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# Functional Characterization of an NADPH Dependent 2-Alkyl-3-ketoalkanoic Acid Reductase Involved in Olefin Biosynthesis in *Stenotrophomonas maltophilia*

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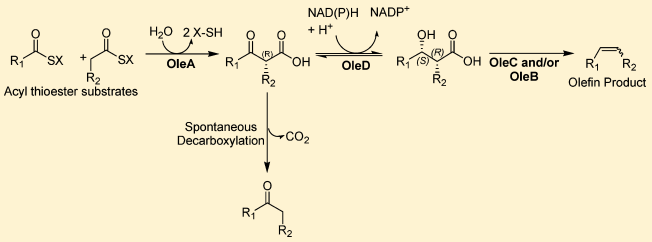
Supporting Information

**ABSTRACT:** OleD is shown to play a key reductive role in the generation of alkenes (olefins) from acyl thioesters in *Stenotrophomonas maltophilia*. The gene coding for OleD clusters with three other genes, *oleABC*, and all appear to be transcribed in the same direction as an operon in various olefin producing bacteria. In this study, a series of substrates varying in chain length and stereochemistry were synthesized and used to elucidate the functional role and substrate specificity of OleD. We demonstrated that OleD, which is an NADP(H) dependent reductase, is a homodimer which catalyzes the reversible stereospecific reduction of 2-alkyl-3-ketoalkanoic acids. Maximal catalytic efficiency was observed with *syn*-2-decyl-3-hydroxytetradecanoic acid, with a $k_{cat}/K_m$ 5- and 8-fold higher than for *syn*-2-octyl-3-hydroxydodecanoic acid and *syn*-2-hexyl-3-hydroxydecanoic acid, respectively. OleD activity was not observed with *syn*-2-butyl-3-hydroxyoctanoic acid and compounds lacking a 2-alkyl group such as 3-ketodecanoic and 3-hydroxydecanoic acids, suggesting the necessity of the 2-alkyl chain for enzyme recognition and catalysis. Using diastereomeric pairs of substrates and 4 enantiopure isomers of 2-hexyl-3-hydroxydecanoic acid of known stereochemistry, OleD was shown to have a marked stereochemical preference for the (2*R*,3*S*)-isomer. Finally, experiments involving OleA and OleD demonstrate the first 3 steps and stereochemical course in olefin formation from acyl thioesters; condensation to form a 2-alkyl-3-ketoacyl thioester, subsequent thioester hydrolysis, and ketone reduction.




Biochemical pathways in which alkanes or alkenes are generated from the CoA activated fatty acids are known and are of significant interest for generation of diesel and gasoline type products. Alkanes can be generated from an acyl-CoA thioester, a well characterized process which involves reduction to the corresponding aldehyde and a subsequent decarbonylation step.[1] There have also been reports dating back over 40 years of alkenes (olefins) with varying chain length ($C_{23}$−$C_{33}$) being produced by various organisms such as *Stenotrophomonas maltophilia*, *Kineococcus radiotolerans*, *Shewanella oneidensis*, *Xanthomonas campestris*, and various *Micrococcus*, *Arthrobacter*, and *Chloroflexus* species.[2−9] The formation of olefins is thought to occur through a process involving the head-to-head condensation of two activated fatty acids followed by $\beta$-keto group reduction. The resulting olefin contains a double bond near the middle of the chain, presumably located at the sight of condensation.[2−6,10−15] This reductive condensation process holds a potential for generating renewable petroleum from microbial fermentation, but is poorly understood.

A series of genetic and biochemical investigations of the olefin pathway resulted in the identification of four genes, annotated *oleABCD*, to be responsible for long chain alkene biosynthesis in olefin producing organisms.[7−10,16] Approximately, 70 bacterial genomes contain all four *ole* synthesis genes, 7 of which contained a fused *oleBC* gene pair.[10,16] In all cases, the four *ole* genes are clustered together and appear to be transcribed in the same direction as an operon.

Initial *in vitro* and *in vivo* analysis demonstrated that OleA catalyzes a decarboxylative condensation of two activated fatty acids to give a ketone.[7,9,10,16] More recently, OleA from *X. campestris* was shown to catalyze the nondecarboxylative condensation of two CoA activated fatty acids, releasing two molecules of CoA and providing a 2-alkyl-3-ketoalkanoic acid as the likely final product [9] (Figure 1). This acid then decarboxylates either *in vitro* or under GC−MS analysis conditions to the corresponding ketone.[9] *In vitro* and *in vivo* experiments involving OleC and OleD demonstrated their involvement in the subsequent steps following the OleA catalyzed condensation. While these studies have provided the first clear biochemical insight into the role of OleA in the processes of olefin generation, the specific role of the remaining proteins OleB−D, remains unknown.

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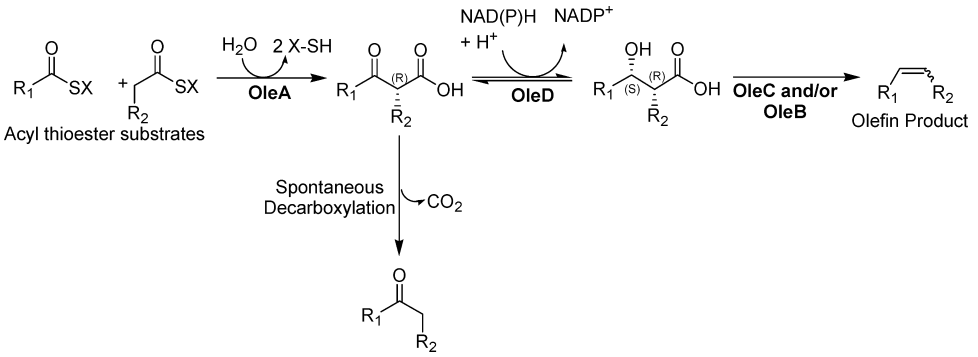



**Figure 1.** Working hypothesis for the roles of OleA–D in catalyzing olefin formation from two thioester activated fatty acids. $R_1$ and $R_2$ are variable; X = CoA or acyl carrier protein.

Our efforts have been focused on the functional and biochemical characterization of OleD, which has been annotated as an NAD(P)H dependent enzyme and belongs to the short chain dehydrogenase/reductase superfamily (SDR). Members of this superfamily catalyze various $NAD(P)^+$/NAD(P)H dependent reactions including oxidation, reduction, epimerization, dehydrogenation, dehydration, and synthase reactions.[17−21] On the basis of bioinformatic and preliminary biochemical analysis,[9,10] OleD is believed to function as a reductase and therefore potentially responsible for the reduction of 2-alkyl-3-ketoalkanoic acids (Figure 1). To test this hypothesis, a series of compounds were generated and used to probe the functionality and substrate specificity, including stereochemistry and carbon chain length, of OleD. The results of these analyses and the roles of OleA and OleD in the first two steps of the olefin biosynthetic pathway are presented.

## EXPERIMENTAL PROCEDURES

**Materials.** All chemicals were from Sigma or Fisher unless otherwise noted. β-NADH, β-$NAD^+$, β-NADPH, β-$NADP^+$, and dithiothreitol (DTT) were from Research Products International Corp.; (3*R*/*S*)-3-hydroxydecanoic acid (**45**) was from Matreya. ThioGlo-1, [10-(2,5-dihydro-2,5-dioxo-1*H*-pyrrol-1-yl)-9-methoxy-3-oxo-methyl ester], was purchased either from Calbiochem or Covalent Associates.

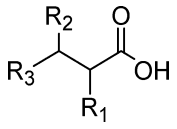
**Substrate Synthesis and Characterization.** The synthetic strategies employed in the generation of various racemic stereoisomers of 2-alkyl-3-ketoalkanoic acids and 2-alkyl-3-hydroxyalkanoic acids, *N*-acetylcysteamine (SNAC) thioesters, and methyl esters are outlined in the Supporting Information. Using Evans aldol methodology, we have also synthesized all four stereoisomers of 2-hexyl-3-hydroxydecanoic acid (**17a**, **17b**, **17c**, and **17d**).[22,23] All compounds were characterized by NMR and liquid chromatography–mass spectrometry (LC–MS) analysis and a summary of their structures and activity are summarized in Table 1.

**Expression and Purification of Recombinant *K. radiotolerans* OleA and *S. maltophilia* OleD $His_6$-Tag Fusion Proteins.** Expression plasmids carrying the *oleD* gene from *S. maltophilia* and the *oleA* gene from *K. radiotolerans* cloned into pET-21b vector system (Novagen) were a kind gift from the renewable petroleum company LS9, Inc. (San Francisco, CA). Each of the recombinant $His_6$-tagged fusion proteins were overproduced in *Escherichia coli* BL21(DE3) cells and purified by $Ni^{2+}$-agarose affinity chromatography using standard protocols. Protein concentrations were determined using the Qubit Fluorometer from Invitrogen. The resulting proteins were >90% pure, based on SDS–PAGE analysis and were stored in 50 mM Tris·HCl (pH 8.0), 100 mM NaCl, 10% glycerol, and 1 mM TCEP at −80 °C.

**Spectrophotometric Assay for OleD Activity.** Enzymatic activity of OleD was monitored by change in absorbance at 340 nm, due to the formation or consumption of NAD(P)H ($\varepsilon_{340}$ = 6220 $M^{-1}$ $cm^{-1}$). Assays were performed in the presence of 100 mM sodium phosphate (pH 7.5), 0.5% Tween 20, 1 mM TCEP, 0.5–1 mM substrate (suspended in DMSO to give a final concentration of 1%), and either 0.2 mM NAD(P)H or 1 mM $NAD(P)^+$ in a final volume of 0.5 mL. The reactions were initiated upon the addition of protein (0.05–1 μM) and allowed to proceed for 10 min at 25 °C. All assays were performed in triplicate.

**Fluorescence Assay for OleD Activity.** A modified method for the fluorescence detection of alkaline $NAD(P)^+$

**Table 1. Summary of the Compounds Utilized in the Biochemical Analysis of *S. maltophilia* OleD and Their Relative Activity**



| compd[a] | $R_1$ | $R_2$ | $R_3$ | stereochemistry[b] | relative activity (%)[c] |
|---|---|---|---|---|---|
| **9** | n-$C_6H_{13}$ | =O | n-$C_7H_{15}$ | (2*R*/*S*) | 13 |
| **16** | n-$C_4H_9$ | −OH | n-$C_5H_{11}$ | *syn* | 0 |
| | | | | *anti* | 0 |
| **17** | n-$C_6H_{13}$ | −OH | n-$C_7H_{15}$ | *syn* | 15 |
| | | | | *anti* | <1 |
| **18** | n-$C_8H_{17}$ | −OH | n-$C_9H_{19}$ | *syn* | 20 |
| | | | | *anti* | <1 |
| **19** | n-$C_{10}H_{21}$ | −OH | n-$C_{11}H_{23}$ | *syn* | 100 |
| | | | | *anti* | <1 |
| **20** | n-$C_{12}H_{25}$ | −OH | n-$C_{13}H_{27}$ | *syn* | ND[d] |
| | | | | *anti* | ND[d] |
| **26** | H | =O | n-$C_5H_{11}$ | - | 0 |
| **27** | H | =O | n-$C_7H_{15}$ | - | 0 |
| **45** | H | −OH | n-$C_7H_{15}$ | (3*R*/*S*) | 0 |

[a]For compounds **9** and **16–20** where the carboxylic acid was either a NAC thioester or a methyl ester, no activity was observed. [b]Stereochemical assignment of *syn* ((2*R*,3*S*)- and (2*S*,3*R*)-enantiomeric pair) and *anti* ((2*S*,3*S*)- and (2*R*,3*R*)-enantiomeric pair) was based on NMR analysis and LC–MS comparison to enantiomerically pure standards. [c]Percent relative activity based on the comparison of $k_{cat}/K_m$ values. [d]ND denotes not determined due to solubility issues.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in a 96 well plate was also used to examine OleD activity.[24−26] Reactions were conducted under conditions described above except concentrations of NAD(P)H up to 1 mM were used for reduction reactions. At specific time points, 25 μL samples were quenched with an equal volume of 0.3 M HCl and incubated for 15 min at room temperature. Following the addition of 45 μL of 10 M NaOH, the samples were incubated for an additional 2 h at room temperature in the dark. After the addition of 180 μL of water, fluorescence was measured with an excitation wavelength of 360 nm and an emission wavelength of 455 nm. A standard curve of $NAD(P)^+$ was used to determine product concentration. Sequential hydrolytic reductive activity was also examined by fluorescence when 2 μM OleD, 1 μM OleA, 0.25 mM (2*R*/*S*)-2-hexyl-3-ketodecanoyl-SNAC (**10**), and 1 mM NADPH were incubated together.

**Effects of DMSO and Nonionic Detergents on OleD Activity.** The effects of DMSO on enzyme activity was examined by UV-spectroscopy in the presence of 100 mM sodium phosphate (pH 7.5), 1 mM TCEP, 0.5 mM *syn*-2-hexyl-3-hydroxydecanoic acid (**17**), 1 μM OleD, 1 mM $NADP^+$, and varying concentrations of DMSO (0.2−3%). Enzyme activity was also examined in the presence of Tween 20 and Triton X-100 (0−4%) under similar conditions described above except in the presence of 1% DMSO.

**Molecular Mass Determination of OleD.** The molecular mass of $His_6$-tag *S. maltophilia* OleD was estimated by HPLC on a BioSep-SEC-S4000 (300 mm × 7.8 mm, Phenomenex) gel filtration column. The protein (100 μg) was eluted with 20 mM monobasic sodium phosphate (pH 7.2) and 100 mM NaCl at a flow rate of 0.5 mL/min. β-Amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa) were used as molecular weight standards. The void and total column volume were determined from the elution volume of blue dextran and DNP-aspartate (299 Da). Elution of the proteins from the column was monitored by the absorbance at 280 nm. A calibration curve was generated by plotting the retention time against the log of the native molecular mass for the protein standards.

**Steady-State Kinetic Analysis of OleD.** Steady-state kinetic measurements were conducted under initial velocity conditions by UV spectroscopy or fluorescence while having the substrate or cofactor held at a constant concentration and varying the other. Kinetic parameters were calculated from the average of minimally 2 sets of triplicates with the Michaelis−Menten equation using the curve fitting software Kaleidagraph 4.0 (Synergy Software, Reading, PA).

**LC−MS Analysis of Reaction Products.** Assays were performed at 25 °C in the presence of 100 mM sodium phosphate (pH 7.5), 1 mM TCEP, 0.5% Tween 20, 0.2 mM (2*R*/*S*)-2-hexyl-3-ketodecanoic acid (**9**) or *syn*-2-hexyl-3-hydroxydecanoic acid (**17**) (in the presence of DMSO to give a final concentration of 1%), 0.5 mM NADP(H), and 1 μM OleD. Samples were taken at specific time points (0−60 min) and analyzed by LC−MS in the negative mode on a Bruker MicroTOF-Q equipped with an Agilent 1200 Series LC system using a Discovery HS C18 (250 × 2.1, 5 μm, Supelco) column at a flow rate of 0.3 mL/min. Buffer A was 5 mM ammonium acetate in water and Buffer B was 5 mM ammonium acetate in acetonitrile. The following solvent gradient was used to in the analysis (time, % Buffer B): 0 min, 5%; 30 min, 100%; 35 min, 100%; 38 min, 5%; and 45, min 5%.

Assays involving OleA (5 μM) were performed at 25 °C in the presence of 100 mM sodium phosphate (pH 7.5), 1 mM TCEP, 0.5% Tween 20, 1% DMSO, and either 0.2 mM octanoyl-CoA, octanoyl-SNAC (**44**), (2*R*/*S*)-2-hexyl-3-ketodecanoyl-SNAC (**10**), and/or sodium decanoate. Additional analyses included addition of 0.5 mM NADPH and 2 μM OleD. Samples were incubated for 90 min and analyzed by LC−MS in the negative mode on a ThermoElectron LTQ-Orbitrap high resolution mass spectrometer with a dedicated Accela HPLC system using a Discovery HS C18 (250 × 2.1, 5 μm, Supelco) column at a flow rate of 0.3 mL/min. Buffer A was 10 mM ammonium acetate in water and Buffer B was 10 mM ammonium acetate in acetonitrile. The following solvent gradient was used in the analysis (time, % Buffer B): 0 min, 10%; 30 min, 100%; 35 min, 100%; 38 min, 10%; and 45 min, 10%.

**Fluorescence Based Assay for OleA Hydrolytic Activity.** A time course analysis of OleA hydrolytic activity resulting in the formation of a free thiol (CoA or *N*-acetylcysteamine (SNAC) thioester) was carried out at 25 °C in the presence of 100 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$, 0.1 mM TCEP, 1% DMSO, 0.5 μM OleA, and 0.5 mM of either octanoyl-CoA, octanoyl-SNAC (**44**), or (2*R*/*S*)-2-hexyl-3-ketodecanoyl-SNAC (**10**). A series of controls were conducted in parallel, including the use of boiled protein, to ensure all hydrolysis of acyl thioesters were due to enzymatic activity. Aliqouts (50 μL) were withdrawn at specific time points and added to a well of a black 96-well plate containing an equal volume of DMSO to quench the reaction. After the addition of 100 μL of 200 μM ThioGlo-1 solution (in DMSO) to each well, the plate was incubated in the dark at room temperature with gentle shaking for 20 min. All samples were analyzed by fluorescence (excitation 378 nm and emissions at 480 nm) using a Gemini XPS microplate spectrofluorometer from Molecular Devices. The relative fluorescent unit (RFU) was converted to concentration using a standard CoA curve.

## RESULTS

**Sequence Alignment Analysis.** A multiple sequence alignment of putative OleD proteins from various organisms was performed using Geneious 5.3 (see Supporting Information Figure S1). The sequence identity among the proteins varied from 40 to 70%. The alignment revealed the presence of the conserved nucleotide binding motif (Rossman fold) in the N-terminal region and catalytic triad $SX_nYXXXK$ of the SDR superfamily, in OleD.[18,21,27−30] The spatial orientation of the Gly residues within nucleotide binding site, GXXGXXG, is characteristic with the pattern displayed by the extended SDR family.[20,21,31] Despite the low sequence identities (∼14−20%) between OleD and SDR proteins, the nucleotide binding motif and catalytic triad suggest a shared catalytic mechanism, which is believed to be initiated by the transfer of a proton from the Tyr residue to the carbonyl moiety on the substrate followed by hydride transfer. The Ser facilitates catalysis by stabilizing the substrate within the active site, whereas the Lys residue forms a hydrogen bond with the ribose hydroxyl group of the cofactor which in turn is hydrogen bonded to the Tyr residue. The resulting proton relay system presumably lowers the $pK_a$ of the Tyr-OH to promote proton transfer.[18,27]

The working hypothesis for OleD which was verified through this biochemical study was that it would utilize 2-alkyl-3-ketoalkanoic acid as a substrate and not the corresponding thioester. β-Hydroxybutyrate dehydrogenase (HBDH) catalyzes the reversible oxidation of 3-hydroxybutyrate to acetoacetate in the presence of $NAD^+$. Studies of this enzyme from

Table 2. Steady-State Kinetic Parameters of *S. maltophilia* OleD[a]

| substrate | $K_m$ ($\mu$M) | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_m$ ($min^{-1}$ $mM^{-1}$) |
|---|---|---|---|
| (2*R*/*S*)-2-Hexyl-3-ketodecanoic acid (**9**) | 161 ± 17 | 58 ± 4 | 362 ± 21 |
| *syn*-2-Hexyl-3-hydroxydecanoic acid (**17**)[b] | 202 ± 17 | 72 ± 3 | 360 ± 33 |
| (2*R*,3*S*)-2-Hexyl-3-hydroxydecanoic acid (**17a**) | 181 ± 26 | 79 ± 3 | 435 ± 12 |
| *syn*-2-Octyl-3-hydroxydodecanoic acid (**18**)[b] | 214 ± 23 | 122 ± 3 | 574 ± 57 |
| *syn*-2-Decyl-3-hydroxytetradecanoic acid (**19**)[b] | 31 ± 10 | 85 ± 7 | 2876 ± 740 |
| $NADP^+$ [c] | 24 ± 2 | | |
| NADPH[d] | 26 ± 4 | | |

[a]Kinetic values were determined with at least three sets of triplicates to calculate the standard deviation. [b]The *syn* compounds are composed of (2*R*,3*S*)- and (2*S*,3*R*)-enantiomeric pair. [c]Kinetic parameters were obtained in the presence of saturating concentrations of *syn*-**17** and determined by UV-spectroscopy. [d]Kinetic parameters were obtained in the presence of saturating concentrations of **9** and determined by fluorescence.

*Pseudomonas putida* have shown that a strictly conserved tetrad composed of a His, Lys, and two conserved Gln residues coordinate the carboxylate group of 3-hydroxybutyrate.[32] In the human type 2 hydroxybutyrate dehydrogenase, three conserved Arg residues, referred to as a "triple R" motif, are shown to be coordinated to the carboxylate group of the substrate.[33] There are a number of highly conserved Arg, Asn, and His residues found in OleD proteins (see Supporting Information Figure S1); however, with the exception to Arg179 (based on SmOleD numbering), none are conserved in positions of known carboxylate coordinating residues found in *P. putida* and human 3-hydroxybutyrate dehydrogenase proteins.

**Protein Expression and Purification Molecular Mass Determination.** OleD from *S. maltophilia* and OleA from *K. radiotolerans* were successfully overproduced in *E. coli* BL21(DE) as soluble N-terminal $His_6$-tag fusion proteins and purified to greater than 95% purity by a single step nickel metal affinity chromatography. Analysis of purified OleD by SDS–polyacrylamide gel electrophoresis shows a single band with an apparent molecular weight of ~36 kDa, and when analyzed by MS, the recombinant $His_6$-tag protein had a mass of 36 472 Da, consistent with the molecular mass calculated by the amino acid sequence plus the mass of the N-terminal fusion. An apparent native molecular mass of 77 124 ± 1753 Da for the $His_6$-tag OleD was estimated by size-exclusion chromatography on a calibrated BioSep-SEC-S4000 column, indicating that the protein is a homodimer.

**Effects of DMSO and Nonionic Detergents on OleD Activity.** Potential OleD substrates were generally poorly water-soluble but readily soluble in DMSO. OleD assay conditions with DMSO were therefore optimized using *syn*-2-hexyl-3-hydroxydecanoic acid (**17**). Maximum relative activity was observed at 0.5% and slowly decreased with increasing DMSO (see Supporting Information Table S1). Only a 6% decrease from maximal activity was observed with 1% DMSO, and this was selected for all assays to minimize any potential solubility issues associated with longer chain substrates.

Preparing fatty acids in a solution of cyclodextrin[34] and/or carrying out the assays in the presence of nonionic detergents, such as Tween 20 or Triton X-100, are strategies that have also been used to improve solubility of poorly water-soluble compounds in an aqueous environment.[35−41] The effects of nonionic detergents and 1% DMSO on OleD activity are summarized in Supporting Information Table S2. An increase in enzyme activity with increasing Tween 20 concentration was observed with maximum activity at 0.5%. A similar increase in activity was observed when assayed in the presence of Triton X-100; however, the relative activity was lower to that observed in the presence of Tween 20. On the basis of these findings, all subsequent analyses were carried out in the presence of 1% DMSO and 0.5% Tween 20.

**Substrate Specificity and Steady-State Kinetic Analyses.** Through a nonstereospecific synthetic route, four enantiomers of 2-alkyl-3-hydroxyalkanoic acid which could be separated into diastereomers were generated. The *syn*-2-alkyl-3-hydroxyalkanoic acids consist of the (2*R*,3*S*)- and (2*S*,3*R*)-enantiomeric pair, whereas the *anti*-2-alkyl-3-hydroxyalkanoic acids are composed of the (2*S*,3*S*)- and (2*R*,3*R*)-enantiomeric pair. Subsequent work allowed for the stereochemical synthesis of each isomer of 2-hexyl-3-hydroxydecanoic acid (**17a**, **17b**, **17c**, and **17d**). In addition, (2*R*/*S*)-hexyl-3-ketodecanoic acid (**9**) and 3-ketodecanoic acid (**27**) were prepared via nonstereospecific routes.

Each of the synthetic compounds, as shown in Table 1, were screened by UV spectroscopy, fluorescence, and/or LC–MS as potential substrates for purified OleD. In the oxidative direction using $NADP^+$, the 2-alkyl-3-hydroxyalkanoic acids were generally observed to be substrates and significantly higher activity was seen in each case with the *syn* pair. Activity was only observed with the free acids and not with the *N*-acetylcysteamine (SNAC) thioester or methyl ester derivatives (Table 1). Similarly, no activity was observed using $NAD^+$. No activity was seen for (3*R*/*S*)-3-hydroxydecanoic acid (**45**) or *syn*-2-butyl-3-hydroxyhexanoic acid (**16**). In the reductive direction, (2*R*/*S*)-2-hexyl-3-ketodecanoic acid (**9**) was a substrate, whereas 3-ketodecanoic acid (**27**) was not a substrate. This preliminary data demonstrated that OleD catalyzes a stereospecific reversible reaction with substrate specificity for free acids and a C2-alkyl chain.

A more detailed kinetic analyses of the OleD oxidative reaction was made using *syn* isomers of 2-hexyl-3-hydroxydecanoic acid (**17**), 2-octyl-3-hydroxydodecanoic acid (**18**), and 2-decyl-3-hydroxydodecanoic acid (**19**) (Table 2). A steady-state kinetic analysis with **17** and a fixed concentration of $NADP^+$ provided an apparent $k_{cat}$ of 72 ± 3 $min^{-1}$ and a $K_m$ of 202 ± 17 $\mu$M. The analysis with variable $NADP^+$ in the presence of fixed concentration of **17** provided a $K_m$ of 24 ± 4 $\mu$M (Table 2). The catalytic efficiency increased with increasing chain length with *syn*-2-decyl-3-hydroxytetradecanoic acid (**19**) being the preferred substrate with a $k_{cat}/K_m$ 8- and 5-fold higher than for the *syn*-2-hexyl-3-hydroxydecanoic acid (**17**) and *syn*-2-ocyl-3-hydroxydodecanoic acid (**18**), respectively (Table 2). Solubility issues precluded analysis of potential substrates with longer alkyl chain lengths, such as 2-decyl-3-hydroxyhexadecanoic acid (**20**). A steady-state kinetic analysis was also carried out for the reductive reaction using a presumed mixture of (2*R*/*S*)-2-hexyl-3-ketodecanoic acid (**9**), the 3-keto

analogue of **17**, and provided the following: $k_{cat}$ 58 ± 4 $min^{-1}$, $K_m$ 161 ± 18 μM (**9**) and 26 ± 4 μM (NADPH) (Table 2).

The *syn* isomers of the OleD substrates **17**−**19** comprise both the (2*R*,3*S*)- and (2*S*,3*R*)-enantiomers. To determine the stereospecificity of the OleD reaction and in turn the stereochemical course of the olefin biosynthetic pathway, a stereochemical synthesis was used to generate each of the four isomers of 2-hexyl-3-hydroxydecanoic acid (**17**). As shown in Table 3, OleD

**Table 3. Relative Activity of *S. maltophilia* OleD with 2-Hexyl-3-hydroxydecanoic Acid Isomers**

| substrate | relative activity (%) |
|---|---|
| (2*R*,3*S*)-**17a** | 100.0 |
| (2*S*,3*R*)-**17b** | 0.5 |
| (2*R*,3*R*)-**17c** | 1.5 |
| (2*S*,3*S*)-**17d** | 0.3 |

shows high stereoselectivity toward the oxidation of (2*R*,3*S*)-2-hexyl-3-ketodecanoic acid (**17a**), with a greater than 98% reduction for the other *syn* isomer (**17b**) or either *anti* isomer (**17c** and **17d**). Steady-state kinetic analysis with the enantiomer **17a** revealed comparable $k_{cat}$ and $K_m$ values to those observed for the **17** *syn* pair (**17a** and **17b**) (Table 2).

Confirmation of the OleD reaction products in both the oxidative and reductive direction was achieved by LC−MS analyses. As shown in Figure 2, two new peaks eluting at 30 min (major) and 32 min (minor) were observed in the total ion chromatogram (TIC) when a presumed mixture of

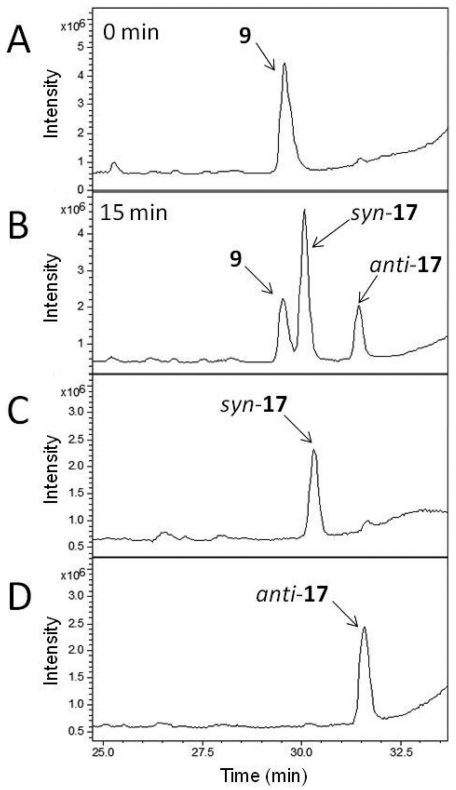


**Figure 2.** TIC of OleD incubated in the presence of (2*R*/*S*)-2-hexyl-3-ketodecanoic acid (**9**). Panel (A) represents time point 0 min, (B) is the TIC of the reaction after 15 min, (C) *syn*-2-hexyl-3-hydroxydecanoic acid (**17**) synthetic standard, and (D) *anti*-2-hexyl-3-hydroxydecanoic acid (**17**) synthetic standard. LC−MS analysis was conducted in the negative mode.

(2*R*/*S*)-hexyl-3-ketodecanoic acid (**9**) (elution time ∼29 min) was incubated in the presence of OleD and NADPH. The new peaks had a *m*/*z* of 271, consistent with the formation of 2-hexyl-3-hydroxydecanoic acid ($[M - H]^-$) and coeluted with synthetic *syn*- and *anti*-2-hexyl-3-hydroxydecanoic acid (**17**) standard, respectively (data not shown). For the oxidative reaction, the LC−MS analysis (Figure 3) revealed the

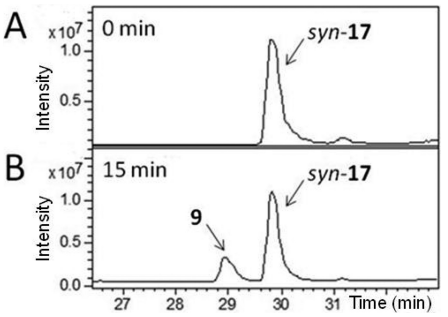


**Figure 3.** TIC of OleD incubated in the presence of *syn*-2-hexyl-3-hydroxydecanoic acid (**17**). Panel (A) represents time point 0 min and (B) is the TIC of the reaction after 15 min. LC−MS analysis was conducted in the negative mode.

conversion of *syn*-2-hexyl-3-hydroxydecanoic acid (**17**) (elution time ∼30 min) to a peak with the retention time (∼29 min) and MS properties (*m*/*z* 269, $[M - H]^-$; *m*/*z* 225, $[M - CO_2]^-$) of (2*R*/*S*)-2-hexyl-3-ketodecanoic acid (**9**).

**OleA Activity and Product Identification.** Our working hypothesis for OleA was that it catalyzed condensation of two acyl thioesters to generate a 2-alkyl-3-ketoacyl thioester. The specificity of OleD for the 2-alkyl-3-ketoalkanoic acid indicated either that OleA or another protein must first catalyze a hydrolytic step. A series of assays using the *K. radiotolerans* OleA and the *S. maltophilia* OleD were carried out to address this question, and to determine the overall stereochemical course of the first steps of the olefin biosynthetic pathway.

OleA hydrolytic activity, as measured by CoA or *N*-acetylcysteamine (SNAC) thioester release and fluorescence assay involving ThioGlo-1, was examined in the presence of either octanoyl-CoA, octanoyl-SNAC (**44**), or (2*R*/*S*)-2-hexyl-3-ketodecanoyl-SNAC (**10**). In all cases, there was a time-dependent release of free thiol. Octanoyl-CoA was the preferred substrate with a 4- to 5-fold higher hydrolytic rate compared to octanoyl-SNAC (**44**), and (2*R*/*S*)-2-hexyl-3-ketodecanoyl-SNAC (**10**) (Table 4). Thioester release from

**Table 4. Acyl-thioester Hydrolysis Rates of *K. radiotolerans* OleA[a]**

| substrate | rate(μM/min) |
|---|---|
| Octanoyl-CoA | 1.61 |
| Octanoyl-SNAC (**44**) | 0.42 |
| (2*R*/*S*)-2-hexyl-3-kecodecanoyl-SNAC (**10**) | 0.33 |

[a]Rates were determined by fluorescence based assay using ThioGlo-1.

**10** would provide 2-hexyl-3-ketodecanoic acid, an OleD substrate. Consistent with this prediction, coincubation of **10**, NADPH, OleA, and OleD was shown to lead to $NADP^+$ generation, through an increase in fluorescence (see Supporting Information Figure S2). This increase was not observed in control experiments where the substrate, cofactor, or either enzyme was absent.


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A series of LC−MS experiments were used to verify that OleA catalyzed both the condensation and hydrolytic steps and that the OleA product was processed by OleD to provide the *syn* isomer of 2-alky-3-hydroxyalkanoic acid. When OleA was incubated with octanoyl-CoA, an ion with an *m*/*z* corresponding to 2-hexyl-3-ketodecanoic acid (**9**) (*m*/*z* 269, $[M - H]^-$; *m*/*z* 225, $[M - CO_2]^-$) eluting at ∼25 min was observed under the conditions implemented (see Supporting Information Figure S3). An ion with a *m*/*z* of 1018 ($[M - H]^-$) corresponding to 2-hexyl-3-ketodecanoyl-CoA eluting ∼20 min was also observed. Upon addition of OleD and $NADP^+$, the ion peak eluting at ∼25 min disappeared and a single new peak emerged at ∼27 min, which had an *m*/*z* of 271 ($[M - H]^-$) consistent with the formation of 2-hexyl-3-hydroxydecanoic acid (**17**) (see Supporting Information Figure S4), and coeluted with synthetic standard of *syn*-2-hexyl-3-hydroxydecanoic acid (**17**) (data not shown). Replacing octanoyl-CoA with octanoyl-SNAC (**44**) thioester resulted in similar findings, formation of 2-hexyl-3-ketodecanoic acid (**9**) with OleA alone (see Supporting Information Figure S5) and conversion to *syn*-2-hexyl-3-hydroxydecanoic acid (**17**) upon addition of OleD and NADPH (see Supporting Information Figure S6). In this case, a peak with an *m*/*z* of 370 ($[M - H]^-$) corresponding to and having the same retention time (∼31.4 min) as 2-hexyl-3-ketodecanoyl-SNAC thioester (**10**) was also observed (see Supporting Information Figure S5). Assays with octanoyl-CoA and octanoyl-SNAC were also conducted in the presence of decanoic acid and resulted in the same findings. The absence of any new products generated using the decanoic acid demonstrated that both substrates for the initial reaction catalyzed by OleA must be thioesters.

Incubating OleA in the presence of a presumed (2*R*/*S*)-2-hexyl-3-ketodecanoyl-SNAC (**10**) resulted in the formation of an ion that had an *m*/*z* and retention time (∼25 min) consistent with 2-hexyl-3-ketodecanoic acid (**9**) (*m*/*z* 269, $[M - H]^-$; *m*/*z* 225, $[M - CO_2]^-$) (see Supporting Information Figure S7). In this case, however, the addition of OleD resulted in the formation of two new compounds eluting at 27 and 28 min, consistent with the formation of both *syn*- and *anti*-2-hexyl-3-hydroxydecanoic acid (**17**) ($[M - H]^-$) (see Supporting Information Figure S8). The same observation of *syn* and *anti* products was made using (2*R*/*S*)-2-hexyl-3-ketodecanoic acid (**9**) with OleD and NADPH alone, and this result suggests that both the 2*R* and 2*S* isomers of **10** are substrates for OleA hydrolytic activity.

## DISCUSSION

Four genes annotated as *oleA*, *oleB*, *oleC*, and *oleD* have now been identified as being responsible for generation of olefins from activated fatty acid thioesters in a number of organisms, including *S. maltophilia* and *K. radiotolerans*.[2−9] Despite the exciting potential for the olefin pathway in the generation of biofuels and other specialty chemicals, most of the individual biochemical steps catalyzed by these the *ole* gene products have not been delineated experimentally.

This work has shown that OleD from *S. maltophilia* (SmOleD) catalyzes the reversible NADPH reduction of 2-alkyl-3-ketoalkanoic acids. No activity is observed with NAD(H), which is consistent with the observation that the conserved Asp residue found in all NAD(H) preferring enzymes is absent in a comparable position in OleD proteins.[17,20,21,42,43] Furthermore, the presence of a basic Arg residue located ∼19 residues from the Gly-rich motif involved in cofactor binding is conserved within the OleD proteins inferring NADP(H) specificity.[17,20,21,42] The *S. maltophilia* OleD catalytic efficiency was greatest with longer alkyl chain substrates, consistent with the observation that saturated and unsaturated acyclic hydrocarbons in the typical range $C_{22}$−$C_{31}$ are made by *S. maltophilia* and constitute approximately 0.25−2% of cellular dry weight.[2,3,5−8,16] Shorter chain length alkenes are observed in *K. radiotolerans* ($C_{19}$−$C_{24}$) and the OleD from this organism might be expected to have different chain length specificity. A sequence analysis revealed a 40% sequence identity and subtle differences in hydrophobicity between OleD proteins from *S. maltophilia* and *K. radiotolerans*. However, the analysis fails to provide insight into the predicted differences in chain length specificity for the two proteins. The enhancement of the SmOleD catalytic efficiency with longer alkyl chains arose predominantly from lower $K_m$ values, suggesting that the difference arise mostly from stronger binding and not the subsequent catalytic steps. The presence of the 2-alkyl chain is crucial for enzyme recognition and catalysis as reflected by the lack of OleD activity toward 3-ketodecanoic (**27**) and 3-hydroxydecanoic acids (**45**).

The OleD reaction product in the reductive direction (Figure 1) contains two stereogenic centers at C2 and C3. In principle, in the olefin biosynthetic process, the stereochemical pattern of the 2-alkyl chain is the direct consequence of the condensation step catalyzed by OleA whereas the stereochemistry of the 3-hydroxy group is established by OleD. The highly stereoselective nature of OleD toward (2*R*,3*S*)-2-hexyl-3-hydroxydecanoic acid (**17a**) in the oxidative direction suggests the stereochemistry of the 2-alkyl group as a result of OleA catalysis is in the *R*-configuration and that OleD catalyzes the stereospecific reduction of the 3-keto group to give a (3*S*)-hydroxy group. Additional support for this stereochemical assignment was the observation that a coincubation of OleA, OleD, and NADPH and either octanoyl CoA or octanoyl-SNAC (**44**) provided a *syn* isomer of **17** (which would be either **17a** or **b**). Conversely, when OleD was incubated with a (2*R*/*S*)-2-hexyl-3-ketodecanoic acid (**9**), both *syn* and *anti* products of **17** were observed. Thus, the OleA reaction must provide a single 2*R* enantiomer of **9** which is converted to **17a** (the (2*R*,3*S*)-isomer) by OleD hydride addition to the *re* face at C3. Previous preliminary deuterium labeling investigations of the olefin pathway studies have demonstrated that the reductive step in the process involves transfer of the 4-*pro*-*R* hydride from NADPH to the substrate.[10] At this point, the stereochemical components of the OleD reaction have thus been elucidated. The production of an *anti* isomer of **17** (**17c** or **d**) from racemic mixture of **9** logically dictates that OleD can also process the 2*S* isomer to generate an *anti* product, which must be **17d** (the (2*S*,3*S*)-isomer). In this case, the hydride addition occurs again to the *re* face at C3. While the processing of a 2*S* isomer by OleD does not appear to be physiologically relevant (this is not the product generated by OleA), it does indicate potential use as a biocatalyst having a tolerance not only for different alkyl chain lengths, but also for the stereochemical configuration of the C2 alkyl substituent.

Our work on the *K. radiotolerans* OleA described herein and recent independent work on the *X. campestris* OleA[9] not only demonstrates that they catalyze a condensation of two acyl thioesters to generate a 2*R*-acyl-3-keto acyl thioester, but that there is a subsequent hydrolysis step to generate the corresponding free acid. Two pieces of evidence suggest that the condensation step occurs fast relative to the second hydrolysis

step: (1) using either the CoA or NAC thioesters, the intermediate 2*R*-acyl-3-keto acyl thioester was observed in reactions with OleA; and (2) similar hydrolytic rates are observed between octanoyl-SNAC (**44**) and (2*R*/*S*)-2-hexyl-3-ketodecanoyl-SNAC (**10**). In the latter case, a faster rate of hydrolysis with **10** would be expected if condensation were the slow step.

Together, these observations have elucidated the role and stereochemical course of OleA and OleD in the first steps of the olefin pathway, leading to a 2-alkyl-3-hydroxyalkanoic acid pathway intermediate (Figure 1). Attaining the olefin from this intermediate would require decarboxylation and dehydration steps. These might occur separately or as a combined decarboxylative−elimination step. There is strong genetic evidence for the role of the *oleC* gene in the olefin pathway,[7,9,10,16] and we hypothesize that it is involved in these final steps. Biochemical investigations of OleC will be reported in due course.

## ■ ASSOCIATED CONTENT

### Supporting Information

The detailed experimental procedures, characterization data of all synthesized compounds, and the LC−MS extracted ion chromatograms (EIC) of OleA reactions associated with this article. This material is available free of charge via the Internet at http://pubs.acs.org.

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## ■ ABBREVIATIONS

SDR, short chain dehydrogenase; HBDH, 3-hydroxybutyrate dehydrogenase; Tris-HCl, tris(hydroxymethyl)-aminomethane-hydrochloric acid; SNAC, *N*-acetylcysteamine; TCEP, tris(2-carboxyethyl)phosphine; ThioGlo-1, 10-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)-9-methoxy-3-oxo-methyl ester; EIC, extracted ion chromatogram; TIC, total ion chromatogram.

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