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 (*Sa*0102) 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(9Z)) 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(9Z) 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(9Z) challenge, and increasing *Sa*OhyA expression using a plasmid system prevented the initial growth arrest. Challenging *S. aureus* with sapienic acid (16:1(6Z)), 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 9Z double bonds, whereas the *Streptococcus pyogenes* OhyA catalyzes hydration of both (9Z) and (12Z) double bonds (9). *E. meningoseptica* OhyA catalyzes the reversible hydration of the cis double bond of oleate (18:1(9Z))⁴ to 10(R)-hydroxy-18:0 (h18:0), which may also be converted to 18:1(10E) or 18:1(9Z) by a reverse dehydration (7, 8). The OhyA family member from *Lactobacillus plantarum* also functions as an isomerase that forms 18:2(9Z,11E) (conjugated linoleic acid) in a four-enzyme pathway for polyunsaturated fatty acid saturation (10).

*Staphylococcus aureus* oleate hydratase (SaOhyA) 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(9Z) is the most potent antimicrobial fatty acid produced by the skin.

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⁴ Fatty acids are designated by number of carbons:number of double bonds:(double bond location/configuration).
Antimicrobial fatty acids permeabilize the cells, leading to the leakage of low-molecular-weight solutes and proteins into the medium (12). However, human sebum is unique in that it is the only documented place where sapienic acid (16:1(6Z)) is produced in the animal kingdom where it is deployed instead of 16:1(9Z) 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 SaOhyA 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, SaOhyA*). An N-terminal His-tagged version of *SaOhyA* was cloned, expressed in *Escherichia coli*, and purified to determine its biochemical properties. *SaOhyA* was found in the cytosol of the *E. coli* expression system and was purified using affinity chromatography on Ni<sup>2+</sup>-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. 2A). The elution position in gel filtration chromatography indicated that *SaOhyA* was a homodimer (Fig. 2A), a configuration that is characteristic of bacterial oleate hydratases (4, 5, 7, 8).

The activity of *SaOhyA* was analyzed in biochemical assays using [1-<sup>14</sup>C]oleate as the substrate. *SaOhyA* required FAD for activity and catalyzed the hydroxylation of 18:1(9Z) to h18:0 (Fig. 2B). FMN did not substitute for FAD, and the presence of oxidized or reduced nicotinamide adenine dinucleotides did not influence enzyme activity either alone (Fig. 2B) or when added with FAD (not shown). The *SaOhyA* protein preparations were not visibly colored, but the activity of purified *SaOhyA* in the absence of FAD was ~6% of the maximum rate with FAD present (Fig. 2B). We determined whether the FAD-independent *SaOhyA* activity (Fig. 2B) was due to the presence of FAD in the protein preparation or FAD-independent enzyme activity by measuring the amount of FAD in the *SaOhyA* 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...
showed that the FAD level was sufficient to occupy 3.5% of the SaOhyA active sites. Thus, the residual activity in the absence of added FAD was attributed to a small amount of FAD that copurified with SaOhyA. The apparent $K_m$ for FAD was $2.1 \pm 0.2 \mu M$ (Fig. 3A), consistent with the significant loss of the FAD cofactor during the purification of SaOhyA. The apparent $K_m$ for oleate was $5.9 \pm 0.6 \mu M$ (Fig. 3B). The kinetics with respect to $18:1(9Z)$ were highly cooperative with a Hill number of $2.2 \pm 0.3$.

**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. 4A). The reaction product from the in vitro reaction was identified as $h18:0$ based on its characteristic mass spectrum (Fig. 4B). The parent mass of the SaOhyA product formed from $18:1(9Z)$ had a $m/z = 299.1$ consistent with the addition of water to $18:1(9Z)$, and the fragmentation pattern was characteristic for $h18:0$ primarily based on the $m/z = 185.1$ fragment that arises from cleavage adjacent to the hydroxyl as diagramed in Fig. 4B (inset). Similarly, the product of SaOhyA action on $18:2(9Z,12Z)$ was identified as 10-hydroxy-$18:1$ ($h18:1, m/z = 297.1$) based on its mass spectrum (Fig. 4C), and the presence of the $m/z = 185.1$ that is derived from cleavage adjacent to the hydroxyl group at carbon 10 (Fig. 4C, inset). These mass spectra exactly match the published mass spectra of $h18:0$ and $h18:1$.

The LC-MS SaOhyA assay was used to examine the substrate specificity of the enzyme toward a panel of unlabeled mamma-
S. aureus oleate hydratase

Table 1
Unsaturated fatty acids as substrates of SaOhyA

| Fatty acid | Product               |
|------------|-----------------------|
| 16:1(6Z)   | Not detected          |
| 16:1(9Z)   | 10-Hydroxy-16:0 (h16:0) |
| 16:1(9E)   | Not detected          |
| 18:1(9Z)   | 10-Hydroxy-18:0 (h18:0) |
| 18:1(9E)   | Not detected          |
| 18:1(11Z)  | Not detected          |
| 18:2(9Z,12Z) | 10-Hydroxy-18:1 (12Z) (h18:1) |
| 18:3(9Z,12Z,15Z) | 10-Hydroxy-18:2 (15Z) |
| 18:3(9Z,12Z) | 10-Hydroxy-18:2 (12Z,12Z) |
| 20:4(5Z,8Z,11Z,14Z) | Not detected         |
| 22:6(4Z,7Z,10Z,13Z,16Z,19Z) | Not detected         |

Our laboratory has published the molecular species composition of S. aureus membrane phospholipids derived from cultures grown with 18:1(9Z) or 16:1(9Z) 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 SaOhyA products are released into the growth medium. Strain JLB2 (ΔfakA)/pPJ490 was used in this experiment to prevent the incorporation of 18:1(9Z) into phospholipid by deleting fakA (the kinase component of fatty acid kinase) and to elevate cellular SaOhyA activity to more clearly determine the fate of hydroxy-fatty acids. Strain JLB2 (ΔfakA)/pPJ490 was grown to mid-log phase and labeled with [14C]18:1(9Z), 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 [14C]h18:0 with a corresponding decrease in [14C]18:1(9Z) (Fig. 5D). These data show that [14C]18:1(9Z) was taken up by the cells and converted to [14C]h18:0, and the [14C]h18:0 was then released into the medium. Similarly, h18:1(12Z), the product of SaOhyA action on 18:2(9Z,12Z), 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 ΔohyA knockout strain PDJ68. The inactivation of the ohyA gene was achieved by the deletion of the ohyA coding sequence as diagramed in Fig. 6A, and the ΔohyA gene deletion was verified by PCR analysis (Fig. 6A). 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(9Z) was determined by LC-MS (Fig. 6B). Strain AH1263/ pPJ480 (WT) formed h16:0 from 16:1, whereas h16:0 formation was not detected in the supernatant of strain PDJ68 (ΔohyA)/ pPJ480. The complemented mutant strain PDJ68 (ΔohyA)/ pPJ490 produced increased amounts of h16:0, 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(9Z) and...
16:1(6Z) 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(9Z,12Z) 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. 7A). Linoleate (18:2(9Z,12Z)) 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(9Z) 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(9Z) 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(9Z), and growth resumed at the normal rate after a lag of 2 h (Fig. 7B). Strain PDJ68 (∆ohyA)/pPJ480 lacked OhyA and did not recover from 16:1(9Z) growth arrest triggered by 24 μM 16:1(9Z) (Fig. 7B). Introduction of the *ohyA* gene into the knockout strain (PDJ68/pPJ490) eliminated the lag phase, rendering the cells refractory to 16:1(9Z) growth inhibition by 24 μM 16:1(9Z). 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(9Z,12Z) also resulted in transient growth arrest (Fig. 7C). Elevated *Sa*OhyA expression rendered cells refractory to growth arrest by 16 μM 18:2(9Z,12Z), whereas the ∆ohyA knockout strain PDJ68 was unable to recover from the 18:2(9Z,12Z) challenge (Fig. 7C). However, when the three
In the cells, and there was no evidence for \( h16:0 \) in the membrane phospholipids (12). \( 16:1(9Z) \) is incorporated into phospholipid by PlsY, following its activation by fatty acid kinase, transfer to ACP by PlsX, and elongation to \( 18:1(11Z) \) and \( 20:1(13Z) \) by FASII (12). However, \( 16:1(9Z) \) 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. \( SaOhyA \) 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 (\( 16:1(9Z) \)) is the principle antimicrobial fatty acid that protects against \( S. aureus \) skin infections in mammals. Human skin has deployed a counter-countermeasure to \( SaOhyA \) by uniquely producing the equally effective \( 16:1(6Z) \) as a substitute for \( 16:1(9Z) \) (13, 14). \( 16:1(6Z) \) is not an \( SaOhyA \) substrate, making human skin lipids a more effective deterrent against \( S. aureus \) infection than the skin lipids of other mammals (13, 14). \( SaOhyA \) 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 \( SaOhyA \) 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 (\( 18:2(9Z,12Z) \)) is an abundant host fatty acid that is hydrated by \( SaOhyA \), although this fatty acid does not have a defined role in innate skin defense. It was realized decades ago that \( 18:2(9Z,12Z) \) metabolites, like \( 18:2(9Z,11E) \) (conjugated \( 18:2 \)) and \( h18:1 \), 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 \( h18:1 \) to educate the immune system 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 \( SaOhyA \) 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.

This work establishes \( SaOhyA \) as an active countermeasure against \( 16:1(9Z) \) through its conversion to \( h16:0 \) 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 \( 16:1(9Z) \) that do not inhibit cell growth (<20 \( \mu \)M), free fatty acids were not detected in the cells, and there was no evidence for \( h16:0 \) in the membrane phospholipids (12). \( 16:1(9Z) \) is incorporated into phospholipid by PlsY, following its activation by fatty acid kinase, transfer to ACP by PlsX, and elongation to \( 18:1(11Z) \) and \( 20:1(13Z) \) by FASII (12). However, \( 16:1(9Z) \) 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. \( SaOhyA \) 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 (\( 16:1(9Z) \)) is the principle antimicrobial fatty acid that protects against \( S. aureus \) skin infections in mammals. Human skin has deployed a counter-countermeasure to \( SaOhyA \) by uniquely producing the equally effective \( 16:1(6Z) \) as a substitute for \( 16:1(9Z) \) (13, 14). \( 16:1(6Z) \) is not an \( SaOhyA \) substrate, making human skin lipids a more effective deterrent against \( S. aureus \) infection than the skin lipids of other mammals (13, 14). \( SaOhyA \) 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 \( SaOhyA \) 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 (\( 18:2(9Z,12Z) \)) is an abundant host fatty acid that is hydrated by \( SaOhyA \), although this fatty acid does not have a defined role in innate skin defense. It was realized decades ago that \( 18:2(9Z,12Z) \) metabolites, like \( 18:2(9Z,11E) \) (conjugated \( 18:2 \)) and \( h18:1 \), 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 \( h18:1 \) to educate the immune system to create a tolerant environment for the bacterium (23, 25). Commensal bacteria produce PPAR-\( \gamma \)-activating ligands that dampen the innate immune response against bacteria (26–28), and hydroxy fatty acids potently activate PPAR-\( \gamma \) (29, 30). The anti-inflammatory action of \( h18:1 \) blocks tumor necrosis factor–induced intestinal barrier impairment (31, 32) and activates GPR40 (32). The discovery that \( SaOhyA \) 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, \( SaOhyA \) 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 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 \( h16:0 \) following a challenge with 20 \( \mu \)M \( 16:1(9Z) \). Strains AH1263/pPJ480 (blue trace), PDJ68 (ΔohyA)/pPJ480 (red trace), and PDJ68/pPJ490 (green trace) were grown to \( A_{opt} \) of 0.5 and exposed to \( 16:1(9Z) \) for 1 h. The cell supernatants were harvested, and the formation of \( h16:0 \) was detected by LC-MS as described under “Experimental procedures.”
**Experimental procedures**

**Materials**

FAD, FMN, NAD, NADH, NADP, NADPH, and 18:1(9Z) were purchased from Sigma–Aldrich. Antibiotics, high density nickel resin, and DTT were purchased from GoldBio (St. Louis, MO). [1-14C]Oleic acid (59 mCi/mmol, 0.1 mCi/ml) was purchased from PerkinElmer Life Sciences. 16:1(9Z), 16:1(6Z), 16:1(9E), 18:1(9E), 18:1(6Z), 18:1(11Z), and 18:2(9Z,12Z) 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. 6A). 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 Bio-labs) yielding pPJ523. Plasmid pPJ523 was transformed into AH1263 by electroporation, and the knockout was generated as previously described (34, 35). The deletion was confirmed by PCR using primers Ohy-F3 and OhyA-R.

**Figure 7. Effect of SoOhyA on antimicrobial activity.** A, concentration-dependent growth inhibition of strain AH1263/pPJ480 when challenged with either 16:1(9Z), 18:2(9Z,12Z), or 16:1(6Z). Strain AH1263/pPJ480 was grown to an A600 of 0.3, and the indicated concentrations of fatty acids were added. The A600 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 (ΔohyA)/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 (A600 = 0.5) and then challenged with the indicated antimicrobial fatty acids. B, strains were challenged with 24 μM 16:1(9Z). C, strains were challenged with 16 μM 18:2(9Z,12Z). D, strains were challenged with 24 μM 16:1(6Z). 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(9Z)) 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(9Z); 1) SoOhyA hydroxylates and inactivates 16:1(9Z) to h16:0, 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(9Z) can also be inactivated by incorporation into phospholipid by the PIsX and PIsY enzymes. The data show that most of the 16:1(9Z) incorporated into phospholipid (PL) is first converted to acyl-ACP by PIsX and elongated by FASII before it is utilized for phospholipid synthesis by PIsY (12). Human skin produces 16:1(6Z), which is not an SoOhyA substrate, to circumvent the OhyA-dependent resistance mechanism.

**Experimental procedures**

**Materials**

FAD, FMN, NAD, NADH, NADP, NADPH, and 18:1(9Z) were purchased from Sigma–Aldrich. Antibiotics, high density nickel resin, and DTT were purchased from GoldBio (St. Louis, MO). [1-14C]Oleic acid (59 mCi/mmol, 0.1 mCi/ml) was purchased from PerkinElmer Life Sciences. 16:1(9Z), 16:1(6Z), 16:1(9E), 18:1(9E), 18:1(6Z), 18:1(11Z), and 18:2(9Z,12Z) 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.
S. aureus olate hydratase

R6. The DNA sequence of locus tag Sa0102 was synthesized with the appropriate restriction sites (Invitrogen) and cloned into the NdeI and Xhol sites of pET28a to construct pPJ520 for the purification of SaOhyA with an N-terminal His tag from E. coli. The SaOhyA expression vector for strain AH1263 (36) was constructed by first modifying plasmid pCS119 (37) using QuickChange Lightning multisite-directed mutagenesis kit (Agilent Technologies) using specific primers pCS119-a2398t-F and pCS119-g3141c-F to delete the Ncol 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' GGAAACGCTATGACATGATTCAAAATTGATATTTTGACCTAAAACCATGCTAACCCGAGAAT-ACTTACGTGTCGCTAATGAATAATTTTATTAA-CACCTTTTTGTTCTCATATTATTTAATATTATAATTAGCGTGAAGGAGGTGATCATGTCGACAGCGC-GCACTGACGACACCACCCACACCATGAGATA-GCCATTATAATGAGCCAAA) and was moved into the digested plasmid via Gibson assembly to obtain plasmid pPJ490. All plasmids were confirmed by sequencing.

Cloning, expression, and purification of SaOhyA

The expression of N-terminal His-tagged SaOhyA was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside in strain BL21(DE3) harboring pPJ520 for 3 h and purified using Ni²⁺-affinity chromatography. The supernatant was loaded on to a Ni²⁺-nitrolotriacetic 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 SaOhyA 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 SaOhyA were thyroglobulin (669 kDa), IgG (150 kDa), BSA (68.6 kDa), and myoglobin (17 kDa).

SaOhyA assay

The SaOhyA 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 [14C]18:1(9Z), and 0.05 mg/ml of SaOhyA 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 chloroform: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 \( K_m \), [14C]18:1(9Z) was varied from 0 to 25 μM.

Mass spectrometry of OhyA products

The substrate specificity of SaOhyA was determined using LC-MS to measure the formation of hydroxy products. SaOhyA reactions contained 2.5 μg of SaOhyA, 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 Q Trap 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 Q Trap 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, −25 V; and collision energy, −35 V. The system was controlled by Analyst® software (Sciex).

The phenotype of the ΔohyA strains was confirmed by analyzing the formation of h16:0 from 16:1(9Z) in the medium. Strains AH1263/pPJ480, PD68/pPJ480, and PD68/pPJ490 were grown to an A600 value of 0.5 in Tryptone broth containing 1% DMSO. 16:1(9Z) 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 h16:0.

FAD measurement by LC/MS/MS

To duplicate samples of 100 μl of 5 mg/ml SaOhyA, 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 Q Trap 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 A600 0.05 and grown to an A600 of 0.5. Fatty acids in DMSO were added, and the A600 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 mg/ml chloramphenicol.

The concentration of the antimicrobial fatty acids that inhibited growth was determined by growing strain AH1263/pPJ480 or 16:0 was purified from an A600 of 0.6 in LB containing 10 mg/ml BSA, and [14C]18:1(9Z) 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 chloroform:methanol (90/10, v/v). The distributions of radioactivity on the dried plates and extent of product formation were quantified using a Typhoon PhosphorImager.

**Efflux of hydroxy-fatty acids**

Strain JLB2 (37) (Δfaka/pPJ490) was grown to an A600 of 0.6 in LB containing 10 mg/ml BSA, and [14C]18:1(9Z) 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 chloroform: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**

h16:0 was purified from an SaOhyA 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 h16:0 were dried under nitrogen, and to remove any residual water, h16:0 was suspended in 100% ethanol and dried under nitrogen. The mass of the h16:0 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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