***Staphylococcus aureus* prophages are gatekeepers of exogenous lipid utilization to control membrane flexibility and bacterial fitness**

Byang Zhou^a, Amit Pathania^a, Deepak Pant^a, Philippe Gaudu^a, Patrick Trieu-Cuot^b, Alexandra Gruss^a and Karine Gloux^a.

^aUniversité Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, Jouy en Josas, France,

^b Institut Pasteur, Université de Paris, CNRS UMR 2001, Unité de Biologie des Bactéries Pathogènes à Gram-Positif, Paris Cedex 15, France.

For correspondence: karine.gloux@inrae.fr

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Significance

Staphylococcus aureus is a major human health challenge. Its lipases activities are crucial for virulence and immune response escape. Prophages inserted in lipases are common in *S. aureus* but the selective benefits of their maintenance is little understood. We demonstrate that two prophages, inserted in triglyceride and sphingomyelin lipase genes, interactively modulate fatty acid (FA) incorporation into bacterial membranes. The triglyceride lipase facilitates elongation of incoming FAs and impacts membrane composition. Saturated and unsaturated FAs compete for entry *via* binding protein FakB1. The events dictated by prophage status illustrate how competitive processes impact membrane flexibility and bacterial fitness in lipid-rich

environments. These findings highlight the importance of assessing prophage status and lipid environments when evaluating infection risk and therapy efficiency.

Abstract

Lipids are essential components of bacterial membranes and phages are quasi-ubiquitous in bacteria. Both are crucial mediators of bacteria-host interactions. In the major pathogen *Staphylococcus aureus*, two common prophages, $\phi 11.1$ and $\phi 13$, are preferentially integrated in lipases genes: respectively encoding Geh, a triglyceride lipase, and Hlb, a sphingomyelinase. Here we discovered that $\phi 11.1$ and $\phi 13$ prophage statuses interactively determine bacterial growth, fatty acid (FA) incorporation and elongation *via* their synthesis pathway (FASII), and membrane composition. Remarkably, FASII-mediated elongation of C18:2 issued from triglycerides was stimulated by intact Geh, *i.e.*, in non- $\phi 11.1$ lysogens. Secondly, while *hlb* has no direct role in triglyceride metabolism, it inhibited C18:2 incorporation from di- and tri-glycerides into bacterial membrane as observed in non- $\phi 13$ lysogens. We show that these interactions are based on competition between Geh-generated fatty acids from tri- and di-glycerides, and Hlb-generated C16, for binding the fatty acid kinase FakB1 subunit. These findings lead to a mechanistic model in which prophage regulates the dynamics of lipid utilization, which in turn dictates FA toxicity, FASII activity and bacterial fitness. We suggest that testing new therapeutics against *S. aureus* should consider prophage status and environmental lipids for accurate prediction of efficiency.

Introduction

Environmental lipids comprise an integral, but often overlooked, aspect of bacterial pathogenesis. Lipids are crucial for bacterial fitness and are front-line players in modulating interactions with the host (1-4). Lipids range in structure from simple short hydrocarbon chains to complex molecules, including triglycerides (TGs), phospholipids (PLs), sterols and their esters. Fatty acids (FAs), the basic constituents of complex lipids, vary in chain length, side chains, and number and position of double bonds. A vast array of bacteria scavenge FAs, which they incorporate into their membranes (5-11). This quasi-ubiquity suggests a great potential of the host lipid environment to modulate invading pathogen behavior. The environmental lipid composition depends on human genetics and diet and is correlated to infection risk (2, 3, 12, 13). *Staphylococcus aureus*, a major human pathogen, incorporates exogenous FAs, such as those present in plasma lipids (6, 14). Incorporation involves a FA kinase, FakA, whose preference for saturated and unsaturated FA is reportedly respectively conferred by the FA binding proteins FakB1 and FakB2 (15-17). While *S. aureus* does not synthesize unsaturated and polyunsaturated FAs (UFAs and PUFAs), their incorporation has documented effects on bacterial membrane functions and interactions with the host (18-22).

Remarkably, two commonly found *S. aureus* prophages, ϕ 11-1 and ϕ 13, are inserted in *geh* and *hly* genes encoding lipases, respectively Geh glycerol ester hydrolase (EC 3.1.1.3) and Hly neutral sphingomyelinase C (EC 3.1.4.12) (23, 24) (Fig. 1A). About 15% of *S. aureus* isolates carry ϕ 11.1 into *geh* and at least 90% of human clinical isolates carry ϕ 13 into *hly* (1, 25). In contrast ϕ 13 prophage was quasi-absent in livestock isolates. ϕ 11-1 and ϕ 13 prophage excision is induced by environmental signals (such as SOS response or biocide/H₂O₂ respectively), leading to reactivation of Geh and Hly activities with consequent impacts on *in vivo* inflammatory responses

(1, 26-31). In contrast consequences of lipase genes inactivation by $\phi 11.1$ and $\phi 13$ on *S. aureus* metabolism and physiology have been little explored (14). Here we show that these two widespread prophages interactively affect membrane phospholipid composition and growth response to toxic UFA C18:2. The FA binding protein FakB1 is identified as a control point for phage-dependent dynamic competition between FAs issued from triglycerides and sphingomyelins.

Results

$\phi 13$ lysogeny increases C18:2 incorporation from serum into *S. aureus* phospholipids. We analyzed FA profiles from two isogenic Newman strains (WT and $\Delta\phi 13$), which differ by the presence of $\phi 13$ prophage (21). The $\phi 13$ -lysogenized WT and $\Delta\phi 13$ strains showed identical FA profiles in control BHI medium,, indicating that FASII-synthesis was unaffected by $\phi 13$ (Fig. S1). In contrast, addition of 10% mouse serum to medium distinguished the two strains by their incorporation of UFAs. The $\Delta\phi 13$ strain had approximately two times less UFAs than the WT ($6 \pm 1\%$ versus $13 \pm 1\%$), including oleic acids C18:1 $\Delta 9$, C18:1 $\Delta 11$, and the PUFA linoleic acid C18:2 (Fig. 1B). Serum was the only UFA source for *S. aureus*, which lacks UFA synthesis and desaturase functions (32). These results thus suggested an unexpected link between $\phi 13$ lysogenic status and UFA incorporation from a serum source.

$\phi 11.1$ lysogeny decreases trilinolein-derived C18:2 incorporation, elongation and toxicity; $\phi 13$ effects depend on $\phi 11.1$ status. Trilinolein (TNL), a TG with three C18:2, is a substrate for Geh lipase (33). We asked how the *geh*/ $\phi 11.1$ status impacts breakdown of TNL to C18:2, and whether $\phi 13$ lysogeny affects this process. For this, we analyzed FA profiles of isogenic Newman strains lysogenized or not with $\phi 11$ (including the three $\phi 11.1$ to $\phi 11.3$ prophages) and $\phi 13$ prophages

(21). In BHI medium, the $\phi 11$ prophage status had no detectable effect on FA profiles which reflect endogenous FA synthesis (Fig. S1). In contrast, TNL addition led to marked differences in FA profiles and growth: the $\phi 11$ lysogenized strains showed markedly less C18:2 incorporation from TNL and improved growth compared to non- $\phi 11$ lysogens (Fig. 1C). This result is in accordance with *geh* inactivation by $\phi 11.1$ and C18:2 toxicity on *S. aureus* (34). The remaining 18:2 incorporation in the $\phi 11$ lysogens may indicate residual Geh activity despite the C-terminal truncation, or C18:2 release by other lipases. Strikingly, $\phi 11$ lysogeny correlated with a quasi-total inhibition of C18:2 elongation by FASII (Fig. 1C) suggesting that Geh facilitates accessibility of TNL-derived C18:2 to FASII pathway. Furthermore, the $\phi 13$ lysogeny had effects depending on the $\phi 11$ status. Indeed when $\phi 11.1$ was present, $\phi 13$ lysogeny led to higher C18:2 incorporation from TNL ($11 \pm 1\%$ in WT *versus* $4 \pm 0.5\%$ in $\Delta\phi 13$ strain). In contrast, in absence of $\phi 11.1$ (intact *geh*), it did not impact the C18:2 incorporation ($28 \pm 4\%$ in $\Delta\phi 11$ *versus* $28 \pm 1\%$ in $\Delta\phi 11\Delta\phi 13$ strain, Fig. 1C) but increases elongation of both C18:2 (from 50 to 70%) and C20:2 (from 14 to 28%). These observations point to an unexpected interconnection between *S. aureus* $\phi 11$ and $\phi 13$ prophages for both incorporation and elongation of the PUFA C18:2 from TNL.

A *geh* mutant shows decreased incorporation of trilinolein-derived C18:2 and its elongation.

We asked whether $\phi 11$ prophage family effects were due to *geh* gene interruption by $\phi 11.1$. We analyzed the strain USA300 which lacks $\phi 11.1$ (intact *geh*) and its isogenic *geh::tn* mutant. While TNL-derived C18:2 was incorporated and efficiently elongated in both these strains, incorporation and particularly elongation were markedly lowered in the USA300 *geh::tn* mutant (Fig. 1D). Both *geh::tn* mutant and $\phi 11$ Newman lysogens encode C-terminal truncations of Geh (at respective amino acid [aa] positions 517 and 643 from a full length protein of 690 aa). Furthermore, orlistat, an inhibitor which binds to Geh (35), inhibited both incorporation and elongation of C18:2 from TNL (Fig. 1D). These results indicate that the Geh state, rather than an

intrinsic property of $\phi 11.1$, determines *S. aureus* capacity to both incorporate and elongate TNL-derived C18:2.

1,3-diglyceride-derived C18:2 is efficiently incorporated and toxic for growth. In TGs, the UFA position on the glycerol backbone affects its absorption and metabolism by cells (36-38). Triglyceride lipases display positional selectivity, which can be discriminated using 1,2-dilinoleoylglycerol (1.2DG) and 1,3-dilinoleoylglycerol (1.3DG) as substrates (39-41). Geh reportedly cleaves TNL in both positions 1 and 2 whereas most lipases prefer position 1 (40, 41). Consequently, Geh activity liberates 1.3DG from TNL, in contrast to other lipases. We therefore examined C18:2 incorporation from 1.2DG and 1.3DG in WT, $\Delta\phi 11$, $\Delta\phi 13$ and $\Delta\phi 11\Delta\phi 13$ Newman isogenic strains. Addition of 30 μ M 1.2DG led to low-level C18:2 incorporation whatever the prophage status. A growth retardation was observed in the Geh⁺ strains (non- $\phi 11.1$ lysogens) where both incorporation and elongation were favored (Fig. 1E). The same concentration of 1.3DG lead to lysis of non- $\phi 11.1$ lysogens (data not shown). Lowering 1.3DG addition to 10 μ M avoided lysis. Under this condition, very high incorporation and elongation of C18:2 clearly occurred only when Geh is intact (in non- $\phi 11.1$ lysogens). TG hydrolysis product, particularly attributed to Geh activity, leads therefore to high PUFA incorporation and elongation in *S. aureus*.

Serum-derived C16 incorporation is increased in $\Delta\phi 13$ and *hlyB*⁺ strains. We focused on possible mechanisms involved in $\phi 13$ impact on UFAs incorporation. In the presence of 10% mouse serum, the percentage of C16 and its derived FAs (C18 + C20) was decreased in Newman WT strain compared to the $\Delta\phi 13$ strain in favor of incorporated serum UFAs (Fig. 2A). Serum sphingomyelins are dominant circulating HlyB substrates that contain mainly C16 as FA moieties (42, 43). To further understand how *hlyB* affects UFAs utilization, we complemented the USA300 *hlyB::tn* mutant with a plasmid carrying an intact *hlyB* gene. HlyB complementation increased C16

and elongated derived FAs whereas it decreased incorporated serum UFAs (Fig. 2B). These results indicate that *h/b* contributes to C16 liberation and its further incorporation whereas ϕ 13 lysogeny increases serum UFAs incorporation. Based on these observations, we hypothesized that the ϕ 13 lysogenic/excised status likely creates a dynamic competition between C16 and UFA from serum TGs for incorporation in membrane phospholipids.

Incorporation of 1.3DG-derived C18:2 requires *fakB1*. The FakA kinase preference for saturated and unsaturated FA incorporation is reportedly conferred by FakB1 and FakB2, respectively (15-17). We first confirmed the reported phenotypes in USA300 derivative *fakA*, *fakB1*, and *fakB2* mutants in keeping with a minor role of FakB1 in UFAs incorporation, including that of free C18:2 PUFA (Fig. S2 and S3)(15, 17)). We then challenged USA300 derivative *fakA*, *fakB1*, and *fakB2* mutants with TNL. The *fakA* mutant failed to incorporate TNL-derived C18:2 (data not shown). Unexpectedly, *fakB1* and *fakB2* mutants failed to show selectivity when TNL was used as C18:2 precursor; both mutants displayed the capacity to incorporate C18:2 from TNL (Fig. S4). Thus, while free C18:2 incorporation was FakB2-dependent (see Fig. S3), incorporation from TNL-derived C18:2 was permissive. The strong effects of 1.3DG on non- ϕ 11.1 lysogens growth and on C18:2 incorporation, compared to 1.2DG (Fig. 2), led us to examine incorporation of these substrates in *fakA*, *fakB1*, and *fakB2* backgrounds. *fakA* was essential for incorporation of both substrates, reflecting the phosphorylation requirement ((15); data not shown). Incorporation of 1.2DG-derived C18:2 was in accordance with the reported FakB2 binding phenotype (15-17) (Fig. S5). In contrast, incorporation of 1.3DG-derived C18:2 was remarkably greater in *fakB2* than in *fakB1* mutant ($20 \pm 7\%$ versus $13 \pm 3\%$) and *fakB1* inactivation results in markedly lower incorporation compared to the USA300 wild type ($13 \pm 3\%$ and $31 \pm 5\%$ respectively, Fig. 3A).

To confirm the direct role of *fakB1*, we used RN450 strain, cured of phage lysogens and with a natural *fakB1* in-frame deletion (483-bp within the 867-bp functional gene, Fig. S6) and the isogenic RN450-R strain, repaired for *fakB1*. The RN450-R repaired strain incorporated more 1.3DG-derived C18:2 than RN450 strain ($35 \pm 3\%$ and $27 \pm 3\%$ respectively, Fig. 4A). Furthermore 1.3DG impairs more growth of *fakB1* defective strains (the USA300 *fakB1* mutant and RN450 strain) compared to their respective *fakB1*⁺ controls (WT USA300 and RN450-R, Fig. 3; Fig. 4). These observations identified therefore FakB1 binding as the unconventional preferred pathway for efficient incorporation of 1.3DG-derived C18:2 into PL and as a contributor of growth protection.

1,3-diglyceride-derived C18:2 and C16 compete for FakB1. The unexpected role of FakB1 in C18:2 incorporation from tri- and di-glycerides (Fig. S4A, 3A, 4A) led us to ask whether C16, favored in *hfb*⁺ strains, competes for FakB1. To test this, C16 was also added in experiments discussed above. We first established that, as reported, free C16 and free C18:2 were poorly competitive ((17), Fig. S3). In the *fakB1* background (USA300 WT and *fakB2* mutant), addition of free C16 led to a slight decrease in C18:2 incorporation from either TNL or 1.3DG (Fig. S4, Fig 3). The 1.3DG-derived C18:2 incorporation is little affected in the WT ($22 \pm 1\%$ compared to $31 \pm 5\%$) and unaffected in the *fakB1* mutant ($14 \pm 4\%$ compared to $13 \pm 3\%$). In contrast, when only FakB1 is available (in the *fakB2* mutant), C16 addition led to a quasi-total arrest of C18:2 incorporation (Fig. S4, Fig 3). As example, *fakB2* mutant has more than three times less 1.3DG-derived C18:2 in the presence of C16 ($6 \pm 3\%$ compared to $20 \pm 7\%$). In RN450 (naturally *fakB1* defective), *fakB2* can compensate for C18:2 incorporation from 1.3DG but C16 had no competitive effect (Fig. 4). A direct role for *fakB1* was confirmed using RN450-R, repaired for *fakB1*. Notably, total incorporation of 1.3DG-derived C18:2 is highly increased in this strain and

quasi-totally inhibited ($80 \pm 10\%$ decreased) in the presence of free C16 (Fig. 4). FakB1 is therefore a key determinant responsible for competition between C16 and 1.3DG-derived C18:2.

Discussion

S. aureus metabolism of environmental lipids is modulated by two common prophages, $\phi 11.1$ and $\phi 13$, which interrupt the lipases encoding genes *geh* and *hlb*. The present work reveals a more complex role of these prophages on exogenous lipid utilization than previously supposed. We identified three interactive factors that determine the bacterial responses: the environmental lipids offered, which are highly variable according to the biotope; the status of prophages, which toggle the lipase switches and control FAs integration in the membrane; and the FA kinase FakB1, which controls competitive entry of PUFA and saturated FAs released from lipids. Expanded roles for Geh, Hlb and FakB1 in *S. aureus* physiology were thus uncovered. Our work demonstrates that lysogeny by $\phi 11.1$ and $\phi 13$ protects bacteria from lipid toxicity and expands membrane flexibility, thus conferring a selective benefit for prophage maintenance. We propose a model for two-level control of FA incorporation and elongation, first by prophages, and then by FakB1 (Fig. 5).

The first control is determined by $\phi 11.1$ lysogeny, which protects *S. aureus* from PUFA toxicity. When TG is available, $\phi 11.1$ lysogens carrying truncated Geh show decreased PUFA C18:2 incorporation and limited elongation. To our knowledge this is the first report of the impact of Geh on FASII elongation efficiency. PUFAs provoke membrane permeabilization and lysis (44-49). Unlike most lipases, Geh cleaves the TG acyl group at both positions on the glycerol backbone liberating both 1,2- and 1,3-diglycerides (40, 41). Direct 1.3DG addition led to greater C18:2

toxicity than did 1.2DG. By inactivating Geh, ϕ 11.1 lysogeny exerts a protective role on *S. aureus* by limiting PUFA incorporation in membrane phospholipids, and likely improves bacterial survival in natural lipid-rich environments. Considering ϕ 13 lysogeny, its prevalence among human clinical *S. aureus* isolates (25) suggests a functional benefit of preventing Hlb activity. As expected, *hly*, intact in the absence of ϕ 13, led to increased saturated FA incorporation from serum. Remarkably however, intact *hly*, which is not implicated in PUFA metabolism, also reduced PUFA incorporation and elongation.

FakB1 defines the second level of control. While FakB1 is reportedly associated with saturated FA binding (15-17), it also mediated efficient incorporation of C18:2 issued from 1.3DG. Entry of 1.3DG -derived C18:2 contrasts with both free and 1.2DG-derived C18:2, which involved mainly FakB2. Thus FakB1 binding is more permissive than initially reported, in accordance with recent studies (15, 17, 50). FakB1 may act as a chaperone that controls free C18:2, toxic on FASII (34), prior to membrane incorporation. It may also promote FakA-mediated phosphorylation of FAs, which is required for C18:2 efflux *via* FarE (51). Further investigation is needed to identify the TG intermediate recognized by FakB1 and Geh contribution to these binding specificities.

The unexpected role of FakB1 in facilitating PUFA incorporation revealed a newly identified role of *hly* in an interference with unsaturated FAs incorporation. Hlb activity in ϕ 13 non-lysogens increases entry of saturated FAs, which bind FakB1, and compete with PUFA. FakB1 is a gatekeeper that discriminates between PUFA and saturated FA incorporation in membrane phospholipids. If PUFA-FakB1 binding is out-competed by saturated FAs, *S. aureus* survival could be compromised due to deficient FakB1 and FarE protecting effects. We propose that the combined effects of prophages and FakB1 on FA incorporation are relevant to *S. aureus* infection. Both ϕ 11.1 and ϕ 13 phage may be maintained episomally and reintegrated, implying transient

activation of *geh* and *hly* expression; these events may help adjust bacterial membrane lipids during infection (25-30, 52). PUFAs may in turn impact the life cycle of these prophages.

The composition and levels of TGs and SMs vary among individuals and according to dietary intake. These factors are determinants of infection risk and predisposition to bacterial resistance (43, 53-57). TGs are major reservoirs of long chain UFAs and PUFAs liberated by *Geh* activity and incorporation of these FAs alters bacterial membrane functions and host interactions (6, 18, 19, 22, 58). Cholesterol was recently reported to be a *Geh* substrate (18); its effects on *S. aureus* fitness according to prophage status remains to be investigated. Interestingly, intact *Geh* contributes to masking *S. aureus* from the immune surveillance by TLR2 receptor (1, 59) and 1,2-diglycerols moieties are involved in the interaction TLR2 (60). Roles of position 2 hydrolysis and ϕ 11.1 lysogeny in immune escape merit further investigation.

The *in vivo* significance of ϕ 13 impacts on PUFAs incorporation and elongation is intriguing. Specific interference by *Hly* in PUFA incorporation is an interesting new avenue to investigate ϕ 13 maintenance in the *hly* locus specifically in healthy human host (25,61). Indeed sphingomyelins and their metabolites vary between animals and in disease associated to atypical ϕ 13 integration (62, 63).

In conclusion, ϕ 11.1 and ϕ 13 are interactive regulators of *S. aureus* membrane phospholipid composition in response to environmental TGs and SMs. Our findings lead us to suggest that prophage lysogeny contributes to protecting bacteria from lipid toxicity. Prophage instability also offers greater membrane flexibility, which contribute to *S. aureus* adaptation in different host biotopes. In particular, while *Hly* (β toxin) is required for *S. aureus* virulence in humans, we showed that the associated production of C16 results in an increase of free 1.3DG-derived C18:2 that is toxic for bacteria. Thus, by controlling the cytoplasmic availability of C18:2 and C16,

respectively, $\phi 11.1$ and $\phi 13$ exert a fine tuning between metabolism and toxicity. These results provide new mechanistic insights of *S. aureus* lipid physiology and indicate the need to assess mechanisms of prophage/lipase switches in developing strategies against *S. aureus* infection.

Materials and methods

Bacterial strains. *S. aureus* Newman, USA300 FPR3757 JE2 (called here USA300), and NCTC 8325 derivative strains RN450 and RN450-R, were used in this study. Isogenic Newman strains differentiated by the presence of prophages were provided by Dr O. Schneewind (Department of Microbiology, University of Chicago). Newman strain has 4 prophages which are from Siphoviridae family: $\phi 11.1$, $\phi 11.2$ and $\phi 11.3$ from Sfi 11/Sa6 subfamily and $\phi 13$ from Sfi 21/Sa3 subfamily (24). Methicillin-resistant USA300 and the Nebraska library of transposon insertions (University of Nebraska Medical Center) were generously supplied by BEI resources (64). USA300 derivatives from this library used in this work contained insertions in the following genes: SAUSA300_0320 (*geh*), SAUSA300_1973 (*hlb*), SAUSA300_1119 (*fakA*), SAUSA300_0733 (*fakB1*), SAUSA300_1318 (*fakB2*); all mutants were verified by PCR. RN450 is a phage-free derivative of the NCTC 8325 strain (65). It was repaired for a natural *fakB1* deletion as described for another strain (66).

Construction of *hlb* complemented strain. A PCR fragment containing the *S. aureus* subsp. *aureus* COL *hlb* promoter and open reading frame from SACOL_RS10470 (-355 from ATG start codon and +40 after TAG stop codon) was cloned in EcoRI and SmaI sites of modified pAW8 (67). The primers pairs used for the PCR are 5'-TTGCCGGAATTCTGCAACTTAATTATAGCCAGACTTTC-3' and 5'-CATCAACCCGGGCGTCCTTTTAGAACGAAGCAAG-3'. Genomic co-ordinates of the 1388 bp cloned segment are 2063371-2064758, as present on plasmid phlb.

Construction of *fakB1*-repaired strain. The *fakB1* gene of *S. aureus* RN450, as the entire NCTC 8325 lineage, displays a 483-bp deletion removing 56% of the 867-bp functional gene (Fig. S6). To repair this deletion, a 1,939-bp DNA fragment containing a functional *fakB1* was amplified as described for other NCTC 8325 derivatives (66). The *fakB1*-repaired strain is designated as RN450-R.

Growth media. Brain heart infusion (BHI) was the base medium for *S. aureus* cultures unless specified. Cultures were grown aerobically at 37°C and the starting OD_{600nm} was 0.02 from a preculture at OD_{600nm} between 1 and 5. Mouse serum (Clinisciences, France) was added at 10% final concentration. Stocks of trilinolein (TNL, 50 mM), 1,2-dilinoeoylglycerol (1.2DG, 50 mM), 1,3-dilinoeoylglycerol (1.3DG, 50 mM), palmitic acid (C16, 100 mM) (Larodan Fine Chemicals, Sweden) and orlistat (38 mM, MedChemExpress) were prepared in DMSO.

Determination of *S. aureus* fatty acid profiles. FA profiles were performed as described (7). For each condition, profiles were determined from three independent cultures. In these experiments culture aliquots were routinely taken under exponential growth at OD₆₀₀ between 0.5 and 2. After sample preparation (7), 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 mm; Phenomenex, France). Data were recorded and analyzed by TotalChrom Workstation (Perkin-Elmer). FA peaks were detected between 12 and 40 min of elution, and identified by comparing to retention times of purified esterified FA standards (Mixture ME100, Larodan, Sweden). Results are shown as representative gas chromatograph profiles, or as percent of specific FAs as calculated from their proportions compared to total peak areas (TotalChrom Workstation; Perkin Elmer).

Statistics analysis. Graphs were prepared using Microsoft Excel software. Means and standard deviations are presented for culture growth (OD_{600nm}/h) and FA percentages. Statistical significance was determined by unpaired, nonparametric Mann Whitney tests (U test calculator, Wilcoxon rank-sum, <https://www.statskingdom.com>), as recommended for small sample sizes.

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FIG 1 *S. aureus* lysogenic status impacts incorporation and elongation of C18:2 derived from acylglycerides.

(A) Upper, site of $\phi 13$ insertion in *hlb*, encoding sphingomyelinase (Hlb). $\phi 13$ insertion results in enzyme N-terminal truncation (position 56 aa of the 330 aa protein). Lower, preferred site of $\phi 11.1$ insertion is *geh*, encoding triacylglycerol lipase (Geh). $\phi 11.1$ insertion results in truncation of the encoded triglyceride Geh (position 643 aa of the 690 aa protein).

(B) $\phi 13$ lysogen displays greater C18:2 incorporation from mouse serum than non-lysogen. The Newman strain (wild type, WT), a $\phi 13$ lysogen, and an isogenic $\Delta \phi 13$ derivative were grown 2h in BHI medium containing 10% mouse serum. FAs were extracted and analyzed by gas

chromatography. The y axis represents relative FA abundance (mV response) at indicated peak positions. Black arrows indicate FAs that can be endogenously produced. Red arrows indicate FAs not synthesized by *S. aureus* but incorporated from serum. Representative FA profiles from four determinations are shown.

(C) ϕ 13 and ϕ 11 prophages interfere to modulate C18:2 incorporation and elongation from trilinolein. Four isogenic Newman strains (NM) vary in prophage content, including ϕ 11.1 and ϕ 13 (21). These isogenic strains were grown 2h in BHI medium supplemented with 30 μ M trilinolein (comprising 3 oleic acid moieties). Growth is presented as the absorbance increase in one hour (OD_{600nm}/h). Proportions of C18:2 and elongated forms (C20:2 and C22:2) were determined from the FA profiles as in (A) and presented as percent of the total FA areas for each profile (n=5).

(D) *geh* truncation decreases both incorporation and elongation of C18:2 from trilinolein. C18:2 incorporation from trilinolein is favored when *geh* is intact. USA300 (*geh*⁺) and the *geh*::tn, were grown in media supplemented with TNL. The Geh inhibitor orlistat (orli; 30 μ M) was added as indicated. The confirmed *geh*::tn mutant has a C-terminal truncated Geh (position 517 aa/690aa). The percent of C18:2 and its elongated forms were determined as in (B). The OD/h of the *geh*::tn mutant and the wild type were comparable (0.8 ± 0.3 for both cultures), (n=3).

(E) Acylated positions in a diglyceride determine C18:2 incorporation into *S. aureus* membrane phospholipids. The four isogenic Newman strains, which differ in ϕ 11.1 and ϕ 13 prophages (21) were grown 2h in BHI medium supplemented with 30 μ M diglycerides comprising linoleic acid moieties; 1,2-dilinoleoylglycerol (1.2DG) or 10 μ M 1,3-dilinoleoylglycerol (1.3DG), (n=4). Note that 1.3DG was more incorporated and more toxic than 1.2DG; 30 μ M 1.3DG generated cell lysis and FA profiles of unrepresentative non-lysed subpopulation (not shown). Studies were therefore with 10 μ M. Percent of C18:2 and its elongated forms were determined as in (B). FA profiles and percent of C18:2 and elongated forms are described as in (B).

p-values were determined by Mann Whitney test on percent total incorporated C18:2, including elongated forms ('i'), and on percent elongation of C18:2 ('e1'), and C20:2 ('e2'); n.s., not significant.

FIG 2 Intact *hlb* favors incorporation of saturated FAs and decreases incorporation of UFAs from serum. (A) Newman (NM) WT and $\Delta\phi 13$ cultures were grown for 2 hours in BHI medium supplemented with 10% mouse serum. The proportion of UFAs (left) and SFAs (right) are shown. $\phi 13$ lysogeny increases unsaturated FAs while lowering incorporation of C16 and elongated forms. (B) Plasmid-driven expression of *hlb* increases incorporation of C16 and elongated forms, while lowering incorporation of unsaturated FAs. Cultures of the USA300 (USA) *hlb::tn p∅* strain bearing an empty plasmid or a plasmid carrying intact *hlb* (*pHlb*, derived from the COL strain) were grown for 2.5 h in BHI supplemented with 10% mouse serum. FA extractions were performed and FA profiles and area of FAs were determined as in Fig. 1. Proportions of saturated FAs (C16, C18 and C20) and unsaturated FAs (C18:1 $\Delta 9$, C18:1 $\Delta 11$, C18:2 and C20:1) were compared to total FAs (shown as percent of total). Data presented are means $\pm$ standard deviations from three independent experiments. p-values were determined by Mann Whitney test.

FIG 3 *fakB1* and C16 modulate 1.3DG-derived C18:2 incorporation and its toxicity on growth. (A) Involvement of *S. aureus fakB1* in 1.3DG-mediated C18:2 incorporation. USA300, *fakB1* and *fakB2* strains were grown 2 hours in BHI supplemented with 10μM 1.3DG. (B) 1.3DG-derived C18:2 and C16 compete for FakB1. Effects of C16 (20 μM) addition on C18:2 incorporation from 1.3DG were assessed in USA300, *fakB1*, and *fakB2* strains, as in (A). Representative FA profiles are shown in (A) and (B), (n=5). (C) Growth of WT and *fakB* mutants under conditions described in (A) and (B), presented as OD_{600nm} per hour. Representative profiles (A, B) or means (C) from four independent experiments are presented. p-values were determined by Mann Whitney test for percent incorporated C18:2, including elongated forms and OD/h.

FIG 4 Competition between 1.3DG-derived C18:2 and C16 at *fakB1* shapes incorporation into phospholipid. (A) FA profiles of RN450 (*fakB1*⁻) and RN450-R (*fakB1*⁺) grown 2.5 h in BHI supplemented with 10μM 1.3DG without or with 40μM C16. (B) Growth of RN450 and RN450-R cultures from (A). Data presented are representative profiles (A) or means (C) from five independent experiments. p-values were determined by the Mann Whitney test for percent incorporated C18:2 (including elongated forms (A) and OD/h (B)).

FIG 5 A mechanistic model for environmental lipid utilization by *S. aureus*; $\phi 11.1$, $\phi 13$ and *fakB1* are lipid toxicity and membrane flexibility checkpoints.

$\phi 11.1$ lysogeny protects *S. aureus* from toxic C18:2 incorporation from triglyceride. $\phi 11.1$ in *geh* prevents TG hydrolysis, so that *S. aureus* incorporates low amounts of C18:2. In the absence of $\phi 11.1$, or upon stress-induced excision, intact Geh allows TG hydrolysis to 1.3 DG which promotes C18:2 incorporation. 1.3DG-derived C18:2 (C18:2^{1,3DG}) is incorporated via the FakA-FakB kinase system and the phosphorylated product (C18:2^{1,3DG}-P) may then be incorporated in phospholipids or cycled towards FASII for rounds of elongation before incorporation. The latter pathway is favored by intact Geh by an unknown mechanism.

FakB1 binds C18:2^{1,3DG} and protects from its toxicity. Exogenous FAs are phosphorylated via FakAB system comprising FakA kinase and FakB1 or FakB2 acyl binding proteins, initially reported as being specialized in saturated and unsaturated FAs respectively (15, 16). Despite this bias, we showed in this study that C18:2 from TG or 1.3DG interacts with FakB1 binding protein.

Intact *hbl* interferes with binding of C18:2^{1,3DG} to FakB1, with consequences on toxicity. The absence of $\phi 13$ (or oxidative stress condition) enables Hlb activity and liberation of C16 from serum SMs. This C16 competes with C18:2^{1,3DG} for FakB1 binding and increases pools of free C18:2, which may be toxic. In contrast, $\phi 13$ lysogeny inactivates *hbl* and prevent sphingomyelins (SMs) hydrolysis, thus reducing competition. The entry of C18:2^{1,3DG} via FakB1 may prevent intracellular toxicity and lead to FA elongation via FASII. $\phi 13$ lysogeny thus fine-tunes membrane flexibility via both C18:2^{1,3DG} incorporation and elongation.

C18:2^{1,3DG} represents C18:2 issued from 1.3DG. Impact of $\phi 11.1$, green arrows; impact of $\phi 13$, purple arrows; unknown mechanism of fatty acid diffusion, gray arrows. Experimental results of this study are indicated by the star (*).

Supplementary information titles and legends

Supplementary FIG S1 ϕ 11.1 or ϕ 13 lysogeny has no effect on fatty acid (FA) profiles of *S. aureus* grown in BHI medium. Four isogenic Newman strains, lysogenized or not by ϕ 11.1 and ϕ 13 prophages (1) were grown in BHI medium. FA were extracted at OD₆₀₀ = 1 and analyzed by gas chromatography as described in material and methods. Representative FA profiles from three determinations are shown.

Supplementary FIG S2 Validation of free fatty acids (FAs) incorporation in USA300 (WT), and *fakA*, *fakB1* and *fakB2* mutant derivatives. The indicated strains were grown in BHI in the presence of 0.17 mM each free FAs, C14:0, C16:0, and C18:1. FAs were extracted and analyzed as in Fig. S1. The three exogenous FAs and elongated forms are indicated in red. Data presented are representative profiles from three independent experiments.

Supplementary FIG S3 Incorporation of free C18:2 in the *fakB2* mutant without and with C16 supplementation. The *fakB2* mutant was grown in BHI in the presence 10 μ M free C18:2 without or with 40 μ M C16, and fatty acids (FAs) were extracted and analyzed as in Fig. S1. C16 in upper panel corresponds to endogenous synthesis; the higher C16 peak in lower panel reflects exogenous FA addition. Exogenous FAs or their elongated forms are represented in red. Data presented are representative profiles from three independent experiments.

Supplementary FIG S4 *fakB1* and *fakB2* mutants both mediate C18:2 incorporation from trilinolein, but only the *fakB2* mutant is affected by C16 addition. (A) C18:2 incorporation from trilinolein in USA300 wild type and *fakB* mutants. Strains were grown two hours in BHI supplemented with 30 μ M trilinolein, and fatty acid (FA) profiles were determined as in Fig. S1. The y-axis represents relative fatty acid (FA) abundance (mV response). Exogenous FA incorporated products (C18:2, C20:2, and C22:2) are in red. (B) Cultures were prepared as in (A), except that 20 μ M C16 was further added to medium. The *fakB2* mutant, which expresses FakB1, is totally inhibited for C18:2 incorporation. Green and red arrows indicate changes due to C16 addition on peaks of C16, and C18:2 + elongated products, respectively. (C) Growth of WT, *fakB1* and *fakB2* strains in the presence of trilinolein supplemented or not with C16. Samples correspond to those presented in (A) and (B); results are presented as OD_{600nm} per hour. Data are representative profiles, (A and B) or means (C) from three independent experiments.

Supplementary FIG S5 *fakB2*, and not *fakB1*, affects C18:2 incorporation from 1,2-dilinoeoylglycerol (1.2DG). C18:2 incorporation from 1.2DG was assessed in USA300 WT, *fakB1*, and *fakB2* strains. Strains were grown two hours in BHI supplemented with 30 μ M 1.2DG, and fatty acids (FA) profiles were determined as in Fig. S1. Exogenous FA incorporated products (C18:2, C20:2, and C22:2) are in red. The *fakB2* mutant shows significant inhibition of the cumulative percentage of C18:2 and elongated forms (10 $\pm$ 3 %) compared to wild type (34 $\pm$ 5%). Data are representative profiles from three independent experiments.

Supplementary FIG S6 FakB1 defect in NCTC 8325 lineage, comprising RN450. Alignment of FakB1 sequences from Newman (non-NCTC 8325 lineage) and NCTC 8325 strains. The non-deleted region of NCTC 8325 FakB1 shares 99% identity with the synonymous region in Newman. Red arrows indicate amino acids required for FA binding and for FakA-mediated phosphotransfer (1).

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