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Terminal Olefin (1-Alkene) Biosynthesis by a Novel P450 Fatty Acid Decarboxylase from *Jeotgalicoccus* Species▽†

Mathew A. Rude, Tarah S. Baron, Shane Brubaker,‡ Murtaza Alibhai,§ Stephen B. Del Cardayre, and Andreas Schirmer*

LS9, Inc., 600 Gateway Boulevard, South San Francisco, California 94080

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Terminal olefins (1-alkenes) are natural products that have important industrial applications as both fuels and chemicals. However, their biosynthesis has been largely unexplored. We describe a group of bacteria, *Jeotgalicoccus* spp., which synthesize terminal olefins, in particular 18-methyl-1-nonadecene and 17-methyl-1-nonadecene. These olefins are derived from intermediates of fatty acid biosynthesis, and the key enzyme in *Jeotgalicoccus* sp. ATCC 8456 is a terminal olefin-forming fatty acid decarboxylase. This enzyme, *Jeotgalicoccus* sp. OleT ($OleT_{JE}$), was identified by purification from cell lysates, and its encoding gene was identified from a draft genome sequence of *Jeotgalicoccus* sp. ATCC 8456 using reverse genetics. Heterologous expression of the identified gene conferred olefin biosynthesis to *Escherichia coli*. $OleT_{JE}$ is a P450 from the cyp152 family, which includes bacterial fatty acid hydroxylases. Some cyp152 P450 enzymes have the ability to decarboxylate and to hydroxylate fatty acids (in α- and/or β-position), suggesting a common reaction intermediate in their catalytic mechanism and specific structural determinants that favor one reaction over the other. The discovery of these terminal olefin-forming P450 enzymes represents a third biosynthetic pathway (in addition to alkane and long-chain olefin biosynthesis) to convert fatty acid intermediates into hydrocarbons. Olefin-forming fatty acid decarboxylation is a novel reaction that can now be added to the catalytic repertoire of the versatile cytochrome P450 enzyme family.

The efficient conversion of carbon dioxide either directly or through biomass into "drop-in compatible" hydrocarbon fuels and renewable chemicals is the ultimate goal of "biorenewable" research and development (4, 6, 19, 46). A key step in this process is the biocatalytic conversion of metabolic intermediates into hydrocarbons such as alkanes or olefins (alkenes). The fatty acid biosynthesis pathway is ideally suited to providing biofuel precursors because of its high efficiency and high energy conservation, and natural metabolic pathways exist that convert fatty acid intermediates into alkanes and/or olefins (17, 31, 52, 54).

The genes for two such pathways—the head-to-head condensation of fatty acids to long-chain olefins (C_{23}-C_{33}) and the decarbonylation of fatty aldehydes to alkanes—have been described recently. The first hydrocarbon biosynthesis genes were discovered in a genetic screen for *Stenotrophomonas maltophilia* mutants unable to synthesize long-chain olefins (20). Based on this work, the homologous genes from *Micrococcus luteus* (7) and *Shewanella oneidensis* (49) were characterized. The key enzyme of the long-chain olefin biosynthesis, OleA, is a homolog of the condensing enzyme FabH (3-oxo-acyl-ACP ketosynthase III), which likely acts through a thiolase reaction mechanism. Subsequently, alkane biosynthesis genes were discovered in cyanobacteria by using a comparative genomics approach (47). The key enzyme of alkane biosynthesis, aldehyde decarbonylase (ADC), is a novel member of the nonheme diiron oxygenases.

The OleA-based mechanism of long-chain olefin biosynthesis apparently occurs only in bacteria because orthologs of OleABCD could not be identified in archaea or eukaryota (48). However, the presence of olefins (medium and long chain) is known in many eukaryotes. This is most easily explained by decarbonylation of unsaturated fatty aldehydes, which has been widely reported in eukaryotes (11, 17, 30). On the other hand, some eukaryotes produce terminal olefins (also referred to as α-olefins or 1-alkenes) derived from fatty acid precursors that cannot be explained by any mechanism mentioned above. Examples are 1-pentadecene in beetles of the genus *Tribolium* (25, 53), 1-heptadecene and related higher unsaturated polyenes in plants such as *Carthamus tinctorius* (24, 42), and C_{23}-C_{33} dienes and trienes in the green microalga *Botryococcus braunii* race A (50, 51). The last is the only natural organism known to overproduce true hydrocarbons and therefore has been intensively studied in recent years as a source for advanced biofuels from algae (39). However, the enzymes that convert fatty acid derivatives into terminal olefins and the genes that encode them have never been described.

The terminal olefins described here represent unique biological products that have direct application as both fuels and industrial chemicals (32). In an attempt to better understand the biochemistry and genetics of terminal olefin biosynthesis as possible tools for future metabolic engineering, terminal olefin production from the genus *Jeotgalicoccus* was investigated. By taking a reverse genetic approach, the biochemical activity

* Corresponding author. Mailing address: LS9, Inc., 600 Gateway Blvd., South San Francisco, CA 94080. Phone: (650) 243-5412. Fax: (650) 243-5401. E-mail: aschirmer@ls9.com.

‡ Present address: Solazyme Inc., 225 Gateway Blvd., South San Francisco, CA 94080.

§ Present address: Life Technologies Inc., 2130 Woodward Street, Austin, TX 78744.

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responsible for terminal olefin biosynthesis was isolated and used to identify the responsible gene from a partially sequenced genome of the producing organism. Heterologous expression of the identified gene in *E. coli* demonstrated the new gene, *Jeotgalicoccus oleT* (*oleT*$_{JE}$), was sufficient to confer olefin biosynthesis and enabled a partial biochemical characterization. The responsible enzyme catalyzes the decarboxylation of fatty acids and is a cytochrome P450 (CYP) similar to P450s that catalyze fatty acid hydroxylation in the α- and/or β-position.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* Top 10 (Invitrogen, Carlsbad, CA) was used for general cloning, C41 (DE3) (Novagen, Gibbstown, NJ) was used for protein purification, and the MG1655 Δ*fadE* strain, which has the acyl-coenzyme A (CoA) dehydrogenase gene deleted, was used for recombinant olefin production. *Jeotgalicoccus* sp. ATCC 8456 and *Bacillus subtilis* (ATCC 33608) were obtained from the American Type Culture Collection (ATCC 8456 is listed as *Micrococcus candicans* [see Results section]). *Jeotgalicoccus halotolerans* DSMZ 17274, *Jeotgalicoccus psychrophilus* DSMZ 19085, *Jeotgalicoccus pinnipedialis* DSMZ 17030, and *Corynebacterium efficiens* YS-134 (DSMZ 44549) were obtained from the German Collection of Microorganisms and Cell Cultures. Plasmids used were pET-15b (Novagen), OP80, and OP183. OP80 contains the pSC101 origin of replication, the aminoglycoside 3′ adenylyltransferase gene from pCL-1920 (34), and the P_{trc} promoter and multicloning site from pTrcHis2 (Invitrogen). OP183 contains the p15A origin of replication from pACYC177 (New England BioLabs, Beverly, MA) and the β-lactamase gene, the P_{trc} promoter, and the multicloning site from pTrcHis2. The multicloning site of OP183 was modified (the NcoI restriction site was replaced with NdeI, and an AvrII restriction site was introduced).

Media and culture conditions. *E. coli* was grown at 37°C in Luria-Bertani (LB) medium to support molecular genetics constructions. All other strains were grown at 30°C in tryptic soy broth supplemented with 0.5% yeast extract (TSBYE). For recombinant olefin production, *E.* coli MG1655 Δ*fadE* with plasmids encoding P450 genes was grown in a modified M9 mineral medium of the following composition: 6g/liter Na_2HPO_4, 3 g/liter KH_2PO_4, 0.5 g/liter NaCl, 1 g/liter NH_4Cl, 0.25 g/liter $MgSO_4 \cdot 7H_2O$, 11 mg/liter $CaCl_2$, 27 mg/liter $Fe_3Cl \cdot 6H_2O$, 2 mg/liter $ZnCl \cdot 4H_2O$, 2 mg/liter $Na_2MoO_4 \cdot 2H_2O$, 1.9 mg/liter $CuSO_4 \cdot 5H_2O$, 0.5 mg/liter H_3BO_3, 1 mg/liter thiamine, 200 mM Bis-Tris (pH 7.25), 0.5 mM δ-aminolevulinic acid, and 0.1% (vol/vol) Triton X-100. The cultures contained 1% glucose as a carbon source and 100 μg/ml spectinomycin or 100 μg/ml carbenicillin for plasmid maintenance. Overnight seed cultures were propagated in the same medium, inoculated (1/40) into production medium, and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the optical density at 600 nm (OD_{600}) reached 0.6 to 0.8. The cultures were grown for an additional 20 to 40 h at 25°C or 30°C before they were evaluated for olefin production.

Molecular biology techniques. All cyp152 genes described here were either PCR amplified from genomic DNA (*oleT*$_{JE}$, *B. subtilis ybdT*, and *C. efficiens* open reading frame [ORF] CE1459) or chemically synthesized (*Kocuria rhizophila* ORF KRH21570, *Methylobacterium populi* ORF Mpop1292, *Bacillus clausii cypC*, and *Sphingomonas paucimobilis* AB006957). The DNAs were cut with NdeI/XhoI and ligated into OP183 and pET-15b for *in vivo* hydrocarbon production and His-tagged protein production, respectively. *oleT*$_{JE}$ was also cloned into the NcoI/XhoI sites of OP80. Site-directed mutagenesis on *B. subtilis ybdT* was carried out using a QuickChange mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). The *fadE* gene of *E. coli* was deleted using the lambda red system following standard protocols (14). The 16S rRNA gene of ATCC 8456 was cloned using universal primers (16). All oligonucleotide primers were synthesized by Integrated DNA Technologies (San Diego, CA) and are listed in Table S1 in the supplemental material. DNA amplification was carried out using Phusion DNA polymerase (Finnzymes, Woburn, MA), and DNA sequencing was performed by Sequetech (Mountain View, CA). Synthetic genes were synthesized by DNA2.0 (Mountain View, CA).

Genome sequencing. A nearly complete genomic sequence of *Jeotgalicoccus* sp. ATCC 8456 was generated at the Center for Genomic Sciences (Allegheny Singer Research Institute, Pittsburg, PA) using a first-generation 454 Life Sciences GS-20 sequencer as described previously (27). Briefly, high-molecular-weight genomic DNA was fragmented by nebulization to an average size of 3 to 500 bp in length. Oligonucleotide adapters were then ligated to the fractionated DNA to produce a fragment library. The fragment library was attached to beads, and the bead-attached library was then used to support an emulsion PCR (emPCR). The emPCR functions through the creation of a water-in-oil emulsion producing individual micelles for each DNA, which provides for the simultaneous amplification in a single tube of hundreds of thousands of individual DNAs. After emPCR, the emulsion is broken, and each bead with its amplified products is placed in a separate picoliter well of a picotiter plate that contains 700,000 wells for pyrosequencing. Assembly was accomplished using the Newbler *de novo* assembler. Open reading frame identification and annotation of the ATCC 8456 genome was carried out using the program GLIMMER (15). The annotated genome was visualized in ARTEMIS (10).

***In vitro* assays with cell extracts.** For the preparation of *Jeotgalicoccus* sp. ATCC 8456 cell extracts, the strain was grown in TSBYE medium at 30°C for 24 h. The cells were recovered by centrifugation (3,700 × *g* for 20 min), resuspended to 0.1g/liter in 50 mM Tris buffer, pH 7.5, containing 0.1 M NaCl and 200 units/ml of lysostaphin (Sigma, St. Louis, MO), and incubated on ice for 30 min. The cells were disrupted by sonication and centrifuged at 13,000 × *g* for 10 min. *In vitro* olefin biosynthesis was evaluated by monitoring the conversion of eicosanoic acid to 1-nonadecene, stearic acid to 1-heptadecene, or palmitic acid to 1-pentadecene. Six microliters of substrate (5% [wt/vol] of the potassium or sodium salt of the fatty acid in 1% tergitol) was added to 1 ml of the cell extract (above) such that the final substrate concentration was 1 mM, and the mixture was incubated at ambient temperature for 3 h. The samples were extracted with 200 μl of ethyl acetate, and the organic phase was analyzed for hydrocarbons by gas chromatography/mass spectrometry (GC/MS). This assay was also used to monitor fatty acid decarboxylase activity in fractions during protein purification from ATCC 8456.

Protein purification methods. The *in vitro* olefin production assay was used to develop the following purification scheme for the enzyme responsible for olefin biosynthesis from *Jeotgalicoccus* sp. ATCC 8456. Six 1-liter cultures were grown in TSBYE medium at 30°C for 24 h. The cells were recovered by centrifugation, and the cell pellet was resuspended in 100 ml of 50 mM Tris, pH 8.0, containing 0.1 M NaCl, 2 mM dithiothreitol (DTT), and bacterial Protease Arrest (100×; G Biosciences, Maryland Heights, MO). The cell slurry was passed once through a French press at a pressure of 30,000 lb/in^2 and then sonicated. The lysate was centrifuged at 11,000 × *g* for 60 min at 4°C to remove cell debris. Ammonium sulfate was added to the cell-free lysate to a final concentration of 50% saturation. The mixture was gently stirred at 4°C for 60 min and then centrifuged at 11,000 × *g* for 30 min. To the supernatant additional ammonium sulfate was added to 65% saturation. The mixture was stirred again for 60 min at 4°C and centrifuged under the same conditions. The supernatant was discarded, and the remaining pellet was resuspended in 50 ml of 50 mM Tris, pH 8.0, containing 2 mM DTT. The mixture was then fractionated by cation exchange chromatography. The sample was loaded (3 ml/min) onto a 5-ml HiTrap SP column (GE Healthcare, Waukesha, WI) equilibrated with 50 mM Tris, pH 8.0, containing 2 mM DTT. The column was then washed with the same buffer containing 0.4 M NaCl (buffer A) followed by a 20-min gradient (3 ml/min) from 0.4 M NaCl to 1 M NaCl in the same buffer. Five-milliliter fractions were collected. Fractions containing olefin-forming activity, typically eluting between 600 and 750 mM NaCl, were pooled and dialyzed against buffer A. The dialyzed sample was then fractionated by anion exchange chromatography. The sample was loaded (4 ml/min) onto a 1-ml Resource Q column (GE Healthcare) equilibrated with the same buffer as before. The column was washed and eluted with a 7-min gradient from 0 to 0.25 M NaCl in the same buffer (4 ml/min). Fractions (1.5 ml) were collected, and active fractions, usually eluting between 150 and 200 mM NaCl, were pooled and concentrated using an Amicon protein concentrator (Millipore, Bedford, MA) with a 10-kDa exclusion size to about 50 μl. Protein concentration was determined by a Bradford assay (Bio-Rad, Hercules, CA), and the purity of the recovered protein was determined by SDS-PAGE.

For purification of recombinant His-tagged proteins, *E. coli* was grown in LB medium supplemented with 100 μg/ml carbenicillin and 0.5 mM δ-aminolevulinic acid. Enzyme production was induced by adding 1 mM IPTG at an OD_{600} of 0.6, and the cultures were grown for an additional 18 h at 25C. The cells were lysed as described above, and the His-tagged proteins were purified using a Ni-nitrilotriacetic acid (NTA) resin (Qiagen, Valencia, CA) according to the manufacturer's instructions.

***In vitro* assays with purified His-tagged proteins.** Fatty acid decarboxylation and hydroxylation assays with purified P450 enzymes were carried out as 500-μl reaction mixtures containing 100 mM sodium phosphate buffer (pH 7.2), 200 μM potassium palmitate, 500 μM hydrogen peroxide (H_2O_2), and 0.5 μM P450 enzyme at room temperature. To determine initial rates, time points were taken at 1, 2, 3, and 5 min by quenching the reactions with 50 μl of 10 M HCl followed by extraction with 200 μl of ethyl acetate. Fifty microliters of the extracts was

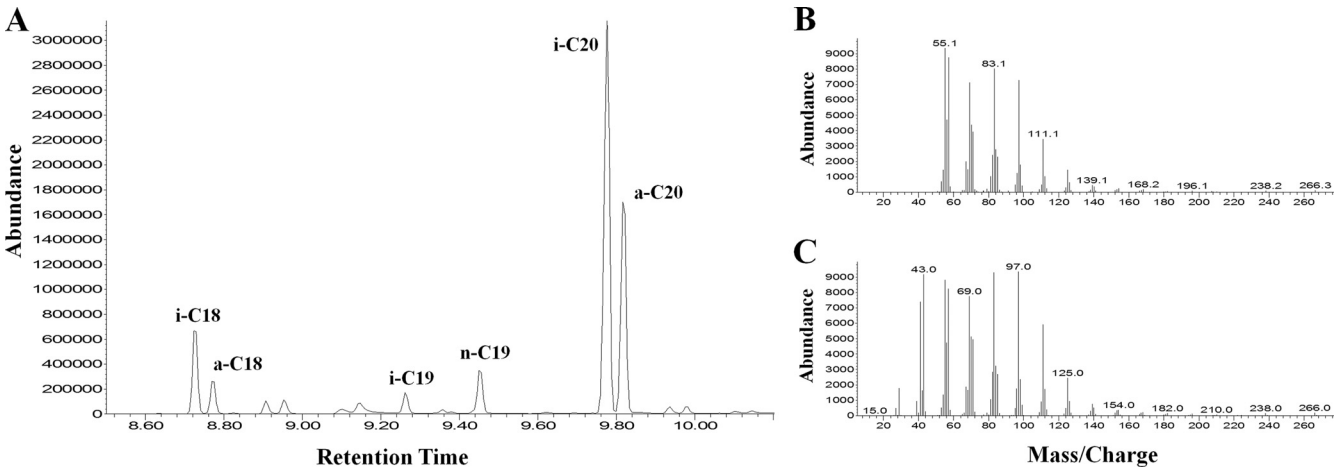


FIG. 1. Terminal olefins identified in *Jeotgalicoccus* sp. ATCC 8456. (A) GC/MS trace of hexane-extracted *Jeotgalicoccus* cells. (B) Mass spectrum of the peak at 9.45 min, which was identified as 1-nonadecene. (C) Mass spectrum of the authentic 1-nonadecene standard. For designations, see Table 1.

derivatized with an equal volume of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (Sigma). Samples were then analyzed using GC/MS. Standard curves were generated using authentic references from Sigma. In some experiments H_2O_2 was replaced with 200 μM DTT.

Peptide analysis by liquid chromatography/mass spectroscopy. To determine the identity of the terminal olefin-forming enzyme, the purified protein bands were excised from an SDS-PAGE gel after electrophoresis and digested with trypsin as follows. Gel pieces were immersed in 110 ml of 4.5 mM DTT and 100 mM Tris, pH 7.8, for 30 min at 55°C and then soaked in 110 ml of 10 mM acrylamide and 100 mM Tris, pH 7.8, for 30 min at ambient temperature. Next, the gel pieces were incubated in 500 ml of 50% acetonitrile and 50 mM Tris, pH 7.8, for 30 min at ambient temperature. The gel pieces were then covered with 25 mM Tris (pH 7.8), 5 pmol of trypsin (Promega, Madison, WI) was added, and the covered pieces were incubated overnight at 37°C. This solution was then removed and injected into a liquid chromatography-tandem mass spectrometry (LC-MS/MS) instrument equipped with a peptide trap for online desalting, followed by separation with a 0.75- by 100-mm C_{18} column. Material eluting from the column was analyzed using an electrospray ionization (ESI)-ion trap mass spectrometer (Thermo Deca XP Plus). This work was carried out at the Vincent Coates Foundation Mass Spectrometry Laboratory (Stanford University Mass Spectrometry, Palo Alto, CA). The data were then compared with the open reading frames identified from the ATCC 8456 genome using the program Mascot (35).

Fatty acid extraction and transesterification. Total lipids were extracted using the methods of Bligh and Dyer (9). Briefly, 1 ml of cell suspension was mixed with 3.75 ml of chloroform-methanol (1:2). After a 15-min sonication, 1.25 ml of chloroform was added, and the mixture was vortexed; this was followed by the addition of 1.25 ml of H_2O, and this mixture was also vortexed. The suspension was centrifuged (4,000 × *g* for 5 min), the lower phase was transferred to a fresh tube, and the solvent was evaporated in a Speedvac. The dried-down samples were redissolved in 1 ml of dimethyl ether, and 40 μl of methyl acetate and 20 μl of sodium methoxide were added for transesterification. After incubation for 15 min at ambient temperature, the reaction was quenched with 30 μl of a saturated solution of oxalic acid in dimethyl ether. The transesterified samples were analyzed by GC/MS.

Hydrocarbon extraction. To evaluate hydrocarbon production, cell pellets from 5-ml *Jeotgalicoccus* cultures were extracted by resuspending them in 1 ml of methanol followed by sonication for 30 min. Then, 4 ml of hexane was added, and the suspension was mixed thoroughly and centrifuged (4,000 × *g* for 5 min). The upper phase was transferred, and after solvent evaporation, hydrocarbons were resuspended in 0.1 ml of hexane and analyzed by GC/MS. Hydrocarbons were isolated from *E. coli* by extracting culture broth with an equal volume of ethyl acetate. After centrifugation (13,000 × *g* for 5 min), the organic phase was directly subjected for GC/MS.

Analytical methods. All hydrocarbon and fatty acid samples were subjected to GC/MS analysis using the following method. An HP-5-MS column (30 m by 0.25 mm; 0.25-μm film) was used with a helium flow rate set to 1.3 ml/min. Injections of 1μl were carried out under splitless injection conditions with the inlet set to 300°C. The temperature profile was as follows. The oven temperature was held at 100°C for 5 min, and the temperature was then ramped up to 320°C at a rate of 20°C/min. The oven was then held for 5 min at 320°C. The mass spectrometer detector temperature was set to 300°C and scanned at 50 to 500 *m*/*z* in the electron impact mode. To aid peak identification, authentic references were used when available, and their retention times and fragmentation patterns were compared with the ones from extracted hydrocarbons. Branch point locations in hydrocarbons were determined by the presence of characteristic secondary C_nH_{2n-1} fragment ions as previously described (29). The location of the double bond in terminal olefins was verified by derivatization with dimethyl disulfide as described previously (5). α- And β-hydroxy fatty acids thermally degrade in the inlet of a GC, forming terminal olefins as a degradation product. To prevent this, samples were derivatized with an equal volume of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (Sigma). This reagent silylates the hydroxyl group, preventing thermal degradation.

Nucleotide accession numbers. The sequence of $oleT_{JE}$ (ORF 880) was deposited in GenBank under accession number HQ709266, and that for the *Jeotgalicoccus* sp. ATCC 8456 16S rRNA gene was deposited under accession number HQ709267.

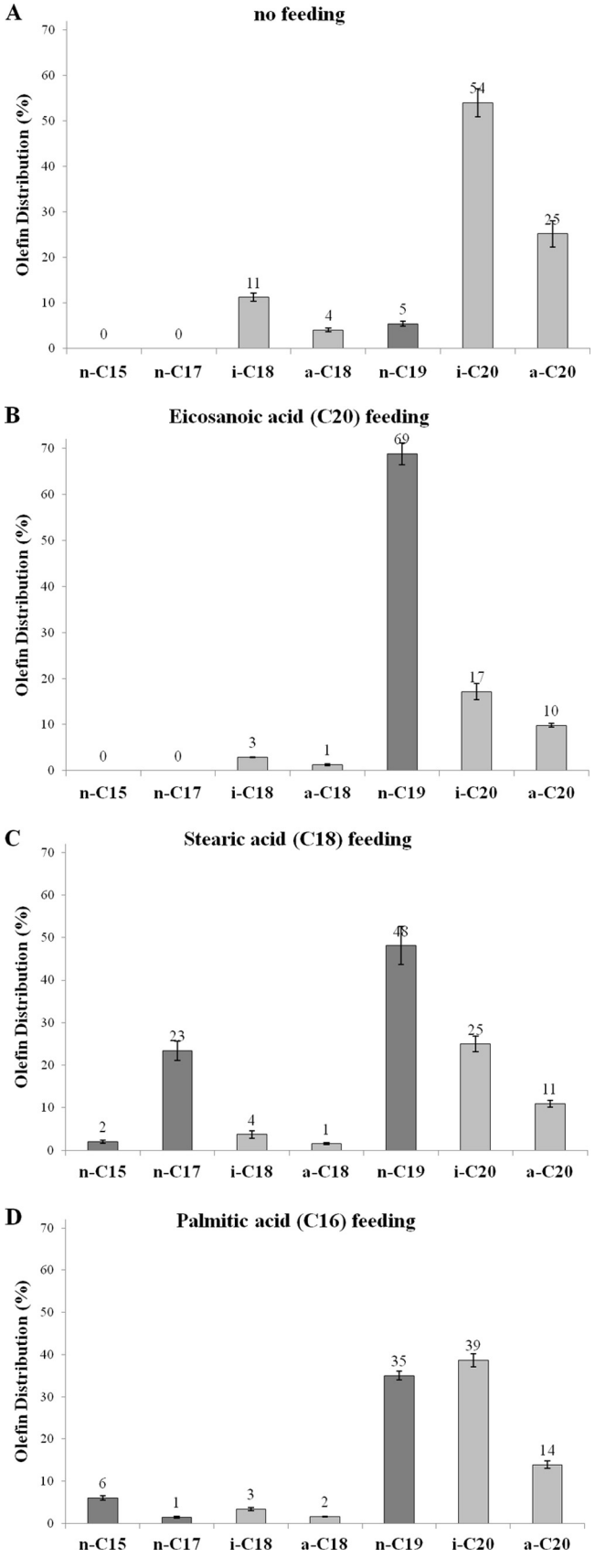
RESULTS

Identification of terminal olefins in strain ATCC 8456. In an effort to identify terminal-olefin-producing microorganisms, a number of micrococci were reanalyzed which had previously been reported to produce olefins (1, 2, 40). Whereas most

TABLE 1. Terminal olefins detected in *Jeotgalicoccus* strains

Terminal olefin observed	Distribution (%) in *Jeotgalicoccus* sp. strain[a]			
	ATCC 8456	DSMZ 17274	DSMZ 19085	DSMZ 17030
1-Heneicosadecene (n-C_{21})	0	5	6	8
19-Methyl-1-eicosadecene (i-C_{21})	0	2	2	2
18-Methyl-1-nonadecene (i-C_{20})	56	36	42	31
17-Methyl-1-nonadecene (a-C_{20})	22	38	43	56
1-Nonadecene (n-C_{19})	5	13	1	3
17-Methyl-octadecene (i-C_{19})	2	4	4	0
16-Methyl-1-heptadecene (i-C_{18})	11	1	1	0
15-Methyl-1-heptadecene (a-C_{18})	4	1	1	0

[a] All strains were grown in TSBYE medium at 30°C for 24 h. Data represent the percent distribution of total olefins (i, iso; a, anteiso; and n, straight).



strains produced long-chain olefins (C_{23} to C_{33}) with internal double bonds, a strain designated *Micrococcus candicans* ATCC 8456 produced shorter olefins (C_{18} to C_{20}). This was recognized previously (40), but the position of the double bond in this strain's olefins had not been elucidated. A detailed analysis (see Materials and Methods) revealed that all olefins isolated from strain ATCC 8456 have terminal, not internal, double bonds. Seven different terminal olefins were detected in strain ATCC 8456, with 18-methyl-1-nonadecene (i-C_{20}) being the most abundant olefin (Fig. 1 and Table 1).

Classification of strain ATCC 8456 as *Jeotgalicoccus* sp. *Micrococcus candicans* ATCC 8456 was described as having a low G+C content of <40% (40), suggesting that it was misclassified (micrococci belong to the class of high-G+C-content Gram-positive bacteria). Therefore, the phylogenetic classification of ATCC 8456 was reassessed by amplifying and sequencing its 16S rRNA gene. The sequence was classified using the Ribosomal Database Project website (http://rdp.cme.msu.edu/) with a 100% confidence threshold as *Jeotgalicoccus* spp. It showed 99% sequence identity to the 16S rRNA genes from *Jeotgalicoccus marinus* JSM 076033 (12) and *Jeotgalicoccus* sp. strain NY-2 (26). As it may relate to olefin biosynthesis, the fatty acid composition of *Jeotgalicoccus* sp. ATCC 8456 lipids was also determined. The lipids contained mainly anteiso- and iso-branched fatty acids, which is common for many Gram-positive bacteria including other jeotgalicocci (55). The major fatty acids were 13-methyl- and 12-methyl-tetradecanoic acid (a-C_{15}, i-C_{15}), 14-methyl pentadecanoic acid (i-C_{16}), and 15-methyl- and 16-methyl hexadecanoic acid (a-C_{17}, i-C_{17}). Palmitic acid (n-C_{16}) and stearic acid (n-C_{18}) were minor components.

Terminal olefin production in other *Jeotgalicoccus* strains. In order to determine if terminal olefin biosynthesis is specific to ATCC 8456 or common to the genus *Jeotgalicoccus*, three other strains, *J. halotolerans* DSMZ 17274, *J. psychrophilus* DSMZ 19085, and *J. pinnipedialis* DSMZ 17030 (28, 55) were evaluated. As shown in Table 1, all three strains produced similar terminal olefins, suggesting that the ability to synthesize terminal olefins is likely widespread in the genus *Jeotgalicoccus*.

Impact of fatty acid feeding on terminal olefin biosynthesis of *Jeotgalicoccus* sp. ATCC 8456. To gain insight into the mechanism of olefin biosynthesis, the impact of fatty acids provided in the culture medium on olefin composition was evaluated for *Jeotgalicoccus* sp. ATCC 8456. As shown in Fig. 2B, ATCC 8456 converted added eicosanoic acid (C_{20}) exclusively to 1-nonadecene (C_{19}) (a 20-fold increase over the control was observed). Palmitic acid (C_{16}) and stearic acid (C_{18}) feeding led to a significant increase in C_{19} as well, but C_{15} and C_{17} terminal olefins were also observed (Fig. 2C and D). While the C_{20} feeding data supported a direct decarboxylation mechanism, the production of the higher olefins from the C_{16} and C_{18}

FIG. 2. Fatty acid feeding of *Jeotgalicoccus* sp. ATCC 8456 cultures. The changes in olefin distribution upon feeding of three different fatty acids (0.5%, wt/vol) are shown. Straight-chain olefins are in dark gray and branched-chain olefins in light gray. For designations, see Table 1. The values are averages of five and two independent experiments for the unfed and fed cultures, respectively.

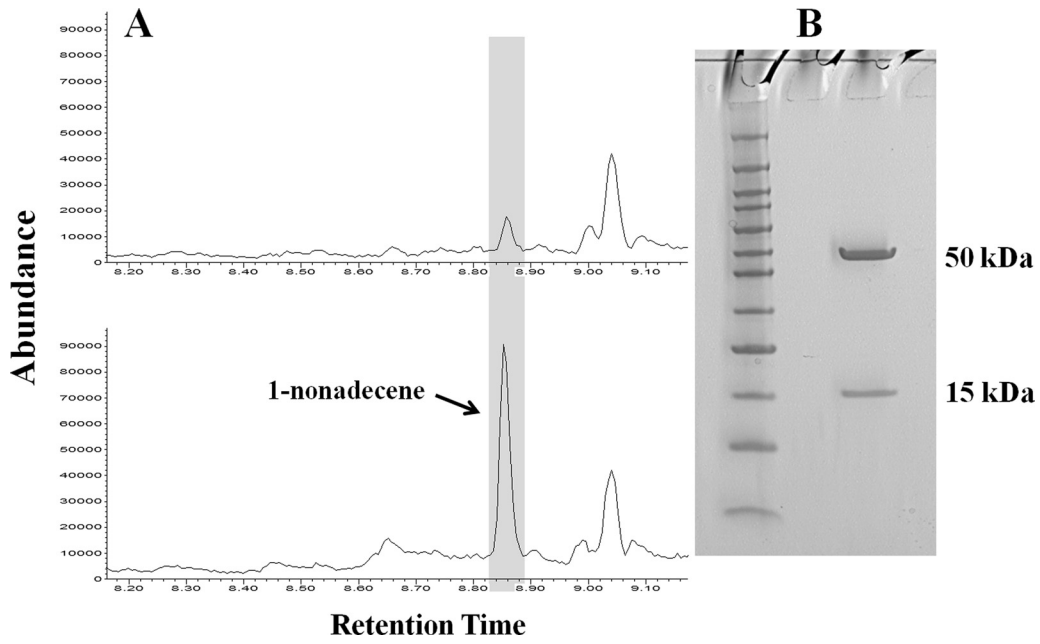


FIG. 3. Demonstration of *in vitro* activity and purification of a fatty acid decarboxylase from *Jeotgalicoccus* sp. ATCC 8456. (A) GC/MS trace of *in vitro* reactions of ATCC 8456 cell-free lysate without (top) and with (bottom) eicosanoic acid as a substrate. The peak at 8.85 min was identified as 1-nonadecene. (B) Coomassie-stained SDS-PAGE of the active enzyme fraction after a three-step purification protocol.

feeds suggested that other reactions, such as elongation, could precede decarboxylation of the C_{16} and C_{18} fatty acids.

***In vitro* fatty acid decarboxylation by *Jeotgalicoccus* sp. ATCC 8456 extracts.** *Jeotgalicoccus* sp. ATCC 8456 cell extracts were evaluated for their ability to convert fatty acids to terminal olefins. As shown in Fig. 3A, eicosanoic acid was converted to 1-nonadecene *in vitro*. When stearic acid was used as a substrate, the predominant product was 1-heptadecene; however, small amounts of 1-nonadecene were also observed (data not shown). These data further suggested that *Jeotgalicoccus* sp. ATCC 8456 contains a novel enzymatic activity that decarboxylates fatty acids to terminal olefins.

Identification of the fatty acid decarboxylase gene from *Jeotgalicoccus* sp. ATCC 8456. The ability to follow the conversion of free fatty acids to terminal olefins allowed us to use a reverse genetic strategy to identify the responsible olefin biosynthetic genes. Starting from an ATCC 8456 cell extract, a three-step purification protocol was developed that resulted in the isolation of the fatty acid decarboxylase activity in a fraction that contained only two major proteins with approximate molecular masses of 50 kDa and 15 kDa according to SDS-PAGE (Fig. 3B). LC-MS/MS evaluation of the trypsin digests of the protein bands provided protein sequence information that could be used to probe the ATCC 8456 genome. Therefore, a draft genome sequence of ATCC 8456 was obtained. Assembly of the sequence data resulted in 21 contigs that suggested that the *Jeotgalicoccus* ATCC 8456 genome was approximately 2.1 Mbp with a 36.7% G+C content. Open reading frame (ORF) analysis identified 1,584 ORFs in the draft genome, and the predicted peptide fragments from the 50-kDa protein gave a high Mascot score of 919 with open reading frame 880, which had a predicted molecular mass of 48,367 Da, matching the active protein's experimentally determined value. The predicted peptide fragments from the 15-kDa protein could not be assigned.

To evaluate whether ORF 880 was sufficient to support terminal olefin biosynthesis, the gene was cloned into an expression vector and transformed into *E. coli* (MG1655 *fadE*). As can be seen from Fig. 4A, *E. coli* expressing the putative fatty acid decarboxylase produced 1-pentadecene and 1,10-heptadecadiene, while control cells not expressing the ATCC 8456 ORF 880 did not. Further, when the expressing cells were fed stearic acid, 1-heptadecene formation was observed. These results demonstrate that ORF 880 is sufficient for recombinant terminal olefin production in *E. coli* and further support the notion that terminal olefin biosynthesis occurs via the decarboxylation of free fatty acids. ORF 880 was named *oleT*$_{JE}$.

Assignment of OleT$_{JE}$ (ORF 880) to the cyp152 enzyme family of P450 peroxygenases. The ORF 880 gene product, OleT$_{JE}$, is predicted to be a cytochrome P450 enzyme. The closest ortholog to OleT$_{JE}$ is a P450 from *Macrococcus caseolyticus* with unknown function (59% amino acid sequence identity) (see also Fig. S1 in supplemental material for the genomic context of *oleT*$_{JE}$). P450s form a large superfamily of multifunctional proteins (8) and are divided into different CYP families according to their sequence similarity (43). Based on sequence comparison, OleT$_{JE}$ was assigned to the cyp152 family (see Fig. S2). The cyp152 family consists of P450s from a variety of bacteria. The best-studied family members, *Bacillus subtilis* P450 (P450$_{BS\beta}$) (38) and *Sphingomonas paucimobilis* P450 (P450$_{SP\alpha}$) (37), catalyze the hydroxylation of fatty acids in the β- and/or α-position. In contrast to most other P450 monooxygenases, which require O_2, NADPH, and redox partners, P450$_{BS\beta}$ (cyp152A1) and P450$_{SP\alpha}$ (cyp152B1) are highly active with hydrogen peroxide (H_2O_2) as the sole electron and oxy-

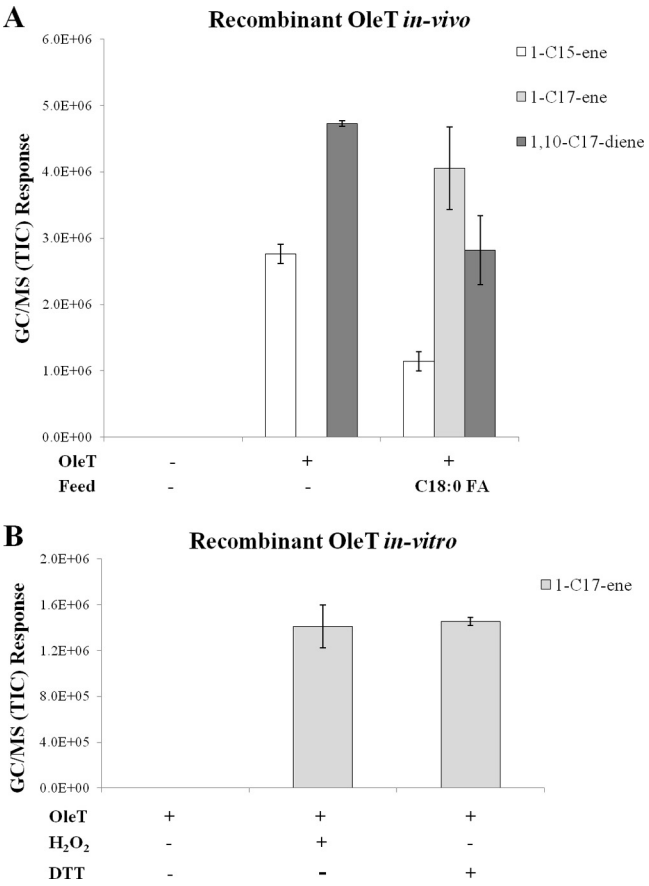


FIG. 4. Analysis of recombinant $OleT_{JE}$. (A) *In vivo* activity upon expression in *E. coli* with and without stearic acid feeding. (B) *In vitro* activity of purified $OleT_{JE}$ with stearic acid as substrate. 1-C_{15}-ene, 1-pentadecene; 1-C_{17}-ene, 1-heptadecene; 1,10-C_{17}-diene, 1,10-heptadecadiene. TIC, total ion count.

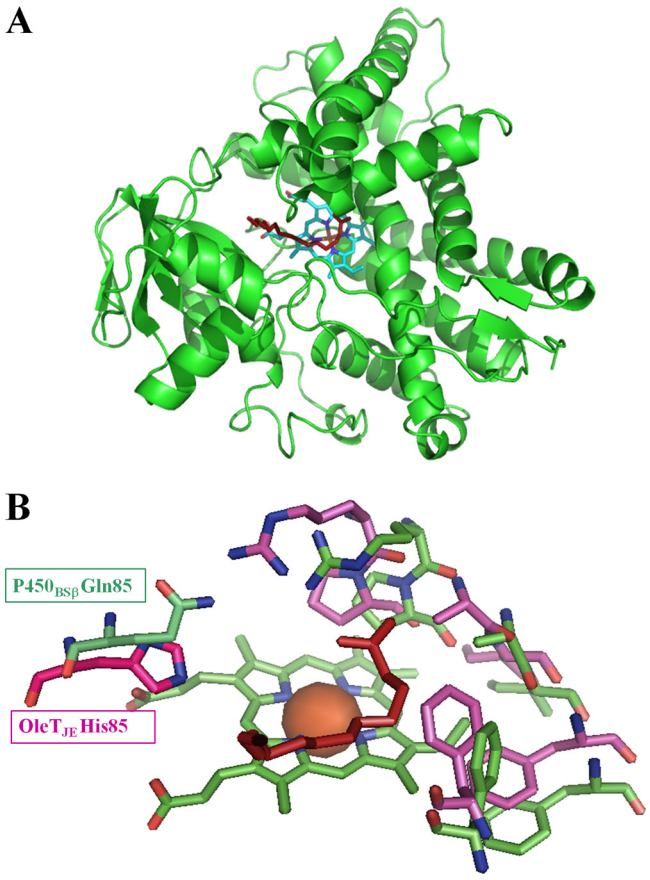


FIG. 5. Homology model of $OleT_{JE}$. (A) Overview of homology model of $OleT_{JE}$ obtained by threading the $OleT_{JE}$ sequence with the crystal structure of $P450_{BS\beta}$ from *B. subtilis* (Protein Data Bank entry 1IZO). (B) A view of the active sites of $OleT_{JE}$ (purple) overlaid with the one of $P450_{BS\beta}$ (green). In red is the free fatty acid cocrystallized with $P450_{BS\beta}$. Most amino acid residues in the active site are conserved between the two enzymes, except for His85 in $OleT_{JE}$, which corresponds to Gln in $P450_{BS\beta}$.

gen donor and are referred to as peroxygenases (36). To examine if $OleT_{JE}$ catalytic activity requires H_2O_2, a His-tagged version of the enzyme was purified from *E. coli*, and *in vitro* assays with different cofactors were carried out. Indeed, $OleT_{JE}$ was unable to catalyze stearic acid decarboxylation alone, but 1-heptadecene was produced when H_2O_2 was added to the reaction mixture (Fig. 4B). This result is consistent with our earlier data in which DTT had been added to all decarboxylation assays. Previous studies have shown that DTT in the presence of oxygen and iron—the metal center found in P450s—creates hydrogen peroxide (41). As can be seen in Fig. 4B, the recombinant protein converted stearic acid to 1-heptadecene when H_2O_2 was replaced with DTT. The enzyme also formed olefins from myristic acid, palmitic acid, and eicosanoic acid, but no olefins were formed from β-hydroxy fatty acids or fatty aldehydes (data not shown). However, α- and β-hydroxy fatty acids were detected as minor products in some *in vitro* fatty acid decarboxylation reactions.

Homology modeling of $OleT_{JE}$ based on the $P450_{BS\beta}$ structure. Most members of the cyp152 family are of unknown function, but at least three members of the family have been shown to hydroxylate fatty acids in the α- or β-position (23, 37, 38). The discovery of $OleT_{JE}$ as a terminal olefin-forming fatty acid decarboxylase raised the question as to whether there are structural determinants that distinguish P450 fatty acid hydroxylases from decarboxylases. The only crystal structure of a cyp152 enzyme available is the one of $P450_{BS\beta}$ (33), the product of the *B. subtilis ybdT* gene, which has 41% amino acid sequence identity with $OleT_{JE}$. A homology model of $OleT_{JE}$ was generated using Swiss-Model (3) with $P450_{BS\beta}$ as the template (Fig. 5A). The most notable difference in the substrate binding channel near the P450 heme was the presence of a histidine (position 85) in $OleT_{JE}$ in place of glutamine in $P450_{BS\beta}$ (Fig. 5B). This glutamine residue is conserved in most cyp152 P450s (see Fig. S3 in the supplemental material) and is approximately 8 to 9 Å from the β-position of the bound fatty acid substrate (Fig. 5B). No significant additional amino acid differences in the active site between $OleT_{JE}$ and $P450_{BS\beta}$ were apparent.

Analysis of other members of the cyp152 P450 enzyme family for terminal olefin-forming activity. Based on the homology model, six cyp152 P450s were tested for their ability to synthesize terminal olefins from palmitic acid *in vivo* (heterologously expressed in *E. coli*) and *in vitro* (purified enzymes): the two

TABLE 2. Analysis of terminal olefin-forming fatty acid decarboxylase activity of selected cyp152 P450s

Organism	Accession no.	Function	% Identity	Position 85 residue[b]	Product from palmitic acid[a]		
					1-PE	α-OH PA	β-OH PA
Jeotgalicoccus sp.	HQ709266	Fatty acid decarboxylase	100	His	+	+	+
C. efficiens	NP_739069	Unknown	27	His	+	(−)[c]	(−)[c]
K. rhizophila	YP_001856010	Unknown	29	His	+	−	+
B. clausii	YP_176535	Unknown	37	Gln	−	+	−
M. populi	ZP_02200540	Unknown	31	Met	+	−	−
B. subtilis	NP_388092	Fatty acid hydroxylase	41	Gln	+	+	+
S. paucimobilis	BAA22987	Fatty acid hydroxylase	36	Gln	−	+	−

[a] Detection in *E. coli* cells expressing heterologous enzymes (*in vivo*) and measurement of *in vitro* activity using purified enzyme; *in vivo* and *in vitro* results correlated well for all analyzed P450s. 1-PE, 1-pentadecene; α-OH PA, α-hydroxy palmitic acid; β-OH PA, β-hydroxy palmitic acid.
[b] Position 85 in *Jeotgalicoccus* sp. ATCC 8456 was hypothesized to be important for olefin formation (see text).
[c] Analyzed only in an *in vivo* experiment; *in vitro* activity was not determined as His_6-P450 could not be purified.

most intensively studied cyp152 P450s ($P450_{BS\beta}$ and $P450_{SP\alpha}$), three P450s of unknown function with amino acid residues other than glutamine in position 85 (from *Kocuria rhizophila*, *Corynebacterium efficiens*, *Methylobacterium populi*), and a P450 from *Bacillus clausii*. None of these organisms had been reported to produce terminal olefins. As shown in Table 2, four of the P450s were able to form 1-pentadecene *in vivo* as well as *in vitro*, which coincided with their ability to form β-hydroxy palmitic acid as the major product. The two P450s without olefin-forming activity catalyzed fatty acid hydroxylation only in the α-position. Consequently, there is a correlation between the ability of a P450 enzyme to hydroxylate fatty acids in the β-position and the ability to decarboxylate fatty acids to the terminal olefin.

Terminal olefin-forming activity of *B. subtilis* $P450_{BS\beta}$ (cyp152A1). Except for $P450_{BS\beta}$ all three additional olefin-forming P450s identified had amino acids other than glutamine in position 85, suggesting that this position may be important for fatty acid decarboxylation. To test the impact of replacing glutamine 85 with histidine, a Q85H variant of $P450_{BS\beta}$ was created and analyzed for its catalytic conversion of palmitic acid. As shown in Fig. 6A, $OleT_{JE}$ and $P450_{BS\beta}$ had similar rates of 1-pentadecene formation *in vitro*, while $OleT_{JE}$ formed 25 and 15 times less α-hydroxy- and β-hydroxy palmitic acid, respectively. This suggests that, unlike $P450_{BS\beta}$, $OleT_{JE}$ is more selective for decarboxylating than hydroxylating fatty acids (Fig. 6B). Furthermore, $P450_{BS\beta}$ Q85H showed a decreased rate of α-hydroxy palmitic acid formation and an increased rate of 1-pentadecene and β-hydroxy palmitic acid formation compared to wild-type $P450_{BS\beta}$ (Fig. 6A and B), confirming that this amino acid residue is important for the catalytic mechanism of these P450 enzymes.

DISCUSSION

Terminal olefin (or 1-alkene) production has been reported mainly in eukaryotic organisms (25, 39, 42). Here, we report that terminal olefin biosynthesis is also common in bacteria of the genus *Jeotgalicoccus*, a novel genus within the low-G+C-content Gram-positive firmicutes often associated with sea animals found in seawater (28, 55). The ability to synthesize olefins has not been reported for other low-G+C-content Gram-positive bacteria such as *Bacillus* or *Staphylococcus* (40). Strain ATCC 8456, which was the main focus of this study, was originally classified as *Micrococcus candicans* (40), but sequencing its 16S rRNA gene unequivocally identified it as *Jeotgalicoccus* sp.

To evaluate the mechanism of olefin biosynthesis, fatty acid feeding studies were carried out. The *in vivo* bioconversion studies along with cell-free *in vitro* olefin biosynthesis studies suggest that *Jeotgalicoccus* utilizes fatty acid intermediates as substrates for olefin biosynthesis and that fatty acid decarbox-

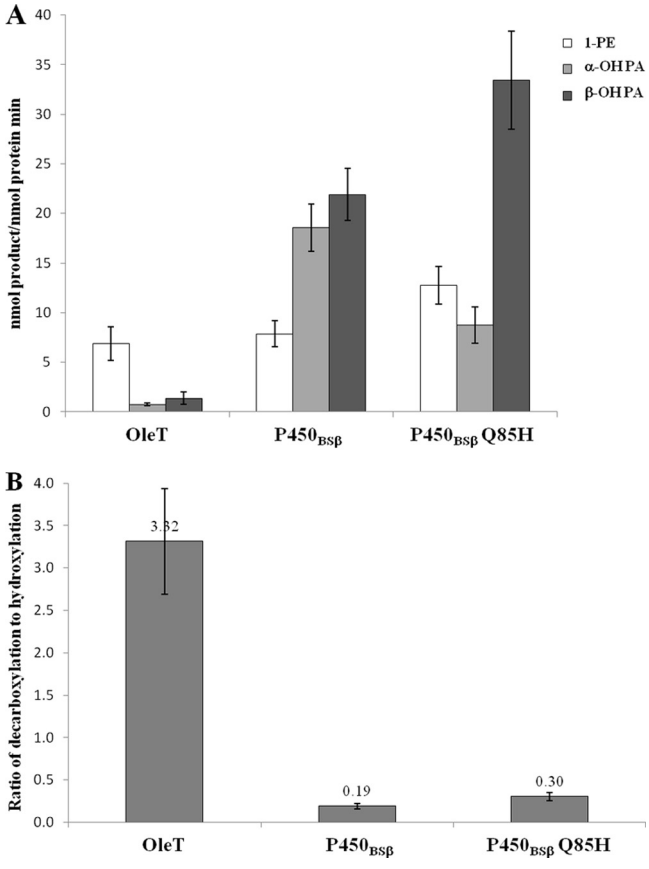


FIG. 6. *In vitro* hydroxylation and decarboxylation of palmitic acid by purified $OleT_{JE}$, $P450_{BS\beta}$, and $P450_{BS\beta}$ Q85H proteins. (A) Rate of product formation. (B) Ratio of fatty acid decarboxylation over hydroxylation. 1-PE, 1-pentadecene; α-OH PA, α-hydroxy palmitic acid; β-OH PA, β-hydroxy palmitic acid.

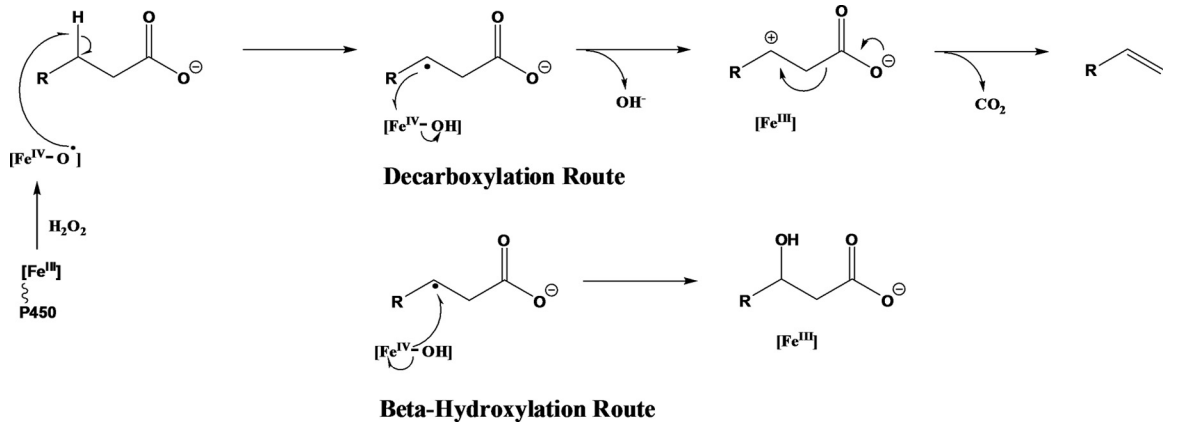


FIG. 7. Proposed mechanism for fatty acid decarboxylation and α- or β-hydroxylation carried out by $OleT_{JE}$ and related cyp152 P450 enzymes.

ylation is a primary mechanism of terminal olefin production. It should be noted that the *in vivo* bioconversion of C_{16} and C_{18} fatty acids was primarily to C_{19} terminal olefins. Thus, additional reactions, such as fatty acid elongation, may precede decarboxylation of shorter chain length fatty acids in *Jeotgalicoccus*. Since the appearance of the n-1 terminal olefins was consistent for all substrates, albeit at lower levels than the C_{19} olefin, it is possible that the shorter substrates were naturally elongated before decarboxylation occurred and may reflect the preference of the decarboxylase for longer chain substrates.

The strongest evidence for a direct fatty acid decarboxylation mechanism came from experiments with *Jeotgalicoccus* sp. ATCC 8456 cell-free lysates, in which eicosanoic acid was exclusively converted to 1-nonadecene and stearic acid was converted mainly to 1-heptadecene. Using this *in vitro* assay, the terminal-olefin-forming fatty acid decarboxylase was purified, and its encoding gene was identified by reverse genetics. The gene, named *oleT*, encoded a P450 enzyme. Heterologously expressed $OleT_{JE}$ converted fatty acid to terminal olefins both *in vivo* (in *E. coli*) and *in vitro*. Although *oleT* could not be deleted in *Jeotgalicoccus* sp. ATCC 8456 for lack of a genetic system, its gene product is the most likely source for terminal olefin biosynthesis in this strain. Interestingly, the ATCC 8456 genome contained an *oleT* paralog with 38% amino acid sequence identity, but overexpression of this P450 in *E. coli* did not lead to terminal olefin formation (data not shown).

$OleT_{JE}$ belongs to the cyp152 family of peroxygenases, which includes certain fatty acid hydroxylases (36). The presence of α- and β-hydroxy fatty acids as minor products in the $OleT_{JE}$ *in vitro* reaction was therefore not completely unexpected (see below for the proposed reaction mechanism). However, the ability of $P450_{BS\beta}$ to decarboxylate fatty acids in a standard *in vitro* assay was surprising as it had not been reported previously. Therefore, extra care was taken to avoid any analytical artifacts by fully derivatizing all samples (α- and β-hydroxy fatty acids can undergo thermal degradation to terminal olefins in the inlet of a gas chromatograph). The discrepancy may be explained by the methods used in a previous study (38) to detect *in vitro* conversion of myristic acid (C_{14}) by $P450_{BS\beta}$: derivatization with a fluorescent probe and product analysis with high-performance liquid chromatography (HPLC), which would not have detected the nonfluorescent, underivatized olefin side product, or GC/MS with a temperature program that may not have observed the volatile olefin (i.e., 1-tridecene).

As expected from a member of the cyp152 family, it was confirmed that $OleT_{JE}$ uses H_2O_2 as its main source of electrons (36). Figure 7 provides a possible mechanism describing how this class of P450s is able to carry out both fatty acid hydroxylation (in α- and β-position) and decarboxylation chemistries. First, the iron center of the P450 is oxidized by H_2O_2 to the high-energy oxo-ferryl heme via the hydrogen peroxide shunt. Next, the iron abstracts a hydrogen atom from either the α- or β-position of the free fatty acid, giving rise to a carbon radical on the substrate. Two possible reaction pathways are possible from here. Oxygen rebound can take place, resulting in hydroxylation at the α- or β-position forming a hydroxy fatty acid. Alternatively, an additional proton can be abstracted from the β-position, forming water and a carbocation, which can readily decarboxylate to form the terminal olefin. This mechanism would explain why fatty acid hydroxylases that only hydroxylate at the α-position do not form terminal olefins because a radical at the α-position cannot readily decarboxylate. The role of His85 in $OleT_{JE}$ is not apparent, but its importance for catalysis was demonstrated by an increased decarboxylase and β-hydroxylase activity observed in the $P450_{BS\beta}$ Q85H variant. More detailed experimental work is needed to confirm the proposed mechanism.

Cytochrome P450s are incredibly versatile biocatalysts (8). Olefin-forming fatty acid decarboxylation can now be added to the impressive list of enzymatic reactions carried out by P450 enzymes. Mechanistically, $OleT_{JE}$ shows similarities to a P450 from the yeast *Rhodotorula minuta*, $P450_{Rm}$, which has been shown to form isobutene from isovalerate via a decarboxylative mechanism that involves hydrogen removal from the β-carbon (22). $P450_{Rm}$ is a monooxygenase (Cyp53B) and it also catalyzes the hydroxylation of benzoate (21). $OleT_{JE}$ activity on the other hand is quite distinct from P450-catalyzed deformylation of aldehydes, which also forms olefinic molecules, for example, the deformylation of terpenoids such as citronellal to 2,6-dimethyl-1,5-heptadiene by cyp2B4 (45). In contrast to $OleT_{JE}$,

these olefin-forming P450s act on carbonyl and not carboxyl groups and release formate as opposed to carbon dioxide (13). Furthermore, branching at the α- or β-position seems to be an important feature of the substrates for deformylation. Consequently, the deformylation of fatty aldehydes to terminal olefins has not been reported. OleT-type P450s also need to be distinguished from another class of P450s that catalyze the oxidative decarbonylation of fatty aldehydes to alkanes, which have been described so far only in insects (18). A mechanism has been proposed for this reaction (44), but so far no P450 gene has been associated with this putative alkane-forming activity.

In an attempt to identify the most efficient routes for the direct and selective conversion of renewable sugar to fuel and chemical grade hydrocarbons, we have sought to indentify and characterize the major mechanisms by which such compounds are produced in nature. Since fatty acid metabolism is most efficient in natural hydrocarbon biosynthesis (46), fatty acid derivatives have been specifically investigated. We previously described the discovery and engineering of the genes responsible for internal olefin (20) and alkane (47) biosynthesis. Here, we report for the first time the discovery and engineering of genes responsible for terminal olefin biosynthesis. The further understanding of these and other mechanisms of natural hydrocarbon biosynthesis will support growing efforts to apply industrial biotechnology toward renewable fuel and chemical production and a move away from fossil feedstock dependence.

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