Biosynthesis of isoprenoid wax ester in *Marinobacter hydrocarbonoclasticus* DSM 8798: Identification and characterization of isoprenoid CoA-synthetase and wax ester synthases

Running title: Isoprenoid wax ester biosynthesis in *Marinobacter hydrocarbonoclasticus* 8798

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ABSTRACT

*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 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 (WS/DGAT) recently described from *Acinetobacter* 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 towards acyl substrates than any previously characterized acyl-WS and may reflect adaptations to available carbon sources in their environments.
INTRODUCTION

Neutral lipid biosynthesis is ubiquitous in nature and occurs in animals, plants, and microbes. Microorganisms have been reported to synthesize triacylglycerols (TAG’s) (27), polyhydroxyalkonates (PHA’s) (22) and wax esters (WE’s) (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. PHA’s 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 is well described (22). The enzymes involved in microbial TAG biosynthesis and WE have only very recently been identified (4, 11, 29, 30).

Microbial WE’s have been found in Mycobacterium (4), Rhodococcus (1), Acinetobacter (2) and Marinobacter (15, 17) strains that grow in environments where a carbon source (i.e. petroleum hydrocarbons (9), gluconate (11)) may be abundant relative to other nutrients such as phosphorous and nitrogen. Acyl WE’s are synthesized from long chain fatty alcohol and fatty acyl-CoA substrates. Another class of WE’s is isoprenoid WE’s that are made from branched, long-chained isoprenoyl alcohol and isoprene fatty acid substrates. Isoprenoid WE’s 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 (from here on called 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 WE’s and/or TAG’s 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 submission of this 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 (in this paper referred to 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 intermediate 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, like in acyl WE synthesis, involve the
condensation of a CoA-activated isoprenoid acid with an isoprenoid alcohol. Based on this hypothesis, we describe in this paper the isolation and characterization of an isoprenoid CoA-synthetase as well as two isoprenoid WE synthetases from strain 8798 (7) capable of producing an isoprenoid wax from phytanoyl-CoA and phytol.

EXPERIMENTAL PROCEDURES

Chemicals and materials

Coenzyme A 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 (C16:0), stearoyl-CoA (18:0), arachidoyl-CoA (C20:0), C18 linolenoyl-CoA (C18:3), myristoyl-CoA (C14:0), lauroyl (C12:0) and 5,5’-dithio-bis(2-nitrobenzoic acid) (DNTB), 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 (HPLC grade) were purchased through 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 (NEB, 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 Dr. Nicholas Ornston at Yale University (New
Haven, CT). *Pseudomonas putida* strain U was kindly given to us by Dr. José M. Luengo at University of Léon (Léon, Spain). Cloning and heterologous gene expression was carried out in *E. coli* strains DH5α 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 Luria-Bertani (LB) media 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 rDNA oligonucleotides (TPU1 5′-AGAGTTTGATCMTGGCTCAG and RTU8 5′-AAGGAGGTGATCCANCCRCA (6)) were used to amplify the 16s rDNA 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* DH5α using standard molecular biology techniques described in Sambrook et al. (18). Genes encoding WS1, WS2, WS3, Acs1, Acs2, Acs3, and Acs4 were PCR amplified from strain 8798 genomic DNA 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 6XHis tag and WS genes have a N-terminal 6XHis 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**

100 mL cultures of *E. coli* JM109 transformed with pUCmod expressing putative 6XHis tagged CoA-synthetases or WS’s were grown in LB media supplemented with 100 µg/mL of ampicillin at 30 °C overnight in 500 mL unbaflled 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 seconds on and 30 seconds off for 10 cycles. Cell lysates were spun down at a centrifugal force of 13,763 × g in 50 ml Oakridge tubes in a Beckman J2-HS floor centrifuge equipped with a JA-17 rotor for 30 minutes at 4 °C. The supernatant was applied to immobilized metal affinity chromatography (IMAC) 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 Vivaspin (Vivascience, Hannover, Germany) 10,000 Da columns. Protein concentrations were determined using the BCA (bicinchoninic acid) protein assay method.
using bovine serum albumin (BSA) as a protein standard (Pierce Biotechnology Inc.,
Rockford, Il).

CoA synthetase assay

In vitro reactions were performed as in (24) with some modifications. CoA synthetase assays were carried out in 250 µL 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 CoASH, 10 mM ATP, and 0.5 µg of purified Acs (either 1, 2, 3, or 4) protein. The reactions were incubated for 20 minutes at 37 °C and stopped with 25 µL of 5% acetic acid followed by HPLC analysis of reaction products.

HPLC and LC/ESI-MS analysis of CoA synthetase reactions

25 µL of CoA-synthetase assay samples 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 reverse phase Eclipse XDB-C8 column (Agilent Technologies, Palo Alto, CA) 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-methanol (85:15, v/v). Acyl-CoA and phytanoyl-CoA products were eluted using the following conditions: from 0-5 min 65:35 A:B followed by a gradient from 65:35 A:B to 100% solvent B in 30 minutes. LC-MS analyses of reaction products were done with a LCQ mass spectrophotometer equipped with an electrospray ionization source (ESI)
(Thermo Finnigan, USA). Mass fragmentation spectra were monitored in a mass range of
m/z 400-1500 with a negative electron spray ionization (ESI) interface.

**Preparation of phytanoyl-CoA**

Commercially unavailable phytanoyl-CoA for WS assays was synthesized
enzymatically with Acs2 using the CoA synthetase assay conditions described above with
phytanic acid as the substrate. Enzymatically-derived phytanoyl-CoA was purified by
preparative HPLC: 100 µL *in vitro* reaction samples were separated using 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/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 µg mL⁻¹ BSA, 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 CoA-synthetases Asc1 and Asc2 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 µL reactions containing in 50 mM Tris-
HCl buffer pH 8.0, 12.5 µL of each acid and alcohol substrate stock solution (final
concentrations of each substrate 250 µM), 10 mM MgCl₂, 10 mM CoASH, 10 mM ATP, 5 mM TCEP, 0.1 U of inorganic pyrophosphatase, 0.25 µg of Acs1 and Acs2 CoA synthetase and 0.5 µg of WS to be tested. Assays were incubated at 37 °C overnight before thin-layer chromatography (TLC) analysis of reaction products.

Thin layer chromatography

In vitro WS assay samples were extracted with 500 µL chloroform:methanol (1:1, v/v) 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, v/v/v). TLC plates were stained with either iodine vapor or anisaldehyde solution as described in (10). Palmitoyl palmitate and triolein were used as wax ester and TAG reference compounds, respectively.

Diacylglycerol acyltransferase (DGAT) assay

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 250 µM) and 0.25 µg Acs2 isoprenoid/acyl-CoA synthetase to generate CoA activated oleic acid.

GC-MS analysis of wax ester

Gas chromatography electron impact mass spectrometry 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 ID by 1.5 µm
coated with 5% phenyl methyl silicone) with the injector temperature set to 250 °C. The oven was set to a temperature gradient of 30 °C min⁻¹ from 60 °C to 130 °C and then slowing the gradient from 130 to 300 °C 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 40-600 at 1 second 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 (ε =13,600 M⁻¹ 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, 1-250 µM hexadecanol and 0.5 µg of WS enzyme. Assay reactions were preincubated at 37 ºC for 5 minutes before the reactions were started by the addition of enzyme. Heat-denatured enzyme (99 °C for 15 minutes) 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, while palmitoyl-CoA was used as acyl donor to determine alcohol specificity. Isoprenoid WS activity of WS2 was determined with 250 µM of HPLC-purified phytanoyl-CoA and 250 µM phytol as substrates.
RESULTS

Identification and cloning of putative isoprenoid wax ester biosynthetic genes

*M. hydrocarbonoclasticus* DSM 8798 (strain 8798) was shown previously to synthesize isoprenoid wax ester from phytol (17). Based on the recent characterization of a fatty acid wax ester synthase (WS/DGAT) from *Acinetobacter baylyi* ADP1 that condenses a CoA activated fatty acid and a fatty alcohol to make fatty acid wax ester (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 *Marinobacter aquaeolei* VT8 strain (8, 13) (strain VT8 hereafter) for the identification and cloning of isoprenoid WE biosynthetic genes in strain 8798. Sequence analysis of the 16s rDNA of strain 8798 showed it to be 99.4% identical to that of strain VT8, suggesting a high degree of genomic conservation among 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) (Fig. 2). Two of the homologues, WS 1 and WS2, were located in two putative alkane degradation gene clusters that share some sequence similarity with a known alkane degradation cluster described from *Ps. oleovorans* (26). However, while this manuscript was under review,
the sequence of *Marinobacter aquaeolei* VT8 genome was reassembled and a final draft
of the genome sequence was released December 28, 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
supplementary Fig. 1 which maintains the gene organization in the first genome assembly
but now also shows the new gene localizations). WS1 and 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
supplementary Fig. 1, gene cluster 2) as 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 ORF’s annotated as medium chain (Acs1) and long chain (Asc2, 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
supplementary Fig. 1), 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
homologues from strain 8798. PCR products were obtained for Acs1-4 and WS1-4 ORF’s 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 greater than 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, while the corresponding ORF of WS4 from strain VT8 appears to be intact based on the released draft genome sequence. Acs1-4 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 (C10, C12, C14, C16, C18 and C20) 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 C10 to C16, while Acs2, 3, and 4 were found to be long-chain acyl-
CoA synthetases that act on fatty acids with chain lengths ranging from C12 to C20 (data not shown). The long-chain acyl-CoA synthetases (Acs2, 3, and 4) showed the most activity when palmitic acid (C16) was given as a substrate. Only Acs2 converted phytanic acid into phytanoyl-CoA, which was 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 alkanoic acids (13). The broad substrate range of FadD prompted us to investigate whether this enzyme would also accept phytic acid. We cloned FadD and assayed the purified recombinant protein with phytic acid. However, unlike the *Marinobacter* enzyme, FadD does not synthesize phytanoyl-CoA (data not shown).

### Substrate profiles of *Marinobacter* WS’s

Activities of the three cloned putative WS with various CoA-activated fatty acids, phytic acid and primary alcohols, including the isoprenoid alcohols farnesol and phytol, were investigated using a coupled enzyme assay. Fifty-four *in vitro* reactions containing fatty acids and alcohols with varying 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 analyzed by TLC. CoA-activation of medium-chain fatty acids (C10-C14) in these assays was conducted with Acs1, while long-chain fatty acids (C16-C20) and phytic 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.

Table 2 summarizes wax ester product formation detected on TLC plates for the three tested putative WS from strain 8798. The TLC substrate profiles show that WS1 and WS2 catalyzed ester bond formation between various activated fatty acid 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 (C10 and C12) substrates were only detected using the overnight assay conditions. No wax ester 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 wax esters (see supplementary Fig. 2). However, fatty alcohols were not condensed to phytanoyl-CoA by either enzyme. Synthesis of hybrid isoprenoid wax ester was therefore only possible between an activated fatty acid and an isoprenoid alcohol. Notably, both enzymes produced isoprenoid wax esters 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 wax ester was confirmed by GC mass spectrometry. Figure 5 shows 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 matches those of the isoprenoid
wax ester 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 wax ester storage compounds in \textit{M. hydrocarbonoclasticus} DSM 8798. Because the two \textit{Marinobacter} WS's displayed novel wax ester synthase activity, the previously described \textit{Acinetobacter} WS/DGAT was also tested but did not show isoprenoid wax ester formation using phytanic acid and phytol as substrates.

\textbf{WS/DGAT activity of \textit{Marinobacter} WS's}

It has been reported that \textit{Acinetobacter} WS/DGAT has diacylglycerol acyltransferase (DGAT) activity (11). To test whether any of the three \textit{Marinobacter} WS's 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 products were resolved on TLC plates. TAG products were only detected for WS1, while WS2 did not show DGAT activity (see supplementary Fig. 3).

\textbf{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 using Ellman’s reagent (5,5'-dithio-bis(2-nitrobenzoic acid), DTNB)(5). Specific activities of WS1, WS2 and of \textit{Acinetobacter} WS/DGAT were measured with palmitoyl-CoA and hexadecanol, palmitoyl-CoA and phytol, and with phytanoyl-CoA and phytol. Phytanoyl-CoA for these assays 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\(^{-1}\) compared to 1.3 mmol mg\(^{-1}\) min\(^{-1}\) for WS1 and 0.38 mmol mg\(^{-1}\) min\(^{-1}\) for *Acinetobacter* WS/DGAT. WS2 was approximately 20-fold more active than WS1 or *Acinetobacter* WS/DGAT in creating the hybrid wax ester 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 4794 sec\(^{-1}\) (Fig. 6A and B).

**Acyl-CoA and fatty/isoprenoid alcohol specificity of WS 2**

Acyl-CoA and fatty/isoprenoid alcohol specificity of WS2 was determined using the developed spectrophotometric assay. Acyl-CoA specificity of this enzyme was investigated using acyl-CoA’s with various acyl chain lengths (C12-C20) and
hexadecanol as substrates (Fig. 6C). Long chain fatty acyl-CoA derivatives arachidoyl-CoA (C20) and stearoyl-CoA (C18:0) were readily accepted as substrates by WS2 as was the polyunsaturated acyl-CoA linolenoyl-CoA (C18:3). 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.

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 various chain length alcohols. Decanol and dodecanol were more readily taken up for wax ester synthesis than the equivalent chain length acyl-CoA carbon chain.

**DISCUSSION**

*M. hydrocarbonoclasticus* DSM8798 has previously been shown to accumulate isoprenoid wax ester 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 isoprenoid wax ester synthase shown in Fig. 1. In this report, 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 WS’s (WS1 and WS2) in strain 8798 and characterized their enzymatic activities. These previously undescribed enzymes are capable of synthesizing 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 WS’s as it enabled synthesis of 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 *Mycobacterium* was shown to activate fatty acid derivatives with methyl-groups at α- or β-positions (3, 14), while 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 where they are involved in the metabolism of phytol (21, 31).

Two (WS1 and WS2) of the three WS’s cloned from *M. hydrocarbonoclasticus* were capable of synthesizing isoprenoid WE, while 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 this manuscript was under review, none of the WS genes are clustered with these alkane-utilizing genes (see supplementary Fig. 1). 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 is non-functional. The highly conserved acyltransferase domain HHXXXDG (30) found in ADP1 DGAT/WS, WS1, WS2 and in NRPS’s 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 wax ester from phytanoyl-CoA and farnesol or phytol. Long chain acyl-CoA’s (>C14) 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 towards 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 WS’s described in this study, 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 possess WS and CoA synthetase activity for bulky substrates. Characterization of these enzyme functions is not only important for understanding metabolic processes in microorganisms, but could also yield useful enzymes for biocatalytic applications such as the synthesis of novel wax ester polymers.
ACKNOWLEDGMENTS

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FOOTNOTES

The abbreviations used are DGAT, acyl-CoA:diacylglycerol acyltransferase; PHA, poly(hydroxyalkanoic acid); TAG, triacylglycerol; WE, wax ester; WS, wax ester synthase.
REFERENCES


Figure 1: Proposed pathway for isoprenoid wax ester biosynthesis. Phytol is transported into *Marinobacter hydrocarbonoclasticus* by an unknown system and is reduced to phytenal by an alcohol dehydrogenase. Phytenal is further oxidized into phytanic acid by an aldehyde dehydrogenase. Concurrently, phytanic acid (saturated by an unknown reductase (indicated as “reductase?”) a saturated form of phytanic acid) is activated to phytanoyl-CoA by an isoprenoid/acyl-CoA synthetase. Phytanoyl-CoA and phytol are substrates for a wax ester synthase to form the isoprenoid wax ester product. The isoprenoid/acyl-CoA synthetase (CoA synthetase) and isoprenoid WE synthase are the focus of this research (boxed region).

Figure 2: Multiple protein sequence alignment of *Acinetobacter WS/DGAT (ADP1)* with four putative WE synthase cloned from *M. hydrocarbonoclasticus* strain 8798 (WS1-4). 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 *). The region outlined by a black box corresponds to the putative conserved acyltransferase catalytic domain, HHXXDG (11).

Figure 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 minutes and the corresponding mass spectrum of the product peak (inset). The observed parent ion (1062 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.

**Figure 4:** Thin layer chromatography (TLC) plate with iodine-stained wax ester products. TLC analysis of coupled *in vitro* reactions containing isoprenoid CoA-synthetase (Asc2) and wax ester synthase (WS1, 2, or 3) and palmitic acid and hexadecanol or phytanic acid and phytol as substrates. A wax standard consisting of palmitoyl palmitate (marked “wax”) shows the position of the expected acyl and isoprenoid wax ester products (marked with an arrow). Products are observed only with WS1, WS2, while no products are observed with WS3.

**Figure 5:** GC-MS analysis of isoprenoid wax ester 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 wax ester product peak at a retention time of 41.9 min (arrow). B) Electron impact mass spectrum of isoprenoid wax ester 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).

**Figure 6:** Kinetic measurement of WS2 activity using spectrophotometric assay. A) Plot of reaction velocity versus hexadecanol concentration (0, 10, 25, 50, 150 and 250 µM) with palmitoyl-CoA (250 µM) and B) corresponding double reciprocal plot of WS2 activity. Comparison of WS2 specific activities for C) various chain lengths of CoA-
activated fatty acids (C12-C20) and hexadecanol and D) various fatty/isoprenoid alcohols (C10-C18, F: farnesol, P: phytol) and palmitoyl-CoA. Values are averages of three experiments; error bars correspond to one standard deviation.
Table 1: Cloned *M. hydrocarbonoclasticus* DSM 8798 acyl-CoA synthetase and wax ester synthases. Shown are peptide sequence identities of cloned enzymes to homologues in *M. aquaeolei* strain VT8 and other related enzymes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Peptide length (aa)</th>
<th>GenBank Accession no.</th>
<th>ORF# in strain VT8 and % ID (^a)</th>
<th>Peptide sequence identity to related enzymes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WS1</td>
<td>455</td>
<td>EF219376</td>
<td>168 (99)</td>
<td>45% (*Acinetobacter baylyi ADP1 WS/DGAT(11))</td>
</tr>
<tr>
<td>WS2</td>
<td>473</td>
<td>EF219377</td>
<td>3067 (99)</td>
<td>38% (*A. baylyi ADP1 WS/DGAT (11))</td>
</tr>
<tr>
<td>WS3</td>
<td>508</td>
<td>EF219378</td>
<td>851 (99)</td>
<td>27% (*A. baylyi ADP1 WS/DGAT (11))</td>
</tr>
<tr>
<td>WS4</td>
<td>349</td>
<td>EF219379</td>
<td>3371 (98)</td>
<td>25% (*A. baylyi ADP1 WS/DGAT (11))</td>
</tr>
<tr>
<td>Acs1</td>
<td>544</td>
<td>EF219372</td>
<td>438 (99)</td>
<td>62% (<em>Pseudomonas putida AlkK</em> (GenBank #AJ245436))</td>
</tr>
<tr>
<td>Acs2</td>
<td>558</td>
<td>EF219373</td>
<td>1593 (99)</td>
<td>64% (<em>Ps. putida FadD</em> (13))</td>
</tr>
<tr>
<td>Acs3</td>
<td>555</td>
<td>EF219374</td>
<td>1090 (99)</td>
<td>55% (<em>Ps. aeruginosa FadD</em> (GenBank #ABJ10798.1))</td>
</tr>
<tr>
<td>Acs4</td>
<td>560</td>
<td>EF219375</td>
<td>2888 (97)</td>
<td>51% (<em>Ps. aeruginosa FadD</em> (GenBank #ABJ10798.1))</td>
</tr>
</tbody>
</table>

\(^a\) Homologous ORF’s in *M. aquaeolei* VT8 genome sequence and peptide sequence identity (% ID) to corresponding enzymes cloned from *M. hydrocarbonoclasticus* DSM 8798.
Table 2: Substrate profiles of WS1 and WS2. Wax ester formation was determined by TLC after 12 hrs at 37 °C for reactions containing different combinations of fatty acids or phytanic acid and fatty alcohols, phytol, or farnesol. Spot intensity of wax ester products on TLC plates was used for qualitative description of WS activities: ranging from (-) no activity to (+++) most active.

<table>
<thead>
<tr>
<th>Acids</th>
<th>Alcohols</th>
<th>WS1</th>
<th>WS2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C10</td>
<td>C12</td>
</tr>
<tr>
<td>C20</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C18Δcis9,12,15</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C18Δcis9</td>
<td></td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>C18</td>
<td></td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>C16Δcis9</td>
<td></td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>C16</td>
<td></td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>C14</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C12</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C10</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>phytanic acid</td>
<td></td>
<td>-</td>
<td>-</td>
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</tbody>
</table>
Table 3: Spectrophotometric analysis of the specific activities of *Marinobacter 8798* WS1, WS2 and *Acinetobacter WS/DGAT*

<table>
<thead>
<tr>
<th>Substrates</th>
<th><em>Marinobacter</em> WS1 (mmol min(^{-1}) mg(^{-1}))</th>
<th><em>Marinobacter</em> WS2 (mmol min(^{-1}) mg(^{-1}))</th>
<th><em>Acinetobacter</em> WS/DGAT (mmol min(^{-1}) mg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>palmitoyl-CoA (C16) + hexadecanol (C16)</td>
<td>1.338±0.5</td>
<td>61.323±1.79</td>
<td>0.389±0.039</td>
</tr>
<tr>
<td>palmitoyl-CoA (C16) + phytol</td>
<td>0.152±0.011</td>
<td>28.872±2.13</td>
<td>0.138±0.003</td>
</tr>
<tr>
<td>phytanoyl-CoA + phytol</td>
<td>ND</td>
<td>0.397±0.099</td>
<td>ND</td>
</tr>
</tbody>
</table>
Figure 1

1. Phytol

2. Alcohol dehydrogenase

3. Phytenal (reductase?)

4. Aldehyde dehydrogenase

5. Phytanic acid

6. Isoprenoid acyl CoA-synthetase

7. Phytanoyl-CoA

8. Isoprenoid wax ester synthase

9. Isoprenoid wax ester
Figure 3

[Graph showing signal (2.59 nm) vs. time (minutes). Peaks at m/z 664.4, 708.3, 752.3, 1062.1, and 1063.1 labeled with Triton X-100 and Phytanoyl-CoA.]
Figure 4

WS1  WS2  WS3  WS1  WS2  WS3  Wax

 substrates

wax

palmitoyl-CoA/hexadecanol
phytanoyl-CoA/phytol
Figure 5

A

Retention Time (minutes)

Abundance %

B

Abundance %

\[ m/z \]

590

278

365

311

123

207

278

355

365

311

57

100

50

0

100 200 300 400 500
Figure 6

A

Specific activity [mmol (mg min)^{-1}] vs [µM Hexadecanol]

B

1/velocity (min mM^{-1}) vs 1/[µM Hexadecanol]

C

Specific activity [mmol (mg min)^{-1}] vs Acyl-CoA chain length

D

Specific activity [mmol (mg min)^{-1}] vs Fatty/Isoprenoid alcohol