, which discloses examples of yeast in Table 26 and other fungi in Table 27 and which is hereby incorporated by reference for its teachings regarding same), and algae (see, e.g., U.S. Pat. App. Pub. No. 2011/0294174, which discloses examples of algae in Table 1 and which is hereby incorporated by reference for its teachings regarding same; also, see U.S. Pat. No. 7,935,515 and U.S. Pat. App. Pub. No. 2012/0164700, which disclose methods of expressing enzymes, specifically thioesterases, in microalgae and examples of microalgae and which are hereby incorporated by reference for their teachings regarding same; see, also, U.S. Pat. App. Pub. No. 2009/0317878, which is hereby incorporated by reference for its teachings regarding expression of genes in algae) can be preferred. A preferred bacterium is *Escherichia coli*, in particular the strain K27. A preferred yeast is *Saccharomyces cerevisiae*. Alternatively, a crop plant (e.g., maize, canola, and others), such as an oilseed crop plant or a seed cell thereof, can be preferred (see, e.g., U.S. Pat. No. 7,504,563, which discloses expression of a nucleic acid encoding an enzyme, specifically a thioesterase, in soybean seed and which is incorporated herein for its teachings regarding same). See, also, U.S. Pat. App. Pub. No. 2010/0154293, which discloses other examples of host cells in paragraph [0080] and which is incorporated herein by reference for its teachings regarding same.

[00113] Fatty acids can be harvested, or otherwise collected (e.g., isolation from media containing bacteria that secrete the fatty acids), from host cells or organisms by any convenient method. Cells can be lysed/disrupted (e.g., heat, enzymes, ultrasound, mechanical lysis, osmotic shock, acid/base addition, or infection with a lytic virus), and fatty acids can be separated from cell mass by centrifugation and extraction (e.g., extraction with hydrophobic solvent, liquefaction, supercritical CO₂ extraction, or hexane extraction after freeze-drying and pulverization) and further processed/refined as necessary. See, e.g., U.S. Pat. No. 7,935,515 and U.S. Pat. App. Pub. No. 2012/0135479, which are incorporated specifically by reference for their teachings regarding same.

[00114] In view of the above, a method of producing bi-functional fatty acids in a host cell or organism is also provided. The method comprises introducing into a host cell or organism, which comprises one or more ω -functionalized acyl-CoAs, and expressing therein a nucleic acid molecule comprising a nucleotide sequence encoding a KASIII (or a mutant or chimera thereof, such as a mutant or chimera with an altered substrate specificity or an altered level of activity), such as a KASIII from family KS1 (ketoacyl synthase 1) from www.enzyme.cbirc.iastate.edu (incorporated herein by reference for its teachings regarding KASIII), e.g., a KASIII from *A. acidocaldarius*, which can use one or more of the ω -functionalized acyl-CoAs as a substrate. The one or more ω -functionalized acyl-CoAs can be functionalized at the ω position with a moiety comprising a hydroxyl group, a benzoyl group, a cyclic group, a branched-chain group, an amino group, or a halogen group. The host cell or organism can be a mutant *R. rubrum*, such as a mutant *R. rubrum* described above. A preferred host cell or organism can be a bacterium, such as *E. coli*, an alga, or a plant. Preferably, the host cell or organism, e.g., *E. coli*, has been mutated so that it does not express endogenous KASIII (*fabH* gene). More preferably, the host cell or organism, e.g., *E. coli*, has been modified so that it overproduces acyl-CoA starter substrate, does not degrade fatty acid, does not terminate fatty acid elongation, and/or secretes fatty acids, such as into the surrounding medium. For example, the *fadD* gene, which codes for acyl-CoA synthetase, which initiates degradation of fatty acids (Kelin et al., European Journal of Biochem/FEBS 19:442-450 (1971)), can be deleted. Acyl-ACP thioesterase, which results in secretion of fatty acids, can be expressed (Li et al., Metabolic engineering 14: 380-387 (2012); Zhang et al., Metabolic engineering 13: 713-722 (2011); Jing et al., BMC Biochem 12: 44 (2011)). The PHA biosynthetic operon, specifically *phaA* and *phaB* without *phaC*, can be expressed/over-expressed, in an effort to produce enantiopure (R) and (S)-3-hydroxybutyrate (Tseng et al., Applied and Environmental Microbiology 75: 3137-3145 (2009)).

[00115] The bi-functional fatty acids produced in accordance with the above methods can be used in a variety of different applications, such as surfactants, which can vary somewhat in the length of the carbon chain of the fatty acid, lubricants, which also can vary somewhat in the length of the carbon chain of the fatty acid, and polymers (e.g., polyester), which preferably do not vary in the length of the carbon

chain of the fatty acid. The provision of novel bi-functional, bio-based feedstocks (e.g., hydroxy fatty acids and amino fatty acids, such as for polyamides) to the emerging biorenewable chemical industry can lead to the production of novel "green" plastics and specialty chemicals. Such chemical products can act as substitutes for petroleum-based chemical products, and be precursors for novel bio-based products. Such chemical feedstocks can be used to manufacture polymers, specifically polyesters, as well as being utilized in detergents, surfactants, solvents, paints, varnishes, lubricants, cosmetics, and specialty chemical synthesis. Thus, further provided is a composition comprising a bi-functional fatty acid produced in accordance with a method described herein. The composition can be a feedstock, for example. Still further provided is a method of using the feedstock to manufacture a composition, such as a polymer, such as a polyester, a detergent, a surfactant, a solvent, a paint, a varnish, a lubricant, a cosmetic, and the like. Even still further provided is a composition, such as a polymer, e.g., a polyester, a detergent, a surfactant, a solvent, a paint, a varnish, a lubricant, a cosmetic, and the like, produced by the method. See, e.g., Nikolau et al., *Plant J.* 54: 536-545 (2008), which is hereby incorporated by reference for its teachings regarding same.

[00116] Further provided is an *in vitro*, high-throughput spectrophotometric method of assaying KASIII activity. The method comprises (i) incubating holo-ACP, malonyl-CoA, acyl-CoA, NADPH, and malonyl-CoA:ACP transacylase (FabD), (ii) adding KASIII and 3-ketoacyl-ACP reductase (FabG), and (iii) measuring the change in absorbance at 340 nm when NADPH is converted to NADP⁺ during reduction of 3-ketoacyl-ACP to 3-hydroxyl-acyl ACP by FabG. The acyl-CoA can be acetyl-CoA, propionyl-CoA, isobutyryl-CoA and/or hydroxybutyryl-CoA. Such acyl-CoAs are commercially available, such as from Sigma-Aldrich, and can be synthesized by a mixed anhydride reaction in accordance with methods known in the art. Preferably, incubating in (i) is for about two minutes, e.g., two minutes, in the presence of a buffer, such as sodium phosphate buffer, at slightly alkaline pH, e.g., pH 7.2. Preferably, a disulfide-reducing agent, such as dithiothreitol (DTT), is used in step (i). The acyl-CoA can be a straight-chain-CoA, a branched-chain-CoA, or a hydroxylated-CoA. The method can be used to assess enzymes and evaluate/compare the catalytic efficiency of

enzymes with different acyl-CoA substrates. The rate of change in absorbance can be used to calculate directly the rate of the KASIII-catalyzed reaction.

EXAMPLES

[00117] The following examples serve to illustrate the present disclosure. The examples are not intended to limit the scope of the claimed invention in any way.

Example 1

[00118] This example describes the selection of KASIII enzymes based on substrate diversity.

[00119] As many as 2,308 KASIII sequences collected in the ThYme database (www.enzyme.cbirc.iastate.edu; Cantu et al., Nucleic Acids Res. 39: D342-D346 (2011)) were analyzed phylogenetically in collaboration with Dr. Peter Reilly (Iowa State University). Protein sequences were aligned, and a minimum evolution method with 250 bootstrap iterations was employed. Sequences were grouped into 12 sub-families. KASIII gene sequences were selected based on fatty acid profiles of the host organisms, occurrence of the same strain in multiple sub-families, and statistical analysis of clades, for which no fatty acid data are available. KASIII gene sequences from the following organisms were chemically synthesized with codon optimization for *E. coli*: *Aeromonas hydrophila*, *Erythrobacter* sp SD-21, *Haliangium ochraceum*, *Myxococcus xanthus* (three sequences), *Capnocytophaga gingivalis* (three sequences), *Brevibacterium linens* (two sequences), *Renibacterium salmoninarum*, *Legionella pneumophila* (four sequences), *Nocardiopsis dassonvillei*, *Desulfovibrio vulgaris* st., *Stappia aggregata*, *Methylosinus trichosporium*, *Escherichia coli* (the KASIII of which acts on straight-chain primers), *Aeromonas hydrophila* (two sequences), *Physcomitrella patens* subsp *patens*, *Synechococcus* sp CC9605, *Bacteroides vulgatus*, *Alicyclobacillus acidocaldarius* (the KASIII of which acts on cyclic primers), *Thermus aquaticus*, *Bacillus licheniformis* bFabHA, *Bacillus licheniformis* bFabHB, *Bacillus subtilis* subsp *spizizenii*, *Bacillus subtilis* (the KASIII of which acts on branched-chain primers) bFabHA, and *Bacillus subtilis* bFabHB. While not included, *Mycobacterium tuberculosis* KASIII prefers long-chain primers, whereas *Dichapetalum toxicarium* KASIII prefers halogenated primers.

Example 2

[00120] This example describes the generation and characterization of *B. subtilis* KASIII single and double deletion mutants.

[00121] --Bacterial strains and growth conditions.

[00122] *B. subtilis* strain 168 was obtained from the *Bacillus* Genetic Stock Center (www.bgsc.org). *Escherichia coli* strains DH5 α and BL21(DE3) were obtained from Invitrogen Corporation (Carlsbad, CA).

[00123] *E. coli* and *B. subtilis* were routinely grown in LB medium at 37 °C. *B. subtilis* minimal medium was composed of Spizizen salts (Spizizen, PNAS USA 44: 1072-1078 (1958)), supplemented with 0.5 % glucose and amino acids (Sueoka et al., Cold Spring Harbor Symp. Quant. Biol. 33: 695-705 (1968)). As needed, media were supplemented with erythromycin (1 μ g/ml) and ampicillin (100 μ g/ml). IPTG and X-gal were used at concentrations of 0.4-1mM and 40 μ g/ml, respectively. As needed, media were supplemented with 10-100 μ M individual fatty acids suspended in 0.01 % (v/v) Brij 58P detergent. Fatty acids were obtained from Sigma-Aldrich Corporation (St. Louis, MO). The purity of the commercial sources of anteiso-C16:0, iso-C16:0 and palmitoleic acid were determined by GC-MS analysis (see Table 1). Cell density was determined by monitoring A₆₀₀ using a Spectronic 20D+ spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) or in a 96-well plate using an ELx808 Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, Vermont). Doubling time (T_d) was determined from the log-phase time points of cultures.

Table 1. The purity of the commercial sources of anteiso-C16:0, iso-C16:0 and palmitoleic acid

Composition	<i>a</i> -C16	<i>i</i> -C16	<i>n</i> -C16:1(<i>n</i> -7)
n-C12:0	0.04%		
n-C14:0	0.24%		
i-C15:0	0.74%		
a-C15:0	0.02%		
n-C15:0	0.08%		
i-C16:0		99.90%	
a-C16:0	96.73%		
n-C16:1(9)			99.94%
n-C16:0	1.53%	0.10%	0.06%
a-C17:0	0.01%		
n-C18:0	0.61%		

[00124] --DNA manipulation.

[00125] DNA manipulation techniques, such as PCR amplification, plasmid preparation, restriction endonuclease digestion, agarose gel electrophoresis, and genetic transformation, were carried out by standard methods (Sambrook et al., *Molecular Cloning: A laboratory manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (2001)). Transformation of *B. subtilis* was conducted on modified competent medium (Kunst et al., "Signal transduction network controlling degradative enzyme synthesis and competence in *bacillus subtilis*," In: *Regulation of Bacterial Differentiation*, pp. 1-20, Piggot, editor, American Society for Microbiology, Washington, DC (1994)). All oligonucleotide sequences used are listed in Table 2.

Table 2. Primers used

Fragment amplified for purpose	Primer name	Primer sequence
The <i>bfabHA</i> gene for construction of pET30a-bfabHA	Af1- <i>Nco</i> I	CATGCCATGGTAATGAAAGCTGGAATAC [SEQ ID NO:1]
	Ar1- <i>Eco</i> RI	GCGGGATCCGGAGATAATGCTCCAAG [SEQ ID NO:2]
The <i>bfabHB</i> gene for construction of pET30a-bfabHB	Bf1- <i>Bam</i> HI	CGCGGATCCATTCATATGTCAAAAGC [SEQ ID NO:3]
	Br1- <i>Hin</i> dIII	AGGGAAGCTTCAGAAGAAGACAGCCGG [SEQ ID NO:4]
323-bp fragment of the <i>bfabHA</i> gene (nucleotide position from 191 to 513) for construction of vector pM4A	Af2- <i>Hin</i> dIII	AAGCTTAACAAGCTGAAGTGGCTGCT [SEQ ID NO:5]
	Ar2- <i>Bam</i> HI	GGATCCATCACTGACTGGCCCGACTA [SEQ ID NO:6]
429-bp fragment of the <i>bfabHB</i> gene (nucleotide position from 415 to 843) for construction of vector pM4B	Bf2- <i>Hin</i> dIII	AAGCTTGCCGGAGAGACGTTATCAAA [SEQ ID NO:7]
	Br2- <i>Bam</i> HI	GGATCCCGTGTTCCGTAGTGCTCAA [SEQ ID NO:8]
897-bp upstream fragment of the <i>bfabHA</i> ORF for construction of vector pMU4A	AUf- <i>Pac</i> I	TTAATTAATATTAACCATCACGGTGCAA [SEQ ID NO:9]
	AUr- <i>Sal</i> I	GTCGACGAATGTAACGTCCAACACCA [SEQ ID NO:10]
799-bp downstream fragment of the <i>bfabHA</i> ORF for construction of vector pMU4A	ADf- <i>Sal</i> I	GTCGACTGGAAGCCGGTAAAATCAA [SEQ ID NO:11]
	ADr- <i>Pst</i> I	CTGCAGGCCGACAATTTCTCCGTAAA [SEQ ID NO:12]
836-bp upstream fragment of the <i>bfabHB</i> ORF for construction of vector pMU4B	BUf- <i>Pst</i> I	CTGCAGATATAAAACCGCCGGGACAT [SEQ ID NO:13]
	BUr- <i>Sal</i> I	GTCGACGCATAGGTGCCGATAGCTGTA [SEQ ID NO:14]
802-bp downstream fragment of the <i>bfabHB</i> ORF for construction of vector pMU4B	BDf- <i>Sal</i> I	GTCGACTCAAATCGTTTTGCTTTTCG [SEQ ID NO:15]
	BDr- <i>Pac</i> I	TTAATTAACCAACAGGAGATATCGATGC [SEQ ID NO:16]
836-bp upstream fragment of the <i>bfabHB</i> ORF for construction of vector pUCB-	BUf2- <i>Eco</i> RI	GAATTCATATAAAACCGCCGGGACAT [SEQ ID NO:17]
	BUr2- <i>Sal</i> I	GTCGACGCATAGGTGCCGATAGCTGTA

erm		A [SEQ ID NO:18]
738-bp downstream fragment of the <i>bfabHB</i> ORF for construction of vector pUCB-erm	Bdf2- <i>Sal</i> I	<i>GTCGACTCAAATCGTTTTGCTTTTCG</i> [SEQ ID NO:19]
	BDr2- <i>Hin</i> dIII	<i>AAGCTTCCAAAGATGATGCCATTCA</i> [SEQ ID NO:20]
<i>erm</i> gene fragment for construction of vector pUCB-erm	ermf	<i>GTCGACCAAATTTACAAAAGCGACTCA</i> [SEQ ID NO:21]
	ermr	<i>GTCGACGAGGCCCTTTCGTCTTCAA</i> [SEQ ID NO:22]
Verification of the <i>bfabHA::pM4A</i> allele	RB	GACAGTATCGGCCTCAGGAA [SEQ ID NO:23]
	AL	TGCTGTTCCCTCCTCCTTCTC [SEQ ID NO:24]
Verification of the <i>bfabHA::pM4B</i> allele	RB	GACAGTATCGGCCTCAGGAA [SEQ ID NO:25]
	BL	GGAGTGATTCATATGTCAAAAAGCA [SEQ ID NO:26]
Verification of <i>bfabHA</i> deletion	lofAf	GCATACGCCTCCTTTCCATA [SEQ ID NO:27]
	lofAr	TTTGCCGGATATTCTTCAGC [SEQ ID NO:28]
Verification of <i>bfabHB</i> deletion	lofBf	CAATGTTAAGCCGGAAGGAA [SEQ ID NO:29]
	lofBr	AGCAGCCGTAAATGCCATAC [SEQ ID NO:30]

^a Restriction sites designed into the nucleotide sequences are indicated in bold and italics.

[00126] KASIII-coding genes were expressed in *E. coli* strain BL21 (DE3) using pET-based vectors (Novagen, Madison, WI). The *bfabHA* gene was PCR-amplified with the primers Af1-*Nco* I and Ar1-*Eco* RI. The resulting fragment was cloned into the *Nco* I and *Eco* RI restriction sites of the pET30a vector, resulting in expression vector pET30a-bfabHA. By the analogous procedure, the *bfabHB* gene was also cloned into the pET30a vector, resulting in the expression vector pET30a-bfabHB.

[00127] *B. subtilis* KASIII-coding genes were disrupted by the insertion of the pMUTIN4 vector (Vagner et al., Microbiology 144(Pt 11): 3097-3104 (1998)) via homologous recombination. A fragment of the *bfabHA* gene (nucleotides 191-513) was PCR-amplified with the primers Af2-*Hin* dIII and Ar2-*Bam* HI. This *bfabHA* PCR-fragment was cloned into the *Hin* dIII and *Bam* HI restriction sites of pMUTIN4 vector (Vagner et al. (1998), *supra*). By the analogous procedure, a *bfabHB* fragment (from nucleotide from 415 to 843) was also cloned into the pMUTIN4 vector. The recombinant pMUTIN4 plasmids carrying the *bfabHA* and *bfabHB* gene fragments were named pM4A and pM4B, respectively.

[00128] The two *B. subtilis* KASIII-coding genes were deleted by using the vectors pMU4A and pMU4B, respectively, which are derivatives of pMUTIN4 (Vagner et al. (1998), *supra*). The pMU4A vector contained two *bfabHA*-derived fragments, one 5' of the open reading frame (ORF), and the other 3' of the ORF. The 5'-, 897-bp DNA fragment spanned from 860 bp upstream of the *bfabHA* ORF to 37 bp within the *bFabHA* ORF, and the 3'-, 897-bp DNA fragment spanned from 799 bp downstream to 98 bp within the 3'-end of the *bfabHA* ORF. These two fragments were initially PCR-amplified with the primer pair AUf-*Pac* I and AUr-*Sal* I, and the primer pair ADf-*Sal* I and ADr-*Pst* I, and both fragments were cloned into pMUTIN4, at the *Pac* I and *Pst* I sites. The resulting pMU4A vector contained an in-frame 135-bp *bfabHA* fragment missing 804 bp from the middle of the ORF; the fact that this deletion allele carried an in-frame ORF avoided any polar effect on the downstream genes of the *bfabHA*-containing transcription unit. The vectors pMU4B and pUCB-*erm*, which were used to generate the deletion allele for *bfabHB*, were constructed by an analogous procedure, except that the vector pUCB-*erm* was constructed from plasmid pUC19 and the erythromycin-resistant gene *erm* was inserted between the downstream and upstream DNA fragments of *bfabHB* ORF.

[00129] --Construction of *B. subtilis* mutants.

[00130] *B. subtilis* gene-insertion mutant alleles were generated by transforming *B. subtilis* strain 168 with the plasmids pM4A and pM4B, and the desired mutants were selected by virtue of their ability to grow in lethal doses of erythromycin. PCR reactions with primers that would support amplification only from appropriately recombined alleles were conducted to verify that the single cross-over recombination-mediated integration of the vectors occurred as expected. A genomic specific primer AL and a vector specific primer RB were used to confirm the *bfabHA::pM4A* allele. A genomic specific primer BL and a vector specific primer RB were used to confirm the *bfabHB::pM4B* allele (see Table 2).

[00131] Two single-deletion strains of *B. subtilis*, each lacking one or the other KASIII-coding gene, were generated by homologous recombination via a two-step procedure using the vector pMU4A and pMU4B, respectively. Briefly, plasmid pMU4A or pMU4B was transformed into wild-type strain 168, followed by selection for erythromycin resistance. The recovered integrant colonies were grown in LB liquid

medium without erythromycin, the overnight cultures were diluted 1:10⁷, and about 100 µl of the diluted culture were plated on LB medium with IPTG and X-gal and screened for white colonies, indicating the loss of the *lacZ*-containing pMUTIN4 sequence, which would result in the deletion of the appropriate KASIII-coding gene. The nature of the deletion mutants was confirmed by PCR amplification of a specific sequence of each deletion allele.

[00132] The double-deletion mutant strain, *AbfabHA AbfabHB::erm*, was generated by homologous recombination via a one-step procedure, by transforming the mutant *AbfabHA* strain with the *Eco* RI-linearized plasmid pUCB-erm followed by selection for erythromycin resistance on media containing anteiso-C16:0 fatty acid. Primers lofAf and lofAr were used to confirm the *AbfabHA* allele. Primers lofBf and lofBr were used to confirm the *AbfabHB* and *AbfabHB::erm* alleles (see Table 2).

[00133] --Assays of β-galactosidase.

[00134] β-galactosidase activity, expressed as Miller units (Miller, *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1992)), was assayed as described (Cutting et al., "Genetic Analysis," In: *Molecular Biological Methods for Bacillus*, pp. 27-74, Harwood and Cutting, eds., John Wiley, Chichester (1990)) using o-nitrophenyl-β-D-galactoside as substrate.

[00135] --Protein purification and preparation of antibodies.

[00136] The two *B. subtilis* KASIII proteins were expressed in *E. coli* BL21 (DE3) using the plasmid pET30a-bfabHA or pET30a-bfabHB (Novagen, San Diego, CA), and cultures were grown in LB medium containing the appropriate antibiotic. When the culture reached an OD₆₀₀ of 0.9, 1 mM IPTG was used to induce expression. Cells were harvested, and the proteins were purified by affinity chromatography on a Ni-NTA agarose column (Qiagen, Valencia, CA) according to the manufacturer's instructions. Antibodies directed against the KASIIIA and KASIIIB proteins were generated by injecting each recombinant protein into a rabbit using Freund's Complete and Incomplete Adjuvants (Pierce, Rockford, IL).

[00137] --Protein Analysis.

[00138] Protein extracts were prepared from bacterial cells collected by centrifugation of a 3-ml aliquot of early stationary-phase growth culture. The pellet

was suspended in 0.3 ml extraction buffer, consisting of 30 mM Tris-HCl, pH 8, 10 mM EDTA, and 0.5 mg/ml lysozyme, and incubated at 37 °C for 30 minutes. The lysate was further disrupted by sonication. Following centrifugation for 10 minutes at 16,100 g, the supernatant was retained for analysis. Protein concentration was determined by Bradford's method (Bradford, Anal. Biochem. 72: 248-254 (1976)) using bovine serum albumin to generate a standard curve. Immunoblot analysis was performed as described previously (Li et al., Plant Physiol. 155: 293-314 (2011)).

[00139] --Fatty acid analysis.

[00140] A 0.5 ml aliquot of an overnight *B. subtilis* culture normalized for equal cell density was collected by centrifugation at 13,200×g for 30 seconds. The cell pellet was suspended in 50 ml minimal medium or LB rich medium. Cell cultures were shaken at 250 rpm. Cells were collected at late log phase by centrifugation at 5,000×g for 10 minutes. The collected cell pellets were lyophilized and stored at -20 °C until analysis. Lipids were extracted from lyophilized bacterial cell pellets using chloroform/methanol (Ways et al., J. Lipid Res. 5: 318-328 (1964)), and fatty acids were then converted to picolinyl ester (lipidlibrary.aocs.org/ms/ms02/index.htm) (Harvey, Biomed. Mass Spectrom. 9: 33-38 (1982)) or methyl esters using methanolic-HCl at 80 °C for 60 minutes (Broekman et al., J. Bacteriol. 116: 285-289 (1973); and Broekman et al., J. Bacteriol. 117: 971-977 (1974)). The recovered picolinyl ester or fatty acid methyl esters were concentrated as needed under a stream of nitrogen gas and analyzed with GC-MS interfaced with a Mass Detector 5973 (Agilent Technologies, Santa Clara, CA). The double bond positions in unsaturated fatty acids were determined by GC-MS analysis of dimethyl disulfide adducts (Buser et al., Anal. Chem. 55: 818-822 (1983)).

[00141] --Growth characteristics of single gene mutant strains.

[00142] The wild-type *B. subtilis* strain 168 and the isogenic mutant strains *ΔbfabHA* and *ΔbfabHB*, which lacked KASIIIA and KASIIIB, respectively, were grown at 37 °C and 16 °C (Fig. 3). At 37 °C there was no difference in the growth rate between the wild-type and either of the mutants (Fig. 3a). When these strains were cultured at the lower temperature (16 °C), however, the log-phase growth rate of the *ΔbfabHA* mutant was faster than that of the wild-type and the *ΔbfabHB* mutant (Fig. 3b) (doubling time was 13.3 ± 0.4 hours as compared to 14.4 ± 0.4 hours for the wild-type strain and 14.5

± 0.3 hours for $\Delta bfabHB$ mutant, p-value = 0.04). In contrast, the log-phase growth rate of $\Delta bfabHB$ mutant was indistinguishable from that of the wild-type.

[00143] --Expression of *bfabHA* and *bfabHB* genes.

[00144] Protein extracts from the wild-type, $\Delta bfabHA$ and $\Delta bfabHB$ mutant strains grown at 37 °C and 16 °C were analyzed by Western blot to confirm the nature of the mutant growth phenotype of the $\Delta bfabHA$ mutant strain. The KASIII A protein, but not the KASIII B protein, accumulated to detectable levels in the wild-type strain, and, as expected, the KASIII A protein was not detected in the $\Delta bfabHA$ mutant. In contrast, the KASIII B protein, which is undetectable in the wild-type strain, was induced in the $\Delta bfabHA$ mutant.

[00145] Because the finding that the expression of the *bfabHB* gene is conditional on the absence of the *bfabHA*-encoded KASIII was unexpected, corroborative evidence for this finding was gained with an independent approach, i.e., promoter-*lacZ* fusion reporters. In this experiment these reporter alleles were generated by a single-crossover recombination event between a KASIII-encoding genomic locus (either *bfabHA* or *bfabHB*) and the plasmid pMUTIN4 (Vagner et al., Microbiology 144(Pt. 11): 3097-3104 (1998)), which carries one or the other KASIII-encoding fragment, fused to the *lacZ* gene. The resulting recombination event generated a transgenic new locus that expressed β -galactosidase under the transcriptional regulation of either of the *bfabHA* promoter or the *bfabHB* promoter. Figure 4 shows that the *bfabHA* promoter was considerably more active than the *bfabHB* promoter, by a factor of at least 10-fold. Activity of the *bfabHA* promoter was similar, irrespective of the growth temperature, and maximal expression occurred at early to mid-log phase of growth, coincident with maximal need for membrane lipid deposition. Hence, the β -galactosidase reporter assays were consistent with the Western blot data and showed that the *bfabHB* gene was minimally expressed, while the *bfabHA* gene accounted for the major form of KASIII that was expressed under normal growth conditions.

[00146] These data therefore, indicate that, while the *bfabHA* gene is expressed in the wild-type, the *bfabHB* gene is normally silent, and its expression is induced in the absence of a functional *bfabHA* gene. Moreover, the growth characteristics of the two mutants $\Delta bfabHA$ and $\Delta bfabHB$ described in Fig. 3B indicate that the *bfabHB*-encoded KASIII confers a growth advantage at 16 °C.

[00147] --Effect of *bfabHA* and *bfabHB* gene deletions on fatty acid composition.

[00148] Fatty acid compositions of the $\Delta bfabHA$ and $\Delta bfabHB$ mutants were compared to the wild-type strain, and these comparisons were conducted on strains that were grown at either 37 °C or 16 °C. Fatty acids were chemically identified by a combination of comparing retention indices to commercial standards, MS-fragmentation of picolinyl esters (Harvey, Biomed. Mass Spectrom. 9: 33-38 (1982)), and determination of double bond positions in unsaturated fatty acids by MS-fragmentation of DMDS adducts (Buser et al., Anal. Chem. 55: 818-822 (1983)).

[00149] These analyses indicated that, at both growth temperatures, the fatty acid composition of the $\Delta bfabHB$ mutant was nearly identical to that of wild-type (Fig. 5A and 5D). This is consistent with the fact that the *bfabHB* gene was not expressed in the wild-type; thus, the deletion of this gene was functionally inconsequential. However, there were significant alterations in the fatty acid composition caused by the $\Delta bfabHA$ deletion (Fig. 5A and 5D). Some of these genetic effects on the fatty acid composition were independent of the growth temperature, whereas the growth temperature further modulated the $\Delta bfabHA$ deletion effect on fatty acid composition. Specifically, at both growth temperatures, most of the fatty acids were similarly affected by the $\Delta bfabHA$ deletion (Fig. 5A and 5D); for example, there was a significant increase in the proportion of even-numbered iso- and odd-numbered normal fatty acids at the expense of odd-numbered iso-fatty acids at both growth temperatures (Fig. 5G and 5H). In addition, there was a significant increase in the ratio of anteiso:iso BCFAs in the $\Delta bfabHA$ mutant (Fig. 5B and 5E). Finally, $\Delta bfabHA$ mutant accumulated significantly more C16 fatty acids (both normal and iso-branched saturated and unsaturated form) (Fig. 5C and 5F). In contrast, the genetic effect of deleting the *fabHA* gene on the anteiso-fatty acids was unique in that it expressed at the lower temperature, whereas the accumulation of these fatty acids was not altered by the $\Delta bfabHA$ deletion at 37 °C (Fig. 5G and 5H). Furthermore, at 37 °C the $\Delta bfabHA$ mutant accumulated significantly lesser amounts of shorter-chain fatty acids (<C16) than the wild-type strain (Fig. 5C), and this difference was not apparent at 16 °C (Fig. 5F). Rather, at the lower growth temperature, the $\Delta bfabHA$ strain expressed a lower level of the longer-chain fatty acids (>C16) (Fig. 5F). Finally, the genetic manipulations of the KASIII-

coding genes did not affect the degree of fatty acid unsaturation; rather, in all three genotype strains, unsaturated fatty acids increased at the lower growth temperature (Fig. 5A and 5D), consistent with the role of a $\Delta 5$ desaturase in the temperature adaptation of *B. subtilis* (Aguilar et al., J. Bacteriol. 180: 2194-2200 (1998)).

[00150] --Rescue of KASIII deficiency in *B. subtilis* by anteiso-, iso- and unsaturated fatty acids.

[00151] A KASIII-deficient strain was developed in the *AbfabHA* strain by inserting the *erm* gene into the *bfabHB* locus via a homologous double-crossover recombination event. The resulting *AbfabHA AbfabHB::erm* double-deletion mutant proved to be lethal, but could be rescued by the inclusion of BCFAs in the media, specifically anteiso-16:0. The absence of either KASIII proteins in the rescued double-mutant strain was confirmed by Western blot analysis. These findings established that KASIII is essential for *B. subtilis* cellular metabolism.

[00152] Although anteiso-16:0 could rescue the KASIII deficiency, the rescued strain grew considerably slower than the wild-type strain. Therefore, whether or not other fatty acids could improve the growth capabilities of the KASIII-deficient strain was examined. Rescue of the KASIII-deficient strain was attempted by the inclusion of anteiso- and iso-BCFAs, and normal saturated and unsaturated fatty acids of different chain lengths. These complementation experiments were conducted by providing these fatty acids in the media at three different concentrations (10, 30 and 100 μ M each). At the lowest concentration tested, only anteiso-fatty acids supported the growth of the KASIII-deficient strain, and of all the chain-lengths attempted (anteiso-C5:0, C6:0, C7:0, C8:0, C9:0, C10:0, C12:0, C13:0 and C16:0), only C13:0 and C16:0 anteiso-fatty acids were successful in complementing the KASIII deficiency. At the higher concentration level (30 μ M), iso-C16:0 was able to rescue growth, but none of the other shorter chain length iso-fatty acids that were attempted (i.e., iso-C4:0, C5:0, C6:0, C7:0, C9:0, and C10:0) could rescue the KASIII deficiency, even at 100 μ M levels. None of the tested normal saturated chain fatty acids (n-C8:0, C10:0, C12:0, C14:0, C16:0 and C18:0) could rescue growth (at any of the tested concentrations). Rescue of this strain was also attempted with monounsaturated fatty acids, and of the three that were attempted, palmitoleic acid, n-C17:1(10) could rescue the KASIII deficiency when supplied at 30 μ M, but oleic acid could not rescue the strain even at 100 μ M.

These results indicate that BCFAs, either iso- or anteiso, and mono-unsaturated fatty acids are important constituents that provide an essential function to *B. subtilis* membranes, most probably associated with maintaining membrane fluidity.

[00153] Because anteiso-16:0 is not a fatty acid that *B. subtilis* normally synthesizes, fatty acid analysis of the rescued strain provided novel insights into the fatty acid metabolism of this organism. For example, the anteiso-C16:0 rescued KASIII-deficient strain not only incorporated the exogenously provided fatty acid into the membrane lipids, but it metabolized this fatty acid by two rounds of elongation, indicated by the presence of anteiso-C18:0 and anteiso-C20:0, and one round of β -oxidation, indicated by the presence of anteiso-C14:0 (Fig. 6a). In total about 50% of the fatty acids recovered in the rescued strain was accounted by these metabolic derivatives of the exogenously provided anteiso-BCFAs (Fig. 6b).

[00154] Interestingly, this anteiso-C16:0-rescued KASIII-deficient strain still had the ability to synthesize significant amounts of straight-chain fatty acids (Fig. 6a), and these accounted for about 50% of the recovered fatty acids (Fig. 6b). This observation therefore, indicates the occurrence of an alternative, KASIII-independent mechanism for initiating normal fatty acid biosynthesis. This mechanism is likely to involve the decarboxylation of malonyl-ACP to generate acetyl-ACP (Alberts et al., J. Biol. Chem. 247: 3190-3198 (1972); McGuire et al., Biochem. 40: 9836-9845 (2001); and Kaneda, Microbiol. Rev. 55: 288-302 (1991)), and subsequently the condensation of acetyl-ACP with malonyl-ACP to form 3-ketobutryl-ACP by KASII (encoded by *yjaY*), overcoming the need for KASIII function (Butterworth et al., Eur. J. Biochem. 12: 496-501 (1970)).

[00155] --Comparative efficacy of rescue of KASIII deficiency by anteiso-, iso- and unsaturated fatty acids at different growth temperatures.

[00156] Presupposing that the chemo-physical properties of fatty acids determine their capacity to affect membrane function, the KASIII-deficient strain, which grew only in the presence of exogenously provided fatty acids that affect membrane fluidity, provided an excellent bio-system to test the relative ability of anteiso-, iso- and unsaturated fatty acids to rescue the KASIII deficiency by modulating membrane fluidity and, hence, supporting growth. Moreover, by conducting these tests at different growth temperatures, the degree to which these different types of fatty acids

maintained membrane fluidity in response to thermal tolerance was assessed (Suutari et al., Crit. Rev. Microbiol. 20: 285-328 (1994)).

[00157] Four different types of 16-carbon fatty acids (anteiso-C16:0, iso-C16:0, palmitoleic acid, and palmitic acid) were supplied in the medium to evaluate the capacity of these different fatty acids to rescue the KASIII-deficient strain at four different temperatures between 16 °C and 37 °C. The rescued strains grew at different rates depending upon the type of fatty acid that was provided, which was interpreted as an indication of each fatty acid's ability to contribute to membrane functionality, by maintaining membrane fluidity as the strain was challenged with lower temperatures. At all temperatures tested, anteiso-C16:0 was most efficient in supporting growth. The second most efficient fatty acid to support growth depended on the growth temperature. At 37 °C iso-C16:0 was better able to support growth than n-C16:1(9), but at lower temperatures n-C16:1(9) was more efficient than iso-C16:0. Indeed, at or below 20 °C iso-C16:0 was incapable of supporting growth, whereas anteiso-C16:0 and n-C16:1(9) rescued the KASIII deficiency, with the former being considerably more efficient (Fig. 7).

[00158] With the exception of the experiment conducted at 16 °C, fatty acid analysis of the rescued strains indicated that the exogenously provided fatty acids and their metabolic derivatives accounted for about 90% of the cellular fatty acids (Fig. 7). The exception at 16 °C, the exogenously provided anteiso-16:0 and its derivatives accounted for over 98% of the cellular fatty acids but, in contrast, the exogenously provided n-C16:1(9) and its derivatives accounted for only 75% of the cellular lipids at this temperature. At all temperatures tested and with all three types of fatty acids that rescued growth, the metabolic derivatives were the result of three types of conversions of the exogenously provided fatty acids: 1) elongation by one or two cycles of fatty acid synthase, resulting in the accumulation of 18- or 20-carbon fatty acids; 2) chain shortening, probably by one cycle of β -oxidation to generate 14-carbon fatty acids; and 3) desaturation, via the $\Delta 5$ -desaturase (Aguilar et al. (1998), *supra*), generating mono-unsaturated fatty acids.

Example 3

[00159] This example describes the structural analysis of wild-type and mutant KASIII enzymes from *B. subtilis* and *E. coli*.

[00160] --Tertiary structure prediction of *B. subtilis* KASIII enzymes.

[00161] Tertiary structures of KASIII homologs found in *B. subtilis* (i.e., KASIIIA and KASIIIB) were predicted using homology modeling. An NCBI BlastP search against the PDB database was used to identify sequences that shared >40% sequence identity with KASIIIA and KASIIIB protein sequences. For KASIIIA, *S. aureus* KASIII (PDB ID – 1ZOW; chain A) showed maximum sequence identity (58%), followed by *Aquifex aeolicus* KASIII (PDB ID – 2EBD; chain A) with 52% sequence identity. These two sequences were used as template sequences for KASIIIA, and each of these was aligned with the KASIIIA sequence using ClustalW alignment software (www.ebi.ac.uk). The target-template sequence alignments were used to model tertiary structures of KASIIIA using the alignment mode of Swiss Model (swissmodel.expasy.org). The two different models obtained were assessed for their quality using Verify3D, Gromos and Anolea, and the best model was chosen for further analysis. A similar approach was used for predicting the tertiary structure of *B. subtilis* KASIIIB using *Thermus thermophilus* KASIII (PDB ID – 1UB7; chain A), which showed maximum sequence identity (44%), and *S. aureus* KASIII, which had 42% identity, as template sequences. The PDB files of selected models were analyzed using PyMol software (www.pymol.org).

[00162] --Gene cloning.

[00163] The *E. coli fabH* gene that encodes KASIII was PCR-amplified from *E. coli* strain MG1655 (*E. coli* Genetic Stock Center, New Haven, CT) and cloned into pDEST17 vector using Gateway cloning (Invitrogen, Carlsbad, CA), resulting in the pDEST_KASIII construct. The *yhfb* and *yjaX* genes encoding *B. subtilis* KASIIIA and KASIIIB, respectively, were PCR-amplified from *B. subtilis* strain 168 (*Bacillus* Genetic Stock Center, Columbus, OH). These genes were cloned into pET30a expression vector (Novagen, Merck, Germany) to construct pET30_KASIIIA and pET30_KASIIIB, and also were cloned into the pDEST17 expression vector using Gateway cloning to generate pDEST_KASIIIA and pDEST_KASIIIB. Each pDEST17

and pET30a construct encoded an N-terminal His-tag. The resulting plasmids were confirmed by sequencing.

[00164] --Expression and purification of recombinant wild-type and mutant KASIII proteins.

[00165] *E. coli* OverExpress™ C41 (Lucigen, Middletown, WI) strain was used for expression of KASIII proteins from constructs pDEST_KASIII, pET30_KASIIIA, pET30_KASIIIB, pDEST_KASIIIA and pDEST_KASIIIB. The transformants were grown at 37°C in 50ml Luria-Bertani medium, supplemented with 100 µg/ml ampicillin (Research Products International Corps., Mount Prospect, IL) for pDEST_KASIII, pDEST_KASIIIA, and pDEST_KASIIIB or 50 µg/ml kanamycin (RPI Corps.) for pET30_KASIIIA and pET30_KASIIIB. The cultures were induced by the addition of IPTG (Gold Biotechnology, Olivette, MO) to a final concentration of 0.4 mM when the OD₆₀₀ was 0.6-0.8. After incubation for another 16-18 hours at 25°C, cells were harvested by centrifugation (10,000 X g, 4°C, 10 minutes). Soluble proteins were extracted by first suspending the cell pellet in lysis buffer (0.5 M NaCl, 5 mM imidazole, 20 mM Tris-HCl, pH 8.0, 0.1 mg/ml phenylmethylsulfonyl fluoride, and 0.1% Triton-X 100), followed by sonication (10-second pulses separated by three-second intervals for a total of three minutes) and centrifugation (10,000 X g, 4°C, 30 minutes). The resulting supernatant (soluble protein fraction) was analyzed by running on SDS-PAGE gel. Based on the small-scale expression experiments that optimized the conditions for obtaining the highest yield of soluble recombinant KASIII proteins, the constructs pDEST_KASIII, pET30_KASIIIA, and pDEST_KASIIIB were used for large-scale expression and purification of wild-type and mutant proteins. Cultures were grown, induced, and harvested, and soluble protein was extracted as described in small-scale expression methods. The soluble protein fraction was filtered through a 0.45 µ filter (Corning, the Netherlands) and applied to 8 ml Ni-NTA His-bind resin. After washing the unbound protein with wash buffers I and II (0.5M NaCl, 20 mM Tris-HCl, pH 8.0) supplemented with 20 mM and 40 mM imidazole, respectively, the proteins of interest were eluted with the same buffer containing 250 mM imidazole. The purified His-tagged KASIII proteins were dialyzed against sodium phosphate buffer, pH 7.4, and concentrated using 10,000 molecular weight cut-off ultrafiltration centrifugation filters (Millipore, Billerica, MA) at 4°C. The concentrated proteins were either

supplemented with 16% glycerol and stored at -80°C or immediately used for Saturation Transfer Difference NMR experiments. Protein purity was assessed by Coomassie-staining SDS-PAGE gels, which showed the presence of near-homogenous, pure proteins (greater than 95% purity). Protein concentrations were determined by Bradford's assay (BioRad, Hercules, CA).

[00166] --Site-directed mutagenesis of *E. coli* and *B. subtilis* KASIII enzymes.

The QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used for introducing point mutations in the pDEST_KASIII, pET30_KASIIIA, and pDEST_KASIIIB constructs. Four mutants were generated from each of these constructs as indicated in Table 3 using the listed primers and their complements.

Table 3. Primers for site-directed mutagenesis of <i>E. coli</i> and <i>B. subtilis</i> KASIII enzymes				
Gene	Construct	Mutation	QuikChange Forward Primer	QuikChange Reverse Primer
<i>E. coli</i> <i>fabH</i> (KASIII)	pDEST_K ASIII	V215F	5'- GCAACGAAGTCTTCAAGTTGCG GTAACGGAACG-3' [SEQ ID NO:31]	5'- CAGTTCCGTTACCGCAAACCTGAAGA CTTCGTTGC-3' [SEQ ID NO:32]
		L220M	5'- TTCAAGGTTGCGGTAACGGAAT GGCGCACATC-3' [SEQ ID NO:33]	5'- GATGTGCGCCATTTCCGTTACCGCAAC CTTGAA-3' [SEQ ID NO:34]
		V215F_L2 20M	5'- GCAACGAAGTCTTCAAGTTGCG GTAACGGAACG-3' [SEQ ID NO:35] 5'- TTCAAGGTTGCGGTAACGGAAT GGCGCACATC-3' [SEQ ID NO:37]	5'- CAGTTCCGTTACCGCAAACCTGAAGA CTTCGTTGC-3' [SEQ ID NO:36] 5'- GATGTGCGCCATTTCCGTTACCGCAAC CTTGAA-3' [SEQ ID NO:38]
		F304A	5'- GGTTCTGCTTGAAGCCGCTGGCG GTGGATTCACC-3' [SEQ ID NO:39]	5'- GGTGAATCCACCGCCAGCGGCTTCAA GCAGAACC-3' [SEQ ID NO:40]
<i>B. subtilis</i> <i>yjaX</i> (KASIIIA)	pET30_KA SIIIA	F208V	5'- GAATGGACGAGAAGTTTCAAAG TTGCAGTCCGCC-3' [SEQ ID NO:41]	5'- GGCGGACTGCAACTTTGAAAACCTCTC GTCCATTC-3' [SEQ ID NO:42]
		M213L	5'- CAAATTTGCAGTCCGCCAATTGG GAGAATCATGCG-3' [SEQ ID NO:43]	5'- CGCATGATTCTCCCAATTGGCGGACTG CAAATTTG-3' [SEQ ID NO:44]
		F208V_M2 13L	5'- GAATGGACGAGAAGTTTCAAAG TTGCAGTCCGCC-3' [SEQ ID NO:45] 5'- CAAAGTTGCAGTCCGCCAATTGG GAGAATCATGCG-3' [SEQ ID NO:47]	5'- CGCATGATTCTCCCAATTGGCGGACTG CAAATTTG-3' [SEQ ID NO:46] 5'- CGCATGATTCTCCCAATTGGCGGACTG CAACTTTG-3' [SEQ ID NO:48]
		F297A	5'- GGTCGTTATGGTAGGGCCGCG GAGGACTAACA-3' [SEQ ID NO:49]	5'- TGTTAGTCCTCCGCCGCCCTACCAT AACGACC-3' [SEQ ID NO:50]
<i>B. subtilis</i> <i>yhjB</i> (KASIIIB)	pDEST_K ASIIIB	W221V	5'- GCAAAACGACGCGAGGTATATA AAGTGGCCGCAAGAACC-3' [SEQ ID NO:51]	5'- GGTTCCTGCGGCCACTTTATATACCTC GCGTCCGTTTTC-3' [SEQ ID NO:52]
		V226L	5'- GGCCGCAAGAACCCTCCCTGGCG AATT-3' [SEQ ID NO:53]	5'- AATTCGCCAGGGAGGGTTCTTGCGGC C-3' [SEQ ID NO:54]
		W221VV2	5'-	5'-

		26L	GCAAAACGGACGCGAGGTATATA AAGTGGCCGCAAGAACC-3' [SEQ ID NO:55] 5'- GGCCGCAAGAACCCTCCCTGGCG AATT-3' [SEQ ID NO:57]	GGTTCTTGCGGCCACTTTATATACCTC GCGTCCGTTTGC-3' [SEQ ID NO:56] 5'- AATTCGCCAGGGAGGGTTCTTGCGGC C-3' [SEQ ID NO:58]
		F310A	5'- AATCGTTTTGCTTTTCGGGGCTGG CGGCGGATTAACCTAT-3' [SEQ ID NO:59]	5'- ATAGGTTAATCCGCCGCCAGCCCCGA AAAGCAAAACGATT-3' [SEQ ID NO:60]

[00167] --Circular Dichroism (CD) spectroscopy of KASIII mutants.

[00168] All CD spectra of purified KASIII proteins (0.1–0.25 mg/ml in 10 mM sodium phosphate buffer, pH 7.4) were collected with Jasco J-710 Spectropolarimeter, in a 0.1 cm cell at 25°C. Far-UV spectra were recorded with a bandwidth of 1.0 nm and a time response of eight seconds with a total of two data accumulations.

[00169] --Saturation Transfer Difference NMR experiments.

[00170] NMR experiments were performed at 25°C on a Bruker AV700 MHz spectrometer equipped with a 5 mm HCN cryoprobe. Samples for STD NMR experiments were prepared in 0.1 M sodium phosphate buffer (pH 7.4) with 5% D₂O. The protein/ligand ratio was set as 1:100 with KASIII concentration in the 20–25 µM range. Saturation was applied as CW pulse with a power of 58–60 Hz, with on-resonance pulses at 0.62 ppm and 6.86 ppm for upfield and downfield, respectively. The off-resonance pulse was applied at 45 ppm. 3-9-19 WATERGATE suppression was used with a T1-ρ filter. Saturation time of five seconds with an additional delay of 5.1 seconds was used. A set of three replicates was acquired, with 32 scans in each replicate, for each STD NMR experiment of KASIII wild-type or mutated enzyme with a ligand, which was either acetyl-CoA or isobutyryl-CoA (Sigma-Aldrich, St. Louis, MO). The reference and the saturated spectra were obtained in an interleaved manner. Resonance assignments of ¹H NMR spectra of free ligands were completed using 1D ¹H and COSY.

[00171] Control experiments were carried out by applying on- and off-resonance saturation pulses either to the ligand in the absence of protein, or to the ligand in the presence of denatured protein. Time dependence of the saturation transfer was calculated by varying the saturation times from 0.1 milliseconds to 100 seconds, which showed that five seconds were sufficient to transfer the saturation from protein to the ligand. Topspin (Bruker Biospin Corp., Billerica, MA) was used for processing the

reference and saturated spectra and integrating areas of the peaks that showed STD effect.

[00172] Relative STD effects (A_{STD}) were calculated according to the equation $A_{STD} = (I_o - I_{sat})/I_o$ where I_{sat} is the signal intensity of the saturated spectrum and I_o is the signal intensity of the reference spectrum (Mayer et al., J. Am. Chem. Soc. 123: 6108-6117 (2001)). STD amplification factor was calculated according to the equation: $STD_{af} = A_{STD} \times \text{molar ligand excess}$.

[00173] Competition binding experiments were performed under the conditions described above, using a first ligand at constant concentration of 10mM, and a competing ligand present at molar ratios of 1, 2, 4, 8 and 10-fold higher with respect to the first ligand.

[00174] --Differences in active site residues of *E. coli* and *B. subtilis* KASIII based on homology modeling.

[00175] The tertiary structures of *B. subtilis* KASIII homologs (KASIIIA encoded by *yjaX*, and KASIIIB encoded by *yhfB*) were predicted via homology modeling using the methods described above. Superposition of the *E. coli* KASIII crystal structure (PDB code 3IL9) with predicted *B. subtilis* KASIIIA and KASIIIB structures allowed the identification of active site cleft residues that have different conformations in KASIII enzymes from the two organisms. A previous hypothesis suggests that the rotamer conformation of a conserved Phe (Phe-304 in *E. coli*) may impact substrate specificity (Gaijwala et al. (2009), *supra*). In accordance with this hypothesis, we observed that, whereas in Gram-negative *E. coli* KASIII, the side chain of this conserved Phe-304 residue is oriented away from the active site (i.e., its rotamer is in an active-site distal conformation) (Fig. 8a), in the predicted structure of Gram-positive *B. subtilis* KASIIIA, the side chain of Phe-297 faces towards the active site (i.e., it exhibits an active-site proximal conformation). Similarly, in the predicted structure of *B. subtilis* KASIIIB, Phe-310 rotamer shows the active-site proximal conformation. The conformation of this Phe residue correlates with the substrate specificity of the three KASIII enzymes, with the *E. coli* enzyme, which shows a narrow substrate specificity, having the active-site distal rotamer conformation, and the two *B. subtilis* KASIIIs, which show a broader substrate specificity, having the active-site proximal rotamer conformation for this Phe residue. Consistent with this correlation is the

observation that KASIIIs of Gram-positive organisms that produce branched-chain fatty acids, and presumably have a KASIII with a broader substrate specificity, have bulky residues neighboring this Phe residue, and these bulky residues are assumed to force Phe's conformation to the active-site proximal rotamer conformation and orient the Phe side chain towards the active site cleft (Gajiwala et al. (2009), *supra*; Pereira et al. (2012), *supra*). These bulky residues were found to be Phe-208 and Met-213 in *B. subtilis* KASIIIA (Fig. 9a), and Trp-221 and Val-226 in *B. subtilis* KASIIIB, corresponding to smaller residues in *E. coli* KASIII, i.e., Val-215 and Leu-220. These bulky residues in the *B. subtilis* KASIIIs were postulated to cause the conserved Phe active-site proximal rotamers to orient differently from the *E. coli* KASIII Phe-304's active-site distal rotamer, thereby affecting the size and hydrophobicity of active site pockets, and subsequently imparting broad substrate specificity to the *B. subtilis* KASIIIs.

[00176] To verify these hypotheses, Val-215 and Leu-220 of *E. coli* KASIII were mutated to the corresponding residues in *B. subtilis* KASIIIA, i.e., Phe and Met, respectively (Fig. 8b). Two single mutants (V215F (nucleotide sequence [SEQ ID NO:111]; amino acid sequence [SEQ ID NO:112]) and L220M (nucleotide sequence [SEQ ID NO:113]; amino acid sequence [SEQ ID NO:114])) and one double mutant (V215F_L220M (nucleotide sequence [SEQ ID NO:115]; amino acid sequence [SEQ ID NO:116])) were obtained. Similarly, residues in *B. subtilis* KASIIIA were mutated to equivalent residues in *E. coli* KASIII to generate two single mutants (F208V (nucleotide sequence [SEQ ID NO:119]; amino acid sequence [SEQ ID NO:120]) and M213L (nucleotide sequence [SEQ ID NO:121]; amino acid sequence [SEQ ID NO:122])) and a double mutant (F208V_M213L, Fig. 9b; nucleotide sequence [SEQ ID NO:123]; amino acid sequence [SEQ ID NO:124]). In *B. subtilis* KASIIIB, similar set of mutations generated two single mutants (W221V (nucleotide sequence [SEQ ID NO:127]; amino acid sequence [SEQ ID NO:128]) and V226L (nucleotide sequence [SEQ ID NO:129]; amino acid sequence [SEQ ID NO:130])) and a double mutant (W221V_V226L; nucleotide sequence [SEQ ID NO:131]; amino acid sequence [SEQ ID NO:132]). If substrate specificity is governed by the orientation of the conserved Phe and its orientation is determined by the residues listed above, we hypothesized that *E. coli* KASIII, when mutated to resemble *B. subtilis* KASIII at these sites, would have

broadened substrate specificity for both straight- and branched-chain ligands (Fig. 8b). In contrast, *B. subtilis* KASIIIA and KASIIIB, when mutated to resemble *E. coli* KASIII at these sites, would have narrowed substrate specificity and be able to bind to only straight-chain substrates (Fig. 9b).

[00177] Each of the wild-type and mutated enzymes were purified to near homogeneity, with the exception of *B. subtilis* KASIIIB double mutant, which formed inclusion bodies and could not be purified. CD spectra of all purified proteins were obtained to ensure that the mutants folded the same as the wild-type KASIII proteins.

[00178] STD NMR experiments elucidated interactions of these purified enzymes (both wild-type and mutated) with straight-chain substrate (acetyl-CoA) and branched-chain substrate (isobutyryl-CoA) and enabled mapping of binding epitopes on the two substrates. Relative saturation transfer to each of the binding epitopes was measured and converted to the STD amplification factor (STD_{af}), which is an indicator of ligand binding (Mayer et al., J. Am. Chem. Soc. 123: 6108-6117 (2001)). Ligand epitopes with high STD_{af} values are assumed to receive higher saturation transfer from the enzyme, and thus are in close contact with the enzyme. Comparison of STD_{af} values revealed differences in interaction of wild-type and mutated enzymes with straight- and branched-chain ligands.

[00179] --Ligand binding epitopes for *E. coli* and *B. subtilis* KASIII enzymes.

[00180] From STD NMR experiments with *E. coli* KASIII, binding epitopes were found to be quite similar in the acetyl-CoA and isobutyryl-CoA ligands, irrespective of the wild-type or mutant state of KASIII tested. In both ligands the H_{AP} proton, positioned at C-2 of the adenine ring of CoA, received the maximum saturation transfer (Fig. 10a). This indicates that H_{AP} is in close contact with the KASIII enzyme. This result is in agreement with previous crystallographic data (Choi et al. (2000), *supra*; and Revill et al. (2001), *supra*) which showed the adenine ring of CoA sandwiched between Trp-32 and Arg-151 of *E. coli* KASIII. The second highest saturation was received by the terminal proton, H_T (Fig. 10a), in acetyl-CoA, whereas proton H_B , which is located on C-2 of the isobutyryl portion of the ligand and is close to the H_T protons, received the second highest saturation transfer in isobutyryl-CoA (Fig. 10b). Saturation transfer to all other protons in both acetyl-CoA and isobutyryl-CoA was very low, less than 38% relative to H_{AP} whose STD_{af} was normalized to 100%. Also,

H_{AP} is on the conserved pantethiene arm in each of the acyl-CoA ligands, whereas H_T is on the variable acyl end that has been shown to interact with the active site Cys of KASIII (specifically, with Cys-112 of *E. coli* KASIII (Revill et al. (2001), *supra*)).

[00181] Similar to *E. coli* KASIII, for *B. subtilis* KASIIIA, the binding epitopes of acetyl-CoA were the methyl group carrying H_T and the adenine moiety bearing H_{AP}, which received the largest saturation transfer (Fig. 10d), whereas other protons received very little saturation transfer (less than 35% relative to H_{AP}, which received the maximum saturation, i.e., 100%). Isobutyryl-CoA, surprisingly, had H_B with maximum saturation, H_{AP}, and H_T with the second and the third highest saturation transfer, respectively (Fig. 10e). Interestingly, for *B. subtilis* KASIIIB, binding epitopes were slightly different from its homolog KASIIIA and its ortholog *E. coli* KASIII. In addition to H_T, H_{AP} in acetyl-CoA, and H_T, H_B and H_{AP} in isobutyryl-CoA, a new binding epitope, H_R, was identified that received strong saturation transfer in both acetyl and isobutyryl-CoA (Figs. 10f and 10g). H_R is located on C-5 of the ribose moiety of coenzyme A (Fig. 10a).

[00182] For *E. coli* KASIII and *B. subtilis* KASIIIA, because H_T, H_{AP} protons in acetyl-CoA and H_T, H_B and H_{AP} in isobutyryl-CoA received strong saturation transfer, and consequently had the highest STD_{af}, focus was placed on differences in saturation transfer to these protons. As H_T is on the acyl end of the ligand, the amount of saturation transferred to it indicated the extent of KASIII active site's interaction with the ligand. In *B. subtilis* KASIIIB, in addition to H_T, H_{AP} in acetyl-CoA and H_T, H_B and H_{AP} in isobutyryl-CoA, focus also was placed on H_R for comparing the effect of mutations on substrate binding because it received strong saturation transfer in both acetyl-CoA and isobutyryl-CoA.

[00183] --Val215Phe and Leu220Met mutations improve branched-chain substrate binding in *E. coli* KASIII but decrease catalytic activity.

[00184] Wild-type *E. coli* KASIII showed binding with acetyl-CoA (Fig. 10b) with STD_{af} values of ~5.0 and ~6.0 for the H_T and H_{AP} protons, respectively. In contrast, STD NMR of *E. coli* KASIII with isobutyryl-CoA as the ligand resulted in relatively lower saturation transfer, STD_{af} of 2.5 to the H_T proton and ~4.0 to the H_B proton, indicating lower binding with branched-chain ligand (Fig. 10c). The *E. coli* double mutant V215F_L220M, on the other hand, exhibited increased STD_{af} for H_T, H_B and

H_{AP} as compared to wild-type *E. coli* KASIII for both acetyl and isobutyryl-CoA. This suggests that the double mutant, which was mutated to resemble the broad substrate specificity enzyme *B. subtilis* KASIIIa, is showing enhanced interactions with both straight- and branched-chain ligands.

[00185] To identify which of the two mutations in the *E. coli* double mutant had a stronger effect on substrate binding, we examined STD amplification for each of the single mutants (i.e., *E. coli* V215F and *E. coli* L220M). The V215F mutation resulted in a slight increase in STD_{af} for H_T protons of both acetyl-CoA and isobutyryl-CoA as compared to the wild-type. However, the L220M mutation did not alter STD_{af} for acetyl-CoA proton H_T but significantly increased STD_{af} for H_T of isobutyryl-CoA (by ~66%) as compared to the wild-type (Figs. 10b and 10c), suggesting that the substrate binding of *E. coli* KASIII is affected by Leu-220 tremendously and by Val-215 to a lesser extent. Enzymological assays of *E. coli* wild-type and mutant KASIII enzymes showed that both individual mutations at Leu-220 and Val-215 reduced catalytic activity of the enzyme (see Table 4). The double mutant had a decreased specific activity with both acetyl-CoA and isobutyryl-CoA as compared to the *E. coli* wild-type KASIII, whereas the single mutants showed negligible specific activity. In summary, these two mutations, which are proposed to influence the rotamer conformation of the conserved substrate-determining Phe residue (active site-distal rotamer in *E. coli* KASIII and active site-proximal rotamer in *B. subtilis* KASIIIa as depicted in Fig. 8), increased *E. coli* KASIII interaction with branched-chain substrate in addition to straight-chain acyl-CoA substrate but decreased the catalytic activity of the enzyme.

Table 4. Specific Activity (nmol/min/mg) of wild-type and mutant KASIII with straight-chain and branched-chain acyl-CoA substrates

Enzyme	Mutation	Acetyl-CoA	Isobutyryl-CoA
<i>E. coli</i> KASIII	Wild-type	447 ± 68	36 ± 14
	V215F	n.d.	n.d.
	L220M	n.d.	n.d.
	V215F_L220M	221 ± 104	18 ± 5
	F304A	n.d.	n.d.
<i>B. subtilis</i> KASIIIa	Wild-type	64 ± 32	205 ± 139
	F208V	n.d.	n.d.
	M213L	n.d.	156 ± 47
	F208V_M213L	n.d.	17 ± 7
	F297A	n.d.	56 ± 28
<i>B. subtilis</i> KASIIIb	Wild-type	n.d.	279 ± 8
	W221V	n.d.	n.d.
	V226L	n.d.	134 ± 6

Enzyme (wild-type)	Propionyl-CoA	Isovaleryl-CoA	Acetyl-CoA	Isobutyryl-CoA
<i>C. gingivalis</i> KASIIIa	25 ± 2.7	53.5 ± 6.1	n.d.	166.08 ± 82.3
<i>C. gingivalis</i> KASIIIc	213 ± 70	22 ± 8.3	102.8 ± 5.7	26 ± 9.1
<i>L. pneumophila</i> KASIIIa	83.2 ± 17	90.5 ± 17.7	27.5 ± 9.7	304.9 ± 25.3
<i>M. xanthus</i> KASIIIc	37.4 ± 12.9	7.6 ± 1.8	45.2 ± 10.0	19.8 ± 3.0

n.d. = not detectable

[00186] --Phe208Val and Met213Leu mutations negatively affect substrate binding in *B. subtilis* KASIIIa.

[00187] For wild-type *B. subtilis* KASIIIa, the H_T protons of both acetyl and isobutyryl-CoA had similar STD_{af} (1.5), whereas H_{AP} proton of isobutyryl-CoA received ~28% higher saturation as compared to the acetyl-CoA H_{AP} proton (Figs. 10d and 10e). The double mutant F208V_M213L, which is mutated to resemble *E. coli* KASIII at two residues neighboring Phe-297 (Fig. 9), showed decreased binding with acetyl-CoA and isobutyryl-CoA, as evident by significantly decreased STD_{af} for each ligand (Figs. 10d and 10e). Although the single mutant *B. subtilis* KASIIIa F208V did not impact binding with acetyl-CoA much, it decreased saturation transfer to the isobutyryl-CoA H_T and H_B protons by ~40% in comparison to the wild-type. For the second single mutant, *B. subtilis* KASIIIa M213L, saturation transfer decreased to both acetyl-CoA and isobutyryl-CoA, with 40% decrease in STD_{af} for acetyl-CoA H_T proton, and 60-70% decrease in STD_{af} for isobutyryl-CoA H_T and H_B protons. These data clearly indicate that the M213L mutation has a more pronounced negative effect on *B. subtilis* KASIIIa's interaction with substrates, especially with the branched-chain substrate (isobutyryl-CoA).

[00188] The decreased binding of acetyl-CoA and isobutyryl-CoA ligands by the *B. subtilis* KASIIIa mutants is accompanied by decreases in catalytic activity. Individual mutations of both residues (F208V and M213L) resulted in loss of catalytic activity with acetyl-CoA as the substrate and decreased catalytic activity towards isobutyryl-CoA (see Table 4). These data clearly indicate that both M213L and F208V mutations decrease binding and catalytic activity of *B. subtilis* KASIIIa enzyme with both straight- and branched-chain substrates. However, the M213L mutation has a more pronounced negative effect on the binding of substrates by *B. subtilis* KASIIIa, especially with the branched chain substrate (isobutyryl-CoA). Phe-208 also appears to influence branched-chain substrate binding, but to a lesser extent.

[00189] It can, therefore, be inferred that Met-213 is a critical residue for both straight- and branched-chain substrate recognition in *B. subtilis* KASIIIA. Phe-208 also appears to influence branched-chain substrate binding, but to a lesser extent.

[00190] --Trp-221 and Val-226 are critical for *B. subtilis* KASIIIB's folding, substrate recognition, and catalytic activity.

[00191] The wild-type *B. subtilis* KASIIIB enzyme exhibited very high STD_{af} values for both acetyl-CoA and isobutyryl-CoA, ranging from 5.0 to 10.0, as compared to STD_{af} values of the same ligands with *B. subtilis* KASIIIA which ranged from 0.5 to 2.5 (Figs. 10f and 10g). This could be either due to stronger enzyme-ligand interactions for *B. subtilis* KASIIIB or high k_{off} rate for the ligands from the active site pocket of this enzyme. The double mutant of *B. subtilis* KASIIIB (W221V_V226L), which contains mutations to resemble *E. coli* KASIII at positions 221 and 226, could not be purified due to its poor solubility. The single mutants (*B. subtilis* KASIIIB W221V and *B. subtilis* KASIIIB V226L) could be purified but they did not exhibit efficient saturation transfer to either of the ligands. The W221V mutation appeared to result in complete loss of KASIIIB's interaction with acetyl-CoA and isobutyryl-CoA, as suggested by negligible STD_{af} values (Figs. 10f and 10g). The second mutation, V226L, resulted in less severe reduction of the STD amplification factors. Enzymatic assays of the wild-type and mutant *B. subtilis* KASIIIB enzymes with either acetyl-CoA or isobutyryl-CoA indicated a very high specific activity with isobutyryl-CoA and no activity with acetyl-CoA (see Table 4). The W221V mutation completely eliminated activity with isobutyryl-CoA, whereas the V226L mutation led to an approximately 50% loss of activity with isobutyryl-CoA. These observations, along with poor solubility of the *B. subtilis* KASIIIB double mutant, imply that both Trp-221 and Val-226 are important for substrate recognition by *B. subtilis* KASIIIB. Trp-221 in particular seems to be critical for proper folding, substrate binding, and catalytic functionality of *B. subtilis* KASIIIB.

[00192] --Role of the conserved Phe in substrate binding of *E. coli* KASIII and *B. subtilis* KASIIIA, KASIIIB.

[00193] For each of the three enzymes, mutating the two residues that were predicted to affect the orientation of the conserved Phe clearly impacted substrate binding. However, the putative roles of these mutations in determining the Phe rotamer

conformation (active site-distal or active site-proximal) were not directly assessed by the STD NMR experiments. In order to investigate the role of the Phe itself in substrate binding, the conserved Phe was mutated to Ala in each of the three KASIII enzymes. The *E. coli* KASIII F304A mutant (nucleotide sequence [SEQ ID NO:117]; amino acid sequence [SEQ ID NO:118]) resulted in approximately 60% lower saturation transfer to the H_T protons of acetyl-CoA and isobutyryl-CoA compared to the wild-type (Figs. 11a and 11b). Thus, these mutations eliminate all enzymatic activity of the *E. coli* KASIII (see Table 4) but, in the case of the *B. subtilis* KASIIIA, about 25% of the catalytic activity with the isobutyryl-CoA substrate is retained and all catalytic activity with the acetyl-CoA substrate is eliminated (see Table 4). The *B. subtilis* KASIIIB F310A mutant (nucleotide sequence [SEQ ID NO:133]; amino acid sequence [SEQ ID NO:134]) could not be purified, owing to poor expression and solubility, suggesting that Phe-310 is required for proper folding of *B. subtilis* KASIIIB.

[00194] --Competition binding experiments reveal relative affinities of ligands to KASIII enzymes.

[00195] For *E. coli* KASIII, a low STD_{af}, as seen for the H_T proton of isobutyryl-CoA as compared to acetyl-CoA, could be either because isobutyryl-CoA is a poor substrate for *E. coli* KASIII and does not bind to the active site, or because it is a very tight binder with dissociation constant, K_D, below 10⁻¹⁰, resulting in an extended stay in the binding site and inefficient saturation transfer (Mayer et al., J. Am. Chem. Soc. 123: 6108-6117 (2001); and Meyer et al., Ernst Schering Res. Found. Workshop: 149-167 (2004)). To investigate which of the two scenarios is true, competition binding experiments, in which the concentration of acetyl-CoA was kept constant and isobutyryl-CoA was titrated at increasing concentrations, were performed to see if the latter ligand replaces the former ligand. Competition binding data strongly suggested that acetyl-CoA was the preferred substrate for *E. coli* KASIII, while isobutyryl-CoA competed with acetyl-CoA only when its concentration was at least four times more than that of acetyl-CoA (Fig. 12a).

[00196] For *B. subtilis* KASIIIA, to determine the competition between acetyl-CoA and isobutyryl-CoA, increasing amounts of isobutyryl-CoA were titrated, while acetyl-CoA was held at a constant concentration. The results showed that isobutyryl-CoA was

the preferred substrate for *B. subtilis* KASIII_A and always had a higher STD amplification factor for the observed proton H_T than that of the acetyl-CoA H_T proton (Fig. 12b). Similar results were obtained for competition binding experiments with *B. subtilis* KASIII_B, where isobutyryl-CoA was found to be the preferred substrate for *B. subtilis* KASIII_B and its STD_{af} increased with increasing concentration (Fig. 12c).

Example 4

[00197] This example describes the analysis of KASIII from *Alicyclobacillus acidocaldarius*.

[00198] --Expression and purification of recombinant *A. acidocaldarius* KASIII protein.

[00199] The *A. acidocaldarius* KASIII gene was codon-optimized for expression in *E. coli*, chemically synthesized, and cloned into the pUC57 vector by Genscript USA (Piscataway, NJ, USA). The gene was further cloned into the pDEST-17 vector using Gateway Cloning (Invitrogen, Carlsbad, CA) resulting in the pDEST_AA construct. *E. coli* OverExpress™ C41 (Lucigen, Middletown, WI) strain was used for expression of *A. acidocaldarius* KASIII protein from the construct pDEST_AA. The C41 transformants were grown at 37°C in 2L Luria-Bertani medium and were supplemented with 100 µg/ml ampicillin (Research Products International Corps., Mount Prospect, IL). The cultures were induced by addition of IPTG (Gold Biotechnology, Olivette, MO) to a final concentration of 0.4 mM when the OD₆₀₀ was 0.6-0.8. After incubation for another 16-18 hours at 25°C, cells were harvested by centrifugation (10,000 X g, 4°C, 10 minutes). Soluble proteins were extracted by first suspending the cell pellet in lysis buffer (0.5 M NaCl, 5 mM imidazole, 20 mM Tris-HCl, pH 8.0, 0.1 mg/ml phenylmethylsulfonyl fluoride, and 0.1% Triton-X 100), followed by sonication (10 second pulses separated by 3 second intervals for a total of 3 minutes) and centrifugation (10,000 X g, 4°C, 30 minutes). The resulting supernatant (soluble protein fraction) was analyzed for its homogeneity and purity by running on SDS-PAGE gel, which showed the presence of near-homogenous, pure proteins (greater than 95% purity).

[00200] The soluble protein fraction was filtered through a 0.45 µ filter (Corning, the Netherlands) and applied to 8 ml Ni-NTA His-bind resin. After washing the unbound protein with wash buffers I and II (0.5M NaCl, 20 mM Tris-HCl, pH 8.0)

supplemented with 20 mM and 40 mM imidazole, respectively, the proteins of interest were eluted with the same buffer containing 250 mM imidazole. The purified His-tagged KASIII proteins were dialyzed against sodium phosphate buffer, pH 7.2, and concentrated using 10,000 molecular weight cut-off ultrafiltration centrifugation filters (Millipore, Billerica, MA) at 4°C. The concentrated proteins were either supplemented with 16% glycerol and stored at -80°C or immediately used for KASIII activity assay. Protein concentrations were determined by Bradford's assay (BioRad, Hercules, CA).

[00201] --Purification of recombinant Malonyl-CoA ACP Transacylase (MCAT or FabD), β -ketoacyl ACP reductase (FabG), and holo-Acyl Carrier Protein (ACP).

[00202] FabD, FabG and ACP proteins are required for the spectrophotometric assay of KASIII. Therefore, genes encoding these proteins, namely *fabD*, *fabG* and *acpP*, were obtained from *E. coli* Hub in pCA24N expression vectors. The *acpP* gene was further cloned into pETDUET vector along with *acpS* gene that encodes for ACP synthase. These three recombinant proteins (FabD, FabG and holo-ACP) with N-terminal His-tags were purified to near-homogeneity using the same procedure as described for the purification for *A. acidocaldarius* KASIII. Purity of these proteins was assessed by running an SDS-PAGE gel.

[00203] --Spectrophotometric assay to determine *A. acidocaldarius* activity with different substrates.

[00204] Activity of *A. acidocaldarius* KASIII with different acyl-CoA substrates (acetyl-CoA, isobutyryl-CoA and 3-hydroxybutyryl-CoA (Sigma-Aldrich)) was ascertained by a coupled assay. The assay was performed in 96-well plate format with three replicates for each reaction condition. In a total volume of 100 μ l for each reaction the reaction mix containing 100 μ M holo-ACP, 200 μ M malonyl-CoA, 10mM DTT, 50 μ M acyl-CoA substrate (either acetyl-CoA, isobutyryl-CoA or 3-hydroxybutyryl-CoA) and 200 μ M NADPH in 0.1 M sodium phosphate buffer (pH 7.2) was pre-incubated with 60 μ g of FabD for two minutes. The reaction was started by the addition of 30 μ g of FabG and varying concentrations of *A. acidocaldarius* KASIII (0.5-15 μ g). Change in absorbance of NADPH that was being converted to NADP⁺ during reduction of 3-ketoacyl-ACP to 3-hydroxy-acyl-ACP by FabG was recorded at 340 nm using a Biotek multi-plate reader.

[00205] --Purified *A. acidocaldarius* KASIII, FabD, FabG and ACP.

[00206] Each of the recombinant proteins (*A. acidocaldarius* KASIII, FabD, FabG and holo-ACP) were purified to near-homogeneity.

[00207] --Activity of *A. acidocaldarius* KASIII with acetyl-, isobutyryl-, and 3-hydroxybutyryl-CoA substrates.

[00208] Spectrophotometric assay was used to assess the activity of *A. acidocaldarius* KASIII with various acyl-CoA substrates. The assay coupled the appearance of the KASIII-product (3-ketoacyl-ACP) to the oxidation of NADPH, catalyzed by 3-ketoacyl-ACP reductase (FabG). This latter reaction resulted in a change of absorbance at 340 nm, due to the conversion of NADPH to NADP⁺, and the rate of this change was used to directly calculate the rate of the KASIII-catalyzed reaction. Studies of *A. acidocaldarius* KASIII with different substrates using this spectrophotometric assay established that this KASIII has the ability to utilize a number of different acyl-CoA substrates, particularly with higher preference for hydroxylated acyl-CoA substrate (3-hydroxybutyryl-CoA) and branched chain substrate (i.e., isobutyryl-CoA) as compared to straight chain substrate (acetyl-CoA).

Example 5

[00209] This example describes the cloning of *A. acidocaldarius* KASIII into *Rhodospirillum rubrum*.

[00210] The *A. acidocaldarius* KASIII gene was cloned into the *phaC2* locus in the *R. rubrum* genome via a double-crossover recombination event. The *R. rubrum* recipient strain for this experiment was the *phaC* triple mutant (*ΔphaC1ΔphaC2ΔphaC3*) that lacked any PhaC activity. First, the upstream flanking sequence (922bp) of the *R. rubrum* *phaC2* gene (Aru_2413) was cloned upstream of the *A. acidocaldarius* KASIII sequence, and this chimeric protein was introduced into the *E. coli* strain S17-1. The transformed S17-1 was co-incubated overnight with *R. rubrum* *phaC* triple mutant (*ΔphaC1ΔphaC2ΔphaC3*) on 0.22 μm filter for conjugation. The bacterial mixture was grown on minimal medium plate containing 25 μg/ml gentamicin for one to two weeks. The resulting colonies carry the product of a single recombination crossover event, which integrates the *A. acidocaldarius* KASIII gene at the *phaC2* gene (Aru_2413) locus. These colonies were streaked out on another

minimal medium plate containing 25 µg/ml gentamicin for colony purification. The resulting colonies were cultured in SMN rich medium for two to three days in the light without gentamicin selection. Finally, the culture was plated out on SMN rich medium containing 5% sucrose to screen for double-crossover events. The resulting colonies were PCR sequence-confirmed to carry the *A. acidocaldarius* KASIII gene at the *phaC2* gene (Aru_2413) locus.

[00211] In order to investigate the metabolic functions of the three *phaCs*, single locus deletion mutants (Δ *phaC1*, Δ *phaC2*, Δ *phaC3*), double-loci deletion mutants (Δ *phaC1 Δ *phaC2*, Δ *phaC1 Δ *phaC3*, and Δ *phaC2 Δ *phaC3*), and a triple-loci deletion mutant (Δ *phaC1 Δ *phaC2 Δ *phaC3*) were created, and these mutants were characterized relative to growth, PHA yields and monomer composition of the polymer (Jin et al., J. Bacteriol. 194: 5522-5529 (2012)). Of most significance, the triple *phaC* mutant (Δ *phaC1 Δ *phaC2 Δ *phaC3*) failed to accumulate any PHA polymer, and showed only a slight impact on growth characteristics. This strain, therefore, has the capacity to generate 3-hydroxybutyryl-CoA, which could be used by a KASIII enzyme to produce ω -hydroxy-branched-fatty acids. This hypothesis was tested by recombinantly expressing the *A. acidocaldarius* KASIII in the triple *phaC R. rubrum* mutant (described in Example 6), and then analyzing the fatty acids produced (described in Example 7).*******

Example 6

[00212] This example describes the fermentation-based production of bi-functional fatty acids.

[00213] To generate omega-hydroxy fatty acid in *R. rubrum*, the recombinant *R. rubrum* carrying the *A. acidocaldarius* KASIII was grown on RRNCO medium (but omitting ammonium chloride, hydrogen sulfide, carbon monoxide and carbon dioxide) for five days, and the bacterial pellet was used for fatty acid analysis.

[00214] *A. acidocaldarius* KASIII was cloned into triple *phaC R. rubrum* mutant to evaluate whether this KASIII enzyme has the ability to use *in vivo*-generated hydroxyacyl-CoA starter substrates, and produce ω -hydroxy-fatty acids. After the fermentation process involving growth of recombinant *R. rubrum* cells containing *A.*

acidocaldarius KASIII on RRNCO medium, fatty acids were extracted from the bacterial cells and analyzed as described in Example 7.

Example 7

[00215] This example describes the analysis of produced bi-functional fatty acids by GC-MS.

[00216] The resultant fatty acids in *R. rubrum* were extracted and analyzed using GC-MS. Analysis of the fatty acids from recombinant *R. rubrum* strain containing *A. acidocaldarius* KASIII demonstrated the production of 15-hydroxypalmitate, which was identified by GC-MS analyses conducted at Iowa State University's W. M. Keck Metabolomics Research Laboratory (Figs. 13a and 13b). Thus, this indicates that the substrate hydroxybutyryl-CoA in *R. rubrum* was being utilized by *A. acidocaldarius* KASIII and metabolized via fatty acid synthesis pathway resulting in long-chain ω -hydroxy fatty acids. This gave a proof of concept of production of bi-functional fatty acids in recombinant bacterial hosts.

Example 8

[00217] This example describes the *in vitro* characterization of *Bacteroides vulgatus* KASIII.

[00218] --Expression and purification of recombinant *B. vulgatus* KASIII protein.

[00219] The *B. vulgatus* KASIII gene was codon-optimized for expression in *E. coli*, chemically synthesized, and cloned into the pUC57 vector by Genscript USA (Piscataway, NJ, USA). The gene was further cloned into the pDEST-17 vector using Gateway Cloning (Invitrogen, Carlsbad, CA) resulting in the pDEST_BV construct. The *E. coli* OverExpressTM C41 (Lucigen, Middletown, WI) strain was used for expression of the *B. vulgatus* KASIII protein from the construct pDEST_BV. The *B. vulgatus* KASIII was expressed and purified using the same methods as described in Example 4.

[00220] --Spectrophotometric assay to determine *B. vulgatus* KASIIIA activity with different substrates.

[00221] Activity of *B. vulgatus* KASIIIA with different acyl-CoA substrates (acetyl-CoA, isobutyryl-CoA and 3-hydroxybutyryl-CoA (Sigma-Aldrich)) was ascertained by a coupled assay using the methods described in Example 4.

[00222] --Activity with straight-, branched-, and hydroxy-acyl-CoA substrates.

[00223] Results from the spectrophotometric assay established that *B. vulgatus* KASIII can utilize acetyl-CoA (straight chain substrate), isobutyryl-CoA (branched-chain substrate) and 3-hydroxybutyryl-CoA (hydroxylated substrate) with a higher preference for isobutyryl-CoA and 3-hydroxybutyryl-CoA as compared to acetyl-CoA (Fig. 14).

Example 9

[00224] This example describes the *in vitro* characterization of *Legionella pneumophila* KASIIIA.

[00225] --Expression and purification of recombinant *L. pneumophila* KASIIIA protein.

[00226] The *L. pneumophila* KASIIIA gene was codon-optimized for expression in *E. coli*, chemically synthesized, and cloned into the pUC57 vector by Genscript USA (Piscataway, NJ, USA). The gene was further cloned into the pDEST-17 vector using Gateway Cloning (Invitrogen, Carlsbad, CA) resulting in the pDEST_LP1 construct. The *E. coli* OverExpressTM C41 (Lucigen, Middletown, WI) strain was used for expression of the *L. pneumophila* KASIIIA protein from the construct pDEST_LP1. The *L. pneumophila* KASIIIA protein was expressed and purified using the same methods as described in Example 4.

[00227] --Spectrophotometric assay to determine *L. pneumophila* KASIII activity with different substrates.

[00228] Activity of *L. pneumophila* KASIII with different acyl-CoA substrates (acetyl-CoA, isobutyryl-CoA and 3-hydroxybutyryl-CoA (Sigma-Aldrich)) was ascertained by a coupled assay using the methods described in Example 4.

[00229] --Activity with straight, branched and hydroxy-acyl-CoA substrates.

[00230] Results from the spectrophotometric assay established that *L. pneumophila* KASIIIa can utilize acetyl-CoA (straight-chain substrate), isobutyryl-CoA (branched-chain substrate) and 3-hydroxybutyryl-CoA (hydroxylated substrate) with a higher preference for isobutyryl-CoA and 3-hydroxybutyryl-CoA as compared to acetyl-CoA (Fig. 15).

Example 10

[00231] This example describes *in vivo* characterization of KASIII from various biological sources (*Aeromonas hydrophila*, *Bacteroides vulgatus*, *Brevibacterium linens*, *Capnocytophaga gingivalis*, *Thermus aquaticus*, *Bacillus licheniformis*, *Desulfovibrio vulgaris*, *Bacillus subtilis* subsp. *S*, *Haliangium ochraceum*, *Alicyclobacillus acidocaldarius*) using *B. subtilis* FabH deletion mutant.

[00232] --Complementation of *B. subtilis* FabH deletion mutant with recombinant KASIII genes from various sources.

[00233] The mutant strain $\Delta bfabHA \Delta bfabHB::(erm-Pspac::fabH)$ was generated by homologous recombination via a one-step procedure, by transforming the mutant $\Delta bfabHA$ strain with plasmid pUCB-erm-fabH, followed by selection for erythromycin-resistance on LB media.

[00234] The pUCB-erm-fabH vector was constructed from plasmid pUC19. The erythromycin-resistant gene *erm* and the exogenous fabH gene under the control of the promoter Pspac were inserted between the downstream and upstream DNA fragments of *bfabHB* ORF.

[00235] --Fatty acid analysis of *B. subtilis* strains containing recombinant KASIII genes.

[00236] In these experiments, a 0.5 ml aliquot of an overnight *B. subtilis* culture normalized for equal cell density was collected by centrifugation at 13,200×g for 30 seconds. The cell pellet was suspended in 50 ml minimal medium or LB rich medium. Cell cultures were shaken at 250 rpm. Cells were collected at late log phase by centrifugation at 5000×g for 10 minutes. The collected cell pellets were lyophilized and stored at -20 °C until analysis. Lipids were extracted from lyophilized bacterial cell pellets using chloroform/methanol, and fatty acids were then converted to methyl

esters using methanolic-HCl at 80 °C for 60 minutes. The recovered fatty acid methyl esters were concentrated as needed under a stream of nitrogen gas and analyzed with GC-MS interfaced with a Mass Detector 5973 (Agilent Technologies, Santa Clara, CA).

[00237] --Results.

[00238] The eleven KASIII genes from diverse biological sources (namely, *Aeromonas hydrophila*, *Bacteroides vulgatus*, *Brevibacterium linens*, *Capnocytophaga gingivalis*, *Thermus aquaticus*, *Bacillus licheniformis*, *Desulfovibrio vulgaris*, *Bacillus subtilis* subsp. *S*, *Haliangium ochraceum*, *Alicyclobacillus acidocaldarius*) could rescue the *B. subtilis* FabH deletion mutant that fails to grow by itself, indicating that these KASIII enzymes can synthesize branched-chain fatty acids *in vivo*. Fatty acid content of *B. subtilis* FabH deletion mutants rescued by recombinant KASIII genes from diverse sources is shown in Table 5.

Table 5. Fatty Acid Production by KASIII in *B. subtilis* FabH deletion mutant

Source of KASIII	Fatty acid content (mole %)				
	Anteiso-fatty acids	Even-number iso-fatty acids	Odd-number iso-fatty acids	Even-number normal fatty acids	Odd-number normal fatty acids
KASIIIB from <i>Aeromonas hydrophila</i>	36.64	4.46	6.27	51.4	1.23
KASIII from <i>Bacteroides vulgatus</i>	70.43	1.73	18.24	9.51	0.09
KASIIIA from <i>Capnocytophaga gingivalis</i>	27.24	1.28	58.94	12.46	0.08
KASIIIA from <i>Brevibacterium linens</i>	39.96	0.29	0.5	59.27	0.04
KASIII from <i>Thermus aquaticus</i>	52.1	1.09	26.89	19.84	0.12
KASIIIA from <i>Bacillus licheniformis</i>	63.27	3.3	28.25	5.17	0.04
KASIIIB from <i>Bacillus licheniformis</i>	68.83	8.87	11.59	10.76	0.07
KASIII from <i>Desulfovibrio vulgaris</i>	36.05	0.95	42.59	19.74	0.54
KASIII from <i>Bacillus subtilis</i> subsp. <i>S</i>	68.02	6.83	12.39	12.77	0.06
KASIII from <i>Haliangium ochraceum</i>	53.87	0.98	12.29	32.79	0.07

KASIII from <i>Alicyclobacillus acidocaldarius</i>	48.13	3.64	30.44	17.73	0.05
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Example 11

[00239] This example identifies KASIII enzymes that can process atypical substrates.

[00240] KASIII enzymes from *Alicyclobacillus acidocaldarius* (aaKASIII), *Thermus aquaticus* (taKASIII), and *Capnocytophaga gingivalis* (cgKASIIIa), which rescued the lethal phenotype of the *B. subtilis fabH* deletion mutant were purified to homogeneity and then screened for binding with typical and atypical KASIII substrates. Also purified to homogeneity and screened for binding with KASIII substrates were *Legionella pneumophila* and *Myxococcus xanthus*. Typical substrates included straight-chain and branched-chain acyl-CoA primers, whereas atypical substrates included di-acid (malonyl-CoA and methylmalonyl-CoA), hydroxylated (3-hydroxybutyryl-CoA), unsaturated (crotonyl-CoA), and aromatic (such as benzoyl-CoA and phenylacetyl-CoA) acyl-CoAs. *E. coli* KASIII and *B. subtilis* KASIIIb were included as standards. The ability of the KASIII enzymes to bind to the substrates was measured via a fluorescence-based thermal shift assay, which measured the thermal stability of a protein in the presence and absence of a specific ligand or substrate. A positive shift in melting temperature of the protein (T_m) in the presence of a substrate is correlated with substrate binding and concomitant stabilization of the enzyme, whereas a negative shift in T_m suggests destabilization of the protein by the substrate. Thermal shift analysis showed that the KASIII from *B. subtilis*, *A. acidocaldarius*, and *T. aquaticus* bound a broad range of substrates, whereas *C. gingivalis* KASIIIa and *E. coli* KASIII bound to a relatively narrow range of substrates. KASIIIa from *L. pneumophila* bound straight-chain (C3:0 and C4:0), branched-chain (iso-C4:0 and iso-C5:0), and dicarboxylate (malonyl and methylmalonyl) acyl-CoAs. KASIIIc from *M. xanthus* bound straight-chain acyl-CoAs (C2:0, C4:0 and C6:0) and malonyl-CoA.

[00241] Inherent T_m s in water without any ligand were measured for each of the KASIII (Table 6). These melting temperatures were used as the baseline T_m s to measure the shift in T_m by addition of substrate. The KASIII enzymes from *A. acidocaldarius* and *T. aquaticus* were unusually thermally stable, with baseline T_m s of ~ 73°C and ~84°C, respectively. These melting temperatures are considerably higher

than those for the other KASIIIs that were studied and can be correlated with their ability to survive at extremely high temperatures.

Table 6. Melting temperatures T_m of KASIII enzymes without ligand

Organism	Enzyme	Melting temperature ($^{\circ}\text{C}$)
<i>C. gingivalis</i>	KASIIIa	68.3 ± 0.17
	KASIIIb	67.8 ± 0.03
	KASIIIc	56.8 ± 0.62
<i>A. acidocaldarius</i>	KASIII	73.2 ± 0.05
<i>T. aquaticus</i>	KASIII	84.1 ± 0.11
<i>B. subtilis</i>	KASIIIb	48.7 ± 0.24
<i>E. coli</i>	KASIII	55.0 ± 0.18
<i>L. pneumophila</i>	KASIIIa	54.0 ± 0.36
	KasIIIb	60.2 ± 0.16
	KasIIIc	62.0 ± 0.03
	KasIIId	65.8 ± 0.18
<i>M. xanthus</i>	KASIIIa	64.6 ± 0.13
	KASIIIb	54.5 ± 0.15
	KASIIIc	61.4 ± 0.50

[00242] Consistent with previous reports that *E. coli* KASIII has narrow substrate specificity, ecKASIII bound only short, straight-chain acyl-CoAs (acetyl-CoA and propionyl-CoA). In contrast, *C. gingivalis* KASIIIa bound mainly branched-chain (isovaleryl-CoA and isobutyryl-CoA) and unsaturated (crotonyl-CoA; T_m shift of 2°C) substrates that resulted in statistically significant thermal shifts (see Figs. 16a and 16d).

[00243] *B. subtilis* KASIIIb and *A. acidocaldarius* KASIII exhibited the broadest ranges of substrate specificities. In particular, *B. subtilis* KASIIIb bound the straight-chain substrates (propionyl-CoA and butyryl-CoA), the branched-chain substrates (isobutyryl-CoA and isovaleryl-CoA), and a di-acidic substrate (phenylacetyl-CoA). Each of these substrates induced at least a 6°C increase in the T_m of bsKASIIIb (see Fig. 16d). In contrast, some substrates (e.g., hexanoyl-CoA and benzoyl-CoA) apparently destabilized the protein, as evidenced by a decrease in the T_m of bsKASIIIb.

The binding capacity of *A. acidocaldarius* KASIII was equally broad. aaKASIII bound to the straight-chain acetyl-CoA and hexanoyl-CoA substrates, the branched-chain isobutyryl-CoA and isovaleryl-CoA, a hydroxylated substrate (3-hydroxybutyryl-CoA), an unsaturated substrate (crotonyl-CoA), and the aromatic substrate (benzoyl-CoA), each of which resulted in at least a 4°C increase in the T_m of the enzyme. Malonyl-CoA and phenylacetyl-CoA also bound to aaKASIII, inducing a 2°C increase in T_m .

[00244] *T. aquaticus* KASIII bound with rather a small range of substrates, specifically straight-chain butyryl-CoA and hexanoyl-CoA and branched-chain isobutyryl-CoA and isovaleryl-CoA substrates that resulted in 2-6°C increases in T_m as compared to the baseline T_m (see Fig. 16d).

[00245] Amongst the four KASIIIs from *L. pneumophila*, LpKASIIIa was the only protein that could be thermally stabilized in the presence of potential acyl-CoA ligands (see Fig. 16b); and this protein was stabilized by straight-chain (C3:0 and C4:0), branched-chain (iso-C4:0 and iso-C5:0), and dicarboxylate (malonyl and methylmalonyl) acyl-CoAs. For each reacting ligand, the T_m was increased by 2-4°C relative to the control (p-value < 0.05), suggesting that the LpKASIII enzyme has a broad substrate preference. In contrast, the observation that the remaining three LpKASIIIs are incapable of binding any acyl-CoA derivatives is consistent with the observation that these three proteins lack at least one of the conserved residues important for CoA binding (see Fig. 16b).

[00246] Of the four *M. xanthus* KASIII enzymes, MxKASIIIC was the only protein that was stabilized by binding to acyl-CoA ligands, specifically straight-chain acyl-CoAs (C2:0, C4:0 and C6:0) and malonyl-CoA, each of which increased the T_m of MxKASIIIC by 2-4 °C compared to the control (p-value < 0.05) (see Fig. 16c). Methylmalonyl-CoA and free CoA destabilized MxKASIIIC by 4°C relative to the control. In contrast, MxKASIIIa was the most destabilized by most of the CoA derivatives that were tested, with the exceptions of CoA and 3-hydroxybutyryl-CoA, neither of which significantly affected the T_m of the protein (see Fig. 16c). This could be explained because of lack of the catalytic triad in MxKASIIIa. In contrast to MxKASIIIa and MxKASIIIC, MxKASIIIB remained largely unaffected by each CoA-derivative, suggesting that it does not have affinity for any of the acyl-CoA ligands

tested in this study. These data are supported by the fact that MxKASIIIb lacks some of the conserved CoA binding residues (see Fig. 16c).

Example 12

[00247] This example describes bioengineering of *E. coli* for the production of ω -hydroxy-branched fatty acids (HBFA).

[00248] --Disruption and replacement of endogenous *E. coli* KASIII gene with novel KASIII genes that have capability to utilize hydroxybutyryl-CoA as the substrate.

[00249] Lambda recombinase method is used to create gene knockouts. The *fabH* gene in *E. coli* strain MN1655, a K12 derivative, is deleted using a kanamycin disruption cassette that is flanked by FLP recognition target sites to excise precisely the kanamycin-resistance cassette, creating an in-frame deletion. Afterwards, the PHA biosynthetic operon is overexpressed in the *E. coli* strain, but without the *phaC* gene, namely expressing the *phaA* and *phaB* genes in combination. This modification is coupled with introduction, and expression therein, of a recombinant KASIII gene that has the ability to utilize hydroxybutyryl-CoA as the substrate. This KASIII is introduced into the *E. coli* strain using expression cassettes that include low-copy and high-copy plasmid vectors that utilize constitutive or inducible promoters of different strengths.

[00250] --Bioengineering of *E. coli* KASIII strain to block fatty acid degradation pathway.

[00251] To maximize production of the targeted ω -hydroxy-branched-fatty acids, the β -oxidation pathway can be blocked to result in the secretion of fatty acids into the growth medium. The lambda red recombinase method can be used to delete the *E. coli* *fadD* gene, which codes for acyl-CoA synthetase, the enzyme that initiates the degradation of fatty acids.

[00252] --Production of ω -hydroxy-branched-fatty acids in bioengineered *E. coli* KASIII strain.

[00253] The combination of *phaA*, *phaB* and an exogenous KASIII gene, such as a wild-type gene from another organism, a natural variant thereof, or a mutant thereof, which can use hydroxybutyryl-CoA as a substrate, results in the production of ω -hydroxy-branched-chain fatty acids.

[00254] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[00255] The use of the terms "a," "an," "the," and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to illuminate better the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[00256] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. It should be understood that the illustrated embodiments are exemplary only, and should not be taken as limiting the scope of the invention.

WHAT IS CLAIMED IS:

1. A mutant *Bacillus subtilis*, which does not express a functional 3-ketoacyl-acyl carrier protein (ACP) synthase III (KASIII) selected from the group consisting of KASIIIA and KASIIIB.
2. The mutant *B. subtilis* of claim 1, which does not express a functional KASIIIA and a functional KASIIIB.
3. A method of making the mutant *B. subtilis* of claim 1, which method comprises introducing into wild-type *B. subtilis* a mutation selected from the group consisting of a mutation that prevents expression of a functional KASIIIA and a mutation that prevents expression of a functional KASIIIB.
4. The method of claim 3, which method comprises introducing into wild-type *B. subtilis* a mutation that prevents expression of a functional KASIIIA and a mutation that prevents expression of a functional KASIIIB.
5. A mutant *Rhodospirillum rubrum*, which does not express a functional polyhydroxyalkanoate (PHA) polymerase selected from the group consisting of PhaC1, PhaC2, and PhaC3.
6. The mutant *R. rubrum* of claim 5, which does not express a functional PhaC1, a functional PhaC2, and a functional PhaC3.
7. A method of making the mutant *R. rubrum* of claim 5, which method comprises introducing into wild-type *R. rubrum* a mutation selected from the group consisting of a mutation that prevents expression of a functional PhaC1, a mutation that prevents expression of a functional PhaC2, and a mutation that prevents expression of a functional PhaC3.
8. The method of claim 7, which method comprises introducing into wild-type *R. rubrum* a mutation that prevents expression of a functional PhaC1, a mutation that

prevents expression of a functional PhaC2, and a mutation that prevents expression of a functional PhaC3.

9. A method of characterizing substrate specificity of a KASIII, which method comprises expressing the KASIII, which is not expressed in wild-type *B. subtilis*, in the mutant *B. subtilis* of claim 2 and assessing the production of ω -functionalized fatty acids, whereupon the substrate specificity of a KASIII is characterized.

10. The method of claim 9, wherein the KASIII is derived from an organism, the wild-type of which produces ω -functionalized fatty acids.

11. A method of characterizing substrate specificity of a KASIII, which method comprises expressing the KASIII, which is not expressed in wild-type *R. rubrum*, in the mutant *R. rubrum* of claim 6 and assessing the production of ω -functionalized fatty acids, whereupon the substrate specificity of a KASIII is characterized.

12. The method of claim 11, wherein the KASIII is derived from an organism, the wild-type of which produces ω -functionalized fatty acids.

13. A method of altering the specificity of a KASIII for at least one of its substrates, which method comprises introducing into the KASIII one or more mutations comprising at least one mutation, which causes the rotamer conformation of a phenylalanine in the KASIII corresponding to Phe304 in KASIII from *E. coli* to change, whereupon the specificity of a KASIII for at least one of its substrates is altered.

14. The method of claim 13, wherein the KASIII is from *E. coli* and the one or more mutations comprises a mutation of Leu220, alone or in further combination with Val215.

15. The method of claim 14, wherein the mutation of Leu220 is Leu220Met and the mutation of Val215 is Val215Phe.

16. The method of claim 14 or 15, wherein the rotamer conformation of Phe304 changes from active site-distal to active site-proximal.

17. The method of claim 13, wherein the KASIII is KASIIIA from *B. subtilis* and the one or more mutations comprises a mutation of Met213, alone or in further combination with Phe208.

18. The method of claim 17, wherein the mutation of Met213 is Met213Leu and the mutation of Phe208 is Phe208Val.

19. The method of claim 17 or 18, wherein the rotamer conformation of Phe297 changes from active site-proximal to active site-distal.

20. The method of claim 13, wherein the KASIII is KASIIIB from *B. subtilis* and the one or more mutations comprise a mutation of Trp221 and a mutation of Val226.

21. The method of claim 20, wherein the mutation of Trp221 is Trp221Val and the mutation of Val226 is Val226Leu.

22. The method of claim 20 or 21, wherein the rotamer conformation of Phe310 changes from active site-proximal to active site-distal.

23. The method of claim 13, wherein the KASIII is from *Aeromonas hydrophila*, *Bacteroides vulgatus*, *Brevibacterium linens*, *Capnocytophaga gingivalis*, *Thermus aquaticus*, *Bacillus licheniformis*, *Desulfovibrio vulgaris*, *Bacillus subtilis* subsp. *S*, *Haliangium ochraceum*, *Alicyclobacillus acidocaldarius*, *Staphylococcus aureus*, *Legionella pneumophila*, or *Myxococcus xanthus*.

24. An isolated or purified nucleic acid molecule comprising a nucleotide sequence encoding a mutant KASIII, which comprises one or more mutations comprising at least one mutation, which causes the rotamer conformation of a

phenylalanine in the KASIII corresponding to Phe304 in KASIII from *E. coli* to change, wherein the isolated or purified nucleic acid molecule can be a vector.

25. The isolated or purified nucleic acid molecule of claim 24, wherein the KASIII is from *E. coli* and the one or more mutations comprises a mutation of Leu220, alone or in further combination with Val215.

26. The isolated or purified nucleic acid molecule of claim 25, wherein the mutation of Leu220 is Leu220Met and the mutation of Val215 is Val215Phe.

27. The isolated or purified nucleic acid molecule of claim 23 or 24, wherein the rotamer conformation of Phe304 changes from active site-distal to active site-proximal.

28. The isolated or purified nucleic acid molecule of claim 24, wherein the KASIII is KASIIIA from *B. subtilis* and the one or more mutations comprises a mutation of Met213, alone or in further combination with Phe208.

29. The isolated or purified nucleic acid molecule of claim 28, wherein the mutation of Met213 is Met213Leu and the mutation of Phe208 is Phe208Val.

30. The isolated or purified nucleic acid molecule of claim 28 or 29, wherein the rotamer conformation of Phe297 changes from active site-proximal to active site-distal.

31. The isolated or purified nucleic acid molecule of claim 24, wherein the KASIII is KASIIIB from *B. subtilis* and the one or more mutations comprise a mutation of Trp221 and a mutation of Val226.

32. The isolated or purified nucleic acid molecule of claim 31, wherein the mutation of Trp221 is Trp221Val and the mutation of Val226 is Val226Leu.

33. The isolated or purified nucleic acid molecule of claim 31 or 32, wherein the rotamer conformation of Phe310 changes from active site-proximal to active site-distal.

34. The isolated or purified nucleic acid molecule of claim 24, wherein the KASIII is from the KASIII is from *Aeromonas hydrophila*, *Bacteroides vulgatus*, *Brevibacterium linens*, *Capnocytophaga gingivalis*, *Thermus aquaticus*, *Bacillus licheniformis*, *Desulfovibrio vulgaris*, *Bacillus subtilis subsp. S*, *Haliangium ochraceum*, *Alicyclobacillus acidocaldarius*, *Staphylococcus aureus*, *Legionella pneumophila*, or *Myxococcus xanthus*.

35. A host cell or organism comprising the isolated or purified nucleic acid molecule of any of claims 24-34.

36. An isolated or purified mutant KASIII, which comprises one or more mutations comprising at least one mutation, which causes the rotamer conformation of a phenylalanine in the KASIII corresponding to Phe304 in KASIII from *E. coli* to change.

37. The isolated or purified mutant KASIII of claim 36, wherein the KASIII is from *E. coli* and the one or more mutations comprises a mutation of Leu220, alone or in further combination with Val215.

38. The isolated or purified mutant KASIII of claim 37, wherein the mutation of Leu220 is Leu220Met and the mutation of Val215 is Val215Phe.

39. The isolated or purified mutant KASIII of claim 37 or 38, wherein the rotamer conformation of Phe304 changes from active site-distal to active site-proximal.

40. The isolated or purified mutant KASIII of claim 36, wherein the KASIII is KASIIIA from *B. subtilis* and the one or more mutations comprises a mutation of Met213, alone or in further combination with Phe208.

41. The isolated or purified mutant KASIII of claim 40, wherein the mutation of Met213 is Met213Leu and the mutation of Phe208 is Phe208Val.

42. The isolated or purified mutant KASIII of claim 40 or 41, wherein the rotamer conformation of Phe297 changes from active site-proximal to active site-distal.

43. The isolated or purified mutant KASIII of claim 36, wherein the KASIII is KASIIIB from *B. subtilis* and the one or more mutations comprise a mutation of Trp221 and a mutation of Val226.

44. The isolated or purified mutant KASIII of claim 43, wherein the mutation of Trp221 is Trp221Val and the mutation of Val226 is Val226Leu.

45. The isolated or purified mutant KASIII of claim 43 or 44, wherein the rotamer conformation of Phe310 changes from active site-proximal to active site-distal.

46. The isolated or purified mutant KASIII of claim 36, wherein the KASIII is from *Aeromonas hydrophila*, *Bacteroides vulgatus*, *Brevibacterium linens*, *Capnocytophaga gingivalis*, *Thermus aquaticus*, *Bacillus licheniformis*, *Desulfovibrio vulgaris*, *Bacillus subtilis subsp. S*, *Haliangium ochraceum*, *Alicyclobacillus acidocaldarius*, *Staphylococcus aureus*, *Legionella pneumophila*, or *Myxococcus xanthus*.

47. An *in vitro*, high-throughput spectrophotometric method of assaying KASIII activity, which method comprises:

- (i) incubating holo-ACP, malonyl-CoA, acyl-CoA, NADPH, and malonyl-CoA:ACP transacylase (FabD),
- (ii) adding KASIII and 3-ketoacyl-ACP reductase (FabG), and
- (iii) measuring the change in absorbance at 340 nm when NADPH is converted to NADP⁺ during reduction of 3-ketoacyl-ACP to 3-hydroxyl-acyl ACP by FabG,

whereupon KASIII activity is assayed in an *in vitro*, high-throughput spectrophotometric method.

48. The method of claim 47, wherein acyl-CoA is a straight-chain-CoA, a branched-chain-CoA or a hydroxylated-CoA.

49. A method of producing bi-functional fatty acids in a host cell or organism, which method comprises introducing into a host cell or organism, which comprises one or more ω -functionalized acyl-CoAs, and expressing therein a nucleic acid molecule comprising a nucleotide sequence encoding a KASIII, which can use one or more of the ω -functionalized acyl-CoAs as a substrate, whereupon bi-functional fatty acids in the host cell or organism are produced.

50. The method of claim 49, wherein the one or more ω -functionalized acyl-CoAs is functionalized at the ω position with a moiety comprising a hydroxyl group, a benzoyl group, a cyclic group, a branched chain group, an amino group, or a halo group.

51. The method of claim 49, wherein the host cell or organism is the mutant *R. rubrum* of claim 5 or 6.

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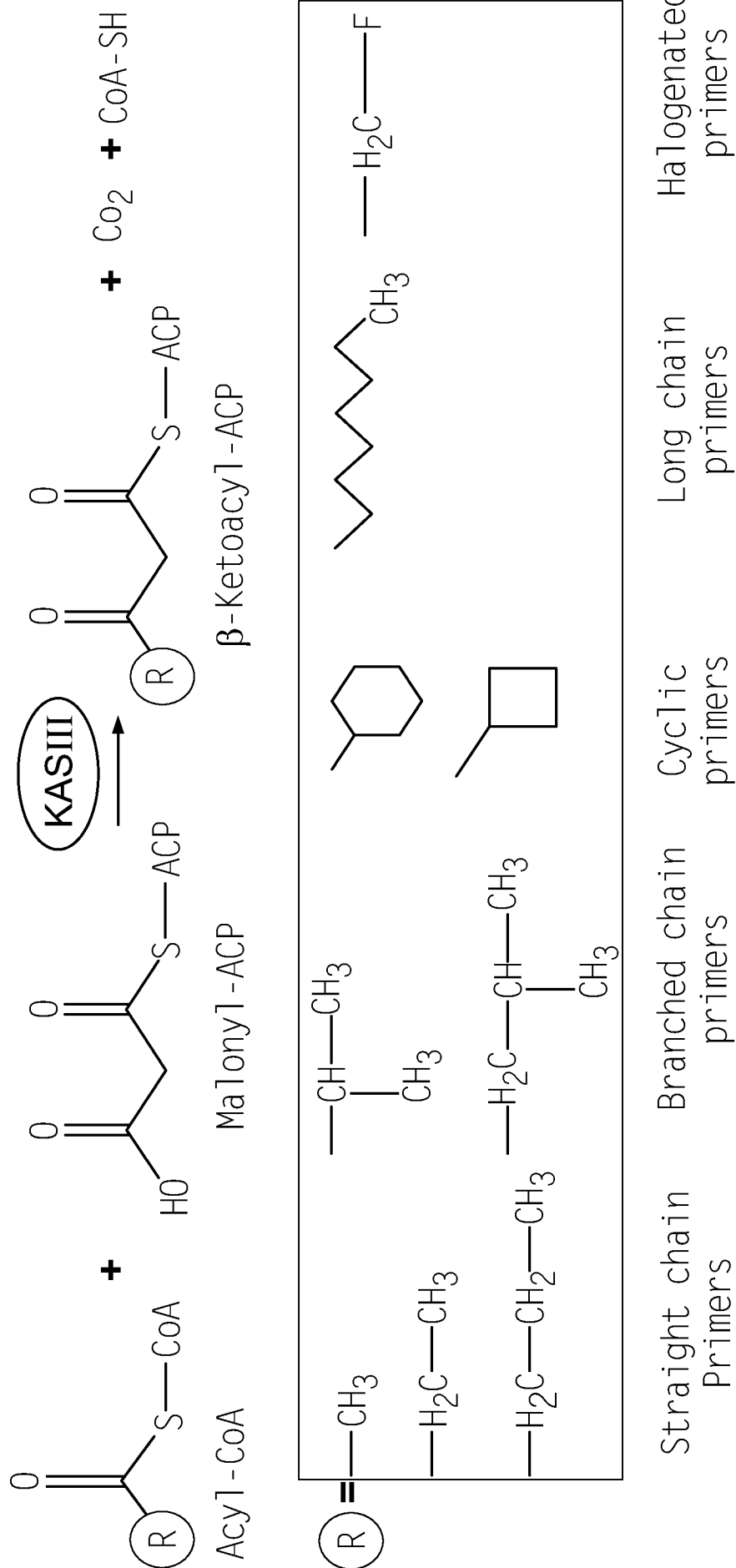


FIG. 1

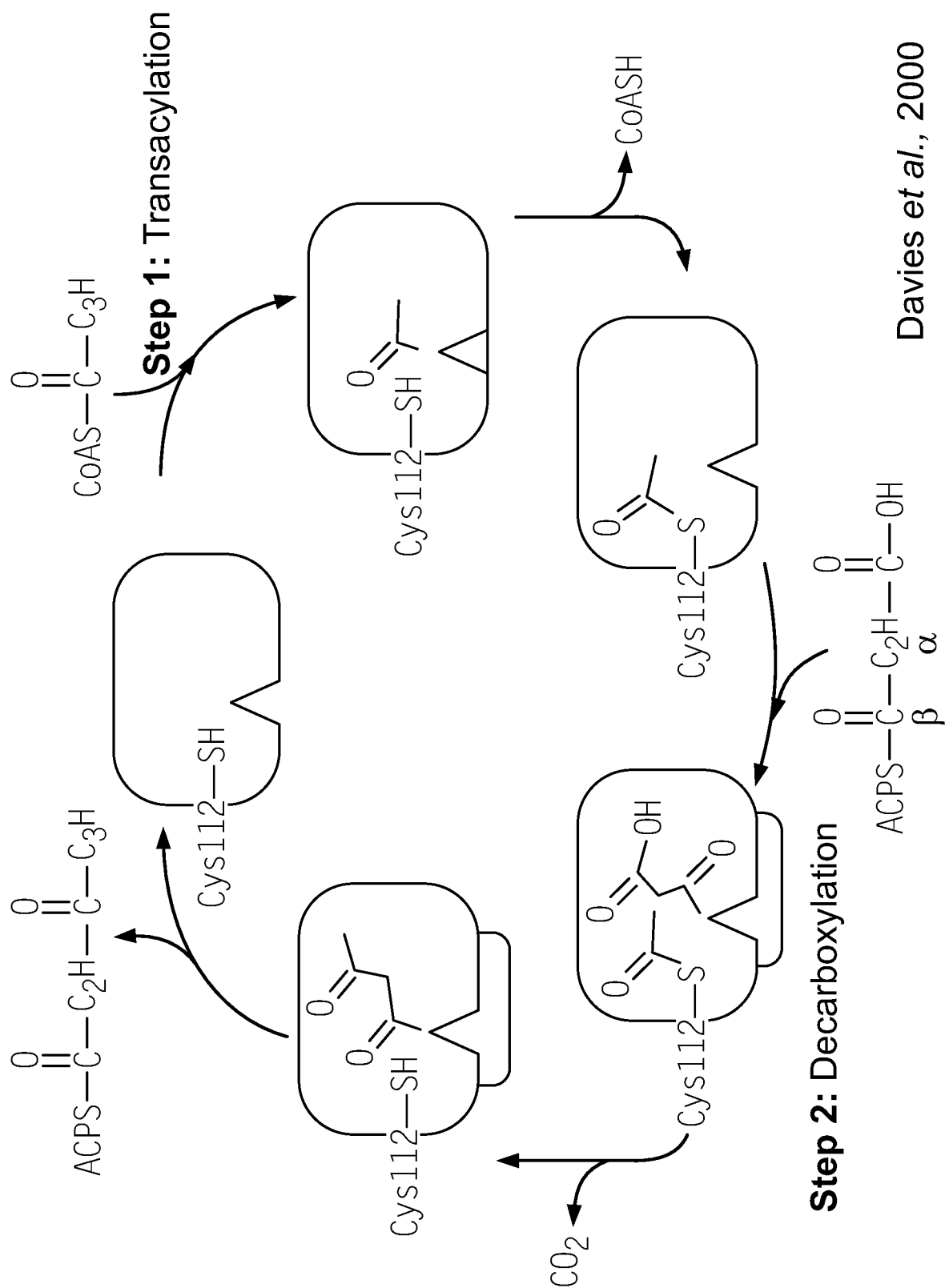


FIG. 2

Davies et al., 2000

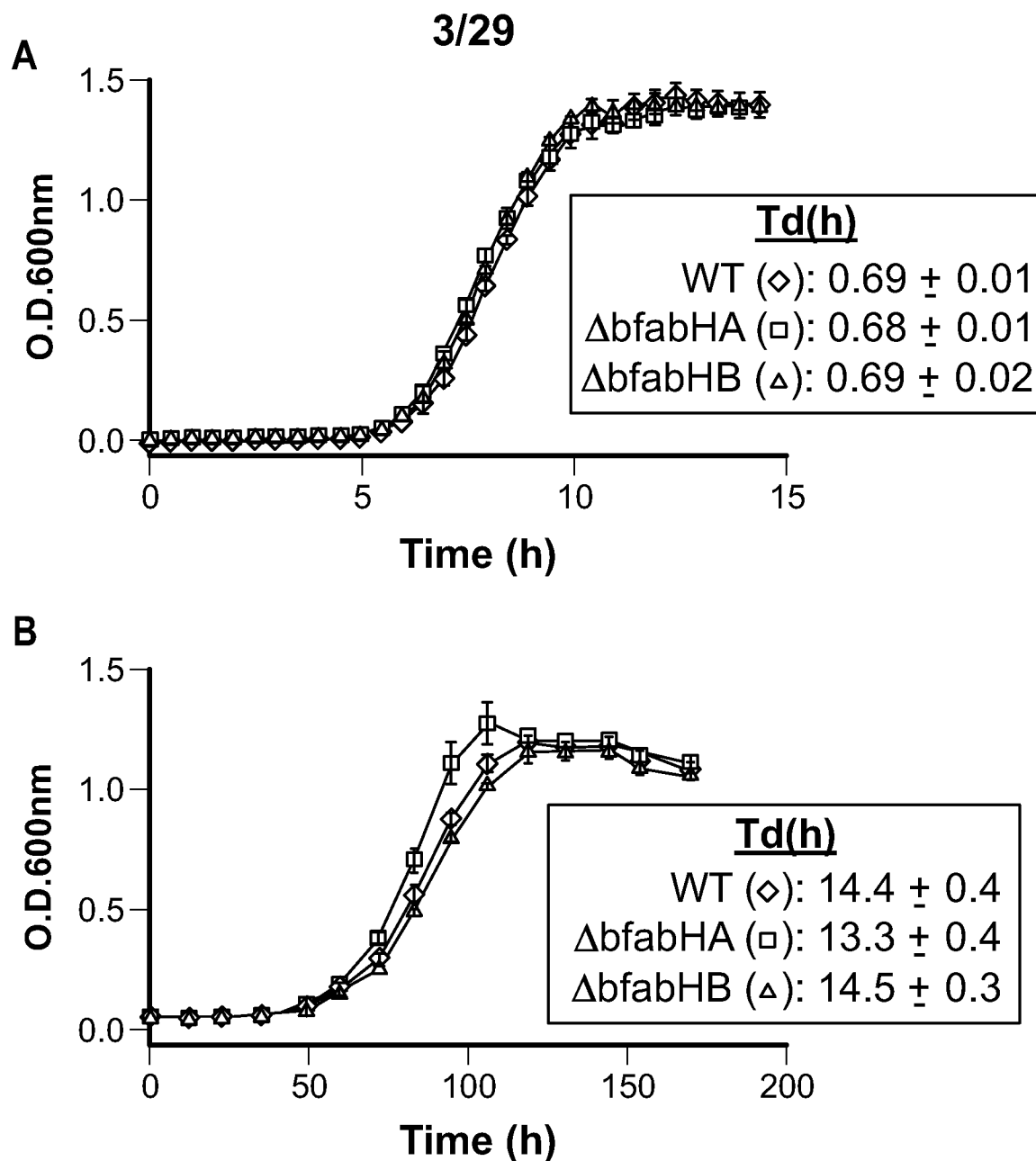


FIG. 3

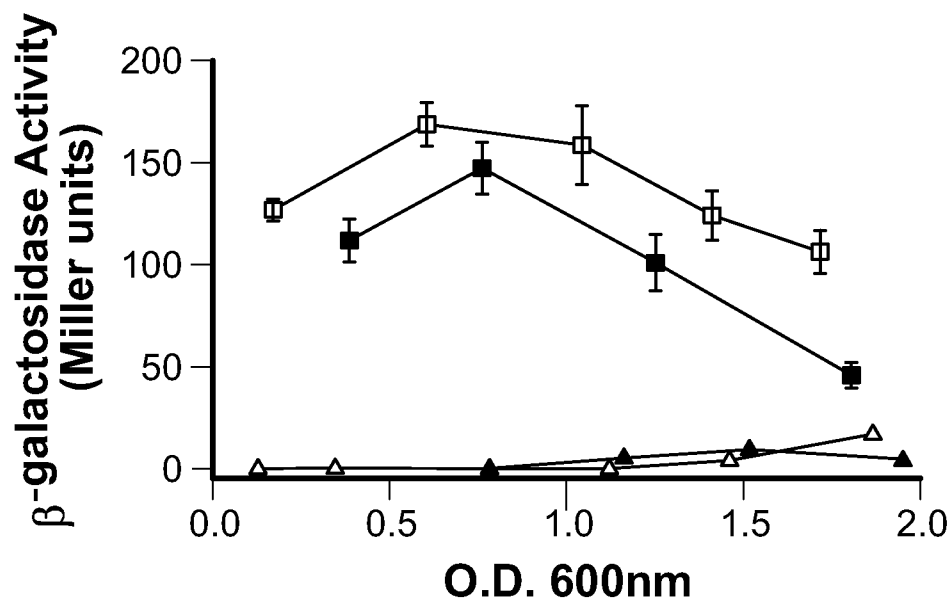


FIG. 4

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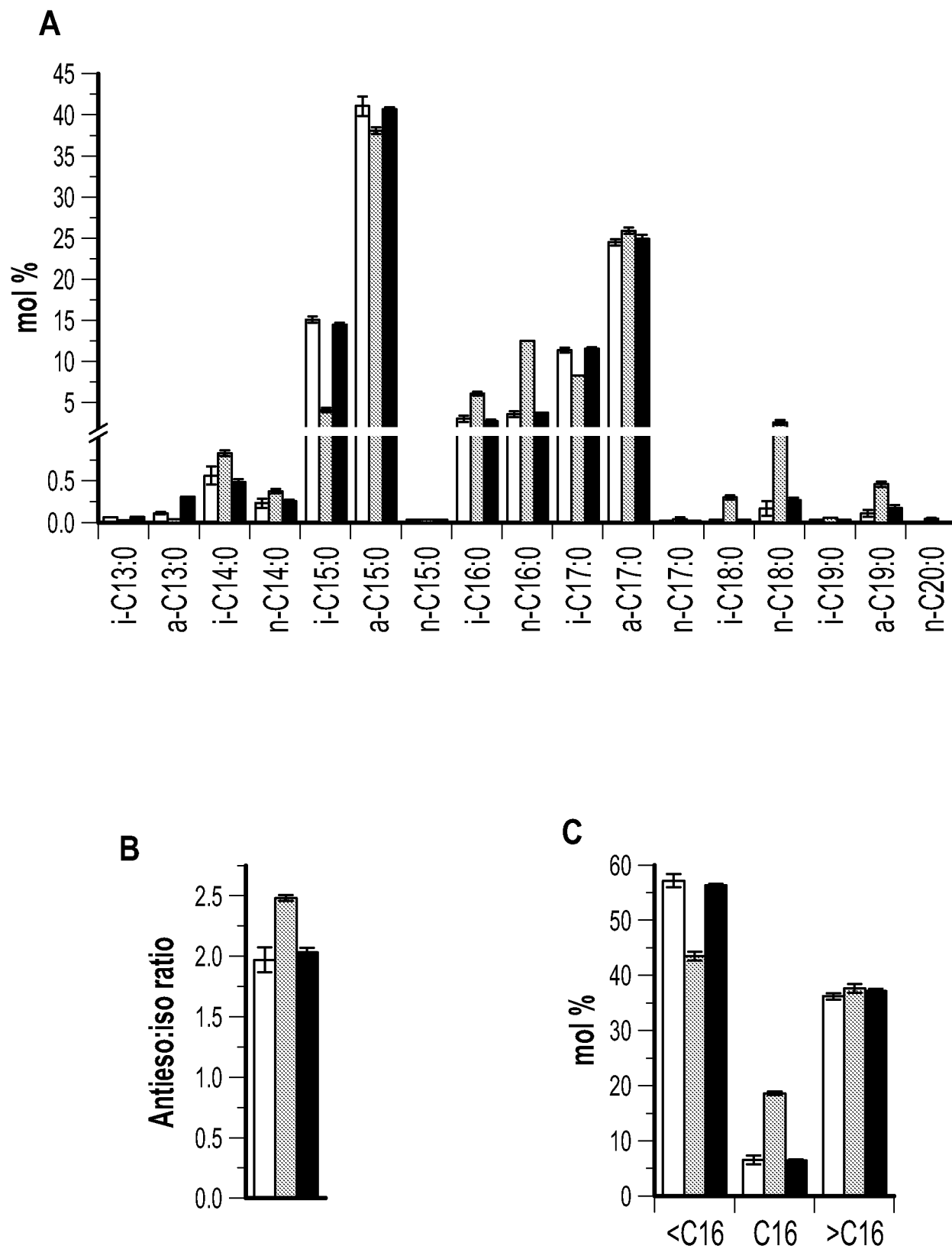


FIG. 5

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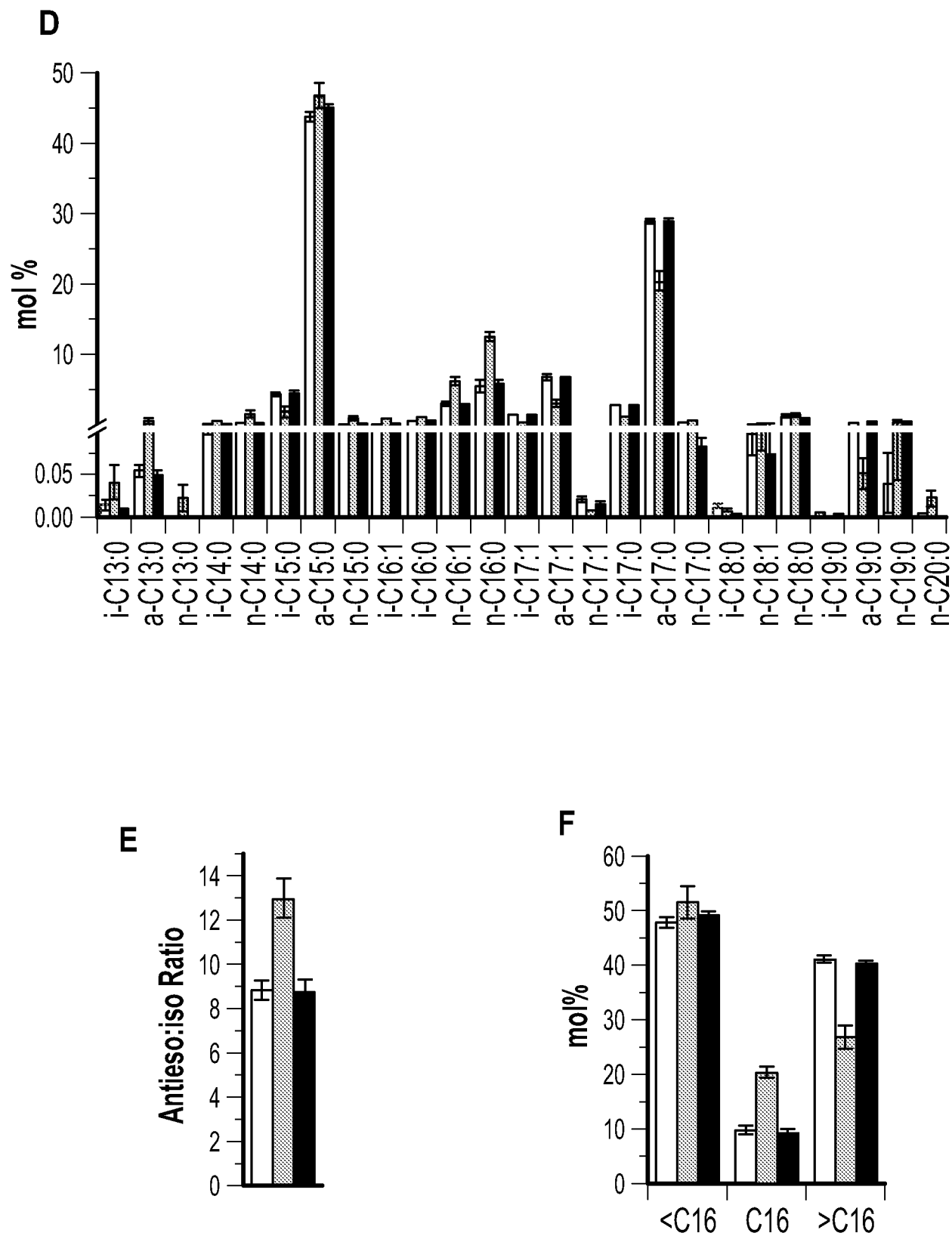


FIG. 5 (Cont.)

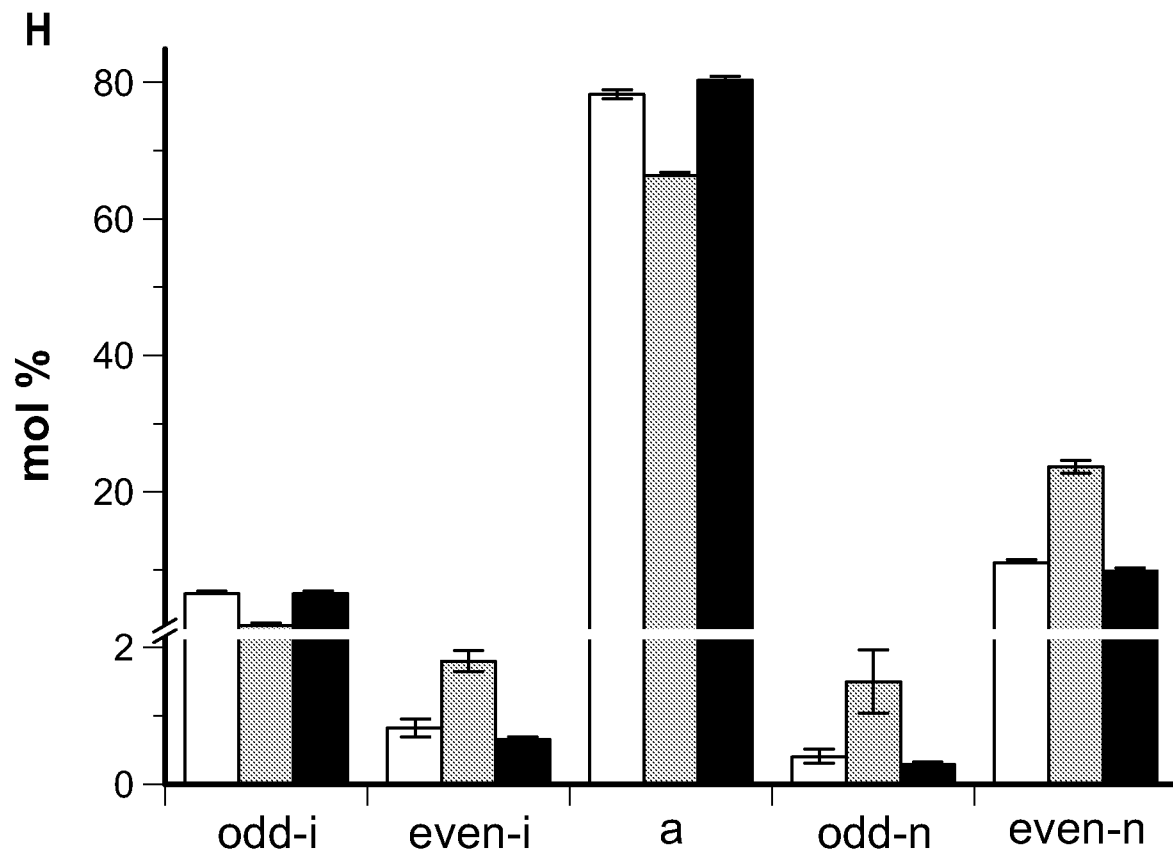
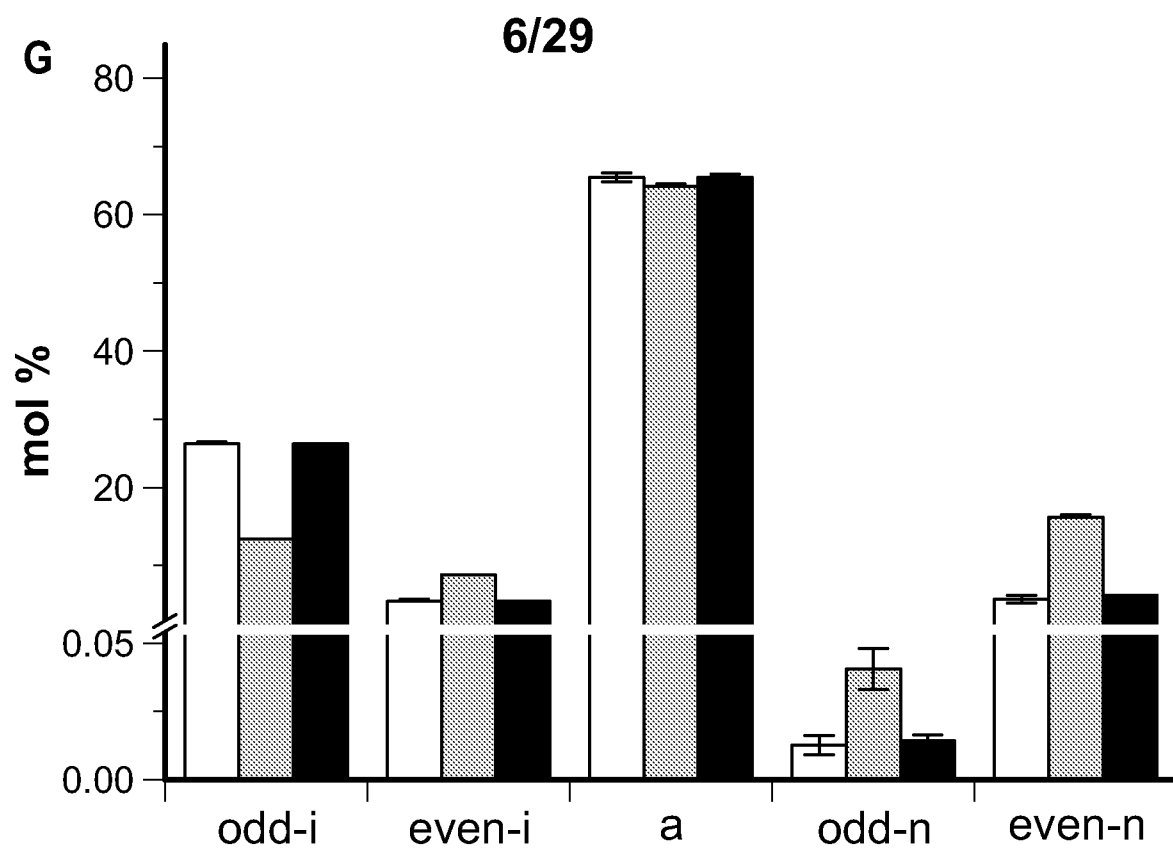
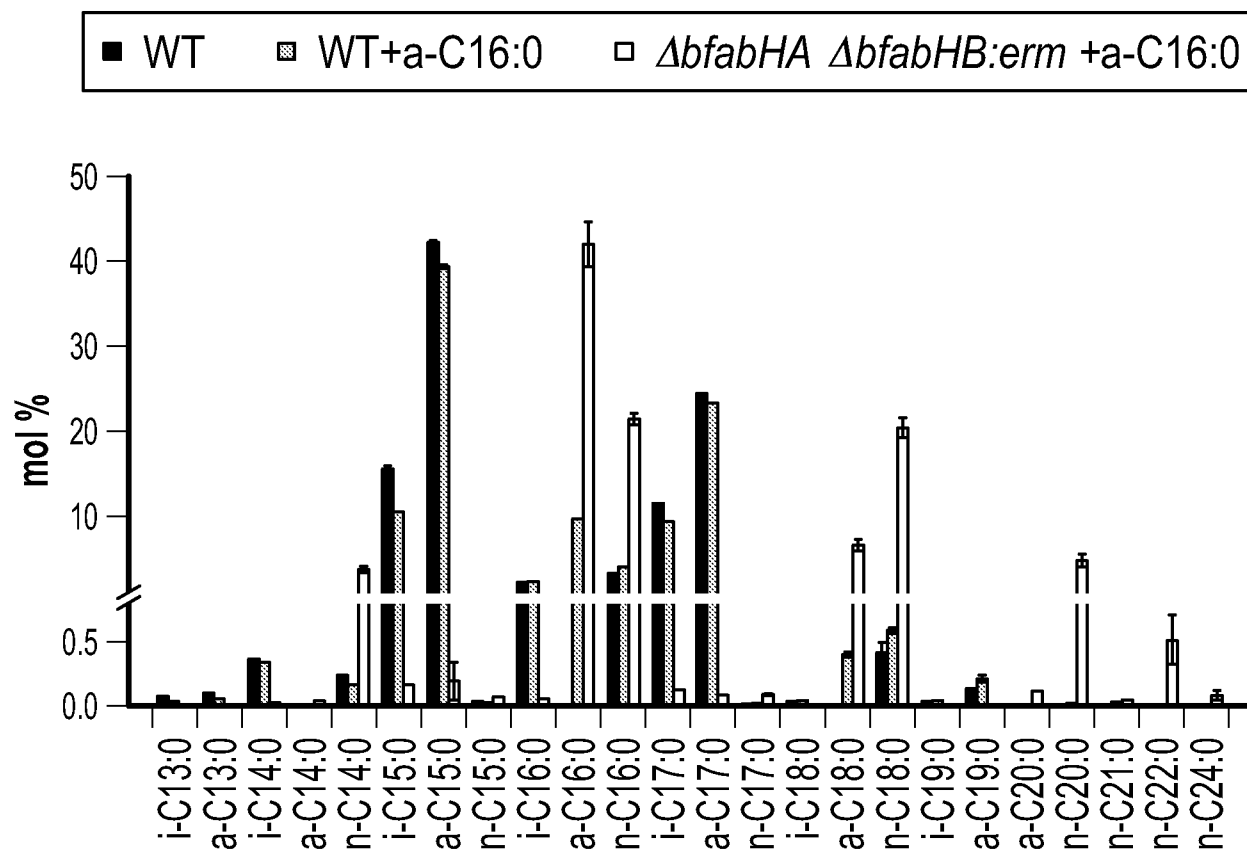
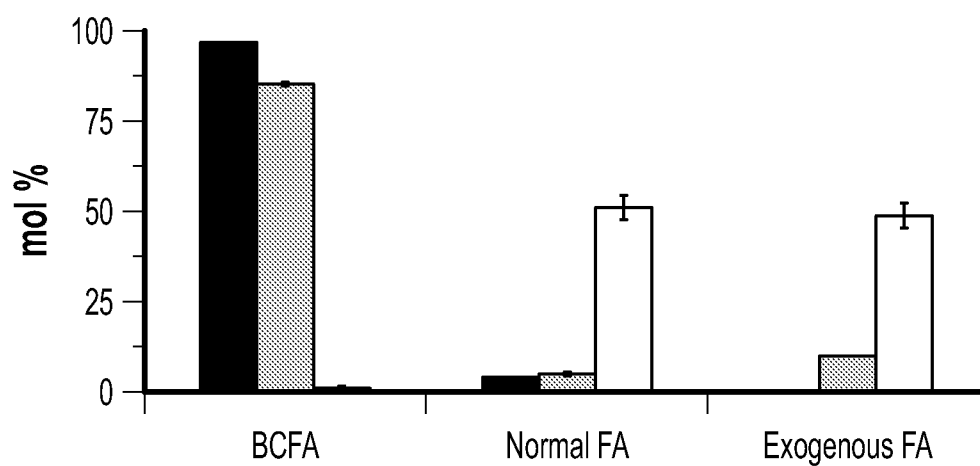


FIG. 5 (Cont.)

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A**B****FIG. 6**

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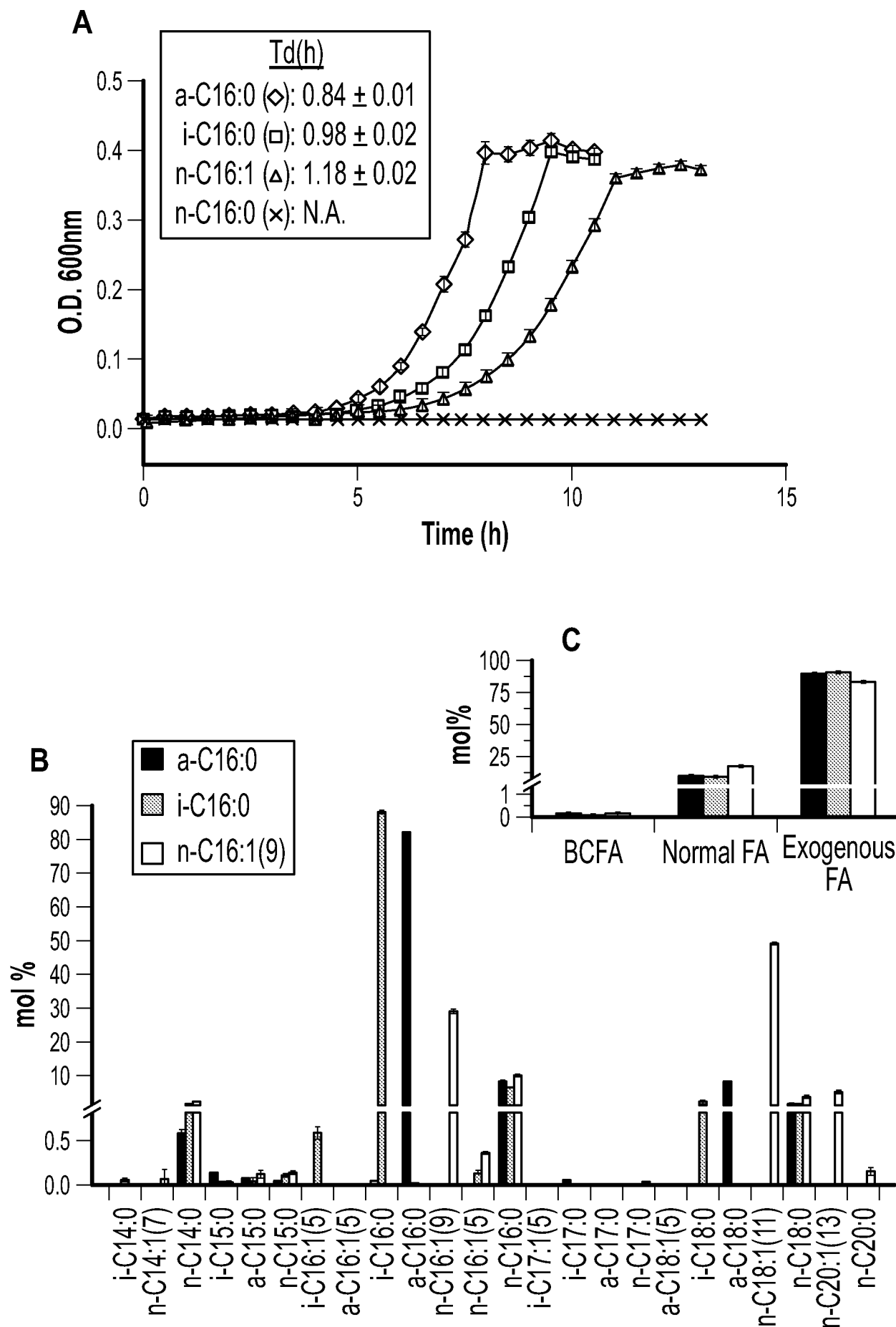
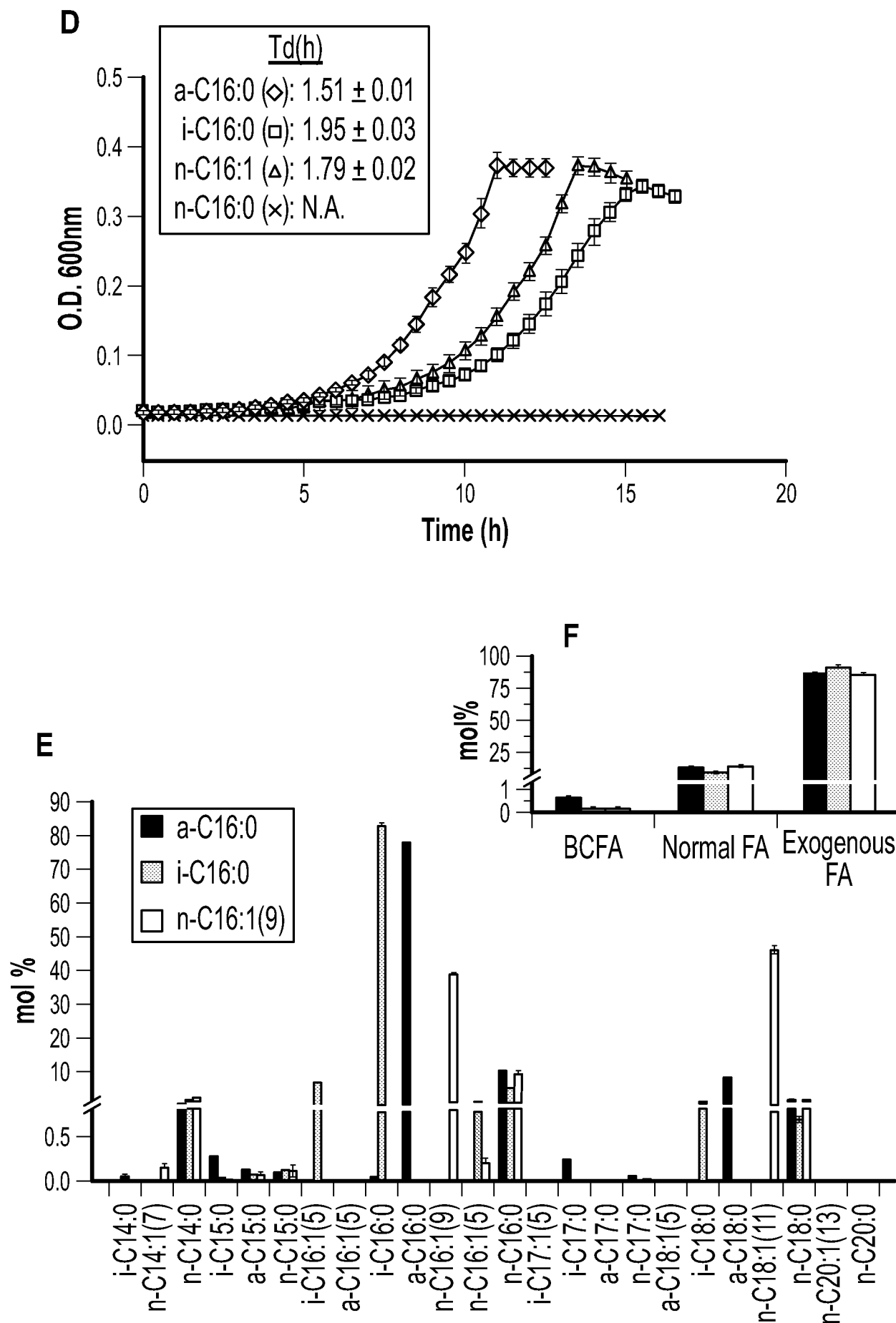


FIG. 7

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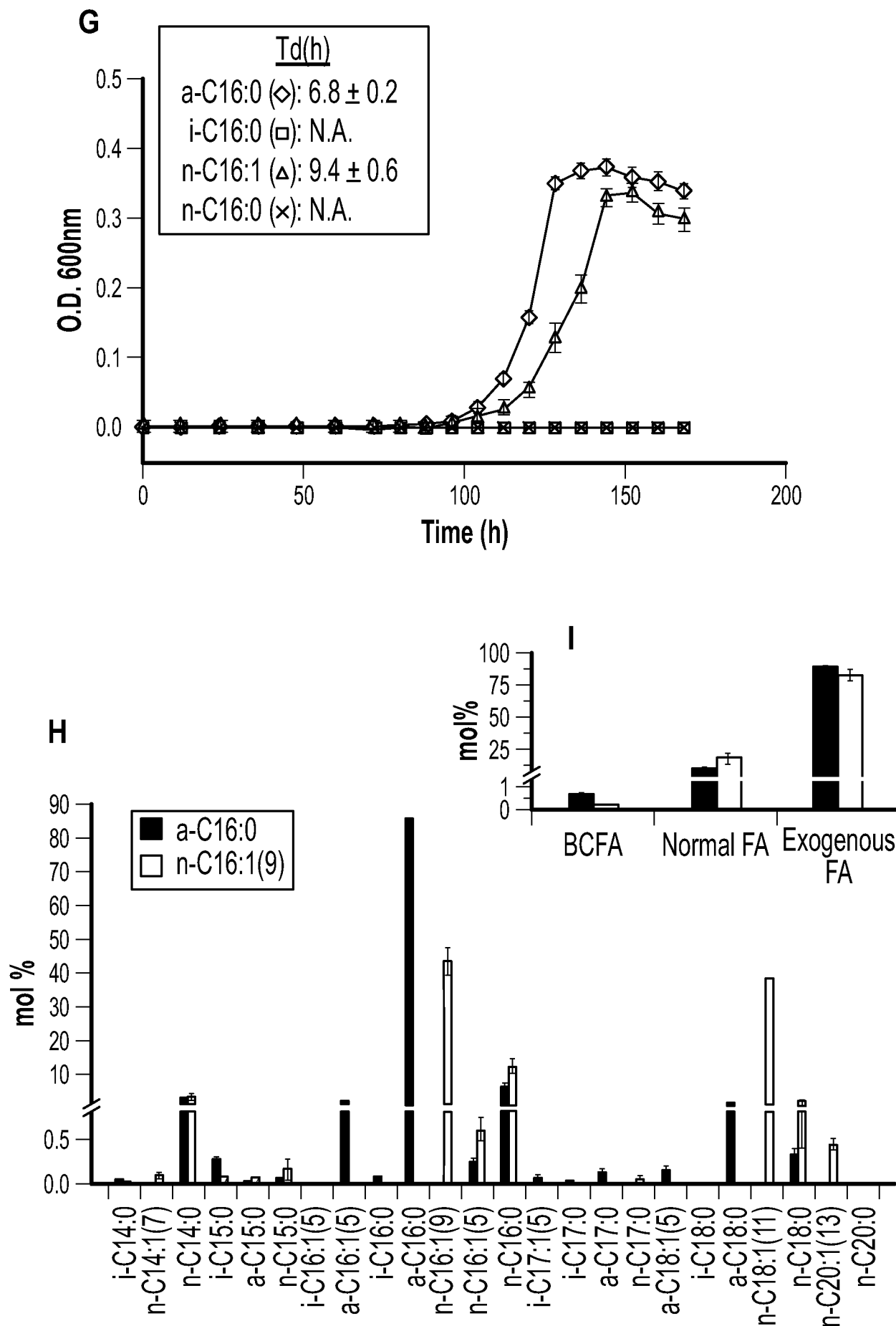
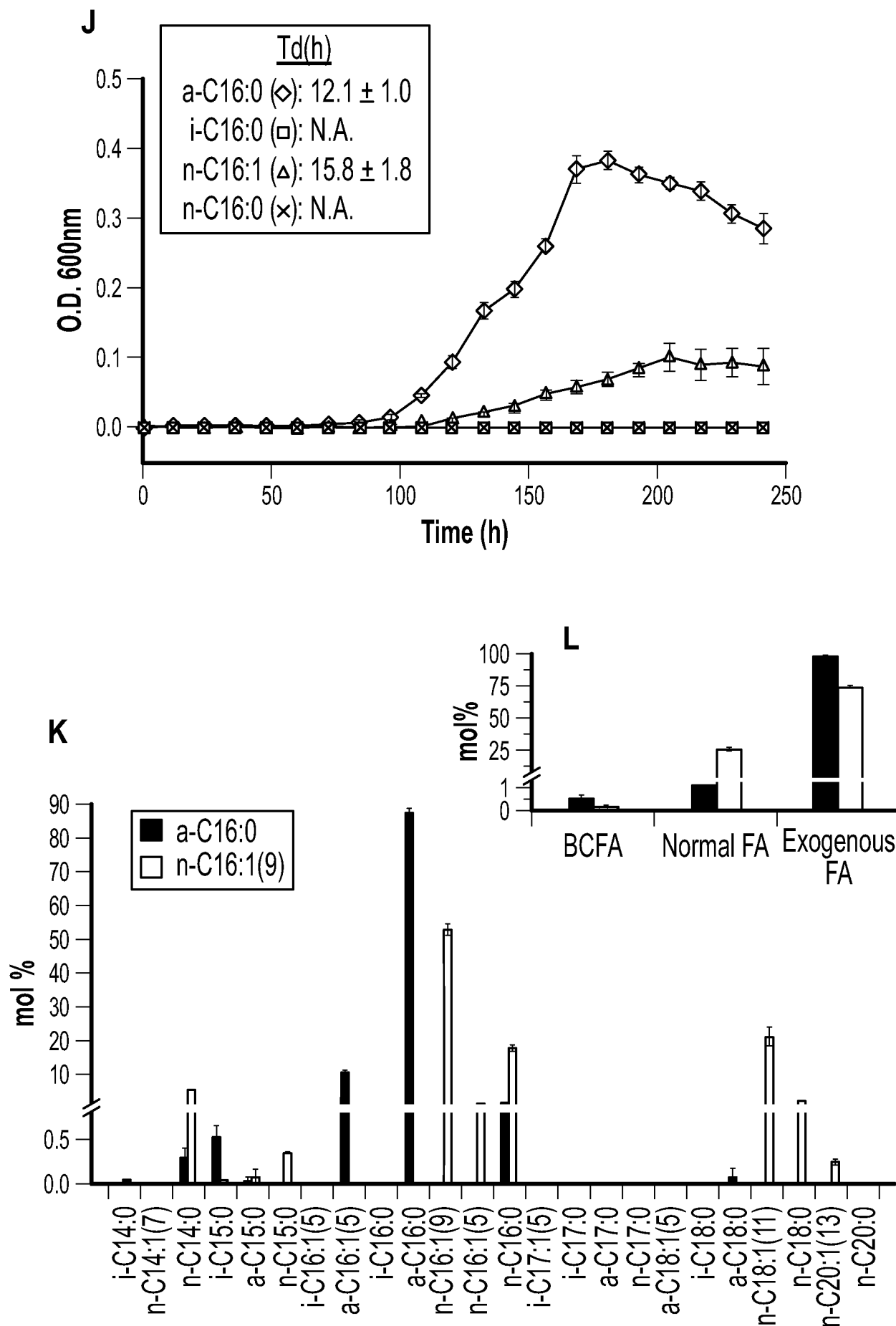


FIG. 7 (Cont.)

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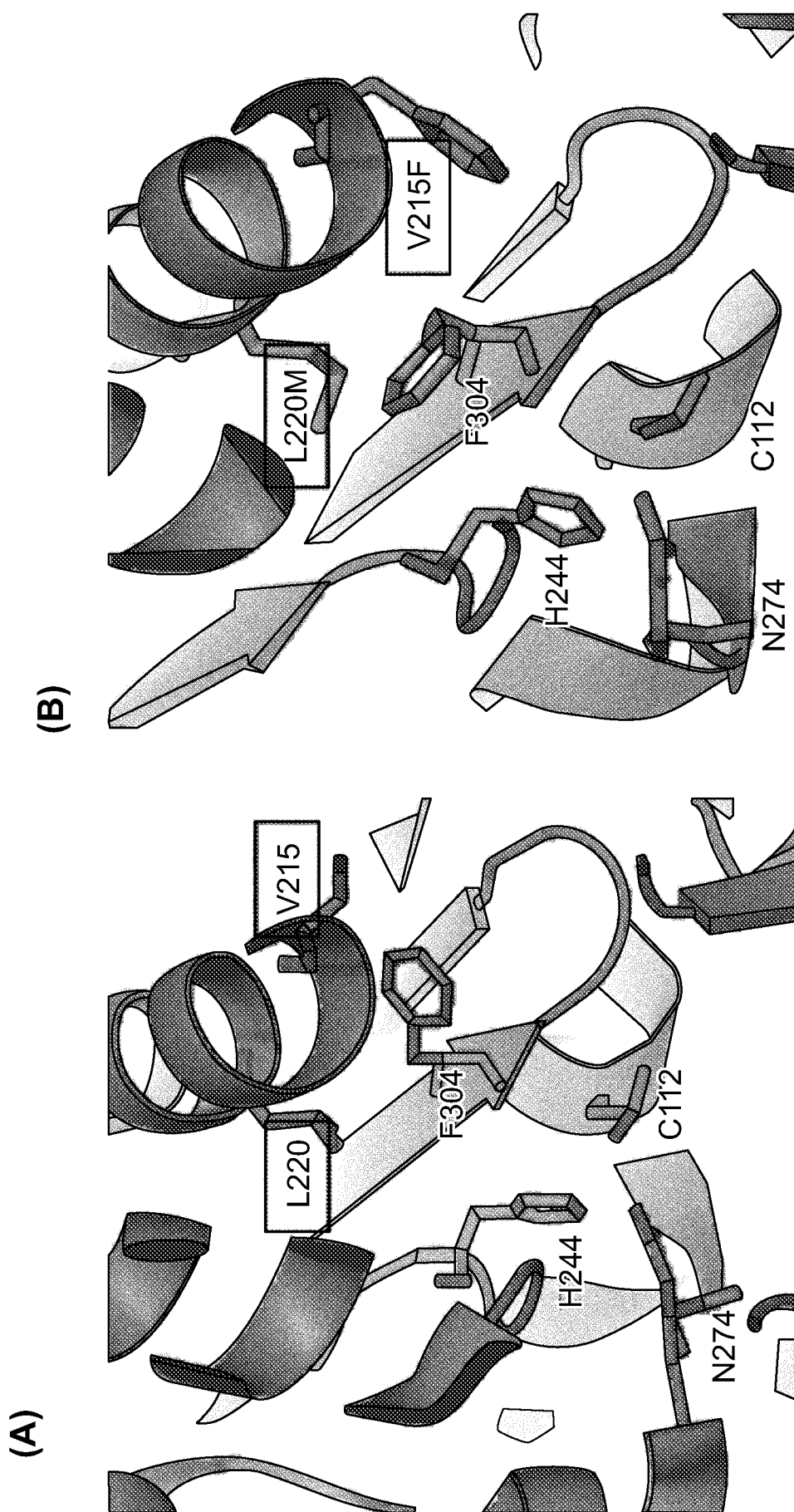


FIG. 8

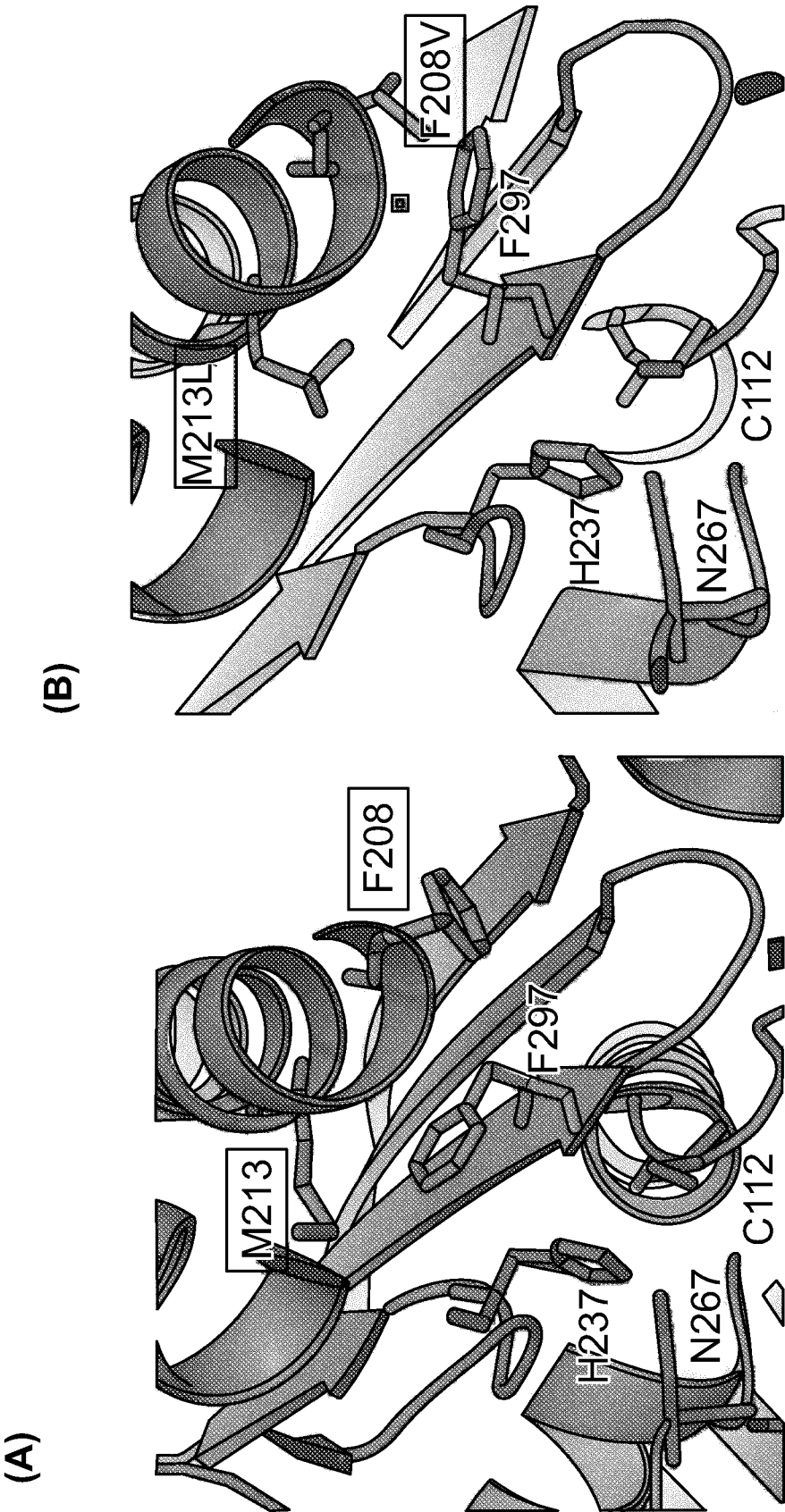


FIG. 9

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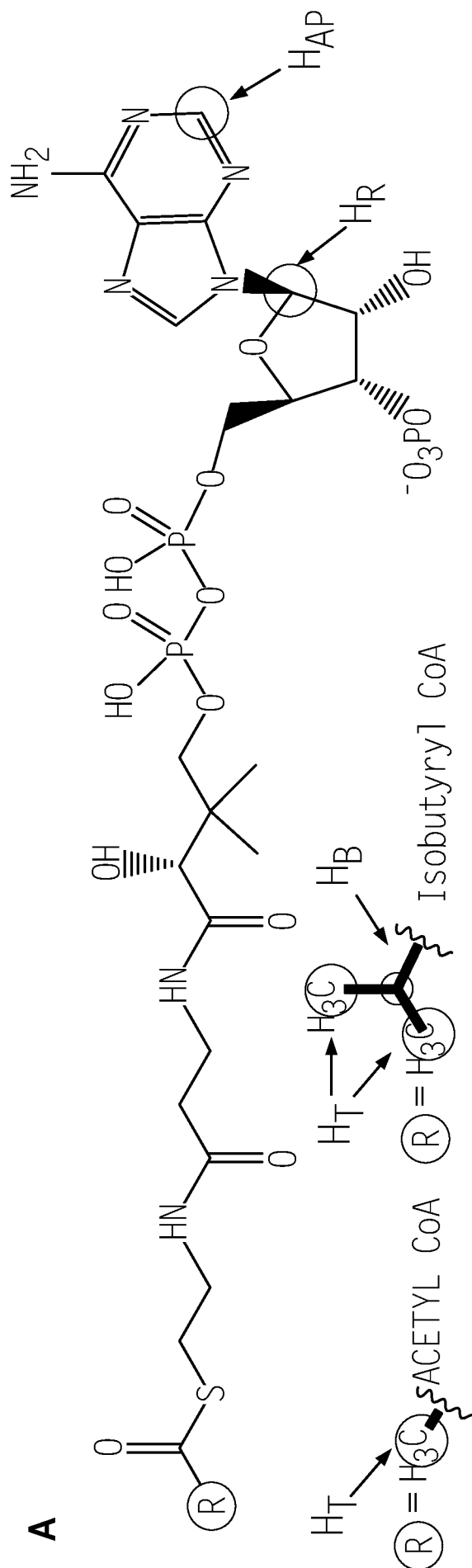


FIG. 10

A

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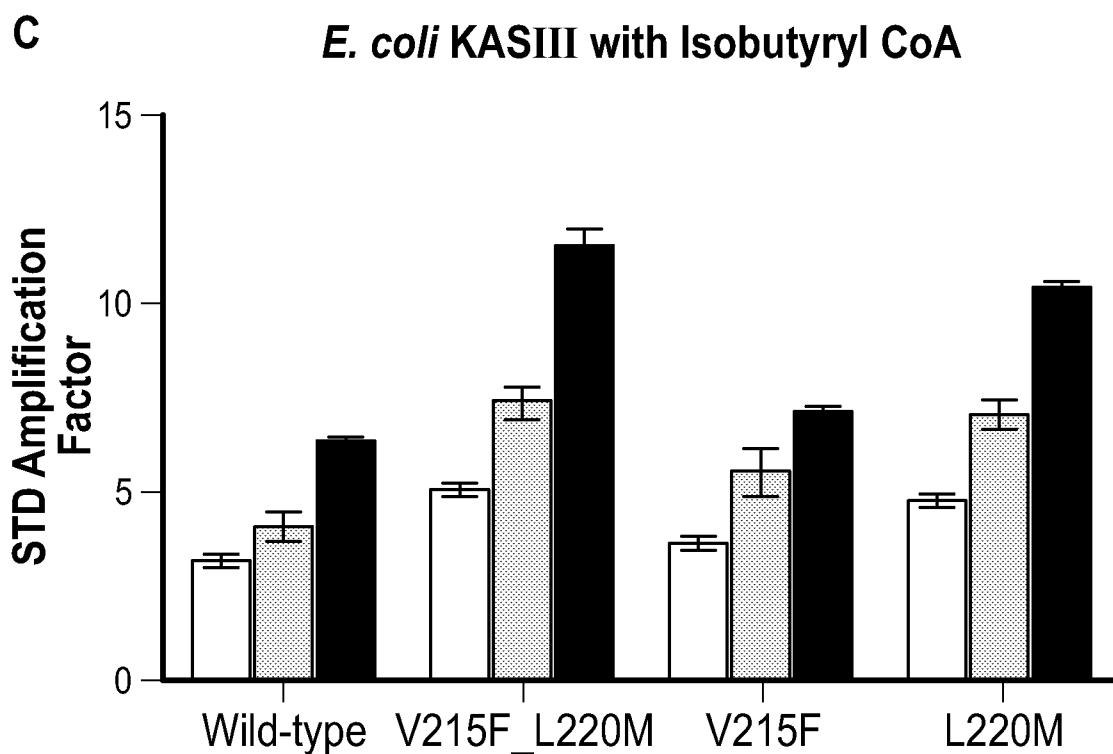
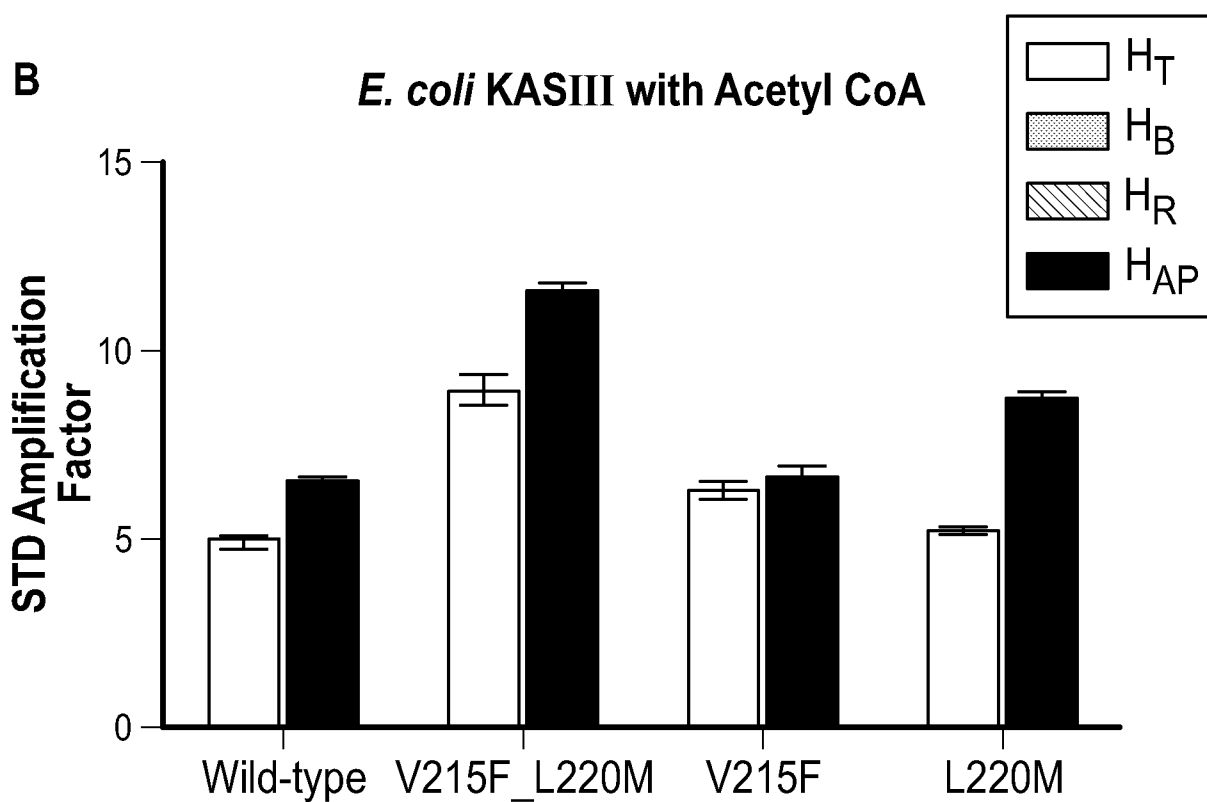


FIG. 10 (Cont.)

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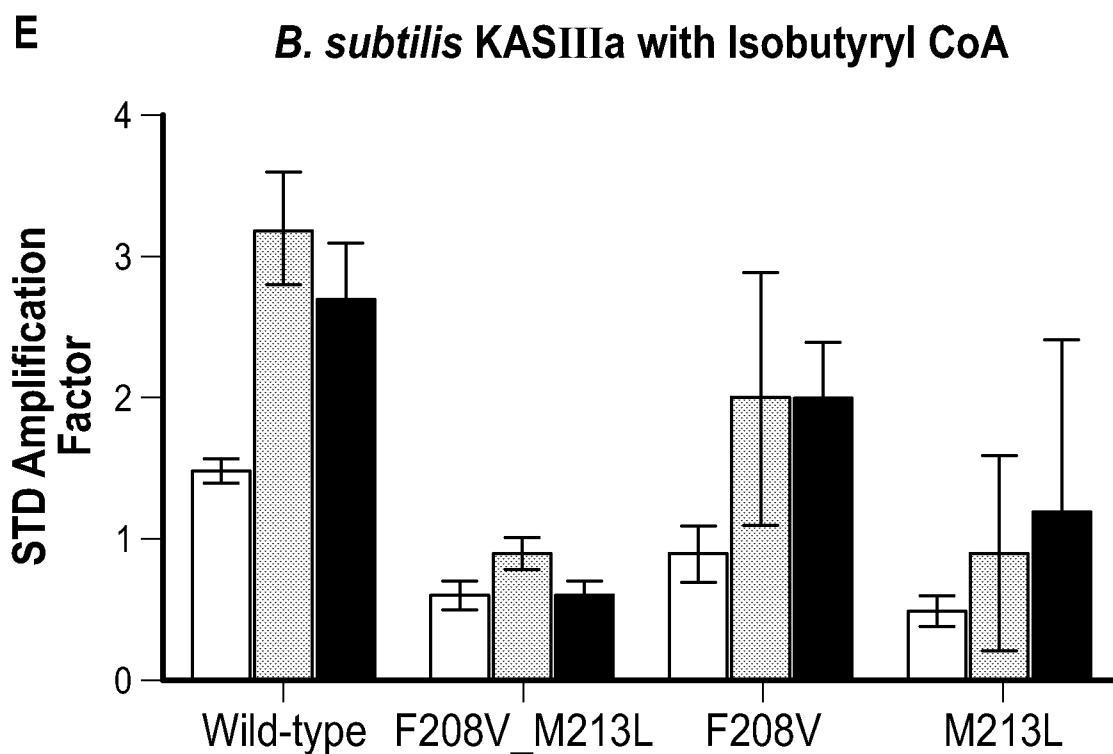
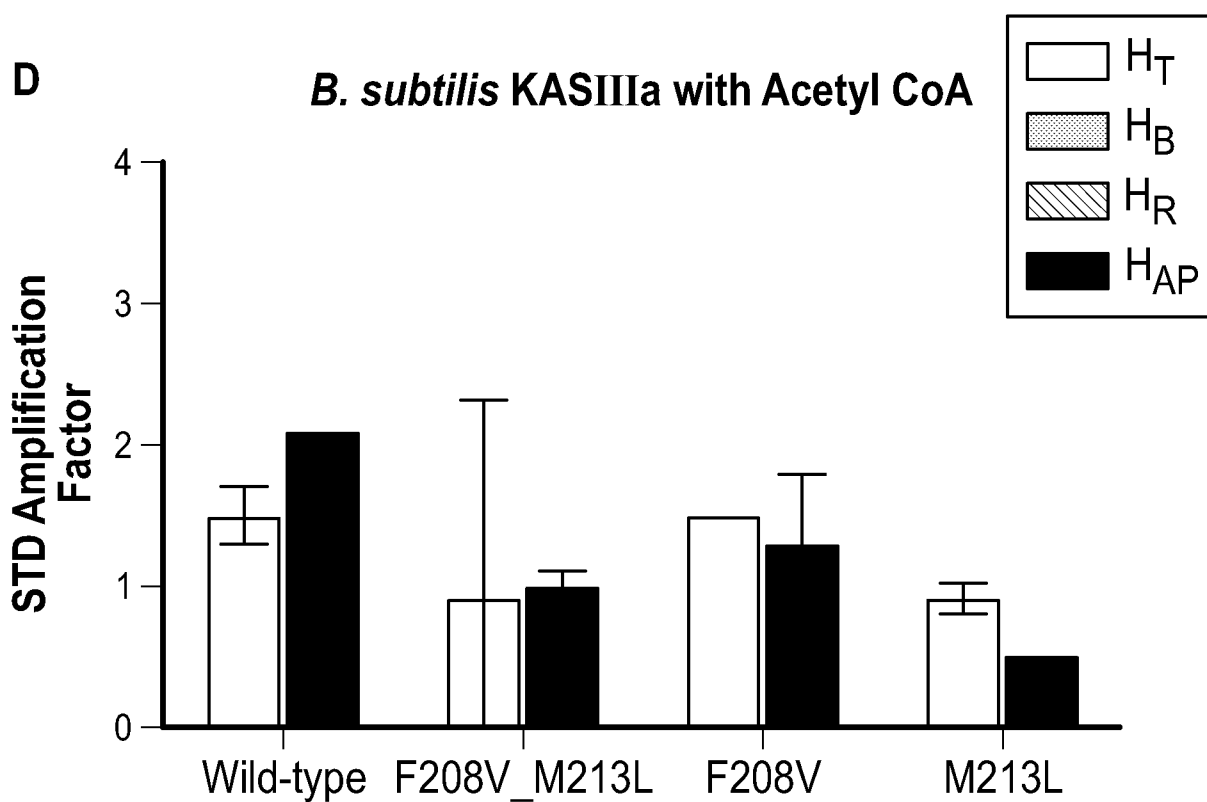


FIG. 10 (Cont.)

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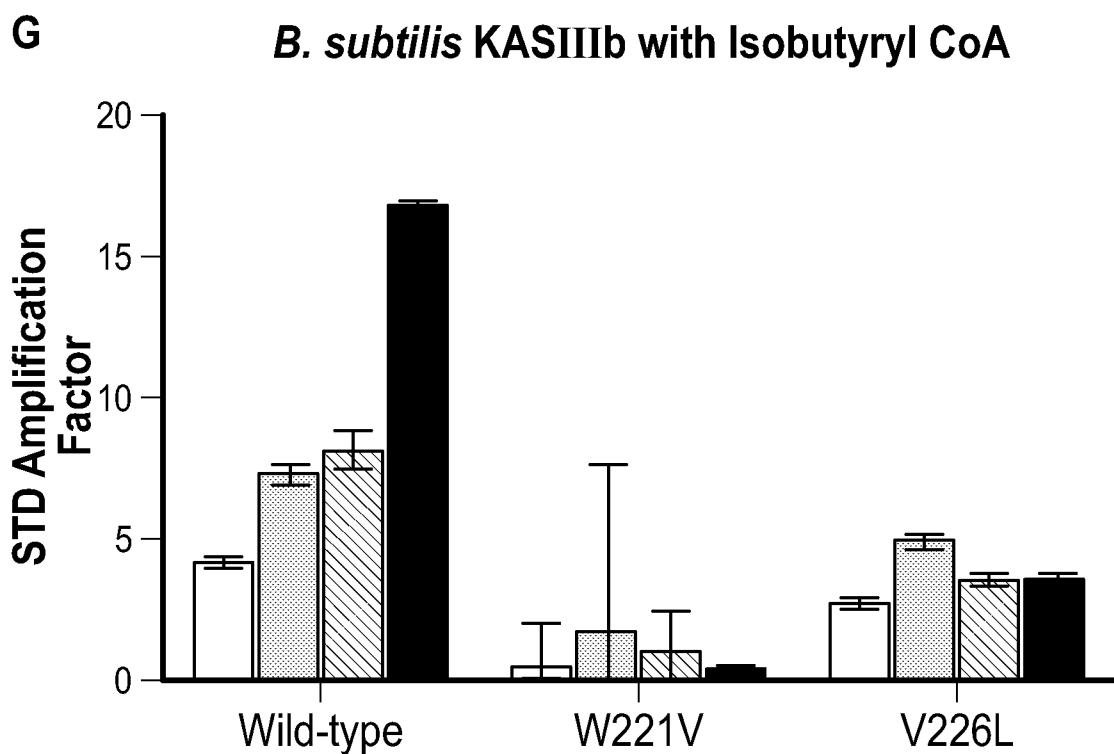
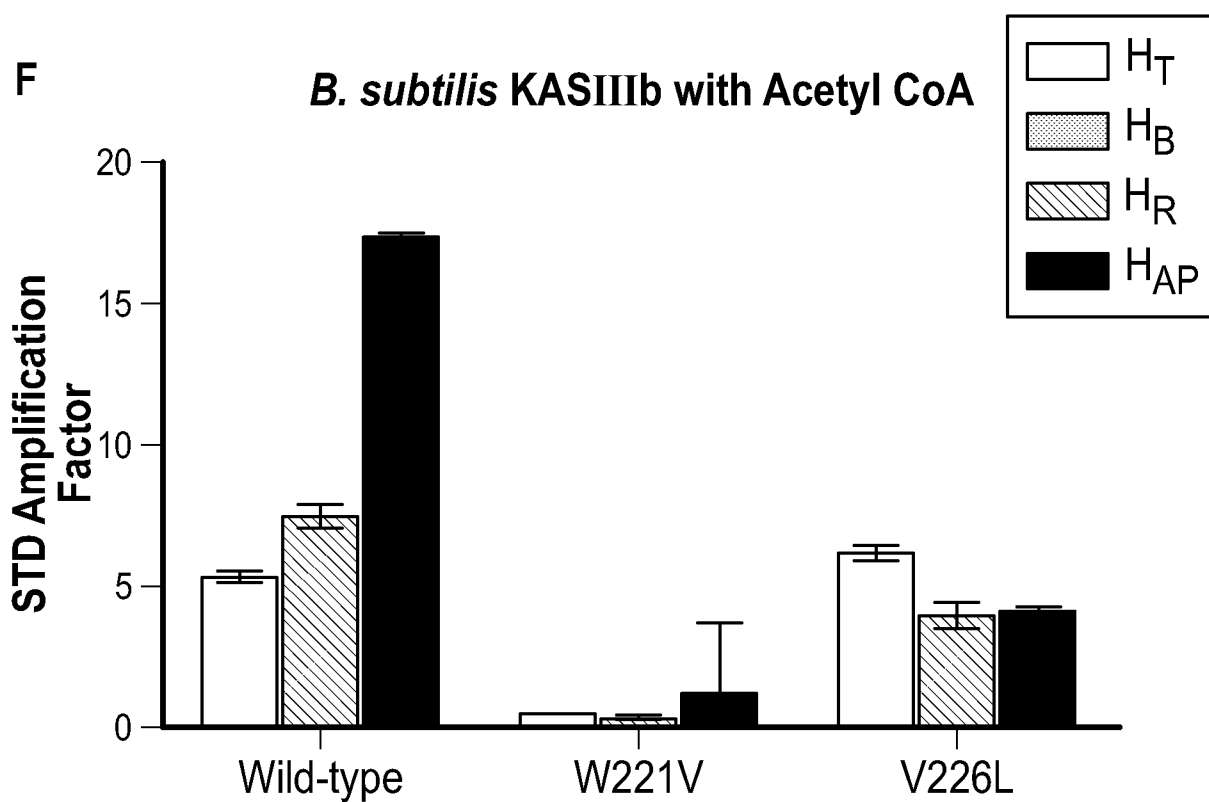


FIG. 10 (Cont.)

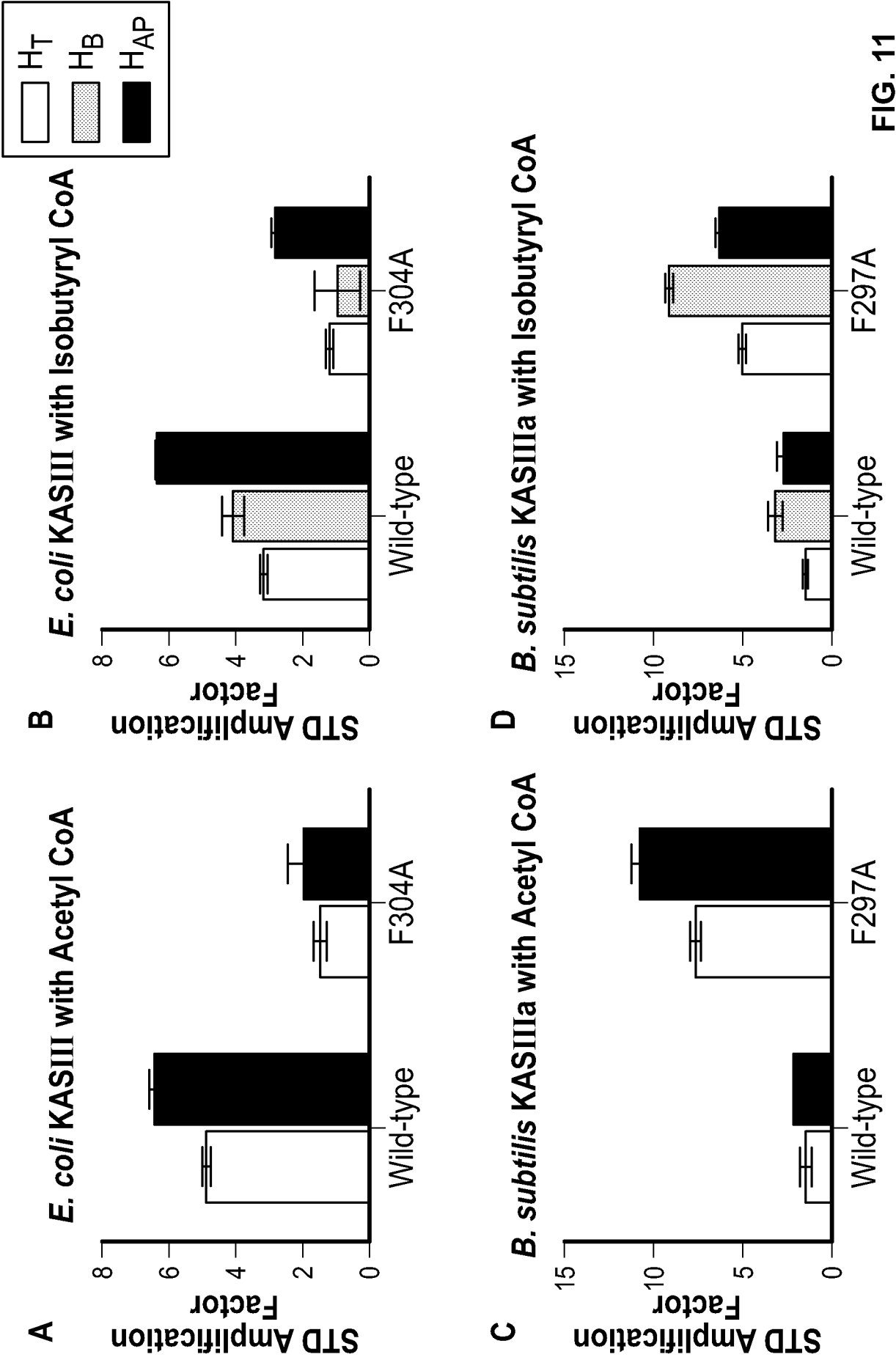


FIG. 11

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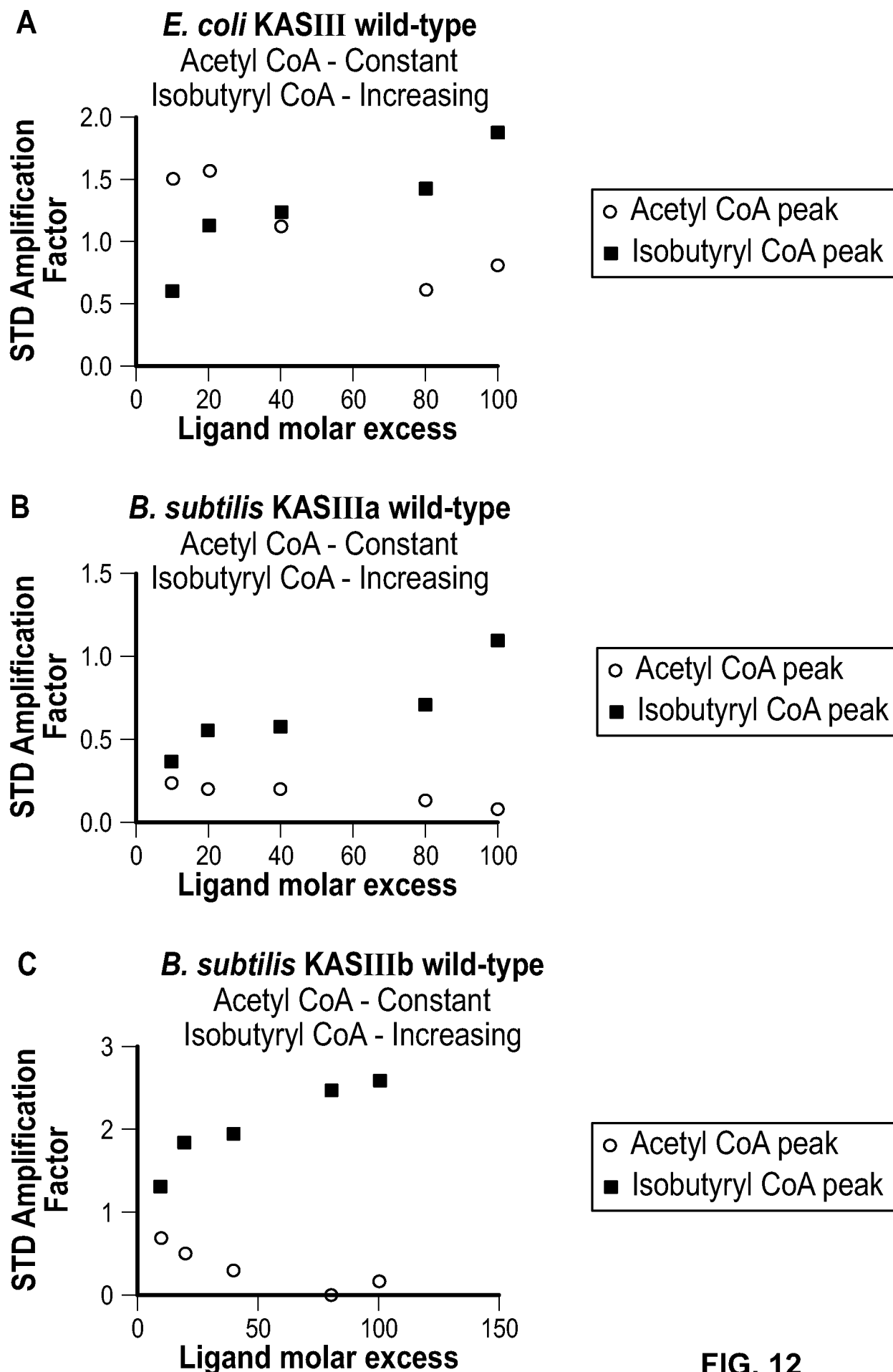
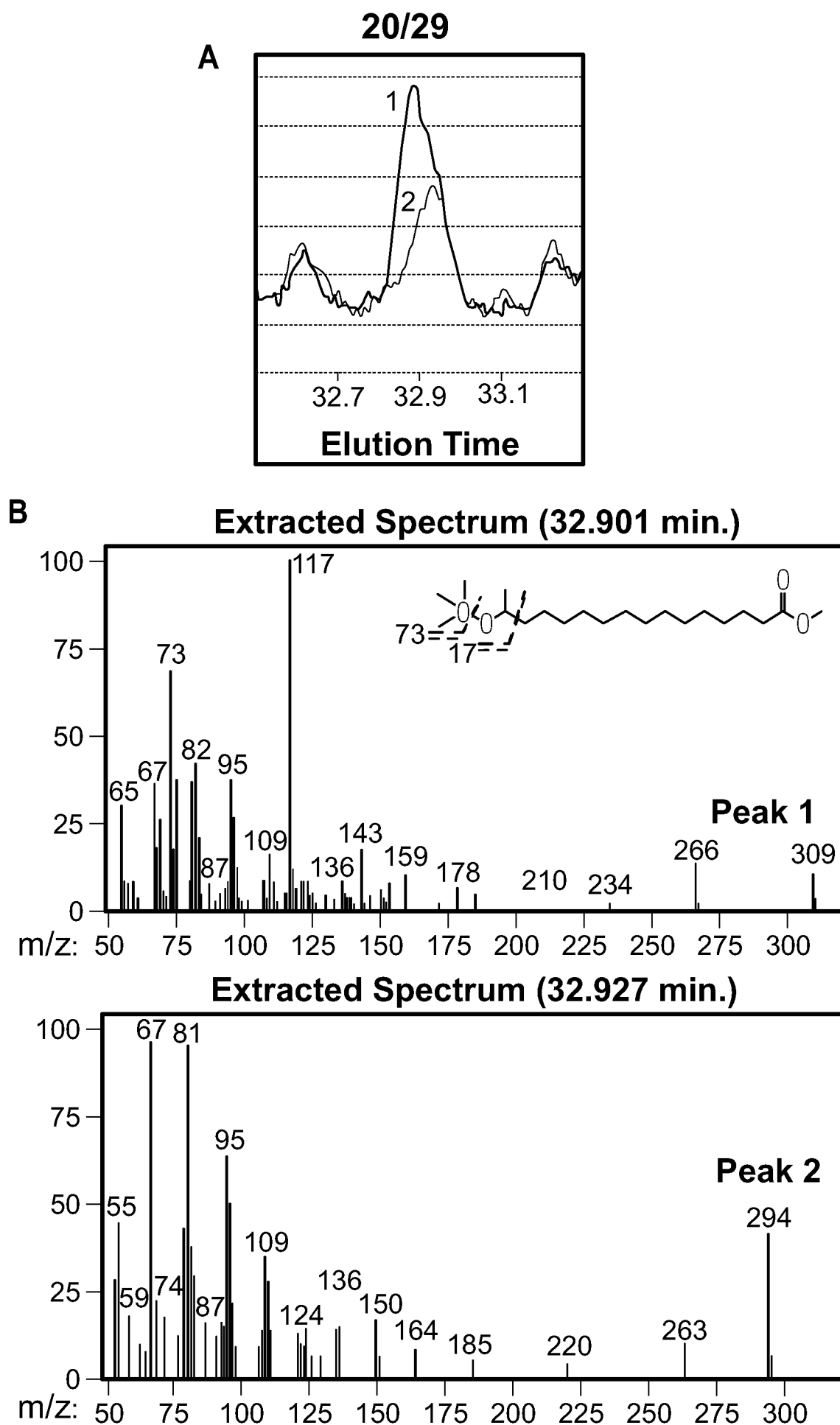
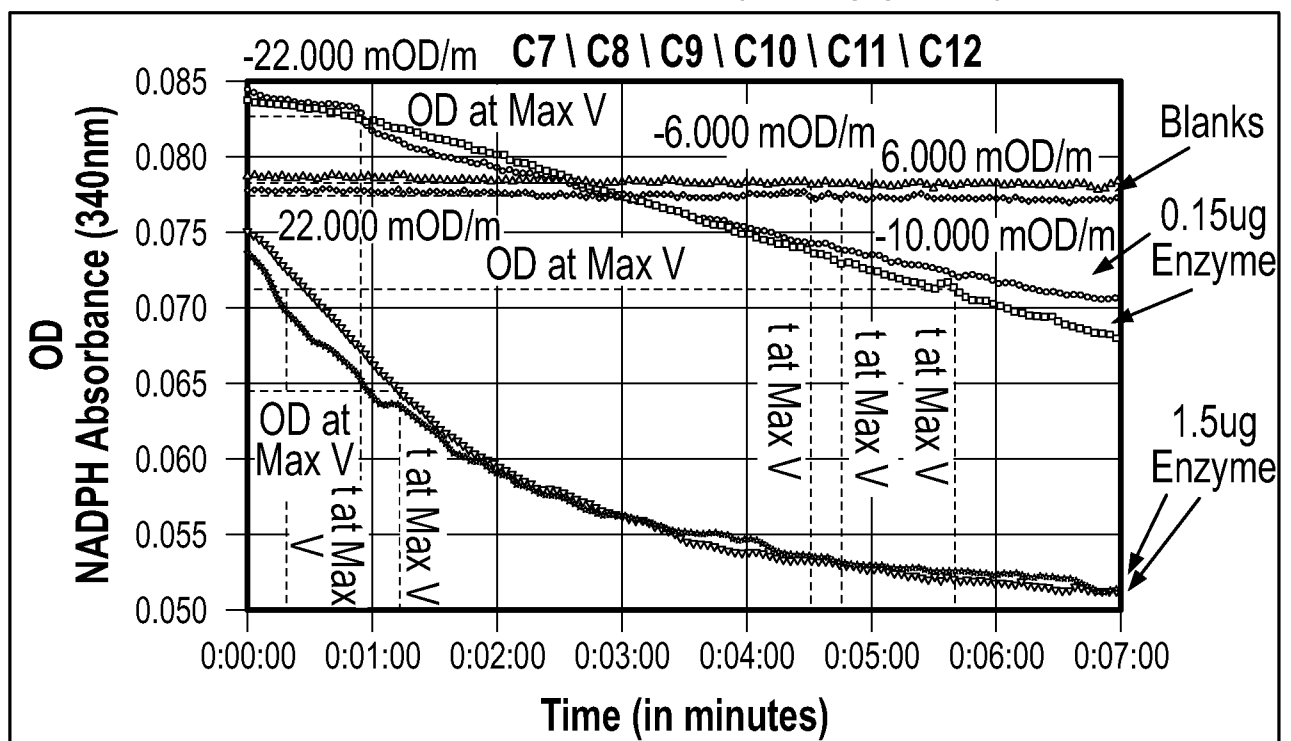
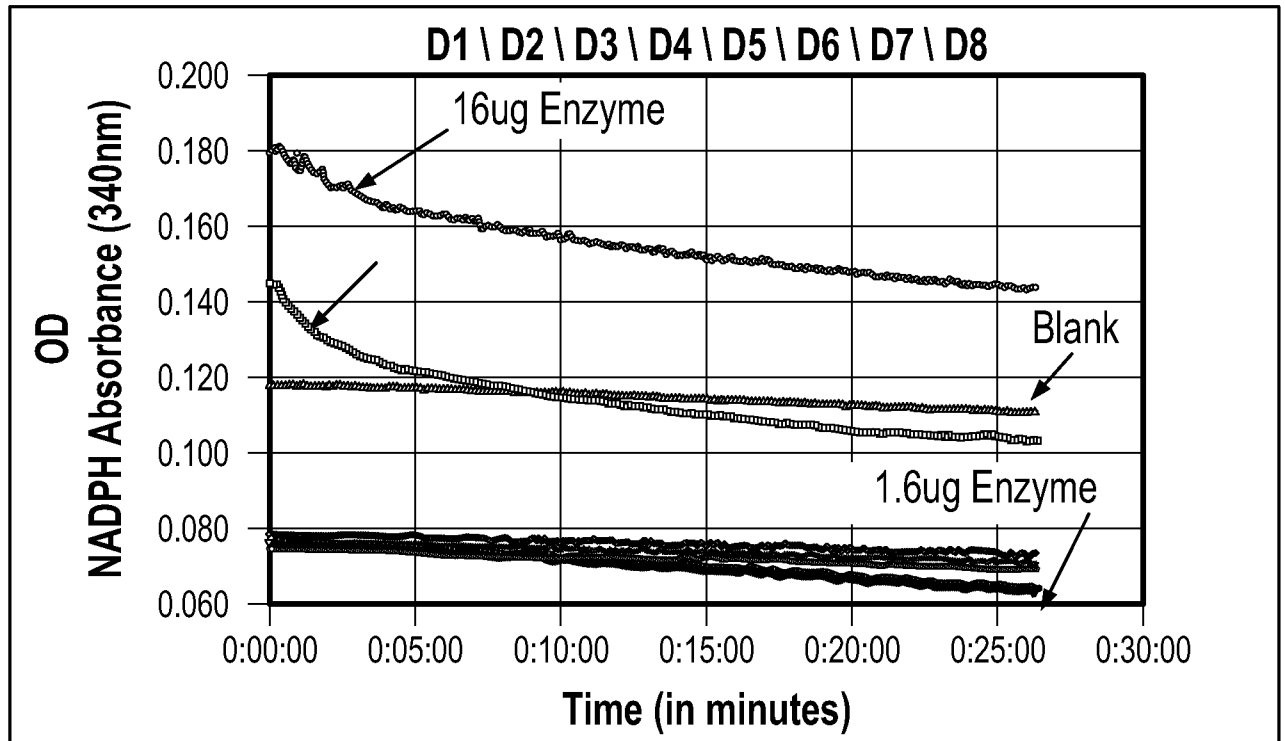


FIG. 12



A



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C

In-vitro *B. Vulgatus* KASIII Activity with
Hydroxylated Substrate (β -hydroxybutyryl CoA)

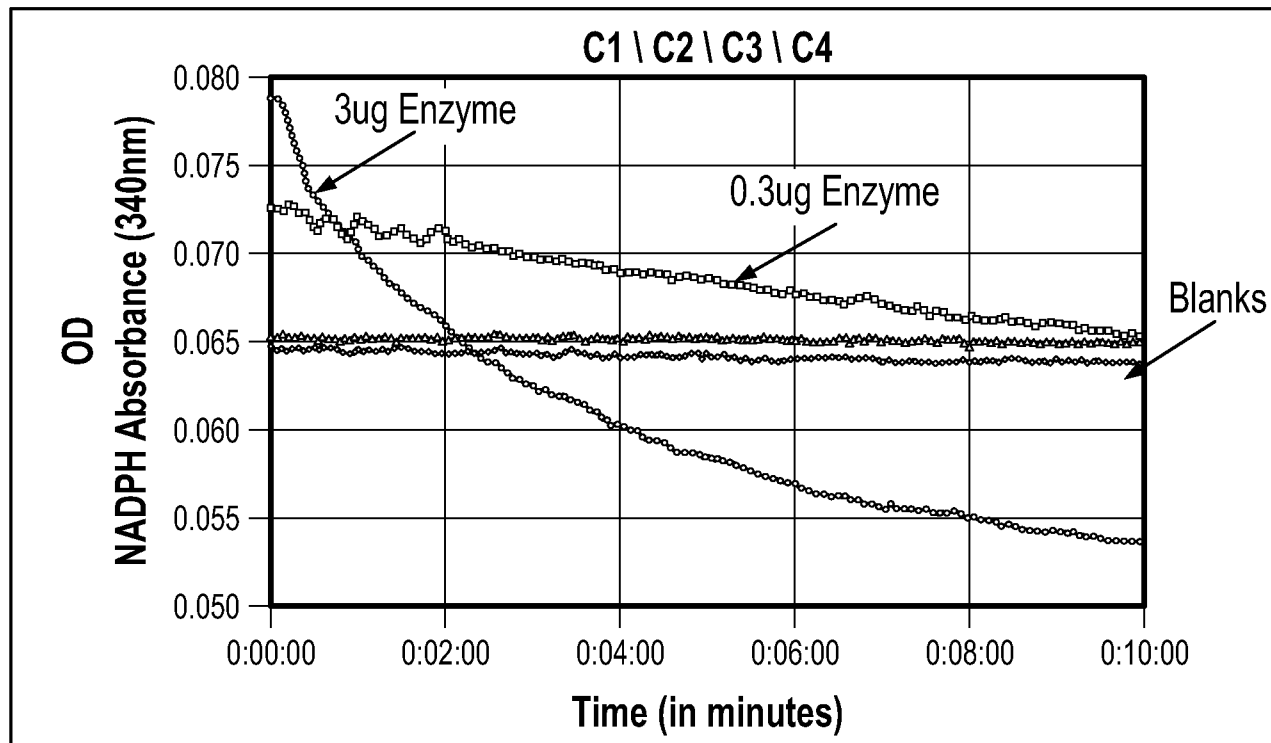


FIG. 14 (Cont.)

A

In-vitro *L. Pneumophila* KASIIIa Activity with
Straight Chain Substrate (Acetyl CoA)

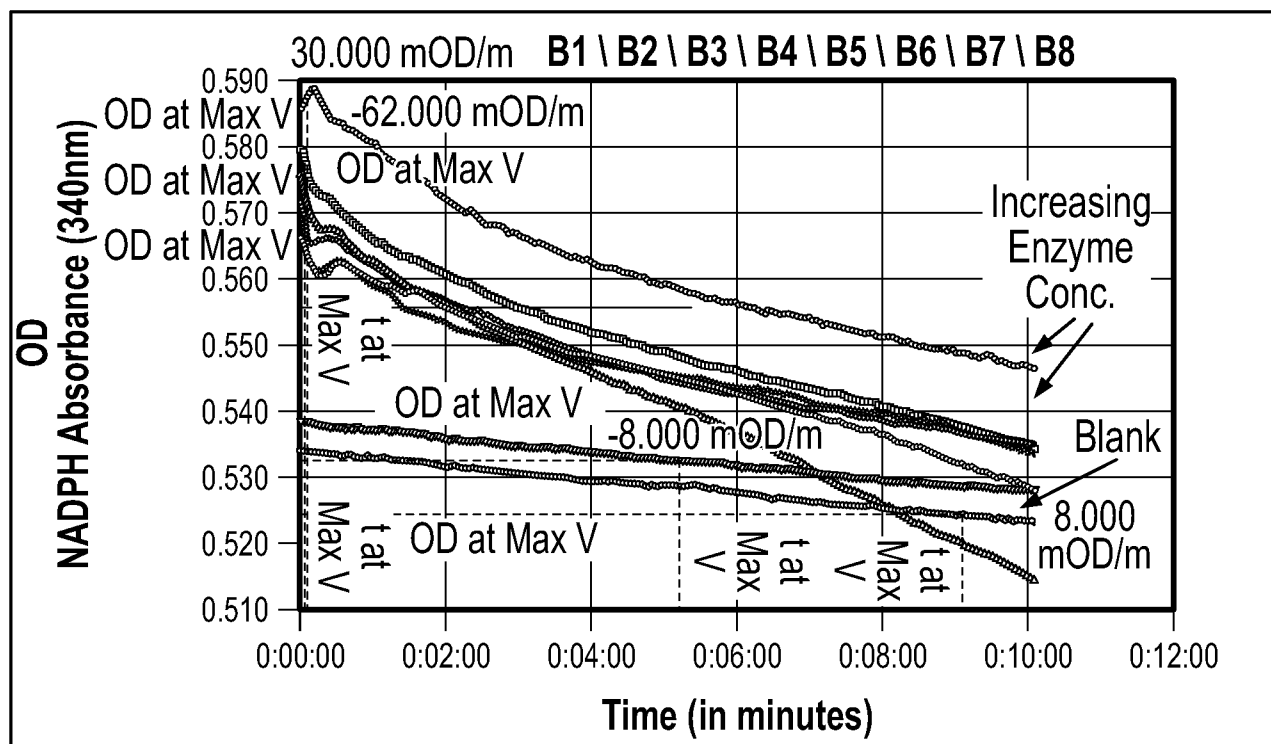
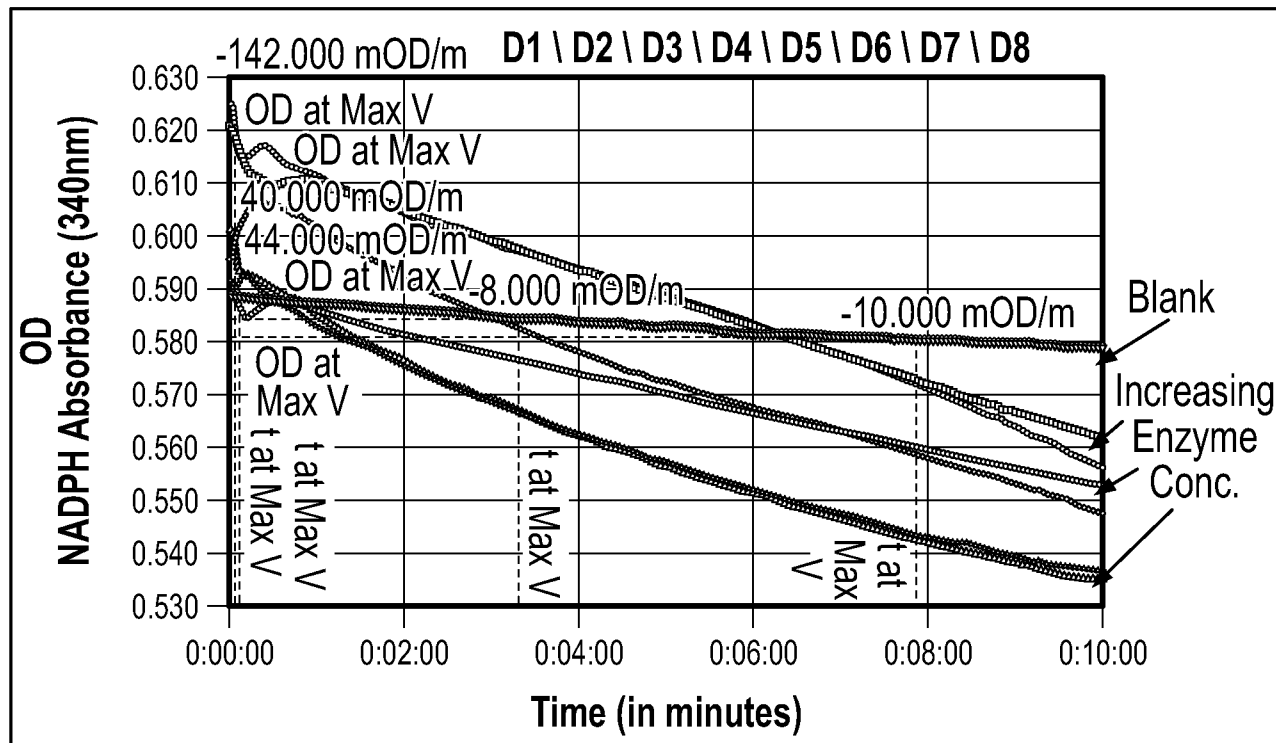


FIG. 15

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B

**In-vitro *L. pneumophila* KASIIIa Activity with
Branched Chain Substrate (Isobutyryl CoA)**



C

**In-vitro *L. Pneumophila* KASIIIa Activity with
Hydroxylated Substrate (β -Hydroxybutyryl CoA)**

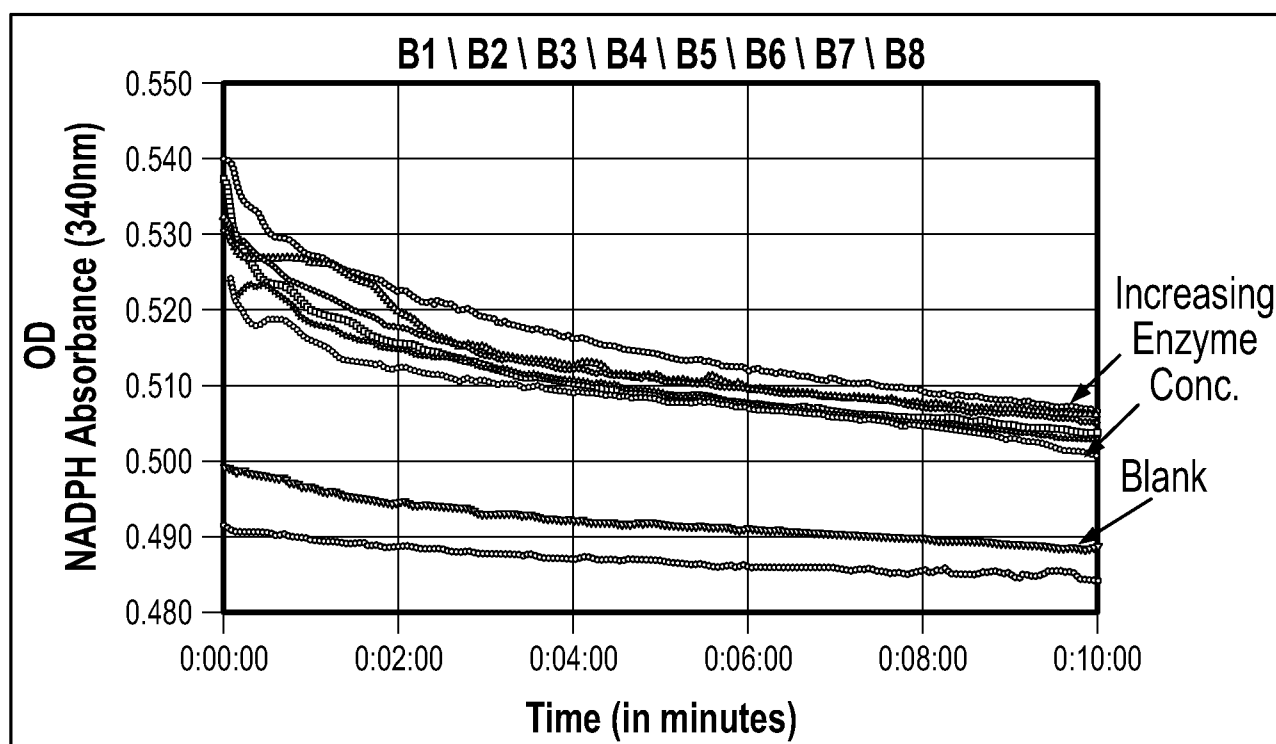


FIG. 15 (Cont.)

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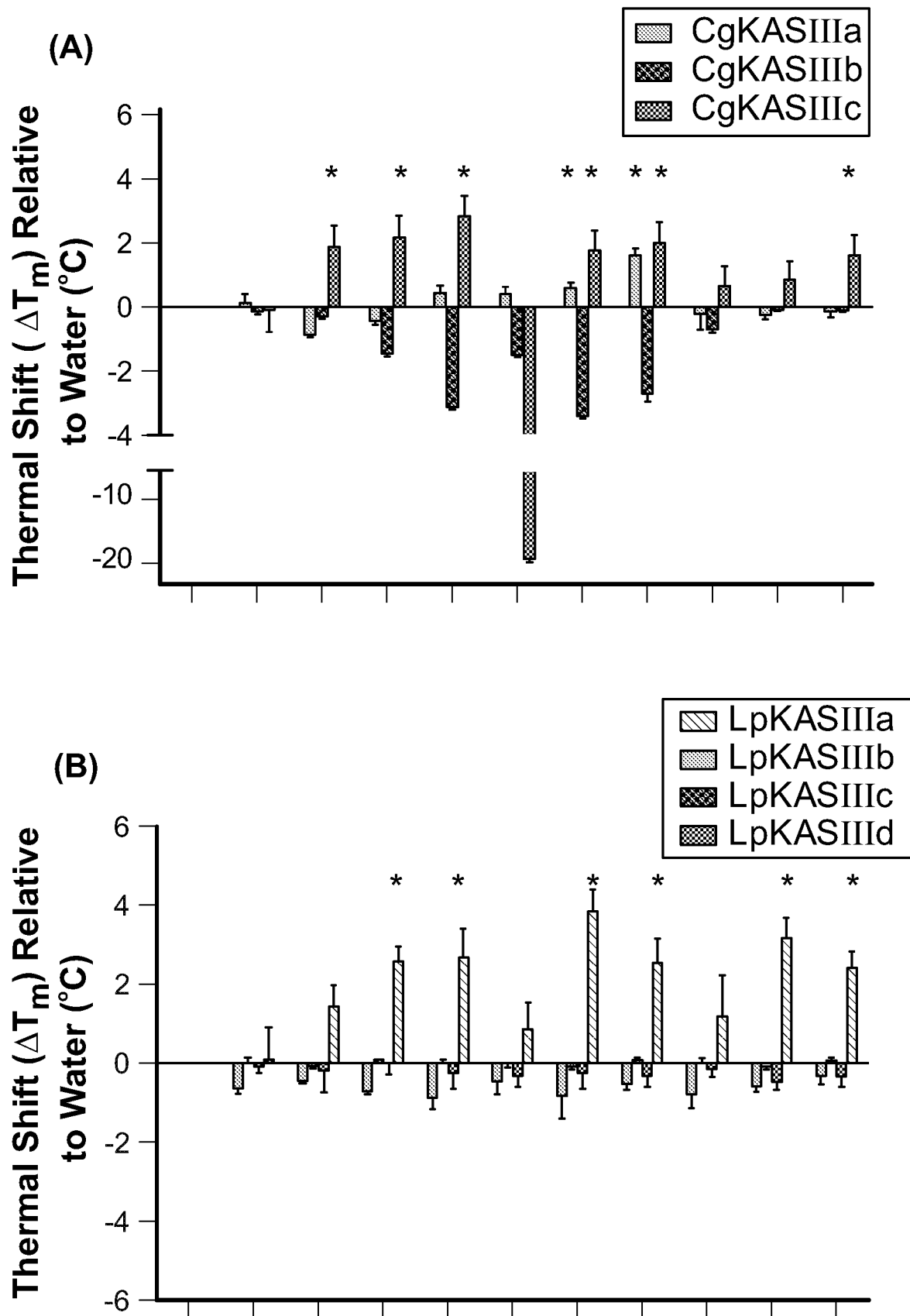
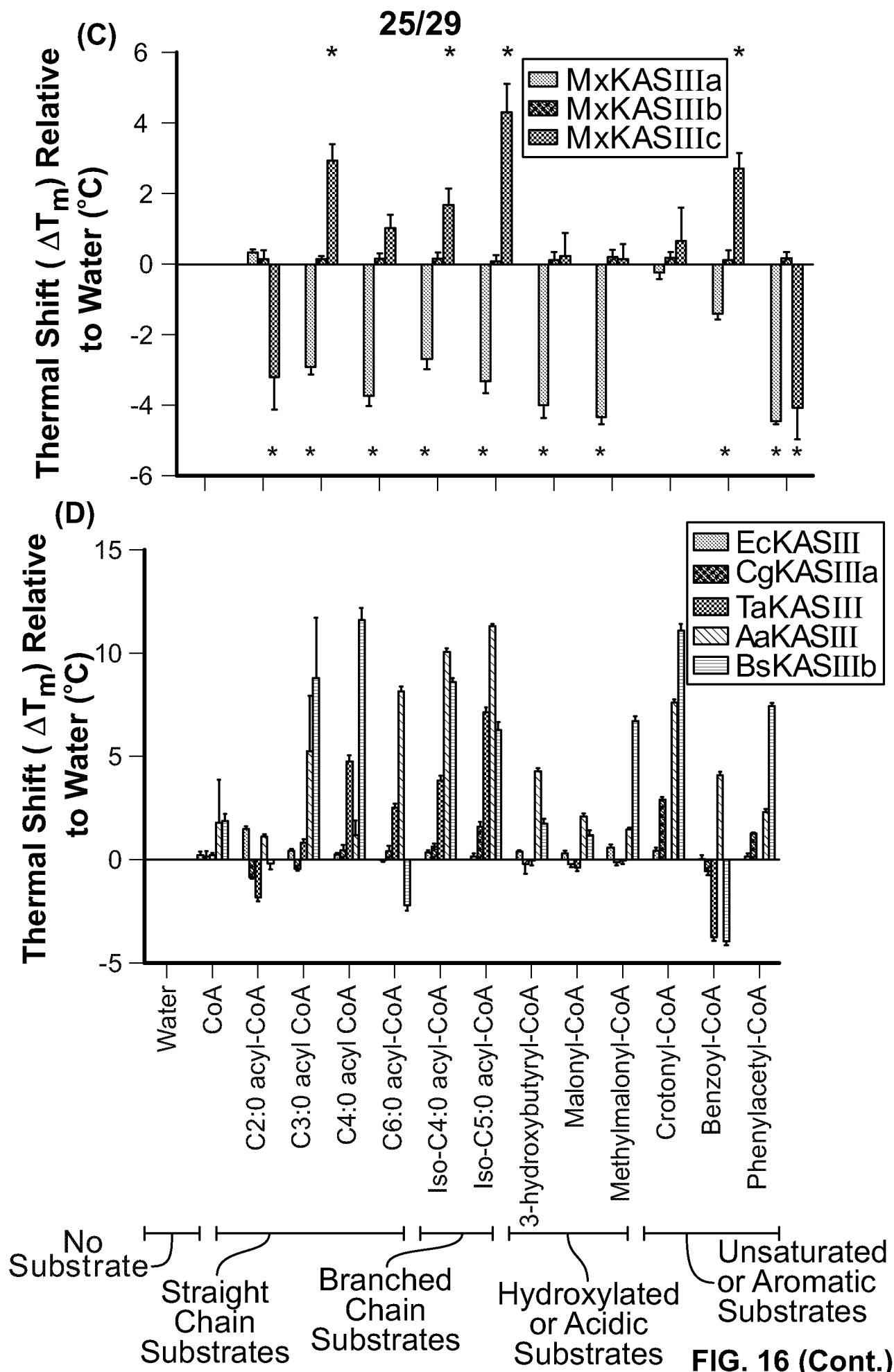


FIG. 16



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GRAM- CHARACTER	BACTERIAL SOURCE OF KASIII	BRANCHED CHAIN FATTY ACID CONTENT (%)	GENBANK ACCESSION	PHYLOGENETIC SUB-FAMILY FROM KASIII TREE	ACTIVE SITE RESIDUES	CoA BINDING RESIDUES	ACP BINDING RESIDUES	RESIDUES AFFECTING ACTIVE SITE CLEFT SHAPE	
					112 244 274	28 32 151	36 213 214 249 253	304 215 220	
GRAM-NEGATIVE BACTERIA	ESCHERICHIA COLI	0	AAG55837.1	12	C H N	T W R	F R R	F K R A	F V L
	HAEMOPHILUS INFLUENZAE	0	AAC21826.1	11	C H N	T W R	R R R	F K R A	F L L
	YERSINIA PESTIS +	0	AAM85324.1	NOT PRESENT	C H N	T W R	R R R	F K R A	F V L
	VIBRIO VULNIFICUS +	2	AAO11338.2	1	C H N	T W R	R R R	F K R A	F V L
	VIBRIO CHOLERAEE +	5	AAF96649.1	NOT PRESENT	C H N	T W R	R R R	F K R T	F R M
	CAPNOCYTOPHAGA GINGIVALIS KASIIIa*	84	EEK13720.1	4	C H N	T W R	R R R	F K R A	F M
	CAPNOCYTOPHAGA GINGIVALIS KASIIIB*	84	EEK14223.1	7	C H N	T W R	R R R	F K R F	F N F
	CAPNOCYTOPHAGA GINGIVALIS KASIIIC*	84	EEK14078.1	10	C H N	W K R	F N R	F N H K	F F T
	LEGIONELLA PNEUMOPHILA KASIIIa*	65	CAH12499.1	1	C H N	T W R	R R R	F K R A	F I M
	LEGIONELLA PNEUMOPHILA KASIIIB*	65	CAH13332.1	9	C H N	K K E	E K E	F V N S	Q V L Y
	LEGIONELLA PNEUMOPHILA KASIIIC*	65	CAH13337.1	10	C H N	S V F	S F S	F V N S	A K M
	LEGIONELLA PNEUMOPHILA KASIIID*	65	CAH12752.1	12	C H N	L S L	F S L	F K L H	T L I
	MYXOCOCCUS XANTHUS KASIIIa*	62	ABF92307.1	8	C T Y	K W R	N V Q	R A S	F F W
	MYXOCOCCUS XANTHUS KASIIIB*	62	ABF89527.1	6	C H N	W R Y	A R	R A V	S M
	MYXOCOCCUS XANTHUS KASIIIC*	62	ABF92876.1	11	C H N	T W R	R R Q	K R A	V F A
GRAM-POSITIVE BACTERIA	STREPTOCOCCUS PNEUMONIAE	0	ACF56437.1	OUTLIER	C H N	T W R	R R F	K R K	F F V
	ENTEROCOCCUS FAECALIS	0	AAO82575.1	9	C H N	T W R	R R F	D R K	F F V
	BACTEROIDES VULGATUS +	55	ABR38167.1	4	C H N	T W R	R R F	D R A	F M
	BACILLUS SUBTILIS KASIIIa	95	CAB12974.1	12	C H N	T W R	R R F	K R A	F F M
	BACILLUS SUBTILIS KASIIIB	95	CAB12857.1	12	C H N	T W R	R R R	K R S	F V
	BACILLUS LICHENIFORMIS KASIIIa +	91	AAU22783.1	6	C H N	T W R	R R F	K R A	F F M
	BACILLUS LICHENIFORMIS KASIIIB +	91	AAU22657.1	11	C H N	T W R	R R R	K R S	F V
	LISTERIA MONOCYTOGENES	93	CAD00280.1	NOT PRESENT	C H N	T W R	R R F	R A	F F M
	MICROCOCCUS LUTEUS	83	ACS30452.1	NOT PRESENT	C H N	W R R	R R F	R E	F M
	STAPHYLOCOCCUS AUREUS	69	BAB57145.1	6	C H N	T W R	M R	F K R S	F F M

FIG. 17

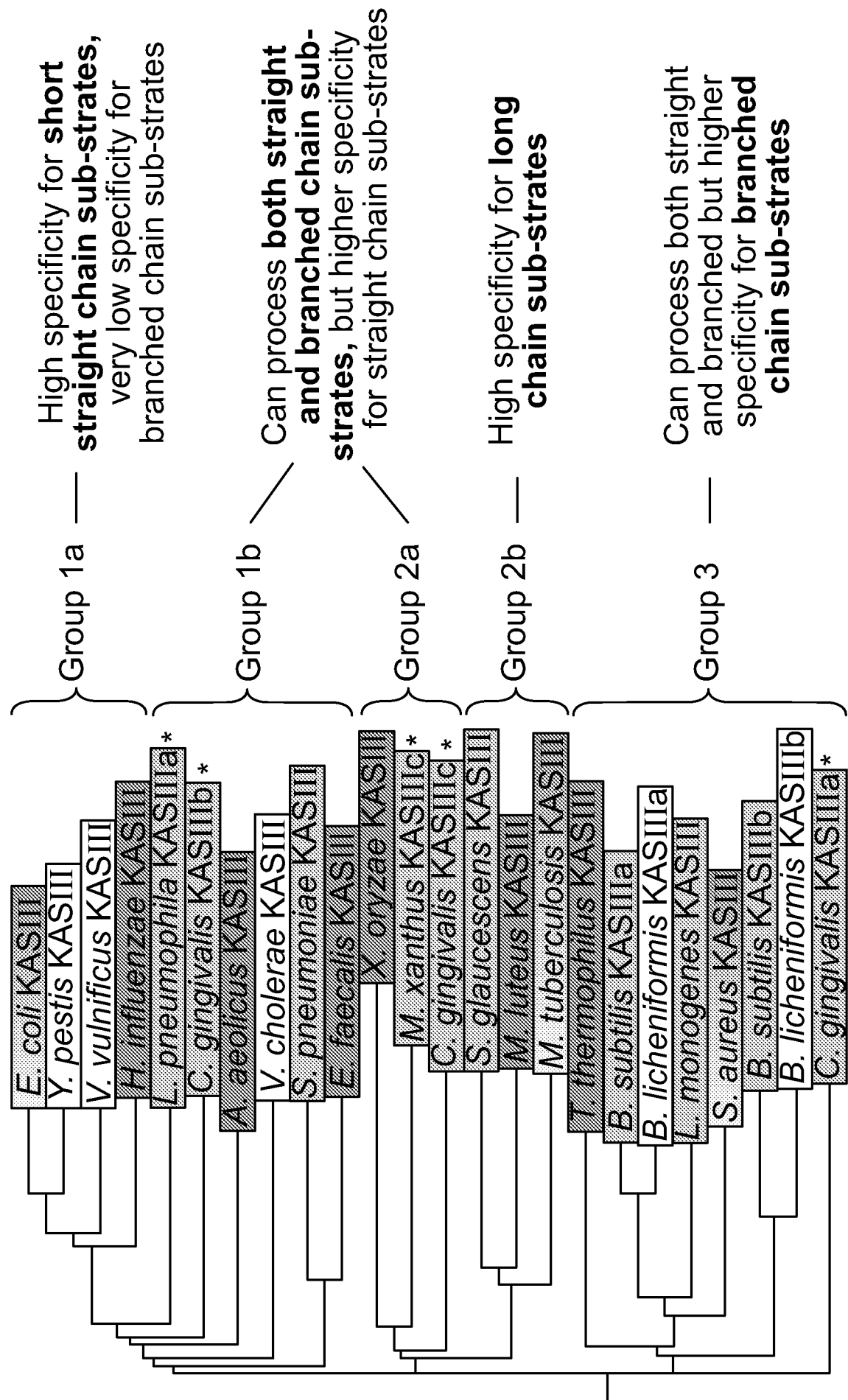


FIG. 18

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Phylogenetic structure-function group	Organismal data		Structural data		Functional data (Specific activity)		KASIII functional class (experimentally determined)
	Bacterial source of KASIII	Branched chain fatty acid content (%)	Residues proposed to affect substrate specificity	Rotamer conformation of the substrate-limiting Phe (from crystal structures)	Straight chain acyl-CoA substrates utilized	Branched chain acyl-CoA substrates utilized	
Group 1a	<i>Escherichia coli</i>	0	F V L	Active site distal	C2:0, C3:0	Very low activity	Narrow
	<i>Yersinia pestis</i>	0	F V L	N.D.	N.D.	N.D.	N.D.
	<i>Vibrio vulnificus</i>	2	F V L	N.D.	N.D.	N.D.	N.D.
	<i>Haemophilus influenzae</i>	0	F L L	Active site distal	N.D.	N.D.	N.D.
Group 1b	<i>Legionella pneumophila</i> KASIIIa*	65	F I M	N.D.	C2:0, C3:0, C4:0	i-C4:0, i-C5:0	Broad
	<i>Capnocytophaga gingivalis</i> KASIIIb*	84	F N F	N.D.	No activity shown	No activity shown	N.D.
	<i>Aquifex aeolicus</i>		M V M	Not applicable	N.D.	N.D.	N.D.
	<i>Vibrio cholerae</i>	5	F R M	N.D.	N.D.	N.D.	N.D.
	<i>Streptococcus pneumoniae</i>	0	F F V	N.D.	C2:0, C3:0, C4:0	i-C4:0, i-C5:0	Broad
	<i>Enterococcus faecalis</i>	0	F F V	Active site proximal	N.D.	N.D.	N.D.
	<i>Xanthomonas oryzae</i>	12	I E A	Not applicable	N.D.	N.D.	N.D.
Group 2a	<i>Myxococcus xanthus</i> KASIIIc*	62	V F A	Not applicable	C2:0, C3:0, C4:0, C6:0	i-C4:0, i-C5:0	Broad
	<i>Capnocytophaga gingivalis</i> KASIIIc*	84	F F T	N.D.	C2:0, C3:0, C4:0	i-C4:0, i-C5:0	Broad

FIG. 19

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Group 2b	<i>Streptomyces glaucescens</i>	90	F	M	N.D.	C2:0, C4:0, C12:0	i-C4:0	Broad
	<i>Micrococcus luteus</i>	83	F	M	Active site proximal		N.D.	Broad
	<i>Mycobacterium tuberculosis</i>	Mycolic acids	Y	M	Active site proximal	C8:0, C10:0, C12:0, C14:0, C16:0, C18:0	N.D.	Broad
			F	M	Active site proximal	N.D.	N.D.	N.D.
Group 3	<i>Thermus thermophilus</i>	99	F	M	Active site proximal			N.D.
	<i>Bacillus subtilis</i> KASIIIa	95	F	M	N.D.	C2:0, C3:0, C4:0, C5:0, C6:0, C7:0, C8:0	i-C4:0, i-C5:0, a-C5:0	Broad
	<i>Bacillus licheniformis</i> KASIIIa	91	F	M	N.D.	N.D.	N.D.	N.D.
	<i>Listeria monocogenes</i>	93	F	M	N.D.	C2:0	i-C4:0, i-C5:0, a-C5:0	Broad
	<i>Staphylococcus aureus</i>	69	F	M	Active site proximal	C2:0, C3:0, C4:0, C6:0, C12:0, C16:0	i-C4:0, i-C5:0	Broad
	<i>Bacillus subtilis</i> KASIIIb	95	F	V	N.D.	C2:0, C3:0, C4:0, C5:0, C6:0, C7:0	i-C4:0, i-C5:0, a-C5:0	Broad
	<i>Bacillus licheniformis</i> KASIIIb	91	F	V	N.D.	N.D.	N.D.	N.D.
	<i>Capnocytophaga gingivalis</i> KASIIIa*	84	F	M	N.D.	C3:0	i-C4:0, i-C5:0	Broad
			F	M				
Non-functional KASIII	<i>Myxococcus xanthus</i> KASIIIa*	62	S	F	Not applicable	No activity shown	No activity shown	
	<i>Myxococcus xanthus</i> KASIIIb*	62	V	S	Not applicable	No activity shown	No activity shown	
	<i>Legionella pneumophila</i> KASIIIb*	65	V	L	Not applicable	No activity shown	No activity shown	
	<i>Legionella pneumophila</i> KASIIIc*	65	A	K	Not applicable	No activity shown	No activity shown	
	<i>Legionella pneumophila</i> KASIIId*	65	T	L	Not applicable	No activity shown	No activity shown	

FIG. 19 (Cont.)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US14/12616

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 1/20; C12P 7/64, 7/44 (2014.01)

USPC - 435/252.31, 134, 7.4

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): C12N 1/20, 9/10, 5/04; C12P 7/64, 7/44, 21/02; G01N 33/53, 33/573; C12Q 1/48, 1/34, 1/68 (2014.01)

USPC: 435/252.31, 142, 252.5, 134, 132, 41, 252.3, 252.1, 243, 7.71, 7.4, 7.1, 15, 18, 963, 968, 975; 800/281; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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

MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google; Google Scholar; ProQuest; PubMed; Search Terms: KASIII, KASIIIa, KASIIIb, subtilis, rubrum, PHA, rotamer, 340nm

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y --- A	NOEL, J. NSF Engineering Research Center For Biorenewable Chemicals, NSF Engineering Research Center for Biorenewable Chemicals, Fourth Annual Report, Volume II - T1.1 - 3-Ketoacyl-ACP Synthase: Characterization of Novel Biocatalysts for Diversifying FAS/PKS Metabolic Pathways. Center for Biorenewable Chemicals Annual Reports. Book 3. Digital Repository at Iowa State University. 02 April 2012; page 20, second paragraph; seventh paragraph.	1-4, 9, 10, 13-15, 16/14, 16/15, 17, 18, 19/17, 19/18, 23, 36-38, 39/37, 39/38, 40, 41, 42/40, 42/41, 46 ----- 11, 12, 19/17, 19/18, 24-36, 27/23, 27/24, 28, 29, 30/28, 30/29, 34, 42/40, 42/41, 49, 51/49/5, 51/49/6 ----- 20, 21, 22/20, 22/21, 31, 32, 33/31, 33/32
X --- Y	JIN, H. Role Of Genetic Redundancy in Polyhydroxyalkanoate (PHA) Polymerases In PHA Biosynthesis In Rhodospirillum Rubrum. Journal of Bacteriology. 03 August 2012, Vol. 194, No. 20; pages 5522-5529; abstract; page 5524, left column, third paragraph to right column, first paragraph; page 5525, Table 2; page 5525, right column, third paragraph. DOI: 10.1128/JB.01111-12.	5-8 ----- 11, 12, 51/49/5, 51/49/6
X --- Y	US 2012/0122193 A1 (BERRY, DA et al.) May 17, 2012; abstract; paragraphs [0011], [0016]-[0022], [0053], [0216]-[0218]	49 ----- 24-36, 27/23, 27/24, 28, 29, 30/28, 30/29, 34, 50, 51/49/5, 51/49/6

☒ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

15 May 2014 (15.05.2014)

Date of mailing of the international search report

23 MAY 2014

Name and mailing address of the ISA/US

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US14/12616

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2012/071439 A1 (STEEN, EJ et al.) May 31, 2012; abstract; paragraph [0020], figure 1	49, 50, 51/49/5, 51/49/6
Y	YU, X et al. In Vitro Reconstitution And Steady-State Analysis Of The Fatty Acid Synthase From Escherichia Coli. PNAS. 15 November 2011, Vol. 108, No. 46; pages 18643-18648; abstract; page 18644, figure 1; page 18644, left column, first paragraph to right column, fourth paragraph. DOI: 10.1073/pnas.111085210.	47, 48
Y	BAYS, NW et al. A Simplified Scintillation Proximity Assay For Fatty Acid Synthase Activity: Development And Comparison With Other FAS Activity Assays. Journal of Biomolecular Screening. 17 July 2009, Vol. 6; pages 636-642; abstract; page 5, right column, second paragraph, NADPH absorbance assay. DOI: 10.1177/1087057109335746.	47, 48
Y	NOMURA, C et al. Expression Of 3-Ketoacyl-Acyl Carrier Protein Reductase (fabG) Genes Enhances Production Of Polyhydroxyalkanoate Copolymer From Glucose In Recombinant Escherichia Coli JM109. Applied and Environmental Microbiology. August 2005, Vol. 71, No. 8; pages 4297-4306; abstract; page 4297, right column, third paragraph; page 4298, figure 1. DOI: 10.1128/AEM.71.8.4297-4306.2005.	47, 48
Y	MATSUBARA, H et al. Alicyclobacillus Acidiphilus sp. nov., A Novel Thermo-Acidophilic, Omega-Alicyclic Fatty Acid-Containing Bacterium Isolated From Acidic Beverages. International Journal of Systematic and Evolutionary Microbiology. September 2002, Vol. 52, pp 1681-1685; entire document. DOI: 10.1099/ijs.0.02169-0.	10, 12
A	WO 2001/51925 A1 (REYNOLDS, KA et al.) July 19, 2001; entire document	1-15, 16/14, 16/15, 17, 18, 19/17, 19/18, 20, 21, 22/20, 22/21, 23-26, 27/23, 27/24, 28, 29, 30/28, 30/29, 31, 32, 33/31, 33/32, 34, 36-38, 39/37, 39/38, 40, 41, 42/40, 42/41, 43, 44, 45/43, 45/44, 46-50, 51/49/5, 51/49/6
A	US 2003/0145350 A1 (SPENER, F et al.) July 31, 2003; entire document	1-15, 16/14, 16/15, 17, 18, 19/17, 19/18, 20, 21, 22/20, 22/21, 23-26, 27/23, 27/24, 28, 29, 30/28, 30/29, 31, 32, 33/31, 33/32, 34, 36-38, 39/37, 39/38, 40, 41, 42/40, 42/41, 43, 44, 45/43, 45/44, 46-50, 51/49/5, 51/49/6
A	US 2009/0075249 A1 (DEHESH, K et al.) March 19, 2009; entire document	1-15, 16/14, 16/15, 17, 18, 19/17, 19/18, 20, 21, 22/20, 22/21, 23-26, 27/23, 27/24, 28, 29, 30/28, 30/29, 31, 32, 33/31, 33/32, 34, 36-38, 39/37, 39/38, 40, 41, 42/40, 42/41, 43, 44, 45/43, 45/44, 46-50, 51/49/5, 51/49/6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US14/12616

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 35
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

-Please See Supplemental Page-

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

---Continued from Box No. III: Observations Where Unity of Invention Is Lacking:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-4, 9 and 10 are directed towards a mutant *Bacillus subtilis* which does not express a functional 3-ketoacyl-acyl-carrier protein (ACP) synthase III (KASIII) selected from the group consisting of KASIIIA and KASIIIB, a method of making said mutant *B. subtilis*; a method of characterizing substrate specificity of a KASIII, which method comprises expressing the KASIII, which is not expressed in wild-type *B. subtilis*, in said mutant *B. subtilis*.

Group II: Claims 5-8, 11, 12 and 51 (in-part) are directed towards a mutant *Rhodospirillum rubrum*, which does not express a functional polyhydroxyalkanoate (PHA) polymerase PhaC1 are directed towards a mutant *Rhodospirillum rubrum*, which does not express a functional polyhydroxyalkanoate (PHA) polymerase selected from the group consisting of PhaC1, PhaC2, and PhaC3; and a method of characterizing substrate specificity of a KASIII, the method comprising expressing the KASIII, which is not expressed in wild-type *R. rubrum*, in said mutant *R. rubrum*.

Group III: Claims 13-34 and 36-46 are directed toward a method of altering the specificity of a KASIII for at least one of its substrates, which method comprises introducing into the KASIII one or more mutations comprising at least one mutation, which causes the rotamer conformation of a phenylalanine in the KASIII corresponding to Phe304 in KASIII from *E. coli* to change, whereupon the specificity of a KASIII for at least one of its substrates is altered; and an isolated or purified nucleic acid molecule comprising a nucleotide sequence encoding a mutant KASIII, which comprises one or more mutations comprising at least one mutation, which causes the rotamer conformation of a phenylalanine in the KASIII corresponding to Phe304 in KASIII from *E. coli* to change, wherein the isolated or purified nucleic acid molecule can be a vector.

Group IV: Claims 47 and 48 are directed towards an in vitro, high-throughput spectrophotometric method of assaying KASIII activity, which method comprises: (i) incubating holo-ACP, malonyl-CoA, acyl-CoA, NADPH, and malonylCoA: ACP transacylase (FabD), (ii) adding KASIII and 3-ketoacyl-ACP reductase (FabG), and (iii) measuring the change in absorbance at 340 nm when NADPH is converted to NADP+ during reduction of 3-ketoacyl-ACP to 3-hydroxyl-acyl ACP by FabG, whereupon KASIII activity is assayed in an in vitro, high-throughput spectrophotometric method.

Group V: Claims 49, 50 and 51 (in-part) are directed toward a method of producing bi-functional fatty acids in a host cell or organism, which method comprises introducing into a host cell or organism, which comprises one or more omega-functionalized acyl-CoAs, and expressing therein a nucleic acid molecule comprising a nucleotide sequence encoding a KASIII, which can use one or more of the omega-functionalized acyl-CoAs as a substrate, whereupon bi-functional fatty acids in the host cell or organism are produced.

The inventions listed as Groups I-V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include a mutant *Bacillus subtilis* which does not express a functional 3-ketoacyl-acyl carrier protein (ACP) synthase III, which are not present in any other Group, the special technical features of Group II including a mutant *Rhodospirillum rubrum*, which does not express a functional polyhydroxyalkanoate (PHA) polymerase, which are not present in any other Group, the special technical features of Group III including a method of altering the specificity of a KASIII for at least one of its substrates, which method comprises introducing into the KASIII one or more mutations which causes the rotamer conformation of a phenylalanine in the KASIII corresponding to Phe304 in KASIII from *E. coli* to change, which are not present in any other Group, the special technical features of Group IV including an in vitro, high-throughput spectrophotometric method of assaying KASIII activity, which are not present in any other Group, the special technical features of Group V including a method of producing bi-functional fatty acids in a host cell or organism.

Groups I-V share the technical features including 3-ketoacyl-acyl-carrier protein (ACP) synthase III (KASIII). Groups I-III share the technical features including KASIII substrate specificity. Groups I, II and V share the technical features including omega-functionalized fatty acids. Groups I and II share the technical features including a method of characterizing substrate specificity of a KASIII, which method comprises expressing the KASIII, and assessing the production of omega-functionalized acids, whereupon the substrate specificity of a KASIII is characterized.

However, these shared technical features are previously disclosed by the publication entitled 'Probing The Mechanism Of The Mycobacterium Tuberculosis Beta-Ketoacyl-Acyl Carrier Protein Synthase III mtFabH' by Brown, et al. (hereinafter 'Brown') in view of US 2012/0122193 A1 to Berry et al. (hereinafter 'Berry'). Brown discloses 3-ketoacyl-acyl-carrier protein (ACP) synthase III (KASIII) (beta-ketoacyl-acyl carrier protein (ACP) synthase III (3-ketoacyl-acyl-carrier protein (ACP) synthase III (KASIII)); abstract); KASIII substrate specificity (mtFabH (KASIII) substrate chain length specificity (KASIII substrate specificity); abstract); and a method of characterizing substrate specificity of a KASIII (assessment of ACP-dependent Acyl-CoA chain length preference of mtFabH (a method of characterizing substrate specificity of a KASIII); page 32541, column 2, paragraph 3), including the characterization of KASIII mutants (characterization of KASIII mutants; abstract). Brown does not disclose omega-functionalized fatty acids; and a method of characterizing substrate specificity of a KASIII, which method comprises expressing the KASIII, and assessing the production of omega-functionalized acids, whereupon the substrate specificity of a KASIII is characterized. Berry discloses engineered microorganisms capable of producing desired compounds (engineered microorganisms capable of producing desired compounds; abstract), including the utilization of branched chain (branched chain; paragraph [0248]) omega-functionalized fatty acids (omega-cyclic fatty acids (omega-functionalized fatty acids); paragraph [0250]) by enzymes including KASIII (fabH (KASIII); paragraphs [0248], [0250]) with specificity for branched chain fatty acids (specificity for branched chain fatty acids; paragraph [0248]) or omega-functionalized fatty acids (omega-cyclic fatty acids (omega-functionalized fatty acids); paragraph [0250]). It would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the procedures disclosed by Brown, in order to have provided for the characterization of substrate specificity for one or more omega-modified fatty acids, provided the previous disclosure of Berry regarding the capacity of an enzyme to utilize this type of substrate, in order to better determine the range of omega-functionalized fatty acids substrates which may be used efficiently by mtKASIII, as well as implementing the use of mutagenesis, as previously disclosed by Brown, for determining which parts of the enzyme contribute to the specificity, for mutating or altering the portions of the enzyme, and enabling the generation of desired products, through routine experimentation and testing, provided the previous disclosure of Berry.

Since none of the special technical features of the Groups I-V inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Brown and Berry references, unity of invention is lacking.