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Staphylococcus aureus Adaptation to FASII-Targeted Antibiotics in Host Environments and Strategies to Potentiate Antibiotic Activities

Paprapach Wongdontree

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Staphylococcus aureus Adaptation to FASII-Targeted Antibiotics in Host Environments and Strategies to Potentiate Antibiotic Activities

Adaptation de *Staphylococcus aureus* aux antibiotiques ciblant FASII dans l'hôte et stratégies de potentialisation des activités antibiotiques

Thèse de doctorat de l'université Paris-Saclay

École doctorale n° 581, agriculture, alimentation, biologie, environnement et santé (ABIES)

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Graduate School : Biosphera. Référent : AgroParisTech

Thèse préparée dans l'UMR **Micalis Institute** (Université Paris-Saclay, INRAE, AgroParisTech),
sous la direction de **Jamila ANBA-MONDOLONI**, Chargée de recherche (HDR),
et la co-direction d'**Alexandra GRUSS**, Directrice de recherche

Thèse soutenue à Paris-Saclay, le 18 décembre 2023, par

Paprapach WONGDONTREE

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Membres du jury avec voix délibérative

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Titre : Adaptation de *Staphylococcus aureus* aux antibiotiques ciblant FASII dans l'hôte et stratégies de potentialisation des activités antibiotiques

Mots clés : *Staphylococcus aureus*, synthèse des acides gras, stress oxydatif, virulence, acide lipotéichoïque

Résumé : *Staphylococcus aureus* est un pathogène opportuniste responsable d'infections difficiles à traiter dues à l'inefficacité des antibiotiques actuels. En 2019, cette bactérie a causé le taux de mortalité mondial le plus élevé. La nécessité de recherche de nouveaux antibiotiques a permis de développer de nouvelles molécules inhibant la synthèse des acides gras de type II (FASII). Cependant, ces molécules anti-FASII présentent une faiblesse: *S. aureus* et les pathogènes Firmicutes sont capables de contourner l'inhibition de FASII par incorporation d'acides gras environnementaux présents dans l'hôte (eFA).

L'objectif de ma thèse est d'élucider et comprendre comment le contournement des anti-FASII permettent un regain de développement bactérien. Ceci a permis d'identifier les points faibles de l'adaptation de *S. aureus* aux anti-FASII afin de développer des traitements en bi-thérapie associant d'autres molécules. Deux principales pistes sont développées.

I. Une approche protéomique couplée aux études physiologiques ont permis de mettre en évidence que l'anti-FASII provoque une résistance accrue au stress oxydant associée à une baisse de la virulence via une reprogrammation des fonctions bactériennes. Les données *in vitro* sont confirmées *in vivo* dans un modèle d'insecte montrant que la mortalité des bactéries adaptées à l'anti-FASII est plus lente que les bactéries non traitées. Un pré-traitement par H₂O₂ avant l'ajout des anti-FASII accélérerait l'adaptation. Par ailleurs, il a été montré qu'un tel traitement active l'efflux des antibiotiques. Ce prétraitement stimulerait l'incorporation des eFA permettant une adaptation rapide à l'anti-FASII. En réponse à l'infection les cellules hôte se défendent en produisant du H₂O₂ suggérant que les bactéries adaptées à l'anti-FASII pourrait présenter un avantage sélectif dans de tels biotopes engendrant un état de persistance des bactéries.

II. Les analyses des données protéomiques ont révélé que UgtP, enzyme impliquée dans la synthèse de l'acide lipotéichoïque (LTA), n'était plus produite dans les bactéries adaptées à l'anti-FASII. Après son adaptation à l'anti-FASII, le LTA n'est plus détectable par immunoprécipitation. Ce phénomène est indépendant de la nature des acides gras présents dans les phospholipides membranaires. Les fonctions du LTA dans la division et la croissance sont décrites comme essentielles, comment la bactérie prolifère en son absence? Contrairement au LTA, l'acide teichoïque pariétal (WTA) et la cardiolipine sont surproduits. L'une ou l'autre molécule peut compenser la perte d'expression du LTA. L'absence simultanée du LTA et du WTA est létale pour la bactérie. Ainsi la synergie d'un anti-FASII couplé à un inhibiteur de la synthèse du WTA (le targocil) a été étudiée.

Tous ces résultats montrent que les bactéries adaptées à un anti-FASII sont moins virulentes et plus résistantes au stress oxydatif. Un lien entre le stress oxydatif et l'incorporation des acides gras est pour la première fois démontré. Un élément du métabolisme des acides gras (FASII) impacte la synthèse du LTA. Ce travail souligne l'importance des lipides membranaires dans la pathogénie de *S. aureus* et ouvre de nouvelles perspectives de traitements en bi-thérapie.

Title : *Staphylococcus aureus* Adaptation to FASII-Targeted Antibiotics in Host Environments and Strategies to Potentiate Antibiotic Activities

Keywords : *Staphylococcus aureus*, fatty acid synthesis, oxidative stress, virulence, lipoteichoic acid

Abstract : *Staphylococcus aureus* is a common opportunist pathogen causing infections that are notoriously difficult to treat due to antibiotic failure. In 2019, *S. aureus* caused the highest mortality among pathogens. The need for new treatments spurred the development of a novel pipeline class of antibiotics targeting the fatty acid synthesis (FASII) pathway. However, the use of FASII antibiotics has an inherent flaw: *S. aureus*, and several Firmicute pathogens, are able to compensate FASII inhibition by incorporating environmental fatty acids (eFAs) as present in the host.

The aim of my thesis project is to elucidate the factors contributing to this FASII antibiotic adaptation and to analyze the consequences on bacterial fitness. This information will be used to identify the weak points of anti-FASII-adapted *S. aureus*, and to develop a bi-therapy treatment coupled with anti-FASII. Two main findings are reported.

I. Using combined omics and physiological approaches, we show that anti-FASII adaptation reprograms *S. aureus* to be more oxidative stress resistant, but less virulent. These *in vitro* results were in accordance with studies in an insect model, showing that anti-FASII-adapted *S. aureus* killed insects slowly compared to non-treated *S. aureus*. Priming *S. aureus* with H₂O₂ accelerated FASII-antibiotic adaptation. Previously, H₂O₂ priming was reported to stimulate antibiotic efflux. In contrast, H₂O₂ priming stimulates fatty acid (FA) incorporation, thereby accelerating anti-FASII adaptation *via* a unique mechanism. As the host produces H₂O₂ as part of its antibacterial defense mechanism, FASII-antibiotic-adapted *S. aureus* may have a fitness advantage in some host biotopes.

Collectively, reduced virulence and greater oxidative stress resistance may provide *S. aureus* a means to escape detection and persist in the host. Noted, this is the first report showing the link between oxidative stress and FA incorporation.

II. Our proteomics analyses revealed that a lipoteichoic acid (LTA) synthesis enzyme, "UgtP", was turned off in anti-FASII-treated bacteria. Remarkably, after *S. aureus* adapts to anti-FASII, LTA was nearly non-detectable. This loss was observed regardless of the phospholipid FA composition. In view of LTA reported essentiality for *S. aureus* cell division and growth, we then questioned how *S. aureus* compensates LTA depletion. In contrast to LTA, wall teichoic acid (WTA) and cardiolipin (CL) pools increased in anti-FASII-adapted bacteria. This suggests that WTA and/ or CL might compensate LTA depletion and enable *S. aureus* to grow. Simultaneous removal of both TAs is reportedly lethal. Consequently, the LTA depletion observed in anti-FASII-adapted bacteria enabled us to demonstrate synergistic inhibition between anti-FASII and the WTA inhibitor, targocil, in *S. aureus*.

Our work reveals that anti-FASII-adapted bacteria produce less virulence factors and are more oxidative stress-resistant. These findings reveal a link between oxidative stress and FA incorporation. They further suggest that an element of the FASII pathway exerts control on LTA synthesis. Altogether this work underlines the importance of membrane lipids in *S. aureus* pathogenesis and opens perspectives for designing bi-therapy treatments.

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ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ACP	Acyl carrier protein
AD	Anti-FASII-adapted <i>S. aureus</i>
AFN/A	AFN-1252
Agr	Accessory gene
ai	Anteiso
AIP	Auto inducing peptide
BCAA	Branched-chain amino acid
BCFA	Branched-chain fatty acid
BHI	Brain heart infusion medium
C-di-AMP	Cyclic diadenosine monophosphate
CFU	Colony forming unit
CL	Cardiolipin
ClfA	Clumping factor A
ClfB	Clumping factor B
CWA	Cell wall-anchored proteins
CWG	Cell wall glycopolymer
CRlg	Complement Receptor of Immunoglobulin superfamily
DAG	Diacylglycerol
DG-DAG	Diglucoyl diacylglycerol (lipid anchored)
eFA	Environmental/ exogenous fatty acid
eLtaS	Extracellular LtaS moiety
FA	Fatty acid
FabD	Malonyl CoA-ACP transacylase
FabG	β -ketoacyl-ACP reductase
FabI	Enoyl-ACP reductase
Fad	FA degradation
FadM	Putative acyl-CoA thioesterase
Fak	FA kinase
FAS	Fatty acid synthesis
FASI	Type I fatty acid synthesis
FASII	Type II fatty acid synthesis
Fig.	Figure
G3P	Glycerol-3-phosphate
GdpP	Cyclic di-AMP phosphodiesterase
GluNAc	<i>N</i> -acetylglucosamine
GroP	Glycerolphosphate
h	Hours
Hla	α -toxin/ α -hemolysin
H₂O₂	Hydrogen peroxide
i	Iso
iltaS	IPTG-inducible <i>ltaS</i> LAC strain

IPTG	Isopropyl-D-thiogalactopyranoside
IsdA	Iron-regulated surface determinant A
Lpp	Lipoprotein
LTA	Lipoteichoic acid
LtaA	Glycolipid flippase
LtaS	LTA synthase
Lyso-PA	Lyso phosphatidic acid
Lysyl-PG	Lysyl phosphatidylglycerol
ManNAc	<i>N</i> -acetylmannosamine
MIC	Minimal inhibitory concentration
MRSA	Methicillin-resistant <i>S. aureus</i>
MSCRAMM	Microbial surface components recognizing adhesive matrix molecule
MurNAc	<i>N</i> -acetylmuramic acid
NT	Non-treated <i>S. aureus</i>
P	Platensimycin
PA	Phosphatidic acid
PBP2a	Penicillin-binding protein 2a
PDG	Peptidoglycan
PG	Phosphatidyl glycerol
PlsX	Glycerol-3-phosphate acyltransferase
PlsC	1-acyl-sn-glycerol-3-phosphate acyltransferase
WT	Wild-type
WTA	Wall teichoic acid
RhoP	Ribitolphosphate
SCFA	straight-chain fatty acid
SD	Standard deviation
SdrD	Serine-aspartate repeat-containing protein D
SEM	Standard error mean
SFA/SerFA	BHI + 10% Serum + eFAs
SgtB	Glycosyltransferase
T7SS	Type VII secretion system
TA	Teichoic acid
TLR	Toll-like receptor
Tric/T	Triclosan
UDP-Glc	Uridine diphosphate glucose
UFA	Unsaturated fatty acid
UgtP/YpfP	Glucosyltransferase

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BEFORE READING THE TEXT

In this section, I would like to explain how I have constructed the thesis to make it easy to follow. The thesis begins with an introduction of my work; **Chapters 1-4**. This part is made up of four chapters: *Staphylococcus aureus*, *S. aureus* as an effective pathogen, fatty acid and lipid metabolism in *S. aureus*, and lipoteichoic acid: an essential bacterial structure?, respectively. Subsequently, there will be the result of my thesis work, which will be separated into two sections for **Chapters 5** and **6**. In **Chapter 5**, I will describe the global proteomic changes upon exposure to the FASII inhibitor, in particular virulence factors and oxidative stress. In **Chapter 6**, I will show the main changes in bacterial envelope structure after adaptation to anti-FASII. These two parts will be presented in manuscript form. I will then conclude my work and my perspectives in **Chapter 7**.

In addition, I have also attached my side work that I carried out in collaboration with an M2 student, Milya Palmier, in the **Annex**. This work will focus on $\Delta plsX$ suppressors that allow the *plsX* mutant to grow without fatty acid supplementation.

At the end of the thesis, I will also attach my varolization of the thesis; grant and awards, conferences, and courses during my PhD.

Lastly, I hope you enjoy reading my thesis and see how passionate I've been over the last three years and three months

INTRODUCTION

CHAPTER 1

Staphylococcus aureus

CHAPTER 1

Staphylococcus aureus

1.1 Introduction

Staphylococcus aureus is a Gram-positive opportunist pathogen that was first described by the Scottish surgeon Alexander Ogston in a surgical abscess in 1881, and classified as a "micrococcus". According to its morphology and its appearance, he later named it *Staphylococcus*, derived from Greek words (Greek staphyle, "a bunch of grapes"; kokkos, "grain or berry") (Ogston, 1881). In 1884, *S. aureus* was isolated and cultured by German physician Friedrich Rosenbach from humans and due to its characteristics, produced golden or yellowish colonies, it was assigned the species *-aureus* (from Latin aurum, "gold") (for review, (Rasheed, Hussein, Region, Polytechnic, & Region, 2021)). *S. aureus* belongs to the phylum Firmicutes, class Bacilli, order Bacillales, family Staphylococcaceae, and genus *Staphylococcus* (*Staphylococcus aureus* subsp. *aureus* (gbif.org) access August 2023).

S. aureus both colonizes and infects its hosts. Around 20% of the human population are persistent carriers, while 30% and 50% are intermittent carriers and noncarriers, respectively (Belkum et al., 2009). This carriage may constitute a reservoir that increases risk of disease or transmission to other individuals. *S. aureus* infections range from mild to life-threatening, and the severity of disease is multifactorial (for review, (Salgado-Pabón & Schlievert, 2014)). *S. aureus* infections are of primary concern to both the healthcare sector and community.

We point out three features of *S. aureus* that may contribute to its success as a pathogen: 1- it encodes toxins and numerous secreted and surface factors enabling host infection (see **Chapter 2**) (review (Clarke & Foster, 2006; Heilmann, 2011; Otto, 2014; Rasheed et al., 2021)); 2- It is adaptable to different biotopes, both in non-animal and host environments, e.g., high salinity environments (Tsai et al., 2011), human skin (Burian et al., 2021) and host serum (Kénanian et al., 2019), which will be discuss in **Chapter 3**; 3- it can acquire antibiotic resistance, and is currently a primary cause of untreatable bacterial infections in hospitals. In view of the present antibiotic treatment crisis, the studies we will present will use a methicillin-resistant *S. aureus* (MRSA) isolated from a jailed patient called "USA300" (Fey et al., 2013). Before going into the detailed, we will discuss the general information and importance of *S. aureus* in this chapter.

1.2 Morphology and Habitat of *S. aureus*

S. aureus is spherical in shape with a diameter between 0.5 -1.5 μm . It is non-motile and non-spore-forming and is seen microscopically as a single coccus, diplococcus, or in grape-like clusters (**Fig. 1**). *S. aureus* obtains energy for growth by aerobic or anaerobic respiration, or fermentation, with an optimal growth range between 18-40 $^{\circ}\text{C}$ (review (Rasheed et al., 2021)).

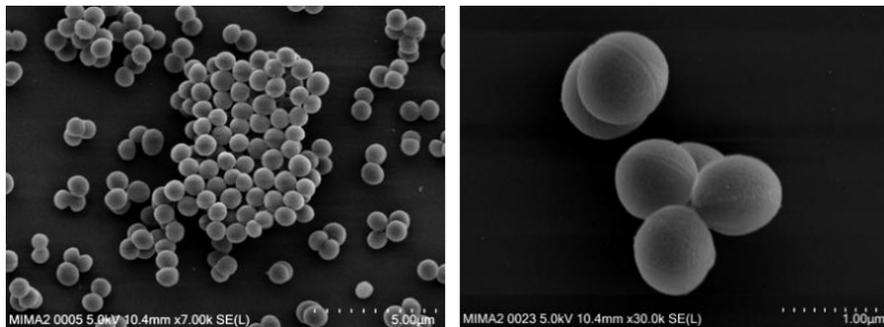


Figure 1. *S. aureus* morphology under scanning electron microscopy (SEM). *S. aureus* can grow as a cluster or as individual cells. Scanning microscopy MIMA2 platform (T. Meylheuc).

S. aureus is described as having numerous natural habitats and is often viewed as a colonizer of the moist squamous epithelium in the anterior nares (review (Foster & Geoghegan, 2015; Williams, 1963)). However, *S. aureus* is also found in the posterior nasal cavity which involves different attachment mechanisms, which correspond to different epithelial cell types (Krismer, Weidenmaier, Zipperer, & Peschel, 2017). To colonize, *S. aureus* uses different cell wall adhesins to bind to host surface cells including adhesin clumping factor B (ClfB), iron-regulated surface determinant A (IsdA), serine-aspartate repeat-containing protein D (SdrD) and wall teichoic acid (WTA) (Krismer et al., 2017).

In addition *S. aureus* also colonizes numerous other body parts, both in the absence or presence of disease, including various skin surfaces (atopic dermatitis) (for review, (Byrd, Belkaid, & Segre, 2018; Geoghegan, Irvine, & Foster, 2018)), perineum (Dancer & Noble, 1991; Ridley, 1959), axillary (Dancer & Noble, 1991)), vagina (Chiaruzzi et al., 2020) and intestine (for review (Acton, Plat-sinnige, Wamel, Groot, & Belkum, 2009)). Moreover, the skin is a non-homogenous surface, having variations in moisture and oiliness, which may require specialized bacterial features for development (Grice & Segre, 2011). Nevertheless, *S. aureus* can colonize various ecological niches of the human body.

1.3 *S. aureus* as a Major Cause of Infections

S. aureus can cause a mild disease to life threatening such as toxic shock syndrome, pneumonia, sepsis and endocarditis (for review, (Keim & Horswill, 2023; Salgado-Pabón & Schlievert, 2014)) (**Fig. 2**). These infections can lead to substantial morbidity and mortality, and high healthcare costs (for review, (Lowy, 1998; Rasigade & Vandenesch, 2014; Salgado-Pabón & Schlievert, 2014)). Indeed, *S. aureus* remains a dominant cause of mortal infections in humans (for review, (Lowy, 1998)), attributed to its capacity to elude treatment by commonly used antimicrobial agents (penicillins, macrolides, aminoglycosides, tetracyclines) (for review, (Foster, 2017)).

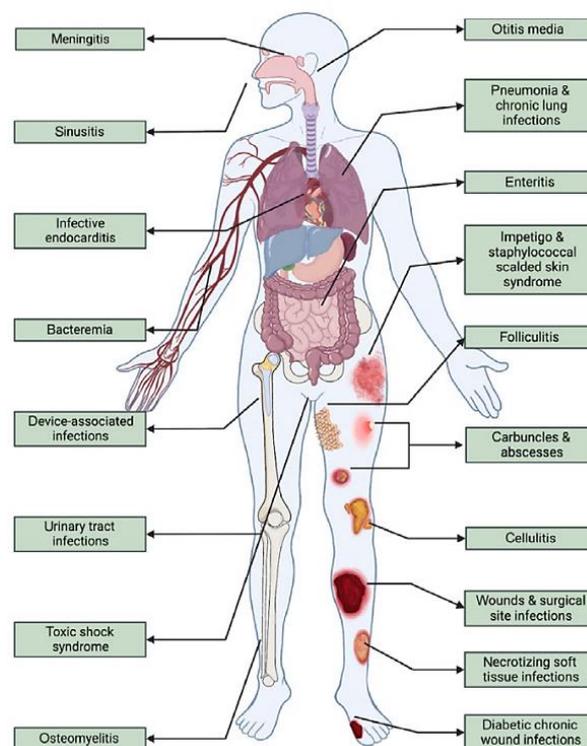


Figure 2. Infection in humans caused by *S. aureus*. *S. aureus* can cause a wide range of diseases in humans from mild disease to life threatening such as pneumonia, sepsis, osteomyelitis and skin infection from (Keim & Horswill, 2023).

While some recent studies report that in some regions, the incidence of staphylococcal infection has decreased, *S. aureus* still poses a serious clinical threat, with high morbidity and mortality, especially antimicrobial resistance bacteria (for review, (Turner et al., 2019)). Methicillin-resistant *S. aureus*, MRSA, is a main type of antimicrobial resistance. Resistance is mediated by a chromosomally carried *mecA* gene, which encodes the penicillin-binding protein 2a (PBP2a), and is acquired by horizontal transfer of a mobile genetic element designated staphylococcal cassette chromosome *mec* (SCC*mec*). PBP2a mediates

peptidoglycan crosslinking. Mutation in *mecA* causes a low affinity of PBP2a for β -lactams, and resulting in resistance to this entire class of antibiotics such as penicillin, methicillin, and oxacillin. (for review, (Turner et al., 2019; Uehara, 2022)). In 2019, MRSA are listed as serious threats due to antibiotic resistance by the CDC (Centers for Disease Control and Prevention) ([Biggest Threats and Data | Antibiotic/Antimicrobial Resistance | CDC](#)). In 2022, a study on global mortality associated with 33 bacterial pathogens showed that *S. aureus* caused the highest mortality in 2019 among 33 inventoried pathogens (GBD 2019 Antimicrobial Resistance Collaborators, 2022) (**Fig. 3**). Altogether, this information alerted researchers about the urgent need for novel treatments against *S. aureus* infection.

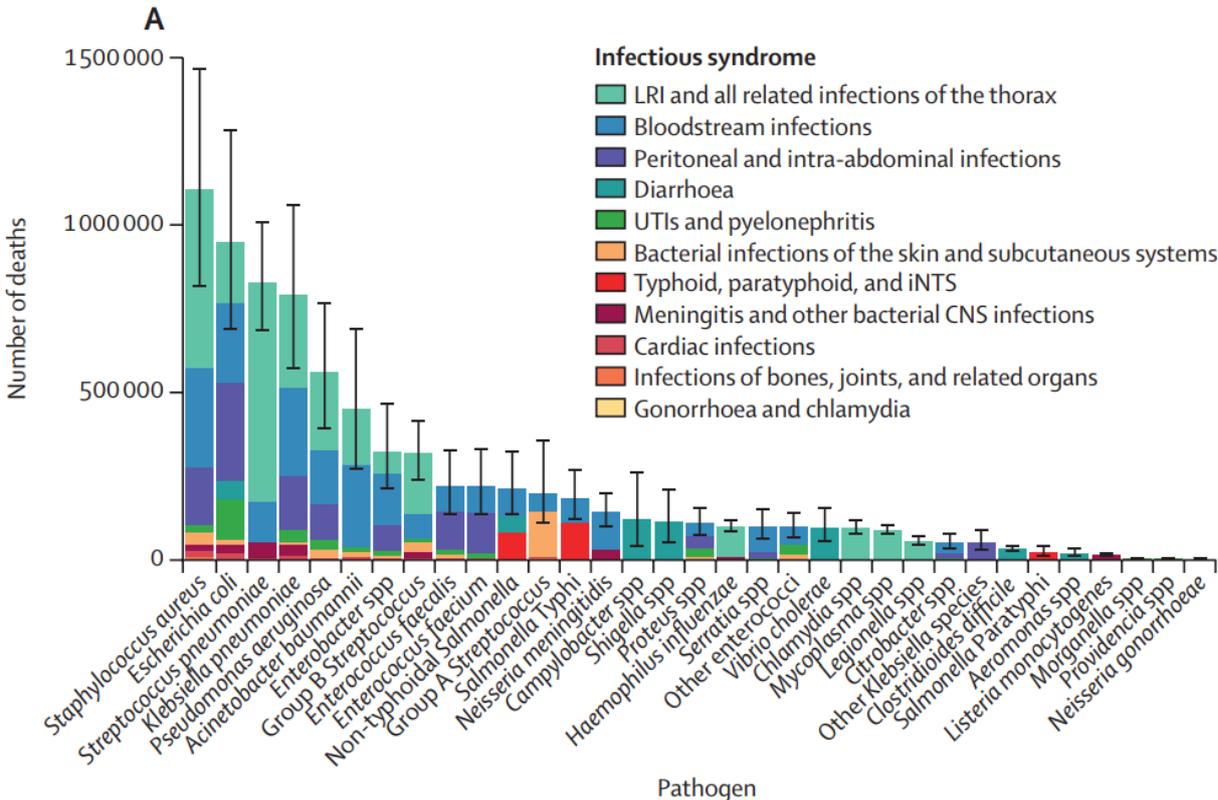


Figure 3. *S. aureus* is a leading cause of human deaths in 2019 among 33 major bacterial pathogens. Column shows total number of deaths for each pathogen, with error bars showing 95% confidence intervals, with the bars segmented into infectious syndromes. LRI=lower respiratory infection. iNTS=invasive non-typhoidal *Salmonella*. *Salmonella* Typhi=*Salmonella enterica* serotype Typhi. *Salmonella* Paratyphi=*Salmonella enterica* serotype Paratyphi. UTI=urinary tract infection. From GBD 2019 Antimicrobial Resistance Collaborators, 2022.

CHAPTER 2

S. aureus as a Successful Pathogen

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S. aureus as a Successful Pathogen

2.1 Introduction

In this section, we will discuss the mechanisms that make *S. aureus* an effective pathogen, focusing on 1- virulence factors, which *S. aureus* uses to adhere to and damage the host tissue, and 2- adaptability to the host environment, which enables bacteria to remain or persist in the host conditions.

2.2 Staphylococcal Virulence Factors

Here, we define “virulence factors” by factors that facilitate the ability of a pathogen to either infect or damage host tissues. *S. aureus* contains a plethora of virulence factors, which contribute to its success as a pathogen (Fig. 4). These virulence factors may mediate colonization, or allow the bacterium to evade the host immune system. Among such factors are 1) cell surface adhesins, 2) secreted factors, and 3) others, which will be discussed below.

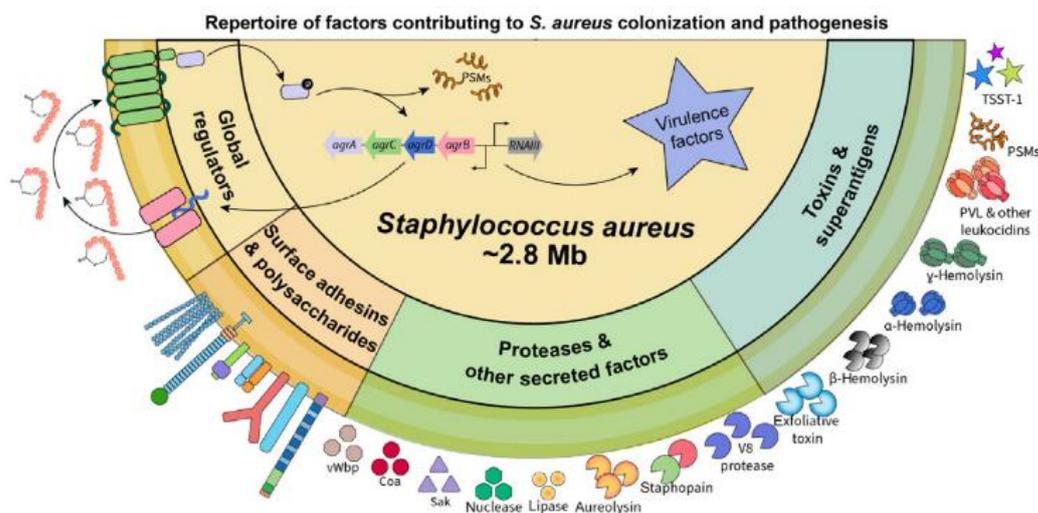


Figure 4. *S. aureus* virulence factors. Examples of *S. aureus* virulence factors include surface adhesins, toxins and secreted proteins, which help mediate infection (from (Keim & Horswill, 2023)).

These virulence factors undergo complex control *via* numerous regulator functions. A major characterized regulator is called Agr (for accessory gene regulator; (Recsei et al., 1986)), which is a global regulator of staphylococcal virulence. The Agr system involves a quorum sensing peptide, auto inducing peptide (AIP) that regulates a large number of virulence-

related factors and regulators including secreted virulence factors and surface proteins (**Fig. 5**). Overall, Agr assures that adhesion factors expressed during exponential phase growth are repressed during the transition to stationary phase, and that secreted toxins and virulence factors are then activated (Traber et al., 2008). Note that numerous regulatory systems have been characterized since the discovery of Agr, which are outside the scope of this study.

In this chapter, virulence factors produced by *S. aureus*, which confer bacteria with the capacity to enter and to evade its host, are categorized into three groups: 1- adhesins that mediate bacterial binding to host tissue, 2- secreted toxins that cause tissue damage and interfere with host immunity, and 3- others (Nikolic & Mudgil, 2023)

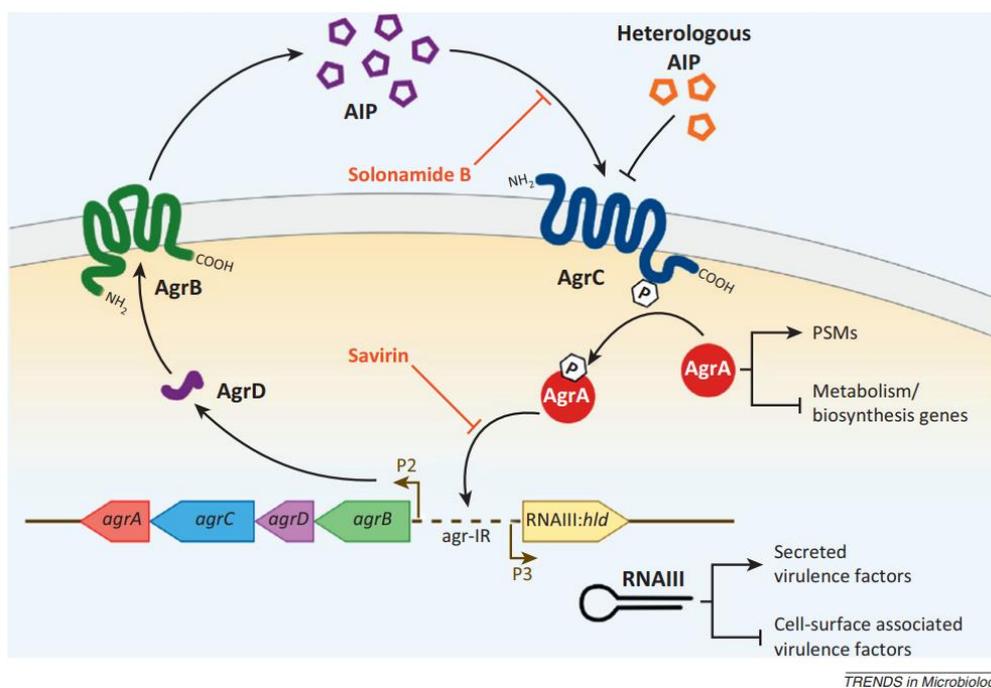


Figure 5. *S. aureus* accessory gene regulatory Agr system. This system comprises two divergent transcription units, RNAII and RNAIII driven by promoters P2 and P3, respectively. The P2 promoter produces components of a quorum-sensing system (AgrBDCA), whereas expression from P3 produces the *agr* effector molecule RNAIII. The P2 operon encodes a two-component signalling module AgrA and AgrC, where AgrC is the histidine kinase receptor and AgrA is the response regulator. The P2 operon also encodes two proteins, AgrB, an integral membrane endopeptidase and AgrD, precursor of autoinducing peptide (AIP). AgrB converts the precursor AIP to mature AIP and exports it outside the cell. AIP is recognized by histidine kinase AgrC, which subsequently phosphorylates AgrA in the cytosol. AgrA activates transcription from its own promoter and from the P3 promoter, which drives RNAIII synthesis. RNAIII is responsible for posttranscriptional regulation of several virulence factors, mediating the shift from expression of cell-surface associated proteins to the production of secreted toxins and δ -haemolysin. From (Painter, Krishna, Wigneshweraraj, & Edwards, 2014).

2.2.1 Surface adhesins

S. aureus surface adhesins bind to human extracellular matrix components and serum proteins, as needed for host colonization and subsequent dissemination (Clarke & Foster, 2006; Heilmann, 2011). Many, but not all cell surface adhesins are covalently bound to the cell-wall peptidoglycan by sortase SrtA, and are also known as cell wall-anchored proteins (CWAs) (**Table. 1**) (Clarke & Foster, 2006; Heilmann, 2011). *S. aureus* can express up to 24 CWAs (McCarthy & Lindsay, 2010).

CWAs are required during the process of infection and abscess formation. This was shown by genetic experiments where adhesin attachment to bacterial peptidoglycan was disabled by inactivating *srtA*. The *srtA* defective mutant showed lower bacterial burden and a significantly lessened pathology compared to the wild-type strain in a murine skin infection model (Kwiecinski, Jin, & Josefsson, 2014), and also led to reduced virulence in a murine kidney abscess model (Mazmanian, Liu, Jensen, Lenoy, & Schneewind, 2000).

Among the CWA proteins, the MSCRAMM (microbial surface components recognizing adhesive matrix molecule) family contributes to *S. aureus* infection and colonization. MSCRAMMs comprise two tandemly arrayed IgG-like folded domains that bind to the host in initial steps of colonization and infection. The best characterized MSCRAMMs are clumping factor A (ClfA) and clumping factor B (ClfB) (for review, (Foster, 2019; Foster, Geoghegan, Ganesh, & Höök, 2014; Foster & Höök, 1998)). Both ClfA and ClfB bind fibrinogen, but to different chains: ClfB reacts with the α - and β -chains of fibrinogen whereas ClfA binds exclusively to the γ -chain in Western ligand blots (Eldhin et al., 1998; Mcdevitt et al., 1997). Besides, ClfB reportedly binds to cytokeratin 10 and loricrin of nasal epithelial cell, indicating a role in colonization (Mulcahy et al., 2012; O'Brien, Walsh, Massey, Peacock, & Foster, 2002). In *in vivo* studies, both *clfA* and *clfB* mutants are attenuated in endocarditis, skin, and soft tissue infection models (Entenza et al., 2000; Lacey et al., 2019, Moreillon et al., 1995). ClfB was recently reported as being required for *S. aureus* virulence in a sepsis model (Dyzenhaus et al., 2023).

As may be expected, CWA proteins are also implicated in biofilm formation *e.g.*, ClfB (under calcium depleted conditions) (Abraham & Jefferson, 2012), SdrC (Barbu, Mackenzie, Foster, & Höök, 2014), IsdA (Corrigan, Miajlovic, & Foster, 2009), and protein A (spA) (Merino et al., 2009). These above examples illustrate the important roles of surface adhesin proteins for *S. aureus* colonization and pathogenesis.

Table 1. Cell surface adhesins of *S. aureus*. Factors were ordered as covalently-linked surface proteins and non-covalently-linked surface-associated proteins (from (Heilmann, 2011)).

Gene	Product	Ligand specificity/Function
Covalently-linked surface proteins		
<i>bbp</i>	Bbp	Bone sialoprotein
<i>bap</i>	Bap	Attachment to polystyrene; intercellular adhesion; prevents binding to Fg, Fn, and host tissue and internalization
<i>clfA</i>	ClfA	Fg; platelet aggregation
<i>clfB</i>	ClfB	Fg, cytokeratin 10, desquamated nasal epithelial cells; platelet aggregation
<i>cna</i>	Cna	collagen
<i>fnbA</i>	FnBPA	Fn, Fg, elastin; intercellular adhesion
<i>fnbB</i>	FnBPB	Fn, elastin; intercellular adhesion
<i>isdA</i>	IsdA	Fg, Fn, fetuin, haemoglobin, transferrin, haemin, desquamated nasal epithelial cells
<i>isdB</i>	IsdB	haemoglobin, haemin, platelet integrin GPIIb/IIIa
<i>isdC</i>	IsdC	Haemin
<i>isdH</i>	IsdH	Haptoglobin, haptoglobin-haemoglobin complex
<i>sasA (sraP)</i>	SasA (SraP)	Binding to platelets
<i>sasB</i>	SasB	unknown
<i>sasC</i>	SasC	Attachment to polystyrene; intercellular adhesion
<i>sasD</i>	SasD	unknown
<i>sasF</i>	SasF	unknown
<i>sasG</i>	SasG	Binding to nasal epithelial cells; intercellular adhesion; prevents binding to Fg, Fn, cytokeratin 10, IgG
<i>sasK</i>	SasK	unknown
<i>sasH</i>	SasH	unknown
<i>sdrC</i>	SdrC	Binding to nasal epithelial cells and β -neurexin
<i>sdrD</i>	SdrD	Binding to nasal epithelial cells
<i>sdrE</i>	SdrE	Platelet aggregation
<i>spa</i>	Protein A (Spa)	IgG, IgM, von Willebrand Factor, platelet receptor α IIb β 3; bacterial cell aggregation
<i>pls</i>	Pls	Promotes binding to nasal epithelial cells, glycolipids; intercellular adhesion; prevents binding to IgG and Fn and internalization
Non covalently-linked surface-associated proteins		
<i>atl</i>	Atl	Attachment to polystyrene, Fg, Fn, Vn, endothelial cells
<i>aaa</i>	Aaa	Fg, Fn, Vn
<i>eap</i>	Eap (Map, P70)	Fg, Fn, Vn, collagen, ICAM-1, eukaryotic cell surfaces, staphylococcal cells; promotes uptake of <i>S. aureus</i> by eukaryotic cells
<i>emp</i>	Emp	Fg, Fn, Vn, collagen
<i>ebh</i>	Ebh	Fn
<i>ebps</i>	Ebps	Elastin

2.2.2 Secreted virulence factors

The apparent primary roles of many secreted virulence factors are to manipulate the host innate and adaptive immune responses such as α -toxin, lipases and exfoliative toxins (for review, (Nikolic & Mudgil, 2023; Otto, 2014; Tam & Torres, 2019)) (**Figure 6**).

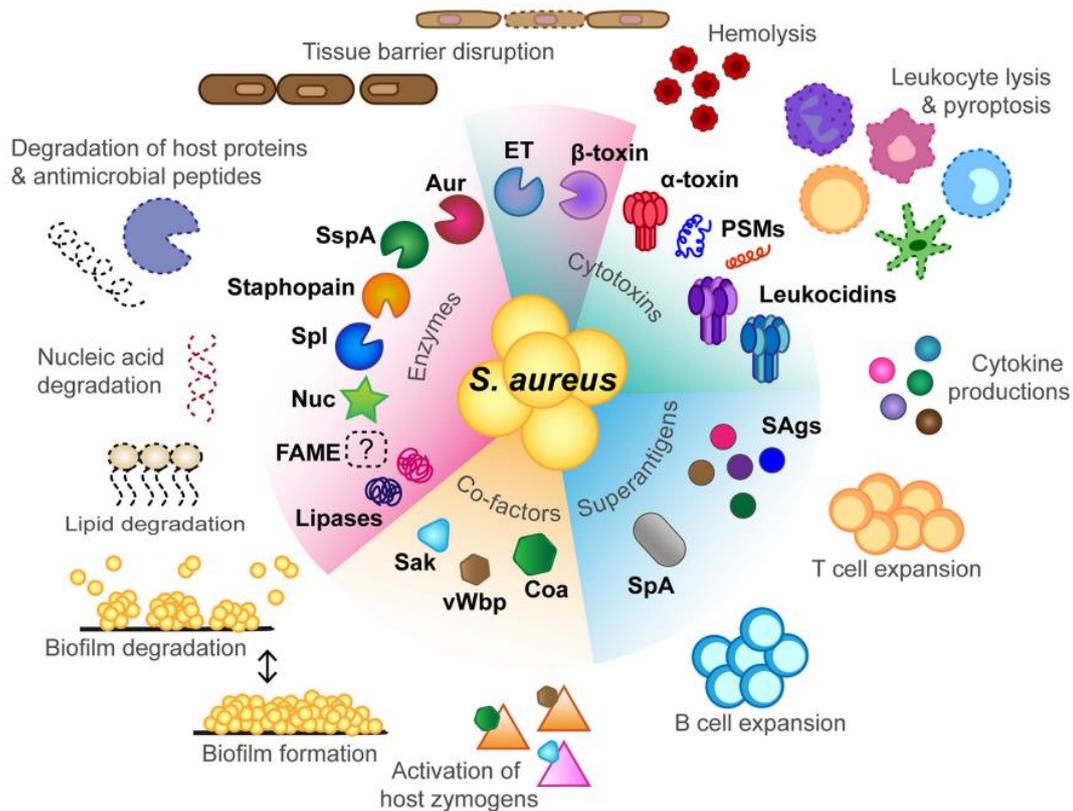


Figure 6. Secreted virulence factors of *S. aureus*. Principal secreted virulence factors produced by *S. aureus* include cytotoxins, superantigens, co-factors and enzymes such as nuclease (Nuc) and lipase. Their main functions are schematized in the outer ring of the diagram. ET: exfoliative toxins; PSM: phenol soluble modulins; SAGs: T cell superantigens; Spa: staphylococcal protein A; Coa: coagulase; vWbp: von Willebrand factor binding protein; Sak: staphylokinase; FAME: fatty acid-modifying enzyme; Nuc: nuclease; Spl: serine protease-like proteins A; SspA: V8 protease; Aur: aureolysin. From (Tam & Torres, 2019).

Secreted toxins or exotoxins represent about 10% of the total secretome (for review, (Kusch & Engelmann, 2014)). Several of them are highly inflammatory and can cause leukocyte cell death by cytolysis or clonal deletion (removal of immature lymphocytes that interact with antigens by apoptosis). These exotoxins are categorized into three main groups due to their functions: cytotoxins, superantigens, and enzymes (**Table 2**).

Table 2. Major exotoxins produced by *S. aureus*. From (Tam & Torres, 2019).

Exotoxin(s)	Gene(s)	Function(s)	
α -Toxin	<i>hla</i>	Pore-forming toxin	Cytotoxins
PVL (LukSF-PV)	<i>lukS, lukF</i>	Pore-forming toxin	
HlgAB	<i>hlgA, hlgB</i>	Pore-forming toxin	
HlgCB	<i>hlgC, hlgB</i>	Pore-forming toxin	
LukED	<i>lukE, lukD</i>	Pore-forming toxin	
LukAB/HG	<i>lukA/H, lukB/G</i>	Pore-forming toxin	
LukMF'	<i>lukM, lukF'</i>	Pore-forming toxin	
LukPQ	<i>lukP, lukQ</i>	Pore-forming toxin	
PSMa1 to PSMa4	<i>psma1 to psma 4</i>	Phenol soluble modulins	
PSM β 1, PSM β 2	<i>psmβ1, psmβ2</i>	Phenol soluble modulins	
δ -Toxin	<i>hld</i>	Phenol soluble modulins	
PSM-mec	<i>psm-mec</i>	Phenol soluble modulins	
ϵ -Toxin	<i>cytE</i>	Cytotoxin	
SEA to SEE, SEG	<i>sea to see, seg</i>	Enterotoxins, T cell superantigens	
SE-I H to SE-I Y	<i>selh to sely</i>	T cell superantigens	
TSST-1	<i>tst</i>	T cell superantigen	
SpA	<i>spa</i>	B cell superantigen	
β -Toxin	<i>hlb</i>	Sphingomyelinase, biofilm ligase	Cytotoxic enzymes
Exfoliative toxin A	<i>eta</i>	Serine protease	
Exfoliative toxin B	<i>etb</i>	Serine protease	

The main roles of cytotoxins secreted by *S. aureus* is to lyse host cells, presumably for nutrient acquisition. Indeed, the different secreted toxins can lyse various cell types. The most well-known cytotoxins are α -toxin/ α -hemolysin (Hla) and leukocidins, which can lyse several types of cells including erythrocytes, platelets, endothelial cells, epithelial cells, and leukocytes (for review, (Vandenesch, Lina, & Henry, 2012)). Superantigens produced by *S. aureus* can cause T and B cell expansions, which lead to clonal deletion and massive cytokine production, as occurs in response to toxic shock syndrome toxins and enterotoxins. Other staphylococcal enzymes, such as β -toxin and exfoliative toxins can also damage the host. Some enzymes mediate degradation of host proteins (for review, (Tam & Torres, 2019)); for example, the secreted nuclease was proposed to degrade neutrophil DNA traps meant to limit bacterial dissemination (Berends et al., 2010; Brinkmann et al., 2004)

2.2.3 Other virulence factors

In addition to surface adhesins and secreted virulence factors, there are other structures that contribute to *S. aureus* virulence. Here, we discuss membrane-associated structures involved in staphylococcal infection, including staphyloxanthine (*S. aureus* pigment), and lipoproteins (Lpps). Most *S. aureus* produce the carotenoid 'golden' pigment known as staphyloxanthin, including USA300, whose biosynthesis genes are encoded by the *crtOPQMN* operon (Pelz et al. 2005). Staphyloxanthin reportedly protects *S. aureus* from oxidative stress and neutrophil killing (Clauditz et al. 2006; Liu et al. 2005). Disruption of this

pigment led to reduced virulence in a mouse subcutaneous abscess model (Liu et al. 2005). Increased staphyloxanthin production also raised cell membrane rigidity and reduce bacterial susceptibility to cationic peptides (Mishra et al. 2011). There are also Lpps that contribute to virulence of *S. aureus*, which will be discussed in **Chapter 4**. Then, we have a type VII secretion system (T7SS), which mediates the secretion of two small proteins; EsxA (6 kDa) and EsxB (10 kDa). These products are involved in *S. aureus* abscess formation during infection, and are implicated in bacterial persistence (for review, (Bowman & Palmer, 2021)). Interestingly, the T7SS is induced by environmental fatty acids (Lopez et al., 2017). The proteins secreted by T7SS are implicated in assuring membrane integrity from lipid stress, although these mechanisms remain to be elucidated (Kengmo Tchoupa et al., 2020).

Altogether, These show that *S. aureus* contains several virulence factors, which confers *S. aureus* during host infection. Here we illustrated that successful *S. aureus* infection involves expression of surface adhesins that bind host cells for colonization, of secreted virulence factors that provide access to nutrients and/or prevent destruction by the host immune system, and others (e.g. Lpps and Staphyloxanthin), which contribute to *S. aureus* virulence. Coordinated expression of these factors is essential for infection. This information relates to the results of our research work (**Chapter 5**) showing that alterations in expression of virulence, as occurs during bypass of the fatty acid synthesis pathway, leads to altered infection dynamics.

2.3 Staphylococcal Adaptability in the Host: Importance of Lipid Homeostasis

The capacity of *S. aureus* to cause a wide variety of diseases depends not only on the controlled expression of virulence factors, but also on the pathogen's ability to adapt to distinct and changing environmental niches during infection. In view of the numerous niches colonized by *S. aureus*, we speculate that expression of various factors is needed according to bacterial localization in different host environments, which help adaptation and/or prevent bacterial elimination. Major stress factors encountered in the host include metal starvation (called "nutritional immunity") (for review, (van Dijk, de Kruijff, & Hagedoorn, 2022)), oxidative and pH stress (for review, (Beavers & Skaar, 2016; C. Zhou & Fey, 2020)), and lipid-related stresses. Here, we describe mainly adaptation related to lipid-rich host environments, as relevant to the thesis project.

The animal host is rich in lipids, which are found to different extents in all *S. aureus* sites of infection. The lipid-rich environment varies according to i- the site on the host (*i.e.*, organs and fluids differ in lipid composition), ii- individual differences (*e.g.*, genetics, diet, and drug use). As lipids can be toxic (Chen & Alonzo, 2019) as discussed below, bacterial adaptation involves the capacity to avoid toxicity. In this part, we will discuss separately for each condition.

1- Skin and fatty acid efflux: Commensal and pathogenic *S. aureus* can colonize human skin even though the healthy human epidermis provides physical protection and is impenetrable for pathogenic microbes. *S. aureus* that colonize the skin produce lower levels of virulence factors, while surface adhesins increase upon initial contact with skin (Burian et al., 2021). Moreover, during colonization and infection *S. aureus* are exposed to some antimicrobial fatty acids (FAs) such as palmitoleic acids (C16:1), linoleic (C18:2), and arachidonic (C20:4), however, the main FAs found in host during normal or abscess periods are palmitic (C16), oleic (C18:1) and linoleic (C18:2), which is not exclusively toxic FAs (Shryock & Kapral, 1992). *S. aureus* possess mechanisms conferring FA resistance by increased expression of *farE* (FA resistance), which encodes a pump that effluxes linoleic acids (Alnaseri et al., 2015). Additionally, bacterial surface structures, such as wall teichoic acid (WTA) (Parsons, Yao, Frank, Jackson, & Rock, 2012) and surface protein IsdA (Clarke et al., 2007) also show to protect *S. aureus* from FAs toxicity as the mutation in these gene makes the bacteria more sensitive to FAs.

2- Host immune system and bacterial lipases: *S. aureus* might escape from, or defend itself against, the host immune system. For example, lipases may give *S. aureus* access to free FAs and contribute to disruption of lipid barriers from host. Interestingly, bacterial Geh lipase masks bacteria from host immune recognition by inactivating bacterial lipoproteins (Lpps) (Chen & Alonzo, 2019).

Overall, this chapter demonstrated different strategies used by *S. aureus* to overcome the host during infection, either by producing virulence factors such as adhesins and secreted virulence factors, or by adapting to host environmental stresses and/or escaping from the host immune system.

Thinking Box 1

To date, *S. aureus* infections are still difficult to treat even though antibiotics are available, due largely to antimicrobial resistance to available drugs. In addition, *S. aureus* produces a wide range of virulence factors and is capable of surviving in numerous host biotopes. The bacterial flexibility could cause severe infection, especially in a compromised host, if existing treatments fail. Consequently, novel treatments are urgently required for killing *S. aureus*.

In **Chapter 3** we will discuss a much-considered antimicrobial target for use in *S. aureus*, enzymes in the fatty acid synthesis (FASII) pathway. The consequences of anti-FASII adaptation with related to virulence and stress is also the subject of **Chapter 5**.

CHAPTER 3

Fatty Acid and Lipid Metabolism in *S. aureus*

CHAPTER 3

Fatty Acid and Lipid Metabolism in *S. aureus*

3.1 Introduction

Membrane biogenesis is a vital aspect of bacterial physiology, as bacterial survival depends on the capacity of bacteria to adapt their lipid composition to changing environments (for review (Albanesi, Arabolaza, Gramajo, & Mendoza, 2020)). Fatty acid (FA) metabolism is expectedly a crucial step in membrane homeostasis, as the biophysical properties of membranes are largely determined by the composition of FAs. FA availability is thought to rely on *de novo* synthesis (for review, (Albanesi et al., 2020; Y. M. Zhang & Rock, 2008)). Added to this, bacteria are often subject to abrupt changes in their extracellular environment, requiring the modification of existing FAs to rapidly adjust to new conditions (J. E. Cronan & Gelmann, 1975). While the enzymology of fatty acid synthesis (FAS) has been under study for over 70 years, the ways that enzymes interact, and how they impact cell physiology are still under study.

FAS is classified into two types. While the components of both FAS pathways share common features, there are considerable differences between bacterial (FASII) and eukaryotic (FASI) systems (**Fig. 7**). The FASII system is commonly found in bacteria, but is also present in specialized eukaryotic organelles such as mitochondria and plastids in plants (for review, (A. P. Brown, Slabas, & Rafferty, 2009; Hiltunen et al., 2010)). FASII comprises discrete enzymes, each of which is encoded by a different gene and exists as a soluble monofunctional protein. Conversely, FASI is typically found in fungi and animal cells. In those cases, one or two large proteins integrate all catalytic steps (for review (Albanesi et al., 2020)). Thanks to these differences, unique target sites can be considered for selective bacterial inhibition by antimicrobials (for review, (Marrakchi, Zhang, & Rock, 2002)), as will also be discussed. FASII has therefore long been an attractive target for drug development for prospective treatment of infections due to bacterial pathogens. In this study, we will focus on the effects of anti-FASII against the major pathogen *S. aureus*, which is related to our results (**Chapter 5 & 6**).

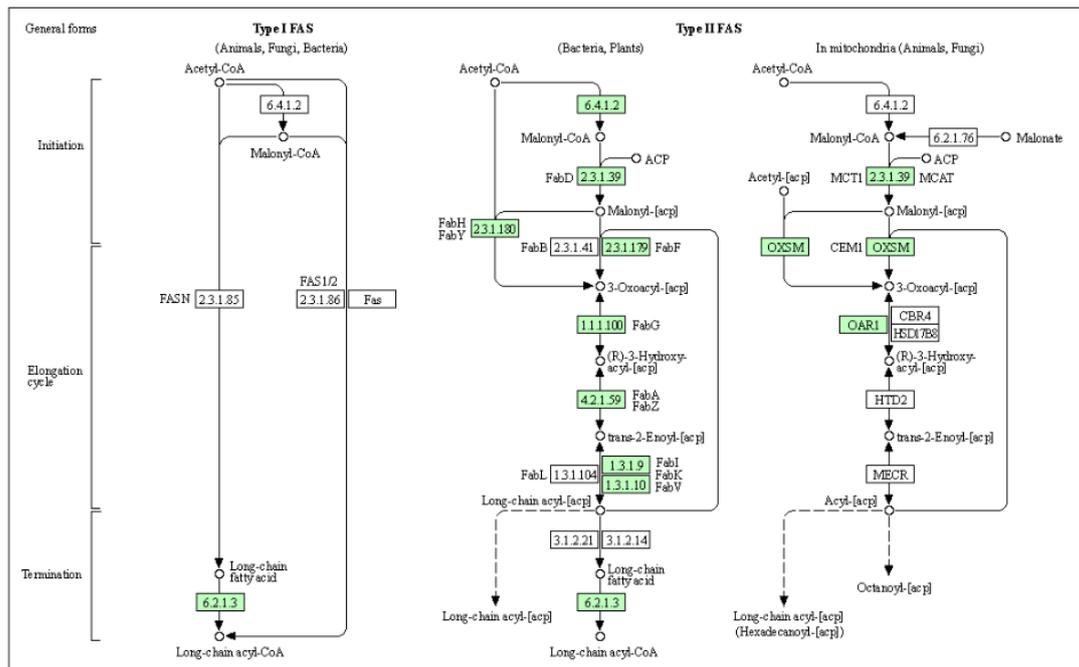


Figure 7. Schematics of FASI and FASII synthesis, as generally found in eukaryotes and bacteria.

FASI notably utilizes a long single multifunctional enzyme (left), whereas each reaction of FASII is performed by a separate enzyme (right). Green box represents the genes utilized in each FAS. Diagram source is from KEGG pathway: [KEGG PATHWAY: Fatty acid biosynthesis - *Staphylococcus aureus subsp. aureus* USA300 FPR3757 \(CA-MRSA\)](#). Note that there are exceptions to this general rule. For example, *Bifidobacterium* seems to encode a single polypeptide that assures FAS functions ([KEGG PATHWAY: Fatty acid biosynthesis - *Bifidobacterium animalis subsp. lactis* BLC1 \(genome.jp\)](#)). *Mycoplasma* and some lactobacilli lack a fully functional FASII pathway and rely on exogenous lipids for growth (Boekhorst et al., 2004; Himmelreich et al., 1996).

3.2 FASII and Phospholipid Synthesis Pathways in *S. aureus*

Most known bacteria synthesize their own FAs, which are used to produce membrane phospholipids. To our knowledge, saturated straight chain FAs are always synthesized. To counterbalance membrane rigidity, some bacteria (*e.g.*, streptococci) also synthesize unsaturated FAs (UFAs) (for review, (Lambert, Poyart, Gruss, & Fouet, 2022)), while others (*e.g.*, *S. aureus*, *Listeria*, and *Bacilli*) synthesize branched chain saturated FAs (H. Oku & Kaneda, 1988; Sun & O’Riordan, 2010). In addition, some bacteria (*e.g.*, *Bacillus subtilis*), encode a desaturase that generates UFAs from the saturated FAs in membrane phospholipids (Aguilar, Cronan, & De Mendoza, 1998). Finally, species such as *mycoplasma* and some lactobacilli lack an intact FASII pathway, and rely on exogenous FAs (eFAs) to produce their membranes (Boekhorst et al., 2004; Himmelreich et al., 1996). These differences illustrate the variability of how bacteria produce their membranes. Here, we will focus on FASII as studied in *S. aureus*, which will be shown below.

In *S. aureus*, FASII begins from acetyl-CoA and branched-chain amino acids (BCAAs), which are substrates for producing the building blocks used for phospholipid synthesis. FA products are then used for membrane lipid formation in the next step, as discussed below (Marrakchi et al., 2002). A key component of the FASII pathway is the acyl carrier protein (ACP), which in its activated form carries all steps of FASII pathway intermediates from enzyme to enzyme (Prescott & Vagelos, 1972). Recent studies suggest that the affinity of ACP intermediates for FASII enzymes varies according to the FA features and the enzyme. These differences, as shown for FabF, likely affect the dynamics of FASII activity (Mindrebo, Misson, Johnson, Noel, & Burkart, 2020). It is notable that in *S. aureus*, phospholipid FAs differ in chain lengths usually between 14 and 20 carbons. They are all saturated FAs, that are either straight chain, or which contain *iso* (*i*) or *anteiso* (*ai*) methyl branches (branched chain FAs; BCFA) (**Table 3**). In addition to *S. aureus*, *Listeria* and *Bacillus sp.*, also synthesize BCFA as major FA components of membrane phospholipids (H. Oku & Kaneda, 1988; Sun & O’Riordan, 2010). BCFA in bacteria are synthesized from branched-chain α -keto acids derived from valine, leucine, and isoleucine substrates. The BCFA products exist in either *anteiso* or *iso* forms (Kaneda, 1991; Zhu, Bayles, Xiong, Jayaswal, & Wilkinson, 2005). Interestingly, the *S. aureus* FabH enzyme shows preferred substrate preferences, with isobutyryl- > hexanoyl- > butyryl- > isovaleryl- >> acetyl-CoA, leading to synthesis respectively of *iso* → *anteiso* → straight-chain FAs (SCFAs) (Qiu et al., 2005). The substrate preference differs from that of *E. coli*, where FabH preferentially uses acetyl-CoA to synthesize SCFA. Another means of synthesizing BCFA is by using branched short-chain carboxylic acids as substrates, which are exogenously supplied; these are used by a small proportion of bacteria, including ruminal bacteria and mutants of *Bacillus subtilis* that need branched short-chain carboxylic acids for growth (Kaneda, 1991). In *S. aureus*, the main FA is *ai*15:0 (Morvan et al., 2016; O’Donnell, Nahaie, Goodfellow, Minnikin, & Hájek, 1985). The steps of FA synthesis is shown below.

a. FA initiation

Acetyl-CoA is a “hub” substrate for numerous reactions, including the Krebs cycle, pigment formation, and FASII synthesis (Khemici et al., 2020; Tiwari, Gatto, & Wilkinson, 2018) (**Fig. 8**). It is carboxylated to form malonyl-CoA by the first dedicated FASII enzyme, acetyl-CoA carboxylase (ACC). ACC contains four subunits encoded by four genes, *accA*, *accB*, *accC* and *accD*. Malonyl-CoA is then transferred to ACP by the malonyl-CoA:ACP transacylase (FabD) to form malonyl-ACP (**Fig. 9**).

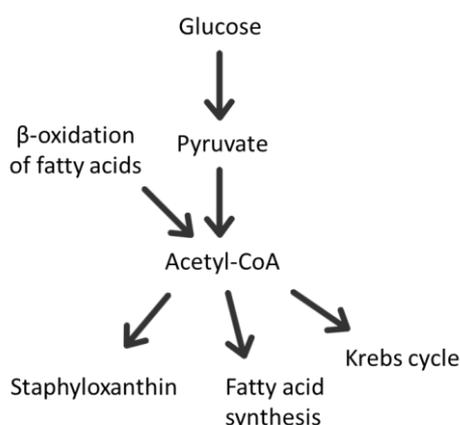


Figure 8. Acetyl-CoA in *S. aureus* is at the crossroads of different reactions. Acetyl-CoA is a substrate for many reactions, including the Krebs cycle (for energy production), pigment formation (staphyloxanthin), and fatty acid synthesis (FASII).

Table 3. Structures of two major FAs present in membrane phospholipids of *S. aureus*.

From (Sen et al., 2016).

BCFA	Anteiso C15:0	
	Iso C15:0	
SCFA	C18:0 (Stearic acid)	

b. FA elongation

FabH condenses an acyl-CoA with malonyl-ACP to generate β -ketoacyl-ACP, which primes the elongation cycle. FA elongation is then catalyzed by four enzymes to synthesize the acyl-ACP end-product. Each turn adds two carbons to the growing chain (John E. Cronan & Thomas, 2009) (**Fig. 9**). FabF or FabH provides the priming FA to be elongated to produce

β -ketoacyl-ACP, which then undergoes NADPH-dependent reduction to β -hydroxyacyl-ACP by β -ketoacyl-ACP reductase (FabG). The β -hydroxyacyl-ACP product is dehydrated to *trans*-2-enoyl-ACP which is catalyzed by β -hydroxyacyl-ACP dehydratase (FabZ) in *S. aureus*. Interestingly, some bacteria produce a FabZ analogue, FabA, or alternatively FabM, both of which mediate UFA synthesis; these enzymes are lacking in *S. aureus* (Richard J. Heath & Rock, 1996; Marrakchi et al., 2002). Importantly, dehydratases are readily reversible, and cycles of elongation must be driven to completion by an enoyl-ACP reductase (FabI) (R. J. Heath & Rock, 1995). Unlike other bacterial species that have multiple isoforms of enoyl-ACP reductase (FabI/K/L/V), *S. aureus* encodes only FabI, which may explain the considerable interest in using this enzyme as antibiotic target (Schiebel et al., 2012).

According to the fatty acyl chain length, and possibly other factors affecting the bacterial state, the acyl-ACP may have limited access to FabF, and may then be utilized by the acyltransferase module to generate phosphatidic acid (PA).

c. Phosphatidic acid formation

The initial step in membrane lipid formation involves the transfer of FAs from the acyl-ACP FASII product to the glycerol-3-phosphate (G3P) backbone. PlsX, a glycerol-3-phosphate acyltransferase, is a conditionally membrane-associated protein that catalyzes formation of acyl-PO₄ (Sastre et al., 2020). This product is used by PlsY, an integral plasma-membrane protein (Y. J. Lu, Zhang, Grimes, Lee, & Rock, 2007), to acylate the 1-position of glycerol-3-P, thereby forming lysophosphatidic acid (Lyso-PA). Lyso-PA is a substrate for PlsC (1-acyl-sn-glycerol-3-phosphate acyltransferase), a membrane-associated protein that acylates the 2-position of Lyso-PA to form PA (**Fig. 11**), a universal precursor of phospholipid species. Of note, PlsC of *E. coli* uses either acyl-ACP or acyl-CoA as acyl donors, but the PlsC of Gram-positive bacteria uses only acyl-ACP as substrate (Y. J. Lu et al., 2006).

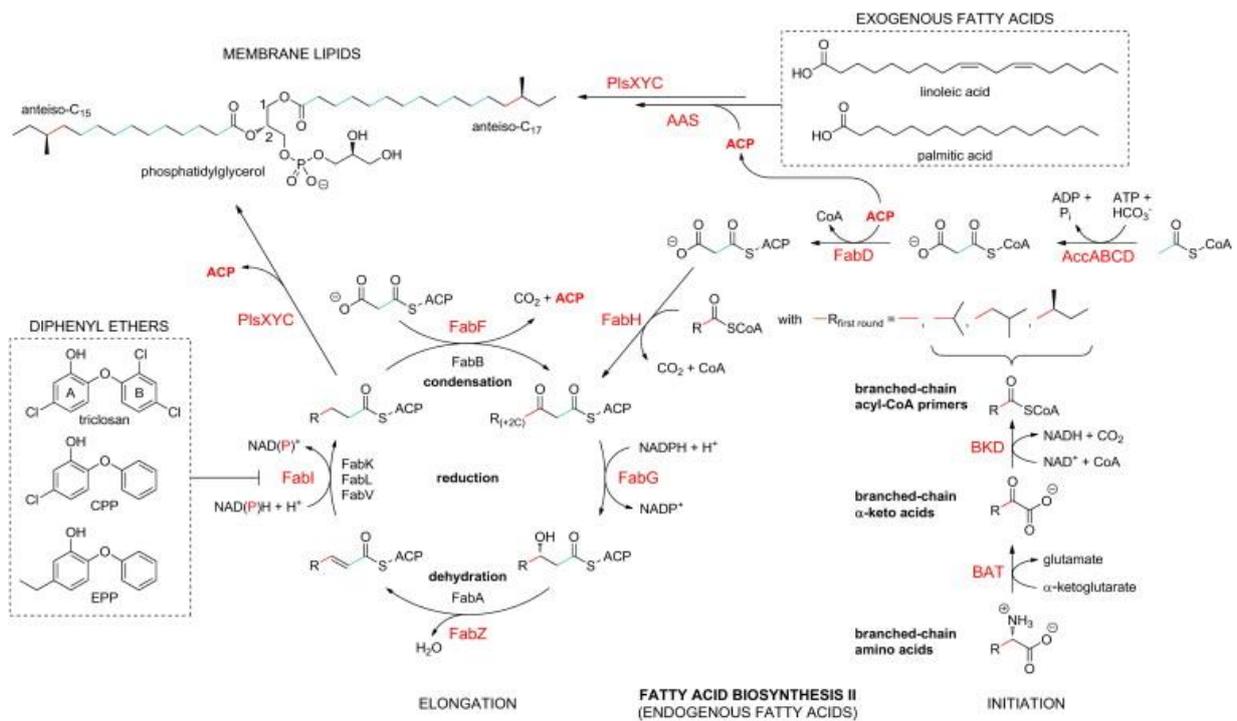


Figure 9. Fatty acid synthesis pathway in *S. aureus*. Three steps for synthesizing FAs and PA; (a) initiation of FASII (AccBCDA, FabD, FabH), (b) FA elongation (FabG, FabZ, FabI and FabF) and (c) phosphatidic acid (PlsXYC). From (Schiebel et al., 2012).

3.3 FASII Regulation in *S. aureus*

Gram-positive bacterial regulation seems to be distinguished into two groups, feedback based on a FabT-like repressor (for streptococcae), and feedforward based on a FapR-like repressor (for staphylococci, bacilli, and listeria sp) (for review, (Albanesi & de Mendoza, 2016; Lambert et al., 2022)). In the first group, FabT repression is activated by eFAs, so that FASII is turned off and exogenous FAs are incorporated. In the second group, FapR repression is alleviated when FASII precursors (malonyl-CoA, and malonyl-ACP) accumulate, which activates FASII pathway and PA synthesis genes (Albanesi et al., 2013). Interestingly, FapR regulates many but not all the FASII genes, and also regulates PlsX directly, and PlsC indirectly (Pathania et al., 2021). Since FASII bypass requires PlsX and PlsC activity, FapR status may also affect the outcome of this process (Kénanian et al., 2019; Morvan et al., 2016).

3.4 Utilization of Exogenous Fatty Acids in *S. aureus* for Energy and for FASII Bypass.

To our knowledge, all cell types are capable of incorporating environmental FAs (eFAs), even when they synthesize their own. FA synthesis by FASII is energetically and materially costly; thus, incorporation of eFAs, which are abundant in animal and food environments, makes sense. For example, synthesis of the 16 carbon, saturated palmitoyl-CoA needs 8 acetyl-CoA molecules, 14 NADPH reducing equivalents, and the hydrolysis of 7 ATP molecules (for review, (Yao & Rock, 2017)).

eFAs can be used in two different ways by bacteria:

i- as an energy source *via* FAD, the FA degradation pathway. FAD was characterized in different species (Fujita, Matsuoka, & Hirooka, 2007). A *fad* operon is present on the *S. aureus* genome (*fadX,E,D,B,A*), but was reported to be inactive (Parsons, Frank, Subramanian, Saenkham, & Rock, 2011). However, recent studies, and omics analyses, give evidence that Fad is indeed active, likely in growth-limiting conditions (Kuiack et al., 2023; Mäder et al., 2016).

ii- for direct FA incorporation in phospholipids: eFA incorporation into cell membrane phospholipids is well documented. The enzymes responsible for eFA incorporation in phospholipids were more recently identified as FA kinase (Fak), a conserved system that comprises FakA, FakB1 and FakB2 in *S. aureus* (Parsons, Broussard, et al., 2014; Parsons, Frank, Jackson, Subramanian, & Rock, 2014). Fak generates an acyl-PO₄ molecule, which is also the product of PlsX (Parsons, Broussard, et al., 2014).

The Fak system in *S. aureus* relies on the FakA kinase (originally named VfrB, and shown to be implicated in protease and hemolytic activity (Bose, Daly, Hall, & Bayles, 2014)). FakA is associated with an FA binding protein (FakB). *S. aureus* encodes two such proteins: FakB1 is reportedly selective for SCFAs, whereas FakB2 prefers UFAs (Parsons, Broussard, et al., 2014). Recently, this view was revised by evidence that FakB1 also catalyzes uptake of C18:2 from tri- and di-glycerides (B. Zhou et al., 2023). The underlying *S. aureus* Fak functions were uncovered in a *plsX* knockout mutant (Parsons, Frank, et al., 2014). Inactivation of *plsX* leads to FA auxotrophy, but also requires *de novo* FASII synthesis for growth. In a *plsX* mutant, eFAs only incorporated into the 1-position, and endogenous acyl groups were channeled into the 2-position of PA. Moreover, eFAs were not elongated, as their incorporation was disconnected from FASII. Extracts from the Δ *plsX* strain exhibited ATP-

dependent Fak activity. Moreover, when purified PlsX was added, the acyl-PO₄ was converted to acyl-ACP, adding evidence for the reverse activity of PlsX. These data, which identified the enzyme complex involved in eFA incorporation into *S. aureus* phospholipids, were instrumental in characterizing the shift from FASII to eFA utilization (Parsons, Frank, et al., 2014) (**Fig. 10**).

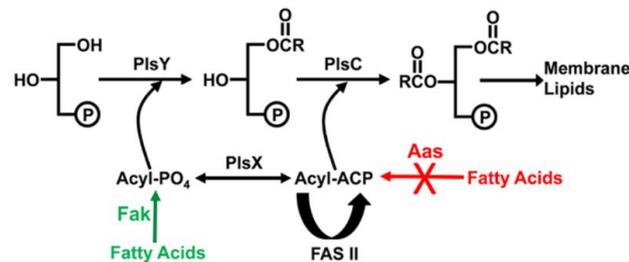


Figure 10. Utilization of eFAs in *S. aureus*. (A) eFAs are flipped to the inner leaflet and interact with FakB. This is then phosphorylated by FakA to form acyl-PO₄ (green), which can be used by either PlsY or converted by PlsX to generate acyl-ACP, which can be used by PlsC or elongation. In *plsX* inactivation of *S. aureus*, the bacteria need both eFAs and *de novo* synthesis, which rule out the existence of an acyl-ACP synthetase (Aas, red). From Parsons, Frank, et al. (2014).

3.5 FASII Enzymes as Targets for Staphylococcal Antibiotic Development

Increasing drug resistance amongst major human pathogens has led to an urgent call for new types of antibiotic treatments. As FASII was considered as an essential pathway, its enzymes were proposed as priority targets for drug discovery, specifically against *S. aureus* infection (Balemans et al., 2010; Marrakchi et al., 2002) (for review, (Foster, 2017)). Numerous anti-FASII drugs targeting FabI or FabF have been proposed, but with limited proof of efficacy in host conditions, which will be discussed below (Balemans et al., 2010; Bibens, Becker, Dassonville-Klimpt, & Sonnet, 2023; Escaich et al., 2011; Parsons & Rock, 2011; Wang et al., 2006). Several other inhibitors of FASII targets were reported, but generally not developed (summarized in **Fig. 11**) (Morvan et al., 2017).

FabI is functionally unique in only some bacteria, notably in *S. aureus*; for example, *B. subtilis* contains the enoyl-ACP reductase FabL in addition to a FabI (Richard J. Heath, Su, Murphy, & Rock, 2000). It was therefore considered a good narrow spectrum target for antibiotic development, which would not disturb the gut microbiome during treatment (Karlowsky, Kaplan, Hafkin, Hoban, & Zhanel, 2009; Tsuji, Harigaya, Lesse, Forrest, & Ngo, 2013; Yao & Rock, 2016).

Triclosan is the first described FabI inhibitor, which is specific at low concentrations; at high concentrations, triclosan has nonspecific killing effects on cells including those lacking FabI, explaining its widespread use as a biocide (McMurry, Oethinger, & Levy, 1998). Triclosan was shown more recently to damage host cells and is implicated in tumor formation and muscle cell damage (Yueh et al., 2014). In the context of bacterial growth, a complicating factor in triclosan use as a broadly used biocide is that *S. aureus* undergoes mutations and/or acquired genes to become resistant triclosan. Mutations mapped to *fabI*, indicating that triclosan no longer inhibited its target (Grandgirard et al., 2015). A novel resistance mechanism was linked to the acquisition of an *sh-fabI* allele derived from *Staphylococcus haemolyticus* via horizontal gene transfer, indicating that this biocide could exert selective pressure that may drive the spread of a resistance determinant in a human pathogen (Ciusa et al., 2012).

A new class of anti-FabI was developed, whose basic structures differ from that of triclosan (**Fig. 12**). The most developed example is AFN-1252, whose reported minimal inhibitory concentration (MIC) against *S. aureus* is 0.004-0.008 µg/ml (Banevicius, Kaplan, Hafkin, & Nicolau, 2013). Unlike triclosan, AFN-1252 (AFN) shows high specificity for FabI, and low toxicity in pharmacological tests (Hunt, Kaplan, & Hafkin, 2016). AFN-1252 was therefore proposed as an effective treatment to treat staphylococcal infections. During the thesis work, both anti-FabI were used for experiments.

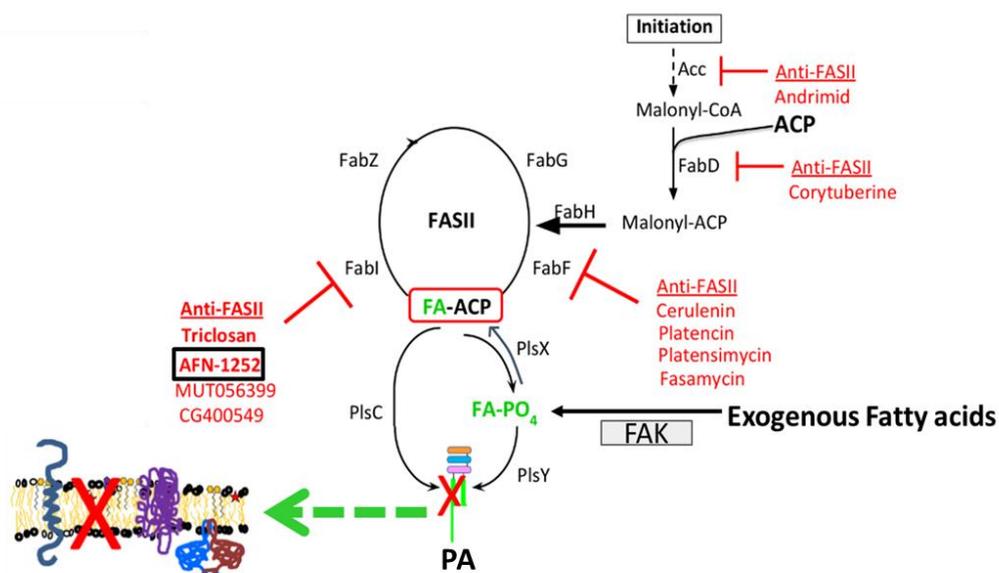


Figure 11. FASII inhibitors. Inhibitors of FASII were developed to target enzymes in FASII pathway. Some chemicals target at the initial steps of FASII; Acc, FabD and some target the elongation step; FabF or FabI. (See, (Morvan et al., 2017), for review). Adapted from (Kénanian et al., 2019).

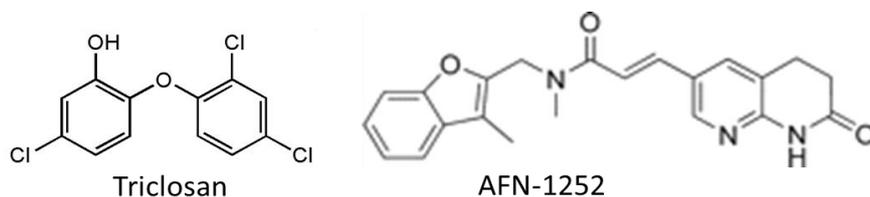


Figure 12. Structures of FabI inhibitors triclosan and AFN-1252.

3.6 FASII Bypass in Lipid-Rich Conditions Compromises Anti-FASII Use to Treat Firmicute Pathogen Infection

The use of anti-FASII as antibiotic was questioned when numerous Firmicute pathogens, as shown for streptococci, enterococci, and staphylococci, were shown to totally bypass inhibitions when external FA sources were available, including those supplied in human fluids (Brinster et al., 2009; Gloux et al., 2017; Kénanian et al., 2019; Morvan et al., 2016, 2017). These findings challenged early reports of FASII efficacy (Wang et al., 2006), which could be understood in retrospect: 1- many *in vitro* efficacy tests were conducted in the absence of eFAs, failing to take into account lipid-richness of the host. 2- in numerous *in vivo* anti-FASII challenge tests, the antibiotic was injected shortly (within 30 minutes to a few hours) after bacterial infection, *i.e.*, before the pathogen could reach its niche and adapt to environmental FAs (Brinster et al., 2009; Kénanian et al., 2019).

In depth analyses of FASII bypass in *S. aureus* revealed three strategies by which bacteria adapt to anti-FASII treatment, shown below.

a. Acquired mutations affecting the FabI target

Anti-FASII-resistant *S. aureus* strains were first identified using triclosan, correlating with a mutation in *fabI*, FA elongation (Brenwald & Fraise, 2003). FabI enzyme was shown to be the direct target of triclosan (McMurry et al., 1998). Moreover, screening of hospital strains revealed that mutations in *fabI* genes were common, suggesting that selective pressure was established due to widespread triclosan use in hygiene products (Brenwald & Fraise, 2003).

b. Acquired mutations in FASII initiation enzymes promote FASII bypass

Laboratory selection in the presence of anti-FASII and exogenous FAs led to emergence of *fabD* point mutants that also grew in non-supplemented medium (Morvan et al., 2016). Similarly, naturally occurring *fabD* polymorphisms were identified among *S. aureus*

hospital isolates (Gloux et al., 2017; Morvan et al., 2016). The *fabD* mutants showed cross-resistance to other FASII inhibitors and were infectious in mice (Morvan et al., 2016). Mutations mapping to *acc* showed similar phenotypes, indicating that disturbance of FASII initiation functions enabled eFA incorporation (Gloux et al., 2017; Morvan et al., 2016; Parsons et al., 2011) (**Fig. 13**).

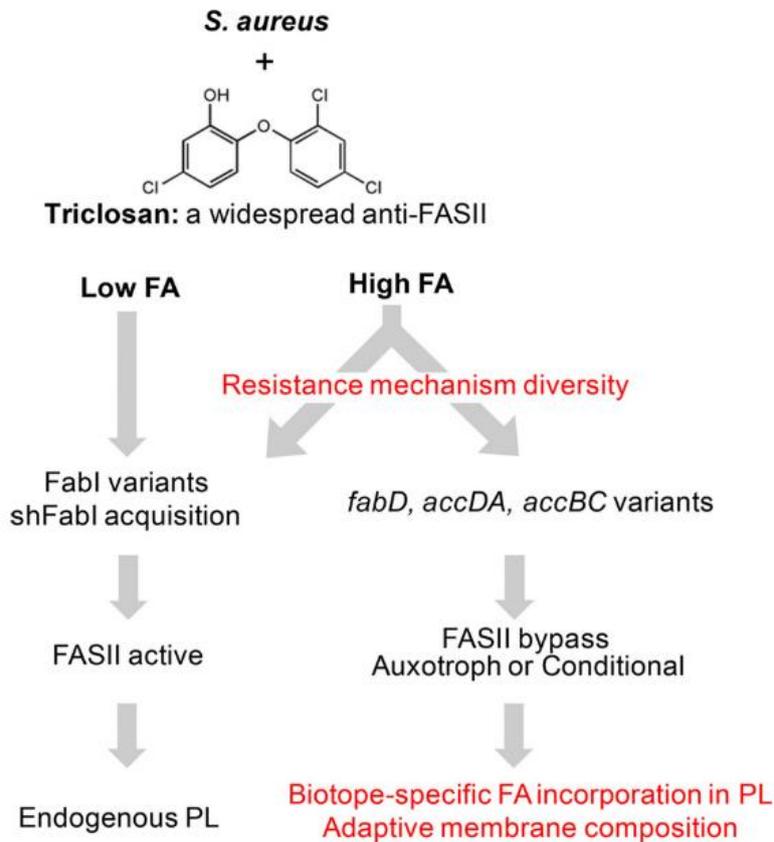


Figure 13. Diversity of anti-FASII resistance in *S. aureus* upon fatty acid (FA) medium. (Left) Poor eFA availability led to emergence of triclosan-resistant *fabI* variants, and FASII was active. (Right) In contrast, high-FA environments select for diverse resistance mechanisms, including those that require eFAs; in this condition, mutations in *fabD* and/or *accDA accBC* facilitated FASII bypass. From Gloux et al. (2017).

c. *S. aureus* adapts to anti-FASII without mutation in low-stress lipid containing environments

S. aureus are able to adapt to anti-FASII by a non-mutational adaptation mechanism involving incorporation of eFAs (**Fig. 14**). In this condition FASII enzymes are dispensable for growth as long as FAs are available (Brinster et al., 2009; Kénanian et al., 2019). Anti-FASII-adaptation likely occurs in the host, where numerous lipid sources are available (Kénanian et al., 2019). FA binding to serum, and notably albumin (~5% of total serum protein), decreases

FA toxicity, and facilitates anti-FASII adaptation (**Fig. 15**) within ~8 h post-treatment, followed by robust growth (**Fig. 16**). Growth of anti-FASII-treated *S. aureus* relies on incorporation of available eFAs. Results showed that anti-FASII reaches its target but does not efficiently kill *S. aureus* (Kénanian et al., 2019).

S. aureus encodes numerous lipases, which liberate FAs attached to complex lipids as produced by the host (Delekta et al. 2018; Zhou et al. 2023). Notably *S. aureus* encodes Sal1 and Geh (which respectively hydrolyze short- and long-chain FAs from tri- or di-glycerides and from low-density-lipoprotein), and Hlb (a sphingomyelinase that releases mainly saturated FAs) (Delekta, Shook, Lydic, Mulks, & Hammer, 2018; Hines et al., 2020; B. Zhou et al., 2023)

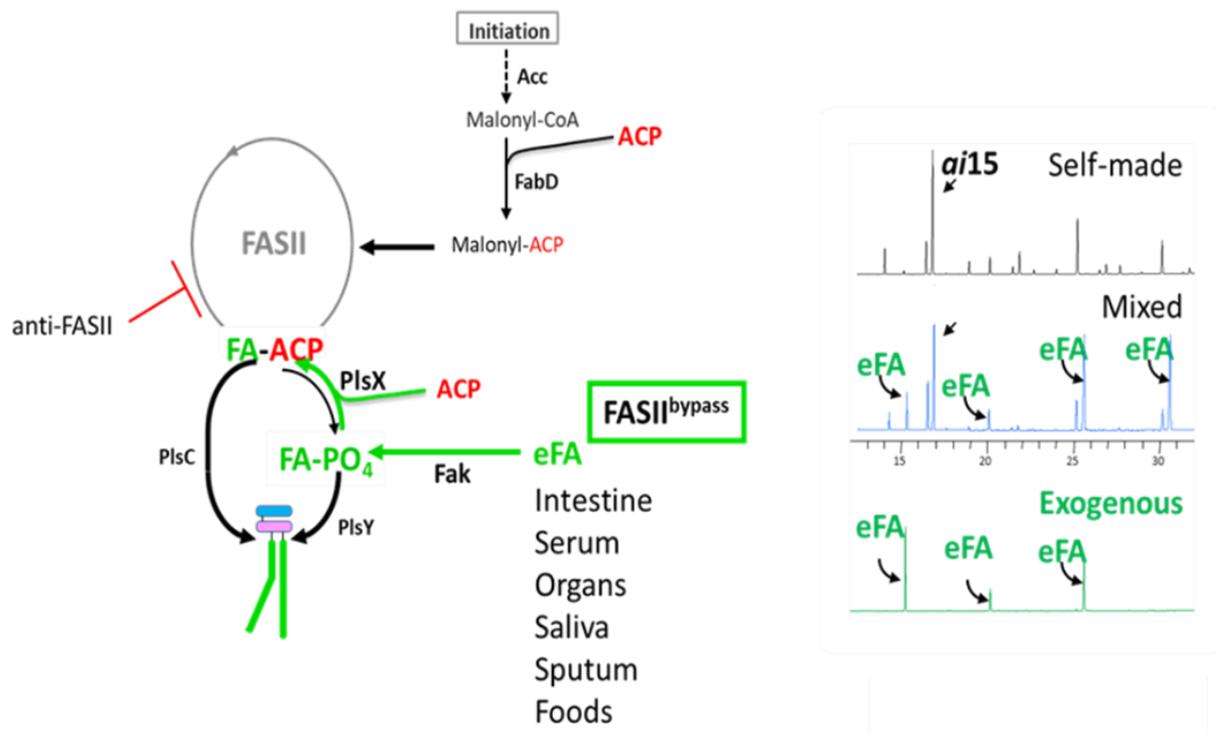


Figure 14. FASII bypass in *S. aureus* when eFAs are available. (Left) *S. aureus* bypasses anti-FASII inhibition (red cross-bar) by incorporating eFAs available in host environments. Fatty acid kinase (Fak) phosphorylates entering eFAs. PlsY assures FA placement at position 1 of glycerophosphate backbone; bidirectionality of PlsX allows FA to also occupy position 2 to complete phospholipid synthesis. (Right) FA profiles from phospholipid extractions. The most abundant FASII product in *S. aureus* is usually *ai15* (upper arrow, self-made FAs). Upon eFA addition, the FA profile contains both endogenous and exogenous FAs (eFA in green). During anti-FASII treatment, *S. aureus* incorporate available eFAs; here, C14, C16 and C18:1, (respectively numbered 1, 2, and 3) were added. Adapted from (Brinster et al., 2009; Gloux et al., 2017; Kénanian et al., 2019; Morvan et al., 2016; Pathania et al., 2021).

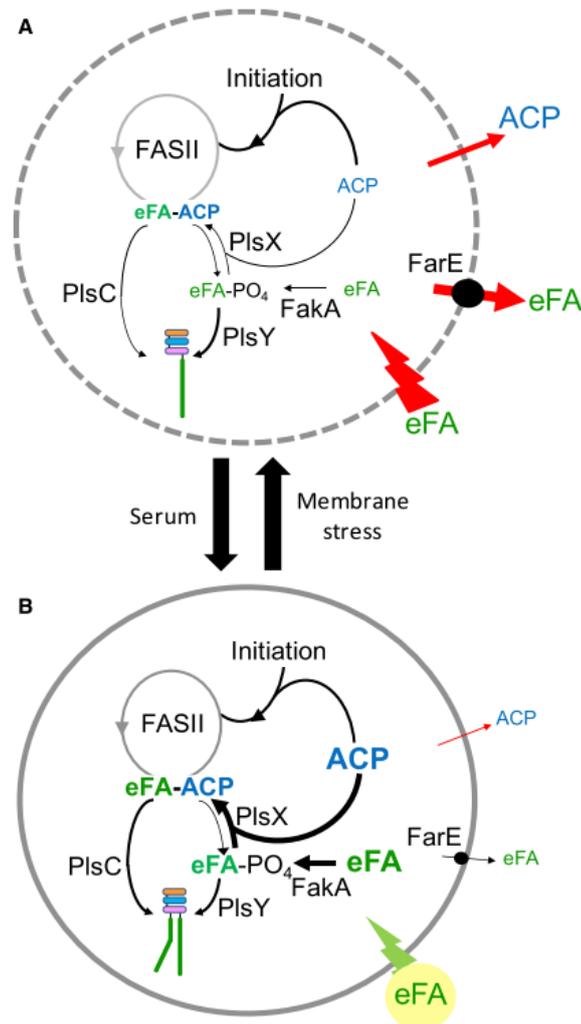


Figure 15. Model for the protective role of serum for *S. aureus* adaptation to FASII antibiotics. (A) The membrane stress state impacts eFA and ACP intracellular pools and dictates FASII antibiotic adaptation. Without serum, free eFAs permeabilize membranes. FA efflux *via* FarE, and ACP leakage (Alnaseri et al., 2015) lead to depletion of FASII-bypass substrates. (B) Serum binds eFAs (yellow disk), which reduces their toxicity, improves membrane integrity, and favors eFA uptake. Higher intracellular ACP and/or eFA-PO₄ pools in this condition drive PlsX reversal to produce eFA-ACP. eFA-ACP and endogenous acyl-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. eFA (green) and ACP (blue) abundance is represented by font size; phospholipids are shown as 'pi'-form shapes; thick arrows, favored reactions; thin arrows, decreased reactions. Gray dashed circle, permeable membrane; gray solid circle, intact membrane. From (Kénanian et al., 2019).

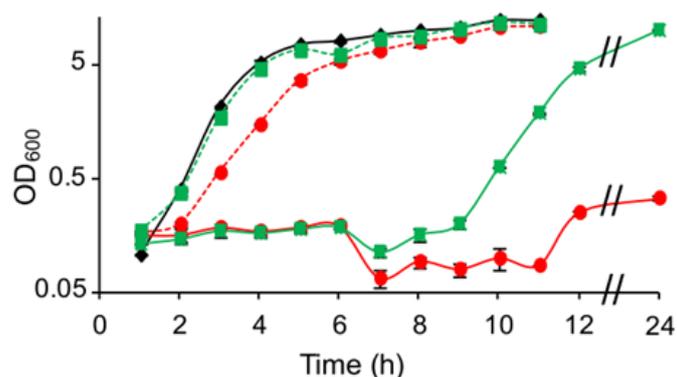


Figure 16. Positive effect of serum on anti-FASII adaptation in *S. aureus* USA 300. Growth (measuring OD₆₀₀) was compared in media containing or not an anti-FASII (Triclosan). Line in black, BHI medium. In red, bacteria grown in medium containing free FAs without triclosan (dashed lines), or with triclosan (solid lines). In green, bacteria grown in medium containing eFAs and supplemented with 10% serum, without triclosan (dashed lines), or with triclosan (solid lines). Adaptation to anti-FASII in the presence of serum occurs within 6 to 8 hours; the duration depends on the state of bacteria prior to anti-FASII treatment. Similar results were obtained using AFN-1252 as anti-FASII. From (Kénanian et al., 2019).

Thinking Box 2

FASII bypass enables *S. aureus* growth when eFAs are available in the environment. This indicates that single treatments using antibiotics targeting FASII would not be sufficient to stop *S. aureus* growth *in vivo*. Understanding the mechanisms of *S. aureus* FASII bypass, and consequences after adaptation could reveal bacterial weaknesses during anti-FASII adaptation. Such studies could uncover strategies for killing *S. aureus* by a combinatorial approach, which is the objective of this thesis.

Changes in FA composition may also impact phospholipid synthesis. In the next part, we will discuss phospholipid synthesis in *S. aureus*.

3.7 Phospholipid Species in *S. aureus*

a. Phospholipids species found in *S. aureus*

Phospholipids are major components of most bacterial membranes, which protect bacteria from the surrounding environment (*n.b.* some bacteria produce sulfolipids in phosphate-limiting conditions (Van Mooy, Rocap, Fredricks, Evans, & Devol, 2006) (for review (López-Lara & Geiger, 2017)). Bacterial Firmicutes use phosphatidic (PA), the PlsX, PlsY, PlsC product, to produce phosphatidyl glycerol (PG), phosphatidylethanolamine, or phosphatidylcholine (Hines et al., 2020).

However, *S. aureus* only synthesizes PG, and its derivatives (Nahaie, Goodfellow, Minnikin, & Hajek, 1984), which we consider below (**Fig. 17**). The derivatives include three main phospholipids, phosphatidyl glycerol (PG), cardiolipin (CL) and lysyl-phosphatidylglycerol (lysyl-PG), as well as two membrane-linked structures; lipoprotein (Lpp) and lipoteichoic acid (LTA). Since all these structures contain FAs, changing the FA composition may impact their production and/or their properties.

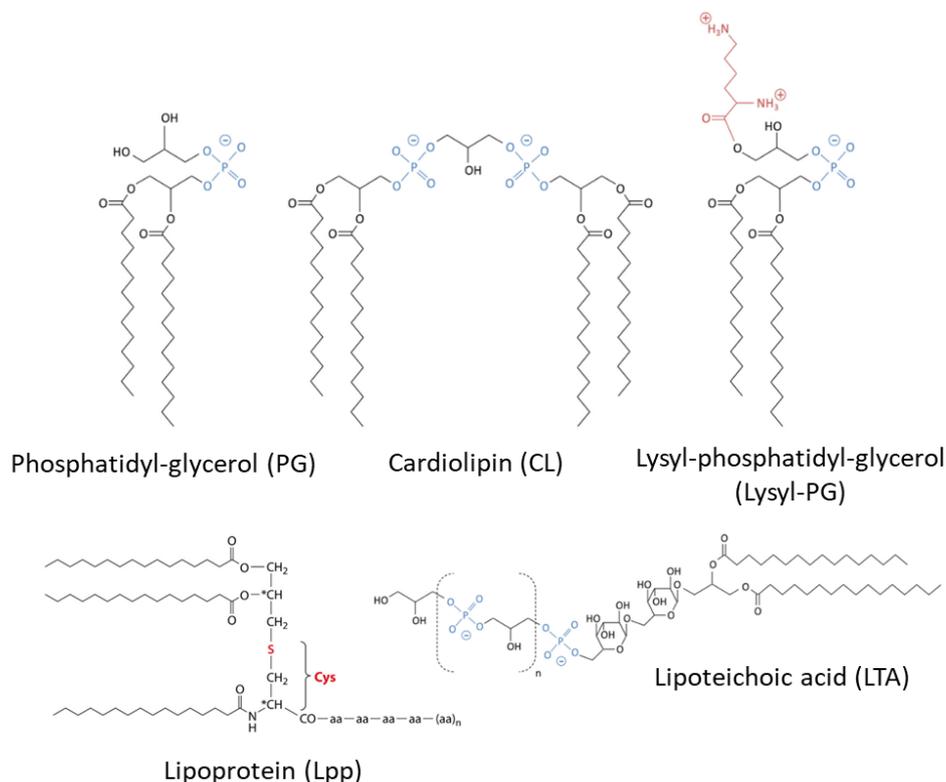


Figure 17. Chemical structures of major staphylococcal phospholipids and membrane-associated structures. Adapted from (Kuhn, Slavetinsky, & Peschel, 2015; M. T. Nguyen & Götz, 2016).

b. Lipid synthesis enzymes and lipid functions

PA, the first phospholipid synthesized by *S. aureus*, is produced by PlsX and PlsY enzymes, which catalyze synthesis of the lysophosphatidic acid (Lyso-PA) intermediate, and PlsC, which uses Lyso-PA and acyl-ACP to produce PA. Three lipid intermediates generated from PA lead to synthesis of PG, which is negatively charged (**Fig. 18**). PG itself is the “hub” for synthesis of the major phospholipid-derived species. CL, lysyl-PG, Lpp, and LTA (**Fig. 17**). Each phospholipid species may vary in its FA composition (for review, (Werner Fischer, 1994)); this variability may help modulate bacterial responses to different environmental conditions.

CL is produced by cardiolipin synthases Cls1 and Cls2, which catalyze joining of two PG molecules, with the release of glycerol. The CL head group is negatively charged (Short & White, 1972) (**Fig. 17**). Cls2 is the major CL synthase under ‘normal’ growth conditions, whereas Cls1 is used for CL synthesis under stress conditions (Ohniwa, Kitabayashi, & Morikawa, 2013; Tsai et al., 2011). CLs are implicated in membrane curvature (Renner & Weibel, 2011) and cell division (Labajová et al., 2021) (for review, (Arias-Cartin, Grimaldi, Arnoux, Guigliarelli, & Magalon, 2012)). *S. aureus* CL pools are higher in stationary phase than in exponential phase cells (Tsai et al., 2011), and are also known to increase upon treatment with a cell wall antibiotic (Kariyama, 1982). CL-rich domains were also suggested as targets for *S. aureus* antibiotics (Sautrey et al., 2016). While *cls* genes are not essential for *S. aureus* growth, they are reportedly required for long-term survival under high-salt conditions, and also for L-form bacteria (Tsai et al., 2011). A *cls* double mutant is impaired for virulence (Yeo, Dyzenhaus, Torres, Brinsmade, & Bae, 2023).

PG can be also aminoacylated with an L-lysine group from a lysyl-tRNA to generate Lysyl-PG by the “multiple peptide resistance factor” MprF (Peschel et al., 2001; Staubitz, Neumann, Schneider, Wiedemann, & Peschel, 2004) (**Fig. 17**). An *mprF* mutant, which fails to produce Lysyl-PG, displays greater sensitivity to host defense peptides, and is less virulent *in vivo* (Peschel et al., 2001; Weidenmaier et al., 2005). The lysyl group; therefore, confers *S. aureus* resistance to cationic antimicrobials produced as part of host defense systems. In addition, the *mprF* mutation has been shown to reduce sensitivity to daptomycin, but the mechanism involved remains to be investigated (Friedman, Alder, & Silverman, 2006; Jones et al., 2008).

PG provides the lipid moiety required for synthesis of Lpps, which are directly implicated in virulence (for review, (M. T. Nguyen & Götz, 2016)). There are 67 Lpps found in *S. aureus* USA300, which are distinguished by their N-terminal lipid moiety used for anchoring in the outer leaflet of the cytoplasmic membrane. Triacylated Lpp are also produced in *S. aureus*, and are produced *via* a supplementary acylation *via* LnsAB enzymes (Gardiner et al., 2020) (for review, (Mohammad et al., 2022; M. T. Nguyen, Matsuo, Niemann, Herrmann, & Götz, 2020)) (**Fig. 17**). Compared to diacylated Lpp, the triacylated molecules stimulate TLR2 (Toll-like receptor) more weakly than diacylated Lpp, which suggests that they are important for staphylococci pathogens to escape TLR2 immune surveillance (Gardiner et al., 2020) (for review, (M. T. Nguyen & Götz, 2016)). Approximately 40% of *S. aureus* USA300 Lpp proteins are transporters of molecules such as iron, cations, anions, and amino acids. Several of them, particularly those involved in iron acquisition (*i.e.*, FhuD1, FhuD2, SirA and IsdE) are important for infection (Shahmirzadi, Nguyen, & Götz, 2016). Strains defective for Lpp maturation are impaired for growth, immune activation, and virulence (Shahmirzadi et al., 2016; Stoll, Dengjel, Nerz, & Götz, 2005). Lpps and lipoprotein-like (Lpl) have also been proposed as potential candidates for a vaccine against *S. aureus* as many of them share a highly conserved core region of amino acids such as Lpl-1, Lpl-2 and Csa1A. A vaccine targeting this domain would therefore interact with several antigens. Moreover, tandem Lpps are also highly present only in *S. aureus* (Shahmirzadi et al., 2016), suggesting a narrow target. Overall, Lpp make up a significant part of the membrane lipid identity in *S. aureus*, and are crucial for virulence.

LTA is also produced using PG, which provides the scaffold, and the multiple GroP units that make up the PG head group (Percy & Gründling, 2014). Features of LTA will be discussed more fully in the **Chapter 4**, as this molecule is affected by FASII bypass.

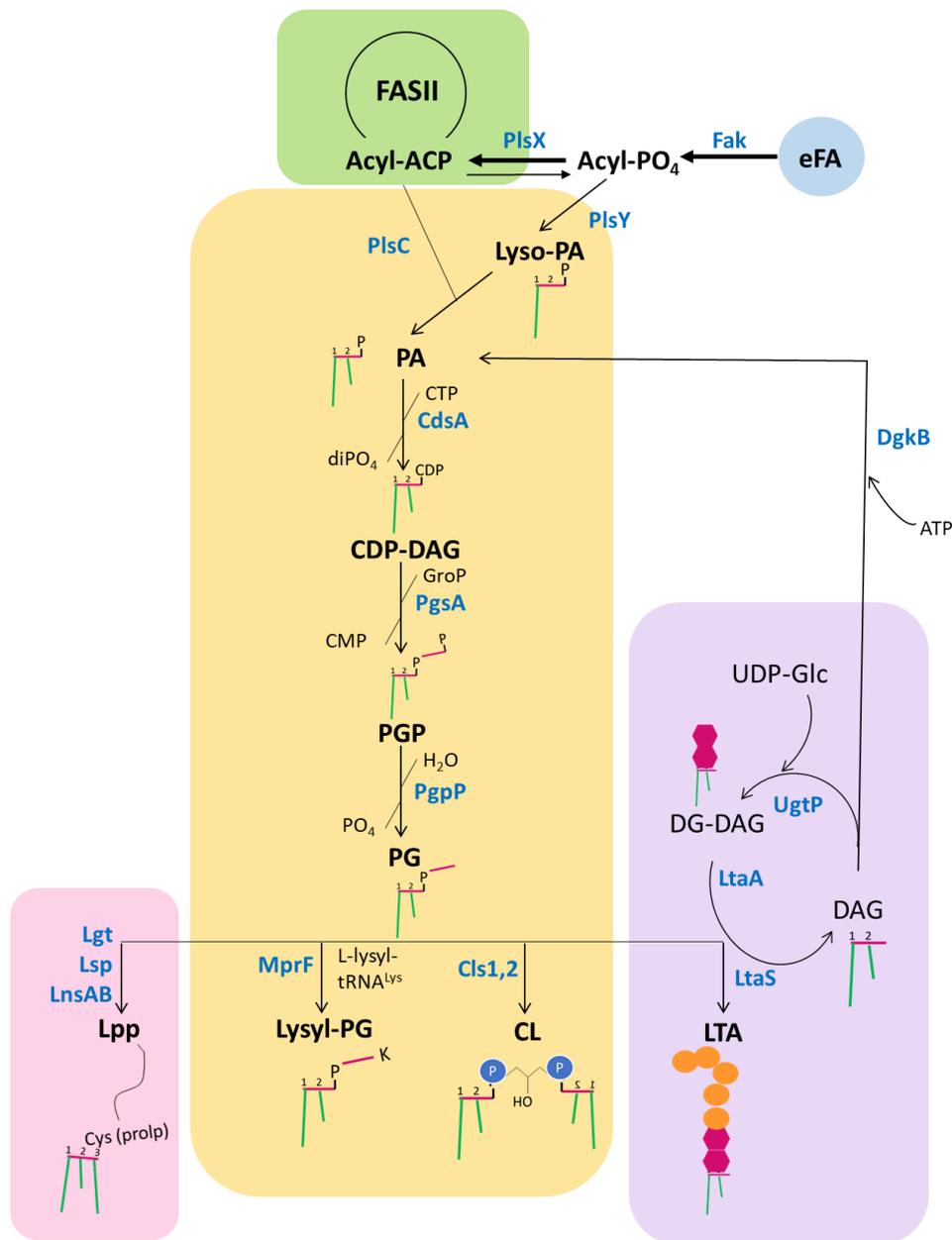


Figure 18. Enzymes and reactions of staphylococcal lipid biosynthesis and anchored-membrane structures. Schematics of major products of lipid synthesis and lipid-anchored structures in *S. aureus*. Enzymes are in blue. Boxed shaded pathways are involved in producing different lipid types; pink, lipoprotein (Lpp); green, fatty acid synthesis (FASII); yellow, lipid synthesis; purple, LTA. Staphylococcal phospholipid synthesis starts with the acylation of glycerol-3-phosphate (G3P) with fatty acids (using acyl-ACP from FASII) to first generate phosphatidic acid (PA) by using PlsX, PlsY, and PlsC. eFAs (blue circle) are phosphorylated by Fak. The main *S. aureus* membrane lipid is phosphatidyl glycerol (PG), which is produced from phosphatidic acid (PA) in 3 consecutive steps involving CdsA, PgsA, and PgpP, as illustrated. PG is the “hub” lipid substrate for synthesizing lipoproteins (Lpp), cardiolipin (CL), lysyl-PG, and lipoteichoic acid (LTA), which will be described in **Chapter 4**. This schematic summarizes known information gathered from articles specialized in each lipid product (Gardiner et al., 2020; Gründling & Schneewind, 2007b, 2007a; Morvan et al., 2016; Parsons, Broussard, et al., 2014; Peschel et al., 2001; Sastre et al., 2020; Short & White, 1972; Staubitz et al., 2004; Stoll et al., 2005) (for review, (Kuhn et al., 2015; M. T. Nguyen & Götz, 2016; Percy & Gründling, 2014; Short & White, 1972; Staubitz et al., 2004)).

3.8 Link Between Membrane FA Composition and *S. aureus*

Physiology and Virulence

Adjustment of membrane composition according to growth conditions appears to contribute to bacterial adaptation. For example, bacterial phospholipids produce greater proportions of rigidifying FAs in response to acid or high temperature stress, upon transition to stationary phase or when in biofilms (Dubois-Brissonnet, Trotier, & Briandet, 2016) (for review, (Denich, Beaudette, Lee, & Trevors, 2003)). More rigid membranes appear to protect bacteria from daptomycin, a membrane targeting antibiotic (Boudjemaa et al., 2018).

However, the question of how changes in membrane FAs affect *S. aureus* metabolism or fitness has received little attention. One study examined the changes in bacterial transcription in response to addition of free oleic or linoleic FAs, which were toxic to bacteria (Kenny et al., 2009). The first result (**Chapter 5**) of the thesis work aims to define how a major shift in membrane composition impacts *S. aureus* protein expression, which to our knowledge, has not been previously examined. Using eFAs has additional energetic advantages (Kénanian et al., 2019; Morvan et al., 2016; Tiwari et al., 2018), but the overall consequences may be double-edged, as suggested by the following examples:

1- Some eFAs are toxic for *S. aureus* and other pathogens, and are considered to contribute to host skin defense against infection (e.g. C18:2 and C20:4) (for review, (Wille & Kydonieus, 2003)). Their incorporation contributes to toxicity (B. Zhou et al., 2023);

2- Incorporation of C18:2 into Lpp can disequilibrate the innate immune-stimulation activity due to elevated proinflammatory cytokine production, which is dependent on Toll-like receptor 2 (TLR2) (M. T. Nguyen, Hanzelmann, Härtner, Peschel, & Götz, 2015).

3- Supplementation with eFA activates expression of the type VII secretion system (T7SS), which exports virulence factors. Functional T7SS promotes bacterial survival and long-term persistence in abscesses (Lopez et al., 2017). A strain defective for T7SS is more sensitive to C18:2, and produces more ROS compared to its wild type strain (Kengmo Tchoupa et al., 2020). Nevertheless, T7SS is not induced by eFA in the presence of serum, which reduces toxicity (Kénanian et al., 2019); this may suggest that membrane perturbation by eFA leads to T7SS induction.

4- Culturing *S. aureus* in the presence of various UFAs lowers expression of the two-component system SaeR SaeS, thus turning down expression of virulence factors (n.b., this appeared to occur independently of eFA incorporation in phospholipids, and was suggested

to involve FA inhibition of SaeS (DeMars et al., 2021; Ericson, Subramanian, Frank, & Rock, 2017)). Nevertheless, decreased virulence might favor bacterial persistence in the host (Tuchscher, Löffler, Buzzola, & Sordelli, 2010).

Altogether, the outcome of eFA supplementation is complex, and depends on the site of infection, and the lipid biotope. These various effects could alter staphylococcal pathogenicity during bacterial host infection. We will examine in depth the consequences of altering membrane eFA composition in the course of this thesis work.

3.9 Relevance to Thesis Work: Membrane Phospholipid Flexibility and the Link to *S. aureus* Fitness and Virulence

The compiled work in the introduction illustrate how membrane dynamics may have major roles in *S. aureus* physiology and virulence. This may reflect direct roles of lipids or lipid-linked products *e.g.*, Lpp and LTA, or also staphyloxanthin, which contains a FA chain (**Fig. 17**). Membrane lipid composition may also have indirect roles, by altering the activities of membrane-bound proteins affecting virulence.

This will be also discuss in the **Chapter 5**, *S. aureus* depends on eFAs during adaptation to FASII inhibitors. Phospholipid composition shifts from endo- to totally exogenous FAs.

Numerous studies in the lab prior to starting my thesis project led to the hypothesis that production of membrane proteins or membrane-linked structures would be impacted by altering membrane FAs after *S. aureus* adaptation to FASII inhibitor. The aims of the thesis project were to explore the links between membrane FA composition, which is altered by anti-FASII-adaptation, and how *S. aureus* fitness and virulence are affected.

CHAPTER 4

Lipoteichoic Acid: An Essential Bacterial Structure?

*This chapter will be included as part of a Review Article to
be submitted for publication.*

CHAPTER 4

Lipoteichoic Acid: An Essential Bacterial Structure?

4.1 Abstract

Lipoteichoic acid (LTA) is a major cell wall glycopolymer (CWG) present in most Gram-positive bacteria. LTA is reportedly indispensable in the opportunist pathogen *Staphylococcus aureus*, making this structure an attractive target for vaccines and antibiotic drug development, particularly for the methicillin-resistant *S. aureus* (MRSA). In this review, we discuss the known roles of LTA, with a focus on conditions that allow *S. aureus* to survive without LTA. A main finding of our work is that LTA depletion is a consequence of anti-FASII use (**Chapter 6**).

Keywords Bacterial cell wall, cell wall glycopolymers (CWGs), teichoic acid (TA), lipoteichoic acid (LTA), wall teichoic acid (WTA)

4.2 Introduction

The bacterial cell envelope acts as a barrier that maintains cell shape and intracellular turgor pressure, and also negotiates all transactions between the cytoplasm and the environment. In Gram-positive bacteria, such as *S. aureus*, the envelope comprises the cytoplasmic membrane and a thick peptidoglycan layer that is decorated with numerous proteins and polymers important for viability and virulence (A. L. Koch, 2006). Cell wall glycopolymers (CWG), particularly teichoic acids (TA; from the Greek *teichos*, for “wall”), are major cell envelope components (A. R. Archibald, Armstrong, Baddiley, & Hay, 1961; A. L. Koch, 2006). The TA refers to all glycerolphosphate (GroP)- or ribitolphosphate (RhoP)-containing polymers in the cell wall, membrane, and capsule of bacteria (for review, (Baddiley, 1989; Percy & Gründling, 2014)). Their phosphate-rich composition accounts for approximately 40% of total phosphorus in *S. aureus* (for review, (Werner Fischer, 1994)).

Firmicute bacteria usually have two types of TAs based on their subcellular localization (**Figure 19**, Left, edited from (Xia, Kohler, & Peschel, 2010)): 1- **Wall TA (WTA)** is covalently linked to the peptidoglycan by phosphodiester bonds between *N*-acetylmuramic acid [MurNAc] and a disaccharide (often *N*-acetylglucosamine [GluNAc] and *N*-acetylmannosamine

[ManNAc]). WTA usually contains a glycerol or ribitol group. 2- **Lipoteichoic acid (LTA)**, the focus of this review, is anchored in the cytoplasmic membrane *via* a glycolipid anchor (diglucosyl-diacylglycerol; DG-DAG). LTA polymers are generally formed by glycerolphosphate (GroP) repeating units.

In *S. aureus*, the enzymes responsible for WTA and LTA synthesis involve distinct pathways. However, they share a modification in common, which is D-alanylation. The WTA polymer of *S. aureus* is made up of RboP repeats, whereas LTA contains GroP repeats (Gründling & Schneewind, 2007b; Meredith, Swoboda, & Walker, 2008) (**Figure 19**, Right). In some bacteria, such as *Streptococcus pneumoniae*, LTA and WTA have the same repeating unit (RboP) (Brown, Santa Maria, & Walker, 2013; Fischer, et al., 1993). Other species, such as *Bacillus subtilis*, may express three or four different TA types (Schirner, Marles-Wright, Lewis, & Errington, 2009).

Though TAs have been studied for decades, their exact functions are still being explored. LTA is reportedly indispensable for cell viability in *S. aureus*, making it an attractive target for antimicrobial development to kill *S. aureus* (Chee Wezen et al., 2022; Fedtke et al., 2007; Gründling & Schneewind, 2007b; Oku et al., 2009), and for antibody-based treatments (A. Kumar, Ray, Kanwar, Sharma, & Varma, 2005). Remarkably, there are growth conditions and mutations that make LTA, and/or the entire cell wall, dispensable for *S. aureus* growth and survival, raising questions about the LTA requirement during infection (Bæk et al., 2016; Corrigan et al., 2011; Karinou et al., 2019; Kawai et al., 2015; Leaver et al., 2009). In this review, we summarize the main known roles of LTA, growth conditions, and compensatory mutations that allow bacteria to grow without LTA, particularly in *S. aureus*, a major pathogen and first cause of death due to antimicrobial resistance (GBD 2019 Antimicrobial Resistance Collaborators, 2022). The choice of LTA as an antibiotic target is discussed in view of these considerations.

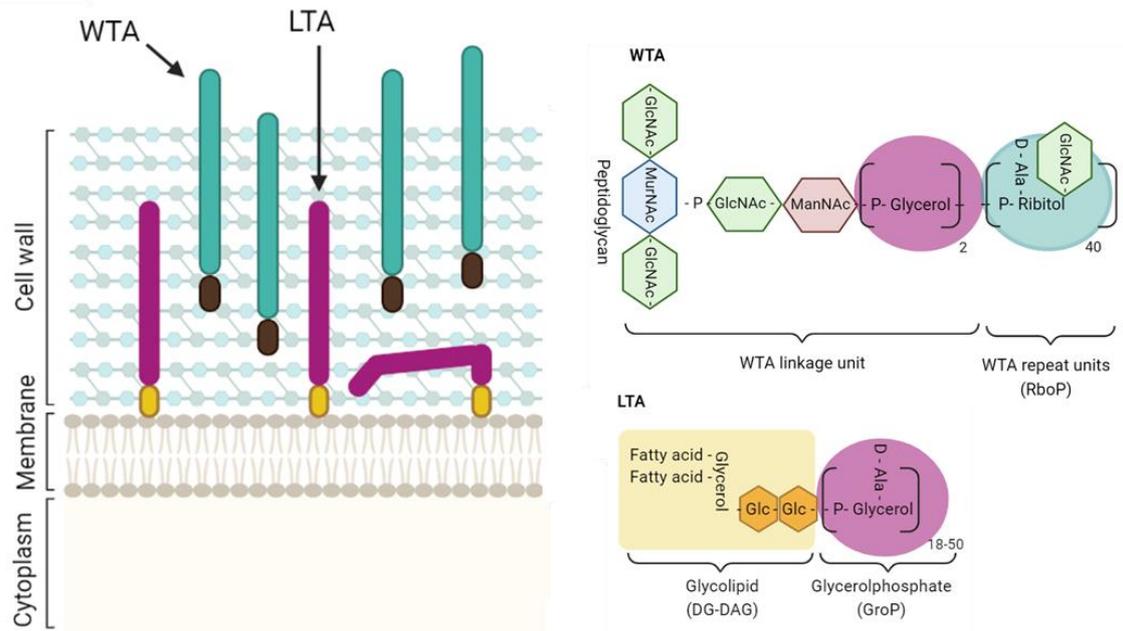


Figure 19. Cartoon of TA localization and structures in *S. aureus*. Left: *S. aureus* produces two types of TA, LTA and WTA. Right: LTA is membrane bound *via* a glycolipid anchor (DG-DAG) with a glycerolphosphate (GroP) repeat unit. WTA is and peptidoglycan-linked *via* the 'WTA linkage unit', with a ribitol-phosphate (RboP) repeat unit. Figure is made using BioRender.

4.3 Composition and localization of LTA

LTA is composed of a repeat units attached on the bacterial surface by a cytoplasmic membrane anchor, DG-DAG. Both the polymer and the anchors may differ according to the species, leading to LTA classification into five groups having different chemical structures (Types I-V) (for review, (W. Fischer, 1988; Percy & Gründling, 2014; Schneewind & Missiakas, 2019)). *S. aureus*, *B. subtilis*, and *Listeria monocytogenes* express are Type I LTA, which contains simple unbranched 1-3 linked GroP repeat units, and is the best characterized LTA class (for review (Percy & Gründling, 2014)). Three main enzymes are needed for LTA synthesis in *S. aureus*; UgtP/YpfP, LtaA and LtaS (discussed below). LtaS is preferentially localized at the cell division site while UgtP and LtaA are distributed throughout the bacterial membrane (Reichmann et al., 2014) (**Fig. 20**). The LTA polymer is suggested not to be surface polymer; using immunofluorescence, it can be detected only when peptidoglycan is digested (Reichmann et al., 2014).

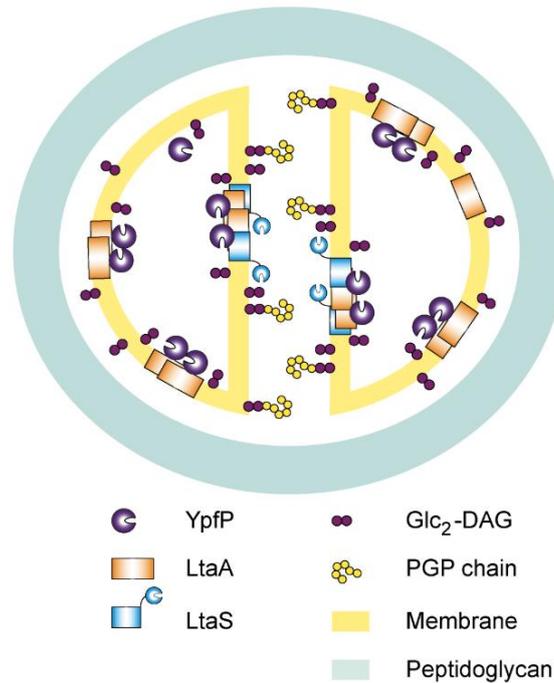


Figure 20. Model of LTA synthesis machinery localization in *S. aureus*. YpfP and LtaA are located throughout the membrane, whereas LtaS is accumulated at the cell division site. From (Reichmann et al., 2014).

4.4 LTA Synthesis in *S. aureus*

Here, we focus on Type I LTA synthesis, which is the best characterized and found in most Gram-positive bacteria belonging to the phylum *Firmicutes* (H. U. Koch, Haas, & Fischer, 1984). We separated enzymes for LTA synthesis into two parts; *i*-LTA synthesis; glycolipid anchor (PgcA, GtaB, UgtP), glycolipid flipping (LtaA and CozE), and LTA polymerization (LtaS and MprF), and *ii*-Lipid recycling (DgkB, CdsA, PgsA and PgpB); **Fig. 21**.

a. Glycolipid anchor (DG-DAG)

The DG-DAG lipid anchor for LTA is synthesized on the inner membrane leaflet by the sequential action of three enzymes. UDP-Glc (uridine diphosphate glucose) is generated by the phosphoglucose mutase PgcA and the UTP-glucose-1-phosphate uridylyltransferase GtaB. The glucosyltransferase UgtP (also called YpfP), then generates the lipid anchor by sequentially joining two UDP glucose molecules to diacylglycerol (DAG), which is located in the bacterial membrane (Gründling & Schneewind, 2007a; Jorasch, Wolter, Zähringer, & Heinz, 1998; Michael Y Kiriukhin, Debabov, Shinabarger, & Neuhaus, 2001) (**Fig. 21**).

b. Glycolipid flipping to the outer leaflet membrane

S. aureus lipid anchor is translocated from inner to outer leaflet membrane *via* the LtaA flippase (Gründling & Schneewind, 2007b; B. Zhang et al., 2020) (**Fig. 21**). LtaA is reported to be a proton-coupled antiporter-flippase that is essential for *S. aureus* survival under acidic stress. Authors suggest LtaA as an antimicrobial target for treating *S. aureus* infection (B. Zhang et al., 2020). Interestingly, the recent research shows that CozE, which is membrane proteins related to cell division regulates the flipping activity of glycolipid by LtaA *in vitro*. Inactivation of LtaA in double mutant of CozEa and CozEb is also alleviated the growth (Barbuti et al., 2023), suggesting the relationship between CozE and LTA synthesis.

*Noted** *S. aureus* mutants in which *ltaA* or *ugtP* is inactivated display an abnormal shape; in both cases, LTA is still detected, but with higher molecular weight in SEJ1 (a derivative of RN4220 strain), RN4220, and USA300 (Gründling & Schneewind, 2007a; Hesser, Matano, et al., 2020), except *ugtP* in SA113, which is shorter chain compared to parental strain (Fedtke et al., 2007). This LTA polymer is polymerized on DAG instead of DG-DAG as described by using mass spectrometry and nuclear magnetic (Fedtke et al., 2007) or by thin-layer chromatography and MALDI-TOF mass spectrometry (Gründling & Schneewind, 2007a). However, the recent research also shows that LTA polymer of *ugtP* mutant can polymerize on PG by using proteoliposomes system containing different types of lipids (Hesser, Schaefer, Lee, & Walker, 2020).

c. LTA polymerization and lipid turn over

S. aureus encodes a unique LTA synthase LtaS, which hydrolyzes GroP from PG and then polymerizes it on the glycolipid anchor, leaving DAG as a byproduct (Gründling & Schneewind, 2007b). This contrasts with other Firmicutes that encode more than one LTA synthase, but with varying repeat units; *L. monocytogenes* encodes two LTA synthases (LtaP and LtaS) (Webb, Karatsa-dodgson, & Gründling, 2009), and *B. subtilis* contains four LTA synthase (LtaS, YfnI, YvgJ, and YqgS) (Wörmann, Corrigan, Simpson, Matthews, & Gründling, 2011). The extracellular LtaS moiety, called eLtaS, mediates GroP hydrolysis from PG; the full LtaS mediates polymerization on the glycolipid anchor (Gründling & Schneewind, 2007b; D. Lu et al., 2009). A recent report implicates MprF, which is required for Lysyl-PG synthesis of both *B. subtilis* and *S. aureus* (SA113 strain), in LTA production. MprF was proposed to be involved in anchoring, capping, and potentially localizing the LTA repeat structure (Guyet,

Alofi, & Daniel, 2023). In *S. aureus*, deletion of *mprF* led to faster LTA migration (Low molecular weight) as based on results of immunoblotting using antibody specific of LTA (Guyet et al., 2023). In *S. aureus*, the average GroP length is 25 residues, with some variation for individual molecular species. GroP is linked to the C-6 position of the nonreducing glycosyl of the glycolipid (for review, (Werner Fischer, 1994; Schneewind & Missiakas, 2019)).

The DAG liberated during PG hydrolysis by LtaS is recycled to re-synthesize PG *via* diacylglycerol kinase (DgkB), CDP-diacylglycerol (CdsA), and phosphatidyl glycerophosphate (PgsA) and possibly PgpB (*Staphylococcus aureus aureus* NCTC 8325 *pgpB* (biocyc.org)), which could restore PG in the inner membrane leaflet in *S. aureus* (Jerga, Lu, Schujman, Mendoza, & Rock, 2007; Miller, Jerga, Rock, & White, 2008; Tong et al., 2016) (**Fig. 21**). However, recent research shows that in *ltaS* mutant, DAG is still detectable ranging from approximately 20% to the same level of wild type, which suggests that DAG production might be partially disconnected from LTA synthesis (Hesser, Matano, et al., 2020).

Of note, LTA synthesis is energetically costly. Considering that one LTA requires 25 units of GroP, 25 DAGs must be regenerated to 25 PGs per LTA molecule [DGDG + 25 PG → LTA (25 GroP) + 25 DAG]. To resynthesize PG from DAG and GroP, bacteria need to spend a high energy [25DAG + 25 GroP + 75ATP → 25PG +75ADP +75Pi] (for review, (Werner Fischer, 1994)). Moreover, to synthesize LTA, the PG pool has to be turned over three times (H. U. Koch et al., 1984). This suggests that LTA may have an important role in membrane function. Indeed, an *ltaS* knockout is lethal at 37°C, unless bacteria are cultured in high-osmolarity growth medium (Oku et al., 2009). This phenotype correlates with the absence of detectable LTA (Gründling & Schneewind, 2007b). We will see below that while LTA appears to be essential or nearly so, some genetic backgrounds tolerate, and even select for *ltaS* mutations.

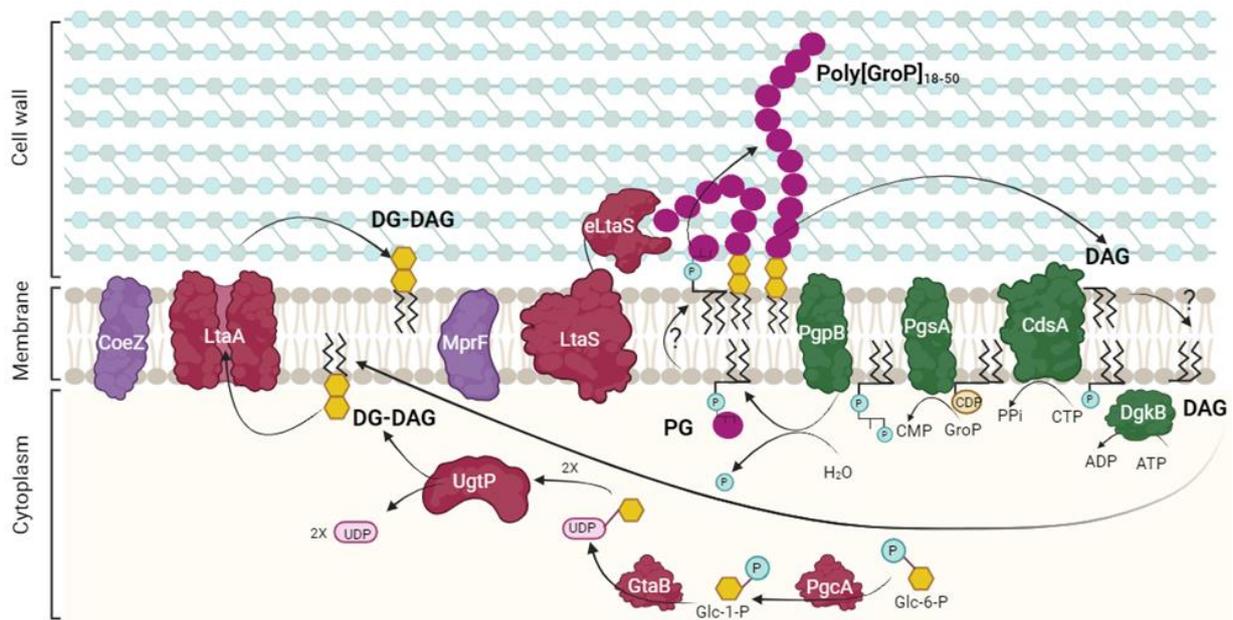


Figure 21. LTA synthesis and recycling of DAG in *S. aureus*. *S. aureus* synthesizes the simple type I LTA. In an early step of LTA synthesis, bacteria synthesize UDP glucose *via* PgcA and GtaB. The glycolipid anchor (DG-DAG) produced by UgtP/YpfP is then transported to the outer leaflet *via* LtaA. The LtaS enzyme hydrolyzes GroP from PG in the outer leaflet and then polymerizes it on the DG-DAG glycolipid anchor. Simultaneously, DAG generated by LtaS hydrolysis for GroP liberation is recycled to PG *via* DgkB, CdsA, PgsA, and possibly PgpB. DAG is then used as the substrate for producing the glycolipid anchor. LTA synthesis proteins are represented in red, and lipid recycling enzymes for LTA synthesis are in green. The '?' indicates that unknown functions are involved. Proteins CoeZ and MprF (in purple) were recently implicated in LtaA activity and GroP anchoring and capping respectively (Barbuti et al., 2023; Guyet et al., 2023). The Figure is mainly based on (Gründling & Schneewind, 2007a, 2007b) and made using BioRender.

4.5 Decoration of LTA with D-alanine

LTA is modified with D-alanine residues, as first elucidated by Neuhaus and coworkers in *Lactobacillus casei*, which expresses a Type IV LTA (Childs & Neuhaus, 1980) to make LTA zwitterionic polymers. Deficiency of this decoration causes increased susceptibility to host defense molecules and cationic antimicrobial peptides (Collins et al., 2002; Peschel et al., 1999). D-alanylation of TAs is mediated by the *dltABCD* operon (M. Y. Kiriukhin & Neuhaus, 2001). This process begins from the cytoplasmic D-alanine-D-alanyl carrier protein ligase DltA activates D-alanine by using ATP to generate a high-energy D-alanyl-AMP intermediate. The D-alanyl moiety is then transferred to the D-alanine carrier protein of DltC (Lee et al., 2022; Perego et al., 1995) (for review, (Percy & Gründling, 2014)) (**Fig. 22**). Of note, the DltC sequence and structure are similar to those of acyl carrier protein (ACP), which is required for

fatty acid biosynthesis (FASII) (Volkman et al., 2001). Moreover, both ACP and DltC are abundant, and are pantotheinylated by AcpS (Elovson & Vagelos, 1968; Nikolopoulos et al., 2022), possibly suggesting competition during their activation step. For the next step of D-alanylation, two models were proposed for the role of DltB and DltD; I) Fischer model (Perego et al., 1995) and II) Neuhaus and Baddiley model (Debabov, Kiriukhin, & Neuhaus, 2000) (**Fig. 22**). Recent work supports the Fischer model, in which DltC does not cross the bacterial membrane, and DltD is anchored outside the cell, thus differing from the model proposed by Neuhaus and Baddiley model (Reichmann, Cassona, & Gründling, 2013) (**Fig. 22a**). Interestingly, WTA isolated from an LTA-negative *S. aureus* strain reveals a drastic decrease in D-alanine, suggesting that LTA is important for D-alanylation of WTA (Reichmann et al., 2013). This result fits with previous work suggesting that LTA-alanine is the donor for WTA alanine (Haas, Koch, & Fischer, 1984).

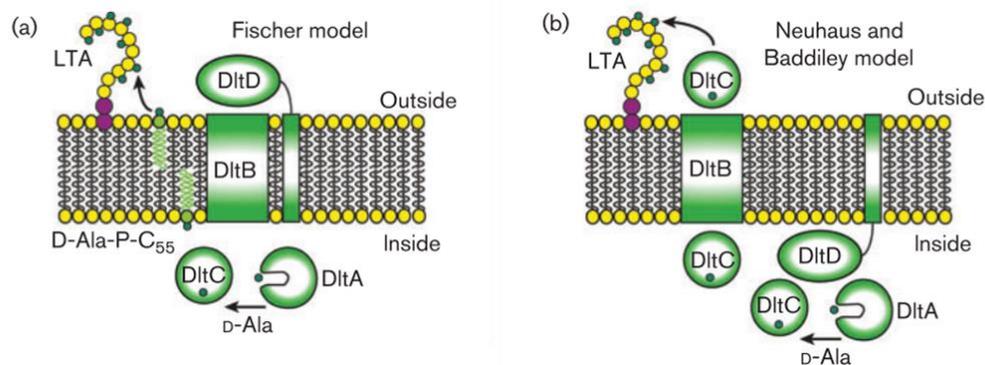


Figure 22. Models of D-alanine substitution of LTA. (a) Fischer model: D-alanine (small green circle) is ligated by DltA onto the carrier protein DltC. D-alanine is then transferred from DltC to undecaprenyl phosphate (C55-P) and traverses the membrane *via* DltB; the existence of a membrane-linked intermediate remains to be demonstrated. In the final step, DltD facilitates D-alanine transfer to LTA on the outside of the cell. (b) Neuhaus and Baddiley model: DltD functions intracellularly to enhance the efficiency of DltA-mediated ligation of D-alanine to DltC. The charged DltC protein then crosses the membrane assisted by DltB and then D-alanine is transferred onto LTA. From (Reichmann et al., 2013).

4.6 Roles of LTA

CWGs are essential for Gram-positive cell envelope homeostasis (for review, (Weidenmaier & Peschel, 2008)). Interestingly, WTA and LTA play partially redundant roles in cell envelope integrity and cannot be inactivated simultaneously (Oku et al., 2009; Santa Maria et al., 2014; Schirner et al., 2009). However, while *S. aureus* can grow under standard

laboratory conditions without WTA (Elia et al., 2006), cells lacking LtaS are non-viable and rapidly acquire suppressor mutations. These defective mutants give insight into the roles of LTA, as discussed below (**Fig. 25**).

Cell division: Mutations in LTA enzymes are responsible for cell morphology defects (Hesser, Matano, et al., 2020; Michael Y Kiriukhin et al., 2001; Matsuoka et al., 2011) (**Fig. 23**). Inactivation of enzymes required for glycolipid anchor, UgtP, synthesis led to distended and bent shapes in *B. subtilis*: bacteria showed decreased MreB protein. They suggest that glucolipids play a role in MreB isoforms to mediate discrete MreB dots and shape bacteria into straight rods, which need to be defined (Matsuoka et al., 2011). Mutations in *ugtP* or *ltaA* lead to glycolipid anchor depletion; in this case, LTA is still produced in RN4220 strain (Gründling & Schneewind, 2007a; Hesser, Schaefer, et al., 2020), but not in *ugtP* SA113 (Fedtke et al., 2007). However, these phenotypes were corrected by mutations in *ltaS* that reduce polymer length (Hesser, Matano, et al., 2020). LtaS appears to be indispensable for *S. aureus* growth and cell division, as suggested by a requirement for LtaS and its preferred localization at the septum (Reichmann et al., 2014). Moreover, enzymes involved in LTA synthesis interact with numerous peptidoglycan synthesis and cell division proteins such as EzrA, FtsA, DivIB and PBP (Reichmann et al., 2014). Both the localization of LTA, and the LtaS enzyme interactants point to a role for LTA in cell division and cell growth. Nevertheless, we will give evidence that LtaS, but not LTA, may be required for these functions (**Chapter 6**).

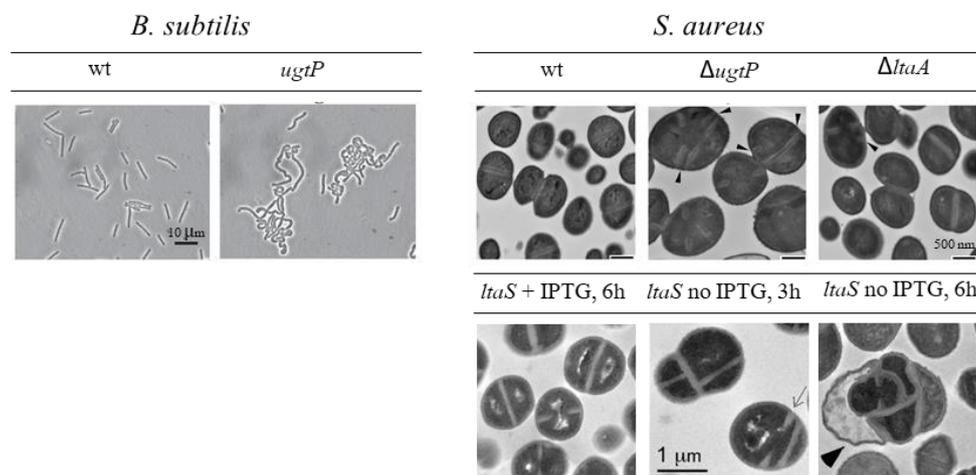


Figure 23. Defective cell shape in LTA mutants. Left, a *ugtP* mutant, defective in glycolipid anchor synthesis in *B. subtilis* demonstrates distended and bent bacterial cell shapes. From (Matsuoka et al., 2011) (left). TEM of *S. aureus* mutants defective for LTA synthesis, showing abnormal morphology; *ugtP* and *ltaA* of RN4220 strains (upper right); from (Hesser, Matano, et al., 2020), *ltaS* ± IPTG of 3h and 6h; (ANG499; RN4220 with IPTG-inducible *ltaS* expression, Erm^r) (bottom right); from (Gründling & Schneewind, 2007b).

A role for LTA in bacterial integrity during cell division was suggested based on physical properties of LTA and WTA (Erickson, 2021). Negatively-charged LTA and WTA were proposed to interact to make a stiff repulsive brush, creating a barrier that maintains turgor pressure on the cell exterior. One might speculate that the high negatively charged components are held together by cations. This barrier would protect bacteria from the potentially high internal pressure, particularly during the division process (Erickson, 2021) (**Fig. 24**).

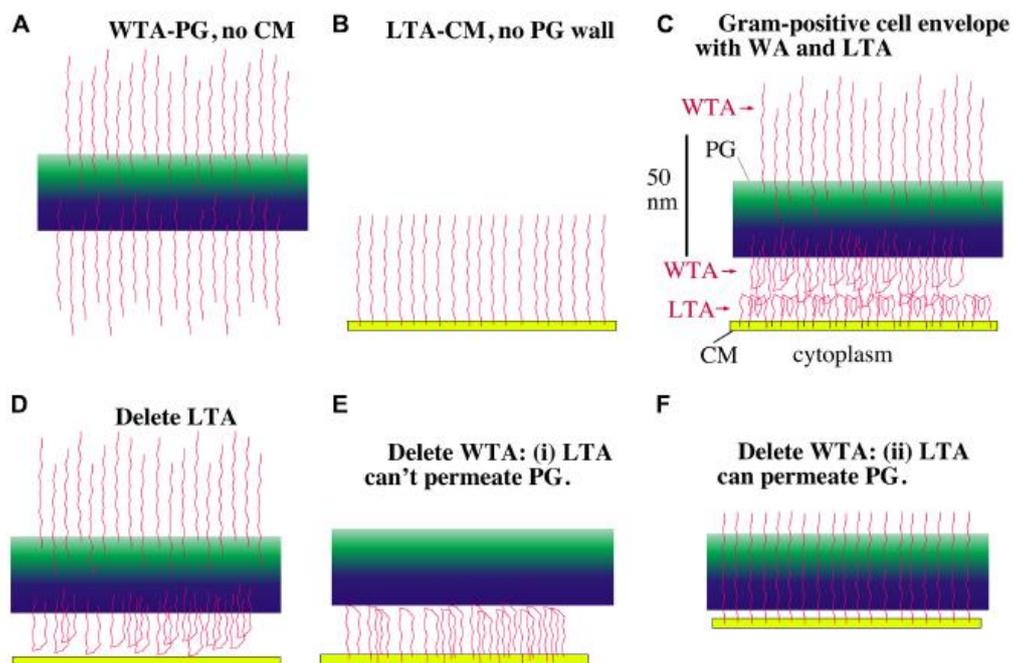


Figure 24. Model by Erickson 2021 for the roles of LTA and WTA in generating a periplasmic space and maintaining turgor pressure outside the cell cytoplasm. Peptidoglycan, PG green; cytoplasmic membrane, CM, yellow; LTA, WTA, red. Different scenarios are schematized to show WTA or LTA structures according to the available supports (PG and/or membrane are present). Notably, in (C), Erickson depicts the gram-positive cell envelope, where LTA and WTA interact to form a periplasm, and “compress each other until their electrostatic repulsion matches the turgor pressure of the cytoplasm”. (D) If LTA is lost, Erickson suggests that “WTA facing the CM can still support a periplasm”. (E,F) The presence of LTA alone might lead to a reduced (E) or totally squashed (F) periplasm.

Autolysis: Three independent studies led to the suggestion that autolysin activity is linked to LTA. 1- a *ugtP* mutant displayed strongly reduced autolytic activity, while autolytic enzyme levels were at wild-type levels (Fedtke et al., 2007). 2- An *ltaS* mutant showed decreased autolysis in the cell wall fraction. Activity was restored by complementation with an *ltaS* expression plasmid (Oku et al., 2009). 3- Similarly, *ltaS* suppressors found in *gdpP* show lower amounts of cell wall-associated hydrolytic enzymes, which is restored by complementation

with *ltaS* expression plasmid, as shown by zymogram assays. LTA was also restored in these strains when *ltaS* complementation was introduced (Corrigan et al., 2011).

These results led to the suggestion that LTA has a role in maintaining autolysin levels on the bacterial cell envelope, which may be linked to cell division. Interestingly, early studies in *S. pneumoniae* indicated that LTA binds autolysin, and could inhibit activity when added exogenously. Authors evoked the possibility that LTA decoration with phosphocholine residues could be involved in autolysin inhibition by LTA (Holtje & Tomasz, 1975). A parallel may be drawn with *S. aureus*, where autolysis was related to alanine decoration of LTA: Using LTAs with different molar ratios of alanine/phosphorus, it was shown that increased alanine ester substitution correlates with a decreased inhibitory effect of LTA on *S. aureus* autolysins; here, LTA charge could be important for autolytic activity (Werner Fischer, Rosel, & Koch, 1981). It is notable that in addition to LTA, cardiolipin, which is negatively charged, also reportedly binds autolysins ((Suginaka, Shimatani, Ohno, & Yano, 1979); see **Chapter 6**).

Cation homeostasis: TAs have a reported affinity for bivalent cations; they were shown to scavenge Mg^{2+} , and were proposed as an Mg^{2+} ion-storage strategy, as studied in *S. aureus* (Heptinstall, Archibald, & Baddiley, 1970). Membrane-bound Mg^{2+} -requiring enzymes include those required to synthesize envelope components (e.g., membrane, peptidoglycan, capsule and wall polysaccharides, glycolipids and TAs), and also oxidative phosphorylation for energy generation (Brooks & Baddiley, 1969; Chatterjee & Park, 1964; Heptinstall et al., 1970). The bivalent cations stocked in the membrane might thus satisfy the requirements of these enzymes. Of note, Mg^{2+} scavenging by TAs is reportedly decreased by high levels of alanine ester substituents (A. Ronald Archibald, Baddiley, & Heptinstall, 1973).

Biofilm: Bacterial surface hydrophobicity varies according to envelope component properties and conditions. For example, the loss of LTA (87%) in an *S. aureus* *ugtP* mutant increases hydrophobicity, which reduces the ability to form biofilms on plastic, but not glass (Fedtke et al., 2007). In *Streptococcus mutans*, the loss of LTA D-alanylation correlates with impaired adhesion capacity and reduced biofilm formation (Wu et al., 2022). Overall, altering the net charge of LTA could affect adhesion and biofilm formation.

Antimicrobial resistance: Decoration of the LTA backbone with D-alanine protects bacteria against cationic antimicrobial peptides, likely by reducing the negative charge to the LTA structure (Collins et al., 2002; Peschel et al., 1999; Weidenmaier & Peschel, 2008). LTA length may also contribute to antibiotic sensitivity: in the methicillin-resistant *S. aureus* (MRSA) COL strain, increased LTA chain length in *ltaA* and *ugtP* COL strains, show 8-fold and 64-fold greater sensitivity to the β -lactam antibiotic oxacillin than their parental strain, respectively (Hesser, Matano, et al., 2020). The impact of LTA structure on antimicrobial resistance thus varies according to decoration and chain length; the mechanisms driving these effects deserve further study.

Virulence: The LTA glycolipid anchor is implicated in *in vivo* infection. Indeed, infection by *S. aureus* lacking *ltaA* (encoding the DG-DAG flippase) results in a 2-log reduction in CFU in lung and kidney tissues of mice compared to the parental strain, while *in vitro* growth of these strains is equivalent (Gründling & Schneewind, 2007a). Moreover, the nature of LTA membrane-anchoring also affects host cell binding of *S. aureus*, as inactivation of DG-DAG synthesis reduces the ability of *S. aureus* to penetrate the blood–brain barrier (human brain microvascular endothelial cells) (Sheen et al., 2010), although LTA should nonetheless be envelope bound *via* DAG or PG, as above (Fedtke et al., 2007; Gründling & Schneewind, 2007a; Hesser, Schaefer, et al., 2020)). Intradermal injections of LTA in mice reveals that LTA is sufficient to damage the mice skin barrier (Brauweiler, Goleva, & Leung, 2019). The above studies suggest that LTA is implicated in virulence and antimicrobial resistance, but that the way in which LTA is attached to bacteria, as well as the length and modifications of LTA, all modulate its different functions.

Immune evasion and stimulation: A first line of defense against bacterial infection involves stimulation of host inflammatory responses (Beutler, 2004). Toll-like receptors (TLRs) are a central point in early immune recognition of the pathogen as part of the host defense system (Abreu & Arditi, 2004; H. Kumar, Kawai, & Akira, 2009). LTA was reportedly sensed by TLR2 in *S. aureus* (Schwandner et al., 1999; Seo, Michalek, & Nahm, 2008). However, subsequent studies showed that this stimulatory effect was attributed to lipoproteins that contaminated LTA preparations, and *not* LTA itself (Hashimoto et al., 2007, 2006; M.-T. Nguyen, Peisl, Barletta, Luqman, & Götz, 2018). LTA was also suggested to act as an anti-inflammatory molecule on keratinocytes by suppressing TLR-3-mediated responses due to skin injury; TLR-3 mediates activation of the inflammation pathway (Lai et al., 2009).

A host-protective effect of LTA was also observed: Specialized liver macrophages known as Kupffer cells express a Complement Receptor of Immunoglobulin superfamily (CRlg) for pathogen capture. CRlg directly recognizes Gram-positive bacteria *via* LTA, which the host uses for clearance of circulating bacteria (Zeng et al., 2016). The absence of LTA might thus allow bacteria to evade clearance by the Kupffer cells.

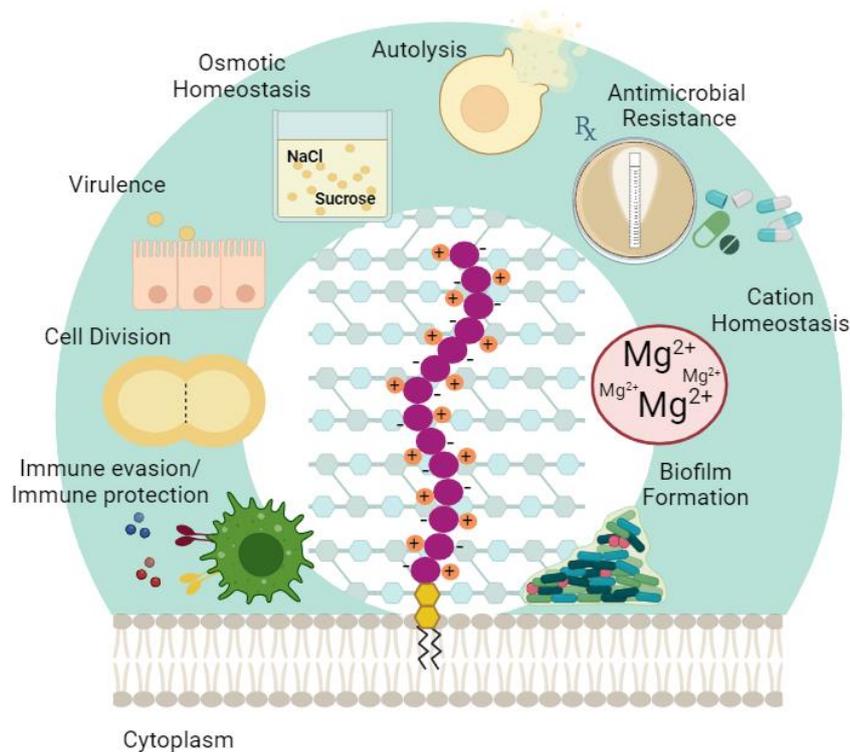


Figure 25. Summary of the physiological roles of *S. aureus* LTA. The zwitterionic LTA is shown by negatively charged phosphates; this charge is lowered by the presence of alanines. The roles of LTA are illustrated. This is made by BioRender.

4.7 When is LTA not Essential for Growth?

LTA is crucial for staphylococcal survival, and depletion of this polymer leads to mispositioning of cell division septa and even cell lysis (Gründling & Schneewind, 2007a, 2007b). These features led to the proposed use of LTA as a target for antimicrobial development. (Chee Wezen et al., 2022; Fedtke et al., 2007; Gründling & Schneewind, 2007b; Oku et al., 2009). However, in recent years, mutants without LTA have become available, which elucidated LTA functions, and also the conditions where LTA is dispensable. Here we summarize the conditions in which LTA is not needed for *S. aureus* growth (**Fig. 26**). To facilitate reading, we separated conditions of LTA absence into two groups; I) mutational changes and II) environmental changes.

I. LTA absence by mutational changes

Generally, LTA-negative strains are unable to grow in standard laboratory medium unless bacteria acquire suppressors. Several compensatory mutations in *S. aureus* were recently reported, which enable bacteria to grow in the absence of LTA (using *ltaS* mutants). We note that LTA rescue by mutations might complicate its use as a target for antimicrobial development. Here we discuss compensatory mutations allowing for *ltaS* survival.

a- *gdpP*: An *ltaS* deletion strain is compensated by a mutation in *gdpP*, a cyclic diadenosine monophosphate (c-di-AMP) phosphodiesterase (Corrigan et al., 2011). In *S. aureus*, c-di-AMP is produced by the *S. aureus* DacA protein (Corrigan et al., 2011; Tosi et al., 2019). Both a *gdpP* mutant, and the *ltaS gdpP* suppressor, produced high levels of c-di-AMP. Increased c-di-AMP pools may have two consequences in the cell: first, it leads to lower intracellular potassium, an important ion in osmotic homeostasis (Bai et al., 2014; Corrigan et al., 2013; Schuster et al., 2016). Second, increased c-di-AMP in the *gdpP* mutant correlates with increased amounts of cross-linked peptidoglycan with slightly decreased hydrolytic enzymes from zymogram assays (Corrigan et al., 2011). Altogether, lower Intracellular potassium, and increased peptidoglycan cross-linking could respectively adjust turgor pressure (Erickson, 2021) and peptidoglycan rigidity to compensate the *ltaS* mutation and allow *S. aureus* survival.

b- *clpX*: Inactivation of *clpX* results in a drastic growth defect at 30°C. However, *clpX* mutant lethality is suppressed by *ltaS* mutations, which restore growth at low temperature (Bæk et al., 2016). Noted, LTA is reported to be essential for *S. aureus* growth at 37°C, not at 30°C unless a high osmolarity medium is used (Oku et al., 2009). Surprisingly, *clpX ltaS* double mutants also survive at 37°C, but not at 44°C (Bæk et al., 2016). Moreover, the absence of ClpX partly improved the septum placement defects of an LTA-depleted strain (Bæk et al., 2016). Moreover, *clpX ltaS* double mutants showed reduced autolytic activity compared to *clpX* mutant. In a reciprocal study starting with an *ltaS* mutant, the suppressor allowing growth mapped to *clpX* (Karinou et al., 2019). The above studies led to the suggestion that *ltaS* and *clpX* are intimately linked in their roles in cell division as mutation in *clpX* improves the misplaced septum of *ltaS* strain.

c- *sgtB*: The growth defect of an *ltaS* mutant is suppressed by a secondary mutation in *sgtB*, encoding a glycosyltransferase. Inactivation of SgtB results in increased peptidoglycan cross-

linking. This phenotype may compensate a decrease in cross-linking in the *ltaS* (i.e., LTA-defective) strain (Karinou et al., 2019). This interaction might suggest a role for autolysin modulation by LTA (see above) as being a cause for cell growth arrest when *ltaS* is mutated.

d- *vraT*: Growth deficiency of the *ltaS* mutant was suppressed by a mutation in *vraT* (Karinou et al., 2019), encoding a membrane protein (VraT) that positively regulates what is described as a VraTSR three-component regulatory system. VraSR is implicated in tolerance to cell wall antibiotics including methicillin; VraT inactivation resensitizes bacteria to methicillin (Boyle-Vavra, Yin, Jo, Montgomery, & Daum, 2013). While the *vraT* mutant suppressor improves growth of *ltaS*, the strain remains sensitive to Congo red, an LtaS inhibitor (Karinou et al., 2019). This led authors to suggest that *vraT* is not a true suppressor. This is surprising, as the initial strain is *ltaS* minus, possibly suggesting that the *ltaS* negative phenotype was reverted.

e- *mazE*: MazE is an antitoxin component of a type II toxin-antitoxin system, where the MazF toxin is an RNase (Schuster et al., 2015). The *mazE* suppressor mutation seemed to improve positioning of the septum and resistance to Congo red (Karinou et al., 2019). A *mazE mazF* deletion mutant shows greater β -lactam sensitivity (Schuster et al., 2015). However, no phenotype for a *mazE* single mutation was characterized, and its impact on MazE/MazF expression is unclear. Nevertheless, the P_{mazE} promoter upstream of *mazEF* is reported to respond to environmental and antibiotic stresses by controlling expression of the alternative sigma factor gene *sigB* (Donegan & Cheung, 2009). How *mazE* rescues *ltaS* strain is still unclear, but might be, like other suppressors, linked to effects on cell wall integrity.

f- *cshA* : A *cshA* mutant (dead box helicase) cannot grow at low temperature (25°C) in the absence of secondary mutations, one of which is in *ltaS*, resulting in the loss of LTA (Khemicic et al., 2020). LTA may play a role in bacterial membrane rigidity and its loss may contribute to fluidifying the membrane at low temperature (Khemicic et al., 2020). This interpretation is in accordance with findings that LTA is not required at 30°C (Oku et al., 2009).

g- *pgl*: A *S. aureus pgl* encoding 6-phosphogluconolactonase mutant was recently shown to abolish LTA synthesis, and grows normally. This mutant demonstrated a smaller cell size, but thicker cell wall compared to wild type, and a greater resistance to β -lactam antibiotics when glucose is present (Zeden et al., 2023). However, LtaS seems to be continuously produced, as suggested by *pgl* sensitivity to Congo Red, which is similar to *vraT* (Karinou et al., 2019;

Zeden et al., 2023). It is possible that a substrate for LtaS is limiting or rerouted in this mutant. Alleviation of the LTA requirement could relate to the smaller cell size and thicker cell envelope reported in the *pgl* mutant.

h- *fabD*: We will show below (**Chapter 6**) that inactivation of malonyl CoA-acyl carrier protein transacylase *fabD*, a FASII initiation enzyme, fails to produce LTA.

Collectively, we described several mutants that compensate for an LTA deficiency, implying that inhibition of LTA production alone may not be sufficient as an antimicrobial target. Several mutant suppressors implicate their roles in regulating autolysin; interestingly, this goes back to studies of Tomasz and colleagues showing that LTA inhibits autolysin activity (Holtje & Tomasz, 1975).

Interestingly, these *ltaS*-suppressor mutants are expected to be more resistant to Congo red, an anti-LtaS (Vickery et al., 2018); however, some mutants (*vraT* and *pgl*) are susceptible to Congo red. This raises the possibility that LtaS may be required in some cases independently of LTA production, which still remains in question.

II. LTA depletion by environmental changes

In addition to suppressor mutations, two growth conditions allow the LTA-deficient strain to grow without detectable genetic changes. These examples further question the efficacy of LTA inhibitors in stopping *S. aureus* infection.

a- High osmolarity: The *S. aureus* LtaS requirement was alleviated in high osmolarity growth medium (7.5% NaCl or 40% glucose) at 37°C or below, but not at higher temperatures (43°C) (Oku et al. 2009). Although this condition allows the *ltaS* mutant to grow, cells still show morphological defaults, aberrant cell division septa, increased cell size, and clumping (Oku et al., 2009). Interestingly, at low temperature (30°C) allows *ltaS* mutant growth without highly osmotically protective medium. However, bacteria still display abnormal cell division as well as decreased autolysis and levels of peptidoglycan hydrolases. These persisting defects could favor the choice of LtaS as an antibiotic target, although subsequent suppressor mutants might lead to bacterial persistence.

b- FASII antibiotic-adapted bacteria: A second condition where LTA is not required will be revealed in the research project. Briefly, FASII bypass conditions alleviate the LTA

requirement, while bacterial growth is robust. The phenotypes and consequences of LTA depletion will be discussed (**Chapter 6**).

c- Balance between LTA and WTA: LTA and WTA were proposed to play overlapping roles in the regulation of cell division and cell envelope homeostasis (Erickson, 2021; Hesser, Matano, et al., 2020; Santa Maria et al., 2014). Both polymers also have the same negative charge on the bacterial cell envelope, which are both described as "continuum of negative charge". (Neuhaus & Baddiley, 2003). Oku et al. (2009) has shown that *ltaS* mutant could grow at 30°C, but double mutant of both *ltaS* (no LTA) and *tagO* (no WTA) leads to mortality at 30°C. Simultaneous inactivation of both LTA and WTA, *S. aureus* lacks ability to form Z ring and leads to stop of cell division (Santa Maria et al., 2014), which indicates that LTA and WTA have at least partially redundant roles. Furthermore, pools of WTA are enhanced in the *ltaS* suppressor while amounts of LTA are significantly reduced, suggesting compensation between the two structures. (Hesser, Matano, et al., 2020). Altogether, these results suggest that WTA could compensate the loss of LTA. Combination inhibition of both these structures are suggested to effectively kill *S. aureus* (Santa Maria et al., 2014).

Overall, these results highlight compensatory mutations that could allow *S. aureus* to survive in specific conditions in the absence of LTA. It suggests a dialog between the need for LTA and the membrane and cell wall states. The ability of bacteria to survive without LTA raises questions on the use of LTA as a mono-therapeutic treatment against *S. aureus*.

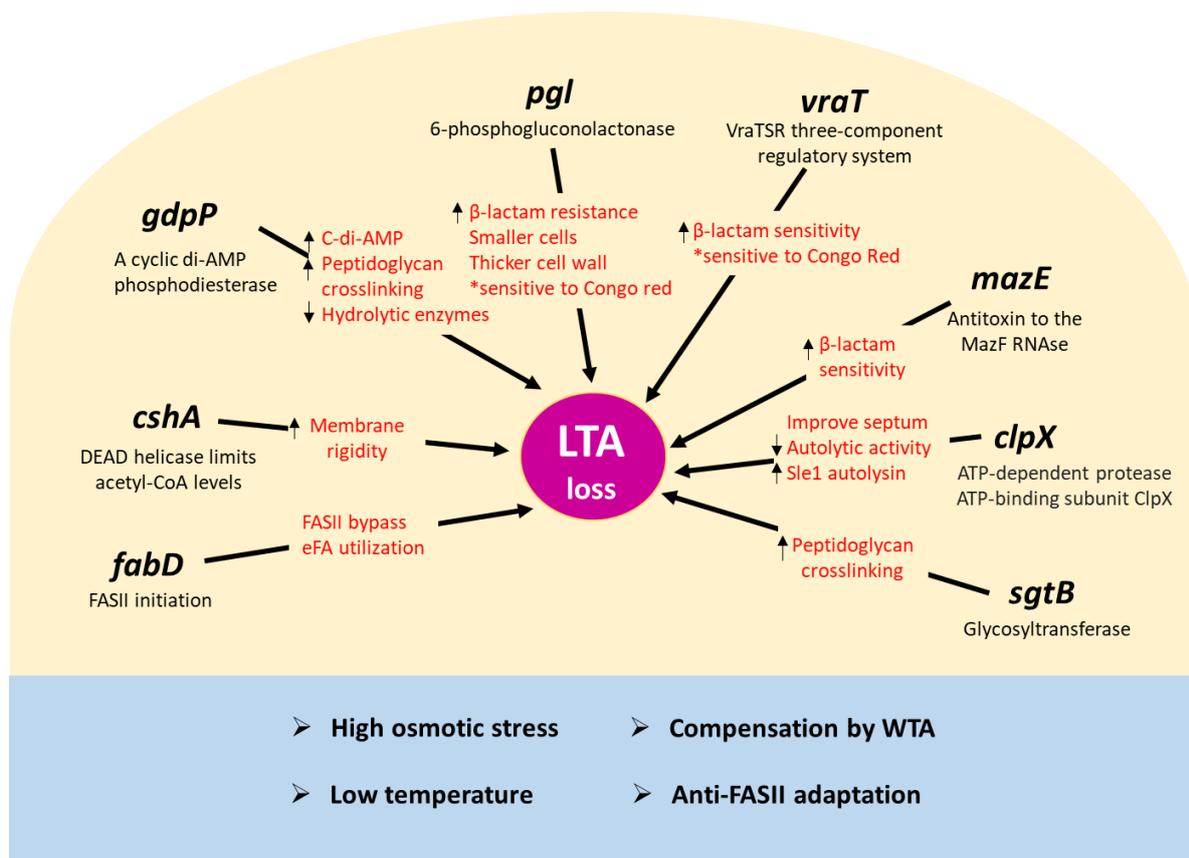


Figure 26. Conditions in which LTA is not crucial for *S. aureus* growth and survival. Mutations in the genes surrounding 'LTA loss' (center) alleviate the requirement for LTA or more specifically, for LtaS. Outside gene names in black indicate functional roles of the indicated genes. Inner information in red indicates the consequences of mutating these genes. The blue box below shows conditions in which LTA depletion is tolerated by *S. aureus*.

4.8 Conclusion

LTA is related to several physiological functions, and importantly in cell division. This makes LTA, or LTA synthesis enzymes as potential antimicrobial targets. On the other hand, several studies have shown that bacteria do not need LTA when a compensatory mutations occurs (Bæk et al., 2016; Corrigan et al., 2011; Karinou et al., 2019; Khemici et al., 2020; Zeden et al., 2023) or when cultivating bacteria in specific conditions like high osmotic conditions or at low temperature (Bæk et al., 2016; Oku et al., 2009). It might also be of interest to try introducing a gene overexpression library in an *ltaS* mutant strain to identify other functions that compensate for growth in the absence of LtaS. In conditions of FASII bypass, bacteria survive with little detectable LTA, possibly by increased production of WTA (**Chapter 6**).

RESULTS

This chapter will be included as part of a Research Article.

CHAPTER 5

Manuscript I:

Oxidative Stress is Intrinsic to Staphylococcal Adaptation to Fatty Acid Synthesis Antibiotics

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1 **Oxidative Stress is Intrinsic to Staphylococcal Adaptation to Fatty Acid Synthesis**
2 **Antibiotics**

3

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17

18 **Key words:** Fatty acid synthesis (FASII) pathway/antibiotic adaptation/peroxide priming/fitness/
19 virulence.

20

21

22 **Short title:** FASII bypass redirects *S. aureus* fitness

23

24

25 **Abstract**

26 Antibiotics inhibiting the fatty acid synthesis (FASII) pathway of the major pathogen
27 *Staphylococcus aureus* reach their enzyme targets, but bacteria continue growth by using
28 environmental fatty acids (eFAs) to produce phospholipids. We assessed how extreme changes
29 in membrane phospholipids provoked by FASII-antibiotics affect global *S. aureus* physiology.
30 Anti-FASII provoked massive lasting expression changes without genomic rearrangements.
31 Several regulators, rather than one master switch, contributed to the timing of anti-FASII
32 adaptation. Numerous virulence and adhesion factors showed decreased levels and/or activity.
33 Conversely, stress response protein levels increased, and correlated with greater tolerance to
34 peroxides. Notably, peroxide priming stimulated eFA incorporation efficiency and facilitated
35 adaptation to FASII inhibition. These findings establish a link between oxidative stress and FA
36 incorporation. Consistent with major shift in protein expression, anti-FASII-adapted *S. aureus*
37 killed an insect host more slowly but continued multiplying. Thus, while anti-FASII-adapted
38 populations are less equipped to damage the host, they may be better fit for long term survival,
39 and could constitute a reservoir for re-infection.

40

41 **Introduction**

42 *Staphylococcus aureus* is a Gram-positive opportunistic bacterium that remains a major cause
43 of disease and mortality in humans and animals. The unsolved crisis of non-treatable infections
44 due to multidrug-resistance notably methicillin-resistant *S. aureus* (MRSA) underlines the need
45 for alternative treatments, especially in compromised patients (Lowy, 1998, Rasigade and
46 Vandenesch, 2014, Lakhundi and Zhang, 2018, Cusumano et al., 2020).

47 Enzymes of the fatty acid (FA) synthesis pathway (FASII) are longtime candidate targets for
48 drug development against *S. aureus* infections (Wang et al., 2006, Wright and Reynolds, 2007).
49 FabI, enoyl-ACP reductase, was a preferred target as a narrow spectrum inhibitor that would
50 not disrupt the gut microbiome during treatment (Karlowsky et al., 2009). However, while anti-
51 FASII drugs targeting FabI effectively reached their targets in *S. aureus*, bacterial growth
52 continued in *in vitro* or mouse bacteremia models; this is because bacteria incorporate
53 environmental FAs (eFAs), which are abundant in host biotopes and are used directly to
54 produce bacterial membrane phospholipids; the process is termed FASII bypass (Brinster et al.,
55 2009, Gloux et al., 2017, Kenanian et al., 2019, Morvan et al., 2016) (**Fig. S1** schematizes FASII
56 and FASII bypass pathways). Exposure to host lipids during septicemic infection thus favors *S.*
57 *aureus* adaptation to anti-FASII treatment (Kenanian et al., 2019). Despite these limitations of
58 FASII antibiotics in treating streptococcal, enterococcal, and staphylococcal infections, new
59 anti-FASII drugs are in development (Pishchany et al., 2018, Yao and Rock, 2016, Fage et al.,
60 2020, Parker et al., 2022). Characterizing the anti-FASII-adapted *S. aureus* populations that
61 might persist in the host after treatment is thus crucial for future use.

62 We report here that anti-FASII treatment results in the emergence of a novel *S. aureus*
63 fitness state. Anti-FASII-adapted bacteria produce less virulence factors. In contrast, stress
64 responses are activated, and adapted bacteria show greater peroxide tolerance. Pre-exposure
65 to peroxides accelerates anti-FASII adaptation by enhancing fatty acid incorporation, thus
66 identifying a new strategy by which peroxide priming facilitates antibiotic survival. These

67 findings elucidate the state of anti-FASII-adapted bacteria that persist in the host after anti-
68 FASII treatment.

69 **Results**

70 **Anti-FASII treatment leads to long-term *S. aureus* adaptation without detectable**
71 **chromosomal rearrangements.** Our previous studies ruled out a role for point mutations or
72 indels in *S. aureus* adaptation to anti-FASII in serum-containing medium; studies were
73 performed using either of two potent FabI inhibitors, triclosan (McMurry et al., 1998) or AFN-
74 1252, a pipeline antimicrobial with high FabI specificity (Karlowsky et al., 2009), giving equivalent
75 results (Kenanian et al., 2019). As large chromosomal rearrangements are not detected by mi-
76 Seq (Kenanian et al., 2019), we resorted to nanopore sequencing of four anti-FASII-adapted
77 cultures. Tests were performed using AFN-1252 and two non-treated cultures. No
78 rearrangements specific to anti-FASII adaptation were detected, showing that anti-FASII-
79 adaptation occurs in the absence of chromosomal rearrangements or mutations. Once adapted
80 to anti-FASII, growth was robust in SerFA liquid medium (BHI supplemented with serum and
81 fatty acids); however, bacteria grew poorly in FA-free medium (**Fig. S2**). These results indicated
82 that phenotypic alterations were responsible for anti-FASII adaptation.

83 **Anti-FASII adaptation involves massive protein reprogramming.** A proteomics approach was
84 used to elucidate changes occurring during anti-FASII adaptation. FASII inhibition is overcome
85 by eFAs provided in the presence of serum. Serum protects bacteria from toxic free FAs, and
86 promotes FASII bypass (Kenanian et al., 2019). A complete kinetics study on *S. aureus* USA300
87 used the anti-FASII triclosan, both without and with added serum, at 2, 4, 6, 8, and 10 h post-
88 anti-FASII addition (**Fig. 1A, B, C** respectively show strategy, growth kinetics, and proteome
89 results; **Table S1, tabs 1 and 2**, give complete results according to cluster analyses). As
90 expected, protein abundance was mainly lower in triclosan medium (FA-Tric) without serum,
91 where adaptation occurs by mutation emergence (Morvan et al., 2016). In contrast, massive
92 protein level changes in adaptation (SerFA-Tric) medium suggest metabolic activity in latency

93 phase prior to full outgrowth, with transient or lasting expression differences compared to non-
94 treated bacteria (compare SerFA and SerFA-Tric, **Fig. 1C**).

95 We first exploited the data to screen for proteins whose levels selectively increased in the
96 presence of specific signals related to FAs and/or anti-FASII (**Fig. S3**). Highest expression of
97 several Type 7 secretion system proteins (comprised between SAUSA300_0278 and
98 SAUSA300_0289) occurred in FA conditions, as reported (Lopez et al., 2017). The FarE fatty acid
99 efflux pump was also up-regulated, presumably to avoid accumulation and toxicity of free FAs
100 (Alnaseri et al., 2015); as reported, induction was alleviated in serum, which lowers toxicity
101 (Kenanian et al., 2019). Overall, serum markedly affects *S. aureus* responses to anti-FASII, thus
102 highlighting its crucial role in adaptation.

103 Proteins related to FASII and phospholipid metabolism showed striking differences
104 according to conditions (**Fig. S4**). Levels of most FASII- and phospholipid-related enzymes
105 increased in SerFA-Tric as compared to those in all other conditions, including FA-Tric without
106 serum. Notably, PlsC (1-acyl-sn-glycerol-3-phosphate acyltransferase), is required for
107 phospholipid synthesis and anti-FASII adaptation. After an initial decrease, PlsC levels are then
108 restored during adaptation outgrowth, in accordance with results using a reporter fusion
109 (Pathania et al., 2021). This result highlights the importance of serum in enabling FASII bypass.

110 We focus below on the changes occurring in serum-supplemented medium containing anti-
111 FASII (SerFA), in which FASII bypass occurs without genetic modification. Three categories of
112 proteins, regulators, stress response proteins, and virulence factors, are analyzed.

113 **Anti-FASII adaptation triggers shifts in regulatory protein expression and phosphorylation.**

114 Regulatory proteins are likely implicated in the massive changes caused by anti-FASII
115 treatment. Proteomics revealed 22 hypothetical or confirmed proteins implicated in regulation
116 whose expression decreased (9) or increased (13) during anti-FASII adaptation; they affect
117 stress response, virulence, nutritional immunity and metabolism, or combinations of these
118 factors (**Fig. 2A**). As phosphorylation is a frequent post-translational modification that alters

119 functions of numerous regulatory proteins (Garcia-Garcia et al., 2016, Mijakovic et al., 2016,
120 Derouiche et al., 2013), we performed a phosphoproteomics analysis, focusing here on
121 regulatory proteins. Five regulators showed altered phosphorylation patterns during transition
122 or upon adaptation to anti-FASII (**Fig. 2B** and **Fig. S5**; **Table S2** for full results). Altogether, we
123 selected CcpE, CshA, HrcA, Rot, and XdrA as showing high expression and/or phosphorylation
124 changes at or just prior to adaptation outgrowth (8-10 h) (**Fig. 2A and B**) for further study.

125 To determine how these regulators affected anti-FASII adaptation, we compared growth
126 kinetics of USA300 and the corresponding mutant strains (Fey et al., 2013) (**Fig. 2C**). The
127 absence of *cshA* led to ~2 h delays in anti-FASII adaptation time. CshA controls the supply of
128 acetyl-CoA; its interruption results in phospholipids enriched in saturated FAs (Khemici et al.,
129 2020). We propose that this membrane alteration might interfere with eFA incorporation.

130 The *xdrA* mutant also showed a 2 h delay in adaptation. Both the amounts and
131 phosphorylation of XdrA decreased during anti-FASII treatment. The putative fatty acid
132 degradation gene *fadX* is reportedly upregulated when XdrA pools are lower (McCallum et al.,
133 2010), which might transiently compete for eFAs and limit their availability for FASII bypass.
134 Alternatively, XdrA DNA binding activity reportedly overlaps that of the CodY regulator (Lei and
135 Lee, 2018). We recently showed that a *codY* mutant is delayed in adaptation, which we
136 proposed relates to a positive regulatory effect of CodY on Acc expression (Pathania et al.,
137 2021). The delay in anti-FASII adaptation of the *xdrA* mutant might relate to a coordinate role
138 between XdrA and CodY. *hrcA* and *ccpE* mutants showed minor changes in anti-FASII
139 adaptation kinetics, while *rot* had no detectable change (**Fig. 2C**).

140 Thus, while regulatory mutants individually affect anti-FASII-adaptation efficacy, no single
141 tested regulator acted as a master controller. In *Escherichia coli*, stress response regulators
142 respond to discrete extracytoplasmic signals, each of which independently contributes to stress
143 adaptation (Bury-Mone et al., 2009). Similarly, differentially expressed regulators may each

144 respond to different stimuli consequent to membrane perturbation by anti-FASII, which
145 cooperatively promote adaptation.

146 **Reduced levels and activities of virulence factors in anti-FASII-adapted *S. aureus*.** Levels of 10
147 out of 16 detected virulence-related proteins decreased transiently or durably during anti-FASII
148 adaptation compared to non-treated *S. aureus* cultures (**Fig. 3A**). Levels of several adhesins
149 were markedly decreased, including ClfB, which is required for virulence (Dyzenhaus et al.,
150 2022); iron receptor protein and toxin levels were also overall reduced. In contrast, two
151 peptidoglycan hydrolases, Atl and IsaA, showed increased levels. Hydrolases are implicated in
152 housekeeping, and also in virulence (Stapleton et al., 2007); their increased expression upon
153 adaptation outgrowth might resolve cell wall thickening in the latency phase (PW, AG, JAM, in
154 preparation). We asked whether decreased amounts of adhesion factors SdrD, SdrE, EbpS, and
155 ClfB in anti-FASII-adapted *S. aureus* might impact adhesion to host cells. Anti-FASII-adapted *S.*
156 *aureus* (using AFN-1252) adhered poorly to human THP-1 macrophages compared to non-
157 adapted bacteria (20% versus 44%, $p < 0.01$; **Fig. 3B**). We also evaluated major secreted
158 virulence factors not detected by proteomics: lipase, nuclease, protease, and hemolysin in non-
159 treated versus anti-FASII-adapted *S. aureus* cultures (**Fig. 3C**). Activities of these exoproteins
160 were visibly lower in anti-FASII adapted cultures obtained using triclosan or AFN-1252.
161 Altogether these results indicated that anti-FASII-adapted *S. aureus* populations may be less fit
162 for virulence.

163 **Anti-FASII-adapted *S. aureus* produce increased levels of stress response proteins and show**
164 **increased resistance to an oxidative stress.** Levels of 26 out of 28 assessed stress response
165 proteins showed transient or lasting increases in anti-FASII-adapted *S. aureus* compared to the
166 non-treated control. Among them, 18 were pronounced in during adaptation outgrowth.
167 Proteins are implicated in pH, oxidative, osmotic, and unfolded protein responses (**Fig. 4A**).

169 Host production of reactive oxidative species (ROS) is a documented response to infection
170 as a means to eliminate the pathogen (Liu et al., 2005). We asked whether increased levels of

171 stress-related factors in anti-FASII-adapted *S. aureus* affected bacterial tolerance to ROS. This
172 was tested by challenging non-treated and anti-FASII-adapted cultures with 0.5 mM H₂O₂ for 5
173 h, followed by survival assessments by colony forming unit (CFU) determinations. Anti-FASII-
174 adapted *S. aureus* CFUs were ~20-fold higher than for non-treated bacteria ($p < 0.01$; **Fig. 4B**).
175 Greater peroxide tolerance might thus confer a survival advantage to the anti-FASII-adapted,
176 oxidative stress-resistant populations.

177 **Priming with peroxides prior to anti-FASII challenge stimulates adaptation by enhancing eFA**
178 **incorporation, and requires PerR.** Stress response induction by anti-FASII led us to ask the
179 contrary, whether stress priming would stimulate anti-FASII bypass. A ROS priming effect
180 reportedly increased resistance to various antibiotics in *E. coli*, including oxolinic acid,
181 ampicillin, kanamycin (Mosel et al., 2013), and fluoroquinolones (Gerstel et al., 2020). The
182 effects of ROS priming on anti-FASII adaptation in *S. aureus* were tested by subjecting cultures
183 to overnight priming with sub-lethal concentrations of H₂O₂ (0.5 mM) or phenazine-
184 methosulfate (PMS, a redox cycling compound; 20-50 μ M (Gerstel et al., 2020)). Cultures were
185 diluted, and kinetics of anti-FASII (AFN-1252) adaptation was followed. H₂O₂ or PMS priming
186 shortened the time of *S. aureus* adaptation to FASII antibiotics by 1.5 – 1.7 h compared to non-
187 primed WT cultures (**Fig. 4C upper, Table 1**). A *katA* mutant lacks catalase and would
188 accumulate H₂O₂; anti-FASII adaptation in *katA* was ~1 h shorter than in the WT parental strain.
189 Priming *katA* with H₂O₂ as above further shortened the adaptation time by ~2 h as compared
190 to WT without peroxide (**Table 1**). Conversely, treating the WT strain with reducing agents (Na
191 citrate 10 mM, or vitamin C 5.7 mM) retarded adaptation by 3.5 and 1.7 h respectively (**Table**
192 **1**). *S. aureus* oxidation state is thus implicated in anti-FASII adaptation.

193 ROS priming induces antibiotic efflux in *E. coli*, leading to antibiotic tolerance (Gerstel et al.,
194 2020, Mosel et al., 2013, Wu et al., 2012). Induction of the *E. coli* Fe-S repair system Suf matures
195 the SoxR regulator, which in turn activates the AcrAB efflux pump to eliminate
196 fluoroquinolones (Gerstel et al., 2020). We asked whether peroxide similarly induces an efflux

197 mechanism that would accelerate anti-FASII adaptation. If this were the case, FASII activity
198 would be restored, and bacteria would be anti-FASII-sensitive. However, examination of FA
199 profiles revealed the contrary: at 6 h post anti-FASII treatment (using AFN-1252), *i.e.*, during
200 the latency phase, H₂O₂ priming resulted in more efficient eFA incorporation compared to that
201 in non-primed cultures (respectively 82% *versus* 54% eFAs; **Fig. 4C**). Moreover, a transposon
202 insertion inactivating the *suf* homolog in *S. aureus* (Roberts et al., 2017) did not abolish the
203 H₂O₂ priming effect (**Table 1**). These results show that stimulated eFA incorporation and hence
204 more rapid adaptation, rather than anti-FASII efflux, explains the priming effects of peroxide
205 on anti-FASII adaptation.

206 PerR (SAUSA300_1842) is a conserved highly sensitive metal-dependent peroxide sensor
207 and regulator protein (Ji et al., 2015). PerR was not detected in proteomics of anti-FASII
208 adaptation (**Table S1**). However, as PerR senses H₂O₂, we examined its implication in H₂O₂-
209 accelerated anti-FASII adaptation. Unlike the USA300 parent, *perR* mutant adaptation time and
210 FA incorporation efficiency were unaffected by peroxide (proportions of eFA incorporation at
211 6 h were 52% and 51%, with and without H₂O₂ priming; **Fig. 4C**). Altogether these results
212 indicate the existence of a unique H₂O₂ priming system that requires PerR. They reveal a novel
213 link between peroxide and FA incorporation that facilitates adaptation to the FASII class of
214 antibiotics independently of antibiotic efflux.

215 **Anti-FASII-adapted *S. aureus* are less virulent, yet persist in a *Galleria mellonella* infection**
216 **model.** Lower virulence factor production but greater stress resistance due to anti-FASII
217 adaptation raises questions on the outcome of anti-FASII-adapted *S. aureus* infection. We
218 compared anti-FASII-adapted *versus* non-treated *S. aureus* in an insect *G. mellonella* model,
219 which allows the use of a large cohort. Moreover, larval hemocoel, like blood serum, is lipid-
220 rich (Kazek et al., 2021). AFN-1252, as chosen for this study, was pharmacologically vetted for
221 non-toxicity in the host (Hunt et al., 2016, Parsons et al., 2011); we showed previously that
222 AFN-1252 treatment did not stop infection in a mouse model (Kenanian et al., 2019). Non-

223 treated and AFN-1252-adapted *S. aureus* USA300 showed equivalent growth kinetics in SerFA
224 *in vitro* (**Fig. S2**). Insects infected by 10^6 anti-FASII-adapted bacteria were killed more slowly
225 than those infected by equivalent CFUs of untreated bacteria, as monitored over 72 h post-
226 infection (**Fig. 5A**). At T_{48} , 95% of insects were killed by untreated *S. aureus*, compared to 30%
227 killing with anti-FASII-adapted *S. aureus*. At T_{72} , when all larvae infected by untreated *S. aureus*
228 were dead, anti-FASII-adapted *S. aureus* killed over 60% of the insects. Thus, killing by anti-
229 FASII-adapted *S. aureus* was delayed but not stopped.

230 Bacterial CFUs determined from infected insects at 24 h were about 3-fold lower in those
231 infected by anti-FASII-adapted compared to non-treated bacteria, and then decreased in
232 surviving insects (**Fig. 5B left**). In contrast, CFUs in dead larvae at T_{48} were comparable for both
233 groups, indicating that anti-FASII-adapted bacteria multiplied as well as non-treated bacteria
234 in the insects they killed (**Fig. 5B right**).

235 To determine whether anti-FASII-adapted bacteria remained adapted during infection, CFU
236 platings as above were done in parallel on SerFA and SerFA-AFN solid media. Only anti-FASII-
237 adapted bacteria formed colonies on SerFA-AFN solid medium (**Table S3**). Among surviving
238 insects infected with adapted bacteria, only one contained bacteria that returned to the non-
239 adapted state at 72 h; this indicates that bacteria remained mainly adapted during insect
240 infection. Altogether, these results show that anti-FASII-adapted *S. aureus* are less virulent,
241 while populations still multiply in the insect host. Greater stress resistance capacity may help
242 withstand host conditions and can explain bacterial persistence in the insect host.

243

244 **Discussion**

245 eFA incorporation during FASII-antibiotic-induced bypass redesigns *S. aureus* membrane
246 phospholipids, leading to massive shifts in protein expression, and a prolonged adaptation
247 state without detectable genomic rearrangements or mutations (summarized in **Fig. 6** model).
248 These changes, which begin before bacterial outgrowth, alter the *S. aureus* fitness state

249 towards less damage to the host, and more tolerance to host-generated toxicity. Peroxide
250 priming accelerates adaptation and eFA incorporation, establishing a link between ROS and
251 FASII bypass. The *in vitro* phenotypes deduced from proteomic and *in vitro* findings are
252 consistent with the differences observed in insect infection kinetics between non-treated and
253 anti-FASII-treated *S. aureus*.

254 **Regulator changes during anti-FASII treatment.** Various regulators appear to be implicated in
255 anti-FASII adaptation, *e.g.*, XdrA or CshA, and to lesser extents HrcA or CcpE. However, no single
256 regulator was found to have a “master” role. In addition to alterations in protein abundance
257 and phosphorylation levels as assessed here, we hypothesize that membrane perturbations, as
258 caused by anti-FASII, alter the intracellular environment, to liberate signaling lipids that
259 modulate regulator activities (**Fig. 6**). For example, FAs and cardiolipin reportedly affect
260 activities of the *S. aureus* two-component system SaeRS (Schurig-Briccio et al., 2020, Yeo et al.,
261 2023). Virulence factor activity may be blocked by FA binding as shown in *Vibrio cholerae*
262 (Lowden et al., 2010). Interaction of regulators with lipid species released during anti-FASII
263 treatment may be a rapid means to adjust regulator functions for FASII bypass.

264 **Link between FASII-antibiotic adaptation and *S. aureus* fitness against peroxide stress.** We
265 showed that ROS priming stimulates FASII bypass by increasing eFA incorporation (**Fig. 4**). To
266 our knowledge, this is the first report establishing a connection between oxidative stress and
267 stimulation of FASII antibiotic adaptation, and defines a novel paradigm. This mechanism
268 contrasts to that described in *E. coli*, where ROS priming either lowered proton-motive-force-
269 mediated antibiotic entry and/or induced efflux pump expression, *via* a Suf-controlled Fe-S
270 repair system (Gerstel et al., 2020). In contrast, the H₂O₂-stimulated anti-FASII adaptation is
271 mediated by greater, rather than less, eFA incorporation and precocious adaptation, ruling out
272 a role for efflux. A *suf* mutant remained sensitive to peroxide activation, further distinguishing
273 the two mechanisms.

274 We hypothesize that H₂O₂ has a direct role in stimulating FASII bypass: H₂O₂-mediated
275 oxidation of FASII initiation enzymes could diminish competition with the FASII bypass system
276 (**Fig. 6**). We show here that a *katA* mutant, which presumably accumulates H₂O₂, shortens
277 adaptation time, while a *perR* mutant, which activates KatA to degrade H₂O₂, lengthens
278 adaptation time. Direct or indirect targets of H₂O₂ could be involved in disabling FASII to favor
279 FASII bypass; this hypothesis is currently under study.

280 Host macrophages and neutrophils generate peroxides presumably as a means to control
281 infection (Cole et al., 2014, Mayer-Scholl et al., 2004). *S. aureus* adaptation to anti-FASII is
282 potentiated in these same conditions, which needs to be considered for future use of FASII
283 antibiotics.

284 **Potential advantages of a fitness reset in FASII-antibiotic-adapted *S. aureus*.** Lower virulence,
285 greater stress response, and exclusive eFA utilization may be advantageous to *S. aureus*
286 lifestyle. We note that streptococci and enterococci use FASII bypass even without antibiotics,
287 favoring eFA incorporation during infection (Brinster et al., 2009, Lambert et al., 2023).
288 Although these species employ distinct FASII regulation system from that of *S. aureus* (feedback
289 versus feedforward in *S. aureus* (Lambert et al., 2022, Albanesi et al., 2013)) and differ in their
290 *in vivo* lifestyles, they have common infection biotopes, raising the possibility that FASII bypass
291 is advantageous in the course of infection.

292 Parallels also exist between anti-FASII-adapted *S. aureus*, and small colony variants (SCVs):
293 both emerge more efficiently in oxidative stress (Peyrusson et al., 2022, Painter et al., 2015),
294 and both produce less virulence factors (Tuchscher et al., 2020, Sendi and Proctor, 2009),
295 which might facilitate bacterial escape from host immune surveillance. In both cases,
296 uneradicated reservoirs of these bacteria might be sources of chronic infection (**Fig. 6**).

297 **Potential applications of FASII antibiotics.** Although anti-FASII do not eliminate *S. aureus* when
298 compensatory eFAs are available, the present study demonstrates that it reduces *S. aureus*
299 virulence factor production. Anti-FASII synergy with other treatments that prevent adaptation

300 may potentiate its efficacy (Pathania et al., 2021). Here, anti-oxidants delayed anti-FASII
301 adaptation, which may offer perspectives for a bi-therapy approach to eliminate *S. aureus* with
302 reduced virulence.

303

304 **Materials and Methods**

305 **Strains, media, and growth conditions.** Experiments were performed using *S. aureus*
306 SAUSA300_FPR3757 JE2 strain, referred to as USA300, and transposon insertion derivative
307 strains from the Nebraska mutant library ((Fey et al., 2013); generously supplied by BEI
308 Resources NIAID, NIH, USA). All strains were confirmed for transposon insertion by PCR (see
309 **Table S4** for strains and primers used). Cultures were grown aerobically at 37°C. Solid and liquid
310 growth media were based on BHI as follows: no additives (BHI), containing 0.5 mM FAs (BHI-
311 FA, with an equimolar mixture of 0.17 mM each C14, C16, C18:1 [Larodan, Sweden]), and BHI-
312 FA containing 10% newborn calf serum (Ser-FA; Eurobio Scientific, France [Fr]). Where
313 specified, the anti-FASII triclosan (McMurry et al., 1998) was added at 0.25 µg/ml in media
314 without serum, and at 0.5 µg/ml in media containing serum, to respectively give BHI-Tric, FA-
315 Tric, or SerFA-Tric, as described (Morvan et al., 2017, Kenanian et al., 2019, Morvan et al.,
316 2016). H₂O₂ (0.5 mM final concentration) was added to SerFA precultures when indicated. The
317 anti-FASII AFN-1252 (Karlowsky et al., 2009) was used in SerFA at 0.5 µg/ml (SerFA-AFN) as
318 described (Kenanian et al., 2019). For most experiments, *S. aureus* USA300 was streaked on
319 solid BHI medium, and independent colonies were used to inoculate overnight BHI pre-
320 cultures. For proteomic and phosphoproteomic studies, cultures were inoculated at a starting
321 OD₆₀₀ = 0.1.

322 **Nanopore sequencing.** Six single colonies of USA00 were resuspended in SerFA and grown
323 overnight. Cultures were then diluted in SerFA, SerFA-AFN-1252, 10% mouse serum, or 10%
324 mouse serum containing AFN-1252, and grown to OD₆₀₀ = 1. Whole chromosomal DNA was
325 prepared from the independent cultures as described, and outsourced for nanopore

326 sequencing (Eurofins, Germany). Whole genome DNA sequences were presented as
327 circularized genomes, and entered in the Mendeley database doi:10.17632/grt4htck9k.1.
328 Sequences were compared by performing full genome alignments
329 <https://dgenies.toulouse.inra.fr/> 09/02/2023.

330 **Proteomics preparation.** Adaptation to anti-FASII varies with growth media: BHI-Tric-grown
331 and FA-Tric-grown *S. aureus* do not adapt in the time periods tested, (high frequency adaptive
332 mutations arise with a delay in FA-Tric; (Morvan et al., 2016)), whereas SerFA-Tric-grown *S.*
333 *aureus* adapt without mutation after an initial latency period (6-8 hours, depending on growth
334 conditions) (Kenanian et al., 2019). Kinetics experiments were performed on USA300 to
335 determine the protein changes associated with FA-Tric and SerFA-Tric anti-FASII adaptation.
336 Cultures for each condition were prepared as independent quadruplicates. For each sample,
337 BHI precultures were diluted and shifted to the specified medium starting cultures at $OD_{600} =$
338 0.1. Control cultures in BHI, BHI-FA and BHI-SerFA were grown to $OD_{600} = \sim 1$. BHI-Tric cultures
339 (no added FAs) were collected at 6 h. USA300 samples grown in FA-Tric and SerFA-Tric were
340 collected at 2, 4, 6, 8 and 10 h post-antibiotic addition (see **Fig. 1A** and **Table S1** for growth
341 conditions and complete data). For each sample, 20 OD_{600} units culture equivalent was
342 collected and centrifuged for 10 min at 4°C at 8000 rpm. Pellets were washed twice in Tris
343 10mM pH7.0 containing 0.02% TritonX-100, and Halt™ Protease & Phosphatase Inhibitor
344 Cocktail (100X) (Thermo Scientific, Fr). Pellets were then resuspended in 650 μ l washing buffer,
345 mixed with 0.1 mm silica beads and subjected to 3 cycles of vigorous shaking (Fast-Prep-24,
346 MP-Bio, Fr). After 10 min centrifugation at 10000 rpm, supernatants were recovered and stored
347 at -80°C prior to analyses.

348 Protein extractions, LC-MS/MS analyses, and bioinformatics, and statistical data analyses
349 were done as described in detail (Bednarz et al., 2021). The reference genome GenBank
350 Nucleotide accession code NC_007793.1 was used for protein annotation. The bioinformatic
351 tools used for proteomics analysis (X!TandemPipeline C++, MassChroQ, MCQR) are open and

352 free resources available in the following repository: <https://forgemia.inrae.fr/pappso>. The
353 mass spectrometry proteomics data was deposited to the ProteomeXchange Consortium
354 (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository with the
355 dataset identifier PPXD034256.

356 **Proteome data analyses.** Protein abundance differences were detected by ANOVA tests for all
357 methods used (spectral counting, SC; extracted ion chromatograms, XIC; and peak counting,
358 PC). The abundance of a protein was considered significantly variable when the adjusted p-
359 value was <0.05. Proteins showing significant statistical abundance differences were
360 represented on heat maps normalized by using GraphPad Prism 9.5.1 (Bednarz et al., 2021). K-
361 means clustering analysis was performed using RStudio. Proteins whose abundancies were
362 significantly different in one or more conditions were manually curated and classified according
363 to functional groups.

364 **Phosphoproteome preparation.** USA300 cultures were prepared in BHI and SerFA and
365 harvested at $OD_{600} = \sim 1$. SerFA-Tric cultures were harvested at 6h and 10 h post-treatment.
366 Samples were prepared independently from those in the proteomics study, as independent
367 biological triplicates, and treated as in proteomics studies, except that we collected the
368 equivalent of 50 OD_{600} units. Bacteria were processed as for proteome extraction except that
369 Tris was replaced by triethylammonium bicarbonate (50 mM; *n.b.* Tris interferes with dimethyl
370 tag labelling) containing antiprotease and antiphosphatase at the recommended
371 concentrations. Lysed bacteria after Fast-Prep were centrifuged 15 min at 12000 rpm
372 supernatants containing soluble proteins were kept at -80°C before use. Protein concentrations
373 were determined by the Bradford method.

374 One mg protein samples were evaporated and resuspended in 1 ml 5 % formic acid and
375 dimethyl-tag labeled as described (Boersema et al., 2009). Briefly, differential on-column
376 labeling of peptide amine groups (NH_2) created dimethyl labels leading to mass shifts of
377 +28.0313 Da for the peptides from SerFA 3 h samples, +32.0564 Da for the peptides from SerFA-

378 Tric 6 h samples and +36.0757 for the peptides from SerFA-Tric 10 h samples respectively. The
379 three samples were mixed and then submitted to six rounds of phosphopeptide enrichment
380 with 5mg TiO₂ beads/mg of protein (Titansphere Phos-TiO, GL Sciences Inc., Netherlands) as
381 described (Soufi et al., 2018).

382 LC-MS/MS analyses of samples were done using an Ultimate 3000 nano-RSLC coupled on
383 line with a Q Exactive HF mass spectrometer (Thermo Scientific, San Jose California). 1 µL of
384 each sample was loaded on a C18 Acclaim PepMap100 trap-column 300 µm inner diameter (ID)
385 x 5 mm, 5 µm, 100Å, (Thermo Scientific) for 3.0 minutes at 20 µL/min with 2% acetonitrile
386 (ACN), 0.05% TFA in H₂O and then separated on a C18 Acclaim Pepmap100 nano-column, 50
387 cm x 75 µm ID, 2 µm, 100 Å (Thermo Scientific) with a 100 minute linear gradient from 3.2% to
388 20% buffer B (A: 0.1% FA in H₂O, B: 0.1% FA in ACN), from 20 to 32% of B in 20 min and then
389 from 32 to 90% of B in 2 min, hold for 10 min and returned to the initial conditions. The flow
390 rate was 300 nL/min.

391 Labeled peptides were analyzed with top15 higher energy collisional dissociation (HCD)
392 method: MS data were acquired in a data dependent strategy selecting the fragmentation
393 events based on the 15 most abundant precursor ions in the survey scan (m/z range from 350
394 to 1650). The resolution of the survey scan was 120,000 at m/z 200 Th and for MS/MS scan the
395 resolution was set to 15,000 at m/z 200 Th. For HCD acquisition, the collision energy = 27 and
396 the isolation width is of 1.4 m/z. The precursors with unknown charge state, charge state of 1
397 and 5 or greater than 5 were excluded. Peptides selected for MS/MS acquisition were then
398 placed on an exclusion list for 20 s using the dynamic exclusion mode to limit duplicate spectra.
399 The mass spectrometry proteomics data have been deposited to the Center for Computational
400 Mass Spectrometry repository (University of California, San Diego) *via* the MassIVE tool with
401 the dataset identifier MassIVE MSV000089781 (accessible at
402 <http://massive.ucsd.edu/ProteoSAFe/status.jsp?task=db3f5002e32e4a47a71996358bc6ae8c>
403).

404 **Phosphoproteome data analyses.** Proteins were identified by database searching using
405 SequestHT with Proteome Discoverer 2.5 software (Thermo Scientific, Fr) against the Uniprot
406 *S. aureus* USA300 database (2020-01 release, 2607 sequences). Precursor mass tolerance was
407 set at 10 ppm and fragment mass tolerance was set at 0.02 Da, and up to 2 missed cleavages
408 were allowed. Oxidation (M), acetylation (Protein N-terminus), and phosphorylation (S, T, Y)
409 were set as variable modifications. The differentially dimethyl-labeled peptides in primary
410 amino groups K and N-ter (see above), and carbamidomethylation (C) were set as fixed
411 modifications. Peptides and proteins were filtered with a false discovery rate (FDR) at 1% using
412 the Percolator tool (The et al., 2016). Protein quantitation was performed with precursor ions
413 quantifier node in Proteome Discoverer 2.5 software, peptide and protein quantitation based
414 on pairwise ratios and t-test statistical validation.

415 **Macrophage adhesion.** Confluent cell lawns of THP-1 macrophages were prepared as
416 described to obtain confluent cell lawns of 3×10^5 cells per well (Bourrel et al., 2022). *S. aureus*
417 was pre-cultured in SerFA medium and then subcultured overnight in SerFA medium without
418 or with AFN-1252. The next day, bacteria were subcultured in the same corresponding fresh
419 media to $OD_{600} = 1-2$. Bacteria were then washed twice with PBS buffer, and diluted in RPMI
420 GluMax (Gibco, France) to obtain an MOI = 1. One ml of the dilution was added in each well in
421 24-well plates. Plates were centrifuged at 1000 rpm for 5 minutes to sediment bacteria, and
422 then incubated at 4°C for 1h. After incubation, wells were washed 3 times with PBS, then 1ml
423 of sterile cold water was added to each well and left for 5min at room temperature. Finally, the
424 cells and bacteria were scraped from the surface and CFU were determined on SerFA agar
425 plates. Controls without macrophage were done in parallel. Results are derived from 5
426 biological replicates, and are presented as the mean \pm standard error of the mean (SEM) using
427 GraphPad Prism 9.5.1 (San Diego, Ca). CFU counts were compared by paired t-test ($P < 0.05$).

428 **Exoprotein activity assays.** SerFA day precultures were diluted into SerFA without or with
429 either triclosan (SerFA-Tric) and AFN-1252 (SerFA-AFN). Solid medium as prepared for

430 exoprotein detection were then spotted with resulting overnight saturated cultures or culture
431 supernatants as indicated. For nuclease detection, DNase agar (Oxoid, Thermo Scientific, Fr)
432 containing toluidine blue O 0.05 g/L (Sigma, Fr) was prepared as described (Zierdt and Golde,
433 1970). Culture supernatants from test samples were heated to 80°C for 10 m, and then spotted
434 (10 µl) on plates. Photographs were taken after overnight incubation at 37°C. Contrast was
435 uniformly enhanced by Photoshop to visualize pink halos indicating nuclease activity. To
436 measure protease activity, powdered skim milk 50 g/L was added to autoclaved 1% non-
437 nutrient agar (Invitrogen) as described (Park et al., 2012). Cultures were spotted (10 µl) on
438 plates and allowed to dry, then incubated at 37°C for 72 h and photographed. Lipase activity
439 was assayed in medium comprising 1% non-nutrient agar (Invitrogen) to which was added 2.5%
440 olive oil and 0.001% Rhodamine B (starting from a Rhodamine B stock solution of 1 mg per ml
441 in water; Sigma-Aldrich, Fr) as described (Araiza-Villanueva et al., 2019). To improve olive oil
442 emulsification, NaCl was added to medium (1M final concentration), followed by vigorous
443 shaking just prior to plate preparation. Supernatants from overnight cultures were sterile-
444 filtered through a 0.2 µm membrane syringe filter (Pall Corporation, Michigan). Supernatants
445 (10 µl) were deposited in holes pierced in solid medium. After 24 h incubation at 37°C plates
446 were visualized under UV light at 312 nm and photographed. Hemolytic activity was assayed
447 on *S. aureus* sterile-filtered supernatants that were prepared from overnight cultures. Aliquots
448 (10 µl) were spotted on 5% sheep blood agar plates (BioMérieux SA, Fr). After drying, plates
449 were incubated at 37°C overnight, and photographed.

450 **H₂O₂ resistance of non-treated and anti-FASII-adapted *S. aureus*.** *S. aureus* non-treated or
451 AFN-adapted cells (see above) were diluted to OD₆₀₀= 0.005 in SerFA and grown for 1 h at 37°C,
452 after which H₂O₂ (0.5mM final concentration) was added or not to cultures, and allowed to
453 grow for 5 h. Growth kinetics was followed by OD₆₀₀ determinations, and CFUs were
454 determined at the final time point. Plates were photographed after overnight incubation at
455 37°C.

456 **H₂O₂ and PMS pre-treatment.** For pre-adaptation in hydrogen peroxide or in PMS, *S. aureus*
457 were precultured in SerFA and then subcultured in SerFA containing or not 0.5 mM H₂O₂ (final
458 concentration) or PMS (20-50 μM) for 16 h at 37°C. Cultures were diluted to OD₆₀₀ = 0.1 in SerFA
459 or SerFA medium containing AFN-1252 (SerFA-AFN), and growth was monitored.

460 ***G. mellonella* infection by anti-FASII-adapted and untreated *S. aureus*.** Killing capacities and
461 CFUs of anti-FASII-adapted and untreated *S. aureus* USA300 were compared in the *G.*
462 *mellonella* insect model (Sheehan et al., 2019). *G. mellonella* larvae were reared on beeswax
463 and pollen in sealed containers with a wire mesh lid permitting aeration. The rearing container
464 was stored at 27°C temperature in a humidified incubator in our laboratory facilities. Fifth
465 instar larvae weighing ~250 mg were subjected to starvation for 24 h at 27°C and 1-2 h at 37°C
466 prior to infection. *S. aureus* SerFA cultures were subcultured in SerFA, and SerFA plus AFN-1252
467 (0.5 μg/ml; SerFA-AFN) for overnight growth. Resulting cultures were then diluted to 0.1 and
468 grown in respective media to OD₆₀₀ = 1. Based on previous CFU determinations, each culture
469 was pelleted, washed once in PBS, and then resuspended in PBS to obtain 10⁸ CFU/ml. Ten μl
470 (10⁶ CFUs) was administered by injection in the fourth proleg. To follow larvae mortality
471 following *S. aureus* infection, 20 larvae were inoculated using 3 independent cultures per
472 culture condition (totaling 60 insects injected with SerFA, and 60 with SerFA-AFN), and 20
473 larvae were inoculated with PBS buffer for the control. Surviving larvae were counted every 24
474 h for 72 hours. To monitor *S. aureus* bacterial counts post-infection, the experiment above was
475 repeated, and surviving larvae (9 total per condition, treated in 3 groups from independent
476 biological replicates) were sacrificed until there were no surviving larvae left. CFUs were also
477 determined from newly-dead larvae. To determine *S. aureus* CFUs, surviving larvae were chilled
478 in ice for 30 min, and then crushed in 1 ml sterile deionized water. The resulting content was
479 vortexed for ~30 sec. Five μl of 10-fold dilutions were prepared in sterile water and spotted on
480 SerFA and SerFA-AFN solid media. CFUs were determined after overnight incubation at 37°C.
481 The detection threshold was 10³ CFU per insect. Results of the 3 biologically independent

482 experiments were pooled for presentation of insect mortality, and similarly for CFUs as done
483 in the second independent experiment. Results are presented as the mean \pm standard
484 deviation (SD) using GraphPad Prism 9.5.1 (San Diego, Ca). CFU counts were compared by
485 nonparametric t-test (Mann-Whitney U) ($P < 0.05$). Note that *G. mellonella* naturally harbor
486 enterococci (Allonsius et al., 2019). In contrast to *S. aureus*, enterococci are catalase-negative
487 on routine plate tests (*n.b.*, some enterococci produce catalase upon heme addition). *S. aureus*
488 were thus differentiated from enterococci on plates used for CFU enumeration by their
489 catalase-positive response (*i.e.*, bubble formation) upon application of a 3% hydrogen peroxide
490 solution. Enterococci did not produce bubbles.

491

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511

512 **Data Availability**

513 All relevant data are within the manuscript, supporting information files, and depositories.

514

515 **Competing interests**

516 The authors declare that no competing interests exist.

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References

- 520 ALBANESI, D., REH, G., GUERIN, M. E., SCHAEFFER, F., DEBARBOUILLE, M., BUSCHIAZZO, A.,
521 SCHUJMAN, G. E., DE MENDOZA, D. & ALZARI, P. M. 2013. Structural basis for feed-forward
522 transcriptional regulation of membrane lipid homeostasis in *Staphylococcus aureus*. *PLoS*
523 *Pathog*, 9, e1003108.
- 524 ALLONSIUS, C. N., VAN BEECK, W., DE BOECK, I., WITTOUCK, S. & LEBEER, S. 2019. The
525 microbiome of the invertebrate model host *Galleria mellonella* is dominated by
526 *Enterococcus*. *Anim Microbiome*, 1, 7.
- 527 ALNASERI, H., ARSIC, B., SCHNEIDER, J. E., KAISER, J. C., SCINOCCA, Z. C., HEINRICH, D. E. &
528 MCGAVIN, M. J. 2015. Inducible Expression of a Resistance-Nodulation-Division-Type Efflux
529 Pump in *Staphylococcus aureus* Provides Resistance to Linoleic and Arachidonic Acids. *J*
530 *Bacteriol*, 197, 1893-905.
- 531 ARAIZA-VILLANUEVA, M., AVILA-CALDERON, E. D., FLORES-ROMO, L., CALDERON-AMADOR, J.,
532 SRIRANGANATHAN, N., QUBLAN, H. A., WITONSKY, S., AGUILERA-ARREOLA, M. G., RUIZ-
533 PALMA, M. D. S., RUIZ, E. A., SUAREZ-GUEMES, F., GOMEZ-LUNAR, Z. & CONTRERAS-
534 RODRIGUEZ, A. 2019. Proteomic Analysis of Membrane Blebs of *Brucella abortus* 2308 and
535 RB51 and Their Evaluation as an Acellular Vaccine. *Front Microbiol*, 10, 2714.
- 536 BEDNARZ, B., MILLAN-OROPEZA, A., KOTOWSKA, M., SWIAT, M., QUISPE HARO, J. J., HENRY, C.
537 & PAWLIK, K. 2021. Coelimidin Synthesis Activatory Proteins Are Key Regulators of
538 Specialized Metabolism and Precursor Flux in *Streptomyces coelicolor* A3(2). *Front Microbiol*,
539 12, 616050.
- 540 BOERSEMA, P. J., RAIJMAKERS, R., LEMEER, S., MOHAMMED, S. & HECK, A. J. 2009. Multiplex
541 peptide stable isotope dimethyl labeling for quantitative proteomics. *Nat Protoc*, 4, 484-94.
- 542 BOURREL, A. S., PICART, A., FERNANDEZ, J. C., HAYS, C., SAUBAMEA, B., MIGNON, V., POYART,
543 C., TAZI, A. & GUIGNOT, J. 2022. Specific interaction between Group B *Streptococcus* CC17
544 hypervirulent clone and phagocytes. *BioRxiv*.
- 545 BRINSTER, S., LAMBERET, G., STAELS, B., TRIEU-CUOT, P., GRUSS, A. & POYART, C. 2009. Type II
546 fatty acid synthesis is not a suitable antibiotic target for Gram-positive pathogens. *Nature*,
547 458, 83-6.
- 548 BURY-MONE, S., NOMANE, Y., REYMOND, N., BARBET, R., JACQUET, E., IMBEAUD, S., JACQ, A. &
549 BOULOC, P. 2009. Global analysis of extracytoplasmic stress signaling in *Escherichia coli*. *PLoS*
550 *Genet*, 5, e1000651.
- 551 COLE, J., ABERDEIN, J., JUBRAIL, J. & DOCKRELL, D. H. 2014. The role of macrophages in the innate
552 immune response to *Streptococcus pneumoniae* and *Staphylococcus aureus*: mechanisms
553 and contrasts. *Adv Microb Physiol*, 65, 125-202.
- 554 CUSUMANO, J. A., KLINKER, K. P., HUTTNER, A., LUTHER, M. K., ROBERTS, J. A. & LAPLANTE, K. L.
555 2020. Towards precision medicine: Therapeutic drug monitoring-guided dosing of
556 vancomycin and beta-lactam antibiotics to maximize effectiveness and minimize toxicity. *Am*
557 *J Health Syst Pharm*, 77, 1104-1112.
- 558 DECOURSEY, T. E. 2004. During the respiratory burst, do phagocytes need proton channels or
559 potassium channels, or both? *Sci STKE*, 2004, pe21.
- 560 DEROUICHE, A., BIDNENKO, V., GRENHA, R., PIGONNEAU, N., VENTROUX, M., FRANZ-WACHTEL,
561 M., NESSLER, S., NOIROT-GROS, M. F. & MIJAKOVIC, I. 2013. Interaction of bacterial fatty-
562 acid-displaced regulators with DNA is interrupted by tyrosine phosphorylation in the helix-
563 turn-helix domain. *Nucleic Acids Res*, 41, 9371-81.
- 564 DYZENHAUS, S., SULLIVAN, M. J., ALBURQUERQUE, B., BOFF, D., VAN DE GUCHTE, A., CHUNG,
565 M., FULMER, Y., COPIN, R., ILMAN, J. K., O'KEEFE, A., ALTMAN, D. R., STUBBE, F. X.,
566 PODKOWIK, M., DUPPER, A. C., SHOPSIN, B., VAN BAKEL, H. & TORRES, V. J. 2022. MRSA
567 lineage USA300 isolated from bloodstream infections exhibit altered virulence regulation.
568 *Cell Host Microbe*.

- 569 FAGE, C. D., LATHOUWERS, T., VANMEERT, M., GAO, L. J., VRANCKEN, K., LAMMENS, E. M., WEIR,
570 A. N. M., DEGROOTE, R., CUPPENS, H., KOSOL, S., SIMPSON, T. J., CRUMP, M. P., WILLIS, C. L.,
571 HERDEWIJN, P., LESCRIER, E., LAVIGNE, R., ANNE, J. & MASSCHELEIN, J. 2020. The
572 Kalimantacin Polyketide Antibiotics Inhibit Fatty Acid Biosynthesis in *Staphylococcus aureus*
573 by Targeting the Enoyl-Acyl Carrier Protein Binding Site of FabI. *Angew Chem Int Ed Engl*, 59,
574 10549-10556.
- 575 FEY, P. D., ENDRES, J. L., YAJJALA, V. K., WIDHELM, T. J., BOISSY, R. J., BOSE, J. L. & BAYLES, K. W.
576 2013. A genetic resource for rapid and comprehensive phenotype screening of nonessential
577 *Staphylococcus aureus* genes. *MBio*, 4, e00537-12.
- 578 GARCIA-GARCIA, T., PONCET, S., DEROUCHE, A., SHI, L., MIJAKOVIC, I. & NOIROT-GROS, M. F.
579 2016. Role of Protein Phosphorylation in the Regulation of Cell Cycle and DNA-Related
580 Processes in Bacteria. *Front Microbiol*, 7, 184.
- 581 GERSTEL, A., ZAMARRENO BEAS, J., DUVERGER, Y., BOUVERET, E., BARRAS, F. & PY, B. 2020.
582 Oxidative stress antagonizes fluoroquinolone drug sensitivity via the SoxR-SUF Fe-S cluster
583 homeostatic axis. *PLoS Genet*, 16, e1009198.
- 584 GLOUX, K., GUILLEMET, M., SOLER, C., MORVAN, C., HALPERN, D., POURCEL, C., VU THIEN, H.,
585 LAMBERET, G. & GRUSS, A. 2017. Clinical Relevance of Type II Fatty Acid Synthesis Bypass in
586 *Staphylococcus aureus*. *Antimicrob Agents Chemother*, 61, 61:e02515-16.
- 587 HUANG, L., MATSUO, M., CALDERON, C., FAN, S. H., AMMANATH, A. V., FU, X., LI, N., LUQMAN,
588 A., ULLRICH, M., HERRMANN, F., MAIER, M., CHENG, A., ZHANG, F., OESTERHELT, F.,
589 LAMMERHOFER, M. & GOTZ, F. 2022. Molecular Basis of Rhodomyrtone Resistance in
590 *Staphylococcus aureus*. *mBio*, e0383321.
- 591 HUNT, T., KAPLAN, N. & HAFKIN, B. 2016. Safety, tolerability and pharmacokinetics of multiple
592 oral doses of AFN-1252 administered as immediate release (IR) tablets in healthy subjects. *J*
593 *Chemother*, 28, 164-71.
- 594 JI, C. J., KIM, J. H., WON, Y. B., LEE, Y. E., CHOI, T. W., JU, S. Y., YOUN, H., HELMANN, J. D. & LEE,
595 J. W. 2015. *Staphylococcus aureus* PerR Is a Hypersensitive Hydrogen Peroxide Sensor using
596 Iron-mediated Histidine Oxidation. *J Biol Chem*, 290, 20374-86.
- 597 KARLOWSKY, J. A., KAPLAN, N., HAFKIN, B., HOBAN, D. J. & ZHANEL, G. G. 2009. AFN-1252, a FabI
598 inhibitor, demonstrates a *Staphylococcus*-specific spectrum of activity. *Antimicrob Agents*
599 *Chemother*, 53, 3544-8.
- 600 KAZEK, M., KACZMAREK, A., WRONSKA, A. K. & BOGUS, M. I. 2021. Dodecanol, metabolite of
601 entomopathogenic fungus *Conidiobolus coronatus*, affects fatty acid composition and
602 cellular immunity of *Galleria mellonella* and *Calliphora vicina*. *Sci Rep*, 11, 15963.
- 603 KENANIAN, G., MORVAN, C., WECKEL, A., PATHANIA, A., ANBA-MONDOLONI, J., HALPERN, D.,
604 GAILLARD, M., SOLGADI, A., DUPONT, L., HENRY, C., POYART, C., FOUET, A., LAMBERET, G.,
605 GLOUX, K. & GRUSS, A. 2019. Permissive Fatty Acid Incorporation Promotes *Staphylococcal*
606 Adaptation to FASII Antibiotics in Host Environments. *Cell Rep*, 29, 3974-3982 e4.
- 607 KHEMICI, V., PRADOS, J., PETRIGNANI, B., DI NOLFI, B., BERGE, E., MANZANO, C., GIRAUD, C. &
608 LINDER, P. 2020. The DEAD-box RNA helicase CshA is required for fatty acid homeostasis in
609 *Staphylococcus aureus*. *PLoS Genet*, 16, e1008779.
- 610 LAKHUNDI, S. & ZHANG, K. 2018. Methicillin-Resistant *Staphylococcus aureus*: Molecular
611 Characterization, Evolution, and Epidemiology. *Clin Microbiol Rev*, 31.
- 612 LAMBERT, C., BACHMANN, C., GAILLARD, M., HAUTCOEUR, A., GLOUX, K., GUILBERT, T.,
613 MÉHATS, C., PROST, B., SOLGADI, A., ABREU, S., ANDRIEU, M., POYART, C., GRUSS, A. &
614 FOUET, A. 2023. *Streptococcus pyogenes* FASII regulator FabT is required for human tissue
615 infection but is a negative fitness determinant in lipid-rich biotopes. *Submitted*.
- 616 LAMBERT, C., POYART, C., GRUSS, A. & FOUET, A. 2022. FabT, a Bacterial Transcriptional
617 Repressor That Limits Futile Fatty Acid Biosynthesis. *Microbiol Mol Biol Rev*, 86, e0002922.
- 618 LEI, M. G. & LEE, C. Y. 2018. Repression of Capsule Production by XdrA and CodY in
619 *Staphylococcus aureus*. *J Bacteriol*, 200.

- 620 LIU, G. Y., ESSEX, A., BUCHANAN, J. T., DATTA, V., HOFFMAN, H. M., BASTIAN, J. F., FIERER, J. &
621 NIZET, V. 2005. *Staphylococcus aureus* golden pigment impairs neutrophil killing and
622 promotes virulence through its antioxidant activity. *J Exp Med*, 202, 209-15.
- 623 LOPEZ, M. S., TAN, I. S., YAN, D., KANG, J., MCCREARY, M., MODRUSAN, Z., AUSTIN, C. D., XU, M.
624 & BROWN, E. J. 2017. Host-derived fatty acids activate type VII secretion in *Staphylococcus*
625 *aureus*. *Proc Natl Acad Sci U S A*, 114, 11223-11228.
- 626 LOWDEN, M. J., SKORUPSKI, K., PELLEGRINI, M., CHIORAZZO, M. G., TAYLOR, R. K. & KULL, F. J.
627 2010. Structure of *Vibrio cholerae* ToxT reveals a mechanism for fatty acid regulation of
628 virulence genes. *Proc Natl Acad Sci U S A*, 107, 2860-5.
- 629 LOWY, F. D. 1998. *Staphylococcus aureus* infections. *N Engl J Med*, 339, 520-32.
- 630 MAYER-SCHOLL, A., AVERHOFF, P. & ZYCHLINSKY, A. 2004. How do neutrophils and pathogens
631 interact? *Curr Opin Microbiol*, 7, 62-6.
- 632 MCCALLUM, N., HINDS, J., ENDER, M., BERGER-BACHI, B. & STUTZMANN MEIER, P. 2010.
633 Transcriptional profiling of XdrA, a new regulator of spa transcription in *Staphylococcus*
634 *aureus*. *J Bacteriol*, 192, 5151-64.
- 635 MCMURRY, L. M., OETHINGER, M. & LEVY, S. B. 1998. Triclosan targets lipid synthesis. *Nature*,
636 394, 531-2.
- 637 MIJAKOVIC, I., GRANGEASSE, C. & TURGAY, K. 2016. Exploring the diversity of protein
638 modifications: special bacterial phosphorylation systems. *FEMS Microbiol Rev*, 40, 398-417.
- 639 MORVAN, C., HALPERN, D., KENANIAN, G., HAYS, C., ANBA-MONDOLONI, J., BRINSTER, S.,
640 KENNEDY, S., TRIEU-CUOT, P., POYART, C., LAMBERET, G., GLOUX, K. & GRUSS, A. 2016.
641 Environmental fatty acids enable emergence of infectious *Staphylococcus aureus* resistant to
642 FASII-targeted antimicrobials. *Nat Commun*, 7, 12944.
- 643 MORVAN, C., HALPERN, D., KENANIAN, G., PATHANIA, A., ANBA-MONDOLONI, J., LAMBERET, G.,
644 GRUSS, A. & GLOUX, K. 2017. The *Staphylococcus aureus* FASII bypass escape route from FASII
645 inhibitors. *Biochimie*, 141, 40-46.
- 646 MOSEL, M., LI, L., DRILICA, K. & ZHAO, X. 2013. Superoxide-mediated protection of *Escherichia*
647 *coli* from antimicrobials. *Antimicrob Agents Chemother*, 57, 5755-9.
- 648 PAINTER, K. L., STRANGE, E., PARKHILL, J., BAMFORD, K. B., ARMSTRONG-JAMES, D. & EDWARDS,
649 A. M. 2015. *Staphylococcus aureus* adapts to oxidative stress by producing H₂O₂-resistant
650 small-colony variants via the SOS response. *Infect Immun*, 83, 1830-44.
- 651 PARK, J. H., LEE, J. H., CHO, M. H., HERZBERG, M. & LEE, J. 2012. Acceleration of protease effect
652 on *Staphylococcus aureus* biofilm dispersal. *FEMS Microbiol Lett*, 335, 31-8.
- 653 PARKER, E. N., CAIN, B. N., HAJIAN, B., ULRICH, R. J., GEDDES, E. J., BARKHO, S., LEE, H. Y.,
654 WILLIAMS, J. D., RAYNOR, M., CARIDHA, D., ZAINO, A., SHEKHAR, M., MUNOZ, K. A., RZASA,
655 K. M., TEMPLE, E. R., HUNT, D., JIN, X., VUONG, C., PANNONE, K., KELLY, A. M., MULLIGAN,
656 M. P., LEE, K. K., LAU, G. W., HUNG, D. T. & HERGENROTHER, P. J. 2022. An Iterative Approach
657 Guides Discovery of the FabI Inhibitor Fabimycin, a Late-Stage Antibiotic Candidate with In
658 Vivo Efficacy against Drug-Resistant Gram-Negative Infections. *ACS Cent Sci*, 8, 1145-1158.
- 659 PARSONS, J. B., BROUSSARD, T. C., BOSE, J. L., ROSCH, J. W., JACKSON, P., SUBRAMANIAN, C. &
660 ROCK, C. O. 2014. Identification of a two-component fatty acid kinase responsible for host
661 fatty acid incorporation by *Staphylococcus aureus*. *Proc Natl Acad Sci U S A*, 111, 10532-7.
- 662 PARSONS, J. B., FRANK, M. W., SUBRAMANIAN, C., SAENKHAM, P. & ROCK, C. O. 2011. Metabolic
663 basis for the differential susceptibility of Gram-positive pathogens to fatty acid synthesis
664 inhibitors. *Proc Natl Acad Sci U S A*, 108, 15378-83.
- 665 PATHANIA, A., ANBA-MONDOLONI, J., GOMINET, M., HALPERN, D., DAIROU, J., DUPONT, L.,
666 LAMBERET, G., TRIEU-CUOT, P., GLOUX, K. & GRUSS, A. 2021. (p)ppGpp/GTP and Malonyl-
667 CoA Modulate *Staphylococcus aureus* Adaptation to FASII Antibiotics and Provide a Basis for
668 Synergistic Bi-Therapy. *mBio*, 12.
- 669 PEYRUSSON, F., NGUYEN, T. K., NAJDOVSKI, T. & VAN BAMBEKE, F. 2022. Host Cell Oxidative
670 Stress Induces Dormant *Staphylococcus aureus* Persists. *Microbiol Spectr*, 10, e0231321.

- 671 PISHCHANY, G., MEVERS, E., NDOUSSE-FETTER, S., HORVATH, D. J., JR., PALUDO, C. R., SILVA-
672 JUNIOR, E. A., KOREN, S., SKAAR, E. P., CLARDY, J. & KOLTER, R. 2018. Amycomycin is a potent
673 and specific antibiotic discovered with a targeted interaction screen. *Proc Natl Acad Sci U S*
674 *A*, 115, 10124-10129.
- 675 RASIGADE, J. P. & VANDENESCH, F. 2014. *Staphylococcus aureus*: a pathogen with still
676 unresolved issues. *Infect Genet Evol*, 21, 510-4.
- 677 ROBERTS, C. A., AL-TAMEEMI, H. M., MASHRUWALA, A. A., ROSARIO-CRUZ, Z., CHAUHAN, U.,
678 SAUSE, W. E., TORRES, V. J., BELDEN, W. J. & BOYD, J. M. 2017. The Suf Iron-Sulfur Cluster
679 Biosynthetic System Is Essential in *Staphylococcus aureus*, and Decreased Suf Function
680 Results in Global Metabolic Defects and Reduced Survival in Human Neutrophils. *Infect*
681 *Immun*, 85.
- 682 SCHURIG-BRICCIO, L. A., PARRAGA SOLORZANO, P. K., LENCINA, A. M., RADIN, J. N., CHEN, G. Y.,
683 SAUER, J. D., KEHL-FIE, T. E. & GENNIS, R. B. 2020. Role of respiratory NADH oxidation in the
684 regulation of *Staphylococcus aureus* virulence. *EMBO Rep*, 21, e45832.
- 685 SENDI, P. & PROCTOR, R. A. 2009. *Staphylococcus aureus* as an intracellular pathogen: the role
686 of small colony variants. *Trends Microbiol*, 17, 54-8.
- 687 SHEEHAN, G., DIXON, A. & KAVANAGH, K. 2019. Utilization of *Galleria mellonella* larvae to
688 characterize the development of *Staphylococcus aureus* infection. *Microbiology (Reading)*,
689 165, 863-875.
- 690 SOUFI, B., TAUMER, C., SEMANJSKI, M. & MACEK, B. 2018. Phosphopeptide Enrichment from
691 Bacterial Samples Utilizing Titanium Oxide Affinity Chromatography. *Methods Mol Biol*, 1841,
692 231-247.
- 693 STAPLETON, M. R., HORSBURGH, M. J., HAYHURST, E. J., WRIGHT, L., JONSSON, I. M.,
694 TARKOWSKI, A., KOKAI-KUN, J. F., MOND, J. J. & FOSTER, S. J. 2007. Characterization of IsaA
695 and SceD, two putative lytic transglycosylases of *Staphylococcus aureus*. *J Bacteriol*, 189,
696 7316-25.
- 697 THE, M., MACCOSS, M. J., NOBLE, W. S. & KALL, L. 2016. Fast and Accurate Protein False
698 Discovery Rates on Large-Scale Proteomics Data Sets with Percolator 3.0. *J Am Soc Mass*
699 *Spectrom*, 27, 1719-1727.
- 700 TUCHSCHERR, L., LOFFLER, B. & PROCTOR, R. A. 2020. Persistence of *Staphylococcus aureus*:
701 Multiple Metabolic Pathways Impact the Expression of Virulence Factors in Small-Colony
702 Variants (SCVs). *Front Microbiol*, 11, 1028.
- 703 WANG, J., SOISSON, S. M., YOUNG, K., SHOOP, W., KODALI, S., GALGOCI, A., PAINTER, R.,
704 PARTHASARATHY, G., TANG, Y. S., CUMMINGS, R., HA, S., DORSO, K., MOTYL, M.,
705 JAYASURIYA, H., ONDEYKA, J., HERATH, K., ZHANG, C., HERNANDEZ, L., ALLOCCO, J., BASILIO,
706 A., TORMO, J. R., GENILLOUD, O., VICENTE, F., PELAEZ, F., COLWELL, L., LEE, S. H., MICHAEL,
707 B., FELCETTO, T., GILL, C., SILVER, L. L., HERMES, J. D., BARTIZAL, K., BARRETT, J., SCHMATZ,
708 D., BECKER, J. W., CULLY, D. & SINGH, S. B. 2006. Platensimycin is a selective FabF inhibitor
709 with potent antibiotic properties. *Nature*, 441, 358-61.
- 710 WRIGHT, H. T. & REYNOLDS, K. A. 2007. Antibacterial targets in fatty acid biosynthesis. *Curr Opin*
711 *Microbiol*, 10, 447-53.
- 712 WU, Y., VULIC, M., KEREN, I. & LEWIS, K. 2012. Role of oxidative stress in persister tolerance.
713 *Antimicrob Agents Chemother*, 56, 4922-6.
- 714 YAO, J. & ROCK, C. O. 2016. Resistance Mechanisms and the Future of Bacterial Enoyl-Acyl Carrier
715 Protein Reductase (FabI) Antibiotics. *Cold Spring Harb Perspect Med*, 6, a027045.
- 716 YEO, W. S., DYZENHAUS, S., TORRES, V. J., BRINSMAD, S. R. & BAE, T. 2023. Regulation of
717 Bacterial Two-Component Systems by Cardiolipin. *BioRxiv*.
- 718 ZHANG, Y. M. & ROCK, C. O. 2008. Membrane lipid homeostasis in bacteria. *Nat Rev Microbiol*,
719 6, 222-33.
- 720 ZIERDT, C. H. & GOLDE, D. W. 1970. Deoxyribonuclease-positive *Staphylococcus epidermidis*
721 strains. *Appl Microbiol*, 20, 54-7.

722 **Table 1. Pre-treatments with ROS-generating versus reducing agents respectively accelerate**
 723 **and retard adaptation to anti-FASII.**
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Strain ^a	Additive ^b	Time (h) to exit	Δ anti-FASII exit (h) compared to WT (SD) ^c	N
WT	-	8	-	8
WT	H ₂ O ₂	6.3	+ 1.68 (\pm 0.45)	8
WT	PMS	6.5	+ 1.50 (\pm 0.35)	5
<i>katA</i>	-	7	+ 1.00 (\pm 0.00)	3
<i>katA</i>	H ₂ O ₂	5.8	+ 2.17 (\pm 0.58)	3
<i>perR</i>	-	9	- 1.00 (\pm 0.00)	3
<i>perR</i>	H ₂ O ₂	9	- 1.00 (\pm 0.00)	3
<i>sufD</i> *	-	7.3	+ 0.75 (\pm 0.50)	4
<i>sufD</i> *	H ₂ O ₂	6.7	+ 1.25 (\pm 0.29)	4
WT	Na citrate	11.5	- 3.50 (\pm 0.71)	2
WT	Vit C	9.8	- 1.75 (\pm 0.35)	2

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^a Strains USA300 (WT) or mutant derivatives *katA* SAUSA300_1232, *perR* SAUSA300_1842, or *sufD** intergenic between *sufC* (SAUSA300_0818) and *sufD* (SAUSA300_0819), at position 898566 (Fey et al., 2013, Roberts et al., 2017), are used. ^b Oxidizing agents were added only to precultures: H₂O₂, 0.5 mM; phenazine-methosulfate (PMS), 20 (n=2), 30 (n=1), 40 (n=1), 50 (n=1) μ g/ml, gave equivalent results. Reducing agents were added at the same time as anti-FASII, here AFN-1252: sodium citrate, 10 mM; vitamin C (VitC), 5.7 mM. ^c Bacterial outgrowth for USA300 in SerFA-AFN is observed at 8 h, and is used as reference. Differences (Δ) in time of outgrowth are relative to that of the WT. +, early exit from latency (h); -, delayed exit (h). SD, standard deviation; N, number of independent experiments.

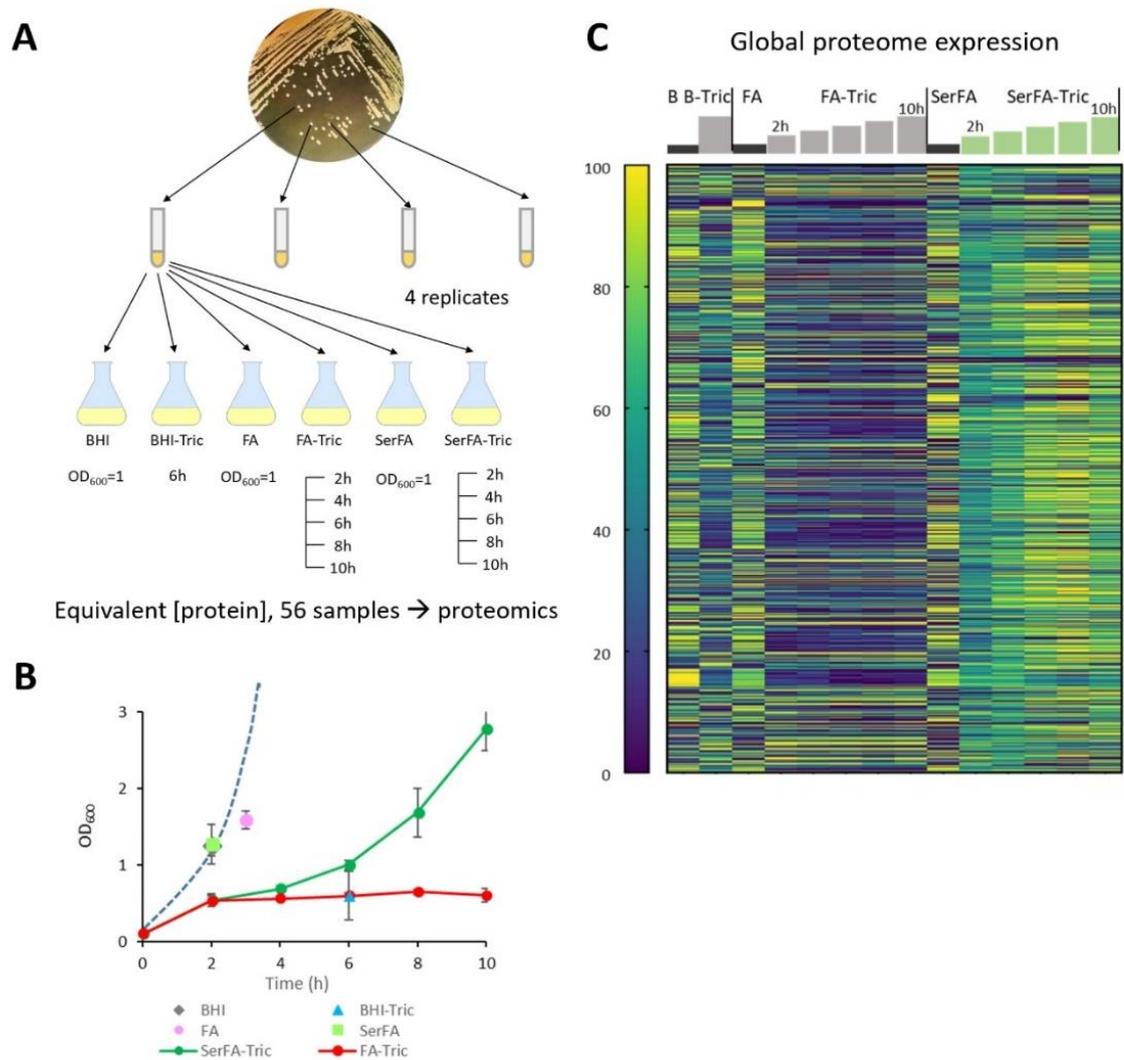


Fig. 1. Proteomic kinetics and global expression changes related to *S. aureus* USA300 strain adaptation to anti-FASII. A. Schematics of sample preparation. Samples (4 biological replicates) were grown and prepared in conditions and harvesting times (or OD_{600} for controls) as indicated below flasks. **B. Sample growth.** Means and standard deviations are indicated. Points correspond to sampling times. See **Table S1** for details. Dashed blue line represents typical growth in SerFA medium determined separately. *S. aureus* adapts to anti-FASII within 10h on SerFA-Tric, but not on FA-Tric, after a latency period (double arrow). **C. Heat map of proteins showing altered levels in at least 1 condition relative to other samples.** All sample conditions are shown. Sampling times (h) above steps correspond to 2, 4, 6, 8, and 10 h for FA-Tric and SerFA-Tric. The heat map shows global protein changes, and is determined relative to weighted value for each protein, as on scale at left (navy, down-represented; yellow, up-represented).

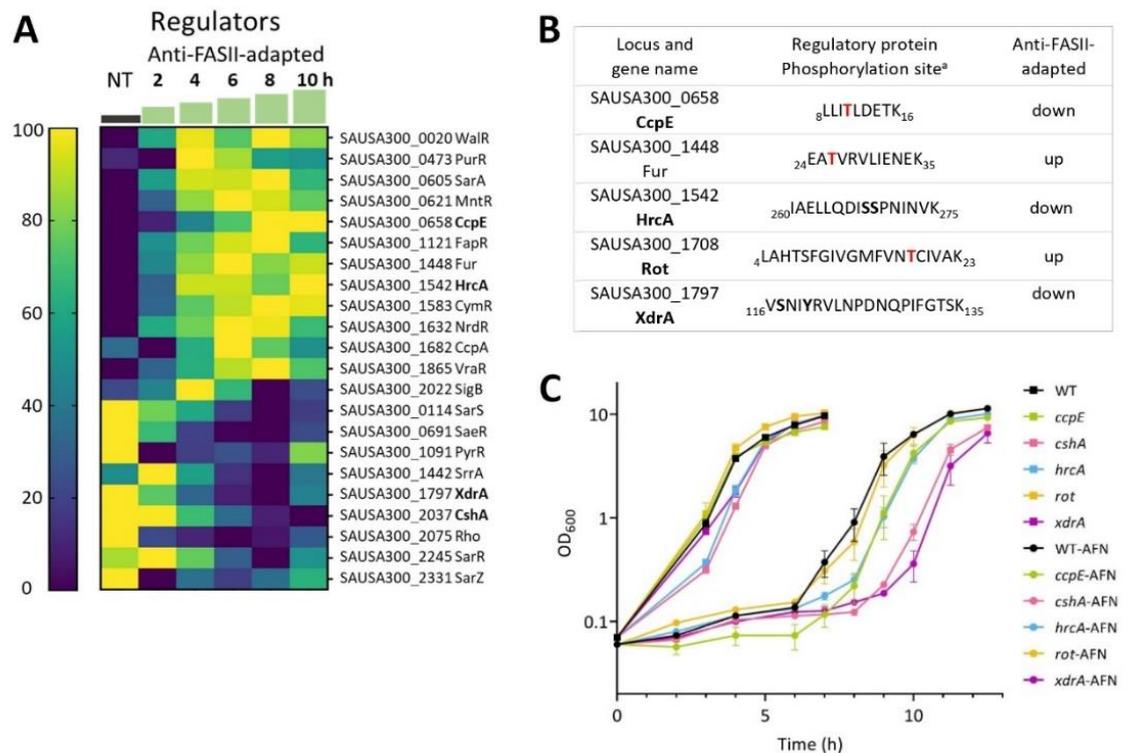


Fig. 2. Anti-FASII affects pools and phosphorylation status of regulatory proteins. A. Expression of known or putative regulators that differ in at least one time point during anti-FASII adaptation (SerFA-Tric) compared to non-treated (NT) SerFA sample. Changes in putative regulatory protein levels are shown in anti-FASII adaptation SerFA medium (see **Table S1** for all test conditions). Gene loci and names are at right. Times (h) of sampling are indicated above green steps. Heat map (scale at left) is determined relative to weighted value for each protein (navy, down-represented; yellow, up-represented). **B. Phosphorylated regulatory proteins with altered expression during anti-FASII adaptation.** Peptides showing differential phosphorylation at 10 h post anti-FASII adaptation are indicated (see **Table S2** for complete information). ^a Peptide positions in respective protein sequences are indicated. Phosphorylated amino acids are in bold red or in bold black when there is an ambiguity. **C. Effects of regulator gene inactivation on anti-FASII adaptation.** Kinetics of anti-FASII-adaptation of USA300 (WT) and insertional mutants, based on regulators with altered protein and/or phosphorylation levels (**A** and/or **B**; selected loci in bold), were compared to that of the parental strain. Cultures were grown without (squares) and with anti-FASII AFN-1252 (circles). Growth curves and standard deviations are based on biological triplicates.

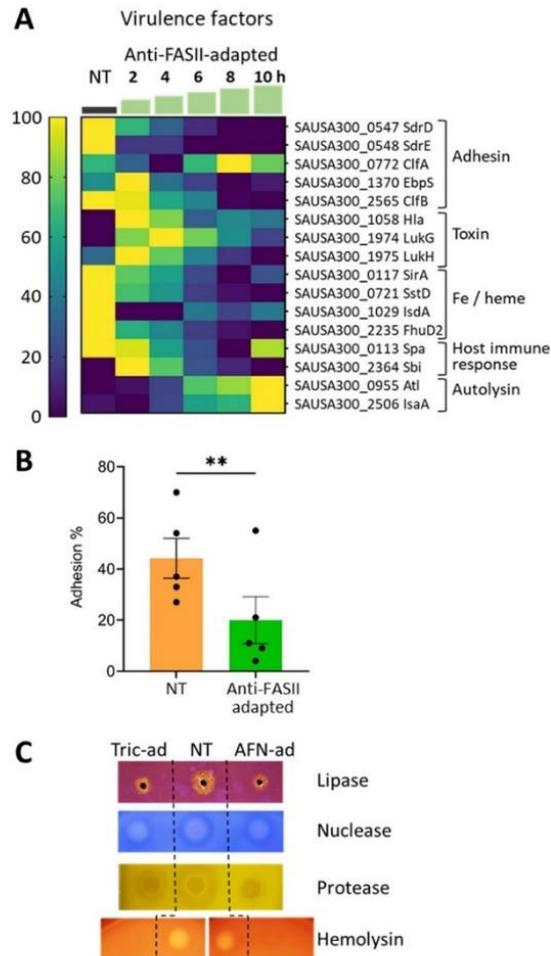


Fig. 3. Expression changes during anti-FASII treatment. A. Heat maps of known or putative virulence factors. Samples shown are in anti-FASII adaptation medium (SerFA; see **Table S1** for results in all test conditions). Kinetics (in h) of sampling is indicated above green steps. Gene loci, protein names, and functional categories are at right. Correspondence between color and expression in heat map (scale at left) is determined relative to weighted value for each protein (navy, down-represented; yellow, up-represented). **B. Differential NT and anti-FASII-adapted *S. aureus* adhesion to macrophage.** Non-treated (NT) or AFN-1252-treated *S. aureus* USA300 (3×10^5) were added to of THP-1 macrophage monolayers (3×10^5 cells per well), and incubated for 1 h at 4° C. Colony forming units (CFU) of bacteria that adhered to macrophage were determined on five independent bacterial samples ($P = 0.003$; see Materials and Methods). **C. Secreted virulence factor activities.** Non-treated (NT) and anti-FASII-adapted cultures treated with triclosan (Tric-ad) or AFN-1252 (AFN-ad) were grown overnight, and reached similar OD_{600} values (= 13, 9 and 9 respectively for NT, Tric-ad, and AFN-ad). Cultures (for protease detection) and culture supernatants (lipase, nuclease, and hemolysin detection) were prepared (see Materials and Methods) and spotted on appropriate detection medium. Representative results of 3 biologically independent replicates are shown.

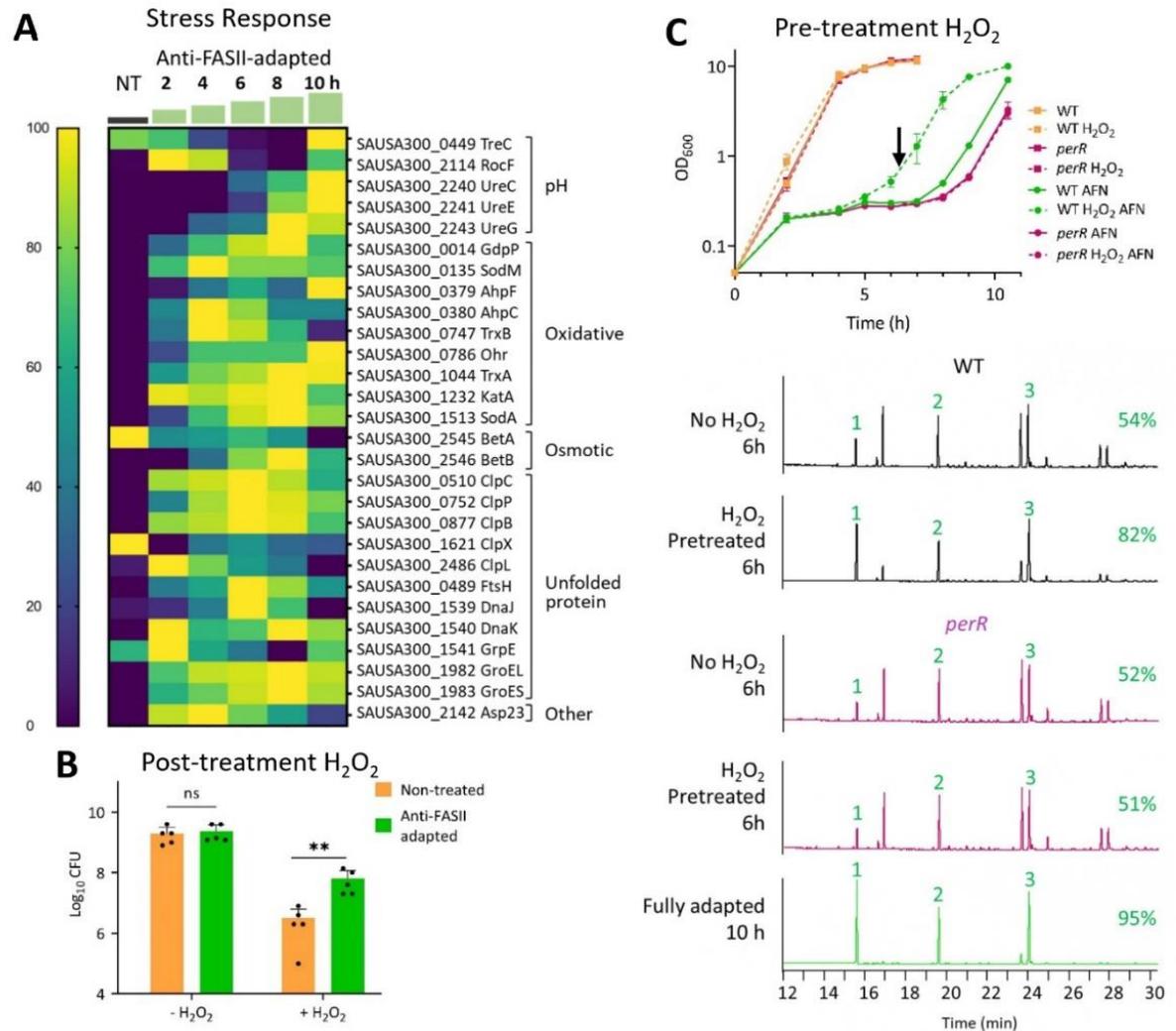


Fig. 4. Anti-FASII adaptation confers oxidative stress resistance, and is accelerated by prior exposure to peroxide stress. **A. Stress response heatmap.** Results are shown for anti-FASII adaptation medium (SerFA; see Table S1 for all test conditions). Sampling times (h) are indicated above green steps. Gene names and functional categories are at right. Heat map (scale at left) is determined relative to weighted value for each protein (navy, down-represented; yellow, up-represented). **B. *S. aureus* anti-FASII adaptation confers increased H₂O₂ resistance.** USA300 non-treated (NT, orange bar) and AFN-1252-adapted overnight cultures (green bar) were challenged with 0.5 mM H₂O₂ for 5 h, and CFUs were determined. **, p < 0.01. **C. Priming *S. aureus* with H₂O₂ accelerates anti-FASII adaptation and requires PerR.** **Upper:** USA300 (WT) and *perR* (SAUSA300_1842) mutant SerFA cultures were grown overnight without or with 0.5 mM H₂O₂. Cultures were diluted (OD₆₀₀ = 0.1) in SerFA without or with 0.5 µg/ml AFN-1252, and growth was monitored. Results are shown for biological triplicates. H₂O₂ – primed samples, without or with AFN addition at T0 are as indicated. **Lower:** FA profiles of indicated strains harvested after 6 h anti-FASII treatment (arrow in ‘C’). In green, FA profile of fully adapted H₂O₂-pretreated cultures at 10 h, shown here for *perR* and non-distinguishable from WT. 1, 2, and 3, eFAs present in SerFA medium (respectively C14, C16, and C18:1). At left of each profile, proportion of incorporated eFAs (%) are the average of two independent measurements (<3% difference between replicates).

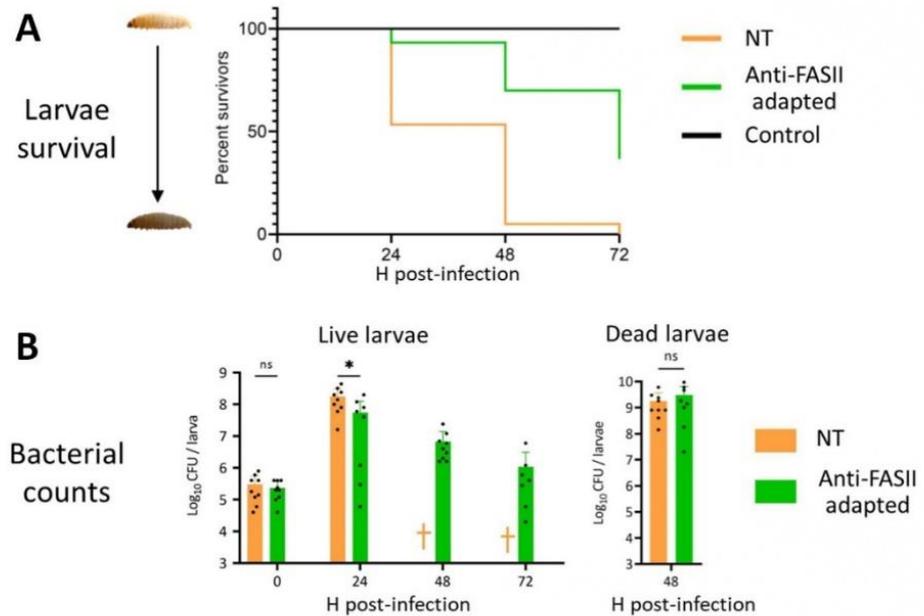


Fig 5. Comparison of untreated and anti-FASII-treated *S. aureus* in a *G. mellonella* infection model. Insects were injected with 10^6 CFU *S. aureus* USA300 that were either non-treated (NT) or preadapted to anti-FASII (anti-FASII adapted) (see Materials and Methods). **A. Insect mortality in NT and anti-FASII-adapted *S. aureus*.** Data was analyzed using Kaplan-Meier with pooled values of biologically independent triplicates (60 insects per condition). Results excluding PBS controls (insects injected with equivalent volumes of PBS, black line) were analyzed by Mantel-Cox, and showed a significance value of $p < 0.0001$. **B. Bacterial CFUs in surviving insects.** Insects were infected as in 'A'. †, no surviving insects. **C. Bacterial CFUs in dead infected insects at 48 h.** 'B' and 'C' analyses were done using the non-parametric Mann Whitney test (Graphpad prism software). *, $p = 0.02$; ns, non-significant.

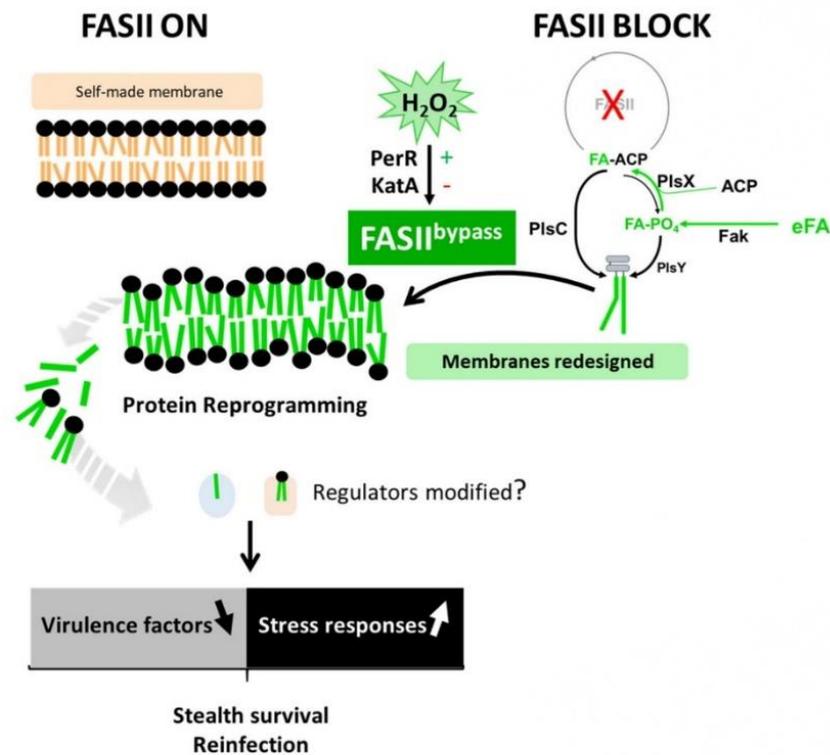


Fig 6. Summary model of anti-FASII adaptation and *S. aureus* fitness. *S. aureus* synthesizes fatty acids (FAs) to produce membrane phospholipids (upper left, FAs in orange). Anti-FASII treatment in SerFA medium promotes FASII bypass, during which exogenous FAs (eFAs, in green) are incorporated and constitute the membrane phospholipid FAs (Kenanian et al., 2019). FASII bypass is accelerated by H₂O₂ priming, which requires PerR, but is lower if KatA is present. Anti-FASII adaptation is accompanied by massive changes in protein expression. Membrane perturbation in the new phospholipid environment is proposed to signal protein reprogramming; as reported, membrane FAs or phospholipids may shed internally and bind to regulatory proteins to modulate their function (Schurig-Briccio et al., 2020, Yeo et al., 2023, Lowden et al., 2010, Huang et al., 2022). Decreased virulence factor production may help bacteria escape host immune surveillance (Tuchscherer et al., 2020). Up-regulation of stress response protein levels confers greater ROS tolerance, and could facilitate survival during infection (DeCoursey, 2004). Peroxide priming accelerates FA incorporation and anti-FASII adaptation by a novel process. Anti-FASII treatment would favor emergence of *S. aureus* populations that are at least transiently less infectious, but that may persist in the host.

CHAPTER 6

Manuscript II:

Fatty Acid Synthesis Arrest Provokes Lipoteichoic Acid Depletion in *Staphylococcus aureus*

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*This chapter will be included as part of a Research Article, which
will be submitted*

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Keywords : anti-FASII adaptation, bi-therapy, fatty acid synthesis (FASII), lipoteichoic acid (LTA), wall teichoic acid (WTA), *S. aureus*, lipidomics

Abstract

Staphylococcus aureus is a Gram-positive pathogenic bacterium causing a high mortality rate, as mainly attributed to multi-antibiotic-resistance emergence. Enzymes of the fatty acid synthesis (FASII) pathway are proposed as essential targets. However, the use of FASII antibiotics is flawed, as *S. aureus* compensates FASII inhibition by incorporating environmental fatty acids (eFAs) and reprogramming its expression. We report that lipoteichoic acid (LTA), a main structural surface factor, is nearly non-detectable in anti-FASII-adapted *S. aureus*. LTA is depleted regardless of the eFAs supplied for growth. Wall teichoic acid (WTA) and cardiolipin (CL) pools are increased in anti-FASII-adapted bacteria, suggesting WTA and/or CL may compensate for LTA depletion and allow *S. aureus* to grow. Inactivation of both LTA and WTA is lethal. Consequently, LTA depletion in anti-FASII-adapted bacteria led to synergistic inhibition between anti-FASII and an anti-WTA, here targocil, against *S. aureus*. We conclude that depletion of LTA by anti-FASII treatment leads to increases in cardiolipin and WTA, and suggest strategies of bi-therapy against a major pathogen.

Introduction

The Gram-positive pathogen *Staphylococcus aureus* is a major cause of human mortality mainly due to resistance to antibiotics, and mainly to methicillin (GBD 2019 Antimicrobial Resistance Collaborators, 2022; Lowy, 1998). The high rate of treatment failure has underlined the need for novel therapies.

The fatty acid (FA) synthesis pathway (FASII) has been considered as essential, and FASII enzymes have been primary drug development targets to treat *S. aureus* infection (Foster, 2017; Y. Wang & Ma, 2013). The recursive FASII pathway comprises four enzymes: FabF (β -ketoacyl-ACP synthase), FabG (3-oxoacyl-acyl-carrier-protein (ACP) reductase), FabZ ((3R)-hydroxymyristoyl-ACP dehydratase), and FabI (enoyl-ACP reductase)), all of which were considered as potential antibiotic targets (Wright & Reynolds, 2007); An inhibitor of FabH (3-oxoacyl-(acyl carrier protein) synthase III), a FASII initiation enzyme was also reported (Pishchany et al., 2018). Among them, FabI is a target of choice, as the enzyme is not present or not unique in the majority of bacteria of the intestinal microbiota, making inhibitors “narrow host range” such as triclosan and AFN-1252 (AFN) (Karlowsky, Kaplan, Hafkin, Hoban, & Zhanel, 2009; McMurry, Oethinger, & Levy, 1998). Other anti-FASII that received wide attention targeted FabF (platencin, platensimycin and fasamycins A and B) (Feng, Chakraborty, Dewell, Reddy, & Brady, 2012; J. Wang et al., 2007, 2006).

Nevertheless, although anti-FASII drugs effectively reached their targets, bacterial growth is not stopped: *S. aureus* bypasses a FASII block by utilizing exogenous FAs (eFAs), which compensate *de novo* synthesis (Brinster et al., 2009; Gloux et al., 2017; Kénanian et al., 2019; Morvan et al., 2016) (**Fig. S1**). This bypass occurs without detectable mutations (Kénanian et al., 2019; Wongdontree et al., 2023). Human biotopes are rich in FAs, which explains anti-FASII treatment failure *in vivo* (Delekta, Shook, Lydic, Mulks, & Hammer, 2018; Kénanian et al., 2019; Teoh, Chen, Laczkovich, & Alonzo, 2021). *In vitro*, *S. aureus* adapts to anti-FASII within about 6-8 h, with marked changes in expression in the presence of FAs (Kénanian et al., 2019; Wongdontree et al., 2023). Mutations in initiation enzymes *acc* and *fabD* further accelerate FASII bypass, and were identified in clinical isolates (Gloux et al., 2017; Morvan et al., 2016).

Our objectives are to elucidate *S. aureus* fitness changes upon adaptation to anti-FASII. Our recent proteomics study revealed that this adaptation improved oxidative stress survival, but lowered virulence factor production (Wongdontree et al., 2023). Here we report

that lipoteichoic acid (LTA), a membrane-anchored surface structure involved in cell division and virulence (Gründling & Schneewind, 2007b, 2007a), is strongly depleted in anti-FASII-adapted *S. aureus*. Compensatory increased expression of cardiolipin (CL) and wall teichoic acid (WTA) point to strategies for combination treatments using anti-FASII. Our characterization of this phenotype reveals a novel link showing that FASII activity dictates LTA production.

Results

1. Anti-FASII induces *S. aureus* cell envelope changes during adaptation. *S. aureus* undergoes an adaptive process when exposed to FASII inhibitors that involve FA incorporation. After a first latent phase (~6 to 8 h), bacteria proliferate exponentially without detectable mutation as verified by whole genome mi-Seq and nanopore sequencing (Kénanian et al., 2019; Wongdontree et al., 2023). Membrane phospholipids comprise solely eFAs (Kénanian et al., 2019). We investigated the changes in bacterial morphology during anti-FASII treatment, by transmission electron microscopy (TEM). Cultures of *S. aureus* USA300 were prepared in SerFA (BHI+10%serum + three eFAs; Materials and Methods) as a control, and the same medium supplemented with anti-FASII; SerFA-AFN (AFN-1252) or SerFA-Tric (triclosan). Samples were analyzed during latency (6 hours) and adaptation outgrowth (10 hours) stages (Materials and Methods). Distinct morphological changes were observed during anti-FASII adaptation (**Fig. 1**). During latency, bacteria were mainly in tetrads and septa were often incomplete as reported (Kénanian et al., 2019). Cell envelopes appeared thicker (black arrow in **Fig. 1B**), suggesting that anti-FASII treatment induces substantial alterations in the cell envelope during latency. Upon outgrowth, bacterial envelopes remained thicker compared to the control group, but overall appeared as diploids, in keeping with resumed cell division. Overall, cell envelope thickening during the latency phase and even after adaptation suggests a significant impact of anti-FASII treatment on bacterial envelope structure, potentially contributing to the adaptive response of *S. aureus*.

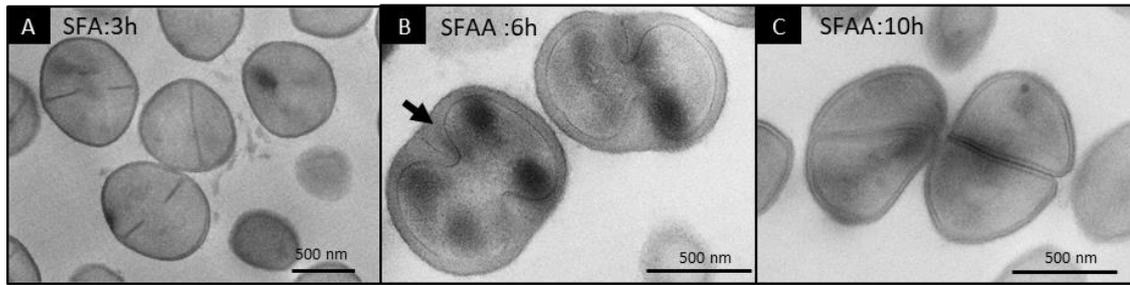


Figure 1. Cell envelope changes of *S. aureus* during anti-FASII treatment. Bacteria are visualized in the following conditions: **(A)** non-treated exponential growth (in SerFA [SFA] at 3h; **(B)** SerFA-AFN (SFAA) during latency period at 6h. Black arrow, region of cell envelope thickening and incomplete septum; **(C)** SerFA-AFN (SFAA) during outgrowth at 10h.

2. **Anti-FASII alters cell envelope synthesis protein levels.** We used a recent inventory of protein and phosphorylation modifications during anti-FASII adaptation of the USA300 JE2 strain (Wongdontree et al., 2023) to focus on proteins related to cell envelope biogenesis whose expression is altered during adaptation in SerFA-Tric (Materials and Methods) (**Fig. 2**). UgtP (YpfP) (diacylglycerol glucosyltransferase; SAUSA300_0918), which mediates a step in glycolipid anchor synthesis, which serves as the base for glycerolphosphate (GroP) polymerization (Gründling & Schneewind, 2007a; Kiriukhin, Debabov, Shinabarger, & Neuhaus, 2001), was turned off in anti-FASII. In contrast, proteins associated with wall teichoic acid (WTA), and peptidoglycan (PDG) were overall increased. These differences show that FASII antibiotic treatment induces cell envelope changes in the adapted population.

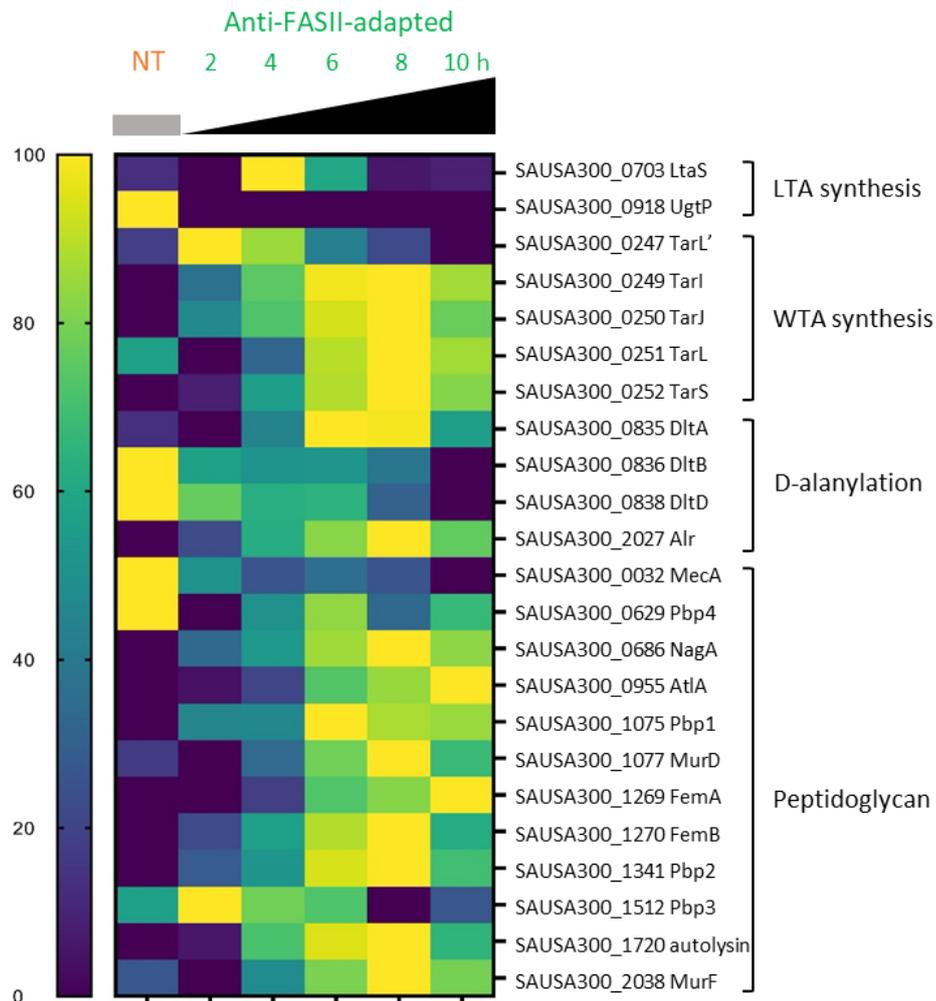


Figure 2. Kinetic heatmap of cell envelope proteins induced in anti-FASII adapted *S. aureus* USA300 JE2 strain. Changes in levels of proteins related to cell wall metabolism during anti-FASII treatment are highlighted. The conditions shown are SerFA for non-treated *S. aureus* (NT) and during the anti-FASII treatment in SerFA-Tric containing triclosan. Sampling times (h) above steps correspond to 2, 4, 6, 8, and 10 h for anti-FASII-treated *S. aureus*. The heat map shows global protein changes, and is determined relative to weighted values for each protein, as on scale at left (navy, down-represented; yellow, up-represented). This heatmap represents results from four biological replicates (peptide e value= 0.01). Data is derived from (Wongdontree et al., 2023).

3. FASII inhibitors provoke LTA depletion. The disappearance of UgtP upon anti-FASII treatment of USA300 prompted us to examine LTA expression. Immunoblotting was performed using LTA-directed antibodies (Clone55; (Reichmann et al., 2014)), on both non-treated (NT) and anti-FASII-adapted *S. aureus* (AD) samples. For this, whole-cell samples of USA300 *S. aureus* were collected from cultures in SerFA \pm FASII inhibitor, here using the anti-FabI; AFN1252 (SerFA-AFN). We also tested the effects of the FabF inhibitor platensimycin (SerFA-Plat) (see Materials and Methods). Remarkably, LTA

was nearly non-detectable or undetectable in *S. aureus* USA300 after adaptation to both FASII inhibitors compared to the control without inhibitors (**Fig. 3**). This phenotype, as tested with AFN-1252, persisted even after prolonged adaptation (Supplementary, **Fig. S2**). Similar results were obtained in RN4220-R and HG1-R, which unlike USA300, are 8325 derivatives (tested with AFN-1252; **Fig. S3**). We conclude that inhibition of FASII causes LTA depletion.

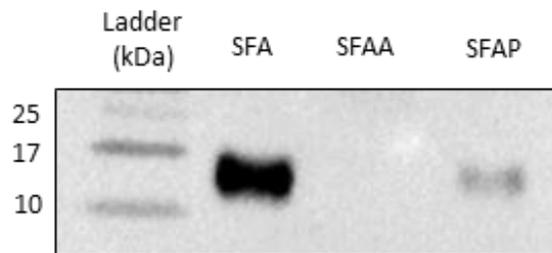


Figure 3. LTA detection in different FASII inhibitors. LTA was detected by immunoblotting with anti-LTA antibody between non-treated *S. aureus* in SerFA (SFA) and anti-FASII-adapted *S. aureus*. *S. aureus* adapted to anti-FabI, AFN-1252 (SerFA-AFN; SFAA) and to anti-FabF, platensimycin (SerFA-Plat; SFAP) compared to control (SerFA; SFA) are shown. This result is representative of three biological replicates.

- 4. LtaS is required for anti-FASII adaptation, but its overproduction does not restore LTA.** The loss of LTA in anti-FASII-adapted bacteria led us to ask whether LTA would be restored by overexpressing the LTA biosynthetic enzyme LtaS. This enzyme polymerizes GroP on the LTA anchor. To test this, an IPTG-inducible *ltaS* (*iltaS*) LAC strain (ANG2505; kindly provided by Dr. A. Gründling, Imperial College, UK) was used. Bacteria were precultured in SerFA with IPTG for *ltaS* induction and then subcultured in SerFA with IPTG, and without or with AFN for overnight growth. The next day, both cultures were subcultured in fresh SerFA-AFN medium in the presence or absence of IPTG, and assessed for growth and LTA production (see Materials and Methods). Poor or no growth in cultures without IPTG in all tested conditions suggested that LtaS is still essential as reported (Gründling & Schneewind, 2007b), even after anti-FASII adaptation. Addition of IPTG restored normal growth for both cultures (**Fig. 4A**). Remarkably, however, LtaS induction did not restore LTA to the antibiotic-adapted strain (**Fig. 4B**). This observation indicates that factors other than LtaS are responsible for LTA loss in AFN-treated *S. aureus*.

To further investigate the LTA-defective phenotype, we compared NT and AD *S. aureus* growth on medium containing Congo red, an inhibitor of LTA synthase (LtaS) (Vickery et al., 2018). AD *S. aureus* strain exhibited ~10-fold increased resistance to Congo red compared to the NT bacteria (**Fig. 5**). This information supports the notion that the LtaS requirement is lower in anti-FASII-adapted *S. aureus*. However, it seems to be in contradiction with the need for LtaS for growth **Fig. 4A**. Thus, while these results suggest that LtaS has an essential function aside from LTA polymerization, the precise roles of LtaS need to be explored.

Congo red is also used to detect exopolysaccharide production, detected as a black color surrounding bacteria (Ferreira, Souza Tette, Santos Mendonça, De Souza Soares, & De Carvalho, 2015; Freeman, Falkiner, & Patrick, 1989). A pronounced black zone surrounded AD, but not NT bacteria, suggesting that anti-FASII-adapted *S. aureus* may produce increased amounts of exopolysaccharide (**Fig. 5**).

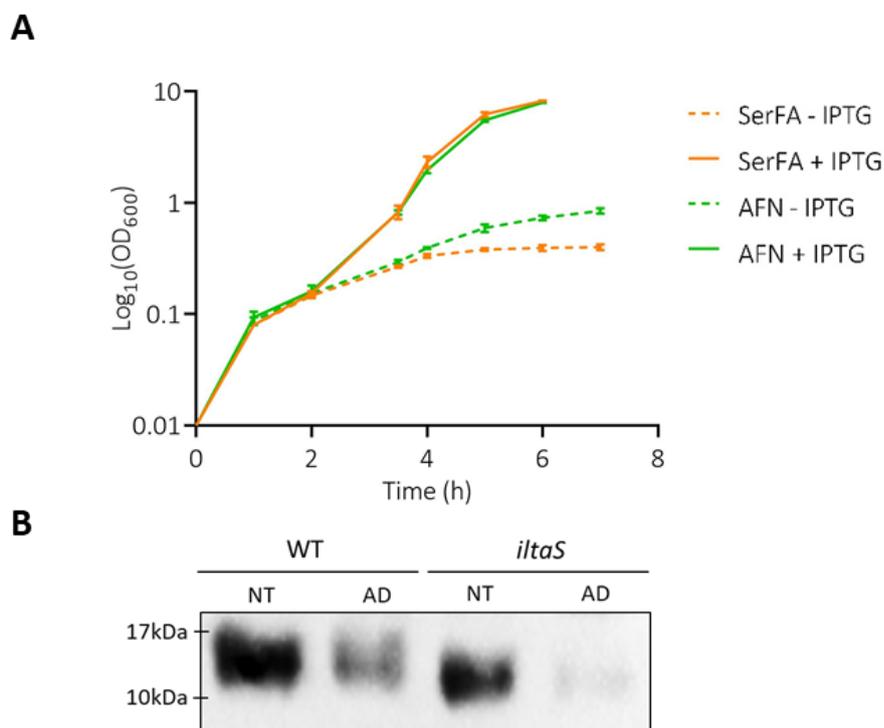


Figure 4. Growth curves and LTA detection between non-treated and anti-FASII-adapted *itaS* *S. aureus*. (A) Growth curves of non-treated (NT) and anti-FASII-adapted (AD) *itaS* (IPTG-inducible expression of *ltaS*) without (dash line) or with IPTG (straight line). Bacteria were grown in SerFA, or the same medium containing anti-FASII (AFN-1252; AFN). Results were generated by Graphpad, and show means \pm SD for three biological replicates. (B) LTA immuno-detection with anti-LTA antibody between NT and AD in WT USA300 and *itaS* with IPTG (*itaS*) compared to parental strain. Results are representative of biological triplicate experiments.

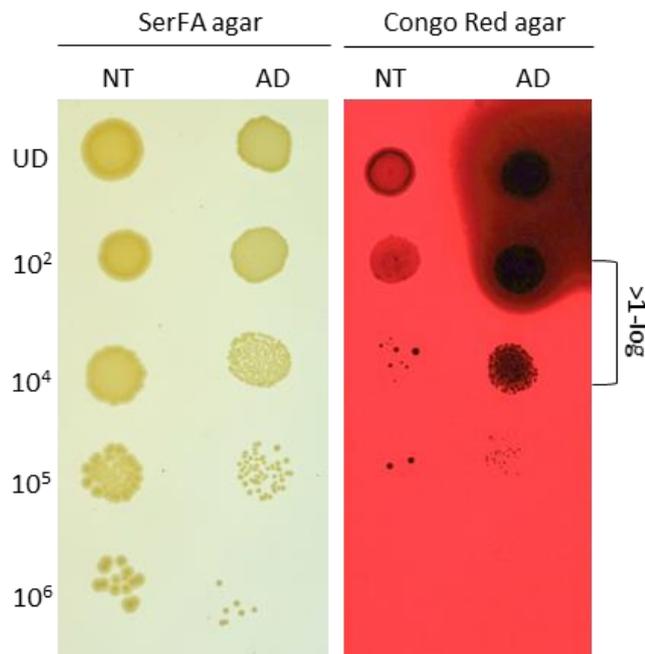


Figure 5. Congo red resistance. Spot test dilution of non-treated (NT) and anti-FASII adapted (AD) cultures are shown. Solid medium contains SerFA, without (left), or with (right) 2.5mg/ml Congo red (an LtaS inhibitor (Vickery et al., 2018)). The black stain on the Congo red plate presumably indicates the production of exopolysaccharide. Results shown are representative of biological triplicate experiments.

5. Variations in membrane phospholipid FAs do not trigger LTA loss. *S. aureus* anti-FASII adaptation can lead to efficient incorporation of numerous different FAs in membrane phospholipids (Kénanian et al., 2019). Our initial studies showing LTA loss were performed using a three-FA mixture (C14:0, C16:0, and C18:1) (e.g., **Fig. 6**, lower). These FAs differ from the predominant FA found in *S. aureus*, *anteiso* 15 (*ai15*), which is also a component of LTA (Fischer, 1994; O'Donnell, Nahaie, Goodfellow, Minnikin, & Hájek, 1985). We, therefore, conducted the same experiment using a proportionately balanced mixture of FAs synthesized by *S. aureus* (C14, *ai15*, C16:0, C18:0, and C20:0; called 'Natural FA mix', see Materials and Methods). LTA pools were significantly decreased in AD *S. aureus* even in these FAs (**Fig. 6**, bottom). Thus, supplementation with *ai15* or other specific FAs are insufficient to compensate LTA loss. These results seem to rule out a major role for membrane FA composition as causing this phenotype.

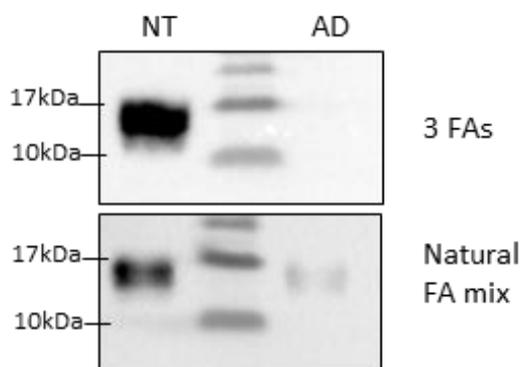


Figure 6. Western blotting of LTA detection between non-treated and anti-FASII adapted *S. aureus* USA300 JE2 strain with different FAs. LTA immuno-detection with anti-LTA antibody. Upper, in SerFA medium containing 3 FAs; Lower the “natural FA mix”, whose FA composition and proportions are similar to those synthesized by *S. aureus* (prepared in delipidated serum). Cells are non-treated (NT) or anti-FASII-adapted (AD). Cultures were grown overnight \pm anti-FASII AFN-1252, and then subcultured in same medium until $OD_{600} \sim 4$. Results are representative of biological triplicate experiments.

6. FASII inactivation is a signal for LTA depletion. Above results indicate that LTA depletion is not related to membrane FA composition. We then asked whether LTA depletion is instead linked to FASII pathway inhibition. Since phospholipids are essential precursors for LTA synthesis (Percy & Gründling, 2014), we considered that FASII inactivation could transmit a signal for LTA synthesis arrest. To test this, we used two means of making *S. aureus* rely on eFAs for growth. As above, FASII inhibition *via* FabI- or FabF-targeted antibiotics led to diminished LTA (**Fig. 3**). We then used a deletion mutant of *fabD* (constructed by V. Leguillier, in preparation), which encodes a malonyl CoA-acyl carrier protein transacylase that mediates FASII initiation (**Fig. S1**). *fabD* inactivation leads to FA auxotrophy (Morvan et al., 2016; Leguillier et al., in preparation). No LTA was detected in the *fabD* mutant when supplemented with different eFAs at $OD_{600} = \sim 3$ (**Fig. 7A**). We conclude that FASII inactivation by various means and in conditions of eFA utilization leads to LTA depletion.

A means of generating an *S. aureus* FA auxotroph while leaving FASII intact is by inactivating *plsX* (encoding a glycerol-3-phosphate acyltransferase). PlsX uses the FASII product, acyl-ACP, to produce acyl- PO_4 , which is then used as substrate for lysophosphatidic acid (PA) synthesis (i.e., FA joined at the 1 position of glycerolphosphate) (Sastre et al., 2020). *plsX* inactivation leads to FA auxotrophy in *S. aureus* (Parsons, Frank, Jackson, Subramanian, & Rock, 2014). Thus, both $\Delta fabD$ and

$\Delta plsX$ mutants require eFA supplementation for growth. We constructed a *plsX* deletion mutant in the USA300 background. NT, $\Delta plsX$, $\Delta fabD$, and AD *S. aureus* USA300 were grown in medium supplemented with *ai15*, and then performed FA and LTA determinations. In the tested condition, FA profiles of $\Delta plsX$, $\Delta fabD$, and AD strains were nearly identical (**Fig. 7A**). In sharp contrast, while LTA pools in $\Delta fabD$ AD were not detectable, the $\Delta plsX$ mutant produced abundant LTA, like the NT WT at $OD_{600} = \sim 3$ (**Fig. 7B**). These observations further exclude a role of FA composition as affecting LTA pools (**Fig. 6**). We conclude that phospholipid composition changes are not responsible for LTA depletion. However, blocking the FASII pathway is a main factor leading to LTA depletion. These findings lead us to propose that metabolites whose levels are altered by FASII bypass might interfere with LTA production.

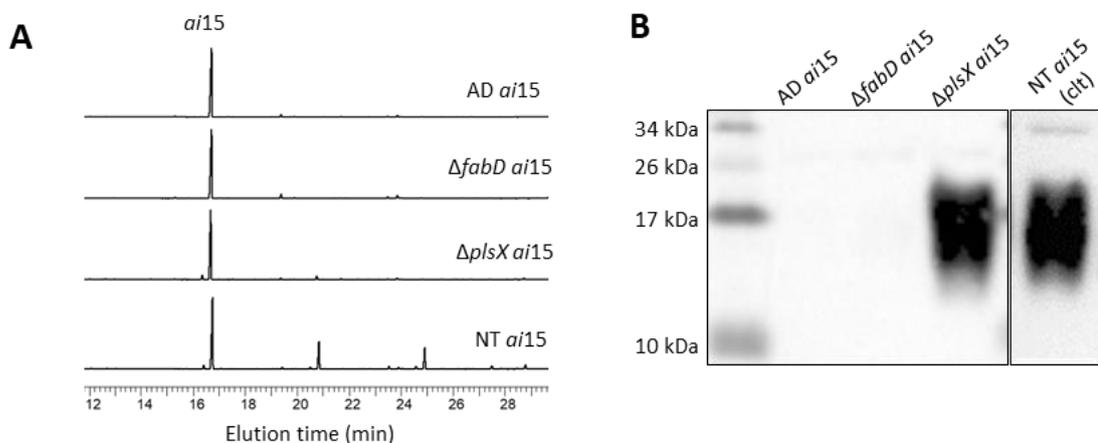


Figure 7. FA profiles do not predict LTA detection. (A) FA profiles of anti-FASII-adapted (AD), $\Delta fabD$, $\Delta plsX$, and non-treated (NT) *S. aureus* grown to $OD_{600} = \sim 3$ in delipidated serum containing *ai15*. Strains are USA300 or derivatives as indicated. (B) LTA immuno-detection with anti-LTA antibody, of extracts from AD, $\Delta fabD$, $\Delta plsX$, and NT *S. aureus* cultures grown as in (A). Note that the NT *ai15* sample at right was analyzed on the same gel, and shown with the same exposure as the other samples.

- 7. LTA depletion increases membrane permeability in stationary phase cultures.** We asked whether LTA loss contributes to changes in bacterial fitness during anti-FASII-adaptation. As LTA is an abundant membrane-anchored molecule (*via* diglucoyl-diacylglycerol; DG-DAG) (Gründling & Schneewind, 2007a), it was possible that LTA depletion could affect membrane fluidity and/ or permeability. These possibilities were assessed using Laurdan dye for membrane fluidity (Scheinpflug, Krylova, & Strahl, 2017), and propidium iodide for permeability (Albaayit, Maharjan, Abdullah, & Noor, 2022). No difference of membrane fluidity was found between NT and AD *S.*

aureus, when tested on both exponential and stationary phase cultures (Supplementary, **Fig. S4**). In contrast, membrane permeability was greater in AD *S. aureus* compared to NT *S. aureus* in stationary phase; this difference was not observed in exponential phase (**Fig. 8A**). To evaluate a possible contribution of LTA in permeability, we compared behavior of the *ltaS* strain ANG2505 in the absence and presence of its inducer. Without IPTG, the *ltaS* strain showed greater membrane permeability than in the presence of IPTG, supporting a role for LTA in membrane integrity (**Fig. 8B**). These results suggest, but do not prove, that LTA may be implicated in the greater membrane permeability of anti-FASII-adapted *S. aureus* in the stationary phase.

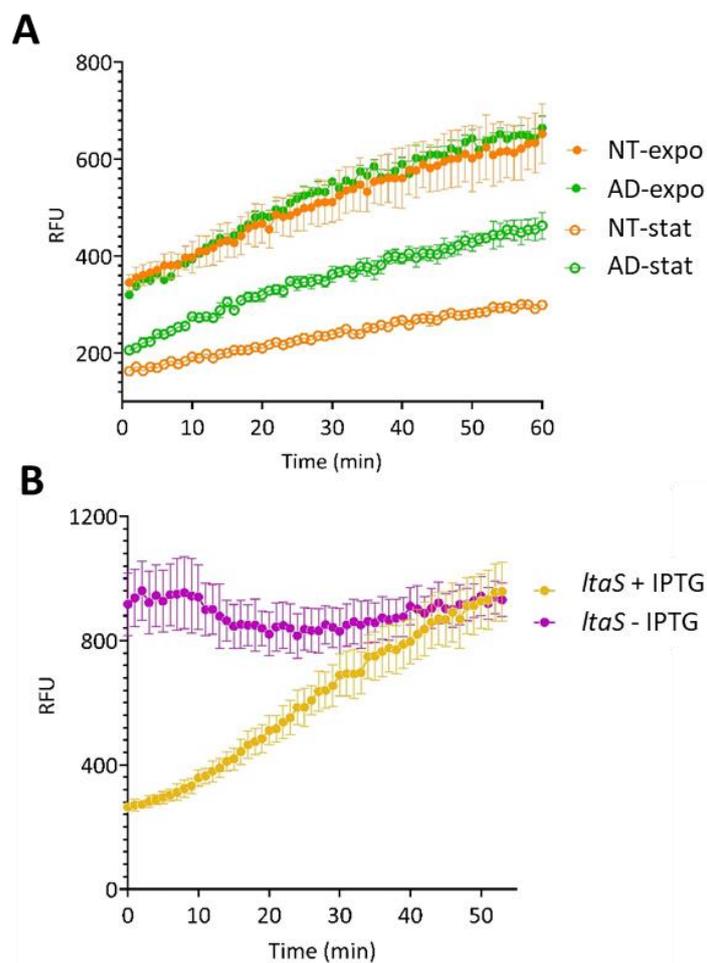


Figure 827. Stationary-phase cell membrane permeability is higher in anti-FASII-adapted cultures than in untreated cultures. (A) Membrane permeability of non-treated (NT) and anti-FASII-adapted (AD) *S. aureus*. **(B)** Membrane permeability of inducible *ltaS* \pm IPTG. RFU = relative fluorescence unit. Figures were prepared using Graphpad.

8. Greater cardiolipin and reduced LTA glycolipid pools in anti-FASII-adapted

***S. aureus*.** As anti-FASII-adapted *S. aureus* are depleted for both UgtP enzyme and LTA, we asked whether the UgtP product DG-DAG, and possibly other lipid pools, would be altered. Lipid analyses were performed on NT and AD *S. aureus*. Three FA conditions, namely 'Natural FA mix', *ai15* (the main *S. aureus* FA), and *ai15*+C18:0 (main *S. aureus* FAs and predominant moieties of LTA), were compared in NT and AD conditions. The main identified *S. aureus* lipids were phosphatidylglycerol (PG), DG-DAG, and cardiolipin (CL). Proportions of PG were similar between NT and AD in all three FA-supplemented conditions (**Fig. 9A**). DG-DAG pools were lower in AD *S. aureus*, in keeping with the absence of detectable UgtP (**Fig. 9B**). In contrast, AD bacteria showed higher proportions of CL (**Fig. 9C**). Both trends were observed across the tested FA conditions, suggesting a possible link between DG-DAG loss and concomitant increases in CL. The most pronounced effects were seen in cultures supplemented with only *ai15*, the shortest FA used in experiments. Previous work showed that CL is increased to 53.9% of total lipids in Newman *S. aureus* L-form, wall-less bacteria, compared to 22.5% in normal bacteria (Hayami, Okabe, Kariyama, Abe, & Kanemasa, 1979). This suggested that CL may contribute to osmotic stability of the L-form. We speculate that similarly, greater CL production in anti-FASII-adapted *S. aureus* may improve membrane integrity to compensate the loss of LTA.

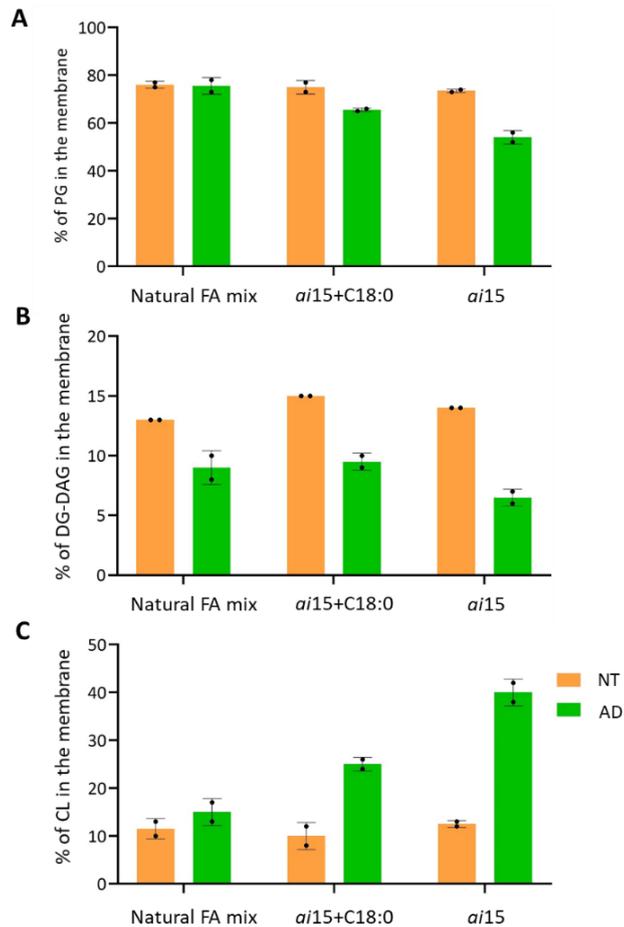


Figure 9. Proportion of lipid species in non-treated and anti-FASII adapted *S. aureus* cultured with different FAs. (A) phosphatidylglycerol (PG), (B) diglucosyl-diacylglycerol (DG-DAG) and (C) cardiolipin (CL). Data represents the mean \pm SD of two biological replicates. NT = non-treated *S. aureus*, AD = anti-FASII adapted *S. aureus*. Generated by Graphpad.

- 9. Cell envelope thickening upon FASII bypass does not correspond to peptidoglycan (PDG) changes.** The increased size and thicker cell envelope of staphylococci during adaptation to anti-FASII led us to question whether other envelope changes might compensate the loss of LTA (**Fig. 1**). Proteomic data (Wongdontree et al., 2023) showed upregulation of proteins involved in PDG synthesis during adaptation (**Fig. 2**). Amounts of PDG in NT, AD, and anti-FASII-treated (prior to outgrowth) *S. aureus* were evaluated by flow cytometry using HADA dye, which specifically labels PDG in live bacteria (Kuru et al., 2013). AD and latency samples appeared to produce higher PDG levels compared to NT (**Fig. 10A**). However, cell sizes varied among samples, likely due to increased cell clumping in anti-FASII conditions (Kénanian et al., 2019) (**Fig. 10B**). With this consideration, the corrected value indicated that the PDG to cell size

ratios were actually lower in AD and latency cells (**Fig. 10C**). These findings indicate that increased cell envelope thickness during and after anti-FASII adaptation (**Fig. 1**) is not primarily due to increased PDG content. Other factors or structural changes were therefore searched to reconcile the observed alterations in cell morphology and greater envelope thickness of the anti-FASII-treated *S. aureus*.

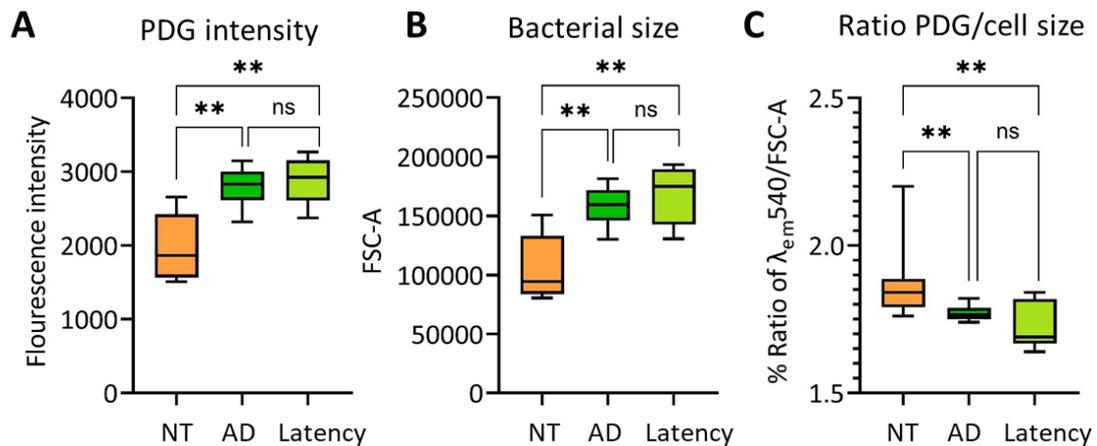


Figure 10. Detection of peptidoglycan (PDG) in *S. aureus* NT, AD, and anti-FASII-treated bacteria at 6 h. PDG was evaluated by staining with HADA (Weihs et al., 2018) and performing flow cytometry. Events were counted for each measurement by flow cytometry. **(A)** Fluorescence intensity reflects HADA incorporation. **(B)** Bacterial size (FSC-A). **(C)** Ratio of PDG intensity over bacterial size. Graphs (generated by Graphpad) represent the means \pm standard mean error and maximum and minimum of each data group. Orange = non-treated *S. aureus* (NT); dark green = anti-FASII-adapted *S. aureus* (AD); light green = anti-FASII treated *S. aureus* 6h post-treatment, in latency period prior to outgrowth (Latency).

10. WTA is increased in anti-FASII-adapted *S. aureus*. The observed LTA depletion in AD *S. aureus* raised the question of whether other structures compensate the absence of this key structure, if not PDG. Specifically, PDG-bound WTA is required when LTA is absent (Hesser et al., 2020; Santa Maria et al., 2014). We asked whether WTA compensates LTA loss in adaptation. WTA was prepared from NT or AD *S. aureus*. The WTA extracts were loaded onto a native polyacrylamide gel electrophoresis (PAGE) gel and then visualized using the alcian blue and silver staining technique. AD *S. aureus* showed greater amounts of WTA compared to NT *S. aureus* (**Fig. 11**). We speculate that increased WTA contributes to *S. aureus* survival during anti-FASII treatment by compensating for the loss of LTA.

Taken together with above results, AD bacteria show three cell envelope shifts: loss of LTA with a concomitant decrease in the lipid anchor, DG-DAG, increased CL, and increased WTA.

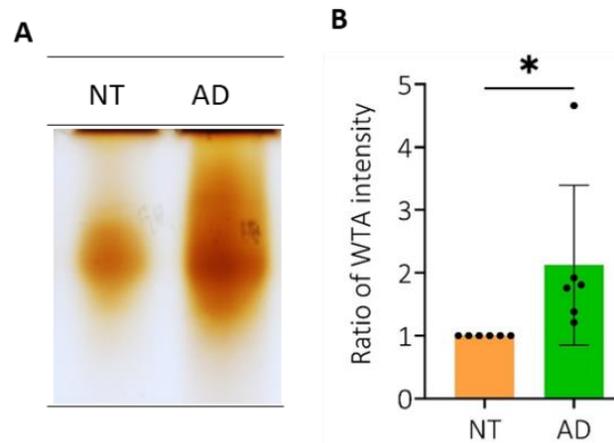


Figure 11. Detection of WTA in non-treated and anti-FASII adapted *S. aureus*. WTA in non-treated (NT) and anti-FASII-adapted (AD) *S. aureus* was compared using the alcian blue and silver nitrate staining technique on native-PAGE gel (n=6). (A) Representative result. (B) Mean and standard deviation of 6 independent samples tested as in (A). Generated by Graphpad.

11. Bi-therapy using combinations of FASII and envelope inhibitors act synergistically to eliminate *S. aureus*. Increased cardiolipin in AD membranes (Fig. 9) is expected to confer a negative charge to membranes. We asked whether a positively charged membrane-active antimicrobial would thus have increased toxicity if combined with an anti-FASII (here AFN-1252). Nisin (net +4 charge) forms pores and binds lipid II to inhibit cell wall synthesis (Breukink & Kruij, 1999; Wiedemann, Benz, & Sahl, 2004). We therefore tested whether nisin synergizes with anti-FASII AFN-1252. At the tested concentrations (0.1, 0.25, and 0.5 $\mu\text{g/ml}$), nisin alone did not prevent USA300 growth. However, a synergistic effect was observed in the presence of AFN-1252 (Fig. 12). This synergy is consistent with a role for cardiolipin in permitting access to nisin, although this remains to be formally demonstrated.

Interestingly, inactivation of both teichoic acid structures is reportedly lethal in *S. aureus* (Santa Maria et al., 2014). Since anti-FASII induces LTA depletion, we tested whether it would be synergistic with an antibiotic targeting WTA synthesis. Our results indicate that co-administration of AFN-1252 as anti-FASII, and targocil as anti-WTA, has a biostatic effect (Fig. 13).

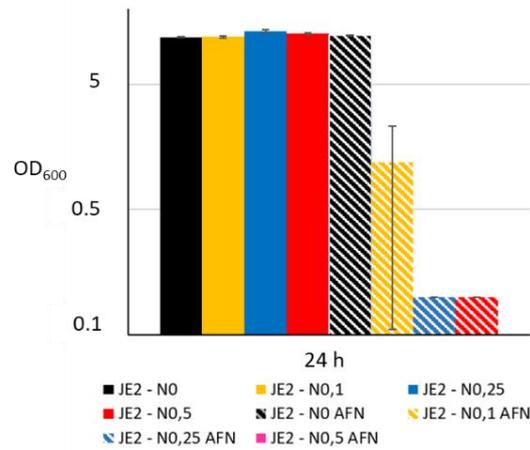


Figure 12. Bi-therapy of anti-FASII and a cationic membrane disrupter. USA300 was grown in SerFA without (solid bars) and with an anti-FASII (0.5 μ g/ml AFN-1252; AFN, hatched bars) for 24h. N0, N0.25, N0.5 indicates in μ g/ml the amount of nisin added in samples. Cultures were incubated 24 h and OD₆₀₀ were determined. Experiments show results of three biological replicates (mean \pm SD). Note that no growth was observed even after 40 h of incubation in bi-therapy treatments including 0.5 μ g/ml nisin and higher.

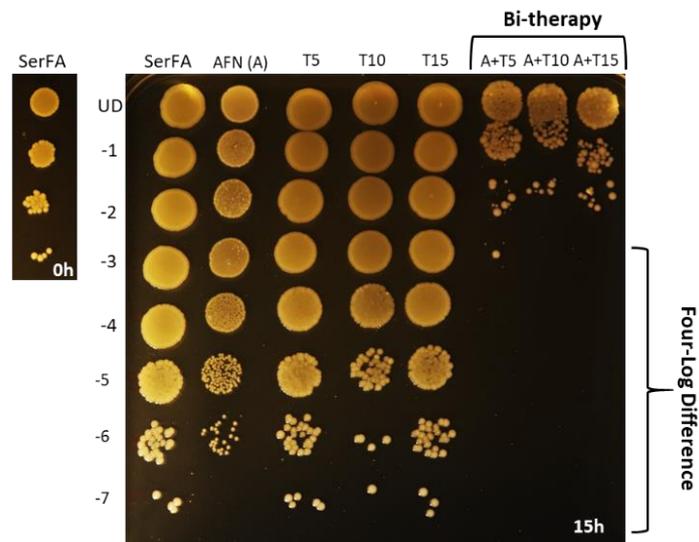


Figure 13. Synergy between an anti-FASII (AFN-1252) and anti-WTA (targocil). Left, a SerFA (SFA) USA300 culture was used as inoculum: CFUs correspond to the culture at 0 h. Right, after inoculation, cultures were grown 15 h in the indicated conditions. Dilutions of cultures grown in the indicated media were then spotted on solid SerFA medium. SerFA media contained: T5= 5 μ g/ml targocil, T10= 10 μ g/ml targocil, T15= 15 μ g/ml targocil. AFN (A)= 0.5 μ g/ml AFN-1252. Bi-therapy treatments used AFN (A) at 0.5 μ g/ml. Plates are representative of three biological replicates.

Discussion

1. Is LTA required for *S. aureus* growth?

LTA was shown to be indispensable for *S. aureus* growth and division (Gründling & Schneewind, 2007b). Nevertheless, exceptions are reported (see **Chapter 4**). Notably, specific conditions including high osmotic medium and low temperature allow *S. aureus* survival without LTA (Oku et al., 2009). These results may suggest that LTA might not be essential as previously proposed. Moreover, growth defects of *ltaS* mutants are suppressed by secondary mutations (e.g. in *gdpP*, *clpX*, and *sgtB*) (Bæk et al., 2016; Corrigan, Abbott, Burhenne, Kaever, & Grundling, 2011; Karinou, Schuster, Pazos, Vollmer, & Gründling, 2019).; a growth defect of *cshA* is suppressed by the loss of LTA (Khemici et al., 2020); and a *pgl* mutation leads to low LTA pools (Zeden et al., 2023).

Previous studies showed that cell wall antibiotic treatment of *S. aureus* provoked loss of LTA. However, bacteria did not survive such treatment (Kariyama, 1982). Here we showed that FASII antibiotics provoke the loss of LTA, but in this case bacteria grow vigorously. This effect is independent of bacterial mutations. Thus, an intimate link is established between FASII bypass and the capacity to withstand LTA loss, which occurs independently of membrane FA composition. These findings showing that bacteria depleted for LTA can continue growth raises questions on the use of LTA as a candidate target for drug development.

2. Why is LTA lost after anti-FASII treatment?

A change in membrane phospholipid composition is ruled out as a cause for LTA loss: two different strategies of phospholipid manipulation led to comparable FA profiles, but widely different LTA pools (**Fig. 7B**). We conclude that FASII activity is a requisite for full LTA presence. To explain this, we hypothesize that a shift in metabolites in anti-FASII-treated *S. aureus* could account for LTA loss. Notably, LTA synthesis is energetically costly. Bacteria spend 75 ATP to resynthesize PG from DAG and glycerol phosphate (GroP) after LTA polymerization, if one considers that one LTA comprises 25 units of GroP (Fischer, 1994). Interestingly, we observed an increase in membrane permeability in anti-FASII adapted cells, which is visible particularly in late growth phase (**Fig. 8A**). It is tempting to speculate that depleted ATP pools due to leakage from anti-FASII-adapted membranes would slow down LTA synthesis compared to synthesis of the other PG products (**Fig. 14**). Thus, a slight shift in

ATP balance could favor synthesis of CL over LTA. Metabolite leakage might occur in numerous situations, and could be driving factors in cell homeostasis in different conditions.

Another possibility is that intermediates of FASII and phospholipid synthesis, or of LTA, in anti-FASII-adapted bacteria could inhibit steps leading to LTA synthesis. An acyl-glycerol, dodecylglycerol (DDG), provoked increased accumulation of Lyso-PA, PA, and CL, and inhibited LTA production in *Streptococcus mutans* (Brissette et al., (1986) . We found that a *fabD* mutant, which bypasses FASII directly, accumulates long-chain acyl-ACP (Morvan et al., 2016); since acyl-ACP and lyso-PA are both substrates of PlsC, one can speculate that both substrates accumulate, and that PlsC activity is slowed. In this case, a consequence of lyso-PA accumulation, or slowed PlsC activity, would alter membrane dynamics and be responsible for reducing LTA synthesis (**Fig. 14**).

A third possibility is based on the observation that expression of UgtP was turned off by anti-FASII treatment (**Fig. 2**), which might disable LTA synthesis. However, LTA depletion in *ugtP* mutants occurs in some but not all *S. aureus* backgrounds (Fedtke et al., 2007); note that USA300 *ugtP* mutant continues to produce LTA with a higher molecular weight (Hesser et al., 2020), making this hypothesis less likely.

The above hypotheses to explain LTA loss in anti-FASII-adaptation conditions have to be explored and constitute one of the perspectives of this study.

3. What compensates LTA depletion?

We found that CL and WTA were increased in anti-FASII-adapted *S. aureus*. Since LTA plays a role in both membrane and periplasmic homeostasis, both these molecules may be needed to compensate LTA depletion.

CL reportedly shares common properties and/or functions with LTA: Like LTA, CL adds negative charged head groups on the membrane. Both structures are implicated in binding autolysin, and might compensate for each other in regulating bacterial lysis (Suginaka, Shimatani, Ohno, & Yano, 1979). The increased production of CL could replace this role of LTA, and also contribute to structuring the membrane by reducing membrane leakage and thereby reducing sensitivity to antimicrobial peptides (Hernández-villa et al., 2018; Rocha-Roa et al., 2021). Increasing CL also decreases lipid headgroup spacing (Hernández-villa et al.,

2018), suggesting greater membrane rigidity. These features could contribute to bacterial fitness in the absence of LTA.

Previous studies suggested that LTA and WTA structures play redundant roles for cell division and cell viability, and may at least partially compensate for each other (Hesser et al., 2020; Oku et al., 2009; Santa Maria et al., 2014). WTA and LTA are both described as “continuum of negative charge” (Neuhaus & Baddiley, 2003). Studies on bacterial turgor pressure suggest that the intertwining LTA and WTA head groups may help maintain envelope integrity with a stiff negatively charged “brush” barrier separating membrane and peptidoglycan; the loss of LTA might be at least partially compensated by WTA (Erickson, 2021). In addition, similarly to LTA, WTA possesses cation binding functions, and is involved in cell elongation and division and also biofilm formation and host tissue adhesion (Swoboda, Campbell, Meredith, & Walker, 2010). Simultaneous inactivation of both LTA and WTA structures is lethal (Santa Maria et al., 2014), which motivated our use of combinatorial treatment between anti-FASII (to eliminate LTA), and anti-WTA (to eliminate WTA). Compensation of LTA with WTA supports the hypothesis that these two structures have some overlapping roles.

4. Is anti-FASII a feasible choice for bi-therapy in view of our findings?

Anti-FASII as a single-treatment antibiotic does not kill bacteria (Kénanian et al., 2019). While the surviving populations show diminished virulence, the presence of persisting adapted bacteria runs the risk of chronic infections, especially as adapted bacteria show tolerance to oxidative stress (Wongdontree et al., 2023). Bi-therapy treatments based on synergistic drugs could be a promising strategy for *S. aureus* elimination (Pathania et al., 2021). Anti-FASII, coupled to a cationic membrane disrupter, or to an anti-WTA, as shown here using respectively nisin or targocil (**Figs. 12 and 13**) may be promising strategies for treating infection.

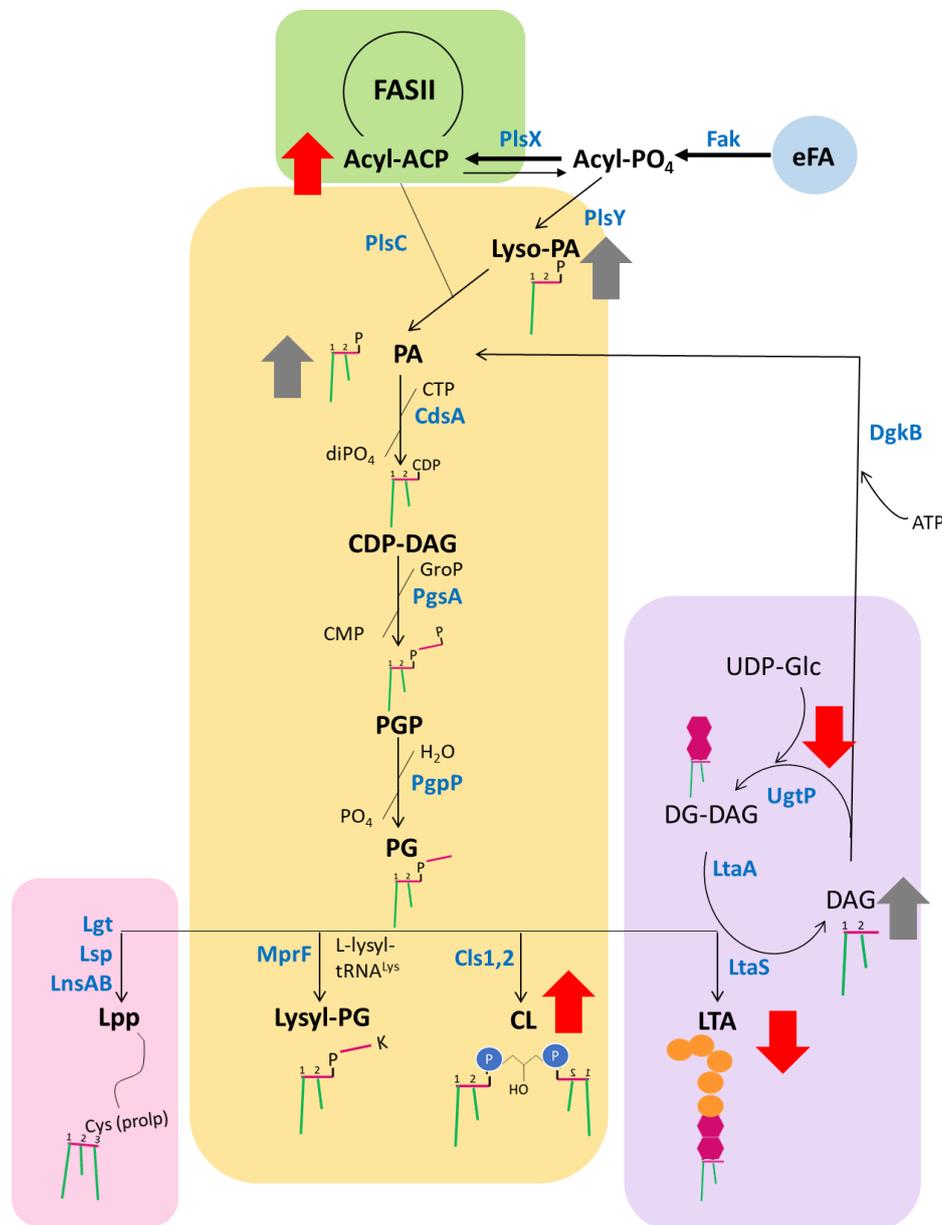


Figure 14. Schematic diagram for phospholipid synthesis and proposed role for lipid intermediates in decreased LTA. The diagram is adapted from **Chapter 3 Fig. 28**. It schematizes the enzymes and reactions of staphylococcal lipid biosynthesis and anchored-membrane structures. Enzymes are in blue. Boxed shaded pathways are involved in producing different lipid types. See **Fig. 18 legend p. 47** for detailed descriptions based on articles specialized in each lipid product (Gardiner et al., 2020; Gründling & Schneewind, 2007b, 2007a; Kuhn, Slavetinsky, & Peschel, 2015; Morvan et al., 2016; Nguyen & Götz, 2016; Parsons, Broussard, et al., 2014; Percy & Gründling, 2014; Peschel et al., 2001; Sastre et al., 2020; Short & White, 1972; Staubitz, Neumann, Schneider, Wiedemann, & Peschel, 2004; Stoll, Dengjel, Nerz, & Götz, 2005)). We hypothesize that intermediates in the FASII or LTA synthesis pathways such as lysylphosphatidylglycerol (Lyso-PG), phosphatidic acid (PA) or diacylglycerol (DAG) may inhibit LTA production. This remains to be proven. Red arrow = result based on the present work; gray arrow = expected. Notably, DAG intermediates might increase due to UgtP decrease and potentially due to lower ATP pools. CDP-DAG = cytidine diphosphate diacylglycerol. PGP = phosphatidyl glycerolphosphate. Lpp = lipoprotein. Lysyl-PG = lysylphosphatidylglycerol. DG-DAG = diglucosyl diacylglycerol. UDP-Glc = uridine diphosphate glucose.

Materials and Methods

Strains, media, and growth conditions. Community-associated methicillin-resistant *S. aureus* (CA-MRSA), specifically the *S. aureus* SAUSA300_FPR3757 JE2 strain, referred to as USA300, was used in this work as a wild-type strain. A mutant strain of *fabD*, malonyl CoA-ACP transacylase inactivation, was constructed in our laboratory and referred to as a FASII bypass strain (Vincent Leguillier and AG, in preparation). The LAC strain with isopropyl-D-thiogalactopyranoside (IPTG)-inducible expression of *ltaS* (ANG2505) was kindly provided by Dr. Angelika Gründling Imperial College London, UK. All cultures were grown aerobically at 37°C and 200 rpm. Liquid or agar growth media were based on BHI (Brain-heart infusion medium) and SerFA, which contained BHI with 10% newborn calf serum (Eurobio Scientific, France) and 0.5 mM FA. The main FAs used in this work were a 0.5 mM final concentration of a mixture of three FAs (0.17 mM each of C14:0, C16:0, C18:1 [Larodan, Sweden]), referred as SerFA, and a natural FA mix contained (C14:0, *ai*15, C16:0, C18:0, and C20:0). Specific FA mixes were used, such as *ai*15 and C18:0, or a single FA alone in some experiments. To test the FASII bypass in *S. aureus*, AFN-1252 (anti-FabI) (Karlowsky et al., 2009) or platensimycin (anti-FabF) (J. Wang et al., 2006) was added at 0.5 µg/ml in SerFA as described and called SerFA+AFN and SerFA+Plat, respectively (Kénanian et al., 2019). For most experiments, *S. aureus* USA300 was streaked on solid BHI medium, and independent colonies were used to inoculate overnight SerFA pre-cultures. The bacteria cultured in SerFA were referred to non-treated (NT) bacteria, while the bacteria that exited from SerFA containing anti-FASII (AFN-1252 or platensimycin) were referred to as anti-FASII adapted bacteria (AD).

Electron microscopy. Bacteria were fixed with 2% glutaraldehyde in 0.1 M Na cacodylate buffer pH 7.2, for 3 hours at room temperature. Samples were then contrasted with Oolong Tea Extract (OTE) 0.2% in cacodylate buffer, postfixed with 1% osmium tetroxide containing 1.5% potassium cyanoferrate, gradually dehydrated in ethanol (30% to 100%) and substituted gradually in mix of ethanol-epon and embedded in Epon. (Delta microscopie –France). Thin sections (70 nm) were collected onto 200 mesh copper grids, and counterstained with lead citrate. Grids were examined with Hitachi HT7700 electron microscope operated at 80kV (Elexience – France), and images were acquired with a charge-coupled device camera (AMT).

LTA detection by Western blotting. LTA was evaluated in *S. aureus* USA300, and *iltaS* was evaluated in the absence and presence of anti-FASII agents, AFN-1252 or platensimycin, in SerFA (control) or SerFA+AFN. Cultures were inoculated in SerFA and then subcultured overnight in SerFA with or without anti-FASII. The following day, each culture was subsequently changed to fresh medium with or without anti-FASII until it reached an OD₆₀₀ of approximately 1 or in the early of exponential phase (OD, optical density). The cultures were collected at 10 units of OD₆₀₀ for each condition to check the LTA levels. Bacteria were spun at 8000 rpm for 5 minutes and then washed with buffer (phosphate-buffered saline (PBS) with 0.02% Triton) once, using the same speed and time of spinning, to eliminate the SFA, and then washed twice with PBS. Pellets were stored at -80 °C until use. FastPrep (program 2; 6.5 m/s, 50s x 3) was applied to all samples before performing the Bradford protein assay to determine the protein amount. 10 µg of protein were used for loading in 15% polyacrylamide-SDS gels. The proteins on the gels were then transferred to a PVDF membrane (BIO-RAD) using a power blot cassette (Thermo Scientific) and the "medium" setting molecular weight program for 7 minutes. PVDF-Membranes were subsequently incubated with specific primary antibodies against LTA (clone 55; HyCult Biotechnology) and horseradish peroxidase-linked anti-mouse secondary antibody with dilutions of 1:2,500 and 1:5,000, respectively. The anti-LTA antibodies reportedly detect polyglycerolphosphate of LTA (Gründling & Schneewind, 2007a). Finally, immunoreactive LTA species were detected by enhanced chemiluminescence and analyzed using Image Lab 5.0 program. Experiments were independently performed more than 10 times for LTA detection in wild-type USA300 in NT and AD *S. aureus*, and 3 times independently in the mutant strains.

FA extraction. One unit of optical density at 600nm (OD₆₀₀) of bacterial culture was collected in SerFA with or without anti-FASII. The cultures were then centrifuged at 8,000 rpm for 5 minutes and washed once in 0.9% NaCl containing 0.02% Triton X-100, followed by two additional washes in 0.9% NaCl at the same speed and time. The cell pellets were then subjected to extraction of the membrane lipid as previously described, using sodium methoxide in methanol and heptane with methyl-10-undecenoate (Kénanian et al., 2019). Gas chromatography was 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 program (Perkin-Elmer). Fatty acid peaks of *S. aureus* were determined between

12 and 32 minutes of elution and identified based on their retention times compared to purified esterified fatty acid standards.

Membrane fluidity. *S. aureus* USA300 were inoculated in SerFA during the day at 37°C, and then subcultured overnight in the fresh media SerFA ± AFN-1252 to obtain non-treated and anti-FASII adapted *S. aureus* (after regrowth). The next day, both cultures were subcultured again in fresh medium and grown until OD₆₀₀ = 1. 0.5 ml of bacterial culture was collected as the exponential phase culture, centrifuged, and resuspended in PBS. Overnight cultures were also collected. Bacterial membrane fluidity was assessed using Laurdan, a fluorescent probe that intercalates into the membrane bilayer. Dye was added at a final concentration of 10 µM and incubated at 30°C for 10 min (see (Wenzel, 2018) for protocol). Samples were then washed 4 times in pre-warmed Laurdan buffer (30°C). Next, 200 µl of OD₆₀₀ = 0.4 culture was transferred to a pre-warmed black 96-well plate (30°C) and subsequently the fluorescence was measured using a Tecan Spark plate reader (Tecan, France). Then, the generalized polarization (GP) was calculated according to the formula: $GP = (I_{460} - I_{500}) / (I_{460} + I_{500})$ to compare the membrane fluidity. Three independent replicates were performed.

Membrane permeability. *S. aureus* USA300 was precultured in SerFA and then transferred to the fresh SerFA with or without AFN-1252 overnight. The next day, bacterial samples of non-treated and anti-FASII adapted bacteria were collected to measure membrane permeability during stationary phase. Simultaneously, both overnight culture with or without AFN-1252 were subcultured again to obtain samples from exponential phase (OD₆₀₀ = 1). 0.5 unit of OD₆₀₀ were collected for each sample and then washed twice with PBS buffer. Then, 100 µl of each washed sample was transferred to a black 96-well plate, and 1-10 µl of ethidium bromide (1 µg/ml) was added. Membrane permeability was monitored by Tecan at 37°C for 1 hour. In addition, ANG2505 (IPTG-inducible *ltaS* expression) was also used to access membrane permeability. The *ltaS* was cultured with or without IPTG for 2h, and the membrane permeability was monitored (N=3 for each condition).

Lipid extraction and profile. The bacteria were grown until OD₆₀₀ = ~3, and 100 units OD₆₀₀ was collected for lipid extraction. The bacterial cells were washed as described. The samples were weighed (wet weight) using pre-weighed empty tubes to calculate the concentration later. Subsequently, the bacterial pellets were dried by lyophilization overnight and weighed again (dry weight). The samples were stored at -80°C before lipid extraction. For lipid

extraction, all processes were performed in glass tubes to avoid contamination from plastic. The freeze-dried pellet was extracted with 4.75 ml of extraction buffer (chloroform, methanol, and 0.3% NaCl (0.9% NaCl stock solution) in a ratio of 1:2:0.8) without shaking at 80°C for 15 minutes, followed by vortexing for 1 hour (repeat this step, but vortex only for 30 minutes for second time). Then, 2.5 ml each of chloroform and 0.3% NaCl were added, and phase separation was achieved by centrifugation at 4000 rpm for 15 minutes. The lower phase was collected, and then was evaporated in the next step under nitrogen gas. The samples were stored at -20°C. The treated samples were then injected into a mass spectrometer for analysis. The data were analyzed using Thermo Xcalibur Qual Browser, and each lipid type was identified based on their respective standard peaks. The data were analyzed by using three main lipids found in this work; PG, DGDG and CL. The proportion of each lipid was shown by mean \pm SD of two replicates using GraphPad Prism 9.5.1.

Measurement of peptidoglycan. The bacteria were precultured in erSFA and then subcultured overnight in the medium with/ without AFN-1252 to obtain non-treated and anti-FASII adapted *S. aureus*. On the subsequent day, all the cultures were subcultured into fresh medium with/ without AFN-1252, supplemented with 25 μ M HADA dye (7-hydroxycoumarin-3-carboxylic acid-amino-D-alanine), which stains the peptidoglycan in live bacteria (Weihs et al., 2018). Non-treated and anti-FASII adapted *S. aureus* were grown with HADA for 2h, whereas the latency phase grown for 4-6h. The latency phase was subcultured from SerFA overnight and then added AFN-1252 during the day. One unit of cultures ($OD_{600}=1$) were spun at 6000 rpm for 5 min and washed once with PBS before measuring the amount of peptidoglycan using flow cytometry.

WTA detection by Alcian blue and silver staining. Non-treated and anti-FASII adapted *S. aureus* USA300 were grown until OD_{600} reached approximately 2-3. Sixty or 120 units of bacterial cultures were collected for WTA extraction and processed as previous described (Covas, Vaz, Henriques, Pinho, & Filipe, 2016) by loading the samples onto a 15% native polyacrylamide gel electrophoresis (PAGE) gel and visualizing using Alcian blue and silver nitrate staining.

Combinatorial approaches between anti-FASII and identified candidates. Anti-FASII (0.5 μ g/ml AFN-1252) was combined with two potential candidates: there are a positively charged agent, nisin (0.1, 0.25 and 0.5 μ g/ml), and a WTA inhibitor (5, 10, and 15 μ g/ml

targocil). For anti-WTA, the combination was tested in USA300 at starting $OD_{600} = 0.05$ or 0.1 and bacterial growth was monitored. The bi-therapy using AFN-1252 and targocil was incubated for 15h in liquid SerFA, and 10-fold dilutions were spotted ($5\mu\text{l}$ / spot) on SerFA plates. Plates were incubated at 37°C overnight before determining the colony forming units (CFUs). Treatment of *S. aureus* by AFN-1252 and nisin was done as follows: USA300 was precultured in SerFA, and then diluted to a starting $OD_{600} = 0.005$ in SerFA containing or not different antibiotics as described in **Fig. 12**. Cultures were incubated for 24 h and OD_{600} values were recorded.

Resistance to Congo red and targocil. Non-treated and anti-FASII adapted *S. aureus* USA300 were grown until $OD_{600} \sim 2$ and then adjusted to $OD_{600} = 1$ to initiate the spot test dilution (undiluted=UD). For each dilution, $5\mu\text{l}$ of bacteria were spotted onto SFA, SFA+2.5 mg/ml Congo red and/or SFA+10 μg /ml targocil plates. These plates were then left overnight at 37°C before being photographing.

Supplementary

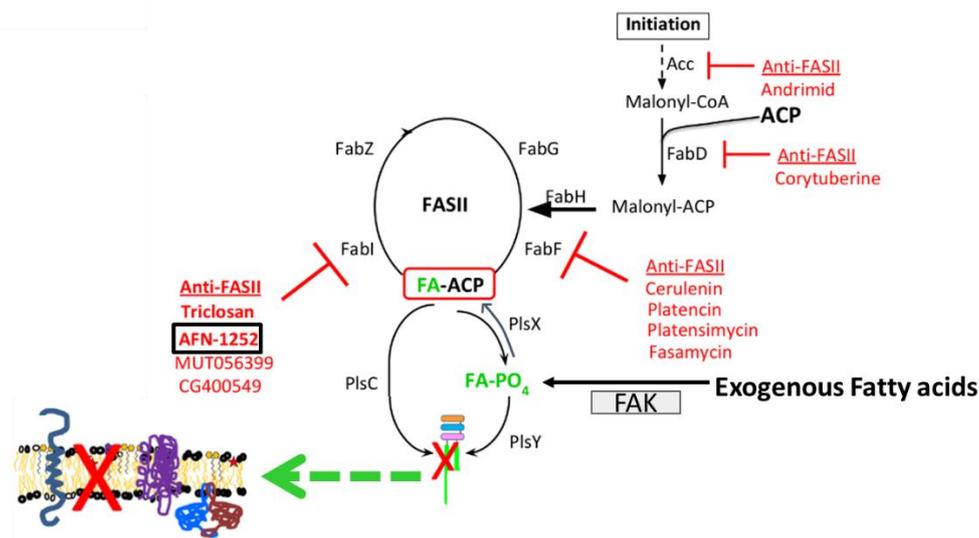


Figure S1. Inhibitors of FASII. The FASII pathway enzymes and known inhibitors are indicated. FASII inhibitors may target initiation enzymes ACC and FabD, or elongation enzymes, particularly FabF or FabI. (See, (Morvan et al., 2017), for review). Adapted from (Kénanian et al., 2019). Note that the fatty acid kinase complex (Fak) is required for phosphorylation of eFAs (Parsons, Broussard, et al., 2014), which may then be extended by FASII, and/or be used in FASII bypass to produce phospholipids.

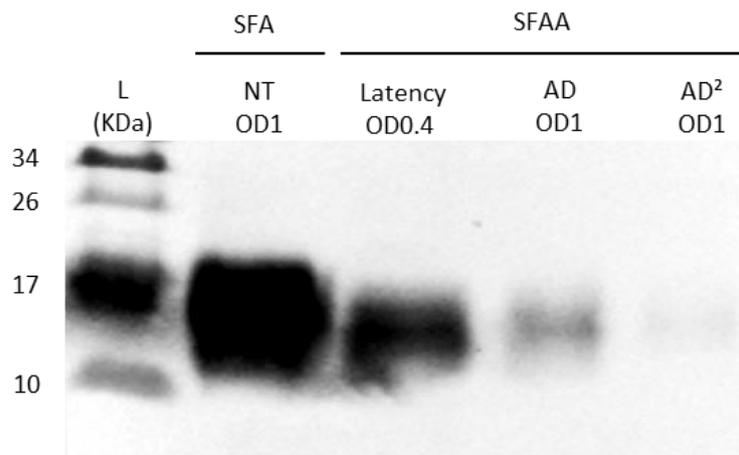


Figure S2. LTA depletion after prolonged anti-FASII adaptation. LTA production in USA300 JE2 was detected by immunoblotting with anti-LTA antibodies. Samples were taken in SerFA (SFA) medium, and in SerFA-AFN-1252 (SerFAA) medium during latency period around ($OD_{600} = 0.4$), upon outgrowth at $OD_{600} = 1$ (AD) and after subculturing (1:100 dilution) in the same medium until $OD_{600} = 1$ (AD²). Results are representative of 3 independent experiments.

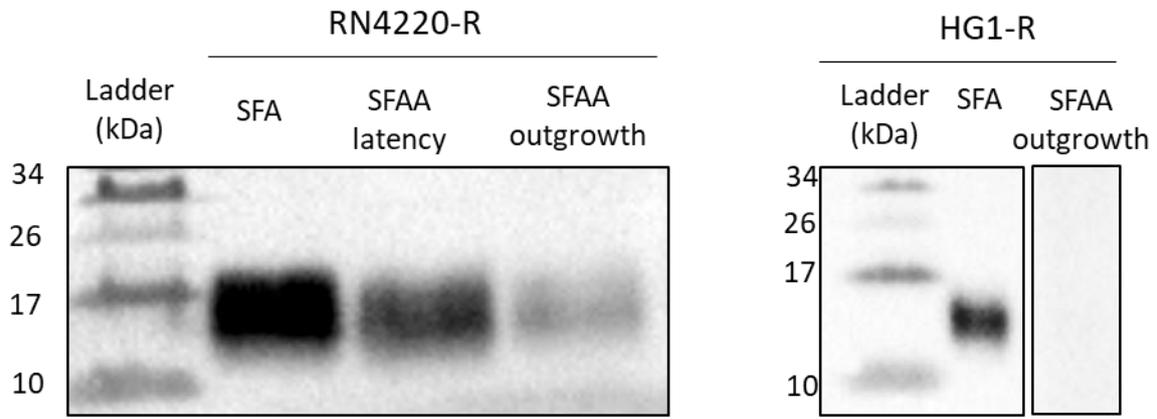


Figure S3. LTA detection in different strain backgrounds. LTA production was detected by immunoblotting with anti-LTA antibodies, comparing non-treated (NT) in SerFA (SFA) and anti-FASII-adapted (AD) in SerFA-AFN-1252 (SFAA). (Right) LTA was detected in the RN4220-R strain and in HG1-R strain. Both these strains derive from 8325 lineage and were repaired to obtain an intact *fakB1* gene (Pathania et al., 2021). Presented immunoblot is representative of three biological replicates. HG1-R LTA samples were run and detected from the same gel.

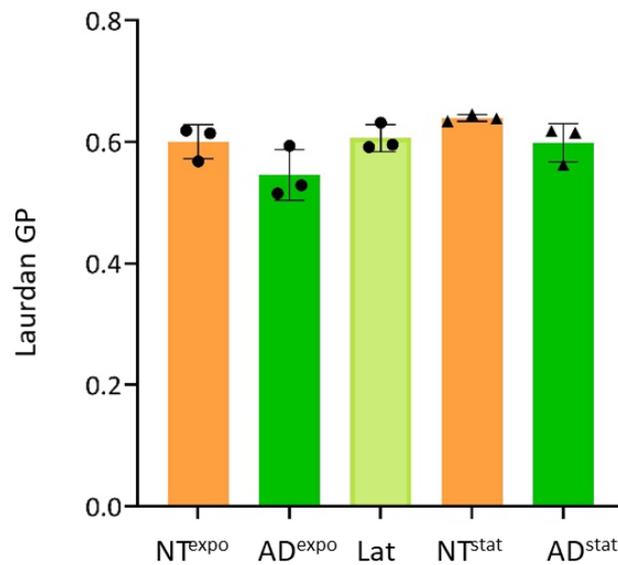


Figure S4. Membrane fluidity. Membrane fluidity was measured using Laurdan dye (see Materials and Methods). No differences were observed between non-treated (NT) and anti-FASII-adapted (AD) *S. aureus* USA300 strain, as tested in exponential (expo) and stationary phase (stat) cultures, and also latency period during anti-FASII treatment. The graph summarizes results of three biological replicates.

References

- Albaayit, S. F. A., Maharjan, R., Abdullah, R., & Noor, M. H. M. (2022). Evaluation of Anti-Methicillin-Resistant *Staphylococcus aureus* Property of Zerumbone. *Journal of Applied Biomedicine*, *20*, 15–21.
- Bæk, K. T., Bowman, L., Millership, C., Søgaaard, D., Kaeffer, V., Siljamäki, P., ... Frees, D. (2016). The Cell Wall Polymer Lipoteichoic Acid Becomes Nonessential in *Staphylococcus aureus* Cells Lacking the ClpX chaperone. *MBio*, *7*, 1–11.
- Breukink, E., & Kruij, B. De. (1999). The Lantibiotic Nisin, a Special Case or Not? *Biochimica et Biophysica Acta* *1462*, *1462*, 223–234.
- Brinster, S., Lamberet, G., Staels, B., Trieu-Cuot, P., Gruss, A., & Poyart, C. (2009). Type II Fatty Acid Synthesis is not a Suitable Antibiotic Target For Gram-Positive Pathogens. *Nature*, *458*, 83–86.
- Brissette, J. L., Cabacungan, E. A., & Pieringer, R. A. (1986). Studies on the Antibacterial Activity of Dodecylglycerol. Its limited Metabolism and Inhibition of Glycerolipid and Lipoteichoic Acid Biosynthesis in *Streptococcus mutans* BHT. *Journal of Biological Chemistry*, *261*, 6338–6345.
- Corrigan, R. M., Abbott, J. C., Burhenne, H., Kaeffer, V., & Grundling, A. (2011). C-Di-AMP is a New Second Messenger in *Staphylococcus aureus* With a Role in Controlling Cell Size and Envelope Stress. *PLoS Pathogens*, *7*, 1–16.
- Covas, G., Vaz, F., Henriques, G., Pinho, M. G., & Filipe, S. R. (2016). Analysis of Cell Wall Teichoic Acids in *Staphylococcus aureus*. *Methods in Molecular Biology*, *1440*, 201–213.
- Delekta, P. C., Shook, J. C., Lydic, T. A., Mulks, M. H., & Hammer, N. D. (2018). *Staphylococcus aureus* Utilizes Host-Derived Lipoprotein Particles as Sources of Fatty Acids. *Journal of Bacteriology*, *200*, 1–22.
- Erickson, H. P. (2021). How Teichoic Acids Could Support a Periplasm in Gram-Positive Bacteria , and Let Cell Division Cheat Turgor Pressure. *Frontiers in Microbiology*, *12*, 1–11.
- Fedtko, I., Mader, D., Kohler, T., Moll, H., Nicholson, G., Biswas, R., ... Peschel, A. (2007). A *Staphylococcus aureus* *ypfP* Mutant With Strongly Reduced Lipoteichoic Acid (LTA) Content: LTA Governs Bacterial Surface Properties and Autolysin Activity. *Molecular Microbiology*, *65*, 1078–1091.
- Feng, Z., Chakraborty, D., Dewell, S. B., Reddy, B. V. B., & Brady, S. F. (2012). Environmental DNA-Encoded Antibiotics Fasamycins A and B Inhibit FabF in Type II Fatty Acid Biosynthesis. *Journal of the American Chemical Society*, *134*, 2981–2987.
- Ferreira, A. A., Souza Tette, P. A., Santos Mendonça, R. C., De Souza Soares, A., & De Carvalho, M. M. (2015). Detection of Exopolysaccharide Production and Biofilm-Related Genes in *Staphylococcus spp.* Isolated from A Poultry Processing Plant. *Food Science and Technology (Brazil)*, *34*, 710–716.
- Fischer, W. (1994). Lipoteichoic Acid and Lipids in the Membrane of *Staphylococcus aureus*. *Med Microbiol Immunol*, *183*, 61–76.
- Foster, T. J. (2017). Antibiotic Resistance in *Staphylococcus aureus*. Current Status and Future Prospects. *FEMS Microbiology Reviews*, *41*, 430–449.
- Freeman, D. J., Falkiner, F. R., & Patrick, S. (1989). New Method For Detecting Slime Production By Coagulase Negative Staphylococci. *Journal of Clinical Pathology*, 872–874.
- Gardiner, J. H., Komazin, G., Matsuo, M., Cole, K., Götz, F., & Meredith, T. C. (2020). Lipoprotein N-Acylation in *Staphylococcus aureus* is Catalyzed by a Two-Component Acyl Transferase System. *mBio*, *11*, 1–18.
- GBD 2019 Antimicrobial Resistance Collaborators. (2022). Global Mortality Associated with 33 Bacterial Pathogens in 2019: a Systematic Analysis for the Global Burden Of Disease Study 2019. *Lancet (London, England)*, *6736*, 1–28.
- Gloux, K., Guillemet, M., Soler, C., Morvan, C., Halpern, D., Pourcel, C., ... Gruss, A. (2017). Clinical Relevance of Type II Fatty Acid Synthesis Bypass in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, *61*, 1–10.
- Grundling, A., & Schneewind, O. (2007a). Genes Required for Glycolipid Synthesis and Lipoteichoic Acid Anchoring in *Staphylococcus aureus*. *Journal of Bacteriology*, *189*, 2521–2530.
- Grundling, A., & Schneewind, O. (2007b). Synthesis of Glycerol Phosphate Lipoteichoic Acid in *Staphylococcus aureus*. *PNAS*, *104*, 8478–8483.

- Hayami, M., Okabe, A., Kariyama, R., Abe, M., & Kanemasa, Y. (1979). Lipid Composition of *Staphylococcus aureus* and Its Derived L-forms. *Microbiol. Immunol.*, *23*, 435–442.
- Hernández-villa, L., Manrique-moreno, M., Leidy, C., Jemioła-Rzemińska, M., Ortiz, C., & Strzałka, K. (2018). Biophysical Chemistry Biophysical Evaluation of Cardiolipin Content as a Regulator of the Membrane Lytic Effect of Antimicrobial Peptides. *Biophysical Chemistry*, *238*, 8–15.
- Hesser, A. R., Matano, L. M., Vickery, C. R., Wood, B. M., Santiago, G., Morris, H. G., ... Losick, R. (2020). The Length of Lipoteichoic Acid Polymers Controls *Staphylococcus aureus* Cell Size and Envelope Integrity. *Journal of Bacteriology*, *202*.
- Karinou, E., Schuster, C. F., Pazos, M., Vollmer, W., & Gründling, A. (2019). Inactivation of the Monofunctional Peptidoglycan Glycosyltransferase *sgtb* Allows *Staphylococcus aureus* to Survive in the Absence of Lipoteichoic Acid. *Journal of Bacteriology*, *201*, 1–47.
- Kariyama, R. (1982). Increase of Cardiolipin Content in *Staphylococcus aureus* by the Use of Antibiotics Affecting the Cell Wall. *The Journal of Antibiotics*, *35*, 1700–1704.
- Karlowsky, J. A., Kaplan, N., Hafkin, B., Hoban, D. J., & Zhanel, G. G. (2009). AFN-1252, a FabI inhibitor, Demonstrates a *Staphylococcus*-Specific Spectrum of Activity. *Antimicrobial Agents and Chemotherapy*, *53*, 3544–3548.
- Kénanian, G., Morvan, C., Weckel, A., Pathania, A., Anba-Mondoloni, J., Halpern, D., ... Gruss, A. (2019). Permissive Fatty Acid Incorporation Promotes *Staphylococcal* Adaptation to FASII Antibiotics in Host Environments. *Cell Reports*, *29*, 3974-3982.e4.
- Khemici, V., Prados, J., Petrignani, B., Nolfi, B. Di, Berge, E., Manzano, C., ... Linder, P. (2020). The DEAD-box RNA Helicase CshA is Required for Fatty Acid Homeostasis in *Staphylococcus aureus*. *PLoS Genetics*, *16*, 1–27.
- Kiriukhin, M. Y., Debabov, D. V, Shinabarger, D. L., & Neuhaus, F. C. (2001). Biosynthesis of the Glycolipid Anchor in Lipoteichoic Acid of *Staphylococcus aureus* RN4220: Role of YpfP , the Diglycosyldiacylglycerol Synthase. *JOURNAL OF BACTERIOLOGY*, *183*, 3506–3514.
- Kuhn, S., Slavetinsky, C. J., & Peschel, A. (2015, February 1). Synthesis and Function of Phospholipids in *Staphylococcus aureus*. *International Journal of Medical Microbiology*, Vol. 305, pp. 196–202. Elsevier GmbH.
- Kuru, E., Hughes, H. V., Brown, P. J., Hall, E., Tekkam, S., Cava, F., ... VanNieuwenhze, M. S. (2013). In Situ Probing of Newly Synthesized Peptidoglycan in Live Bacteria with Fluorescent D-Amino Acids. *Angew Chem Int Ed Engl*, *51*, 12519–12523.
- Lowy, F. D. (1998). *Staphylococcus aureus* infections. *The New England Journal of Medicine*, *339*, 520–532.
- McMurry, L. M., Oethinger, M., & Levy, S. B. (1998). Triclosan Targets Lipid Synthesis. *Nature*, *394*, 531–532.
- Morvan, C., Halpern, D., Kénanian, G., Hays, C., Anba-Mondoloni, J., Brinster, S., ... Gruss, A. (2016). Environmental Fatty Acids Enable Emergence of Infectious *Staphylococcus aureus* Resistant to FASII-Targeted Antimicrobials. *Nature Communications*, *7*.
- Morvan, C., Halpern, D., Kénanian, G., Pathania, A., Anba-Mondoloni, J., Lamberet, G., ... Gloux, K. (2017). The *Staphylococcus aureus* FASII Bypass Escape Route from FASII Inhibitors. *Biochimie*, *141*, 40–46.
- Neuhaus, F. C., & Baddiley, J. (2003). A Continuum of Anionic Charge: Structures and Functions of D-Alanyl-Teichoic Acids in Gram-Positive Bacteria. *Microbiology and Molecular Biology Reviews*, *67*, 686–723.
- Nguyen, M. T., & Götz, F. (2016). Lipoproteins of Gram-Positive Bacteria: Key Players in the Immune Response and Virulence. *Microbiology and Molecular Biology Reviews*, *80*, 891–903.
- O'Donnell, A. G., Nahaie, M. R., Goodfellow, M., Minnikin, D. E., & Hájek, V. (1985). Numerical Analysis of Fatty Acid Profiles in the Identification of *Staphylococci*. *Journal of General Microbiology*, *131*, 2023–2033.
- Oku, Y., Kurokawa, K., Matsuo, M., Yamada, S., Lee, B. L., & Sekimizu, K. (2009). Pleiotropic Roles of Polyglycerolphosphate Synthase of Lipoteichoic Acid in Growth of *Staphylococcus aureus* cells. *Journal of Bacteriology*, *91*, 141–151.
- Parsons, J. B., Broussard, T. C., Bose, J. L., Rosch, J. W., Jackson, P., Subramanian, C., & Rock, C. O. (2014). Identification of a Two-Component Fatty Acid Kinase Responsible for Host Fatty Acid

- Incorporation by *Staphylococcus aureus*. *PNAS*, *111*, 10532–10537.
- Parsons, J. B., Frank, M. W., Jackson, P., Subramanian, C., & Rock, C. O. (2014). Incorporation of Extracellular Fatty Acids by a Fatty Acid Kinase-Dependent Pathway in *Staphylococcus aureus*. *Molecular Microbiology*, *92*, 234–245.
- Pathania, A., Anba-Mondoloni, J., Gominet, M., Halpern, D., Dairou, J., Dupont, L., ... Gruss, A. (2021). (P)ppGpp/GTP and Malonyl-coA Modulate *Staphylococcus aureus* Adaptation to FASII Antibiotics and Provide a Basis for Synergistic Bi-Therapy. *MBio*, *12*, 1–15.
- Percy, M. G., & Gründling, A. (2014). Lipoteichoic Acid Synthesis and Function in Gram-Positive Bacteria. *Annual Review of Microbiology*, *68*, 81–100.
- Peschel, A., Jack, R. W., Otto, M., Collins, L. V., Staubitz, P., Nicholson, G., ... Van Strijp, J. A. G. (2001). *Staphylococcus aureus* Resistance to Human Defensins and Evasion of Neutrophil Killing via the Novel Virulence Factor MprF is Based on Modification of Membrane Lipids with L-lysine. *J. Exp. Med.*, *193*, 1067–1076.
- Pishchany, G., Mevers, E., Ndousse-Fetter, S., Horvath, D. J., Paludo, C. R., Silva-Junior, E. A., ... Kolter, R. (2018). Amycomycin is a Potent and Specific Antibiotic Discovered with a Targeted Interaction Screen. *PNAS*, *115*, 10124–10129.
- Reichmann, N. T., Piçarra Cassona, C., Monteiro, J. M., Bottomley, A. L., Corrigan, R. M., Foster, S. J., ... Gründling, A. (2014). Differential Localization of LTA Synthesis Proteins and Their Interaction with the Cell Division Machinery in *Staphylococcus aureus*. *Molecular Microbiology*, *92*, 273–286.
- Rocha-Roa, C., Orjuela, J. D., Leidy, C., Cossio, P., & Camilo Aponte-Santamaria. (2021). Cardiophilin Prevents Pore Formation in Phosphatidylglycerol Bacterial Membrane Models. *FEBS Letters*, *595*, 2701–2714.
- Santa Maria, J. P., Sadaka, A., Moussa, S. H., Brown, S., Zhang, Y. J., Rubin, E. J., ... Walker, S. (2014). Compound-Gene Interaction Mapping Reveals Distinct Roles for *Staphylococcus aureus* Teichoic Acids. *PNAS*, *111*, 12510–12515.
- Sastre, D. E., Pulschen, A. A., Basso, L. G. M., Benites Pariente, J. S., Marques Netto, C. G. C., Machinandiana, F., ... Gueiros-Filho, F. J. (2020). The Phosphatidic Acid Pathway Enzyme PlsX Plays Both Catalytic and Channeling Roles in Bacterial Phospholipid Synthesis. *Journal of Biological Chemistry*, *295*, 2148–2159.
- Scheinflug, K., Krylova, O., & Strahl, H. (2017). Measurement of Cell Membrane Fluidity by Laurdan GP: Fluorescence Spectroscopy and Microscopy. *Antibiotics: Methods and Protocols*, *1520*, 85–106.
- Short, S. A., & White, D. C. (1972). Biosynthesis of Cardiophilin from Phosphatidylglycerol in *Staphylococcus aureus*. *Journal of Bacteriology*, *109*, 820–826.
- Staubitz, P., Neumann, H., Schneider, T., Wiedemann, I., & Peschel, A. (2004). MprF-Mediated Biosynthesis of Lysylphosphatidylglycerol, an Important Determinant in Staphylococcal Defensin Resistance. *FEMS Microbiology Letters*, *231*, 67–71.
- Stoll, H., Dengjel, J., Nerz, C., & Götz, F. (2005). *Staphylococcus aureus* Deficient in Lipidation of Prelipoproteins is Attenuated in Growth and Immune Activation. *Infection and Immunity*, *73*, 2411–2423.
- Suginaka, H., Shimatani, M., Ohno, Y., & Yano, I. (1979). Effects of Bacterial Lipids and Lipoteichoic Acid on Extracellular Antilysozyme Activity from *Staphylococcus aureus*. *FEMS Microbiology Letters*, *353*–355.
- Swoboda, J. G., Campbell, J., Meredith, T. C., & Walker, S. (2010). Wall Teichoic Acid Function, Biosynthesis, and Inhibition. *ChemBiochem*, *11*, 35–45.
- Teoh, W. P., Chen, X., Laczkovich, I., & Alonzo, F. (2021). *Staphylococcus aureus* Adapts to the Host Nutritional Landscape to Overcome Tissue-Specific Branched-Chain Fatty Acid Requirement. *PNAS*, *118*.
- Vickery, C. R., Wood, B. M. K., Morris, H. G., Losick, R., & Walker, S. (2018). Reconstitution of *Staphylococcus aureus* Lipoteichoic Acid Synthase Activity Identifies Congo Red as a Selective Inhibitor. *Journal of the American Chemical Society*, *140*, 876–879.
- Wang, J., Kodali, S., Sang, H. L., Galgoci, A., Painter, R., Dorso, K., ... Singh, S. B. (2007). Discovery of Platencin, a Dual FabF and FabH Inhibitor with In Vivo Antibiotic Properties. *PNAS*, *104*, 7612–7616.
- Wang, J., Soisson, S. M., Young, K., Shoop, W., Kodali, S., Galgoci, A., ... Singh, S. B. (2006). Platensimycin is a Selective FabF Inhibitor with Potent Antibiotic Properties. *Nature*, *441*, 358–361.

- Wang, Y., & Ma, S. (2013). Recent Advances in Inhibitors of Bacterial Fatty Acid Synthesis Type II (FASII) System Enzymes as Potential Antibacterial Agents. *ChemMedChem*, *8*, 1589–1608.
- Weihs, F., Wacnik, K., Turner, R. D., Culley, S., Henriques, R., & Foster, S. J. (2018). Heterogeneous Localisation of Membrane Proteins in *Staphylococcus aureus*. *Scientific Reports*, 1–12.
- Wenzel, M. (2018).
- Wiedemann, I., Benz, R., & Sahl, H. (2004). Lipid II-Mediated Pore Formation by the Peptide Antibiotic Nisin: a Black Lipid Membrane Study. *Journal of Bacteriology*, *186*, 3259–3261.
- Wongdontree, P., Millan-oropeza, A., Upfold, J., Lavergne, J.-P., Halpern, D., Lambert, C., ... Gruss, A. (2023). Oxidative Stress is Intrinsic to Staphylococcal Adaptation to Fatty Acid Synthesis Antibiotics. *BioRxiv*.
- Wright, H. T., & Reynolds, K. A. (2007). Antibacterial Targets in Fatty Acid Biosynthesis. *Curr Opin Microbiol.*, *10*, 447–453.
- Zeden, M. S., Gallagher, L. A., Bueno, E., Nolan, A. C., Ahn, J., Shinde, D., ... O’Gara, J. P. (2023). Metabolic Reprogramming and Altered Cell Envelope Characteristics in a Pentose Phosphate Pathway Mutant Increases MRSA Resistance to β -Lactam Antibiotics. *PLoS Pathogens*, *19*, 1–35.

CHAPTER 7

Discussion and Perspectives

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Environmental FAs enable *S. aureus* to bypass FASII inhibitors without genetic changes (Kénanian et al. (2019) and this work). The transition from endogenous to totally exogenous FAs in phospholipids contents by treatment with antibiotics targeting FASII (anti-FASII) induces massive changes in protein expression, particularly stress response and virulence factors, which affect the kinetics of *in vivo* infection in the insect model. In addition to overall reprogramming in response to anti-FASII, oxidative stress conditions actually favor anti-FASII adaptation by faster FA incorporation (**Chapter 5**). Remarkably, bacteria adapted to FASII antibiotics showed a major loss in an important cell surface structure, lipoteichoic acid (LTA). As a possible compensation, other cell envelope components were increased, notably cardiolipin (CL) and wall teichoic acid (WTA) (**Chapter 6**). We propose that the increases in these two pools contribute to normal growth of anti-FASII-adapted *S. aureus* in the presence of serum, so that they remain overall competitive for growth. We uncovered a link between FASII and LTA synthesis; indeed, LTA is drastically depleted when FASII activity is blocked despite availability of phospholipid precursors. Finally, using a positive selection, we showed that an “essential” function involved in phospholipid synthesis (encoded by *plsX*) can be compensated by specific point mutations in two different enzymes: FabF, which is required for FA elongation, and FadM, which encodes an acyl-CoA thioesterase. For both these enzymes, it appears that their mutated forms may liberate FAs that are then used to overcome the absence of PlsX (see **Annex**).

Open questions for future work:

The present work leads to questions about how bacteria adjust their metabolism to FASII synthesis arrest, and what insights can be gained about bacterial lifestyle.

1- What is the effect of oxidative stress on FA incorporation? We showed that oxidative priming stress promotes faster FA incorporation, and consequently faster adaptation to anti-FASII (**Chapter 5**). We hypothesized that oxidation might impact FASII gene transcription, or inactivate proteins involved in FASII. In a previous study, a short-term (10 or 20 min) exposure to H₂O₂ led to decreased transcription of genes involved in *S. aureus* FA and phospholipid metabolism (e.g. *accC*, *accB*, *plsC* and *fabH*) (Chang, Small, Toghrol, & Bentley, 2006). Lower ex-

pression of initiation functions (encoded by *acc*) might favor FA incorporation (Gloux et al., 2017; Morvan et al., 2017; Parsons et al., 2011). Proof of oxygen sensitivity of FASII enzymes would be best addressed by a biochemical approach.

2- What triggers LTA loss in anti-FASII-adapted *S. aureus*? We would like to test our hypotheses (**Chapter 6**) that a metabolite or an accumulated intermediate actually blocks LTA synthesis. To this end, we could try to overproduce *plsC* as a means of reducing pools of intermediates (*i.e.*, acyl-ACP or lysophosphatidic acid), which might restore LTA pools. Alternatively, we could add a lyso-PA intermediate (Brissette, Cabacungan, & Pieringer, 1986) to test whether this addition would provoke LTA loss. We also considered it possible that anti-FASII leads to lower cytoplasmic ATP pools, which in turn would lower LTA synthesis due to its need for large ATP pools (Werner Fischer, 1994). This could be tested by comparing ATP pools under the different growth conditions.

3- What compensates LTA loss in anti-FASII-adapted *S. aureus*? Two candidate functions might separately or jointly compensate LTA loss: WTA, and/or CL. We showed that targocil blocks growth in anti-FASII-adapted bacteria, indicating that WTA is required for growth in this condition (**Chapter 6**). As CL pools are increased, and CL, like LTA, is negatively charged, it is possible that CL compensates LTA depletion. A CL requirement after anti-FASII adaptation may be tested in double mutants of *cls1 cls2* (encoding cardiolipin synthases), or by using an agent that alters amounts of CL, while verifying pools of LTA.

Possible applications to develop based on our findings:

Anti-FASII alone does not inhibit *S. aureus* growth when eFAs are present (Kénanian et al., 2019; Morvan et al., 2016). However, this work revealed that anti-FASII-adapted *S. aureus* populations display reduced virulence both *in vivo* and *in vitro*, giving some promise for using anti-FASII. We considered that combinations of anti-FASII with other treatments could prevent *S. aureus* adaptation and potentiate its efficacy. Pathania et al. (2021) showed that an anti-FASII (triclosan or AFN-1252) and a (p)ppGpp inducer (mupirocin) blocks *S. aureus* growth. In this work, we demonstrate or suggest other couples that would act in synergy with anti-FASII.

1- We gave evidence that antioxidants delayed anti-FASII adaptation, which evokes the possibility of using antioxidants to favor elimination of anti-FASII-adapted populations (**Chapter 5**). While administering large doses of antioxidants might not be a feasible bi-therapy treatment, supplementing the diet with vitamin C could favor elimination of remaining bacterial anti-FASII-adapted populations if a second bi-therapy treatment is identified.

2- Suppression of both TAs is lethal (Santa Maria et al., 2014). The anti-FASII (AFN-1252), which reduces LTA production, proved synergistic with the anti-WTA, targocil, in eliminating *S. aureus* (**Chapter 6**). On the other hand, our and others' results may suggest that *S. aureus* can survive when LTA is inhibited, thus questioning the choice of LTA for treatment of *S. aureus* infection.

3- We observed an increase in CL in anti-FASII-adapted *S. aureus*, but did not determine whether CL is required for growth in adapted conditions. Increased CL in anti-FASII-adapted *S. aureus* might lead to greater bacterial sensitivity to cationic antibiotics. Indeed, the potential synergy between nisin, a cationic antimicrobial, and anti-FASII was demonstrated (**Chapter 6, Fig. 12**). A role for CL in synergistic killing by nisin remains to be proven.

4- Based on the present results, we suggest two other targets whose inhibitors could work synergistically with anti-FASII:

- a-** FakA: while to date no inhibitor exists, it would be a choice combination with anti-FASII, as such an inhibitor would block FA incorporation, and entirely stop FA metabolism as we observed in *fakA* inactivated strain where no bypass was possible (unpublished result). Such molecule will probably work in combination with anti-FASII and will stop growth of *S. aureus*, and FASII by-pass *in vitro*. However, FakA is not a narrow target as it is present in almost all bacteria in the microbiota with a homology between 56% to 84% (ncbi blast) to *S. aureus* FakA
- b-** PlsX: no inhibitor exists against this target. We demonstrated that a $\Delta plsX$ *S. aureus* mutant cannot grow in anti-FASII, even if FA is available (**Annex**). A PlsX inhibitor in combination with anti-FASII would also stop FA metabolism.

To pursue development based on the proposed targets, we would use bioinformatics approaches based on ligand predictions and 3-D protein structures.

References for Introduction

- Abraham, N. M., & Jefferson, K. K. (2012). *Staphylococcus aureus* Clumping Factor B Mediates Biofilm Formation in the Absence of Calcium. *Microbiology (United Kingdom)*, *158*, 1504–1512.
- Abreu, M. T., & Arditi, M. (2004). Innate Immunity and Toll-Like Receptors: Clinical Implications Of Basic Science Research. *Medical Progress*, 421–429.
- Acton, D. S., Plat-sinnige, M. J. T., Wamel, W. van, Groot, N. de, & Belkum, A. van. (2009). Intestinal Carriage of *Staphylococcus aureus*: How Does its Frequency Compare with that of Nasal Carriage and What is its Clinical Impact? *Eur J Clin Microbiol Infect Dis*, *28*, 115–127.
- Aguilar, P. S., Cronan, J. E., & De Mendoza, D. (1998). A *Bacillus subtilis* Gene Induced by Cold Shock Encodes a Membrane Phospholipid Desaturase. *Journal of Bacteriology*, *180*, 2194–2200.
- Albanesi, D., Arabolaza, A., Gramajo, H., & Mendoza, D. de. (2020). Homeostatic Control of Membrane Lipid Biosynthesis in Bacteria.
- Albanesi, D., & de Mendoza, D. (2016). FapR: From Control of Membrane Lipid Homeostasis to a Biotechnological Tool. *Frontiers in Molecular Biosciences*, *3*, 1–9.
- Albanesi, D., Reh, G., Guerin, M. E., Schaeffer, F., Debarbouille, M., Buschiazzo, A., ... Alzari, P. M. (2013). Structural Basis for Feed-Forward Transcriptional Regulation of Membrane Lipid Homeostasis in *Staphylococcus aureus*. *PLoS Pathogens*, *9*, 1–11.
- Alnaseri, H., Arsic, B., Schneider, J. E. T., Kaiser, J. C., Scinocca, Z. C., Heinrichs, D. E., & McGavin, M. J. (2015). Inducible Expression of a Resistance-Nodulation-Division-Type Efflux Pump in *Staphylococcus aureus* Provides Resistance to Linoleic and Arachidonic Acids. *Journal of Bacteriology*, *197*, 1893–1905.
- Archibald, A. R., Armstrong, J. J., Baddiley, J., & Hay, J. B. (1961). Teichoic Acids And the Structure of Bacterial Walls. *Nature*, *191*, 570–572.
- Archibald, A. Ronald, Baddiley, J., & Heptinstall, S. (1973). The Alanine Ester Content and Magnesium Binding Capacity of Walls of *Staphylococcus aureus* H Grown at Different pH Values. *Biochimica et Biophysica Acta*, *291*, 629–634.
- Arias-Cartin, R., Grimaldi, S., Arnoux, P., Guigliarelli, B., & Magalon, A. (2012). Cardiolipin Binding in Bacterial Respiratory Complexes: Structural and Functional Implications. *Biochimica et Biophysica Acta*, *1817*, 1937–1949.
- Baddiley, J. (1989). *Bacterial Cell Walls and Membranes. Discovery of the Teichoic Acids*. *10*, 207–210.
- Bæk, K. T., Bowman, L., Millership, C., Søggaard, D., Kaever, V., Siljamäki, P., ... Frees, D. (2016). The Cell Wall Polymer Lipoteichoic Acid Becomes Nonessential in *Staphylococcus aureus* Cells Lacking the ClpX chaperone. *MBio*, *7*, 1–11.
- Bai, Y., Yang, J., Zarrella, T. M., Zhang, Y., Metzger, D. W., & Bai, G. (2014). Cyclic Di-AMP Impairs Potassium Uptake Mediated by a Cyclic Di-AMP Binding Protein in *Streptococcus pneumoniae*. *Journal of Bacteriology*, *196*, 614–623.
- Balemans, W., Lounis, N., Gilissen, R., Guillemont, J., Simmen, K., Andries, K., & Koul, A. (2010). Essentiality of FASII Pathway for *Staphylococcus aureus*. *Nature*, *463*, 2009–2011.
- Banevicius, M. A., Kaplan, N., Hafkin, B., & Nicolau, D. P. (2013). Pharmacokinetics, Pharmacodynamics and Efficacy of Novel FabI Inhibitor AFN-1252 Against MSSA and MRSA in the Murine Thigh Infection Model. *Journal of Chemotherapy*, *25*, 26–31.
- Barbu, E. M., Mackenzie, C., Foster, T. J., & Höök, M. (2014). SdrC Induces Staphylococcal Biofilm Formation Through a Homophilic Interaction. *Mol Microbiol*, *94*, 172–185.
- Barbuti, M. D., Lambert, E., Myrbråten, I. S., Ducret, A., Stamsås, G. A., Wilhelm, L., ... Kjos, M. (2023). The Function of CozE Proteins is Linked to Lipoteichoic Acid Biosynthesis in *Staphylococcus aureus*. *BioRxiv Preprint*, 1–37.
- Beavers, W. N., & Skaar, E. P. (2016). Neutrophil-Generated Oxidative Stress and Protein Damage in *Staphylococcus aureus*. *Pathogens and Disease*, *74*, 1–15.
- Belkum, A. Van, Verkaik, N. J., Vogel, C. P. De, Boelens, H. A., Verveer, J., Nouwen, J. L., ... Wertheim, H. F. L. (2009). *Reclassification of Staphylococcus aureus Nasal Carriage Types*. *199*.
- Berends, E. T. M., Horswill, A. R., Haste, N. M., Monestier, M., Nizet, V., & Von Kückritz-Blickwede, M. (2010). Nuclease Expression by *Staphylococcus aureus* Facilitates Escape From Neutrophil Extracellular Traps. *J Innate Immun*, *2*, 576–586.
- Beutler, B. (2004). Innate Immunity: an Overview. *Molecular Immunology*, *40*, 845–859.
- Bibens, L., Becker, J. P., Dassonville-Klimpt, A., & Sonnet, P. (2023). A Review of Fatty Acid Biosynthesis Enzyme Inhibitors as Promising Antimicrobial Drugs. *Pharmaceuticals*, *16*.
- Boekhorst, J., Siezen, R. J., Zwahlen, M. C., Vilanova, D., Pridmore, R. D., Mercenier, A., ... Desiere, F. (2004). The Complete Genomes of *Lactobacillus plantarum* and *Lactobacillus johnsonii* Reveal Extensive Differences in Chromosome Organization and Gene Content. *Microbiology*, *150*, 3601–3611.
- Bose, J. L., Daly, S. M., Hall, P. R., & Bayles, K. W. (2014). Identification of the *Staphylococcus aureus* *vfrAB* Operon, a

- Novel Virulence Factor Regulatory Locus. *Infection and Immunity*, *82*, 1813–1822.
- Boudjemaa, R., Dubois-brissonnet, F., Bourg, N., Dupuis, G., Gruss, A., Briandet, R., & Steenkeste, K. (2018). Impact of Bacterial Membrane Fatty Acid Composition on the Failure of Daptomycin to Kill *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, *62*, 1–12.
- Bowman, L., & Palmer, T. (2021). The Type VII Secretion System of *Staphylococcus*. *Annual Review of Microbiology*, *75*, 471–494.
- Boyle-Vavra, S., Yin, S., Jo, D. S., Montgomery, C. P., & Daum, R. S. (2013). VraT/YvqF is Required for Methicillin Resistance and Activation of the VraSR Regulon in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, *57*, 83–95.
- Brauweiler, A. M., Goleva, E., & Leung, D. Y. M. (2019). *Staphylococcus aureus* Lipoteichoic Acid Damages the Skin Barrier. *J Invest Dermatol*, *139*, 1753–1761.
- Brenwald, N. P., & Fraise, A. P. (2003). Triclosan Resistance in Methicillin-Resistant *Staphylococcus aureus* (MRSA). *Journal of Hospital Infection*, *55*, 141–144.
- Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., ... Zychlinsky, A. (2004). Neutrophil Extracellular Traps Kill Bacteria. *Science*, *303*, 1532–1535.
- Brinster, S., Lamberet, G., Staels, B., Trieu-Cuot, P., Gruss, A., & Poyart, C. (2009). Type II Fatty Acid Synthesis is not a Suitable Antibiotic Target for Gram-Positive Pathogens. *Nature*, *458*, 83–86.
- Brissette, J. L., Cabacungan, E. A., & Pieringer, R. A. (1986). Studies on the Antibacterial Activity of Dodecylglycerol. Its limited Metabolism and Inhibition of Glycerolipid and Lipoteichoic Acid Biosynthesis in *Streptococcus mutans* BHT. *Journal of Biological Chemistry*, *261*, 6338–6345.
- Brooks, D., & Baddiley, J. (1969). The Mechanism of Biosynthesis and Direction of Chain Extension of a Poly-(N-Acetylglucosamine 1-Phosphate) from the Walls of *Staphylococcus lactis* N.C.T.C. 2102. *Biochem. J.*, *118*, 635–642.
- Brown, A. P., Slabas, A. R., & Rafferty, J. B. (2009). *Chapter2: Fatty Acid Biosynthesis in Plants — Metabolic Pathways, Structure and Organization*.
- Brown, S., Santa Maria, J. P., & Walker, S. (2013). Wall Teichoic Acids of Gram-Positive Bacteria. *Annual Review of Microbiology*, *67*, 313–336.
- Burian, M., Plange, J., Schmitt, L., Kaschke, A., Marquardt, Y., Huth, L., ... Yazdi, A. S. (2021). Adaptation of *Staphylococcus aureus* to the Human Skin Environment Identified Using an ex vivo Tissue Model. *Frontiers in Microbiology*, *12*.
- Byrd, A. L., Belkaid, Y., & Segre, J. A. (2018). The Human Skin Microbiome. *Nature Reviews Microbiology*, *16*, 143–155.
- Chang, W., Small, D. A., Toghrol, F., & Bentley, W. E. (2006). Global Transcriptome Analysis of *Staphylococcus aureus* Response to Hydrogen Peroxide. *Journal of Bacteriology*, *188*, 1648–1659.
- Chatterjee, A. N., & Park, J. T. (1964). Biosynthesis of Cell Wall Mucoprotein by a Particulate Fraction From *Staphylococcus aureus*. *Biochemistry*, *51*, 9–16.
- Chee Wezen, X., Chandran, A., Eapen, R. S., Waters, E., Bricio-Moreno, L., Tosi, T., ... Rahman, T. (2022). Structure-Based Discovery of Lipoteichoic Acid Synthase Inhibitors. *Journal of Chemical Information and Modeling*.
- Chen, X., & Alonzo, F. (2019). Bacterial Lipolysis of Immune-Activating Ligands Promotes Evasion of Innate Defenses. *PNAS*, *116*, 3764–3773.
- Chiaruzzi, M., Barbry, A., Muggeo, A., Tristan, A., Jacquemond, I., Badiou, C., ... Lina, G. (2020). Vaginal Tampon Colonization by *Staphylococcus aureus* in Healthy Women. *Applied and Environmental Microbiology*, *86*, 1–9.
- Childs, W. C., & Neuhaus, F. C. (1980). Biosynthesis of D-Alanyl-Lipoteichoic Acid: Characterization of Ester-Linked D-Alanine in the In Vitro-Synthesized Product. *Journal of Bacteriology*, *143*, 293–301.
- Ciusa, M. L., Furi, L., Knight, D., Decorosi, F., Fondi, M., Raggi, C., ... Oggioni, M. R. (2012). A Novel Resistance Mechanism to Triclosan That Suggests Horizontal Gene Transfer and Demonstrates a Potential Selective Pressure for Reduced Biocide Susceptibility in Clinical Strains of *Staphylococcus aureus*. *International Journal of Antimicrobial Agents*, *40*, 210–220.
- Clarke, S. R., & Foster, S. J. (2006). Surface Adhesins of *Staphylococcus aureus*. In *Advances in Microbial Physiology* (Vol. 51).
- Clarke, S. R., Mohamed, R., Bian, L., Routh, A. F., Kokai-kun, J. F., Mond, J. J., ... Foster, S. J. (2007). The *Staphylococcus aureus* Surface Protein IsdA Mediates Resistance to Innate Defenses of Human Skin. *Cell Host & Microbe*, *1*, 199–212.
- Collins, L. V., Kristian, S. A., Weidenmaier, C., Faigle, M., Kessel, K. P. M. Van, Strijp, J. A. G. Van, ... Peschel, A. (2002). *Staphylococcus aureus* Strains Lacking D -Alanine Modifications of Teichoic Acids are Highly Susceptible to Human Neutrophil Killing and are Virulence Attenuated in Mice. *The Journal of Infectious Diseases*, *186*, 214–219.
- Corrigan, R. M., Abbott, J. C., Burhenne, H., Kaever, V., & Gründling, A. (2011). C-Di-AMP is a New Second Messenger in *Staphylococcus aureus* with a Role in Controlling Cell Size and Envelope Stress. *PLoS Pathogens*, *7*, 1–16.

- Corrigan, R. M., Campeotto, I., Jeganathan, T., Roelofs, K. G., Lee, V. T., & Gründling, A. (2013). Systematic Identification of Conserved Bacterial C-Di-AMP Receptor Proteins. *PNAS*, *110*, 9084–9089.
- Corrigan, R. M., Miajlovic, H., & Foster, T. J. (2009). Surface Proteins that Promote Adherence of *Staphylococcus aureus* to Human Desquamated Nasal Epithelial Cells. *BMC Microbiology*, *9*, 1–10.
- Cronan, J. E., & Gelmann, E. P. (1975). Physical Properties of Membrane Lipids: Biological Relevance and Regulation. *Bacteriological Reviews*, *39*, 232–256.
- Cronan, John E., & Thomas, J. (2009). Bacterial Fatty Acid Synthesis and Its Relationships with Polyketide Synthetic Pathways John. *Methods Enzymol*, *459*, 395–433.
- Dancer, S. J., & Noble, W. C. (1991). Nasal, Axillary, and Perineal Carriage of Identification of Strains Producing Epidermolytic Toxin. *J Clin Pathol*, *44*, 681–684.
- Debabov, D. V., Kiriukhin, M. Y., & Neuhaus, F. C. (2000). Biosynthesis of Lipoteichoic Acid in *Lactobacillus rhamnosus*: Role of DltD in D-alanylation. *Journal of Bacteriology*, *182*, 2855–2864.
- Delekta, P. C., Shook, J. C., Lydic, T. A., Mulks, M. H., & Hammer, N. D. (2018). *Staphylococcus aureus* Utilizes Host-Derived Lipoprotein Particles as Sources of Fatty Acids. *Journal of Bacteriology*, *200*, 1–22.
- DeMars, Z. R., Krute, C. N., Ridder, M. J., Gilchrist, A. K., Menjivar, C., & Bose, J. L. (2021). Fatty Acids can Inhibit *Staphylococcus aureus* SaeS Activity at the Membrane Independent of Alterations in Respiration. *Molecular Microbiology*, *116*, 1378–1391.
- Denich, T. J., Beaudette, L. A., Lee, H., & Trevors, J. T. (2003). Effect of Selected Environmental and Physico-Chemical Factors on Bacterial Cytoplasmic Membranes. *Journal of Microbiological Methods*, *52*, 149–182.
- Donegan, N. P., & Cheung, A. L. (2009). Regulation of the *mazEF* Toxin-Antitoxin Module in *Staphylococcus aureus* and Its Impact on *sigB* Expression. *Journal of Bacteriology*, *191*, 2795–2805.
- Dubois-Brissonnet, F., Trotier, E., & Briandet, R. (2016). The Biofilm Lifestyle Involves an Increase in Bacterial Membrane Saturated Fatty Acids. *Frontiers in Microbiology*, *7*, 1–8.
- Dyzenhaus, S., Sullivan, M. J., Albuquerque, B., Boff, D., van de Guchte, A., Chung, M., ... Torres, V. J. (2023). MRSA Lineage USA300 Isolated From Bloodstream Infections Exhibit Altered Virulence Regulation. *Cell Host and Microbe*, *31*, 228-242.e8.
- Eldhin, D. N., Perkins, S., Francois, P., Vaudaux, P., Höök, M., & Foster, T. J. (1998). Clumping Factor B (ClfB), a New Surface-Located Fibrinogen-Binding Adhesin of *Staphylococcus aureus*. *Molecular Microbiology*, *30*, 245–257.
- Elia, M. A. D., Pereira, M. P., Chung, Y. S., Zhao, W., Chau, A., Kenney, T. J., ... Brown, E. D. (2006). Lesions in Teichoic Acid Biosynthesis in *Staphylococcus aureus* Lead to a Lethal Gain of Function in the Otherwise Dispensable Pathway. *Journal of Bacteriology*, *188*, 4183–4189.
- Elovson, J., & Vagelos, P. R. (1968). Acyl Carrier Protein: X. Acyl Carrier Protein Synthetase. *Journal of Biological Chemistry*, *243*, 3603–3611.
- Entenza, J. M., Foster, T. J., Eidhin, D. N., Vaudaux, P., Francioli, P., & Moreillon, P. (2000). Contribution of Clumping Factor B to Pathogenesis of Experimental Endocarditis due to *Staphylococcus aureus*. *Infection and Immunity*, *68*, 5443–5446.
- Erickson, H. P. (2021). How Teichoic Acids Could Support a Periplasm in Gram-Positive Bacteria, and Let Cell Division Cheat Turgor Pressure. *Frontiers in Microbiology*, *12*, 1–11.
- Ericson, M. E., Subramanian, C., Frank, M. W., & Rock, C. O. (2017). Role of Fatty Acid Kinase In Cellular Lipid Homeostasis And SaeRS-Dependent Virulence Factor Expression in *Staphylococcus aureus*. *MBio*, *8*, 1–14.
- Escaich, S., Prouvensier, L., Saccomani, M., Durant, L., Oxoby, M., Gerusz, V., ... Soulama-Mouze, C. (2011). The MUT056399 Inhibitor of Fabi is a New Antistaphylococcal Compound. *Antimicrobial Agents and Chemotherapy*, *55*, 4692–4697.
- Fedtke, I., Mader, D., Kohler, T., Moll, H., Nicholson, G., Biswas, R., ... Peschel, A. (2007). A *Staphylococcus aureus* *ypfP* Mutant with Strongly Reduced Lipoteichoic Acid (LTA) Content: LTA Governs Bacterial Surface Properties and Autolysin Activity. *Molecular Microbiology*, *65*, 1078–1091.
- Fey, P. D., Endres, J. L., Yajjala, V. K., Yajjala, K., Widhelm, T. J., Boissy, R. J., ... Bayles, K. W. (2013). A Genetic Resource for Rapid and Comprehensive Phenotype Screening of Nonessential *Staphylococcus aureus* Genes. *MBio*, *4*, e00537-12.
- Fischer, W. (1988). Physiology of Lipoteichoic Acids in Bacteria. In *Advances in Microbial Physiology* (Vol. 29).
- Fischer, W., Behr, T., Hartmann, R., Peter-Katalinic, J., & Egge, H. (1993). Teichoic Acid and Lipoteichoic Acid of *Streptococcus pneumoniae* Possess Identical Chain Structures. A Reinvestigation of Teichoid Acid (C Polysaccharide). *Eur. J. Biochem.*, *215*, 851–857.
- Fischer, Werner. (1994). Lipoteichoic Acid and Lipids in the Membrane of *Staphylococcus aureus*. *Med Microbiol Immunol*, *183*, 61–76.
- Fischer, Werner, Rosel, P., & Koch, H. U. W. E. (1981). Effect of Alanine Ester Substitution and Other Structural Features of Lipoteichoic Acids on Their Inhibitory Activity Against Autolysins of *Staphylococcus aureus*. *Journal of Bacteriology*, *146*, 467–475.
- Foster, T. J. (2017). Antibiotic Resistance in *Staphylococcus aureus*. Current Status and Future Prospects. *FEMS Microbiology Reviews*, *41*, 430–449.

- Foster, T. J. (2019). The MSCRAMM Family of Cell-Wall-Anchored Surface Proteins of Gram-Positive Cocci. *Trends in Microbiology*, 27, 927–941.
- Foster, T. J., & Geoghegan, J. A. (2015). *Staphylococcus aureus*. *Molecular Medical Microbiology*. Elsevier Ltd.
- Foster, T. J., Geoghegan, J. A., Ganesh, V. K., & Höök, M. (2014). Adhesion, Invasion and Evasion: The Many Functions of the Surface Proteins of *Staphylococcus aureus*. *Nature Reviews Microbiology*, 12, 49–62.
- Foster, T. J., & Höök, M. (1998). Surface Protein Adhesins of *Staphylococcus aureus*. *Trends in Microbiology*, 6, 484–488.
- Friedman, L., Alder, J. D., & Silverman, J. A. (2006). Genetic Changes that Correlate with Reduced Susceptibility to Daptomycin in *Staphylococcus aureus*. *Antimicrob. Agents Chemother*, 50, 2137–2145.
- Fujita, Y., Matsuoka, H., & Hirooka, K. (2007). Regulation of Fatty Acid Metabolism in Bacteria. *Molecular Microbiology*, 66, 829–839.
- Gardiner, J. H., Komazin, G., Matsuo, M., Cole, K., Götz, F., & Meredith, T. C. (2020). Lipoprotein N-Acylation in *Staphylococcus aureus* is Catalyzed by a Two-Component Acyl Transferase System. *MBio*, 11, 1–18.
- GBD 2019 Antimicrobial Resistance Collaborators. (2022). Global Mortality Associated With 33 Bacterial Pathogens in 2019: a Systematic Analysis for the Global Burden of Disease Study 2019. *Lancet (London, England)*, 6736, 1–28.
- Geoghegan, J. A., Irvine, A. D., & Foster, T. J. (2018). *Staphylococcus aureus* and Atopic Dermatitis: A Complex and Evolving Relationship. *Trends in Microbiology*, 26, 484–497.
- Gloux, K., Guillemet, M., Soler, C., Morvan, C., Halpern, D., Pourcel, C., ... Gruss, A. (2017). Clinical Relevance of Type II Fatty Acid Synthesis Bypass in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 61, 1–10.
- Grandgirard, D., Furi, L., Ciusa, M. L., Baldassarri, L., Knight, D. R., Morrissey, I., ... Oggioni, M. R. (2015). Mutations Upstream of *fabI* in Triclosan Resistant *Staphylococcus aureus* Strains are Associated with Elevated *fabI* Gene expression. *BMC Genomics*, 16.
- Grice, E. A., & Segre, J. A. (2011). The Skin Microbiome. *Nature Reviews Microbiology*, 9, 244–253.
- Gründling, A., & Schneewind, O. (2007a). Genes Required For Glycolipid Synthesis and Lipoteichoic Acid Anchoring in *Staphylococcus aureus*. *Journal of Bacteriology*, 189, 2521–2530.
- Gründling, A., & Schneewind, O. (2007b). Synthesis of Glycerol Phosphate Lipoteichoic Acid in *Staphylococcus aureus*. *PNAS*, 104, 8478–8483.
- Guyet, A., Alofi, A., & Daniel, R. A. (2023). Insights into the Roles of Lipoteichoic Acids and MprF in *Bacillus subtilis*. *MBio*, 14, 1–25.
- Haas, R., Koch, H. U., & Fischer, W. (1984). Alanin Turnover from Lipoteichoic Acid to Teichoic Acid in *Staphylococcus aureus*. *FEMS Microbiology Letters*, 21, 27–31.
- Hashimoto, M., Furuyashiki, M., Kaseya, R., Fukada, Y., Akimaru, M., Aoyama, K., ... Suda, Y. (2007). Evidence of Immunostimulating Lipoprotein Existing in the Natural Lipoteichoic Acid Fraction. *Infection and Immunity*, 75, 1926–1932.
- Hashimoto, M., Tawaratsumida, K., Kariya, H., Kiyohara, A., Suda, Y., Krikae, F., ... Götz, F. (2006). Not Lipoteichoic Acid but Lipoproteins Appear to Be the Dominant Immunobiologically Active Compounds in *Staphylococcus aureus*. *The Journal of Immunology*, 177, 3162–3169.
- Heath, R. J., & Rock, C. O. (1995). Enoyl-Acyl Carrier Protein Reductase (*fabI*) Plays a Determinant Role in Completing Cycles of Fatty Acid Elongation in *Escherichia coli*. *Journal of Biological Chemistry*, 270, 26538–26542.
- Heath, Richard J., & Rock, C. O. (1996). Roles of the FabA and FabZ β -hydroxyacyl-Acyl Carrier Protein Dehydratases in *Escherichia coli* Fatty Acid Biosynthesis. *Journal of Biological Chemistry*, 271, 27795–27801.
- Heath, Richard J., Su, N., Murphy, C. K., & Rock, C. O. (2000). The Enoyl-[Acyl-Carrier-Protein] Reductases FabI and FabL from *Bacillus subtilis*. *Journal of Biological Chemistry*, 275, 40128–40133.
- Heilmann, C. (2011). *Adhesion Mechanisms of Staphylococci*.
- Heptinstall, S., Archibald, A. R., & Baddiley, J. (1970). Teichoic Acids and Membrane Function in Bacteria. *Nature*, 225, 519–521.
- Hesser, A. R., Matano, L. M., Vickery, C. R., Wood, B. M., Santiago, G., Morris, H. G., ... Losick, R. (2020). The Length of Lipoteichoic Acid Polymers Controls *Staphylococcus aureus* Cell Size and Envelope Integrity. *Journal of Bacteriology*, 202.
- Hesser, A. R., Schaefer, K., Lee, W., & Walker, S. (2020). Lipoteichoic Acid Polymer Length is Determined by Competition Between Free Starter Units. *PNAS*, 117, 29669–29676.
- Hiltunen, J. K., Autio, K. J., Schonauer, M. S., Kursu, V. A. S., Dieckmann, C. L., & Kastaniotis, A. J. (2010). Mitochondrial Fatty Acid Synthesis and Respiration. *Biochimica et Biophysica Acta*, 1797, 1195–1202.
- Himmelreich, R., Hubert, H., Plagens, H., Pirkel, E., Li, B. C., & Herrmann, R. (1996). Complete Sequence Analysis of the Genome of the Bacterium *Mycoplasma pneumoniae*. *Nucleic Acids Research*, 24, 4420–4449.
- Hines, K. M., Alvarado, G., Chen, X., Gatto, C., Pokorny, A., Alonzo, F., ... Xu, L. (2020). Lipidomic and Ultrastructural Characterization of the Cell Envelope of *Staphylococcus aureus* Grown in the Presence of Human Serum. *MSphere*, 5.

- Holtje, J. V., & Tomasz, A. (1975). Lipoteichoic Acid: A Specific Inhibitor of Autolysin Activity in *Pneumococcus*. *PNAS*, *72*, 1690–1694.
- Hunt, T., Kaplan, N., & Hafkin, B. (2016). Safety, Tolerability and Pharmacokinetics of Multiple Oral Doses of AFN-1252 Administered as Immediate Release (IR) Tablets in Healthy Subjects. *Journal of Chemotherapy*, *28*, 164–171.
- Jerga, A., Lu, Y., Schujman, G. E., Mendoza, D. De, & Rock, C. O. (2007). Identification of a Soluble Diacylglycerol Kinase Required for Lipoteichoic Acid Production in *Bacillus subtilis*. *Journal of Biological Chemistry*, *282*, 21738–21745.
- Jones, T., Yeaman, M. R., Sakoulas, G., Yang, S. J., Proctor, R. A., Sahl, H.-G., ... Bayer, A. S. (2008). Failures in Clinical Treatment of *Staphylococcus aureus* Infection with Daptomycin are Associated with Alterations in Surface Charge, Membrane Phospholipid Asymmetry, and Drug Binding. *Antimicrob. Agents Chemother*, *52*, 269–278.
- Jorasch, P., Wolter, F. P., Zähringer, U., & Heinz, E. (1998). A UDP Glucosyltransferase from *Bacillus subtilis* Successively Transfers up to Four Glucose Residues to 1,2-Diacylglycerol: Expression of *ypfP* in *Escherichia coli* and Structural Analysis of its Reaction Products. *Molecular Microbiology*, *29*, 419–430.
- Kaneda, T. (1991). Iso- and Anteiso-Fatty Acids in Bacteria: Biosynthesis, Function, and Taxonomic Significance. *Microbiological Reviews*, *55*, 288–302.
- Karinou, E., Schuster, C. F., Pazos, M., Vollmer, W., & Gründling, A. (2019). Inactivation of the Monofunctional Peptidoglycan Glycosyltransferase *sgtb* Allows *staphylococcus aureus* to Survive in the Absence of Lipoteichoic Acid. *Journal of Bacteriology*, *201*, 1–47.
- Kariyama, R. (1982). Increase of Cardiolipin Content in *Staphylococcus aureus* by the Use of Antibiotics Affecting the Cell Wall. *The Journal of Antibiotics*, *35*, 1700–1704.
- Karlowsky, J. A., Kaplan, N., Hafkin, B., Hoban, D. J., & Zhanel, G. G. (2009). AFN-1252, a FabI Inhibitor, Demonstrates a *Staphylococcus*-Specific Spectrum Of Activity. *Antimicrobial Agents and Chemotherapy*, *53*, 3544–3548.
- Kawai, Y., Mercier, R., Wu, L. J., Domínguez-Cuevas, P., Oshima, T., & Errington, J. (2015). Cell Growth of Wall-Free L-Form Bacteria is Limited by Oxidative Damage. *Current Biology*, *25*, 1613–1618.
- Keim, K. C., & Horswill, A. R. (2023). *Staphylococcus aureus*. *Trends in Microbiology*, 1–2.
- Kénanian, G., Morvan, C., Weckel, A., Pathania, A., Anba-Mondoloni, J., Halpern, D., ... Gruss, A. (2019). Permissive Fatty Acid Incorporation Promotes *Staphylococcal* Adaptation to FASII Antibiotics in Host Environments. *Cell Reports*, *29*, 3974–3982.e4.
- Kengmo Tchoupa, A., Watkins, K. E., Jones, R. A., Kuroki, A., Alam, M. T., Perrier, S., ... Unnikrishnan, M. (2020). The Type VII Secretion System Protects *Staphylococcus aureus* Against Antimicrobial Host Fatty Acids. *Scientific Reports*, *10*, 1–16.
- Kenny, J. G., Ward, D., Josefsson, E., Jonsson, I., Hinds, J., Rees, H. H., ... Horsburgh, M. J. (2009). The *Staphylococcus aureus* Response to Unsaturated Long Chain Free Fatty Acids: Survival Mechanisms and Virulence Implications. *PLoS ONE*, *4*, 1–29.
- Khemic, V., Prados, J., Petrignani, B., Nolfi, B. Di, Berge, E., Manzano, C., ... Linder, P. (2020). The DEAD-box RNA Helicase CshA is Required for Fatty Acid Homeostasis in *Staphylococcus aureus*. *PLoS Genetics*, *16*, 1–27.
- Kiriukhin, M. Y., & Neuhaus, F. C. (2001). D-alanylation of Lipoteichoic acid: Role of the D-alanyl carrier protein in acylation. *Journal of Bacteriology*, *183*, 2051–2058.
- Kiriukhin, Michael Y., Debatov, D. V., Shinabarger, D. L., & Neuhaus, F. C. (2001). Biosynthesis of the Glycolipid Anchor in Lipoteichoic Acid of *Staphylococcus aureus* RN4220: Role of YpfP, the Diglucoxydiacylglycerol Synthase. *Journal of Bacteriology*, *183*, 3506–3514.
- Koch, A. L. (2006). The Exocytoskeleton. *J Mol Microbiol Biotechnol*, *11*, 115–125.
- Koch, H. U., Haas, R., & Fischer, W. (1984). The Role of Lipoteichoic Acid Biosynthesis in Membrane Lipid Metabolism of Growing *Staphylococcus aureus*. *European Journal of Biochemistry*, *138*, 357–363.
- Krismer, B., Weidenmaier, C., Zipperer, A., & Peschel, A. (2017). The Commensal Lifestyle of *Staphylococcus aureus* and Its Interactions with the Nasal Microbiota. *Nature Publishing Group*, *15*, 675–687.
- Kuhn, S., Slavetinsky, C. J., & Peschel, A. (2015, February 1). Synthesis and Function of Phospholipids in *Staphylococcus aureus*. *International Journal of Medical Microbiology*, Vol. 305, pp. 196–202. Elsevier GmbH.
- Kuiack, R. C., Tuffs, S. W., Dufresne, K., Flick, R., McCormick, J. K., & McGavin, M. J. (2023). The *fadXDEBA* Locus of *Staphylococcus aureus* is Required for Metabolism of Exogenous Palmitic Acid and In Vivo Growth. *Molecular Microbiology*, *120*, 425–438.
- Kumar, A., Ray, P., Kanwar, M., Sharma, M., & Varma, S. (2005). A Comparative Analysis of Antibody Repertoire Against *Staphylococcus aureus* antigens in Patients with Deep-Seated Versus Superficial staphylococcal Infections. *International Journal of Medical Sciences*, *2*, 129–136.
- Kumar, H., Kawai, T., & Akira, S. (2009). Toll-Like Receptors and Innate Immunity. *Biochemical and Biophysical Research Communications*, *388*, 621–625.
- Kusch, H., & Engemann, S. (2014). Secrets of the Secretome in *Staphylococcus aureus*. *International Journal of*

Medical Microbiology, 304, 133–141.

- Kwiecinski, J., Jin, T., & Josefsson, E. (2014). Surface Proteins of *Staphylococcus aureus* Play an Important Role in Experimental Skin Infection. *Apmis*, 122, 1240–1250.
- Labajová, N., Baranova, N., Jurásek, M., Vácha, R., Loose, M., & Barák, I. (2021). Cardiolipin-Containing Lipid Membranes Attract the Bacterial Cell Division Protein DivIVA. *Int. J. Mol. Sci.*, 22, 1–21.
- Lacey, K. A., Mulcahy, M. E., Towell, A. M., Geoghegan, J. A., & McLoughlin, R. M. (2019). Clumping Factor B is an Important Virulence Factor during *Staphylococcus aureus* Skin Infection and a Promising Vaccine Target. *PLoS Pathogens*, 15.
- Lai, Y., Nardo, A. Di, Nakatsuji, T., Leichtle, A., Yang, Y., Cogen, A. L., ... Gallo, R. L. (2009). Commensal Bacteria Regulate Toll-like Receptor 3 – Dependent Inflammation After Skin Injury. *Nature Medicine*, 15, 1377–1383.
- Lambert, C., Poyart, C., Gruss, A., & Fouet, A. (2022). FabT, a Bacterial Transcriptional Repressor That Limits Futile Fatty Acid Biosynthesis. *Microbiology and Molecular Biology Reviews*, 86, 1–15.
- Leaver, M., Domínguez-Cuevas, P., Coxhead, J. M., Daniel, R. A., & Errington, J. (2009). Life Without a Wall or Division Machine in *Bacillus subtilis*. *Nature*, 457, 849–853.
- Lee, I.-G., Song, C., Yang, S., Jeon, H., Park, J., Yoon, H.-J., ... Lee, B.-J. (2022). Structural and Functional Analysis of the D-Alanyl Carrier Protein Ligase DltA from *Staphylococcus aureus* Mu50. *Acta Crystallographica Section D: Structural Biology*, 78, 424–434.
- López-Lara, I. M., & Geiger, O. (2017). Bacterial Lipid Diversity. *Biochimica et Biophysica Acta*, 1862, 1287–1299.
- Lopez, M. S., Tan, I. S., Yan, D., Kang, J., McCreary, M., Modrusan, Z., ... Brown, E. J. (2017). Host-Derived Fatty Acids Activate Type VII Secretion in *Staphylococcus aureus*. *PNAS*, 114, 11223–11228.
- Lowy, F. D. (1998). *Staphylococcus aureus* Infections. *The New England Journal of Medicine*, 339, 520–532.
- Lu, D., Wö Rmann B, M. E., Zhang, X., Schneewind, O., Gründling B, A., & Freemont, P. S. (2009). Structure-Based Mechanism of Lipoteichoic Acid Synthesis by *Staphylococcus aureus* LtaS. *PNAS*, 106, 1584–1589.
- Lu, Y. J., Zhang, F., Grimes, K. D., Lee, R. E., & Rock, C. O. (2007). Topology and Active Site of PlsY: The bacterial Acylphosphate:Glycerol-3-Phosphate Acyltransferase. *Journal of Biological Chemistry*, 282, 11339–11346.
- Lu, Y. J., Zhang, Y. M., Grimes, K. D., Qi, J., Lee, R. E., & Rock, C. O. (2006). Acyl-Phosphates Initiate Membrane Phospholipid Synthesis in Gram-Positive Pathogens. *Molecular Cell*, 23, 765–772.
- Mäder, U., Nicolas, P., Depke, M., Pané-Farré, J., Debarbouille, M., van der Kooi-Pol, M. M., ... van Dijl, J. M. (2016). *Staphylococcus aureus* Transcriptome Architecture: From Laboratory to Infection-Mimicking Conditions. *PLoS Genetics*, 12, 1–32.
- Marrakchi, H., Zhang, Y.-M., & Rock, C. O. (2002). Mechanistic Diversity and Regulation of Type II Fatty Acid Synthesis. *Biochemical Society Transactions*, 30, 1050–1055.
- Matsuoka, S., Chiba, M., Tanimura, Y., Hashimoto, M., Hara, H., & Matsumoto, K. (2011). Abnormal Morphology of *Bacillus subtilis* *ugtP* Mutant Cells Lacking glucolipids. *Genes Genet. Syst.*, 88, 295–304.
- Mazmanian, S. K., Liu, G., Jensen, E. R., Lenoy, E., & Schneewind, O. (2000). *Staphylococcus aureus* Sortase Mutants Defective in the Display of Surface Proteins and in the Pathogenesis of Animal Infections. *PNAS*, 97, 5510–5515.
- McCarthy, A. J., & Lindsay, J. A. (2010). Genetic Variation in *Staphylococcus aureus* Surface and Immune Evasion Genes is Lineage Associated: Implications for Vaccine Design and Host-Pathogen Interactions. *BMC Microbiology*, 10.
- Mcdevitt, D., Nanavaty, T., Housh-Pompeo, K., Bell, E., Turner, N., Mcintire, L., ... Höök, M. (1997). Characterization of the Interaction Between the *Staphylococcus aureus* Clumping Factor (ClfA) and Fibrinogen. *European Journal of Biochemistry*, 247, 416–424.
- McMurry, L. M., Oethinger, M., & Levy, S. B. (1998). Triclosan Targets Lipid Synthesis. *Nature*, 394, 531–532.
- Meredith, T. C., Swoboda, J. G., & Walker, S. (2008). Late-Stage Polyribitol Phosphate Wall Teichoic Acid Biosynthesis in *Staphylococcus aureus*. *Journal of Bacteriology*, 190, 3046–3056.
- Merino, N., Toledo-Arana, A., Vergara-Irigaray, M., Valle, J., Solano, C., Calvo, E., ... Lasa, I. (2009). Protein A-Mediated Multicellular Behavior in *Staphylococcus aureus*. *Journal of Bacteriology*, 191, 832–843.
- Miller, D. J., Jerga, A., Rock, C. O., & White, S. W. (2008). Analysis of the *Staphylococcus aureus* DgkB Structure Reveals a Common Catalytic Mechanism for the Soluble Diacylglycerol Kinases. *Structure*, 16, 1036–1046.
- Mindrebo, J. T., Misson, L. E., Johnson, C., Noel, J. P., & Burkart, M. D. (2020). Activity Mapping the Acyl Carrier Protein: Elongating Ketosynthase Interaction in Fatty Acid Biosynthesis. *Biochemistry*, 59, 3626–3638.
- Mohammad, M., Ali, A., Nguyen, M.-T., Götz, F., Pullerits, R., & Jin, T. (2022). *Staphylococcus aureus* Lipoproteins in Infectious Diseases. *Frontiers in Microbiology*, 13, 1–17.
- Moreillon, P., Entenza, J. M., Francioli, P., McDevitt, D., Foster, T. J., Francois, P., & Vaudaux, P. (1995). Role of *Staphylococcus aureus* Coagulase and Clumping Factor in Pathogenesis of Experimental Endocarditis. *Infection and Immunity*, 63, 4738–4743.
- Morvan, C., Halpern, D., Kénanian, G., Hays, C., Anba-Mondoloni, J., Brinster, S., ... Gruss, A. (2016). Environmental Fatty Acids Enable Emergence of Infectious *Staphylococcus aureus* Resistant to FASII-Targeted Antimicrobials. *Nature Communications*, 7.

- Morvan, C., Halpern, D., Kénanian, G., Pathania, A., Anba-Mondoloni, J., Lamberet, G., ... Gloux, K. (2017). The *Staphylococcus aureus* FASII Bypass Escape Route from FASII Inhibitors. *Biochimie*, *141*, 40–46.
- Mulcahy, M. E., Geoghegan, J. A., Monk, I. R., O’Keeffe, K. M., Walsh, E. J., Foster, T. J., & McLoughlin, R. M. (2012). Nasal Colonisation by *Staphylococcus aureus* Depends Upon Clumping Factor B Binding to the Squamous Epithelial Cell Envelope Protein Loricrin. *PLoS Pathogens*, *8*.
- Nahaie, M. R., Goodfellow, M., Minnikin, D. E., & Hajek, V. (1984). Polar Lipid and Isoprenoid Quinone Composition in the Classification of *Staphylococcus*. *Journal of General Microbiology*, *130*, 2427–2437.
- Neuhaus, F. C., & Baddiley, J. (2003). A Continuum of Anionic Charge: Structures and Functions of D-Alanyl-Teichoic Acids in Gram-Positive Bacteria. *Microbiology and Molecular Biology Reviews*, *67*, 686–723.
- Nguyen, M.-T., Peisl, L., Barletta, F., Luqman, A., & Götz, F. (2018). Toll-Like Receptor 2 and Lipoprotein-Like Lipoproteins Enhance *Staphylococcus aureus* Invasion in Epithelial Cells. *Infection and Immunity*, *86*, 1–11.
- Nguyen, M. T., & Götz, F. (2016). Lipoproteins of Gram-Positive Bacteria: Key Players in the Immune Response and Virulence. *Microbiology and Molecular Biology Reviews*, *80*, 891–903.
- Nguyen, M. T., Hanzelmann, D., Härtner, T., Peschel, A., & Götz, F. (2015). Skin-Specific Unsaturated Fatty Acids Boost the *Staphylococcus aureus* Innate Immune Response. *Infection and Immunity*, *84*, 205–215.
- Nguyen, M. T., Matsuo, M., Niemann, S., Herrmann, M., & Götz, F. (2020). Lipoproteins in Gram-Positive Bacteria: Abundance, Function, Fitness. *Frontiers in Microbiology*, *11*, 1–15.
- Nikolic, P., & Mudgil, P. (2023). The Cell Wall, Cell Membrane and Virulence Factors of *Staphylococcus aureus* and Their Role in Antibiotic Resistance. *Microorganisms*, *11*.
- Nikolopoulos, N., Matos, R. C., Courtin, P., Ayala, I., Akherraz, H., Simorre, J. P., ... Grangeasse, C. (2022). DltC Acts as an Interaction Hub for AcpS, DltA and DltB in the Teichoic Acid D-Alanylation Pathway of *Lactiplantibacillus plantarum*. *Scientific Reports*, *12*, 1–17.
- O’Brien, M. M., Walsh, E. J., Massey, R. C., Peacock, S. J., & Foster, T. J. (2002). *Staphylococcus aureus* Clumping Factor B (ClfB) Promotes Adherence to Human Type I Cytokeratin 10: Implications for Nasal Colonization. *Cellular Microbiology*, *4*, 759–770.
- O’Donnell, A. G., Nahaie, M. R., Goodfellow, M., Minnikin, D. E., & Hájek, V. (1985). Numerical Analysis of Fatty Acid Profiles in the Identification of Staphylococci. *Journal of General Microbiology*, *131*, 2023–2033.
- Ogston, A. (1881). Micro-Organisms in Surgical Diseases. *The British Medical Journal*, 369–375.
- Ohniwa, R. L., Kitabayashi, K., & Morikawa, K. (2013). Alternative Cardiolipin Synthase CIs1 Compensates for Stalled CIs2 Function in *Staphylococcus aureus* Under Conditions of Acute Acid Stress. *FEMS Microbiology Letters*, *338*, 141–146.
- Oku, H., & Kaneda, T. (1988). Biosynthesis of Branched-chain Fatty Acids in *Bacillus subtilis*. A Decarboxylase is Essential for Branched-chain Fatty Acid Synthetase. *Journal of Biological Chemistry*, *263*, 18386–18396.
- Oku, Y., Kurokawa, K., Matsuo, M., Yamada, S., Lee, B. L., & Sekimizu, K. (2009). Pleiotropic Roles of Polyglycerolphosphate Synthase of Lipoteichoic Acid in Growth of *Staphylococcus aureus* cells. *Journal of Bacteriology*, *91*, 141–151.
- Otto, M. (2014). *Staphylococcus aureus* Toxins. *Curr Opin Microbiol.*, 32–37.
- Painter, K. L., Krishna, A., Wigneshweraraj, S., & Edwards, A. M. (2014). What Role Does the Quorum-Sensing Accessory Gene Regulator System Play During *Staphylococcus aureus* Bacteremia? *Trends in Microbiology*, *22*, 676–685.
- Parsons, J. B., Broussard, T. C., Bose, J. L., Rosch, J. W., Jackson, P., Subramanian, C., & Rock, C. O. (2014). Identification of a Two-Component Fatty Acid Kinase Responsible for Host Fatty Acid Incorporation by *Staphylococcus aureus*. *PNAS*, *111*, 10532–10537.
- Parsons, J. B., Frank, M. W., Jackson, P., Subramanian, C., & Rock, C. O. (2014). Incorporation of Extracellular Fatty Acids by a Fatty Acid Kinase-Dependent Pathway in *Staphylococcus aureus*. *Molecular Microbiology*, *92*, 234–245.
- Parsons, J. B., Frank, M. W., Subramanian, C., Saenkham, P., & Rock, C. O. (2011). Metabolic Basis for the Differential Susceptibility Of Gram-Positive Pathogens To Fatty Acid Synthesis Inhibitors. *PNAS*, *108*, 15378–15383.
- Parsons, J. B., & Rock, C. O. (2011). Is bacterial Fatty Acid Synthesis a Valid Target for Antibacterial Drug Discovery? *Current Opinion in Microbiology*, *14*, 544–549.
- Parsons, J. B., Yao, J., Frank, M. W., Jackson, P., & Rock, C. O. (2012). Membrane Disruption by Antimicrobial Fatty Acids Releases Low- Molecular-Weight Proteins from *Staphylococcus aureus*. *Journal of Bacteriology*, *194*, 5294–5304.
- Pathania, A., Anba-Mondoloni, J., Gominet, M., Halpern, D., Dairou, J., Dupont, L., ... Gruss, A. (2021). (P)ppGpp/GTP and Malonyl-coA modulate *Staphylococcus aureus* Adaptation to FASII Antibiotics and Provide a Basis for Synergistic Bi-Therapy. *MBio*, *12*, 1–15.
- Percy, M. G., & Gründling, A. (2014). Lipoteichoic Acid Synthesis and Function in Gram-Positive Bacteria. *Annual Review of Microbiology*, *68*, 81–100.
- Perego, M., Glaser, P., Minutello, A., Strauch, M. A., Leopold, K., & Fischer, W. (1995). Incorporation of D-alanine

- into Lipoteichoic Acid And Wall Teichoic Acid in *Bacillus subtilis*: Identification of genes and regulation. *Journal of Biological Chemistry*, 270, 15598–15606.
- Peschel, A., Jack, R. W., Otto, M., Collins, L. V., Staubitz, P., Nicholson, G., ... Van Strijp, J. A. G. (2001). *Staphylococcus aureus* Resistance to Human Defensins and Evasion of Neutrophil Killing Via the Novel Virulence Factor MprF Is Based on Modification of Membrane Lipids with L-lysine. *J. Exp. Med.*, 193, 1067–1076.
- Peschel, A., Otto, M., Jack, R. W., Kalbacher, H., Jung, G., & Gotz, F. (1999). Inactivation of the *dlt* Operon in *Staphylococcus aureus* Confers Sensitivity to Defensins, Protegrins, and Other Antimicrobial Peptides. *The Journal of Biological Chemistry*, 274, 8405–8410.
- Prescott, D. J., & Vagelos, P. R. (1972). Acyl Carrier Protein. *Advances in Enzymology and Related Areas of Molecular Biology*, 36, 269–311.
- Qiu, X., Choudhry, A. E., Janson, C. A., Grooms, M., Daines, R. A., Lonsdale, J. T., & Khandekar, S. S. (2005). Crystal Structure and Substrate Specificity of The β -Ketoacyl-Acyl Carrier Protein Synthase III (FabH) from *Staphylococcus aureus*. *Protein Science*, 14, 2087–2094.
- Rasheed, N. A., Hussein, N. R., Region, K., Polytechnic, D., & Region, K. (2021). *Staphylococcus aureus*: An Overview of Discovery, Characteristics, Epidemiology, Virulence Factors and Antimicrobial Sensitivity. *European Journal of Molecular & Clinical Medicine*, 08, 1160–1183.
- Rasigade, J. P., & Vandenesch, F. (2014). *Staphylococcus aureus*: A Pathogen with Still Unresolved Issues. *Infection, Genetics and Evolution*, 21, 510–514.
- Recsei, P., Kreiswirth, B., O'Reilly, M., Schlievert, P., Gruss, A., & Novick, R. P. (1986). Regulation of Exoprotein Gene Expression in *Staphylococcus aureus* by *agr*. *Mol Gen Genet*, 202, 58–61.
- Reichmann, N. T., Cassona, C. P., & Gründling, A. (2013). Revised Mechanism of D-Alanine Incorporation Into Cell Wall Polymers in Gram-Positive Bacteria. *Microbiology (United Kingdom)*, 159, 1868–1877.
- Reichmann, N. T., Piçarra Cassona, C., Monteiro, J. M., Bottomley, A. L., Corrigan, R. M., Foster, S. J., ... Gründling, A. (2014). Differential Localization of LTA Synthesis Proteins and Their Interaction With The Cell Division Machinery in *Staphylococcus aureus*. *Molecular Microbiology*, 92, 273–286.
- Renner, L. D., & Weibel, D. B. (2011). Cardiolipin Microdomains Localize to Negatively Curved Regions of *Escherichia coli* Membranes. *PNAS*, 108, 6264–6269.
- Ridley, M. (1959). Perineal Carriage of *Staph. aureus*. *British Medical Journal*, 270–273.
- Salgado-Pabón, W., & Schlievert, P. M. (2014). Models Matter: The Search for an Effective *Staphylococcus aureus* vaccine. *Nature Reviews Microbiology*, 12, 585–591.
- Santa Maria, J. P., Sadaka, A., Moussa, S. H., Brown, S., Zhang, Y. J., Rubin, E. J., ... Walker, S. (2014). Compound-Gene Interaction Mapping Reveals Distinct Roles for *Staphylococcus aureus* Teichoic Acids. *PNAS*, 111, 12510–12515.
- Sastre, D. E., Pulschen, A. A., Basso, L. G. M., Benites Pariente, J. S., Marques Netto, C. G. C., Machinandiarena, F., ... Gueiros-Filho, F. J. (2020). The Phosphatidic Acid Pathway Enzyme Plsx Plays Both Catalytic and Channeling Roles in Bacterial Phospholipid Synthesis. *Journal of Biological Chemistry*, 295, 2148–2159.
- Sautrey, G., El Khoury, M., Giro Dos Santos, A., Zimmermann, L., Deleu, M., Lins, L., ... Mingeot-Leclercq, M. P. (2016). Negatively Charged Lipids as a Potential Target for New Amphiphilic Aminoglycoside Antibiotics: A biophysical study. *Journal of Biological Chemistry*, 291, 13864–13874.
- Schiebel, J., Chang, A., Lu, H., Baxter, M. V., Tonge, P. J., & Kisker, C. (2012). *Staphylococcus aureus* FabI: Inhibition, Substrate Recognition and Potential Implications for In Vivo Essentiality. *Structure*, 20, 802–813.
- Schirner, K., Marles-Wright, J., Lewis, R. J., & Errington, J. (2009). Distinct and Essential Morphogenic Functions for Wall- and Lipo-Teichoic Acids in *Bacillus subtilis*. *EMBO Journal*, 28, 830–842.
- Schneewind, O., & Missiakas, D. (2019). Lipoteichoic Acid Synthesis and Function in Gram-Positive Bacteria. In *Hydrocarbon and Lipid Microbiology*.
- Schuster, C. F., Bellows, L. E., Tosi, T., Campeotto, I., Corrigan, R. M., Freemont, P., & Gründling, A. (2016). The Second Messenger C-Di-AMP Inhibits the Osmolyte Uptake System OpuC in *Staphylococcus aureus*. *Sci Signal*, 9, 1–31.
- Schuster, C. F., Mechler, L., Nolle, N., Krismer, B., Zelder, M.-E., Götz, F., & Bertram, R. (2015). The MazEF Toxin-Antitoxin System Alters the β -Lactam Susceptibility of *Staphylococcus aureus*. *PLoS ONE*, 10, 1–22.
- Schwandner, R., Dziarski, R., Wesche, H., Rothe, M., & Kirschning, C. J. (1999). Peptidoglycan- and Lipoteichoic Acid-induced Cell Activation is Mediated by Toll-like. *Journal of Biological Chemistry*, 274, 17406–17409.
- Sen, S., Sirobhusanam, S., Johnson, S. R., Song, Y., Tefft, R., Gatto, C., & Wilkinson, B. J. (2016). Growth-Environment Dependent Modulation of *Staphylococcus aureus* Branched-Chain to Straight-Chain Fatty Acid Ratio and Incorporation of Unsaturated Fatty Acids. *PLoS ONE*, 11, 1–17.
- Seo, H. S., Michalek, S. M., & Nahm, M. H. (2008). Lipoteichoic Acid is Important in Innate Immune Responses to Gram-Positive Bacteria. *Infection and Immunity*, 76, 206–213.
- Shahmirzadi, S. V., Nguyen, M. T., & Götz, F. (2016). Evaluation of *Staphylococcus aureus* Lipoproteins: Role in Nutritional Acquisition and Pathogenicity. *Frontiers in Microbiology*, 7, 1–15.
- Sheen, T. R., Ebrahimi, C. M., Hiemstra, I. H., Barlow, S. B., Peschel, A., & Doran, K. S. (2010). Penetration of the

- Blood – Brain Barrier by *Staphylococcus aureus*: Contribution of Membrane-Anchored Lipoteichoic Acid. *J Mol Med*, 88, 633–639.
- Short, S. A., & White, D. C. (1972). Biosynthesis of Cardiolipin from Phosphatidylglycerol in *Staphylococcus aureus*. *Journal of Bacteriology*, 109, 820–826.
- Shryock, T. R., & Kapral, F. A. (1992). The Production of Bactericidal Fatty Acids from Glycerides in Staphylococcal Abscesses. *J. Med. Microbio*, 36, 288–292.
- Staubitz, P., Neumann, H., Schneider, T., Wiedemann, I., & Peschel, A. (2004). MprF-Mediated Biosynthesis of Lysylphosphatidylglycerol, an Important Determinant in Staphylococcal Defensin Resistance. *FEMS Microbiology Letters*, 231, 67–71.
- Stoll, H., Dengjel, J., Nerz, C., & Götz, F. (2005). *Staphylococcus aureus* Deficient in Lipidation of Prelipoproteins is Attenuated in Growth and Immune Activation. *Infection and Immunity*, 73, 2411–2423.
- Suginaka, H., Shimatani, M., Ohno, Y., & Yano, I. (1979). Effects of Bacterial Lipids and Lipoteichoic Acid on Extracellular Antilysin Activity from *Staphylococcus aureus*. *FEMS Microbiology Letters*, 353–355.
- Sun, Y., & O’Riordan, M. X. D. (2010). Branched-Chain Fatty Acids Promote *Listeria monocytogenes* Intracellular Infection and Virulence. *Infection and Immunity*, 78, 4667–4673.
- Tam, K., & Torres, V. J. (2019). *Staphylococcus aureus* Secreted Toxins and Extracellular Enzymes. *Microbiology Spectrum*, 7, 1–34.
- Tiwari, K. B., Gatto, C., & Wilkinson, B. J. (2018). Interrelationships Among Fatty Acid Composition, Staphyloxanthin Content, Fluidity, and Carbon Flow in the *Staphylococcus aureus* Membrane. *Molecules*, 23, 3–5.
- Tong, S., Lin, Y., Lu, S., Wang, M., Bogdanov, M., & Zheng, L. (2016). Structural Insight Into Substrate Selection and Catalysis Of Lipid Phosphate Phosphatase PgpB in the Cell Membrane. *Journal of Biological Chemistry*, 291, 18342–18352.
- Tosi, T., Hoshiga, F., Millership, C., Singh, R., Eldrid, C., Patin, D., ... Gründling, A. (2019). Inhibition of the *Staphylococcus aureus* C-Di-AMP Cyclase DacA by Direct Interaction With The Phosphoglucosamine Mutase GlmM. *PLoS Pathogens*, 15, 1–28.
- Traber, K. E., Lee, E., Benson, S., Corrigan, R., Cantera, M., Shopsis, B., & Novick, R. P. (2008). *agr* Function in Clinical *Staphylococcus aureus* Isolates. *Microbiology*, 154, 2265–2274.
- Tsai, M., Ohniwa, R. L., Kato, Y., Takeshita, S. L., Ohta, T., Saito, S., ... Morikawa, K. (2011). *Staphylococcus aureus* Requires Cardiolipin for Survival Under Conditions Of High Salinity. *BMC Microbiology*, 11.
- Tsuji, B. T., Harigaya, Y., Lesse, A. J., Forrest, A., & Ngo, D. (2013). Activity of AFN-1252, a Novel FabI Inhibitor, Against *Staphylococcus aureus* in an In Vitro Pharmacodynamic Model Simulating Human Pharmacokinetics. *Journal of Chemotherapy*, 25, 32–35.
- Tuchscher, L., Löffler, B., Buzzola, F. R., & Sordelli, D. O. (2010). *Staphylococcus aureus* Adaptation to the Host and Persistence: Role of Loss of Capsular Polysaccharide Expression. *Future Microbiol*, 5, 1823–1832.
- Turner, N. A., Sharma-Kuinkel, B. K., Maskarinec, S. A., Eichenberger, E. M., Shah, P. P., Carugati, M., ... Fowler, V. G. (2019). Methicillin-Resistant *Staphylococcus aureus*: an Overview of Band Clinical Research. *Nature Reviews Microbiology*, 17, 203–218.
- Uehara, Y. (2022). *Current Status of Staphylococcal Cassette*. 1–12.
- van Dijk, M. C., de Kruijff, R. M., & Hagedoorn, P. L. (2022). The Role of Iron in *Staphylococcus aureus* Infection and Human Disease: A Metal Tug of War at the Host—Microbe Interface. *Frontiers in Cell and Developmental Biology*, 10, 1–7.
- Van Mooy, B. A. S., Rocap, G., Fredricks, H. F., Evans, C. T., & Devol, A. H. (2006). Sulfolipids Dramatically Decrease Phosphorus Demand by Picocyanobacteria in Oligotrophic Earine environments. *PNAS*, 103, 8607–8612.
- Vandenesch, F., Lina, G., & Henry, T. (2012). *Staphylococcus aureus* Hemolysins, Bi-Component Leukocidins , and Cytolytic Peptides: a Redundant Arsenal of Membrane-Damaging Virulence Factors? *Frontiers in Cellular and Infection Microbiology*, 2, 1–15.
- Vickery, C. R., Wood, B. M. K., Morris, H. G., Losick, R., & Walker, S. (2018). Reconstitution of *Staphylococcus aureus* Lipoteichoic Acid Synthase Activity Identifies Congo Red as a Selective Inhibitor. *Journal of the American Chemical Society*, 140, 876–879.
- Volkman, B. F., Zhang, Q., Debatov, D. V., Rivera, E., Kresheck, G. C., & Neuhaus, F. C. (2001). Biosynthesis of D-Alanyl-Lipoteichoic Acid: The Tertiary Structure Of Apo-D-Alanyl Carrier Protein. *Biochemistry*, 40, 7964–7972.
- Wang, J., Soisson, S. M., Young, K., Shoop, W., Kodali, S., Galgoci, A., ... Singh, S. B. (2006). Platensimycin is a Selective FabF Inhibitor With Potent Antibiotic Properties. *Nature*, 441, 358–361.
- Webb, A. J., Karatsa-dodgson, M., & Gründling, A. (2009). Two-Enzyme Systems for Glycolipid and Polyglycerolphosphate Lipoteichoic Acid Synthesis in *Listeria monocytogenes*. *Molecular Microbiology*, 74, 299–314.
- Weidenmaier, C., & Peschel, A. (2008, April). Teichoic Acids and Related Cell-Wall Glycopolymers in Gram-positive Physiology and Host Interactions. *Nature Reviews Microbiology*, Vol. 6, pp. 276–287.
- Weidenmaier, C., Peschel, A., Kempf, V. A. J., Lucindo, N., Yeaman, M. R., & Bayer, A. S. (2005). DltABCD- and MprF-

- Mediated Cell Envelope Modifications of *Staphylococcus aureus* Confer Resistance to Platelet Microbicidal Proteins and Contribute to Virulence in a Rabbit Endocarditis Model. *Infection and Immunity*, 73, 8033–8038.
- Wille, J. J., & Kydonieus, A. (2003). Palmitoleic Acid Isomer (C16:1 Δ 6) in Human Skin Sebum is Effective against Gram-Positive Bacteria. *Skin Pharmacology and Applied Skin Physiology*, 16, 176–187.
- Williams, R. E. O. (1963). Healthy Carriage of *Staphylococcus aureus*: Its Prevalence and Importance. *Bacteriological Reviews*, 27, 56–71.
- Wörmann, M. E., Corrigan, R. M., Simpson, P. J., Matthews, S. J., & Gründling, A. (2011). Enzymatic Activities and Functional Interdependencies of *Bacillus subtilis* Lipoteichoic Acid Synthesis Enzymes. *Molecular Microbiology*, 79, 566–583.
- Wu, M., Huang, S., Du, J., Li, Y., Jiang, S., Zhan, L., & Huang, X. (2022). D-alanylation of Lipoteichoic Acid Contributes to Biofilm Formation and Acidogenesis Capacity of *Streptococcus mutans*. *Microbial Pathogenesis*, 169.
- Xia, G., Kohler, T., & Peschel, A. (2010, February). The Wall Teichoic Acid and Lipoteichoic Acid Polymers of *Staphylococcus aureus*. *International Journal of Medical Microbiology*, Vol. 300, pp. 148–154.
- Yao, J., & Rock, C. O. (2016). Resistance Mechanisms and the Future of Bacterial Enoyl-Acyl Carrier Protein Reductase (FabI) Antibiotics. *Cold Spring Harbor Perspectives in Medicine*, 6, 1–11.
- Yao, J., & Rock, C. O. (2017). Exogenous Fatty Acid Metabolism in Bacteria. *Biochimie*, 141, 30–39.
- Yeo, W., Dyzenhaus, S., Torres, V. J., Brinsmade, S. R., & Bae, T. (2023). Regulation of Bacterial Two-Component Systems by Cardiolipin. *Infection and Immunity*, 91, 1–12.
- Yueh, M.-F., Taniguchi, K., Chen, S., Evan, R. M., Hammock, B. D., Karin, M., & Tukey, R. H. (2014). The Commonly Used Antimicrobial Additive Triclosan is a Liver Tumor Promoter. *PNAS*, 111, 17200–17205.
- Zeden, M. S., Gallagher, L. A., Bueno, E., Nolan, A. C., Ahn, J., Shinde, D., ... O’Gara, J. P. (2023). Metabolic Reprogramming And Altered Cell Envelope Characteristics In a Pentose Phosphate Pathway Mutant Increases MRSA Resistance to β -lactam Antibiotics. *PLoS Pathogens*, 19, 1–35.
- Zeng, Z., Surewaard, B. G. J., Wong, C. H. Y., Geoghegan, J. A., Jenne, C. N., & Kubes, P. (2016). CRIg Functions as a Macrophage Pattern Recognition Receptor to Directly Bind and Capture Blood-Borne Gram-Positive Bacteria. *Cell Host and Microbe*, 20, 99–106.
- Zhang, B., Liu, X., Lambert, E., Mas, G., Hiller, S., Veening, J. W., & Perez, C. (2020). Structure of a Proton-Dependent Lipid Transporter Involved in Lipoteichoic Acids Biosynthesis. *Nat Struct Mol Biol*, 27, 561–569.
- Zhang, Y. M., & Rock, C. O. (2008). Membrane Lipid Homeostasis in Bacteria. *Nature Reviews Microbiology*, 6, 222–233.
- Zhou, B., Pathania, A., Pant, D., Halpern, D., Gaudu, P., Trieu-Cuot, P., ... Gloux, K. (2023). *Staphylococcus aureus* Prophages are Gatekeepers of Lipid Utilization to Control Membrane Plasticity, Bacterial Fitness, and Antibiotic Adaptation. *BioRxiv*.
- Zhou, C., & Fey, P. D. (2020). The Acid Response Network of *Staphylococcus aureus*. *Curr Opin Microbiol.*, 55, 67–73.
- Zhu, K., Bayles, D. O., Xiong, A., Jayaswal, R. K., & Wilkinson, B. J. (2005). Precursor and Temperature Modulation of Fatty Acid Composition and Growth of *Listeria monocytogenes* Cold-sensitive Mutants with Transposon-interrupted Branched-chain α -keto Acid Dehydrogenase. *Microbiology*, 151, 615–623.

ANNEX

Manuscript III:

Gain-of-function mutations in FabF or in an acyl-CoA thioesterase rescue growth of *plsX* deletion mutants of *Staphylococcus aureus*

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This chapter will be included as part of a Research Article.

Gain-of-function mutations in FabF or in an acyl-CoA thioesterase rescue growth of *plsX* deletion mutants of *Staphylococcus aureus*

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Keywords : *Staphylococcus aureus*, fatty acid, FASII, phospholipid synthesis, *plsX* deletion suppressors, FabF, acyl-CoA thioesterase, acyl-ACP thioesterase.

Abstract

PlsX is a reversible enzyme that converts acyl-acyl carrier protein (acyl-ACP) to acyl-PO₄. This enzyme was considered essential for phospholipid synthesis in the major pathogen *Staphylococcus aureus*. However, growth of a *plsX* deletion strain was rescued by adding exogenous fatty acids (eFAs), which are phosphorylated to acyl-PO₄ by the Fak fatty acid kinase. Here, we generated *plsX* deletion mutants in two *S. aureus* backgrounds. As expected, both $\Delta plsX$ strains failed to grow in non-FA-containing medium. However, we discovered two classes of suppressor mutants of the $\Delta plsX$ strain that emerged on solid medium lacking FAs: First, a specific point mutation in *fabF* encoded a FabF^{A119E} mutant. Second, several mutations mapped to a putative acyl-CoA thioesterase (named FadM). Membrane FAs derived from FabF^{A119E} were shorter, whereas those from *fadM* suppressor strains were similar to those of the parental strains. The $\Delta plsX$ suppressor strains both grew more slowly than an RN4220-derivative parent, but grew normally in a USA300-JE2 background. Evidence is given that both FabF and FadM suppressor loci lead to the release of FA from acyl-ACP substrates, by different mechanisms, *i.e.*, respectively leaky activity and greater substrate permissivity. We hypothesize that FAs are released by leaky activities of the $\Delta plsX$ suppressor loci, and are then phosphorylated by Fak to produce acyl-PO₄, the PlsX product. Unlike the WT parents, neither the $\Delta plsX$ strain, nor its suppressors grew in the presence of an anti-FASII, indicating that FASII activity is essential in the $\Delta plsX$ background.

Introduction

PlsX, a glycerol-3-phosphate acyltransferase, is the first dedicated enzyme of the phospholipid synthesis pathway in Firmicute bacteria. This membrane-associated enzyme produces acyl-phosphate (Acyl-PO₄) from acyl-acyl carrier protein (acyl-ACP), thus linking FASII to phospholipid synthesis. Acyl-PO₄ is used by the integral membrane protein PlsY to attach the acyl group to the glycerophosphate backbone at "position 1". But being a reversible enzyme, PlsX also produces acyl-ACP from an acyl-PO₄. This "backwards" reaction is useful, for example, in conditions where exogenous fatty acids (eFAs) are available, as in the lipid-rich host. eFAs are phosphorylated by the fatty acid kinase (Fak) to produce an acyl-PO₄ (Parsons et al., 2014a). This product could then be used by PlsY for phospholipid synthesis as above, or it could be a substrate for the reverse PlsX reaction to produce acyl-ACP, which then may either reenter the FASII pathway, or be used by PlsC to complete synthesis of phosphatidic acid (**Fig. 1**). These complex events rely on PlsX activity (**Fig. 1**). Interestingly, studies of PlsX in *Bacillus subtilis* indicate that it is a conditionally membrane-bound protein, which may contribute to its reversibility (Sastre et al., 2020).

Previous studies in *S. aureus* showed that despite its importance, PlsX can be deleted when eFAs are supplied. In this case, acyl-PO₄ is produced from eFAs by Fak, which compensates the absence of PlsX (Parsons et al., 2014a; Parsons et al., 2014b). In *Streptococcus pneumoniae*, *plsX* deletion is achieved without adding eFAs, thanks to the presence of TesS (or FatA; SP1408), an acyl-ACP thioesterase that liberates FAs, which are then phosphorylated by Fak (Parsons et al., 2015). Since *S. aureus* did not encode an acyl-ACP thioesterase to liberate FAs, the source of FAs had to be exogenous (Parsons et al., 2014b).

Using positive genetic selections is a powerful means of uncovering genetic links, or of revealing secondary gain-of-function mutations that compensate genetic loss. Such selections reveal novel networks, as well as previously unknown repurposing capacities of known genes. In the context of our studies on fatty acid metabolism, we constructed *plsX* deletion mutants in two *S. aureus* backgrounds. We show that growth of the $\Delta plsX$ mutants on solid medium lacking eFAs gives rise to suppressors. The suppressor loci are identified and map to 2 alternative genes, each unrelated to PlsX. Suppressor phenotypes are characterized, and models are presented to explain their compensatory roles. Both appear to liberate free FAs at different steps, which would then be phosphorylated by Fak to compensate *plsX* deletion.

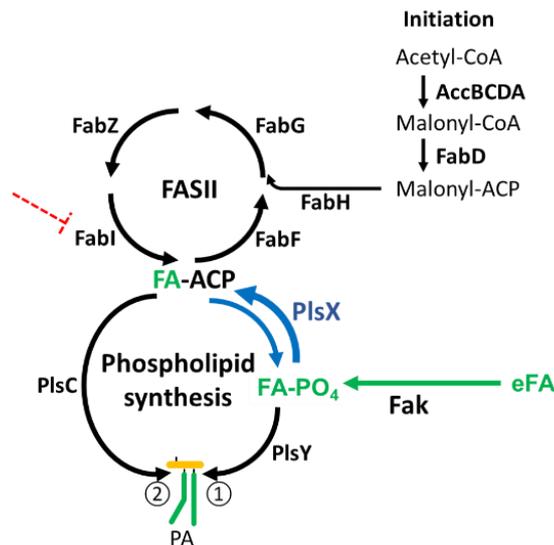


Fig. 1: Dual functional role of PlsX, a reversible enzyme, in phospholipid synthesis.

PlsX (in blue) uses acyl-ACP (FA-ACP) as a substrate to produce acyl-PO₄, which in turn is a substrate for PlsY to join the acyl group to a glycerol phosphate backbone in position 1. This product, called lysophosphatidic acid, is a substrate for PlsC, which then uses FA-ACP to add an acyl group at position 2, generating phosphatidic acid (PA). In the presence of eFAs (in green), PlsX can display reverse activity: eFA phosphorylated by Fak produces FA-ACP. In the presence of a FASII inhibitor (dashed red T), eFA is the sole source of FAs, and FA-PO₄ is used i- as a substrate for PlsY, and ii- as a substrate for reverse PlsX activity, to produce PA. Note: in a *plsX* mutant, FASII remains active, but charging FAs on position 1 and position 2 for PA synthesis are dissociated.

Materials and methods

Strains and plasmids. The *S. aureus* strains used in this work are USA300_FPR3757 (designated JE2; (Fey et al., 2013)) and RN4220-R (called here RN-R; (Pathania et al., 2021)). RN-R is a readily transformable strain derived from RN4220, of the NCTC 8325 line (Herbert et al., 2010). It was repaired to restore a functional *fakB1* gene, which is defective in the entire NCTC 8325 lineage (Pathania et al., 2021)). $\Delta plsX$ mutants were grown on BHI medium containing C18:1 fatty acid at a concentration of 0.25 mM (Larodan, Sweden). Parental strains and $\Delta plsX$ -suppressor mutants were grown in BHI medium (Brain Heart Infusion; Gibco, France) at 37°C with shaking.

***plsX* mutant construction.** The $\Delta plsX$ mutants were constructed in RN-R (RN4220 repaired for a defective *fakB1* gene in the NCTC8325 lineage; (Pathania et al., 2021), and USA300 JE2 backgrounds. The preparatory plasmid for *plsX* deletion was constructed using the Gibson assembly method to insert DNA segments flanking the *plsX orf* into Sma1-linearized pMAD, a thermosensitive plasmid (Arnaud et al., 2004; Biswas et al., 1993). Oligos for $\Delta plsX$ construction are in **Table S1**. Constructions were verified by DNA sequencing of PCR-

amplified fragments. The double cross-over gene replacements were obtained as described in RN-R and JE2 strains. The resultant strains were submitted to whole genome sequencing (Eurofins, Germany). Sequences will be submitted to the EBI database.

Cloning and complementation of the $\Delta plsX$ mutant in RN-R and JE2 strains. To confirm that strain characteristics were due to the *plsX* deletion, the intact *plsX* gene was cloned on a multicopy plasmid (pIMAY derivative kindly provided by P. Bouloc, I2BC, France (Coronel-Tellez et al., 2022) Cloning was performed by Gibson assembly (**Table S1**).

Table S1. Oligonucleotides used for chromosomal and plasmid constructions*.

Plasmid for $\Delta plsX$ construction on pMAD linearized by SmaI		
Oligonucleotides	sequence 5' to 3'	
pMAD_FapR_F	TCTATCGATGCATGCCATGGTACCCCTTCATCACAGACCATGAACTAAGC	FapR fragment
FapR_FabD_R	CGGAAAAATAATTGCTGTTTTACTCATAATTTTATCCTCGCTTATCATAAAAC	
FapR_FabD_F	GTTTTATGATAAGCGAGGATAAAATTATGAGTAAACAGCAATTATTTTTCCG	FabD fragment
FabD_pMAD_R	GCAGAAGCTTCTAGAATTCGAGCTCCCTGTGACCTGAAACAACAATTTGACC	
Verifications for pMAD constructions		
pMAD_forward oligo	GTTACGTTACACATTAAGTAGACAG	
pMAD_reverse oligo	TGGACAGCATGGCCTGCAACGC	
Cloning <i>plsX</i> on pIMAY-derived locus1 plasmid (kindly provided by P. Bouloc, lab collection)		
Oligonucleotides	sequence 5' to 3'	
pIMAY_FapR_F	AGTTTGTGGTTAATGGGGTTGACACTTGAACATTTTAAA	FapR fragment
FapR_plsX_R	TCAATTGCTAATTTAACCATTTTTAGTACCTAGTCTTAA	
FapR_plsX_F	TTAAGACTAGGTAATAAAAAATGGTTAAATTAGCAATTGA	<i>plsX</i> fragment
<i>plsX</i> _pIMAY_R	TTGTTGCTATAATGAAATGCGTTCCTACTCATTGATTCACCTA	
pIMAY_FapR_R	TTTAAATAGTTCAAGTGCAACCCCATTAACCACAAACT	pIMAY linearized by reverse PCR
<i>plsX</i> _pIMAY_F	TAGGTGAATCAAATGAGTAAGAACGCATTTTCATTATAGCAACAA	

* Chromosomal mutations and plasmids were established in RN-R and USA300 JE2 strains.

Isolation of suppressor mutants. JE2 $\Delta plsX$ and RN-R $\Delta plsX$ strains were first streaked on solid BHI medium containing C18:1, an FA that promotes growth. For each strain, a single colony was then used to inoculate a liquid culture (in BHI+C18:1). When the culture reached exponential phase, it was diluted to $OD_{600} = 0.1$. Serial dilutions were plated on solid medium to obtain $\sim 10^5$ to $\sim 10^7$ colony forming units per plate on solid BHI medium without FAs. Colonies were scored after two or more days of incubation at 37°C, and selected at random for restreaking on solid BHI medium. Liquid cultures were prepared in BHI, and glycerol was added at a final concentration of 20% for storage at -70°C. The stored mutant clones were analyzed by DNA sequencing to identify mutated loci.

PCR amplification and whole genome sequencing. Selected candidate colonies were suspended in 100 μ L of 0.9% NaCl with 1 μ L of lysostaphin from a 5 mg/mL stock. Tubes con-

taining suspensions are shaken for 45 min at 37°C. Glass beads are then added and samples are shaken at high speed for 1 min using FastPrep (FP120; MP Biomedical). Primers ((reverse and forward respectively) used for PCR amplification of **1-** *fabF* DNA: 5' GGAATCAGCTAGAGAACGC 3' and 5' CATTTCACACCCACAAGAGG 3'; **2-** SAUSA300_0150 (designated 0150): 5' GGTCCTTGTCGATACCTATCA 3' and 5' TTGGCAGTAATTGACTGAAC 3'; **3-** *csa1A* DNA: 5' GAT-TATCCTGAGCTACTTCG 3' and 5' CGTGGCCTGGATATGATGAT 3'; **4-** thioesterase gene (SAUSA_1247 or SAOUHSC_01348): 5' ATCTTCACGCTAATTACATT 3' and 5' AATCGGTATTTGAGGCAC 3'. PCR reactions were carried out in a SimpliAmp Thermal Cycler (ThermoFisher Scientific, France) as follows: 30 s at 98°C, 30 cycles consisting of 10 s at 98°C, 30 s at 48°C, 1 min 30 s at 72°C, and 4 min at 72°C. PCR products were analyzed on a 0.8% agarose gel prepared in Tris (0.02M) EDTA (0.5 mM) acetate (0.01M) containing 0.5 µg/mL ethidium bromide. Gels were run at 100 V for 33 min in an electrophoresis chamber (Mupid ONE; Dutscher, France), and photographed under UV light using the EBOX VX5 imaging system (Vilber Lourmat, France). PCR samples were purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, France). Samples were then sent for DNA sequencing (Eurofins Genomics, France).

Genomic DNA preparation for whole genome sequencing. Bacterial cell pellets, prepared from 3 mL liquid exponential phase cultures, were centrifuged at 8,000 rpm for 5 min. Pellets were re-suspended in 800 µL TE buffer (10 mM Tris- HC1, pH 8.0/1 mM EDTA) plus 20 µL lysostaphin 5 mg/mL, and incubated for 45 min at 37°C. We then added 2.7 µL RNAse (40 mg/mL stock) and samples were incubated again at 37°C for 1 hr. 34 µL 20% SDS and 4.3 µL proteinase K were added, and tubes were incubated 1 h at 55°C. 150 µL of 5 M NaCl is then added and gently mixed, followed by addition of 130 µL of CTAB/NaCl (from a stock prepared as 10 g cetrimonium bromide and 4.09 g NaCl in 100 mL H₂O). Tubes were then incubated in a water bath for 20 min at 65°C. 1.14 mL of chloroform:isoamyl alcohol (24:1) was added and the solution was mixed, and then centrifuged 30 min at room temperature. The upper phase was recovered, and 850 µL isopropanol was added. The tube was inverted several times for gentle mixing, and the solution was centrifuged at 13,000 g for 30 min at 4°C. The supernatant is discarded and 100 µL of 10 mM Tris buffer pH 7 was added to resuspend the DNA pellet.

Growth kinetics. 3 mL precultures were prepared in BHI for parental and suppressor strains, or in BHI containing 7.5 μ L C18:1 for Δ *pIsX* strains. After overnight growth, OD₆₀₀ measurements were determined, and cultures were diluted in BHI medium \pm C18:1 to start growth at OD₆₀₀ = 0.05. Growth was monitored by OD₆₀₀ measurements every two hours.

Determination of fatty acid composition. The equivalent of OD₆₀₀ = 2 of bacterial cultures was used for extractions. Bacterial cultures were centrifuged, and pellets were washed once in 700 μ L of 0.02% Triton in 0.9% NaCl, then twice with 700 μ L of 0.9% NaCl. FA extractions were performed as described (Kenanian et al., 2019). Briefly, esterification was performed using sodium methanolate at 0.3% NaCl. Extracts were analyzed by gas chromatography (AutoSystem XL gas chromatograph; Perkin-Elmer) with a ZB-Wax capillary column (30 m x 0.25 mm x 0.25 mm; Phenomenex, France) in split-splitless injection mode. The program used to record and process the data was TotalChrom Workstation (Perkin-Elmer). *S. aureus* FA peaks were identified using purified esterified FA standards.

Detection of acyl carrier protein (ACP). Pellets from OD₆₀₀=10 equivalent of bacterial cultures were collected by centrifugation (8000 rpm for 5 min). They were then washed twice with 0.1% PBS (Phosphate Buffer Saline)-Triton X100, then washed once with PBS. The pellets are then re-suspended in 600 μ L PBS supplemented with 6 μ L antiprotease (100X), 50 μ g/mL lysostaphin and 50 μ g/mL DNase. Samples were then shaken for 25 min at 37°C. Protein concentrations were determined as described (Bradford, 1976).

Protein extracts (10 μ g) were loaded onto non-denaturing gels containing 2.5 M urea and 17% acrylamide gel prepared as described (Morvan et al., 2016). The separated proteins were transferred to a PVDF membrane (Biorad, France) by electroblotting using a power blotter semi-dry transfer system (Invitrogen, France). The primary anti-ACP antibody (Morvan et al., 2016) was used at 1:500 dilution and secondary anti-rabbit IgG conjugated with peroxidase at a 1:5000 dilution (Covalabs). Revelation of ACP-reactive bands was done using the ECF substrate (Perkin Elmer, France) and the fluorescent signal recorded using a ChemiDoc (Biorad, France). Protein transfer is realized using a current of 25 V and 1.3 A Power Blotting Station (Invitrogen) during 7 min. The membrane is incubated with 1/1500 anti-ACP antibody then with goat anti-rabbit IgG coupled to horseradish peroxidase (Invitrogen). The membrane is impregnated for 1 min in luminescence reagent (Western Lightning Pro, Chemiluminescent Substrate, Perkin Elmer). Membranes were photographed using ChemiDoc (Biorad) and analyzed using Image Lab 5.0 program.

FASII bypass growth experiments. Strains are grown overnight in liquid SerFA medium (SerFA, BHI containing 10 % newborn calf serum and a mixture of free fatty acids, an equimolar mixture of 0.17 mM each C14, C16, C18:1 [Larodan, Sweden]). Cultures are then diluted to OD₆₀₀=0.05 in SerFA supplemented or not with the anti-FASII AFN-1252 (here called AFN; (Karlowsky et al., 2009); MedChemExpress, France). Growth was monitored after 16h by OD₆₀₀ measurements.

Results and Discussion

1-Construction and phenotypic confirmation of a Δ *plsX* in-frame mutant. *plsX* is the second gene in a multicistronic operon (**Fig. S1**). We constructed an in-frame Δ *plsX* mutant, which was established in RN-R (an RN4220 strain repaired for a defective *fakB1* gene in the 8325 lineage; (Pathania et al., 2021), and in the USA300 JE2 strain. Both Δ *plsX* mutants were confirmed by full genome sequencing.

To confirm that strain characteristics were due to the *plsX* deletion, the intact *plsX* gene fused upstream to the *fapR* promoter was cloned on a multicopy plasmid (Materials and Methods), and established in RN-R and JE2. The complemented strain displayed normal growth on medium lacking eFAs.

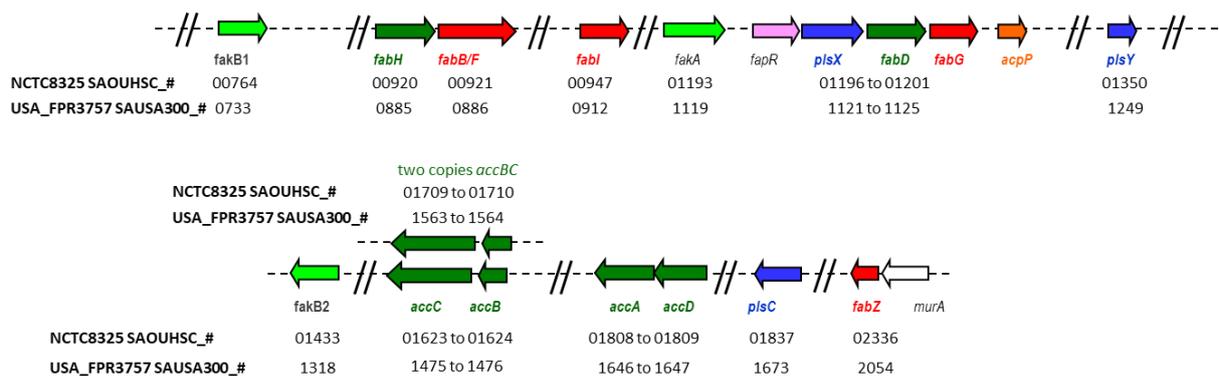


Fig. S1. Organization of genes involved in FASII and FASII bypass. Dark green FASII initiation; red, FASII elongation; orange, acyl carrier protein (*acpP*) involved in all processes; blue, phospholipid synthesis; light green, FASII bypass fatty acid kinase; pink, regulatory. Strain names and corresponding loci for the given functions are indicated.

2-FA auxotrophy of *S. aureus* Δ *plsX* mutants is suppressed by secondary mutations.

During phenotypic characterizations of the purified confirmed mutants derived from the USA300 parental strain, we noted the emergence of colonies on BHI medium without FA. Colonies were reisolated and stocked. Three suppressors (designated Sup 1, Sup2, and Sup3) were characterized by whole genome sequencing. Results confirmed the in-frame deletion of

the entire *pIsX* gene. In addition, point mutations were found in each of the suppressor strains (**Table S1**). Sup 1 and Sup 2 each had a different mutation in *fabF* (SAUSA300_0886) encoding the 3-oxoacyl-(acyl-carrier-protein) synthase II (FabF) protein of the FASII elongation cycle (**Fig. 1, Fig. S1**). These led to FabF^{A119E} (alanine to glutamic acid) and FabF^{D266A} (aspartic acid to alanine) substitutions. However, the latter mutant (Sup2) could not be recovered from the initial stocked suppressor strain, and was excluded from this study. We noted that Sup1 and Sup2 also carried the same point mutation in *csa1A* (SAUSA300_0100), encoding a tandem lipoprotein. Suppressor 3 carried a mutation in the SAUSA300_0150 gene, which is annotated as encoding a transposase, but is not conserved among *S. aureus* strains. This mutation was not further studied.

To identify which loci were relevant to $\Delta pIsX$ suppression, we isolated 31 other $\Delta pIsX$ suppressors, starting from $\Delta pIsX$ constructs in both RN-R and USA300 JE2 backgrounds. We selected 16 and 15 suppressors, respectively, and screened for those carrying *fabF* mutations by PCR and DNA sequence analyses (**Table S1**). Of the 31 suppressors sequenced (16 RN-R and 15 JE2), overall, 14 carried *fabF* mutations that all mapped to the same nucleotide (genome position 972590), resulting in a FabF^{A119E} alteration as in Sup1. Alanine is a neutral to positive amino acid, and glutamic acid adds a negative charge. This protein modification may impact its interactions with its acyl-ACP substrates.

The remaining 17 suppressors carried a wild-type (WT) *fabF* sequence, suggesting that mutations conferring growth on BHI occurred elsewhere.

3-Whole genome sequencing identifies a second $\Delta pIsX$ target. Four strains were randomly selected among the $\Delta pIsX$ suppressors that were not mutated in *fabF* (two derived from JE2 $\Delta pIsX$ and two from RN-R $\Delta pIsX$). Genomic DNA sequencing identified a single common gene target when compared to the sequence of the reference strains (using the respective $\Delta pIsX$ strains): SAUSA300_1247, which encodes a putative 155 amino acid acyl-CoA thioesterase, was mutated in every suppressor whose complete genome was sequenced (**Table S1**). We noted that a 99% identical *S. aureus* protein was previously crystallized, and assigned the name FadM (PBD database protein 6FDG_A; the name derives from a 39% homologous FadM protein from *E. coli*). Following this result, the *S. aureus* *fadM* gene was sequenced in all the other suppressors lacking mutations in *fabF*. All of them carried mutations in *fadM*. Three discrete FadM mutations were observed among the JE2 $\Delta pIsX$ suppressors (FadM^{I38T},

FadM^{Y90F}, and FadM^{Y133F}), and a single mutation (FadM^{Y90F}) was identified in the four isolates selected from RN-R Δ *plsX* (**Table S1; Table S1a and S1b**).

Table S1. Compilation of mutations found in Δ *plsX* suppressors. The red 'X' indicates that the mutation is present in the tested isolate. NT, not tested. Suppressors identified from total genome sequencing are in bold. The green letter of each strain name is used for following the growth curve.

Strain name	Mutation						
	SAUSA300_0886		SAUSA300_0150	SAUSA300_0100	SAUSA300_1247 (Acyl-CoA thioesterase) FadM		
	FabF A119E	FabF D266A	0150 P71S	Csa1A K185Q	FadM Y133F	FadM I38T	FadM Y90F
RN4220 5.1	X		NT		NT	NT	NT
RN4220 5.2	X		NT		NT	NT	NT
RN4220 5.3	X		NT		NT	NT	NT
RN4220 5.4	X		NT		NT	NT	NT
RN4220 5.5	X		NT		NT	NT	NT
RN4220 5.6				NT			X
RN4220 5.7							X
RN4220 5.8				NT			X
RN4220 6.1	X		NT		NT	NT	NT
RN4220 6.2	X		NT		NT	NT	NT
RN4220 6.3	X		NT		NT	NT	NT
RN4220 6.4	X		NT		NT	NT	NT
RN4220 6.5	X		NT		NT	NT	NT
RN4220 6.6	X		NT		NT	NT	NT
RN4220 6.7							X
RN4220 6.8	X		NT		NT	NT	NT
JE2 7.1				NT			X
JE2 7.2				NT		X	
JE2 7.3				NT			X
JE2 7.4				NT		X	
JE2 7.5						X	
JE2 7.7	X		NT		NT	NT	NT
JE2 7.8	X		NT		NT	NT	NT
JE2 8.1				NT		X	
JE2 8.2				NT			X
JE2 8.3				NT		X	
JE2 8.4				NT		X	
JE2 8.5				NT		X	
JE2 8.6				NT		X	
JE2 8.7					X		
JE2 8.8				NT	X		
JE2 Sup 1	X			X			
JE2 Sup 2		X		X			
JE2 Sup 3			X				

Table S1A. Incidence of *fabF* mutations among Δ *plsX* suppressors.

RN-R	Total	Mutated in <i>fabF</i>	Not mutated in <i>fabF</i>
Number of isolates	16	12	4
Percent of total	100	75	25

USA300_FPR3757 JE2	Total	Mutated in <i>fabF</i>	Not mutated in <i>fabF</i>
Number of isolates	15	2	13
Percent of total	100	13	87

Table S1B. Δ *plsX* suppressors mapping in thioesterase gene, SAUSA300_1247 (*fadM*).

PCR sequencing of SAUSA300_1247 (*fadM*) was done on 17 Δ *plsX* suppressors grown on BHI, which were not mutated in *fabF*. These include 4 RN-R Δ *plsX* and 13 JE2 Δ *plsX* isolates. All carried a *fadM* mutation.

strains	Total	Mutation	Mutation	Mutation
		I38T	Y90F*	Y133F
RN-R Δ <i>plsX</i>	4	-	4	-
JE2 Δ <i>plsX</i>	13	8	3	2
Number of isolated	17	8	7	2
Percent total	100	47	41	12

* All 4 RN-R Δ *plsX* isolated carry the Y90F mutation.

4-Impact of Δ *plsX* suppressors on bacterial growth. Growth of representative Δ *plsX* suppressors was determined in the RN-R and JE2 strain backgrounds (**Fig. 2**). As expected the Δ *plsX* deletion strain failed to grow in BHI medium in both RN-R and JE2 backgrounds. However, while the *fabF* and *fadM* suppressor mutations were identical in the two strains, the growth phenotypes differed according to strain backgrounds: In the RN-R background, growth of both suppressors was intermediate between the WT strain and the Δ *plsX* mutant. In contrast, all suppressors in the JE2 background grew in BHI essentially as the WT. Thus, the suppressor mutations, whether in *fabF* or in *fadM*, restore normal growth to JE2 Δ *plsX*, where-

as in RN-R, these same mutations only partially restore growth. Other bacterial factors may contribute to restoring growth, which distinguish the two background strains used here. For example, JE2, but not RN-R (derived from RN4220), produces staphyloxanthin pigment, which comprises an acyl moiety (*ai15*) (Pelz et al., 2005). Staphyloxanthin production in $\Delta plsX$ mutant suppressor strains might contribute to growth and fitness. This hypothesis remains to be tested.

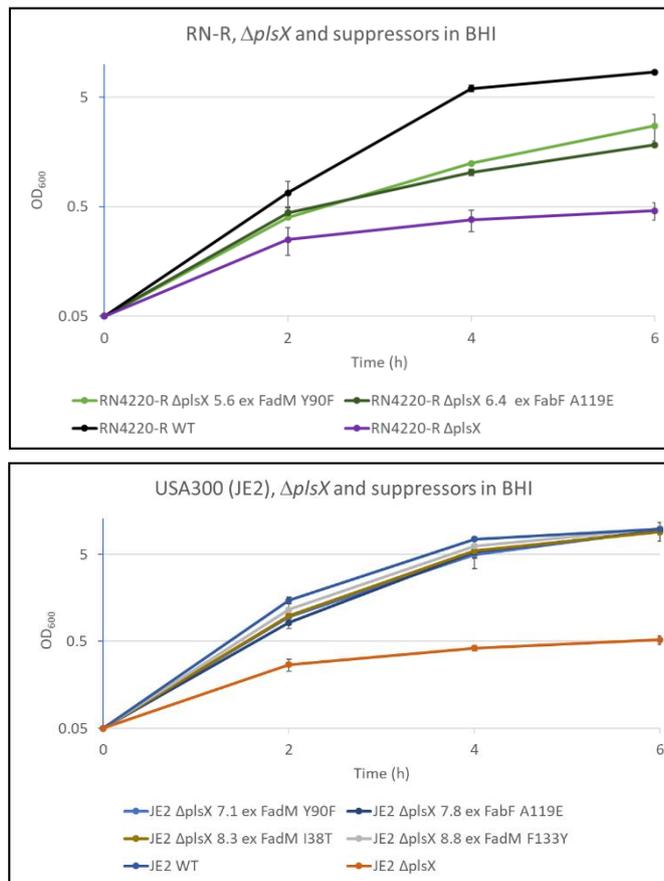


Fig. 2. Growth of *S. aureus* WT, $\Delta plsX$ and $\Delta plsX$ suppressors in the RN-R and JE2 backgrounds. Upper: Strain RN-R $\Delta plsX$ 6.4 is representative of 12 isolates whose mutations led to a $FabF^{A119E}$ modification. Strain RN-R $\Delta plsX$ 5.6 is representative of 4 isolates with an $FadM^{Y90F}$ modification. **Lower:** The JE2 $\Delta plsX$ 7.8 strain is representative of 2 isolates carrying the a $FabF^{A119E}$ mutation. JE2 $\Delta plsX$ 8.3 is representative of 8 isolates with a $FadM^{I38T}$ mutation. Strain JE2 $\Delta plsX$ 7.1 is representative of 3 isolates that possess a $FadM^{Y90F}$ mutation. Strain JE2 $\Delta plsX$ 8.8 is representative of 2 isolates carrying a $FadM^{Y133F}$ mutation. Growth curves are the mean and range of biologically independent duplicates. See **Table S1** for strain designations.

5-Impact of $\Delta plsX$ suppressors on membrane FA composition. We assessed membrane FA composition, comparing WT, $\Delta plsX$, and $\Delta plsX fabF$ and $\Delta plsX fadM$ suppressor mutants in both RN-R and USA300 JE2 backgrounds. We note that the suppressors show two distinct FA profiles. The *fabF* suppressor strains produce membrane FAs of shorter overall length than both wild-type RN-R and USA300 JE2 parents. The longest FA significantly detected in the *fabF* suppressor was *ai17* (anteiso17). Neither C18:0 nor C20:0, which are straight-chain FAs commonly produced in the WT background, were detected in the *fabF* suppressor strain. This could be explained if the mutated $FabF^{A119E}$ suppressor allele is partially defective for elongating the acyl-ACP substrate.

FA profiles in *fadM* suppressor strains were closer to parental profiles than those in the *fabF* suppressor strain. Of note however, both RN-R and USA300 JE2 *fadM* suppressors produced proportionately less C18:0 and C20:0. The differences between the FA profiles between the two suppressor loci underline the differences between their modes of action.

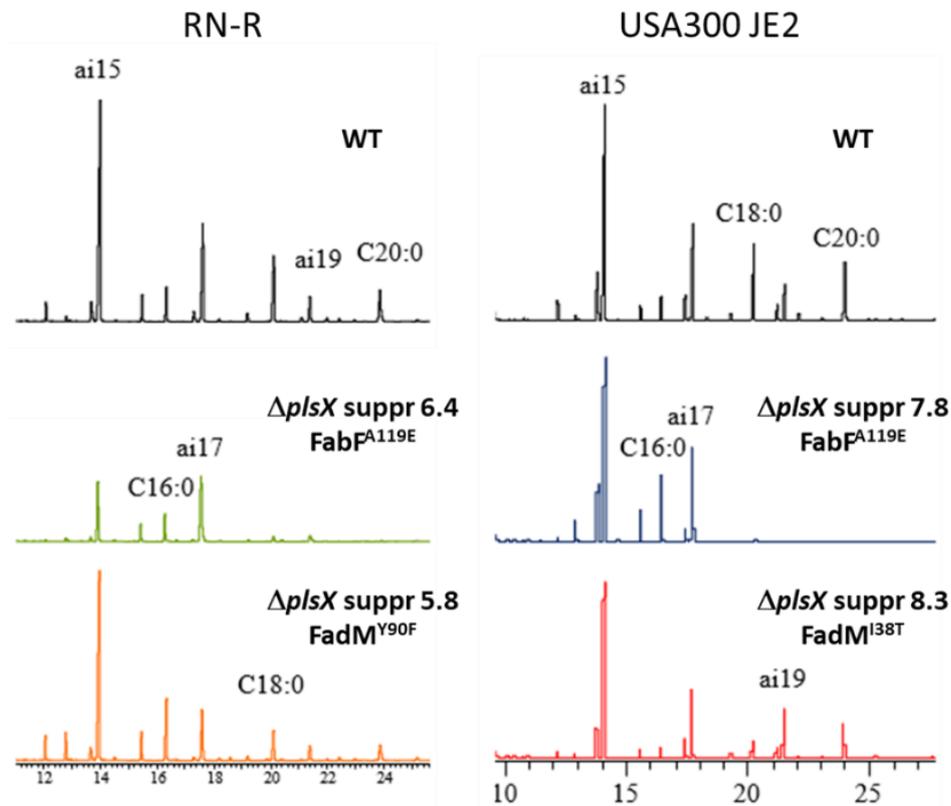


Fig. 3. FA profiles of $\Delta plsX$ suppressors in RN-R (left column) and JE2 (right column) grown on BHI. The suppressor identities are indicated directly in the figure. *ai* represents anteiso: *ai15* is the main FA synthesized by *S. aureus*. Note that the FA lengths in *fabF* suppressors are globally shorter than those in the WT parental strain. Noted, $\Delta plsX$ doesn't elongate C18:1.

6-ACP distribution in JE2 WT, $\Delta plsX$, and $\Delta plsX$ suppressors. ACP accompanies all FA intermediates of the FASII cycle, and is also involved in reverse PlsX activity (**Fig. 2**). We performed immunoblots on gels run under non-denaturing conditions (Cronan and Thomas, 2009) to follow the distribution of FA intermediates linked to ACP in the JE2 parental strain, $\Delta plsX$, and $\Delta plsX$ suppressors (**Fig. 4**).

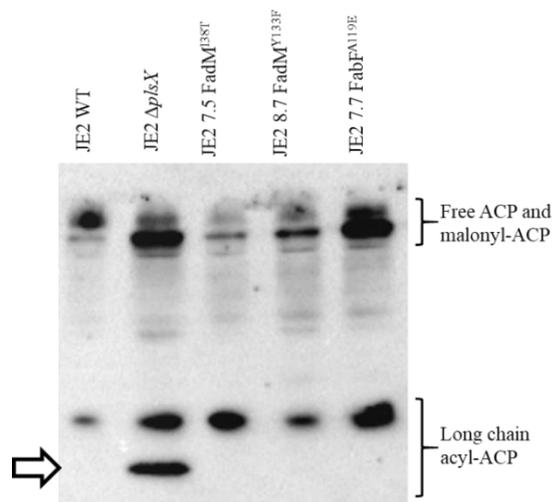


Fig. 4. ACP species in *S. aureus* JE2, $\Delta plsX$, and $\Delta plsX$ suppressor strains. The indicated strains were grown in BHI. The $\Delta plsX$ mutant was first grown in BHI+C18:1 medium, and then shifted for 2h in BHI prior to harvesting. JE2 7.5 and 8.7 strains respectively containing FadM^{138T} or FadM^{Y133F} (encoded by SAUSA300_1247). JE2 7.7 carries FabF^{A119E} (encoded by SAUSA300_0886). On this non-denaturing acrylamide gel, acyl-ACP species migrate faster with longer acyl moieties (attributed to tighter protein folding; (Cronan and Thomas, 2009)). The $\Delta plsX$ strain accumulates long acyl-ACP (left arrow) as reported (Parsons et al., 2014b). This species likely corresponds to a pileup of intermediates that cannot be used by PlsC at position 2 until position 1 is filled (**Fig. 1**). In contrast, the long acyl-ACP species is absent in the WT strain and the 3 suppressors. The immunoblot is representative of biological duplicates.

The $\Delta plsX$ mutant was distinguished by the presence of a fast-migrating band, corresponding to ACP bound to long-chain FAs. Its presence only in the $\Delta plsX$ sample, indicates that FASII is active, and produces long-chain FAs that accumulate that are not used to produce phosphatidic acid. This is expected, because the PlsC substrate (lysophosphatidic acid) is unavailable until position 1 is filled by PlsX; in the $\Delta plsX$ strain, this reaction cannot occur (**Fig. 1**; (Parsons et al., 2014b)).

In $\Delta plsX$ *fabF* or *fadM* suppressor strains, the absence of the long acyl-ACP species, and good growth suggest that FA is released from the acyl-ACP intermediate, and is then processed to occupy position 1. We propose that FabF and FadM mutations facilitate hydrolysis of the acyl-ACP species. Subsequent phosphorylation of the released FA to acyl-PO₄ would require coordination with Fak. FadM suppressor mutants showed lower detectable pools of free and/or malonyl-ACP to WT levels.

7- $\Delta plsX$ suppressors do not restore *S. aureus* ability to bypass the FASII pathway. We asked whether the suppressor strains also complemented the reverse PlsX activity (i.e., to form acyl-ACP from acyl-PO₄) (**Fig. 2**). To test this, we grew WT, $\Delta plsX$, and $\Delta plsX$ *fabF* and $\Delta plsX$ *fadM* suppressor strains in SerFA medium (containing eFA) and AFN-1252, a FASII inhibitor. All strains grew to equivalent densities in the absence of anti-FASII in this eFA-supplemented medium (SerFA). In contrast, only the JE2 WT strain grew in the presence of AFN-1252, as reported (Kenanian et al., 2019), while the $\Delta plsX$ mutant and $\Delta plsX$ suppressor

strains failed to grow. We conclude that $\Delta plsX$ and its suppressor strains lack the ability to bypass anti-FASII inhibitor in the presence of eFA, indicating that an active FASII pathway is required for their growth. We conclude that the reverse reaction catalyzed by PlsX is not compensated by suppressor mutations.

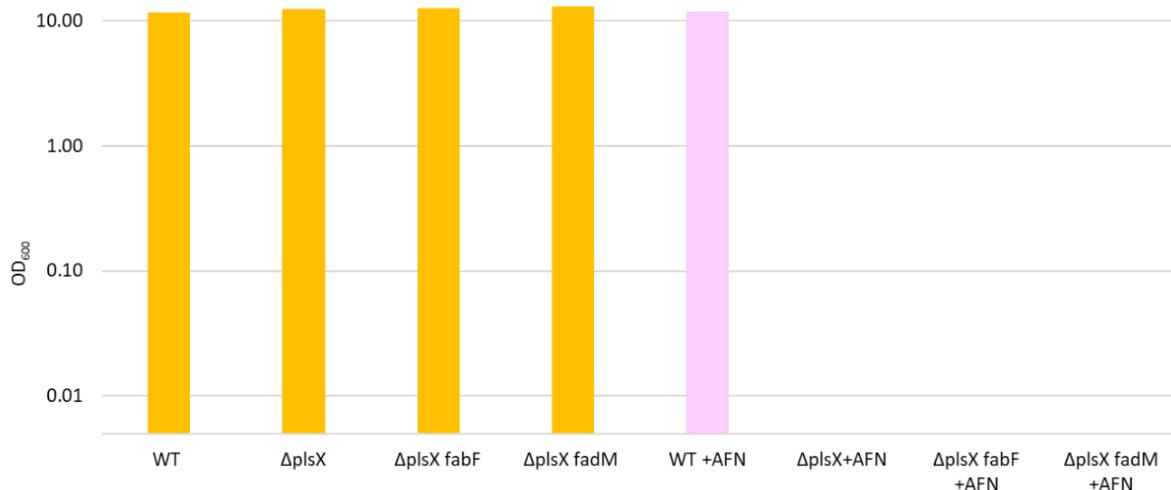


Fig. 5. Effects of anti-FASII treatment on JE2, $\Delta plsX$, and $\Delta plsX$ suppressor strain growth in eFA-supplemented medium. The strains indicated above were precultured in SerFA (Materials and Methods), and then grown overnight (15 h) in SerFA without (yellow) or with (pink) addition of anti-FASII AFN-1252 (AFN). OD₆₀₀ values after overnight growth are presented. *fabF* sup, FabF^{A119E} suppressor; *fadM* sup, FadM^{Y133F} (SAUSA300_1247) suppressor. In the presence of AFN, WT *S. aureus* uses eFA to constitute membrane phospholipids, thus bypassing inhibition. Since $\Delta plsX$ and its suppressors cannot catalyze the reverse reaction needed for FASII bypass, they remain sensitive to anti-FASII. Results represent independent triplicates.

8-Model for FabF^{A119E} suppressor rescue of $\Delta plsX$. We propose a working hypothesis to explain FabF^{A119E} suppressor rescue of $\Delta plsX$, based on a previously published structure-function study of FabF (Mindrebo et al., 2020). Native FabF catalyzes a 2-step reaction in which its acyl-ACP substrate undergoes cleavage of ACP. The free FA is then joined to malonyl-ACP, which adds two carbons to the intermediate. We propose that FabF^{A119E} correctly positions acyl-ACP and catalyzes release of the ACP moiety. However, the FabF^{A119E} mutant releases part of its FA cargo at this stage, before malonyl-ACP addition (**Fig. 6 upper**; (Mindrebo et al., 2020)). The released FA would then be phosphorylated by Fak to rescue $\Delta plsX$, and acyl-PO₄ can then be used by PlsY to initiate phosphatidic acid synthesis (**Fig. 6 lower**).

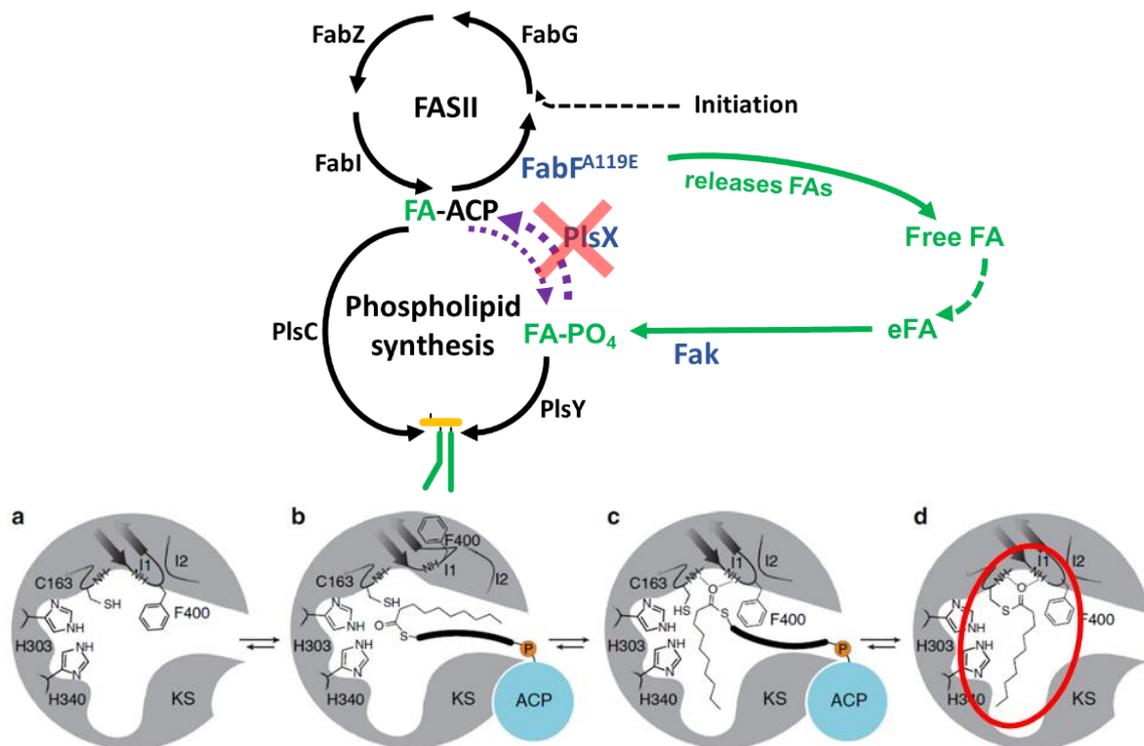


Fig. 6. Upper: Model of molecular interactions between FabF (β -acetoacyl-ACP synthase) and its acyl-ACP substrate, which produces a free FA intermediate (from Mindrebo et al., 2020, as modeled for the *E. coli* enzyme). Upper: The first steps of FabF interactions with its acyl-ACP substrate are schematized (from (Mindrebo et al., 2020)). Acyl-ACP enters the cavity (b) and undergoes a conformational shift (c), which leads to hydrolysis and release of the ACP moiety and the presence of free FA in the cavity (d). The red circle in (d) indicates the FA just before it is bound to entering malonyl-ACP. **Lower: Model for $FabF^{A119E}$ suppression of the $\Delta plsX$ mutant.** We propose that $FabF^{A119E}$ releases FA after acyl-ACP cleavage reaction due to a defect in the subsequent step of malonyl-ACP attachment. The free FA is then phosphorylated by the fatty acid kinase (Fak; (Parsons et al., 2014a)), thus restoring the PlsX product. Phospholipid synthesis can then proceed. The *fabF* ($FabF^{A119E}$) suppressor compensates the PlsX forward reaction, but not the reverse reaction.

9-Model for the impact of acyl-CoA thioesterase mutations on PlsX compensation. The ability of *fadM* mutants to suppress $\Delta plsX$ raises the question of how this mechanism works. We hypothesize that the amino acid changes in FadM suppressor mutants may increase enzyme substrate permissiveness. While FadM is predicted to hydrolyze acyl-CoA, we suggest that altered structure due to point mutations might facilitate cleavage of acyl-ACP. This hypothesis remains to be proven. However, an *E. coli* thioesterase (TesA) of the "SGNH" family preferentially hydrolyses acyl-CoA, but also has a 3-4-log lower activity on acyl-ACP (Spencer et al., 1978). Further supporting this possibility, FadM shares weak amino acid homology with the *S. pneumoniae* acyl-ACP thioesterase TesS (or FatA; SP1408; (Parsons et al., 2015)), and appears to share structural features (**Fig. 7 upper**). The FadM mutants are thus proposed to cleave acyl-ACP (the products of FASII), to liberate FA. As with the *fabF*

suppressors, the free FAs would be phosphorylated by Fak and utilized by PlsY to initiate phospholipid synthesis (**Fig. 7 lower**). This hypothesis remains to be proven.

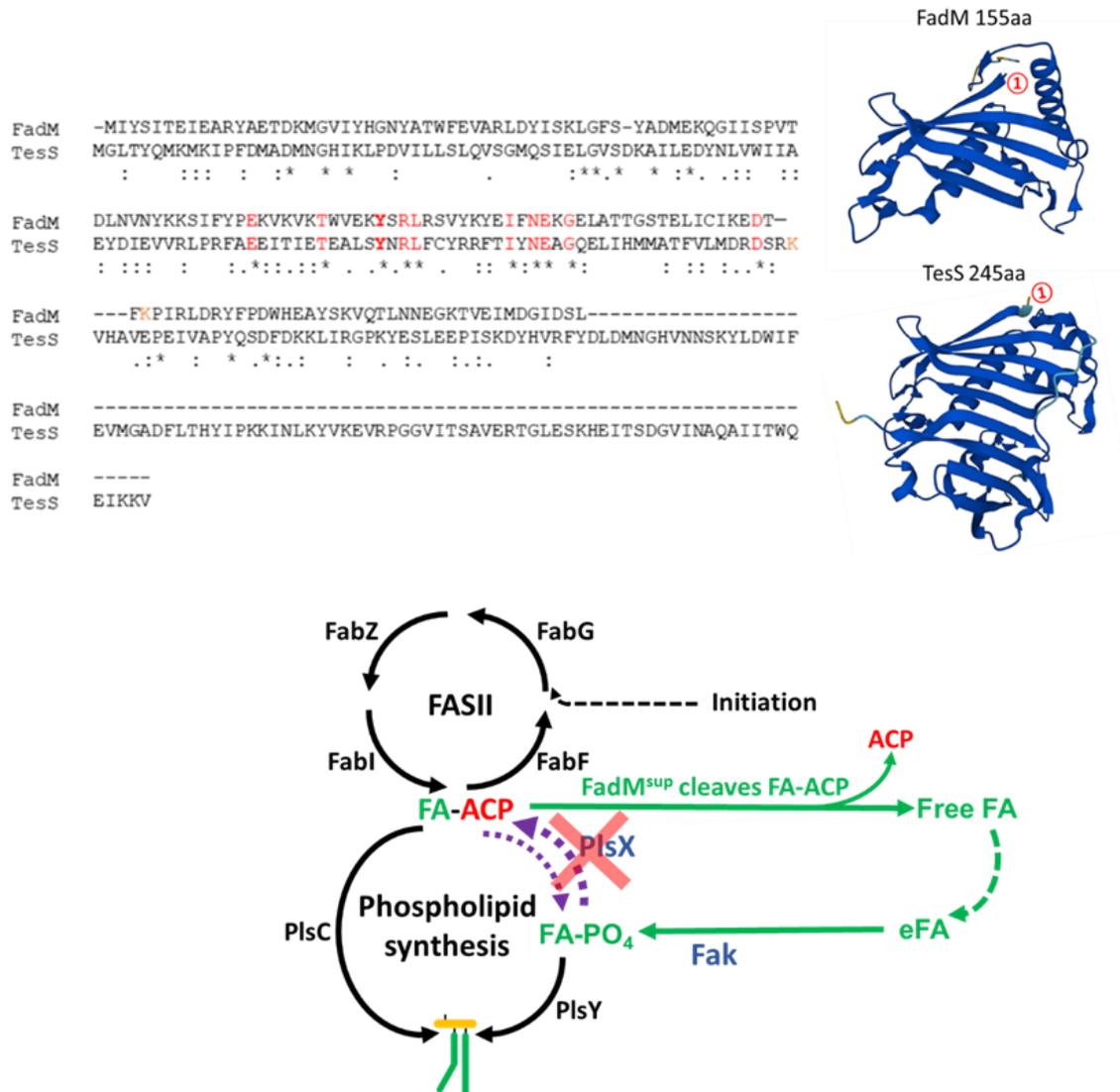


Fig. 7. Upper: Similarities between *S. aureus* FadM a putative acyl-CoA thioesterase, and *S. pneumoniae* TesS, the *S. pneumoniae* acyl-ACP and acyl-CoA thioesterase. Left, Amino acid homologies using ClustalW. Amino acids in red point out identities. Right, AlphaFold representations of FadM and TesS suggest structural similarities between the FadM and the N-terminal part of TesS. The circled "1" represents the N-terminal methionine positions. **Lower: Model for $\Delta plsX$ suppression by *fadM* mutations.** We propose that point mutations in the putative acyl-CoA thioesterase (*FadM^{sup}*, SAUSA300_1247 in USA300 JE2) leads to greater substrate permissiveness, and acyl-ACP cleavage. Free FAs are then phosphorylated by Fak to rescue $\Delta plsX$ growth. As for *fabF* suppressors, the *fadM* mutants compensate the PlsX forward reaction, but not the reverse reaction.

Conclusions

We identified *S. aureus* gain-of-function mutations that restore growth when *plsX*, a gene considered essential for phospholipid synthesis and bacterial growth, is deleted. PlsX-

deficient *S. aureus* strains were shown previously to have a strict requirement for eFAs (Khun et al., 2015). The suppressors identified here allow growth without eFA supplementation. These findings underline a remarkable enzyme flexibility, which is revealed by selective pressure for their emergence.

The two suppressor loci are distinct, and not directly implicated in phospholipid synthesis: FabF is a FASII elongation enzyme, while FadM is a putative acyl-CoA thioesterase. The activity of each mutated enzyme is proposed to provide free FAs, which are substrates for Fak phosphorylation. Consistently, the long chain acyl-ACP intermediate detected in the $\Delta plsX$ mutant is absent in suppressor strains, and is likely used to restart phospholipid synthesis.

An explanation for the suppressor function of FabF^{A119E} is based on the reported mechanism of FabF activity (Mindrebo et al., 2020): as free FAs are briefly generated from ACP hydrolysis in the FabF cavity, we suggest that the FabF^{A119E} mutant fumbles the FA intermediate, so that the next FabF step, malonyl-ACP joining, is inefficient. Fak would phosphorylate the FAs released by FabF^{A119E}, thus providing acyl-PO₄ in the absence of PlsX. Less efficient FabF^{A119E} activity would impair FASII activity, explaining why this strain produces shorter chain FAs in membrane phospholipids (**Fig. 6**).

In the case of suppressors mapping to FadM acyl-CoA thioesterase, we hypothesize that the mutations would reduce FadM specificity to include acyl-ACP as a substrate. Weak amino acid homology and structural similarities between the *S. aureus* acyl-CoA thioesterase FadM and the *S. pneumoniae* acyl-ACP thioesterase TesS (SP1048; (Parsons et al., 2014b) give some support for this model. Consistent with this model, *fadM* mutant suppressors compensate the *plsX* defect without greatly altering FA composition, compared to *fabF* suppressors (**Fig. 7**).

Growth restoration of both suppressor classes was partial in the RN-R (RN4220) background, but quasi-complete in JE2 (**Fig. 2**). Possibly, full growth of suppressors in JE2 could reflect production of other molecules that might stabilize the membrane. For instance, staphyloxanthin is not produced in the RN-R strain, but could contribute to membrane homeostasis and fitness of JE2 mutants.

FASII and phospholipid synthesis proteins were proposed as potential targets for new antibiotic inhibitors. The present study argues against the use of PlsX as a single target for antibiotic development: first, growth is restored by FAs as commonly found in the host. Second, suppressors readily appear in PlsX-depleted conditions (*e.g.*, by PlsX-inhibition), which promote outgrowth. However, *in vitro* experiments (**Fig. 5**) indicate that PlsX-depleted strains

would not survive in the presence of an anti-FASII, suggesting that PlsX could be used as a second target in combination with an anti-FASII. Indeed, even when suppressor mutations emerge, they would not allow *S. aureus* outgrowth in the presence of an anti-FASII.

Perspectives

Proof that suppressor activity is due to *fabF* and *fadM* alleles.

The presence of *fabF* or thioesterase mutations in all the suppressor strains (31 screened) gives evidence that these specific mutations are responsible for the suppressor phenotypes. This could be further confirmed by introducing the mutated *fabF* locus into the $\Delta plsX$ mutant, or possibly, by expressing the mutated or unmutated *fabF* gene on a plasmid. We cloned both versions of the gene onto a plasmid (pCM11). After initial cloning in *Escherichia coli*, constructions were established in *S. aureus* strains RN-R and USA300 JE2.

Acknowledgements

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References

- Arnaud, M., Chastanet, A., and Debarbouille, M. (2004). New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. *Appl Environ Microbiol* 70, 6887-6891.
- Biswas, I., Gruss, A., Ehrlich, S.D., and Maguin, E. (1993). High-efficiency gene inactivation and replacement system for gram-positive bacteria. *J Bacteriol* 175, 3628-3635.
- Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248-254.
- Coronel-Tellez, R.H., Pospiech, M., Barrault, M., Liu, W., Bordeau, V., Vasnier, C., Felden, B., Sargueil, B., and Bouloc, P. (2022). sRNA-controlled iron sparing response in Staphylococci. *Nucleic Acids Res* 50, 8529-8546.
- Cronan, J.E., and Thomas, J. (2009). Bacterial fatty acid synthesis and its relationships with polyketide synthetic pathways. *Methods Enzymol* 459, 395-433.
- Fey, P.D., Endres, J.L., Yajjala, V.K., Widhelm, T.J., Boissy, R.J., Bose, J.L., and Bayles, K.W. (2013). A genetic resource for rapid and comprehensive phenotype screening of nonessential *Staphylococcus aureus* genes. *MBio* 4, e00537-00512.
- Herbert, S., Ziebandt, A.K., Ohlsen, K., Schafer, T., Hecker, M., Albrecht, D., Novick, R., and Gotz, F. (2010). Repair of global regulators in *Staphylococcus aureus* 8325 and comparative analysis with other clinical isolates. *Infect Immun* 78, 2877-2889.
- Karlowsky, J.A., Kaplan, N., Hafkin, B., Hoban, D.J., and Zhanel, G.G. (2009). AFN-1252, a FabI inhibitor, demonstrates a *Staphylococcus*-specific spectrum of activity. *Antimicrob Agents Chemother* 53, 3544-3548.
- Kenanian, G., Morvan, C., Weckel, A., Pathania, A., Anba-Mondoloni, J., Halpern, D., Gaillard, M., Solgadi, A., Dupont, L., Henry, C., et al. (2019). Permissive Fatty Acid Incorporation Promotes Staphylococcal Adaptation to FASII Antibiotics in Host Environments. *Cell Rep* 29, 3974-3982 e3974.
- Mindrebo, J.T., Patel, A., Kim, W.E., Davis, T.D., Chen, A., Bartholow, T.G., La Clair, J.J., McCammon, J.A., Noel, J.P., and Burkart, M.D. (2020). Gating mechanism of elongating beta-ketoacyl-ACP synthases. *Nat Commun* 11, 1727.
- Morvan, C., Halpern, D., Kenanian, G., Hays, C., Anba-Mondoloni, J., Brinster, S., Kennedy, S., Trieu-Cuot, P., Poyart, C., Lamberet, G., et al. (2016). Environmental fatty acids enable emergence of infectious *Staphylococcus aureus* resistant to FASII-targeted antimicrobials. *Nat Commun* 7, 12944.
- Parsons, J.B., Broussard, T.C., Bose, J.L., Rosch, J.W., Jackson, P., Subramanian, C., and Rock, C.O. (2014a). Identification of a two-component fatty acid kinase responsible for host fatty acid incorporation by *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* 111, 10532-10537.
- Parsons, J.B., Frank, M.W., Eleveld, M.J., Schalkwijk, J., Broussard, T.C., de Jonge, M.I., and Rock, C.O. (2015). A thioesterase bypasses the requirement for exogenous fatty acids in the *plsX* deletion of *Streptococcus pneumoniae*. *Mol Microbiol* 96, 28-41.
- Parsons, J.B., Frank, M.W., Jackson, P., Subramanian, C., and Rock, C.O. (2014b). Incorporation of extracellular fatty acids by a fatty acid kinase-dependent pathway in *Staphylococcus aureus*. *Mol Microbiol* 92, 234-245.
- Pathania, A., Anba-Mondoloni, J., Gominet, M., Halpern, D., Dairou, J., Dupont, L., Lamberet, G., Trieu-Cuot, P., Gloux, K., and Gruss, A. (2021). (p)ppGpp/GTP and Malonyl-CoA Modulate *Staphylococcus aureus* adaptation to FASII antibiotics and provide a basis for synergistic bi-therapy. *mBio* 12.
- Pelz, A., Wieland, K.P., Putzbach, K., Hentschel, P., Albert, K., and Gotz, F. (2005). Structure and biosynthesis of staphyloxanthin from *Staphylococcus aureus*. *J Biol Chem* 280, 32493-32498.
- Sastre, D.E., Pulschen, A.A., Basso, L.G.M., Benites Pariente, J.S., Marques Netto, C.G.C., Machinandiarena, F., Albanesi, D., Navarro, M., de Mendoza, D., and Gueiros-Filho, F.J. (2020). The phosphatidic acid pathway enzyme PlsX plays both catalytic and channeling roles in bacterial phospholipid synthesis. *J Biol Chem* 295, 2148-2159.
- Spencer, A.K., Greenspan, A.D., and Cronan, J.E., Jr. (1978). Thioesterases I and II of *Escherichia coli*. Hydrolysis of native acyl-acyl carrier protein thioesters. *J Biol Chem* 253, 5922-5926.

Résumé de thèse en français

Ma thèse s'intitule "Adaptation de *Staphylococcus aureus* aux antibiotiques ciblant FASII dans l'hôte et stratégies de potentialisation des activités antibiotiques ". Les infections à *S. aureus* vont de modérées à mortelles, et sont l'une des principales causes de décès associés à des pathogènes bactériens résistants à de multiples antibiotiques. Il est donc nécessaire de trouver de nouvelles stratégies thérapeutiques pour arrêter la croissance de *S. aureus*. La voie de synthèse des acides gras (FASII) est une voie dite essentielle pour *S. aureus*. En bloquant cette voie, on s'attendait à ce que la croissance de la bactérie soit stoppée. Cependant, il a été démontré que les antibiotiques inhibant FASII (anti-FASII) atteignent effectivement leur site cible mais ne peuvent pas tuer *S. aureus*, car ce dernier est capable d'incorporer les acides gras environnementaux pour compenser l'inhibition du FASII. Dans cette étude, nous visons à élucider les facteurs contribuant à cette adaptation aux antibiotiques du FASII et à analyser les conséquences sur l'aptitude bactérienne. Ces informations sont ensuite utilisées pour identifier les points faibles du *S. aureus* adapté à l'anti-FASII et pour mettre au point un traitement bi-thérapeutique associé à l'anti-FASII. Les principales techniques que j'ai utilisées au cours de mes travaux sont : l'analyse protéomique, des suivis de la physiologie bactérienne, le Western blotting, l'extraction d'acides gras et le développement des stratégies de la bi-thérapie pour éliminer *S. aureus*. Les résultats de mon travail de thèse sont présentés en trois chapitres, sous forme de manuscrits ; les deux premiers résultats sont directement liés à mon objectif, et le troisième s'agit d'un projet plus fondamental, concernant la flexibilité génétique de *S. aureus* pour le métabolisme des acides gras. Le résumé de chaque manuscrit est présenté ci-dessous.

Manuscrit I : Le stress oxydatif est intrinsèque à l'adaptation des staphylocoques aux antibiotiques de synthèse des acides gras

Les antibiotiques inhibant la voie de synthèse des acides gras (FASII) du principal agent pathogène *Staphylococcus aureus* atteignent leurs cibles enzymatiques, mais les bactéries poursuivent leur croissance en utilisant les acides gras environnementaux (AGE) pour produire des phospholipides. Nous avons évalué comment les changements extrêmes dans les phospholipides membranaires provoqués par les antibiotiques FASII affectent la physiologie globale de *S. aureus*. L'anti-FASII a engendré des changements massifs et durables de l'expression sans rémaniements génomiques. Plusieurs régulateurs, plutôt qu'un seul régulateur, contribuent à la synchronisation de l'adaptation à l'anti-FASII. De nombreux facteurs de virulence et d'adhésion ont vu leur niveau et/ou leur activité diminuer. À l'inverse, les niveaux des protéines de réponse au stress ont augmenté et sont corrélés avec une plus grande tolérance aux peroxydes. Notamment, l'amorçage par les peroxydes a stimulé l'efficacité de l'incorporation des AGE et a facilité l'adaptation à l'inhibition du FASII. Ces résultats établissent un lien entre le stress oxydatif et l'incorporation des AG. Conformément au changement

majeur dans l'expression des protéines, les *S. aureus* adaptés à l'anti-FASII tue un insecte hôte plus lentement, mais ont continué à se multiplier. Ainsi, si les populations adaptées à l'anti-FASII sont moins aptes à endommager l'hôte, elles sont peut-être mieux adaptées à la survie à long terme et pourraient constituer un réservoir pour la réinfection.

Manuscript II : L'arrêt de la synthèse des acides gras provoque une diminution du niveau de l'acide lipoteichoïque chez *Staphylococcus aureus*

Staphylococcus aureus est une bactérie pathogène à Gram positif qui entraîne un taux de mortalité élevé, principalement dû à l'émergence d'une multi-résistance aux antibiotiques. Les enzymes de la voie de synthèse des acides gras (FASII) sont proposées comme cibles essentielles. Cependant, des antibiotiques inhibant la voie FASII est contournée : *S. aureus* compense l'inhibition de FASII en incorporant des acides gras environnementaux (AGE) et en reprogrammant son expression. Nous démontrons que l'acide lipoteichoïque (LTA), un facteur de surface structurel principal, est presque indétectable lorsque les bactéries s'adaptent à l'anti-FASII. Le LTA est diminué indépendamment des AGEs fournis pour la croissance. En revanche, les pools d'acide teichoïque de paroi (WTA) et de cardiolipine (CL) sont augmentés dans les bactéries adaptées à l'anti-FASII, ce qui suggère que le WTA et/ou la CL peuvent compenser l'appauvrissement en LTA et permettre à *S. aureus* de se développer. L'inactivation simultanée de la synthèse du LTA et du WTA est létale. Par conséquent, nous avons observé un effet inhibiteur synergique entre l'anti-FASII, qui épuise le LTA, et un anti-WTA, ici le targocil, contre *S. aureus*. Nous concluons que la déplétion de LTA par un traitement anti-FASII conduit à des augmentations de CL et WTA chez *S. aureus*, et suggérons des stratégies de bi-thérapie contre ce pathogène majeur.

Manuscript III : Des mutations dans les gènes codant FabF ou acyl-CoA thioestérase permettent de rétablir la croissance des mutants de délétion *plsX* de *Staphylococcus aureus*.

PlsX catalyze la conversion de la protéine porteuse d'acyl (acyl-ACP) en acyl-PO₄ dans une réaction réversible. Cette enzyme a été considérée comme essentielle pour la synthèse des phospholipides chez des Firmicutes tels que *Staphylococcus aureus*. Cependant, une souche délétée pour le gène *plsX* est viable lorsque des acides gras exogènes (AGE) sont disponibles ; dans cette condition, la kinase d'acide gras Fak assure la phosphorylation des AGE en acyl-PO₄. Des mutants de délétion *plsX* ont été générés dans deux fonds génétiques de *S. aureus*. Comme prévu, les deux souches $\Delta plsX$ n'ont pas réussi à se développer dans un milieu ne contenant pas d'acide gras, en cohérence avec son auxotrophie pour les acides gras. Néanmoins, nous avons découvert deux classes de mutants suppresseurs de la souche $\Delta plsX$ qui ont émergé sur un milieu solide dépourvu d'AGE : 1- une mutation ponctuelle spécifique dans *fabF* code un mutant FabF^{A119E} ; 2- plusieurs mutations sont associées à une acyl-CoA

thioestérase putative (nommée FadM). Les AG membranaires dérivés de FabF^{A119E} étaient plus courts, tandis que ceux des souches suppressives *fadM* étaient similaires à ceux du sauvage. Les souches suppresseurs Δ *plsX* ont toutes deux une croissance plus lente qu'un parent dérivé de la souche RN4220, mais une croissance normale sur un fond génétique USA300-JE2. Nos résultats indiquent que les deux mutants permettant la croissance de la souche Δ *plsX* (mutés dans *fabF* et *fadM*) conduisent tous deux à la libération d'AG à partir de l'acyl-ACP, par des mécanismes différents. La FabF mutée aurait une activité de fuite qui libère l'AG libre ; la FabM mutée aurait une plus grande permissivité du substrat. Nous supposons que les AG ainsi libérés sont ensuite phosphorylés par Fak pour produire de l'acyl-PO₄, le produit de PlsX. Contrairement aux souches parentales WT, ni la souche Δ *plsX*, ni ses suppresseurs ne se développent en présence d'un anti-FASII, ce qui indique que l'activité FASII est essentielle dans le contexte Δ *plsX*.

En résumé, la membrane bactérienne joue un rôle important dans de nombreuses fonctions telles que la physiologie bactérienne, l'altération de l'enveloppe cellulaire, l'aptitude bactérienne à l'infection. Par conséquent, l'étude de la membrane bactérienne pourrait ouvrir de nouvelles perspectives, non seulement en termes d'informations fondamentales, mais aussi en termes de recherche appliquée pour le traitement des infections provoquées par *S. aureus* ou par d'autres Firmicutes pathogènes.

Valorization of Thesis:

Paprapach WONGDONTREE

Awards and Scholarships:

- ❖ Franco-Thai Scholarship and Khon Kaen University (**2020-2023**): To pursue a PhD, France
- ❖ ABIES Doctoral Day (Oral presentation) (**2022**)
- ❖ ABIES international mobility fellowship (**2022**): To visit and exchange with Grundling lab at Imperial College London, UK

Conferences and Seminars:

- ❖ CSHL Meeting: Microbial Pathogenesis & Host Response (Poster presentation), USA (**2023**)
- ❖ Gordon Research Conference: Bacterial cell surface: Altering the Bacterial Cell Envelope: Shaping Fitness in Response to the Host and Environment (Poster presentation), USA (**2022**)
- ❖ Gordon Research Seminar: A Multidimensional View of the Bacterial Cell Envelope: From Molecular Structures to Intracellular Interactions, USA (**2022**)
- ❖ Journées des Doctorants ABIES (Oral presentation), France (**2022**)
- ❖ Virtual *Staphylococcus aureus* seminar series (27 seminars), online (**2020-2022**)
- ❖ JPIAMR Junior Researchers Workshop, online (**2021**)

Courses (Total 341H/160H):

- ❖ Academic Writing Skills in English – Becoming a Writer
- ❖ Research integrity in Scientific professions (EN and FR)
- ❖ Doctoral Training in basic Statistics
- ❖ Communication oral en française langue étrangère : 2020, 2021, 2022, 2023
- ❖ Journée d'accueil des nouveaux doctorants
- ❖ Discover consulting and NGO sectors & Increase PhD employability
- ❖ Introduction to Open Science
- ❖ Journées des Doctorants ABIES 2021 & 2022
- ❖ Writing and publishing research papers
- ❖ Why and how to develop your post-doc project 1
- ❖ Doctor'Preneuriales
- ❖ Innovation Thérapeutique : Drug Discovery and Design