Oleate hydratase from *Staphylococcus aureus* protects against palmitoleic acid, the major antimicrobial fatty acid produced by mammalian skin

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Oleate hydratases (OhyAs) belong to a large family of bacterial proteins catalyzing the hydration or isomerization of double bonds in unsaturated fatty acids. A *Staphylococcus aureus* gene (*Sa0102*) is predicted to encode an OhyA. Here, we recombinantly expressed and purified *Sa*OhyA and found that it forms a homodimer that requires FAD for activity. *Sa*OhyA hydrates only unsaturated fatty acids containing *cis*-9 double bonds, but not fatty acids with *trans*-9 double bonds or *cis* double bonds at other positions. *Sa*OhyA products were not detected in *S. aureus* phospholipids and were released into the growth medium. *S. aureus* does not synthesize unsaturated fatty acids, and the *Sa*OhyA substrates are derived from infection sites. Palmitoleate (16:1(9*Z*)) is a major mammalian skin–produced antimicrobial fatty acid that protects against *S. aureus* infection, and we observed that it is an *Sa*OhyA substrate and that its hydroxylated derivative is not antimicrobial. Treatment of *S. aureus* with 24 μM 16:1(9*Z*) immediately arrested growth, followed by growth resumption after a lag period of 2 h. The Δ*ohyA* mutant strain did not recover from the 16:1(9*Z*) challenge, and increasing *Sa*OhyA expression using a plasmid system prevented the initial growth arrest. Challenging *S. aureus* with sapienic acid (16:1(6*Z*)), an antimicrobial fatty acid produced only by human skin, arrested growth without recovery in WT, Δ*ohyA*, and *Sa*OhyA-overexpressing strains. We conclude that *Sa*OhyA protects *S. aureus* from palmitoleic acid, the antimicrobial unsaturated fatty acid produced by most mammals, and that sapienic acid, uniquely produced by humans, counters the OhyA-dependent bacterial defense mechanism.

Bacterial oleate hydratase (OhyA)[2] activity (EC 4.2.1.53) was first detected in 1962 (1), and the product was characterized as 10(*R*)-hydroxy-18:0 using soluble enzyme preparations from *Pseudomonas sp.* (2) (Fig. 1). This organism is now called *Elizabethkingia meningoseptica*, and *E. meningoseptica* OhyA is a well-characterized member of a large family of related bacterial genes predicted to encode unsaturated fatty acid hydratases (3, 4). OhyA has utility in the synthesis of hydroxy fatty acids of commercial interest by bioconversion (5, 6). OhyA genes are widely distributed in bacteria, and recently a Hydratase Database was established (https://hyed.biocatnet.de/)[3] to accelerate the characterization of OhyAs as biocatalysts. This bioinformatic analysis sorts sequences of known and putative hydratases into 11 subfamilies (3). Members of the OhyA family belong to the subset of FAD-dependent enzymes that are not oxidoreductases (4) (Fig. 1). FAD oxidation/reduction does not have an active role in substrate conversion, and it is thought that FAD binding promotes organization of the active site or stabilizes the transition state (4, 7, 8). Although the bacterial OhyA proteins are highly related and all use an FAD cofactor (4), there are clear differences in the products formed by the individual enzymes. Some OhyA enzymes are selective for 9*Z* double bonds, whereas the *Streptococcus pyogenes* OhyA catalyzes hydration of both (9*Z*) and (12*Z*) double bonds (9). *E. meningoseptica* OhyA catalyzes the reversible hydration of the *cis* double bond of oleate (18:1(9*Z*))[4] to 10(*R*)-hydroxy-18:0 (*h*18:0), which may also be converted to 18:1(10*E*) or 18:1(9*Z*) by a reverse dehydration (7, 8). The OhyA family member from *Lactobacillus plantarum* also functions as an isomerase that forms 18:2(9*Z*,11*E*) (conjugated linoleic acid) in a four-enzyme pathway for polyunsaturated fatty acid saturation (10).

Staphylococcus aureus oleate hydratase (*Sa*OhyA) is a member of the HF#2 hydratase subfamily that contains 1188 sequences including *Lactobacillus* and *Streptococcus* species (3) (Fig. 1). The substrate specificity and biological function of *Sa*OhyA are unknown. However, *S. aureus* does not synthesize unsaturated fatty acids, and therefore the system is present to metabolize environmental fatty acids that would be encountered during infection. *S. aureus* is the leading cause of skin and soft tissue infections (11), and unsaturated 16-carbon fatty acids are a potent innate immune defense deployed by the skin to prevent infection. In mice (and other mammals), 16:1(9*Z*) is the most potent antimicrobial fatty acid produced by the skin

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This article contains Table S1.

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[2] The abbreviations used are: OhyA, oleate hydratase; *Sa*OhyA, *S. aureus* oleate hydratase; ACP, acyl carrier protein; *h*18:0, 10-hydroxyoctadecanoic acid; *h*18:1, 10-hydroxy-12(*Z*)-octadecenoic acid; qRT-PCR, quantitative real-time PCR.

[3] Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.

[4] Fatty acids are designated by number of carbons:number of double bonds-(double bond location/configuration).

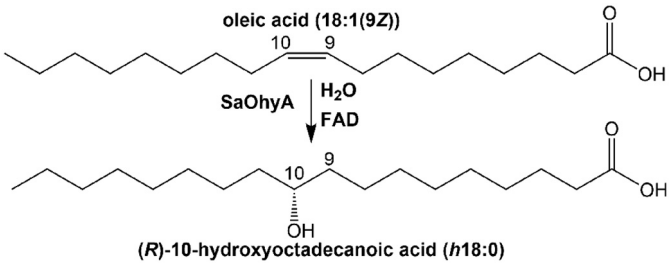

Figure 1. The oleate hydratase reaction. OhyAs are a group of bacterial enzymes that hydrate and/or isomerize the double bonds present in unsaturated fatty acids. *Sa*OhyA catalyzes the addition of water to *cis*-9 double bonds, but not to *trans* double bonds or *cis* double bonds at other positions.

(12). Antimicrobial fatty acids permeabilize the cells, leading to the leakage of low-molecular-weight solutes and proteins <20 kDa into the medium (12). However, human sebum is unique in that it is the only documented place where sapienic acid (16:1(6*Z*)) is produced in the animal kingdom where it is deployed instead of 16:1(9*Z*) as a major skin antimicrobial fatty acid (13, 14). This is because fatty acid desaturase 2 (*FADS2*), the same enzyme that is involved in the formation of polyunsaturated fatty acids, is highly expressed in human skin, leading to the desaturation of palmitate at carbon-6 (15). Humans (16) and mice (17) deficient in the production of these 16-carbon monounsaturated fatty acids are more susceptible to *S. aureus* skin infections. The goal of this project is to biochemically characterize *Sa*OhyA and determine whether it functions as a countermeasure used by *S. aureus* to combat host antimicrobial fatty acids.

Results

Identification and purification of SaOhyA

S. aureus has a single *ohyA* gene homolog in its genome (*Sa0102*, *Sa*OhyA). An N-terminal His-tagged version of *Sa*OhyA was cloned, expressed in *Escherichia coli*, and purified to determine its biochemical properties. *Sa*OhyA was found in the cytosol of the *E. coli* expression system and was purified using affinity chromatography on Ni^{2+}–nitrilotriacetic acid–agarose. The protein was further purified by gel filtration chromatography on a Sepharose S-200 column to yield a protein of >95% purity based on gel electrophoresis (Fig. 2*A*). The elution position in gel filtration chromatography indicated that *Sa*OhyA was a homodimer (Fig. 2*A*), a configuration that is characteristic of bacterial oleate hydratases (4, 5, 7, 8).

The activity of *Sa*OhyA was analyzed in biochemical assays using [1-^{14}C]oleate as the substrate. *Sa*OhyA required FAD for activity and catalyzed the hydroxylation of 18:1(9*Z*) to *h*18:0 (Fig. 2*B*). FMN did not substitute for FAD, and the presence of oxidized or reduced nicotinamide adenine dinucleotides did not influence enzyme activity either alone (Fig. 2*B*) or when added with FAD (not shown). The *Sa*OhyA protein preparations were not visibly colored, but the activity of purified *Sa*OhyA in the absence of FAD was ~6% of the maximum rate with FAD present (Fig. 2*B*). We determined whether the FAD-independent *Sa*OhyA activity (Fig. 2*B*) was due to the presence of FAD in the protein preparation or FAD-independent enzyme activity by measuring the amount of FAD in the *Sa*OhyA preparation by LC-MS/MS. The amount of FAD present was determined following extraction of the purified protein and quantification of the FAD amount using a standard curve as described under "Experimental procedures." This analysis

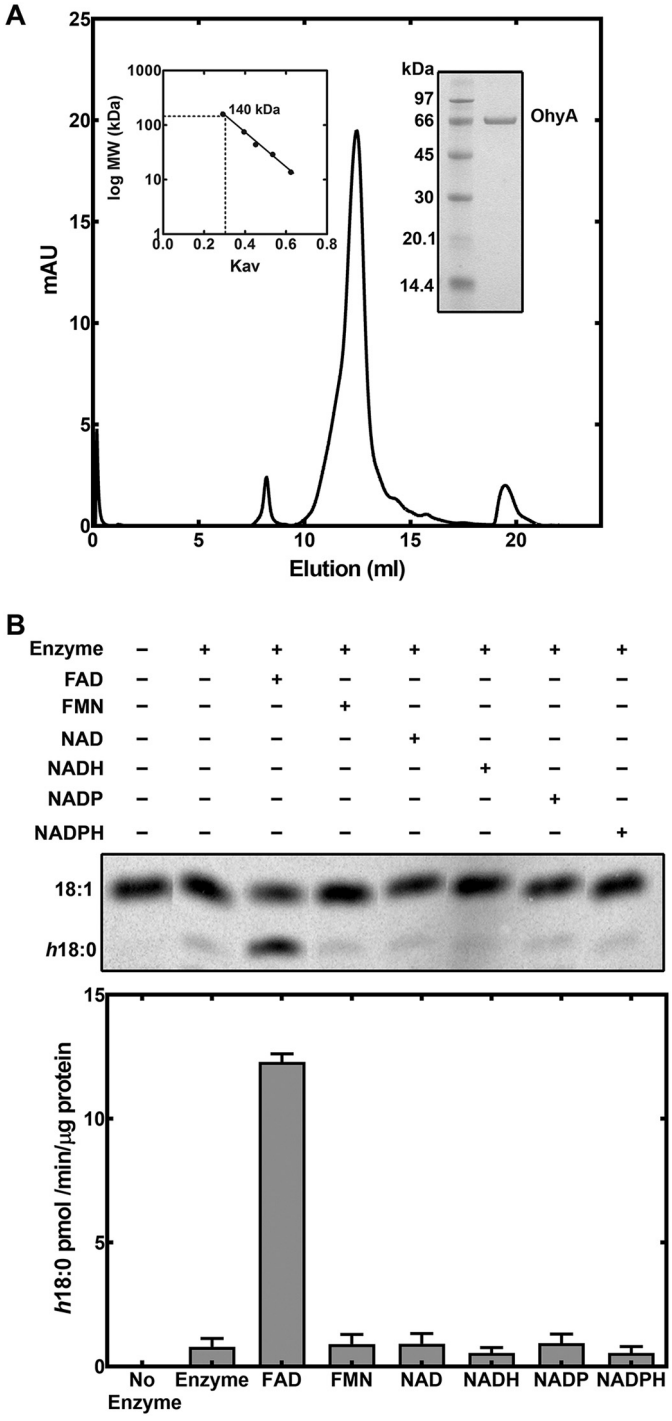

Figure 2. Purification and cofactor requirement for *Sa*OhyA. *A*, His-tagged *Sa*OhyA was purified by Ni^{2+}-affinity chromatography followed by gel filtration chromatography on Sephadex S-200. Elution volume of *Sa*OhyA compared with a standard curve (*left inset*) indicated that *Sa*OhyA is a 140-kDa dimer. *Sa*OhyA was a 68-kDa monomer and was >95% pure based on gel electrophoresis (*right inset*). *B*, the cofactor requirement for *Sa*OhyA. An example showing the separation of [^{14}C]18:1 from [^{14}C]*h*18:0 by TLC. The concentrations of the cofactors were 50 μM, and the quantitation of the reaction rates from three experiments is shown in the *bar graph*. There was no effect of NAD(H) or NADP(H) when added in the presence of 50 μM FAD (not shown). The data presented are the results from triplicate experiments. *Error bars*, S.E.

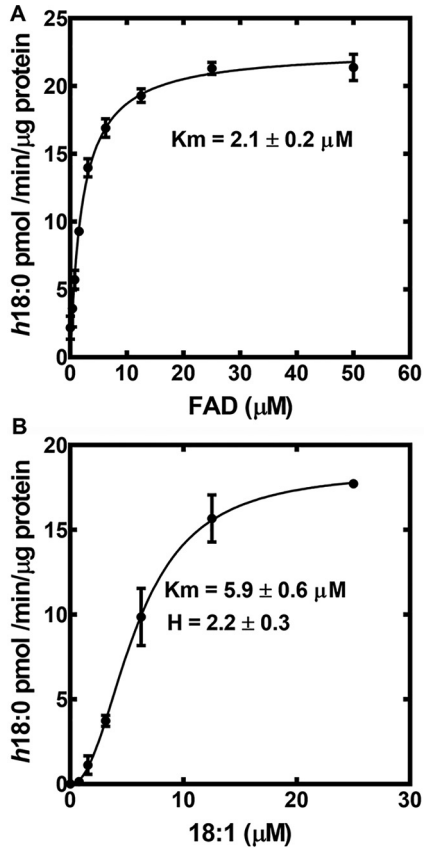

Figure 3. Kinetic analysis of *Sa*OhyA. *A*, the apparent K_m for FAD calculated using 20 μM 18:1(9*Z*) substrate. The *line* represents the fit of the data points to the Michaelis–Menten equation. *B*, the apparent 18:1(9*Z*) K_m determined at 50 μM FAD substrate. The *line* represents the fit of the data points to the Hill equation. The dependence of the reaction on 18:1(9*Z*) was highly cooperative with a Hill number of 2.2 ± 0.3. *Error bars*, S.E.

showed that the FAD level was sufficient to occupy 3.5% of the *Sa*OhyA active sites. Thus, the residual activity in the absence of added FAD was attributed to a small amount of FAD that copurified with *Sa*OhyA. The apparent K_m for FAD was 2.1 ± 0.2 μM (Fig. 3*A*), consistent with the significant loss of the FAD cofactor during the purification of *Sa*OhyA. The apparent K_m for oleate was 5.9 ± 0.6 μM (Fig. 3*B*). The kinetics with respect to 18:1(9*Z*) were highly cooperative with a Hill number of 2.2 ± 0.3.

SaOhyA acts on cis-9 double bonds

An LC-MS system was developed to measure hydroxy-fatty acid formation from unsaturated fatty acids and identify the location of the hydroxyl groups in the product. Hydroxy-fatty acids clearly separated from the parent unsaturated fatty acids in the chromatographic system (Fig. 4*A*). The reaction product from the *in vitro* reaction was identified as *h*18:0 based on its characteristic mass spectrum (Fig. 4*B*). The parent mass of the *Sa*OhyA product formed from 18:1(9*Z*) had a $m/z = 299.1$ consistent with the addition of water to 18:1(9*Z*), and the fragmentation pattern was characteristic for *h*18:0 primarily based on the $m/z = 185.1$ fragment that arises from cleavage adjacent to the hydroxyl as diagramed in Fig. 4*B* (*inset*). Similarly, the product of *Sa*OhyA action on 18:2(9*Z*,12*Z*) was identified as 10-hydroxy-18:1 (*h*18:1, $m/z = 297.1$) based on its mass spectrum

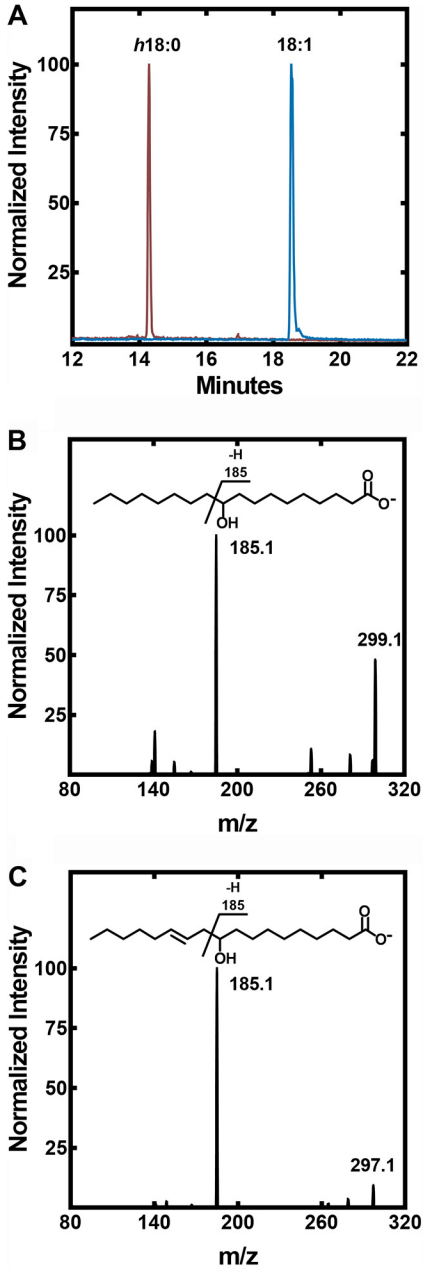

Figure 4. Formation and structure of *Sa*OhyA products. *A*, an example illustrating the LC-MS method for separating and detecting hydroxy-fatty acids in *Sa*OhyA assays (Table 1). The experiments shown are samples from an assay containing 18:1(9*Z*) in the presence (*vermilion trace*) or absence (*blue trace*) of *Sa*OhyA (2.5 μg). There was almost complete conversion of 18:1(9*Z*) to *h*18:0 in this experiment. *B*, mass spectrum of *h*18:0 product peak from an *Sa*OhyA assay containing 18:1(9*Z*). The molecular ion ($m/z = 299.1$) corresponds to the addition of water, and the $m/z = 185.1$ fragment was diagnostic for hydroxylation at carbon-10 as indicated in the fragmentation diagram (*inset*). *C*, mass spectrum of *h*18:1 from an *Sa*OhyA assay containing 18:2(9*Z*,12*Z*). The same $m/z = 185.1$ peak was diagnostic for hydroxylation at carbon-10 in this fatty acid as indicated in the fragmentation diagram (*inset*). Chromatographic conditions and MS parameters are detailed under "Experimental procedures."

(Fig. 4*C*), and the presence of the $m/z = 185.1$ that is derived from cleavage adjacent to the hydroxyl group at carbon 10 (Fig. 4*C*, *inset*). These mass spectra exactly match the published mass spectra of *h*18:0 and *h*18:1 (8, 10).

The LC-MS *Sa*OhyA assay was used to examine the substrate specificity of the enzyme toward a panel of unlabeled mamma-

Table 1
Unsaturated fatty acids as substrates of *Sa*OhyA
*Sa*OhyA assays were performed as described under "Experimental procedures" using LC–MS to detect hydroxy-fatty acid formation and identify the location of the hydroxyl group (see Fig. 4).

Fatty acid	Product
16:1(6*Z*)	Not detected
16:1(9*Z*)	10-Hydroxy-16:0 (*h*16:0)
16:1(9*E*)	Not detected
18:1(9*Z*)	10-Hydroxy-18:0 (*h*18:0)
18:1(9*E*)	Not detected
18:1(11*Z*)	Not detected
18:2(9*Z*,12*Z*)	10-Hydroxy-18:1 (12*Z*) (*h*18:1)
18:3(9*Z*,12*Z*,15*Z*)	10-Hydroxy-18:2 (12*Z*,15*Z*)
18:3(6*Z*,9*Z*,12*Z*)	10-Hydroxy-18:2 (6*Z*,12*Z*)
20:4(5*Z*,8*Z*,11*Z*,14*Z*)	Not detected
22:6(4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)	Not detected

lian unsaturated fatty acids (Table 1). In these experiments, the *Sa*OhyA (2.5 μg) was used to yield ~80–90% conversion of 18:1 to *h*18:0, so that even poor substrates for the reaction would be detected. If a hydroxy-fatty acid was produced by *Sa*OhyA, then the mass spectrum was obtained to identify the location of the hydroxyl group along the acyl chain. The only unsaturated fatty acids that were substrates were those containing a *cis*-9 double bond (Table 1). 16:1(9*Z*) was an excellent substrate, but product formation was not detected with either 18:1(11*Z*) or 16:1(6*Z*) substrates. *Sa*OhyA did not hydroxylate 16:1(9*E*) or 18:1(9*E*) substrates, showing that *trans* double bonds were not substrates. Both α-linolenic (18:3(9*Z*,12*Z*,15*Z*)) and γ-linolenic (18:3(6*Z*,9*Z*,12*Z*)) acids were *Sa*OhyA substrates and hydrated the *cis*-9 double bond in these fatty acids (Table 1). Arachidonic and docosahexaenoic acids are two prevalent mammalian unsaturated fatty acids that lack a 9*Z* double bond, and they were not *Sa*OhyA substrates (Table 1). We did not determine the stereochemistry of the hydroxyl group, but members of the oleate hydratase family exclusively produce the *R*-hydroxy isomer (18). Thus, *Sa*OhyA was specific for the formation of 10-hydroxy fatty acids only from unsaturated fatty acids with *cis*-9 double bonds.

Metabolism of antimicrobial fatty acids

The first step was to determine whether *h*16:0, the *Sa*OhyA product derived from the antimicrobial 16:1(9*Z*), possessed antimicrobial activity. The *Sa*OhyA assay was scaled, and *h*16:0 was purified and quantified as described under "Experimental procedures." Strain AH1263 was grown to mid-log phase and treated with 30 μM of either 16:1(9*Z*) or *h*16:0, and the growth was monitored (Fig. 5*A*). Treatment with 16:1(9*Z*) triggered the abrupt cessation of cell growth, whereas *h*16:0 was without effect. These data illustrate that the hydroxylated products of *Sa*OhyA had attenuated antimicrobial activity, suggesting that *Sa*OhyA may function as a countermeasure against growth inhibition by unsaturated antimicrobial fatty acids.

In our previous work with antimicrobial fatty acids, we analyzed the global gene expression response to 18:1(9*Z*) (12) (accession number GSE36231 in the NCBI Gene Expression Omnibus database). The *ohyA* gene was increased 2.5-fold in the published array experiment, and we validated the result obtained with the microarray using qRT-PCR (Fig. 5*B*). The levels of *ohyA* mRNA were elevated slightly by three fatty acids (18:1(9*Z*), 16:1(6*Z*), and 18:2(9*Z*,12*Z*)). Although we detected induction of *ohyA* expression in the presence of these unsaturated fatty acids, the up-regulation of *ohyA* transcription was not robust compared with the highly regulated *farE* gene that is controlled by the FarR transcriptional regulator (12, 19) (Fig. 5*C*). In the published microarray experiment (12), there were numerous genes that were elevated by 2–3-fold, including many genes encoding ribosomal proteins. These results suggest that the unsaturated fatty acid effect on *ohyA* expression may be tied to the oleate-dependent increase in growth rate and cellular yield (12) rather than a specific transcriptional response to an extracellular *Sa*OhyA substrate.

Our laboratory has published the molecular species composition of *S. aureus* membrane phospholipids derived from cultures grown with 18:1(9*Z*) or 16:1(9*Z*) supplements and has not observed a phosphatidylglycerol molecular species with a mass consistent with the incorporation of hydroxy-fatty acids (12, 20, 21). Thus, metabolic labeling experiments were performed to determine whether *Sa*OhyA products are released into the growth medium. Strain JLB2 (Δ*fakA*)/pPJ490 was used in this experiment to prevent the incorporation of 18:1(9*Z*) into phospholipid by deleting *fakA* (the kinase component of fatty acid kinase) and to elevate cellular *Sa*OhyA activity to more clearly determine the fate of hydroxy-fatty acids. Strain JLB2 (Δ*fakA*)/pPJ490 was grown to mid-log phase and labeled with [^{14}C]18:1(9*Z*), and at the indicated times following fatty acid addition, samples were removed and cells were separated from media by centrifugation. The label remained constant in the cell culture supernatant at all time points, and the small amount of radioactivity associated with the cell pellet was removed from the cells by resuspension and washing (not shown). TLC analysis of the label in the supernatant showed a steady increase in [^{14}C]*h*18:0 with a corresponding decrease in [^{14}C]18:1(9*Z*) (Fig. 5*D*). These data show that [^{14}C]18:1(9*Z*) was taken up by the cells and converted to [^{14}C]*h*18:0, and the [^{14}C]*h*18:0 was then released into the medium. Similarly, *h*18:1(12*Z*), the product of *Sa*OhyA action on 18:2(9*Z*,12*Z*), was found in the culture supernatants and was not incorporated into membrane phospholipids (not shown).

SaOhyA confers resistance to antimicrobial fatty acids

The role of *ohyA* in resistance to antimicrobial fatty acids was evaluated using a Δ*ohyA* knockout strain PDJ68. The inactivation of the *ohyA* gene was achieved by the deletion of the *ohyA* coding sequence as diagramed in Fig. 6*A*, and the Δ*ohyA* gene deletion was verified by PCR analysis (Fig. 6*A*). An expression vector was also prepared containing the *ohyA* gene driven by the *sarA* promoter (pPJ490) to complement the gene deletion as described under "Experimental procedures." The ability of the WT, knockout, and complemented strains to hydroxylate 16:1(9*Z*) was determined by LC-MS (Fig. 6*B*). Strain AH1263/pPJ480 (WT) formed *h*16:0 from 16:1, whereas *h*16:0 formation was not detected in the supernatant of strain PDJ68 (Δ*ohyA*)/pPJ480. The complemented mutant strain PDJ68 (Δ*ohyA*)/pPJ490 produced increased amounts of *h*16:0, showing that *ohyA* expression was the only gene required for *S. aureus* to produce hydroxylated fatty acids.

The major antimicrobial fatty acids produced by the innate immune system to protect skin from infection are 16:1(9*Z*) and

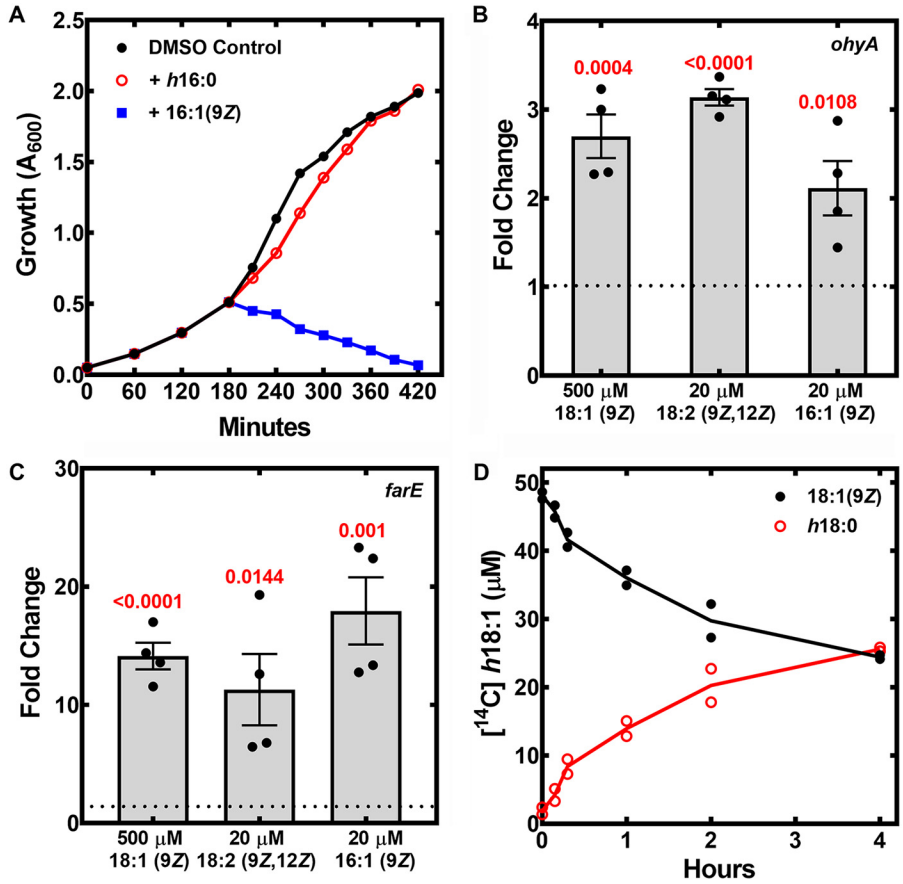

Figure 5. Growth, gene expression, and metabolism of unsaturated fatty acids by *S. aureus*. *A*, strain AH1263 was grown to mid-log phase, treated with 30 μM 16:1(9*Z*), *h*16:0, or vehicle (DMSO), and the growth of the cultures was monitored. *B*, qRT-PCR measurement of *ohyA* mRNA levels in strain AH1263 exposed to the indicated concentrations of fatty acids. The data are representative of four independent cultures. *C*, qRT-PCR measurement of *farE* mRNA in strain AH1263 exposed to the indicated concentrations of fatty acids. The calibrator was *glyA*, and the *p* values calculated using the Student's *t* test (GraphPad) are shown in *red*. The data are graphed as means ± S.E. *D*, the conversion of [^{14}C]18:1(9*Z*) to [^{14}C]*h*18:0 by strain JLB2 (Δ*ohyA*)/pPJ490 and its release into the culture media. We used a Δ*fakA* strain to prevent incorporation of fatty acids into phospholipid and introduced the *Sa*OhyA expression plasmid pPJ490 to amplify product formation. Culture supernatants were sampled at the indicated times and extracted, and [^{14}C]18:1(9*Z*) was separated from [^{14}C]*h*18:0 by TLC and quantitated using a Typhoon PhosphorImager. The results from two experiments are plotted.

16:1(6*Z*) in mice and humans, respectively (16, 17). These two fatty acids differ by the location of their double bond, but they do not have a different minimum inhibitory concentrations for *S. aureus* using a standard microbroth dilution assay method (12). Although 18:2(9*Z*,12*Z*) does not have an established physiological role in innate skin defense, it was included in our study because it is a potent antimicrobial fatty acid (12, 19) and an *Sa*OhyA substrate (Table 1). Antimicrobial fatty acids act to permeabilize the *S. aureus* membrane, resulting in the release of intracellular metabolites (like ATP) and low-molecular-weight proteins (like ACP) into the medium (12). Growth arrest triggered by the three fatty acids occurred over a very narrow concentration range in liquid culture (Fig. 7*A*). Linoleate (18:2(9*Z*,12*Z*)) was slightly more potent than the two equivalently potent 16:1 isomers. At concentrations below the transition concentration, cell growth was not affected, and above the transition point, the cells did not recover. At a 16:1(9*Z*) concentration within the transition range (24 μM), the growth of strain AH1263 (WT) was immediately arrested, but after 2 h of continued incubation, the cells recovered and resumed growth at the same rate as 16:1(9*Z*) concentrations below the transition point (not shown). This observation led us to test the role of OhyA in the growth response of *S. aureus* to antimicrobial fatty acids.

A series of plasmid-bearing strains harboring either the empty control plasmid (pPJ480) or pPJ490, the expression vector containing the *ohyA* gene under the control of the *sarA* promoter (Table S1) was used. Like strain AH1263, the growth of strain AH1263/pPJ480 immediately arrested when challenged with 24 μM 16:1(9*Z*), and growth resumed at the normal rate after a lag of 2 h (Fig. 7*B*). Strain PDJ68 (Δ*ohyA*)/pPJ480 lacked OhyA and did not recover from 16:1(9*Z*) growth arrest triggered by 24 μM 16:1(9*Z*) (Fig. 7*B*). Introduction of the *ohyA* gene into the knockout strain (PDJ68/pPJ490) eliminated the lag phase, rendering the cells refractory to 16:1(9*Z*) growth inhibition by 24 μM 16:1(9*Z*). This result indicates that the combination of the *sarA* promoter and a multicopy plasmid increased *ohyA* expression to higher levels than present in the WT strain. Challenging strain AH1263/pPJ480 with 16 μM 18:2(9*Z*,12*Z*) also resulted in transient growth arrest (Fig. 7*C*). Elevated *Sa*OhyA expression rendered cells refractory to growth arrest by 16 μM 18:2(9*Z*,12*Z*), whereas the Δ*ohyA* knockout strain PDJ68 was unable to recover from the 18:2(9*Z*,12*Z*) challenge (Fig. 7*C*). However, when the three

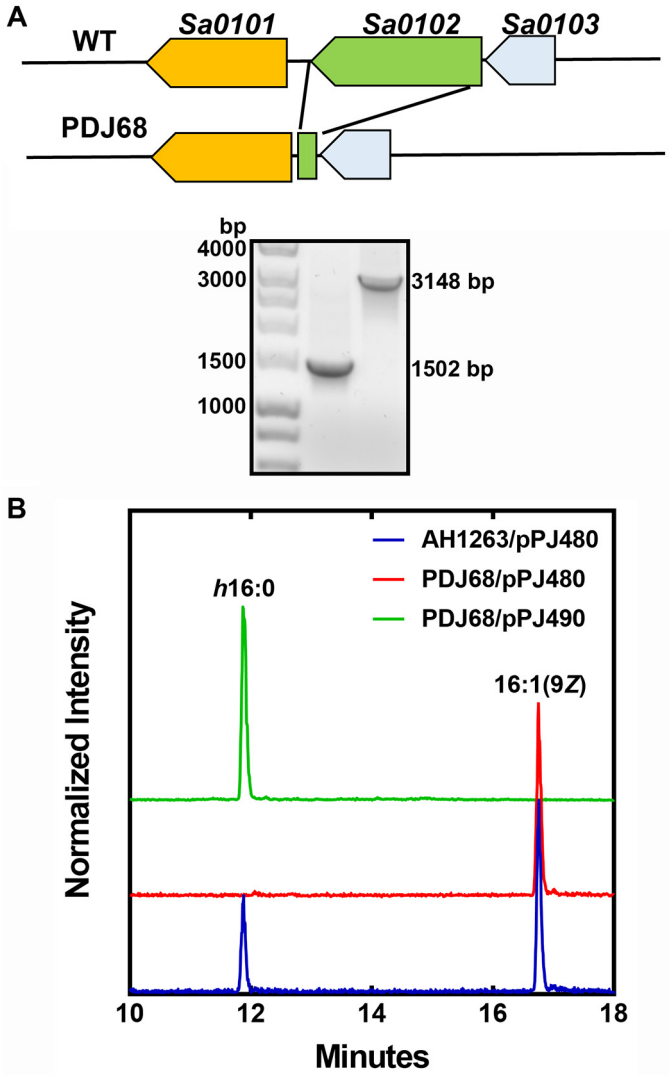

Figure 6. Validation of the *ohyA* gene deletion in *S. aureus* strain AH1263. *A*, *top panel*, a diagram of the genomic region surrounding *ohyA* (locus tag *Sa0102*) and the genomic structure following excision of the *ohyA* coding sequence. *Bottom panel*, PCR verification of the *ohyA* deletion in strain PDJ68 (1502 bp). *B*, analysis of the formation of *h*16:0 following a challenge with 20 μM 16:1(9*Z*). Strains AH1263/pPJ480 (*blue trace*), PDJ68 (Δ*ohyA*)/pPJ480 (*red trace*), and PDJ68/pPJ490 (*green trace*) were grown to A_{600} of 0.5 and exposed to 16:1(9*Z*) for 1 h. The cell supernatants were harvested, and the formation of *h*16:0 was detected by LC-MS as described under "Experimental procedures."

strains were treated with 24 μM 16:1(6*Z*), growth arrest was immediate, and none of the strains recovered from 16:1(6*Z*) (Fig. 7*D*). These results showed that *Sa*OhyA protected cells from the action of 16:1(9*Z*) and 18:2(9*Z*,12*Z*), but not 16:1(6*Z*), consistent with the substrate specificity of *Sa*OhyA (Table 1), and the fact that hydroxy-fatty acids were not antimicrobial (Fig. 5*A*).

Discussion

This work establishes *Sa*OhyA as an active countermeasure against 16:1(9*Z*) through its conversion to *h*16:0 and efflux from the cell (Fig. 8). An incorporation pathway into membrane phospholipids is one mechanism *S. aureus* uses to detoxify antimicrobial fatty acids. At concentrations of 16:1(9*Z*) that do not inhibit cell growth (<20 μM), free fatty acids were not detected in the cells, and there was no evidence for *h*16:0 in the membrane phospholipids (12). 16:1(9*Z*) is incorporated into phospholipid by PlsY, following its activation by fatty acid kinase, transfer to ACP by PlsX, and elongation to 18:1(11*Z*) and 20:1(13*Z*) by FASII (12). However, 16:1(9*Z*) itself is a poor substrate for phospholipid biosynthesis, and the capacity of this pathway to dispose of the antimicrobial fatty acids will be overwhelmed as the concentration of extracellular fatty acids rises. *Sa*OhyA is a FAD-dependent hydratase that acts as a primary line of defense by hydrating unsaturated fatty acids with *cis*-double bonds located at carbon-9. Hydration of the double bond eliminates antimicrobial activity, and the hydroxy products are not used for phospholipid synthesis and are released into the medium. Palmitoleic acid (16:1(9*Z*)) is the principle antimicrobial fatty acid that protects against *S. aureus* skin infections in mammals. Human skin has deployed a counter-countermeasure to *Sa*OhyA by uniquely producing the equally effective 16:1(6*Z*) as a substitute for 16:1(9*Z*) (13, 14). 16:1(6*Z*) is not an *Sa*OhyA substrate, making human skin lipids a more effective deterrent against *S. aureus* infection than the skin lipids of other mammals (13, 14). *Sa*OhyA is not the only mechanism used by *S. aureus* to avoid toxic fatty acids. Antimicrobial fatty acids may also be inactivated by their incorporation into phospholipid following their activation of fatty acid kinase (Fig. 8). Efflux pumps like FarE (19) and potentially Tet38 (22) are additional active countermeasures to prevent the cellular accumulation and toxicity of antimicrobial fatty acids. Thus, the *Sa*OhyA defense against skin antimicrobial fatty acids is one mechanism in a layered defense deployed by *S. aureus* against antimicrobial fatty acids deployed by the innate immune system.

An expanding area of research focuses on how commensal bacteria signal the immune system to tolerate their presence, and OhyA products appear to have an important role in this process (23). Linoleate (18:2(9*Z*,12*Z*)) is an abundant host fatty acid that is hydrated by *Sa*OhyA, although this fatty acid does not have a defined role in innate skin defense. It was realized decades ago that 18:2(9*Z*,12*Z*) metabolites, like 18:2(9*Z*,11*E*) (conjugated 18:2) and *h*18:1, are produced by commensal lactic acid bacteria that inhabit the gut microbiome (10, 24). More recently, it has become clear that commensal bacteria use signaling molecules like *h*18:1 to educate the immune system to create a tolerant environment for the bacterium (23, 25). Commensal bacteria produce PPARγ-activating ligands that dampen the innate immune response against bacteria (26–28), and hydroxy fatty acids potently activate PPARγ (29, 30). The anti-inflammatory action of *h*18:1 blocks tumor necrosis factor–induced intestinal barrier impairment (31, 32) and activates GPR40 (32). The discovery that *Sa*OhyA produces the same immunomodulatory metabolite as commensal bacteria illustrates how this pathogen has coopted a biochemical signalling pathway in symbionts to evade immune surveillance. Thus, *Sa*OhyA joins the growing list of countermeasures deployed by *S. aureus* not only to resist attack by the innate immune system but also to alter host response to infection (33).

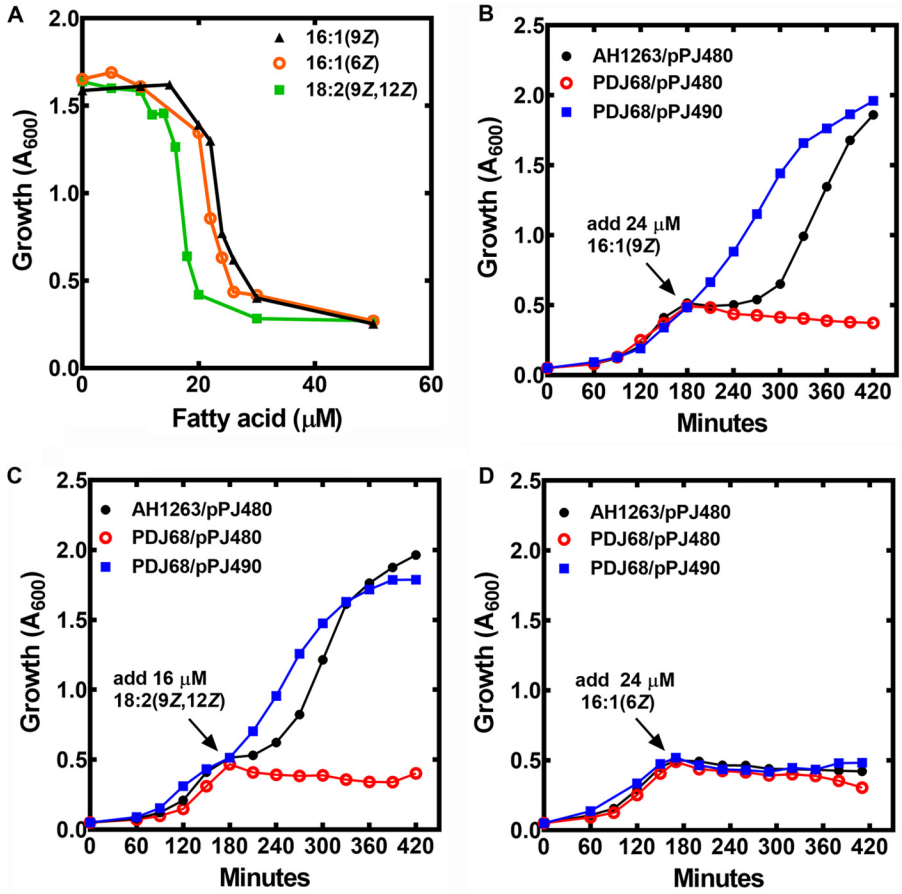

Figure 7. Effect of *Sa*OhyA on antimicrobial activity. *A*, concentration-dependent growth inhibition of strain AH1263/pPJ480 when challenged with either 16:1(9*Z*), 18:2(9*Z*,12*Z*), or 16:1(6*Z*). Strain AH1263/pPJ480 was grown to an A_{600} of 0.3, and the indicated concentrations of fatty acids were added. The A_{600} values of the cultures were then determined after 2 h as described under "Experimental procedures." *B–D*, three strains were used in the following experiments. Strain AH1263/pPJ480 (empty expression vector) was the WT control, strain PDJ68 (Δ*ohyA*)/pPJ480 (empty vector) was the *ohyA* knockout, and PDJ68/pPJ490 was the *ohyA* knockout with the complementing *ohyA* expression vector. Each of the three strains were grown to mid-log phase (A_{600} = 0.5) and then challenged with the indicated antimicrobial fatty acids. *B*, strains were challenged with 24 μM 16:1(9*Z*). *C*, strains were challenged with 16 μM 18:2(9*Z*,12*Z*). *D*, strains were challenged with 24 μM 16:1(6*Z*). The data shown are examples of the growth curves in one experiment, and the results were verified in a second experiment.

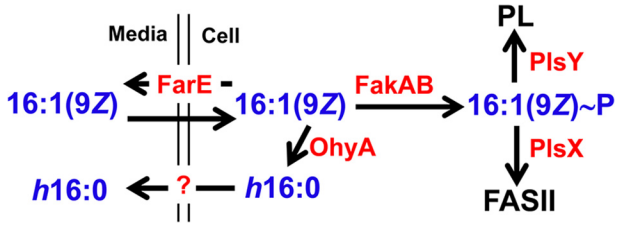

Figure 8. Model illustrating the active defense mechanisms used by *S. aureus* to detoxify antimicrobial fatty acids. Antimicrobial fatty acids (16:1(9*Z*)) are produced by the skin to block the growth of *S. aureus* and are thought to enter the cell by flipping across the membrane bilayer. *S. aureus* deploys three active countermeasures to combat 16:1(9*Z*); 1) *Sa*OhyA hydroxylates and inactivates 16:1(9*Z*) to *h*16:0, which then exits the cell into the medium by an unknown mechanism; 2) FarE is a fatty acid efflux pump that protects against antimicrobial fatty acid by returning them to the medium (19); and 3) 16:1(9*Z*) can also be inactivated by incorporation into phospholipid by the PlsX and PlsY enzymes. The data show that most of the 16:1(9*Z*) incorporated into phospholipid (*PL*) is first converted to acyl-ACP by PlsX and elongated by FASII before it is utilized for phospholipid synthesis by PlsY (12). Human skin produces 16:1(6*Z*), which is not an *Sa*OhyA substrate, to circumvent the OhyA-dependent resistance mechanism.

Experimental procedures

Materials

FAD, FMN, NAD, NADH, NADP, NADPH, and 18:1(9*Z*) were purchased from Sigma–Aldrich. Antibiotics, high density nickel resin, and DTT were purchased from GoldBio (St. Louis, MO). [1-^{14}C]Oleic acid (59 mCi/mmol, 0.1 mCi/ml) was purchased from PerkinElmer Life Sciences. 16:1(9*Z*), 16:1(6*Z*), 16:1(9*E*), 18:1(9*E*), 18:1(6*Z*), 18:1(11*Z*), and 18:2(9*Z*, 12*Z*) were purchased from Matreya (State College, PA). Bacteria media supplies were purchased from BD Medical Technologies (Franklin Lakes, NJ). All chemicals and solvents were reagent grade or better. Bacterial strains, plasmids, and primers used in this study are listed in Table S1.

Molecular biology

Oleate hydratase knockout, in which the first 1646 bases of the coding sequence are deleted, was generated in AH1263 by allelic replacement (Fig. 6*A*). Briefly, 900 bp from either side of ohyA was amplified by PCR using primers OhyA-Up-F, OhyA-Up-R, OhyA-Dn-F, and OhyA-Dn-R. Plasmid vector pJB38 was digested with SacI and SalI and gel-purified. The PCR products were moved into pJB38 by Gibson Assembly (New England Biolabs) yielding pPJ523. Plasmid pPJ523 was transformed into AH1263 by electroporation, and the knockout was generated as previously described (34, 35). The Δ*ohyA* knockout strain PDJ68 was confirmed by PCR using primers Ohy-F3 and OhyA-

R6. The DNA sequence of locus tag *Sa0102* was synthesized with the appropriate restriction sites (Invitrogen) and cloned into the NdeI and XhoI sites of pET28a to construct pPJ520 for the purification of *Sa*OhyA with an N-terminal His tag from *E. coli*. The *Sa*OhyA expression vector for strain AH1263 (36) was constructed by first modifying plasmid pCS119 (37) using QuikChange Lightning multisite-directed mutagenesis kit (Agilent Technologies) using specific primers pCS119-a2398t-F and pCS119-g3141c-F to delete the NcoI and HindIII sites in the body of the plasmid by introducing silent mutations. The resulting plasmid was digested with EcoRI and HindIII and gel-purified. A DNA sequence was synthesized containing the SarAP1 promoter, a ribosome-binding site, and multiple cloning sites (5′-GGAAACAGCTATGACATGATTACGAATTT-GATATTTTTGACTAAACCAAATGCTAACCCAGAAAT-ACAATCACTGTGTCTAATGAATAATTTGTTTTATAAA-CACTTTTTTGTTTACTTCTCATTTTTAATTAGTTATA-ATTAACGCTAGAAAGGAGGTGGATCCATGGCCGCG-GGAATTCGAGCTCCTGCAGCGTCGACAAGCTTGCGG-CCGCACTCGAGCACCACCACCACCACCACTGAGATA-GCTTTTAAAAAGCAAATATGAGCCAAA) and was moved into the digested plasmid via Gibson assembly to obtain plasmid pPJ520. The His-tagged *ohyA* gene was subcloned from pPJ520 to pPJ480 by NcoI and XhoI to yield pPJ490. All plasmids were confirmed by sequencing.

Cloning, expression, and purification of SaOhyA

The expression of N-terminal His-tagged *Sa*OhyA was induced with 0.5 mM isopropyl β-D-thiogalactopyranoside in strain BL21(DE3) harboring pPJ520 for 3 h and purified using Ni^{2+}-affinity chromatography. The supernatant was loaded on to a Ni^{2+}–nitrilotriacetic acid column and was washed with 20 column volumes of each 10, 20, and 40 mM imidazole in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl. The protein was eluted with 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 250 mM imidazole and dialyzed overnight against 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 100 mM EDTA at 4 °C. EDTA was sequentially removed by dialyzing against 20 mM Tris-HCl, pH 7.5, 400 mM NaCl, and 50 mM EDTA; then with 20 mM Tris, pH 7.5, 300 mM NaCl, and 25 mM EDTA; and finally with 20 mM Tris, pH 7.5, and 200 mM NaCl. Size-exclusion chromatography was performed by loading affinity-purified *Sa*OhyA onto a Sephadex S-200 column that was eluted with 20 mM Tris-HCl, pH 7.5, and 200 mM NaCl. Protein standards used for estimating the size of *Sa*OhyA were thyroglobulin (669 kDa), IgG (150 kDa), BSA (68.6 kDa), and myoglobin (17 kDa).

SaOhyA assay

The *Sa*OhyA assay contained 50 mM potassium phosphate buffer, pH 6.0, 10 mM NaCl, 10 mM DTT, 50 μM FAD, 0.2 mg/ml BSA, 20 μM [^{14}C]18:1(9Z), and 0.05 mg/ml of *Sa*OhyA in a final volume of 20 μl. The reactions were incubated at 37 °C for 15 min and were spotted onto silica gel H TLC plates developed with choloroform:methanol (90/10, v/v). The distributions of radioactivity on the dried plates and the extent of product formation were quantified using a Typhoon PhosphorImager. The cofactor FAD was replaced with FMN, NAD, NADH, NADP, and NADPH at final concentrations of 50 μM to determine the cofactor specificity. To determine the FAD K_m, FAD was varied from 0 to 50 μM, and to determine the fatty acid K_m, [^{14}C]18:1(9*Z*) was varied from 0 to 25 μM.

Mass spectrometry of OhyA products

The substrate specificity of *Sa*OhyA was determined using LC-MS to measure the formation of hydroxy products. *Sa*OhyA reactions contained 2.5 μg of *Sa*OhyA, 50 mM potassium phosphate buffer, pH 6.0, 10 mM NaCl, 10 mM DTT, 50 μM FAD, 0.2 mg/ml BSA, and 400 μM fatty acid in a final volume of 20 μl. The reactions were incubated at 37 °C for 10 min and stopped by the addition of 80 μl of acetonitrile followed by centrifugation at 4000 × *g* for 10 min. The supernatant containing the fatty acid substrate and hydroxy-fatty acid product were analyzed with a Shimadzu Prominence UFLC attached to a QTrap 4500 equipped with a Turbo V ion source (Sciex). Samples were injected onto an XSelect® HSS C18, 2.5 μm, 3.0 × 150-mm column (Waters) at 45 °C with a flow rate of 0.4 ml/min. Solvent A was water, and solvent B was acetonitrile. The HPLC program was as follows: starting solvent mixture of 60% B, 0–1 min isocratic with 60% B; 1–16 min linear gradient to 100% B; 16–21 min isocratic with 100% B; 21–23 min linear gradient to 0% B; and 23–28 min isocratic with 0% B. The Sciex QTrap 4500 was operated in the negative mode, and the ion source parameters were: ion spray voltage, −4500 V; curtain gas, 30 p.s.i.; temperature, 320 °C; collision gas, medium; ion source gas 1, 20 p.s.i.; ion source gas 2, 35 p.s.i; and declustering potential, −35 V. The system was controlled by Analyst® software (Sciex).

The Sciex QTrap 4500 mass spectrometer was operated in the negative mode using the product scan to determine the position of the hydroxyl group in the *Sa*OhyA reactions. The source parameters were: ion spray voltage, −4500 V; curtain gas, 15 p.s.i.; temperature, 250 °C; collision gas, high; ion source gas 1, 15 p.s.i.; ion source gas 2, 20 p.s.i.; declustering potential, −25 V; and collision energy, −35 V. The system was controlled by Analyst® software (Sciex).

The phenotype of the Δ*ohyA* strains was confirmed by analyzing the formation of *h*16:0 from 16:1(9Z) in the medium. Strains AH1263/pPJ480, PDJ68/pPJ480, and PDJ68/pPJ490 were grown to an A_{600} value of 0.5 in Tryptone broth containing 1% DMSO. 16:1(9*Z*) in DMSO was added to a final concentration of 20 μM, and the cultures were grown for 1 h at 37 °C with shaking. The cells were separated from media by centrifugation, and the medium was extracted by adding methanol to a final concentration of 80%. Extracts were centrifuged to pellet debris, and the supernatant was analyzed by LC-MS as described above to detect the presence of the *h*16:0.

FAD measurement by LC/MS/MS

To duplicate samples of 100 μl of 5 mg/ml *Sa*OhyA, acetonitrile was added to a final concentration of 80% and incubated on ice for 5 min. Samples were centrifuged at 3500 × *g* for 5 min, and the supernatant was transferred to a glass vial. An FAD standard curve was created by having known amounts of FAD (0.01–50 pmol) in the protein buffer and extracted similarly as described above. FAD was analyzed using a Shimadzu Prominence UFLC attached to a QTrap 4500 equipped with a Turbo V ion source (Sciex). The samples were injected onto an XSelect® HSS C18, 2.5 μm, 3.0 ×

150-mm column at 40 °C using a flow rate of 0.3 ml/min. Solvent A was 100 mM ammonium formate, pH 5.0, 2% acetonitrile, and 0.1% tributylamine, and solvent B was 95% acetonitrile, 50 mM ammonium formate, pH 6.3, and 0.1% tributylamine. The HPLC program was as follows: starting solvent mixture of 0% B, 0–2 min isocratic with 0% B; 2–12 min linear gradient to 5% B; 12–17 min linear gradient to 90% B; 17–25 min isocratic with 90% B; 25–27 min linear gradient to 0% B; and 27–30 min isocratic with 0% B. The Sciex QTrap 4500 was operated in the negative mode, and the ion source parameters were: ion spray voltage, −4500 V; curtain gas, 40 p.s.i.; temperature, 500 °C; collision gas, medium; ion source gas 1, 50 p.s.i.; and ion source gas 2, 50 p.s.i.. The multiple reaction monitoring transition for FAD was 784/437 *m*/*z* with a declustering potential of −20 V and a collision energy of −41 V. The system was controlled by Analyst® software (Sciex) and analyzed with MultiQuant™ 3.0.2 software (Sciex).

Growth strains in the presence of fatty acids

Selected WT, knockout, and plasmid-bearing strains were inoculated in Tryptone broth containing 1% DMSO at A_{600} 0.05 and grown to an A_{600} of 0.5. Fatty acids in DMSO were added, and the A_{600} was monitored every 30 min. For the experiments with the plasmids pPJ480 and pPJ490, the plasmids were electroporated into AH1263 or PDJ68 and selected on 10 μg/ml chloramphenicol.

The concentration of the antimicrobial fatty acids that inhibited growth was determined by growing strain AH1263/pPJ480 in Tryptone broth containing 1% DMSO with 10 μg/ml chloramphenicol to an A_{600} of 0.3. Then, 16:1(9*Z*) or 16:1 (6*Z*) were added at 10, 15, 20, 22, 24, 26, 30, or 50 μM, 18:2 (9*Z*, 12*Z*) was added at 5, 10, 12, 14, 16, 18, 20, 30, or 50 μM, and the A_{600} was measured after growing for 2 h at 37 °C with shaking. DMSO was added at 0.1% as zero fatty acid control.

Quantitative real-time PCR

Strain AH1263 was grown to an A_{600} of 0.6 in 1% Tryptone broth and treated with 500 μM 18:1(9*Z*), 20 μM 16:1(9*Z*), and 20 μM 18:2(9*Z*,12*Z*) for 20 min. RNA was isolated with an Ambion RNAqueous purification kit (Ambion, Austin, TX) according to the manufacturer's specifications. Purified RNA was then mixed with a 0.5 volume of LiCl precipitation solution (Ambion) and left at −20 °C for 30 min. Turbo DNase (Ambion) was added to the precipitated RNA to a final concentration of 1 unit of DNase per 5 μg of RNA. This mixture was incubated at 37 °C for 30 min. Integrity of the RNA was assessed before its use in qRT-PCR by agarose gel electrophoresis using the Agilent Technologies 2100 Bioanlyzer. Primers used are listed in Table S1. Each 20-μl RT reaction contained 500 ng of RNA, 12.5 ng/μl of random hexamers (Invitrogen), 0.5 mM dNTPs (Sigma), 40 units of RNaseout (Invitrogen), and 10 units/μl of Superscript II reverse transcriptase (Invitrogen). After the RT reaction, 10 ng of the cDNA product was added to a RT-PCR with SYBR Green PCR master mixes (Applied Biosystems), 150 nM of each forward and reverse primer. Specific products were detected on an ABI Prism 7700 sequence detection system (Applied Biosystems) using the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and then a final dissociation curve. The samples were processed in 96-well plates, three RT-PCRs were performed on each cDNA sample. cDNA was prepared from four separate cultures for each biological condition with no template, and reactions without reverse transcriptase were run as negative controls. Real-time values were evaluated using the threshold cycle (C_T) method, with genes being normalized to a *glyA* calibrator.

Efflux of hydroxy-fatty acids

Strain JLB2 (37) (Δ*fakA*)/pPJ490 was grown to an A_{600} of 0.6 in LB containing 10 mg/ml BSA, and [^{14}C]18:1(9*Z*) was added to a final concentration of 50 μM. Aliquots (100 μl) were removed at the indicated times and centrifuged to collect the cell pellet and media. An equal volume of methanol was added to the media, and the samples were centrifuged to remove debris. The cells were washed twice with buffered saline and 10 mg/ml fatty acid free BSA, extracted with 200 μl of 50% methanol, and centrifuged to pellet cell debris. Equal volumes (10 μl) were spotted on silica gel H thin-layer plates developed with choloroform:methanol (90/10, v/v). The distributions of radioactivity on the dried plates and extent of product formation were quantified using a Typhoon PhosphorImager.

Purification of hydroxy-fatty acids

*h*16:0 was purified from an *Sa*OhyA reaction using a 2795 Alliance HT (Waters) equipped with the 2424 evaporative light scattering detector and fraction collector III. The sample was injected onto an XSelect® HSS C18, 2.5-μm, 3.0 × 150-mm column (Waters) at 45 °C using a flow rate of 0.25 ml/min. Solvent A was water, and solvent B was acetonitrile. The HPLC program was as follows: starting solvent mixture of 60% B, 0–1 min isocratic gradient with 60% B, 1–26 min linear gradient to 100% B, 26–31 min isocratic gradient with 100% B, 31–35 min linear gradient to 60% B, and 35–40 min isocratic gradient with 60% B. Detector parameters were: detector gain, 20; gas pressure, 50 p.s.i.; nebulizer mode, heating; power level, 50%; and drift tube temperature, 65 °C. Fractions containing *h*16:0 were dried under nitrogen, and to remove any residual water, *h*16:0 was suspended in 100% ethanol and dried under nitrogen. The mass of the *h*16:0 was determined from a standard curve constructed with 10-hydroxy-18:0 (AA Blocks, LLC, San Diego, CA) using the chromatography and detection system described above.

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