Identification of new proteins in Staphylococci involved in bacterial metabolism and adaptation

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Abbreviations and Symbols

Abbreviations

AIM	Auto-inducing peptide
AR	Adrenergic receptor
Des	Desaturase
eFA	Extracellular fatty acids
Fc	Fragment crystallizable region of antibody
h	Hour
HK	Histidine kinase
HPLC	High performance liquid chromatography
kDa	Kilo Dalton
LQ-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
min	Minute
mM	Mili Molar
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
Ni-NTA	Nickel-nitrilotriacetic acid Ni-NTA
OD	Optical density
PGT	Glycosyltransferase
PSM	Phenol-soluble modulin
QS	Quorum sensing
RBS	Ribosomal binding site
RR	Response regulator
SadA	Staphylococcal aromatic amino acid decarboxylase
sdAb	Single-domain antibodies
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SER	Serotonin
SFA	Saturated fatty acid
SNV	Single nucleotide variant
Sp.	Species
Spp.	Species (plural)
TA	Trace amine
TAAR	Trace amines-activated receptors
TP	Transpeptidase
TRY	Tryptamine
TSB	Tryptic soy broth
UFA	Unsaturated fatty acids
V_H	variable heavy-chain domain
V_HH	variable domain of camelid heavy chain antibody
V_L	Variable domain of the antibody light chain
WGS	Whole genome sequencing
WT	Wild type
µM	Micro Molar

Symbols

°C	Degree in Celcius
Δ	Deletion of gene
α	Alpha
β	Beta
%	Percentage
µ	Micro

Summary

Bacteria of the genus *Staphylococcus* are of particular importance because of their widespread presence in the environment and their ability to cause skin and soft tissue infections in humans and animals. These infections are often difficult to treat because staphylococcal strains are or can become resistant to antibiotics. In this work, we studied different aspects of bacterial behavior with a focus on *Staphylococcus aureus* and *Staphylococcus pseudintermedius*.

Single-domain antibodies (V_H/V_HH) have desirable molecular features, such as small size, high tissue penetration, and stability, which make them suitable as potent inhibitors of enzymes and toxins. In chapter 1, we studied the potential of penicillin binding protein 2 (PBP2)-specific V_H/V_HH to sensitize *S. aureus* in combination with β-lactam antibiotics. Our results indicate that the selected antibodies have little inhibitory effect on *S. aureus* under the tested conditions and show no synergistic effect in combination with β-lactams. Hence, the tested V_H/V_HH cannot serve as promising drugs against *S. aureus*. In chapter 2, we observed an effect of the two nuclease genes encoded in *S. aureus* genome on the expression of some virulence factors. In an attempt to study the underlying mechanisms, we found out that the observed effects result from secondary mutations in the genome. In chapter 3, we identified a serotonin N-acetyltransferase (SNAT) in *S. pseudintermedius* based on sequence similarities to known SNAT proteins. In mammals, SNAT catalyze the acetylation of neurochemicals such as serotonin and tryptamine, a reaction that modulates and regulates different functions in the body. The activity of the newly identified SNAT in *S. pseudintermedius* was confirmed *in vitro* using recombinant putative SNAT proteins and *in vivo* using a knockout deletion mutant of the wildtype strain. We also found that SNAT homologues are widely distributed among staphylococci. SNAT products are important antioxidants in bacteria but are also known to induce different responses in the human body. This indicates that SNAT expression in staphylococci could potentially lead to bidirectional signaling with the host. In the final chapter, we aimed to identify the function of the *S. aureus* gene cluster that bears homology to the cold-sensitive *DesKR* operon in *Bacillus subtilis*. In *S. aureus* the cluster encodes the two-component system TCS-7 and two effector proteins. Based on our results, this four-gene cluster plays a role in cold temperature adaptation in *S. aureus* as a cluster deletion mutant does not survive a shift to 20°C. However, the predicted effector proteins of TCS-7 share no similarity

with DesKR which is a membrane-associated fatty acids desaturase. Accordingly, the effects of TCS-7 activation and their underlying mechanism remain to be identified.

The major goal of this work was to gain insight into the physiology of staphylococci and explore new therapeutic avenues against these opportunistic pathogens. The main findings discussed here are the identification of SNAT, the enzyme that enable staphylococci to produce neuromodulating molecules with the potential to interfere with signaling pathways in the colonized host and, revealing the involvement of the proteins encoded in the gene cluster of TCS-7 in *S. aureus* cold adaptation.

Zusammenfassung

Bakterien der Gattung Staphylococcus sind von besonderer Bedeutung, da sie in der Umwelt weit verbreitet sind und Haut- und Weichteilinfektionen bei Menschen und Tieren verursachen können. Diese Infektionen sind oft schwer zu behandeln, da Staphylokokkenstämme gegen Antibiotika resistent sind oder werden können. In dieser Arbeit haben wir verschiedene Aspekte des bakteriellen Verhaltens untersucht, wobei wir uns auf Staphylococcus aureus und Staphylococcus pseudintermedius konzentriert haben.

Single-Domain-Antikörper (VH/VHH) verfügen über wünschenswerte molekulare Eigenschaften wie geringe Größe, hohe Gewebepenetration und Stabilität, die sie als potente Inhibitoren von Enzymen und Toxinen geeignet machen. In Kapitel 1 untersuchten wir das Potenzial von Penicillin-Bindungsprotein 2 (PBP2)-spezifischen VH/VHH zur Sensibilisierung von S. aureus in Kombination mit □-Lactam-Antibiotika. Unsere Ergebnisse zeigen, dass die ausgewählten Antikörper unter den getesteten Bedingungen nur eine geringe hemmende Wirkung auf S. aureus haben und in Kombination mit □-Lactamen keine synergistische Wirkung zeigen. Daher können die getesteten VH/VHH nicht als vielversprechende Medikamente gegen S. aureus dienen. In Kapitel 2 haben wir eine Wirkung der beiden im S. aureus-Genom kodierten Nuklease-Gene auf die Expression einiger Virulenzfaktoren beobachtet. Bei dem Versuch, die zugrunde liegenden Mechanismen zu untersuchen, fanden wir heraus, dass die beobachteten Effekte auf sekundäre Mutationen im Genom zurückzuführen sind. In Kapitel 3 identifizierten wir eine Serotonin-N-Acetyltransferase (SNAT) in S. pseudintermedius aufgrund von Sequenzähnlichkeiten mit bekannten SNAT-Proteinen. Bei Säugetieren katalysiert SNAT die Acetylierung von Neurochemikalien wie Serotonin und Tryptamin, eine Reaktion, die verschiedene Funktionen im Körper moduliert und reguliert. Die Aktivität der neu identifizierten SNAT in S. pseudintermedius wurde in vitro mit rekombinanten mutmaßlichen SNAT-Proteinen und in vivo mit einer Knockout-Deletionsmutante des Wildtyp-Stamms bestätigt. Wir fanden auch heraus, dass SNAT-Homologe unter Staphylokokken weit verbreitet sind. SNAT-Produkte sind wichtige Antioxidantien in Bakterien, aber auch dafür bekannt, dass sie verschiedene Reaktionen im menschlichen Körper hervorrufen. Dies deutet darauf hin, dass die Expression von SNAT in Staphylokokken möglicherweise zu einer

bidirektionalen Signalübertragung mit dem Wirt führen könnte. Im letzten Kapitel haben wir versucht, die Funktion des S. aureus-Genclusters zu identifizieren, das eine Homologie zum kälteempfindlichen DesKR-Operon in Bacillus subtilis aufweist. In S. aureus kodiert der Cluster für das Zwei-Komponenten-System TCS-7 und zwei Effektorproteine. Unsere Ergebnisse deuten darauf hin, dass dieser Vier-Gene-Cluster eine Rolle bei der Anpassung an kalte Temperaturen in S. aureus spielt, da eine Deletionsmutante des Clusters eine Temperaturverschiebung auf 20 °C nicht überlebt. Die vorhergesagten Effektorproteine von TCS-7 haben jedoch keine Ähnlichkeit mit DesKR, einer membranassoziierten Fettsäure-Desaturase. Die Auswirkungen der TCS-7-Aktivierung und die ihnen zugrunde liegenden Mechanismen müssen daher noch ermittelt werden.

Das Hauptziel dieser Arbeit war es, Einblicke in die Physiologie von Staphylokokken zu gewinnen und neue therapeutische Wege gegen diese opportunistischen Krankheitserreger zu erkunden. Die wichtigsten hier diskutierten Ergebnisse sind die Identifizierung von SNAT, dem Enzym, das Staphylokokken in die Lage versetzt, neuromodulierende Moleküle zu produzieren, die das Potenzial haben, die Signalwege im kolonisierten Wirt zu stören, und die Aufdeckung der Beteiligung der im Gencluster von TCS-7 kodierten Proteine an der Kälteanpassung von S. aureus.

General Introduction

The Genus Staphylococcus

The genus *Staphylococcus* was first identified by the Scottish surgeon Alexander Ogston in 1880, when he isolated it from a surgical abscess. He named the genus "*Staphylococcus*" (from the Greek *staphyle*, meaning bunch of grapes and *kokkos*, meaning berry) that describes the morphology of these bacteria (Ogston, 1880) (Licitra, 2013) In 1884, the German physician Rosenbach further classified the genus based on colony pigmentation, naming *Staphylococcus aureus* from the Latin "aurum" meaning "gold" and *Staphylococcus albus* (now known as *S. epidermidis*) from the Latin "albus" meaning "white" (Rosenbach, 1884). Taxonomically, Staphylococci belong to the phylum *Firmicutes*, class *Bacilli*, order Bacillales, and family *Staphylococcaceae*. They are Gram-positive cocci that form irregular, grape-like clusters and are non-motile and non-pore-forming. They have a low DNA G+C content of 33 – 40 mol% and most species are facultative anaerobes (Götz *et al.*, 2006).

S. aureus is the most significant member of the genus *Staphylococcus* in terms of human infections. It is a well-known bacterium that colonizes persistently 20% and intermittently 60% of healthy people as a harmless commensal (Kluytmans *et al.*, 1997; Graham *et al.*, 2006; Miller *et al.*, 2009). Despite its presence as a commensal, *S. aureus* is also an opportunistic pathogen that is capable of causing a wide range of diseases, including life-threatening infections such as endocarditis, osteomyelitis, and sepsis (Gordon and Lowy, 2008). Its ability to evade the human immune system and its resistance to antibiotics have been a focus of research for many years. Of particular concern are the emergence of methicillin-resistant *S. aureus* (MRSA) and more recently, vancomycin-resistant *S. aureus* (VRSA) strains (Chambers and DeLeo, 2009). Despite being less recognized, emerging evidence suggests that other members of the genus also play an important role in human colonization and pathogenicity but are often overlooked or misidentified. Because of their proximity to the host, these species continue to acquire genes for adaptation to and signaling with the host as well as resistance genes, rendering them a new emerging threat (Grundmann *et al.*, 2006; Gajdács, 2019).

Among the most prominent non-aureus staphylococci is *S. pseudintermedius*, which was first identified as a relevant pathogen in veterinary medicine (van Duijkeren *et al.*, 2011; Riegel *et al.*, 2011; Carroll *et al.*, 2021). It was not long until the exposure of

domestic animals to MRSA from close contact with humans contributed to the acquisition of methicillin-resistance in *S. pseudintermedius* as well (Baptiste *et al.*, 2005; Weese and van Duijkeren, 2010). In 2006, the first case of *S. pseudintermedius* infection in humans was recorded (Van Hoovels *et al.*, 2006). Similarly to *S. aureus*, reports of *S. pseudintermedius* infections are being increasingly registered and there is a growing concern about the species' acquisition of resistance genes and evolution within the host (Frank and Loeffler, 2012; Somayaji *et al.*, 2016).

Resistance to β-lactam antibiotics

The cell wall of *S. aureus* is similar to that of other Gram-positive bacteria and is composed mainly of a thick layer of peptidoglycan (60 – 80 nm) in addition to teichoic acids and cell wall-bound proteins. The peptidoglycan layer is composed of long chains of sugar molecules N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) that are cross-linked by short peptides (**Fig. 1**). As it plays an essential role in the mechanical stability of the cell, antibiotics targeting the peptidoglycan biosynthesis such as β-lactams play a major role in fighting against *S. aureus* infections.

The peptidoglycan biosynthesis involves the addition of five glycines to the lysine chain of the precursor Lipid II by the action of three enzymes, FemXAB. After being exported to the cell surface, Gly_5-Lipid II is then polymerized by peptidoglycan glycosyltransferases (PGT) and the formed glycan strands are crosslinked by transpeptidases (TP). PGT and TP domains are parts of penicillin binding proteins (PBP). *S. aureus* harbors four native penicillin binding proteins (PBP1, PBP 2, PBP 3 and PBP4) involved in peptidoglycan metabolism. PGT domains can be found in both bifunctional enzymes, such as PBP2, which also contain transpeptidase (TP) domains, or in monofunctional enzymes. Inhibition of PBPs results in a defect in cell wall metabolism, which leads to growth inhibition or lysis. β-lactam antibiotics bind covalently to the TP domain of PBP. This irreversible inhibition of PBPs blocks the final crosslinking of the nascent peptidoglycan layer, which results in the disruption of cell wall formation (Fisher *et al.*, 2005).

As a first response to β-lactam antibiotics, the exposed *S. aureus* strains produced hydrolyzing enzymes or β-lactamases. Afterwards, methicillin-resistant

Staphylococcus aureus (MRSA) strains emerged upon the introduction of the β-lactamase-resistant methicillin. The β-lactam resistance of MRSA is caused by the acquisition of a fifth low-affinity PBP called PBP2a encoded by the non-native *mecA* (Hartman and Tomasz, 1984). It was assumed for a long time that in presence of β-lactam antibiotics, PBP2a takes over cell wall biosynthesis from the four native staphylococcal PBPs because the latter become rapidly acylated and inactivated at low β-lactam concentrations. However, this model is inaccurate. It has been found that inactivation of the PGT domain of PBP2, but not the TP domain, prevents expression of β-lactam resistance, despite the presence of the low-affinity PBP2a (Pinho *et al.*, 2001). In other words, the cell-wall synthesis in the presence of β-lactam antibiotics requires the cooperative functioning of the native staphylococcal PBPs and the PBP2a (Pinho *et al.*, 2001; Łęski and Tomasz, 2005). Mildly resistant strains of *S. aureus* have also been identified that lack both *mecA* and β-lactamases. In these strains, mutations in the native PBP2 lead to weak binding to penicillin (Georgopapadakou *et al.*, 1982; Hackbarth *et al.*, 1995). Thus, *S. aureus* has developed different strategies to resist β-lactam antibiotics: producing β-lactamases to break down the antibiotic, reducing the binding affinity of its natural PBPs for β-lactams, and most effectively by expressing a new PBP that is not affected by β-lactams (Zapun *et al.*, 2008).

The ability of *S. aureus* to become resistant to all classes of antibiotics that are currently used in clinical settings poses a threat to human health. This resistance occurs through the emergence of *de novo* mutations in chromosomal genes or through the acquisition of resistance determinants that are transferred horizontally between bacteria. Therefore, new therapeutic approaches are constantly under development. (Vestergaard *et al.*, 2019).

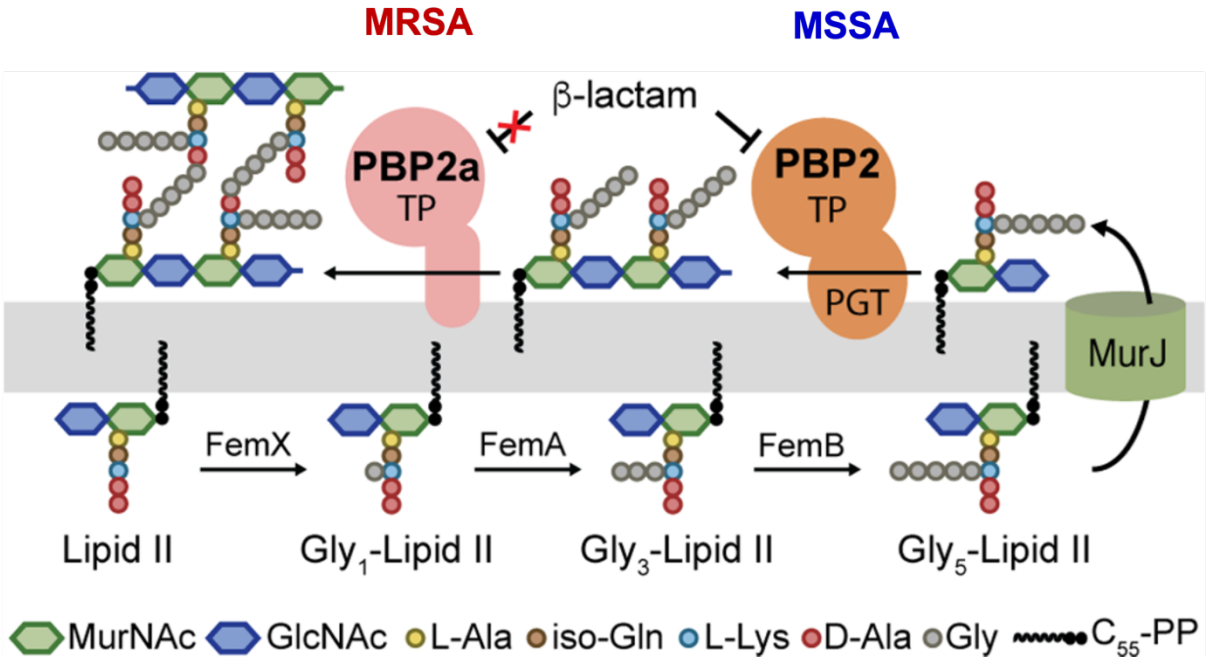

Figure 1. Schematic representation of peptidoglycan biosynthesis in *S. aureus*. PBP2a crosslinks glycan strands when native TPs, such as PBP2, are inhibited by β-lactams. Adapted with permission from (Srisuknimit *et al.*, 2017). Copyright (2017) American Chemical Society.

Staphylococcal membrane phospholipids

Phospholipids play a multitude of roles in the cell, most importantly creating a semi-permeable barrier for the cell that allows the selective uptake of nutrients, defined release of molecules, and prevents harmful substances from entering the cell while maintaining the concentration of intracellular metabolites. Phospholipids are also involved in various cellular processes including the generation of an energy gradient and the scaffolding of membrane-associated processes such as the respiratory electron chain and the synthesis of macromolecules. Additionally, phospholipids play a key role in infection by serving as both a target and barrier for antibiotics and the host immune defense. The composition of phospholipids is therefore crucial for bacterial survival in harsh environments including the host environment during infection. The membrane's lipid composition varies greatly between different bacterial species to accommodate for these functions and is constantly adjusted to changing conditions (Cronan and Gelmann, 1975a; W. Dowhan, 1997). The major membrane lipids in *S. aureus* are phosphatidylglycerol (PG), lysyl-phosphatidylglycerol (L-PG) and cardiolipin (CL) which constitute 98% of the total phospholipids (White and Frerman, 1967).

Fatty acids (FAs) are essential building blocks of cell membranes. They are primarily found in the form of phospholipid acyl constituents. FAs can be divided into two main categories based on their biosynthetic origins: straight-chain and branched-chain. Straight-chain FAs are synthesized from acetyl-CoA and malonyl-CoA, with possible modifications afterwards. Branched-chain FAs, contain one or more alkyl groups attached to the hydrocarbon chain. The most common branched-chain phospholipids found in membranes are monomethylated at the iso- or anteiso- position. They are synthesized from iso, anteiso, or cyclic primer and malonyl-CoA. Methyl iso- and anteiso-branched FAs constitute the majority of fatty acids in the cell membrane of some bacteria, for instance, in *Bacillus subtilis* (93%) (Clejan *et al.*, 1986), *Listeria monocytogenes* (95%) (Annous *et al.*, 1997), and *Staphylococcus aureus* (85%) (Parsons *et al.*, 2011).

Biosynthesis of staphylococcal phospholipids

The biosynthesis of phospholipids is mediated by a complex protein machinery (Sohlenkamp and Geiger, 2016). In staphylococci, it starts with the addition of fatty acids to glycerol-3-phosphate (G3P) through acylation. This process uses a molecule called acyl-acyl carrier protein (acyl-ACP), derived from the dissociated type II fatty acid synthase (FASII) (Lu *et al.*, 2004; Parsons and Rock, 2013).

In *de novo* synthesis of FA via FASII, unlike the mammalian type I fatty acid synthase, each reaction is performed by a different enzyme. The first step, performed by the ACC complex, results in malonyl-CoA. This serves as the starting point for elongation, which involves consecutive reduction, dehydration, reduction, and condensation reactions by the Fab enzymes. The synthesis of branched-chain fatty acids utilizes the same mechanism as the synthesis of straight-chain fatty acids, with malonyl-CoA playing a role in elongation as well. So, the only distinction between the two processes is the starting material and end product (Lu *et al.*, 2004).

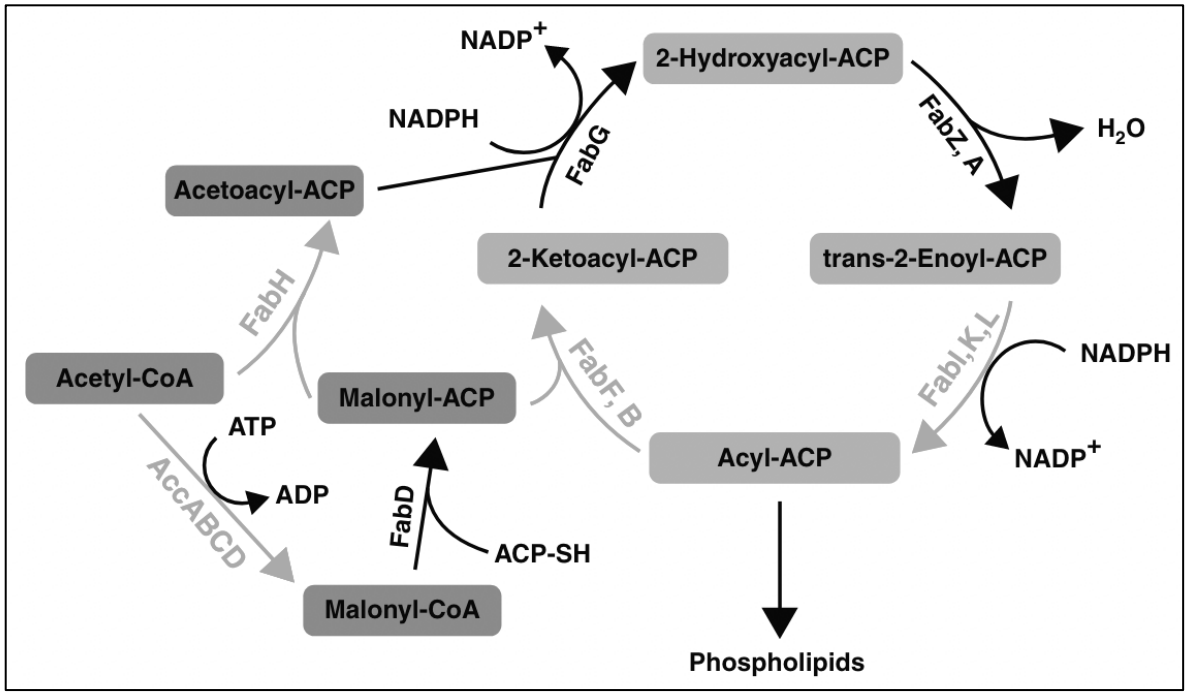

Figure 2. Bacterial FASII cycle. Adapted from (Parsons and Rock, 2011).

Type II fatty acids synthesis was assumed to be non-essential for the survival of staphylococci (Brinster *et al.*, 2009) because these bacteria could obtain extracellular FAs from the environment. However, later studies showed that this is not the case at least in *S. aureus* where a level of heterogeneity in the capacity for FA uptake was observed and the essentiality of FASII was reported (Balemans *et al.*, 2010). Accordingly, various natural products have been found to inhibit enzymes in this pathway and thereby impact bacterial growth (Parsons and Rock, 2011).

The enzyme PlsX converts acyl-ACP obtained from FASII to acyl-phosphate (acyl-PO4), which is then used by the membrane associated G3P acyltransferase PlsY to add a fatty acid to G3P, creating 1-acyl-G3P. The deletion of *plsX* in *S. aureus* results in a strictly fatty-acid auxotroph mutant (Parsons *et al.*, 2014a). PlsY is essential in *S. aureus* (Chaudhuri *et al.*, 2009). A second fatty acid from acyl-ACP is added to the carbon 2 position of acyl-G3P by another membrane associated G3P acyltransferase called PlsC, creating phosphatidic acid (PtdOH), a universal precursor for bacterial phospholipids. This pathway, known as the PlsX/Y pathway, is present in most bacteria including all staphylococci (Lu *et al.*, 2006).

Besides the FAs synthesized by FASII, *S. aureus* can incorporate extracellular fatty acids into PtdOH (Parsons *et al.*, 2014a). In this pathway, FAs enter the cell through

passive diffusion across the membrane and bind to a fatty-acid binding protein FakB1 or FakB2. Saturated fatty acids bind to FakB1, while FakB2 preferentially binds unsaturated fatty acids (Parsons *et al.*, 2014b). The ATP-binding FakA interacts with FakB and converts the bound FA to acyl-PO4 by phosphorylation. The newly formed acyl-PO4 is then incorporated into the membrane. Without FakA, *S. aureus* is unable to incorporate extracellular FA. Additionally, the FakA mutant strain has reduced production of virulence factors, increased resistance to antimicrobial peptides, and no hemolysin production, indicating that FakA depletion results in a general stress response in the cell (Li *et al.*, 2009; Bose *et al.*, 2014).

The synthesis of CDP-diacylglycerol (CDP-DAG) from PtdOH and cytidine triphosphate (CTP) is catalyzed by the phosphatidate cytidylyltransferase Cds. PgsA then replaces the cytidine monophosphate with glycerolphosphate to generate phosphatidylglycerolphosphate (PG-P). PgsA is essential in *S. aureus* (Martin *et al.*, 1999). PG-P is finally dephosphorylated to produce PG, the major phospholipid of *S. aureus* (Yasuhiro *et al.*, 1972).

Some of the PG molecules are processed to CL by the CL synthases Cls1 and Cls2. These enzymes produce CL by catalyzing the fusion of two PG molecules and the release of glycerol (Short and White, 1972). Cls2 plays the major role in CL production under normal conditions while Cls1 is necessary for CL synthesis under acid stress . Cls1 and Cls2 are not essential for *S. aureus* growth, but their absence impacts long-term survival under high salt conditions (Tsai *et al.*, 2011). Another portion of PG can be aminoacylated with an l-lysine group to form L-PG by the multiple peptide resistance factor MprF (Peschel *et al.*, 2001). An *mprF* deletion mutant is still viable but more susceptible to cationic antimicrobial peptides.

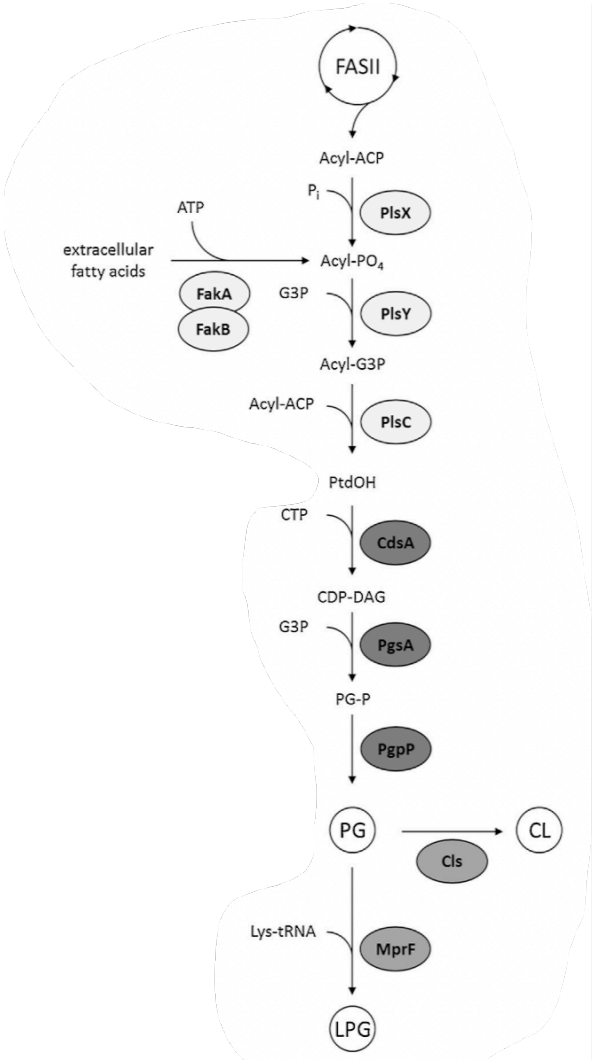

Figure 3. The main enzymes and reactions of staphylococcal phospholipid biosynthesis. Adapted from (Kuhn *et al.*, 2015).

Bacterial membrane fluidity

Biological membranes can exist in distinct physical states, characterized by the organization of their lateral structure, the molecular order, and the mobility of lipid molecules within the bilayer (Eeman and Deleu, 2010). There are three main states of cell membranes: the gel phase, the liquid-crystalline phase, and the liquid membrane phase. The liquid-crystalline phase represents the physiologically optimal fluid membrane state. The gel phase is also known as the solid-ordered phase and is accompanied by growth defect due to disruption of membrane-associated processes, such as electron transport in the respiratory chain, membrane permeability, and substrate transport (Zhang and Rock, 2008). In the liquid phase, also referred to as the liquid-disorder phase, there is a degree of distance and disorder between the

phospholipids which in extreme cases could lead to a disintegration of the lipid bilayer (Vigh *et al.*, 1998).

The fluidity and interfacial properties of the membrane are important in determining the activity of membrane-associated proteins and the regulation of this activity as the membrane needs to provide a suitable environment that stabilizes those proteins in an active conformation (McElhaney, 1982). Membrane lipids play also important roles in transport and enzymatic activities and in solubilizing and dispersing water-insoluble substrates and products. Different types of environmental stresses affect membrane fluidity such as temperature, antibiotics and osmotic stress, among others.

The membrane fluidity is maintained mainly by regulation of its FA components, which differ across bacterial species. Membranes composed of straight-chain fatty acids are regulated by saturation and monounsaturation of fatty acids, while the fluidity of membranes composed of branched-chain fatty acids is primarily restored by modification of the iso and anteiso-FA composition (Annous *et al.*, 1997). As a result, bacteria with straight-chain membrane systems require unsaturated fatty acids (UFA) for growth, while those are nonessential for bacteria with branched-chain membrane systems. In *S. aureus* about 3 to 4% of the total FAs are monounsaturated between C12 and C19 (White and Frerman, 1968; O'Donnell *et al.*, 1985; Parsons *et al.*, 2011). These FAs essentially do not occur free but as part of the complex lipids such as the mono- and diglucosyl diglyceride and the phospholipids PG, L-PG and CL.

The fatty acid composition of membranes is therefore crucial and is shown to be affected by the growth phase, temperature, pH, oxygen availability, carbon source, and an excessive supply of a single primer source (such as isoleucine, leucine, valine, and related substrates) (Kaneda, 1991).

Role of FA in Electron Transport System

Formation of the electron transport system involves the synthesis of protoheme, cytochromes, cytochrome oxidase, phospholipids, glucolipids, and vitamin K2 isoprenologues (Taber and Morrison, 1964; Frerman and White, 1967). The membrane fatty acids composition seems to play an important role in electron transport system assembly as the proportion of fatty acids was found to change remarkably between *S. aureus* grown under aerobic and anaerobic conditions. During

the formation of the electron transport system, there is an increase in the total fatty acids in the membrane. This increase is accompanied by a significant increase in the levels of branched and unsaturated fatty acids of 13, 17, 19, 20, and 21 carbon atoms, which ranges from 3 to 200-fold (White and Frerman, 1968).

Role of FA in *S. aureus* pathogenicity

Staphylococcal membrane fatty acids play also a critical role in the interaction between host and pathogen. Membrane-associated products, such as lipopolysaccharides and lipoproteins are detected by pattern recognition receptors that are encoded in the germline. This detection triggers the activation of various host immune cells (Nguyen and Götz, 2016; Chandler and Ernst, 2017). *S. aureus* produces mainly saturated fatty acids at the human body temperature (37°C) but could incorporate exogenous UFA using the fatty acids kinase (FakA) which remodels the lipid composition of the bacterial membrane (DeMars *et al.*, 2020) and allows it to induce immune stimulatory effects in the host (Nguyen *et al.*, 2015).

Role of FA in stress response

Antibiotics can alter the composition of fatty acids in *S. aureus* in several ways: inhibition of biosynthesis, stimulation of alternative FA production and altering gene expression. The stimulation of alternative FA production could select for antibiotics resistance as it was shown to increase the production of branched-chain FA, which are less susceptible to degradation by antibiotics and host defenses. FA composition is crucial in developing resistance towards membrane-targeting antibiotics such as daptomycin (Boudjemaa *et al.*, 2018). Osmotic stress can also affect the fatty acid composition in *S. aureus* as it was found to increase the proportion of branched-chain FA in cardiolipin (Yasuhiro *et al.*, 1972).

Role of FA in cold adaptation

Bacteria adapt their membrane lipid composition in response to changes in temperature. When the ambient temperature decreases, the fluidity of the bacterial cell membrane decreases as well. In response, bacteria increase the amount of low melting temperature FA in their membranes, such as UFA (Cronan and Gelmann, 1975b). This prevents the transition of the membrane from a fluid-crystalline state to

a solid-ordered gel state and the impairment of physiological functions. This phenomenon, known as homeoviscous adaptation, involves different FA altering mechanisms depending on the bacterial membrane composition.

Bacterial cold shock response

Temperature is one of the most critical environmental stresses that living organisms must cope with. Bacterial physiology is generally reprogrammed through the activation of adaptive systems in response to changes in temperature. These changes are sensed by conformational changes in protein, DNA, and RNA molecules (Klinkert and Narberhaus, 2009). At low temperatures, bacteria produce cold shock proteins (CSP) that seem to be involved in various cellular processes such as transcription, translation, and recombination (Weber and Marahiel, 2003) and mainly work at modulating changes in cell size, wall thickness, and metabolic homeostasis (Onyango *et al.*, 2013; Alreshidi *et al.*, 2015; Barria *et al.*, n.d.). The process of cold adaptation has been extensively studied in *E. coli* as a model for Gram-negative bacteria. *E. coli* encodes nine CSPs and its growth is impaired at low temperatures when four out of these nine variants are simultaneously deleted (Fang *et al.*, 1997; Xia *et al.*, 2001; Uppal *et al.*, 2008). Similar phenomenon has been reported in Gram-positive bacteria such as *Listeria monocytogenes* (Schmid *et al.*, 2009), *Bacillus subtilis* (Willimsky *et al.*, 1992; Graumann *et al.*, 1997), *Lactococcus lactis* (Chapot-Chartier *et al.*, 1997) and *Staphylococcus aureus* (Duval *et al.*, 2010).

Cold temperatures impact the physical state of the bacterial membrane (Zhang and Rock, 2008) and adaptive mechanisms exist in bacteria to maintain an active physiological membrane for membrane-dependent processes. The process of thermal control involves mainly changes in the membrane fatty acid composition. Firmicutes respond to cold shock by incorporating low melting temperature fatty acids in their membrane by desaturation, shortening of FA chain length or alteration of branching from iso to anteiso (Grau and de Mendoza, 1993; Aguilar *et al.*, 1998). This decreases the melting temperature at which the membrane transitions from a solid to a liquid-crystalline state, thus providing the membrane with increased fluidity.

Fatty acid desaturation

Fatty acid desaturases are enzymes that introduce double bonds into previously synthesized fatty acid acyl chains. They play an essential role in modulating the composition of membrane fatty acids and thereby maintaining a proper structure and function of biological membranes under different conditions (Stumpf, 1980; Stubbs and Smith, 1984). Fatty acid desaturases are found in most organisms and a variety of types exists (Los and Murata, 1998). While desaturation targets mainly pre-existing FA synthesized by dissociable fatty acids synthase FASII (Kaneda and Smith, 1980), *de novo* biosynthesis of UFA exists in some bacteria, such as *Escherichia coli* through the activity of FabA (Bloch, 1963; Heath and Rock, 1996).

There are three types of fatty acid desaturase: acyl-CoA, acyl-ACP, and acyl-lipid desaturases. Acyl-lipid desaturases catalyze the desaturation reaction of fatty acids that are in a lipid-bound form in bacteria as well as in some animals and yeast (Murata and Wada, 1995). Those desaturases are hydrophobic proteins typically 300 – 350 amino acid residues long with several (4 – 6) transmembrane domains (Murata and Wada, 1995). The desaturation reaction requires one molecule of oxygen and two electrons. The electrons are donated by different carriers in different types of organisms and organelles. In the case of acyl-lipid desaturases, the electron donor is ferredoxin. The majority of acyl-lipid desaturases recognize a broad range of fatty acyl substrates and introduce double bonds at various positions. The desaturase discussed in the rest of this work is an acyl-lipid desaturase.

Other cold adaptation mechanisms

Shortening of FA chain length and alteration of branching from iso to anteiso are the main cold adaptation strategies seen in bacteria with high branched-chain FA membrane composition (Kaneda, 1991; Annous *et al.*, 1997; Klein *et al.*, 1999). Changing the FA chain length or degree of branching by *de novo* synthesis of the corresponding lipids represents an energy intensive adaptation mechanism. Considering the limiting environmental factors for bacteria during growth, *de novo* synthesis of lipids should be unfavorable especially under cold shock stress. *Listeria monocytogenes* is an example where the mechanism of branched-chain alteration has been studied in detail (Seel *et al.*, 2018).

Two-component systems and sensing the environment

Two-component systems (TCS) are signaling mechanisms that allow living cells to sense and adapt to environmental cues by triggering physiological changes in the cell. The triggered cellular response could be related to various processes such as development, cell division, metabolism, pathogenicity, antibiotic resistance, and taxis (Hoch, 2000; Zschiedrich *et al.*, 2016).

TCSs are capable of detecting and responding to a wide range of chemical and physical stimuli through different perception modes (Mascher *et al.*, 2006; Krell *et al.*, 2010). The stimuli include changes in temperature, pH, oxygen levels, light intensity, osmotic pressure, the redox state of the cell and the presence of specific chemicals or molecules. The stimuli can be detected in the periplasm or extracellular space, within the membrane, or in the cytoplasm (Mascher *et al.*, 2006). The most common response to signal perception and transduction by TCSs is the activation or repression of the transcription of specific genes (Stock *et al.*, 2000).

TCSs typically consist of a histidine kinase (HK) and a corresponding response regulator (RR). HKs are composed of a variety of sensor and signaling domains and therefore display a wide range of architectures (Gushchin and Gordeliy, 2018). According to current sequence databases, the majority of histidine kinases (63%) are homodimeric and associated with the membrane and the rest are cytoplasmic (Mascher *et al.*, 2006). RRs are cytoplasmic and always have a receiver (REC) domain that can be phosphorylated. Many response regulators also have additional domains, such as an output effector domain. The REC domain can bind to proteins in a regulated manner, while effector domains often function as DNA-binding transcription factors. Effector domains may also have RNA-binding, protein-binding, or enzymatic activities (Zschiedrich *et al.*, 2016).

Sensor HKs are bifunctional enzymes that can switch between kinase and phosphatase modes in response to signals. In the kinase mode, a HK undergoes autophosphorylation of a conserved histidine residue and then transfers the phosphoryl group to a conserved aspartate residue on the receiver (REC) domain of its partner RR. In the phosphatase mode, it catalyzes the dephosphorylation of the phosphorylated REC domain. The phosphorylation of the REC domain allosterically modifies its activity (Gao and Stock, 2009; Zschiedrich *et al.*, 2016).

Biochemical data and structural studies on various HKs have provided insight into different signaling models. However, understanding the signal transduction between the sensor and output domains is hugely complex due to the great flexibility of histidine kinases (Hulko *et al.*, 2006; Etzkorn *et al.*, 2008; Albanesi *et al.*, 2009; Casino *et al.*, 2009; Cheung and Hendrickson, 2010; Bhate *et al.*, 2015). Recently, the research on TCS signaling has been significantly advanced, especially due to the availability of X-ray structures of several HKs in distinct states of activity. Most notably, structural data have been elucidated using crystal structures of the histidine kinase DesK in *Bacillus subtilis*, both alone and together with the REC domain of its cognate RR, DesR, in different functional states. This provided the first models for the TCS catalytic reactions (Trajtenberg *et al.*, 2016).

TCSs in *Staphylococcus aureus*

The genome of *S. aureus* encodes a total of 16 conserved TCSs (Bleul *et al.*, 2022). All TCSs are dispensable for *S. aureus* living under constant environmental conditions, with *WalRK* being the only exceptionally essential TCS (Martin *et al.*, 1999; Villanueva *et al.*, 2018). Nevertheless, sensing and responding to environmental conditions through signal transduction is critical for the expression of virulence factors and the development of bacterial pathogenesis (Haag and Bagnoli, 2017a). Hence, some TCSs were considered as suitable targets to inhibit bacterial growth (Gotoh *et al.*, 2010).

These TCSs fall under five main groups according to their function in: global regulation of virulence (AgrCA and SaeRS), response to antimicrobial peptides and cell wall damage (VraSR, GraXSR and BraRS), cell wall metabolism and autolysis (WalRK, ArlRS and LyrSR), respiration, fermentation, and nitrate metabolism (SrrAB, NreCBA and AirRS) and nutrient sensing and metabolism (HssSR, KdpDE and PhoRP). The function of two TCSs remains unidentified, namely TCS-2 and TCS-7. These regulatory networks are often interconnected, influencing each other's expression, allowing for precise control of gene regulation in staphylococcus (Haag and Bagnoli, 2017a).

Quorum sensing and virulence regulation

In most bacteria, the expression of virulence genes is tightly regulated. This allows the bacteria to survive within the host and evade the host's immune system by only turning on these virulence genes under the appropriate conditions. *S. aureus* is no exception, as its virulence factors are specifically activated in response to cell density through quorum sensing (QS), energy availability, and various environmental signals.

The accessory gene regulator (Agr) in *S. aureus* coordinates the switch to an invasive mode by expressing and suppressing different sets of genes. During the establishment phase, genes for adhesins and protective surface proteins such as staphylococcal protein A (Spa) are expressed. During the invasive phase, Agr suppresses the latter and triggers the expression of virulence factors such as phenol-soluble modulins (PSMs), α-hemolysin and secreted proteases that promote host cell and tissue damage (Recsei *et al.*, 1986; Novick, 2003; Quave and Horswill, 2014). These factors disrupt the epithelial barrier, prevent opsonization by antibodies and complement, destroy neutrophils, interfere with neutrophil chemotaxis, and neutralize antimicrobial peptides (Spaan *et al.*, 2013; Foster *et al.*, 2014; Otto, 2014).

There are several other global regulatory systems that control gene expression and help *S. aureus* adapt to changes in its environment. Those include: SarA, which regulates the expression of genes involved in virulence and biofilm formation; Rot, which regulates the expression of genes involved in resistance to oxidative stress and antibiotic tolerance; and SigB which regulates the expression of genes involved in stress response and virulence. These global regulators functions overlap and hence they work together (Booth *et al.*, 1997; Lauderdale *et al.*, 2009). They also enable cross talk between *S. aureus* and other staphylococci (Canovas *et al.*, 2016).

The accessory gene regulator (Agr)

The agr locus encodes two divergent RNA transcripts, RNAII and RNAIII, which are regulated by the promoters P2 and P3, respectively (Novick, 1993; Morfeldt *et al.*, 1995). RNAII encodes the four proteins of the AgrBDCA quorum-sensing module. AgrB, a transmembrane endopeptidase, plays a crucial role in the processing and secretion of AgrD propeptide, resulting in the production of the autoinducing peptide (AIP). There are four known types of AIP produced by *S. aureus*. The strains can be

grouped based on their response to the different AIP, as each type only activates the agr response in strains within the same group. Reciprocally, AIP from one group of *S. aureus* can inhibit the activation of the agr response in other groups (Ji *et al.*, 1995; Ji *et al.*, 1997).

AgrC and AgrA are the sensor kinase and response regulator of a two-component system (TCS), which transmits the quorum-sensing signal once the AIP concentration reaches a threshold level. AIP binds the extracellular sensor domain of the membrane bound AgrC, which undergoes autophosphorylation of its kinase domain. The phosphate is then transferred to the response regulator AgrA. The phosphorylated AgrA regulates this agr autoinduction cycle and binds to the P2 and P3 promoters. It was initially indicated that the regulator AgrA has an exclusive role in *agr* activation, but it was later discovered that AgrA induced the expression of α- and β-phenol-soluble modulins (PSMs) by direct activation with their respective promoters (Queck *et al.*, 2008; Peschel and Otto, 2013).

The transcription of *agr* is further regulated by other transcription factors than AgrA such as SarA and SarR that can bind the P2 promoter region and thereby upregulate and downregulate the transcription from P2, respectively (Reyes *et al.*, 2011). In addition to self-activation, *agr* expression is also known to be affected by environmental stimuli, such as changes in glucose levels and pH (Regassa *et al.*, 1992).

RNAIII is the major effector of *agr* (Novick, 1993). The interaction of RNAIII with target mRNAs modulates the expression of more than a hundred genes encoding surface proteins, excreted toxins, and proteases, among others (Dunman *et al.*, 2001; Haag and Bagnoli, 2017b). RNAIII transcript encodes haemolysin (Hld), however, deletion of the *hld* gene does not result in an agr- phenotype (Janzon and Arvidson, 1990). RNAIII is composed of 14 stem-loop (also known as hairpin) structures and two long helices made from long-range base pairings, which divide into separate structural domains (Benito *et al.*, 2000). Different domains mediate the versatile regulatory activity of RNAIII which takes place at the translational or the transcriptional level. For instance, the 5' end of RNAIII promotes the translation of the *hla* transcript, by competing with the intramolecular secondary RNA structure blocking its ribosomal binding site (RBS) (Morfeldt *et al.*, 1995). On the contrary, hairpins H13, H14, and H7

act as repressors for virulence factors like staphylococcal protein A (*spa*), coagulase (*coa*), and the repressor of toxins (*rot*) , by binding to their mRNAs in an antisense manner and causing RNaseIII-dependent degradation (Huntzinger and Romby, 2005; Boisset *et al.*, 2007; Chevalier *et al.*, 2010).

Agr plays a primordial role in the expression of an invasive phenotype in *S. aureus* and disease development (Jarraud *et al.*, 2002; Yarwood and Schlievert, 2003; Singh and Ray, 2014). Secreted proteins and toxins behind this phenotype include lipases, protease, nucleases, PSMs, haemolysins, leukocidin, toxic shock syndrome toxins and exfoliative toxins (Dunman *et al.*, 2001). The expression of this large number of virulence factors results in a heavy metabolic burden on the bacterium. Accordingly, it was observed that non-stressful conditions, such as the colonization of immunocompromised patients, favor the selection of agr- strains. Reportedly, 15 – 60% of clinical isolates of *S. aureus* are defective in agr (Park *et al.*, 2007; Paulander *et al.*, 2012). Other factors that affect Agr expression include the expression of resistance genes such as PBP2a, presumably also due to its metabolic burden. High levels of PBP2a result in lower Agr expression and deleting PBP2a can restore Agr activity (Rudkin *et al.*, 2012; Pozzi *et al.*, 2012). Interestingly, Agr regulation has also been found to be different between *S. aureus* isolates from hospital-acquired (HA) and community-acquired (CA) infections. The difference was not due to Agr expression in particular but on the level of regulation of genes in the signaling cascade which differed significantly between HA and CA strains (Cheung *et al.*, 2011). Hence, the effect of Agr on protein expression may be affected by the strain background.

The agr quorum-sensing system is a well-studied model for signal transduction and plays a major role in regulating staphylococcal virulence gene expression. Despite extensive research, questions about the Agr and its impact on *S. aureus* infections remain unanswered. Why does the Agr TCS seem to have a negative impact on the bacterium in hospital environments? Are the non-stressful and/or infection-facilitating conditions the only factors contributing to the fitness burden associated with Agr?

Biogenic amines

While QS is known to drive intra- and inter-species communication, there is increasing evidence for interkingdom communication between bacteria and their host. This can be mediated by a plethora of metabolites among which are the biogenic amines.

Biogenic amines are neurotransmitters or neuroactive compounds found in the mammalian central nervous system as well as in other living organisms. They are essential for the proper functioning of the nervous system and are involved in processes such as mood regulation, appetite control, sleep, pain sensation, and cardiovascular function. The type and amount of biogenic amine released, as well as the receptors it activates, determine its specific effects on the body. Examples of biogenic amines include serotonin, dopamine, epinephrine, norepinephrine, and histamine. Deregulation of aminergic signaling has been linked to numerous neurological diseases such as Parkinson's disease, schizophrenia, and depression. Given their significance, the physiological levels of biogenic amines are tightly regulated at various levels of synthesis, storage, and removal by reuptake systems and enzymatic degradation to terminate aminergic signaling. A complex network of enzymatic activities regulates the synthesis of these bioactive amines, which also produces intermediate products.

In addition to the major biogenic amines, there are other amines that are present at low levels in the mammalian central nervous system and are referred to as trace amines (TAs). Typical TAs include compounds like tryptamine, tyramine, phenylethylamine, and octopamine. Both TAs and conventional biogenic amines are produced through a common synthesis network. Early theories suggested that TAs were simply unavoidable byproducts of these biogenic amines synthesis pathways, due to their low concentration and lack of information about specific receptors. This changed recently with the discovery of a specific class of G-protein-coupled receptors called trace amine activated receptors (TAARs) (Borowsky *et al.*, 2001). TAARs can be activated at nanomolar concentrations of TAs. The discovery of TAARs revived the interest in these molecules (Grandy, 2007; Roeder, 2016; Khan and Nawaz, 2016; Gainetdinov *et al.*, 2018).

Serotonin

Serotonin (SER) is a monoamine neurotransmitter. It is synthesized from the amino acid tryptophan in the brain and other parts of the body in a two-step process: first, the enzyme tryptophan hydroxylase converts tryptophan into 5-hydroxytryptophan (5-HTP), 5-HTP is then converted into serotonin by the enzyme aromatic amino acid decarboxylase. SER is synthesized and found in a variety of organisms. SER has also been found in bacteria, where it is thought to play a role in intercellular signaling and communication. In addition, serotonin has been shown to play a role in the interaction between bacteria and their host organisms (Sugiyama *et al.*, 2022). For instance, SER plays an important role in the gut-brain axis (Gershon and Tack, 2007; Fung *et al.*, 2019) Certain bacteria have been shown to produce SER, which can stimulate the host's immune response and contribute to the development of inflammatory bowel diseases such as colitis (Spohn and Mawe, 2017). SER can also regulate the host's immune response by affecting the production of cytokines and other signaling molecules (Baganz and Blakely, 2013; Shajib and Khan, 2015). SER is also involved in the universal melatonin biosynthesis pathway. It can be acetylated into N-acetylserotonin (NAS) which serves as the direct precursor of melatonin (N-acetyl-5-methoxytryptamine). NAS also functions as a melatonin receptor MT3 agonist in the gut, thereby affecting the circadian rhythm (Axelrod and Weissbach, 1960; Jockers *et al.*, 2016). The levels of NAS in the brain and gut are crucial for regulating melatonin synthesis (Klein, 2007; Maronde *et al.*, 2011). Beyond its role in the melatonin pathway, NAS also binds to TrkB receptors, exhibiting anti-depressive, neurotrophic, and cognitive-enhancing properties. NAS also has important antioxidant properties (Wölfler *et al.*, 1999; Oxenkrug, 2005).

Trace Amines (TA)

The trace amines (TAs) tryptamine (TRY), phenethylamine (PEA) and tyramine (TYM) are produced by decarboxylation of aromatic amino acids tryptophan, phenylalanine, and tyrosine respectively. Tryptamine is of particular importance because it is involved in various functions through receptor-mediated or receptor-independent pathways (Jones, 1982). An important role of TRY is the regulation SER’s effects by acting directly on serotonin receptors (Jones, 1982)., The acetylated form N-acetyltryptamine

(NAT) acts as a partial agonist of melatonin receptors and plays also a significant role in the regulation of circadian rhythm.

TA-producing staphylococci

Staphylococci have recently been found to produce serotonin and tryptamine through the action of staphylococcal aromatic amino acid decarboxylase (SadA). Many staphylococcal species, such as *Staphylococcus pseudintermedius* ED99, harbor SadA, which acts as a promiscuous enzyme capable of decarboxylating various biogenic aromatic amino acids. SadA-generated products have different functions and have been found to be advantageous for both bacteria and the host. For instance, they enhance the adherence and internalization of staphylococci into intestinal epithelial cells by activating α-2 adrenergic receptors, leading to increased bacterial colonization and survival in the host (Luqman *et al.*, 2018). On the other hand, SadA products contribute to faster wound healing by acting as β-2 adrenergic receptor inhibitors, which inhibits the negative impact of epinephrine on cell motility and wound healing (Luqman *et al.*, 2020). Different staphylococcal species produce differing amounts of TAs. SadA is distributed among staphylococci and TAs are mainly produced by the members of the staphylococcal cluster groups Intermedius and Simulans-Carnosus.

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Chapter 1 – Sensitizing *Staphylococcus aureus* by PBP2-specific single-domain antibodies and β-lactam antibiotics

Chapter 1

Introduction

Despite the efforts to mitigate its resistance, *S. aureus* remains a threat to human health due to the various antibiotics resistance strategies it can employ. Among those are the production of enzymes that neutralize drugs such as β-lactamases, acquiring mutations that reduce the affinity of antibiotics to their target and, the acquisition of new resistance mechanisms from other bacterial species by horizontal gene transfer. β-lactam antibiotics target the final stages of peptidoglycan biosynthesis in *S. aureus* by inhibiting the transpeptidase domain (TP) of PBPs that crosslink glycan strands in cell wall assembly. In methicillin resistant *S. aureus* (MRSA), the cell-wall synthesis in the presence of β-lactam antibiotics requires the cooperative functioning of the native staphylococcal PBPs and the PBP2a (Pinho *et al.*, 2001; Łęski and Tomasz, 2005). This indicates that targeting PBP2 as well as PBP2a is necessary to inhibit MRSA.

One strategy that has been under development since the emergence of antibiotics resistance is the use of monoclonal antibodies (Ab) against specific bacterial targets (Rennermalm *et al.*, 2001; Conrath *et al.*, 2001). Antibodies are adaptor molecules containing binding sites for antigen at one end and for effector molecules at the other. They can bind to a wide variety of antigens and thereby neutralize some toxins and viruses or, trigger the complement system and cell mediated killing to eliminate the foreign bodies. Using monoclonal antibodies from various animal species showed limited success as an antibiotic's replacement due to immunogenicity problems or the inhibition of antibodies before they reach their target (de Silva *et al.*, 2016). The main protein fulfilling this task is staphylococcal protein A (Spa) which has high binding affinity to the constant fragment of conventional antibodies (Fc) (Hjelm *et al.*, 1972).

The discovery of single-chain antibodies lacking the light chain in the serum of dromedary camel opened up new potential for therapeutic antibodies development (Hamers-Casterman *et al.*, 1993). Those unconventional, naturally occurring heavy chain antibodies (HCAb) consist of a single heavy-chain with only three antigen-binding loops that bind selectively to a specific antigen (Muyldermans *et al.*, 1994; Maass *et al.*, 2007).

Camelid immunoglobulin germlines encode both conventional antibodies and HCAb (Hamers-Casterman *et al.*, 1993; Nguyen *et al.*, 1998). The variable domain in HCAb (V_HH) is similar to conventional human heavy chain variable domain (V_H) but with unique structural characteristics. The difference is caused by the substitution of five

residues in V_HHs of otherwise conserved sequences in the framework region 2 (FR2): Leu11Ser, Val37Phe or Val37Tyr, Gly44Glu, Leu45Arg or Leu45Cys and Trp47Gly. This substitution compensates for the absence of the light chain, improves solubility and hampers the association with the conventional variable domain of the light chain (V_L) (Davies and Riechmann, 1994; Nieba *et al.*, 1997; Vu *et al.*, 1997; Harmsen *et al.*, 2000). Accordingly, the camel blood serum is distinctive for being a source of both conventional Ab and HCAb as well as heavy chain variable domain fragments of those occurring singly in a soluble form, namely V_H and V_HH (Chothia *et al.*, 1992). Unlike V_H in humans that occur singly only in pathological disorders, camelid V_H and V_HH are usually stable and functional in antigen binding (Chothia *et al.*, 1992; Nguyen *et al.*, 1998). Soluble camelid V_H/V_HH (typically 12 – 15 kDa) are significantly smaller as compared to conventional Ab in other mammalian species (150 – 160 kDa). As a result, V_HH possess not only a high epitope specificity but also can bind small active site pockets and inhibit enzymes (Lauwereys *et al.*, 1998; Transue *et al.*, 1998; De Genst *et al.*, 2006; Koch-Nolte *et al.*, 2007).

Phage libraries displaying V_H/V_HH clones have been constructed (Arbabi Ghahroudi *et al.*, 1997; Muyldermans and Lauwereys, 1999; Verheesen *et al.*, 2006; Maass *et al.*, 2007; Thanongsaksrikul *et al.*, 2010) and used to screen for antibodies with potential use as a therapeutic tool against multiple targets (Harmsen *et al.*, 2006; Thanongsaksrikul *et al.*, 2010; Wrapp *et al.*, 2020).

The many favorable drug properties of V_H/V_HH made them widely used in research: their small size, expression of antigen-binding domain as a single peptide facilitating phage display, their structural stability (van der Linden *et al.*, 1999; Dumoulin *et al.*, 2002) and their ease of production (Arbabi Ghahroudi *et al.*, 1997; Gibbs, 2005).

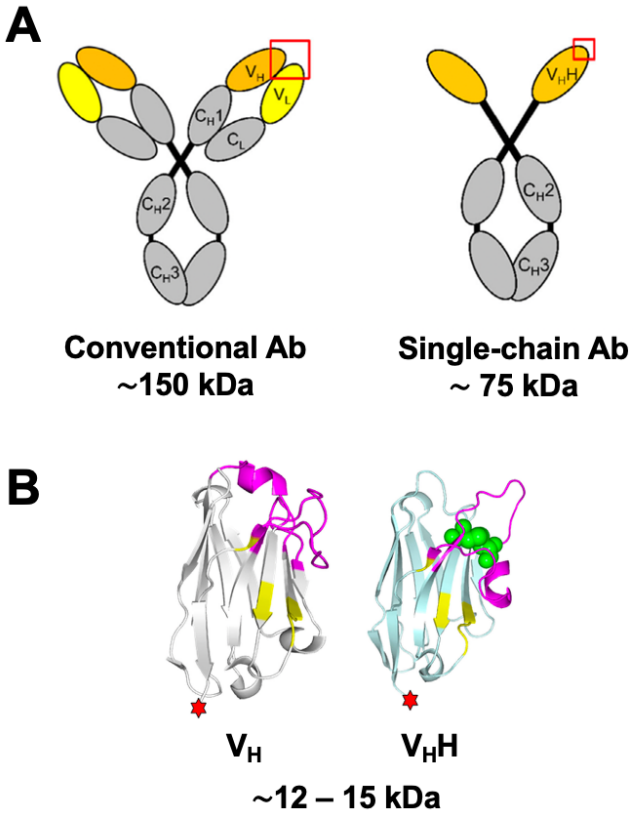

Figure 1.1 A. Conventional vertebrate immunoglobulins or antibodies (Ab) are tetramers of two heavy and two light chains. The variable domains of each chain (VH and VL, respectively) assemble to form the antigen-binding site. Camels produce homodimeric single chain Ab devoid of light chains. **B.** Structures of exemplar variable domains VH and VHH: here human VH (anti-MDM4, PDB ID: 2VYR) and camelid VHH (antilysozyme, PDB ID: 1JTT) are represented. The CDR loops are colored in magenta. The interface with the absent VL domain is marked in yellow. Camelid VHH often contain noncanonical intradomain disulfide linkages (green) sculpting the binding site. The exposed C termini are marked with a red star.

Here, we aimed to produce a single-domain antibody (V_H/V_HH) that can bind to the transpeptidase domain of PBP2 and thereby inhibit the growth of methicillin-sensitive *S. aureus* (MSSA). In that order, we screened a library of camelid single-domain antibodies (V_H/V_HH) for those that can bind the transpeptidase domain of PBP2. The binding activity of selected antibodies was tested and the structural basis for interaction between these antibodies and the transpeptidase domain was analyzed. Additionally, we tested the *in-vitro* activity of selected antibodies alone and in combination with penicillin to inhibit the growth of MSSA. We investigated whether a combination therapy of antibodies and antibiotics could decrease the MIC of β-lactams. This approach could alleviate the selective pressure against a single drug molecule and reduce the usage of antibiotics.

Results

Expression and purification of recombinant PBP2_TP

We started with the production of the antigen that will be used to select antibodies that can bind the transpeptidase (TP) domain of PBP2. The TP-encoding part of the *pbp2* gene of *S. aureus* HG001 was amplified and cloned into the inducible expression vector pET22b(+) with a His-tag added to the C-terminal end. As expected, we found the recombinant PBP2_TP localized in inclusion bodies (IBs) and not the soluble fraction of cell lysates of IPTG-induced *E. coli* cultures. Therefore, the IBs were solubilized, purified, and subjected to Ni-NTA column chromatography under denaturing condition to isolate the his-tagged recombinant PBP2_TP (rPBP2_TP). Then, rPBP2_TP was refolded by stepwise dialysis (**Fig. 1.2**).

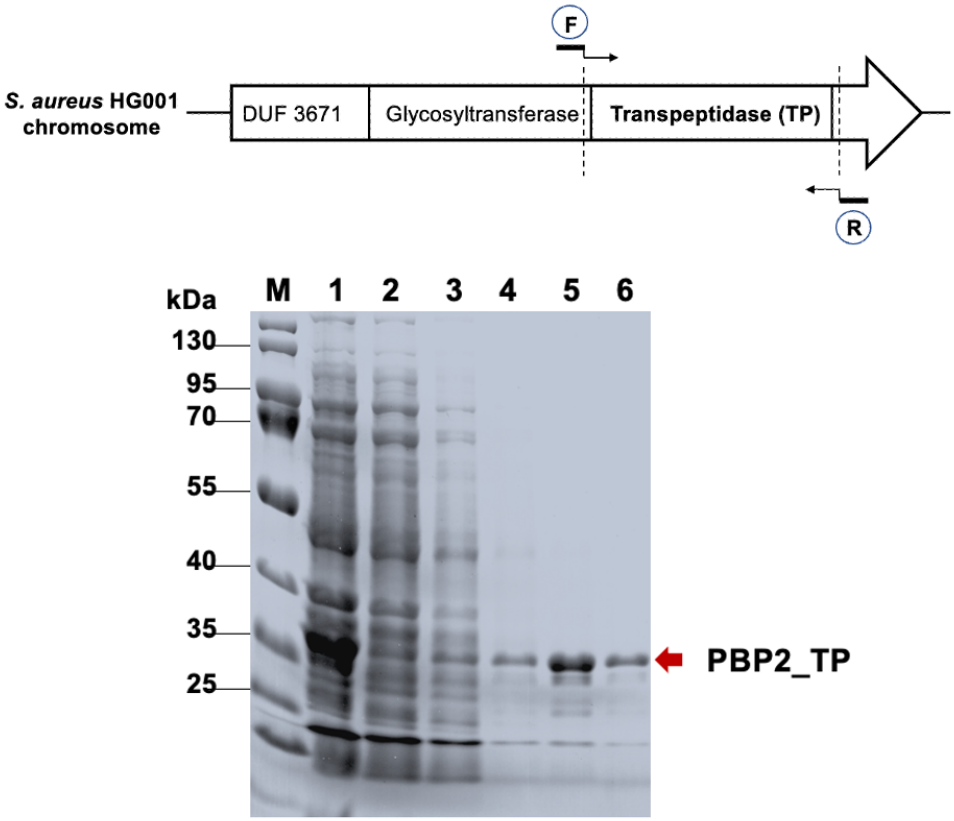

Figure 1.2 Recombinant PBP2_TP (30 kDa) purified from the insoluble fraction of *E. coli* BL21(DE3) carrying the expression plasmid pET22b(+)-*pbp2_TP*. Lane M, protein ladder; lane 1, whole cells lysate of *E. coli* BL21(DE3) (pET22b(+)-*pbp2_TP*); lane 2, purified inclusion bodies of PBP2_TP protein; lane 3, flowthrough of Ni-NTA purification; lane 4-5: elution fractions of purified PBP2_TP protein by Ni-NTA in denaturing condition; lane 6, refolded TP protein.

Phage-biopanning for rPBP2_TP-specific V_H/V_HH

The purified rPBP2_TP was immobilized on a polystyrene surface well and used to screen the V_H/V_HH library for specific antibodies by phage-biopanning method.

Phage bio-panning using the V_H/V_HH phage library of ~ 4 × 10^{11} different single – domain antibodies (Thanongsaksrikul *et al.*, 2010) for those that can bind rPBP2_TP resulted in 37 positive clones among 96 randomly tested *E. coli* HB2151 transformants. The ability of the positive *E. coli* HB2151 clones to express the pCANTABE5 phagemid-encoded V_H/V_HH was confirmed by western blot analysis using an antibody against the E-tag on the phagemid-encoded antibodies. 30 clones could produce the corresponding antibodies, as detected in the IB fraction (**Fig 1.3**).

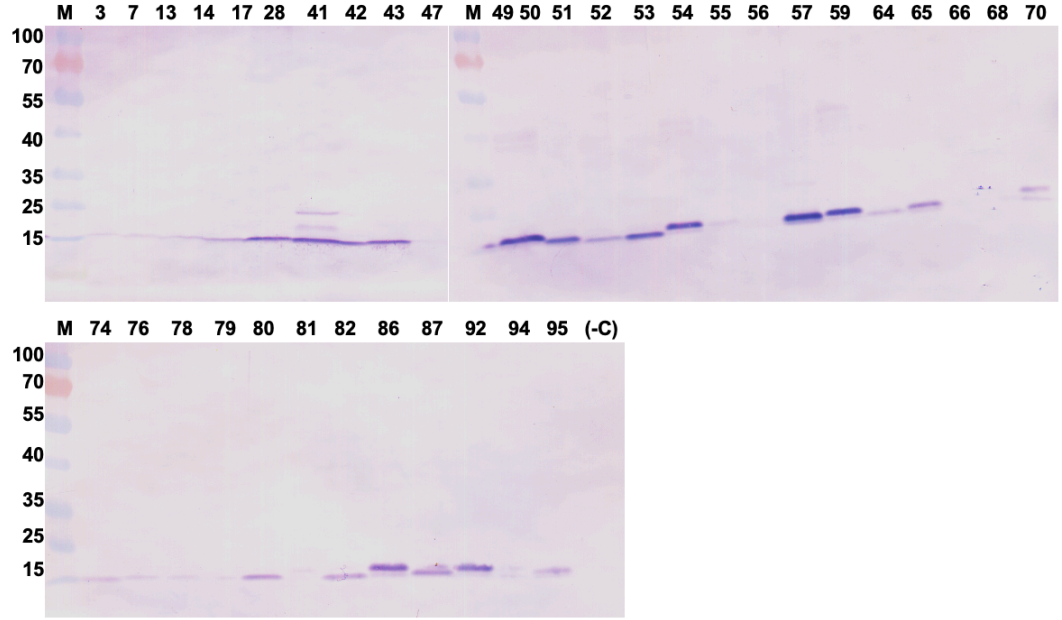

Figure 1.3 Western blot analysis of the PCR-positive clones of *E. coli* HB2151 transformed with V_H/V_HH phagemid. The proteins in the cell lysate of the 37 clones of V_H/V_HH-phagemid transformants were separated by 12% SDS-PAGE (10 µl of cell lysate per well). The proteins were transferred to a nitrocellulose membrane. V_H/V_HHs (~12-15 kDa) were detected using an E-tag specific antibody. Lane M, protein ladder; other lane numbers correspond to the respective *E. coli* clone. 30 out of the 37 clones could express the pCANTABE5 encoded V_H/V_HH.

Binding of V_H/V_HH clones to rPBP2-TP

The purified V_H/V_HH clones were first tested for binding rPBP2_TP by indirect ELISA. All of the 30 clones showed a significant binding to the immobilized rPBP2_TP as compared to binding to BSA as a control. The binding of a VH/V_HHs to *S. aureus*

PBP2a_TP is considered significant when the measured absorbance A_{405nm} is at least two times higher than with the BSA control (**Fig. 1.4**).

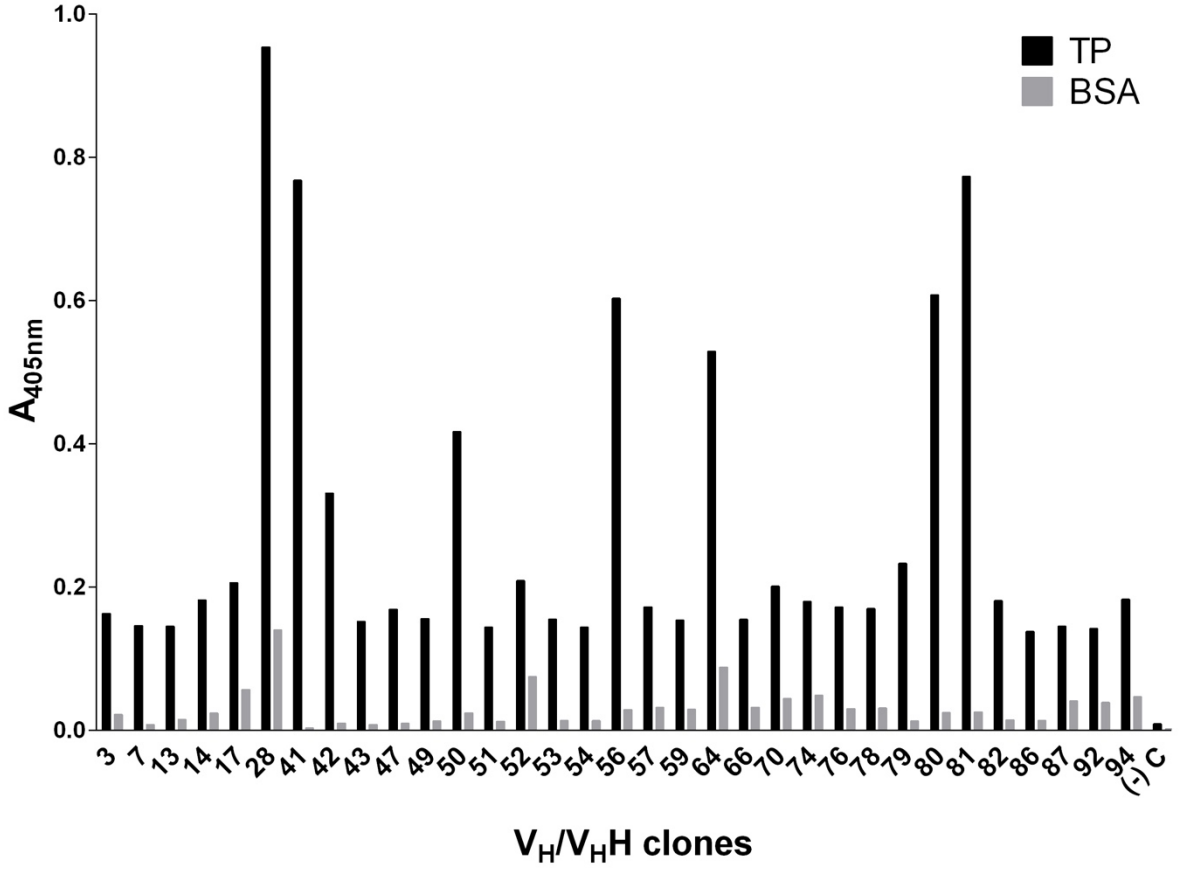

Figure 1.4 Indirect ELISA for determining the binding of V_H/V_HH to rPBP2_TP. Bar plot of the absorbance values (A_{405nm}) of different ELISA wells coated with PBP2_TP or BSA. The binding of a V_H/V_HH to *S. aureus* PBP2a_TP is considered significant when the A_{405nm} is at least two times higher than with the BSA control. The cell lysate from the parent *E. coli* HB2151 was added as a negative control (-C) for binding.

Characterization of the V_H/V_HH clones

To identify the single-domain antibodies as V_H or V_HH, we isolated the pCANTAB5E phagemid from the 30 *E. coli* clones and sequenced the inserted gene. The deduced amino acid sequences were analyzed using the International Immunogenetics Information System server. Sequencing results showed that some of the clones were identical which reduced the clone number to 26. The immunoglobulin frameworks (FRs) and complementarity determining regions (CDRs) of the 26 V_H/V_HH were predicted using TMGT/V-QUEST. The results show that 5 out of the 26 antibodies are V_HH as they possess the hallmark amino acids for V_HH in their FR2, and others are

conventional V_H clones. The V_H/V_HH carried long CDR3 composed of at least 15 amino acids, another characteristic of camelid antibodies (**Fig. 1.5**).

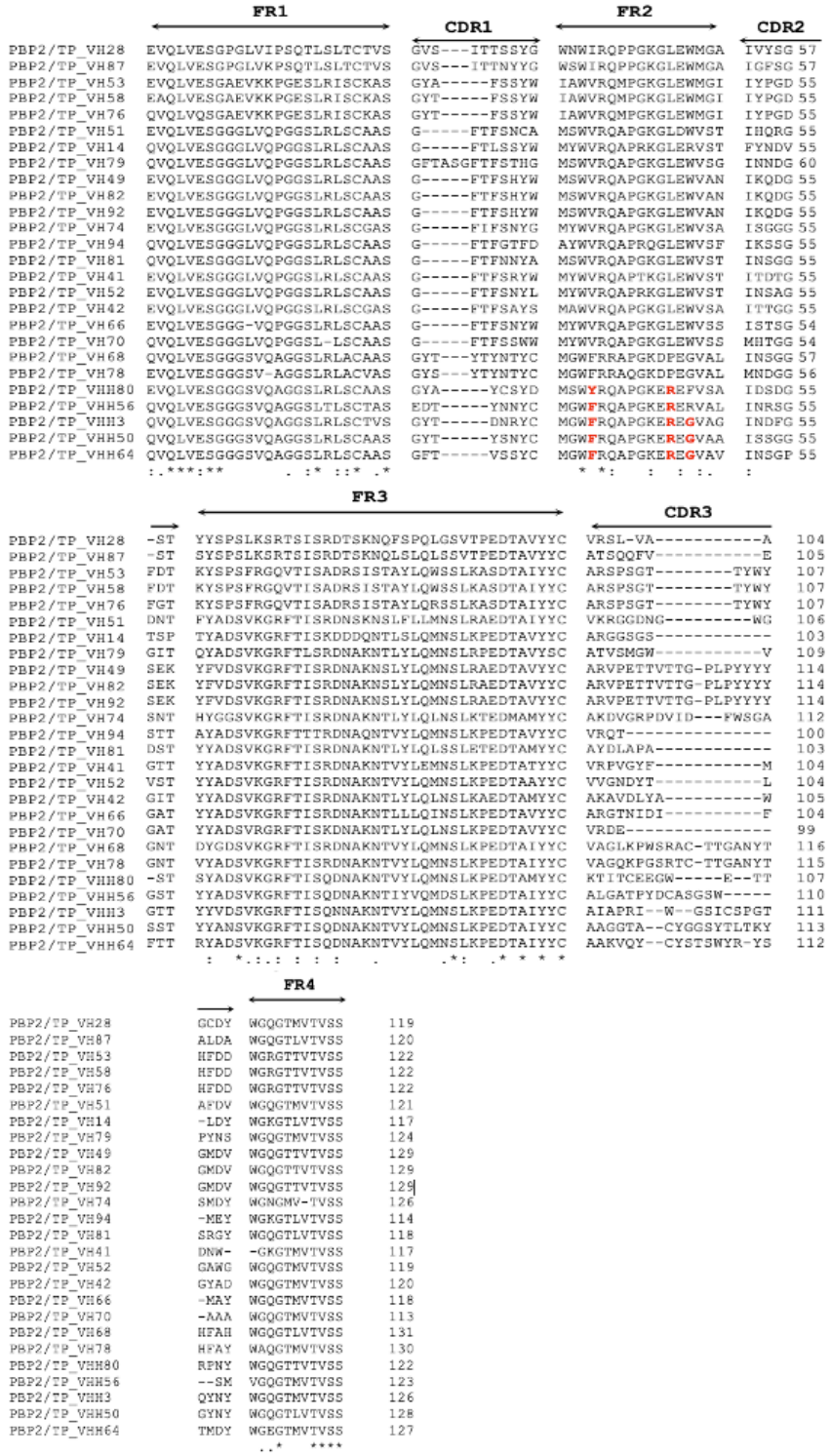

Figure 1.5 Amino acid sequence alignment of the 26 V_H/V_HH clones that could bind rPBP2_TP. Five of the sdAb were camelid V_HH and the rest were conventional V_H antibody fragments. The hallmark amino acids of camelid V_HH located at FR2 are indicated by a red font. Asterisks indicate conserved amino acids; colons indicate conserved amino acid substitutions; and periods indicate semiconserved amino acid substitutions.

Large Scale production of soluble V_H/V_HH

Seven among the tested clones (V_H/V_HH clone 28, 41, 50, 56, 64, 80 and 81) showed significantly higher binding to recombinant PBP2_TP (**Fig 1.6**). We selected those for large-scale production and further analysis. For this, the genes encoding the selected V_H/V_HH were subcloned into the pLATE52 expression system with tightly regulated expression and high yield.

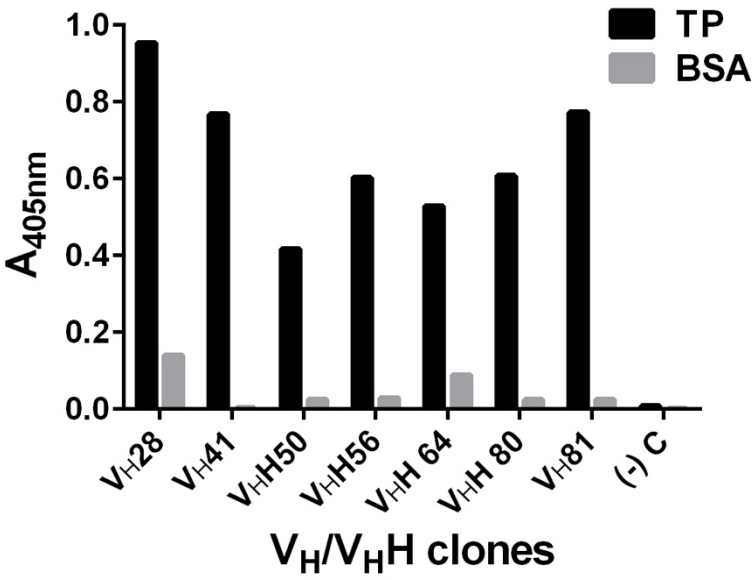

Figure 1.6 Indirect ELISA for the binding of selected V_H/V_HH to rPBP2_TP. Bar plot of the absorbance values (A_{405nm}) of different ELISA wells coated with PBP2_TP or BSA.

Inhibitory activity of V_H/V_HH

After subcloning, expression, purification and solubilization, the activity of the antibodies against *S. aureus* was tested. MIC test using individual soluble antibodies was performed. All of the 7 tested antibodies showed no inhibitory activity against methicillin-sensitive *S. aureus* HG001. Only after testing at very high concentration of 200 μg/mL some impact on bacterial growth was observed. The effect was similar among all of the antibodies. No difference was observed between V_H and V_HH (**Fig. 1.7**).

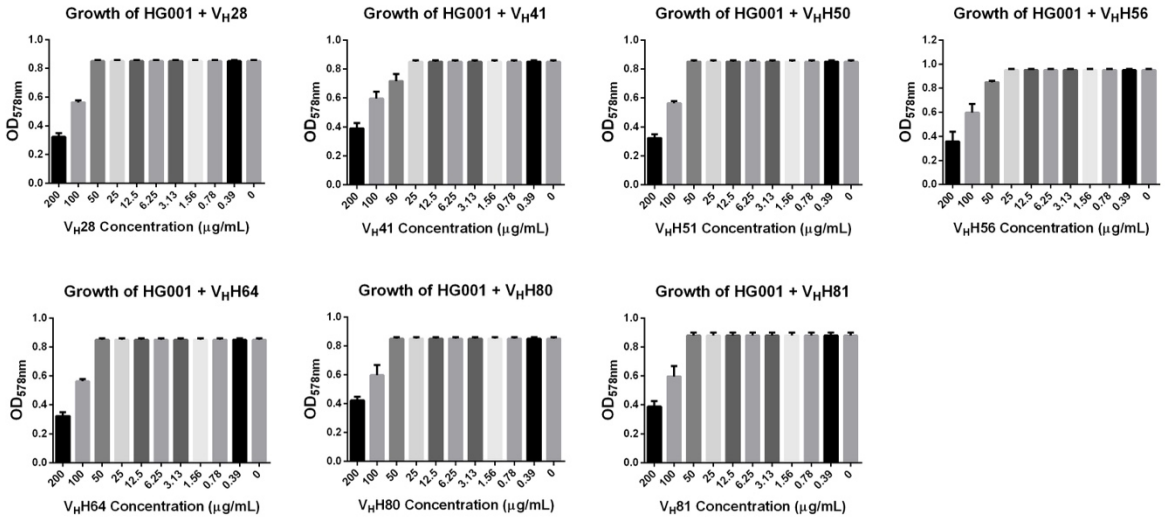

Figure 1.7 Bar plots showing the effect of different concentrations of V_H/V_HH on the growth of *S. aureus* HG001 represented by the OD_{578nm} after overnight incubation with the antibodies.

Synergistic tests of V_H/V_HH and Penicillin

We further tested whether a better activity of V_H/V_HH could be obtained when the antibodies were used in combination with β-lactams. For this, we used a subinhibitory concentration of penicillin G (0.01 μg/mL) in combination with individual antibodies at a concentration of 100 μg/mL. The synergistic effect of V_H/V_HH and the antibiotics was negligible and non-significant (**Fig. 1.8**).

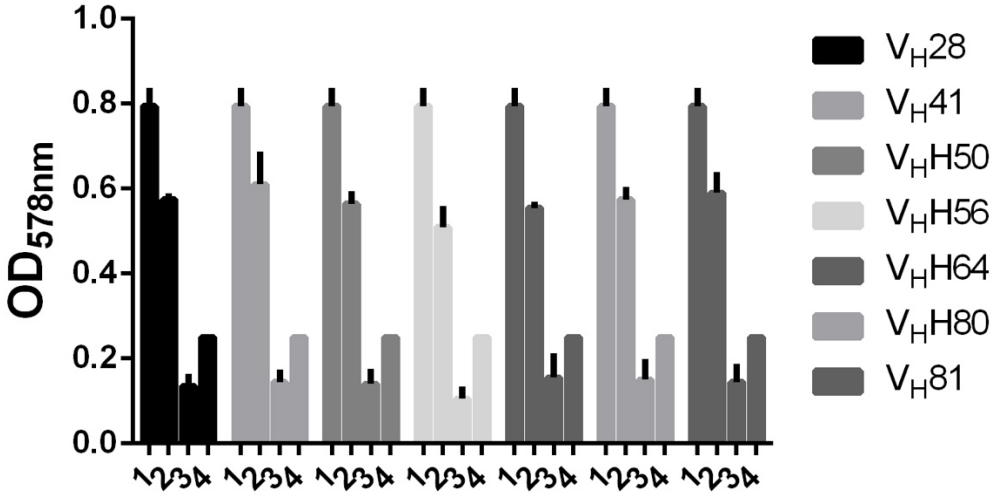

Figure 1.8 Bar plot presenting the growth of *S. aureus* HG001 incubated alone (1), with 100 μg/mL of different V_H/V_HH (2), with combination of V_H/V_HH and penicillin G (0.01 μg/mL) (3) or with penicillin G (0.01 μg/mL) alone.

Discussion and Conclusion

Curbing methicillin resistance is a challenging task and attempts to use antibodies to inhibit *S. aureus* were previously made. One of the earliest studies using antibody fragments specific to β-lactamase showed some binding when the target protein was expressed in *E. coli* (Conrath *et al.*, 2001). Other studies developed antibodies against α-haemolysin, and fibronectin-binding protein among other targets (Rennermalm *et al.*, 2001; Foletti *et al.*, 2013). However, the successful inhibition of *S. aureus* growth by antibodies has been not achieved so far. It was found that better inhibition could be achieved with the use of a combination treatment of antibodies and antibiotics (Slavetinsky *et al.*, 2022).

The use of V_H/V_HH could present various advantages over conventional antibodies. First, the use of phage display technique for the production of single-domain V_H/V_HH reduces their immunogenicity (Vincke *et al.*, 2009). This is a key feature for the therapeutic use of antibodies once they show promising activity *in vitro*. The small antibody fragments can also directly bind the conformational structure of an enzyme's active site, an area that is inaccessible to larger conventional heavy-light chain antibodies. Therefore, targeting the enzymatically active transpeptidase (TP) domain of PBP2 could be achieved by V_H/V_HH. The binding between the recombinant TP and the V_H/V_HHs was confirmed by indirect ELISA. Nevertheless, the interaction between these proteins differed a lot when whole cells were used, and the antibodies did not inhibit the growth of HG001 when used alone or in combination with penicillin G (0.01 μg/mL).

Previous studies in our lab generated V_H/V_HH antibodies against other targets in *S. aureus* cell wall, namely the glycosyltransferases (PGT) domain of PBP2 and the transpeptidase (TP) domain of PBP2a (unpublished data). The PGT domain that polymerizes the peptidoglycan Gly_5-Lipid II units was found to be important for the expression of β-lactam resistance, besides the presence of the low-affinity PBP2a (Pinho *et al.*, 2001). Therefore, it seems more reasonable to target the PGT than the TP domain of PBP2.

Similarly, PBP2a_TP-specifc V_H/V_HH antibodies were produced. The antibodies were tested for their inhibitory activity against methicillin-resistant *S. aureus* (MRSA) alone and in combination with subinhibitory concentration of methicillin (unpublished data). The antibodies failed here also to inhibit MRSA growth.

Despite the identification of essential proteins conferring resistance to *S. aureus* and the development of various methods to produce them, difficulties are met in the process of inhibiting this bacterium. The problem lays in the adaptability of *S. aureus* and the wide range of exoproteins and toxins that it can produce. Although the antibody fragments that we generate here lack the Fc region and are theoretically not a binding target for Spa, they are still inhibited by an unknown mechanism. The reason behind V_H/V_HH inability to inhibit their target in *S. aureus* was not further investigated New approaches against MRSA are moving towards targeting quorum sensing molecules to block signaling and production of virulence factors (Wright *et al.*, 2005; Brown *et al.*, 2020; Piewngam and Otto, 2020).

Material and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table S1.1. For cloning procedures in *S. aureus* strain FPR3757 genome (accession no. CP000255.1) was used as a reference. *S. aureus* was grown in tryptic soy broth (TSB) (unless stated differently) and *E. coli* strains were grown in 2×YT or Luria Bertani (LB) medium. Bacteria were cultivated aerobically (150 rpm) at 37°C. Each experiment was started from an overnight preculture by adjusting the OD_{600nm} to 0.05 – 0.1 in the corresponding medium. The medium was supplemented with a final concentration of 100 μgml−1 ampicillin where applicable.

Cloning and expression of *S. aureus* HG001 PBP2_TP

Expression plasmids were constructed using pET28a (Novagen). pET22b(+) was linearized, using the restriction enzymes *Bam*HI and *Xho* I to add a 6× histidine tag to the C-terminal end of the inserted *pbp2*-TP, amplified from *S. aureus* HG001 genome (oligonucleotides listed in Table S1.2). The ligations were performed using Hi-Fi DNA Assembly Master Mix (New England Biolabs), then transformed into *E. coli* DC10B by heat shock method. The colonies grown on selective agar media containing ampicillin were confirmed by plasmid isolation and sequencing. The plasmids were then transformed into the expression host *E. coli* BL21(DE3). The clones containing the correct plasmid were cultured overnight at 37°C in 2×YT supplemented with ampicillin (2× YT-A). For PBP2a_TP expression, *E. coli* BL21 (DE3) clones were grown in 2× YT-A broth at 37°C with 150 rpm shaking for 3 – 4 hours, protein expression was induced by adding 0.5 mM IPTG. The cells were cultured for 3 hrs. then, harvested and lysed using 0.1 mm glass beads in FastPrep instrument (MP Biomedicals). The cell lysate was centrifuged (15,000 ×g, 30 min, 4°C). For localization of the expressed protein, the proteins in the soluble and insoluble (inclusion bodies, IBs) cell lysate fractions were separated on 12% SDS-PAGE. The presence of the his-tagged PBP2-

TP was verified by western blot analysis using mouse anti-His tag AP-conjugated mAb (Abcam, ab49746) and BCIP/NBT substrate.

Purification of recombinant PBP2a_TP

The *E. coli* BL21 (DE3) clone that could express PBP2_TP was inoculated in 2× YT-A broth at 37°C with 150 rpm shaking for 16 hrs. then, sub-cultured (2%, v/v) into fresh 2× YT-A and grown until OD_{600nm} = 1. IPTG was added at a final concentration 0.5 mM and the cells were further grown for 5 hrs. The cells were harvested by centrifugation at 8,000 ×g, 15 min, 4°C and the supernatant was discarded. The pellet was washed with phosphate buffer saline (PBS, pH 7.4), weighed, and stored at -20°C until used again.

The recombinant his-tagged PBP2-TP was purified from the insoluble IBs following the method described in (Jittavisutthikul *et al.*, 2016). The IBs were purified and solubilized in 8 M urea solution. PBP2a_TP was purified by Ni-NTA from the soluble IBs under denaturing conditions according to the manufacturer's instructions (IBA Life Sciences). The eluted protein was refolded by stepwise dialysis as previously described (Jittavisutthikul *et al.*, 2016). The refolded recombinant protein PBP2_TP (rPBP2_TP) was visualized on 12% SDS-PAGE stained with Coomassie Brilliant Blue (CBB).

Phage bio-panning for V_H/V_HH selection

A humanized nanobody (VH/V_HH) library comprising ~ 4 × 10^{11} V_H/V_HHs (Thanongsaksrikul *et al.*, 2010) was screened by phage bio-panning method for PBP2_TP-binding antibodies. The phage library was constructed by Surasak Jittavisutthikul. The displaying phage in this library is the phagemid pCANTAB5E (Amersham).

To screen for PBP2_TP-specific antibodies by phage display technique, the purified rPBP2-TP (5 μg/well) was used as an immobilized antigen in coating buffer pH 9.4 (BupH (TM) Carbonate – Bicarbonate Buffer pH 9.4) onto a polystyrene well, and incubated at 4°C, overnight. The coated well was washed three times with 0.05% PBST (0.05% Tween 20 in PBS, pH 7.4), 300 μL/well. To confirm the immobilization of PBP2_TP, the OD_{600nm} in two control wells was measured; in the first well a mouse anti-His tag primary antibody was incubated, followed by a goat anti-mouse secondary antibody and ABTS substrate, in the second only the ABTS substrate was added. Then, the unoccupied area in the well was blocked with 3% bovine serum albumin (BSA) in PBST (300 μL/well) and incubated at 25°C for 1 hr in a moist chamber. The wells were washed again three times with 0.05% PBST. Next, the phage library was added (100 μL/well) and the plates were incubated at room temperature (RT) in a moist chamber with slow shaking for 1 hr. The wells were washed intensively with 300 μL 0.5% PBST (20% Tween 20 in PBS). The bound phage was eluted by adding 200 μL of mid-log phase *E. coli* HB2151 (OD_{600nm}=0.5) into the well so the bound phages infect *E. coli* for 15 min, shaking at 150 rpm, RT. The elution step was repeated twice. The eluted phage was diluted in LB broth and spread onto 2×YT-AG agar plates

(2xYT, 100 μg/mL Ampicillin, 2% glucose). The plates were incubated at 37°C, overnight. To screen for the phage-infected *E. coli* HB2151, colony PCR using pCANTAB5E phagemid-specific oligonucleotides (listed in **Table S2)** was performed.

Expression of V_H/V_HH

Western blot analysis was used to confirm phage expression in *E. coli* HB2151 clones harboring pCANTAB5E. The cells were cultivated in 2×YT-AG, then lysed, and the proteins were separated by 12% SDS-PAGE (as described earlier). The proteins separated by SPS-PAGE were transferred to nitrocellulose (NC) membrane. The membrane was blocked with 3% BSA then, rabbit anti-E-tag primary antibody (Abcam, Ab3397) was added, followed by goat anti-rabbit AP-conjugated secondary antibody (Abcam, Ab6722). BCIB/NBT substrate was added until the reacting protein bands appear. The membrane was washed three times with TBS-T after each step.

Binding activity of soluble V_H/V_HH to rPBP2-TP by ELISA

To test the binding activity of the antibodies to rPBP2_TP, ELISA was performed. Briefly, rPBP2a_TP was immobilized on ELISA 96-well plate, the plate was blocked with 3% BSA, the soluble individual V_H/V_HH were added, the plate was washed 3× with PBST, rabbit anti-E-tag primary antibody (Abcam, Ab3397) was added, followed by goat anti-rabbit AP-conjugated secondary antibody (Abcam, Ab6722). BCIB/NBT substrate was added. The binding activities of antibodies to rPBP2-TP were determined by measuring OD_{405nm} of rPBP2-TP and BSA coated wells in Tecan Infinite® 200 plate reader (Tecan Life Sciences).

Binding activity of soluble V_H/V_HH to rPBP2-TP by Western blot

Western blot was performed to confirm the binding of V_H/V_HH to rPBP2-TP. Briefly, rPBP2-TP was loaded into 12% SDS-PAGE wells and transferred onto a nitrocellulose membrane. The membrane was cut into strips that were incubated separately with different clones of V_H/V_HH for 1 hour. The strips were washed and blocked with 3% BSA before being incubated with rabbit anti-E-tag primary antibody (Abcam, Ab3397), followed by goat anti-rabbit AP-conjugated secondary antibody (Abcam, Ab6722) and BCIB/NBT substrate. rPBP2_TP blotted strips probed with lysate of wild type *E. coli* HB2151 were used as a negative control and rPBP2_TP blotted strips probed with mouse anti-6× His Tag as positive control.

Characterization of the V_H/V_HH

The nucleotide sequences of the V_H/V_HH sequences amplified from the selected *E. coli* clones were determined by DNA sequencing. The deduced amino acid sequences of all clones were then aligned with the camelid V_H/V_HH of the International Immunogenetics Information System database. The immunoglobulin frameworks and the CDRs of the individual V_H/V_HH sequences were predicted using TMGT/V-QUEST.

Large-scale production, purification, and refolding of V_H/V_HH

To express the selected antibodies in high amounts, the vector pLATE52 (Thermo Scientific) was used. To clone into pLATE52, the genes encoding the selected antibodies were amplified from pCANTAB5 using the oligonucleotides listed in the **Table S2** and ligated into pLATE52 according to the aLICator^{TM} Ligation Independent Cloning and Expression System product manual (ThermoFisher Scientific). The assembled pLATE52 harboring the different antibody genes was transformed into *E. coli* DC10B by heat-shock method and after confirmation of the plasmid by sequencing, pLATE52 vectors were transformed into the expression host *E. coli* Rosetta2. The positive *E. coli* Rosetta2 clones were confirmed by colony PCR and their ability to express the encoded antibodies was tested by western blot analysis as described above.

The clones harboring pLATE52 encoding the different selected antibodies were cultured in 2×YT-A, the expression of antibodies was induced by IPTG (final concentration = 1 mM) and the antibodies were purified from the IBs, refolded and dialyzed as described (Jittavisutthikul *et al.*, 2016).

Determination of minimal inhibitory concentration (MIC)

MIC values of soluble V_H/V_HH clones were determined in 96-well microtiter plates using TSB medium. Briefly, 50 µl of Rom was serially diluted from 200 µg/ml. Then, 50 µl of bacterial culture (10^6 CFU/ml) was added to each well. An inoculated broth without antibodies was used as positive control and the well without bacteria was used as negative control. The 96-well microtiter plates were incubated at 37°C for 24 h. The OD_{578nm} was measured using a Tecan plate reader as an indicator for bacterial growth.

Synergistic tests of Penicillin G and PBP2_TP-specific V_H/V_HH

To test the effect of the produced antibodies on MSSA inhibition by penicillin, synergy tests were performed in 48-well plates. Briefly, *S. aureus* HG001 inoculum of OD_{578nm} = 0.05 was prepared from overnight bacterial cultures in TSB (250 µL/ well) . The same volume (250 µL) of antibodies in combination with penicillin G (0.01 µg/ml) were added at different indicated concentrations to the wells. The plates were incubated at 37°C under aerobic conditions. After 24 h incubation, the OD_{578nm} values of the wells were measured in Tecan plate reader. The bacteria were grown in TSB alone or in addition of the antibody dialysis buffer as negative control. Penicillin alone at concentration of 1, 0.5, and 0.25 µg/ml was used as positive control for bacterial growth inhibition.

Supplementary Material

Table S1.1 Strains and plasmids used in this study.

Strain	**Description**	**Source**
E. coli BL21 (DE3)	Classic *E. coli* strain for high level protein expression using a T7 RNA polymerase-IPTG induction system	(Studier and Moffatt, 1986)
E. coli HB2151	E. coli expression host used for antibodies production	(Chan *et al.*, 2004)
E. coli DC10B	Classic *E. coli* strain for routine cloning and plasmid maintenance	(Monk *et al.*, 2012)
E. coli Rosetta 2^{TM}	*E. coli* strain designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*.	Novagen
S. aureus HG001	Methicillin-sensitive *S. aureus*, derivative of NCTC8325	(Herbert *et al.*, 2010)
Plasmid	**Description**	**Source**
pET2b(+)a	IPTG-inducible protein expression plasmid	Novagen
pCANTAB5E	*E. coli* M13-derived phagemid, IPTG-inducible protein expression	Amersham
pLATE52	Large-scale IPTG-inducible protein expression, ligation-independent cloning (LIC)	Thermo Scientific

Table S1.2 Oligonucleotides used in this study.

For cloning of PBP2a_TP	
transpeptidase Forward	5'-tggatatcggaattaattcgGGATCCGGTGCAACAATC -3'
transpeptidase Reverse	5'-agtggtggtggtggtggtgcTCGAGACCATATTGTTTAACTTAC -3'
For detection of *E. coli* harboring phage:	
pCANTAB5-R1	5'-CCA TGA TTA CGC CAA GCT TTG GAG CC -3'
pCANTAB5-R2	5'-GCT AGA TTT CAA AAC AGC AGA AAG G -3'
For cloning of antibodies into vector pLATE52	
Forward_VH/VHH_LIC	5'- GGT TGG GAA TTG CAA GCG GCC CAG CCG GCC-3'
Reverse_ESCFV_LIC	5'- GGAGATGGGAAGTCATTA ACG CGG TTC CAG CGG ATC C-3'

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Chapter 2 – Investigating the role of endogenous nucleases in *Staphylococcus aureus* virulence

Chapter 2

Introduction

Staphylococcus aureus harbors two nuclease genes in its genome, namely *nuc1* and *nuc2*. Nuc1 was one of the earliest nuclease enzymes to be discovered and extensively characterized (Heins *et al.*, 1967; Shortle *et al.*, 1990). The 3D structure of the enzyme was resolved in 1969 (3). Nuc1 is a monomeric Ca^{2+} dependent enzyme that catalyzes the hydrolysis of both DNA and RNA at the 5' position of the phosphodiester bond. The full protein is 25.4 kDa in size including a signal peptide (SP) and a pro-peptide domain. The SP functions as an exit signal and is cleaved upon translocation of the enzyme to the extracellular medium. Upon secretion, the pro-peptide domain is cleaved which gives the mature nuclease Nuc1 of 18 kDa in size.

As secreted enzyme Nuc1 is considered as a virulence factor and has been implicated in *S. aureus* pathogenicity and immune evasion. Nuc1 modulates biofilm formation and thereby affects *S. aureus* dissemination (Mann *et al.*, 2009; Tang *et al.*, 2011; Kiedrowski *et al.*, 2011; Beenken *et al.*, 2012; Joo and Otto, 2012). Additionally, Nuc1 plays an important role in infection as it promotes resistance against neutrophil extracellular traps (NET)-mediated killing and contributes to pathogenesis *in vivo* (Berends *et al.*, 2010).

Nuc2 was first reported only after the whole genome sequence of *S. aureus* was made available (Kuroda *et al.*, 2001) based on sequence similarities to *nuc1*. The protein is 20.4 kDa in size and has conserved domains like those found in Nuc1. However, the SP in Nuc2 is not cleaved and the enzyme remains anchored to the extracellular side of the cell membrane. Nuc2 possesses comparable *in vitro* activity to Nuc1 (Tang *et al.*, 2008), in terms of substrate hydrolysis (Hu *et al.*, 2012; Hu *et al.*, 2013), thermostability, and effect on biofilm formation (Kiedrowski *et al.*, 2014). The role of the membrane-anchored Nuc2 in virulence is much less pronounced than that of the abundantly secreted Nuc1 (Kiedrowski *et al.*, 2014).

The two proteins share 60% similarity and 36% identity, a conserved active site composed of the residues Arg114, Glu122 and Arg166 and, a metal binding site composed of the residues Asp 100, Asp119 and Thr120.

The encoding genes *nuc1* and *nuc2* are conserved among *S. aureus* strains and are located in different places in the genome: in *S. aureus* USA300 JE2 (USA300_FPR3757), they are annotated as *SAUSA300_0776* and *SAUSA300_1222*, respectively.

In a previous work in our laboratory, *nuc* genes were deleted in *S. aureus* SA113, which is a derivative of the model strain RN1 and defective in the expression of the accessory gene regulator (Agr) (Herbert *et al.*, 2010). Agr plays a major role in the expression of virulence factors and pathogenicity of *S. aureus*. When activated upon quorum sensing signals, Agr upregulates more than a hundred genes associated with virulence and downregulates more than thirty genes encoding surface and protection proteins. This flips the switch in *S. aureus* from establishment to invasive mode (Recsei *et al.*, 1986; Cheung *et al.*, 2011). When the single and double *nuc* null mutants were tested for DNA hydrolysis, biofilm formation and extracellular proteins (ECP) pattern, the *nuc1* mutant could not hydrolyze DNA on a DNA agar activity test, whereas the *nuc2* mutant showed a DNA hydrolytic activity similar to the wild type (WT). Unexpectedly, the *S. aureus* SA113ΔnucΔnuc2 mutant showed a distinct phenotype that is normally associated to an agr positive strain in regard to the repression of the expression of the staphylococcal protein A (Spa) and overexpression of virulence factors such as haemolysins and phenol-soluble modulins (PSMs) (Recsei *et al.*, 1986). This was visualized by comparing the ECP pattern of the mutant to the WT strain (Fig. 2.1). Given such an impact, in this work we aimed to understand the link between the nuclease genes and *agr* expression in *S. aureus*.

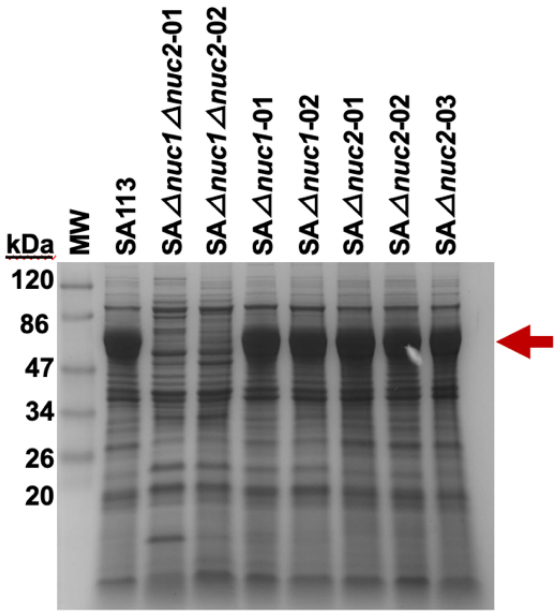


Figure 2.1 The extracellular proteins (ECP) pattern of the WT and the constructed mutants was visualized on 12% SDS-PAGE. The red arrow indicates the band corresponding (Unpublished results from Günther Thumm, 2007).

Results

Deletion of *nuc2* affects agr expression in *S. aureus* strain USA300 JE2

In order to confirm the link between nuclease genes and *agr* expression, we constructed new *nuc* mutants in *agr*+ strains. *S. aureus* USA300 JE2 have a very robust Agr system which makes the strain a good model to study Agr activity (Li *et al.*, 2009). We chose this strain for the generation of new mutants. We constructed the new deletion mutants using markerless allelic replacement. After confirming the genes´ deletion by PCR, we checked for the expression of extracellular proteins by SDS-PAGE.

Our results showed that the ECP pattern in the mutants was different than the WT not only in the double deletion mutant but also in the single *nuc2* mutant. In the *agr*+JE2 strain normally protein A expression is repressed and the expression of virulence factors such as PSMs is upregulated. The deletion of *nuc2* resulted in what seems like an *agr* defective phenotype in terms of ECP expression. This was compared to the constructed *agrBDCA* deletion mutant (JE2Δ*agrBDCA*) ECP pattern on SDS-PAGE (**Fig. 2.2**).

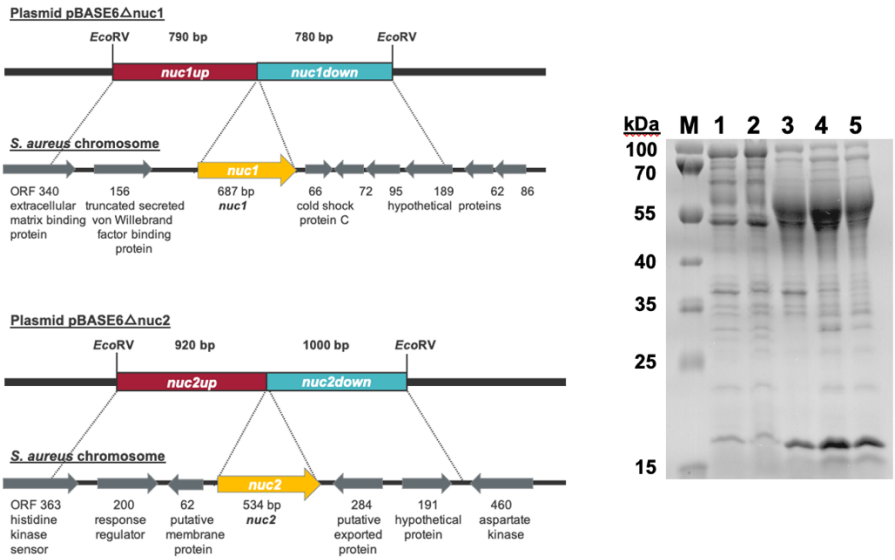


Figure 2.2 Construction maps of *nuc1* and *nuc2* deletion in *S. aureus* JE2. The extracellular proteins (ECP) pattern of the JE2 WT and the corresponding mutants was visualized on 12% SDS-PAGE. Lane 1, JE2 WT; lane 2: JE2Δ*nuc1*; lane 3: JE2Δ*nuc2*; lane 4: JE2Δ*nuc1*Δ*nuc2*; lane 5: JE2Δ*agrBDCA*.

Chapter 2

The identify of extracellular proteins affected by *nuc* deletion was confirmed by LC-MS/MS analysis

The profile of extracellular proteins indicated a change in Agr-regulated proteins. To confirm the identity of the proteins corresponding to the visualized bands in SDS-PAGE, we analyzed the variable protein bands by Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). This analysis revealed that the main affected proteins were those that are expected to be modulated by Agr. The downregulated proteins in the mutant were mainly secreted virulence factors such as PSMs, lipases and proteases, whereas the main upregulated proteins were the surface proteins Spa, IsdB and PBP3. The presence of surface proteins in the ECP on SDS-PAGE has been observed previously (Herbert *et al.*, 2010). Despite not being actively secreted, some of the surface proteins are found to be detached from the cell wall and detected in the supernatant. The identified proteins were limited to proteins present in the two bands of the SDS-PAGE gel that were cut out and analyzed, the one around 55 kDa (which contained mainly Spa) and the other around 10 kDa (which contained mainly PSMs).

Table 2.1 Results of LC-MS/MS analysis showing the difference in the expression of certain proteins in *S. aureus* JE2 and its congenic Δ*nuc1*Δ*nuc2* mutant as presented by their iBAQ (intensity Based Absolute Quantification) values.

Identified protein			iBAQ		Fold change
Protein ID(s)	**Protein**	**MW (kDa)**	**JE2 WT**	**JE2*Δnuc1Δnuc2***	**WT:Mutant**
P02976	Staphylococcal protein A	56,4	681970000	26487000000	0,025
P0C7Y1, P0C7Z3, P0C805, P0C818	PSMs-(alpha)	2,1 - 2,6	2,28E+11	147684000	1541,141
Q2FY21	Penicillin-binding protein 3	77,2	11707000	77391000	0,151
Q2FYV4	Homoserine dehydrogenase	46,8	188000000	1396100	134,660
Q2FZF0	Iron-regulated surface determinant protein B (isdB)	72,1	12922000	241630000	0,053
Q2FZP2	Serine protease HtrA-like	86,4	19334000	764410	25,292
Q2FZR3	Oligopeptide ABC transporter	61,5	2157900	64394000	0,033
Q2FZV7	NADH dehydrogenase-like protein	44,1	0	6413200	0
Q2G0W8	Lipase 3 domain-containing protein	49,2	328600000	1306500	251,511
Q2G2I1	Membrane-associated protein TcaA	52,1	26075000	0	-
Q2G2U3	YycH domain-containing protein	49,0	82115000	0	-

The generated *nuc2* deletion mutants show attenuated virulence in *S. aureus* JE2

To test the virulence of *S. aureus* WT and *nuc* mutants, we used an insect infection model. The model was tested previously with different *S. aureus* strains (Popella *et al.*, 2016; Fan *et al.*, 2019). The infection assay showed that the deletion of *nuc2* and/or *agr* resulted in an attenuated virulence as evidenced by the survival of 50 – 70 % of the larvae 5 days following the infection. When *S. aureus* JE2 WT and Δ*nuc1* mutants were injected, all of the larvae died by 4 days post-infection (**Fig. 2.3**).

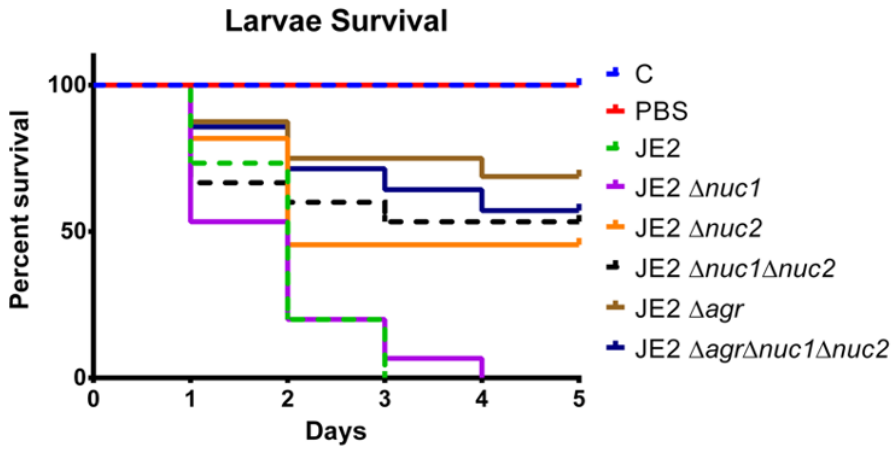


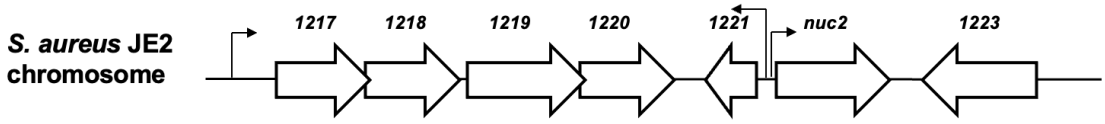
Figure 2.3 *Galleria mellonella* larvae survival experiment. The deletion of *nuc2* and/or *agrBDCA* decreased the virulence of *S. aureus* strain JE2 in larvae. We used 20 larvae per group and injected 10^6 CFU per larvae.

Complementation of *nuc2* expression on a plasmid did not reverse the agr negative phenotype of the mutants

To confirm the role of the *nuc2* deletion in the resulting phenotype, we complemented the mutant with plasmids expressing Nuc2. For this, we used two different plasmids: pRB473 for expression under control of the native promotor and pCX19 for xylose-inducible expression. Of note, *nuc2* insert sequence cloned into pRB473 included the gene's native transcription promoter and terminator sequences. As analysis of the sequence downstream of *nuc2* did not result in the identification of a rho-independent terminator sequence, we opted for including 199 bp downstream of the coding region. The complementation of Nuc2 on neither plasmid did reverse the phenotype to the

agr+ WT strains. We doubted that the nucleotide sequence upstream or downstream *nuc2* is necessary for Agr expression.

The deletion of *SAUSA300_1221* upstream of *nuc2* results in an Agr-defective phenotype



Gene number	Description	Product size	Localization	Occurrence in *S. aureus*
SAUSA300_1217	ABC transporter, ATP-binding protein	34,9 kDa	Intracellular	76%
SAUSA300_1218	ABC transporter, permease protein	27,9 kDa	6 transmembrane domains	76%
SAUSA300_1219	putative sensor histidine kinase	41,6 kDa	6 transmembrane domains	76%
SAUSA300_1220	DNA-binding response regulator, two-component response regulator	22,8 kDa	Cytoplasmic	76%
SAUSA300_1221	conserved hypothetical protein	7,0 kDa	membrane	100%
SAUSA300_1222	thermonuclease (nuc2)	20,3 kDa	membrane-anchored facing out	100%
SAUSA300_1223	conserved hypothetical protein	32,9 kDa	membrane-anchored facing out	100%

Figure 2.4 Neighboring genes of *nuc2*. The occurrence of the genes in staphylococcal strains was retrieved from AureoWiki (Fuchs *et al.*, 2018) based on the amino acid sequences from 33 strains.

Since we could not revert the *agr* phenotype by *nuc2* complementation, we inspected the DNA sequence around the *nuc2* coding region (**Fig. 2.4**). We found a small gene (*SAUSA300_1221*) upstream of *nuc2* encoding a protein of unknown function and about 7 kDa in size. This protein is conserved among *S. aureus* strains and is predicted to be localized in the membrane. We asked whether the expression of this protein could have been affected by *nuc2* deletion and whether this small protein is responsible for the observed role in Agr regulation. As a highly conserved membrane-localized protein, SAUSA300_1221 could play a role in quorum sensing and thereby affect Agr expression. To verify this, we deleted the gene *SAUSA300_1221* and checked the ECP expression of the mutant, which seemed to be also defective in *agr* expression (**Fig. 2.5**).

The role of the downstream gene of *nuc2*, namely *SAUSA300_1223* encoding another hypothetical protein was ruled out. This is because a transposon insertion mutant of this gene was available in the Nebraska library of *S. aureus* JE2 (Fey *et al.*, 2013)

which was tested for Agr expression. The ECP pattern of this mutant was not different from the WT strain. We concluded that *SAUSA300_1223* has no role in Agr expression.

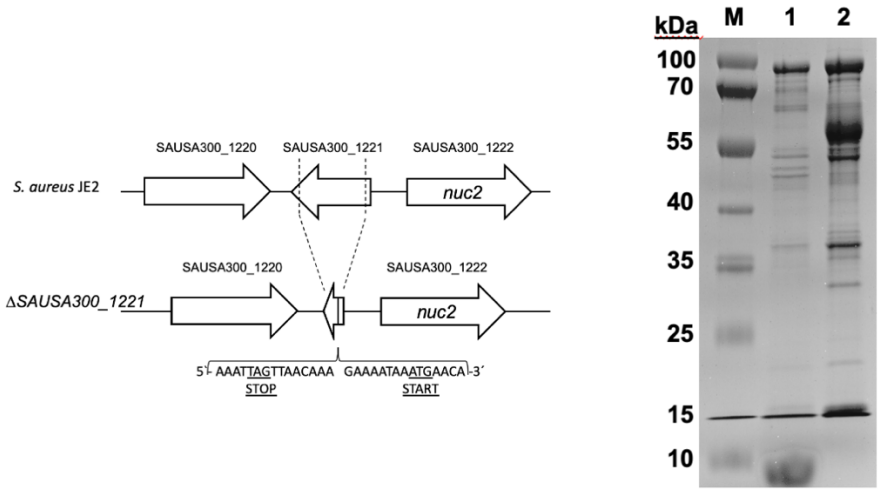


Figure 2.5 Deletion of *SAUSA300_1221* results in an agr– phenotype as shown on 12% SDS-PAGE of ECP. Lane 1: ECP in supernatants from JE2 WT, and lane 2: ECP in supernatants from JE2Δ*SAUSA300_1221* cultures.

Again, to confirm the role of SAUSA300_1221 in Agr expression, we attempted to complement the JE2Δ*SAUSA300_1221* mutant. This also was not achieved by the expression of SAUSA300_1221 on pCX19. To express the protein under control of the native promoter in pRB473, the terminator sequence should be identified. However, no transcription terminator region could be found between *SAUSA300_1221* and the upstream gene cluster. Therefore, we opted to construct pRB473 plasmids with different inserts (**Fig. 2.6**). The listed plasmids were transformed into *S. aureus* JE2Δ*SAUSA300_1221* by electroporation and confirmed by PCR. None of the transformed plasmids could complement the Agr phenotype (results not shown). However, a control for confirmation of the transcription of the cloned genes and the expression of corresponding proteins was missing in our experiments.

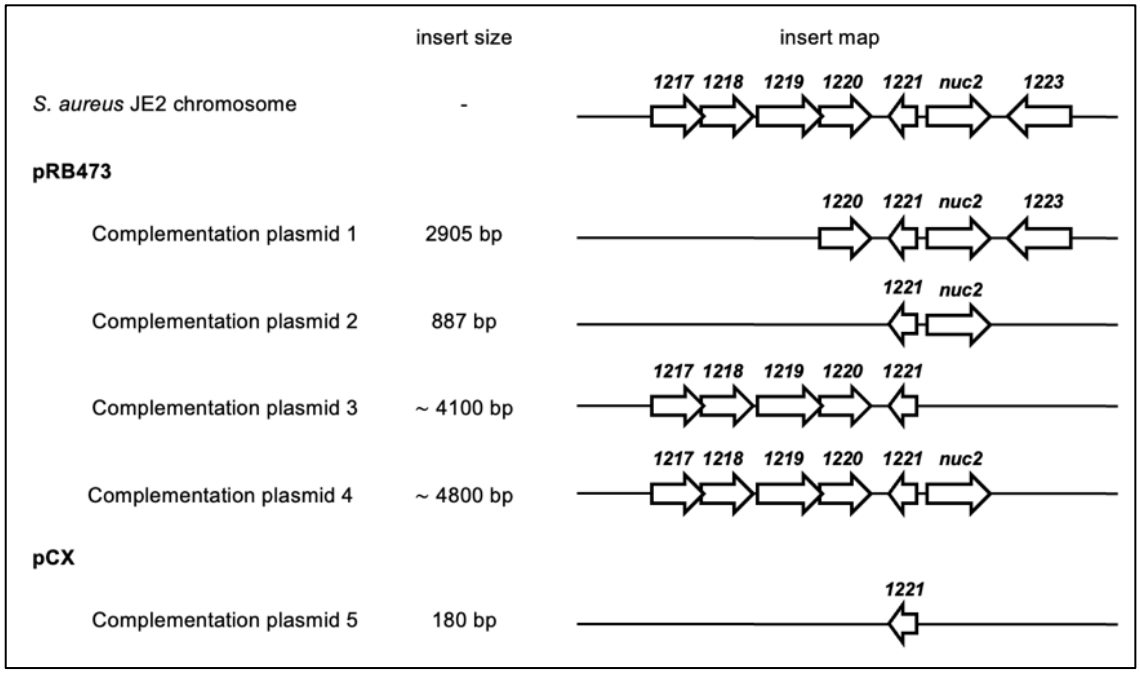


Figure 2.6 Constructed plasmids for complementation of *SAUSA300_1221* in *S. aureus* JE2.

Agr mutant control strain cannot be complemented on a plasmid

After the complementation attempts failed, we tried a different approach to confirm the importance of the deleted region in *agr* expression. Hence, we constructed another deletion mutant strain in which the gene cluster *agrBDCA* as well as *SAUSA300_1221* were deleted. And we attempted to complement Agr using pRB473 in both this newly constructed mutant and the strain JE2Δ*agrBDCA*. The expression of AgrBDCA would then be used as an indicator for the role of *SAUSA300_1221* in Agr expression. However, the complementation of Agr by expression of AgrBDCA using pRB473 was not achieved. Similar results were found later in previous studies on Agr complementation.

Whole genome sequencing reveals a mutation in *agrC* of JE2Δ*nuc2*

Whole genome sequencing (WGS) of the strains of interest was necessary to understand the previous results given the limitations in genetic manipulation of *agr* defective strains. For this, the genomic DNA of *S. aureus* JE2 WT and its congenic Δ*nuc2* deletion mutant was isolated and sequenced. The whole genome sequence of both strains was compared to the refence genome of *Staphylococcus aureus subsp. aureus* USA300_FPR3757 (GenBank: CP000255.1) and analyzed for the presence of

single nucleotide variants (SNV). The list of SNV for each strain are found in the supplementary material.

The comparison of SNV present in the genome of the WT and Δ*nuc2* shows that the latter possess 19 additional SNV dispersed throughout the genome, 5 SNV are found in non-coding sequences and the rest in different proteins listed in **Table 2**, among which is the sensor histidine kinase AgrC. The SNV in *agrC* results in a modification of the amino acid residue 397 in the encoded AgrC from Asp to Asn. This aspartic acid residue is present in one of the β-sheets of the transmembrane protein and its modification into aspartate could affect the integration of AgrC into the cell membrane and subsequently the sensing of AIP molecules and activation of the Agr system (Srivastava *et al.*, 2014). The presence of this secondary mutation in *agrC* which could have resulted from the strain manipulation presents a sound explanation for the Agr- phenotype observed in our constructed mutants.

SNV position	Affected gene ID	Protein product annotation
591684	*SAUSA300_0528*	rpoC
930890	*SAUSA300_0852*	Na(+)/H(+) antiporter subunit D
944692	non-coding	-
1269542	*SAUSA300_1157*	DNA polymerase III, alpha subunit
1286986	*SAUSA300_1169*	DNA translocase FtsK
1295497	*SAUSA300_1177*	competence/damage-inducible protein cinA
1354028	*SAUSA300_1236*	conserved hypothetical protein
1355744	non-coding	-
1357665	*SAUSA300_1239*	transketolase
1533312	non-coding	-
1630757	non-coding	-
1688528	*SAUSA300_1539*	chaperone protein DnaJ
1752707	*SAUSA300_1598*	holliday junction DNA helicase RuvA
1762523	*SAUSA300_1611*	valyl-tRNA synthetase
1765020	*SAUSA300_1611*	valyl-tRNA synthetase
2148909	***SAUSA300_1991***	**accessory gene regulator protein C (AgrC)**
2487639	non-coding	-
2648396	*SAUSA300_2451*	drug transporter
2805278	*SAUSA300_2584*	preprotein translocase, secA protein

Table 2.2 List of SNV found in *S. aureus* JE2Δ*nuc2*. The SNV positions in the reference genome, the affected coding regions and the annotated protein products are presented.

Discussion and Conclusion

In this study, different approaches were used to find a link between the *nuc2* gene and/or its upstream coding region *in agr* expression. Our results showed that the alteration in Agr expression is a result of secondary mutations that were found in the constructed mutants. Genetic manipulation of *S. aureus* has been previously shown to cause random secondary mutations. More specifically, it has been observed that *in vitro* serial passaging of *S. aureus* strains leads to mutations in *agr* (Somerville *et al.*, 2002). Spontaneous *agr* defective mutants arisen in frozen stock cultures have been also reported previously (Herbert *et al.*, 2010). *S. aureus* clinical isolates with dysfunctional *agr* are also prevalent and the natural tendency of *agr* to mutate has been described (Shopsin *et al.*, 2008).

In a recent study, a similar phenotype to what we describe here was obtained after the construction of a WalKR mutant in *S. aureus* (18, retracted). The WalKR mutant showed reduced susceptibility to lysostaphin, loss of hemolytic activity and reduced virulence. Shortly after, whole genome sequencing of the constructed mutant confirmed that the observed phenotype was due to spontaneous secondary mutations in *saeRS* and had no connection to WalKR (Monk *et al.*, 2017). SaeRS is another global regulator that controls the virulence factors expression factors (Jenul and Horswill, 2019). SaeRS also plays a role in *agr* regulation as its expression is required to coordinate the effects of quorum sensing and activation of Agr (Novick and Jiang, 2003).

Based on these studies, it seems plausible that spontaneous mutations lead to secondary phenotypes and misinterpretation of results. This renders studying the function of genes based on mutants' construction more tedious.

Regarding the complementation of agr, previous reports showed that the complementation of agr on a plasmid does not restore the agr- phenotype (Novick, 1993; Booth *et al.*, 1997; Pang *et al.*, 2010) and that complementation by chromosomal insertion is required.

The inconsistency in the effect of *nuc* genes deletion could be another indicator that the obtained phenotype is due to secondary mutations taking place elsewhere in the genome (Helle *et al.*, 2011). It is interesting to find out the type of mutation that resulted in the activation of the originally defective Agr in SA113 however, this was not tested.

In this study, we disregarded the involvement of other regulatory systems in the observed phenotype despite previous reports that Agr plays an important but not exclusive role in the regulation of the virulence factors in question (Li *et al.*, 2009). Another problem was disregarding from the beginning the possibility of secondary mutations despite various reports where this was described.

In conclusion, secondary mutations are a common side effect that results from *S. aureus* manipulation and subculturing in the lab. To avoid this, frequent testing of hemolytic activity of α-hemolysin on blood agar plates to confirm the agr expression in newly constructed mutants is highly recommended.

Chapter 2

Material and Methods

Bacterial strains and growth conditions

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table S2.1. For cloning procedures in *S. aureus*, the USA300_FPR3757 genome sequence (NCBI reference sequence NC_007793) was used as a reference. *S. aureus* and *E. coli* strains were grown in basic medium (BM) containing 1% (w/v) soy peptone, 0.5% yeast extract, 0.5% NaCl, 0.1% K2HPO4 and 0.1% glucose at pH 7.2. Bacteria were cultivated aerobically (200 rpm) at 37°C. The medium was supplemented with the following antibiotics, where applicable at the indicated final concentrations: chloramphenicol at 10 μgml−1 for staphylococcal strains and 100 μgml−1 ampicillin for *E. coli* strains.

Construction of deletion mutants

The deletion mutants were constructed as markerless deletion as previously described (Bae and Schneewind, 2006). Briefly, recombinant knockout plasmids were constructed using the temperature sensitive plasmid pBASE6 (Geiger *et al.*, 2012), ~1000 bp upstream and ~1000 bp downstream fragments of the gene(s) of interest were amplified from the genomic DNA of S. aureus JE2. Then, the fragments were assembled with the EcoRV-linearized pBASE6 by Gibson Assembly (Gibson *et al.*, 2009) using Hi-Fi DNA Assembly Master Mix (New England Biolabs). The resulting plasmids were first introduced into E. coli DC10B (Monk *et al.*, 2012) and then into S. aureus RN4220 via electroporation before transformation of S. aureus JE2. Deletion of the genes were confirmed by PCR and sequence analysis. The mutants were named as described in the Table S2.1. Oligonucleotides used are listed in the Table S2.2.

Analysis of extracellular proteins

Bacterial cultures of the WT and mutant strains were adjusted to OD_{578nm} = 0.1 in BM from an overnight preculture. The cells were grown for 16 hrs then the supernatant was collected by centrifugation. The supernatant was incubated with StrataClean resin to concentrate the proteins. The resin was washed with PBS, resuspended in 6x loading buffer and boiled at 95°C for 7 min. The resin in the suspension was spinned-down and 10μL was loaded on 12% SDS-PAG and stained with Coomassie Brilliant Blue (CBB).

LC-MS/MS analysis of the bands was performed by in-gel digestion with trypsin. LC-MS/MS analysis on a Proxeon Easy-nLC coupled to Exploris, method: 60min, Top12. The date was processed data using MaxQuant software (Version 1.6.7.0.) with integrated Andromeda Peptid search engine). The spectra were searched against a *Staphylococcus aureus* database (UP000008816_93061_complete_2020-10-07.fasta).

Construction of complementation vectors

Complementation of Δ*nuc2* and Δ*agr* was carried out with plasmid pRB473 and pCX19. For construction of complementation vector for *nuc2*, different fragments (listed in Table X) were amplified and then assembled into linearized pRB473 plasmid (HindIII and PacI restriction enzyme) by Gibson assembly. As for construction of complementation vector for agr, the full operon region was amplified and then inserted into linearized pRB473 (EcoRI restriction enzyme). The constructed plasmids were first introduced into *E. coli* DC10B then into *S. aureus* RN4220 before being transformed into *S. aureus* mutant strains.

Larvae infection model

Galleria mellonella larvae were purchased from reptilienkosmos.de, Germany. Ten larvae per group weighing between 300 and 600 mg were infected with either *S. aureus* WT or different mutants. Bacteria were grown in TSB at 37 °C with shaking for 24 h. Cells were washed twice and resuspended in Dulbecco's Phosphate-Buffered Saline (DPBS) (GibcoTM) before being adjusted to an OD_{578nm}, which corresponds to 5×10^8 colony-forming unit (CFU) for each strain. Each larva was injected with 5×10^6 cells. Larvae injected with DPBS served as control group. The larvae were incubated at 37 °C for 5 days after infection and surviving larvae were counted every day starting from 24 h after infection. The experiments were replicated three times. Data collected from each independent experiment were pooled for statistical analysis, resulting in n = 30 for every strain.

Whole genome sequencing

The genomic DNA of *S. aureus* JE2 WT and Δ*nuc2* was isolated using the Quick-DNA Microprep kit (Zymo Research) following bacterial cell lysis by lysostaphin. The quality of DNA was tested by fluorescence-based quantification method. The whole genome sequencing was performed by Cegat (Tübingen, DE) as follows. Demultiplexing of the sequencing reads was performed with Illumina bcl2fastq (2.20). Adapters were trimmed with Skewer (version 0.2.2) (Jiang *et al.*, 2014). Quality trimming of the reads has not been performed. For DNA-Seq, trimmed raw reads were aligned to the reference genomes *Staphylococcus aureus subsp. aureus* USA300_FPR3757 using the Burrows-Wheeler Aligner (BWA-mem version 0.7.17-cegat) (Li and Durbin, 2009). Duplicates were marked. Using a Cegat proprietary tool, reads that aligned to more than one locus with the same mapping score and duplicated reads, which most likely originated from the same PCR amplicon, were discarded. For variant calling, a Cegat proprietary software was used for variant detection. Variant calling was performed in the target regions with +/- 30 base pairs. For FASTQ quality check, the quality of the FASTQ files was analyzed with FastQC (version 0.11.5-cegat).

Supplementary Material

Table S2.1 Strains and plasmids used in this study.

Strain	**Description**	**Source**
E. coli DC10B	Universal staphylococcus cloning host	(Monk *et al.*, 2012)
SA113	*agr* $^{-}$, restriction modification deficient	(Iordanescu and Surdeanu, 1976)
SA113 Δ*nuc1*	*nuc1* deletion, derivative of SA113	Lab strain collection
SA113 Δ*nuc2*	*nuc2* deletion, derivative of SA113	Lab strain collection
SA113 Δ*nuc1* Δ*nuc2*	*nuc1 and nuc2* deletion, derivative of SA113	Lab strain collection
JE2	MRSA, *agr*$^{+}$	
JE2 Δ*nuc1*	*nuc1* deletion, derivative of JE2	This study
JE2 Δ*nuc2*	*nuc2* deletion, derivative of JE2	This study
JE2 Δ*nuc1* Δ*nuc2*	*nuc1 and nuc2* deletion, derivative of JE2	This study
JE2 Δ*agr*	*agr* deletion, derivative of JE2	This study
JE2 Δ*agr* Δ*nuc1* Δ*nuc2*	*agr, nuc1* and *nuc2* deletion, derivative of JE2	This study
JE2 Δ*SAUSA300_1221*	*SAUSA300_1221* deletion, derivative of JE2	This study
Plasmid	**Description**	**Reference**
pBASE6	Allelic replacement in staphylococcus	(Geiger *et al.*, 2012)
pRB473	*E. coli/S. aureus* shuttle vector	(Brückner, 1992)
pCX19	Xylose-inducible plasmid for staphylococcus	(Hussain *et al.*, 2001)

Table S2.2 Oligonucleotides used in this study.

Primer	5'-3'- Sequence
For the construction of pBASE6-nuc1 KO	
Nuc1up-F	cgcgcagatctgtcgacgattctactttgaaagttggaag
Nuc1up-R	attattgaccaagtattctgtcataactaacac
Nuc1down-F	cagaatacttggtcaataatgctcattgtaaaag
Nuc1down-R	tgcaggcatgcaagcttgatttttaatgtttcgccttttaaatg
For the construction of pBASE6-nuc2 KO	
Nuc2up-F	cgcgcagatctgtcgacgataatataatcctaacgttgttttttag
Nuc2up-R	tactccaaattttgacttcatattgccac
Nuc2down-F	tgaagtcaaaatttggagtaaataattaaataggtg
Nuc2down-R	tgcaggcatgcaagcttgatgttgtacttatcattatcattgtag
For the construction of pBASE6-agr KO	
Agrup-F	cgcgcagatctgtcgacgatttttaaccgctggcgaatac
Agrup-R	ctcaccgatgcatagcagacaccactctcctcactgtca
Agrdown-F	cagtgaggagagtggtgtctgctatgcatcggtgagaa
Agrdown-R	tgcaggcatgcaagcttgatcgccatgcaatgtagcttgt
For the construction of SAUSA300_1221 KO	
1221up-F	cgcgcagatctgtcgacgattgatgtagaaaaagtaaaagctg
1221up-R	ataaatgaacaaaattagttaacaaatctaaattgcg
1221down-F	taactaattttgttcatttattttcactccttaac
1221down-R	tgcaggcatgcaagcttgataaggttctgaagaaagag
For the construction of pRB473nuc2strep	
F	attcgagctcggtacccgggcactccttaacaataacattatatc
R	cctgcaggtcgactctagagctaaacacccttaaataaagaac

For the construction of pRB473nuc1nuc2	
Nuc1 F	attcgagctcggtacccgggacataaaagtaagtatagttattttattataattattaaa
Nuc1 R	agcatgatataaatacacttacttttgatactatttac
Nuc2 F	aagtgtatttatatcatgctatagctttcc
Nuc2 R	cctgcaggtcgactctagagctatctaaacacccttaaataaag
For the construction of pRB473(1220-1221-nuc2-1223)	
F	caagcgctcatcgcagtgcgtggcaaaggtatcgatag
R	cctgcaggtcgactctagagcataaattcgacataaacttgaattc
For the construction of pRB473(1221-nuc2)	
F	
R	
For the construction of pRB473(1217-1218-1219-1220-1221)	
F	caagcgctcatcgcagtgcgaatagaccgctgtctgttaaattc
R	cctgcaggtcgactctagagagccatatctacatatttcaatattttg
For the construction of pRB473(1217-1218-1219-1220-1221-nuc2)	
F	caagcgctcatcgcagtgcgaatagaccgctgtctgttaaattc
R	cctgcaggtcgactctagagaatcttcgttacgacgtattattag
For the construction of pCX19-nuc2	
F	tgtaggatccagaagtaggtggcaatatg
R	agataaagcgttcatcattattatttactccaaatatttaatttctgttg
For the construction of pRB473agr	
F	caagcgctcatcgcagtgcgtgtcatgactaaacatagatttatg
R	cctgcaggtcgactctagagtattaatagtttgccaacattac

Table S2.3 Single nucleotide variants (SNV) in *S. aureus* USA300 JE2 wildtype compared to the reference genome (GenBank: CP000255.1)

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CP000255.1	5010	.	C	G	.	.	DP=1605;HET=0;HOM=1;RD=1;AD=1604;AF=0.9994	GT	1/1
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CP000255.1	61025	.	G	A	.	.	DP=1255;HET=0;HOM=1;RD=0;AD=1255;AF=1	GT	1/1
CP000255.1	164912	.	G	A	.	.	DP=1388;HET=0;HOM=1;RD=1;AD=1387;AF=0.9993	GT	1/1
CP000255.1	240358	.	T	A	.	.	DP=1457;HET=0;HOM=1;RD=2;AD=1455;AF=0.9993	GT	1/1
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CP000255.1	292738	.	A	C	.	.	DP=1694;HET=0;HOM=1;RD=2;AD=1692;AF=0.9994	GT	1/1
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CP000255.1	513055	.	G	T	.	.	DP=572;HET=0;HOM=1;RD=88;AD=484;AF=0.8462	GT	1/1
CP000255.1	517837	.	C	T	.	.	DP=743;HET=0;HOM=1;RD=0;AD=743;AF=1	GT	1/1
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CP000255.1	537902	.	A	T	.	.	DP=1488;HET=0;HOM=1;RD=1397;AD=91;AF=0.0618	GT	0/0
CP000255.1	556082	.	T	C	.	.	DP=865;HET=0;HOM=1;RD=0;AD=865;AF=1	GT	1/1
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CP000255.1	658669	.	T	A	.	.	DP=616;HET=0;HOM=1;RD=2;AD=614;AF=0.9968	GT	1/1
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CP000255.1	1997387	.	A	G	.	.	DP=2570;HET=1;HOM=0;RD=1697;AD=873;AF=0.3397	GT	0/1
CP000255.1	1997484	.	A	T	.	.	DP=2415;HET=1;HOM=0;RD=1632;AD=783;AF=0.3246	GT	0/1
CP000255.1	1997489	.	G	C	.	.	DP=2442;HET=1;HOM=0;RD=1685;AD=757;AF=0.3104	GT	0/1
CP000255.1	2002083	.	A	G	.	.	DP=6;HET=0;HOM=1;RD=2;AD=4;AF=0.8333	GT	1/1
CP000255.1	2007223	.	G	T	.	.	DP=1363;HET=0;HOM=1;RD=0;AD=1363;AF=1	GT	1/1
CP000255.1	2143526	.	T	C	.	.	DP=930;HET=0;HOM=1;RD=908;AD=22;AF=0.0237	GT	0/0
CP000255.1	2143527	.	A	T	.	.	DP=932;HET=0;HOM=1;RD=909;AD=23;AF=0.0247	GT	0/0
CP000255.1	2143544	.	C	T	.	.	DP=802;HET=0;HOM=1;RD=726;AD=76;AF=0.096	GT	0/0
CP000255.1	2143545	.	T	C	.	.	DP=831;HET=0;HOM=1;RD=747;AD=84;AF=0.1011	GT	0/0
CP000255.1	2143554	.	T	C	.	.	DP=891;HET=0;HOM=1;RD=757;AD=134;AF=0.1504	GT	0/0
CP000255.1	2143557	.	C	T	.	.	DP=866;HET=0;HOM=1;RD=731;AD=135;AF=0.157	GT	0/0
CP000255.1	2143560	.	G	T	.	.	DP=994;HET=0;HOM=1;RD=798;AD=196;AF=0.1972	GT	0/0
CP000255.1	2164596	.	A	G	.	.	DP=1466;HET=0;HOM=1;RD=0;AD=1466;AF=1	GT	1/1
CP000255.1	2322700	.	T	C	.	.	DP=1592;HET=0;HOM=1;RD=1;AD=1591;AF=0.9994	GT	1/1
CP000255.1	2362341	.	A	T	.	.	DP=1374;HET=1;HOM=0;RD=846;AD=528;AF=0.385	GT	0/1
CP000255.1	2487639	.	A	G	.	.	DP=1431;HET=0;HOM=1;RD=2;AD=1429;AF=0.9993	GT	1/1
CP000255.1	2549677	.	C	T	.	.	DP=1762;HET=0;HOM=1;RD=0;AD=1762;AF=1	GT	1/1
CP000255.1	2744011	.	T	A	.	.	DP=1423;HET=0;HOM=1;RD=1374;AD=49;AF=0.0351	GT	0/0
CP000255.1	2790094	.	A	T	.	.	DP=1482;HET=0;HOM=1;RD=1437;AD=45;AF=0.0304	GT	0/0

Table S2.4 Single nucleotide variants (SNV) in *S. aureus* USA300 JE2 Δ*nuc2* compared to the reference genome (GenBank: CP000255.1)

##fileformat=VCFv4.1
##source=CeGaT GmbH, Paul-Ehrlich-Str. 23, 72076 Tuebingen, Germany
##INFO=<ID=DP,Number=1,Type=Integer,Description="Raw read depth">
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##INFO=<ID=AD,Number=1,Type=Integer,Description="Depth of variant-supporting bases (reads2)">
##INFO=<ID=AF,Number=1,Type=Float,Description="Novel allele frequency (AD/DP)">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">

#CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO	FORMAT	Sample
CP000255.1	12	.	A	T	.	.	DP=199;HET=0;HOM=1;RD=195;AD=4;AF=0.0251	GT	0/0
CP000255.1	19	.	T	A	.	.	DP=203;HET=0;HOM=1;RD=199;AD=4;AF=0.0246	GT	0/0
CP000255.1	20	.	A	C	.	.	DP=204;HET=0;HOM=1;RD=200;AD=4;AF=0.0245	GT	0/0
CP000255.1	21	.	C	T	.	.	DP=206;HET=0;HOM=1;RD=201;AD=5;AF=0.0243	GT	0/0
CP000255.1	317	.	A	G	.	.	DP=1087;HET=0;HOM=1;RD=0;AD=1087;AF=1	GT	1/1
CP000255.1	5010	.	C	G	.	.	DP=1657;HET=0;HOM=1;RD=0;AD=1657;AF=1	GT	1/1
CP000255.1	34179	.	T	A	.	.	DP=1592;HET=0;HOM=1;RD=0;AD=1592;AF=1	GT	1/1
CP000255.1	52549	.	A	G	.	.	DP=1006;HET=0;HOM=1;RD=982;AD=24;AF=0.0239	GT	0/0
CP000255.1	61025	.	G	A	.	.	DP=1316;HET=0;HOM=1;RD=0;AD=1316;AF=1	GT	1/1
CP000255.1	164912	.	G	A	.	.	DP=1488;HET=0;HOM=1;RD=0;AD=1488;AF=1	GT	1/1
CP000255.1	240358	.	T	A	.	.	DP=1434;HET=0;HOM=1;RD=0;AD=1434;AF=1	GT	1/1
CP000255.1	270510	.	T	C	.	.	DP=1685;HET=0;HOM=1;RD=0;AD=1685;AF=1	GT	1/1
CP000255.1	292738	.	A	C	.	.	DP=1612;HET=0;HOM=1;RD=0;AD=1612;AF=1	GT	1/1
CP000255.1	376057	.	C	T	.	.	DP=1508;HET=0;HOM=1;RD=0;AD=1508;AF=1	GT	1/1
CP000255.1	376231	.	T	G	.	.	DP=1219;HET=0;HOM=1;RD=0;AD=1219;AF=1	GT	1/1
CP000255.1	425833	.	A	T	.	.	DP=1664;HET=0;HOM=1;RD=0;AD=1664;AF=1	GT	1/1
CP000255.1	512979	.	C	T	.	.	DP=866;HET=0;HOM=1;RD=748;AD=118;AF=0.1363	GT	0/0
CP000255.1	513055	.	G	T	.	.	DP=599;HET=0;HOM=1;RD=101;AD=498;AF=0.8314	GT	1/1
CP000255.1	517837	.	C	T	.	.	DP=729;HET=0;HOM=1;RD=0;AD=729;AF=1	GT	1/1
CP000255.1	518090	.	T	C	.	.	DP=978;HET=0;HOM=1;RD=0;AD=978;AF=1	GT	1/1
CP000255.1	518102	.	G	A	.	.	DP=1049;HET=0;HOM=1;RD=2;AD=1047;AF=0.9981	GT	1/1
CP000255.1	518359	.	G	A	.	.	DP=1853;HET=0;HOM=1;RD=1538;AD=315;AF=0.17	GT	0/0
CP000255.1	556082	.	T	C	.	.	DP=884;HET=0;HOM=1;RD=0;AD=884;AF=1	GT	1/1
CP000255.1	556291	.	T	C	.	.	DP=626;HET=0;HOM=1;RD=3;AD=623;AF=0.9968	GT	1/1
CP000255.1	558118	.	T	C	.	.	DP=2812;HET=1;HOM=0;RD=1750;AD=1062;AF=0.378	GT	0/1
CP000255.1	591684	.	G	T	.	.	DP=1698;HET=0;HOM=1;RD=0;AD=1698;AF=1	GT	1/1
CP000255.1	656344	.	A	T	.	.	DP=1056;HET=0;HOM=1;RD=0;AD=1056;AF=1	GT	1/1
CP000255.1	656345	.	A	T	.	.	DP=1037;HET=0;HOM=1;RD=0;AD=1037;AF=1	GT	1/1
CP000255.1	656348	.	G	T	.	.	DP=1037;HET=0;HOM=1;RD=0;AD=1037;AF=1	GT	1/1
CP000255.1	658463	.	A	T	.	.	DP=1363;HET=0;HOM=1;RD=42;AD=1321;AF=0.9692	GT	1/1
CP000255.1	658465	.	T	A	.	.	DP=1326;HET=0;HOM=1;RD=43;AD=1283;AF=0.9676	GT	1/1
CP000255.1	658668	.	T	G	.	.	DP=674;HET=0;HOM=1;RD=3;AD=671;AF=0.997	GT	1/1
CP000255.1	658669	.	T	A	.	.	DP=601;HET=0;HOM=1;RD=2;AD=599;AF=0.9967	GT	1/1
CP000255.1	658670	.	T	G	.	.	DP=598;HET=0;HOM=1;RD=2;AD=596;AF=0.9967	GT	1/1
CP000255.1	658671	.	T	C	.	.	DP=589;HET=0;HOM=1;RD=3;AD=586;AF=0.9966	GT	1/1
CP000255.1	658672	.	T	C	.	.	DP=592;HET=0;HOM=1;RD=3;AD=589;AF=0.9966	GT	1/1
CP000255.1	658674	.	T	G	.	.	DP=641;HET=0;HOM=1;RD=2;AD=639;AF=0.9984	GT	1/1
CP000255.1	680376	.	T	G	.	.	DP=12;HET=0;HOM=1;RD=0;AD=12;AF=1	GT	1/1
CP000255.1	684888	.	A	C	.	.	DP=14;HET=0;HOM=1;RD=1;AD=13;AF=0.9286	GT	1/1
CP000255.1	930890	.	T	C	.	.	DP=1662;HET=0;HOM=1;RD=0;AD=1662;AF=1	GT	1/1
CP000255.1	944692	.	A	T	.	.	DP=1666;HET=0;HOM=1;RD=0;AD=1666;AF=1	GT	1/1
CP000255.1	1022578	.	T	A	.	.	DP=1183;HET=0;HOM=1;RD=1159;AD=24;AF=0.0211	GT	0/0
CP000255.1	1022894	.	C	A	.	.	DP=1354;HET=0;HOM=1;RD=1318;AD=36;AF=0.0266	GT	0/0
CP000255.1	1269542	.	A	T	.	.	DP=1065;HET=0;HOM=1;RD=1;AD=1064;AF=0.9991	GT	1/1
CP000255.1	1286986	.	A	G	.	.	DP=1234;HET=0;HOM=1;RD=0;AD=1234;AF=1	GT	1/1
CP000255.1	1295497	.	A	G	.	.	DP=1420;HET=0;HOM=1;RD=0;AD=1420;AF=1	GT	1/1
CP000255.1	1339845	.	T	A	.	.	DP=11;HET=0;HOM=1;RD=0;AD=11;AF=1	GT	1/1
CP000255.1	1354028	.	C	T	.	.	DP=1356;HET=0;HOM=1;RD=1;AD=1355;AF=0.9993	GT	1/1
CP000255.1	1355744	.	T	A	.	.	DP=1454;HET=0;HOM=1;RD=0;AD=1454;AF=1	GT	1/1
CP000255.1	1357665	.	C	T	.	.	DP=1549;HET=0;HOM=1;RD=0;AD=1549;AF=1	GT	1/1
CP000255.1	1533312	.	A	T	.	.	DP=1239;HET=0;HOM=1;RD=1;AD=1238;AF=0.9992	GT	1/1
CP000255.1	1630757	.	T	A	.	.	DP=464;HET=0;HOM=1;RD=0;AD=464;AF=1	GT	1/1
CP000255.1	1688528	.	C	T	.	.	DP=1493;HET=0;HOM=1;RD=2;AD=1491;AF=0.9993	GT	1/1
CP000255.1	1752707	.	G	T	.	.	DP=1331;HET=0;HOM=1;RD=0;AD=1331;AF=1	GT	1/1
CP000255.1	1762523	.	G	T	.	.	DP=1398;HET=0;HOM=1;RD=2;AD=1396;AF=0.9986	GT	1/1
CP000255.1	1765020	.	A	C	.	.	DP=1375;HET=0;HOM=1;RD=0;AD=1375;AF=1	GT	1/1
CP000255.1	1857109	.	A	G	.	.	DP=1927;HET=0;HOM=1;RD=1456;AD=471;AF=0.2449	GT	0/0
CP000255.1	1857117	.	T	C	.	.	DP=1896;HET=1;HOM=0;RD=1334;AD=562;AF=0.2969	GT	0/1
CP000255.1	1857182	.	G	A	.	.	DP=2502;HET=1;HOM=0;RD=1210;AD=1292;AF=0.5164	GT	0/1
CP000255.1	1857188	.	C	T	.	.	DP=2392;HET=1;HOM=0;RD=1163;AD=1229;AF=0.5142	GT	0/1
CP000255.1	1857201	.	T	C	.	.	DP=2250;HET=1;HOM=0;RD=1186;AD=1064;AF=0.4729	GT	0/1
CP000255.1	1857202	.	G	A	.	.	DP=2180;HET=1;HOM=0;RD=1148;AD=1032;AF=0.4734	GT	0/1
CP000255.1	1857213	.	T	C	.	.	DP=2181;HET=1;HOM=0;RD=1101;AD=1080;AF=0.4952	GT	0/1
CP000255.1	1857215	.	T	C	.	.	DP=2134;HET=1;HOM=0;RD=1142;AD=992;AF=0.4649	GT	0/1
CP000255.1	1857232	.	T	C	.	.	DP=2326;HET=1;HOM=0;RD=1133;AD=1193;AF=0.5129	GT	0/1
CP000255.1	1857258	.	C	T	.	.	DP=2200;HET=1;HOM=0;RD=956;AD=1244;AF=0.5659	GT	0/1
CP000255.1	1857276	.	C	T	.	.	DP=1994;HET=1;HOM=0;RD=597;AD=1397;AF=0.7011	GT	0/1
CP000255.1	1857321	.	C	T	.	.	DP=1578;HET=0;HOM=1;RD=1492;AD=86;AF=0.0545	GT	0/0
CP000255.1	1872844	.	T	C	.	.	DP=1640;HET=0;HOM=1;RD=0;AD=1640;AF=1	GT	1/1
CP000255.1	1939443	.	C	G	.	.	DP=1374;HET=0;HOM=1;RD=0;AD=1374;AF=1	GT	1/1
CP000255.1	1957938	.	C	T	.	.	DP=1461;HET=0;HOM=1;RD=0;AD=1461;AF=1	GT	1/1
CP000255.1	1961241	.	G	C	.	.	DP=1377;HET=0;HOM=1;RD=0;AD=1377;AF=1	GT	1/1
CP000255.1	1961354	.	C	T	.	.	DP=1332;HET=0;HOM=1;RD=2;AD=1330;AF=0.9992	GT	1/1
CP000255.1	1988091	.	G	T	.	.	DP=933;HET=0;HOM=1;RD=914;AD=19;AF=0.0204	GT	0/0
CP000255.1	1997060	.	G	T	.	.	DP=2168;HET=1;HOM=0;RD=1447;AD=721;AF=0.333	GT	0/1
CP000255.1	1997102	.	A	T	.	.	DP=2568;HET=1;HOM=0;RD=1491;AD=1077;AF=0.4194	GT	0/1
CP000255.1	1997387	.	A	G	.	.	DP=2461;HET=1;HOM=0;RD=1648;AD=813;AF=0.3304	GT	0/1
CP000255.1	1997484	.	A	T	.	.	DP=2381;HET=1;HOM=0;RD=1565;AD=816;AF=0.3431	GT	0/1
CP000255.1	1997489	.	G	C	.	.	DP=2436;HET=1;HOM=0;RD=1646;AD=790;AF=0.3247	GT	0/1
CP000255.1	2002083	.	A	G	.	.	DP=7;HET=0;HOM=1;RD=0;AD=7;AF=1	GT	1/1
CP000255.1	2007223	.	G	T	.	.	DP=1336;HET=0;HOM=1;RD=0;AD=1336;AF=1	GT	1/1
CP000255.1	2148909	.	G	A	.	.	DP=1300;HET=0;HOM=1;RD=0;AD=1300;AF=1	GT	1/1
CP000255.1	2164596	.	A	G	.	.	DP=1424;HET=0;HOM=1;RD=0;AD=1424;AF=1	GT	1/1
CP000255.1	2322700	.	T	C	.	.	DP=1517;HET=0;HOM=1;RD=0;AD=1517;AF=1	GT	1/1
CP000255.1	2487639	.	A	G	.	.	DP=1382;HET=0;HOM=1;RD=0;AD=1382;AF=1	GT	1/1
CP000255.1	2549677	.	C	T	.	.	DP=1663;HET=0;HOM=1;RD=0;AD=1663;AF=1	GT	1/1
CP000255.1	2648396	.	G	A	.	.	DP=1656;HET=0;HOM=1;RD=0;AD=1656;AF=1	GT	1/1
CP000255.1	2805278	.	G	T	.	.	DP=1424;HET=0;HOM=1;RD=2;AD=1422;AF=0.9986	GT	1/1

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Chapter 3 – Identification of a serotonin N-acetyltransferase (SNAT) from *Staphylococcus pseudintermedius* ED99

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Chapter 3

Abstract

Serotonin N-acetyltransferase (SNAT) catalyzes the biosynthesis of N-acetylserotonin (NAS) and N-acetyltryptamine (NAT), two pleiotropic molecules with neurotransmitter functions. Here, we report the identification of a SNAT protein in the genus *Staphylococcus*. The SNAT gene identified in *Staphylococcus pseudintermedius* ED99, namely *SPSE_0802*, encodes a 140 residues-long cytoplasmic protein. The recombinant protein SPSE_0802 was expressed in *E. coli* BL21 and found to acetylate serotonin (SER) and tryptamine (TRY) as well as other trace amines *in vitro*. The production of the neuromodulators NAS and NAT was detected in the cultures of different members of the genus *Staphylococcus* and the role of SPSE_0802 in this production was confirmed in an ED99 *SPSE_0802* deletion mutant. A search for SNAT homologues showed that the enzyme is widely distributed across the genus which correlated with the SNAT activity detected in 22 out of the 40 *Staphylococcus* strains tested.

The N-acetylated products of SNAT are precursors for melatonin synthesis and are known to act as neurotransmitters and activate melatonin receptors, among others, inducing various responses in the human body. The identification of SNAT in staphylococci could contribute to a better understanding of the interaction between those human colonizers and the host peripheral nervous system.

Introduction

Acetylation of biomolecules is a widespread strategy used for regulation of cellular processes such as protein synthesis, detoxification, and virulence. In higher organisms, acetylation also serves to modify small molecules into hormones or neurotransmitters with diverse biological activities (Hu *et al.*, 2010; Hentchel and Escalante-Semerena, 2015). Serotonin N-acetyltransferases (SNAT) belong to the superfamily of GCN5-related N-acetyltransferases (GNAT) which catalyze the acetylation of various molecules. SNAT acetylate monoamines, such as serotonin (5-hydroxytryptamine), tryptamine and other trace amines (TAs), by transfer of an acetyl moiety from acetyl coenzyme A (acetyl-CoA) onto the target substrate (Burckhardt and Escalante-Semerena, 2020).

Chapter 3

In mammals, serotonin (SER) is an important hormone and neurotransmitter that regulates a myriad of functions in the CNS, including emotion, cognition and stress. In the enteric nervous system, SER is involved in gastrointestinal motility and secretion (Gershon and Tack, 2007). SER can be modified by SNAT into N-acetylserotonin (NAS), another pleiotropic molecule which, in addition to being the direct precursor of melatonin (N-acetyl-5-methoxytryptamine) (Axelrod and Weissbach, 1960), can itself act as an agonist of melatonin receptors MT3, which are predominant in the gut (Jockers *et al.*, 2016) and thereby modulate the circadian rhythm. The amounts of NAS in the brain and the gut are critical for regulating the melatonin biosynthesis pathway (Brown *et al.*, 1983; Brown *et al.*, 1984; Klein, 2007; Maronde *et al.*, 2011). Independently from the melatonin pathway and receptors, NAS also performs potent anti-depressant, neurotrophic and cognition-enhancing effects (Nosjean *et al.*, 2000; Oxenkrug and Ratner, 2012; Tosini *et al.*, 2012). Those effects are shown to be the result of NAS' robust activation of the tyrosine kinase receptor TrkB, presumably by direct binding (Jang *et al.*, 2010). This activation is essential in regulating hippocampal neurogenesis and sensitivity to anti-depressive drugs (Li *et al.*, 2008). In addition to receptor-mediated signaling, NAS possesses important anti-oxidant (Wölfler *et al.*, 1999; Oxenkrug, 2005) and immune-modulating effects (Lotufo *et al.*, 2001; Perianayagam *et al.*, 2005).

Tryptamine (TRY) is a so-called trace amine (TA) that despite being present in trace amounts in the brain, is no less important than SER. TRY can act directly on SER receptors and modulate the effects of the latter (Jones, 1982). Its acetylated form, N-acetyltryptamine (NAT), is similarly to NAS, a partial agonist of melatonin receptors (Dubocovich, 1995).
SER, TRY, and their acetylated forms (NAS and NAT) perform important functions in many organisms via receptor-mediated or receptor-independent signaling pathways. Hence, they are highly conserved, and their synthesis is tightly regulated.

Gram-positive bacteria are thought to be the evolutionary origin of melatonin in vertebrates (Iyer *et al.*, 2004; Coon and Klein, 2006), which suggests that they harbor enzymes involved in melatonin biosynthesis. However, only little is known about enzymes involved in melatonin biosynthesis in these microorganisms. Interestingly, we recently identified the enzyme 'staphylococcal aromatic amino acid decarboxylase'

(SadA) responsible for SER and TRY production in several staphylococcal species. In *Staphylococcus pseudintermedius* ED99, SadA is a promiscuous enzyme decarboxylating all biogenic aromatic amino acids such as 5-hydroxytryptophan (5-HTP) and tryptophan into SER and TRY, respectively (Luqman *et al.*, 2018). SadA products perform different functions and are found to be beneficial for both the producing bacteria and the host. For instance, they promote the adherence and internalization of staphylococci into intestinal epithelial cells by activation of α2-adrenergic receptors. As a result, bacterial colonization and survival in the host is increased (Luqman *et al.*, 2018). In contrast, SadA products play a role in accelerating wound healing by acting as β2-adrenergic receptor inhibitors. This inhibitory activity abrogates the negative effect of epinephrine on cell motility and wound healing (Luqman *et al.*, 2020).

Based on these findings, we aimed to investigate whether staphylococci produce SNAT, the protein responsible for the next step in melatonin biosynthesis, namely the N-acetylation of SadA products. Here, we identified three putative serotonin N-acetyltransferase proteins from *S. pseudintermedius* ED99 (annotated SPSE_0436, SPSE_0802 and SPSE_1761) based on similarity to the cyanobacterium *Synechocystis sp.* SNAT (cSNAT) (Byeon *et al.*, 2013). Among the three proteins, SPSE_0802, sharing 39% similarity with the functional cSNAT, exhibited N-acetyltransferase activity *in-vitro*, catalyzing the conversion of SER and TRY to NAS and NAT, respectively. The biosynthesis of NAS and NAT was confirmed in *S. pseudintermedius* ED99 cells, as well as in other staphylococcal species. The presence of SPSE_0802 protein homologues in other staphylococci correlated with the ability of the species to produce the N-acetylated monoamines, which confirms the role of SPSE_0802 in NAS and NAT production.

Results

Putative SNAT proteins in S. pseudintermedius ED99 genome

We searched for SNAT homologues in *S. pseudintermedius* ED99, as a model of SER-producing staphylococci using cSNAT identified in the cyanobacterium *Synechocystis* sp. PCC 6803 (GenBank accession no. NP_442603) as a reference. cSNAT is a 171 amino acids long protein possessing an N-terminal sequence comprising at least 22

amino acid residues that have no effect on its enzymatic activity (Byeon *et al.*, 2013). The inactive N-terminal domain is typically found in SNATs in higher organisms and usually serves as a translocation signal.

RefSeq database search was performed to identify homologues in ED99 using BLASTP (O'Leary *et al.*, 2016). The three protein hits that were identified and annotated as SPSE_0802, SPSE_0436 and SPSE_1761 are 140, 138 and 142 amino acids long respectively. SPSE_0802, SPSE0_436 and SPSE_1761 are annotated as GNAT (GCN5-related N-acetyltransferases) family peptides and they share 19, 18 and 10% identity (34, 33 and 16% similarity) with cSNAT, respectively (**Table 3.1**). Comparison to the fully functional truncated $cSNAT_{23\text{-}171}$ increases the similarity to 39, 38 and 19% to each of SPSE_0802, SPSE0_436 and SPSE_1761 respectively.

Locus tag	Predicted gene product	Protein ID	Protein size (kDa)	Percent identity to cSNAT	Percent similarity to cSNAT
SPSE 0802	**acetyltransferase, GNAT family**	**ADX76117.1**	**15.9**	**19**	**34**
SPSE 0436	acetyltransferase, GNAT family	ADX75773.1	15.3	18	33
SPSE 1761	acetyltransferase, GNAT family	ADX77015.1	16.4	10	16

Table 3.1 List of putative SNAT proteins in *S. pseudintermedius* ED99 genome. The percent identity and similarity of the putative SNAT proteins to each of the cyanobacterium *Synechocystis* sp. PCC 6803 SNAT (cSNAT) are shown. The percent similarity of SPSE0802, SPSE0436 and SPSE1761 to the fully functional truncated $cSNAT_{23\text{-}171}$ (Byeon *et al.*, 2013) is 39, 38 and 19% respectively.

Secondary structure prediction of the three proteins suggests that they do possess neither a signal peptide nor a transmembrane domain. Therefore, the enzymes were predicted to be localized in the cytoplasm similarly to previously identified SNATs. Tertiary structure prediction shows the presence of a conserved acetyl-CoA binding domain in all of the four aligned proteins **(Fig. 3.1A)**. SPSE_0802 shares notable structural similarity with cSNAT presented by a common fold, comprised of 6–7 antiparallel β-strands (5 present in cSNAT and SPSE_0802) and 4 α-helices in the topology β1- α1- α2- β2- β3- β4- α3- β5- α4- β6- β7 **(Fig. 3.1B)**.

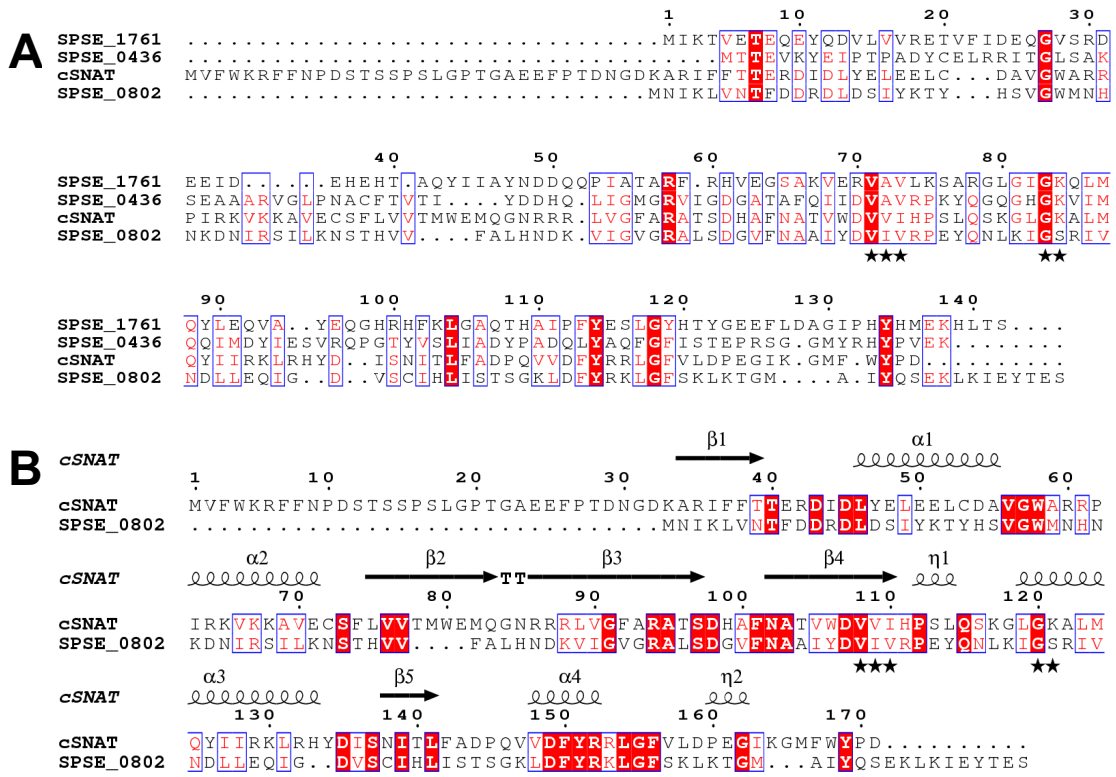

Figure 3.1 Alignment of the predicted SNAT proteins in *S. pseudintermedius* ED99 genome. (A) Protein alignment of the candidate SNATs (SPSE_0802, SPSE_0436 and SPSE_1761) with cSNAT. The acetyl coenzyme A binding pocket indicated with black stars is conserved among the 4 proteins. cSNAT possess an N-terminal sequence comprising at least 22 amino acid residues that have no effect on its enzymatic activity. (B) Alignment of cSNAT and SPSE_0802 sharing the highest similarity (34%). 3-D structure prediction shows that both proteins share a common fold known as the GNAT fold, comprised of 6–7 antiparallel $\boldsymbol{\beta}$-strands (5 present in cSNAT and SPSE_0802) and 4 $\boldsymbol{\alpha}$-helices in the topology $\boldsymbol{\beta}$1- $\boldsymbol{\alpha}$1- $\boldsymbol{\alpha}$2- $\boldsymbol{\beta}$2- $\boldsymbol{\beta}$3- $\boldsymbol{\beta}$4- $\boldsymbol{\alpha}$3- $\boldsymbol{\beta}$5- $\boldsymbol{\alpha}$4- $\boldsymbol{\beta}$6- $\boldsymbol{\beta}$7. Amino acid alignment of the proteins was generated with Clustal Omega and structural information ($\boldsymbol{\beta}$-sheets and $\boldsymbol{\alpha}$-helices) was added with ESPript 3.0 based on cSNAT modeling by Swiss Model. Identical residues are shaded red with white letters, and residues with similar properties are in red font surrounded by blue boxes. Black stars underneath the alignment indicate residues forming the coenzyme A binding pocket.

In-vitro enzymatic activity of recombinant SPSE_1761, SPSE_0802 and SPSE_0436

To determine whether any of the three identified proteins from ED99 are SNAT enzymes, the corresponding genes were cloned into the bacterial expression vector pET28a and expressed as C-terminal His-tagged fusion proteins in *E. coli* BL21. His-tagged SPSE_0802, SPSE_0436 and SPSE_1761 proteins were isolated from the cytoplasmic fraction of *E. coli* BL21 clones and purified by Ni-NTA superflow resin (**Fig. 3.2**).

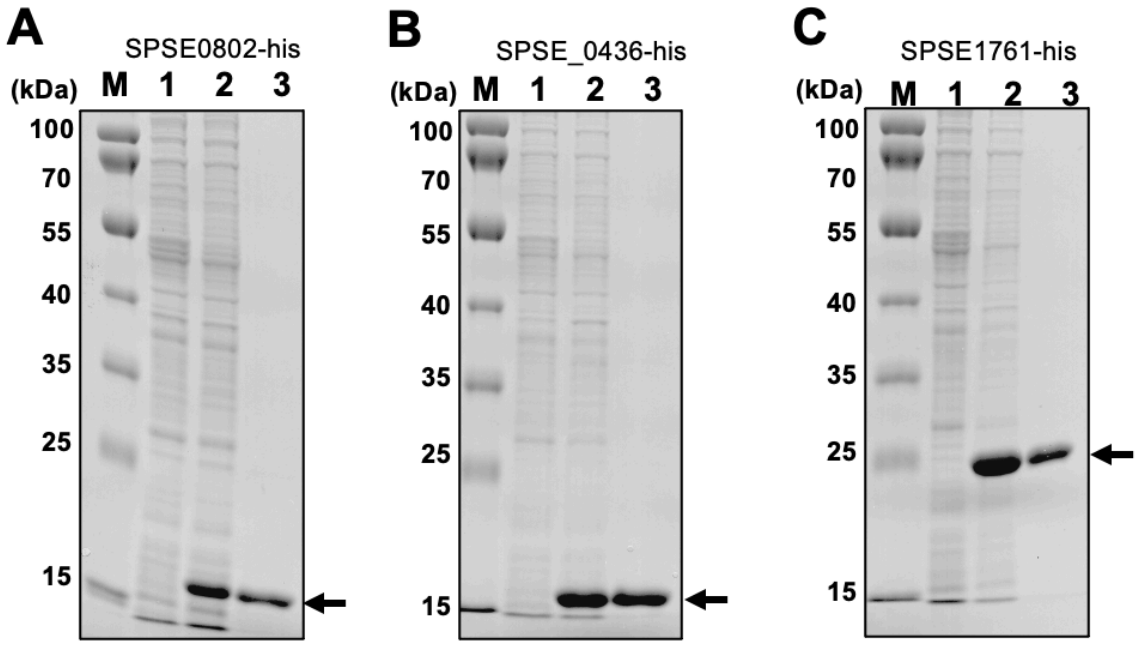

Figure 3.2 Expression of the *S. pseudintermedius* ED99 putative SNAT genes in *E. coli* BL21 harboring (A) SPSE_0802, (B) SPSE_0436 or (C) SPSE_1761 in the vector pET28a. M, molecular marker; Lane 1, total protein in 10-μL aliquots of bacterial cells without IPTG; Lane 2, total cytoplasmic protein after IPTG treatment; Lane 3, 5 μg soluble protein purified by affinity chromatography. Proteins were separated by 14% SDS-PAGE and stained with Coomassie blue. The recombinant proteins SPSE_0802 and SPSE_0436 migrated to roughly their theoretical sizes (15.9 and 15.3 kDa respectively), whereas SPSE_1761 migrates slightly higher than its theoretical size on the gel.

The enzymatic activity of the recombinant proteins was assayed by measuring the N-acetylation of 1 mM SER or tryptamine in presence of 1 mM acetyl-CoA as co-factor at 37°C. SPSE_0802 could acetylate SER and TRY into NAS and NAT, respectively as detected by high performance liquid chromatography (HPLC) analysis (**Fig. 3.3A-B**). At pH 8.0, SPSE_0802 acetylated around 18% of the total SER and 23% of the total tryptamine after 6 hours incubation. At pH 6.8, a lower activity was detected, as only 12% of SER and 16% of tryptamine were acetylated after the same incubation period. In both conditions, however, SPSE_0802 had a slightly higher activity when TRY was used as a substrate. These results match the higher activity at pH 8.0 – 9.0 and the higher specificity using TRY as substrate reported for other SNATs (Byeon *et al.*, 2013; Kang *et al.*, 2013; Lee *et al.*, 2014). HPLC analysis of the enzymatic reactions using recombinant SPSE_1761 and SPSE_0436 under the same conditions did not detect any N-acetylated products, indicating that neither of these two proteins is a functional SNAT enzyme (**Fig. 3.3A**).

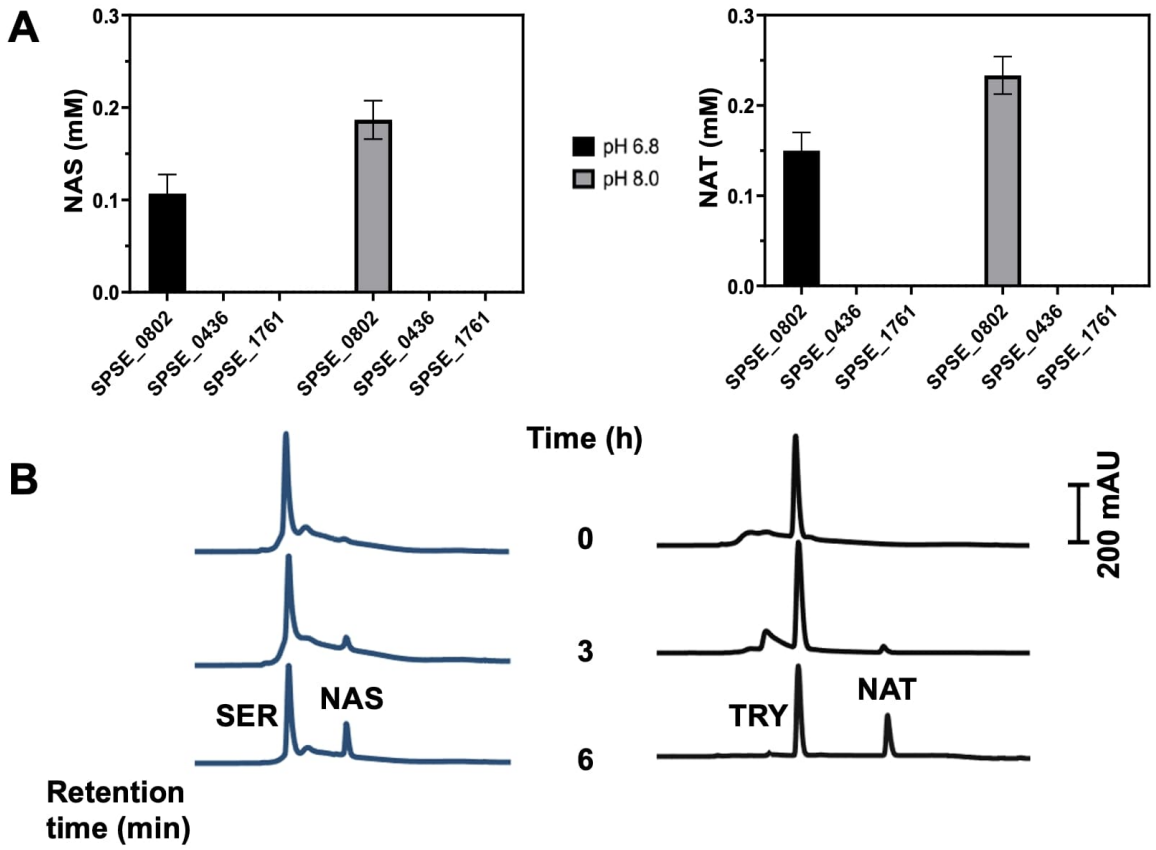

Figure 3.3 *In-vitro* SNAT enzymatic activity of the recombinant proteins SPSE_1761, SPSE_0802 and SPSE_0436. (A) The purified recombinant proteins (200 μg) were incubated in a total volume of 1 mL containing 1 mM serotonin or tryptamine and 1 mM acetyl-CoA in 50 mM potassium phosphate (pH 6.8 or 8.0) at 37°C. The amounts of N-acetylated compounds (NA-SER and NA-TRY) were quantified after 6 hr incubation using HPLC analysis using the standard curve. Only SPSE_0802 could acetylate the substrates. (B) Chromatograms of N-acetylation of serotonin and tryptamine by SPSE_0802 after 6-hr incubation in 50 mM potassium phosphate (pH 8.0) at 37°C.

SPSE_0802 could also acetylate other substrates such as dopamine, phenethylamine, and tyramine (**Fig. 3.4**), which shows the wide spectrum of substrate specificity of the enzyme. There was no acetylation of SER and TRY in absence of SPSE_0802, which rules out the possibility of non-enzymatic acetylation of the compounds. To further confirm the activity of SPSE_0802, the recombinant enzyme was inactivated by incubation at 95°C for 10 minutes before being added to the reaction mixture. In this case, neither NAS nor NAT were detected by HPLC analysis of the reaction after 6 hours.

The N-acetylation reaction catalyzed by SPSE_0802 was acetyl-CoA dependent as no activity was seen in the absence of acetyl-CoA. The reaction was also time-dependent, as more acetylated products accumulated after a longer incubation time.

The acetylated products were quantified using the standard curves (**Fig. S3.1)** and confirmed by comparing their retention time to synthetic standards in HPLC.

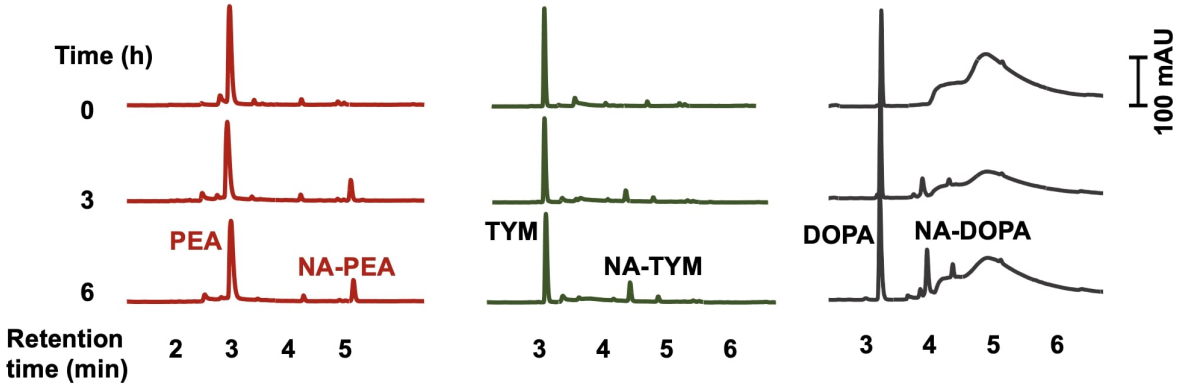

Figure 3.4 HPLC analysis of *in-vitro* enzymatic activity of SPSE_0802 (50 μg) using 0.5 mM phenethylamine (PEA), tyramine (TYM) and dopamine (DOPA) as substrates after 6-hours incubation in phosphate buffer (pH 8.0) at 37°C. All 3 substrates could be acetylated by SPSE_0802.

SNAT activity in *S. pseudintermedius* ED99 WT and SPSE_0802 deletion mutant

To investigate the role of SPSE_0802 in N-acetylation of monoamines in *S. pseudintermedius* ED99, we constructed the deletion mutant ED99Δ*SPSE_0802* and compared both strains' ability to synthesize NAS. Due to its hydrophilic properties, exogenous SER needs to be actively transported into the cell cytoplasm in order to be processed by SPSE_0802. Since ED99 cannot uptake SER from the extracellular medium, we used 5-HTP, the substrate of SadA decarboxylation and precursor of SER. We have previously shown that the decarboxylation reaction by SadA takes place in the cytoplasm where SER is produced (Luqman *et al.*, 2018). Accordingly, we expected 5-HTP to be processed into NAS in a two-step process: decarboxylation to SER by SadA, followed by N-acetylation to NAS by SPSE_0802.

S. pseudintermedius ED99 WT and Δ*SPSE_0802* cells were fed with 5 mM 5-HTP and incubated overnight at 37°C. HPLC analysis of the culture supernatant of cells fed with 5-HTP shows that ED99 WT produced around 24 nM NAS whereas Δ*SPSE_0802* mutant produced around 4 nM NAS (**Fig. 3.5**). These results verify that ED99 can produce NAS and that SPSE_0802 plays an important role in this production. The residual amounts of NAS in the cultures of the ED99 Δ*SPSE_0802* mutant suggest

the presence of another mechanism or enzyme that could produce NAS with a much lower substrate specificity than SPSE_0802.

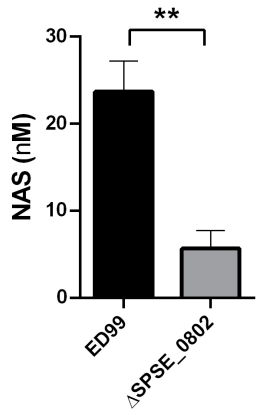

Figure 3.5 NAS biosynthesis in *S. pseudintermedius* ED99. Quantfifcation of NAS using HPLC analysis from cell lysate of *S. pseudintermedius* ED99 and its congenic *SPSE_0802* mutant. The cells were cultured overnight in TSB supplemented with 5 mM 5-HTP as a precursor for NAS production in two reactions; decarboxylation to SER by SadA followed by N-acetylation of SER by SPSE_0802. The cells were incubated for 14 hrs at 37°C, with shaking at 150 rpm. The cells were pelleted and lysed. The cell lysate was collected and stored at -20°C prior to HPLC analysis. Data was analyzed by Mann-Whitney test, **p < 0.01.

SNAT enzymes in plants were recently found to be implicated in an alternative pathway in which they catalyze the conversion of 5-methoxytryptamine (5-MT) directly to melatonin (Back *et al.*, 2016; Tan *et al.*, 2016). To test whether the identified SPSE_0802 could also catalyze this reaction in staphylococci, we searched for melatonin production by *S. pseudintermedius* ED99 and its congenic deletion mutant ED99Δ*SPSE_0802*. No melatonin was detected by HPLC analysis of the cells from overnight cultures fed with 5-MT which indicates that SPSE_0802, unlike plant SNAT, cannot use 5-MT as a substrate and that ED99 does not harbor the enzyme involved in the last step of melatonin biosynthesis from N-acetylserotonin (namely, the acetylserotonin o-methyltransferase ASMT) (results not shown).

SNAT activity in other staphylococcal species

To verify whether other staphylococci possess a SNAT activity like *S. pseudintermedius* ED99, we tested the ability of 40 different staphylococcal strains belonging to 15 cluster groups to synthesize NAT from TRY as a substrate. HPLC analysis of the bacterial culture supernatants revealed that 22 of the 40 tested strains

were able to produce NAT (**Table 3.2**). This indicates that SNAT is widespread in the genus *Staphylococcus*.

As many of the tested staphylococcal strains could produce NAT, we searched for SPSE_0802 homologues in the genus *Staphylococcus* and found 13 hits based on amino acid sequence availability on NCBI. Among the 13 hits, 9 were tested as NAT producers in our experiment. The homologous proteins and accession numbers are listed in **Table 3.3**. The predicted 3D structures of the homologues found in NAT-producing strains are presented with that of cSNAT in **Fig. 3.6**. We note that cSNAT and the homologues in staphylococci share the previously described organization of β-helices and α-sheets. The protein alignment of the SPSE_0802 homologues and the organization of their corresponding genes are presented in **Fig. S3.2** and **Fig. S3.3,** respectively.

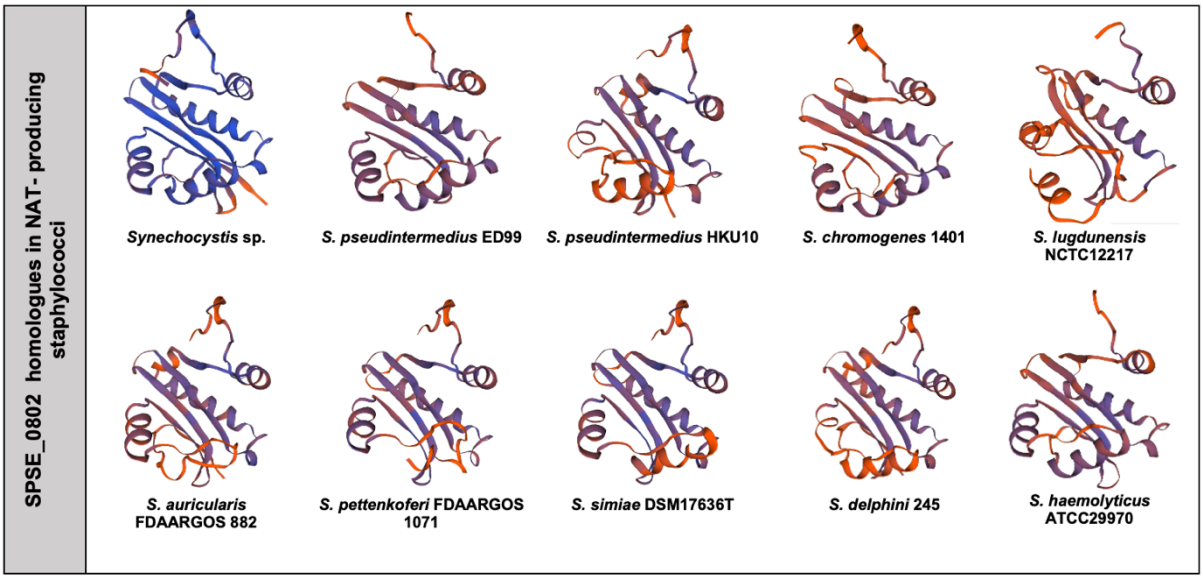

Figure 3.6 SPSE_0802 protein homologues in staphylococci. Predicted 3D structure of cSNAT, SPSE_0802 and its homologues found in the tested NAT-producing staphylococcal species. The 3D structures were modeled using SWISS-MODEL (44).

Table 3.2 Biosynthesis of NAT in staphylococci. N-acetyltryptamine production by different staphylococcal laboratory strains was analyzed by HPLC. Resting bacterial cells were fed with 5 mM tryptophan and incubated overnight at 37°C. 22 out of the 40 strains tested positive for NAT production.

Cluster group	Strain	SNAT activity
Auricularis	*S. auricularis* DSM20609T	+
Muscae	*S. muscae* DSM70687	-
Hyicus	*S. chromogenes* DSM20454T	+
	S. hyicus NCTC10350	-
	S. felis DSM7377T	-
Intermedius	*S. intermedius* CCM5739	+
	S. pseudintermedius ED99	+
	S. delphini DSM220771T	+
	S. schleiferi subsp. schleiferi DSMZ4	+
	S. lutrae DSM10244T	+
Epidemidis	*S. caprae* DSM20608T	-
	S. saccharolyticus DSM20359T	-
	S. capitis LK499	-
	S. epidermidis O47	+
Aureus	*S. aureus* HG003	+
	S. simiae DSM17636T	+
Warneri	*S. wameri* DSM20316T	-
	S. pasteuri ATCC51129	-
Haemlolyticus	*S. haemolyticus* CCM2737	+
	S. hominis DSM20328	+
Lugdunensis	*S. lugdunensis* ATCCA3809	+
Cohnii-Napelensis	*S. cohnii* DSM20260	+
	S. nepalensis DSM15150T	+
Saprophyticus	*S. saprophyticus* NT219	+
	S. equorum LTH5155	-
	S. gallinarum DSM20610T	-
	S. xylosus DSM20266	-
	S. succinus LTH6218/3	+
Arlettae-Kloosi	*S. arlettae* DSM20672T	+
	S. kloosi DSM202676T	+
Pettenkoferi-Masilliensis	*S. pettenkoferi* B3117	+
Simulans-Carnosus	*S. simulans* MK148	-
	S. carnosus TM300	+
	S. piscifermentas LTH3588	+
	S. condiment LTH5866	-
Sciuri	*S. sciuri* SC116	-
	S. vitulinus DSM 15615T	-
	S. fleuretti DSM13212T	-
	S. pulvereri DSM9931	-
	S. lentus	-

Table 3.3 List of SPSE_0802 protein homologues present in other staphylococcal species, their accession number and their percentage of similarity to SPSE_0802. The homologues were found based on prediction by SyntTax. Based on our SNAT activity results, 9 of the listed staphylococcus species, tested positive for NAT production (presented with a + sign) and in four species the SNAT activity was not determined (ND). The proteins SPSE_1761 and SPSE_0436 were also added.

Staphylococcal species	SNAT activity	Protein tag	Protein size (aa)	Accession Number	Similarity to SPSE0802 (%)
S. pseudintermedius ED99	+	SPSE 0802	140	ADX76117.1	-
S. lloydii 2327LY	ND	ISP08_09495	139	QPM74569.1	85
S. haemolyticus ATCC29970	+	EQ029_10910	139	QCY39241.1	77
S. taiwanensis NTUHS172	ND	HYI43_10955	139	UDI79051.1	76
S. agnetis 12B	ND	DWB91_10855	139	QDW99586.1	67
S. chromogenes 1401	+	GJU84_10980	140	QIN27537.1	70
S. pseudoxylosus 14AME19	ND	JMB28_12950	139	QRA18016.1	69
S. delphini 2451	+	IPU21_02605	138	QUM67412.1	45
S. pseudintermedius HKU1003	+	SPSINT_2046	138	ADV06574.1	45
S. simiae NCTC13838	+	SAMEA4384339_02208	133	SNV80616.1	47
S. capitis BN2	+	FRG19_02560	133	QOX60030.1	49
S. lugdunensis NCTC12217	+	NCTC12217_00650	133	SQI89931.1	49
S. auricularis FDAARGOS882	+	I6G39_02390	133	QPT06522.1	48
S. pettenkoferi FDAARGOS1071	+	I6I28_03895	133	QQC38043.1	47
S. pseudintermedius ED99	+	SPSE_1761	142	ADX77015.1	20
S. pseudintermedius ED99	+	SPSE_0436	138	ADX75773.1	30

Discussion

SNATs constitute a universal group of enzymes present in vertebrates, plants, insects, yeast and cyanobacteria (Voisin *et al.*, 1984; Byeon *et al.*, 2013; Kang *et al.*, 2013; Lee *et al.*, 2014). In humans, SNAT is produced as the penultimate enzyme in the classical pathway of melatonin biosynthesis (Klein *et al.*, 1997). New functions of SNAT products are still being discovered, which highlights the importance of these enzymes in fulfilling diverse roles across kingdoms (Coon and Klein, 2006; Zhao *et al.*, 2019). Despite sharing a characteristic protein fold and an acetyl-CoA binding pocket, SNATs show limited primary sequence homology as well as different catalytic residues (De Angelis *et al.*, 1998; Dutnall *et al.*, 1998; Hickman *et al.*, 1999; Dyda *et al.*, 2000; Liao *et al.*, 2021). This variation could be behind the enzyme's wide substrate range and its involvement in alternative pathways in different species (Tan *et al.*, 2016). In addition, the need to be translocated to different parts and organelles contributes to the difference observed on the N-terminal end of the enzymes (Coon

and Klein, 2006). For instance, SNAT in *Oryza sativa* (OsSNAT) possess a long N-terminal sequence (residues 1–83), which corresponds to a chloroplast transit sequence (Kang *et al.*, 2013; Liao *et al.*, 2021). Similarly, the N-terminal residues 1–27 in sheep SNAT have no effect on its activity and most likely function as a signal peptide (Hickman *et al.*, 1999).

A number of SNAT proteins have been recombinantly expressed and purified using a tag fused either to the N- or C-terminal ends (Byeon *et al.*, 2013; Kang *et al.*, 2013; Lee *et al.*, 2014; Back *et al.*, 2021). The catalytically active recombinant SNATs presented a variable activity *in-vitro* at a wide range of temperature (25 – 95°C) and pH (6.5 – 9.0). This range depends on internal characteristics like the isoelectric point, 3-D structure and stability of the protein (Byeon *et al.*, 2013; Kang *et al.*, 2013). Since we were testing SNAT activity in staphylococci, we conducted all the assays at the human body temperature (37°C), despite previous reports on higher optimal temperatures for SNATS in plants and cyanobacteria (Byeon *et al.*, 2013; Kang *et al.*, 2013; Lee *et al.*, 2014). Our results are conforming with the higher SNAT activity reported at pH 8.0 and the higher specificity for TRY as a substrate (Byeon *et al.*, 2013; Kang *et al.*, 2013; Lee *et al.*, 2014). The limited acetylation activity of SPSE_0802, as in other SNATs is associated with their large spectrum of substrates and the resulting low specific binding to a particular substrate (Back *et al.*, 2016).

We focused in our study on *S. pseudintermedius* ED99, as it produces high amounts of SER and TRY (Luqman *et al.*, 2018). ED99 could produce NAS and NAT from 5-HTP and TRY respectively, and the amount was 5 – 6-fold less in its congenic Δ*SPSE_0802* mutant. Those results confirm the activity of SPSE_0802 as a SNAT enzyme with similar properties to those characterized in cyanobacteria and plants (Fig. S3.4 and Fig. S3.5). Since SNAT products are usually found in the cell cytoplasm, we presume that they are produced mainly to act inside the cell, scavenging free radicals to protect the cell from oxidative stress (Wölfler *et al.*, 1999; Oxenkrug, 2005; Álvarez-Diduk *et al.*, 2015).

Next, we checked for the production of N-acetylated products by different staphylococci using TRY as a substrate. Unlike SER, TRY diffuses into the cell and hence, could be used to test SNAT activity in SadA non-producing strains as well. NAT

was detected in the supernatant of ED99 as well as other staphylococcal species (Table 2). Inspection of the genomic locus of *SPSE_0802* homologues revealed that despite their occurrence in many different staphylococcal strains, the upstream and downstream regions seem not to be conserved among the species (Fig. S3). This suggests horizontal gene transfer of *SPSE_0802*, which was also observed for *sadA* (Luqman *et al.*, 2018).

SER and TRY are implicated in bidirectional signaling across species. For instance, gut bacteria in humans regulate the production of SER in the intestinal epithelium and lumen, and thereby modulate host physiology in terms of gastrointestinal motility and blood platelet function (Reigstad *et al.*, 2015; Yano *et al.*, 2015). Inversely, high levels of SER improve the fitness of spore-forming bacteria colonizing the GI tract. This effect was found to be mediated by signaling via SER, which is imported by those bacteria through a serotonin transporter (SERT), homologous to mammalian SERT (Fung *et al.*, 2019). A similar bidirectional signaling has also been described for the interaction between the human skin and TA-producing bacteria. TAs boost the adherence and internalization of *S. pseudintermedius* ED99 into human colon epithelial cells via α2 adrenergic receptors signaling. This renders the bacteria invisible to the immune system. In contrast, TAs benefit the colonized host by acting as β2 adrenergic receptors antagonists on the skin and accelerating wound-healing. Regulating the levels of SER and TRY is hence crucial and can be achieved by different strategies including N-acetylation into NAS and NAT, a modification that redirects them into other signaling pathways.

Members of the genus *Staphylococcus* are common colonizers of the nares, skin and intestine in humans as well as other mammals (Acton *et al.*, 2009; Parlet *et al.*, 2019; Luqman *et al.*, 2020; Carroll *et al.*, 2021). Their proximity to the host might have resulted in their acquisition of the *sadA* gene by horizontal gene transfer, enabling them to produce SER and TRY fulfilling different functions (Luqman *et al.*, 2018). Here, we provide evidence that staphylococcal species harbor another gene, namely *SPSE_0802*, that is responsible for catalyzing NAS and NAT biosynthesis. These products are produced in the cytoplasm and function mainly as antioxidants inside the cell nevertheless, they could also be detected in the culture medium. This implies that

they could be involved in other processes that affect neurotransmission pathways and play another role in signaling with the colonized host.

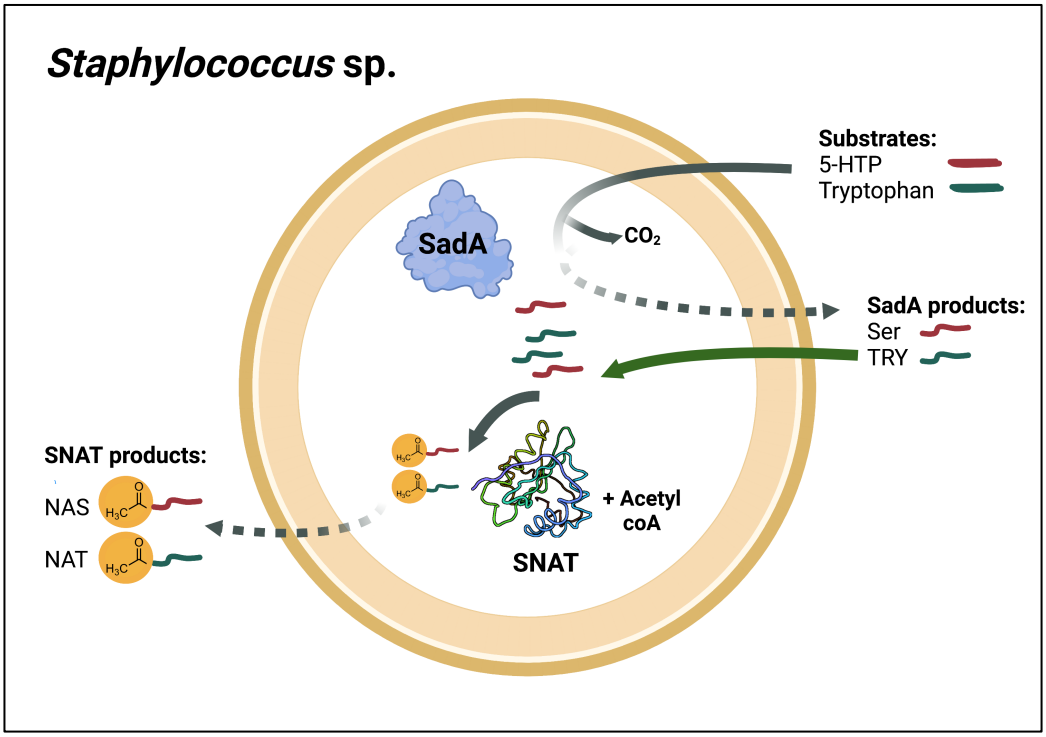

Figure 3.7 Model for the biosynthesis of NAS and NAT by stahylococci. *Staphylococcus* species harboring SadA and SPSE_0802 could produce NAS from 5-HTP as a substrate in two reactions: decarboxylation of 5-HTP into SER by SadA and N-acetylation of SER into NAS by SPSE_0802. Similarly, those staphylococci could synthesize NAT from tryptophan in two reactions: decarboxylation of tryptophan into tryptamine (TRY) by SadA and N-acetylation of TRY into NAT by SPSE_0802. Staphylococci that harbor SPSE_0802 but not SadA could also produce NAT when TRY is available in the environment. SadA- and SPSE_0802- products could be detected in the extracellular medium.

Material and Method

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table S3.1. For cloning procedures in *S. pseudintermedius ED99*, the genome sequence with GenBank accession number NZ_CP065921.1 was used as a reference. *S. pseudintermedius* strains were grown in tryptic soy broth (TSB) and *E. coli* strains were grown in Luria Bertani (LB) medium. Bacteria were cultivated aerobically (200 rpm) at 37°C. Each experiment was started from an overnight preculture by adjusting the OD_{578nm} to 0.05 – 0.1 in the corresponding medium. The medium was supplemented with the following antibiotics, where applicable, at the indicated final concentrations: chloramphenicol at 10 µgml−1 for staphylococcal strains and 30 µgml−1 kanamycin for *E. coli* strains.

BLAST analysis of serotonin N-acetyltransferase of *Synechocystis* sp. against *S. pseudintermedius* ED99

The protein sequence of serotonin N-acetyltransferase of *Synechocystis* sp. (Byeon *et al.*, 2013) (GenBank accession number WP_010873901.1) was used to search the homologous protein in *S. pseudintermedius* ED99 using BLASTP. The protein hits were identified as putative SNAT enzymes.

Bacterial expression and purification of recombinant SNAT candidates

Expression plasmids were constructed using pET28a (Novagen). pET28a was linearized, using the restriction enzymes NotI and NcoI to add a histidine tag to the C-terminal end, and ligated with either of the 3 genes encoding SPSE_1761, SPSE_0802 or SPSE_0436 amplified from *S. pseudintermedius* ED99 genome (oligonucleotides listed in Table S3.2). The ligations were performed using Hi-Fi DNA Assembly Master Mix (New England Biolabs), then transformed into *E. coli* DC10B by heat shock method. The colonies grown on selective agar media containing kanamycin were confirmed by plasmid isolation and sequencing. The plasmids were then transformed into the expression host *E. coli* BL21(DE3). The clones containing the correct plasmid were cultured overnight at 25°C in LB supplemented with kanamycin. The cells were harvested and lysed using 0.1 mm glass beads in FastPrep instrument (MP Biomedicals). The cell lysate was centrifuged (15,000 g, 30 min, 4°C) and the proteins in the supernatant were subjected to protein purification using Ni-NTA superflow resin (IBA). The expression and purification steps were verified by 14% SDS-PAGE. The protein concentration was determined by the Bradford method using a protein assay dye (Bio-Rad, Hercules, CA, USA).

***In-vitro* enzymatic assay**

The purified proteins were subjected to *in-vitro* enzymatic assay. The purified recombinant proteins (200 μg) were incubated in a total volume of 1 mL containing 1 mM substrate and 1 mM acetyl-CoA in 50 mM potassium phosphate (pH 6.8 or 8) at 37 °C. The assay was performed using serotonin as substrate as well as dopamine, tryptamine, phenethylamine, and tyramine. At the indicated time points, the reaction was stopped by adding 250 μL methanol, and the samples were stored at -20°C before being analyzed by HPLC. For the heat inactivation of SPSE_0802, the recombinant protein was incubated at 95°C for 10 min before being added to the reaction mixture.

HPLC analysis

The *in-vitro* enzymatic assays and the bacterial supernatants were analyzed using reversed-phase HPLC (RP-HPLC) as previously described (Luqman, Kharisma, et al., 2020; Luqman, Muttaqin, et al., 2020). Briefly, the HPLC analysis was performed at room temperature with an Eclipse XDB-C18 column (4.6 150 mm; 5 m) (Agilent) and an analytical guard column for Eclipse XDB-C-18 (4.6 12.5 mm; 5 m) (Agilent) with a 15 min linear gradient of 0.1 percent phosphoric acid to acetonitrile and 5 min post time washing with an injection flow rate of 1.5 mL/min, and a sample volume of 10 μl. As a reference, diode array detectors (DAD) were employed at 210 and 360 nm.

Construction of deletion mutant

The null mutant lacking the putative serotonin N-acetyltransferase-encoding gene in *S. pseudintermedius* ED99 was constructed using the plasmid pBASE6 (Geiger *et al.*, 2012). Briefly, pBASE6 was linearized using EcoRV and ~1000 bp upstream and ~1000 bp downstream fragments of the gene of interest were amplified from the genomic DNA of *S. pseudintermedius* ED99. The fragments were fused by Gibson Assembly (Gibson *et al.*, 2009) using Hi-Fi DNA Assembly Master Mix (New England Biolabs). The resulting plasmid was first introduced into *E. coli* DC10B (Monk *et al.*, 2012) and then into ED99. The deletion mutant construction was performed as previously described (Bae and Schneewind, 2006). Deletion of the genes were confirmed by PCR and sequence analysis. The mutants were named as described in the Table S3.1. Oligonucleotides used are listed in the Table S3.2.

NAS production by *S. pseudintermedius* ED99

HPLC analysis of bacterial cell lysates was performed to determine the production of NAS by *S. pseudintermedius* ED99. The strains were cultured in 10 mL TSB supplemented with 5 mM 5-hydroxytryptophan (5-HTP) and incubated overnight at 37°C with shaking at 150 rpm. Then, the cells were harvested, resuspended in 2 mL potassium phosphate buffer (pH 7.2) and lysed using 0.1 mm glass beads in FastPrep instrument (MP Biomedicals). The cell lysate was centrifuged for 30 min, 5000 × g, 4°C. The supernatant was collected and stored at -20°C until analyzed by HPLC.

NAT production screening in *Staphylococcus* strains

HPLC analysis of bacterial cultures supernatants was performed to determine the production of NAT by different *Staphylococcus* strains. The strains were cultured in TSB overnight. Then, the cells were harvested, washed with PBS (pH 7.2), and resuspended in PBS (pH 7.2) supplemented with 1% glucose and 5 mM TRY and incubated overnight at 37°C with shaking at 150 rpm; the cell density was kept relatively high (OD_{578}=50). The cultures were then analyzed using HPLC to determine the presence of NAT.

Statistical significance

For each experiment, the results were expressed as the mean value ± SEM of data from 3–5 replicates. All the statistical analyses were performed using GraphPad Prism software, and a P-value of <0.05 was considered statistically significant. Statistical test choice and significance are indicated in the figure legends.

Author contributions

A.L. and F.G. conceived the idea. A.L. and N.H. designed the experiments. A.L., N.H. and N.L. performed all the experiments.

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Competing interests. None to declare.

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Supplementary Material

Supplementary material of this article may be found at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1073539/ full#supplementary-material

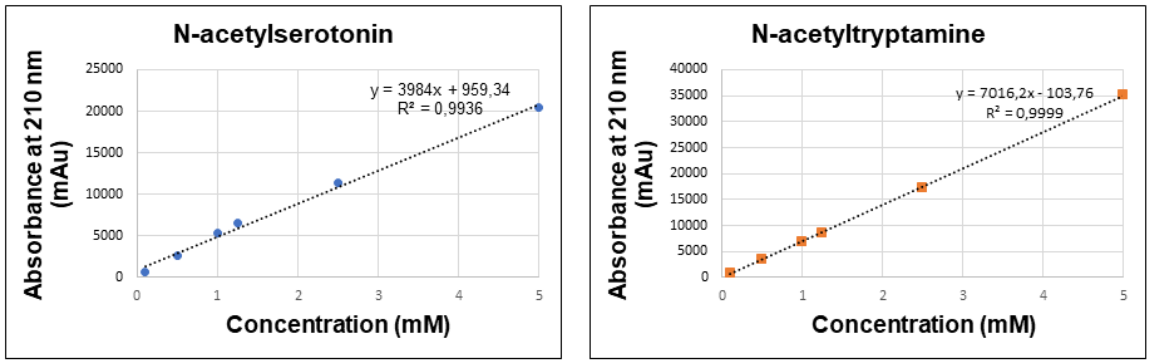

Figure S3.1 Standard curves for quantification of NAS and NAT by HPLC analysis.

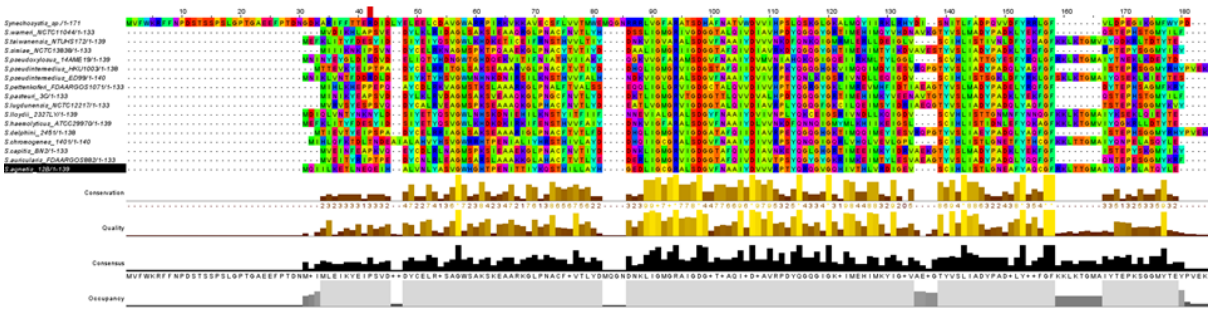

Figure S3.2 Protein alignment of SPSE_0802 homologues in staphylococci. The alignment of putative SNAT in staphylococcal strains was performed using Jalview. The consensus residues and their degree of conservation are noted.

Figure S3.3 Comparison of gene location of SPSE_0802 homologues in some NAS-producing staphylococci. Putative SNAT-encoding genes in staphylococcal strains were predicted using SyntTax.

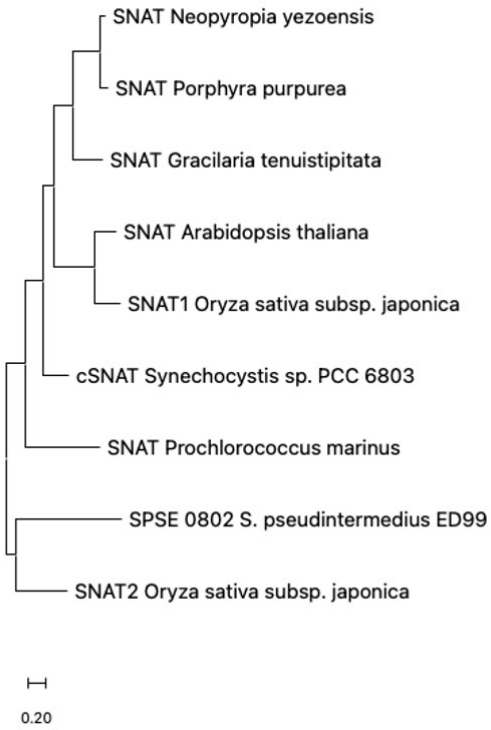

Figure S3.4. Phylogenetic tree of SPSE_0802 and other SNATs from plants and cyanobacteria. The phylogenetic tree was built based on the BLASTP results of SPSE_0802 outside the genus Staphylococcus. The hits were checked for the amino acid sequence on NCBI and were aligned. The multiple sequence alignment was used to construct the phylogenetic tree using maximum likelihood (ML) analysis in Mega11.

Supplementary Tables

Table S3.1. Strains and plasmids used in this study.

Strain	Description	Reference
Staphylococcus		
S. pseudintermedius ED99	Canine bacterial pyoderma clinical isolate	(1)
RN4220	Restriction-deficient *S. aureus* strain	(2)
S. pseudintermedius ED99 Δ*SPSE_0802*	Markerless deletion of *SPSE_0802*	This study
E. coli		
E. coli DC10B	Common laboratory strain for cloning	(3)
E. coli BL21(DE3)	Common protein expression strain	(4)
Plasmids		
pBASE6	Temperature-sensitive plasmid used for knockout	(5)
pBASE-Δ*SPSE_0802*	Plasmid for markerless deletion of *SPSE_0802*	This study
pET28a	IPTG-inducible expression for *E. coli*	Novagen
pET28a-SPSE_0802-his	IPTG-inducible expression of his tagged SPSE_0802	This study
pET28a-SPSE_0436-his	IPTG-inducible expression of his tagged SPSE_0436	This study
pET28a-SPSE_1761-his	IPTG-inducible expression of his tagged SPSE_1761	This study

Table S3.2. Oligonucleotides used in this study.

Primer	Sequence (5'-3')
SPSE0802_fwd	AGAGTTCGAGGAGGTTTAATATGAATATAAAACTTGTTAACACATTT
SPSE0802_rev	TTAAGTACTTCAGCTAATTATTTTTCAAATTGTGGATGTGACCATGATTCAGTATATTCGATTTTCAATTTTTC
SPSE0436_fwd	AGAGTTCGAGGAGGTTTAATATGACAACTGAAGTAAAATATGAAATTC
SPSE436_rev	TTAAGTACTTCAGCTAATTATTTTTCAAATTGTGGATGTGACCACTTTTCAACGGGATAGTG
SPSE1761_fwd	AGAGTTCGAGGAGGTTTAATATGATTAAAACAGTAGAAACGG
SPSE1761_rev	TTAAGTACTTCAGCTAATTATTATTTTTCAAATTGTGGATGTGACCAAGAAGTGAGGTGCTTTTC
SPSE0802 up fwd	CGCGCAGATCTGTCGACGATAACACAGAACTAGAAGTTG
SPSE0802 up rev	TATTCGATTTCAAGTTTTATATTCATACTAGGC
SPSE0802 down fwd	ATAAAACTTGAAATCGAATATACTGAATCATAGTTTGAC
SPSE0802 down rev	TGCAGGCATGCAAGCTTGATAGTTGGCGC

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Chapter 4 – Investigating the function of the two-component system TCS-7 in *Staphylococcus aureus*

Chapter 4

Introduction

Bacteria have evolved mechanisms to sense the environment, respond to changes and hence adapt and survive. To fulfill these functions, they possess two-component systems (TCS) which are bacteria-specific signal transduction systems consisting of a sensory histidine kinase (HK) and a cognate response regulator (RR) (Stock *et al.*, 2000; Capra and Laub, 2012). TCSs are triggered by a specific external signal such as a shift in pH, salinity, temperature, or the presence of antibiotics, and respond by regulating gene transcription.

Staphylococcus aureus is a prominent human pathogen commonly colonizing the skin and anterior nares of healthy individuals (Wertheim *et al.*, 2005) and possessing strategies to cause a variety of infections ranging from relatively mild cutaneous and soft tissue infections to life threatening pneumonia, sepsis, septic arthritis and endocarditis (Tong *et al.*, 2015). The chromosome of *S. aureus* encodes 16 different TCSs (Bleul *et al.*, 2022). TCSs are dispensable for *S. aureus* living under constant environmental conditions, with *WalRK* being the only essential TCS (Villanueva *et al.*, 2018). Nevertheless, sensing and responding to environmental conditions is critical for the expression of virulence factors and the development of bacterial pathogenesis (Haag and Bagnoli, 2017). One of the two still uncharacterized TCSs in *S. aureus* is designated TCS-7. It is encoded in a four-gene cluster including two additional genes encoding unidentified proteins. This operon is referred to as *SAUSA300_1217-1220* based on its annotation in *S. aureus* strain USA300_FPR3757. TCS-7 shows homology with DesKR in *Bacillus subtilis* (Fernández *et al.*, 2020).

Incidentally, DesKR is one of the most well characterized TCSs in Gram-positive bacteria. It comprises DesK, a HK which senses sudden drop in temperature through its transmembrane domain. The cold shock induces a physiological change presented as a decrease in the membrane fluidity. Upon sensing this, activated DesK phosphorylates its cognate RR DesR (Albanesi *et al.*, 2004; Albanesi *et al.*, 2009). In response, DesR binds to a DNA sequence extending from position –28 to –77 from the start site of the temperature-regulated fatty acids (FA) desaturase gene (*des*) (Aguilar et al., 2001) and activates its transcription (Aguilar et al., 2001; Aguilar *et al.*, 1998; Weber *et al.*, 2001; Altabe *et al.*, 2003; Najle *et al.*, 2009). This results in the expression of the encoded Δ5-desaturase protein (Des), which generates unsaturated

fatty acids (UFAs) by introducing a *cis*-double bond at the fifth carbon in the acyl chain of the membrane phospholipids (Fulco, 1969; de Mendoza, 2014). The expression of Des in *Bacillus* spp. is hence upregulated in response to a sudden drop in temperature. The incorporation of UFAs is an important mechanism for restoring membrane fluidity and increasing bacterial survival upon cold shock (Zhang and Rock, 2008).

The TCS DesKR and its effector protein Des are encoded in the same gene cluster which is highly conserved among *Bacillus* spp. The transcription of the *des* and *desKR* mRNA is tightly regulated in cold shock as demonstrated by transcriptional analysis and operon fusion studies (Aguilar *et al.*, 1998; Aguilar *et al.*, 1999). The *desKR* mRNA transcript was shown to be constitutively expressed at 37°C as well as 25°C. In contrast, the *des* mRNA transcript is barely detectable at 37°C and its production transiently increases upon a downshift in temperature to 25°C (Aguilar et al., 2001). This suggests a post-transcriptional downregulation of *des,* which involves the dephosphorylation of DesR by DesK as well as another UFA-dependent mechanism. UFAs, in particular 16:1 Δ5, may downregulate *des* transcription by favoring DesR dephosphorylation or by interacting with an UFA-responsive DNA binding protein that displaces DesR from its binding site (Aguilar et al., 2001).

In *B. subtilis* a paralogue of DesKR exists as a part of another four-gene cluster named *yvfRSTU*. The operon encodes the TCS composed of YvfT and YvfU that share 37% and 58% identity with DesK and DesR, respectively. YvfTU is also activated at low temperature (Fernández *et al.*, 2020). However, the response induced by YvfTU activation has not been studied. Additionally, the function of the two proteins encoded by the upstream genes is unknown. SAUSA300_1217-1220 shares an even higher similarity with YvfRSTU, particularly the effector proteins encoded in the genes upstream of the TCS are more similar between these two operons as compared to Des.

Despite the role of Des in maintaining membrane fluidity in *Bacillus* spp., the deletion of *des* does not have an impact on the bacterial cell growth and morphology. A decrease in bacterial survival during prolonged growth was detected and the cells were not able to sporulate (Aguilar *et al.*, 1998). This suggests that *Bacillus* spp. possess compensatory mechanisms for cold adaptation and that Des is not essential for the survival of bacteria upon cold shock. Probably one of the most important factors

contributing to cold adaptation of *Bacillus* spp. is their membrane FA composition rich in branched chain fatty acids (95% of the total FAs) (Kaneda, 1963; Kaneda, 1966; Kaneda, 1977). Branched-chain FA have lower melting temperature and are normally less susceptible to cold than straight-chain fatty acids (Gounot and Russell, 1999). Therefore, desaturases are essential in cold adaptation of bacteria that produce straight-chain FA exclusively but are not essential for *Bacillus* spp. survival. Des activity is independent of the length of the fatty acid substrate (Fulco, 1969) and straight-chain as well as branched-chain FA can be desaturated (Quint and Fulco, 1973).

Cold adaptation is necessary for the survival of pathogenic bacteria like *S. aureus* during host-to-environment transition which is crucial for their dissemination and transmission. The ability of *S. aureus* to persist at ambient temperature on inanimate surfaces increases the risk of it colonizing new hosts and causing outbreaks (Uhlemann *et al.*, 2011). *S. aureus* was found to survive on the dry surfaces for months (Walther and Ewald, 2004; Kramer *et al.*, 2006; Davis *et al.*, 2012). Nevertheless, the mechanisms that allow *S. aureus* to adapt to cold temperatures outside of the host are not well understood. The cell membrane of *S. aureus* is very similar to that of *B. subtilis* in terms of phospholipids composition. The majority of FAs in *S. aureus* (81%) are branched-chain fatty acids (O'Donnell *et al.*, 1985).

A recent study showed that the HK in staphylococcal TCS-7 could complement the cold shock response (from 37°C to 25°C) in a *B. subtilis desK* mutant. It was also shown that the promoter of TCS-7 is induced by the same shift in temperature when cloned in *B. subtilis* (Fernández *et al.*, 2020). The aim of this work was to verify whether TCS-7 is involved in the cold shock response in *S. aureus*.

Results

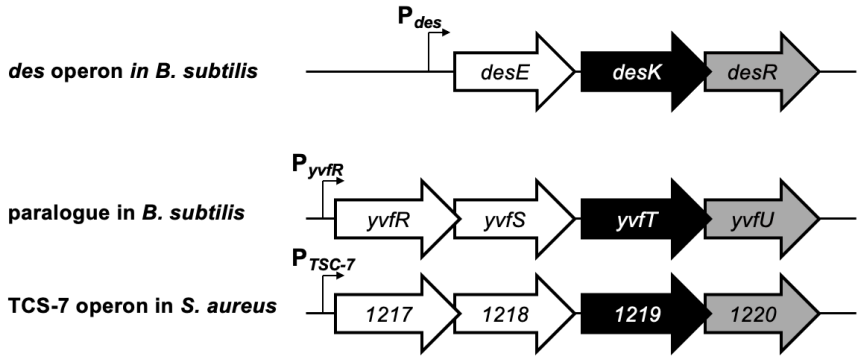
Comparative analysis of the operons of interest

The common element between the Des operon and each of its paralogue in *B. subtilis*, and the homologue in *S. aureus* is mainly the cold-sensitive TCS. DesK shares 40% and 30% identity with YvfT and SAUSA300_1219, respectively. DesR shares 59% and 43% identity with YvfU and SAUSA300_1220, respectively. The effector proteins lack significant similarity to Des but they are similar among each other as YvfR and

SAUSA300_1217 are 34% identical and, YvfS and SAUSA300_1218 are 27% identical (**Table 4.1**). The gene organization of the operons is presented in **Fig 4.1A**. The promoter binding regions of those operons, with an inverted and a direct repeat have high similarity as shown in **Fig 4.1B**. All three HK share the same autophosphorylation residue (His188) (Albanesi *et al.*, 2004; Fernández *et al.*, 2020) located in the conserved active site of phosphorylation comprised in the dimerization and histidine phosphotransfer (DHp) domain (Trajtenberg *et al.*, 2010). The three RR share the same phosphorylatable catalytic aspartate as well (Trajtenberg *et al.*, 2016).

To identify the effector proteins based on the presence of conserved active domains, we searched for their protein family domains (Pfam) in the Integrated Microbial Genomes & Microbiomes (IGM/G) database (Mistry *et al.*, 2021). While the desaturase Des possess the FA desaturase domain Pfam00487, the potential effector proteins within the paralogue and orthologue operons don't. Pfam domain prediction suggests that the first protein in the operon (SAUSA300_1217) is an ATP-binding transporter (Pfam00005) and the second protein (SAUSA300_1218) is a transporter protein involved in the export of drugs and carbohydrates (Pfam12689) (Reizer *et al.*, 1992).

A



B

P_{des}	AGACATGACAAATGTCATATAGGAGGATGATGTGTGCTACTATA
P_{yvfR}	AAAAATGACATTTGTCACTCCATCATCATGACACCCCATACTAAC
P_{TSC-7}	AATAATGACATTTGTCATTTCAAATCTATGACAAAGTGTTCTATT

Figure 4.1 A. Gene organization of the operons *DesEKR* and *YvfRSTU* from *B. subtilis* and *SAUSA300_1217-1220* from *S. aureus*. B. The nucleotide sequence in the upstream region of the start of each of the operons. The promoter binding sites are highlighted in yellow. The nucleotides differing from the promoter binding region of Des are in bold.

	B. subtilis		*S. aureus*	% Identity	% Similarity
Histidine kinase	desK	yvfT		40	57
	desK		1219	30	52
		yvfT	1219	30	50
Response regulator	desR	yvfU		59	76
	desR		1220	43	63
		yvfU	1220	45	61
Upstream/ Effector proteins	desE	yvfR		ns	ns
	desE	yvfS		ns	ns
	desE		1217	ns	ns
	desE		1218	ns	ns
	yvfR		1217	34	57
	yvfR		1218	ns	ns
	yvfS		1217	ns	ns
	yvfS		1218	27	55

Table 4.1 Protein sequence comparison between proteins in the three operons of interest in *B. subtilis* and *S. aureus*. The percent similarity and identity were based on Blast P results.

Desaturase protein sequences vary across species, for instance, Des shows little sequence similarity to membrane desaturases from cyanobacteria and plants. But it contains all three histidine residues conserved in the known membrane desaturases (Aguilar *et al.*, 1998), which are not found in SAUSA300_1217 and 1218. Based on this analysis, we presumed that the effector proteins in SAUSA300_1217-1220 are not desaturases. Nevertheless, the cold sensitivity of the TCS-7 suggests that the two upstream genes could play a role in cold shock response. The potential role of SAUSA300_1217 and 1218 in cold response and potentially FA modification in *S. aureus* needs to be verified.

	Gene symbol	Gene ID	Locus tag	Gene product name	Product size (aa)	Pfam
Effector	***desE***	646318468	BSU19180	fatty acid desaturase	352	pfam00487
	yvfR	646320033	BSU34090	putative ABC efflux transporter (ATP-binding protein)	301	pfam00005 - ABC_tran
	yvfS	646320032	BSU34080	putative ABC transporter (permease)	245	pfam12698 - ABC2_membrane_3
	1217	637887869	SAUSA300_1217	ABC transporter, ATP-binding protein	302	pfam00005 - ABC_tran
	1218	637887870	SAUSA300_1218	ABC transporter, permease protein	243	pfam12698 - ABC2_membrane_3
HK	***desK***	646318469	BSU19190	two-component sensor histidine kinase	370	pfam07730
	yvfT	646320031	BSU34070	two-component sensor histidine kinase	371	pfam02518 - HATPase_c pfam07730 - HisKA_3
	1219	637887871	SAUSA300_1219	putative sensor histidine kinase	363	pfam07730 - HisKA_3
RR	***desR***	646318470	BSU19200	two-component response regulator	199	pfam00072 - Response_reg pfam00196 - GerE
	yvfU	646320030	BSU34060	two-component response regulator	200	pfam00072 - Response_reg pfam00196 - GerE
	1220	637887872	SAUSA300_1220	DNA-binding response regulator, LuxR family	200	pfam00072 - Response_reg pfam00196 - GerE

Table 4.2 Gene products and Pfam domain prediction of the genes of interest (Mistry *et al.*, 2021).

The effect of the deletion of operon *SAUSA300_1217-1220* on *S. aureus* cold shock survival

To prove the role of the operon *SAUSA300_1217-1220* in cold shock response in *S. aureus*, we constructed deletion mutants and monitored the growth of the two *S. aureus* strains USA300 JE2 wildtype (WT) and its congenic operon deletion mutant in TSB with and without cold shock induced at the early exponential growth phase. The results showed that both strains grow similarly in a rich medium. This effect was expected as similar results were reported in experiments using *B. subtilis*. Next, to test the ability of *S. aureus* to recover from cold shock induced at different growth phases, we performed agar plate spotting assays and checked the CFU count. *S. aureus* WT and *SAUSA300_1217-1220* mutant strains were grown in BM at 37°C to the early exponential growth phase (OD_{578nm} = 0.45). Aliquots of the cultures were washed, serially diluted and spotted on two replica BM agar plates; one placed at 37°C and the other 20°C. The results show no growth difference between the WT and mutant at 37°C. The WT strain could grow on agar plates after 48 h incubation at 20°C however, the mutant was not able to grow at the same temperature.

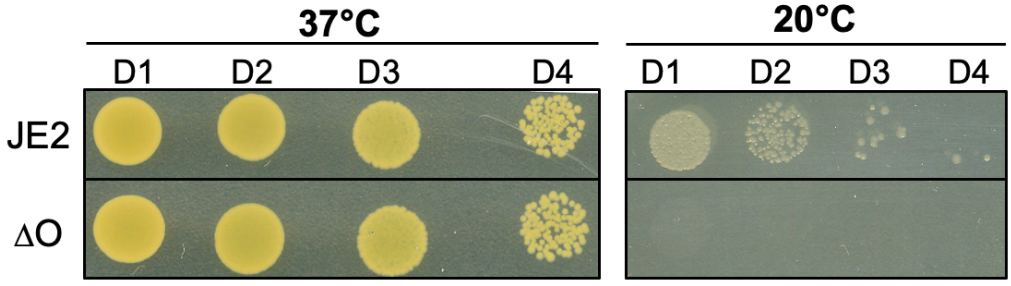


Figure 4.2 Spotting assay of *S. aureus* JE2 WT and *SAUSA3001217-1220* operon mutant (ΔO). The bacterial cultures were washed with PBS, diluted, and spotted on agar plates at OD_{578nm} = 0.45. The plates were incubated overnight at 37°C and for 48 hr at 20°C (until the colonies were visible).

Susceptibility of the *SAUSA300_1217-1220* mutant to membrane disrupting agents

In addition to testing the growth of JE2 and the operon mutant Δ*SAUSA300_1217-1220*, we checked whether the mutant is more susceptible to cell membrane disrupting agents. For this, we performed the spotting assay using agar plates supplemented

with compounds targeting the cell membrane such as polymyxin B, cerulenin and Triton X-100. The plates were prepared also in duplicates and incubated at 37°C and 20°C. We observed no difference in the effects of subinhibitory concentrations of these compounds on the mutant as compared to the WT. Additionally, similar growth between the two strains was also observed when the plates were incubated at 20°C. These results suggest that the mechanism of cold adaptation mediated by the operon is independent from the targets of the tested compounds.

Discussion

The cell membrane plays a crucial role in cellular processes including the uptake of nutrients, release of secretory molecules as well as respiratory energy metabolism, stress response and antibacterial resistance. Hence, maintaining a fluid and functional membrane is of critical importance. In response to cold shock, membrane fluidity can be maintained by different mechanisms including FA acyl chain shortening, branching, and changing the composition of membrane phospholipids to incorporate UFAs (Sinensky, 1971; de Mendoza and Cronan, 1983; de Mendoza *et al.*, 1983; Russell, 1984). These modifications lower the melting temperature of the membrane and protect it from transitioning into a rigid and dysfunctional state. While the first two mechanisms depend on *de-novo* synthesis (Suutari and Laakso, 1994), the desaturation affects pre-existing FAs and could be a faster and less energy intensive response (Fulco, 1969; Fujii and Fulco, 1977).

FAs desaturation in *B. subtilis* was described early in 1969 by Fulco (Fulco *et al.*, 1964). However, the underlying molecular mechanism was not discovered until recently (Aguilar *et al.*, 1999). The membrane lipids composition in *S. aureus* is similar to that of *B. subtilis* (den Kamp *et al.*, 1969; Salzberg and Helmann, 2008). As previously reported, many factors affect the cold-shock response and the types of UFAs produced such as the medium composition, temperature and the growth phase at which the cold shock was induced (Weber *et al.*, 2001). Correspondingly, UFAs are not produced in *B. subtilis* grown in a rich culture medium.

S. aureus produces carotenoids to reestablish membrane fluidity (Chamberlain *et al.*, 1991). The production of the pigment rubixanthin was also found to be increased in *S. aureus* after a downshift in temperature (Joyce *et al.*, 1970). An irreversible defect in

the production of pigments was observed in some *S. aureus* strains upon storage at cold temperatures (Grinsted and Lacey, 1973). Whether the identified operon plays a role in rubixanthin production needs to be investigated.

Confirming the involvement of TCS-7 in cold adaptation is the first step in determining the function of encoding operon. Interestingly, the gene upstream of the operon (*SAUSA300_1216*) encodes a cardiolipin synthase which could indicate a relation of the genes cluster to membrane phospholipids.

The cold shock effect on the cell membrane has been recently studied in *Listeria monocytogenes. L. monocytogenes* belongs to the class Bacilli and the order Bacillales, which also includes Bacillus and Staphylococcus. It has a membrane composition similarly high in branched-chain FA. No evidence for FA desaturation was found in *L. monocytogenes*. However, at least two mechanisms of cold shock adaptation have been verified in this bacterium: a change of branched-chain FA composition from iso to anteiso (Annous *et al.*, 1997) and, increased menaquinones (MK, vitamin K2) in the cell membrane (Seel *et al.*, 2018; Flegler *et al.*, 2021). The genetic basis underlying this mechanism is yet to be elucidated. Interestingly, Staphylococci possess menaquinones in their cytoplasmic membrane as well (Götz *et al.*, 2006). Menaquinone are colorless and are known to play important roles in electron transport and oxidative phosphorylation (White and Frerman, 1967; Marshall and Wilmoth, 1981; Proctor and Peters, 1998). Whether the cold adaptation of *S. aureus* involves a modulation in menaquinone synthesis remains to be investigated.

The membrane lipids of *S. aureus* vary according to the growth phase (Perez-Lopez *et al.*, 2019), oxygen availability (Frerman and White, 1967) and the composition of the medium in which the bacterium grows as it was shown that they produce UFAs when grown in fetal bovine serum (FBS), but not in other media such as TSB, BHI, LB, or MHB (Sen *et al.*, 2016). The composition of membrane lipids following a shift in temperature from 37 to 25°C was studied previously (Joyce *et al.*, 1970) and no UFAs have been detected in the tested samples. However, the cold shock was induced at OD_{750nm}= 8, which is not comparable to other studies and needs to be further verified. The optical density was measured at this wavelength presumably to eliminate the effect of pigments absorbance as *S. aureus* was observed to produce different pigments at 25°C and 37°C. Previous reports as well as our results show that the

growth phase at which the cold shock is induced is critical for the bacterial response. While we have good evidence that the operon *SAUSA300_1217-1220* does not encode a desaturase, the presence of a more complex regulatory mechanism under the control of TCS-7 signaling and affecting FA composition in *S. aureus* should not be ruled out.

The regulatory mechanisms involved in cold adaptation of bacterial membranes have been often overlooked. The common practice of storing and freezing bacterial strains at low temperatures could induce stress adaptive responses in these organisms and affect the study of different processes. This could lead to more subtle phenotypes and difficulties with the identification of the mechanisms involved.
Not all the enzymes involved in FA modifications have phenotypically detectable effects on the bacteria. For instance, *Synechocystis* sp. PCC 6803 harbors four specific desaturases which were found to operate in a very strictly determined order (Higashi and Murata, 1993). It has been shown that the $\Delta 9$ unsaturation, which is the first catalyzed double bond, is essential for conversion of the physical state of a membrane from the gel state to the liquid-crystalline state at physiological temperatures. However, the subsequent introduction of additional unsaturated bonds has progressively smaller effects than the first double bond.

Notably, *S. aureus* does not synthesize UFAs by *de-novo* mechanisms but it does possess genes encoding fatty acid kinases (FakA, FakB1 and FakB2) that incorporate host fatty acids into its membrane phospholipids (Parsons *et al.*, 2014). Extracellular UFAs are incorporated through FakB2 and were found not only to attenuate *S. aureus* virulence mechanisms such as biofilm formation (Yuyama *et al.*, 2020) but also boost the immune response against this bacterium (Nguyen *et al.*, 2015). The incorporation of some free UFAs in *S. aureus* cell membrane has detrimental effects on the bacterium (Butcher *et al.*, 1976; Greenway and Dyke, 1979) by causing protein leakage and disruption of metabolic pathways such as electron transport chain and nutrient uptake (Greenway and Dyke, 1979). The role of extracellular UFAs in *S. aureus* cold shock response has not been studied.

Chapter 4

Conclusion

S. aureus TCS-7 has been shown to sense membrane physiological changes in response to cold temperature when cloned in *B. subtilis*. Here, we present preliminary evidence that a *S. aureus* mutant strain lacking TCS-7 cannot survive a cold shock induced during the early-exponential growth phase. The mechanism behind the role of TCS-7 in cold adaptation needs to be further investigated.

Chapter 4

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table S4.1. For cloning procedures in *S. aureus*, the USA300_FPR3757 genome sequence (NCBI reference sequence NC_007793) was used as a reference. *S. aureus* and *E. coli* strains were grown in basic medium (BM) containing 1% (w/v) soy peptone, 0.5% yeast extract, 0.5% NaCl, 0.1% K_2HPO_4 and 0.1% glucose at pH 7.2. Bacteria were cultivated aerobically (200 rpm) at 37°C. The medium was supplemented with the following antibiotics, where applicable at the indicated final concentrations: chloramphenicol at 10 µgml−1 for staphylococcal strains and 100 µgml−1 ampicillin for *E. coli* strains.

Construction of deletion mutants

The deletion mutants were constructed as markerless deletion as previously described (Bae and Schneewind, 2006). Briefly, recombinant knockout plasmids were constructed using the temperature sensitive plasmid pBASE6 (Geiger *et al.*, 2012), ~1000 bp upstream and ~1000 bp downstream fragments of the gene(s) of interest were amplified from the genomic DNA of *S. aureus* JE2. Then, the fragments were assembled with the EcoRV-linearized pBASE6 by Gibson Assembly (Gibson *et al.*, 2009) using Hi-Fi DNA Assembly Master Mix (New England Biolabs). The resulting plasmids were first introduced into *E. coli* DC10B (Monk *et al.*, 2012) and then into *S. aureus* RN4220 via electroporation before transformation of *S. aureus* JE2 (Monk *et al.*, 2012). Deletion of the genes were confirmed by PCR and sequence analysis. The mutants were named as described in the Table S4.1. Oligonucleotides used are listed in the Table S4.2.

Growth studies of staphylococcal strains

Bacterial strains were cultivated under shaking at 37°C in AAM using a 48-well microplate reader Varioskan Lux (Thermo Scientific). Bacteria were precultured in AAM inoculated into 500 µl of fresh AAM at a starting OD_{578} of 0.1 and growth of JE2 and the constructed mutants was monitored for 48 h.

Spot dilutions

Overnight cultures of each strain grown in TSB were diluted in fresh TSB to an OD_{578nm} of 0.1. Cultures were grown to an OD_{578nm} of 0.45. The cells were washed and resuspended in PBS to an OD_{578nm}=1 then 10-fold dilution series was created, and dilutions were spotted on TSB agar. Duplicate plates were incubated at 37°C and 20°C. Images of the plates were taken after 16 h and 48 h incubation of the plates at 37°C and 20°C respectively. The bacterial strains were always kept at 37°C after retrieval from the glycerol stock (-80°C) until cold shock was induced.

Supplementary Material

Table S4.1 Bacterial strains used in this study.

S. aureus JE2	(Fey *et al.*, 2013)
E. coli DC10B	(Monk *et al.*, 2012)
S. aureus JE2Δ*SAUSA3001217-1220*	This study

Table S4.2 Oligonucleotides used in this study.

For the construction of pBASE6Δ*SAUSA3001217-1220*	
1217-1220 up F	cgcgcagatctgtcgacgataatttcagaatgaataatagaaatcatag
1217-1220 up R	agatccccttgacttgttgatattagatatttgaatc
1217-1220 down F	cgcgcagatctgtcgacgatcagatggaacagttagaaattatac
1217-1220 down R	aatatacaacttctctctagcaagtccc

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Contributions to publications

Publications discussed in this thesis:

Identification of a serotonin N-acetyltransferase from *Staphylococcus pseudintermedius* ED99 (Chapter 2 in the dissertation)

I cloned and purified the three putative SNAT proteins in *E. coli*. I performed *in vitro* enzymatic assays. I performed HPLC analysis of the enzymatic assays and bacterial feeding experiment. I analyzed all the experimental data. I wrote, reviewed, and edited the manuscript (in parts or complete).

Publications not discussed in this thesis:

Nugrahapraja H., Sugiyo P.W.W., Putri B.Q. N., Huang L., Hafza N., Götz F., Santoso H., Wibowo A.T., et al. (2022) Effects of Microplastic on Human Gut Microbiome: Detection of Plastic-Degrading Genes in Human Gut Exposed to Microplastics—Preliminary Study. *Environments*. 9, 140. doi:10.3390/environments9110140

I contributed to the sample preparation, the data analysis and the reviewing of the manuscript.

Fu X., Hafza N., Götz F., Lämmerhofer M. Profiling of Branched Chain and Straight Chain Saturated Fatty Acids by Ultra-High Performance Liquid Chromatography—Mass Spectrometry. *J Chromatogr A*. 1703, 464111. doi:10.1016/j.chroma.2023.464111

I prepared the bacterial samples for lipidomics analysis.