

Biosynthesis of Isoprenoid Wax Ester in *Marinobacter hydrocarbonoclasticus* DSM 8798: Identification and Characterization of Isoprenoid Coenzyme A Synthetase and Wax Ester Synthases^{∇†}

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Marinobacter hydrocarbonoclasticus DSM 8798 has been reported to synthesize isoprenoid wax ester storage compounds when grown on phytol as the sole carbon source under limiting nitrogen and/or phosphorous conditions. We hypothesized that isoprenoid wax ester synthesis involves (i) activation of an isoprenoid fatty acid by a coenzyme A (CoA) synthetase and (ii) ester bond formation between an isoprenoid alcohol and isoprenoyl-CoA catalyzed, most likely, by an isoprenoid wax ester synthase similar to an acyl wax ester synthase, wax ester synthase/diacylglycerol acyltransferase (WS/DGAT), recently described from *Acinetobacter* sp. strain ADP1. We used the recently released rough draft genome sequence of a closely related strain, *M. aquaeolei* VT8, to search for WS/DGAT and acyl-CoA synthetase candidate genes. The sequence information from putative WS/DGAT and acyl-CoA synthetase genes identified in this strain was used to clone homologues from the isoprenoid wax ester synthesizing *Marinobacter* strain. The activities of the recombinant enzymes were characterized, and two new isoprenoid wax ester synthases capable of synthesizing isoprenoid ester and acyl/isoprenoid hybrid ester *in vitro* were identified along with an isoprenoid-specific CoA synthetase. One of the *Marinobacter* wax ester synthases displays several orders of magnitude higher activity toward acyl substrates than any previously characterized acyl-WS and may reflect adaptations to available carbon sources in their environments.

Neutral lipid biosynthesis is ubiquitous in nature and occurs in animals, plants, and microbes. Microorganisms have been reported to synthesize triacylglycerols (TAGs) (27), polyhydroxyalkonates (PHAs) (22), and wax esters (WEs) (28). Neutral lipids accumulate as inclusion bodies within the microbial cell, and their purpose is to serve as carbon and energy storage under growth-limiting conditions. PHAs are composed of aliphatic monomeric unit polyesters, which are the most abundant class of neutral lipids in microbial species (22). It is believed that neutral lipid inclusion bodies not only serve as an energy storage but also remove fatty acids that may cause damage to the bacterial cell membrane (1). Until recently, only microbial PHA biosynthesis has been investigated, and their biochemistry and metabolism has been well described (22). The enzymes involved in microbial TAG biosynthesis and WE have only very recently been identified (4, 11, 29, 30).

Microbial WEs have been found in *Mycobacterium* (4), *Rhodococcus* (1), *Acinetobacter* (2), and *Marinobacter* (15, 17) strains that grow in environments where a carbon source (such as petroleum hydrocarbons [9] and gluconate [11]) may be abundant relative to other nutrients such as phosphorous and nitrogen. Acyl WEs are synthesized from long-chain fatty alcohol and fatty acyl-coenzyme A (CoA) substrates. Another

class of WEs is the isoprenoid WEs that are made from branched, long-chained isoprenoyl alcohol and isoprene fatty acid substrates. Isoprenoid WEs have been identified as a way to provide energy storage in *Marinobacter* species (15–17). *Marinobacter* species grow in marine sediment materials where there is an abundance of recalcitrant acyclic isoprenoid alcohols such as farnesol and phytol, which are derived from (bacterio)chlorophyll molecules (16, 17).

A microbial WE synthase/diacylglycerol acyltransferase (WS/DGAT) capable of catalyzing WE synthesis and, to a lesser degree, TAG synthesis was identified in the gamma proteobacterium *Acinetobacter baylyi* ADP1 (11, 23, 25). The *A. baylyi* ADP1 WS/DGAT (hereafter referred to as WS/DGAT) contains the catalytic motif HHXXXDG that is involved in the acyltransferase reaction (Pfam domain PF00668) (11). This motif has been found in numerous sequenced genomes of microbial strains that are known to make WEs and/or TAGs and is also found in the condensation domain of some non-ribosomal peptide synthetase (NRPS) modules. Mutations within this domain have been shown to abolish NRPS activity (20, 30). Shortly before the submission of the present study, the same group that characterized the WS/DGAT from *A. baylyi* ADP1 (11, 23, 25) reported the identification of two WS/DGAT homologues from the marine hydrocarbonoclastic bacterium *Alcanivorax borkumensis* (12).

The gamma proteobacteria *Marinobacter hydrocarbonoclasticus* DSM 8798 (referred to here as strain 8798) has been shown to synthesize an isoprenoid WE when grown on phytol as the sole carbon source and under nitrogen-limiting conditions (15, 17). It has been proposed that exogenous phytol is transported into the cell, where it is converted into an inter-

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mediate aldehyde (phytenal) that is then further oxidized into the isoprenic fatty acid phytenic acid, which may be hydrogenated into phytanic acid (17). Phytanic acid is then esterified with phytol to form an isoprenoid WE.

The enzymes involved in isoprenoid WE synthesis, however, are not known. We hypothesized that isoprenoid WE synthesis would, as in acyl WE synthesis, involve the condensation of a CoA-activated isoprenoid acid with an isoprenoid alcohol. Based on this hypothesis, we describe here the isolation and characterization of an isoprenoid CoA-synthetase, as well as of two isoprenoid WE synthetases from strain 8798 (7) that are capable of producing an isoprenoid wax from phytanoyl-CoA and phytol.

MATERIALS AND METHODS

Chemicals and materials. CoA trilithium salts were purchased from Roche (Indianapolis, IN). Tris (2-carboxyethyl) phosphine (TCEP) was purchased from EMB Biosciences (La Jolla, CA). Phytanic acid, palmitoyl-CoA ($C_{16:0}$), stearoyl-CoA ($C_{18:0}$), arachidoyl-CoA ($C_{20:0}$), C_{18} linolenoyl-CoA ($C_{18:3}$), myristoyl-CoA ($C_{14:0}$), lauroyl ($C_{12:0}$) and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), inorganic pyrophosphatase, Triton X-100, triolein, and ATP were purchased from Sigma (St. Louis, MO). Tergitol NP-11 was obtained from Dow Chemical Co. (Midland, MI). Gum arabic, sodium taurocholate, and all solvents (high-pressure liquid chromatography [HPLC] grade) were purchased from Fisher Scientific (Pittsburgh, PA). HPLC-grade water was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Restriction endonucleases, polynucleotide kinase, and T4 ligase were purchased from New England Biolabs (Boston, MA).

Strains and growth conditions. *Marinobacter hydrocarbonoclasticus* strain DSM 8798 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). *Acinetobacter baylyi* ADP1 was kindly provided to us by Nicholas Ornston at Yale University (New Haven, CT). *Pseudomonas putida* strain U was kindly given to us by José M. Luengo at the University of León (León, Spain). Cloning and heterologous gene expression was carried out in *Escherichia coli* strains DH5 α and JM109. *M. hydrocarbonoclasticus* DSM 8798 was grown in Luria-Bertani (LB) medium with sterile, synthetic seawater (Ricca Chemical Company, Arlington, TX) instead of distilled water. *E. coli*, *A. baylyi*, and *P. putida* were grown in LB medium at 30°C unless otherwise specified.

Gene cloning. Genomic DNA was isolated from *A. baylyi* ADP1, *M. hydrocarbonoclasticus* DSM 8798, and *P. putida* U using standard phenol-chloroform DNA extraction techniques described in Sambrook et al. (18). Degenerate rRNA oligonucleotides (TPU1, 5'-AGAGTTTGATCMTGGCTCAG; RTU8, 5'-AAG GAGGTGATCCANCCRCA [6]) were used to amplify the 16S rRNA gene sequences from *M. hydrocarbonoclasticus* DSM 8798 (referred to as strain 8798). Gene-specific oligonucleotides for cloning of genes from strain 8798 were designed based on gene sequences identified in the rough-draft genome annotation of *Marinobacter aquaeolei* strain VT8 released by the DOE Joint Genome Institute (<http://www.jgi.doe.gov>).

Cloning and DNA manipulations were carried out in *E. coli* DH5 α using the standard molecular biology techniques described by Sambrook et al. (18). Genes encoding WS1, WS2, WS3, Acs1, Acs2, Acs3, and Acs4 were PCR amplified from strain 8798 genomic DNA by using gene-specific oligonucleotides that introduce XbaI and NotI restriction sites. The XbaI/NotI-digested inserts were ligated into plasmid pUCmod for constitutive expression from a modified *lac* promoter (19). Histidine tags were added to the isolated genes: putative acyl-CoA synthetase genes contain a C-terminal His₆ tag, and WS genes have an N-terminal His₆ tag. Cloned gene sequences were verified by sequencing. The stop codon in the WS4 sequence was verified by sequencing several clones and also the PCR amplification product.

Protein expression and purification. Cultures (100 ml) of *E. coli* JM109 transformed with pUCmod expressing putative His₆-tagged CoA synthetases or WSs were grown in LB media supplemented with 100 μ g of ampicillin/ml at 30°C overnight in 500-ml un baffled flasks. Cells were harvested by centrifugation and resuspended in 10 ml of 50 mM Tris-HCl buffer (pH 8) for CoA synthetase enzymes and in 125 mM sodium phosphate buffer (pH 7.4) for WS enzymes. The cells were lysed by sonication (Branson, Danbury, CT) on ice using a 30% duty cycle consisting of 10 s on and 30 s off for 10 cycles. Cell lysates were spun down at a centrifugal force of $13,763 \times g$ in 50-ml Oakridge tubes in a Beckman J2-HS floor centrifuge equipped with a JA-17 rotor for 30 min at 4°C. The supernatant was applied to immobilized metal affinity chromatography using Talon resin

(Clontech, Mountain View, CA) and washed with 10 mM imidazole in 50 mM Tris-HCl buffer (pH 8) or 125 mM sodium phosphate buffer (pH 7.4). The purified proteins were eluted with 300 mM imidazole in either 50 mM Tris-HCl buffer (pH 8) or 125 mM sodium phosphate buffer (pH 7.4). Elutants were desalted with (Amersham, Piscataway, NJ) PD-10 resin columns to remove excess imidazole. The purified proteins were concentrated to 1 ml using Vivaspins (Vivascience, Hannover, Germany) 10,000-Da columns. Protein concentrations were determined by using the bicinchoninic acid protein assay method with bovine serum albumin as a protein standard (Pierce Biotechnology, Inc., Rockford, IL).

CoA synthetase assay. In vitro reactions were performed as described previously (24) with some modifications. CoA synthetase assays were carried out in 250- μ l reaction volumes containing 50 mM Tris-HCl buffer (pH 7.5), 0.1% Triton X-100, 10 mM MgCl₂, 5 mM TCEP, 0.1 U of inorganic pyrophosphatase, and as substrates 10 mM phytanic or fatty acids, 10 mM reduced coenzyme A (CoASH), 10 mM ATP, and 0.5 μ g of purified Acs (either 1, 2, 3, or 4) protein. The reactions were incubated for 20 min at 37°C and stopped with 25 μ l of 5% acetic acid, followed by HPLC analysis of the reaction products.

HPLC and LC/ESI-MS analysis of CoA synthetase reactions. CoA-synthetase assay samples (25 μ l) were resolved on an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA) equipped with a photodiode array detector set to 259 nm. Samples were separated on a reversed-phase Eclipse XDB-C8 column (Agilent Technologies) at a flow rate of 1 ml min⁻¹. Solvent A consisted of 20 mM ammonium acetate (pH 5.4) in HPLC-grade water, and solvent B contained acetonitrile and methanol (85:15 [vol/vol]). Acyl-CoA and phytanoyl-CoA products were eluted using the following conditions: solvent A-solvent B at 65:35 from 0 to 5 min, followed by a gradient from solvent A-solvent B at 65:35 to 100% solvent B in 30 min. Liquid chromatography-mass spectrometry (LC-MS) analyses of reaction products were done with an LCQ mass spectrophotometer equipped with an electrospray ionization source (ESI) (Thermo Finnigan). Mass fragmentation spectra were monitored in a mass range of *m/z* 400 to 1,500 with a negative ESI interface.

Preparation of phytanoyl-CoA. Commercially unavailable phytanoyl-CoA for WS assays was synthesized enzymatically with Acs2 under the CoA synthetase assay conditions described above with phytanic acid as the substrate. Enzymatically derived phytanoyl-CoA was purified by preparative HPLC: 100 μ l in vitro reaction samples were separated under essentially the same conditions described above for the HPLC analysis of CoA products. Phytanoyl-CoA fractions were collected and dried under nitrogen gas. Phytanoyl-CoA was quantified by comparison to UV/visible (UV/Vis) spectra of lauroyl-CoA, assuming comparable extinction coefficients of the CoA chromophores in phytanoyl- and lauroyl-CoA.

Profiling of WS substrate ranges using a coupled enzyme assay. Stock solutions of various substrates were prepared in 50 mM Tris-HCl buffer (pH 8) containing 1% gum arabic, 12.5 μ g bovine serum albumin ml⁻¹, 0.1% taurocholate, and either 100 mM fatty acid, isoprenoid acid, fatty alcohol, or isoprenoid alcohol. Stock solutions were sonicated to disperse the substrates.

Substrate profiles of the three WS were tested using coupled enzyme in vitro reactions in which the CoA synthetases Acs1 and Acs2 are added to synthesize the CoA-activated fatty acid phytanic acid substrates from corresponding acid precursors for the WS reactions. Assays were carried out in 500- μ l reactions containing in 50 mM Tris-HCl buffer (pH 8.0), 12.5 μ l of each acid, and alcohol substrate stock solution (final concentrations of each substrate of 250 μ M), 10 mM MgCl₂, 10 mM CoASH, 10 mM ATP, 5 mM TCEP, 0.1 U of inorganic pyrophosphatase, 0.25 μ g of Acs1 and Acs2 CoA synthetase, and 0.5 μ g of WS to be tested. Assays were incubated at 37°C overnight before thin-layer chromatography (TLC) analysis of the reaction products.

TLC. In vitro WS assay samples were extracted with 500 μ l of chloroform-methanol (1:1 [vol/vol]), and extracts were analyzed by TLC with Whatman normal phase silica gel 60 plates and developed using hexane-diethyl ether-acetic acid (90:10:1 [vol/vol/vol]). TLC plates were stained with either iodine vapor or anisaldehyde solution as described earlier (10). Palmitoyl palmitate and triolein were used as WE and TAG reference compounds, respectively.

DGAT assay. The DGAT activity of WS enzymes was measured using the coupled WS enzyme assay conditions described above with oleic acid as acyl donor and 1,2 dipalmitoyl-sn glycerol as the acyl acceptor (final concentration of each substrate of 250 μ M) and 0.25 μ g of Acs2 isoprenoid/acyl-CoA synthetase to generate CoA activated oleic acid.

GC-MS analysis of WE. GC electron impact MS analyses were performed with a Hewlett-Packard 6890 series gas chromatograph connected to an HP 5973 mass spectrometer. GC conditions consisted of a column (30 m by 0.25 mm [inner diameter] by 1.5 μ m coated with 5% phenylmethyl silicone) with the injector temperature set to 250°C. The oven was set to a temperature gradient of 30°C min⁻¹ from 60 to 130°C, followed by slowing of the gradient from 130 to 300°C

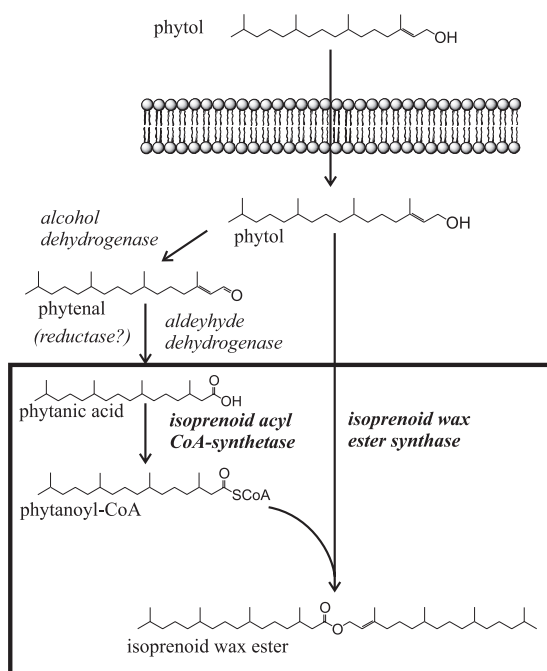


FIG. 1. Proposed pathway for isoprenoid WE biosynthesis. Phytol is transported into *M. hydrocarbonoclasticus* by an unknown system and is reduced to phytanal by an alcohol dehydrogenase. Phytanal is further oxidized into phytic acid by an aldehyde dehydrogenase. Concurrently, phytic acid (saturated form of phytanic acid) is activated to phytanoyl-CoA by an isoprenoid/acyl-CoA synthetase. Phytanoyl-CoA and phytol are substrates for a WS to form the isoprenoid WE product. The isoprenoid/acyl-CoA synthetase (CoA synthetase) and isoprenoid WS are the focus of this research (boxed region).

at 4°C min⁻¹ using helium as a carrier gas. The MS conditions used an electron energy of 70 eV and a source temperature set to 170°C. Mass spectra were scanned in a range of *m/z* 40 to 600 at 1-s intervals.

Kinetic WS assay. WS activity was determined by monitoring CoA release using Ellman's reagent [5,5'-dithio-bis(2-nitrobenzoic acid); DTNB] at 412 nm ($\epsilon = 13,600 \text{ M}^{-1} \text{ cm}^{-1}$) (5). Kinetic *in vitro* assays were performed in triplicate in 125 mM sodium phosphate buffer (pH 7.4) containing 0.1% Tergitol NP-11 detergent, 10 mM MgCl₂, 1 mM DNTB, 250 μM palmitoyl-CoA, 1 to 250 μM hexadecanol, and 0.5 μg of WS enzyme. Assay reactions were preincubated at 37°C for 5 min before the reactions were started by the addition of enzyme. Heat-denatured enzyme (99°C for 15 min) was used as a negative control.

Measurement of acyl-CoA and fatty/isoprenoid alcohol specificity. Acyl-CoA and fatty/isoprenoid alcohol specificity of WS2 was determined in the same

manner as described for the kinetic WS assay with assumed saturating conditions containing both the fatty/isoprenoid alcohol and acyl-CoA substrates at a concentration of 1 mM. Acyl-CoA specificity was measured with hexadecanol as acyl acceptor, whereas palmitoyl-CoA was used as acyl donor to determine alcohol specificity. Isoprenoid WS activity of WS2 was determined with 250 μM HPLC-purified phytanoyl-CoA and 250 μM phytol as substrates.

RESULTS

Identification and cloning of putative isoprenoid WE biosynthetic genes. *M. hydrocarbonoclasticus* DSM 8798 (strain 8798) was shown previously to synthesize isoprenoid WE from phytol (17). Based on the recent characterization of a fatty acid WS (WS/DGAT) from *Acinetobacter baylyi* ADP1 that condenses a CoA activated fatty acid and a fatty alcohol to make fatty acid WE storage compounds, we reasoned that isoprenoid WE synthesis may follow a similar pathway with an isoprenoid specific acyl-CoA synthetase and WS as key enzymes (Fig. 1). Because no sequence information is available for strain 8798, we used a recent draft genome sequence (released by the DOE Joint Genome Institute [http://www.jgi.doe.gov] in October 2005) of the alkane hydrocarbon metabolizing *M. aquaeolei* VT8 strain (8, 13) (referred to as strain VT8 below) for the identification and cloning of isoprenoid WE biosynthetic genes in strain 8798. Sequence analysis of the 16S rRNA gene of strain 8798 showed it to be 99.4% identical to that of strain VT8, suggesting a high degree of genomic conservation between the two strains.

A BLAST homology search of the draft VT8 genome sequence (released in October 2005) using the known WS/DGAT amino acid sequence from *A. baylyi* ADP1 identified four putative WS homologues (WS1, -2, -3, and -4) (Table 1 and Fig. 2). Two of the homologues, WS1 and WS2, were located in two putative alkane degradation gene clusters that share some sequence similarity with a known alkane degradation cluster described from *P. oleovorans* (26). However, while the present study was under review, the sequence of *Marinobacter aquaeolei* VT8 genome was reassembled, and a final draft of the genome sequence was released 28 December 2006. In the previous annotation, WS1 was associated with gene cluster 1, which is no longer the case in the new genome assembly. Now, WS1 is located approximately 250 kb upstream of this cluster (see Fig. S1 in the supplemental material, which maintains the gene organization in the first genome assembly but now also shows the new gene localizations). WS1 and

TABLE 1. Cloned *M. hydrocarbonoclasticus* DSM 8798 acyl-CoA synthetase and WE synthetases^a

Enzyme	Peptide length (amino acids)	GenBank accession no.	ORF no. in strain VT8 (%ID) ^b	Peptide sequence identity to related enzymes	
				%ID	Strain (source or reference)
WS1	455	EF219376	168 (99)	45	<i>A. baylyi</i> ADP1 WS/DGAT (11)
WS2	473	EF219377	3067 (99)	38	<i>A. baylyi</i> ADP1WS/DGAT (11)
WS3	508	EF219378	851 (99)	27	<i>A. baylyi</i> ADP1WS/DGAT (11)
WS4	349	EF219379	3371 (98)	25	<i>A. baylyi</i> ADP1 WS/DGAT (11)
Acs1	544	EF219372	438 (99)	62	<i>P. putida</i> AlkK (GenBank no. AJ245436)
Acs2	558	EF219373	1593 (99)	64	<i>P. putida</i> FadD (13)
Acs3	555	EF219374	1090 (99)	55	<i>P. aeruginosa</i> FadD (GenBank no. ABJ10798.1)
Acs4	560	EF219375	2888 (97)	51	<i>P. aeruginosa</i> FadD (GenBank no. ABJ10798.1)

^a The peptide sequence identities of cloned enzymes to homologues in *M. aquaeolei* strain VT8 and other related enzymes are shown.

^b Homologous ORFs in *M. aquaeolei* VT8 genome sequence and the percent peptide sequence identity (%ID) to corresponding enzymes cloned from *M. hydrocarbonoclasticus* DSM 8798.

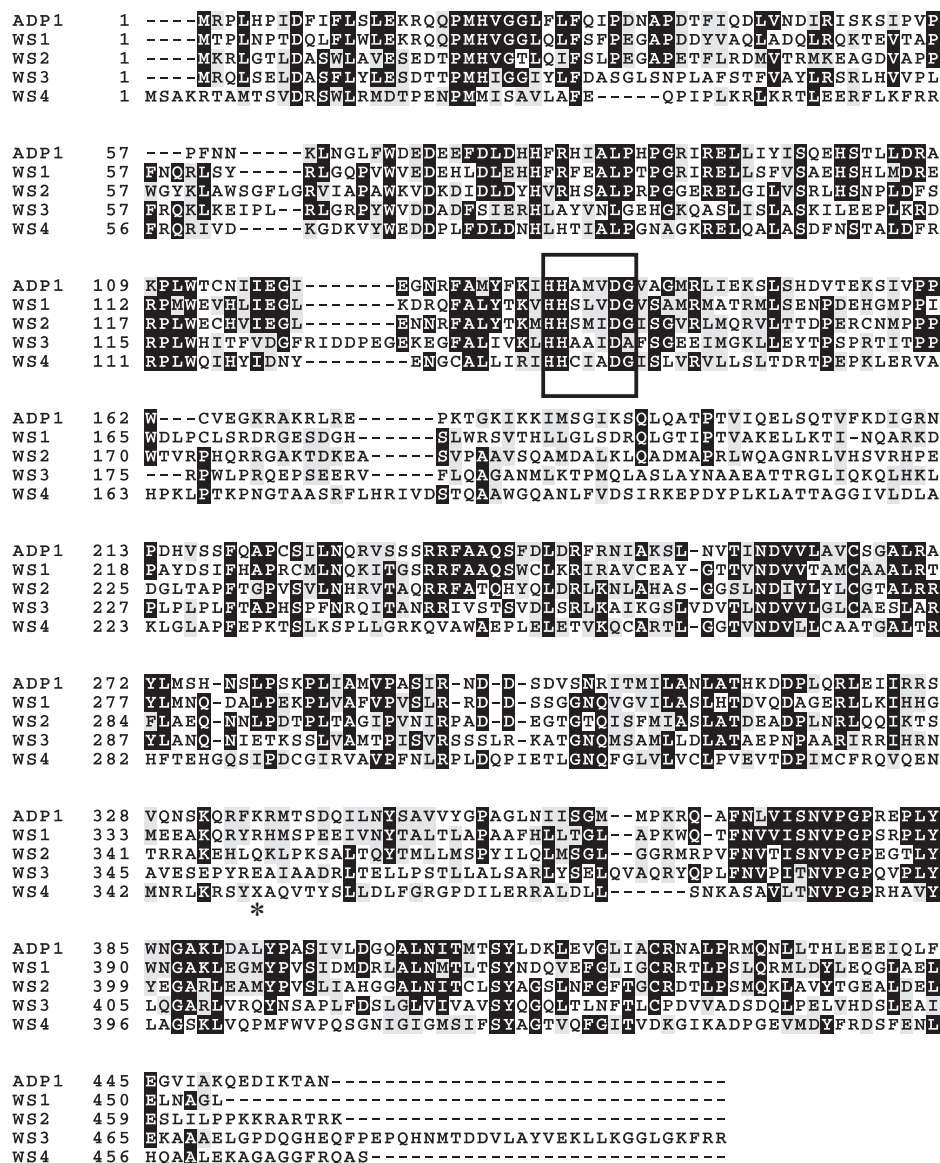


FIG. 2. Multiple protein sequence alignment of *Acinetobacter* WS/DGAT (ADP1) with four putative WS clones from *M. hydrocarbonoclasticus* strain 8798 (WS1 to -4). Identical and similar amino acids are marked black and gray, respectively. The WS4 gene contains a stop codon signified with an "X" at position 350 (denoted with an asterisk). The region outlined by a black box corresponds to the putative conserved acyltransferase catalytic domain, HHXXXDG (11).

alkane gene cluster 1 are each flanked by inverted transposase sequences (of which there are three 100% conserved copies in the genome), resulting in a contiguous assembly in the first draft. Similarly, we hypothesized that WS2 was also associated with an alkane degradation operon (see Fig. S1, gene cluster 2, in the supplemental material) since both were located near a physical sequence gap. This gap has now been closed, and WS2 is not associated with any alkane degradation gene cluster. If the current genome sequence is correct, none of the WS homologues are clustered any longer with any obvious alkane-utilizing metabolic operons.

We searched the draft genome sequence for putative acyl-CoA synthetases and identified four open reading frames (ORFs) annotated as medium-chain (Acs1) and long-chain

(Acs2, -3, and -4) acyl-CoA synthetases (Table 1). Acs2, -3, and -4 are not clustered with any obvious gene functions. Acs1 is part of one of the putative alkane degradation gene cluster 1 (see Fig. S1 in the supplemental material), previously annotated to also include WS1. Gene cluster 2 contained in the first draft genome sequence a partial acyl-CoA synthetase ORF flanking the physical gap. As stated above, this gap has been closed in the new genome assembly, and this ORF is now annotated as a medium-chain CoA synthetase. Oligonucleotides were designed from the VT8 sequences of the putative WS and Acs genes found in the genome annotation and used for PCR amplification and cloning of the homologues from strain 8798. PCR products were obtained for Acs1 to -4 and WS1 to -4 ORFs and cloned into pUCmod for sequencing.

The putative acyl-CoA synthase of gene cluster 2, for which only a partial sequence was available until very recently (see above), could not be amplified using a C-terminal oligonucleotide derived from the Acs1 sequence. All cloned WS and Acs genes from strain 8798 share a >97% peptide sequence identity with those identified in the genome sequence of strain VT8 (Table 1). The peptide sequence identities of the cloned WS homologues to the experimentally characterized WS/DGAT from *A. baylyi* range from 27 to 45%, with WS4 being the least similar and WS1 having the highest identity (Table 1). However, the cloned WS4 from strain 8798 is a pseudogene with a stop codon that truncates its ORF, whereas the corresponding ORF of WS4 from strain VT8 appears to be intact based on the released draft genome sequence. Acs1 to Acs4 show greater than 50% peptide sequence identity to experimentally characterized medium- and long-chain acyl-CoA synthetases from different *Pseudomonas* strains (Table 1).

Identification of *Marinobacter* isoprenoid CoA synthetase.

To determine the substrate specificities and to test whether any of the cloned putative acyl-CoA synthetases can catalyze CoA-activation of isoprenoid acids, purified recombinant enzymes (Acs1, -2, -3, and -4) were assayed with various saturated fatty acid substrates containing different acyl chain lengths (C_{10} , C_{12} , C_{14} , C_{16} , C_{18} , and C_{20}) and with the isoprenoid phytanic acid. HPLC analysis of the reaction products confirmed Acs1 to be a medium-chain acyl-CoA synthetase that accepts fatty acids with chain lengths ranging from C_{10} to C_{16} , while Acs2, -3, and -4 were found to be long-chain acyl-CoA synthetases that act on fatty acids with chain lengths ranging from C_{12} to C_{20} (data not shown). The long-chain acyl-CoA synthetases (Acs2, -3, and -4) showed the most activity when palmitic acid (C_{16}) was the substrate. Only Acs2 converted phytanic acid into phytanoyl-CoA, a finding confirmed by LC-MS (Fig. 3). This enzyme, now referred to as isoprenoid/acyl-CoA synthetase, shows 63% peptide sequence identity with a previously described acyl-CoA synthetase (FadD) found in *P. putida* that accepts aromatic alkanolic acids (13). The broad substrate range of FadD prompted us to investigate whether this enzyme would also accept phytanic acid. We cloned FadD and assayed the purified recombinant protein with phytanic acid. However, unlike the *Marinobacter* enzyme, FadD does not synthesize phytanoyl-CoA (data not shown).

Substrate profiles of *Marinobacter* WSs. The activities of the three cloned putative WS with various CoA-activated fatty acids, phytanic acid, and primary alcohols, including the isoprenoid alcohols farnesol and phytol, were investigated by using a coupled enzyme assay. A total of 54 *in vitro* reactions containing fatty acids and alcohols with various degrees of saturation and carbon chain lengths were arrayed for each WS enzyme. Purified WS proteins and *Marinobacter* CoA synthetases were incubated with CoA and different combinations of acid and alcohol substrates, and product formation was analyzed by TLC. CoA activation of medium-chain fatty acids (C_{10} to C_{14}) in these assays was conducted with Acs1, while long-chain fatty acids (C_{16} to C_{20}) and phytanic acid were esterified with CoA by Acs2. The identification of Acs2 as an isoprenoid/acyl-CoA synthetase made it possible to synthesize commercially unavailable phytanoyl-CoA from available phytanic acid as a substrate for testing with WS1, -2, and -3.

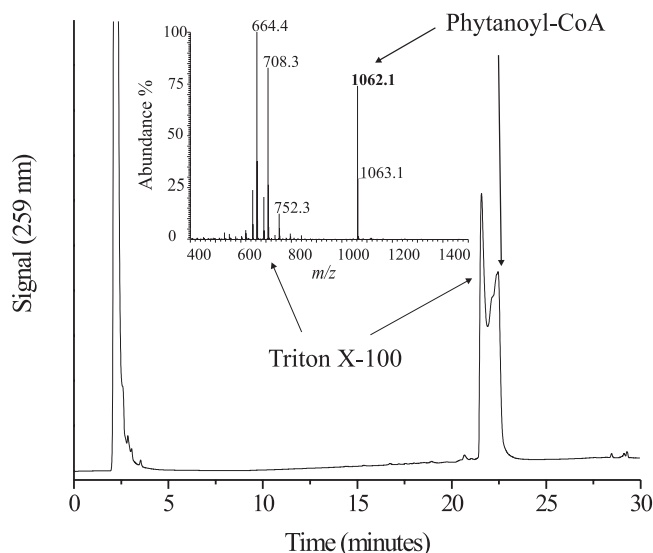


FIG. 3. HPLC-MS analysis of Asc2 *in vitro* reaction with phytanic acid and CoA as substrates. HPLC chromatogram of *in vitro* reaction showing the phytanoyl-CoA product with a retention time of 22.5 min and the corresponding mass spectrum of the product peak (inset). The observed parent ion (1,062 m/z) is consistent with that of the calculated mass of phytanoyl-CoA. Ions of 664, 708, and 752 m/z correspond to Triton X-100 detergent present in the reaction mixture.

Table 2 summarizes WE product formation detected on TLC plates for the tested putative WSs from strain 8798. The TLC substrate profiles show that WS1 and WS2 catalyzed ester bond formation between various activated fatty acids and fatty alcohols or isoprenoid alcohols. Figure 4 shows representative TLC results for reactions with palmitic acid and hexadecanol and with phytanic acid and phytol. WS2 appears to have a broader substrate range and a higher preference for longer-chain fatty alcohols than WS1. WE products derived from short chain acyl-CoA (C_{10} and C_{12}) substrates were only detected using the overnight assay conditions. No WE formation was detected using WS3 with any of the substrate combinations tested.

WS1 and WS2 also esterified activated fatty acids with isoprenoid alcohols (phytol, farnesol), thereby producing hybrid acyl-isoprenoid WEs (see Fig. S2 in the supplemental material). However, fatty alcohols were not condensed to phytanoyl-CoA by either enzyme. Synthesis of hybrid isoprenoid WE was therefore only possible between an activated fatty acid and an isoprenoid alcohol. Notably, both enzymes produced isoprenoid WEs from phytanoyl-CoA and the isoprenoid alcohols phytol and farnesol, although WS2 was considerably more active with these substrates.

The structure of the synthesized isoprenoid WE was confirmed by GC-MS. Figure 5 shows the results of the GC-MS analysis of chloroform extracts of a reaction with WS2 and phytanic acid and phytol as substrates. A product peak with a retention time of 41 min and a mass of 590 m/z was detected that was not present in a control using heat-denatured WS2. Its mass and fragmentation pattern match those of the isoprenoid WE previously isolated from strain 8798 by Rontani et al. (17). Together, these results suggest that WS1 and WS2, along with Acs1, are involved in the synthesis of isoprenoid WE storage

TABLE 2. Substrate profiles of WS1 and WS2

Acid	WS profile ^a											
	WS1						WS2					
	C ₁₀	C ₁₂	C ₁₄	C ₁₆	Phytol	Farnesol	C ₁₀	C ₁₂	C ₁₄	C ₁₆	Phytol	Farnesol
C ₂₀	+	+	+	+	++	++	-	-	+	+	+	+
C _{18Δcis9,12,15}	-	+	+	-	-	+	+	+	+	+	+	-
C _{18Δcis9}	+	++	+++	++	++	++	+++	++	+++	+++	++	++
C ₁₈	++	+	++	+	++	++	+	+	+	+	+	++
C _{16Δcis9}	+	++	++	++	+++	++	++	++	+++	+++	++	++
C ₁₆	+	++	+	+++	+	++	+	+	++	+++	+	++
C ₁₄	-	-	++	+	+	++	++	+	++	+	+	++
C ₁₂	-	+	+	+	-	+++	+	+	+	+	+	++
C ₁₀	+	+	-	+	-	+++	+	+	-	+	+	+
Phytanic acid	-	-	-	-	+	+	-	-	-	-	++	++

^a WE formation was determined by TLC after 12 h at 37°C for reactions containing different combinations of fatty acids or phytanic acid and fatty alcohols, phytol, or farnesol. The spot intensity of wax ester products on TLC plates was used for qualitative description of WS activities: ranging from no activity (-) to most active (+++).

compounds in *M. hydrocarbonoclasticus* DSM 8798. Because the two *Marinobacter* WSs displayed novel WS activity, the previously described *Acinetobacter* WS/DGAT was also tested but did not show isoprenoid WE formation using phytanic acid and phytol as substrates.

WS/DGAT activity of *Marinobacter* WSs. It has been reported that *Acinetobacter* WS/DGAT has DGAT activity (11). To test whether any of the three *Marinobacter* WSs can catalyze this reaction, WS1, WS2, and WS3 were tested with oleoyl-CoA as the acyl donor and dipalmitoyl-glycerol as the acyl acceptor, and the products were resolved on TLC plates. TAG products were only detected for WS1, whereas WS2 did not show DGAT activity (see Fig. S3 in the supplemental material).

Kinetic measurement of WS activities. A spectrophotometric assay was developed to determine the kinetic properties of WS1 and WS2. The concentration of sulfhydryl groups of CoA released during the condensation reaction between fatty/isoprenoid CoA activated acids and alcohols was determined by using Ellman’s reagent (DTNB) (5). The specific activities of WS1, WS2, and *Acinetobacter* WS/DGAT were measured with palmitoyl-CoA and hexadecanol, palmitoyl-CoA and phytol, and phytanoyl-CoA and phytol. Phytanoyl-CoA for these as-

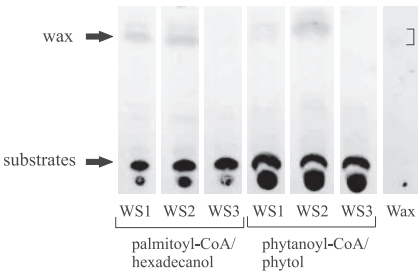


FIG. 4. TLC plate with iodine-stained WE products. TLC analysis of coupled in vitro reactions containing isoprenoid CoA synthetase (Asc2) and WS (WS1, -2, or -3) and either palmitic acid-hexadecanol or phytanic acid-phytol as substrates. A wax standard consisting of palmitoyl palmitate (marked “wax”) shows the position of the expected acyl and isoprenoid WE products (marked with an arrow). Products were observed only with WS1 and WS2; no products were observed with WS3.

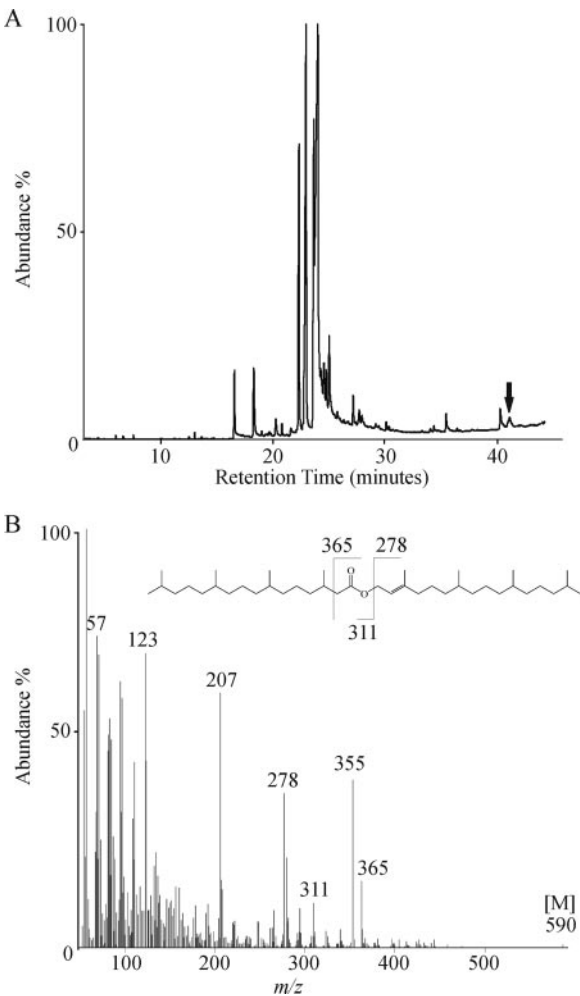


FIG. 5. GC-MS analysis of isoprenoid WE product synthesized in a coupled enzyme reaction with isoprenoid CoA-synthetase Asc2 and WS2 containing phytanic acid and phytol as substrates. (A) Total ion chromatogram showing isoprenoid WE product peak at a retention time of 41.9 min (arrow). (B) Electron impact mass spectrum of isoprenoid WE product peak. The masses of the parent ion at 590 *m/z* and fragment ions (365, 311, and 278 *m/z*) match those reported for the phytanoyl-phytol ester (17).

TABLE 3. Spectrophotometric analysis of the specific activities of *Marinobacter* sp. strain 8798 WS1 and WS2 and *Acinetobacter* WS/DGAT

Substrate	Mean sp act (mmol min ⁻¹ mg ⁻¹) ± SD of:		
	<i>Marinobacter</i>		<i>Acinetobacter</i> WS/DGAT
	WS1	WS2	
Palmitoyl-CoA (C ₁₆) + hexadecanol (C ₁₆)	1.338 ± 0.5	61.323 ± 1.79	0.389 ± 0.039
Palmitoyl-CoA (C ₁₆) + phytol	0.152 ± 0.011	28.872 ± 2.13	0.138 ± 0.003
Phytanoyl-CoA + phytol	ND ^a	0.397 ± 0.099	ND

^a ND, not determined.

says was enzymatically synthesized from phytanic acid and CoA using the above-characterized isoprenoid/acyl-CoA synthetase Acs2. Approximately 5 mM phytanoyl-CoA was purified by preparative HPLC, which was used to determine the specific activity of the most active *Marinobacter* enzyme WS2 (Table 3). Because the *Acinetobacter* WS/DGAT did not show activity with phytanic acid and phytol in the coupled enzyme assay, its specific activity with phytanoyl-CoA and phytol was not measured.

As shown in Table 3, WS2 was more active than either of the other two enzymes tested. The specific activity of WS2 measured with palmitoyl-CoA and hexadecanol as substrates was 61 mmol mg⁻¹ min⁻¹ versus 1.3 mmol mg⁻¹ min⁻¹ for WS1 and 0.38 mmol mg⁻¹ min⁻¹ for *Acinetobacter* WS/DGAT. WS2 was ~20-fold more active than WS1 or *Acinetobacter* WS/DGAT in creating the hybrid WE using palmitoyl-CoA and the

isoprenoid alcohol phytol as substrates. WS2 activity with phytanoyl-CoA and phytol was determined to be 0.397 mmol mg⁻¹ min⁻¹.

The kinetic constants of WS2 with palmitoyl-CoA and hexadecanol as substrates were determined under saturating palmitoyl-CoA conditions (at 250 μM) and various concentrations of hexadecanol. WS2 activity followed typical Michaelis-Menten kinetics with a *K_m* of 44 μM, a *V_{max}* of 10 mmol mg⁻¹ min⁻¹, and a *k_{cat}* of 4,794 s⁻¹ (Fig. 6A and B).

Acyl-CoA and fatty/isoprenoid alcohol specificity of WS2. Acyl-CoA and fatty/isoprenoid alcohol specificity of WS2 was determined by using the developed spectrophotometric assay. Acyl-CoA specificity of this enzyme was investigated using acyl-CoAs with various acyl chain lengths (C₁₂ to C₂₀) and hexadecanol as substrates (Fig. 6C). Long-chain fatty acyl-CoA derivatives arachidoyl-CoA (C₂₀) and stearoyl-CoA (C_{18:0}) were readily accepted as substrates by WS2, as was the polyunsaturated acyl-CoA linolenoyl-CoA (C_{18:3}). Also, WS2 showed a clear preference for palmitoyl-CoA (C₁₆), whereas the shorter acyl-chain substrate myristoyl-CoA (C₁₄) was poorly converted and lauroyl-CoA (C₁₂) was not accepted at all by the enzyme.

The relative substrate activity of WS2 was also tested against various fatty/isoprenoid alcohols and palmitoyl-CoA as substrates (Fig. 6D). Compared to WS2's preference for acyl-CoA substrates with medium and long chains, the enzyme displayed a broad activity with alcohols of various chain lengths. Decanol and dodecanol were more readily taken up for WE synthesis than the equivalent-chain-length acyl-CoA carbon chain.

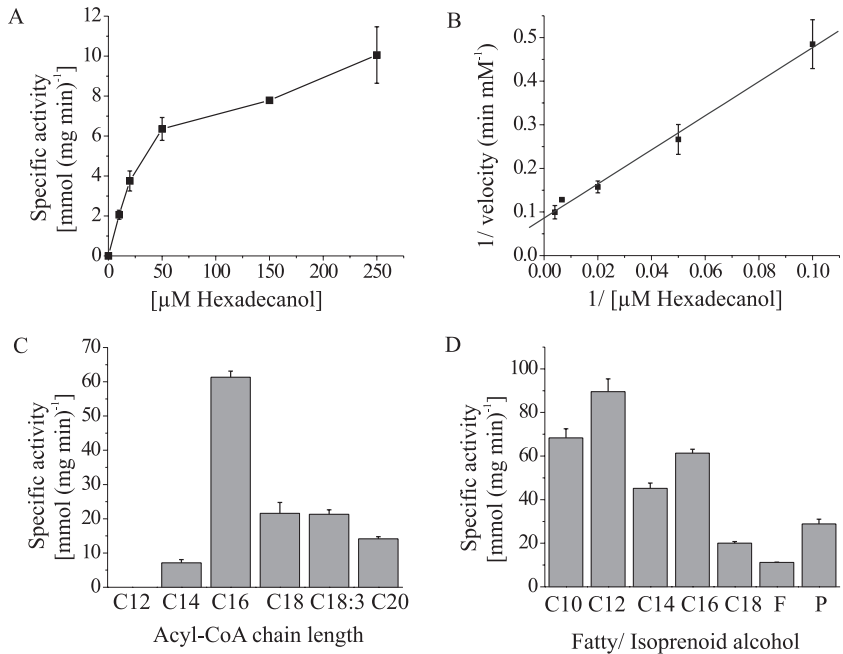


FIG. 6. Kinetic measurement of WS2 activity using a spectrophotometric assay. (A and B) Plot of reaction velocity versus hexadecanol concentration (0, 10, 25, 50, 150, and 250 μM) with palmitoyl-CoA (250 μM) (A) and corresponding double reciprocal plot of WS2 activity (B). (C and D) Comparison of WS2 specific activities for various chain lengths of CoA-activated fatty acids (C₁₂ to C₂₀) and hexadecanol (C) and various fatty/isoprenoid alcohols (C₁₀ to C₁₈; F, farnesol, P, phytol) and palmitoyl-CoA (D). Values are averages of three experiments; error bars correspond to one standard deviation.

DISCUSSION

M. hydrocarbonoclasticus DSM8798 has previously been shown to accumulate isoprenoid WE storage compounds when grown on phytol (17). We hypothesized that the biosynthesis of isoprenoid WE would involve two key enzymes: an isoprenoid acid CoA synthetase and the isoprenoid WS shown in Fig. 1. In the present study, using the draft genome sequence of the very closely related *M. aquaeolei* VT8 (8, 13) strain, we identified an isoprenoid-specific CoA synthetase (Acs2) and two isoprenoid WSs (WS1 and WS2) in strain 8798 and characterized their enzymatic activities. These previously undescribed enzymes can synthesize bulky isoprenoid lipids that are chemically similar to their acyl constituents associated with lipid WE biosynthesis (28).

Isolation of an isoprenoid specific isoprenoid/acyl-CoA synthetase (Acs2) was crucial in the characterization of the isoprenoid WSs since it enabled the synthesis of the commercially unavailable phytanoyl-CoA as a substrate for in vitro enzyme assays. Bulky isoprenoids are usually not accepted as substrates by known acyl-CoA synthetases. Microbial long-chain fatty acid CoA synthetases have been described that utilize unusual acyl acid substrates (3, 14). For example, a CoA synthetase (FadD6) from a *Mycobacterium* sp. was shown to activate fatty acid derivatives with methyl groups at α or β positions (3, 14), whereas another enzyme from *Pseudomonas putida* (FadD1) efficiently activates *n*-phenylalkanoic acids (3, 14). However, FadD1 from *P. putida*, which among experimentally characterized acyl-CoA synthetases is most similar to Acs2, did not activate phytanic acid, suggesting that Acs2 has an unusual specificity for isoprenoid acids. To our knowledge, CoA activation of phytanic acid only has been described for very-long-chain acyl-CoA synthetases from rat and human sources, where they are involved in the metabolism of phytol (21, 31).

Two of the three WSs cloned from *M. hydrocarbonoclasticus* (i.e., WS1 and WS2) were capable of synthesizing isoprenoid WE, whereas WS3 did not show activity with any of the acyl or isoprenoid substrates tested. In the first draft genome sequence, WS1 and WS2 were located in two putative alkane degradation gene clusters. In the final genome assembly released while the present study was under review, none of the WS genes are clustered with these alkane-utilizing genes (see Fig. S1 in the supplemental material). This new gene organization is the result of a new assembly of contigs flanked by 100% conserved, inverted transposase sequences. However, it would require PCR amplifications with oligonucleotides specific to regions flanking these transposase sequences and sequencing of the resulting amplification products to decide whether this new assembly is indeed correct. Furthermore, regions flanked by these transposase sequences may naturally be prone to genomic rearrangements causing strain variations. WS3 is not clustered with any obvious gene functions and may either have an entirely different set of substrates not involved with WE or TAG synthesis or be nonfunctional. The highly conserved acyltransferase domain HHXXXDG (30) found in ADP1 DGAT/WS, WS1, and WS2 and in NRPSs is modified in WS3 (see Fig. 2); substitution of the conserved glycine with alanine may affect the activity of WS3.

Both WS1 and WS2 were found to synthesize isoprenoid WE from phytanoyl-CoA and farnesol or phytol. Long-chain

acyl-CoAs (longer than C_{14}) were preferred by both enzymes and were esterified with a wide range of fatty alcohols and also isoprenoid alcohols (Table 1). The bulky phytanoyl-CoA, however, was only esterified with equally bulky isoprenoid alcohols. An explanation for the observed unidirectional formation of hybrid ester only from acyl-CoA and isoprenoid alcohol substrates will require details on catalytic mechanism and structure of this only recently characterized class of enzymes.

WS2 displays several orders of magnitude higher activity toward acyl substrates than previously characterized acyl-WS (4, 11, 12, 30) (Table 3). Its specific activity with isoprenoid substrates is comparable to specific activities measured for WS/DGAT ADP1 with acyl substrates, suggesting that WS2 under cellular conditions is able to efficiently synthesize isoprenoid WE storage compounds. Only WS1, which has the highest peptide sequence identity to the characterized ADP1 WS/DGAT, has DGAT activity. WS1 also shows similar activity levels and substrate preferences than ADP1 WS/DGAT (11, 23) (Table 3).

The differences in substrate selectivities and activities seen in the isoprenoid WSs described here, reported from *Acinetobacter baylyi* ADP1 (11, 23, 25), and more recently reported from *Alcanivorax borkumensis* (12) may reflect adaptations to available carbon sources in their respective environments (15–17). Identification of additional microbial WE biosynthetic genes will likely yield enzymes with new and interesting substrate selectivities. For example, *Rhodococcus opacus* is known to synthesize WE from phenyldecanoic acid (1) and therefore likely possesses WS and CoA synthetase activity for bulky substrates. Characterization of these enzyme functions not only is important for understanding metabolic processes in microorganisms but also could yield useful enzymes for biocatalytic applications such as the synthesis of novel WE polymers.

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