Acyl-chain selectivity and physiological roles of *Staphylococcus aureus* fatty acid–binding proteins

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Fatty acid (FA) kinase produces acyl-phosphate for the synthesis of membrane phospholipids in Gram-positive bacterial pathogens. FA kinase consists of a kinase protein (FakA) that phosphorylates an FA substrate bound to a second module, an FA-binding protein (FakB). *Staphylococcus aureus* expresses two distinct, but related, FakBs with different FA selectivities. Here, we report the structures of FakB1 bound to four saturated FAs at 1.6–1.93 Å resolution. We observed that the different FA structures are accommodated within a slightly curved hydrophobic cavity whose length is governed by the conformation of an isoleucine side chain at the end of the tunnel. The hydrophobic tunnel in FakB1 prevents the binding of cis-unsaturated FAs, which are instead accommodated by the kinked tunnel within the FakB2 protein. The differences in the FakB interiors are not propagated to the proteins’ surfaces, preserving the protein–protein interactions with their three common partners, FakA, PlsX, and PlsY. Using cellular thermal shift analyses, we found that FakB1 binds FA in vivo, whereas a significant proportion of FakB2 does not. Incorporation of exogenous FA into phospholipid in ΔfakB1 and ΔfakB2 *S. aureus* knockout strains revealed that FakB1 does not efficiently activate unsaturated FAs. FakB2 preferred unsaturated FAs, but also allowed the incorporation of saturated FAs. These results are consistent with a model in which FakB1 primarily functions in the recycling of the saturated FAs produced by *S. aureus* metabolism, whereas FakB2 activates host-derived oleate, which *S. aureus* does not produce but is abundant at infection sites.

Fatty acid (FA) kinase of Gram-positive bacteria is responsible for the activation of FA prior to their incorporation into membrane phospholipids. The enzyme system consists of a kinase module (FakA) that binds to a FA-binding protein (FakB) and phosphorylates FakB(FA) to create an acyl-PO₄ (FakB(FA~P)) (1, 2). The FakB(FA~P) is either utilized by the glycerol-phosphate acyltransferase (PlsY) to initiate phospholipid synthesis or is converted to acyl-acyl carrier protein (ACP) by acyl-phosphate:ACP transacylase (PlsX) to enter the elongation cycle of bacterial FA biosynthesis. FA kinase is the only pathway for FA activation and incorporation into *Staphylococcus aureus* membrane phospholipids and has an important role in activating exogenous FA, but it is also involved in cellular lipid homeostasis (3). FA are generated by metabolism in *S. aureus*, and in the absence of kinase activity, the levels of intracellular FA rise (3). FA kinase-null *S. aureus* are resistant to dermcidin (4) and exhibit increased biofilm formation (5). However, the most striking phenotype of FA kinase knockout strains is the lack of α-hemolysin production indicating a novel role for FA kinase in controlling the production of virulence factors (6). A genome-wide analysis shows FA kinase-null strains are deficient in virulence factor transcription controlled by the SaeRS two-component system (2). The absence of FA kinase leads to a severe depression in the transcription of virulence factor genes due to the inhibitory effect of cellular FA on SaeRS signaling (3). The structure of *S. aureus* FakB2 bound to oleic acid (18:1) is a model for the functional importance of the highly conserved residues in the FakB protein family (7). The acyl chain of FakB2 (18:1) is completely buried in the protein interior with only the carboxyl group exposed at the protein surface. Ser-93, Thr-61, and His-266 form a conserved hydrogen bond network that fixes the position of the FA carboxyl moiety. In addition, Arg-202 is a conserved surface residue that is required for FakB2 to interact optimally with FakA (7).

*S. aureus* has two FakB proteins: FakB1 that binds saturated FA and FakB2 that is specific for monounsaturated FA (2). Other bacteria have between one and four FakB homologs, but the physiological roles of multiple FA-binding proteins and the structural basis for acyl chain selectivity remain open questions. Here, we report the structures of *S. aureus* FakB1 bound to four different straight and branched-chain saturated FA produced.
by the bacterium. FakB1 and FakB2 have acyl chain binding tunnels with distinctly different shapes that account for the acyl chain selectivity of the two proteins, but the protein surfaces are conserved. This allows the two FakBs to not only interact with FakA, but also with the two downstream enzymes that utilize FakB(FA/H11011P) as substrates in bacterial lipid metabolism (PlsX and PlsY). Cellular thermal shift experiments performed with growing S. aureus suggest that FakB1 normally has a bound FA, whereas FakB2 does not. These data lead to a model where FakB1 functions in cellular lipid homeostasis in the absence of exogenous FA, whereas the key function of FakB2 is to activate environmental 18:1/H90049, an abundant host FA that S. aureus does not produce but is incorporated into membrane phospholipids by the FA kinase pathway.

**Results**

**FakB1 structure**

A single FakB1-FA species for crystallization trials was obtained by exchanging 16:0 into purified FakB1 and removing excess FA by gel-filtration chromatography (7). FakB1(16:0) crystallized in space group P1 with two monomers in the asymmetric unit, and the crystal diffracted to 1.83 Å. The FakB1(16:0) structure shows an overall two-domain protein fold very similar to those of other FakB protein family members (Fig. 1A and Table 1) (7–9). The N-terminal domain contains a six-stranded β-sheet flanked by two α-helices on one side and three on the other side (Fig. 1A). The structures of FakB1(16:0) and the previously determined FakB2(18:1) superimpose very well (Fig. 1A). Arg-205 in FakB1 is in the equivalent position to Arg-202 in FakB2 that is required for high-affinity binding to FakA (7) and presumably mediates the same interaction in FakB1. An exposed flexible loop adjacent to the FA carboxyl group was not visible in the FakB2(18:1) structure (residues 174–183), but this loop in FakB1 (16:0) (residues 176–187) is resolved in one of the two monomers of the asymmetric unit (monomer A) (Fig. 1A, Loop). This loop is adjacent to Arg-205 and may also be involved in the interaction with FakA, although its primary sequence is not conserved when compared with the FakB2 loop.

An overlay of the FakB1(16:0) and FakB2(18:1) structures centered on the FA carboxyl group reveals that the carboxyl-binding sites are essentially identical (Fig. 1B). The FA-only calculated alignment was performed with PSCD/MCSALIGN plugin for PyMOL version 1.8 (Schrödinger, LLC). The tunnel residues are presented as semitransparent sticks only and the fatty acids as ball and sticks. Water molecules are single colored spheres. Hydrogen bond networks are presented as dotted lines between atoms. C, positioning of Arg-173 and the hydrogen bond network that connects the guanidinium group to a constellation of structured water molecules and the fatty acid carboxyl group.

![Figure 1](image-url)
Structure and function of *S. aureus* FakBs

Table 1

Data collection statistics for FakB1–fatty acid complexes

| Fatty acids | 14:0 | 15:0 | 16:0 | 17:0 |
|-------------|------|------|------|------|
| PDB codes   | 5WO0 | 6ALW | 5UTO | 6B9I |

Data collection

| Beamline | SER-CAT 22-ID | SER-CAT 22-BM | SER-CAT 22-ID | SER-CAT 22-BM |
|----------|---------------|---------------|---------------|---------------|
| Temperature (K) | 100 | 100 | 100 | 100 |
| Wavelength (Å) | 1.000 | 1.000 | 1.000 | 1.000 |
| Space group | P1 | P1 | P1 | P1 |
| Unit cell parameters (Å) | | | | |
| a, b, c | 33.39, 54.45, 84.91 | 33.35, 54.53, 84.42 | 33.19, 54.29, 84.40 | 32.99, 54.17, 83.90 |
| Resolution range (Å) | 81.73–1.78 | 81.18–1.63 | 48.83–1.83 | 80.87–1.93 |
| Rmerge | 0.086 (0.676) | 0.184 (0.906) | 0.067 (0.641) | 0.135 (0.629) |
| Rfree | 0.051 (0.395) | 0.106 (0.524) | 0.046 (0.445) | 0.080 (0.592) |
| No. of observations | 194,540 (41,418) | 263,005 (13,084) | 141,973 (8479) | 150,843 (5254) |
| No. of unique reflections | 50,586 (2913) | 66,112 (3294) | 46,256 (2815) | 39,271 (2578) |
| Completeness (%) | 96.2 (95.9) | 97.2 (95.3) | 97.9 (96.4) |
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| Completeness (%) | 96.2 (95.9) | 97.2 (95.3) | 97.9 (96.4) |

Model quality

| | 15.1/20.0 | 15.4/19.6 | 15.8/19.1 | 16.6/20.6 |
|---|---|---|---|---|
| Root mean square deviations | | | | |
| Bond lengths (Å) | 0.006 | 0.010 | 0.003 | 0.003 |
| Bond angles (°) | 0.801 | 0.961 | 0.540 | 0.550 |
| Coordinates error (ML, Å) | 0.17 | 0.18 | 0.19 | 0.21 |
| Protein residues | 575 | 569 | 575 | 561 |
| Average B-factor (Å²) | 27.54 | 16.46 | 25.95 | 22.63 |
| All atoms | | | | |
| Protein atoms | 25.65 | 13.58 | 24.62 | 21.43 |
| Fatty acid atoms | 24.15 | 24.23 | 15.68 | 30.86 |
| Solvent atoms | 40.97 | 31.24 | 36.11 | 32.79 |
| Ramachandran plot | 98.59 | 98.22 | 97.36 | 98.92 |
| Favored (%) | 1.23 | 1.42 | 1.76 | 1.08 |
| Allowed (%) | 0.18 | 0.36 | 0.88 | 0.08 |
| Outliers (%) | 6.29 | 6.21 | 3.42 | 2.64 |
| Clashscore | | | | |

Data collection statistics for FakB1–fatty acid complexes

The abbreviations are: myristic acid (14:0), 12-methyltetradecanoic acid (a15:0), palmitic acid (16:0), and 14-methylhexadecanoic acid (a17:0) are shown. Values in parentheses are for the highest resolution shell. ML is maximum likelihood.

Data collection for FakB1 complexes with 14:0, a15:0, and a17:0 FA

Multiple saturated straight and branched-chain FA are found in *S. aureus* phospholipids, which suggests that the FakB1 hydrophobic acyl chain binding tunnel should be flexible enough to accommodate this spectrum of FA. The structures of FakB1 in complex with 14:0, a15:0, and a17:0 FA were obtained by exchanging the indicated FA into FakB1, and re-purifying the protein complexes by gel-filtration chromatography to remove unbound FA and detergent prior to crystallization (7).

The electron densities of all four of the FA in the FakB1 structures are illustrated in Fig. 2A. The FakB1 tunnel is a slightly curved space that expands and contracts to accommodate the different FA chain lengths. This movement arises at the terminus of the FakB1 FA binding tunnel through the re-positioning of the Ile-233 side chain that functions like a “swinging gate” that undergoes a hinge-like motion to either increase or decrease the length of the FA-binding channel (Fig. 2, B and C). This movement of Ile-233 allows the tunnel to easily accommodate the two additional carbons in the FakB1(16:0) structure compared with the FakB1(14:0) structure. Branched-chain anteiso-FA (a15:0 and a17:0) are major constituents of *S. aureus* phospholipids, and they are exclusively the S stereoisomer (11). When a racemic mixture (S and R) of a15:0 was exchanged into FakB1 prior to crystallization, both stereoisomers were represented in the electron density map, although there was a preference for the S isomer (60% S and 40% R). In contrast, only the S isomer was observed in the FakB1 tunnel when the experiment was performed using racemic a17:0. Thus, the additional carbon atoms in a17:0 clearly impose a stricter chain-length and stereospecificity within the FakB1 tunnel. These data show that the binding tunnel in FakB1 is structurally tuned to accommodate the major straight and branched-chain FA produced by *S. aureus*.

There are many FakB structures in the PDB database, but not all are refined with FA in the acyl-chain binding tunnel. This is due in part to the structures being determined prior to the discovery that they are FA-binding proteins in the FA kinase system and that FakBs may bind multiple FA. If care is not taken to exchange a single FA into the pocket, the electron density will reflect the mixture of FA structures that were picked up by the protein during the protein expression. Purified FakBs must have a ligand bound in the pocket to stabilize the protein and allow it to be manipulated *in vitro*. We examined and re-refined two deposited FakB structures, from *Ruminococcus gnavus* (old...
PDB code 3JR7) and Eubacterium eligens (old PDB code 3FDJ), to determine whether there is a FA present and to establish its identity (Table 2). Both structures have PEG modeled in the tunnel. Both structures indeed have a bound FA that makes similar interactions to those described above for the S. aureus FakBs. R. gnavus FakB (new PDB code 5V85) was successfully refined with cis-vaccenic acid (18:1/H9004)11, a major monounsaturated FA of E. coli, and E. eligens was refined with a saturated FA, 17:0 (new PDB code 5UXY). These analyses suggest that all the deposited FakB structures will turn out to have a FA, or a mixture of FA, bound in their tunnels.

Comparison of the FakB1(16:0) and FakB2(18:1) structures

Despite the similarities in the overall structures of FakB1 and FakB2, the two proteins clearly possess different internal organizations that create FA binding tunnels with distinctly different shapes (Fig. 2, D and E). The most significant difference is that, whereas the FakB1 tunnel is slightly curved, the FakB2(18:1/H9004)9 tunnel has a sharp turn at the 9-carbon of the bound FA to accommodate the kink in the acyl chain that is present due to the 9-cis conformation of the double bond. This significant difference is accomplished by amino acid alterations at key locations within the proteins’ hydrophobic cores to create these specific tunnel shapes. Most notably, the FakB1 residues Phe-193, Ile-198, and Phe-263 occupy the region where the hydrocarbon chain following the cis double bond is found in the FakB2 tunnel, whereas FakB2 residues Gly-273, Val-275, and Leu-155 occupy the space where the distal end of the saturated FA tunnel in FakB1 is located. Ile-198 of FakB1 sits under the

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**Figure 2. FakB1 acyl chain binding tunnel.** A, isolated 2Fo − Fc electron densities of the FakB1 FA contoured at the 1σ level. From top to bottom: teal = 14:0; green = a15:0; gray = 16:0; orange = a17:0. In the structures, the a17:0 is only observed in the naturally occurring S enantiomeric form, whereas the a15:0 fatty acid occurs in both enantiomeric forms. B and C, orthogonal views of the FakB1 FA tunnel loaded with C14:0 (teal) and S-a17:0 (orange). The FA are represented as balls and sticks in the middle of both images. C, black dotted line between the CD1 carbon atoms of the two conformations of Ile-233 shows how it moves 1.9 Å to accommodate the longer a17:0 FA. In addition, the structural alignment reveals that Ala-158 and Leu-160 at the end of the tunnel are shifted by ~0.5 Å to fit the longer a17:0 (orange) compared with 14:0 (teal). The FA-only alignment was performed with PSICO/MCSALIGN plugin for PyMOL 1.8 (Schrödinger, LLC). D and E, comparison of the distal ends of the FA tunnels of FakB1(16:0) (PDB code 5UTO) (gray, D) and FakB2(18:1,9) (PDB code 4X9X) (blue, E). The FA are represented as balls and sticks. B–E, the meshes delineate the cavities created within the protein to accommodate the FA and were computed with CAVER/PyMOL.
Table 2
Re-analysis of two PDB entries to identify bound fatty acid
Available data collection statistics from the original PDB entries and refinement of the two PDB entries with heptadecanoic acid (17:0) and cis-vaccenic acid (18:1Δ11) are shown. NA means not applicable.

| Fatty acids | 17:0 | 18:1Δ11 |
|-------------|------|--------|
| PDB codes   | 5UXY (corrected 3FDJ) | 5V85 (corrected 3JR7) |
| Data collection | | |
| Temperature (K) | 100 | 100 |
| Wavelength (Å) | 0.97942–0.97929 | 0.9794 |
| Space group | P 4 1 2 2 | P 3 1 2 1 |
| Unit cell parameters (Å) | | |
| a, b, c | 57.45, 57.45, 186.56 | 96.90, 96.90, 159.92 |
| x, y, z | 90.00, 90.00, 90.00 | 90.00, 90.00, 120.00 |
| Resolution range (Å) | 48.91–1.80 | 83.92–2.00 |
| R/merge | 0.132 (0.804) | (0.352) |
| No. of unique reflections | 30.039 (1990) | 58.785 (4131) |
| Multiplicity | 7.7 (7.8) | |
| Mean I/σ(I) | 19.9 (2.0) | 17.9 (2.1) |
| Mean (I) half-set correlation | NA | NA |
| Completeness (%) | 99.5 (97.2) | 99.4 (99.5) |
| Wilson B-factor (Å²) | 18.71 | 22.56 |

Model quality

| | Rmerge | Rfree (%), value (%) | Root mean square deviations | Bond lengths (Å) | Bond angles (°) | Coordinates error (maximum likelihood, Å) | Protein residues | Average B-factor (Å²) |
|---|---|---|---|---|---|---|---|---|
| All atoms | 15.3/19.6 | 16.5/19.8 | 0.017 / 1.403 | 0.003/0.533 | 0.18 | 0.19 | 0.18 | 276 |
| Favored (%) | 98.54 | 97.81 | 97.81 | 97.81 | 97.81 | 97.81 | 97.81 |
| Allowed (%) | 1.46 | 2.19 | 2.19 | 2.19 | 2.19 | 2.19 | 2.19 |
| Outliers (%) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Clashscore | 3.21 | 4.09 | 4.09 | 4.09 | 4.09 | 4.09 | 4.09 |

FA in Fig. 2D at the position where the kink in the tunnel occurs in FakB2. Whereas the kinked 18:1Δ9 chain cannot fit within the slightly curved hydrophobic tunnel of FakB1, the flexible 16:0 chain should be able to fit into the kinked FakB2 tunnel.

FakB delivery of FA~P to PlsY and PlsX

We have been unable to isolate FakB proteins without a bound FA. The FA remains bound even if there is a compatible FA present in phospholipid vesicles or detergent micelles with which it can exchange (7). FakB that lacks a FA is unstable in vitro and readily precipitates from solution. The importance of the interactions between the protein and the FA carboxyl is critical for the thermal stability of FakB2 (7). The deletion of one hydrogen bond in the FakB2(S93A) mutant lowers the denaturation temperature from 50 to 33 °C (7), and the absence of the FA would result in proteins even more unstable because the entire stabilizing hydrogen bond network would be eliminated. For example, FakB2 expression in *Escherichia coli* with 18:1 in the medium results in high levels of protein expression and the recovery of 100% soluble FakB2(18:1). The same experiment in the absence of 18:1 in the medium achieved the same level of protein expression, but it only results in 50% recovery of soluble FakB2 that contained 18:1 obtained from the *E. coli* host (data not shown). However, in vitro, FA-free FakB proteins must exist because FakB(FA∼P) is used as an acyl donor for at least two downstream enzymes. To confirm this, we first determined whether PlsY utilizes FA∼P from FakB2(FA∼P) (Fig. 3A). Assays containing FakA, FakB2(18:1), [14C]glycerol-phosphate, and membranes derived from *S. aureus* strain PDJ39 (ΔpIsX) containing PlsY resulted in the rapid formation of LPA. The formation of LPA was saturable, suggesting that the transfer of FA∼P occurred by FakB2 binding to PlsY (Fig. 3B). Although these data are consistent with FA∼P bound to FakB2 being directly transferred to PlsY, one cannot completely rule out that the FA∼P bound to FakB2 was first deposited in the membrane where it subsequently encountered the acyltransferase. To rule out this possibility, we next examined the transfer of FA∼P bound to FakB2 to a soluble protein partner, PlsX. We exchanged [14C]18:1 onto FakB2 and then isolated the FakB2([14C]18:1) complex by gel-filtration chromatography to remove unbound [14C]18:1. Assays with PlsX and ACP led to the complete conversion of the 18:1~P from FakB2 to acyl-ACP (Fig. 3C). Gel-filtration chromatography was also used to demonstrate that all the FakB2([14C]18:1) was transferred to [14C]18:1-ACP in a FakA-dependent manner (Fig. 3D). FakA, PlsX, and ACP are all soluble proteins, and there was no detergent in the assay. These data mean that FA-free FakB2 must be a product of the reaction and that FA∼P bound to FakB is removed from the protein through its interaction with downstream metabolic enzymes leaving FA-free FakB.
Status of the FakBs in vivo

We developed a cellular thermal shift assay (CETSA) to investigate whether FakB contains a bound FA in vivo. Ligand binding typically stabilizes proteins to thermal denaturation, and the CETSA technique is commonly used in drug discovery to monitor drug-target engagement inside cells (12, 13). If FakB has a bound FA, it will be more stable to thermal denaturation than in the absence of FA (7). These in vivo experiments required the generation of antibodies that specifically detect either FakB1 or FakB2. We generated polyclonal rabbit antibodies and purified the IgG fraction by affinity chromatography on FakB1- or FakB2-Sepharose. These affinity-purified antibodies only signaled the protein of interest in immunoblots of whole S. aureus extracts (Fig. 4). Anti-FakB1 did not cross-react with FakB2 (Fig. 4A), and anti-FakB2 did not cross-react with FakB1 (Fig. 4B). We began by analyzing FakB2 because this FA-binding protein prefers 18:1, a FA that is abundant in mammalian hosts but is not produced by S. aureus. The addition of 18:1 to the culture medium resulted in a significant stabilization of FakB2 to thermal denaturation (Fig. 5A). The results from three independent replicates showed that there was a shift of 6.2 °C in the stability of FakB2 following exposure to 18:1. These data indicate that a significant proportion of FakB2 does not contain a bound FA unless 18:1 was provided in the medium. We next performed the reverse experiment. Cells were first grown with oleate, and a sample was obtained. Then, the 18:1 was removed from the medium; the cells were grown for 1 h to deplete the cellular 18:1 by incorporation into phospholipid, and the second sample was harvested. The FakB2 population was stabilized to heat denaturation in the presence of 18:1 and returned to its less stable state following the removal of oleate from the medium (Fig. 5B). These data are consistent with the majority of FakB2 existing in the cell without a bound ligand, unless 18:1 was supplied in the medium. FakB1 behaved differently. FakB1 had a high-thermal stability in vivo, and there was no change in the FakB1 status.
when the cells were challenged with 16:0 (Fig. 5C). The lack of a FakB1 response to exogenous 16:0 suggests that a significant proportion of the protein possessed a bound FA even in the absence of an exogenous FA supplement. FakB2 was stabilized by 7.8 °C following a 16:0 challenge (Fig. 5D), consistent with the ability of FakB2 to bind both 18:1 and 16:0 (see below). These data indicated that FakB2 exists mostly in a ligand-free state in vivo, whereas a high proportion of FakB1 contains a bound FA suggesting a role for FakB1 in recycling the saturated FA produced by S. aureus into phospholipid (3).

We next performed the same experiment using a plasmid system to increase the FakB1 cellular concentration. After the addition of 16:0, the FakB1 thermal stability increased by 4.5 °C (Fig. 5E). The stability of overexpressed FakB1 in the presence of 16:0 (Fig. 5E) was equivalent to the stability of normal levels of FakB1 in the absence of a FA supplement (Fig. 5C). The stability of FakB1 was lower in the absence of 16:0 in this experiment suggesting that with the higher levels of FakB1 expression, a proportion of the FakB1 was ligand-free unless 16:0 was added to the medium.

Physiological roles of FakB1 and FakB2 in extracellular FA activation

The physiological roles of FakB1 and FakB2 in exogenous FA activation for phospholipid synthesis were examined by labeling a series of mutant S. aureus strains for 30 min with an equimolar mixture of [D4]16:0 and 18:1, and measuring the synthesis of phosphatidylglycerol (PG) molecular species from these exogenous FA precursors (Fig. 6). Strain AH1263 (WT) exhibited significant incorporation of both FA into PG molecular species (Fig. 6A). [D4]16:0 was elongated to [D4]18:0 and [D4]20:0, whereas 18:1 was elongated to 20:1. Thus, both FA were activated to FA/H2011P and were either used immediately for phospholipid synthesis by PlsY or converted to acyl-ACP by PlsX and elongated by S. aureus FASII prior to being converted again to FA/H2011P for incorporation into the 1-position by PlsY. A few percent of [D4]16:0 was converted to acyl-ACP and used by PlsC to create the new 18:1/[D4]16:0 PG molecular species (Fig. 6A, green peak). In strain JLB27 (∆fakB1), the amount of [D4]16:0 incorporation was decreased and 18:1 incorporation...
increased, consistent with the preference of FakB2 for 18:1 coupled with its ability to also utilize 16:0 (Fig. 6, B and D). Strain JLB27 retained the ability to incorporate [D4]16:0 albeit at lower levels than in the WT strain. In strain JLB28 (ΔfakB2), the incorporation of 18:1 was severely compromised (Fig. 6, C and D). The JLB2 (ΔfakA) and JLB31 (ΔfakB1,ΔfakB2) double knockout strains did not incorporate exogenous FA into phospholipid (Fig. 6D), consistent with FA kinase as the only pathway for exogenous FA activation in S. aureus. FakB2 prefers unsaturated FA (18:1), but can also utilize saturated FA (16:0), whereas FakB1 used saturated FA (16:0), but was severely compromised in the activation of unsaturated FA (18:1). These results confirm the physiological impact of the differing FakB substrate selectivities on the utilization of exogenous FA for phospholipid synthesis.

Biochemical FA kinase assays were used to evaluate the selectivities of the FA kinase system for the two FA in the absence of downstream metabolism (Fig. 7). FakB2 was able to phosphorylate both [14C]16:0 and [14C]18:1 with about equal efficiency under these assay conditions (Fig. 7A), but FakB1 was defective in supporting the phosphorylation of [14C]18:1 (Fig. 7B). The in vivo selectivity for FA type in Fig. 6 was more stringent than observed in these in vitro assays. However, in vivo, FakA may not be the rate-determining step in phospholipid synthesis from exogenous FA. This point was addressed by performing experiments as described in Fig. 6 using a 30 μM concentration of a single FA (Fig. 7C). In this type of experiment, there was no difference between the uptake of [D4]16:0 and 18:1 by strain JLB27 (ΔfakB1) (Fig. 7C), consistent with the biochemistry experiment (Fig. 7, A and B). In strain JLB28 (ΔfakB2), 18:1 incorporation was severely impaired (Fig. 7C), although the amount of 16:0 incorporated in this knockout strain remained robust. These experiments confirmed that unsaturated FA are prevented from interacting with the substrate binding tunnel of FakB1. FakB2 prefers to bind unsaturated FA but did not exclude the flexible saturated FA that can assume the shape of the kinked tunnel.

Discussion

Our results lead to a model for the function of FakBs in S. aureus physiology diagrammed in Fig. 8. FakB1 binds straight, and branched-chain saturated FA that are produced by the S. aureus FA synthase. In the absence of FA kinase activity, the intracellular FA pool rises indicating that one housekeeping function of the kinase is to activate these FA so they can be recycled into phospholipid (3). The metabolic origin of the S. aureus FA pool remains unknown, but they are all saturated FA, and FakB1 has the substrate selectivity consistent with a
role in activating these FA. The CETSA results suggest that a significant proportion of FakB1 may be bound to FA during *S. aureus* growth, consistent with a role for FakB1 in the recycling of saturated FA to maintain lipid homeostasis. *S. aureus* does not synthesize unsaturated FA, and the CETSA results indicate that a significant proportion of FakB2 is not bound to a FA unless 18:1 is supplied in the medium. Oleate (18:1) is an abundant mammalian FA that is present at *S. aureus* infection sites. FA synthesis is an energy-intensive process, and the expression of the two FA-binding proteins enables *S. aureus* to utilize both saturated and unsaturated host resources for the formation of phospholipids during growth at the infection site.

This work shows that distinct differences within the hydrophobic protein cores of bacterial FA-binding proteins account for the FA acyl chain selectivity characteristic of the FakB proteins. FakB1 binds a series of saturated and branched-chain FA that are produced by *S. aureus* within a linear hydrophobic tunnel buried within the FakB1 core. Ile-233 at the end of the binding tunnel acts as a flexible plug that repositions to allow binding of 14–17 carbon chain lengths. Unsaturated FA have a kinked structure due to the *cis* double bond, making it a poor fit for the FakB1 tunnel. This accounts for the low rate of FakB1-dependent FA kinase activity *in vitro* when oleate is the substrate and for the compromised ability of strains that only express FakB1 to incorporate exogenous 18:1 into phospholipid. In contrast, the FakB2 tunnel is almost an exact match for the conformation of 18:1. This tunnel architecture prefers to bind 18:1, but it does not exclude saturated FA because these FA are flexible enough to negotiate the kinked tunnel. Cells expressing only FakB2 incorporate both saturated and mono-unsaturated FA, although FakB2 shows a clear preference for 18:1 when presented in a FA mixture. We conclude that FakB1 is the primary housekeeping FA-binding protein responsible for activating cellular saturated FA that arise from lipid metabolism, and FakB2 functions to activate and exploit unsaturated FA that are available at the infection site (Fig. 8).

The existence of FA-specific binding pockets is a feature of bacterial FakBs that is not shared by the mammalian FA-binding proteins, which bind a range of FA chain lengths and unsaturation (14, 15).

Although the bacterial FA-binding protein family is characterized by diverse hydrophobic protein interiors that accommodate specific FA structures, these variations within the FakB interiors do not extend to the FakB exterior surface. These surface features are critical for protein–protein interactions with FakB-binding partners. All FakB homologs interact with a single FakA kinase domain protein, and a conserved arginine (Arg-205 in FakB1 and Arg-202 in FakB2) has a key role at the docking site (7). In addition, the FakB(FA—P) can donate FA—P to either PlsX or PlsY, illustrating that FakBs have at least two other conserved protein partners in addition to FakA. Some bacteria express three (*Streptococcus pneumoniae*) or four (*Enterococcus faecalis*) FakBs, and it is likely that each of these individual proteins possesses a different organization of interior hydrophobic residues to create tunnels that selectively bind a specific subset of FA structures.

### Experimental procedures

#### Bacterial strains and reagents

The bacterial strains and their origins are listed in Table 3. The FakB1 expression plasmid was constructed by ordering a DNA string from Invitrogen based on the *fakB1* sequence of strain AH1263 that was cloned into BamHI and XbaI sites of plasmid pG164 to yield pPJ464 and sequenced. The expression plasmid was transformed into strain Sa178RI by electroporation and selected on chloramphenicol plates. [14C]16:0 (specific activity, 55 mCi/mol) and [14C]18:1 (specific activity, 59 mCi/mol) were purchased from PerkinElmer Life Sciences. [14C]Glycerol-phosphate (specific activity, 50 mCi/mol) was purchased from American Radiochemicals. FA were purchased from Sigma, and 7,7,8,8- tetradeuteriohexadecanoic acid ([D4]16:0) was obtained from Cambridge Isotope Laboratories, Inc.

#### Protein crystallization and structure determination

Crystals of FakB1(FA), all in space group P1 with very similar cell dimensions, were obtained by mixing equivalent volumes in a sitting drop of protein solution at 10–20 mg/ml concentration (100 mM NaCl, 10 mM Tris, pH 7.5) with the precipitant 0.1M MES, imidazole, pH 6.5, 0.03 M sodium nitrate, 0.03 M disodium hydrogen phosphate, 0.03 M ammonium sulfate, and 12.5% PEG 1000, 12.5% PEG 3350, 12.5% MPD. Crystals typically appeared within 5–7 days. The crystals were cryoprotected with a precipitant mother liquor containing 12.5% glycerol and frozen in liquid nitrogen prior to data collection. X-ray diffraction data were collected on single crystals at SER-CAT beamline ID22 and BM22 at APS Argonne. Diffraction data were integrated with XDS (16, 17) and scaled using AIMLESS/CCP4 (18) to 1.63–1.93 Å resolution depending on the sample. The FakB1(16:0) structure was first determined by molecular replacement using a polyalanine version of the analog PDB database model 4X9X (FakB2) as the search model using PHASER-MR/CCP4 (19). The final refined model of FakB1 contained two monomers per asymmetric unit, each containing 16:0 (Table 1). Subsequent FakB1 structures were determined by molecular replacement with MOLREP (20) using this initial structure (Table 1). For the re-refinement of PDB structures 3JR7 and 3FDJ, the PEG molecules originally modeled in the FakB tunnels were replaced by fatty acid molecules, and the structure factors (.cif files) were obtained from the PDB data-

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**Table 3**

| Description | Ref. |
|-------------|-----|
| IPTG is isopropyl 1-thio-β-d-galactopyranoside |  |
| Strain | |
| AH1263 | USA300 Nebraska collection |
| JLB2 | fakA of strain AH1263 |
| JLB27 | fakB1-Δfak of strain AH1263 |
| JLB28 | fakB2-Δfak of strain AH1263 |
| JLB31 | Derived from RN4220 |
| Sa178RI | ΔpdxX of strain Sa178RI |
| Plasmids | |
| pG164 | IPTG-inducible vector |
| ppJ464 | IPTG-inducible FakB1 expression |

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*Structure and function of S. aureus FakBs*
Expression, purification, and FA exchange

FakA, FakB1, and FakB2 were purified as described previously (2). Purified FakB1 was loaded with a mixture of saturated FA found in the E. coli expression system (mostly 16:0). FakB1 with specific FA bound were prepared as described (7). Briefly, FA were added during cell lysis before protein purification, and the protein isolated after Ni²⁺-affinity purification was subjected to gel-filtration chromatography and concentrated for crystallization studies. S. aureus PlsX was purified as described in Ref. 1. PlsY membranes were purified as described in Ref. 25. Briefly, strain PDJ39 (ΔplsX) was grown to an A₆₀₀ of 2.0 and lysed with 5 mg/ml lysostaphin in 20 mM potassium phosphate buffer, pH 7.5, 5 mM EDTA, 50 mM KCl, 10 mM MgCl₂ with protease inhibitor mixture at 37 °C for 1 h. Cells were further disrupted with a French press, and the cell debris was removed by centrifugation at 10,000 × g for 10 min. Membranes were isolated by centrifuging the supernatant at 200,000 × g for 45 min, and the membrane pellet was resuspended in 20 mM potassium phosphate buffer, pH 7.5, 50 mM KCl, 10 mM MgCl₂ and used for the experiment.

PlsX and PlsY assays

To study the interaction of FakB2 and PlsX, FA kinase assay with 0.5 μM FakA and 5 μM FakB2 exchanged with [¹⁴C]18:1 was added to a reaction mix containing 100 mM Tris, pH 7.5, 20 mM MgCl₂, 10 mM ATP and incubated at 37 °C for 30 min. To this either 25 μM PlsX or 25 μM PlsX and 250 μM S. aureus ACP was added in a final reaction volume of 50 μl and incubated at 37 °C for 20 min. Aliquots (40 μl) were spotted on Whatman 3MM disks and washed twice with chloroform/methanol/acetetic acid (3:6:1, v/v) mixture (20 ml per disk) for 20 min for each wash. The disks were dried and counted by scintillation counting. The experiments were performed twice in duplicate.

Gel filtration

To test the transfer of fatty acid from FakB2 to ACP via PlsX, 10 μM FakB2 exchanged with [¹⁴C]18:1 was added to a reaction mix containing 100 mM Tris, pH 7.5, 20 mM MgCl₂, 10 mM ATP with or without 0.5 μM FakA and incubated at 37 °C for 30 min. To this, 25 μM PlsX and 250 μM ACP was added to a final reaction volume of 100 μl and incubated at 37 °C for 20 min. This was loaded onto a Sephadex S-75 column and eluted with 20 mM Tris, pH 7.5, 200 mM NaCl. 200-μl fractions were collected, and 100 μl was counted by scintillation counting. The experiment was performed twice.

Antibody generation and testing

The antibodies for FakB1 and FakB2 were generated in rabbit against purified His-tagged FakB1 and FakB2 proteins by Rockland Antibodies Inc. The specificity of the FakB1 and FakB2 antibodies were analyzed using extracts from strains AH1263 (WT USA300), JLB27 (ΔfakB1), JLB28 (ΔfakB2), and JLB31 (ΔfakB1 and ΔfakB2). Lysates were resolved using a 10% bis-tris acrylamide SDS gel and transferred to a polyvinylidene difluoride membrane. The blots were blocked for 1 h in 1% milk/TBS-T and then exposed to primary antibody (FakB1 or FakB2 as indicated in Fig. 4) overnight at 1:1000 dilution in 1% BSA/TBS-T followed by secondary antibody (anti-rabbit AP-conjugated, GE Healthcare ECF Western blotting reagent pack) in 1% milk/TBS-T for 1 h at 1:5000 dilution. The blot was washed extensively and exposed to the ECF substrate for 5 min, and the bands on the dried membrane were quantified on the Typhoon FLA9500 using ImageQuant TL software (GE Healthcare).

Cellular thermal shift assay (CETSA)

Strains Sa178RI or AH1263 were grown to an A₆₀₀ of 0.5 in Luria broth containing 10 mg/ml FA-free bovine serum albumin (BSA) and treated with either DMSO or 500 μM FA (18:1 or 16:0) in DMSO as indicated in Fig. 5 for 40 min. Cells were harvested and resuspended in resuspension buffer (20 mM Tris, pH 8.0, 200 mM NaCl, and protease inhibitors) such that the A₆₀₀ is 1.0 in the buffer. Aliquots (100 μl) in 0.5-ml reaction tubes were exposed to various temperatures (room temperature and 42, 45, 48, 51, 54, 57, 60, and 63 °C) in pairs (control and FA-treated) using a thermocycler PCR machine for 3 min, then moved to room temperature for another 3 min before being flash-frozen in liquid nitrogen, and stored at −80 °C. For the FA–pre-loaded experiments, cells were inoculated at an A₆₀₀ of 0.05, and 500 μM FA was added to the media containing 10 mg/ml FA-free BSA. Cells were grown to an A₆₀₀ of 0.5, then either washed with media containing 10 mg/ml FA-free BSA or not washed and grown further for two doublings. Growing cells were harvested and processed as described above. Strain Sa178RI/pPDJ464 was grown; the expression of FakB1 was induced with 200 μM isopropyl 1-thio-β-D-galactopyranoside in the medium, and the cells were grown with FA or DMSO and processed as described above. The frozen cell samples from each thermal denaturation point were lysed by adding 2 μl of lysostaphin (5 mg/ml) followed by incubation on ice for 15 min and then sonicated at 4 °C for 10 s. The lysates were centrifuged at 14,000 × g for 20 min to remove precipitated proteins. Immunoblotting was performed, and the bands were quantified as described above. All the experiments were repeated twice.
and the data shown is a representative graph from one of the blots.

**FA incorporation**

Overnight cultures were used to inoculate 5 ml of fresh Luria broth (LB) plus 0.1% Brij58 to an \( A_{600} \) of 0.04. Cultures were incubated at 37 °C to an \( A_{600} \) of 0.5 and were then split into 0.2% DMSO (control), 30 \( \mu M \) [D4]16:0 or -18:1 individually, or a mix of 15 \( \mu M \) [D4]16:0 + 15 \( \mu M \) 18:1 and incubated at 37 °C for 30 min. Cells were harvested and washed twice with LB and once with PBS, and lipids were extracted using the Bligh and Dyer method (26).

Lipid extracts were resuspended in chloroform/methanol (1:1). Phosphatidylglycerol (PG) was analyzed using a Shimadzu Prominence UFLC attached to a QTrap 4500 equipped with a Turbo V ion source (Sciex). Samples were injected onto an Acquity UPLC BEH HILIC, 1.7 \( \mu M \), 2.1 × 150-mm column (Waters) at 40 °C and a flow rate of 0.2 ml/min. Solvent A was acetonitrile, and solvent B is 15 mM ammonium formate, pH 3. The HPLC program was as follows: starting solvent mixture of 96% A, 4% B, 0–2 min isotropic with 4% B; 2–20 min linear gradient with 80% B; 20–23 min isotropic with 80% B; 23–25 min linear gradient with 4% B; 25–30 min isotropic with 4% B. The QTrap 4500 was operated in the negative mode, and the ion source parameters were as follows: ion spray voltage, -4500 V; curtain gas, 25 p.s.i.; temperature, 350 °C; collision gas, medium; ion source gas 1, 40 p.s.i.; ion source gas 2, 60 p.s.i.; and declustering potential, -40 V. The system was controlled by the Analyst® software (Sciex), and LipidView™ software (Sciex) was used to analyze and quantitate the PG molecular species.

The percentages of each peak in the mass spectra were obtained by calculating the area under each peak and then calculating the percent of the total molecular species that were synthesized from exogenous FA. [D4]16:0 uptake was determined by summing the peaks containing [D4]16:0, [D4]18:0, or [D4]20:0, and 18:1 incorporation were peaks containing either 18:1 or 20:1. The small peak containing both [D4]16:0 and -18:1 was counted as incorporation for both FA.

**Fatty acid kinase assay**

FA kinase assays were performed with 0.2 \( \mu M \) FakA and 20 \( \mu M \) [14C]16:0 or [14C]18:1, 100 \( \mu M \) Tris, pH 7.5, 20 mM MgCl\(_2\), 10 mM ATP, and 1% Triton X-100. After reactions were assembled, FakB1 or FakB2 was added at the indicated concentrations to initiate the reaction and incubated at 37 °C for 20 min. An aliquot (40 \( \mu l \)) of the reaction mixture was spotted onto DE81 Whatman filter paper discs. Discs were washed three times with ethanol, 1% acetic acid mixture for 20 min for each wash. Discs were then dried and counted by scintillation counting. Experiments were each performed two times in duplicate.

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