Permissive Fatty Acid Incorporation Promotes Staphylococcal Adaptation to FASII Antibiotics in Host Environments

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Graphical Abstract

Rationalized antibiotic challenge timeline: symptoms prior to treatment

Day 0  MRSA infection  Bacteria disseminate & adapt  Anti-FASII adapted  Treatment failure

Day 1  Start anti-FASII

S. aureus membrane  FASII fatty acids  Host-scavenged fatty acids  MRSA escape FASII antibiotics

Highlights

- A FASII-targeted antibiotic fails to clear MRSA in a mouse bacteremia model
- Treatment failure correlates with exogenous fatty acid (eFA) incorporation
- Serum favors eFA incorporation at both phospholipid positions even without antibiotic
- S. aureus FASII essentiality, a rationale for antibiotic development, is challenged

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In Brief

FASII antibiotics are emerging as potential alternative therapeutics with the rise of antibiotic resistance; however, their efficacy has been controversial. Kénanian et al. find host fatty acids can compensate MRSA inhibition, thwarting the efficacy of FASII inhibitors. Bacteria can scavenge and incorporate exogenous (host) fatty acids to enable anti-FASII adaptation.
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SUMMARY

The essentiality of fatty acid synthesis (FASII) products in the human pathogen Staphylococcus aureus is the underlying rationale for FASII-targeted antimicrobial drug design. Reports of anti-FASII efficacy in animals support this choice. However, restricted test conditions used previously led us to investigate this postulate in a broader, host-relevant context. We report that S. aureus rapidly adapts to FASII antibiotics without FASII mutations when exposed to host environments. FASII antibiotic administration upon signs of infection, rather than just after inoculation as commonly practiced, fails to eliminate S. aureus in a septicemia model. In vitro, serum lowers S. aureus membrane stress, leading to a greater retention of the substrates required for environmental fatty acid (eFA) utilization: eFAs and the acyl carrier protein. In this condition, eFA occupies both phospholipid positions, regardless of anti-FASII selection. Our results identify S. aureus membrane plasticity in host environments as a main limitation for using FASII antibiotics in monotherapeutic treatments.

INTRODUCTION

Fatty acid synthesis (FASII) pathway enzymes are priority targets for ongoing drug development against methicillin-resistant Staphylococcus aureus (MRSA) (Albanesi et al., 2013; Balemans et al., 2010; Escaich et al., 2011; Fischer et al., 2004; Hunt et al., 2016; Kaplan et al., 2012; Menetrey et al., 2019; Parsons and Rock, 2011; Pishchany et al., 2018; Schiebel et al., 2012, 2014; Yum et al., 2007). However, anti-FASII efficacy remains a critical point of debate (Brinster et al., 2009, 2010; Parsons et al., 2011). Remarkably, S. aureus FASII sensitivity versus tolerance hinges on a single issue: whether environmental fatty acids (eFAs) can occupy the presumably stringent 2-position of membrane phospholipids (the 1-position is permissive) (Parsons et al., 2011; Parsons and Rock, 2011). Anti-FASII resistance due to mutations in the target enzyme or to the horizontal transfer of an antibiotic-resistant gene homolog may occur and are often antibiotic specific (Ciusa et al., 2012; Yao et al., 2013). In contrast, resistant mutants that allow compensatory fatty acid utilization at both phospholipid positions were isolated and also found in clinical strains. These mutants map in FASII initiation genes distinct from the gene encoding the antibiotic target protein, and they confer cross-resistance to various FASII inhibitors (Gloux et al., 2017; Morvan et al., 2016, 2017). Despite the emergence of mutations, continued FASII-targeted drug development is rationalized by the accepted postulate that the general wild-type S. aureus population must synthesize branched-chain fatty acid anteiso 15:0 (a15) to complete membrane phospholipids (Albanesi et al., 2013; Escaich et al., 2011; Hunt et al., 2016; Kaplan et al., 2013a; Parsons et al., 2011; Pishchany et al., 2018; Schiebel et al., 2012, 2014; Yum et al., 2007). We investigated this postulate and report here on an alternative antibiotic adaptation mechanism that is functional in host environments, which enables S. aureus growth in FASII antibiotics.

RESULTS

A FASII Antibiotic Does Not Clear S. aureus Infection in a Septicemia Murine Model

Results of animal tests are decisional checkpoints for antibiotic development. FASII antibiotic challenge tests to date administer treatments within minutes to a few hours post-infection (summarized in Morvan et al., 2017), i.e., prior to bacterial dissemination to host organs and before clinical symptoms would call for antibiotic treatment (Leekha et al., 2011; Surewaard et al., 2016).
No bacterial counts were increased 10-fold in kidneys (to 5 \times 10^6) as an antibiotic, group 2 received AFN-1252 or vancomycin (control for antibiotic efficacy) was administered at T16 h when mice first showed signs of infection (lethargy, ruffled fur) and at regular intervals thereafter until animals were euthanized. (B) CFUs were determined in organs upon sacrifice, at T40 h, and at T88 h. The above data are pooled from two independently performed experiments. In total, n = 18 per type of treatment per experiment. Black lines indicate median CFU corrected to per organ. **p < 0.01; *p < 0.05; ns, non-significant. (C) AFN treatment leads to a selective loss of a S. aureus fakB2 mutant defective for eFA incorporation. Mice (n = 14 per group) were infected with 1 \times 10^7 total CFU of a 1:1 mixture of USA300 and fakB2 strains and were untreated or treated with AFN-1252 starting 16 h post-infection as above. Animals were sacrificed at 88 h, and CFUs were determined in kidney, liver, and spleen. The average ratios of USA300:fakB2 for each organ were determined for the indicated number of mice and shown with p values. Organs without detectable CFUs were excluded from scoring. p values were analyzed using (footnote a) the paired and non-parametric Wilcoxon test and (footnote b) the non-paired non-parametric Mann-Whitney test.

This consideration guided the design of the infection and treatment protocol used here (Figure 1A). MRSA strain USA300 was administered by the intravenous route. Antibiotic treatments were initiated 16 h (T16) post-infection, at which time animals exhibited signs of sickness (lethargy and ruffled fur). Group 1 received no treatment. As an antibiotic, group 2 received AFN-1252, a pipeline FASII inhibitor targeting FabI, an enoyl-acyl-carrier-protein-reductase, following recommended dosing (Kaplan et al., 2013b). Group 3 received vancomycin, which was used to validate that treatment starting at T16 was feasible. At T40 (i.e., 24 h post-antibiotic treatment), bacterial counts were significantly lower in the organs of both antibiotic-treated animals compared to the untreated group, attesting to AFN-1252 activity (Figure 1B). At T88, vancomycin-treated animals were essentially free of bacteria. However, organs from AFN-1252-treated mice still contained S. aureus CFU (colony forming units); bacterial counts were increased 10-fold in kidneys (to 5 \times 10^6; p \leq 0.01), decreased 10-fold in the liver (p \leq 0.05), and unchanged in the spleen. FASII antibiotic treatments thus failed to eliminate S. aureus in a septicemia model. Although single fakB1 or fakB2 mutants may display partial phenotypes (each still incorporates some fatty acids; Cuypers et al., 2019; Parsons et al., 2014), we asked whether such a mutant would be more sensitive than the parental strain to AFN-1252 treatment. This was tested by infecting mice with a 1:1 mix of USA300 and fakB2 following the same antibiotic protocol as above. Erythromycin resistance of fakB2 (Fey et al., 2013) was used to determine the proportion of fakB2 present during infection. The USA300:fakB2 average ratios in untreated mice 88 h post-infection were 1.6, 9.9, and 3.3, respectively, in kidney, liver, and spleen in organs at 88 h (Figure 1C). In contrast, AFN-1252-treated mice showed average USA300:fakB2 ratios of 68.9, 19.2, and 6.8, respectively, in kidney, liver, and spleen. These results indicate that fakB2 is preferentially eliminated by AFN-1252. They support the proposal that fatty acid incorporation occurs during infection and contributes to anti-FASII treatment failure. The underlying mechanisms leading to FASII inhibitor escape in host-relevant conditions were investigated.

**Host Constituents Promote Rapid Staphylococcal Adaptation to FASII Antibiotics**

We reasoned that in septicemic infection, serum and other host constituents bind eFAs and may neutralize FASII inhibitors (Balemans et al., 2010; Hunt et al., 2016; Lacey and Lord, 1981; Litus et al., 2018). The effect of serum on FASII antibiotic activity was
(C) Cell vitality and permeability were evaluated by fluorescence microscopy using, respectively, Syto9™ and propidium iodide (PI) probes. USA300 was grown in FA-Tric and SerFA-Tric for 6 h; mid-exponential phase cultures in BHI, FA, and SerFA were used as references. Proportions of vital and permeable bacteria

(E) Fatty acid profiles were determined on four biological replicates grown as in (C). Proportions of eFAs compared to total fatty acids are shown after 6 h of selection. Note that fatty acid profiles are fully exogenous upon outgrowth.

tested using triclosan, an extensively used biocide that also targets FabI (McMurry et al., 1998). S. aureus triclosan sensitivity was compared in a medium containing a 3-fatty-acid cocktail (FA, containing C14:0, C16:0, and C18:1 cis, 0.17 mM each; with triclosan, FA-Tric) and the same medium supplemented with serum (SerFA-Tric) (Figure 2A). USA300 growth without serum was inhibited by triclosan, with the emergence of FASII mutants usually after 24–48 h of incubation (Morvan et al., 2016). However, serum supplementation markedly shortened latency compared to BHI (brain heart infusion medium) cultures—to 8 h—and was followed by near-normal growth (Figure 2A). Similar results were observed with the unrelated S. aureus Newman strain (Figure S1A). If S. aureus outgrowth were due to triclosan titration by serum, FASII would remain active so that bacterial fatty acid composition would be endogenous. However, the contrary occurred: bacterial fatty acid profiles during outgrowth in the SerFA-Tric medium were totally exogenous (Figures 2B and S1B). As expected, albumin, a major serum constituent, also resulted in FASII bypass (Figure S1C). Similarly, when USA300 was grown with liver or kidney extracts (without added fatty acids) and triclosan, outgrowth kinetics were similar to those of SerFA-Tric cultures, and cells bypassed the FASII block by incorporating organ-derived fatty acids (Figure S1D).

Importantly, pre-incubation in SerFA prior to FASII antibiotic treatments shortened the time prior to S. aureus outgrowth. USA300 was challenged with the AFN-1252, which led to a longer (10 h) latency phase prior to outgrowth than did the triclosan. However, pre-incubation in serum shortened latency to about 6.5 h for both drugs, compared to that in the non-selective medium (Figure S2), indicating that bacterial pre-exposure to the lipid-rich host environment contributes to limiting FASII antibiotic efficacy.

Staphylococcus epidermidis, haemolyticus, and lugdunensis are emerging pathogens that, like S. aureus, synthesize branched-chain fatty acids. Representative strains were grown in SerFA and treated with AFN-1252 as above (Figure S3). All cultures grew after overnight incubation and displayed exogenous fatty acid profiles, indicating that these staphylococcal species also bypass FASII inhibitors.

These results show that in serum, S. aureus and other staphylococcal species escape anti-FASII inhibition and maintain robust growth by replacing endogenously synthesized fatty acids with eFAs. They indicate serum actually enhances, rather than prevents, eFA incorporation by S. aureus.

**Adaptation to FASII Inhibitors Is Not due to FASII Mutations**

Mutations in FASII initiation genes may confer antibiotic resistance (Gloux et al., 2017; Morvan et al., 2016, 2017; Parsons et al., 2011). We monitored mutations in FASII antibiotic-adapted USA300 or Newman strains by DNaseq using FASII inhibitors triclosan or AFN-1252 (Table S1). DNaseq of USA300 grown in BHI and SerFA, and Newman grown in BHI, were used as references. Adaptation was confirmed by exogenous fatty acid profiles of antibiotic-grown samples in 12–14 h cultures (data not shown). Eight of nine genome sequences displayed parental FASII genes (the exception was mutated in SAUSA300_1476 encoding FASII initiation gene accB). One isolate displayed
no detectable genome mutations. The other clones carried SNPs corresponding to commonly found variants and are likely unrelated to FASII antibiotic adaptation (described in Table S1). The absence of FASII mutations distinguishes this adaptation mechanism from resistance due to FASII target or initiator gene mutations (Ciusa et al., 2012; Morvan et al., 2016). The S. aureus evasion of FASII antibiotics in serum identifies a unique strategy of condition-dependent adaptation.

**Serum Lowers Fatty-Acid-Induced Bacterial Membrane Permeability and Improves Fitness**

Numerous fatty acids reportedly perturb bacterial membrane integrity and are a source of stress, while serum albumin neutralizes these effects (Lacey and Lord, 1981; Litus et al., 2018; Nicolaides, 1974; Parsons et al., 2012). Accordingly, serum abolished the eFA-provoked growth lag in the non-selective medium (Figure 2A). Serum effects on S. aureus vitality, permeability, and cell state were examined. Free fatty acids had strong permeabilizing effects on cells from the FA and FA-Tric, as compared to BHI cultures, as evaluated by fluorescence microscopy; these effects were offset by serum in the SerFA and SerFA-Tric cultures (Figures 2C, S4A, and S4B). Plating efficiency was $\sim 10^3$-fold higher after 6 h of growth in SerFA-Tric compared to FA-Tric (Figure 2D). The accumulation of tetrads comprising mixed-stained cells in triclosan-treated cultures correlates with the observed latency prior to outgrowth (Figure S4C).

Importantly, serum facilitates fatty acid incorporation in the latency period, as seen in the 6-h FA-Tric and SerFA-Tric cultures (Figure 2E). The bacterial stress state in FA-Tric supplemented or not supplemented with serum was also assessed by a proteomics approach using the USA300 spa strain (Figure S5). Differences in stress-related protein abundance between anti-FASII-treated and control cultures were, overall, more pronounced when the serum was absent. Serum therefore improves S. aureus fitness in fatty-acid-containing environments and contributes to FASII antibiotic adaptation via eFA incorporation.

**S. aureus Grown in Serum Shows a Greater Retention of the ACP and a Reduced Capacity for eFA Efflux via FarE**

We questioned how serum affects the availability of two key substrates required for FASII antibiotic adaptation: the acyl carrier protein (ACP) and eFAs. ACP is required for both de novo fatty acid synthesis via FASII and eFA incorporation in the phospholipid 2-position (Figure 3A) (Majerus et al., 1964; Morvan et al., 2016). The S. aureus grown in serum shows a greater retention of the ACP and a reduced capacity for eFA efflux via FarE (Figure 3B). The full proteomic study is available at [https://data.mendeley.com/datasets/9292c75797/2](https://data.mendeley.com/datasets/9292c75797/2).
eFAs induce membrane leakage that depletes S. aureus ACP pools and could limit eFA incorporation during FASII inhibition (Parsons et al., 2011, 2012). S. aureus ACP pools were compared by immunoblotting using anti-ACP antibodies in total extracts from cultures grown without and with serum and triclosan (endogenous/endo; in red, PGly species with one endogenous fatty acid and one eFA (endo/eFA); in green, PGly species with eFAs in both positions (eFA/eFA); in pink, PGly species with both endogenous fatty acids in both positions (endo/endo); in red, PGly species with one endogenous fatty acid and one eFA (endo/eFA); in green, PGly species with eFAs in both positions (eFA/eFA). Without the antibiotic, 20%–25% of PGly species comprise eFAs at both positions as estimated from peak heights. See Table S2 for fatty acids comprising major PGly species. Shown is representative result from two independent experiments.

Figure 4. Serum Promotes eFA Replacement in Phospholipids without Antibiotic Selection
S. aureus USA300 was grown for 6 h in 10% delipidated serum containing equimolar C17:1tr-C18:1cis mix (dSer2FA).

(A) FA composition. Fatty acid species are presented as the proportion of endogenous branched-chain fatty acids (BCFAs C15:0, i15; purple), endogenous saturated (straight chain) fatty acids (SFAs C18:0, C20:0; pink), and eFAs (C17:1tr and C18:1cis, green). See Figure S6A for fatty acid profiles. Shown is the average of two independent experiments.

(B) Phosphatidylglycerol (PGly) mass spectrometry (MS) profiles from samples in (A); in black, masses of PGly species with endogenous fatty acids in both positions (endo/endo); in red, PGly species with one endogenous fatty acid and one eFA (endo/eFA); in green, PGly species with eFAs in both positions (eFA/eFA). Without the antibiotic, 20%–25% of PGly species comprise eFAs at both positions as estimated from peak heights. See Table S2 for fatty acids comprising major PGly species. Shown is representative result from two independent experiments.

Figure 5. Kinetics of eFA Incorporation in Both Phospholipid Positions during FASII Antibiotic Selection
S. aureus USA300 dSer2FA pre-cultures and cultures were prepared with 10% delipidated serum (dSer) and C17:1tr and C18:1cis. AFN-1252, 0.5 µg/ml was added at OD600 = 0.1 at the start of kinetics.

(A) Fatty acid composition at indicated time points is shown as the proportion of endogenous branched-chain fatty acids (BCFAs C15:0, i15; purple), endogenous saturated (straight chain) fatty acids (SFAs C18:0, C20:0; pink), and eFAs (C17:1tr and C18:1cis, green). Corresponding profiles are shown in Figure S6B. Gray curve represents OD600 readings at indicated times. Shown is the average of two independent experiments.

(B) Kinetics of phosphatidylglycerol (PGly) profile modifications during FASII bypass in FASII antibiotic. Masses are represented: red, PGly species with one eFA and one endogenous fatty acid; green, PGly species with eFAs in both positions. Fatty acid composition of each major PGly mass is given in Table S3 (dSer2FA-AFN). Shown is representative result from two independent experiments. BHI and dSerFA cultures (Figure 4) were analyzed together with this experiment. The same experiment performed using triclosan in place of AFN-1252 (data not shown) gave comparable results.
Intracellular eFA pools are limited by a recently characterized S. aureus fatty acid efflux pump (FarE), which is induced by unsaturated eFAs (Anaseri et al., 2015, 2019). Proteomics analysis (as above) confirmed high FarE expression in all eFA-containing media without serum. In contrast, nearly no FarE was detected in cells issued from serum-containing cultures, despite the presence of eFAs (Figure 3C). The greater S. aureus intracellular retention of ACP and reduced eFA efflux capacity in serum is consistent with more efficient eFA incorporation and FASII antibiotic adaptation (Figure 2E).

Exogenous Fatty Acids Can Occupy Both Phospholipid Positions in the Absence and Presence of FASII Antibiotics

The accepted rationale for developing S. aureus FASII inhibitors is the presumed stringent requirement for endogenous branched-chain fatty acid a/15 at the phospholipid 2-position, catalyzed by 1-acyl-sn-glycerol-3-phosphate acyltransferase PIsC (Albanesi et al., 2013; Balemans et al., 2010; Parsons et al., 2011; Schiebel et al., 2014; Zhang and Rock, 2008). The above evidence that FASII antibiotic adaptation leads to eFA incorporation at both phospholipid positions (Figures 2B and S1B), and previous unexplained observations (Delekta et al., 2018), gave evidence against the generality of phospholipid stringency. However, eFA incorporation in both S. aureus phospholipid positions could be a last-resort choice when the preferred substrate a/15 is unavailable. Alternatively, the use of eFAs versus a/15 may simply depend on the intracellular substrate availability, which increases in serum. eFA incorporation was assessed in non-selective conditions to discriminate between these alternative hypotheses. To remove ambiguity in distinguishing the endogenous from the exogenous fatty acids in phospholipid identifications, a cocktail of unsaturated eFA 17:1trans (tr) and 18:1cis was used to supplement S. aureus USA300 growth. Although structurally distinct from S. aureus endogenous fatty acids, 17:1trans and 18:1cis did not interfere with growth in the serum-containing medium (optical density [OD]600 = 6.4 for both BHI and dSer2FA cultures at 6 h). In this non-selective growth condition, 17:1 and 18:1cis comprised about 60% of the total fatty acid content (Figures S4A and S6A) at the expense of straight-chain saturated fatty acids, which decreased from 50% in BHI to about 10% in the dSer2FA cultures. Importantly, eFAs occupied both positions in 20%-25% of phosphatidylglycerol (PGly) species after 6 h without the antibiotic (Figure 4B; Table S2). These results prove that wild-type S. aureus incorporates dissimilar fatty acids in both phospholipid positions without the need for FASII antibiotic selection and rules out the previously assumed fatty acid selectivity of phospholipid-synthesizing enzymes.

Fatty acid and PGly profiles were determined 4, 6, 8, 10, and 15 h after USA300 treatment with AFN-1252 or triclosan (not rows, reduced reactions. Dashed circle, permeable membrane; solid circle, intact membrane. PIsY mediates eFA incorporation in the phospholipid 1-position; PIsX and PIsC, catalyze fatty acid insertion in the phospholipid 2-position. Only eFA processing is presented.
S. aureus maintained, as by the host serum, eFA incorporation is not These results show that when S. aureus antibiotic treatments, the predominant phospholipid species at 10 h were totally exogenous (Figure 5B; Table S3). The sharp increase in exclusively eFA-containing phospholipids coincides with the exit from latency, as expected from the coordination between membrane phospholipid synthesis and cell growth (Vadia and Levin, 2015; Vadia et al., 2017). These results show that when S. aureus membrane integrity is maintained, as by the host serum, eFA incorporation is not stringent, and they reflect competition between fatty acids synthesized by S. aureus and those available from the environment. eFAs are incorporated in both phospholipid positions in the absence of selection and completely replace endogenous fatty acids in the presence of FASII antibiotics.

**DISCUSSION**

S. aureus is shown here to adapt to FASII antibiotics in host environments and without FASII mutations. A FASII antibiotic was ineffective in a sepsis model in which the treatment protocol respected the interval between pruto-infection and treatment time. The design of antibiotic challenge tests based on realistic intervals between infection and treatment should improve the predictive value of animal studies, which are decisive for scale-up to clinical trials. Based on this study, AFN-1252 and likely any FASII inhibitor would be ineffective as stand-alone treatments of S. aureus deep infection. S. aureus responses to FASII antibiotics are schematized in a model (Figure 6): greater bacterial membrane integrity and intracellular ACP and eFA pools, as shown here in serum-containing host environments, lead to complete eFA replacement in phospholipids. The rapid kinetics, absence of FASII mutations during antibiotic treatment, and eFA-replete phospholipids in the absence of selection indicate that adaptation impacts a general, non-FASII-mutated, S. aureus population. Serum and other host components promote low stress and FASII adaptation, which links this model to anti-FASII treatment failure. In contrast, skin surfaces producing fatty acids would correspond to high-stress environments unfavorable to FASII bypass except by FASII mutation (Figure 6A; Morvan et al., 2016; Pishchany et al., 2018). Previous in vitro and in vivo studies reporting FASII antibiotic efficacy were based on S. aureus strain 8325 and derivatives (Parsons et al., 2013, 2011). This bacterial lineage bears an atypical 16/288 amino acid deletion in fatty acid kinase subunit FakB1, which may impact saturated eFA entry and FASII antibiotic adaptation (Parsons et al., 2014; data not shown). FASII inhibitors were recently suggested for the elimination of Clostridium difficile and Listeria monocytogenes (Mareddy et al., 2019; Yao et al., 2016). Like S. aureus, these pathogens might show relaxed phospholipid stringency in host environments; niche-dependent antibiotic adaptation in these cases remains to be tested.

Bacterial metabolism and environmental stress play unquestionable roles in the outcome of antimicrobial treatments. For example, reduced metabolism in bacterial persisters is recognized as a major means of escape from antibiotic killing (Chuard et al., 1997; Lewis, 2007). In contrast, FASII antibiotic adaptation involves robust bacterial proliferation concomitant with the compensatory incorporation of host fatty acids. Such metabolic rescue is likely frequent, as bacteria commonly salvage metabolites from their environments. This consideration may set a logical limit when selecting targets for antimicrobial drug development.

As shown here, S. aureus and other firmicute pathogens may have reduced genetic requirements in host biotopes, including a non-essentiality of FASII. Interestingly, a wall-less L-form S. aureus multiplies in the presence of cell wall antibiotics and survives by the oversynthesis of fatty acids (Kawai et al., 2018 and references therein). An intriguing possibility is that such “primitive bacteria” can use the lipid supply of the host, taking their lifestyle one step further toward antibiotic adaptation and parasitism.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.11.071.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Staphylococcus aureus acyl carrier protein polyclonal antibody | Covalabs | [https://doi.org/10.1038/ncomms12944](https://doi.org/10.1038/ncomms12944) |
| **Bacterial and Virus Strains** |        |            |
| S. aureus RN4220    | Laboratory collection | [https://doi.org/10.1128/IAI.00088-10](https://doi.org/10.1128/IAI.00088-10) |
| S. aureus Newman    | Laboratory collection | [https://doi.org/10.1128/IAI.00088-10](https://doi.org/10.1128/IAI.00088-10) |
| USA300 FPR3757 JE2 (referred to as USA300) | BEI Resources | [https://doi.org/10.1128/mBio.00537-12](https://doi.org/10.1128/mBio.00537-12) |
| Transposon insertion strains in USA300 FPR3757 | BEI Resources | [https://doi.org/10.1128/mBio.00537-12](https://doi.org/10.1128/mBio.00537-12) |
| Staphylococcus epidermidis | R. Briandet | ATCC 12228 |
| Staphylococcus haemolyticus | E. Borezée | JCSC1435 |
| Staphylococcus lugdunensis | E. Borezée | N920143 |
| **Biological Samples** |        |            |
| Newborn calf serum  | Sigma-Aldrich | 12023C-500ML |
| Delipidated calf serum | Eurobio | S181L-500 |
| Pork kidney extract | Local butcher | [https://doi.org/10.1038/ncomms12944](https://doi.org/10.1038/ncomms12944) |
| Pork liver extract  | Local butcher | [https://doi.org/10.1038/ncomms12944](https://doi.org/10.1038/ncomms12944) |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Fatty acids C14, C16 | Sigma-Aldrich | CAS # and product #: 544-63-8 & M3128; 57-10-3 & P0500 |
| Fatty acids C18:1cis, C17:1trans | Larodan | CAS # & product #: 112-80-1 & 10-1801; 126761-43-1 & 10-1702-12 |
| Triclosan (Irgasan) | Sigma-Aldrich | CAS # & product #: 3380-34-5 & 72779 |
| AFN-1252 | MedChem Express | CAS # & product #: 620175-39-5 & HY-16911 |
| Syto9™ | Thermo-Fisher | S34854 |
| Propidium iodide | Sigma-Aldrich | CAS # & product #: 25535-16-4 & 79214 |
| **Deposited Data** |        |            |
| Proteomics analysis | Mendeley | [https://data.mendeley.com/datasets//9292c75797/2](https://data.mendeley.com/datasets//9292c75797/2) |
| Genome sequence | EBI | [https://www.ebi.ac.uk/ena/data/view/?PRJEB24433](https://www.ebi.ac.uk/ena/data/view/?PRJEB24433) |
| **Experimental Models: Organisms/Strains** |        |            |
| Mouse: Balb/C 6-week-old female | Janvier Labs | N/A |
| **Oligonucleotides** |        |            |
| spa Fd 5’ | GATGACTTTACAAATACATACAGG | Eurogentec | N/A |
| spa Rv 5’ | GGCGATCAGCTTTTGGAGCTT | Eurogentec | N/A |
| fakB2 Fd 5’ | GTTGTGATTTTATACCCCTAA | Eurogentec | N/A |
| fakB2 Rv 5’ | AGTCAGGCCAGCATAGGTG | Eurogentec | N/A |
| **Software and Algorithms** |        |            |
| Zeiss | ZEN software package | N/A |
| Bowtie Samtools | Patricbrc.org | [https://doi.org/10.1093/nar/gkw1017](https://doi.org/10.1093/nar/gkw1017) |
| GraphPad Prism 5.0 | GraphPad Software | [https://www.graphpad.com/](https://www.graphpad.com/) |
| **Other** |        |            |
| DNeasy® Blood & Tissue Kit | QIAGEN | Cat No./ID: 69504 |
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Alexandra Gruss (alexandra.gruss@inra.fr). This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial strains

*S. aureus* RN4220 and Newman strains are from our laboratory collection. BHI medium was the base medium for all cultures grown at 37 °C in aerobic conditions (200 rpm). *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus haemolyticus* JCSC1435, and *Staphylococcus lugdunensis* N920143 were kindly supplied by R. Briandet and E. Borezée from this institute. Methicillin-resistant USA300 FPR3757 (called here USA300), and the Nebraska library of transposon insertions (University of Nebraska Medical Center) was generously supplied by BEI resources (Fey et al., 2013). Tested USA300 derivatives from the Nebraska library contained insertions in the following genes: USA300_1318 (fakB2), USA300_0113 (spa), and USA300_0226, USA300_0242, USA300_0407, USA300_1177, and USA300_1684 (see Table S1).

Mouse Husbandry

Animals were housed in the Institut Cochin animal facility accredited by the French Ministry of Agriculture. Animal experimentation was performed in compliance with French and European regulations on care and protection of laboratory animals (EC Directive 2010/63, French Law 2013–118, February 6, 2013). All experiments were approved by the Ethics Committee of the Paris-Descartes University (agreement n° 2015032714098562). Animals (6 per cage) were in laminar flow rooms, and water and food were provided ad libitum. Cages were lined with wood shavings and equipped with cubbyholes to improve animal well-being.

METHOD DETAILS

Growth media

Fatty acids (Larodan, Sweden) were prepared as 100 mM stocks in dimethyl sulfoxide (DMSO). The three fatty acid mixture (referred to as ‘FA’) comprising C14:0 (myristic acid), C16:0 (palmitic acid), and C18:1cis (oleic acid) was prepared in a 1:1:1 ratio (at a final concentration in BHI of 0.17 mM each). The two fatty acid mixture comprising C17:1trans and C18:1cis was used at a 1:1 ratio (at a final concentration in BHI of 0.25 mM each); the more rigid 17:1trans species was added to limit membrane fluidity due to C18:1cis. Newborn calf serum (Sigma-Aldrich, France), or delipidated calf serum (Eurobio, France) was added to BHI growth medium (10% final concentration) as indicated. Triclosan (referred to as “Tric”; Irgasan; Sigma-Aldrich) was added at 250 ng/ml and 500 ng/ml in medium without and with serum respectively; this corresponds to 15–30 times the minimum inhibitory concentrations (MIC) as determined on BHI medium (Morvan et al., 2016). AFN-1252 (referred to as “AFN”; MedChem Express) was added at 500 ng/ml in all media, which corresponds to about 100-fold the reported *S. aureus* MIC (~4–8 ng/ml; Kaplan et al., 2012; Parsons et al., 2011). FASII inhibitors were prepared in DMSO (1 mg/ml) for all *in vitro* experiments. Kidney and liver extracts were prepared as described (Morvan et al., 2016). Briefly, we purchased kidney and liver from pigs (6 months) from a local butcher shop, and prepared extracts: 200 g of each organ were cut in 0.5 cm cubes, and resuspended in PBS (1:2 w-v). Samples were homogenized using an ultra-turrax and centrifuged twice (at 4,000 g for 30 min) to remove solid material. Supernatants were filtered through Whatman paper, recentrifuged at 20,000 g, and then sterile-filtered sequentially on 0.45 and 0.2 micron membranes. Sterile extracts were stored at -20 °C and used at 3% final concentration in BHI or LB medium.

Infection and antibiotic treatments in mouse septicemia model

Six-week-old female Balb/C mice (Janvier Labs) were inoculated intravenously in the ophthalmic plexus with exponential phase USA300 (1x10^7 CFU: colony forming units) in 200 μl PBS. Mice were randomized into three groups of at least 18 mice per type of treatment per experiment. Group 1 was mock-treated with excipient (see Group 2); n = 14 and 13 for 40 h and 88 h dissections respectively. Group 2 mice received anti-FASII AFN-1252, administered at 16 h, and every 24 hours until sacrifice, by gavage, as a 2 mg/ml emulsion in 20% PEG 3350; n = 12 and 12 for 40 h and 88 h dissections respectively. Group 3, was treated by intraperitoneal injection at 16 h, and every 12 hours until sacrifice, as described (100 times the MIC determined as 1 μg/ml; Kaku et al., 2016; Lehar et al., 2015); n = 9 and 12 for 40 h and 88 h dissections respectively. This positive control confirmed the capacity to clear infections when treatment is administered 16 h post-infection. Kidney and spleen were dissected and homogenized (‘hard’ setting, 3 times 20 s) in 1 mL saline (Precellys, Bertin Instruments, France); liver was turrexed in 10 ml saline. Sample dilutions were plated on solid BHI medium for CFU determinations.

Competition experiments used a mixture of USA300 and *fakB2* strains grown separately, combined to achieve a 1:1 mixture, with a measured USA300:fakB2 ratio = 0.8, and diluted in PBS to obtain 1x10^7 CFU for inoculation. All other steps were as above, except that: i- 14 mice were used in control and AFN-treated groups that were sacrificed at 88 h, and ii- CFU platings were performed on BHI medium and on BHI containing 5 μg/ml erythromycin. The number of erythromycin resistant bacteria
corresponded to fakB2 mutants. This number was subtracted from the total number of CFUs to determine the number of wild-type USA300 bacteria.

**Determination of *S. aureus* fatty acid profiles**

Aliquots of *S. aureus* cultures (routinely an OD_{600} equivalent ≥ 1 was used) were centrifuged and washed once in 0.9% NaCl containing 0.02% Triton X-100, followed by two washes in 0.9% NaCl. Whole cell esterified fatty acid determinations were then done as described (Yamamoto et al., 2006). Briefly, cell pellets were treated with 0.5 mL of 1 N sodium methoxide in methanol. Heptane (200 μL) was then added, together with methyl-10-undecenoate (Sigma-Aldrich) as internal standard, vortexed for 1 min, and centrifuged. Fatty acid methyl esters were recovered in the heptane phase. Analyses were performed in a split-splitless injection mode on an AutoSystem XL Gas Chromatograph (Perkin-Elmer) equipped with a ZB-Wax capillary column (30 m x 0.25 mm x 0.25 μm; Phenomenex, France). Data were recorded and analyzed by TotalChrom Workstation (Perkin-Elmer). *S. aureus* fatty acid peaks were detected between 12 and 32 min of elution, and identified with retention times of purified esterified fatty acid standards.

**Fluorescence microscopy**

Bacteria were grown in BHI, FA, Ser-FA to OD_{600} = 0.5-1, or FA-Tric and SerFA-Tric for 6 hours. Cells (OD_{600} equivalent of 10-20) were centrifuged and washed once in phosphate buffered saline. Syto9™ (0.5 μL sample of a 5 mM solution in DMSO; Thermo Fischer Scientific) and propidium iodide (3 μL of 1.5 mM solution in water; Sigma, France) were added to 30 μL samples for respectively viable and permeabilized cell visualization. Cells were observed 10 min post-staining by fluorescent microscopy using a Zeiss AxioObserver Z1 inverted fluorescence microscope equipped with a Zeiss AxioCam MRm digital camera and Zeiss fluorescence filters. Images were processed with the Zeiss ZEN software package using a 38 HE Green Fluorescent Protein filter (excitation wavelength 450/490 nm; beam splitter, 495 nm; emission, 500/550 nm) and 45 Texas Red filter (excitation: 540/580 nm; beam splitter, 585 nm; emission, 595-668 nm). The numbers of live (green) and permeabilized (red) cells were counted manually. Dividing cells and tetrads were counted as single entities. Tetrads and clusters containing both viable and permeabilized cells (in FA-Tric and SerFA-Tric samples) were classified in a separate “mixed” category. The proportion of tetrads among total cells was determined by manual counting; 15 micrographs from 3 independent cultures per condition were evaluated, based on a total of ~10 000 cell clusters per condition.

**Genome sequencing**

*S. aureus* USA300 and Newman strains were harvested after exit from latency phase for cultures grown in SerFA-Tric (12 h cultures; 3 independent samples for each strain), or in SerFA-AFN (15 h cultures; 3 independent samples of USA300). Cultures in BHI (one sample per strain) and in SerFA (for USA300) were sequenced as references. DNA extractions were performed using the Qiagen “DNeasy® Blood & Tissue Kit,” following manufacturer’s protocol, except that cell pellets were first resuspended in 0.1 mg lysostaphin/ml Tris 10 mM (Ambi, USA) and incubated 30 minutes at 37°C. Genomic DNA sequencing by Ilumina HiSeq next generation sequencing was outsourced (GATC-Biotech, Konstanz, Germany). Coverage was estimated to be at least 70-fold for USA300 SerFA-Tric samples, and at least 400-fold for all other samples. The 2 × 150 paired-end reads were analyzed using “Variation Analysis” method provided by Patricbrc.org. Bowtie2 (Patricbrc.org) was used to align sequences and SAMtools to identify SNPs (Wat- tam et al., 2017). SNPs that differed in non-antibiotic-treated USA300 and Newman strains from those in the reference sequence (GenBank Nucleotide accession codes NC_007793.1 and NC_009641 respectively) were subtracted prior to variant screening. Variants were identified as representing at least 80% of reads in sequences for which there were at least 10 reads.

**ACP assessment by immunoblotting**

The USA300 spaa::Tn strain (SAUSA300_0113; Fey et al., 2013) was used for immunoblotting to avoid IgG titration by Protein A; this strain was confirmed to behave like its parent with respect to FASII antibiotics. An overnight BHI USA300 spaa pre-culture was used to inoculate BHI, FA, Ser-FA, FA-Tric, or SerFA-Tric media at OD_{600} = 0.1. Cultures were harvested at OD_{600} = ~1 for BHI, FA, and Ser-FA, and after 2 h or 4 h for FA-Tric or SerFA-Tric. All samples were adjusted to equivalent OD_{600} values, and washed twice in TE-protease inhibitor (Complete Tablets, Mini EASYpack Roche, Germany, as per supplier’s instructions), prior to lysis with Fastprep. Samples (20 μg per well as quantified by the Bradford Protein Assay kit (BioRad)) were treated for 3 min at 95°C and then loaded on 12.5% SDS-PAGE gels run at 150 V for 2 h. Gels were then electro-transferred to PVDF membranes (0.2 mm; BioRad; 75mA) for 3 h on a semi-dry transfer unit (Hoefer TE 70). Western blotting and exposure used an ECL kit (Perkin-Elmer) as per supplier’s instructions. Rabbit anti-*S. aureus* ACP antibodies (Morvan et al., 2016) were used at 1:1,300 dilution.

**Proteomic analyses of USA300 responses to anti-FASII**

Four independent overnight BHI precultures of USA300 spaa::Tn strain were used to inoculate BHI, FA, SerFA, FA-Tric, or SerFA-Tric media at OD_{600} = 0.1. Culture extracts were processed for western blotting, and after verification of protein concentration and quality, 10 μg of each protein sample was short-run on SDS-PAGE. Further sample treatment by LC-MS/MS, and bioinformatics and statistical analyses of data are as described (Pérez-Pascual et al., 2017). The reference genome GenBank Nucleotide accession code NC_007793.1 was used for protein annotation. The complete list of proteins expressed in the five growth conditions is available on the Mendeley database (https://doi.org/10.17632/9292c75797.2; https://data.mendeley.com/datasets/9292c75797/draft?a=bb34343b-6314-4421-8b9d-b25e2bbdb0df).
Extraction of polar membrane lipids

Lipid extractions were performed as described with modifications (Bligh and Dyer, 1959; Thiedieck et al., 2006). Briefly, freeze-dried cell material (100 mg) was extracted with 9.5 mL of chloroform-methanol 0.3% NaCl (1:2:0.8 v/v/v) at 80°C for 15 min. All following steps were done at room temperature. Extracts were vortexed for 1 h and centrifuged for 15 min at 4000 rpm. Supernatants were collected and cell debris was re-extracted with 9.5 mL of the same mixture, vortexed 30 min, and centrifuged. Supernatants were then pooled and 5 mL each of chloroform and 0.3% NaCl was added and mixed. Phase separation was achieved by centrifugation at 4000 rpm for 15 min. The upper phase was discarded and the collected chloroform phase was evaporated to dryness under a nitrogen stream and stored at −20°C.

Phosphatidylglycerol identification

The polar membrane lipid samples were injected in chloroform in a chromatographic system (ThermoFisher Scientific) including a Dionex U-3000 quaternary RSLC, a WPS-3000RS autosampler and a column oven. Lipid separation was carried out by liquid chromatography on a PVA-Sil column (150 × 2.1 mm i.d., 120 Å) (YMC Europe GmbH) with a 10x2 mm guard column packed with the same material. Column temperature was thermostatically controlled at 35°C. The chromatographic method separates phospholipids according to class, and was performed as described (Imbert et al., 2012; Moulin et al., 2015).

For phosphatidylglycerol species identification, this system was coupled to an LTQ-Orbitrap Velos Pro (ThermoFisher) equipped with an H-ESI II probe. Spray voltage was set at 3.3 kV. Heater temperature of the probe was set at 200°C. Sheath gas, auxiliary gas and sweep gas flow rates were set at 20, 8 and 0 (arbitrary unit) respectively. Capillary temperature was set at 325°C and S-lens RF level at 60%. Analysis was performed in negative mode to obtain structural information on phosphatidylglycerol fatty chains. The mass spectrometer is equipped with two analyzers: a double linear ion trap (LTQ Velos Pro) for fragmentation at low resolution and an orbital trap (Orbitrap®) for high resolution detection. Detection was performed in full MS Scan with 100,000 resolution and data dependent MS2 and MS3 with collision induced dissociation (CID; collision energy set at 35). Chromatographic retention time was used for polar head identification by comparing to commercial standards. Phospholipid identification was performed using high resolution mass full scan to obtain the formula of the entire PG species (Pulfer and Murphy, 2003), and MS2/MS3 fragmentation to obtain structural information about fatty acid chain composition of each species.

QUANTIFICATION AND STATISTICAL ANALYSIS

Means and standard errors of replicate growth curves and proportions of fatty acids were determined (Excel, Microsoft, USA). For phospholipid determinations, the average of two experiments with the range of duplicates is presented. Data from animal experiments using USA300 only were statistically analyzed by the non-paired non-parametric Mann-Whitney test with GraphPad Prism 5.0 (GraphPad Software, San Diego, California). The same test was used in USA300:fasB2 competition studies to compare results in non-treated and antibiotic-treated animals. In the competition study, the paired and non-parametric Wilcoxon test was used to determine p values in separate organs.

DATA AVAILABILITY

Genome sequence data have been deposited in the European Nucleotide Archive and can be accessed at https://www.ebi.ac.uk/ena/data/view/PRJEB24433. The complete list of proteins from the proteomic study is available on the Mendeley database (https://data.mendeley.com/datasets/9292c75797/2; https://data.mendeley.com/datasets/9292c75797/draft?a=bb34343b-6314-4421-89bd-b25e2bbdb0df). The authors declare that all other data supporting the findings of this study are available within the article and its Supplementary Information files, or from the corresponding author upon request.