Exogenous Fatty Acids Remodel *Staphylococcus aureus* Lipid Composition through Fatty Acid Kinase

Zachary DeMars,[a] Vineet K. Singh,[b] Jeffrey L. Bose[a]

[a]Department of Microbiology, Molecular Genetics and Immunology, University of Kansas Medical Center, Kansas City, Kansas, USA
[b]Department of Microbiology and Immunology, A.T. Still University of Health Sciences, Kirksville, Missouri, USA

ABSTRACT *Staphylococcus aureus* can utilize exogenous fatty acids for phospholipid synthesis. The fatty acid kinase FakA is essential for this utilization by phosphorylating exogenous fatty acids for incorporation into lipids. How FakA impacts the lipid membrane composition is unknown. In this study, we used mass spectrometry to determine the membrane lipid composition and properties of *S. aureus* in the absence of *fakA*. We found the *fakA* mutant to have increased abundance of lipids containing longer acyl chains. Since *S. aureus* does not synthesize unsaturated fatty acids, we utilized oleic acid (18:1) to track exogenous fatty acid incorporation into lipids. We observed a concentration-dependent incorporation of exogenous fatty acids into the membrane that required FakA. We also tested how FakA and exogenous fatty acids impact membrane-related physiology and identified changes in membrane potential, cellular respiration, and membrane fluidity. To mimic the host environment, we characterized the lipid composition of wild-type and *fakA* mutant bacteria grown in mouse skin homogenate. We show that wild-type *S. aureus* can incorporate exogenous unsaturated fatty acids from host tissue, highlighting the importance of FakA in the presence of host skin tissue. In conclusion, FakA is important for maintaining the composition and properties of the phospholipid membrane in the presence of exogenous fatty acids, impacting overall cell physiology.

IMPORTANCE Environmental fatty acids can be harvested to supplement endogenous fatty acid synthesis to produce membranes and circumvent fatty acid biosynthesis inhibitors. However, how the inability to use these fatty acids impacts lipids is unclear. Our results reveal lipid composition changes in response to fatty acid addition and when *S. aureus* is unable to activate fatty acids through FakA. We identify concentration-dependent utilization of oleic acid that, when combined with previous work, provides evidence that fatty acids can serve as a signal to *S. aureus*. Furthermore, using mouse skin homogenates as a surrogate for *in vivo* conditions, we showed that *S. aureus* can incorporate host fatty acids. This study highlights how exogenous fatty acids impact bacterial membrane composition and function.

KEYWORDS MRSA, cell membranes, fatty acids, phospholipids

Despite decades of intense research, *Staphylococcus aureus* remains a tremendous cause of infection and morbidity in the human population (1). Approximately 30% of the population are asymptomatic carriers of *S. aureus* (2); however, this bacterium can cause infection in numerous anatomical sites, including skin and soft tissues, bones, lungs, and the heart, as well as foreign implants, such as catheters and prosthetic joints (3). While *S. aureus* infection was originally characterized as a typically hospital-acquired infection, the incidence of infections in the community has increased concern and awareness of this pathogen, as community-associated strains have become dominant in the United States (4, 5). Thus, a thorough understanding of how *S. aureus* can

establish infection, fend off the immune system, and maintain infection is needed to combat this pathogen.

Phospholipids lie at the interface of the host-pathogen interaction. Membrane-associated products, such as lipopolysaccharides, lipoteichoic acids, and lipoproteins, are sensed by the germ line-encoded pattern recognition receptors that induce the activity of numerous host immune cells (6, 7). In addition to these membrane products, phospholipids themselves can play a role in evading the immune system. For example, lysyl-phosphatidylglycerol (LPG) has been shown to be important for evading neutrophils and antimicrobial peptides (8, 9). The composition of the phospholipid membranes of bacteria can also dictate if antimicrobial treatment during infection is successful. Resistance to daptomycin, a lipopeptide antimicrobial, can result from the mutation of cardiolipin (CL) synthase (cls2) and increased abundance of cardiolipin (10). Microbial lipids can also serve as antigens for the immune system (11), further emphasizing the role that lipids play during the infection process. More recently, the identification of bacterial extracellular vesicles has also become a topic of interest; these vesicles could contribute to host-pathogen interactions (12). These data clearly suggest that the composition of the phospholipid membrane is a vital component of the host-pathogen interface.

Synthesis of lipids is preceded by the production of fatty acids. S. aureus endogenously synthesizes fatty acids via the fatty acid synthesis type II system (FASII) (13). Due to the differences between fatty acid synthesis enzymes of bacteria and humans, FASII has been the subject of antimicrobial targeting (14–17). Bacteria, including S. aureus, can supplement endogenous fatty acid synthesis by utilizing exogenous fatty acids (exoFAs) (18). These exoFAs are predicted to passively diffuse into the phospholipid membrane. In S. aureus, exoFAs are removed from the membrane by fatty acid-binding proteins FakB1 and FakB2 (18). Once removed from the membrane, fatty acid kinase, FakA, then phosphorylates the carboxyl head group of the fatty acid, creating an acyl phosphate (18) that can then be used for lipid synthesis. S. aureus synthesizes predominantly three classes of phospholipids: phosphatidylglycerol (PG), LPG, and CL (8, 19). One interesting caveat to fatty acid and lipid synthesis in S. aureus is the inability of this bacterium to synthesize unsaturated fatty acids (20). Instead, S. aureus utilizes branched-chain fatty acids (BCFAs), derived from the branched-chain amino acids isoleucine, leucine, and valine, to help modulate the membrane in response to environmental stimuli (21, 22). A large portion of the BCFAs produced by S. aureus include odd-numbered iso and anteiso BCFAs, with an acyl chain length of 15 being the most abundant (22, 23).

FakA was first identified as a regulator of virulence due to the decrease in α-hemolysin activity, increased protease activity, and increased dermonecrosis in a murine model of infection (24). Originally named virulence factor regulator B (VfrB) due to this altered virulence, FakA was eventually identified to be a fatty acid kinase (18). Subsequently, the altered virulence factor profile of a fakA mutant was identified to be due, in part, to altered activity of the SaeRS two-component system (25, 26). The current model for the FakA-dependent alteration of SaeRS signaling is due to the accumulation of fatty acids within the cell (26). A mechanism for how these accumulated fatty acids within the cell decrease SaeRS signaling is still undetermined. The absence of FakA affects global metabolism (27) and increases the resistance of S. aureus to toxic fatty acids (28, 29).

How the inability to use exoFAs affects the overall membrane lipid composition has not been evaluated. In the current study, we aimed to determine the changes in membrane lipid composition in the absence of FakA. We tested how these overall membrane lipid changes affect the properties of the membrane itself. Lastly, we provide evidence that S. aureus grown in the presence of host tissue can use unsaturated fatty acids to supplement phospholipid synthesis, extending our observations from standard laboratory media to include fatty acids found in murine skin. These results increase the importance that the fatty acid utilization system has in the presence of host-derived tissues.
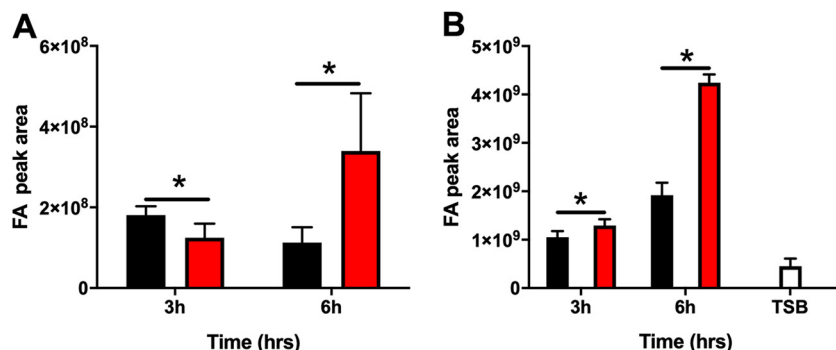
FIG 1 Cellular (A) and supernatant (B) free fatty acids (FA) of the wild type (black) and *fakA* mutant (red) grown for 3 and 6 h in TSB plus 14 mM glucose. The empty bar represents free fatty acids from sterile TSB plus 14 mM glucose. Data represent the means ($n = 4$) with standard deviations. *, $P < 0.05$ by Student's *t* test.

RESULTS

Cellular and extracellular fatty acid profiles of a *fakA* mutant. FakA is necessary for *S. aureus* to incorporate exoFAs into acyl chains of membrane lipids. Thus, it would be expected that the absence of FakA would alter the abundance of FAs in the cell. Indeed, a previous study determined that a *fakA* mutant accumulates fatty acids (26). Our studies are typically performed in tryptic soy broth (TSB), and therefore, we sought to recapitulate this accumulation of fatty acids at different phases of growth in the *fakA* mutant under our growth conditions and in our *S. aureus* strain of choice, AH1263, a derivative of the USA300 strain LAC. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS), we quantified the available free fatty acids in TSB and found that 16:0 and 18:0 constitute the most abundant fatty acids (see Fig. S1 in the supplemental material). Next, we determined how FakA affects fatty acid pools. To this end, the free fatty acid profiles of cellular and extracellular fatty acids for the wild type (WT) and *fakA* mutant was determined. This was tested at two time points representing the early (3 h) and late (6 h) exponential phases of growth, times that we have previously characterized the metabolic changes in the *fakA* mutant (27). In agreement with previous research, we observed a significant increase in cellular free fatty acids in the *fakA* mutant compared to that in the wild type (Fig. 1A) at 6 h. However, the cellular fatty acid levels were slightly, but significantly, decreased at 3 h. The identity of the individual fatty acids was also determined, and we observed differences in several fatty acid species between the wild type and *fakA* mutant after 3 h of growth (Fig. S2A) and 6 h of growth (Fig. S2B). Specifically, we found a significant ($P < 0.05$) increase in the proportion of 15:0, 17:0, 19:0, and 20:0 in the *fakA* mutant during the late exponential (6 h) growth phase. We reasoned that an accumulation of fatty acids in the cell could lead to release of fatty acids into the supernatant. We observed an increased abundance of supernatant fatty acids in both strains over time, which was enhanced in the *fakA* mutant (Fig. 1B). These data demonstrate that the *fakA* mutant accumulates fewer fatty acids during the early exponential phase (3 h) of growth but accumulates more fatty acids during the late exponential phase (6 h) of growth than the wild type. They also show that the *fakA* mutant possesses altered fatty acid pools compared to those of the wild-type strain.

Lipid profile for the wild type and *fakA* mutant in TSB. An in-depth analysis of how FakA affects the composition of the lipid membrane of *S. aureus* has not been undertaken. To do this, we performed a comprehensive analysis of the membrane lipids of *fakA* mutant compared to those of the wild type when grown in TSB. Lipids were extracted using a modified Bligh and Dryer liquid-liquid lipid extraction protocol (30). Since lipid ratios are known to vary by growth phase (31, 32), we examined cells during exponential phase (5 h) and stationary phase (24 h). As expected, PG and LPG were the primary lipids in both strains (Fig. 2A and B). We observed little difference between the
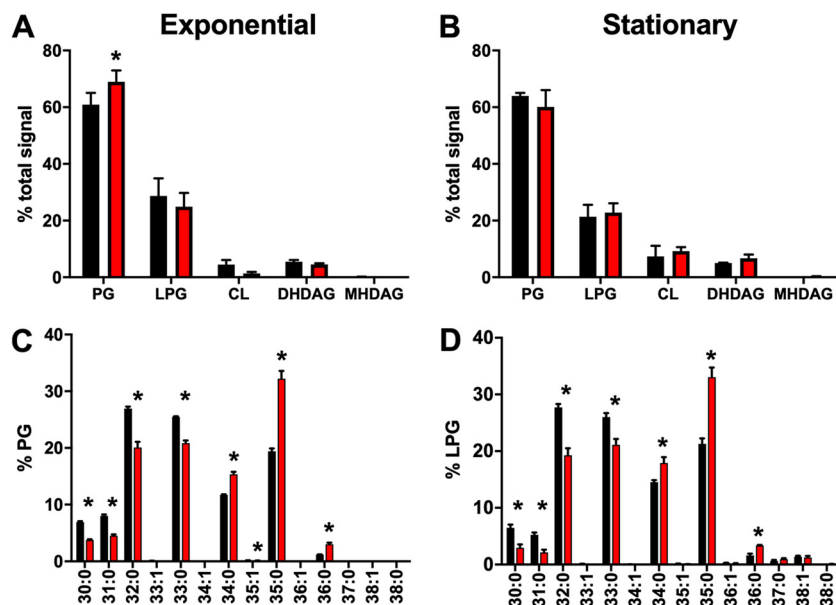
FIG 2 Lipid analysis for the wild type (black) and *fakA* mutant (red). (A and B) Percent total signal for each lipid class was determined at the exponential (A) and stationary (B) phases of growth for phosphatidylglycerol (PG), lysyl-PG (LPG), cardiolipin (CL), dihex-diacylglycerol (DHDAG), and monohex-diacylglycerol (MHDAG). (C and D) Individual PG (C) and LPG (D) species from cells in exponential phase were determined and are presented as percentages of the total PG or LPG signal. Data represent the means (*n* = 4) with standard deviations. *, *P* < 0.05 by Student's *t* test.

major lipid species between the two strains, with only a modest significant increase in PG and a corresponding decrease in CL in the *fakA* mutant during the exponential phase of growth (Fig. 2A). Since LPG and CL are synthesized from PG, the lack of major differences was not unexpected, as FakA is important for insertion of exogenous fatty acids as phospholipid acyl side chains and not, as far as we know, the activity of the enzymes responsible for lipid species generation. Considering this, we determined the total carbon and saturations of the lipid acyl side chains for PG and LPG in the wild type and *fakA* mutant. During growth in TSB, lipids containing an unsaturated acyl chain were near absent at either phase of growth in both strains (Fig. 2C and D). We did identify significant changes in the lipid profiles between the two strains, with the *fakA* mutant tending to contain a higher abundance of longer acyl sides chains, predominantly 35:0, than the parent strain.

Since the data in Fig. 2C and D represent the total carbon length of both acyl side chains, we performed product ion analysis on pooled samples to determine the most abundant acyl side chain pairings that comprise each lipid (Table S1). This analysis revealed that both the wild type and the *fakA* mutant use 15:0 as one acyl side chain, as expected. The second acyl moiety was the same for each lipid species between the two strains, i.e., 35:0 possessed 15:0 and 20:0 fatty acids. Thus, despite using the same fatty acids for membrane generation, the mutant possesses a higher percentage of lipids containing longer acyl chains.

Lipid profiles of the wild type and *fakA* mutant in the presence of exogenous fatty acid. *S. aureus* is unable to endogenously synthesize unsaturated fatty acids but can incorporate them from the environment through FakA. This allows us to use exogenously added fatty acids to track exogenous fatty acid utilization and observe where exoFAs are incorporated. Using this approach, we determined the lipid composition of both the wild type and a *fakA* mutant when grown in the presence of oleic acid (18:1), which was chosen due its relatively low toxicity (Fig. S3) (28). Previously, we demonstrated that the addition of 0.001% (~31 μM) oleic acid elicits a transcriptional response in *S. aureus* (25); therefore, we determined the lipid profiles in TSB supplemented with 0.001% oleic acid. We observed little, if any, incorporation of oleic acid
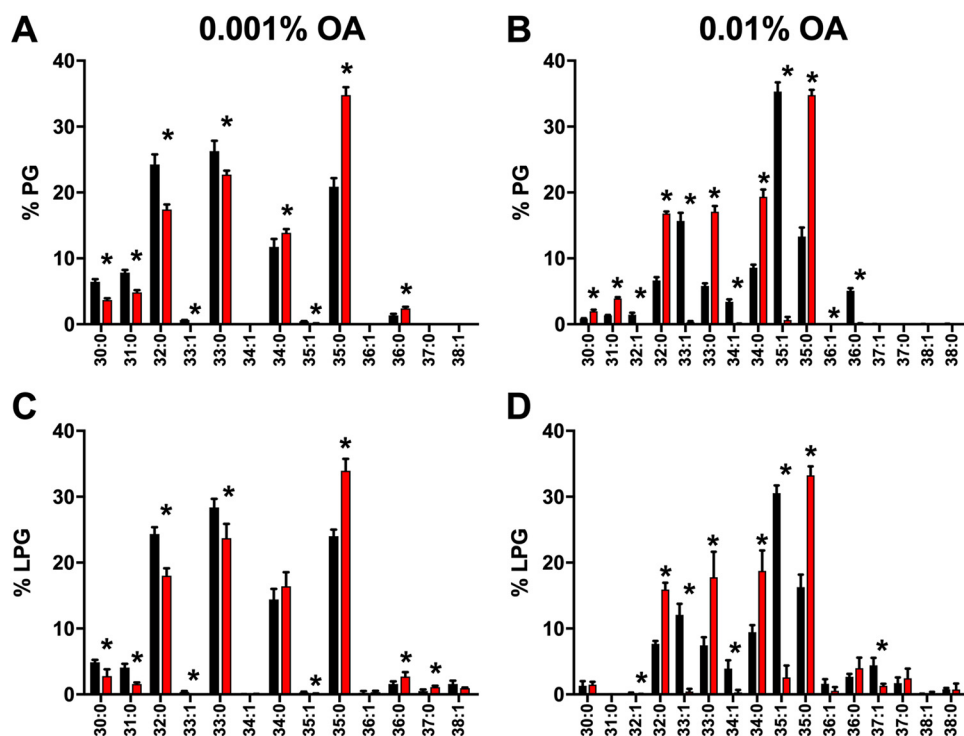
FIG 3 Lipid profile for the wild type (black) and *fakA* mutant (red) of PG (A and B) and LPG (C and D) grown in TSB plus 0.001% oleic acid (A and C) or TSB plus 0.01% oleic acid (B and D). Data represent the means (*n* = 4) with standard deviations. *, *P* < 0.05 by Student's *t* test.

into PG and LPG when grown at this concentration (Fig. 3A and C). Indeed, the lipid profiles of the wild type and *fakA* mutant grown with added 0.001% oleic acid were indistinguishable from growth in TSB alone (Fig. 2). We used fatty acid methyl ester (FAME) analysis followed by gas chromatography to examine the total fatty acid content of the cells, and in agreement with the above-described lipid profiles, we found little oleic acid (18:1) associated with the cells grown in TSB plus 0.001% oleic acid (Table S2). Thus, while *S. aureus* responds transcriptionally to 0.001% additional oleic acid, this concentration was too low to affect incorporation.

Next, we increased the oleic acid concentration to 0.01% (~314 μM). At this concentration, the wild-type strain readily used the oleic acid and was observed as the appearance of a single unsaturated fatty acid chain primarily in 32:1, 33:1, 34:1, and 35:1 lipids (Fig. 3B and D). We had predicted that oleic acid (18:1) would be found primarily paired with 15:0, the most common fatty acid lipid side chain in *S. aureus*. While we did readily detect 33:1, the most abundant lipid was 35:1, which composed >30% of total lipid species. As expected, there was no unsaturated fatty acid in the lipids isolated from the *fakA* mutant. To confirm that the absence of oleic acid in *fakA* mutant lipids is not due to oleic acid not associating with the cell, we performed FAME analysis followed by gas chromatography on the same extracts as used for lipid analysis. Our FAME analysis revealed that in TSB, the wild type and *fakA* mutant have similar total fatty acid profiles, with increased 20:0 in the *fakA* mutant (Fig. 4A). When grown with the additional 0.01% oleic acid, this fatty acid was readily detectable from both strains (Fig. 4B and Table S2). FAME identifies all fatty acids, both free and lipid associated. Since oleic acid was identified by FAME analysis but was not found as a component of the *fakA* lipids, we can conclude that oleic acid associates with the cells but cannot be incorporated into the *fakA* mutant. Thus, the lack of oleic acid in mutant membranes is due to a lack of incorporation and not because oleic cannot interact with or enter the cell.

Our lipid profiling indicated that wild-type cells can pair oleic acid with more than one fatty acid to produce a lipid. Moreover, we had expected 33:1 to be the most
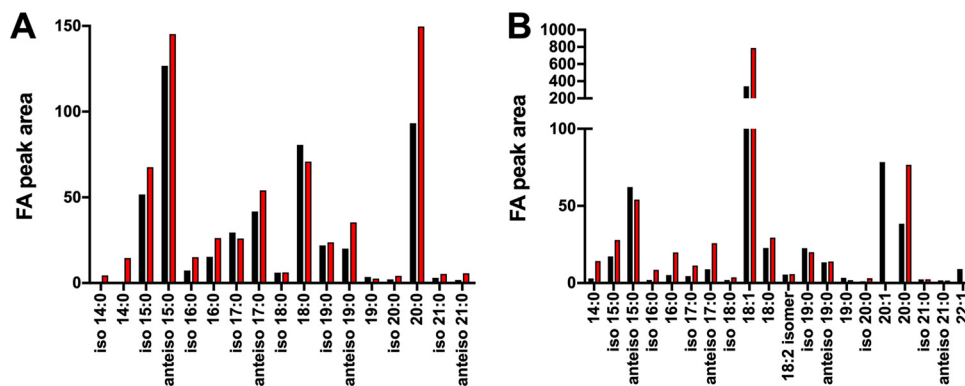
FIG 4 Fatty acid methyl esterase analysis of lipid extracts from the wild type (black) and *fakA* mutant (red) grown in TSB (A) or TSB plus 0.01% oleic acid (B). Data are from pooled replicates (*n* = 4) from each strain and growth environment.

prevalent lipid species by pairing oleic acid with 15:0. We again used product ion analysis to determine the fatty acid pairing in each lipid (Table S1). It was expected that the fatty acid paired with oleic acid would be a saturated fatty acid and that this fatty acid would be equivalent to the total lipid carbon length minus the oleic acid. This prediction was true for 32:1 (14:0 and 18:1) and 33:1 (15:0 and 18:1). Considering that 18:1 was the added fatty acid, we anticipated 35:1 to be comprised of 17:0 and 18:1 fatty acids. This was not the case, as the major 35:1 fatty acid pair consisted of 15:0 and 20:1. The included 20:1 was also the fatty acid associated in the lower-abundance lipid 34:1.

While *S. aureus* membranes comprise both straight-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs), the organism relies on BCFAs to modulate the rigidity of its lipid membrane (23). BCFAs in *S. aureus* are comprised of odd-numbered iso and anteiso fatty acids, and the proportions of BCFAs and SCFAs are highly dependent on the growth environment (21). We analyzed our FAME data to determine if the absence of *fakA* or the addition of oleic acid impacts BCFA or SCFA preference (Table S2). In TSB alone, the wild type and the *fakA* mutant have similar ratios of anteiso and iso fatty acids (~1.5) and BCFAs and SCFAs (1.65 for the wild type and 1.48 for the *fakA* mutant). Not surprising considering our other analysis, when the wild type and *fakA* mutant were grown in TSB plus 0.001% oleic acid, the fatty acid profiles of both were nearly identical to those for growth in TSB. In contrast, growth in TSB plus 0.01% oleic acid resulted in accumulation of oleic acid in both wild-type and *fakA* mutant bacteria. When comparing the BCFA/SCFA ratio under this condition, we observed a dramatic shift, most likely due to the added SCFA oleic acid. For example, wild-type cells grown in TSB contain ~38% SCFA, while growth in 0.01% oleic acid led to the cells altering the fatty acid pools to ~78% SCFA. A similar shift (40% to 84%) was also seen in the *fakA* mutant. As noted in previous studies (33), the wild-type *S. aureus* unsaturated fatty acid pool was comprised of both 18:1 and 20:1. These data, along with our lipid and product ion data, indicate that wild-type *S. aureus* elongates oleic acid (18:1) to 20:1. This was not observed in the *fakA* mutant, as the majority of oleic acid remained oleic acid. In conclusion, the fatty acid composition of the membrane is dramatically altered in the presence of incorporation-level oleic acid seen in the wild type as the incorporation of oleic acid into PG, LPG, and CL.

Membrane function is affected by the inability to utilize exoFAs. The membrane lies at the interface of cellular processes and the external environment. We have now shown that *fakA* influences membrane composition. We previously reported that the *fakA* mutant produces decreased pigment (28), which is associated with membrane fluidity (34–36). Also, our previous metabolomics study found that the *fakA* mutant has a more reduced cellular environment (27). The electron transport chain is embedded in the phospholipid membrane, suggesting that fatty acid accumulation and the compo-
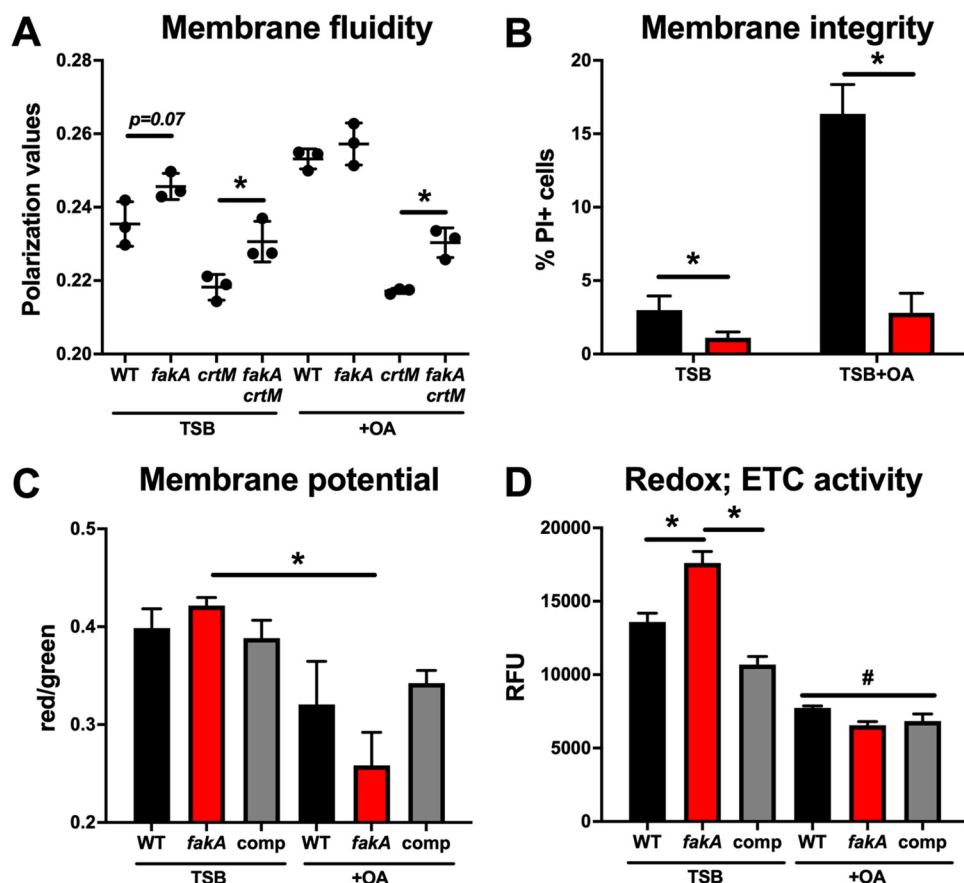
FIG 5 (A) Membrane fluidity in the wild type, *fakA* mutant, *crtM* mutant, and *fakA crtM* mutant grown in the presence (+OA) or absence (TSB) of 0.01% oleic acid. (B) Membrane integrity measured using propidium iodide (PI) as an indicator for the wild type (black) and *fakA* mutant (red). (C) Membrane potential was analyzed using DiOC(3)$_2$ staining of the wild type, *fakA* mutant, and complemented *fakA* mutant (comp). (D) Respiratory activity of the wild type, *fakA* mutant, and complemented *fakA* mutant as measured using CTC as an indicator. Data represent the means ($n = 3$) with SD. *, $P < 0.05$ by Student's t test. "#" indicates that all samples treated with OA differed significantly from the corresponding strain in TSB alone. Data are representative of those from at least three independent experiments.

sition of the membrane may affect respiration. Finally, membrane potential is indicative of metabolic state and membrane function. Therefore, we wanted to determine the role FakA and exoFAs play in membrane fluidity, permeability, membrane potential, and respiratory chain activity.

First, membrane fluidity was determined using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) (23, 34) in the wild type and *fakA* mutant grown to exponential phase (5 h) in TSB with and without incorporation-level oleic acid (0.01%). While the value did not reach significance ($P = 0.07$), the *fakA* mutant had decreased membrane fluidity compared to that of the wild type (Fig. 5A). The Crt-derived pigment staphyloxanthin is known to affect membrane fluidity (37), and *fakA* mutants produce less pigment (28). To remove this complication, we tested a *crtM* mutant with and without *fakA*. We observed that a *crtM* mutant has more membrane fluidity than its isogenic parents, both in the case of the wild type and in the case of the *fakA* mutant. Interestingly, a *fakA crtM* double mutant has significantly decreased membrane fluidity compared to that of the *crtM* mutant. The addition of 0.01% oleic acid decreased membrane fluidity for both the wild type and *fakA* mutant. This was not due to incorporation as part of phospholipids, since the wild type and *fakA* mutants were equivalent. For reasons that are not clear, adding oleic acid to *crtM* mutants had no effect on membrane fluidity (compare the growth of the *crtM* mutant with and without oleic acid), and, again, we observed lower membrane fluidity in the *fakA crtM* mutant

than in the *crtM* mutant. Thus, we determined that the absence of FakA affects membrane fluidity and that this is not due to the altered pigment levels in the mutant.

We next assessed whether the absence of FakA altered membrane integrity using propidium iodide (PI) as an indicator, since this stain cannot diffuse into cells unless membranes are compromised. Cells were grown in TSB and membrane integrity was determined in the presence and absence of 0.01% oleic acid. When grown in TSB alone until mid-exponential phase, the wild type and *fakA* mutant had approximately 3% and 1.1% PI-positive cells, respectively (Fig. 5B). However, when cells were grown in the presence of 0.01% oleic acid, 16.4% of wild-type cells were PI positive, compared to 2.8% for the *fakA* mutant. Thus, when grown in TSB, the *fakA* mutant has less permeable membranes and the permeability is less affected by the presence of oleic acid than for the wild-type strain.

Membrane potential is important for driving ATP production and transporting ions and metabolites into and out of the cell. We determined the membrane potential ($\Delta\Psi$) of wild-type and *fakA* mutant *S. aureus* using the fluorescent reporter 3,3'-diethyloxacarbocyanine iodide [$DiOC_2(3)$] (38, 39). The wild type and *fakA* had similar membrane potentials when grown in TSB alone (Fig. 5C). When grown in the presence of 0.01% oleic acid, both strains had decreased membrane potentials compared to that when grown in TSB alone, but this was enhanced in the *fakA* mutant, with a 40% reduction compared to a 20% reduced potential in the wild type. Thus, under these conditions, oleic acid decreases membrane potential and this may be enhanced in a *fakA* mutant.

Electron transport chain activity was measured using the fluorescent reporter 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (39). This reporter is reduced by respiratory dehydrogenases and is thus indicative of cellular respiration. In TSB alone, the *fakA* mutant had significantly more respiratory activity than that of the wild type (Fig. 5D). However, both the wild type and *fakA* mutant display decreased respiratory activity in the presence of incorporation-level oleic acid, which correlates with reduced growth (Fig. S3). Overall, these studies demonstrate that the *fakA* mutant displays phenotypes that are associated with membrane changes.

Lipid profile is altered in the presence of mouse skin homogenate. The most common infection site of *S. aureus* is the skin, which contains high levels of various fatty acids and lipids (40). These molecules have antimicrobial properties and limit the growth of certain bacteria. It is unknown if *S. aureus* can utilize fatty acids from mouse skin, though it harvests fatty acids from other host molecules, such as low-density lipoprotein (41). To gain insight into this, we determined (i) if *S. aureus* alters its lipids in response to mouse skin and (ii) if *S. aureus* incorporates host fatty acids. To this end, we grew wild-type and *fakA* mutant *S. aureus* in a combination of 75% mouse skin homogenate and 25% TSB. This ratio was chosen based on relatively high bacterial growth (optical density at 600 nm [OD_{600}] of 3.5 to 4.5) to allow for quality extraction of bacterial lipids. After 24 h of growth in 75% mouse skin homogenate, cells were isolated and lipids were extracted and analyzed via LC-MS/MS. To ensure that we did not collect mouse skin lipids without bacterial cells, one sample of 75% mouse skin homogenate and 25% TSB without bacterial cells was treated identically to the bacterial cells we extracted. This sample without bacteria was also analyzed via LC-MS/MS, and we found only trace amounts of lipids (Fig. S4), demonstrating that our data represent lipids isolated from *S. aureus* and not contaminants from the mouse skin. Under this growth condition, we observed equal levels of PG and LPG in the wild-type strain. Unlike in TSB, we saw a pronounced difference in the *fakA* mutant compared to the wild type. In this case, the *fakA* mutant resembled membranes of cells grown in TSB alone, with PG being the most abundant, followed by LPG, and CL being the least abundant lipid. The most abundant saturated PG species were 33:0 and 35:0 in the wild type, while the *fakA* mutant had a relatively even distribution of 32:0, 34:0, and 35:0. Using the natural unsaturated fatty acids found in mouse skin as a marker of host fatty acid utilization, we observed considerable incorporation of unsaturated fatty acids as a component of the wild-type cell lipids (Fig. 6B to D). The unsaturated PG species 33:1,
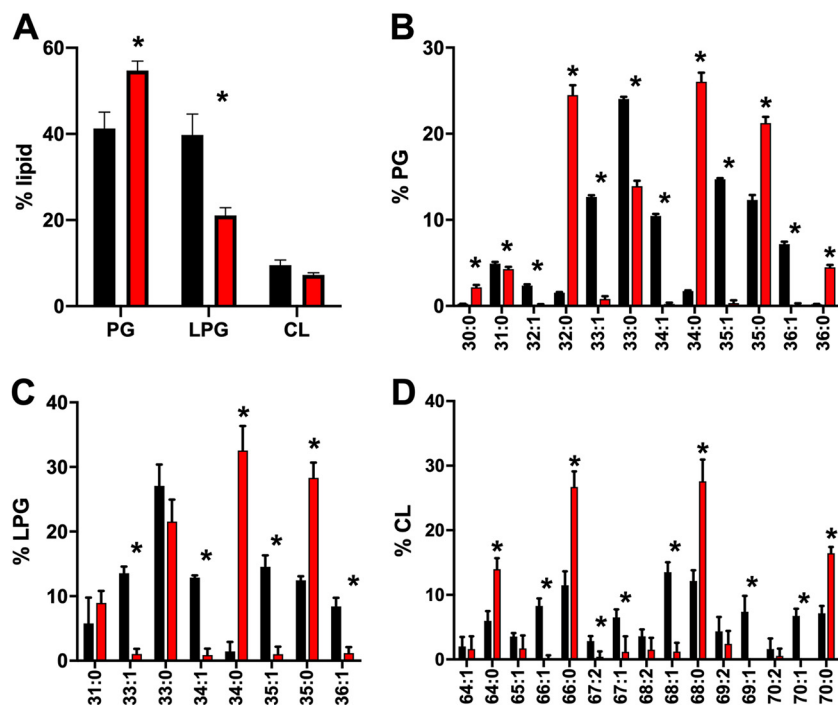
FIG 6 Lipid profile of the wild type (black) and *fakA* mutant (red) grown in 75% mouse skin homogenate. (A) Total percent lipid signal of the wild type and *fakA* mutant by class of lipid. (B to D) Identification of PG (B), LPG (C), and CL (D) represented by the percentage of the total from each class. Data represents the means (*n* = 4) with standard deviations from a representative experiment. *, *P* < 0.05 by Student's *t* test.

34:1, 35:1, and 36:1 were all found in *S. aureus* grown in mouse skin homogenate. Similar trends were observed for LPG and CL species. We did not identify lipids containing more than one fatty acid unsaturation. As expected, we did not find unsaturated fatty acids in the lipids of the *fakA* mutant. In conclusion, we showed that *S. aureus* can utilize unsaturated fatty acids found in mouse skin homogenate for lipid synthesis.

DISCUSSION

The exogenous fatty acid utilization system of *S. aureus* has recently emerged as a new metabolic pathway and is made up of at least the fatty acid kinase FakA and the fatty acid-binding partner proteins FakB1 and FakB2 (18, 20). Despite the characterization of this system, how inactivation of this pathway (via deletion of *fakA*) affects the composition of the phospholipid membrane has remained unknown. *S. aureus* is routinely cultivated in TSB, which is abundant in carbohydrates, protein, and fatty acids, primarily palmitic acid (16:0) and stearic acid (18:0). Not surprisingly, based on a previous report, we identified an accumulation of fatty acids in a *fakA* mutant under our growth conditions. However, our studies revealed that this changes over the course of growth and showed that not only do the *fakA* mutant cells accumulate fatty acids, but also fatty acids are released into the media, where they also increase in abundance. It is unknown whether this accumulation of fatty acids in the extracellular growth environment is an intentional process (i.e., release of fatty acids from the cell), due to cell death and turnover, or whether they are actively released by an efflux system that has been proposed previously (29). Since only free fatty acids were identified in this experiment, the fatty acids found in the supernatant were likely not part of extracellular membrane vesicles produced by *S. aureus*. The consequence of altered fatty acid metabolism in the *fakA* mutant was not a change in the ratio of primary lipid species but a reorganization of the fatty acid phospholipid side chains. Moreover, we demonstrate that the addition of excess 18:1 fatty acid led to its incorporation into wild-type cells but also a change in the fatty acid pools in both the wild type and *fakA* mutant.

S. aureus is known to produce primarily three phospholipids (8, 42–44). Phosphatidylglycerol (PG) is the primary phospholipid and is the precursor for the generation of additional phospholipids. Lysyl-PG (LPG) is produced by MprF, and while S. aureus has two cardiolipin (CL) synthases, cardiolipin is the least abundant species. As expected, our results demonstrated that both wild-type and fakA mutant membranes contained primarily PG and LPG. The amount of CL can vary by strain and condition, and we detected only 4.5% of total phospholipids as cardiolipin in our USA300 S. aureus strain at either the exponential or stationary phase of growth, showing that LAC derivatives grown under our conditions produce little CL.

S. aureus generally prefers endogenously produced 15:0 in the sn2 position of the phospholipids and inserts either a second endogenous fatty acid or exoFA in the sn1 position. Our product ion analysis of the wild-type and fakA mutant phospholipids most often showed a 15:0 fatty acid. However, the fakA mutant paired this 15:0 more frequently with longer-chain fatty acids (Fig. 2) than the parent strain. Phospholipids of lower total carbon abundance (i.e., 30:0, 31:0, 32:0, and 33:0) were more abundant in the wild type than in the fakA mutant. These phospholipids all contained 15:0 in one sn position, with the other acyl chain being 15:0, 16:0, 17:0, and 18:0 in phospholipids 30:0, 31:0, 32:0, and 33:0, respectively. The most abundant phospholipids observed in the wild type were 32:0 and 33:0. In contrast, the fakA mutant tended to have larger fatty acids as part of phospholipids, shifting to 35:0 in both PG and LPG due to the insertion of 20:0 alongside 15:0. This was also reflected in the free fatty acid pools in the fakA mutant (see Fig. S2 in the supplemental material), showing that this mutant possesses and uses a greater abundance of longer fatty acids, such as 19:0 and 20:0. However, the presence of exogenous oleic acid altered the normal relationship of use of 15:0 alongside a varying-length second fatty acid. In this case, the wild type generated lipids with 18:1 oleic acid in combination with not only 15:0 but also commonly 14:0. One observation made from this study, and in agreement with those of Parsons et al. (33) in strain RN4220, is the abundance of fatty acid 20:1 in wild-type S. aureus. Since oleic acid (18:1) was the exogenous fatty acid added to the media, this demonstrates that S. aureus can elongate exoFAs. This was a frequent event, since 35:1 composed of 15:0 and 20:1 was the most abundant lipid species identified in the wild type when grown in the presence of oleic acid (Fig. 3 and Table S1). Our FAME analysis confirmed that the lack of incorporation of 18:1 into the fakA mutant is not due to the inability to associate with the cells and is a result of the inability of the fakA mutant to phosphorylate the fatty acid, as expected. The presence of 20:1 has been observed previously, and while the exact mechanism behind exoFA elongation has not been elucidated, it has been proposed to occur through FabF, part of the fatty acid biosynthesis pathway (45). However, our observation that there was no 20:1 fatty acid in the fakA mutant demonstrates that elongation of exoFAs requires FakA and that only phosphorylated fatty acids can be elongated by S. aureus.

In previous studies, we found that oleic acid can inhibit the Sae two-component system when added exogenously to the medium at 0.001% (25). This was later confirmed by another group (26). Considering this transcriptional response, we anticipated that this concentration would yield membranes containing oleic acid. However, oleic acid-containing phospholipids were not detected when S. aureus was grown in the presence of 0.001% (~31 μM) oleic acid. When the concentration of oleic acid was increased 10-fold to 0.01% (~314 μM), we observed incorporation of oleic acid into lipids. Thus, there is an apparent concentration-dependent incorporation of exogenous unsaturated fatty acids by S. aureus. Moreover, these data revealed that S. aureus can sense and respond transcriptionally to levels of fatty acids that are lower than those used for membrane synthesis, further supporting fatty acids as potent signaling molecules in S. aureus.

The membrane is a dynamic structure that dictates multiple functions of the cell. We identified functional changes between wild-type and fakA mutant S. aureus. In TSB alone, the fakA mutant has more rigid membranes regardless of pigment production. Since pigment impacts membrane fluidity and the fakA mutant produces less pigment,

this could confound membrane fluidity experiments with this strain. Our data confirm that pigment does impact membrane fluidity (Fig. 5), but they also demonstrate that there is a FakA-dependent component. This could possibly be due to increases in the acyl chain length of the phospholipids (Fig. 2C), as longer acyl chains result in a more rigid membrane. Additionally, a slight decrease in BCFA/SCFA ratios in the *fakA* mutant (Table S2) could increase rigidity. To our surprise, addition of 0.01% oleic acid decreased membrane fluidity of both the wild type and *fakA* mutant. While the unsaturated fatty acid linoleic acid (18:2) has been shown to increase membrane fluidity (46), our data clearly demonstrate that growth in the presence of oleic acid does not result in similar membrane fluidity. There may be two explanations for this result. First, this may be due to the difference in structure of the fatty acids. The presence of two unsaturations in linoleic acid may be more disruptive to fluidity than oleic acid. Second, the addition of 0.01% oleic acid dramatically decreases the BCFA/SCFA ratio in both the wild type and *fakA* mutant. Wild-type cells contained 38% and greater than 75% SCFAs when grown in TSB without and with 0.01% oleic acid, respectively. SCFAs, like oleic acid, are known to affect membrane fluidity, and our data are consistent with the increased abundance of SCFAs in the presence of oleic acid decreasing membrane fluidity. It appears that oleic acid impacts membrane fluidity without the requirement of being incorporated, and this is influenced by the presence of the pigment staphyloxanthin. Notably, previous work suggests that pigment levels are not influenced by fatty acid incorporation (47). Dissecting this relationship between fatty acid addition, fatty acid utilization, and *S. aureus* pigmentation will be the focus of future studies.

We have established that the *fakA* mutant has altered metabolism (27). Since the electron transport chain is embedded in the phospholipid membrane, we sought to determine whether the changes in membrane composition in the *fakA* mutant and the presence of 0.01% oleic acid altered cellular respiration. The *fakA* mutant had more respiratory activity than the wild type in TSB alone (Fig. 5D). This agrees with our metabolic study showing that the *fakA* mutant had an increase in $NAD^+/NADH$ and $NADP^+/NADPH$ levels at the same phase of growth. Growth in TSB plus 0.01% oleic acid resulted in decreased respiration in both the wild type and the *fakA* mutant. While the increased respiration of the *fakA* mutant in TSB did not affect membrane potential, growth in TSB plus 0.01% oleic acid depolarized both the wild type and *fakA* mutant; however, the *fakA* mutant was more greatly affected (Fig. 5C). Previous studies indicated that palmitoleic acid (16:1), and not oleic acid (18:1), depolarized membranes (45). It should be noted that we used approximately three times the concentration of oleic acid as the study mentioned, and this can likely account for the differences between experiments. Additionally, the study mentioned above was published prior to the identification of FakA, and therefore, incorporation versus free fatty acid exposure could not be tested. Thus, our data indicate that oleic acid will decrease respiration and depolarize membranes at 0.01% (314 μM) regardless of being incorporated into phospholipids. Why the *fakA* mutant has more respiratory activity in TSB is unknown; however, this observation occurred at a point where we identified enhanced growth in the *fakA* mutant during the switch between glucose and acetate/amino acid utilization (27). Thus, it may be not surprising to see increased respiratory activity during periods of enhanced growth. Why FakA influences metabolism has not been completely elucidated. This could be due to changes in the membrane that may influence nutrient diffusion or transport. Another possibility is that it is the result of the accumulation of fatty acids, which could have different activities or impact levels based on saturation. A recent study indicated that inactivation of type II NADH dehydrogenases (Ndh-2s), particularly NdhC, results in fatty acid accumulation and regulation of the SaeRS two-component system (48). Ndh-2s do not pump ions across the membrane, thus playing an indirect role in membrane potential. In *Escherichia coli*, Ndh-2s are associated with aerobic respiration, while Ndh-1s are associated with anaerobic respiration (49). Since the *fakA* mutant possesses an aeration-dependent growth enhancement and has increased respiratory activity without a significant increase in membrane potential, we predict that Ndh-2s play a pivotal role in altering metabolism when fatty acids are

accumulated within the cell or in the presence of exogenous fatty acids. Studies testing how different types of exogenous fatty acids affect respiration through Ndh-2s, as well as other members of the respiratory chain, will help us understand the mechanism by which fatty acids affect bacterial physiology. It is clear that the inability of *S. aureus* to use fatty acids and/or exposure to exogenous fatty acids impacts physiology. This has been seen by us and others as changes in the glucose, acetate, amino acid metabolism, and energy or redox homeostasis that apparently involve at some level NADH dehydrogenases and regulatory proteins such as CcpA, as well as the transcriptional response in arginine deiminase and urease (27, 28, 45, 48, 50, 51). It is also possible that *S. aureus* identifies unsaturated fatty acids as a stress, as we previously saw that enhanced resistance to unsaturated fatty acids in a *fakA* mutant requires the alternative sigma factor σ^B (28). Future studies will be needed to determine the specific mechanism by which fatty acid addition modulates metabolism in *S. aureus* and whether this is a general fatty acid response or is restricted to certain fatty acid types.

We wanted to expand our observations to more closely mimic what the bacteria may encounter during infection. Since *S. aureus* commonly causes infections in the skin, we wanted to determine if *S. aureus* can actively scavenge unsaturated fatty acids for phospholipid synthesis from host tissue. To do this, we homogenized mouse skin and grew wild-type and *fakA* mutant *S. aureus* in 75% mouse skin homogenate supplemented with 25% TSB. While *S. aureus* can grow relatively well in 100% mouse skin homogenate, we added 25% TSB to ensure high bacterial growth. We confirmed that *S. aureus* can scavenge exogenous fatty acids when grown in mouse skin homogenate. We did observe lipidome changes in wild-type *S. aureus* grown in mouse skin compared to the organism grown in TSB plus 0.01% oleic acid. For example, growth in mouse skin resulted in wild-type *S. aureus* having relatively similar levels of PG 33:0, PG 33:1, PG 35:0, and PG 35:1. This differs from growth in TSB plus 0.01% oleic acid, in which PG 35:1 clearly became the most abundant phospholipid. While other studies have found that *S. aureus* can utilize fatty acids from a host source, specifically low-density lipoprotein (LDL) and pig liver homogenate (41, 52), none have used homogenized skin as a medium. Given the propensity of *S. aureus* to commonly infect the skin, our data provide a framework for future studies utilizing skin as a source of fatty acids and for how bacterial phospholipid membranes adapt to skin tissue. How *S. aureus* scavenges fatty acids from mouse skin and whether they are derived from free fatty acids, lipids, or LDL is one component of ongoing studies.

The exoFA utilization pathway of FakB1, FakB2, and FakA is newly described (18, 24). Despite this pathway being necessary for the incorporation of exoFAs into the membrane, a detailed analysis of how this system alters membrane composition has not been performed. Not surprisingly, our results show no change in the PG/LPG/CL ratio by the absence of FakA, but there was a reorganization of the fatty acid side chains, e.g., change in fatty acid length preference, of *S. aureus* phospholipids in the absence of FakA. These studies provide key details on how membrane composition changes in response to exoFAs regardless of whether they are used for phospholipid synthesis. Furthermore, this work expands our knowledge on how the activity of FakA influences *S. aureus* physiology.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* was grown in tryptic soy broth (TSB) or tryptic soy agar supplemented with chloramphenicol (10 μg ml^{-1}) or erythromycin (5 μg ml^{-1}) when necessary. *S. aureus* cultures were then inoculated at a 1:10 medium-to-flask volume ratio at an initial optical density at 600 nm of 0.1 in TSB and grown at 37°C with shaking at 250 rpm. TSB was supplemented with oleic acid (Alfa Aesar) by adding the desired percentage (volume/volume) of oleic acid to media and vortexed vigorously before being aliquoted into flasks.

Fatty acid analysis. Cell pellets and supernatants were flash frozen in liquid nitrogen and stored at −80°C until processed. The cell pellets were washed three times with 1 ml of 40 mM ammonium formate in water. The washed pellets were resuspended in 50 μl of 5 mM ammonium acetate and homogenized three times for 30 s with a BeadBeater, with 15-min rests on ice in between. Protein concentrations of the homogenates were measured, and each sample was normalized to 500 μg ml^{-1} and aliquoted at 25 μl for extraction. The supernatants were not normalized and aliquoted at 125 μl for extraction. All

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference
AH1263	USA300 CA-MRSA strain LAC without LAC-p03	58
JLB2	AH1263 ΔfakA	24
JLB112	AH1263 crtM::NΣ	28
JLB129	JLB2 crtM::NΣ	28
pJB165	fakA complement plasmid	24

[a]"NΣ" indicates mutations originating from the Nebraska Transposon Mutant Library.

samples were extracted by protein precipitation with 1 ml of 80%:20% methanol-water for 20 min at 4°C. The samples were centrifuged at 20,000 × *g* for 10 min at 4°C. One milliliter of supernatant was collected and dried down under nitrogen gas at 30°C. The samples were reconstituted at 25 μl of 80:20 acetonitrile–5 mM ammonium acetate in water. Each sample was spiked with 1 μl of fatty acid-stable isotope standards.

Analysis of the free fatty acids was performed on a Thermo Q-Exactive Orbitrap mass spectrometer with a Dionex ultrahigh-performance liquid chromatograph (UHPLC) and autosampler. All samples were analyzed in negative heated electrospray ionization in data-dependent MS/MS with a mass resolution of 70,000 in full MS and 17,500 at *m/z* 200 in data-dependent MS/MS. Separation was achieved on an Acquity ultraperformance liquid chromatography (UPLC) high-strength silica T3 2.1- by 150-mm, 1.8-μm column with 1 mM ammonium acetate as mobile phase A and 0.1% acetic acid in acetonitrile as mobile phase B. The flow rate was 500 μl min^{-1}, with a column temperature of 30°C. The injection volume was 4 μl. Peak areas were integrated for each analyte and internal standards using Xcalibur.

ESI-MS/MS lipid profiling. An automated electrospray ionization-tandem mass spectrometry (ESI-MS/MS) approach was used, and data acquisition and analysis and acyl group identification were carried out as described previously (53), with modifications. Each sample was dissolved in 1 ml of chloroform and an aliquot of 2 to 39 μl of extract was used.

For analysis of PG, phosphatidic acid (PA), monogalactosyldiacylglycerol (monohexDAG), and digalactosyldiacylglycerol (dihexDAG), phospholipid and galactolipid internal standards, obtained and quantified as previously described (54), were added in the amounts described by Narayanan et al. (55), except for phosphatidylinositol (PI) (16:0/18:0) (0.28 nmol), PI (18:0/18:0) (0.11 nmol), digalactosyldiacylglycerol (DGDG) (18:0/16:0) (0.44 nmol), DGDG (18:0/18:0) (1.48 nmol), monogalactosyldiacylglycerol (MGDG) (18:0/16:0) (1.67 nmol), MGDG (18:0/18:0) (1.41 nmol), and cardiolipin (CL) (14:0/14:0/14:0/14:0) (0.015 nmol). The sample and internal standard mixture were combined with solvents such that the ratio of chloroform, methanol, and 300 mM ammonium acetate in water was 300/665/35 and the final volume was 1.4 ml. Analysis of PG, PA, monohexDAG, and dihexDAG was performed on unfractionated lipid extracts introduced by continuous infusion into the ESI source on a triple quadrupole MS/MS (API 4000; Applied Biosystems, Foster City, CA). Data were collected and analyzed as previously described for analysis of PG, PA, monohexDAG, and dihexDAG (55).

For CL and LPG analysis, phospholipid standards were added at one-fifth of the amounts indicated above. CL and LPG were analyzed on a triple quadrupole MS/MS (Xevo TQS; Waters Corp., Milford, MA). Samples were introduced to the Xevo TQS using a Waters 2777 Sample Manager autosampler. Lipid species were detected with the following scans: LPG [M-H]$^-$ in negative-ion mode with Precursor 145.0 and cardiolipin in negative ion mode with Precursor 153.0. The scan speed was 50 or 100 u per s. The Xevo TQS in negative-ion mode has the following parameters for CL and LPG: capillary, 2.8 kV; cone voltage, 40 V; source temperature, 150°C; desolvation temperature, 120°C; collision gas, 0.14 ml/min; and nebulizer gas flow, 7 bars. Collision energies on the Xevo TQS, with argon in the collision cell, were −75 V for CL and −35 V for LPG.

Data processing was similar to that described previously, including background subtraction, smoothing, integration of spectral peaks, isotopic deconvolution, correction for chemical or instrumental noise, and quantification (54, 56). Internal standards of the same class were used for PA, PG, and cardiolipin. LPG was quantified against PG (40:0) in negative Precursor mode, and monohexDAG and dihexDAG were quantified against PG (40:0) in positive neutral loss (NL) mode. Finally, the data were corrected for the fraction of the sample analyzed and normalized to signal per CFU to produce data in the units per nanomole per 1e10 CFU.

Product ion analyses were performed on pooled replicates of the treatments on a 4000 QTrap mass spectrometer in enhanced product ion mode. Aliquots of 11 to 14 μl were used. Collision energy was varied as needed for sufficient fragmentation, starting at −45 V. The curtain gas was 10 ml/min, collisionally activated dissociation (CAD) gas was the medium, and the electrospray capillary voltage was −4,500 V.

Membrane fluidity, integrity, potential, and electron transport chain activity. Membrane fluidity for wild-type and *fakA* mutant strains was determined as previously described (57). Membrane integrity was determined using propidium iodide (PI) as an indicator. Wild-type and *fakA* mutant bacteria were grown in TSB with or without 0.01% (314 μM) oleic acid for 5 h. A total of 1.2 ml of culture was spun down and resuspended in 600 μl of phosphate-buffered saline (PBS). The cells were then diluted to an OD$_{600}$ of 0.25 in 500 μl in PBS. As a control, one sample was diluted to an OD$_{600}$ of 0.25 in 500 μl of 70% ethanol. One microliter of PI (50 mg/ml) was added to the cells and allowed to incubate at room temperature for 15 min. PI-positive cells were determined using flow cytometry.

Membrane potential was determined using the BacLight bacterial membrane potential kit (Molecular Probes, Invitrogen) (39). Wild-type and *fakA* mutant bacteria were grown for 5 h. Cultures were diluted to an OD$_{600}$ of 0.1 in 1 ml of PBS. As a negative control, 10 μl of 500 μM carbonyl cyanide

m-chlorophenylhydrazone (CCCP) was added to one sample and vortexed. Ten microliters of 3 mM DiOC$_2$(3) was then added to all samples, excluding a no-stain control, and vortexed. Samples were then allowed to incubate at room temperature for 30 min and analyzed via flow cytometry. The data are presented as the geometric mean ratios of red to green using Texas Red and fluorescein isothiocyanate (FITC) filters, respectively.

Electron transport chain activity (respiratory activity) was determined using the *Bac*Light Redox-Sensor CTC Vitality kit (Molecular Probes, Invitrogen) (39). Wild-type and *fakA* mutant bacteria were grown for 5 h. Cultures were diluted to an OD$_{600}$ of 0.1 in 650 μl of PBS. One sample was diluted in 70% ethanol as a negative control. A total of 65 μl of 50 mM CTC was added to each sample, except for a no-stain control. The samples were then incubated in the dark at 37°C for 30 min and analyzed using a Tecan Spark 10M plate reader (excitation wavelength, 485 $\pm$ 20 nm, and emission wavelength, 645 $\pm$ 40 nm).

Generation of mouse skin homogenates. These studies were conducted under an approved protocol by the Institutional Animal Care and Use Committee (IACUC) of the University of Kansas Medical Center. We used mice of the C57BL/6 background. Following sacrifice, mice were shaved and treated with Nair to remove remaining hair. Next, skin was removed and homogenized in PBS using Lysing Matrix H with a FastPrep-24 5G using the manufacturer's setting. Homogenates were pooled and sequentially passed through 40-μm and 0.45-μm filters.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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