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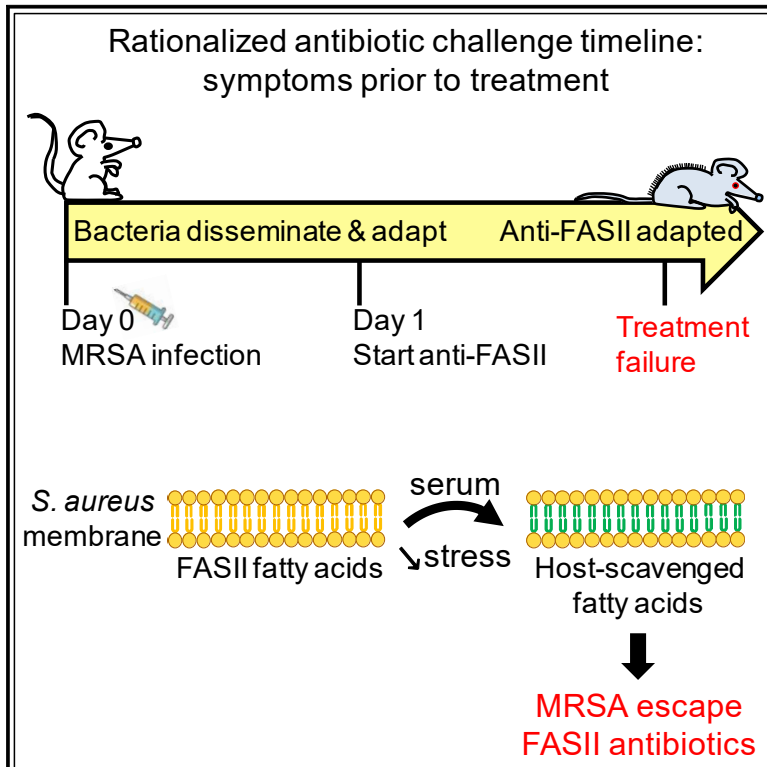
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## Permissive Fatty Acid Incorporation Promotes Staphylococcal Adaptation to FASII Antibiotics in Host Environments

### Graphical Abstract



### Authors

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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.

### 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



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

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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; Bailemans 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

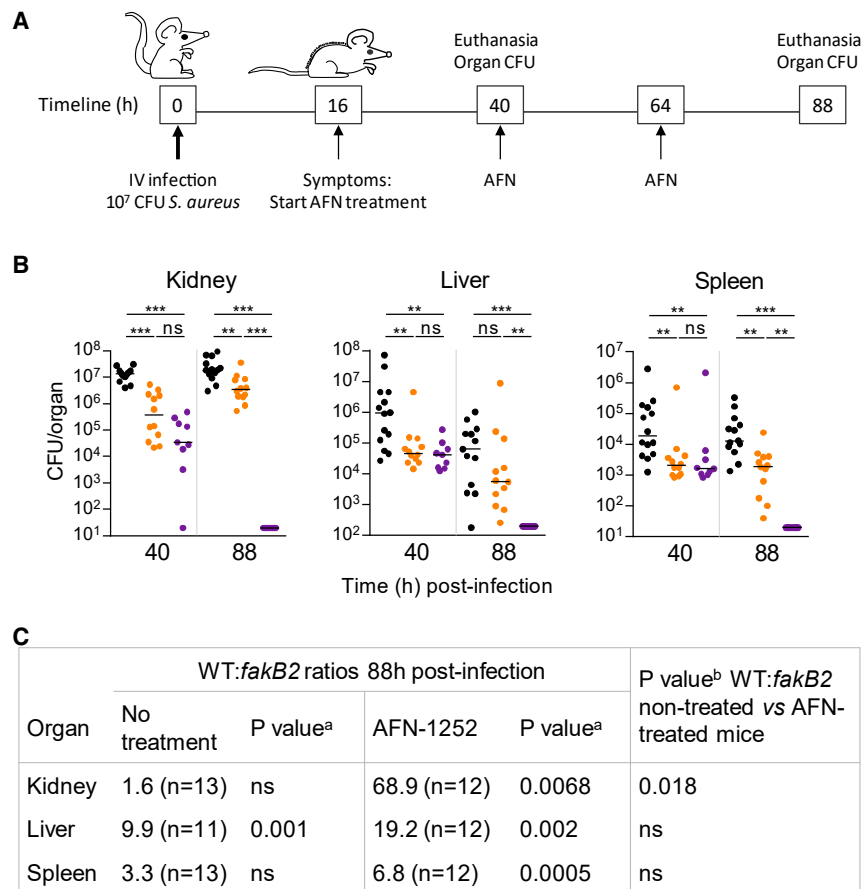
point of debate (Brinster et al., 2009, 2010; Parsons et al., 2011). Remarkably, *S. aureus* FASII sensitivity versus tolerance hinges on a single issue: whether environmental fatty acids (eFAs) can occupy the presumably stringent 2-position of membrane phospholipids (the 1-position is permissive) (Parsons 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 (*ai*15) 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).





**Figure 1. FASII Antibiotic Inefficacy in an *S. aureus* Septicemia Murine Model**

(A) Experimental protocol. Balb/C mice were infected intravenously at T0 with  $1 \times 10^7$  CFU USA300. 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. \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; ns, non-significant. y axis, CFU. Detection limit was 20 CFUs for kidney and spleen and 200 for liver. Mouse groups are as follows: black, untreated control; orange, AFN-1252 treated; purple, vancomycin treated.

(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 are 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.

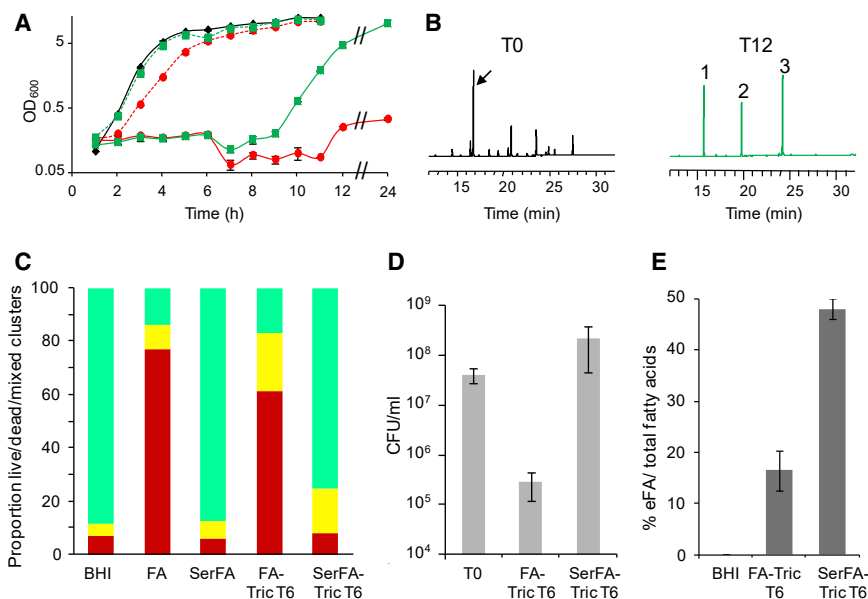
This consideration guided the design of the infection and treatment protocol used here (Figure 1A). MRSA strain USA300 was administered by the intravenous route. Antibiotic treatments were initiated 16 h (T16) post-infection, at which time animals exhibited signs of sickness (lethargy and ruffled fur). 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.

The fatty acid kinase FakA functions with FakB1 or FakB2, which orient specificity, for fatty acid incorporation in *S. aureus* (Parsons et al., 2014). The *fakA* and *fakB1fakB2* mutants show attenuated virulence (Lopez et al., 2017), which could reflect the need for host fatty acid incorporation during infection.

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* and 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



### Figure 2. Positive Effects of Serum on *S. aureus* Adaptation to a FASII Antibiotic

*S. aureus* strain USA300 was cultured in BHI, FA (a 3-fatty acid cocktail of C14:0, C16:0, and C18:1cis, 0.17 mM each), or SerFA (serum-supplemented FA) media containing or not containing triclosan (FA-Tric and SerFA-Tric, respectively). Mean values and standard deviation are shown for replicates in (A), (D), and (E).

(A) Bacterial optical density (OD<sub>600</sub>). Growth in SerFA-Tric resumed after an ~8-h latency period, whereas no growth was observed over 24 h in the absence of serum (FA-Tric). Black line, BHI; red lines, FA (dashed) and FA-Tric (solid); green lines, SerFA (dashed) and SerFA-Tric (solid). Curves are the average of three biological replicates.

(B) Fatty acid profiles. USA300 in BHI (left) displays endogenous fatty acids; SerFA-Tric-grown cells comprise exclusively eFAs. The y axis represents relative response (mV) at indicated peak positions. Black arrow indicates endogenously produced *ai15*. eFA: 1, C14:0; 2, C16:0; 3, C18:1. Representative fatty acid profiles from over 10 determinations are shown.

(C) Cell vitality and permeability were evaluated by fluorescence microscopy using, respectively, Syto9<sup>TM</sup> 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 were determined on ~10,000 bacteria per condition, issued from three independent experiments giving comparable images (Figure S4). Proportions of vital (green), permeable (red), and mixed-cell clusters (yellow) are shown.

(D) CFUs were determined in parallel on three independent cultures per condition: BHI starting culture (T0), FA-Tric, and SerFA-Tric samples (T6 = 6 h).

(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 C18:1cis, 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

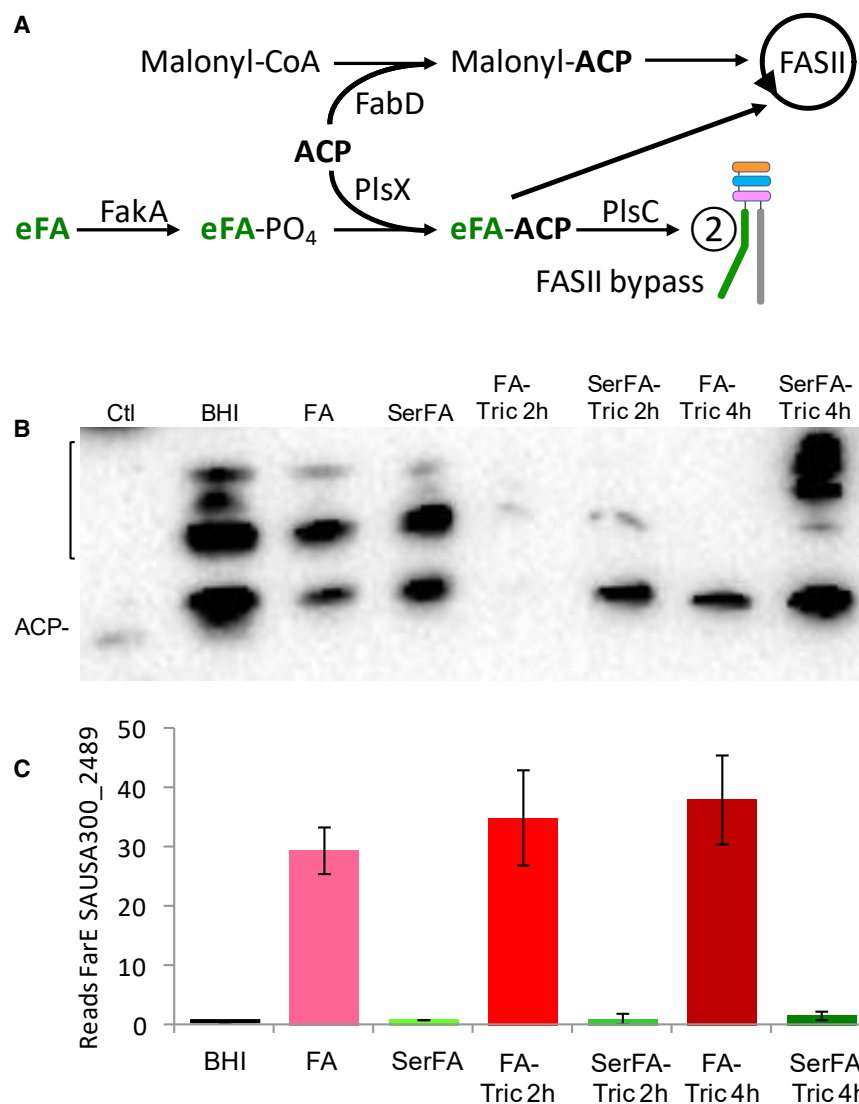
medium (Figure S2), indicating that bacterial pre-exposure to the lipid-rich host environment contributes to limiting FASII antibiotic efficacy.

*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



**Figure 3. Impact of Serum on *S. aureus* Intracellular Retention of ACP and eFAs**

(A) ACP and eFAs (green) are required substrates for FASII bypass via eFA incorporation in the phospholipid 2-position (lower right). FASII and FASII bypass enzymes compete for ACP and eFA-ACP intermediates.

(B) ACP immunodetection. Cell extracts were prepared from *S. aureus* USA300 *spa* strain. BHI, FA, and SerFA cultures were harvested at OD<sub>600</sub> = 1, and triclosan-treated cultures were harvested at 2 h and 4 h. Equivalent protein concentrations were loaded on SDS-PAGE gels. Immunoblotting was performed using an anti-ACP antibody. Migration of purified ACP is shown on the left. Multiple bands (bracket) may correspond to non-dissociable ACP complexes.

(C) Expression of fatty acid efflux pump FarE. Proteomic analyses were performed on *S. aureus* USA300 *spa* treated as in (B). Samples are in the same order as in (B). Mean values and standard deviation are presented. Black, BHI; pink, FA; light green, SerFA; red, FA-Tric 2 h; middle green, SerFA 2 h; burgundy, FA-Tric 4 h; dark green, SerFA 4 h. Quadruplicate independent samples were used for immunoblots and proteomic analyses. The full proteomic study is available at <https://data.mendeley.com/datasets/9292c75797/2>.

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

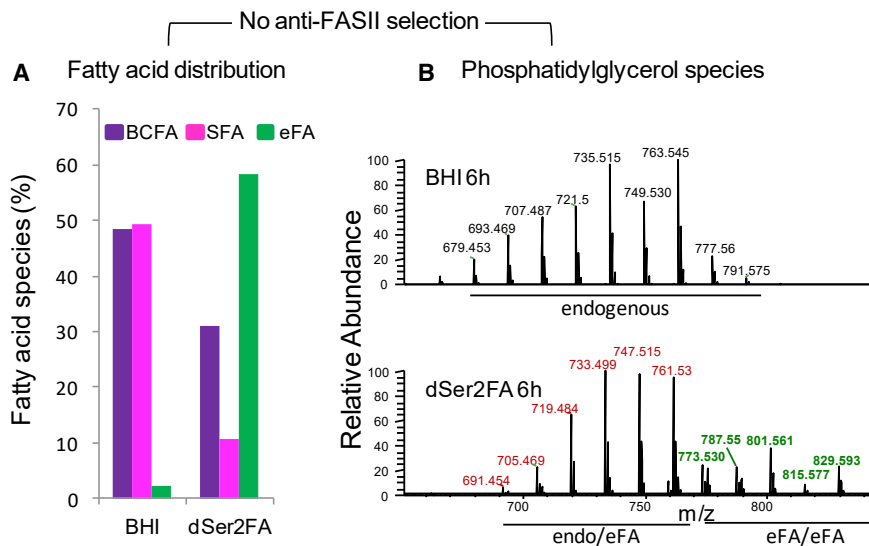
Numerous fatty acids reportedly perturb bacterial membrane integrity and are a source of stress, while serum albumin neutralizes these effects (Lacey and Lord, 1981; Litus et al., 2018; Nicolaidis, 1974; Parsons et al., 2012). Accordingly, serum abolished the eFA-provoked growth lag in the non-selective 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 ~10<sup>3</sup>-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

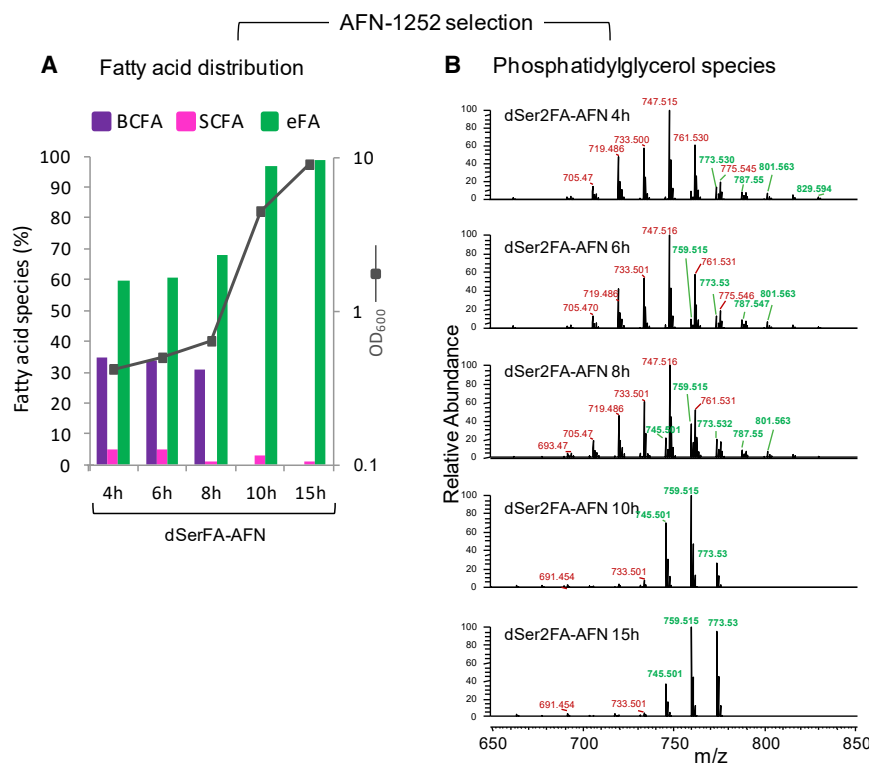


**Figure 4. Serum Promotes eFA Replacement in Phospholipids without Antibiotic Selection**

*S. aureus* USA300 was grown for 6 h in 10% delipidated serum containing equimolar C17:1*tr*-C18:1*cis* mix (dSer2FA). (A) FA composition. Fatty acid species are presented as the proportion of endogenous branched-chain fatty acids (BCFAs C15:0, i15, ai15; purple), endogenous saturated (straight chain) fatty acids (SFAs C18:0, C20:0; pink), and eFAs (C17:1*tr* and C18:1*cis*, 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.

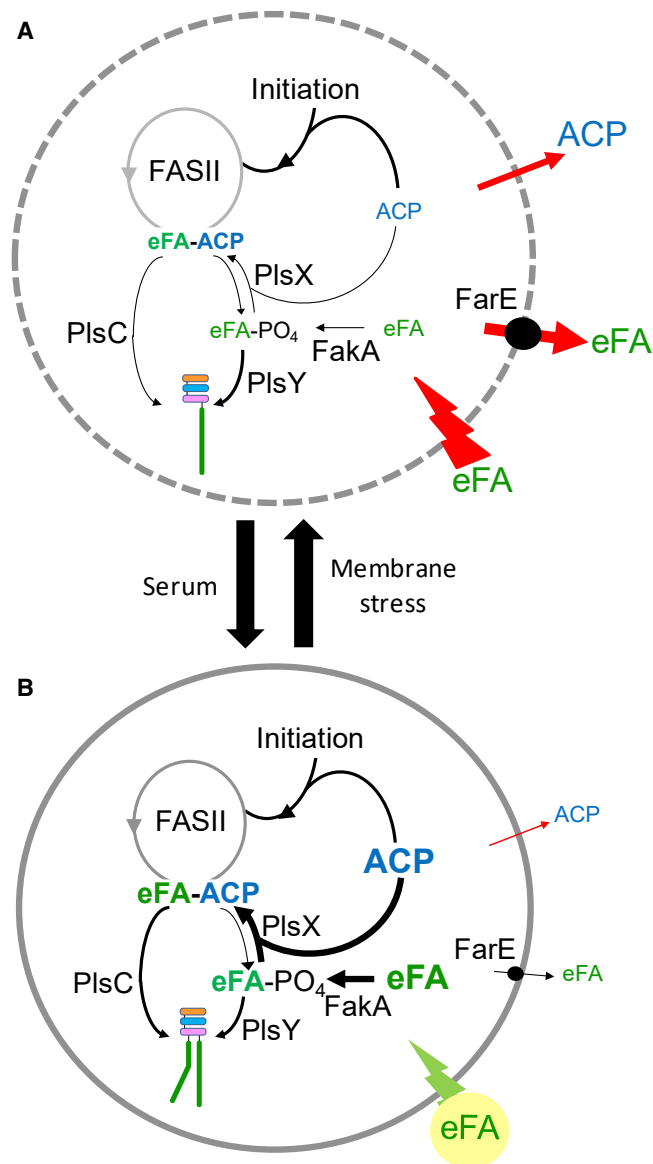
et al., 2016). eFAs induce membrane leakage that depletes *S. aureus* ACP pools and could limit eFA incorporation during FASII inhibition (Parsons et al., 2011, 2012). *S. aureus* ACP pools were compared by immunoblotting using anti-ACP antibodies in total extracts from cultures grown without and with serum and triclosan

(Figure 3B). ACP levels were lower in extracts from cells grown in eFA, compared to the BHI medium, as reported (Parsons et al., 2012). ACP was barely detected in FA-Tric-grown cells after 2 h, and it remained low at 4 h. In contrast, the addition of serum reversed this effect, leading to greater intracellular ACP availability.



**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 C17:1*tr* and C18:1*cis*. AFN-1252, 0.5  $\mu$ g/ml was added at OD<sub>600</sub> = 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, i15, ai15; purple), endogenous saturated (straight chain) fatty acids (SCFAs C18:0, C20:0; pink), and eFAs (C17:1*tr* and C18:1*cis*, green); corresponding profiles are shown in Figure S6B. Gray curve represents OD<sub>600</sub> readings at indicated times. Shown is the average of two independent experiments. (B) Kinetics of phosphatidylglycerol (PGly) profile modifications during FASII bypass in FASII antibiotics. 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.



**Figure 6. Conditional *S. aureus* Adaptation to FASII Antibiotics**  
 The membrane stress state impacts eFA and ACP intracellular pools and dictates FASII antibiotic adaptation as shown in the model.  
 (A) High stress: free eFAs permeabilize membranes. Fatty acid efflux via FarE and ACP leakage (Alnaseri et al., 2015, 2019; Parsons et al., 2012) leads to the depletion of FASII-bypass substrates. In this condition, eFA-PO<sub>4</sub> is used by PlsY to charge the phospholipid 1-position. FASII antibiotics would arrest growth, leading to cell death or the emergence of FASII initiation mutants that bypass FASII (Gloux et al., 2017; Morvan et al., 2016).  
 (B) Low stress: serum (yellow disk) or other host components reduce eFA toxicity (Lacey and Lord, 1981; this report) and improve membrane integrity. Higher ACP and/or eFA-PO<sub>4</sub> pools drive PlsX directionality to eFA-ACP production. eFA-ACP and endogenous ACP (if not blocked by anti-FASII) compete for phospholipid synthesis at the 2-position via PlsC. eFAs occupy both phospholipid positions even without FASII antibiotics (Figure 4B). eFA (green) and ACP (blue) abundance is represented by font size; phospholipids, “π” form; thick arrows, favored reactions; thin ar-

rows, reduced reactions. Dashed circle, permeable membrane; solid circle, intact membrane. PlsY mediates eFA incorporation in the phospholipid 1-position; PlsX and PlsC, catalyze fatty acid insertion in the phospholipid 2-position. Only eFA processing is presented.

### 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 *ai15* at the phospholipid 2-position, catalyzed by 1-acyl-sn-glycerol-3-phosphate acyltransferase PlsC (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 *ai15* is unavailable. Alternatively, the use of eFAs versus *ai15* 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 fatty acids in phospholipid identifications, a cocktail of unsaturated eFA 17:1*trans* (*tr*) and 18:1*cis* prepared in delipidated serum (dSer2FA medium) was used to supplement *S. aureus* USA300 growth. Although structurally distinct from *S. aureus* endogenous fatty acids, 17:1*tr* and 18:1*cis* did not interfere with growth in the serum-containing medium (optical density [OD]<sub>600</sub> = 6.4 for both BHI and dSer2FA cultures at 6 h). In this non-selective growth condition, 17:1*tr* and 18:1*cis* comprised about 60% of the total fatty acid content (Figures 4A 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. PlsY mediates eFA incorporation in the phospholipid 1-position; PlsX and PlsC, catalyze fatty acid insertion in the phospholipid 2-position. Only eFA processing is presented.



shown), in parallel with OD<sub>600</sub> (Figures 5 and S6B). For both FASII antibiotics, bacterial transition from mixed to exclusively exogenous fatty acids was concomitant with outgrowth from latency starting at 8 h post-treatment, and it was completed at 10 h (Figure 5A). For both FASII 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 septicemia model in which the treatment protocol respected the interval between primo-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 161/288 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* (Marreddy 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 recog-

nized 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

Detailed methods are provided in the online version of this paper and include the following:

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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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## AUTHOR CONTRIBUTIONS

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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
<b>Antibodies</b>		
<i>Staphylococcus aureus</i> acyl carrier protein polyclonal antibody	Covalabs	<a href="https://doi.org/10.1038/ncomms12944">https://doi.org/10.1038/ncomms12944</a>
<b>Bacterial and Virus Strains</b>		
<i>S. aureus</i> RN4220	Laboratory collection	<a href="https://doi.org/10.1128/IAI.00088-10">https://doi.org/10.1128/IAI.00088-10</a>
<i>S. aureus</i> Newman	Laboratory collection	<a href="https://doi.org/10.1128/IAI.00088-10">https://doi.org/10.1128/IAI.00088-10</a>
USA300 FPR3757 JE2 (referred to as USA300)	BEI Resources	<a href="https://doi.org/10.1128/mBio.00537-12">https://doi.org/10.1128/mBio.00537-12</a>
Transposon insertion strains in USA300 FPR3757	BEI Resources	<a href="https://doi.org/10.1128/mBio.00537-12">https://doi.org/10.1128/mBio.00537-12</a>
<i>Staphylococcus epidermidis</i>	R. Briandet	ATCC 12228
<i>Staphylococcus haemolyticus</i>	E. Borezée	JCSC1435
<i>Staphylococcus lugdunensis</i>	E. Borezée	N920143
<b>Biological Samples</b>		
Newborn calf serum	Sigma-Aldrich	12023C-500ML
Delipidated calf serum	Eurobio	S181L-500
Pork kidney extract	Local butcher	<a href="https://doi.org/10.1038/ncomms12944">https://doi.org/10.1038/ncomms12944</a>
Pork liver extract	Local butcher	<a href="https://doi.org/10.1038/ncomms12944">https://doi.org/10.1038/ncomms12944</a>
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
Fatty acids C14, C16	Sigma-Aldrich	CAS # and product #: 544-63-8 & M3128; 57-10-3 & P0500
Fatty acids C18:1 <i>cis</i> , C17:1 <i>trans</i>	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
<b>Deposited Data</b>		
Proteomics analysis	Mendeley	<a href="https://data.mendeley.com/datasets/9292c75797/2">https://data.mendeley.com/datasets/9292c75797/2</a>
Genome sequence	EBI	<a href="https://www.ebi.ac.uk/ena/data/view/PRJEB24433">https://www.ebi.ac.uk/ena/data/view/PRJEB24433</a>
<b>Experimental Models: Organisms/Strains</b>		
Mouse: Balb/C 6-week-old female	Janvier Labs	N/A
<b>Oligonucleotides</b>		
spa Fd 5' GATGACTTTACAAATACATACAGG	Eurogentec	N/A
spa Rv 5' GCGCATCAGCTTTTGGAGCTT	Eurogentec	N/A
fakB2 Fd 5' GTGTTGTATTTGATACCCTAAA	Eurogentec	N/A
fakB2 Rv 5' AGTCAGCCAGCCATAGGTG	Eurogentec	N/A
<b>Software and Algorithms</b>		
Zeiss	ZEN software package	N/A
Bowtie Samtools	Patricbrc.org	<a href="https://doi.org/10.1093/nar/gkw1017">https://doi.org/10.1093/nar/gkw1017</a>
GraphPad Prism 5.0	GraphPad Software	<a href="https://www.graphpad.com/">https://www.graphpad.com/</a>
<b>Other</b>		
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](mailto: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 *S. aureus* cultures unless specified. All cultures were 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: SAUSA300\_1318 (*fakB2*), SAUSA300\_0113 (*spa*), and SAUSA300\_0226, SAUSA300\_0242, SAUSA300\_0407, SAUSA300\_1177, and SAUSA300\_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 C18:1*cis* (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 C17:1*trans* and C18:1*cis* was used at a 1:1 ratio (at a final concentration in BHI of 0.25 mM each); the more rigid 17:1*trans* species was added to limit membrane fluidity due to C18:1*cis*. 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, re-centrifuged 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 ( $1 \times 10^7$  CFU: colony forming units) in 200  $\mu$ l 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 in previous work using 5 mg/kg (> 500 times the reported MIC of 4–8 ng/ml; Kaplan et al., 2012; Parsons et al., 2011), delivered in 50  $\mu$ l 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 with vancomycin (100 mg/kg in NaCl 0.9%), and administered in 100  $\mu$ l by intraperitoneal injection at 16 h, and every 12 hours until sacrifice, as described (100 times the MIC determined as 1  $\mu$ g/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 turraxed 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  $1 \times 10^7$  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  $\mu$ g/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<sub>600</sub> equivalent  $\geq 1$  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 1N sodium methoxide in methanol. Heptane (200  $\mu$ l) 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  $\mu$ m; 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<sub>600</sub> = 0.5–1, or FA-Tric and SerFA-Tric for 6 hours. Cells (OD<sub>600</sub> equivalent of 10–20) were centrifuged and washed once in phosphate buffered saline. Syto9<sup>TM</sup> (0.5  $\mu$ l sample of a 5 mM solution in DMSO; Thermo Fischer Scientific) and propidium iodide (3  $\mu$ l of 1.5 mM solution in water; Sigma, France) were added to 30  $\mu$ l 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  $\sim$ 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 for each strain), and in SerFA (for USA300) were sequenced as references. DNA extractions were performed using the QIAGEN “DNeasy<sup>®</sup> 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 Illumina 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 x 150 paired-end reads were analyzed using “Variation Analysis” method provided by [Patricbrc.org](http://Patricbrc.org). Bowtie2 ([Patricbrc.org](http://Patricbrc.org)) was used to align sequences and SAMtools to identify SNPs (Wattam 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 *spa::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 *spa* preculture was used to inoculate BHI, FA, SerFA, FA-Tric, or SerFA-Tric media at OD<sub>600</sub> = 0.1. Cultures were harvested at OD<sub>600</sub> =  $\sim$ 1 for BHI, FA, and SerFA, and after 2 h or 4 h for FA-Tric or SerFA-Tric. All samples were adjusted to equivalent OD<sub>600</sub> values, and washed twice in TE-protease inhibitor (cComplete Tablets, Mini EASYpack Roche, Germany, as per supplier’s instructions), prior to lysis with Fastprep. Samples (20  $\mu$ g 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 *spa::Tn* strain were used to inoculate BHI, FA, SerFA, FA-Tric, or SerFA-Tric media at OD<sub>600</sub> = 0.1. Culture extracts were prepared as described for western blotting, and after verification of protein concentration and quality, 10  $\mu$ g 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-6314-4421-89bd-b25e2bbdb0df>).

### Extraction of polar membrane lipids

Lipid extractions were performed as described with modifications (Bligh and Dyer, 1959; Thedieck 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  $-20^{\circ}\text{C}$ .

### 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 10 × 2 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 MS<sup>2</sup> and MS<sup>3</sup> 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 MS<sup>2</sup>/MS<sup>3</sup> 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:*fakB2* 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.