Bacterial peptidoglycan biosynthesis and recognition by the innate immune system

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Knowledge Creation

"The important thing is to not stop questioning."

Albert Einstein

Aos meus pais.

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Thesis Publications

Atilano, M. L.; Yates J.; Glittenberg M.; Filipe S.R.; Ligoxygakis P (2011). "Wall Teichoic Acids of *Staphylococcus aureus* limit recognition by the Drosophila Peptidoglycan Recognition Protein-SA to promote pathogenicity." PLoS Pathogens 7(12): e1002421

Atilano, M. L.; Pereira P.M.; Yates J.; Reed P.; Veiga H.; Pinho M.G.; Filipe S.R. (2010). "Teichoic acids are temporal and spatial regulators of peptidoglycan cross-linking in *Staphylococcus aureus*." Proceedings of the National Academy of Sciences of the United States of America 107 (44): 18991-18996

Additional Publications

Wang L., Gilbert R. J. C., **Atilano M. L.**, Filipe S. R., Gay N. J. and Ligoxygakis P. (2008). "Peptidoglycan recognition protein-SD provides versatility of receptor formation in Drosophila immunity." Proceedings of the National Academy of Sciences of the United States of America 105(33): 11881-11886

Wang L. H., Weber A. N., **Atilano M. L.**, Filipe S. R., Gay N. J. and Ligoxygakis P. (2006). "Sensing of Gram-positive bacteria in Drosophila: GNBP1 is needed to process and present peptidoglycan to PGRP-SA." EMBO Journal 25(20): 5005-5014

TITLE

Bacterial peptidoglycan biosynthesis and recognition by the innate immune system

COVER IMAGE

S. aureus Δ*tagO* mutant strain labeled with Van-FL (PGN - Green) and Hoescht (DNA- blue). Drosophila fly infected with *S. aureus* Δ*tagO* expressing *DRS-GFP*.

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Abbreviations and Acronyms

Amp	Ampicillin
AMPs	Antimicrobial peptides
CW	Cell wall
Ery	Erythromycin
GlcNAc	*N*-acetylglucosamine
GNBP1	Gram negative binding protein
HMM	High molecular mass
HPLC	High pressure liquid chromatography
IM	Inner membrane
IMD	Immune deficiency
Kan	Kanamycin
LA	Luria-Bertani agar
LB	Luria-Bertani broth
LMM	Lower molecular mass
LTA	Lipoteichoic acid
ManNAC	*N*-acetylmanosamine
MIC	Minimum inhibitory concentration
MurNAc	*N*-acetylmuramic acid
OM	Outer membrane
PAMP	Pathogen associated molecular pattern
PBP	Penicillin binding protein
PGN	Peptidoglycan
PGRP	Peptidoglycan recognition protein
PRR	Pattern recognition receptor
RboP	Ribitol phosphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TA	Teichoic acid
TSA	Tryptic soy agar
TSB	Tryptic soy broth
Van	Vancomycin
WGA	Wheat germ agglutinin
WTA	Wall teichoic acid
X-Gal	5-bromo-4-chloro-3-indolyl-β-galactopyranoside

Abstract

Bacteria have to overcome numerous obstacles in order to invade and infect the host, and therefore have evolved sophisticated mechanisms to prevent their elimination, or detection, by the host immune system. The bacterial cell wall is essential for bacterial survival and central to different interactions between bacteria and the host. It is a dynamic and complex surface structure with a multitude of functions, ranging from acting as a structural scaffold or barrier against osmotic lysis, to being involved in cell adhesion.

Pattern recognition receptors (PRRs) produced by the host can target the peptidoglycan (PGN) macromolecule, a major component of the bacterial cell wall, to trigger an immune response, which culminates in the elimination of the invading bacteria. However, in Gram-positive bacteria such as the pathogen *Staphylococcus aureus*, the PGN backbone is highly modified and it may not be fully exposed on the bacterial cell surface. PGN is found to be covalently linked or tightly associated with glycopolymers, such as teichoic acids or capsular polysaccharides, and surface proteins. Whether or not these modifications and molecules impair PGN accessibility, and consequently recognition, is not yet completely understood. Investigation of these hypotheses forms the basis of this thesis.

The first part of this thesis describes the characterization of the effect of PGN modifications on the accessibility of PGN to fluorescent derivatives of vancomycin and wheat germ agglutinin. Vancomycin is an antibiotic that binds to free D-Alanine-D-Alanine residues of the stem

peptides present in PGN, and wheat germ agglutinin is a lectin capable of binding polymers containing N-acetylglucosamine such as the bacterial PGN. Genes involved in the modification of PGN – *oatA*, *tagO*, *pbpD*, *fmtA*, *atl*, *arlR*, and *dltA* - were deleted from the *S. aureus* chromosome and these mutants were then screened, by fluorescence microscopy, for increased binding of probes to the bacterial surface, indicating increased accessibility of PGN within the cell wall structure. Fluorescence imaging of the labelled *S. aureus* null mutants indicated that the absence of wall teichoic acids (WTAs) in the *tagO* mutant correlated with an increased binding of different probes to the bacterial surface. This analysis revealed that WTAs might play an important role in the prevention of access to, and therefore binding of, PGN by external molecules.

The reason for increased accessibility of PGN to different probes was further investigated as reported in the second part of this thesis. Analysis of the PGN muropeptide composition of the *tagO* mutant by high-pressure liquid chromatography (HPLC) revealed reduced levels of peptidoglycan cross-linking due to the absence of WTAs. Microscopy analysis of bacteria expressing fluorescent derivatives of Penicillin-Binding Proteins (PBPs), but unable to produce WTAs, showed that WTAs attached to PGN regulate the septal localization of penicillin-binding protein 4 (PBP4), a low molecular weight PBP that is responsible for the synthesis of highly cross-linked PGN. Thus, WTAs produced by *S. aureus* were shown to act as temporal and spatial regulators of PGN metabolism and structure.

In the final part of this thesis, the impact of WTA modification on PGN recognition by the host peptidoglycan recognition proteins (PGRPs)

was evaluated. A fluorescent derivative of a PGN receptor from the fruit fly *Drosophila melanogaster*, PGRP-SA (mCherry-PGRP-SA), was constructed and its ability to bind to *S. aureus* mutants producing different amounts of WTAs was determined. In this study we observed that PGRP-SA showed increased binding to the surface of *S. aureus* mutants that lack or produce lower amounts of WTAs comparatively to the *S. aureus* parental strain. Moreover, injection of *S. aureus* lacking WTAs into *D. melanogaster* flies was shown to be associated with a reduction in host susceptibility to infection.

The work described in this dissertation has shown that WTAs are important cell wall components involved in the production of a compact PGN net and in limiting the access of the innate immune receptors to PGN. Therefore we have provided evidence that WTAs are important for bacteria to establish a successful infection and ultimately kill the host.

Resumo

As bactérias têm que ultrapassar diversos obstáculos de modo a infectar o hospedeiro. Assim sendo, as bactéria desenvolveram sofisticados mecanismos para prevenir a sua eliminação ou detecção pelo o sistema imunitário do hospedeiro. A parede celular bacteriana é uma estructura essencial para a sobrevivência das bactérias e para o estabelecimento de interacções com o hospedeiro. Esta estrutura, complexa e dinâmica, possui uma multitude de funções que vão desde ser o suporte estrutural e barreira contra a lise osmótica até contribuir para adesão celular.

O hospedeiro produz receptores de reconhecimento padrão (conhecidas como ''pattern recognition receptors'' - PRRs) capazes de detectar o peptidoglicano (PGN), o maior componente da parede celular das bactérias, e activar uma resposta imunitária que culmina com a eliminação das bactéria invasoras. Contudo, o PGN das bactérias Gram-positivas, como por exemplo o de *Staphylococcus aureus*, é altamente modificado e pode não se encontrar completamente exposto à superficie da bacteria. O PGN nestes microorganismos pode estar covalente ligado a glicopolímeros, tais como os ácidos teicoicos da parede, os polisacarídeos capsulares ou até mesmo proteinas de superfíce. No entanto, ainda não se sabe se a presença destas moléculas interfere com a acessibilidade ao PGN e consequentemente com o seu reconhecimento pelo hospedeiro. O estudo da interferência destas moléculas no reconhecimento do PGN é um dos objectivos desta dissertação.

A primeira parte desta tese caracteriza o efeito das modificações do PGN na sua acessibilidade a moléculas fluorescentes derivadas da

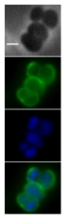
vancomicina e de uma aglutinina da germe do trigo. A vancomicina é um antibiótico que se liga aos aminoácidos D-Alanina-D-Alanina das cadeias peptidicas presentes no PGN. A aglutinina é uma lectina capaz de ligar a polímeros contendo resíduos de N-acetilglucosamina tais como o PGN bacteriano. Os genes envolvidos nas modificações do PGN – *oatA*, *tagO*, *fmtA*, *atl*, *arlR* and *dltA* – foram removidos do cromossoma de *S. aureus* e os mutantes resultantes foram analizados por microscopia de fluorescência. Um aumento da ligação das moléculas fluorescentes ao PGN nestes mutantes corresponderia a um aumento, ou não, do acesso a esta estrutura. A análise das imagens da microscopia de fluorescência dos mutantes nulos de *S. aureus* indicaram que a ausência de acidos teicóicos da parede bacteriana (denominados como ''wall teichoic acids'' – WTAs) no mutante *tagO* está associado a um aumento da ligação de ambas as moléculas fluorescentes à superficie bacteriana. Estes resultados indicam que os WTAs podem ter uma função importante na prevenção do acesso, e consequentemente da ligação, de moléculas externas ao PGN.

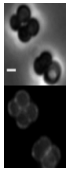
A razão para o aumento da acessibilidade das moléculas fluorescentes ao PGN da bactéria que não produz WTAs, foi mais tarde investigada e os resultados obtidos fazem parte do capitulo dois desta dissertação. A análise da composição muropeptidica do PGN produzido pelo mutante *tagO* por cromatografia liquida de alta pressão (HPLC) revelou uma diminuição da percentagem dos muropeptidos ramificados no PGN. A análise por microscopia de bactérias que expressam derivados fluorescentes de proteínas de ligação à penicilina (denominadas ''penicillin binding proteins'' – PBPs), mas que não produzem WTAs, revelaram que os WTAs associados ao PGN regulam a localização septal

da PBP4. A PBP4 é uma PBP de baixo peso molecular que é responsável pela sintese de PGN altamente ramificado. Desta forma, os WTAs produzidos pela bactéria *S. aureus* funcionam como reguladores espaciais e temporais da síntese do PGN.

Na parte final desta tese avaliámos o impacto da existência de WTAs no reconhecimento do PGN pelas proteinas de reconhecimento do peptidoglicano (conhecidas como ''peptidoglycan recognition proteins'' - PGRPs) do hospedeiro. Para tal construimos e purificamos a proteína mCherry_PGRP-SA, um derivado fluorescente do receptor do PGN da mosca da fruta *Drosophila melanogaster*. A capacidade de ligação desta proteina à superficie bacteriana de mutantes de *S. aureus* que não produzem WTAs, ou que o fazem a niveis mais reduzidos comparativamente com a estirpe parental, foi então determinada. Neste estudo, observámos que a proteína PGRP-SA liga-se com grande intensidade à superficie de mutantes de *S. aureus* que não possuem, ou que produzem uma quantidade reduzida de WTAs. Mais ainda, a injecção destes mutantes em *Drosophila melanogaster* resultou numa redução da susceptabilidade do hospedeiro à infecção por *S. aureus*.

O trabalho apresentado nesta dissertação revelou que os WTAS são componentes importantes da parede celular bacteriana pois estão envolvidos na regulação da produção de um PGN ramificado e compacto, e na limitação do acesso ao PGN pelos receptores do sistema imune inato. Neste trabalho, foram também fornecidas evidências que os WTAs são modificações do PGN que permitem à bactéria estabelecer uma infecção bem sucedida que pode culminar com a morte do hospedeiro.

Study	Aim/Questions	Methods	Main conclusions
General introduction	*S. aureus* cell wall (CW) surface structure and composition. Peptidoglycan (PGN) recognition by the innate immune system of Drosophila.		
Modifications of the *S. aureus* cell wall envelope 	What proteins, involved in the PGN metabolism, contribute to prevent PGN from binding different external probes?	Construction of *S. aureus* mutant strains carrying deletions in genes involved in the PGN metabolism - *oatA*; *pbpD*; *fmtA*; *atl*; *arlR* and *dltA*. Fluorescence microscopy analysis was performed to study binding of CW markers to the surface of the *S. aureus* mutant strains.	1. Enzymes PBP4, FmtA, Atl, TagO and DltA, involved in different steps of PGN biosynthesis, affect PGN accessibility to different cell wall probes. 2. The TagO protein has an important role in protecting PGN from being recognized by different molecules, because tested probes consistently bound with higher affinity to bacterial cells lacking wall teichoic acids (WTAs).
WTAs are temporal and spatial regulators of PGN cross-linking in *S.aureus* 	How does WTA synthesis affect the CW structure?	PGN muropeptide composition of *S. aureus tagO* mutant was analysed by HPLC. PBP4 protein production, in this mutant, was investigated by western blot and the localization of a fluorescent derivative of PBP4 was assessed by fluorescence microscopy.	1. PGN of *tagO* null *S. aureus* mutants, impaired in wall teichoic acids biosynthesis, has a decreased degree of cross-linking, similar to that observed in a PBP4 mutant. 2. PBP4 is normally produced in *tagO* null mutant but it does not localize at the division septum and it is impaired in its function in the absence of wall teichoic acids synthesis. 3. PGN from *tagO* null mutant is more susceptible to the action of lysozyme, a PGN hydrolytic enzyme produced by different hosts.

Study	Aim/Questions	Methods	Main conclusions
WTA of *S. aureus* limit recognition by the Drosophila Peptidoglycan Recognition Protein-SA to promote pathogenicity 	Does WTA play a role in bacteria evasion from the host immune recognition?	A fluorescent derivative of a PGN receptor expressed by Drosophila flies, PGRP-SA (mCherry-PGRP-SA), was purified and its binding to *S. aureus* mutants, producing different amounts of WTA, was evaluated by fluorescence microscopy. Drosophila survival was monitored after injection with *S. aureus* strains that produced different amounts of WTA.	1. Fluorescent PGRP-SA binds more to the surface of *S. aureus* mutants that lack or produce lower amounts of WTA than to the parental strain. 2. Lack of WTA results in lower host susceptibility to infection in a PGRP-SA/GNBP1 manner. 3. PGRP-SD, which is required for sensing wild type *S. aureus*, became redundant when WTA levels were reduced.
Conclusion remarks and future perspectives	A general comment of the results obtained. Future lines of research are suggested in order to evaluate the putative models proposed.		

CHAPTER I

General Introduction

Bacteria

Too small to be seen by the unaided eye, bacteria are thought to be the oldest and structurally simplest living organism on earth [1]. Like other more complex organisms, they possess all of the machinery required to survive and propagate in different hostile environments. Bacteria have diverse genomic contents and metabolic capabilities that permit them to adapt quickly to different surroundings. The ability to synthesize a complex and robust cell wall envelope, ensuring that it encircles the cells, allows the survival of bacteria in many, if not all, ecological niches such as the human body.

A brief history of the bacterial cell wall envelope

Bacteria were unknown until the invention of the microscope and were first observed by Antonie Van Leeuwenhoek in 1676. Leeuwenhoek realized that bacteria could present diverse shapes. Due to the existence of coccal, rod and spiral morphologies, he anticipated that surface structures were responsible for their shape [2], one of the functions of the cell wall (CW) still recognized. The study of bacterial cell surfaces only became possible after Pasteur and Koch started to manipulate bacteria as individual species. Later in the eighteen century, Vincenzy and Fisher tried to characterize the chemical nature of CW by analysing the resistant residues of bacterial cells to plasmolysis, but little information was obtained by their studies, reviewed in [3]. The true nature of the bacterial CW remained unknown until new tools were built in the 1950s. It took more than 300 years, with the use of the newly introduced electron

microscope, for the bacterial CW (after bacteria disruption procedures) became clearly visualized by Mud *et al* in 1941 [4]. Mud and colleagues described the CW as an empty sac retaining the rod shape of the bacteria analysed. These observations were further extended by Dawson in 1949 through studies on the coccal shape of the cell wall produced by *Staphylococcus aureus* (*S. aureus*) [5]. Electron microscopy of thin sections of different bacteria resolved the CW envelope structure as consisting of at least peptidoglycan (PGN) and membranes. In 1951 Salton and Horne, using electron microscopy to assess the purity of their samples, prepared the first pure bacterial CW fractions [6, 7]. This technique allowed Salton in 1953, and Cummins and Harris in 1956, to determine cell wall composition [8, 9]. Later on, Salton proposed that the differences observed by Christian Gram in 1884, regarding the ability of bacterial cells to retain dyes, such as the crystal violet and methylene blue, was due to differences in the cell wall structure and composition of Gram-positive and Gram-negative bacteria [10].

These studies gave rise to a bacterial CW era during which a number of key biochemical discoveries were made such as the identification of the composition and organization of the bacterial cell wall, as well as insights into its roles and mechanisms of synthesis and inhibition.

Different cell wall envelopes

Bacteria were historically divided into two major groups, Gram-positive and Gram-negative bacteria, based on whether or not they are able to retain the crystal violet dye.

The Gram-negative envelope is composed by three principal layers: the outer membrane (OM), the peptidoglycan (PGN), and the inner membrane (IM) [11]. The two membranes, OM and IM confine the periplasm region, which contains a thin layer of PGN (Figure 1). The OM is an asymmetric lipid bilayer contrarily to the IM, because the outer leaflet contains mostly lipopolysaccharide and β-barrel proteins while the inner leaflet is composed of phospholipids.

The cell wall of Gram-positive bacteria has a different organization from the envelopes produced by Gram-negative bacteria (Figure 1).

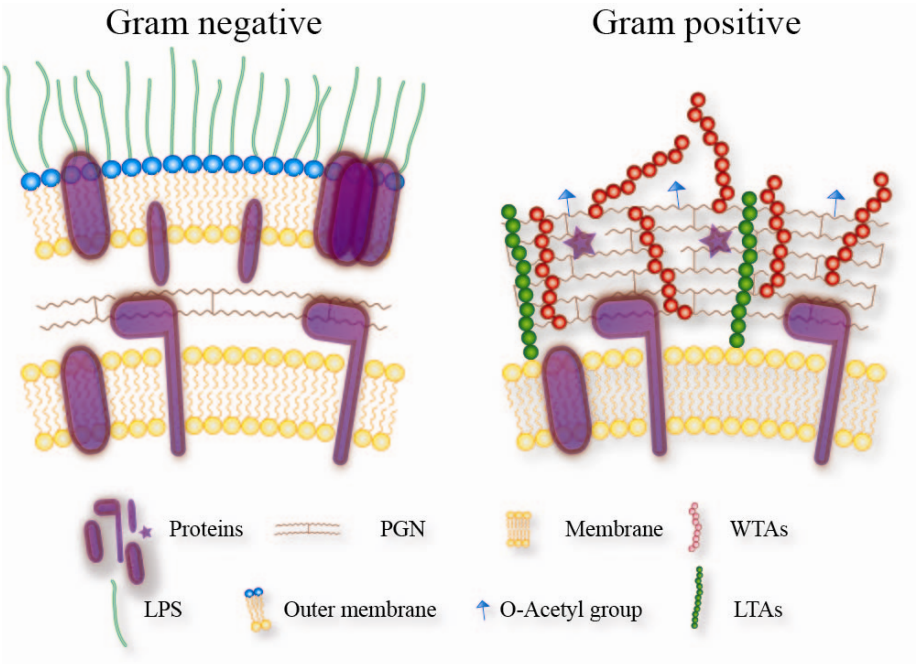


Figure 1. The cell wall envelope of most bacteria can be divided in two major groups: Gram-negative bacteria or Gram-positive bacteria. The Gram-negative bacteria cell wall envelope is composed of a thin layer of PGN between the inner and outer membrane. The Gram-positive bacteria cell wall envelope lacks the outer membrane and it is composed of a thick layer PGN containing polysaccharides polymers.

Gram-positive cell walls typically lack the OM. They contain one membrane and a thick layer of PGN, with attached anionic cell wall polymers such wall teichoic acids (WTAs). Moreover, the cell wall envelope contains a variety of proteins [12] and is often surrounded by a capsular polysacharide.

The cell wall envelope of Gram-positive *S. aureus* bacteria

The cell wall envelope of *S. aureus* shows the typical characteristics of Gram-positive bacteria cell walls. It is composed of a thick PGN layer, which under the electron microscope appears as a homogeneous structure of about 20 to 40 nm, proteins and glycopolymers such as capsule polysacharide or teichoic acids [13, 14]. WTA polymers are present in large quantities in the staphylococcal cell wall [15] and they are thought to extend perpendicularly through the PGN mesh [13] being in contact with the external environment.

Peptidoglycan biosynthesis

The first biochemical insight about the CW synthesis came from the observations made by Park and Johnson [16], that uridine nucleotides accumulated in *S. aureus* in the presence of the antibiotic penicillin,. Further characterization of these uridine nucleotides detected *N*-acetylmuramic acid (MurNAc), D-alanine and D-glutamic acid, components that were very similar to those found in the PGN. Their findings set the background for the unravelling of a series of stages in the biosynthesis of bacterial PGN (Figure 2).

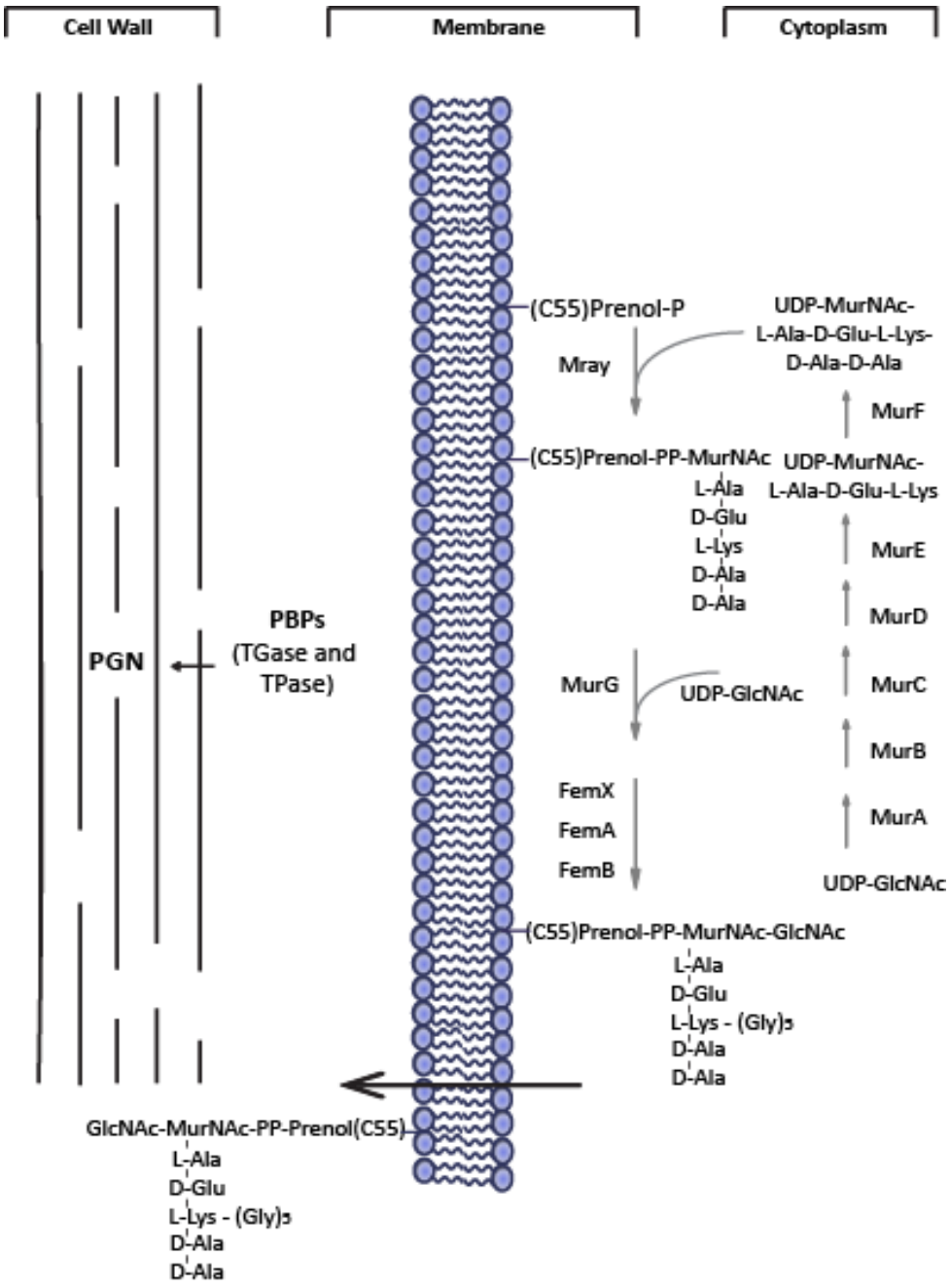


Figure 2. PGN biosynthesis in *S. aureus*. Synthesis of PGN occurs in three stages: assembly of precursor in the cytoplasm, transport of the precursor across the membrane, and polymerization of PGN. The precursor is generated in the

cytoplasm from amino acids and UDP-MurNAc and attached to bactoprenol (lipid I). Transfer of GlcNAc from UDP-GlcNAc to lipid I completes formation of the precursor lipid II. The lipid II is then modified by addition of a pentapeptide bridge and translocated across the membrane. The final stages of peptidoglycan biosynthesis involve polymerization of lipid II through transglycosylation and transpeptidation.

PGN is a heteropolymer built up of alternating β-1,4- linked *N*-acetylglucosamine (GlcNAc) and MurNAc subunits, which are cross-linked through short peptides (reviewed in [17]). Its biosynthesis can be divided into three different stages [18]. The first stage involves the synthesis of the nucleotide sugar-linked precursors UDP-*N*-acetylmuramyl-pentapeptide (UDP-MurNAc-pentapeptide) and UDP-*N*-acetylglucosamine (UDP-GlcNAc) in the cytoplasm. In the second stage the precursor lipid intermediates are synthesised at the cytoplasmic membrane. MraY transfers the phospho-MurNAc-pentapeptide part of UDP-MurNAc-peptide to the bactoprenol, resulting in lipid I (MurNAc-pentapeptide-pyrophosphoryl-undecanoprenol). Then MurG transfers GlcNAc from the UDP-GlcNAc to lipid I, which results in the formation of lipid II intermediate (GlcNAc–β-(1,4)-MurNAc-pentapeptide-pyrophosphoryl-undecanoprenol). This stage also involves the modification of this lipid precursor, through the addition of the pentapetide bridge before translocation of lipid II to the outer side of the cytoplasmic membrane.

The third stage consists in the polymerization of the newly synthesized disaccharide–peptide, and its incorporation into the growing PGN by the transglycosylase and transpeptidase activities of enzymes

such as penicillin-binding proteins and monofunctional transglycosylases.

Penicillin binding proteins

Penicillin binding proteins (PBPs) have been a focus of antibacterial research since the discovery of penicillin, because they are the target of β-lactam antibiotics. The β-lactam ring of the antibiotic penicillin resembles the D-Ala-D-Ala moiety of the PGN precursor [19]. β-lactam antibiotics can therefore bind irreversibly to the active site of PBPs, through the formation of a covalently linked, acyl-enzyme complex, thereby impairing the PGN cross-linking activity of these enzymes [20].

PBPs are membrane-associated proteins that can be classified as multi-modular high molecular mass (HMM) or lower molecular mass (LMM) proteins [21]. LMM PBPs are enzymes that only possess penicillin binding module, which catalyses carboxypeptidation and transpeptidation reactions (formation of the cross-links between the peptides strands). HMM PBPs have an extra N-terminal module connected to the C-terminal penicillin binding module. The identity of this module is used to classify the HMM in classes A and B, according to their ability or not to perform transglycosylation (elongation of the glycan strands) (Figure 3).

S. aureus has only four native PBPs (PBP1 to PBP4) that are responsible for synthesis of PGN. PBP2 is the unique bi-functional HMM class A PBP and its transpeptidation activity is essential for cell viability in methicillin-sensitive strains [22]. The tranglycosylation domain is also important as its inactivation leads to PGN with shorter glycan strands [22]. *S. aureus* has two HMM class B PBPs, PBP1 and PBP3, and only one

LMM PBP, the PBP4 protein. According to Pereira and colleagues, the PBP1 transpeptidase domain has an important role in the initiation of bacterial cell septation and in the production of a signal for cell separation at the end of cell division [23, 24]. The function of PBP3 remains unknown as a PBP3 mutant has no major morphological defects [25] and produces a PGN with similar muropeptide composition to the parental strain [26]. On the other hand, PBP4 is required to achieve the high degree of cross-linking characteristic of the *S. aureus* PGN [26, 27].

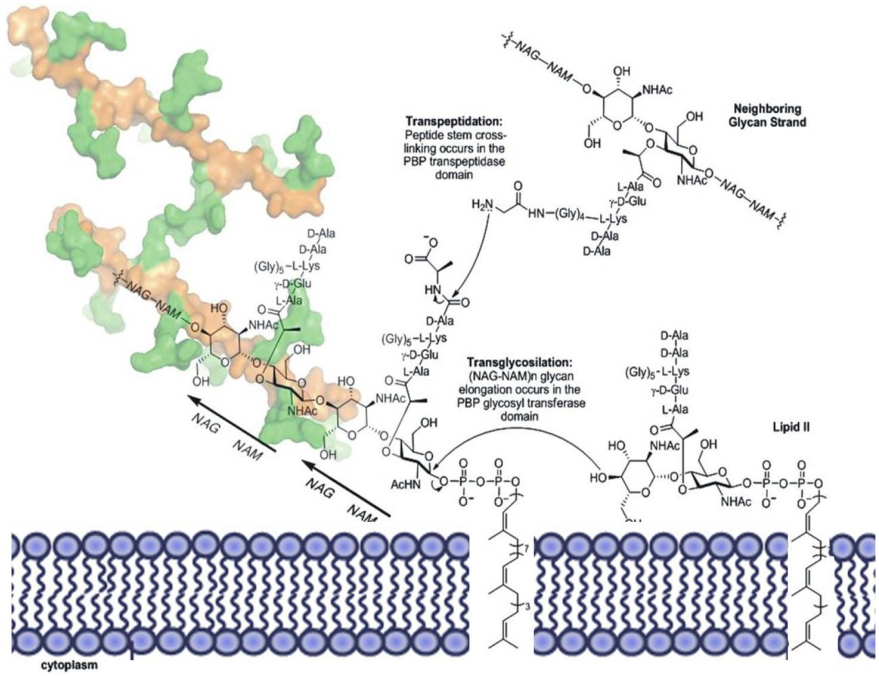


Figure 3. Schematic representation of PGN biosynthesis in *S. aureus*. PBPs that catalyse the transglycosylation and transpeptidation reactions of PGN synthesis assemble at the surface of the cytoplasmic membrane. NAG and NAM stand for *N*-acetylglucosamine and *N*-acetylmuramic acid (adapated from [28]).

Clinical isolates of *S. aureus* resistant to methicillin have acquired an additional PBP, PBP2A, with a transpeptidation domain that exhibits low affinity for penicillin and other β-lactams antibiotics. Therefore, this enzyme can function even in the presence of such inhibitors [29].

Recently, the FmtA protein, a protein that modulates methicillin resistance level and autolysis [30], has been identified as a PBP based on its ability to bind to β-lactams antibiotics and its homology with the conserved motifs of PBPs [31]. FmtA has been hypothesized to participate in PGN biosynthesis under cell wall stress in the presence of antibiotics [31].

Peptidoglycan metabolism

Apart from the extracellular synthesis of PGN by the PBPs, several enzymes are involved in the metabolism of the PGN. This may be important for proper cell wall architecture and integrity against host lytic enzymes. The MurNAc-GlcNAc disaccharide can be modified by adding an acetyl group to C6-OH of MurNAc. This reaction, catalyzed by the membrane protein OatA, has been postulated to involve the transference of the acetate group from the acetyl-coA molecules present in the cytoplasm [32]. The O-acetylation of PGN seems to be important for bacteria as it inhibits lysozyme muramidase activity through a putative steric hindrance mechanism [33].

The structure of PGN is also continuously modified by numerous hydrolytic enzymes, known as autolysins. These enzymes have been shown to cleave pre-existing linkages in the PGN [34] and act in coordination with the PGN biosynthetic enzymes during the

incorporation of the nascent PGN [35] or during the last steps of cell division, in the separation of daughter cells [14, 36]. The major autolysin in *S. aureus* is Atl, a bifunctional enzyme composed of an amidase domain (*N*-acetylmuramoyl-L-amidase, 60kDa) and a glucosaminidase domain (*N*-acetylglucosaminidase, 51 kDa) [37]. The two domains are interconnected by three repeat domains (R1, R2 and R3) responsible for directing the enzyme to the equatorial surface ring [38]. Both amidase and glucosaminidase activities play important roles in cell separation and mutants in *atl* gene are characterized by formation of cell clusters because of defects in cell separation [39, 40].

Due to their potential to compromise cell wall integrity, the expression and activities of autolysins must be tightly regulated. Several two-component regulatory systems are involved in the regulation of the autolysin activity, namely *S. aureus* LytRS [41] and ArlRS [42], which are negative regulators of autolysis.

Teichoic acids

In addition to peptidoglycan, the surface of Gram-positive bacteria contains phosphate rich glypolymers called teichoic acids -TAs (Figure 4). TAs were discovered in 1958 as a result of a search for the role of CDP-glycerol and CDP-ribitol, that had been identified as precursors produced by Gram-positive bacteria [43]. Interestingly, the discovery of TAs nucleotide-linked precursors, by Baddiley and colleagues in 1956, preceded the discovery of the polymers themselves [44, 45].

Conventionally, TAs are classified based on the way they are covalently linked to the surface - either to membrane lipids (lipoteichoic

acids - LTA) or to PGN (wall teichoic acids - WTA) [46]. Nevertheless, both WTA and LTA polymers can create a gradient of anionic charge that extends from the bacterial cell surface beyond the outer most layers of PGN (Figure 4) [15].

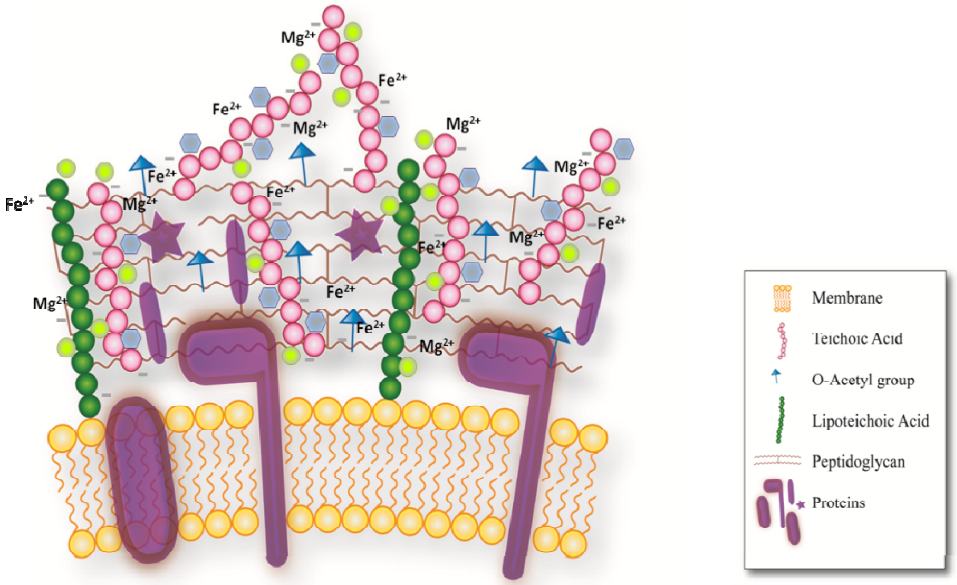


Figure 4 . Teichoic acids at the bacterial cell wall envelope. *S. aureus* and most other Gram-positive bacteria incorporate teichoic acids into their cell wall envelope. Teichoic acids can be covalently linked to PGN (WTA) or to the cytoplasmic membrane (LTA) and create an anionic charge gradient due to their composition.

Wall teichoic acids structure

The chemical structure of WTAs varies among Gram-positive bacteria. The most common structure is composed of β -(1,4)- linked N-acetylmannosamine (ManNAc) and N- acetylglucosamine (GlcNAc),

attached to the C6 hydroxyl of MurNAc residue of PGN via a phosphodiester bond, followed by two glycerol phosphate units which are linked to a chain of glycerol or ribitol phosphate repeating units [47]. The majority of *S. aureus* strains contain polyribitol phosphate (poly-RboP) units with GlcNAc and cationic D-alanine esters substituents at their hydroxyl group (Figure 5) [48]. According to Meredith and colleagues the poly-RboP chain length is variable and it can be regulated by quorum-sensing *agr* system [49].

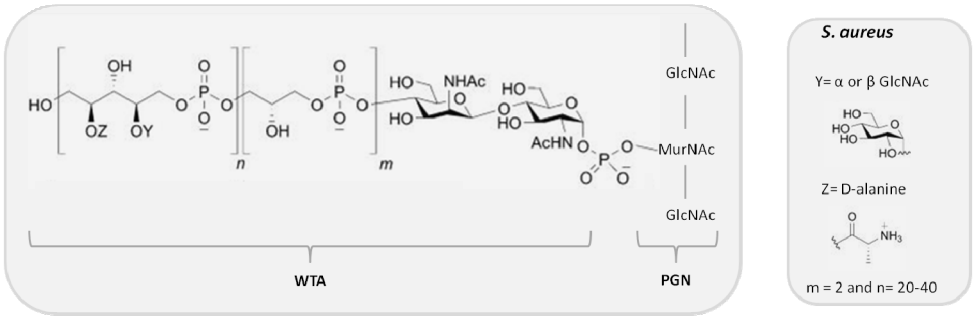


Figure 5. **Structure of WTA from *S. aureus*.** The polyribitol phosphate wall teichoic acid is a polymer composed of a linkage unit made of a disaccharide containing GlcNAc -1-phosphate and ManNAc connected to 2 or 3 units of glycerol-3-phosphate, and a main chain of variable size, made of 20-40 repeating ribitol-5-phosphate units. WTAs are attached to MurNAc residues of the PGN. (adapted from [50]).

Wall teichoic acids biosynthesis

Until recently, not much was known about the genes and proteins involved in the WTA biosynthetic pathway. It was known for sometime that WTAs of *S. aureus* and *Bacillus subtilis* W23 (*B. subtilis* W23), although

differing in structure, are covalently linked to C6 of a MurNAc of the PGN by a linkage unit composed of GlcNAc and residues of glycerol phosphate [51].

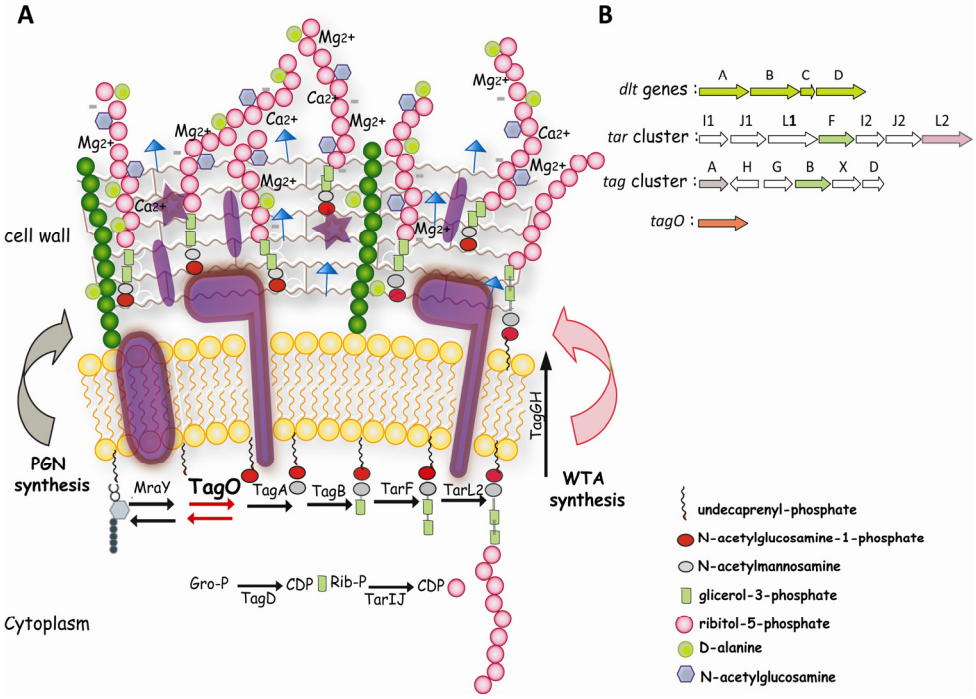


Figure 6. Genes and proteins involved in the *S. aureus* WTA biosynthetic pathway. A) Poly-RboP WTA biosynthesis pathway. Formation of the WTA linkage unit and main chain occurs in the cytoplasm; the polymer is subsequently translocated to the outer side of the membrane and integrated into the PGN. **B)** Genetic organization of the WTA biosynthetic genes. The *tag* and *tar* genes are involved in WTA biosynthesis, whereas the *dlt* genes are involved in the D-alanylation of the WTA.

The WTA biosynthesis pathway has started to be clarified after the discovery and identification of the genes involved – *tagABDEFGHO* - in the production of WTAs in *B. subtilis* 168 [52]. Later on, the pathway for *S.*

aureus WTA biosynthesis models (Figure 6) began to emerge due to large sequencing projects and comparative genomics.

The biosynthesis of WTAs is initiated in the cytoplasm with the transfer of GlcNAc phosphate from UDP-GlcNAc to the undecanoprenyl pyrophosphate lipid carrier, which is also used for the biosynthesis of PGN, anchored in the bacterial membrane. The enzyme involved in this reversible reaction, N- acetylglucosamine-1-phosphate transferase (TagO), belongs to the family of glycosyltransferases that also includes the enzyme MraY, required for PGN biosynthesis [53]. The first evidence of this reaction came from the observation from Yokoyama and colleagues, in 1989, that radiolabelled UDP-GlcNAc is incorporated as a substrate for subsequent WTA reactions, catalysed other enzymes [54]. TagA, an N-acetylmannosaminyl transferase, transfers the ManNAc residue from the UDP-ManNAc to the GlcNAc forming a β-linked disaccharide [54, 55] where two glycerol-3-phosphate units are linked, by TagB and TarF glycerolphosphate transferases [47]. The glycerol-3-phosphate and ribitol-5-phosphate are derived from CDP-glycerol and CDP-ribitol nucleotide-activated precursor molecules by TagD [56] and TarIJ [57] respectively. The synthesis of the WTA main chain in *S. aureus* requires a bi-functional poly-ribitol primase/polymerase (TarK or TarL). Meredith and colleagues proposed that TarK makes a secondary polymer (K-WTA) while TarL makes a primary WTA polymer (L-WTA) depending on the bacterial density and environmental changes [49].

After synthesis in the cytoplasm, the polymer is further glycosylated through addition of α-GlcNAc, by TarM [58] and β-GlcNAc, by not yet identified proteins, before being flipped to the external side of the

membrane by the ABC transporter complex composed of TarG and TarH [59]. The WTA is incorporated into the PGN by an unknown protein, presumably a member of LCP family proteins [60], and D-alanylated with the help of proteins encoded by the *dltABCD* operon. Although the mechanism of D-alanylation reaction is not completely understood, it is believed that the D-alanyl carrier protein ligase encoded by *dltA*, activates D-alanine in an ATP dependent manner and together with the membrane-associated DltD protein, encoded by *dltD*, facilitates the ligation of D-alanine to the carrier protein encoded by *dltC*, DCP. The DltB protein, encoded by *dltB* gene, is predicted to be a transmembrane protein and is thought to be involved in the passage of the D-alanyl-Dcp complex across the cytoplasmic membrane, where D-alanine is then transferred to the WTA backbone [15].

Functions of teichoic acids

Originally, WTAs were thought to be essential as deletions of the genes involved in the later steps of the synthetic pathway were found to be lethal. Later it was proposed that this was due to the accumulation of a toxic intermediates [61], as the inactivation of *tagO* gene results in viable but severely compromised cells devoid of WTAs [62, 63]. Therefore, despite being major constituents of bacterial cell walls, WTAs are not required for cell viability.

The complete three-dimensional structure of WTAs, their molecular interactions and functions are not fully understood**.** During the past few decades, several studies have contributed to unraveling the role of WTAs within a variety of processes, including: physicochemical properties of the

cell surface; cation homeostasis; resistance to antimicrobial peptides and lytic enzymes; phage receptors; cell division; biofilm formation and host adhesion.

Physicochemical properties of the cell wall. The presence of phosphate groups in the WTA polymer backbone creates a negative charge that results in the formation of repulsive electrostatic forces at the bacterial cell surface. To minimize such interactions, the polymer binds to mono- and divalent metal cations. An alteration in the cell surface charge balance can therefore affect the structure of the WTAs and consequently the rigidity, porosity and morphology of the cell wall envelope [64, 65].

Cation Homeostasis. WTA polymers have been implicated in maintaining cation homeostasis. Due to their particular affinity for bivalent cations, WTAs have been proposed to be a cation reservoir (e.g. magnesium - Figure 6A) that might be required for enzyme activity of proteins such as the ones involved in the synthesis of PGN and WTAs, electron transport and oxidative phosphorylation [66]. The binding of cations to WTAs may be modulated by WTA modifications such as D-alanylation, as the introduction of basic amino groups can reduced the net negative charge of the polymer [66].

Resistance to lytic enzymes and antimicrobial peptides. It has been shown that WTA modifications affect the interaction of the bacterial cell wall envelope with external molecules and to host cells. *S. aureus* strains lacking D-alanine esters are more susceptible to lytic enzymes (e.g. lysostaphin, lysozyme and group IIa secretory phospholipase A2), glycopeptides antibiotics (e.g. vancomycin) and antimicrobial cationic peptides [67-70].

Phage receptors. WTAs have been identified as important ligands for many bacteriophages. Changes in their structure are important criteria for phage specificity. Many staphylococcal bacteriophages use WTA polymers as receptors by lectin-mediated binding to the GlcNAc residues on WTAs [71, 72].

Cell division. WTAs are also implicated in targeting autolytic enzymes, involved in the cell wall metabolism, to the septum region [73]. Schlag and colleagues showed that the amidase hydrolase, obtained from the proteolytic processing of the major autolysin Atl, which seems to bind to the divisium septa, can not localize at the septa of a *S. aureus* mutant that is unable to produce WTAs [73]. Moreover, Campbell and co-workers [74] recently proposed that the WTAs, by interfering with the binding and localization of proteins involved in the cell wall assembly, may control the position of the ribs suggested to be key determinants for the placement of future division planes [75].

Biofilm formation and adhesion to host tissues. *S. aureus* mutants deficient in WTAs are impaired in biofilm formation and show reduced ability to adhere to artificial surfaces such as polystyrene and glass [63]. WTAs also affect the cell wall surface interaction with the host tissues. Weidenmaier and colleagues showed that *S. aureus* lacking WTAs are reduced in their ability to adhere to nasal epithelial cells of rats and were not able to colonize that host [62].

Bacterial recognition

Being unique to bacteria, PGN is not only an important target for antibacterial chemotherapy but also an important target for recognition by

the eukaryotic innate immune system. In eukaryotes, PGN recognition is thought to be mediated by pattern recognition receptors (PRRs) such as: Toll-like receptor proteins (TLR) [76, 77]; nucleotide binding oligomerization domain proteins (NOD) [77, 78]; Lysin-motif domain proteins (LysM) [79, 80]; and peptidoglycan recognition proteins (PGRPs) [81-83]. Following the recognition event, PRRs activate several complex signalling cascades, which ultimately regulate transcription of target genes that encode effectors and regulators of the immune response.

Peptidoglycan recognition proteins: structure and specificity

Peptidoglycan recognition proteins (PGRPs) were first discovered in 1996 by Yoshida and colleagues, who purified a 19 KDa protein (PGRP-S) from the haemolymph of a silkworm (*Bombyx mori*) that was able to bind to bacterial PGN and activate a prophenoloxidase cascade involved in the melanization, a host immune defense mechanism [81]. Since this initial discovery a whole family of PGRPs has emerged and found to be highly conserved in many organisms, from insects to mammals.

PGRPs are innate immune proteins that have at least one PGRP domain, a conserved PGN binding type 2 amidase domain of approximately 160 amino acids structurally similar to bacteriophage T7 lisozyme and bacterial type 2 amidase. The protein structure is composed of three peripheral α-helices and a central β-sheet containing several β-strands, reviewed in [84]. The crystal structures of PGRPs alone and in complex with muramyl pentapeptide or muramyl tetrapeptide PGN fragments have revealed the existence of a PGN binding groove, which is

the structural basis of the discrimination between different amino acids in the peptide chain or the discrimination between the PGN MurNAc and its anhydro form [85, 86].

Drosophila PGRPs

Drosophila melanogaster has been used as a key model to study PGN recognition by PRRs. In contrast with humans that have fewer PGRPs (PGRP-S, PGRP-L, PGRP-Iα and PGRP-Iβ) *Drosophila* has 19 transcribed proteins of this family [84]. The multiple PGRPs have been classified into short (PGRP-S) and long (PGRP-L) proteins.

PGRPs can be divided in two major groups defined by their ability to hydrolyze PGN (Figure 7). Catalytic PGRPs containing amidase activity, i.e. capable of cleaving the amide bond between MurNAc and the stem peptide, have a conserved Zn^{2+} binding site [87, 88]. These catalytic PGRPs have been shown to down regulate the immune response [89]. By contrast the non-catalytic PGRPs lack some of the conserved residues and have been proposed to be involved in the recognition of PGN by the innate immune system [90]. PGRP-SA, SD, LC and LE are non-catalytic PGRPs while PGRP-LB, SC1A, SC1B, SC2, SB1 and SB2 are catalytic PGRPs with amidase activity.

The PGRP-SC1A/B, LB and SB1 amidase activity is responsible for removing the peptides from PGN glycan strands, thus reducing or eliminating its immunological activity [87-89, 91, 92].

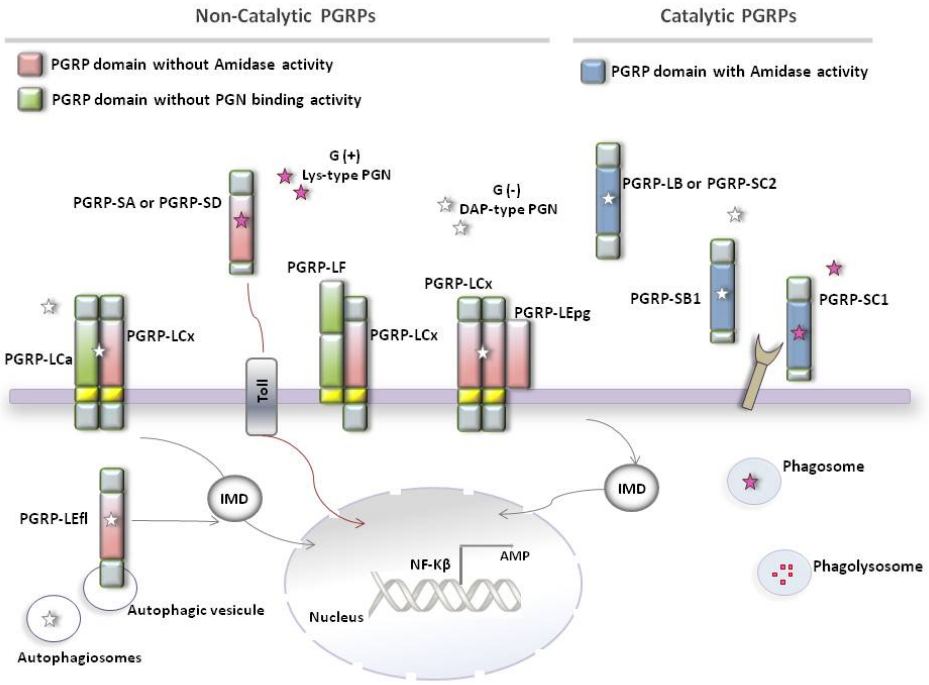


Figure 7. Schematic representation of PGRPs and their functions. *Drosophila* PGRPs can be divided in two major groups: non-catalytic and catalytic PGRPs.

PGRP-LC and PGRP-LE act as pattern-recognition receptors upstream the immune deficiency (IMD) pathway [93-96]. PGRP-LC is a transmembrane receptor with an extracellular PGRP domain involved in recognition of DAP-type PGN from Gram-negative bacteria or *Bacillus subtilis*. The cytoplasmic domain of PGRP-LC interacts with the IMD protein to activate a downstream signaling pathway [93]. Different PGRP-LC isoforms are implicated in the recognition of monomeric and polymeric DAP-type PGN [97, 98]. Beside its role in recognizing extracellular DAP-type PGN, PGRP-LE also has an essential role in detecting intracellular bacteria with DAP-type PGN to induce autophagy [99].

The two non-catalytic PGRPs (Figure 6), PGRP-SA and PGRP-SD are secreted proteins circulating in the hemolymph that have been implicated in the recognition of lysine type PGN from Gram-positive bacteria and in the activation of the Toll pathway [100-102].

Recognition of Gram-positive bacteria

Genetic studies have shown that the Lys-type PGN, or its components, from some Gram-positive bacteria is sensed through PGRP-SA with concomitant activation of Toll pathway [100, 103]. The crystal structure of PGRP-SA revealed a hydrophilic groove involved in binding to Lys-type muropeptide [90, 104]. Protein binding assays have shown that PGRP-SA binds strongly to Lys-type PGN and binds weakly to DAP-type PGN. Although PGRP-SA does not contain amidase activity, the protein has a carboxypeptidase activity specific for DAP-type PGN that has been suggested to explain why DAP-type can not activate the Toll pathway [104].

The precise function of PGRP-SD is not completely understood. While genetics studies associate PGRP-SD with Lys-type PGN recognition of some Gram-positive bacteria such as *S. aureus* and *Streptococcuss pyogenes* [102], the crystal structure suggests that it binds to DAP-type PGN [105].

Recognition of the Lys-type PGN also requires the Gram-negative binding protein (GNBP1). This protein was first identified in silkworms, as a protein that binds to Gram-negative PGN β-(1,3)-glucan [81]. In previous work, we showed that GNBP1 has muramidase-like activity and it is able to hydrolyze Lys-type PGN into muropeptide dimers, suggesting

that its action is required for optimal recognition by PGRP-SA [106]. Moreover, even though PGRP-SD does not bind strongly to Lys-type PGN we showed that GNBP1 together with PGRP-SA and PGRP-SD form one large complex upon PGN recognition required to sense some Gram-positive bacteria such as *S. aureus*, which then leads to activation of the Toll pathway [107].

Toll Pathway

Upon infection, *Drosophila* mounts a rapid antimicrobial response [108]. The *Drosophila* innate immune system is capable of recognizing different microorganisms and capable of responding accordingly. The *Drosophila* antimicrobial response to Gram-positive bacteria is mediated by the Toll pathway (Figure 8). PGN from these bacteria is recognized by the PRRs and causes activation of a serine protease cascade that ultimately cleave Spätzle protease (Spz) [109, 110]. Cleaved Spz serves as a ligand for the Toll receptor in the cytoplasmic membrane of the fat body cells. Ligand stimulation of the transmembrane receptor Toll leads to the activation of a highly conserved signalling pathway as Toll interacts with dMyD88, Tube and Pelle to form a complex that targets a NF-kB-equivalent complex, Dorsal, DIF/Cactus [111, 112].

Phosphorylation of the inhibitory protein Cactus releases Dif and/or Dorsal that are then free to enter in the nucleus and activate transcription of antimicrobial peptides genes such as *Drosomycin*. These proteins are homologous to the mammalian MyD88, IkB, IRAK and NF-kB that are involved in the Toll-like receptor, IL-1 and TNF-receptor signalling [113].

Thus, conserved pathways are employed in *Drosophila* and mammals to regulate innate immunity [114].

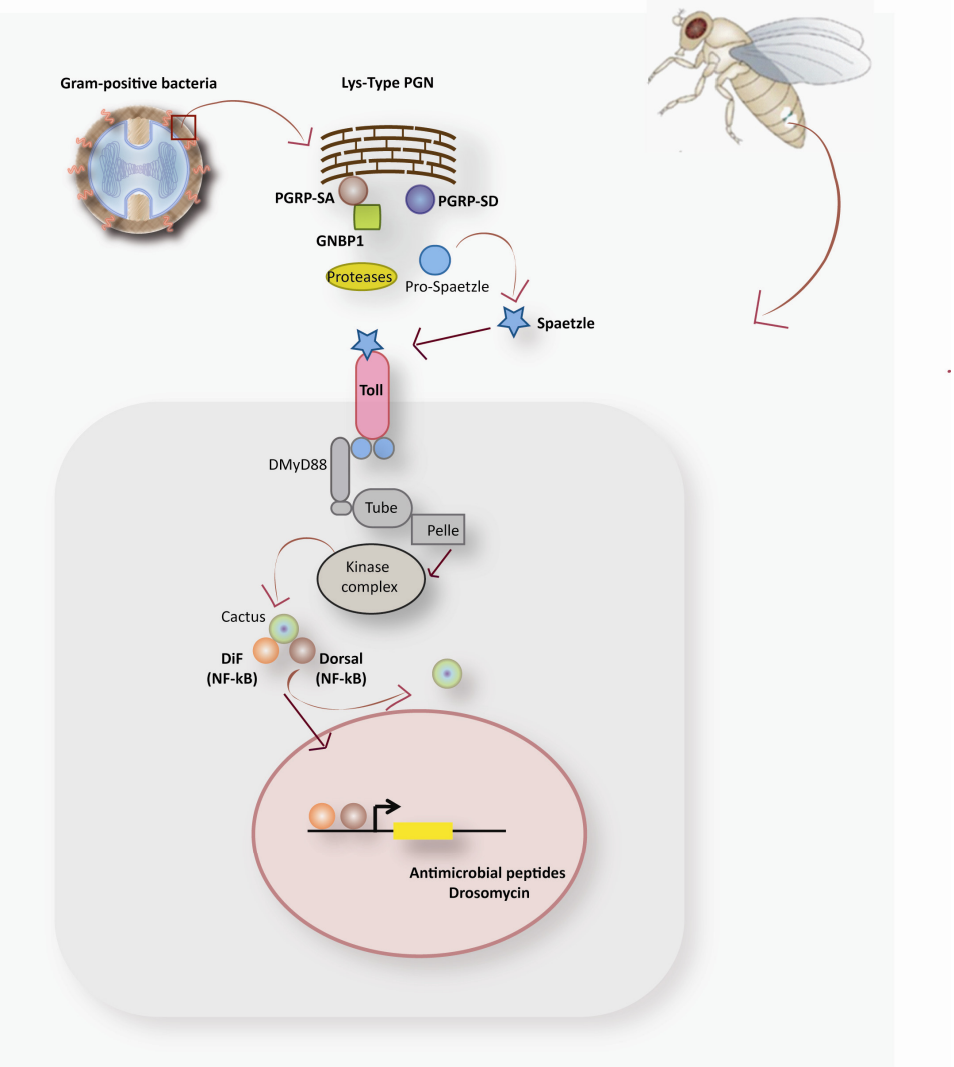


Figure 8. ***Drosophila melanogaster*** **Toll pathway activation**. Upstream of the Toll pathway, PGRP-SA, PGRP-SD and GNBP1 act as PRRs that recognize Lys-type PGN from Gram-positive bacteria. PGN recognition induces a proteolytic cascades that lead to Toll activation and ultimately to the production of antimicrobial peptides (AMPs).

Immune evasion by *S. aureus*

S. aureus is a leading agent of bacterial infections causing a large burden in terms of morbidity and mortality globally, in both hospital and community settings [115]. This pathogen is a frequent inhabitant of the human skin and nares which, after crossing the epithelial barriers and avoiding the host immune system, can cause a wide range of infections and diseases. Among these are osteomyelitis, septic arthritis, heart endocarditis and pneumonia [116].

Table 1. Bacterial resistance mechanisms to the host innate response

Host defense mechanism	Bacterial CW modification	Reference
AMPs	D-alanylation of teichoic acids	[67]
	Capsular polysacharide	[117]
Bacteriolytic enzymes	D-alanylation of WTAs	[118, 119]
	PGN *O*-acetylation	[120, 121]
	WTA	[122]
Respiratory burst	D-Alanylation of teichoic acids	[69]
Phagocytosis	LTA	[123]
	D-Alanylation of teichoic acids	[124]
	Capsular polysaccharides	[125]

The basis for the pathogenicity of *S. aureus* is complex and it is the subject of intensive research. To proliferate and establish an invasive infection *S. aureus* should have mechanisms that reduce host recognition and protect bacteria from elimination by the immune response. Although several studies have shown that *S. aureus* bacteria modify their cell wall surface to resist the host innate immune system (Table 1, reviewed in [126, 127]), the impact of such modifications on host recognition remains largely unknown.

Understanding how bacteria build and use the CW structure to evade the host immune system should bring novel insights into the molecular mechanisms of bacterial pathogenesis.

BIBLIOGRAPHY

1. Donoghue, P.C. and J.B. Antcliffe, *Early life: Origins of multicellularity.* Nature, 2010. **466**(7302): p. 41-2.

2. Porter, J.R., *Antony van Leeuwenhoek: tercentenary of his discovery of bacteria.* Bacteriol Rev, 1976. **40**(2): p. 260-9.

3. Salton, M.R., *The anatomy of the bacterial surface.* Bacteriol Rev, 1961. **25**: p. 77-99.

4. Mudd, S., K. Polevitzky, T.F. Anderson, and L.A. Chambers, *Bacterial Morphology as Shown by the Electron Microscope: II. The Bacterial Cell-wall in the Genus Bacillus.* J Bacteriol, 1941. **42**(2): p. 251-64.

5. Cooper, P.D., D. Rowley, and I.M. Dawson, *Location of radioactive penicillin in Staphylococcus aureus after contact with the drug.* Nature, 1949. **164**(4176): p. 842.

6. Salton, M.R. and R.W. Horne, *Studies of the bacterial cell wall. I. Electron microscopical observations on heated bacteria.* Biochim Biophys Acta, 1951. **7**(1): p. 19-42.

7. Salton, M.R. and R.W. Horne, *Studies of the bacterial cell wall. II. Methods of preparation and some properties of cell walls.* Biochim Biophys Acta, 1951. **7**(2): p. 177-97.

8. Salton, M.R., *Studies of the bacterial cell wall. IV. The composition of the cell walls of some Gram-positive and Gram-negative bacteria.* Biochim Biophys Acta, 1953. **10**(4): p. 512-23.

9. Cummins, C.S. and H. Harris, *The chemical composition of the cell wall in some gram-positive bacteria and its possible value as a taxonomic character.* J Gen Microbiol, 1956. **14**(3): p. 583-600.

10. Salton, M.R., *The relationship between the nature of the cell wall and the Gram stain.* J Gen Microbiol, 1963. **30**: p. 223-35.

11. Glauert, A.M. and M.J. Thornley, *The topography of the bacterial cell wall.* Annu Rev Microbiol, 1969. **23**: p. 159-98.

12. Navarre, W.W. and O. Schneewind, *Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope.* Microbiol Mol Biol Rev, 1999. **63**(1): p. 174-229.

13. Umeda, A., S. Yokoyama, T. Arizono, and K. Amako, *Location of peptidoglycan and teichoic acid on the cell wall surface of Staphylococcus aureus as determined by immunoelectron microscopy.* J Electron Microsc (Tokyo), 1992. **41**(1): p. 46-52.

14. Giesbrecht, P., T. Kersten, H. Maidhof, and J. Wecke, *Staphylococcal cell wall: morphogenesis and fatal variations in the presence of penicillin.* Microbiol Mol Biol Rev, 1998. **62**(4): p. 1371-414.

15. Neuhaus, F.C. and J. Baddiley, *A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria.* Microbiol Mol Biol Rev, 2003. **67**(4): p. 686-723.

16. Park, J.T. and M.J. Johnson, *Accumulation of labile phosphate in Staphylococcus aureus grown in the presence of penicillin.* J Biol Chem, 1949. **179**(2): p. 585-92.

17. Schleifer, K.H. and O. Kandler, *Peptidoglycan types of bacterial cell walls and their taxonomic implications.* Bacteriol Rev, 1972. **36**(4): p. 407-77.

18. Esko, J.D., T.L. Doering, and C.R.H. Raetz, *Eubacteria and Archaea.* 2009.

19. Tipper, D.J. and J.L. Strominger, *Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine.* Proc Natl Acad Sci U S A, 1965. **54**(4): p. 1133-41.

20. Yocum, R.R., J.R. Rasmussen, and J.L. Strominger, *The mechanism of action of penicillin. Penicillin acylates the active site of Bacillus stearothermophilus D-alanine carboxypeptidase.* J Biol Chem, 1980. **255**(9): p. 3977-86.

21. Ghuysen, J.M., *Serine beta-lactamases and penicillin-binding proteins.* Annu Rev Microbiol, 1991. **45**: p. 37-67.

22. Pinho, M.G., S.R. Filipe, H. de Lencastre, and A. Tomasz, *Complementation of the essential peptidoglycan transpeptidase function of penicillin-binding protein 2 (PBP2) by the drug resistance protein PBP2A in Staphylococcus aureus.* J Bacteriol, 2001. **183**(22): p. 6525-31.

23. Pereira, S.F., A.O. Henriques, M.G. Pinho, H. de Lencastre, and A. Tomasz, *Role of PBP1 in cell division of Staphylococcus aureus.* J Bacteriol, 2007. **189**(9): p. 3525-31.

24. Pereira, S.F., A.O. Henriques, M.G. Pinho, H. de Lencastre, and A. Tomasz, *Evidence for a dual role of PBP1 in the cell division and cell separation of Staphylococcus aureus.* Mol Microbiol, 2009. **72**(4): p. 895-904.

25. Pinho, M.G., H. de Lencastre, and A. Tomasz, *Cloning, characterization, and inactivation of the gene pbpC, encoding penicillin-binding protein 3 of Staphylococcus aureus.* J Bacteriol, 2000. **182**(4): p. 1074-9.

26. Memmi, G., S.R. Filipe, M.G. Pinho, Z. Fu, and A. Cheung, *Staphylococcus aureus PBP4 is essential for beta-lactam resistance in community-acquired methicillin-resistant strains.* Antimicrob Agents Chemother, 2008. **52**(11): p. 3955-66.

27. Leski, T.A. and A. Tomasz, *Role of penicillin-binding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of Staphylococcus aureus: evidence for the cooperative functioning of PBP2, PBP4, and PBP2A.* J Bacteriol, 2005. **187**(5): p. 1815-24.

28. Llarrull, L.I., J.F. Fisher, and S. Mobashery, *Molecular basis and phenotype of methicillin resistance in Staphylococcus aureus and insights into new beta-lactams that meet the challenge.* Antimicrob Agents Chemother, 2009. **53**(10): p. 4051-63.

29. Pinho, M.G., H. de Lencastre, and A. Tomasz, *An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci.* Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10886-91.

30. Komatsuzawa, H., M. Sugai, K. Ohta, T. Fujiwara, S. Nakashima, J. Suzuki, C.Y. Lee, and H. Suginaka, *Cloning and characterization of the fmt gene which affects the methicillin resistance level and autolysis in the presence of triton X-100 in methicillin-resistant Staphylococcus aureus.* Antimicrob Agents Chemother, 1997. **41**(11): p. 2355-61.

31. Fan, X., Y. Liu, D. Smith, L. Konermann, K.W. Siu, and D. Golemi-Kotra, *Diversity of penicillin-binding proteins. Resistance factor FmtA of Staphylococcus aureus.* J Biol Chem, 2007. **282**(48): p. 35143-52.

32. Weadge, J.T., J.M. Pfeffer, and A.J. Clarke, *Identification of a new family of enzymes with potential O-acetylpeptidoglycan esterase activity in both Gram-positive and Gram-negative bacteria.* BMC Microbiol, 2005. **5**: p. 49.

33. Bera, A., R. Biswas, S. Herbert, and F. Gotz, *The presence of peptidoglycan O-acetyltransferase in various staphylococcal species correlates with lysozyme resistance and pathogenicity.* Infect Immun, 2006. **74**(8): p. 4598-604.

34. Vollmer, W., B. Joris, P. Charlier, and S. Foster, *Bacterial peptidoglycan (murein) hydrolases.* FEMS Microbiol Rev, 2008. **32**(2): p. 259-86.

35. Antignac, A., K. Sieradzki, and A. Tomasz, *Perturbation of cell wall synthesis suppresses autolysis in Staphylococcus aureus: evidence for coregulation of cell wall synthetic and hydrolytic enzymes.* J Bacteriol, 2007. **189**(21): p. 7573-80.

36. Yamada, S., M. Sugai, H. Komatsuzawa, S. Nakashima, T. Oshida, A. Matsumoto, and H. Suginaka, *An autolysin ring associated with cell separation of Staphylococcus aureus.* J Bacteriol, 1996. **178**(6): p. 1565-71.

37. Oshida, T., M. Takano, M. Sugai, H. Suginaka, and T. Matsushita, *Expression analysis of the autolysin gene (atl) of Staphylococcus aureus.* Microbiol Immunol, 1998. **42**(9): p. 655-9.

38. Baba, T. and O. Schneewind, *Targeting of muralytic enzymes to the cell division site of Gram-positive bacteria: repeat domains direct autolysin to the equatorial surface ring of Staphylococcus aureus.* EMBO J, 1998. **17**(16): p. 4639-46.

39. Takahashi, J., H. Komatsuzawa, S. Yamada, T. Nishida, H. Labischinski, T. Fujiwara, M. Ohara, J. Yamagishi, and M. Sugai, *Molecular characterization of an atl null mutant of Staphylococcus aureus.* Microbiol Immunol, 2002. **46**(9): p. 601-12.

40. Biswas, R., L. Voggu, U.K. Simon, P. Hentschel, G. Thumm, and F. Gotz, *Activity of the major staphylococcal autolysin Atl.* FEMS Microbiol Lett, 2006. **259**(2): p. 260-8.

41. Brunskill, E.W. and K.W. Bayles, *Identification and molecular characterization of a putative regulatory locus that affects autolysis in Staphylococcus aureus.* J Bacteriol, 1996. **178**(3): p. 611-8.

42. Liang, X., L. Zheng, C. Landwehr, D. Lunsford, D. Holmes, and Y. Ji, *Global regulation of gene expression by ArlRS, a two-component signal transduction regulatory system of Staphylococcus aureus.* J Bacteriol, 2005. **187**(15): p. 5486-92.

43. Armstrong, J.J., J. Baddiley, J.G. Buchanan, and B. Carss, *Nucleotides and the bacterial cell wall.* Nature, 1958. **181**(4625): p. 1692-3.

44. Baddiley, J., J.G. Buchanan, B. Carss, and A.P. Mathias, *Cytidine diphosphate ribitol.* Biochim Biophys Acta, 1956. **21**(1): p. 191-2.

45. Baddiley, J., J.G. Buchanan, B. Carss, A.P. Mathias, and A.R. Sanderson, *The isolation of cytidine diphosphate glycerol, cytidine diphosphate ribitol and mannitol 1-phosphate from Lactobacillus arabinosus.* Biochem J, 1956. **64**(4): p. 599-603.

46. Knox, K.W. and A.J. Wicken, *Immunological properties of teichoic acids.* Bacteriol Rev, 1973. **37**(2): p. 215-57.

47. Brown, S., Y.H. Zhang, and S. Walker, *A revised pathway proposed for Staphylococcus aureus wall teichoic acid biosynthesis based on in vitro reconstitution of the intracellular steps.* Chem Biol, 2008. **15**(1): p. 12-21.

48. Endl, J., H.P. Seidl, F. Fiedler, and K.H. Schleifer, *Chemical composition and structure of cell wall teichoic acids of staphylococci.* Arch Microbiol, 1983. **135**(3): p. 215-23.

49. Meredith, T.C., J.G. Swoboda, and S. Walker, *Late-stage polyribitol phosphate wall teichoic acid biosynthesis in Staphylococcus aureus.* J Bacteriol, 2008. **190**(8): p. 3046-56.

50. Swoboda, J.G., J. Campbell, T.C. Meredith, and S. Walker, *Wall teichoic acid function, biosynthesis, and inhibition.* Chembiochem. **11**(1): p. 35-45.

51. Coley, J., E. Tarelli, A.R. Archibald, and J. Baddiley, *The linkage between teichoic acid and peptidoglycan in bacterial cell walls.* FEBS Lett, 1978. **88**(1): p. 1-9.

52. Mauel, C., M. Young, and D. Karamata, *Genes concerned with synthesis of poly(glycerol phosphate), the essential teichoic acid in Bacillus subtilis strain 168, are organized in two divergent transcription units.* J Gen Microbiol, 1991. **137**(4): p. 929-41.

53. Soldo, B., V. Lazarevic, and D. Karamata, *tagO is involved in the synthesis of all anionic cell-wall polymers in Bacillus subtilis 168.* Microbiology, 2002. **148**(Pt 7): p. 2079-87.

54. Yokoyama, K., H. Mizuguchi, Y. Araki, S. Kaya, and E. Ito, *Biosynthesis of linkage units for teichoic acids in gram-positive bacteria: distribution of related*

enzymes and their specificities for UDP-sugars and lipid-linked intermediates. J Bacteriol, 1989. **171**(2): p. 940-6.

55. Ginsberg, C., Y.H. Zhang, Y. Yuan, and S. Walker, *In vitro reconstitution of two essential steps in wall teichoic acid biosynthesis.* ACS Chem Biol, 2006. **1**(1): p. 25-8.

56. Park, Y.S., T.D. Sweitzer, J.E. Dixon, and C. Kent, *Expression, purification, and characterization of CTP:glycerol-3-phosphate cytidylyltransferase from Bacillus subtilis.* J Biol Chem, 1993. **268**(22): p. 16648-54.

57. Pereira, M.P. and E.D. Brown, *Bifunctional catalysis by CDP-ribitol synthase: convergent recruitment of reductase and cytidylyltransferase activities in Haemophilus influenzae and Staphylococcus aureus.* Biochemistry, 2004. **43**(37): p. 11802-12.

58. Xia, G., L. Maier, P. Sanchez-Carballo, M. Li, M. Otto, O. Holst, and A. Peschel, *Glycosylation of wall teichoic acid in Staphylococcus aureus by TarM.* J Biol Chem. **285**(18): p. 13405-15.

59. Schirner, K., L.K. Stone, and S. Walker, *ABC transporters required for export of wall teichoic acids do not discriminate between different main chain polymers.* ACS Chem Biol. **6**(5): p. 407-12.

60. Kawai, Y., J. Marles-Wright, R.M. Cleverley, R. Emmins, S. Ishikawa, M. Kuwano, N. Heinz, N.K. Bui, C.N. Hoyland, N. Ogasawara, R.J. Lewis, W. Vollmer, R.A. Daniel, and J. Errington, *A widespread family of bacterial cell wall assembly proteins.* EMBO J, 2011. **30**(24): p. 4931-41.

61. D'Elia, M.A., M.P. Pereira, Y.S. Chung, W. Zhao, A. Chau, T.J. Kenney, M.C. Sulavik, T.A. Black, and E.D. Brown, *Lesions in teichoic acid biosynthesis in Staphylococcus aureus lead to a lethal gain of function in the otherwise dispensable pathway.* J Bacteriol, 2006. **188**(12): p. 4183-9.

62. Weidenmaier, C., J.F. Kokai-Kun, S.A. Kristian, T. Chanturiya, H. Kalbacher, M. Gross, G. Nicholson, B. Neumeister, J.J. Mond, and A. Peschel, *Role of teichoic acids in Staphylococcus aureus nasal colonization, a major risk factor in nosocomial infections.* Nat Med, 2004. **10**(3): p. 243-5.

63. Vergara-Irigaray, M., T. Maira-Litran, N. Merino, G.B. Pier, J.R. Penades, and I. Lasa, *Wall teichoic acids are dispensable for anchoring the PNAG exopolysaccharide to the Staphylococcus aureus cell surface.* Microbiology, 2008. **154**(Pt 3): p. 865-77.

64. Ou, L.T. and R.E. Marquis, *Electromechanical interactions in cell walls of gram-positive cocci.* J Bacteriol, 1970. **101**(1): p. 92-101.

65. Doyle, R.J., M.L. McDannel, U.N. Streips, D.C. Birdsell, and F.E. Young, *Polyelectrolyte nature of bacterial teichoic acids.* J Bacteriol, 1974. **118**(2): p. 606-15.

66. Heptinstall, S., A.R. Archibald, and J. Baddiley, *Teichoic acids and membrane function in bacteria.* Nature, 1970. **225**(5232): p. 519-21.

67. Peschel, A., M. Otto, R.W. Jack, H. Kalbacher, G. Jung, and F. Gotz, *Inactivation of the dlt operon in Staphylococcus aureus confers sensitivity to defensins, protegrins, and other antimicrobial peptides.* J Biol Chem, 1999. **274**(13): p. 8405-10.

68. Peschel, A., C. Vuong, M. Otto, and F. Gotz, *The D-alanine residues of Staphylococcus aureus teichoic acids alter the susceptibility to vancomycin and the activity of autolytic enzymes.* Antimicrob Agents Chemother, 2000. **44**(10): p. 2845-7.

69. Collins, L.V., S.A. Kristian, C. Weidenmaier, M. Faigle, K.P. Van Kessel, J.A. Van Strijp, F. Gotz, B. Neumeister, and A. Peschel, *Staphylococcus aureus strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice.* J Infect Dis, 2002. **186**(2): p. 214-9.

70. Herbert, S., A. Bera, C. Nerz, D. Kraus, A. Peschel, C. Goerke, M. Meehl, A. Cheung, and F. Gotz, *Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci.* PLoS Pathog, 2007. **3**(7): p. e102.

71. Chatterjee, A.N., *Use of bacteriophage-resistant mutants to study the nature of the bacteriophage receptor site of Staphylococcus aureus.* J Bacteriol, 1969. **98**(2): p. 519-27.

72. Park, J.T., D.R. Shaw, A.N. Chatterjee, D. Mirelman, and T. Wu, *Mutants of staphylococci with altered cell walls.* Ann N Y Acad Sci, 1974. **236**(0): p. 54-62.

73. Schlag, M., R. Biswas, B. Krismer, T. Kohler, S. Zoll, W. Yu, H. Schwarz, A. Peschel, and F. Gotz, *Role of staphylococcal wall teichoic acid in targeting the major autolysin Atl.* Mol Microbiol. **75**(4): p. 864-73.

74. Campbell, J., A.K. Singh, J.P. Santa Maria, Jr., Y. Kim, S. Brown, J.G. Swoboda, E. Mylonakis, B.J. Wilkinson, and S. Walker, *Synthetic lethal compound combinations reveal a fundamental connection between wall teichoic acid and peptidoglycan biosyntheses in Staphylococcus aureus.* ACS Chem Biol. **6**(1): p. 106-16.

75. Turner, R.D., E.C. Ratcliffe, R. Wheeler, R. Golestanian, J.K. Hobbs, and S.J. Foster, *Peptidoglycan architecture can specify division planes in Staphylococcus aureus.* Nat Commun. **1**: p. 26.

76. Yoshimura, A., E. Lien, R.R. Ingalls, E. Tuomanen, R. Dziarski, and D. Golenbock, *Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2.* J Immunol, 1999. **163**(1): p. 1-5.

77. Muller-Anstett, M.A., P. Muller, T. Albrecht, M. Nega, J. Wagener, Q. Gao, S. Kaesler, M. Schaller, T. Biedermann, and F. Gotz, *Staphylococcal peptidoglycan co-localizes with Nod2 and TLR2 and activates innate immune response via both receptors in primary murine keratinocytes.* PLoS One. **5**(10): p. e13153.

78. Girardin, S.E., I.G. Boneca, J. Viala, M. Chamaillard, A. Labigne, G. Thomas, D.J. Philpott, and P.J. Sansonetti, *Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection.* J Biol Chem, 2003. **278**(11): p. 8869-72.

79. Zhang, X.C., X. Wu, S. Findley, J. Wan, M. Libault, H.T. Nguyen, S.B. Cannon, and G. Stacey, *Molecular evolution of lysin motif-type receptor-like kinases in plants.* Plant Physiol, 2007. **144**(2): p. 623-36.

80. Willmann, R., H.M. Lajunen, G. Erbs, M.A. Newman, D. Kolb, K. Tsuda, F. Katagiri, J. Fliegmann, J.J. Bono, J.V. Cullimore, A.K. Jehle, F. Gotz, A. Kulik, A. Molinaro, V. Lipka, A.A. Gust, and T. Nurnberger, *Arabidopsis lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection.* Proc Natl Acad Sci U S A. **108**(49): p. 19824-9.

81. Yoshida, H., K. Kinoshita, and M. Ashida, *Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, Bombyx mori.* J Biol Chem, 1996. **271**(23): p. 13854-60.

82. Kang, D., G. Liu, A. Lundstrom, E. Gelius, and H. Steiner, *A peptidoglycan recognition protein in innate immunity conserved from insects to humans.* Proc Natl Acad Sci U S A, 1998. **95**(17): p. 10078-82.

83. Dziarski, R. and D. Gupta, *The peptidoglycan recognition proteins (PGRPs).* Genome Biol, 2006. **7**(8): p. 232.

84. Royet, J., D. Gupta, and R. Dziarski, *Peptidoglycan recognition proteins: modulators of the microbiome and inflammation.* Nat Rev Immunol. **11**(12): p. 837-51.

85. Guan, R., A. Roychowdhury, B. Ember, S. Kumar, G.J. Boons, and R.A. Mariuzza, *Structural basis for peptidoglycan binding by peptidoglycan recognition proteins.* Proc Natl Acad Sci U S A, 2004. **101**(49): p. 17168-73.

86. Royet, J., D. Gupta, and R. Dziarski, *Peptidoglycan recognition proteins: modulators of the microbiome and inflammation.* Nat Rev Immunol, 2011. **11**(12): p. 837-51.

87. Kim, M.S., M. Byun, and B.H. Oh, *Crystal structure of peptidoglycan recognition protein LB from Drosophila melanogaster.* Nat Immunol, 2003. **4**(8): p. 787-93.

88. Mellroth, P., J. Karlsson, and H. Steiner, *A scavenger function for a Drosophila peptidoglycan recognition protein.* J Biol Chem, 2003. **278**(9): p. 7059-64.

89. Zaidman-Remy, A., M. Herve, M. Poidevin, S. Pili-Floury, M.S. Kim, D. Blanot, B.H. Oh, R. Ueda, D. Mengin-Lecreulx, and B. Lemaitre, *The Drosophila amidase PGRP-LB modulates the immune response to bacterial infection.* Immunity, 2006. **24**(4): p. 463-73.

90. Reiser, J.B., L. Teyton, and I.A. Wilson, *Crystal structure of the Drosophila peptidoglycan recognition protein (PGRP)-SA at 1.56 A resolution.* J Mol Biol, 2004. **340**(4): p. 909-17.

91. Mellroth, P. and H. Steiner, *PGRP-SB1: an N-acetylmuramoyl L-alanine amidase with antibacterial activity.* Biochem Biophys Res Commun, 2006. **350**(4): p. 994-9.

92. Zaidman-Remy, A., M. Poidevin, M. Herve, D.P. Welchman, J.C. Paredes, C. Fahlander, H. Steiner, D. Mengin-Lecreulx, and B. Lemaitre, *Drosophila immunity: analysis of PGRP-SB1 expression, enzymatic activity and function.* PLoS One, 2011. **6**(2): p. e17231.

93. Kaneko, T., T. Yano, K. Aggarwal, J.H. Lim, K. Ueda, Y. Oshima, C. Peach, D. Erturk-Hasdemir, W.E. Goldman, B.H. Oh, S. Kurata, and N. Silverman, *PGRP-LC and PGRP-LE have essential yet distinct functions in the*

drosophila immune response to monomeric DAP-type peptidoglycan. Nat Immunol, 2006. **7**(7): p. 715-23.

94. Takehana, A., T. Katsuyama, T. Yano, Y. Oshima, H. Takada, T. Aigaki, and S. Kurata, *Overexpression of a pattern-recognition receptor, peptidoglycan-recognition protein-LE, activates imd/relish-mediated antibacterial defense and the prophenoloxidase cascade in Drosophila larvae.* Proc Natl Acad Sci U S A, 2002. **99**(21): p. 13705-10.

95. Gottar, M., V. Gobert, T. Michel, M. Belvin, G. Duyk, J.A. Hoffmann, D. Ferrandon, and J. Royet, *The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein.* Nature, 2002. **416**(6881): p. 640-4.

96. Choe, K.M., T. Werner, S. Stoven, D. Hultmark, and K.V. Anderson, *Requirement for a peptidoglycan recognition protein (PGRP) in Relish activation and antibacterial immune responses in Drosophila.* Science, 2002. **296**(5566): p. 359-62.

97. Kaneko, T., W.E. Goldman, P. Mellroth, H. Steiner, K. Fukase, S. Kusumoto, W. Harley, A. Fox, D. Golenbock, and N. Silverman, *Monomeric and polymeric gram-negative peptidoglycan but not purified LPS stimulate the Drosophila IMD pathway.* Immunity, 2004. **20**(5): p. 637-49.

98. Mellroth, P., J. Karlsson, J. Hakansson, N. Schultz, W.E. Goldman, and H. Steiner, *Ligand-induced dimerization of Drosophila peptidoglycan recognition proteins in vitro.* Proc Natl Acad Sci U S A, 2005. **102**(18): p. 6455-60.

99. Yano, T., S. Mita, H. Ohmori, Y. Oshima, Y. Fujimoto, R. Ueda, H. Takada, W.E. Goldman, K. Fukase, N. Silverman, T. Yoshimori, and S. Kurata, *Autophagic control of listeria through intracellular innate immune recognition in drosophila.* Nat Immunol, 2008. **9**(8): p. 908-16.

100. Michel, T., J.M. Reichhart, J.A. Hoffmann, and J. Royet, *Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein.* Nature, 2001. **414**(6865): p. 756-9.

101. Gobert, V., M. Gottar, A.A. Matskevich, S. Rutschmann, J. Royet, M. Belvin, J.A. Hoffmann, and D. Ferrandon, *Dual activation of the Drosophila toll pathway by two pattern recognition receptors.* Science, 2003. **302**(5653): p. 2126-30.

102. Bischoff, V., C. Vignal, I.G. Boneca, T. Michel, J.A. Hoffmann, and J. Royet, *Function of the drosophila pattern-recognition receptor PGRP-SD in the detection of Gram-positive bacteria.* Nat Immunol, 2004. **5**(11): p. 1175-80.

103. Filipe, S.R., A. Tomasz, and P. Ligoxygakis, *Requirements of peptidoglycan structure that allow detection by the Drosophila Toll pathway.* EMBO Rep, 2005. **6**(4): p. 327-33.

104. Chang, C.I., S. Pili-Floury, M. Herve, C. Parquet, Y. Chelliah, B. Lemaitre, D. Mengin-Lecreulx, and J. Deisenhofer, *A Drosophila pattern recognition receptor contains a peptidoglycan docking groove and unusual L,D-carboxypeptidase activity.* PLoS Biol, 2004. **2**(9): p. E277.

105. Leone, P., V. Bischoff, C. Kellenberger, C. Hetru, J. Royet, and A. Roussel, *Crystal structure of Drosophila PGRP-SD suggests binding to DAP-type but not lysine-type peptidoglycan.* Mol Immunol, 2008. **45**(9): p. 2521-30.

106. Wang, L., A.N. Weber, M.L. Atilano, S.R. Filipe, N.J. Gay, and P. Ligoxygakis, *Sensing of Gram-positive bacteria in Drosophila: GNBP1 is needed to process and present peptidoglycan to PGRP-SA.* EMBO J, 2006. **25**(20): p. 5005-14.

107. Wang, L., R.J. Gilbert, M.L. Atilano, S.R. Filipe, N.J. Gay, and P. Ligoxygakis, *Peptidoglycan recognition protein-SD provides versatility of receptor formation in Drosophila immunity.* Proc Natl Acad Sci U S A, 2008. **105**(33): p. 11881-6.

108. Lemaitre, B., J.M. Reichhart, and J.A. Hoffmann, *Drosophila host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms.* Proc Natl Acad Sci U S A, 1997. **94**(26): p. 14614-9.

109. Lemaitre, B., E. Nicolas, L. Michaut, J.M. Reichhart, and J.A. Hoffmann, *The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults.* Cell, 1996. **86**(6): p. 973-83.

110. Ligoxygakis, P., N. Pelte, J.A. Hoffmann, and J.M. Reichhart, *Activation of Drosophila Toll during fungal infection by a blood serine protease.* Science, 2002. **297**(5578): p. 114-6.

111. Yang, J. and R. Steward, *A multimeric complex and the nuclear targeting of the Drosophila Rel protein Dorsal.* Proc Natl Acad Sci U S A, 1997. **94**(26): p. 14524-9.

112. Minakhina, S. and R. Steward, *Nuclear factor-kappa B pathways in Drosophila.* Oncogene, 2006. **25**(51): p. 6749-57.

113. Hargreaves, D.C. and R. Medzhitov, *Innate sensors of microbial infection.* J Clin Immunol, 2005. **25**(6): p. 503-10.

114. Hoffmann, J.A., *The immune response of Drosophila.* Nature, 2003. **426**(6962): p. 33-8.

115. Elston, D.M., *Status update: hospital-acquired and community-acquired methicillin-resistant Staphylococcus aureus.* Cutis, 2007. **79**(6 Suppl): p. 37-42.

116. Gordon, R.J. and F.D. Lowy, *Pathogenesis of methicillin-resistant Staphylococcus aureus infection.* Clin Infect Dis, 2008. **46 Suppl 5**: p. S350-9.

117. Campos, M.A., M.A. Vargas, V. Regueiro, C.M. Llompart, S. Alberti, and J.A. Bengoechea, *Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides.* Infect Immun, 2004. **72**(12): p. 7107-14.

118. Koprivnjak, T., A. Peschel, M.H. Gelb, N.S. Liang, and J.P. Weiss, *Role of charge properties of bacterial envelope in bactericidal action of human group IIA phospholipase A2 against Staphylococcus aureus.* J Biol Chem, 2002. **277**(49): p. 47636-44.

119. Hunt, C.L., W.M. Nauseef, and J.P. Weiss, *Effect of D-alanylation of (lipo)teichoic acids of Staphylococcus aureus on host secretory phospholipase A2 action before and after phagocytosis by human neutrophils.* J Immunol, 2006. **176**(8): p. 4987-94.

120. Davis, K.M., H.T. Akinbi, A.J. Standish, and J.N. Weiser, *Resistance to mucosal lysozyme compensates for the fitness deficit of peptidoglycan modifications by Streptococcus pneumoniae.* PLoS Pathog, 2008. **4**(12): p. e1000241.

121. Bera, A., S. Herbert, A. Jakob, W. Vollmer, and F. Gotz, *Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of Staphylococcus aureus.* Mol Microbiol, 2005. **55**(3): p. 778-87.

122. Bera, A., R. Biswas, S. Herbert, E. Kulauzovic, C. Weidenmaier, A. Peschel, and F. Gotz, *Influence of wall teichoic acid on lysozyme resistance in Staphylococcus aureus.* J Bacteriol, 2007. **189**(1): p. 280-3.

123. Hashimoto, Y., Y. Tabuchi, K. Sakurai, M. Kutsuna, K. Kurokawa, T. Awasaki, K. Sekimizu, Y. Nakanishi, and A. Shiratsuchi, *Identification of lipoteichoic acid as a ligand for draper in the phagocytosis of Staphylococcus aureus by Drosophila hemocytes.* J Immunol, 2009. **183**(11): p. 7451-60.

124. Shiratsuchi, A., K. Shimizu, I. Watanabe, Y. Hashimoto, K. Kurokawa, I.M. Razanajatovo, K.H. Park, H.K. Park, B.L. Lee, K. Sekimizu, and Y. Nakanishi, *Auxiliary role for D-alanylated wall teichoic acid in Toll-like receptor 2-mediated survival of Staphylococcus aureus in macrophages.* Immunology, 2010. **129**(2): p. 268-77.

125. O'Riordan, K. and J.C. Lee, *Staphylococcus aureus capsular polysaccharides.* Clin Microbiol Rev, 2004. **17**(1): p. 218-34.

126. Foster, T.J., *Immune evasion by staphylococci.* Nat Rev Microbiol, 2005. **3**(12): p. 948-58.

127. Kraus, D. and A. Peschel, *Staphylococcus aureus evasion of innate antimicrobial defense.* Future Microbiol, 2008. **3**(4): p. 437-51.

CHAPTER II

Modifications of the *Staphylococcus aureus* cell wall envelope

This chapter contains unpublished data, part of a manuscript in preparation.

The author of this dissertation performed all experiments described in this chapter.

ABSTRACT

The bacterial cell wall envelope is a robust and dynamic structure that protects bacterial cells from different extracellular environments. In Gram-positive bacteria, it is mainly composed of peptidoglycan (PGN) and associated macromolecules. The PGN produced by the pathogenic *Staphylococcus aureus* bacteria is highly modified through the addition of glycopolymers such as wall teichoic acids or capsular polysaccharides, *O*-acetyl groups connected to the N-acetylmuramic acid (MurNAc), and secondary cross-linking. PGN is a major target for lytic enzymes, produced by the host or bacteria, and recognition by the innate immune system. It is therefore expected that alterations in cell wall macromolecule structure might not only interfere with the viability of bacteria but also with recognition by host immune receptors.

In order to study the impact of structural modifications of *S. aureus* PGN on accessibility of this structure for recognition, we deleted genes directly or indirectly involved in PGN metabolism (*oatA*, *tagO*, *pbpD*, *fmtA*, *atl*, *arlR* and *dltA*). The resulting null mutant strains were then studied regarding the level of PGN accessibility to different cell wall fluorescents probes.

We found that *S. aureus* mutants in which *pbpD*, *fmtA*, *atl* or *dltA* had been deleted produce a cell wall surface that was better recognized by the fluorescent derivative of vancomycin (Van-FL). The *S. aureus tagO* null mutant lacked WTA and had an increased PGN exposure not only to Van-FL but also to a fluorescent derivative of wheat germ agglutinin (WGA-FL). This observation indicates that wall teichoic acids may play an

important role in the bacteria cell wall structure and might represent a bacterial strategy to reduce PGN recognition.

INTRODUCTION

In order to survive, bacteria have evolved a complex and robust cell envelope that, in Gram-positive bacteria, is mainly composed of PGN. PGN is a macromolecule of carbohydrates chains - *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) - cross-linked by flexible peptide bridges that provides a physical protection from the external environment [1, 2].

The cell wall of bacteria is an extremely dynamic structure that is continuously being synthesized and modified to enable cells to enlarge and divide. It has to be subjected to a controlled hydrolysis to allow the insertion of new PGN, and presumably addition of molecules such as proteins and glycopolymers. The PGN synthesis pathway can be divided into three major steps: (I) synthesis of UDP – MurNAc – pentapeptide in the cytoplasm, (II) attachment of this precursor, and its modification at the cytoplasmic membrane, followed by its translocation to the outer surface of the cell and (III) assembly of PGN, which is then subjected to additional maturation and modification enzymatic reactions [1].

Different proteins in *S. aureus*, a major pathogen responsible for community and hospital acquired infections, have been shown to participate in the metabolism and modification of PGN. Among these are proteins such as OatA, TagO, DltA, PBP4, FmtA, ArlR and Atl (Table 1).

The OatA protein, the major determinant for lysozyme resistance in *S. aureus*, is an O-acetyltransferase responsible for O-acetylation of the MurNAc residue [3].

The TagO protein is an N-acetylglucosamine-1-phosphate transferase, which catalyzes the first committed step of synthesis of the wall teichoic acid (WTA) polymer, attached to the MurNAc residue of mature PGN. WTAs have different functions in bacteria. Recently, it was found that WTAs are essential for the correct localization of different proteins involved in cell wall metabolism such as the major autolysin Atl [4], a lytic enzyme involved in the separation of daughter cells, and are important virulence factors required for host tissue adhesion [5].

The DltA protein is one of the proteins involved in the D-alanylation of the polyribitol-phosphate backbone of WTAs in *S. aureus*. This WTA modification results in the reduction of the negative charges of WTA polymers and in bacteria resistance to lysozyme and antimicrobial peptides [6].

S. aureus has a high degree of PGN cross-linking [7] – increased number of connections between the stem peptides from different glycan strands - which is mainly due to the action of the penicillin binding protein PBP4 [8]. Moreover, FmtA has been described as a penicillin binding protein [9] that can also mediate the production of highly crosslinked PGN [10]. FmtA, that together with PBP4 has been shown to play important roles in β-lactams antibiotic resistance [11, 12].

The metabolism of PGN in *S. aureus* is tightly controlled by numerous regulators, including several two-component signal transduction systems. ArlR, a regulatory protein of the two-component

system ArlRS, has been shown to be important for pathogenesis and cellular physiology of bacteria. The ArlRS system is involved in the regulation of autolysis and adhesion, multidrug resistance and virulence [13, 14].

In this study, the genes encoding for the proteins described above were deleted from the *S. aureus* chromosome and characterized the regarding the accessibility of the resulting PGN to the cell wall markers, fluorescent labeled vancomycin (Van-FL) and wheat germ agglutinin (WGA-FL). Our results revealed that binding of both markers to PGN is largely reduced by the presence of WTAs. Inhibition of WTA synthesis, due to the deletion of *tagO* gene, resulted in viable bacteria with an enhanced Van-FL and WGA-FL binding to their surface. This suggests that attachment of WTA glycopolymers to the PGN may be advantageous to *S. aureus* bacteria as it might conceal its PGN from recognition of external factors, such as receptors in the infected host.

MATERIALS AND METHODS

Strains and growth conditions The strains and plasmids used in this study are listed in Table 1. Primers sequences are listed in table 2. *Escherichia coli* (*E. coli*) strains were grown in Luria-Bertani (LB) or Luria-Bertani agar (LA) medium; when needed antibiotics were added to the medium at the following concentrations: ampicillin (Amp, 100 μg/ml), kanamycin (Kan, 50 μg/ml) and erythromycin (Ery, 100 μg/ml). All *S. aureus* strains were grown at 30 ºC in tryptic soy broth (TSB; Difco) or in tryptic soy agar (TSA; Difco). Medium was supplemented when required

with Ery (10μg/ml) and/or 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal, 100 μg/ml).

Construction of *S. aureus* strains Genes *oatA*, *fmtA*, *tagO*, *pbpD*, *atl*, *arlR*, and *dltA* were deleted from *S. aureus* chromosome. The *oatA* and *fmtA* null mutants were constructed in the background of strain NCTC8325-4 using the integrative vector pORI280. To delete the *oatA* gene from the genome of *S. aureus* NCTC8325-4 we amplified two 1.3 Kb DNA fragments from NCTC8325-4 chromosomal DNA, corresponding to the upstream (primers P1_oatA and P2_oatA) and downstream (primers P3_oatA and P4_oatA) regions of the *oatA* gene. The upstream fragment was digested with *BamH*I and *Nco*I and cloned into pORI280 [15]. The downstream PCR fragment was digested with *Nco*I and *Bgl*II and then cloned into the previous plasmid, which already contained the upstream fragment, producing the plasmid pΔ*oatA*.

To delete *fmtA* we amplified two DNA fragments of approximately 0.9 Kb from *S. aureus* NCTC8325-4 chromosomal DNA, corresponding to the upstream (primers P7_fmtA and P8_fmtA) and downstream region (primers P5_fmtA and P6_fmtA) regions of the *fmtA* gene. These fragments were joint by overlap PCR (Figure S1A) using primers P5 and P8_fmtA and the resulting PCR product was digested with *BamH*I and *Bgl*II then cloned in the pORI280, digested with *BamH*I, producing the plasmid pΔ*fmtA*. Both plasmids pΔ*oatA* and pΔ*fmtA* were sequenced and electroporated into the transformable strain RN4220 at 37 ºC (using Ery selection, 10μg/ml) as previously described [16]. The plasmids were then transduced to NCTC8325-4 using the phage 80*α* [17]. The resulting strains, carrying the plasmids integrated into the chromosome, were used to

inoculate liquid media without antibiotic and incubated at 37°C for several generations. The absence of selecting antibiotic allowed for the appearance of bacteria that had lost the plasmid and consequently, the *lacZ* and *erm* genes (Figure S1B).

Different dilutions of the cultures were plated on TSA containing X-Gal without Ery and incubated at 37ºC overnight. Several white colonies were isolated and their Ery sensitivity confirmed. The *oatA* and *fmtA* null mutants (NCTCΔ*oatA* and NCTCΔ*fmtA*) were obtained, and gene deletions were verified by PCR and sequence analysis.

All other *S. aureus* null mutants described here were constructed using the thermosensitive vector pMAD [18]. In order to delete the *tagO* gene, PCR products containing the upstream and downstream regions of the gene were amplified from NCTC8325-4 chromosomal DNA, using the primer pairs P9_tagO/P10_tagO and P11_tagO/P12_tagO DNA. The two fragments were joined in a second overlap PCR reaction, using primers P9_tagO/P12_tagO. The resulting PCR product was digested with *Bgl*II and *Nco*I and cloned into the pMAD vector, giving rise to the plasmid pΔ*tagO*.

To delete the *pbpD* gene from the chromosome of *S. aureus* NCTC8325-4 we amplified by PCR two DNA fragments of approximately 0.7 Kb, corresponding to the upstream (primers P15_pbpD/P16_pbpD) and downstream region (primers P13_pbpD/ P14_pbpD) regions of the *pbpD* gene. The two fragments were joined by overlap PCR using primers P13_pbpD and P16_pbpD. The resulting PCR product was digested with *Bgl*II and *Nco*I and cloned into pMAD vector, originating the plasmid pΔ*pbpD*.

To generate the *arlR* null mutant, 0.8 Kb PCR fragments of the upstream and downstream regions of the *arlR* gene were amplified from chromosomal DNA of NCTC8325-4, using primer pairs P19_arlR/P20_arlR and P17_arlR/P18_arlR. These two PCR products were joined in a second overlap PCR reaction, using the primers P17_arlR/P20_arlR. The resulting product was digested with *Nco*I and *Bgl*II and cloned into pMAD, giving rise to the p∆*arlR* plasmid.

The p∆*atl* plasmid was constructed by amplifying 1Kb from the upstream and downstream regions of the *atl* gene, using the primer pairs P23_atl/P24_atl and P21_atl/P22_atl. The fragments were joined by PCR using the primers P23_atl and P21_ atl and the PCR product obtained was digested with *Bgl*II and *Eco*RI then cloned into the pMAD vector.

The p∆*tagO*, p∆*pbpD*, p∆*arlR* and p∆*atl* plasmids were sequenced and electroporated into *S. aureus* RN4220 strain at 30 ºC (using Ery selection, 10 μg/ml) and then transduced to NCTC8325-4 using phage 80*α*. The deletions of the genes (*tagO*, *pbpD*, *arlR* and *atl*) from the NCTC8325-4 chromosome were completed after a two-step homologous recombination process outlined in Figure S1A. In the first step, the recombinant strains were obtained by integration of the plasmids (p∆*tagO*, *p*∆*pbpD*, p∆*arlR* or p∆*atl*) into the chromosome by incubating strains at 43 ºC, a non-permissive temperature for pMAD replication. In the second step, the recombinant strains that had lost the integrative vectors (and therefore the *lacZ* and *erm* genes) were selected at the permissive temperature of 30 ºC and without antibiotic selection (Figure S1B). The NCTC∆*pbpD*, NCTC∆*arlR* and NCTC*∆atl* null mutants were identified by the appearance of white colonies and sensitivity to Ery at 43ºC, with the

exception of the NCTCΔ*tagO* strain where this last step was performed at 30 ºC due to the thermosensitive nature of cells lacking the *tagO* gene. All gene deletions were confirmed by PCR.

To delete the *dltA* gene from the chromosome of *S. aureus* RN4220 two 0.55 Kb DNA fragments were amplified by PCR from the genome of *S. aureus* NCTC 8325-4, corresponding to the upstream (primers P25_dltA/P26_dltA) and downstream (primers P27_dltA/ P28_dltA) regions of the *dltA* gene. The two fragments were joined by overlap PCR (Figure S1A) using primers P25_dltA and P28_dltA and the resulting PCR product was digested with *Bgl*II and *Eco*RI and cloned into the pMAD vector, producing the plasmid pΔ*dltA*. This plasmid was sequenced and electroporated into *S. aureus* RN4220. Insertion and excision of pΔ*dltA* into the chromosome of RN4220 was performed as previously described for the *tagO* gene. Deletion of *dltA* was confirmed by PCR, and the resulting strain was named RNΔ*dltA*

Wall Teichoic acid inhibition and extraction. Tunicamycin minimum inhibitory concentration (MIC) of *S. aureus* NCTC8325-4 (MIC of 12 μg/ml) was determined as previously described [19]. Overnight cultures of NCTC8325-4 were grown in varying sub-inhibitory concentrations of tunicamycin (0.04, 0.08 and 0.16 μg/ml). The culture grown at the highest concentration of tunicamycin was also diluted in fresh TSB medium without antibiotic and grown for 16 hours to revert the antibiotic effect. WTAs were extracted by alkaline hydrolysis from overnight cultures grown in the presence or absence of tunicamycin, analyzed by native polyacrylamide gel electrophoresis and visualized by combined alcian blue silver staining, as previously described [20]. The

overnight cultures were also diluted to an optical density of 0.05 in fresh TSB containing the same tunicamycin concentrations (0.04, 0.08 and 0.16 μg/ml) and were grown until mid-exponential phase (optical density 0.6-0.8). These cells were then harvested, labeled with WGA and examined by fluorescence microscopy as described below.

Bacterial cell wall binding assay. Bacterial cells were grown overnight at 30 ºC, diluted 1/1000 in fresh medium and then incubated until mid-exponential phase (O.D 0.8). A volume of 1 ml was harvested, washed in PBS and incubated for 5 minutes, with different fluorescent probes: cell wall GlcNAc dye wheat germ agglutinin Alexa Fluor 488 (WGA; 1 μg/ml; Invitrogen), the DNA dye Hoechst (1μg/ml; Molecular Probes) and the cell wall D-Alanine-D-Alanine (D-Ala-D-Ala) dye BODIPY FL conjugate of vancomycin (Van-Fl; 1μg/ml; Molecular Probes). Cells were washed twice and resuspended in 50 μl of PBS. A drop of this culture was observed on a thin film of 1% agarose in PBS under a Zeiss Axio observer.Z1 microscope. Image acquisition was performed using a Photometrics Cool SNAP HQ2 camera (Roper Scientific) and Metamorph 7.5 software (Molecular Devices). Image analysis was made using Image J software. Fluorescence signal was determined by quantifying the mean fluorescence of at least 50 cells. Background fluorescence was subtracted from the mean fluorescence signal.

Table1. Strains and plasmids

Strains	Relevant characteristics	Origin
S. aureus		
NCTC8325-4	*S. aureus* reference strain	R. Novick
NCTCΔ*oatA*	NCTC8325-4 *oatA* null mutant	This study
NCTCΔ*tagO*	NCTC8325-4 *tagO* null mutant	[21]
NCTCΔ*pbpD*	NCTC8325-4 *pbpD* null mutant	[21]
NCTCΔ*fmtA*	NCTC8325-4 *fmtA* null mutant	This study
NCTCΔ*atl*	NCTC8325-4 *atl* null mutant	This study
NCTCΔ*arlR*	NCTC8325-4 *arlR* null mutant	This study
RN4220	Restriction deficient derivative of *S. aureus* NCTC8325-4	R. Novick
RNΔ*dltA*	RN4220 *dltA* null mutant	[22]
E. coli		
EC101	*E. coli* cloning strain with *repA* from pWVO1 integrated in the chromosome	Lab stock
DH5α	*E. coli* Cloning strain	Lab stock
Plasmids		
pMAD	*E. coli* – *S. aureus* shuttle vector with a thermosensitive origin of replication for Gram-positive bacteria; Amp^r; Ery^r; $LacZ^+$	[18]
pORI280	Integrative vector which replicates in strains containing RepA; Ery^r; $LacZ^+$	[15]
pΔ*fmtA*	pOri with *arlR* up and downstream regions	This study
pΔ*oatA*	pOri with *oatA* up and downstream regions	This study
pΔ*tagO*	pMAD with t*agO* up and downstream regions	[21]
pΔ*pbpD*	pMAD with *pbpD* up and downstream regions	[21]
pΔ*atl*	pMAD with *atl* up and downstream regions	This study
pΔ*arlR*	pMAD with *arlR* up and downstream regions	This study
pΔ*dltA*	pMAD with *dltA* up and downstream regions	[22]

Table 2. Primers used in this study

Primer	Primer Sequence (5′-3′)
P1_oatA	GAGGATCCCAAAGCACAAGGTTTAGGTG
P2_oatA	TGCCATGGTCCATGTTAATAAACGCCC
P3_oatA	TGCCATGGACGATGGAAACACATGCTAC
P4_oatA	GCGAGATCTGATCAGTGAATAAACCGCTC
P5_fmtA	TATGGATCCGCTTTCATATTGACTCTCAC
P6_fmtA	GCCATATACATGTTATATCTTCTATATCATTTGATAATTGCCTCAC
P7_fmtA	GTGAGGCAATTATCAAATGATATAGAAGATATAACATGTATATGGC
P8_fmtA	TGGAGATCTTTGGGTTTTCATCTTTACTG
P9_tagO	TGGAGATCTCGAGTGAGAAGAAATGCC
P10_tagO	CAGCTATGCTTTCATTCCCTATTATTCACCTTCATCGATATT
P11_tagO	AATATCGATGAAGGTGAATAATAGGGAATGAAAGCATAGCTG
P12_tagO	CATGCCATGGCAAACGATTTATAGTCATGTC
P13_pbpD	GCGAGATCTGAGAAATATACGAATTGTGGCG
P14_pbpD	GGGAAGATTAACGCTTTTAAAACATACTAAAAACGG
P15_pbpD	CCGTTTTTAGTATGTTTTAAAAGCGTTAATCTTCCC
P16_pbpD	CATGCCATGGGATACCACCAAATAATGCG
P17_arlR	TGGAGATCTGTTCGTAATTCATGTGACG
P18_arlR	GTAAAATCACCATATGGAATCATATTAAGTCTCCCTCATTAGAACTC
P19_arlR	GTCACGATCGTATGGTTTTAACTTTAAAATTTGCGTCATTTGTACACC
P20_arlR	CATGCCATGGCCTAAAGTGTCGTAAGGG
P21_atl	CGGAATTCCGCATATAAGGTACTATCAAACG
P22_atl	GTTAGGAGTAATAAATAGAGCAACATGAACATAGGATC
P23_atl	GATCCTATGTTCATGTTGCTCTATTTATTACTCCTAAC
P24_atl	GGAAGATCTTCCGAAACAATGAAATCACTTAGC
P25_dltA	GCAGATCTGAATGTATATATTTGCGCTGATG
P26_dltA	GTAAAATCACCATATGGAATCATATTAAGTCTCCCTCATTAGAACTC
P27 _dltA	GAGTTCTAATGAGGGAGACTTAATATGATTCCATATGGTGATTTTAC
P28 _dltA	GCGAATTCCGAAACGTTTGTAACGATCG

Data analysis. For the Van-FL and WGA-FL binding to bacteria, data (n = 50) was non-normal but with equal variance, therefore nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison was applied.

RESULTS

Deletion of non essential genes involved in cell wall metabolism led to differences in PGN accessibility to external molecules

PGN may not be fully exposed at the cell surface of bacteria. In Gram-positive bacteria it is integrated into a complex net that also includes O-acetylation groups, proteins, glycopolymers such as WTA, and capsule polysaccharide. In order to build this complex cell wall, bacteria require several non-essential proteins such as those listed in Table 3.

To examine whether such proteins could have a role in protecting PGN from external factors such as host receptors and lytic enzymes, we quantified the binding of fluorescent labeled vancomycin (Van-FL) and wheat germ agglutinin (WGA) to the following bacterial mutants: *oatA*, *tagO*, *pbpD*, *fmtA*, *dltA*, *arlR*, and *atl* (Table 1).

In order to eliminate the variability of the effect of each mutant associated with particular *S. aureus* genetic backgrounds, we constructed all deletion mutants of these genes in *S. aureus* NCTC8325-4 strain by removing the entire gene from the bacterial chromosome, leaving no resistance marker, and thus minimizing possible alterations in the transcription of neighbouring genes. We were unable to produce the dltA null mutant in the NCTC8325-4, and therefore it was only made in the RN4220 background.

Table 3. Genes in this study and respective roles in PGN metabolism

Gene	Function	Function	Reference
oatA	*O*-actetylation	Lysozyme resistance	[3]
tagO	WTA synthesis	Lysozyme resistance Cation binding Phage infection Adhesion and biofilm formation	[23] [24] [25] [26]
pbpD	PG cross-linking	Resistance to B-lactams	[27]
fmtA	PG cross-linking	Resistance to B-lactams PG Autolysis	[9] [28]
atl	Bifunctional precursor autolysin	Separation of daughter cells Adhesion and biofilm formation Excretion of cytoplasmic proteins	[29] [30] [31]
arlR	Response regulator of two component system ArlRS	Biofilm formation Regulation of transcription of virulence genes	[14] [13]
dltA	*D* – alanylation of TAs	Resistance to cationic antimicrobial peptides Autolysis regulation	[32] [33]

To visualize the cell wall produced by the different mutants we labeled exponentially growing cells with Van-Fl; a fluorescent derivative of vancomycin that specifically binds to the D-alanyl-D-alanine residues present in the carboxyl-terminal of the PGN precursor [34]. Although none of the genes deleted were essential to *S. aureus* viability, some of the deletions resulted, as expected in bacteria with morphological changes (Figure 1). Microscopic examination of the mutants showed that the main morphological effects observed were cell cluster formation, such as that observed in NCTCΔ*atl* mutant strain, and increased cell size, such as that observed in NCTCΔ*tagO* mutant strain (Figure 1).

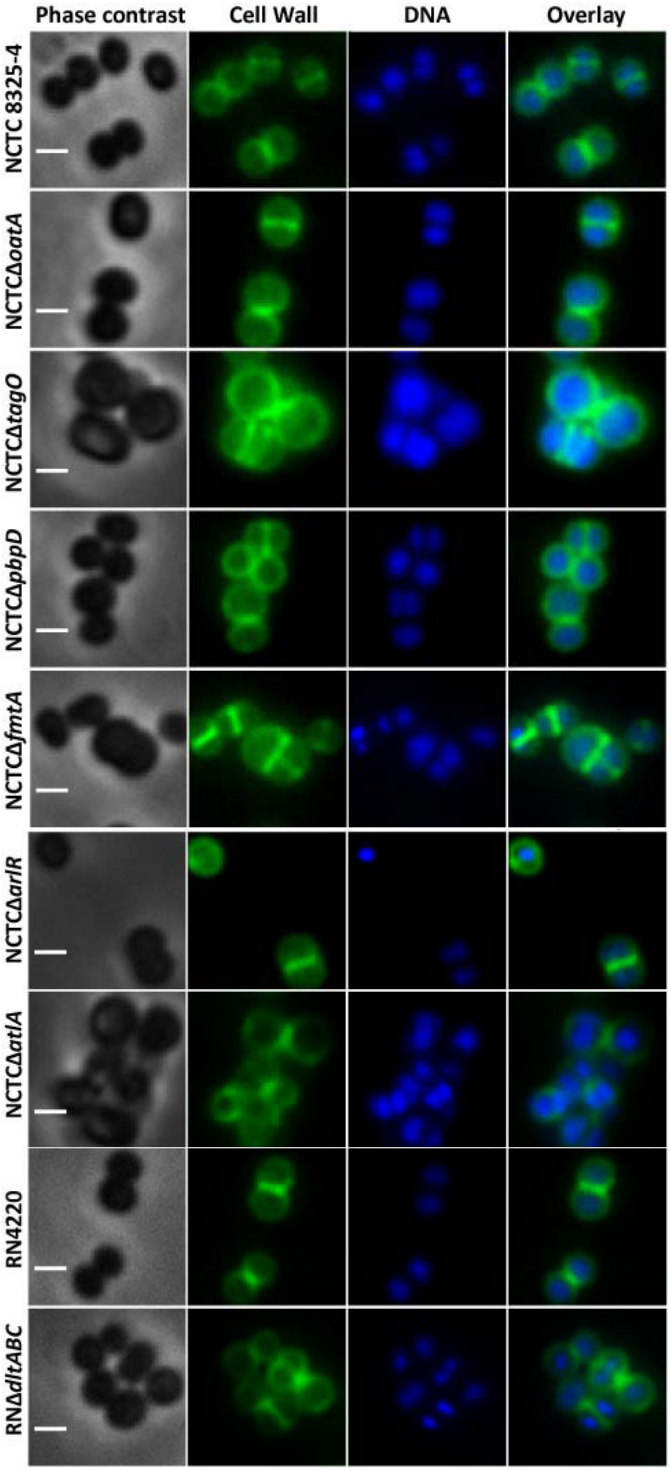


Figure 1. Microscopy analysis of the effects of the gene deletions in *S. aureus*.

Cells were resuspended in 1 ml of PBS and incubated with Van-FL dye (1 μg/mL)

and with Hoechst (1 μg/ml). Grey panels are phase-contrast images of bacterial cells (white scale bar represents 1 μm), and black panels show the Van-FL binding to cell wall, Hoechst binding to DNA and the overlay of both channels. Representative cells are shown. Scale bar corresponds to 1μm.

This is in agreement to what has been previously reported [35, 36]. The *arlR* mutant showed a significantly reduced cell diameter and asymmetric DNA segregation, sometimes with the presence of septa over the DNA (Figure 1).

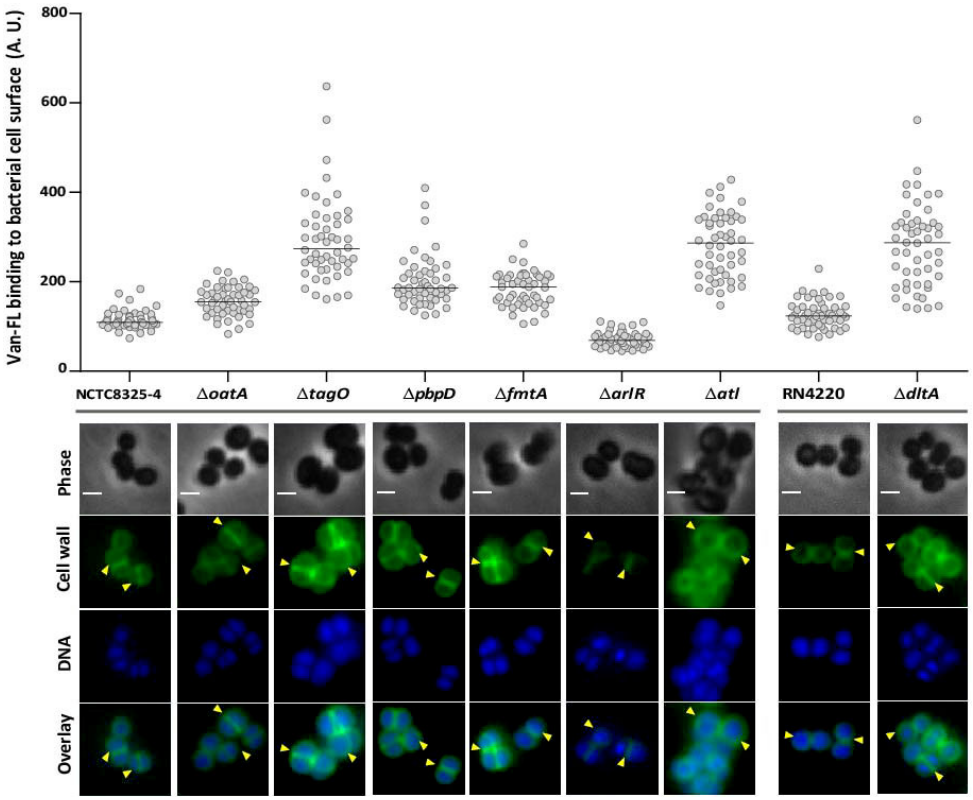


Figure 2. Van-Fl binding to the surface of *S. aureus* mutants. *S. aureus* mutants (*oatA, tagO, pbpD, fmtA, arlR, atl and dltA*) and parental strains (NCTC8325-4 and RN4220) in exponential phase (OD 0.6-0.8) were incubated with Van-FL, and

Hoechst staining markers. Bacterial cells were washed with PBS and visualized by fluorescence microscopy. Grey panels are phase-contrast images of bacterial cells (white scale bar represents 1 μm), and black panels show the stained bacterial cell wall (Van-Fl), labeled DNA (Hoechst) and the overlay of both channels. The average fluorescence of Van-FL bound to a bacterium was quantified for each strain (n = 50), and represented as the median (with 25% and 75% inter-quartile range). Kruskal-Wallis analysis revealed significant difference ($P < 0.05$) between Van-FL binding to *tagO, pbpD, fmtA, atl* and the remaining to the remaining parental and mutant strains.

Deletion of the *oatA*, *pbpD*, *fmtA* and *dltA* genes did not result in observable morphological changes when compared with the *S. aureus* parental strains. Despite the morphological differences found in some of the mutants, approximately equal amounts of Van-FL were bound to the cell surface of the NCTC8325-4 wild type strain and the *oatA* and *arlR* mutants (Figure 2). However, deletion of *tagO*, *pbpD*, *fmtA* and *atl* genes from the NCTC8325-4 parental strain chromosome resulted in a slight increase in binding of Van-Fl to bacteria (Figure 2). This result suggested that TagO, PBP4 FmtA and Atl proteins might function to restrict the access of Van-Fl to the PGN or that the PGN could be modified in such way that the number of D-Ala-D-Ala (recognized by Van-FL) available was increased.

PGN from S. aureus ΔtagO mutant is more accessible to both WGA and Van-FL molecules

To ask whether the increased binding observed with these mutants was specific for Van-FL or a consequence of a reduced access to PGN by the

external probes, we quantified the binding of WGA-FL to the bacterial cell surface of each mutant (Figure 3). WGA is a lectin that preferentially recognizes GlcNAc residues of the cell wall, and binds glycan residues of both peptidoglycan and wall teichoic acids [37]. As shown in Figure 3, the binding of WGA to the various bacterial strains was different to that observed with Van-Fl: whereas Van-Fl bound evenly and at higher levels to *tagO*, *pbpD*, *fmtA* and *atl* null mutants relative to wild type, WGA binding to bacterial cells was only statistically increased in strains lacking the WTAs (NCTCΔ*tagO*).

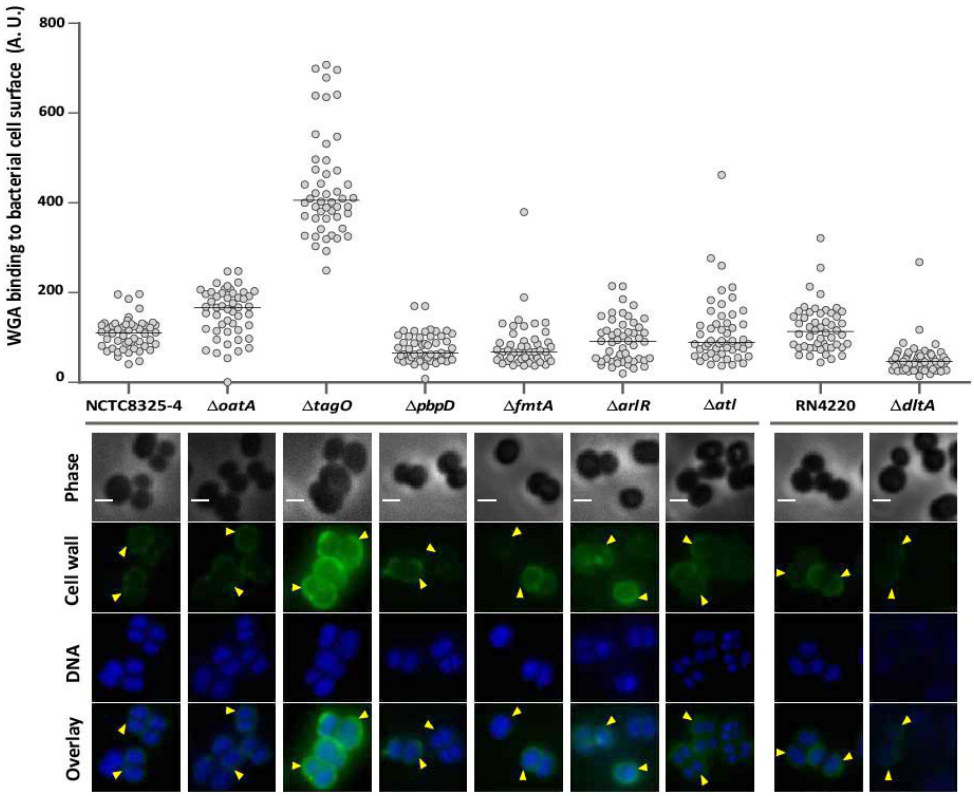


Figure 3. Increased binding of WGA to the surface of *S. aureus* NCTCΔ*tagO* mutant. (**A**) WGA and Hoechst were added to growing cultures of *S. aureus* mutants (*oatA, tagO, pbpD, fmtA, arlR, atl and dltA*) and parental strains

(NCTC8325-4 and RN4220) harvested at the exponential phase (OD_{600nm} 0.6-0.8). Bacterial cells were washed with PBS and visualized using fluorescence microscopy. Grey panels are phase-contrast images of bacterial cells (white scale bar represents 1 μm), and black panels show the WGA-FL binding to bacterial surface, Hoechst binding to bacterial chromosome (DNA) and the overlay of both channels: yellow arrowheads highlight binding to the cell wall. The average of fluorescence of WGA bound to a bacterium was quantified for each strain (n = 50), and represented as the median (with 25% and 75% inter-quartile range). Kruskal-Wallis analysis revealed significant difference (P < 0.05) between WGA-FL binding to NCTCΔ*tagO* and to the remaining wild type and mutant strains.

Synthesis of WTAs, and not TagO protein, reduces binding to external molecules

To verify if WTAs synthesis, or the TagO protein itself, was required to reduce the binding of WGA to the cell surface, we blocked it with tunicamycin and determined the amount of WGA bound in this condition (Figure 4). Tunicamycin is an uridine nucleoside analog that specifically binds to and blocks the first membrane-associated step of WTA biosynthesis and thus, actively inhibits its synthesis [38-40]. When wild type NCTC8325-4 cells were grown in increased concentrations of tunicamycin and visualized by fluorescence microscopy, a dramatic change in their cell shape and increased WGA binding was readily detected under the microscope (Figure 4A). Alcian blue silver staining PAGE analysis of the WTAs extracted from *S. aureus* NCTC8325-4 grown in increasing concentrations of tunicamycin revealed that WTA production is virtually abolished at tunicamycin concentrations of 0.16 μg/ml or higher (Figure 4B).

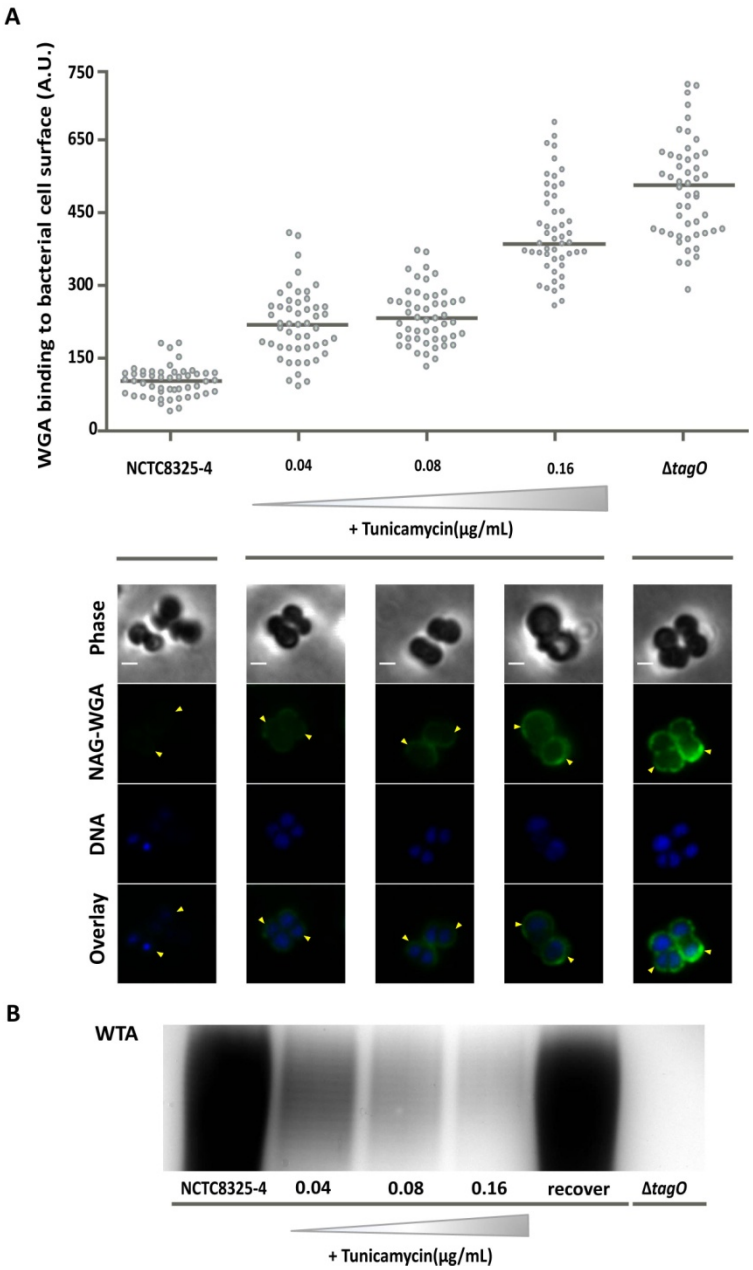


Figure 4. Synthesis of WTA is required to limit WGA binding to *S. aureus* bacterial surface. (**A**) WGA binding to *S. aureus* NCTC8325-4 strain grown without or with varying concentrations of tunicamycin (0.04, 0.08 and 0.16 μg/ml), an inhibitor of WTA synthesis that specifically binds to TagO. The binding of lectin to individual bacterial cells (n = 50) was quantified, and

represented as the median (with 25% and 75% inter-quartile range). WGA binding to bacteria treated with increased tunicamycin concentrations increases as the levels of WTA are reduced. Grey panels are phase-contrast images of bacterial cells (white scale bar represents 1 μm), and black panels show WGA (WGA-Alexa Fluor 488 conjugate) binding to the bacteria surface (PGN) GlcNAc residue, DNA (stained with Hoechst 33342) and a merge of the last channels (Overlay); yellow arrowheads highlight lectin WGA binding to the cell surface of the bacteria. Kruskal-Wallis analysis followed by Dunn's multiple comparison post-test, revealed significant differences in the binding for treated and untreated bacteria ($P < 0.05$**) (B)** Electrophoresis gel analysis of WTAs extracted from overnight culture of *S. aureus* NCTC8325-4 strain treated with varying tunicamycin concentrations and NCTCΔ*tagO*, WTA production is virtually abolished with 0.16 μg/ml tunicamycin. Treated cells with 16 μg/ml can recover WTA synthesis if cells were grown in fresh TSB without tunicamycin.

Consistent with the finding that tunicamycin reproduces the characteristic *tagO* phenotype, wild type cells treated with tunicamycin 0.16 μg/ml and, consequently affected in WTA production displayed WGA binding levels similar to those exhibited by *tagO* mutant cells (Figure 3 and 4). Moreover, the results demonstrated that the signal of WGA binding is dependent on the amount of WTAs produced (Figure 4). Overall the results described above suggested that WTAs reduce PGN accessibility to different fluorescent compounds, Van-Fl and WGA

DISCUSSION

Bacterial PGN is a major target of hydrolytic enzymes and host pattern recognition receptors [41]. In this study, we examined the binding of cell wall markers to *S. aureus* mutants in important but not essential

proteins of PGN metabolism to understand their relevance in limiting PGN accessibility.

Our work revealed that Van-FL binding to the *S. aureus* mutant's bacterial surface was substantially different from the WGA binding. Van-FL bound significantly more to the *tagO*, *pbpD*, *fmtA* and *atl* mutants, whereas WGA bound with higher affinity only to *tagO* mutant. One possible explanation for this difference is that the cell wall markers, WGA and Van-Fl, probe different epitopes in the PGN and the mutations created affect differently the structure and composition of the PGN.

Van-Fl binds to the D-Ala-D-Ala residues present in the carboxyl-terminal of the PGN precursor. When the PGN precursor is incorporated into the cell wall net via transpeptidation, a reaction catalyzed by the PBPs, the D-Ala-D-Ala bond is cleaved and the terminal D-Ala is released to form the cross-bridge. The mature PGN of *S. aureus* is highly cross-linked but it still contains a considerable number of free D-Ala-D-ala residues [42]. Deletion of the *pbpD* gene, which encodes for PBP4, leads to a decrease in the PGN cross-linking [8] and to an increase in the amount of the epitope D-Ala-D-Ala, thus contributing to an enhancement of Van-FL binding. A similar effect might occur in the *fmtA* mutant as Komatsuzawa and colleagues [10, 43] described FmtA as a penicillin binding protein that is also involved in production of highly crosslinked PGN.

A strong Van-FL labeling was also observed in the *S. aureus atl* null mutant. It has already been shown that down-regulation of autolysis in *S. aureus* increases the thickness of cell wall [44, 45]. Inhibition of the autolytic machinery by deletion of the major autolysin gene *atl* may

increase cell wall thickness and as a consequence trap more Van-FL molecules in the net.

Both markers WGA and Van-FL bound strongly to *tagO* mutant. Deletion of *tagO* gene in *S. aureus*, which encodes for the first enzyme of the WTA biosynthesis, impairs WTA production. Furthermore, we showed that it is the synthesis of WTA and not the presence of TagO that is required to prevent the binding of WGA. The decreased production of WTAs in *S. aureus* through chemical inhibition of TagO by tunicamycin was directly correlated with enhanced binding of WGA. WTAs are large polymers made of approximately 40 ribitol phosphate units modified by GlcNAc and D-ala residues, that are covalently linked to PGN [5]. Since this polysaccharide is located outside of the cell it may sterically hinder the access of different molecules to PGN. Thus loss of WTAs may increase accessibility of the PGN and therefore result in enhanced binding of the cell wall markers Van-Fl and WGA. Similar effects have already been reported in *S. aureus* where it was shown that WTAs interfered with the interaction between hydrolytic enzymes (lysostaphin and lysozyme) and the PGN surface [23, 46]. An apparent contradiction contained within our data is that loss of WTAs and the associated GlcNAc residues, the WGA epitope, did not result in decrease of WGA binding comparatively to the wild type *S. aureus*. One possible explanation for this contradiction is that the amount of GlcNAc residues present in the PGN is higher than the amount present in the WTA glycopolymers.

Our studies also revealed that loss of D-ala residues on the surface of WTAs, as a result of the *dltA* mutation, increased the Van-Fl binding, consistent with the previously study [33] but decreased the WGA binding

to PGN. Increase of the cell wall surface negative charge in this mutant may affect the binding of these probes to PGN. Similar effects were reported by Peschel and colleagues [6], as it was shown that increased negative surface charge increased susceptibility to cationic antimicrobial peptides and decreased the binding of negatively charged proteins.

In conclusion, our work revealed that the activity of *S. aureus* PGN modifying enzymes PBP4, FmtA, Atl, DltA and mainly TagO affect PGN accessibility to different cell wall probes. Nevertheless, further studies are required to clarify the impact of these enzymes on PGN composition or its accessibility to lytic enzymes and to recognition by the host immune system.

BIBLIOGRAPHY

1. Navarre, W.W. and O. Schneewind, *Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope.* Microbiol Mol Biol Rev, 1999. **63**(1): p. 174-229.

2. Scott, J.R. and T.C. Barnett, *Surface proteins of gram-positive bacteria and how they get there.* Annu Rev Microbiol, 2006. **60**: p. 397-423.

3. Bera, A., S. Herbert, A. Jakob, W. Vollmer, and F. Gotz, *Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of Staphylococcus aureus.* Mol Microbiol, 2005. **55**(3): p. 778-87.

4. Schlag, M., R. Biswas, B. Krismer, T. Kohler, S. Zoll, W. Yu, H. Schwarz, A. Peschel, and F. Gotz, *Role of staphylococcal wall teichoic acid in targeting the major autolysin Atl.* Mol Microbiol, 2010. **75**(4): p. 864-73.

5. Weidenmaier, C. and A. Peschel, *Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions.* Nat Rev Microbiol, 2008. **6**(4): p. 276-87.

6. Peschel, A., M. Otto, R.W. Jack, H. Kalbacher, G. Jung, and F. Gotz, *Inactivation of the dlt operon in Staphylococcus aureus confers sensitivity to defensins, protegrins, and other antimicrobial peptides.* J Biol Chem, 1999. **274**(13): p. 8405-10.

7. Gally, D. and A.R. Archibald, *Cell wall assembly in Staphylococcus aureus: proposed absence of secondary crosslinking reactions.* J Gen Microbiol, 1993. **139**(8): p. 1907-13.

8. Leski, T.A. and A. Tomasz, *Role of penicillin-binding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of Staphylococcus aureus: evidence for the cooperative functioning of PBP2, PBP4, and PBP2A.* J Bacteriol, 2005. **187**(5): p. 1815-24.

9. Fan, X., Y. Liu, D. Smith, L. Konermann, K.W. Siu, and D. Golemi-Kotra, *Diversity of penicillin-binding proteins. Resistance factor FmtA of Staphylococcus aureus.* J Biol Chem, 2007. **282**(48): p. 35143-52.

10. Komatsuzawa, H., K. Ohta, H. Labischinski, M. Sugai, and H. Suginaka, *Characterization of fmtA, a gene that modulates the expression of methicillin*

resistance in Staphylococcus aureus. Antimicrob Agents Chemother, 1999. **43**(9): p. 2121-5.

11. McAleese, F., S.W. Wu, K. Sieradzki, P. Dunman, E. Murphy, S. Projan, and A. Tomasz, *Overexpression of genes of the cell wall stimulon in clinical isolates of Staphylococcus aureus exhibiting vancomycin-intermediate- S. aureus-type resistance to vancomycin.* J Bacteriol, 2006. **188**(3): p. 1120-33.

12. Llarrull, L.I., J.F. Fisher, and S. Mobashery, *Molecular basis and phenotype of methicillin resistance in Staphylococcus aureus and insights into new beta-lactams that meet the challenge.* Antimicrob Agents Chemother, 2009. **53**(10): p. 4051-63.

13. Fournier, B., A. Klier, and G. Rapoport, *The two-component system ArlS-ArlR is a regulator of virulence gene expression in Staphylococcus aureus.* Mol Microbiol, 2001. **41**(1): p. 247-61.

14. Fournier, B. and D.C. Hooper, *A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic activity of Staphylococcus aureus.* J Bacteriol, 2000. **182**(14): p. 3955-64.

15. Leenhouts, K., A. Bolhuis, J. Boot, I. Deutz, M. Toonen, G. Venema, J. Kok, and A. Ledeboer, *Cloning, expression, and chromosomal stabilization of the Propionibacterium shermanii proline iminopeptidase gene (pip) for food-grade application in Lactococcus lactis.* Appl Environ Microbiol, 1998. **64**(12): p. 4736-42.

16. Veiga, H. and M.G. Pinho, *Inactivation of the SauI type I restriction-modification system is not sufficient to generate Staphylococcus aureus strains capable of efficiently accepting foreign DNA.* Appl Environ Microbiol, 2009. **75**(10): p. 3034-8.

17. Oshida, T. and A. Tomasz, *Isolation and characterization of a Tn551-autolysis mutant of Staphylococcus aureus.* J Bacteriol, 1992. **174**(15): p. 4952-9.

18. Arnaud, M., A. Chastanet, and M. Debarbouille, *New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria.* Appl Environ Microbiol, 2004. **70**(11): p. 6887-91.

19. Campbell, J., A.K. Singh, J.P. Santa Maria, Jr., Y. Kim, S. Brown, J.G. Swoboda, E. Mylonakis, B.J. Wilkinson, and S. Walker, *Synthetic lethal compound combinations reveal a fundamental connection between wall teichoic acid and peptidoglycan biosyntheses in Staphylococcus aureus.* ACS Chem Biol, 2011. **6**(1): p. 106-16.

20. Meredith, T.C., J.G. Swoboda, and S. Walker, *Late-stage polyribitol phosphate wall teichoic acid biosynthesis in Staphylococcus aureus.* J Bacteriol, 2008. **190**(8): p. 3046-56.

21. Atilano, M.L., P.M. Pereira, J. Yates, P. Reed, H. Veiga, M.G. Pinho, and S.R. Filipe, *Teichoic acids are temporal and spatial regulators of peptidoglycan cross-linking in Staphylococcus aureus.* Proc Natl Acad Sci U S A, 2010. **107**(44): p. 18991-6.

22. Atilano, M.L., J. Yates, M. Glittenberg, S.R. Filipe, and P. Ligoxygakis, *Wall teichoic acids of Staphylococcus aureus limit recognition by the drosophila peptidoglycan recognition protein-SA to promote pathogenicity.* PLoS Pathog, 2011. **7**(12): p. e1002421.

23. Bera, A., R. Biswas, S. Herbert, E. Kulauzovic, C. Weidenmaier, A. Peschel, and F. Gotz, *Influence of wall teichoic acid on lysozyme resistance in Staphylococcus aureus.* J Bacteriol, 2007. **189**(1): p. 280-3.

24. Hughes, A.H., I.C. Hancock, and J. Baddiley, *The function of teichoic acids in cation control in bacterial membranes.* Biochem J, 1973. **132**(1): p. 83-93.

25. Xia, G., R.M. Corrigan, V. Winstel, C. Goerke, A. Grundling, and A. Peschel, *Wall teichoic Acid-dependent adsorption of staphylococcal siphovirus and myovirus.* J Bacteriol, 2011. **193**(15): p. 4006-9.

26. Weidenmaier, C., J.F. Kokai-Kun, S.A. Kristian, T. Chanturiya, H. Kalbacher, M. Gross, G. Nicholson, B. Neumeister, J.J. Mond, and A. Peschel, *Role of teichoic acids in Staphylococcus aureus nasal colonization, a major risk factor in nosocomial infections.* Nat Med, 2004. **10**(3): p. 243-5.

27. Memmi, G., S.R. Filipe, M.G. Pinho, Z. Fu, and A. Cheung, *Staphylococcus aureus PBP4 is essential for beta-lactam resistance in community-acquired methicillin-resistant strains.* Antimicrob Agents Chemother, 2008. **52**(11): p. 3955-66.

28. Komatsuzawa, H., M. Sugai, K. Ohta, T. Fujiwara, S. Nakashima, J. Suzuki, C.Y. Lee, and H. Suginaka, *Cloning and characterization of the fmt gene which affects the methicillin resistance level and autolysis in the presence of triton X-100 in methicillin-resistant Staphylococcus aureus.* Antimicrob Agents Chemother, 1997. **41**(11): p. 2355-61.

29. Yamada, S., M. Sugai, H. Komatsuzawa, S. Nakashima, T. Oshida, A. Matsumoto, and H. Suginaka, *An autolysin ring associated with cell separation of Staphylococcus aureus.* J Bacteriol, 1996. **178**(6): p. 1565-71.

30. Houston, P., S.E. Rowe, C. Pozzi, E.M. Waters, and J.P. O'Gara, *Essential role for the major autolysin in the fibronectin-binding protein-mediated Staphylococcus aureus biofilm phenotype.* Infect Immun, 2011. **79**(3): p. 1153-65.

31. Pasztor, L., A.K. Ziebandt, M. Nega, M. Schlag, S. Haase, M. Franz-Wachtel, J. Madlung, A. Nordheim, D.E. Heinrichs, and F. Gotz, *Staphylococcal major autolysin (Atl) is involved in excretion of cytoplasmic proteins.* J Biol Chem, 2010. **285**(47): p. 36794-803.

32. Herbert, S., A. Bera, C. Nerz, D. Kraus, A. Peschel, C. Goerke, M. Meehl, A. Cheung, and F. Gotz, *Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci.* PLoS Pathog, 2007. **3**(7): p. e102.

33. Peschel, A., C. Vuong, M. Otto, and F. Gotz, *The D-alanine residues of Staphylococcus aureus teichoic acids alter the susceptibility to vancomycin and the activity of autolytic enzymes.* Antimicrob Agents Chemother, 2000. **44**(10): p. 2845-7.

34. Reynolds, P.E., *Structure, biochemistry and mechanism of action of glycopeptide antibiotics.* Eur J Clin Microbiol Infect Dis, 1989. **8**(11): p. 943-50.

35. Biswas, R., L. Voggu, U.K. Simon, P. Hentschel, G. Thumm, and F. Gotz, *Activity of the major staphylococcal autolysin Atl.* FEMS Microbiol Lett, 2006. **259**(2): p. 260-8.

36. Vergara-Irigaray, M., T. Maira-Litran, N. Merino, G.B. Pier, J.R. Penades, and I. Lasa, *Wall teichoic acids are dispensable for anchoring the PNAG exopolysaccharide to the Staphylococcus aureus cell surface.* Microbiology, 2008. **154**(Pt 3): p. 865-77.

37. Wagner, M., *Interaction of wheat-germ agglutinin with streptococci and streptococcal cell wall polymers.* Immunobiology, 1979. **156**(1-2): p. 57-64.

38. Brandish, P.E., K.I. Kimura, M. Inukai, R. Southgate, J.T. Lonsdale, and T.D. Bugg, *Modes of action of tunicamycin, liposidomycin B, and mureidomycin A: inhibition of phospho-N-acetylmuramyl-pentapeptide translocase from Escherichia coli.* Antimicrob Agents Chemother, 1996. **40**(7): p. 1640-4.

39. Hancock, I.C., G. Wiseman, and J. Baddiley, *Biosynthesis of the unit that links teichoic acid to the bacterial wall: inhibition by tunicamycin.* FEBS Lett, 1976. **69**(1): p. 75-80.

40. Pooley, H.M. and D. Karamata, *Incorporation of [2-3H]glycerol into cell surface components of Bacillus subtilis 168 and thermosensitive mutants affected in wall teichoic acid synthesis: effect of tunicamycin.* Microbiology, 2000. **146 (Pt 4)**: p. 797-805.

41. Koprivnjak, T. and A. Peschel, *Bacterial resistance mechanisms against host defense peptides.* Cell Mol Life Sci, 2011. **68**(13): p. 2243-54.

42. de Jonge, B.L., Y.S. Chang, D. Gage, and A. Tomasz, *Peptidoglycan composition in heterogeneous Tn551 mutants of a methicillin-resistant Staphylococcus aureus strain.* J Biol Chem, 1992. **267**(16): p. 11255-9.

43. Komatsuzawa, H., G.H. Choi, T. Fujiwara, Y. Huang, K. Ohta, M. Sugai, and H. Suginaka, *Identification of a fmtA-like gene that has similarity to other PBPs and beta-lactamases in Staphylococcus aureus.* FEMS Microbiol Lett, 2000. **188**(1): p. 35-9.

44. Nannini, E., B.E. Murray, and C.A. Arias, *Resistance or decreased susceptibility to glycopeptides, daptomycin, and linezolid in methicillin-resistant Staphylococcus aureus.* Curr Opin Pharmacol, 2010. **10**(5): p. 516-21.

45. Boyle-Vavra, S., M. Challapalli, and R.S. Daum, *Resistance to autolysis in vancomycin-selected Staphylococcus aureus isolates precedes vancomycin-intermediate resistance.* Antimicrob Agents Chemother, 2003. **47**(6): p. 2036-9.

46. Grundling, A., D.M. Missiakas, and O. Schneewind, *Staphylococcus aureus mutants with increased lysostaphin resistance.* J Bacteriol, 2006. **188**(17): p. 6286-97.

Supplementary Information

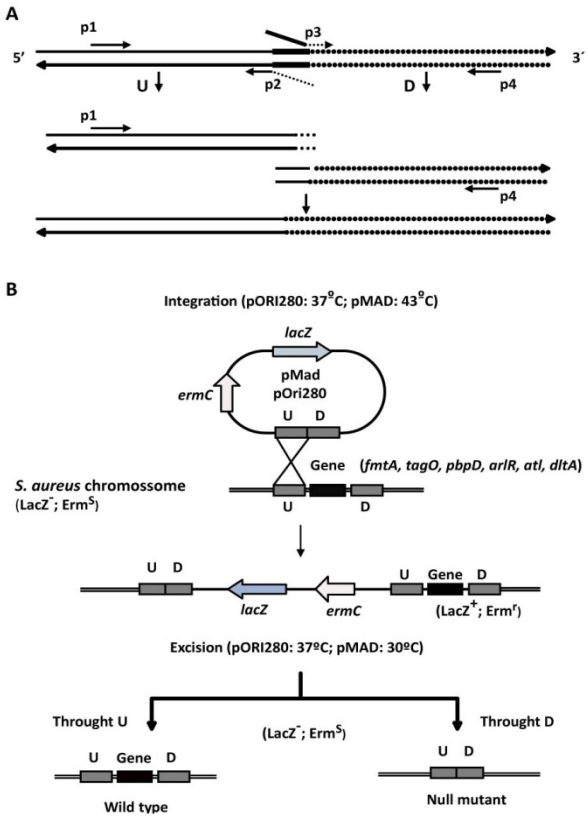


Figure S1. General mechanism used to generate gene deletion in *S. aureus* strains chromosome. **(A)** Schematic diagram of the overlap extension method. In separate amplifications two fragments of the target gene are amplified. One of the PCR uses primers p1 and p2, whereas the other PCR uses p3 and p4. In a second PCR the denatured fragments anneal at the region of overlap and are amplified using primers p1 and p4**(B)** Schematic representation of a two-step procedure for gene replacement using pORI280 or pMAD. Integration of the plasmids via homologous recombination can take place via the upstream (example shown in the figure) or downstream region. After a second recombination event, the gene will either remain in the chromosome - if the second recombination occurs through the upstream region, as in the given example - or be excised along with the plasmid (if the second recombination occurs through the downstream region).

CHAPTER III

Teichoic acids are temporal and spatial regulators of peptidoglycan cross-linking in *Staphylococcus aureus*

This chapter contains data published in:

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The author of this dissertation participated in all experiments described in this chapter, except in the PBPs localization and the bacterial to hybrid studies.

ABSTRACT

The cell wall of *Staphylococcus aureus* is characterized by an extremely high degree of cross-linking within its peptidoglycan (PGN). Penicillin- binding protein 4 (PBP4) is required for the synthesis of this highly cross-linked PGN. We found that wall teichoic acids, glycopolymers attached to the peptidoglycan and important for virulence in Gram-positive bacteria, act as temporal and spatial regulators of PGN metabolism, controlling the level of cross-linking by regulating PBP4 localization. PBP4 normally localizes at the division septum, but in the absence of wall teichoic acids synthesis, it becomes dispersed throughout the entire cell membrane and is unable to function normally. As a consequence, the PGN of TagO null mutants, impaired in wall teichoic acids biosynthesis, has a decreased degree of cross-linking, which renders it more susceptible to the action of lysozyme, an enzyme produced by different host organisms as an initial defense against bacterial infection.

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INTRODUCTION

The cell wall of Gram-positive bacteria is a highly complex network composed mainly of peptidoglycan (PGN) and teichoic acids (TAs), both essential to the maintenance of the structural integrity and shape of the bacterial cell. PGN is a heterogeneous polymer of glycan chains cross-linked by short peptides of variable length and amino acid composition [1]. TAs are phosphate-rich glycopolymers that can be either covalently linked to PGN (wall teichoic acids, or WTAs) or anchored to the cytoplasmic membrane (lipoteichoic acids, or LTAs) [2, 3].

Despite decades of study, it is still not well understood why pathogenic and non-pathogenic bacteria have TAs at their cell surface. TAs contribute to a variety of processes including resistance to environmental stresses, such as heat [4] or low osmolarity [5], to antimicrobial peptides [6], antimicrobial fatty acids [7], cationic antibiotics [8], and lytic enzymes produced by the host, including lysozyme [9, 10]. TAs also act as receptors for phage particles [11], and they can bind cationic groups (particularly magnesium ions), providing a reservoir of ions close to the bacterial surface that may be important for the activity of different enzymes [12]. More recently TAs have been proposed to be involved in cell division and morphogenesis [13]. Lack of LTA synthesis in rod shaped *Bacillus subtilis* cells causes defects in formation of the division septum and in cell separation [14], whereas lack of WTAs results in round cells [15]. Moreover, enzymes involved in LTA synthesis localize predominantly at the division sites of bacteria [14] and enzymes involved in the synthesis of WTAs localize in helical patterns [16] similar to the

previously described patterns of PGN synthesis observed during elongation of *B. subtilis* cells [17]. The observation that mutants lacking LTAs have altered autolysis rates [5, 18] or that interference with the synthesis of WTA in *B. subtilis* triggers the transcription of several genes involved in PGN synthesis [19], constitutes additional, indirect evidence that suggests a link between TAs and cell morphogenesis through an effect on the biosynthesis of PGNs, the correct assembly of which is required for proper cell division and morphogenesis.

To investigate whether the synthesis of WTAs is directly required for building a PGN macromolecule with the correct structure, we used *Staphylococcus aureus* as a model organism. *S. aureus* is a Gram-positive bacteria and a prominent pathogen in the community and healthcare settings, well known for its virulence and antibiotic resistance [20, 21]. The main advantage of using *S. aureus* for studying PGNs is that it has only four penicillin-binding proteins (PBPs 1-4) instead of 12 or 16 present in the traditional model organisms *Escherichia coli* and *B. subtilis*, respectively [22]. PBPs are enzymes involved in the final stages of PGN biosynthesis, which synthesize glycan chains (via transglycosylation reactions) and cross-link different glycan chains through short peptides (via transpeptidation reactions). The level of PGN cross-linking varies between bacterial species, and *S. aureus* has an unusually high degree of cross-linking [23], which is due mainly to the action of PBP4 [24, 25], and seems to require the long and flexible pentaglycine crossbridge that *S. aureus* cells use to connect two stem peptides from different glycan strands [23, 26]. PBP4 is not essential for *S. aureus* viability. However, it has an important role in antibiotic resistance, as it is essential for the expression

of beta-lactam resistance in community-acquired methicillin-resistant strains [25]. Interestingly, inactivation of PBP4 has been reported in laboratory step mutants and clinical isolates with intermediate vancomycin resistance [27-29], which have decreased levels of PGN cross-linking. This suggests that modulation of the degree of cross-linking is important for resistance to different antibiotics.

In this study, we describe uncharacterized link between WTA biosynthesis and PGN biosynthesis. We found that WTAs act as temporal and spatial regulators of PGN metabolism, controlling the level of cross-linking by directing PBP4 to the division septum. We also showed that the highly cross-linked PGN that results from the regulated action of PBP4 is more resistant to enzymatic degradation by lysozyme. This increased resistance may be advantageous to *S. aureus* bacteria during interactions with host organisms that produce lysozyme as an initial defense against bacterial infection.

Material and Methods

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Primers used in this study are listed in Table 2. *S. aureus* strains were grown at 30ºC in Tryptic soy broth medium (TSB; Difco) supplemented with antibiotics when required (erythromycin, Ery 10 μg/ml or kanamycin, Kan 50μg/ml and neomycin, Neo 50μg/ml; Sigma-Aldrich) and transformed by electroporation as previously described [30]. *E. coli* strains were grown at 37ºC in Luria-Bertani medium

(LB; Difco) supplemented with 100 μg/ml of ampicillin, Amp (Sigma-Aldrich).

Table 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Origin
NCTC8325-4	*S. aureus* reference strain	R. Novick
NCTCΔ*tagO*	NCTC8325-4 *tagO* null mutant	This study
NCTCΔ*tagO*Δ*spa*	NCTC8325-4 *tagO* null mutant with inactivated *spa* gene	This study
NCTCΔ*pbpD*	NCTC8325-4 *pbpD* null mutant	This study
RN4220	Restriction deficient derivative of *S. aureus* NCTC8325-4	R. Novick
RNΔ*tagO*	RN4220 *tagO* null mutant	This study
RNΔ*tagO* pMAD	RNΔ*tagO* transformed with pMAD vector, Erm^r	This study
RNΔ*tagO* p*tagO*	RNΔ*tagO* transformed pMAD*tagO*, Erm^r	This study
RNΔ*tagO* p$tagO^{mut*}$	RNΔ*tagO* transformed with pMAD$tagO^{mut}$, Erm^r	This study
RNΔ*dltABCD*	RN4220 *dltABCD* null mutant	This study
RNTagO^{wt}GFP	RN4220 with pSG*tagO* integrated in the chromosome, Erm^r	This study
RNTagO^{G152A}GFP	RN4220 with pSG$tagO^{G152A}$ integrated in the chromosome, Erm^r	This study
RNPBP4YFP	RN4220 with p*pbpDyfp* integrated in the chromosome, Kan^r	This study
RNΔ*tagO*PBP4YFP	RNΔ*tagO* with p*pbpDyfp* integrated in the chromosome, Kan^r	This study
RNΔ*tagO*PBP4YFP pMAD	RNΔ*tagO*PBP4YFP transformed with pMAD vector, Erm^r	This study
RNΔ*tagO*PBP4YFP p*tagO*	RNΔ*tagO*PBP4YFP transformed with pMAD*tagO*, Erm^r	This study
RNΔ*tagO*PBP4YFP p$tagO^{mut}$	RNΔ*tagO*PBP4YFP transformed with pMAD$tagO^{mut}$, Erm^r	This study

RNΔ*dltABCD*PBP4-YFP	RNΔ*dltABCD* with p*pbpDyfp* integrated in the chromosome, Kan^r	This study
RNTagOPBP4	RN4220 with pSG*tagO* and pBCB7-CHKPBP4 integrated in the chromosome, Erm^r, Kan^r	This study
Plasmids		
pMAD	*E. coli – S. aureus* shuttle vector with a thermosensitive origin of replication for Gram-positive bacteria	[31]
pΔ*tagO*	pMAD with t*agO* up and downstream regions	This study
pΔ*dltABCD*	pMAD with *dltABCD* up and downstream regions	This study
p*tagO*	pMAD encoding *tagO* gene and its promoter region	This study
p*tagO*^{mut}	pMAD encoding TagO protein with mutations D87A, D88A, D87/88A, G152A or N198A	This study
pSG5082	*S. aureus* integrative vector that allows C-termini GFP fusions	[32]
pSG*tagO*	pSG5082 encoding GFP fusion to the C-terminus of TagO	This study
pSG*tagO*^{G152A}	pSG5082 encoding GFP fusion to the C-terminus of TagO^{G152A}	This study
pMutinYFPKan	*S. aureus* integrative vector that allows C-terminus YFP fusions	This study
p*pbpDyfp*	pMutinYFPKan encoding YFP fusion to the C-terminus of PBP4	This study
pBCB7-CHKPBP4	pBCB7-CHK encoding mCherry fusion to the C-terminus of PBP4	[33]
pΔ*pbpD*	pMAD with *pbpD* up and downstream regions	This study
pMutinYFP	*B. subtilis* integrative vector for C-termini YFP fusions, Amp^r, Ery^r	[34]
pDG792	Plasmid containing a kan resistance gene	[35]

*mut indicates mutations D87A, D88A, D87/88A, G152A and N198A

Construction of *S. aureus* strains. To delete the *tagO* gene from the chromosome of *S. aureus* RN4220 we started by amplifying two 0.9Kb DNA fragments from the genome of *S. aureus* NCTC8325-4 strain, corresponding to the upstream (primers P1 and P2, see Table 2) and downstream (primers P3 and P4) regions of the *tagO* gene. The two fragments were joined by overlap PCR using primers P1 and P4 and the resulting PCR product was digested with *Bgl*II and *Nco*I and cloned into the pMAD vector [36], producing the plasmid p*ΔtagO*. This plasmid was sequenced and electroporated as previously described [30], into *S. aureus* RN4220 strain. Insertion and excision of p*ΔtagO* into the chromosome of RN4200 was performed as previously described [31] with the exception of the incubation temperature after excision of the plasmid, which was 30ºC (instead of 43ºC) due to the thermosensitive nature of cells lacking *tagO*. Deletion of *tagO* was confirmed by PCR and sequencing, and the resulting strain was named RN*ΔtagO*. The NCTC*ΔtagO* and NCTC*ΔtagOΔspa* mutants were obtained by transducing the plasmid p*ΔtagO* from RN4220 into NCTC8325-4 and NCTC8325-4*Δspa*, respectively, using phage 80*α*, as previously described [37]. Deletion of *tagO* from the chromosome of these strains was performed as described above for RN4220. To complement the deletion mutant RN*ΔtagO*, a DNA fragment of 1.3 Kb containing a full copy of the *tagO* gene and its native promoter was amplified by PCR using primers P5 and P6, digested with *Bgl*II and *Eco*RI and cloned into the pMAD vector. The resulting plasmid, named p*tagO*, was sequenced and electroporated into RN*ΔtagO* and RN*ΔtagO*PBP4YFP.

Four different point mutations were individually introduced into the *tagO* gene by PCR mutagenesis to change TagO amino acids D87, D88,

G152 or N198 into alanines. To generate the D87A mutation, an upstream region containing the *tagO* promoter and the 5′end of the gene, up to the codon encoding the mutated amino acid (primers P5 and P8) and a downstream region from the codon encoding the mutated amino acid to the 3′end of the *tagO* gene (primers P7 and P6) were amplified by PCR. Joining of the up and downstream fragments by overlap PCR (primers P5 and P6) resulted in the amplification of the mutated *tagO* gene, which was cloned into pMAD vector by using *Bgl*II and *Eco*RI restriction enzymes to generate p*tagO*^{D87A} plasmid. Similarly, primer pairs P5 and P10, P9 and P6 were used for the D88A mutation (resulting in plasmid p*tagO*^{D88A}), primer pairs P5 and P14, P13 and P6 for G152A (plasmid p*tagO*^{G152A}) and primer pairs P5 and P16, P15 and P6 for N198A (plasmid p*tagO*^{N198A}). A double mutant D87A/D88A was also made, using primers P5 and P12, P11 and P6 (generating plasmid p*tagO*$^{D87A/D88A}$). After sequencing, the resulting plasmids were individually electroporated into RNΔ*tagO* and RNΔ*tagO*PBP4YFP strains.

To delete the *dltABCD* operon from the chromosome of *S. aureus* RN4220 we started by amplifying two 0.55Kb DNA fragments from the genome of *S. aureus* NCTC 8325-4 strain, corresponding to the upstream (primers P33 and P34) and downstream (primers P35 and P36) regions of the *dltABCD* operon. The two fragments were joined by overlap PCR using primers P33 and P36 and the resulting PCR product was digested with *Bgl*II and *Eco*RI and cloned into the pMAD vector, producing the plasmid p*ΔdltABCD.* This plasmid was sequenced and electroporated into *S. aureus* RN4220 strain. Insertion and excision of p*ΔdltABCD* into the chromosome of RN4220 was performed as previously described [31]with

the exception of the incubation temperature after excision of the plasmid, which was 30ºC (instead of 43ºC). Deletion of *dltABCD* was confirmed by PCR, and the resulting strain was named RN*ΔdltABCD*.

To generate a strain expressing TagO fused to GFP, we amplified by PCR, from *S. aureus* NCTC8325-4 chromosomal DNA, a 1.0 kb DNA fragment containing the *tagO* gene, using primers pair P17 and P18. The PCR product was digested with *Kpn*I and *Xho*I and cloned into pSG5082 [38], upstream of and in frame with the *gfp* gene, encoding GFP+ a variant of the wild-type GFP exhibiting increased fluorescence [39]. The resulting plasmid, named pSG*tagO*, was sequenced and electroporated into *S. aureus* RN4220 strain where it integrated into the chromosomal *tagO* locus. The resulting strain, RNTagO^{wt}GFP, carried the *tagO-gfp* fusion under the control of the native *tagO* promoter and a copy of the native *tagO* gene without an upstream promoter.

The G152A mutation was introduced into *tagO-gfp* fusion by PCR mutagenesis of pSG*tagO* plasmid using primers P13 and P14. After sequence confirmation, the plasmid named pSG*tagO*^{G152A} was electroporated into *S. aureus* RN4220 strain where it integrated into the chromosomal *tagO* locus (strain RNTagO^{G152A}GFP). Total cell extracts were prepared from RNTagO^{wt}GFP and RNTagO^{G152A}GFP cells, separated by SDS-PAGE without boiling the samples and the fluorescence corresponding to the TagO-GFP band was visualized using a 532nm laser in a Fuji FLA-5100 reader.

To delete the *pbpD* gene from the chromosome of *S. aureus* NCTC8325-4 we amplified by PCR two DNA fragments of approximately 0.7 Kb from the *S. aureus* NCTC8325-4 genome, corresponding to the

upstream (primers P15/P16_pbpD) and downstream region (primers P13/ P14_pbpD) regions of the *pbpD* gene. The two fragments were joined by overlap PCR using primers P13 and P16. The resulting PCR product was digested with BglII and NcoI and cloned into pMAD vector, originating plasmid p∆*pbpD*.

To produce a strain expressing PBP4 fused to YFP, we amplified by PCR a 1.3Kb DNA fragment from *S. aureus* NCTC8325-4 chromosomal DNA, containing a full copy of the *pbpD* gene (which encodes for PBP4), using primers P19 and P20. The fragment was digested with *Kpn*I and cloned into pMutinYFPKan (see below) upstream of and in frame with the *yfp* gene. After confirming the correct orientation of the insert, the resulting plasmid, named p*pbpDyfp*, was sequenced and electroporated into RN4220 and RN*∆tagO,* where it integrated into the chromosome, and transduced into RN*∆dltABCD.* As a result, the *pbpD-yfp* fusion was placed under the control of the native *pbpD* promoter, while the native *pbpD* gene was placed under the control of the P*spac* promoter.

To construct pMutinYFPKan, the erythromycin resistance cassette of pMutinYFP ([34], was replaced by a kanamycin resistance cassette obtained from pDG792 plasmid [35]. For that purpose we used primers P25 and P26 to amplify, by PCR, the entire pMutinYFP plasmid, excluding the erythromycin marker, and the resulting product was digested with *Nco*I and *Bgl*II. Plasmid pDG792 was also digested with NcoI and BglII, and a 1.5kb band containing the kanamycin resistance marker was isolated, ligated with the previous PCR product and used to transform DH5*α*,

resulting in plasmid pMutinYFPKan. To generate a strain expressing both PBP4 fused to mCherry [a red fluorescent protein isolated by Shaner et al [40]] and TagO-GFP fusions, plasmid pBCB7-CHKPBP4 [33], was electroporated into RNTagO^{wt}GFP, where it integrated into the chromosome, at the *pbpD* locus.

Table 2 Primers used in this study

Primer	Sequence
P1	Tggagatctcgagtgagaagaaatgcc
P2	Cagctatgctttcattccctattattcaccttcatcgatatt
P3	Aatatcgatgaaggtgaataatagggaatgaaagcatagctg
P4	Catgccatggcaaacgatttatagtcatgtc
P5	Gcgagatctaccactagctattgtaagtg
P6	Gcgaattcctattcctctttatgagatga
P7	Cgtacttgggcttgtagctgatatctacgatt
P8	Aatcgtagatatcagctacaagcccaagtacg
P9	Cgtacttgggcttgtagatgctatctacgatt
P10	Aatcgtagatagcatctacaagcccaagtacg
P11	Cgtacttgggcttgtagctgctatctacgatt
P12	Aatcgtagatagcagctacaagcccaagtacg
P13	Gcaattaacttaattgatgctctcgatggtttgg
P14	Ccaaaccatcgagagcatcaattaagttaattgc
P15	Ggtttttattttacgctttccatcctgc
P16	Gcaggatggaaagcgtaaaataaaaacc
P17	Ggggtaccatggttacattattactag
P18	Ccgctcgagttcctctttatgagatgac
P19	Cgcggtaccggaaaagggaagattaacgc
P20	Gctgcggtaccggaggcgccgcaggattttctttttctaaataaacg
P21	Gcgagatctgagaaatatacgaattgtggcg
P22	Gggaagattaacgcttttaaaacatactaaaaacgg

P23	Ccgtttttagtatgttttaaaagcgttaatcttccc
P24	Catgccatgggataccaccaaataatgcg
P25	gactacgccatgggttcatgtaatcactccttc
P26	gcgagatctggaaataattctatgagtcgc
P27	Acgttggatccggttacattattac
P28	Ctgcgaattcctattcctctttatg
P29	Agcgtggatccatggttacattattac
P30	Tcgtcgaattctcctctttatgag
P31	Cgcaagcttgatgaaaaatttaatatctatta
P32	Ctcggtacccgttttctttttctaaataaacg
P33	Gcagatctgaatgtatatatttgcgctgatg
P34	gttgagttatgtgctatttgtattattaagtctccctcattagaactc
P35	gagttctaatgagggagacttaataatacaaatagcacataactcaac
P36	Gcgaattctcatctctcgaaaggagacttgc

Wall teichoic acids analysis. WTAs were extracted by alkaline hydrolysis from overnight cultures, analyzed by native polyacrylamide gel electrophoresis and visualized by combined alcian blue silver staining, as previously described [41]. ImageJ software was used to quantify the percentage of WTAs produced by each strain [42]. The signal intensity of each lane was quantified and normalized against the corresponding value for the wild type (considered as 100%).

Peptidoglycan purification and analysis. PGN from NCTC8325-4, NCTCΔ*tagO* and NCTCΔ*pbpD* was prepared from exponentially growing cells as previously described [43]. The purified PGN was digested with mutanolysin (Sigma), an *N*-acetylmuramidase that cuts glycan strands between the *N*-acetylmuramic and *N*-acetylglucosamine residues of both

O-acetylated and unmodified PGN. The resulting muropeptides were reduced with sodium borohydride (Sigma) and analyzed by reverse-phase HPLC using a Hypersil ODS column (Thermo Electron Corporation). The eluted muropeptides were detected and quantified by determination of their UV absorption at 206 nm, using the Shimadzu LC solution software.

For measurement of lysozyme susceptibility the purified PGN was incubated in 80mM NaOH at 37ºC for 3 hours, resuspended in 80mM sodium phosphate buffer/ 0.85% NaCl (pH 6.5) to an optical density of 1.0 and digested with 300 μg/ml lysozyme. The decrease in the absorbance at OD_{600} nm was monitored at 30-minute intervals for 400 minutes.

Purification and analysis of Membrane Peptidoglycan Precursors. The isolation and analysis of the lipid-linked peptidoglycan precursors were carried out by adapting a procedure previously used for *Streptococcus pneumonia* [44]. Cultures (1L) were grown until mid-exponential phase (OD_{600} of 0.5-0.6). An additional culture of the parental strain NCTC8325-4 was incubated with vancomycin (Sigma-Aldrich) at a final concentration of 10 μg/ml (10 x minimum inhibitory concentration) for 30 minutes to induce the accumulation of lipid II (positive control). Immediately after harvesting the cells (centrifugation at 11,000 x g for 10 minutes at 4ºC), bacteria were washed in ice-cold Tris buffer (50 mM Tris-Hcl, pH 8) and then with 2 ml of H_2O. Cells were resuspended in 1.5 ml butanol/6M pyridine-acetate buffer (pH 6) and disrupted with glass beads in a FastPrep® FP120 cell disrupter apparatus (Thermo Electron Corporation). Broken cells and glass beads were removed by centrifugation at 16,100 x g for 10 min at 4ºC. The supernatant,

corresponding to the organic phase containing the lipid-linked precursors, was collected and was washed three times with an equal volume of H_2O. The organic solvent was full evaporated under vacuum in a Büchi Rotavapor R-114, and the pellet was resuspended in 120 µl 0.1 M HCl. Incubation at 95ºC resulted in the acidic hydrolysis of the lipid II and the extraction into the aqueous phase of the GlcNAc-MurNAc-peptide previously linked to the bactoprenol molecule. This muropeptide was then reduced with 10 µl sodium borohydride (50 mg/ml) in 0.5 M borate buffer pH 9 and analyzed by HPLC in a Cromolith RP-8 column (Merck) using a 3 mL linear gradient from 0.05% of trifluoracetic acid (TFA) to a 0.05% TFA buffer with 10% acetonitrile for 26 min.

Detection of PBPs. Membrane protein extracts were prepared from exponentially growing cells as previously described [28]. Membranes (100 µg) were labeled with 100 µM Bocillin-FL (Molecular Probes) for 10 min at 30ºC and the reaction was stopped by adding 5X SDS-PAGE sample buffer (500mM DTT; 10% SDS; 250mMTris-HCL pH 6.8; 30% glycerol; 0.02% bromophenol blue). Samples were separated on 10% SDS-PAGE and the labeled proteins were detected using a 532 nm laser in a Fuji FLA-5100 reader. For western blot analysis, membrane proteins were separated by SDS-PAGE, transferred onto a PDVF membrane (GE) and immunostained using specific anti-PBP4 rabbit antibodies.

Fluorescence microscopy. *S. aureus* strains were grown to mid-exponential phase and observed by fluorescence microscopy on a thin layer of 1% agarose in phosphate buffered saline. When necessary, cells

were stained with membrane dye Nile Red (3 ug/ml; Molecular Probes). Images were obtained using a Zeiss Axio Observer.Z1 microscope equipped with a Photometrics CoolSNAP HQ2 camera (Roper Scientific), using Metamorph software (Meta Imaging series 7.5) and analyzed using Image J software [42]. Fluorescence ratio (FR) was determined by quantifying the fluorescence at the center of the division septa (only cells with closed septa were considered for this analysis) divided by the fluorescence at the lateral wall. Average background fluorescence was subtracted from both values. Quantification was performed in at least 100 cells with complete septa for each strain. The percentage of PBP4 localized at the division septa (Figure 4C) was calculated from the ratio of the FR value obtained for each RNΔ*tagO*PBP4YFPp*tagO*^{mut} strain divided by the FR value obtained for RNΔ*tagO*PBP4YFPp*tagO*^{wt}.

Bacterial-Two Hybrid studies. Plasmids and strains used in the bacterial-two hybrid studies are described in Table 3. The *tagO* and *pbpD* genes were amplified from *S. aureus* NCTC8325-4 genomic DNA by PCR. Primers P27 and P28, and P29 and P30 were used to PCR amplify *tagO* and the resulting products were fused in-frame to the 3′ or 5′ end of the *cyaA* gene fragments in the plasmids pUT18c and pKT25 or pUT18 and pKNT25 [45], respectively. PBP4 is a membrane-associated protein with a cytoplasmatic C-terminal region and an external N-terminal region. Therefore the *pbpD* gene was only fused to the 3′ end of the *cyaA* gene in plasmids pUT18c and pKT25, using PCR primers P31 and P32, in order to make a functional fusion. The resulting plasmids produce the following

fusion proteins: T25-TagO, TagO-T25, T18-TagO, TagO-T18, PBP4-T25 and PBP4-T18.

Table 3. Strains and plasmids used in the bacterial two-hybrid studies

Strain/Plasmid	Description	Origin/reference
Strain		
E. coli DH5α	Cloning strain	Lab stock
E. coli BTH101	Reporter strain for BTH system; *cya*$^-$	[45]
Plasmid		
pKT25	BTH plasmid; N-term *cyaA*T25 fusion; Kan^r	[45]
pKNT25	BTH plasmid; C-term *cyaA*T25 fusion; Kan^r	[45]
pUT18	BTH plasmid; C-term *cyaA*T18 fusion; Amp^r	[45]
pUT18c	BTH plasmid; N-term *cyaA*T18 fusion; Amp^r	[45]
p25Zip	BTH control plasmid; Kan^r	[45]
p18Zip	BTH control plasmid; Amp^r	*[45]*
p25TagO	BTH plasmid containing *cyaA*T25-*tagO* fusion	This work
pN25TagO	BTH plasmid containing *tagO-cyaA*T25 fusion	This work
p18TagO	BTH plasmid containing *tagO-cyaA*T18 fusion	This work
p18cTagO	BTH plasmid containing *cyaA*T18-*tagO* fusion	This work
p25PBP4	BTH plasmid containing *pbpD-cyaA*T25 fusion	This work
p18PBP4	BTH plasmid containing *pbpD-cyaA*T18 fusion	This work

All combinations of these fusions were cotransformed into the reporter strain *E. coli* BTH101. Cotransformants were grown on Luria-Bertani (LB) agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, 40 μg/ml), 100 μg/ml Amp and 50 μg/ml Kan or on MacConkey agar plates supplemented with 1% maltose, 100 μg/ml Amp and 50 μg/ml Kan in order to qualitatively detect the production of adenylate cyclase from the *cya*-deficient *E. coli* strain BTH101 transformants. Plasmids p18Zip and

p25Zip, which contain two leucine zipper domains, were also cotransformed into *E. coli* strain BTH101 and used as positive control. The β-galactosidase activity was measured essentially as previously described [45] using cell extracts from liquid cultures grown in LB medium in the presence of 0.5 mM (isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 h at 30ºC.

RESULTS

TagO null mutants have decreased levels of PGN cross-linking

To test whether the presence of WTAs is required to build a PGN macromolecule with the correct structure, we deleted the *tagO* gene from the chromosome of *S. aureus* NCTC8325-4 strain, leaving no antibiotic resistance markers, to produce the strain NCTCΔ*tagO*. TagO is the first enzyme in WTAs biosynthetic pathway, catalyzing the transfer of GlcNAc-1-phosphate from cytoplasmic UDP-linked precursors to the C55-P lipid anchor bactoprenol [46]. NCTCΔ*tagO* did not produce detectable levels of WTAs (Figure 1B) and showed several phenotypes previously described for staphylococcal mutants impaired in WTAs biosynthesis [4] such as larger cell diameter than wild type cells, aggregation in clusters (Figure 1A), temperature sensitivity and resistance to infection by phage 80α.

As a first approach to detect changes in PGN structure due to the deletion of *tagO,* we purified PGN from NCTCΔ*tagO* and its parental strain NCTC8325-4 and tested its susceptibility to the action of lysozyme,

an enzyme that cuts PGN between the *N*-acetylmuramic acid and *N*-acetylglucosamine residues of the glycan chains.

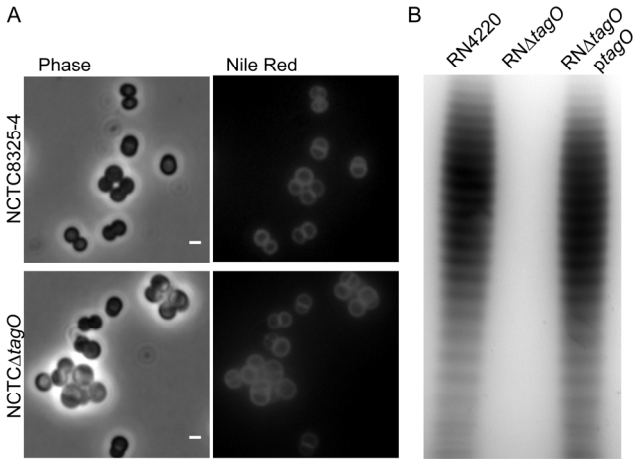


Figure 1. Cell morphology and wall teichoic acid production is altered in *S. aureus* strains lacking *tagO*. (A) Exponentially growing cells of parental strain NCTC8325-4 and NCTCΔ*tagO* were imaged under phase contrast (Phase) and stained with membrane dye (Nile Red). NCTCΔ*tagO* mutant strain is characterized by the presence of larger cells that aggregate in clusters. Scale bar: 1 μm. (B) WTAs were isolated from the transformable *S. aureus* RN4220 and its derivative strains, RNΔ*tagO* and RNΔ*tagO*p*tagO*, and analyzed by native PAGE stained with alcian blue/silver stain. The *tagO* null mutant does not produce detectable levels of WTAs, which are present in the parental strain RN4220 and in RNΔ*tagO*p*tagO* (*tag*O null mutant complemented with *tagO* expressed from a plasmid).

S. aureus is known for its intrinsic ability to resist lysozyme due to modifications of its PGN, such as *O*-acetylation of *N*-acetylmuramic acid residues and attachment of WTAs, which prevent access of the enzyme to

its substrate [10, 47]. We treated cell walls from both the wild type and the TagO mutant with hydrofluoric acid, which removes WTAs and *O*-acetyl groups, and incubated the resulting "naked" PGN with lysozyme. The PGN of NCTCΔ*tagO* was more susceptible to lysozyme than that of NCTC8325-4 (Figure 2D), suggesting differences in PGN structure between the two strains.

The PGN muropeptide composition of NCTCΔ*tagO* and NCTC8325-4 was then analyzed by HPLC, and revealed a reduced level of PGN cross-linking in NCTCΔ*tagO* when compared to the parental strain NCTC8325-4, with the concomitant accumulation of monomeric and dimeric muropeptides (Figure 2A and 3). This result suggests that WTAs may be involved in the later stages of PGN maturation, which include the introduction of extra cross-links between the glycan strands.

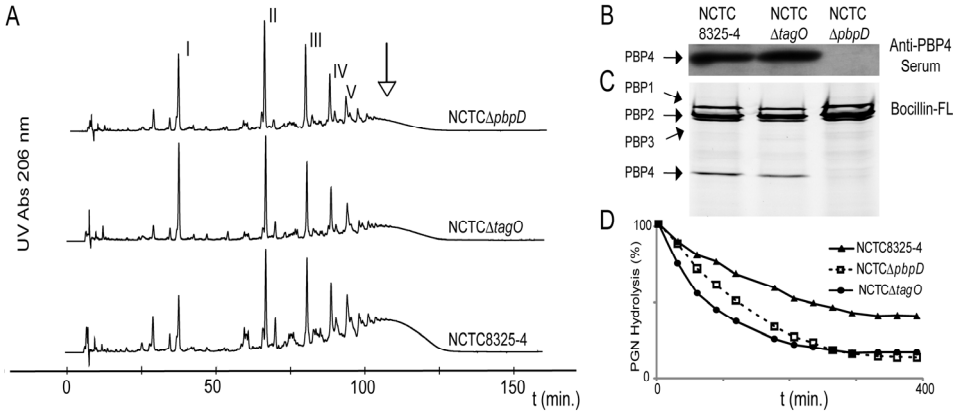
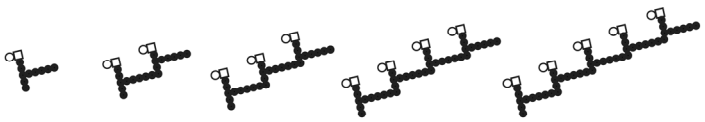


Figure 2. Highly cross-linked muropeptides, resulting from PBP4 activity, are less abundant in the PGN of a *ΔtagO* mutant. (A) HPLC analysis of mutanolysin-digested PGN of the parental strain NCTC8325-4 and mutants NCTCΔ*tagO* and NCTCΔ*pbpD*. Arrow points to highly cross-linked muropeptide species, which are less abundant in the mutants lacking TagO and PBP4. (I to V)

Muropeptide species from monomers to pentamers. **(B)** Western blot analysis, using a specific anti-PBP4 antibody, of membrane proteins from the same three strains showing that PBP4 is expressed at wild type levels in the NCTCΔ*tagO* background. **(C)** Analysis of membrane proteins labeled with Bocillin-FL and separated by SDS-PAGE, showing that PBP4 is able to bind Bocillin-FL in the NCTCΔ*tagO* background. **(D)** Lysozyme hydrolysis of purified PGN from NCTC8325-4, NCTCΔ*tagO* and NCTCΔ*pbpD*, followed by monitoring the decrease in absorbance at OD_{600nm}, and showing that PGN with lower degree of cross-linking had an increased susceptibility to lysozyme.

The secondary (high-level) cross-linking of *S. aureus* results mainly from the activity of PBP4, as inactivation of *pbpD* gene, encoding PBP4, leads to the disappearance of the highly cross-linked muropeptide species [24, 25] that typically elute as a broad peak at the end of the HPLC chromatogram (Figure 2A, arrow). Interestingly, the composition of PGN purified from NCTCΔ*tagO* was very similar to that of the PGN from NCTCΔ*pbpD* (Figure 2A, Figure 3).



	Monomer (I)	Dimer (II)	Trimer (III)	Tetramer (IV)	Pentamer (V)	> Pentamer (> V)
NCTCΔ*pbpD*	9,5	12,0	12,4	9,1	7,7	49,3
NCTCΔ*tagO*	10,5	9,5	9,3	7,2	7,3	56,2
NCTC8325-4	3,3	5,6	7,5	7,5	6,8	69,2

Figure 3. PGN composition is altered in *S. aureus* strains lacking *tagO* and *pbpD*. Mutanolysin-digested PGN of the parental strain NCTC8325-4 and mutants NCTCΔ*tagO* and NCTCΔ*pbpD* was analyzed by HPLC (also Fig 2). The

area of eluted UV-absorbing peaks, corresponding to the different muropeptides, was quantified and is shown as a percentage of the total identified peaks. A schematic representation of the composition of the different muropeptides is also shown: GlcNAc (open circles), MurNAc (open squares), aminoacids (closed circles).

Decreased levels of PGN cross-linking in TagO null mutant result from delocalization of PBP4

This observation led us to test if PBP4 was altered in the Δ*tagO* null mutant. Sequencing of the *pbpD* gene (including its promoter region) in NCTCΔ*tagO* did not identify any mutations and Western blot analysis with specific anti-PBP4 antibodies showed that levels of PBP4 were identical in NCTCΔ*tagO* and in its parental strain NCTC8325-4 (Figure 2B). Furthermore, binding of Bocillin-FL (a fluorescent derivative of penicillin V and a substrate analog for PBPs) to the staphylococcal PBPs showed that the binding of PBP4 to this substrate analog was not altered in NCTCΔ*tagO* cells when compared to the parental strain NCTC8325-4 (Figure 2C).

We have previously shown that inhibition of cell wall synthesis in *S. aureus* by β-lactam antibiotics results in delocalization of PBP2 [48]. Therefore, we decided to test whether the lack of high-level PGN cross-linking in the Δ*tagO* background was the result of incorrect localization of PBP4. For that purpose we constructed *S. aureus* RN4220 strains expressing a C-terminal YFP fusion to PBP4 from its native chromosomal locus and under the control of its native promoter. RN4220 background was used because it is the only *S. aureus* strain that can be efficiently

transformed with foreign DNA. The transformation efficiency of NCTC8325-4 is extremely low, and the Δ*tagO* mutation renders it resistant to phages such as 80α, preventing transduction, and impairing the introduction of additional constructs into this background. When the PBP4-YFP fusion was expressed in the RN4220 parental background (RNPBP4YFP), it localized to the division septum (Figure 4), where cell wall synthesis has been reported to take place in *S. aureus* [49].

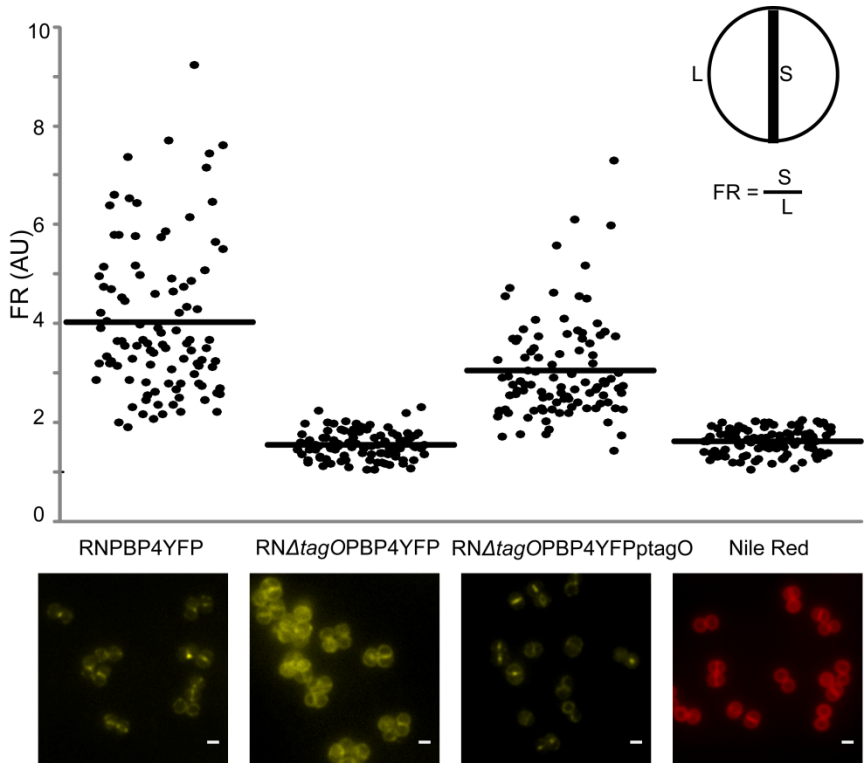


Figure 4. Septal localization of PBP4 is lost in a *tagO* null mutant. Microscopy images and quantification of septum versus lateral membrane fluorescence (fluorescence ratio, FR) of PBP4-YFP in a wild type background (RNPBP4YFP), a Δ*tagO* background (RNΔ*tagO*PBP4YFP), and a Δ*tagO* mutant complemented with

plasmid-encoded *tagO* (RNΔ*tagO*PBP4YFPp*tagO*). Also shown are RNPBP4YFP cells labelled with membrane dye Nile Red, which is homogeneously distributed in the cell membrane. Quantification was performed with 100 cells displaying closed septa for each strain. Horizontal lines correspond to average FR values. FR values over 2 indicate preferential septal localization whereas FR values equal to or under 2 indicate that a protein is dispersed over the cell surface. *p* values $<10^{-7}$. Scale bar: 1μm.

However, when the same fusion was expressed in the Δ*tagO* background (RNΔ*tagO*PBP4YFP), PBP4 was observed all around the cellular membrane, with no specific accumulation at the division septum (Figure 4). This effect was not general to all staphylococcal PBPs, as PBP1 did not lose its septal localization in a Δ*tagO* background (Figure 5).

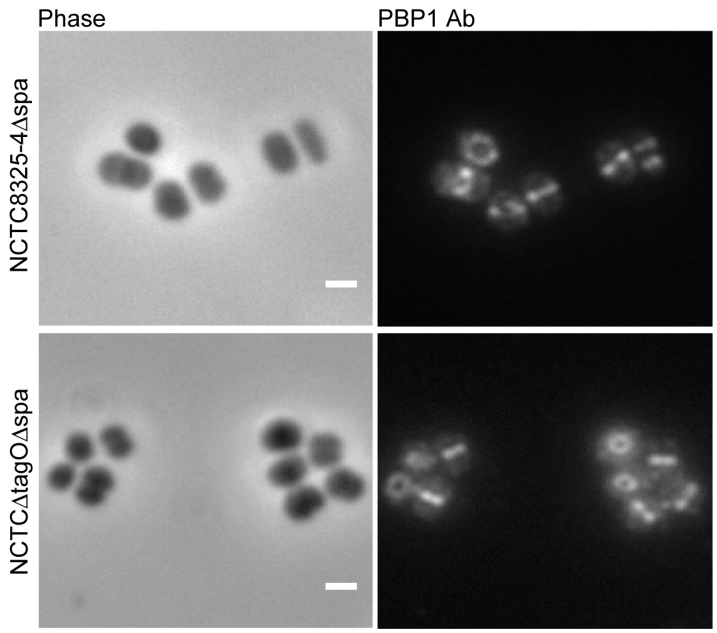


Figure 5: **PBP1 localizes to the division septa in the absence of the TagO protein.** PBP1 localization by immunofluorescence was performed as previously

described [49] with NCTC8325-4Δ*spa* (*Upper Left* and *Right*) and NCTCΔ*tagO*Δ*spa* (*Lower Left* and *Right*), using a specific anti-PBP1 antibody. (*Left*)Phase contrast images. (*Right*) Fluorescence images. Fluorescent images show PBP1 localized to the septum (rings or lines across the cells) in both wild type and the TagO mutant. Scale bar: 1 μm.

To quantify the extent of delocalization of PBP4 in the absence of the TagO protein, we calculated the ratio of fluorescence measured at the septum versus the fluorescence measured at the "lateral" wall (Figure 4). If a fluorescent protein or dye (such as Nile Red membrane stain) is homogeneously distributed over the entire cell membrane, the intensity of the fluorescent signal at the septum (which contains two membranes) should be approximately twice the fluorescence at the lateral membrane. It follows that, if a fluorescent protein is specifically accumulated at the division septum, then the ratio of fluorescence at the septum versus the lateral wall should be higher than two. When this ratio was calculated for PBP4-YFP in the parental strain RNPBP4YFP, we obtained an average value of 4.0±1.52 whereas a value of 1.6±0.27 was obtained for the Δ*tagO* mutant RNΔ*tagO*PBP4YFP, indicating that the specific accumulation of PBP4 at the septum in wild-type cells was completely lost if the TagO protein was absent. Furthermore, complementation of RNΔ*tagO*PBP4YFP, with plasmid encoded TagO (but not with the empty plasmid vector), resulted in the correct localization of PBP4 at the septum (Figure 4).

PBP4 is recruited to the division septum later than TagO

The simplest explanation for the dependence of PBP4 on TagO for septal localization would be that TagO localizes to the division septum and recruits PBP4 by protein-protein interaction. In accordance, we found that a TagO-GFP fusion localized at the division septum (Figure 6A and 8D). To study the dynamics of PBP4 and TagO recruitment to the septum, namely to determine if they arrived at the septum at the same time, we constructed strain RNTagOPBP4, which expressing both TagO-GFP and PBP4-mCherry fusion proteins.

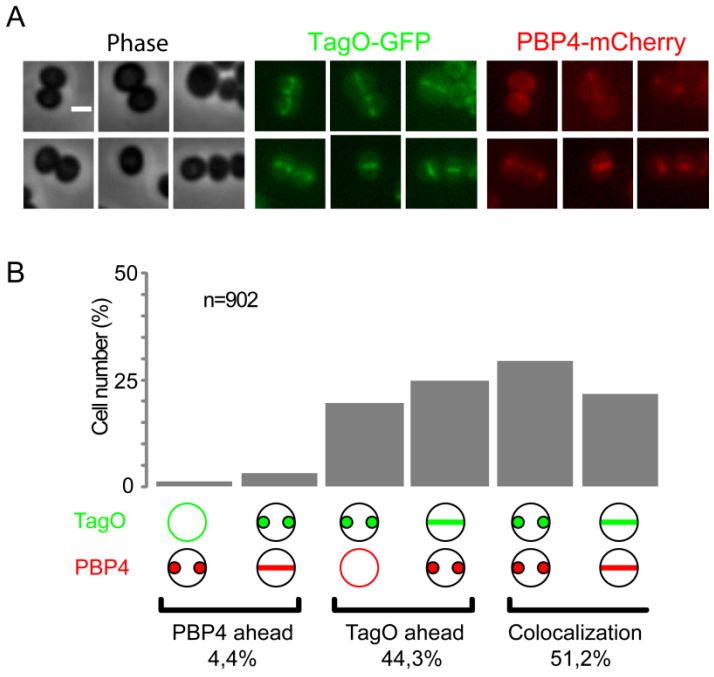


Figure 6: TagO protein is recruited to the division septum before PBP4. (A) The strain RNTagOPBP4, expressing simultaneously TagO-GFP and PBP4-mCherry protein fusions, was analyzed by fluorescence microscopy. (*Top*) Results showing

that TagO reaches the division septum before PBP4 in several cells. (*Bottom*) Cells in which PBP4 and TagO colocalize at the septum. Scale bar: 1 μm. **(B)** Localization of TagO-GFP and PBP4-mCherry was analyzed in 902 cells in early stages of septum formation. Localization of each protein was assigned to three sequential stages: scattering around the entire membrane; localization in a ring around the division plane, usually seen as two spots; and localization over the entire closed septum, usually seen as a line across the cell. In 44% of the cells, TagO was found at the septum "ahead" of PBP4, meaning that either TagO is already at the septum, seen as two spots, whereas PBP4 is still scattered around the cell membrane or TagO is already across the entire septum whereas PBP4 is still in a ring around the division septum.

We analyzed over 3,000 cells, and determined the localization of TagO and PBP4 in approximately 900 cells that were in the initial stages of septum synthesis. In this subpopulation, TagO arrived at the septum before PBP4 (TagO was seen as two septal spots corresponding to a septal ring while PBP4 was not yet present at the septum) or was found "ahead" of PBP4 (TagO found across the entire septum whereas PBP4 was still seen as two septal spots) in 44.3% of the cells (Figure 6B).

PBP4 arrived at the septum before TagO or was found ahead of TagO in only 4.4% of the cells. In 51.2% of the cells, both proteins had the same localization. The fact that TagO and PBP4 recruitment to the septum occurs at different times suggests that PBP4 is not recruited to the septum by direct protein-protein interaction with TagO. Further indication came from bacterial two-hybrid system [50] studies, which failed to detect any interaction between PBP4 and TagO (Figure 7).

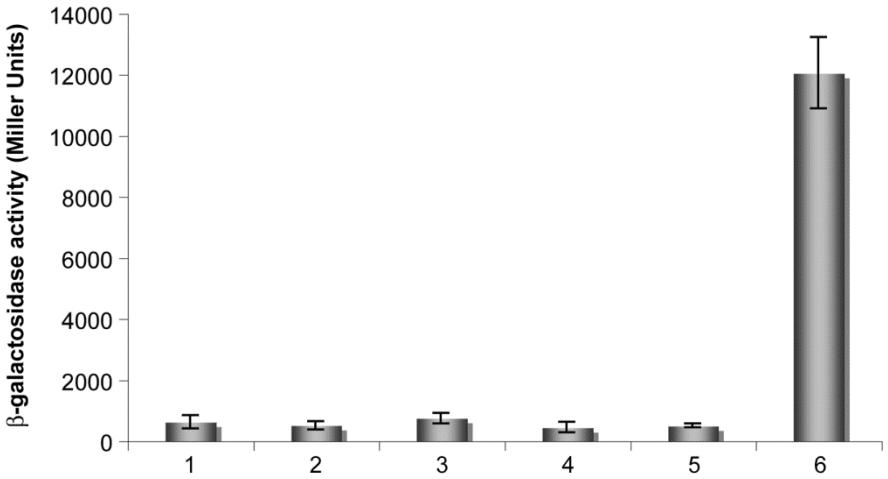


Figure 7. TagO and PBP4 do not interact in bacterial two-hybrid assays. β-Galactosidase activity was determined using cell extracts of *E. Coli* BTH101 strains transformed with (1) p18cTagO and p25PBP4, (2) p18TagO and p25PBP4, (3) p25TagO and p18PBP4, (4) pN25TagO and p18PBP4, (5) pUT18c and pKt25, and (6) p18Zip and p25Zip. Results show the average of three independent experiments and error bars indicate the SD. The results indicate that a direct interaction between TagO and PBP4 was not detected.

Synthesis of WTAs, and not TagO protein itself, is required for PBP4 recruitment to the division septum

To elucidate whether it was the presence of the TagO protein at the septum or the activity of TagO protein (also implying the presence of WTAs) at the septum that was required for PBP4 recruitment, we constructed a series of strains expressing TagO proteins with single amino acid changes, with the aim of selecting mutants with loss of TagO activity (i.e., lack of WTAs production), but with the ability to correctly localize at the septum when fused to GFP protein (suggesting that the protein may

be correctly folded). Four conserved residues of TagO (D87, D88, G152, N198) were individually substituted with alanine residues, and a double mutant in which D87 and D88 were simultaneously substituted with alanines was also constructed. The different TagO proteins (wild-type and mutants) were expressed from the replicative pMAD plasmid [36], under the control of the native *tagO* promoter in the RNΔ*tagO* background and tested for their ability to catalyze WTAs synthesis (Figure 8B). Expression of $TagO^{D87A}$ and $TagO^{D87A/D88A}$ did not result in the production of detectable amounts of WTAs; expression of $TagO^{D88A}$ and $TagO^{G152A}$ led to the production of significantly reduced amounts of WTAs (less than 25% of wild type levels); and expression of $TagO^{N198A}$ result in a small decrease in the amount of WTAs produced when compared to expression of wild-type TagO protein (74% of wild type levels). The localization of PBP4-YFP was then determined in cells expressing either $TagO^{wt}$ or the different TagO mutants, and we found that PBP4 was unable to localize correctly at the division septum in the four mutants with significantly reduced levels of WTAs (Figure 8A). Moreover, there is a direct correlation between the amount of WTAs production and the fraction of PBP4 recruited to the division septum (Figure 8C).

It was possible that PBP4 delocalization in strains expressing TagO proteins with lower or no activity was not due to the lack of WTAs at the septum, but to the fact that mutated TagO proteins were degraded or had lost their septal localization. We therefore selected $TagO^{G152A}$ for further localization studies, as it was still able produce small amounts of WTAs, implying that the protein was probably correctly folded. However, $TagO^{G152A}$ showed some of the phenotypes characteristic of *tagO* mutants

A
B
C
D
RNAtagO
PPPAYEP btagO
PPPAYEP btagO D87A
PPPAYEP btagO D88A
PPPAYEP btagO D88A/D87A
PPPAYEP btagO G125A
PPPAYEP btagO N189A
FL (UA)
0
5
10
wt
D87A
D88A
D88A\D87A
G125A
N189A
ATW %
GBP-AEP %
RNTagO wt GFP
RNTagO G125A GFP

Figure 8. Synthesis of WTAs, and not TagO protein itself, is required for PBP4 recruitment to the division septum. (A) Quantification of septum versus lateral membrane fluorescence (fluorescence ratios, FR) and fluorescence microscopy images for PBP4-YFP protein fusion in Δ*tagO* mutants complemented with wild-type TagO protein (RNΔ*tagO*PBP4YFPp*tagO*^{wt}) or with different TagO mutants (RNΔ*tagO*PBP4YFPp*tagO*$^{D87A,\ D88A,\ D87A/D88A,\ G152A\ and\ N198A}$). Quantification was performed upon 100 cells displaying closed septa for each strain. Horizontal lines correspond to average FR values. FR values over 2 indicate septal localization, and FR values equal to or under 2 indicate that a protein is dispersed over the cell surface. *p* values < 10^{-7}. Scale bar: 1 μm. **(B)** WTAs were isolated from RNΔ*tagO*p*tagO*, RNΔ*tagO*p*tagO*$^{D87A,\ D88A,\ D87A/D88A,\ G152A\ and\ N198A}$ and analyzed by native PAGE stained with alcian blue/silver stain. Mutations of the aspartic acids and glycine residues lead to a decrease or absence of the WTAs. **(C)** Comparison between the levels of WTA and the degree of PBP4 localization to the division septa (calculated as described in *Material and Methods*) indicates a strong correlation between the amount of WTA present in the cell and the ability of PBP4 to localize at the septum. (D) Fluorescence microscopy images of RNTagO^{wt}GFP and RNTagO^{G152A}GFP showing that the TagO^{G152A}-GFP fusion localizes to the division septum, similarly to the GFP fusion to the wild-type TagO protein. Scale: 1 μm.

such as phage resistance, decreased cross-linking, or cell clustering (Figure 9), implying that the amount of WTAs produced was not sufficient to fully complement the phenotype of RNΔ*tagO*. When TagO^{G152A} was fused to GFP and expressed in RN4220, the protein correctly localized to the septum, similarly to TagO^{wt} (Figure 8D). Furthermore, introduction of G152A mutation in TagO-GFP did not result in reduced levels of expression of the fluorescent protein (Figure 9). The fact that TagO^{G152A}

had lower activity but maintained correct folding and localization, and was unable to recruit PBP4 to the septum, strongly suggests that the presence of WTA, and not the presence of the TagO protein itself, was required for PBP4 localization.

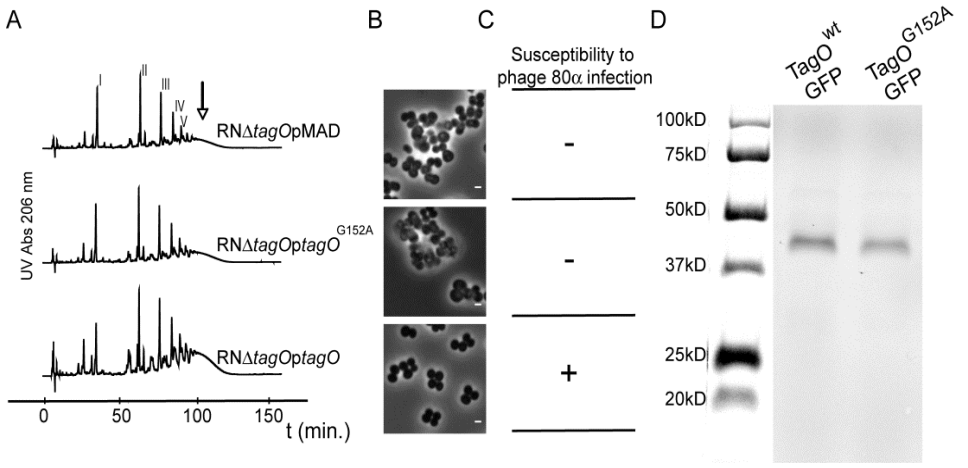


Figure 9. TagO^{G152A} is expressed at wild-type levels, but it is not fully functional. (A) HPLC analysis of mutanolysin-digested PGN of the RNΔ*tagO* mutant strain complemented with the empty pMAD vector or with pMAD encoding TagO or TagO^{G152A}. Arrow points to highly cross-linked muropeptides species, wich are less abundant in the mutants lacking TagO or expressing the non-functional TagO^{G152A}. (I-V) Muropeptide species from monomers to pentamers. **(B)** Exponentially growing cells of the threes strains mentioned above were imaged under phase contrast. RNΔ*tagO*pMAD mutant strain is characterized by the presence of larger cells and clusters of aggregated cells. These phenotypes are complemented by the presence of the wild-type TagO protein but not of the TagO^{G152A} protein. Scale bar: 1 μm. **(C)** The three strains mentioned above were incubated with phage 80*α*, which uses WTAs as a receptor for the infection process. Phage 80*α* infected RNΔ*tagOptagO* but not

RNΔ*tagO*pMAD or RNΔ*tagO*p *tagO*^{G152A}. **(D)** Total protein extract of cells expressing TagO^{WT}-GFP (RNTagO^{WT}GFP) and TagO^{G152A}-GFP (RNTagO^{G152A}GFP) were separated by SDS/PAGE (12%) and visualized using a 532-nm laser in a Fuji Fluorescent Analyser TLA-5100. TagO^{G152A}-GFP fusion protein is expressed at similar levels as TagO^{WT}-GFP.

DISCUSSION

Teichoic acids have been reported to be involved in cell growth, cell division and morphogenesis [4,5, 51], but their exact role in these processes remains unknown. In this study we show that WTAs have a fundamental role in PGN metabolism as they modulate the degree of cross-linking by temporally and spatially regulating the recruitment of PBP4 to the site of cell wall synthesis, the division septum.

The synthesis of PGN in *S. aureus* occurs mainly through the action of PBPs 1-4. PBP1 is a monofunctional transpeptidase, essential for cell viability and required for septation and cell separation at the end of cell division [52]. It localizes to the division septum through a mechanism that is independent of its ability to bind its substrate [53]. PBP2 is an essential bifunctional transglycosylase and transpeptidase that plays a central role in the ability of bacteria to express their resistance to antibiotics [54] and localizes to the division septum in a way that is dependent on its ability to recognize the translocated substrate [48]. PBP3 and PBP4 are non-essential, monofunctional transpeptidases whose localization has not yet been studied in detail [22].

We have shown here that in wild type cells PBP4 can be found at the septum of *S. aureus*, similarly to PBP1 and PBP2. However, in a different

way from these two proteins, recruitment of PBP4 to the septum is dependent upon the synthesis of WTAs. In *S. aureus* strains lacking TagO, the first enzyme in the teichoic acid biosynthesis pathway, PBP4 no longer accumulates specifically at the division septum, but instead is dispersed over the entire cell membrane. Concomitantly with PBP4 delocalization, the level of PGN cross-linking in Δ*tagO* mutants is severely decreased, a phenotype also observed by Schlag et al [55] while this manuscript was in preparation. Recruitment of PBP4 to the septum does not seem to occur via direct protein-protein interaction with TagO because (*i*) PBP4 and TagO do not interact in a bacterial two-hybrid screening, (ii) the two proteins do not colocalize in 49% of the cells in early stages of septum synthesis, and (iii) the presence of (inactive) TagO protein properly localized at the septum is not sufficient to keep PBP4 at that location. Instead, recruitment of PBP4 to the septum seems dependent on the septal synthesis of WTAs. If this synthesis is abolished, either by complete removal of TagO or by generating TagO point mutants, which lose their activity while maintaining correct localization at the septum, then PBP4 loses its septal localization and becomes unable to perform its function in the synthesis of highly cross-linked PGN. The fact that an intact PBP4 is unable to perform its function when incorrectly localized may be due to the substrate being found only at the septum or to the lateral PGN exhibiting a different structure when compared with the septal PGN, which may not allow the addition of further cross-links between the glycan strands.

On the basis of the results described in this work, we propose that teichoic acids function not only as a spatial cue, but also as a temporal

cue, for PBP4 recruitment to the division septum. Figure 10 illustrates a model in which the initial cell wall synthetic machinery is recruited to the division septum in early stages of its formation. TagO, and most likely the remaining enzymes involved in WTAs biosynthesis, are recruited to the septum and initiate WTAs biosynthesis, which functions as a temporal indication that early PGN biosynthesis is complete and that PGN can be further processed to become highly cross-linked. PBP4 (which, as we have shown, arrives at the septum later than TagO) is then recruited to the septum, where it takes over the last steps of PGN synthesis, performing the final weaving of the PGN mesh.

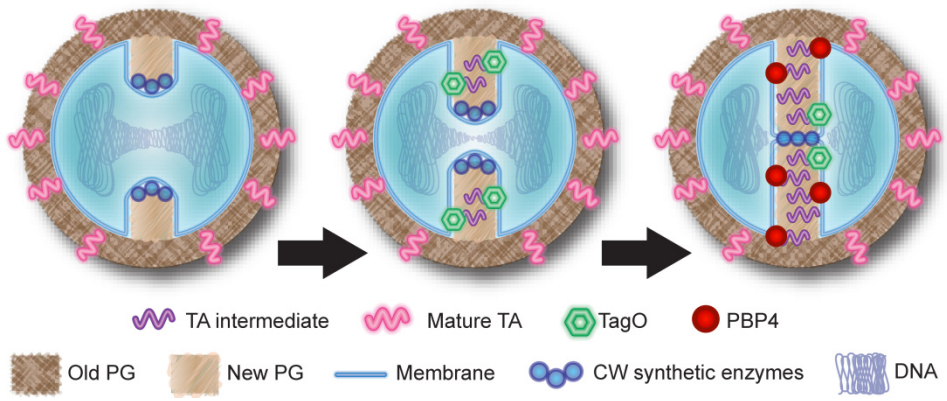


Figure 10. Model for the role of WTAs in PBP4 recruitment to the septum. The early cell wall synthetic machinery assembles at the division site, leading to the synthesis of new PGN, with low levels of crosslinking (left). TagO (together with other WTA synthetic enzymes) is recruited to the septum by an unknown mechanism, leading to the synthesis of intermediate molecules in TA biosynthesis (middle). These intermediates (or another cellular component dependent on TA biosynthesis) function as a temporal and spatial cue for PBP4 recruitment to the

division septum, allowing the synthesis of highly crosslinked PGN to occur in a regulated manner (right).

Importantly, it is likely that recruitment of PBP4 is not mediated by the fully synthesized/mature WTAs, which is present throughout entire surface of *S. aureus* (but may not yet be present at the septum), but rather by an immature form of WTAs corresponding to an intermediate of WTAs biosynthesis, which is only encountered at the septum. One hypothesis would be that addition of D-alanyl to the WTA backbone, catalyzed by the enzymes encoded in the *dltABCD* operon, would be essential for binding of PBP4 to the WTA because the natural substrate of PBP4 is the D-alanyl-D-alanine terminus of the peptidoglycan muropeptide precursor. However, we have deleted the *dltABCD* operon from RN4220 and shown that it has no significant effect on localization of PBP4 (Figure 11).

The fact that synthesis of WTAs and PGN share the same lipid carrier-bactoprenol-for their precursors led us to think of an alternative model for PBP4 delocalization.

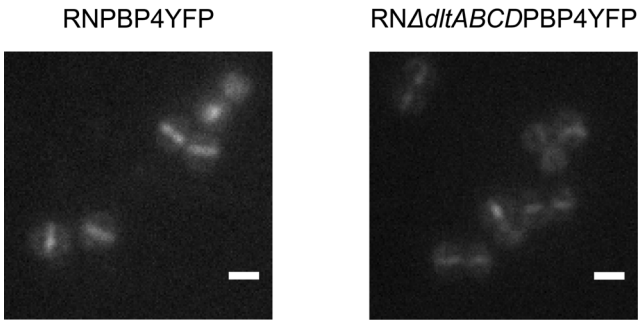


Figure 11. Septal localization of PBP4 is maintened in the Δ*dltABCD* null mutant. Fluorescence microscopy images showing the similar localization of

PBP4-YFP in a wild-type background (RNPBP4YFP) and a Δ*dltABCD* background (RNΔ*dltABCD*PBP4YFP. Scale bar: 1 μm.

Bactoprenol is usually found in limiting amounts in the cell. Therefore, inhibition of WTAs synthesis could increase the availability of bactoprenol for PGN synthesis, leading to an increase in the metabolic flux toward PGN synthesis. This could then result in the increased synthesis and possibly delocalization, of lipid II (bactoprenol linked to a dissacharide-pentapeptide with a pentaglycine crossbridge), the substrate of PBPs, which could be the driving force for PBP4 delocalization. We have tested this hypothesis by purifying the lipid-linked PGN precursors (Figure 12).

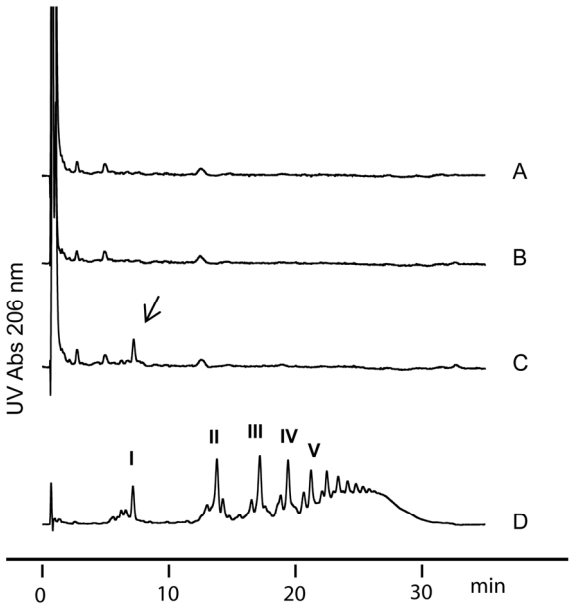


Figure 12. Composition of the lipid-linked precursors in *S. aureus* strains. PGN lipid-linked precursors are composed of a muropeptide connected to bactoprenol. The muropeptides from lipid-linked precursors of the parental NCTC8325-4 strain (*A*) and its *tagO* null mutant NCTCΔ*tagO* strain (*B*) were cleaved, purified, and analyzed by HPLC. No accumulation of cleaved muropeptide was observed,

indicating that there was no accumulation of lipid II in these strains. (*C*) The same procedure was applied to the parental NCTC8325-4 parental strain incubated with vancomycin, known to lead to lipid II accumulation. (*D*) Muropeptides were purified from PGN of the NCTC8325-4 parental strain to allow the identification of the monomeric muropeptides released from lipid precursors. (I-V) Muropeptide species from monomers to pentamers.

The fact that there was no detectable accumulation of lipid II in the *ΔtagO* mutant led us to rule out changes in lipid II concentrations as the cause of PBP4 delocalization. Therefore, although we cannot formally rule out the possibility that delocalization of PBP4 results not from the absence of WTAs intermediates, but from other cellular changes that are themselves caused by the depletion of WTAs intermediates, we currently favour the model depicted in Figure 10.

Interestingly, while this manuscript was in preparation, Schlag and colleagues [55], reported that WTAs are involved in targeting the bifunctional autolysin Atl to the septum. The authors propose an exclusion strategy in which mature WTAs, which are present throughout the mature cell wall but absent (or in lower concentration) at the septum, would prevent binding of Atl to the old cell wall but not to the septal region. Therefore, WTAs may play a key role not only in the regulation of the secondary cross-linking of PGN, but also in regulating the cleavage of the PGN macromolecule, coordinating (or temporally and spatially restricting) both its synthesis and degradation/autolysis.

Why does *S. aureus* require such fine-tuning of the level of cross-linking of its PGN? One possibility may be that careful regulation of the

timing of PGN cross-linking may be required to ensure the covalent attachment different molecules to the PGN. Delaying the production of highly cross-linked PGN would permit the introduction of bulky glycopolymers, such as WTAs or large proteins, through the assembled PGN. Afterward, staphylococcal cells would promote cross-linking of PGN to high levels, which, as we have shown, renders it more resistant to lysozyme, an enzyme produced by infected hosts as a defense against bacterial pathogens.

BIBLIOGRAPHY

1. Schleifer, K.H. and O. Kandler, *Peptidoglycan types of bacterial cell walls and their taxonomic implications.* Bacteriological reviews, 1972. **36**(4): p. 407-77.

2. Neuhaus, F.C. and J. Baddiley, *A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria.* Microbiol Mol Biol Rev, 2003. **67**(4): p. 686-723.

3. Weidenmaier, C. and A. Peschel, *Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions.* Nat Rev Micro, 2008. **6**(4): p. 276-287.

4. Vergara-Irigaray, M., T. Maira-Litrán, N. Merino, G.B. Pier, J.R. Penadés, and I. Lasa, *Wall teichoic acids are dispensable for anchoring the PNAG exopolysaccharide to the Staphylococcus aureus cell surface.* Microbiology (Reading, Engl), 2008. **154**(Pt 3): p. 865-877.

5. Oku, Y., K. Kurokawa, M. Matsuo, S. Yamada, B. Lee, and K. Sekimizu, *Pleiotropic roles of poly-glycerolphosphate synthase of lipoteichoic acid in the growth of Staphylococcus aureus cells.* J Bacteriol, 2009. **191**(1): p. 141-151.

6. Peschel, A., M. Otto, R.W. Jack, H. Kalbacher, G. Jung, and F. Götz, *Inactivation of the dlt operon in Staphylococcus aureus confers sensitivity to defensins, protegrins, and other antimicrobial peptides.* J Biol Chem, 1999. **274**(13): p. 8405-8410.

7. Kohler, T., C. Weidenmaier, and A. Peschel, *Wall teichoic acid protects Staphylococcus aureus against antimicrobial fatty acids from human skin.* J Bacteriol, 2009. **191**(13): p. 4482-4484.

8. Peschel, A., C. Vuong, M. Otto, and F. Götz, *The D-alanine residues of Staphylococcus aureus teichoic acids alter the susceptibility to vancomycin and the activity of autolytic enzymes.* Antimicrob Agents and Chemother, 2000. **44**(10): p. 2845-2847.

9. Collins, L.V., S.A. Kristian, C. Weidenmaier, M. Faigle, K.P.M. Van Kessel, J.A.G. Van Strijp, F. Götz, B. Neumeister, and A. Peschel, *Staphylococcus aureus strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice.* J Infect Dis, 2002. **186**(2): p. 214-219.

10. Bera, A., R. Biswas, S. Herbert, E. Kulauzovic, C. Weidenmaier, A.

Peschel, and F. Götz, *Influence of wall teichoic acid on lysozyme resistance in Staphylococcus aureus.* J Bacteriol, 2007. **189**(1): p. 280-283.

11. Chatterjee, A.N., *Use of bacteriophage-resistant mutants to study the nature of the bacteriophage receptor site of Staphylococcus aureus.* J Bacteriol, 1969. **98**(2): p. 519-27.

12. Heptinstall, S., A.R. Archibald, and J. Baddiley, *Teichoic acids and membrane function in bacteria.* Nature, 1970. **225**(5232): p. 519-521.

13. Xia, G., T. Kohler, and A. Peschel, *The wall teichoic acid and lipoteichoic acid polymers of Staphylococcus aureus.* Int J Med Microbiol, 2010. **300**(2-3): p. 148-154.

14. Schirner, K., J. Marles-Wright, R. Lewis, and J. Errington, *Distinct and essential morphogenic functions for wall- and lipo-teichoic acids in Bacillus subtilis.* EMBO J, 2009. **28**(7): p. 830-842.

15. D'Elia, M.A., K.E. Millar, T.J. Beveridge, and E.D. Brown, *Wall teichoic acid polymers are dispensable for cell viability in Bacillus subtilis.* J Bacteriol, 2006. **188**(23): p. 8313-8316.

16. Formstone, A., R. Carballido-López, P. Noirot, J. Errington, and D.-J. Scheffers, *Localization and interactions of teichoic acid synthetic enzymes in Bacillus subtilis.* J Bacteriol, 2008. **190**(5): p. 1812-1821.

17. Daniel, R.A. and J. Errington, *Control of cell morphogenesis in bacteria: two distinct ways to make a rod-shaped cell.* Cell, 2003. **113**(6): p. 767-776.

18. Fedtke, I., D. Mader, T. Kohler, H. Moll, G. Nicholson, R. Biswas, K. Henseler, F. Götz, U. Zähringer, and A. Peschel, *A Staphylococcus aureus ypfP mutant with strongly reduced lipoteichoic acid (LTA) content: LTA governs bacterial surface properties and autolysin activity.* Molecular Microbiology, 2007. **65**(4): p. 1078-91.

19. D'Elia, M.A., K.E. Millar, A.P. Bhavsar, A.M. Tomljenovic, B. Hutter, C. Schaab, G. Moreno-Hagelsieb, and E.D. Brown, *Probing teichoic acid genetics with bioactive molecules reveals new interactions among diverse processes in bacterial cell wall biogenesis.* Chem Biol, 2009. **16**(5): p. 548-556.

20. Foster, T.J., *Immune evasion by staphylococci.* Nat Rev Micro, 2005. **3**(12): p. 948-58.

21. de Lencastre, H., D. Oliveira, and A. Tomasz, *Antibiotic resistant Staphylococcus aureus: a paradigm of adaptive power.* Current Opinion in Microbiology, 2007. **10**(5): p. 428-35.

22. Scheffers, D.-J. and M.G. Pinho, *Bacterial cell wall synthesis: new insights from localization studies.* Microbiol Mol Biol Rev, 2005. **69**(4): p. 585-607.

23. Gally, D. and A.R. Archibald, *Cell wall assembly in Staphylococcus aureus: proposed absence of secondary crosslinking reactions.* J Gen Microbiol, 1993. **139**(8): p. 1907-13.

24. Łeski, T.A. and A. Tomasz, *Role of penicillin-binding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of Staphylococcus aureus: evidence for the cooperative functioning of PBP2, PBP4, and PBP2A.* J Bacteriol, 2005. **187**(5): p. 1815-1824.

25. Memmi, G., S.R. Filipe, M.G. Pinho, Z. Fu, and A. Cheung, *Staphylococcus aureus PBP4 is essential for beta-lactam resistance in community-acquired methicillin-resistant strains.* Antimicrobial Agents and Chemotherapy, 2008. **52**(11): p. 3955-3966.

26. Stranden, A.M., K. Ehlert, H. Labischinski, and B. Berger-Bachi, *Cell wall monoglycine cross-bridges and methicillin hypersusceptibility in a femAB null mutant of methicillin-resistant Staphylococcus aureus.* J Bacteriol, 1997. **179**(1): p. 9-16.

27. Sieradzki, K. and A. Tomasz, *Gradual alterations in cell wall structure and metabolism in vancomycin-resistant mutants of Staphylococcus aureus.* J Bacteriol, 1999. **181**(24): p. 7566-7570.

28. Sieradzki, K., M.G. Pinho, and A. Tomasz, *Inactivated pbp4 in highly glycopeptide-resistant laboratory mutants of Staphylococcus aureus.* J Biol Chem, 1999. **274**(27): p. 18942-6.

29. Sieradzki, K. and A. Tomasz, *Alterations of cell wall structure and metabolism accompany reduced susceptibility to vancomycin in an isogenic series of clinical isolates of Staphylococcus aureus.* J Bacteriol, 2003. **185**(24): p. 7103-7110.

30. Veiga, H. and M. Pinho, *Inactivation of the Sau1 Type I restriction-modification system is not sufficient to generate Staphylococcus aureus strains capable of efficiently accepting foreign DNA.* Appl Environ Microbiol, 2009. **75**(10): p. 3034-3038.

31. Arnaud, M., A. Chastanet, and M. Debarbouille, *New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria.* Appl Environ Microbiol, 2004. **70**(11): p. 6887-91.

32. Pinho, M.G. and J. Errington, *A divIVA null mutant of Staphylococcus aureus undergoes normal cell division.* FEMS Microbiol Lett, 2004. **240**(2): p. 145-9.

33. Pereira, P.M., H. Veiga, A.M. Jorge, and M.G. Pinho, *Fluorescent reporters for studies of cellular localization of proteins in Staphylococcus aureus.* Appl Environ Microbiol, 2010. **76**(13): p. 4346-53.

34. Kaltwasser, M., T. Wiegert, and W. Schumann, *Construction and application of epitope- and green fluorescent protein-tagging integration vectors for Bacillus subtilis.* Appl Environ Microbiol, 2002. **68**(5): p. 2624-8.

35. Guerout-Fleury, A.M., K. Shazand, N. Frandsen, and P. Stragier, *Antibiotic-resistance cassettes for Bacillus subtilis.* Gene, 1995. **167**(1-2): p. 335-6.

36. Arnaud, M., A. Chastanet, and M. Débarbouillé, *New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria.* Appl Environ Microbiol, 2004. **70**(11): p. 6887-6891.

37. Oshida, T. and A. Tomasz, *Isolation and characterization of a Tn551-autolysis mutant of Staphylococcus aureus.* J Bacteriol, 1992. **174**(15): p. 4952-9.

38. Pinho, M.G. and J. Errington, *A divIVA null mutant of Staphylococcus aureus undergoes normal cell division.* FEMS Microbiology Letters, 2004. **240**(2): p. 145-9.

39. Scholz, O., A. Thiel, W. Hillen, and M. Niederweis, *Quantitative analysis of gene expression with an improved green fluorescent protein. p6.* Eur J Biochem, 2000. **267**(6): p. 1565-70.

40. Shaner, N.C., R.E. Campbell, P.A. Steinbach, B.N. Giepmans, A.E. Palmer, and R.Y. Tsien, *Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein.* Nat Biotechnol, 2004. **22**(12): p. 1567-72.

41. Meredith, T.C., J.G. Swoboda, and S. Walker, *Late-stage polyribitol phosphate wall teichoic acid biosynthesis in Staphylococcus aureus.* J Bacteriol, 2008. **190**(8): p. 3046-3056.

42. Abramoff, M., P. Magelhaes, and S. Ram, Biophoton Itl, 2004. **11**: p. 36-42.

43. Filipe, S.R., A. Tomasz, and P. Ligoxygakis, *Requirements of peptidoglycan structure that allow detection by the Drosophila Toll pathway.* EMBO Rep, 2005. **6**(4): p. 327-333.

44. Filipe, S.R., E. Severina, and A. Tomasz, *Functional analysis of Streptococcus pneumoniae MurM reveals the region responsible for its specificity in the synthesis of branched cell wall peptides.* J Biol Chem, 2001. **276**(43): p. 39618-28.

45. Karimova, G., J. Pidoux, A. Ullmann, and D. Ladant, *A bacterial two-hybrid system based on a reconstituted signal transduction pathway.* Proc Natl Acad Sci U S A, 1998. **95**(10): p. 5752-6.

46. Soldo, B., V. Lazarevic, and D. Karamata, *tagO is involved in the synthesis of all anionic cell-wall polymers in Bacillus subtilis 168.* Microbiology (Reading, Engl), 2002. **148**(Pt 7): p. 2079-87.

47. Bera, A., S. Herbert, A. Jakob, W. Vollmer, and F. Götz, *Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of Staphylococcus aureus.* Mol Microbiol, 2005. **55**(3): p. 778-787.

48. Pinho, M.G. and J. Errington, *Recruitment of penicillin-binding protein PBP2 to the division site of Staphylococcus aureus is dependent on its transpeptidation substrates.* Mol Microbiol, 2005. **55**(3): p. 799-807.

49. Pinho, M.G. and J. Errington, *Dispersed mode of Staphylococcus aureus cell wall synthesis in the absence of the division machinery.* Mol Microbiol, 2003. **50**(3): p. 871-881.

50. Karimova, G., J. Pidoux, A. Ullmann, and D. Ladant, *A bacterial two-hybrid system based on a reconstituted signal transduction pathway.* Proc Natl Acad Sci USA, 1998. **95**(10): p. 5752-5756.

51. Gründling, A. and O. Schneewind, *Synthesis of glycerol phosphate lipoteichoic acid in Staphylococcus aureus.* Proc Natl Acad Sci USA, 2007. **104**(20): p. 8478-8483.

52. Pereira, S.F.F., A.O. Henriques, M.G. Pinho, H. de Lencastre, and A. Tomasz, *Role of PBP1 in cell division of Staphylococcus aureus.* J Bacteriol, 2007. **189**(9): p. 3525-3231.

53. Pereira, S., A. Henriques, M. Pinho, H. de Lencastre, and A. Tomasz,

Evidence for a dual role of PBP1 in the cell division and cell separation of Staphylococcus aureus. Molecular Microbiology, 2009. **72**(4): p. 895_904.

54. Pinho, M.G., H. de Lencastre, and A. Tomasz, *An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci.* Proc Natl Acad Sci USA, 2001. **98**(19): p. 10886-10891.

55. Schlag, M., R. Biswas, B. Krismer, T. Kohler, S. Zoll, W. Yu, H. Schwarz, A. Peschel, and F. Götz, *Role of staphylococcal wall teichoic acid in targeting the major autolysin Atl.* Mol Microbiol, 2010.

CHAPTER IV

WTAs of *Staphylococcus aureus* limit recognition by the Drosophila Peptidoglycan Recognition Protein-SA to promote pathogenicity

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The author of this dissertation participated in all experiments, except in the protein purification experiments.

ABSTRACT

The cell wall of Gram-positive bacteria is a complex network of surface proteins, capsular polysaccharides and wall teichoic acids (WTAs) covalently linked to Peptidoglycan (PGN). The absence of WTAs has been associated with a reduced pathogenicity of *Staphylococcus aureus*. Here, we assessed whether this was due to increased detection of PGN, an important target of innate immune receptors. Antibiotic-mediated or genetic inhibition of WTA production in *S. aureus* led to increase binding of the non-lytic PGN Recognition Protein-SA (PGRP-SA), and this was associated with a reduction in host susceptibility to infection. Moreover, PGRP-SD, another innate sensor required to control wild type *S. aureus* infection, became redundant. Our data imply that by using WTAs to limit access of innate immune receptors to PGN, under-detected bacteria are able to establish an infection that ultimately overwhelm the host. We propose that different PGRPs work in concert to counter this strategy.

INTRODUCTION

The complex cell surface of bacteria has been directly or indirectly associated with different strategies that bacterial pathogens use to interact with the host. These include acquisition of specific adhesion factors, formation of biofilms, adaptation to an intracellular environment, production of a protective capsular polysaccharide or evasion of innate immune defences (e.g. lysozyme) [1]. The host counters these strategies by targeting conserved molecules (pathogen associated molecular patterns, (PAMPS), unique in bacteria, that are either present at the bacterial surface or are released by bacteria as they attempt to establish infection. Bacterial PAMPs include Peptidoglycan (PGN), a heterogeneous polymer of glycan chains cross-linked by short peptides of variable length and amino acid composition [2]. Although PGN recognition is essential to trigger an inflammatory response, this macromolecule may not be easily accessible for recognition at the surface of bacteria.

In Gram-positive bacteria, PGN is buried within a complex cell surface consisting of different molecules [3-5]. Such molecules include surface proteins, covalently linked or tightly associated with PGN, capsular polysaccharides, usually required for the ability of different bacteria to cause disease [6] and wall teichoic acids (WTAs), phosphate-rich glycopolymers involved in the resistance of bacteria to environmental stress and regulation of bacterial division [7]. Therefore, it is not clear therefore, how the host would be able to sense bacterial PGN buried within such complex structures. One hypothesis is that the innate immune system recognises soluble PGN fragments that are released from the

bacterial cell surface through the activity of enzymes produced by bacteria (such as autolysins) or by the host (such as lysozyme) [2, 8]. However, certain bacteria have the ability to modify their PGN, turning it more resistant to the action of such enzymes [9], thus preventing the release of small soluble fragments capable of triggering an innate immune response in the host. This may be the case for *Listeria monocytogenes* that has the ability to de-*N*-acetylate its PGN allowing them to survive the action of lysozyme and evade the host innate immune system [10]. Another hypothesis is that the components of the host innate immune system are able to bind directly to PGN present within the bacterial cell surface. As discussed earlier, PGN is decorated with a variety of large molecules that may sterically block access of host receptors to the underlying PGN. In Gram-positive bacteria, cell wall glycopolymers, including WTAs may play this role [1]. The role of WTAs in protecting the PGN from recognition would have important implications regarding the onset of infection by major human pathogens such as *Staphylococcus aureus* [1].

Recently, it has been shown that different components, present at the cell wall of *S. aureus* bacteria, may determine the survival of infected *Drosophila*. Specifically, *S. aureus* strains impaired in the expression of enzymes involved with the metabolism of cell wall components were unable to kill flies [11]. Moreover, it has been proposed that D-alanylation of the WTAs produced by *S. aureus* may inhibit the recognition of PGN by host receptors. This inhibitory effect was observed *in vitro* not only when WTAs was covalently attached to polymeric PGN but, surprisingly, also when WTAs was covalently attached to monomeric PGN [12].

The fruit fly *Drosophila melanogaster* recognises Gram-positive bacteria by either direct binding to PGN or its smallest components [13]. Based on *in vitro* data [14] and infection studies of mutants [14, 15], the current working hypothesis is that a flexible system of pattern recognition receptors (PRRs) can be deployed by the host immune system to detect Lysine-type (Lys-type) PGN from different Gram-positive bacterial pathogens. Two Peptidoglycan Recognition Proteins (PGRPs), namely PGRP-SA and PGRP-SD are major components of this system [15, 16]. Depending on the bacterium, each, or both of these PGRPs – along with Gram-Negative Binding Protein1 (GNBP1) [17] – interacts with PGN and activate a downstream proteolytic cascade, which culminates in Toll receptor signalling. The signal reaches the cytoplasmic NF-κB/I-κB complex via a receptor/adaptor complex comprising dMyD88, Tube and the IRAK homologue Pelle. At that point the I-κB homologue Cactus is phosphorylated and targeted for degradation while the NF-κB homologue Dif is free to enter the nucleus of host cells and regulate target genes [18]. Prominent among these genes, is a group of potent antimicrobial peptides (AMPs), which are synthesised by the fat body and secreted into the haemolymph. An AMP frequently used as a read-out for the Toll pathway is *Drosomycin* (*Drs*). AMPs and local melanization, along with the phagocytic activity of haemocytes constitute respectively the humoral and cellular arm of the fruit fly response to infection [18].

Here, we report for the first time that Drosophila PGRP-SA, a non-lytic PGRP was able to bind intact live bacteria *in vivo*. Access to PGN was limited by the presence of WTAs: binding of PGRP-SA to various live Gram-positive bacteria was minimal, but binding to purified PGN,

stripped of covalent modifications including WTAs, was far greater. Through inhibiting WTAs synthesis, either by the addition of an antibiotic or genetically, we were able to potentiate detection of these bacteria by PGRP-SA. For *S. aureus*, this correlated with a reduced ability of the bacteria to proliferate within the host, and a reduced susceptibility of the host to infection in a PGRP-SA / GNBP1 dependent manner. We also observed that PGRP-SD, essential for sensing wild type *S. aureus*, became redundant as WTAs levels were reduced. Overall, our results suggest that WTAs may be part of a general mechanism used by Gram-positive bacteria, which limits the access of innate receptors to PGN, thereby enabling bacteria to evade detection and establish infection.

MATERIALS AND METHODS

Microbial and Fly strains. Isogenic wild type flies (Bloomington #25174) were used as the wild type control. For the survival and bacterial Colony Forming Unit (CFU) experiments, and *DD1* flies for assaying *Drs* levels visually or via qPCR; the latter carries a *Drs-GFP* and a *Diptericin-lacz* reporter [19]. The PGRP-SA and PGRP-SD mutant backgrounds are, respectively: flies with the *semmelweis* mutation in *PGRP-SA* [16] and a 1499 bp deletion in *PGRP-SD* (*PGRP-SD*$^{\Delta 3}$) [15]. The *spz*^{rm7} [20] and *spz*^{1} [21] Toll pathway mutant backgrounds, and the *Dif*^{1}*-key*^{1} [22] Toll-IMD pathways double mutant background, were used to assess survival of flies deficient for AMPs. All fly stocks were reared at 25^{o}C. Bacterial strains are listed below. *S. aureus* strains were grown in tryptic soy broth medium (TSB; Difco) supplemented with antibiotic (erythromycin, Ery 10μg/ml;

Sigma-Aldrich) when required. *E. faecalis* was grown in brain heart infusion medium (BHI; Fluka). *M. luteus* was grown in Luria-Bertani medium (LB; Difco). Growth of all bacteria cultures were done at 30°C as *S. aureus* mutants impaired in the synthesis of WTAs are thermosensitive [23].

***S. aureus* strains.** NCTC8325-4 (*S. aureus* reference strain from R. Novick); NCTCΔ*tagO (*NCTC8325-4 *tagO* null mutant [24]); RN4220 (Restriction deficient derivative of *S. aureus* NCTC8325-4 that can be electroporated); RNΔ*tagO* (RN4220 *tagO* null mutant [24]); RNΔ*tagO*pMAD (RNΔ*tagO* transformed with pMAD [24] – shuttle vector with a thermosensitive origin of replication for Gram-positive bacteria); RNΔ*tagO* p*tagO (*RnΔ*tagO* transformed with p*tagO* [24]); RNΔ*tagO* p$tagO^{D87A/D88A}$ (RNΔ*tagO* transformed p$tagO^{D87A/D88A}$ [24]); RNΔ*tagO* p$tagO^{G152A}$; RNΔ*tagO* transformed with p$tagO^{G152A,}$ [24]); RNΔ*dltA* (RN4220 *dltA* null mutant [24] RNΔ*dltABCD* (RN4220 *dltABCD* null mutant [24]); RNΔ*dltABCD* (RN4220 *dltABCD* null mutant [24]); RNΔ*dltA* (RN4220 *dltA* null mutant, this study). *M. luteus* strain**:** DMS20030 [25]; *E. faecalis* strain**:** JH2-2 [26] ; *B. subtilis* strain MB24 [27].

Survival experiments and determination of CFUs. Overnight 10 ml cultures of bacteria were washed and resuspended in an equal volume of sterile phosphate buffered saline (PBS), and further diluted 1/1000. Healthy looking adult flies from uncrowded bottles, 2-4 days old, were injected in the thorax with 32nl of a bacterial cell suspension or PBS using a nanoinjector (Nanoject II, Drummond Scientific). For determination of

CFUs, injected flies (6 females) were crushed immediately in media appropriate for the bacteria injected and the homogenates were diluted and plated on tryptic soy agar-media (TSA). The plates were incubated at 30ºC for 20-30 hours and the colony forming units (CFUs) per fly were measured by counting the number of colonies on each plate, the CFUs per fly were used to adjust the initial dose of bacteria injected to approximately 100 CFUs per fly. For the time course (0, 6, 17 hours) determination of CFUs, each value represents an arithmetic average derived from three biological repeat experiments (n = 3). Flies for survival and PGRP-SA mutant rescue assays were inoculated concurrently with those for determining CFUs, with ten or fifteen flies of each sex injected per bacteria-fly strain combination (or PBS-fly strain); each combination being repeated independently three times (n = 3). Following injection, flies were transferred to 30ºC and survival assessed every 24 hours over a period of 3 days. Since the trends in survival were the same (i.e. survival curves were positioned similarly relative to one another) for each independent biological repeat, the data for each bacteria-fly strain combination was added (n = 60 or n = 90) and estimates of survival curves constructed. Flies injected with PBS were mostly unaffected for all fly backgrounds.

Purification of recombinant rPGRP-SA and mCherry-PGRP-SA from *E.coli*. A truncated version of PGRP-SA (in which the N-terminal sorting sequence was replaced with a T7 tag, and a poly-histidine tag was added to the C-terminus) was expressed in *E. coli* and purified using cobalt affinity resin (Talon; BD Biosciences) under denaturing conditions.

A mCherry tagged derivative, mCherry-PGRP-SA was produced using the same procedure. Proteins were stored in 20mM Tris-HCl pH 8.0 and 150mM NaCl.

Protein functionality assays. Functionality assays of the rPGRP-SA and mCherry-PGRP-SA proteins were performed as previously described [14]. *Drs-GFP* expression was monitored after 24 hours of the *M. luteus* infection through the production of fluorescent signal produced by the infected flies; and by qPCR using as template RNA extracted from 6 infected female flies, similar to what was previously described [28].

Purification of peptidoglycan. Peptidoglycan was prepared from exponentially growing cultures of *S. aureus*, *B. subtilis*, *M. luteus*, and *E. faecalis* as previously described [13].

PGRP-SA-peptidoglycan co-precipitation assay. 50μg of recombinant PGRP-SA was incubated with 0.2mg of peptidoglycan and 17μg of BSA (New England Biolabs) in 20mM Tris-HCL pH 8.0 and 300mM NaCl in a final volume of 300μl. Incubation was at 25°C with agitation for 30 minutes. Peptidoglycan and co-precipitated proteins were harvested by centrifugation, washed twice with 20mM Tris-HCl pH 8.0, 300mM NaCl and then resuspended in 1x SDS loading buffer, boiled for 5 minutes and run on 12% SDS PAGE mini gels. An aliquot of the supernatant, representing unbound protein, was also run. Gels were stained with Coommasie stain, destained and imaged using an ImageScanner (Amersham Biosciences/GE Healthcare). Quantifications of bands performed using ImageJ software [29]; each value represents an

arithmetic average derived from three biological repeat experiments (*n* = 3).

mCherry-PGRP-SA binding to bacteria. Bacteria were grown to mid-exponential phase. Washed cell cultures in PBS (500µl) were incubated with 50µl of mCherry-PGRP-SA (2mg/ml in 150mM NaCl, 20mM Tris pH 8.0) for 5 minutes on ice. The cells were washed twice with PBS and harvested at 4ºC (3000 rpm, 10 minutes). Finally the bacteria were resuspended in 20µl PBS. A drop of this culture was placed on a PBS, 1% agarose slide and visualised. Images were obtained using a Zeiss Axio ObserverZ1 microscope equipped with a Photometrics CoolSNAP HQ2 camera (Roper Scientific using Metamorph software, Meta Imaging series 7.5) and analyzed using ImageJ software.

WTAs extraction. WTAs were extracted as described before.

WTAs inhibition. Tunicamycin minimum inhibitory concentration (MIC) assays were performed as previously described [30]. Overnight cultures of bacteria were grown in antibiotic free medium or in the presence of a subinhibitory concentration of tunicamycin (0.8ug/ml for *E. faecalis* – 17x less than the MIC - and 0.4ug/ml for *S. aureus* and *S. saprophyticus* – 32x less than MIC), that doesn't interfere with the bacterial growth rate. For mCherry-PGRP-SA binding assays, overnight cultures were diluted 1:100 into fresh medium, with or without tunicamycin at the appropriate concentration, and were grown until mid-exponential phase. For survival experiments, we used *S. aureus* overnight culture grown with tunicamycin as above described.

Data analysis. As nonparametric tests lack statistical power with small samples, when required, data sets with three biological repeats (n = 3) were transformed to give a normal distribution (Lilliefors test, $P > 0.05$) and then checked for equal variance (Levene's test, $P > 0.05$); subsequently, data was analysed using parametric tests.

Binding assays. Data for the PGRP-SA-peptidoglycan co-precipitation assay was normal with equal variance, thus not transformed; One-way ANOVA was applied to the data. For the mCherry-PGRP-SA binding to bacteria assays, data (n = 50) was non-normal but with equal variance, therefore nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison was applied.

CFUs. The complete CFU data set exhibited neither normality nor equal variance, and attempts to rectify this by transforming the data failed. Therefore, the data was separated into 6 groups, which were independently transformed via a Box-Cox transformation (Box-Cox returns a λ number, where a transformed data-point = data-point$^{\lambda}$ – 1 / λ) to give a normal distribution with equal variance, and statistical analysis performed as described. Firstly, for each bacterial strain (groups 1 – 3, graphical representations not shown), Repeated Measures Two-way ANOVA was used to look for differences over time and between the fly backgrounds. However, due to interactions between these two factors, Repeated Measures One-way ANOVA with 95% Tukey's HSD Intervals was used to look for differences over time for each particular bacteria strain and fly background combination (i.e. 9 separate tests, data for each was normally distributed with equal variance). Secondly, at each time point (groups 4 – 6, Fig 3), Two-Way ANOVA was used to look for

differences between the bacterial strains and between the fly backgrounds; where there was an interaction between these two factors, One-way ANOVA with 95% Tukey's HSD Intervals was used to look for differences between the fly backgrounds for a particular bacterial strain.

Fly survival. Estimated survival curves were constructed from the raw data sets and the Log-rank (Mantel-Cox) test used to determine statistical significance between the curves. For clarity in display, 95% confidence intervals have been omitted from the graphs. All data was plotted and analyzed using GraphPad Prism 5 (GraphPad Software, Inc.) or MATLAB R2009a.

RESULTS

PGRP-SA binds differently to peptidoglycan than to live cells

To address the question of whether Gram-positive bacteria counter host recognition by limiting access of innate sensors to PGN, we constructed a fluorescent derivative of the fruit fly Lys-type PGN receptor, PGRP-SA (mCherry-PGRP-SA). This construct and an untagged version (rPGRP-SA) were expressed in *Escherichia coli* and the resulting proteins were purified. As shown in the Figure 1A, injection of mCherry-PGRP-SA, or rPGRP-SA, into PGRP-SA deficient flies restored *Drs-GFP* production induced by infection with *Micrococcus luteus* (*M. luteus*). Endogenous *Drs* expression was also restored as confirmed by qPCR (Figure 1B). These observations were consistent with our previous results when using a recombinant PGRP-SA expressed in the lepidopteran cell line Sf9 [31]. Taken together, these results showed that the fluorescently tagged PGRP-

SA and the untagged versions are functional and capable of restoring an innate immune response in PGRP-SA deficient flies.

Initially, we used rPGRP-SA and mCherry-PGRP-SA in co-precipitation experiments in order to study binding to PGN from different Gram-positive bacteria. Both proteins bound with similar affinity to PGN purified from *M. luteus*, *Enterococcus faecalis* (*E. faecalis*), and *S. aureus* (data not shown and Figure 2A, respectively). For details of PGN composition of these bacteria see Figure 3. Importantly, this indicated that the mCherry-tag appeared not to interfere with PGRP-SA binding, and thus, demonstrated that both proteins were able to bind Lys-type PGN of different composition. We therefore assessed *in vitro*, the binding of mCherry-PGRP-SA to the surface of live bacteria harvested during exponential growth phase. Notably, the binding of the recombinant protein to live bacteria exhibited a range of different affinities in contrast to their respective purified PGN. Although, the binding levels of PGRP-SA to the purified PGN from these bacteria were similar (Figure 2A), binding to live *E. faecalis* and *S. aureu*s was significantly reduced, when compared to binding to *M. luteus* (Fig 2B). We also noticed that while mCherry-PGRP-SA was capable of binding the entire surface of *M. luteus* cells, it bound at specific sites at the surface of *S. aureus* cells, similar to what has been described recently for mammalian bactericidal PGRPs [32]. These results suggested that even though the three types of bacterial PGN were similarly recognized by PGRP-SA, the presence of other components found at the surface of live bacteria might have prevented PGRP-SA from finding its PGN ligand.

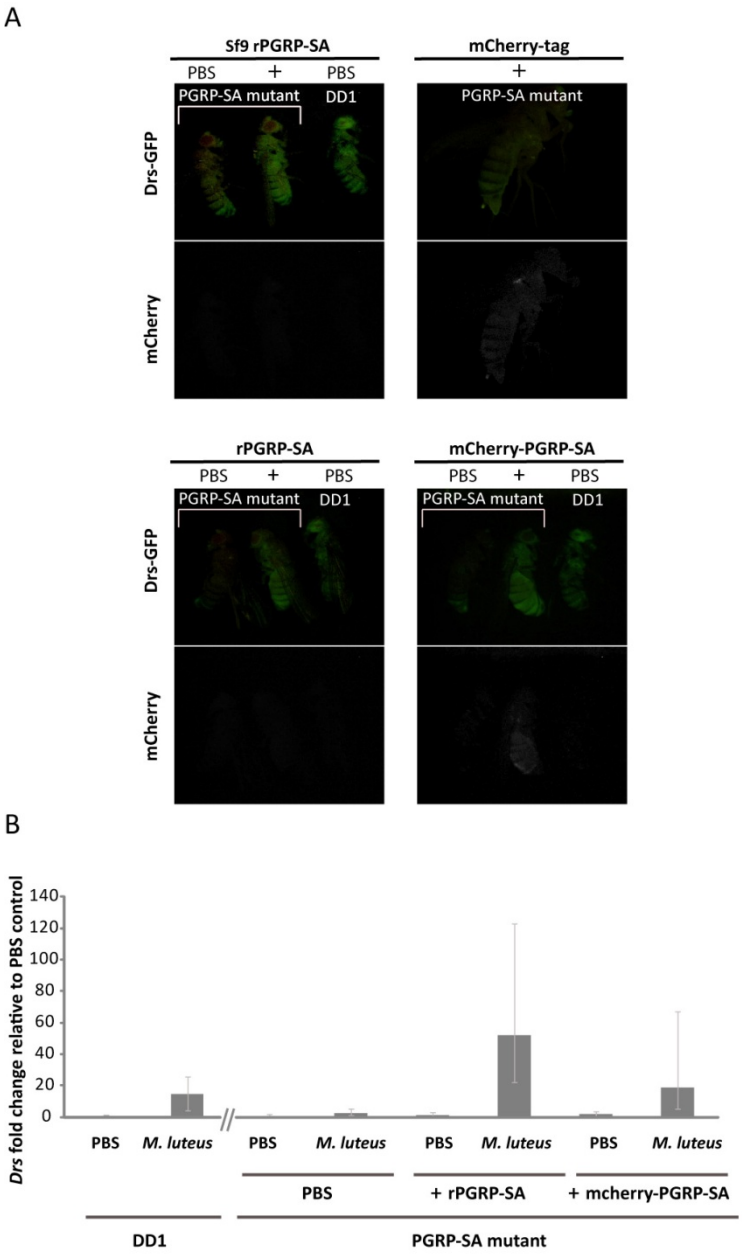


Figure 1. Recombinant PGNPR-SA proteins rescue *Drs* expression in *D. melanogaster*. *D. melanogaster* recombinant PGRP-SA proteins were produced in *E. coli*, except Sf9 rPGRP-SA, which was produced in an insect cell line. Flies carrying a *Drs-GFP* reporter were firstly injected with either 10ng of a recombinant PGRP-SA (+), 10ng of the fluorescent mCherry-tag (+), or an

equivalent volume of sterile PBS when protein was not injected (PBS); after 2 hours the same flies were infected with *M. luteus*. (A) *Drs-GFP* expression was observed after 24 hours (Drs-GFP), and likewise mCherry fluorescence (mCherry). DD1 flies were used as a wild type control for *Drs-GFP* expression upon infection; all recombinant PGRP-SA proteins rescued *Drs-GFP* expression in the PGRP-SA mutant background, whereas the mCherry-tag or sterile PBS did not. (B) The pooled *Drs* mRNA levels (normalised to the non-immune ribosomal gene *RP49*) from 12 female flies was determined 24 hours post-infection via qPCR. For each fly background, the *Drs* mRNA levels induced by *M. luteus* were expressed as fold-change relative to the PBS injection (comparative CT method). Each column represents the mean value for three independent sets of injection ($n = 3$), and the error bars 95% confidence intervals. One-Way ANOVA and 95% Tukey HSD Intervals were used to analyse the data for PGRP-SA mutant flies: significant differences were not found between flies injected with PBS, *M. luteus*, or with only the recombinant proteins. However, the combination of a recombinant PGRP-SA with *M. luteus* greatly enhanced the levels of *Drs* mRNA ($P < 0.05$).

Binding of PGRP-SA to bacteria is reduced by the presence of wall teichoic acids

The cell surface of a Gram-positive bacterium is a complex structure consisting of a thick layer of PGN, surface proteins and glycopolymers such as capsular polysaccharides and WTAs. As previous studies had shown certain PGN-binding proteins, such as bacterial autolysins, have a higher affinity for the surface of bacterial strains lacking WTAs [33-35]. Therefore, we decided to investigate whether presence of WTAs could be preventing PGRP-SA from binding to the surface of live bacteria.

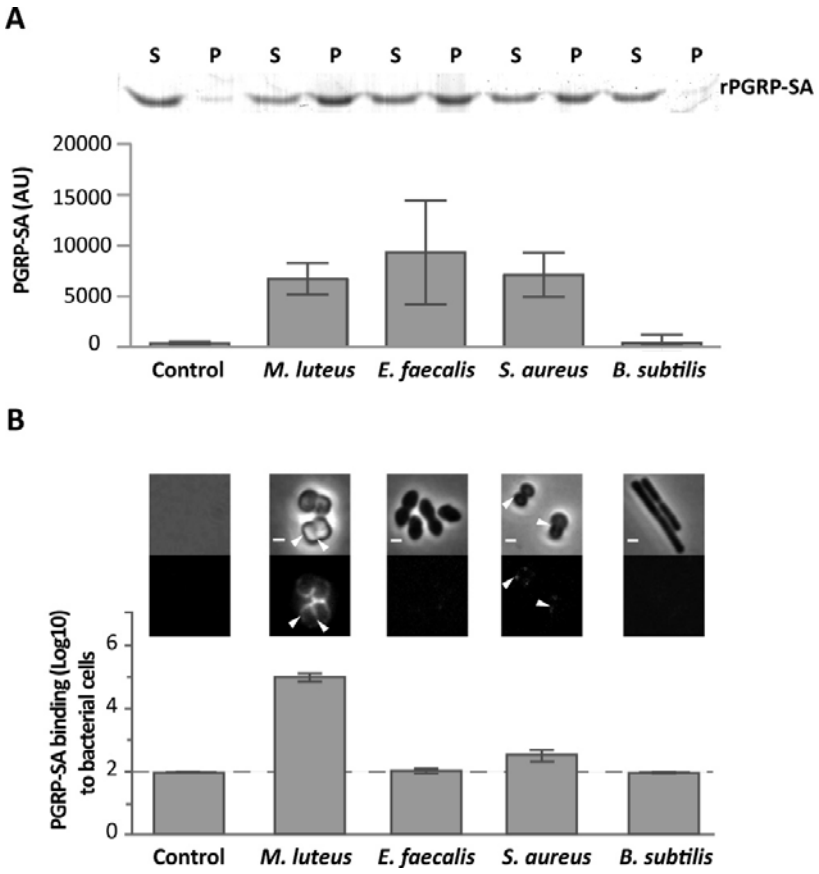


Figure 2. Differential binding of PGRP-SA to the surface of live Gram-positive bacteria (A) PGRP-SA and PGN co-precipitation assay. Lys-type PGN from *M. luteus*, *E. faecalis*, *S. aureus*, and DAP-type PGN from *B. subtilis* (this acts as a negative substrate control for PGRP-SA binding, which recognizes Lys-type PGN), was incubated with rPGRP-SA for 30 minutes. Unbound rPGRP-SA remained in the supernatant fraction upon centrifugation (S). rPGRP-SA bound to the insoluble PGN was co-precipitated and found in the pellet fraction (P). Quantified data (performed using ImageJ software) was plotted as mean values with 95% confidence limits: very little co-precipitation of rPGRP-SA occurred in the absence of PGN (labelled Control) or in the presence of *B. subtilis* DAP-type PGN; however, PGRP-SA was co-precipitated similarly (One-way ANOVA, $P > 0.05$) and at higher levels with the PGN from *M. luteus*, *E. faecalis*, or *S. aureus*. The

data shown (mean with 95% confidence intervals) was obtained from 4 independent co-precipitation experiments. **(B)** mCherry-PGRP-SA was incubated with bacteria cells harvested in exponential phase, washed with PBS and visualized using fluorescence microscopy. Grey panels are phase-contrast images of bacterial cells (white scale bar represents 1 μm), and black panels mCherry-PGRP-SA binding: white arrowheads highlight binding to the lateral cell surface or the region of cell division. The total fluorescence of mCherry-PGRP-SA bound to a bacterium was quantified for each species (n = 50), and represented as the median (with 25% and 75% inter-quartile range). Dashed-line indicates the level of the background signal, control. Kruskal-Wallis analysis with Dunn's multiple comparison post-test did not reveal significant differences ($P > 0.05$) between mCherry-PGRP-SA binding to *E. faecalis* and *B. subtilis*, which were indistinguishable from the control. However, the protein bound more to *S. aureus* and *M. luteus* relative to the control, with the latter exhibiting highest binding ($P < 0.05$ in all cases).

Further support for the choice of WTAs came from the fact that different Gram-positive bacteria can produce WTAs with a variable composition [36-38]. *M. luteus*, for which mCherry-PGRP-SA displayed the highest affinity, does not produce WTAs [36, 39]**,** (Figure 3C).

To test whether WTAs mediated the differential binding of PGRP-SA, we cultured bacteria in the presence of tunicamycin, thereby inhibiting their ability to synthesise WTAs. At lower, sub-inhibitory concentrations as those used in this study, tunicamycin specifically inhibits TagO [30]. TagO is a glycosyltransferase that specifically localizes to the division septum of *S.* aureus [24] and is required for the initial step of WTAs biosynthesis, namely, the transfer of GlcNAc to the C55-P lipid anchor bactoprenol.

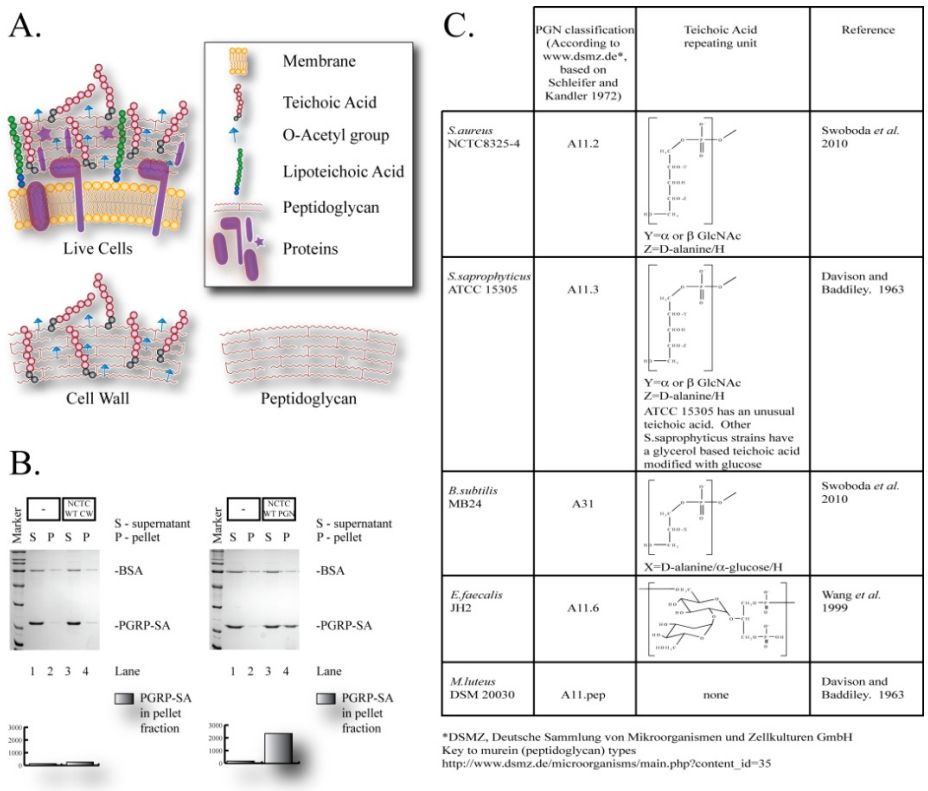


Figure 3. Substrates used in this study. (A) Substrates used in this study. A schematic representation of the different substrates used in binding reactions in this study. The surface of live cells is very complex and consists of Peptidoglycan (PGN) with attached proteins (purple ovoids), large polymers (such as teichoic acids, red spheres) and other covalent modifications (including O-acetylation, blue triangles). The surface of live cells will also be influenced by the presence of other molecules that are not covalently attached to the PGN such as lipoteichoic acids (green spheres) which are anchored in the cell membrane and extra cellular proteins which are not covalently linked to the surface or are anchored in the cell membrane (purple stars and purple shapes in the membrane). It should also be noted that the surfaces of live cells are constantly undergoing remodelling processes and that the PGN will be growing and dividing. Cell wall (CW) is

produced from live cells by a treatment that subjects the cells to mechanical stress followed by boiling in detergent and treatment with proteases, DNases and RNases. CW consists of PGN with covalently attached modifications such as teichoic acids and O-acetylation but free of protein, membrane and nucleic acids. PGN is produced from CW by treatment with hydrofluoric acid that removes teichoic acids and O-acetylation, leaving just the naked PGN mesh. CW and PGN are metabolically inert, the structures should not change with time. (B) PGRP-SA co-precipitation assay in the presence of CW and PGN. Binding of PGRP-SA to CW produced from NCTC8325-4 is very low (left panel, lane 4). On the other hand, binding of PGRP-SA to PGN produced from NCTC8325-4 is high (right panel, lane 4). The difference between CW and PGN is the presence or absence of O-acetyl groups and teichoic acids. Removal of these from CW makes the resulting PGN a far better substrate for binding of PGRP-SA. (C) PGN type and structure of the repeating unit of teichoic acids found in the strains used in this study. Note that the *S. saphrophyticus* strain used here (ATCC 15305) has a similar or identical teichoic acid composition to *S. aureus*. Most other strains of *S. saprophyticus* contain a teichoic acid based around a glycerol repeating unit. This glycerol repeating unit is modified by the addition of glucose.

We observed higher levels of mCherry-PGRP-SA binding to the newly synthesize cell wall material, located at the division septum, when Gram-positive bacteria cells were treated with tunicamycin (Figure 4A). *S. aureus* and *S. saprophyticus* exhibited a similar increase in binding, 63x and 84x respectively, whilst *E. faecalis* binding increased 8x. It should be noted that the effect of tunicamycin in these bacteria was not the same. While addition of the antibiotic resulted in binding of mCherry-PGRP-SA to the entire cell surface of *S. aureus*, binding was observed predominantly at the division septum in *S. saprophyticus* and exclusively at this region in *E.*

faecalis. We attribute these differences to how and where the new cell wall synthesis occurs in these bacteria. Nevertheless, the results described above suggested that WTAs in different bacteria might protect PGN from exposure to host receptors.

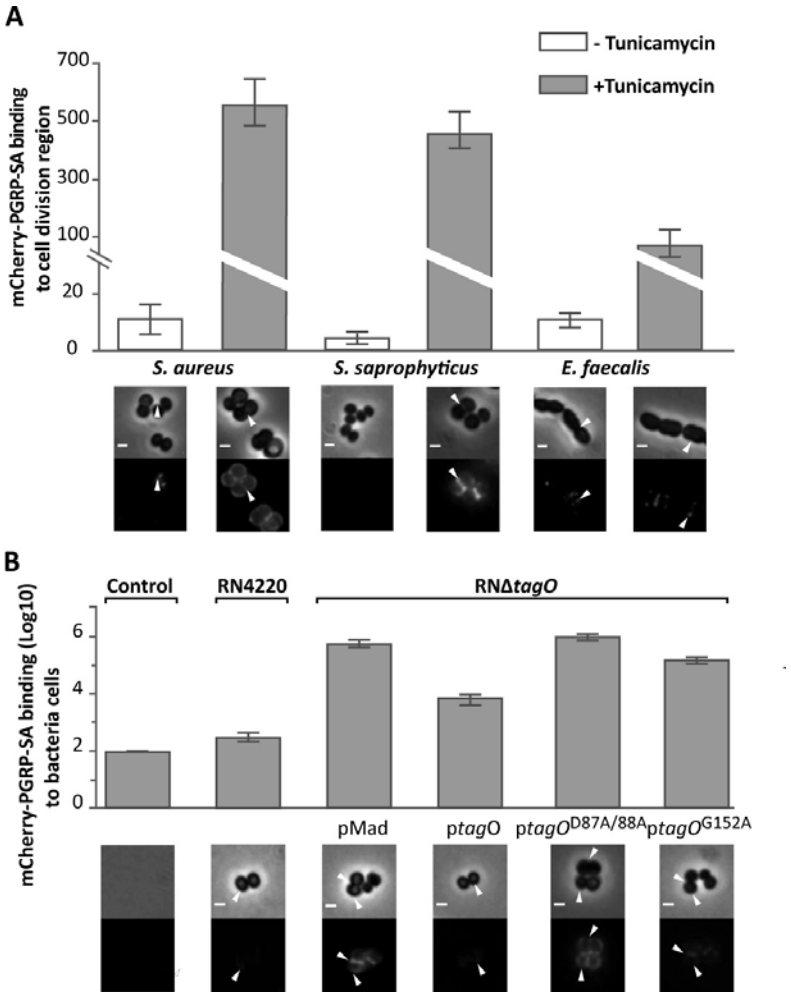


Figure 4. WTAs reduce PGRP-SA binding at the bacterial cell surface. Grey panels are phase-contrast images of bacterial cells (white scale bar represents 1 μm), and black panels mCherry-PGRP-SA binding; white arrowheads highlight binding to the lateral cell surface or region of cell division. The binding of mCherry-PGRP-SA to individual bacterial cells (n = 50) was quantified, and

represented as the median (with 25% and 75% inter-quartile range). (A) mCherry-PGRP-SA binding to Gram-positive bacteria grown with or without tunicamycin, an inhibitor of WTAs synthesis. mCherry-PGRP-SA binding to the cell division region, rather than total binding, was measured because binding at the former was consistently enhanced for all treated bacteria species. Mann-Whitney U tests were used to compare differences for treated and untreated between each type of bacteria ($P < 0.05$ in all cases). (B) RNΔ*tagO* mutant background rescued with variants of the *tagO* gene – expressed from a replicative pMAD vector – produce varying levels of WTAs, given as a % relative to the wild type RN4220: pMAD vector (0%), p*tagO* (90%), ptagOD87A/D88A (0%), ptagOG152A (22%). Total binding of mCherry-PGRP-SA to the surface of live bacteria increases as the levels of WTAs are reduced. Kruskal-Wallis analysis followed by Dunn's multiple comparison post-test, revealed significant differences for all comparisons ($P < 0.05$) except for that between PGRP-SA binding to pMAD and ptagOD87A/D88A.

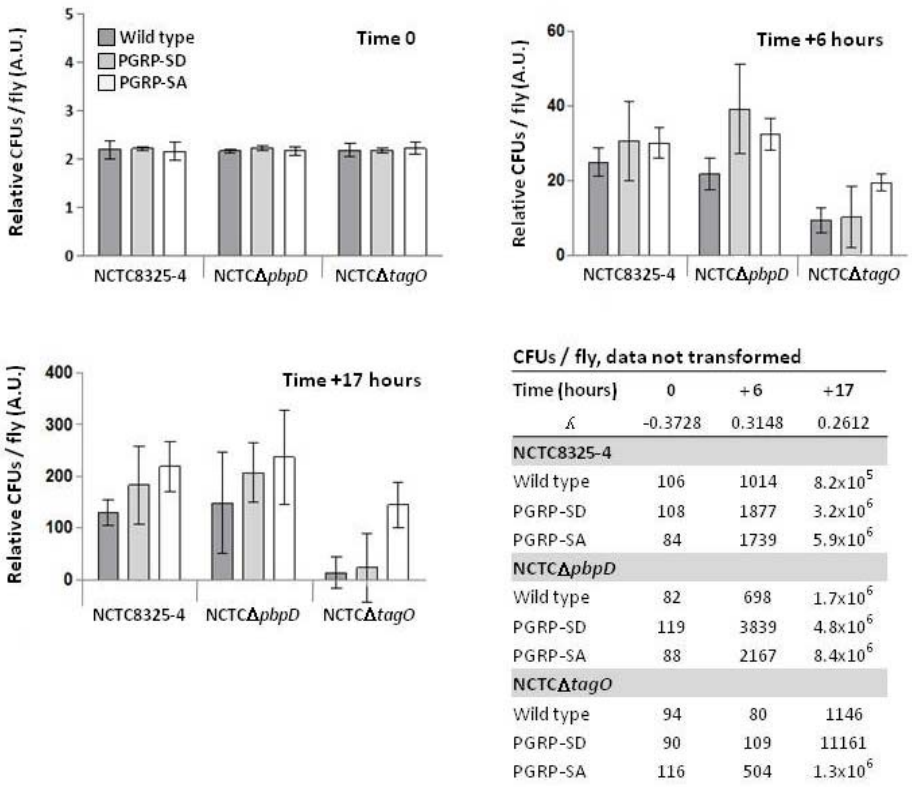
To confirm that WTAs were indeed required to reduce access of PGRP-SA at the cell surface, we quantified the binding of mCherry-PGRP-SA to *S. aureus* mutants that produced varying amounts of WTAs due to mutations in the *tagO* gene [24]. We chose *S. aureus* because it is a major human pathogen with a well-characterised WTAs synthetic pathway [40, 41]. A complete absence of WTAs, which occurs when *tagO* is entirely deleted (RNΔ*tagO* pMAD), or when two highly conserved residues have been mutated (RNΔ*tagO* p*tagO*$^{D87A/D88A}$), resulted in equivalently enhanced levels of mCherry-PGRP-SA binding, when compared to the wild type strain (~$2x10^3$ and ~$3.3x10^3$-fold respectively, Figure 4B). To verify that the observed result was indeed due to the loss of WTAs, we expressed wild type *tagO* in the RNΔ*tagO* background (RNΔ*tagO* p*tagO*): this rescued the

loss of WTAs (WTAs levels restored to 90% of wild type levels) [24], and reduced mCherry-PGRP-SA binding to levels close to those observed for the wild type strain (Figure 2B). A *tagO* mutant that could only support production of a reduced amount of WTAs (RNΔ*tagO* p*tagO*^{G152A}; 24% levels of WTAs compared to wild type) exhibited an intermediate level of mCherry-PGRP-SA binding relative to all strains ($6x10^2$-fold increase relative to the wild type strain, Figure 4B). Overall, our data indicated that WTAs found in the cell wall of different live Gram-positive bacteria restricted PGRP-SA from binding their PGN, and in *S. aureus* this occurs in a dose dependent manner.

Wall teichoic acids allow S. Aureus to propagate inside Drosophila flies

We next wanted to examine whether increased PGRP-SA binding – due to a lack of WTAs – affected the ability of bacteria to survive in an *in vivo* system. We chose *D. melanogaster* because it is a well-established model for dissecting pattern recognition in innate immunity [18]. We know for example that *in vitro*, three PRRs – PGRP-SD / PGRP-SA / GNBP1 – form a ternary complex for binding to the PGN of *S. aureus* [14]. As a first approach wild type and mutant *S. aureus* strains were injected into wild type flies and also into flies defective for PGRP-SD or PGRP-SA. We then determined the number of CFUs 6 and 17 hours post-infection; the latter time point being when the first flies succumb to infection (Figure 5 and 6). All flies were inoculated with low and statistically identical numbers of bacteria (~10^2 CFUs per fly; Figure 5, Time 0). Our rationale was to induce infections that were comparable and that could evolve over

time. For example, flies generally succumb to bacterial infection when their numbers increase beyond 10^6 CFUs per fly [18, 42], and therefore, high initial loads (e.g. 10^4 – 10^5 CFUs per fly) may overwhelm the host and consequently may not be informative regarding the course of an infection. We observed that wild type *S. aureus* (NCTC8325-4) strain CFUs increased in all fly backgrounds over the period of infection to numbers that were statistically separable, with PGRP-SA deficient flies carrying the heaviest load (Figure 5).



CFUs / fly, data not transformed

Time (hours)	0	+6	+17
λ	-0.3728	0.3148	0.2612
NCTC8325-4			
Wild type	106	1014	8.2×10^5
PGRP-SD	108	1877	3.2×10^6
PGRP-SA	84	1739	5.9×10^6
NCTCΔ*pbpD*			
Wild type	82	698	1.7×10^6
PGRP-SD	119	3839	4.8×10^6
PGRP-SA	88	2167	8.4×10^6
NCTCΔ*tagO*			
Wild type	94	80	1146
PGRP-SD	90	109	11161
PGRP-SA	116	504	1.3×10^6

Figure 5. PGRP-SA is fundamental for controlling bacterial numbers in flies infected with a *S. aureus* mutant that lacks WTAs. Wild type flies, and those lacking or expressing mutated PGRP-SD, PGRP-SA or GNBP1 proteins, were

infected with different *S. aureus strains*: NCTC8325-4 is the parental strain; NCTCΔ*pbpD* is a *S. aureus* mutant strain that produces WTAs but has a PGN similar to NCTCΔ*tagO* strain, both exhibiting reduced cross-linking; NCTCΔ*tagO* lacks WTAs. The table gives the mean CFUs per fly (from 3 independent experiments). For each time point, the CFUs per fly data set was transformed via a Box-Cox transformation (which returns a λ number, where data-point = data-point$^{\lambda}$ – 1 / λ) and represented as means with 95% confidence intervals. Flies were inoculated with a low (~100 CFUs per fly) and comparable number of bacteria (Time 0; Two-way ANOVA did not reveal significant differences, $P > 0.05$), and CFUs per fly were determined at 6 and 17 hours post-infection. In contrast to NCTC8325-4 and NCTCΔ*pbpD*, the number of NCTCΔ*tagO* bacteria did not significantly increase in the wild type or PGRP-SD mutant background during the period of infection (Table); however, in the PGRP-SA mutant the number of bacteria increased significantly for all strains ($P < 0.05$, Repeated Measures One-way ANOVA). Two-way ANOVA of the CFUs data at Time +17 hours revealed a significant interaction ($P < 0.05$) between the bacteria and fly strains, which was due to the large increase of NCTCΔ*tagO* CFUs in the PGRP-SA mutant, whilst differences in CFUs were similar for NCTC8325-4 and NCTCΔ*pbpD*. One-way ANOVA and 95% Tukey's HSD intervals were used to look for factor differences at this time. For each fly background NCTC8325-4 and NCTCΔ*pbpD* CFUs were equivalent ($P > 0.05$). NCTCΔ*tagO* CFUs in the wild type and PGRP-SD backgrounds were similar ($P > 0.05$), but separated from all other values ($P < 0.05$). In the PGRP-SA mutant, NCTCΔ*tagO* CFUs reached levels seen with the other bacteria in wild type and PGRP-SD flies.

In contrast, the numbers of the *S. aureus* mutant strain, which lacked WTAs (NCTCΔ*tagO*) [24], did not significantly increase in the wild type or PGRP-SD mutant background. However, the number of NCTCΔ*tagO*

bacteria in the PGRP-SA mutant was significantly higher at both the 6 and 17 hours time points (Figure 5). Two-way ANOVA revealed a significant interaction between the bacteria and fly strains, which was due to the large increase of NCTCΔ*tagO* bacteria in the PGRP-SA mutant. Together, these data indicated that WTAs were fundamental for *S. aureus* to counter recognition by PGRP-SA, and consequently, the bacteria were able to increase their number during the initial course of infection.

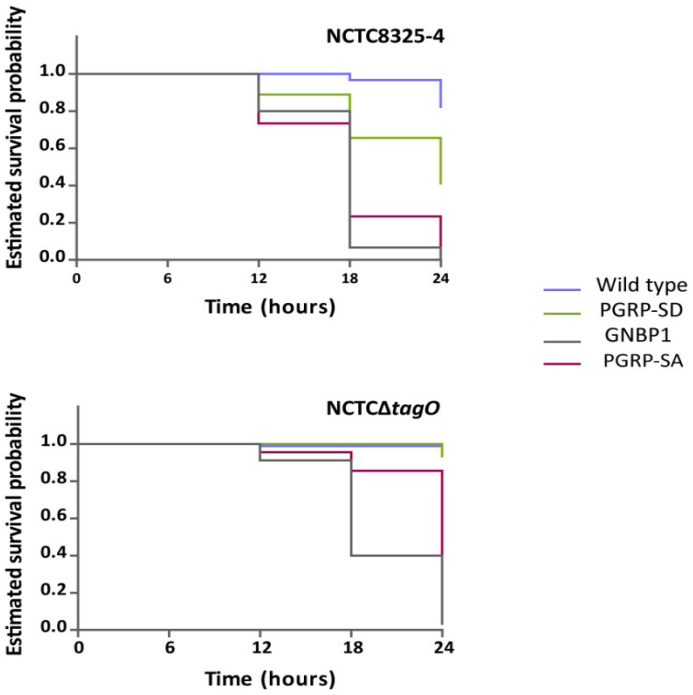


Figure 6. Survival dynamics prior to 24 hours post-infection. As previously performed, the given fly strains (*n*= 90) were infected with either *S. aureus* NCTC8325-4 or NCTCΔ*tagO* strains, and survival monitored every 6 hours. This revealed that PGRP-SA and GNBP1 mutants succumb almost completely to NCTC8325-4 infection after approximately 18 hours, whereas for NCTCΔ*tagO*,

this occurs after 24 hours. Similar survival curves of wild type and PGRP-SD mutant flies were obtained when infected with NCTCΔ*tagO* strain.

We have previously observed that PGN produced by NCTCΔ*tagO* bacteria has reduced levels of cross-linking relative to the wild type strain [24]. To evaluate whether this contributed to the inability of NCTCΔ*tagO* bacteria to increase their number in wild type or PGRP-SD mutant flies, we assessed mCherry-PGRP-SA binding to NCTCΔ*pbpD* and determined CFUs at 6 and 17 hours. NCTCΔ*pbpD* is a derivative of NCTC8325-4 in which *pbpD* (the gene encoding to penicillin binding protein 4, PBP4) has been deleted. Deletion of *pbpD* results in a strain that produces PGN with a similar level of cross-linking to that found in NCTCΔ*tagO* [24], but which still produces WTAs. The inability of NCTCΔ*pbpD* and NCTCΔ*tagO* to produce a highly crosslinked PGN did not interfere with bacteria growth in culture, as its duplication time at 30°C was very similar to the parental NCTC8325-4 strain (Figure 7B). In both experiments, NCTCΔ*pbpD* behaved as the wild type bacteria. Firstly, binding of mcherry-PGRP-SA to the surface of both strains was similar (Figure 7C) and secondly, for each fly background bacteria attaining numbers were statistically inseparable from those for NCTC8325-4 (Figure 5, Time +17 hours).

Wall teichoic acids are required to counter host immunity and reduce host survival

To assess whether the developing trend in bacterial numbers at 17 hours post-infection resolved into differences in how flies survive, we

monitored the number of flies alive at 24 hour intervals over 3 days. In addition, we infected GNBP1 mutant flies, because GNBP1 has been postulated to work as part of a complex with PGRP-SA [14, 17].

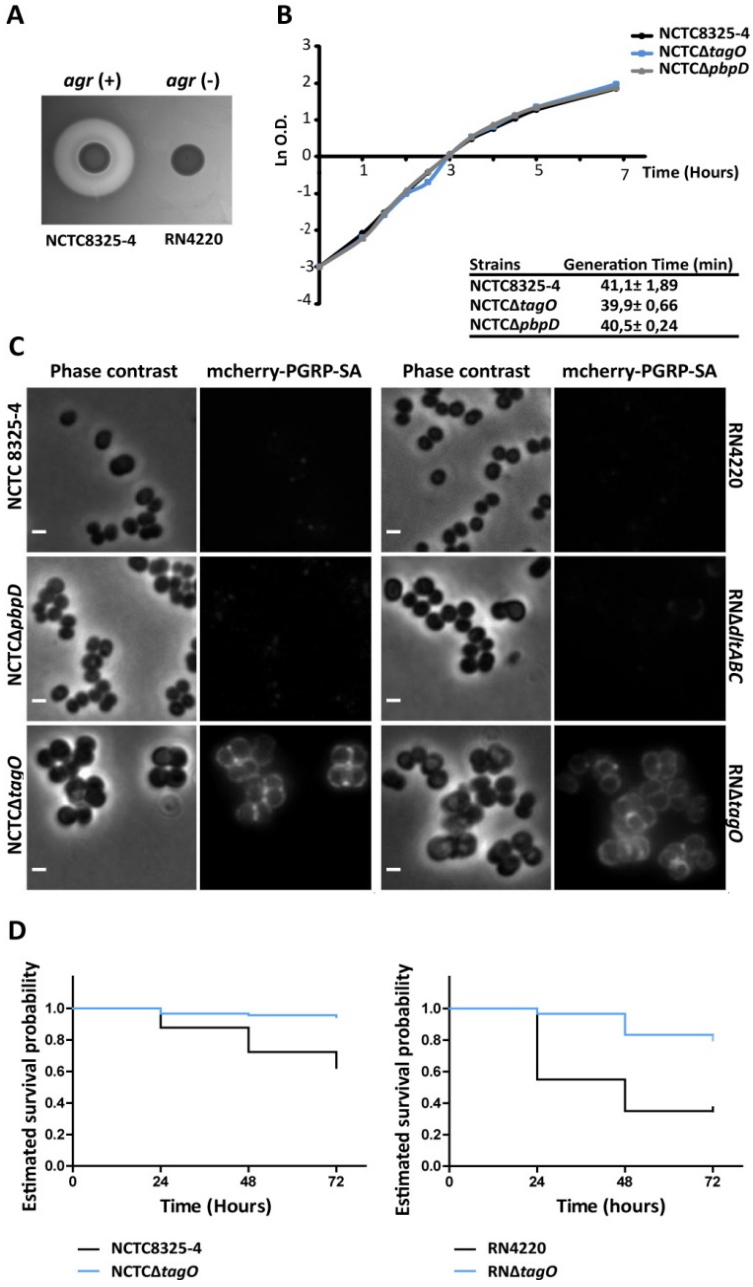


Figure 7. Absence of WTAs, rather than reduced cross-linking or D-alanylation of WTAs, enhances PGRP-SA binding to the surface of S. aureus. (A) Secretion of hemolysins was assayed on TSA blood agar plates to determine the *agr* phenotype of the parental *S. aureus* strains, NCTC8325-4 and RN4220, used in this study. The formation of an inner halo of clearing in the plates is due to the action of the δ-hemolysin, only produced by *agr* positive strains. According to this NCTC8325-4 is an *agr* positive (+) strain while RN4220 is an *agr* negative (-) strain. (B) Growth curves of *S. aureus* wild type and mutants strains in TSB. Overnight cultures were diluted to a starting optical density (O.D. 600) of 0.05, and absorbance measurements were taken every 30 minutes. Representative growth curves of the experiments conducted in triplicate are shown. Generation times are presented in the table as arithmetic averages with standard deviations. NCTCΔ*tagO* and NCTCΔ*pbpD* showed similar generation times to the NCTC8325-4 wild type strain. (C) Exponentially growing cells of NCTC8325-4, NCTCΔ*tagO* and NCTCΔ*pbpD* were incubated with mCherry-PGRP-SA. In addition to lacking WTA, NCTCΔ*tagO* produces a PGN with a reduced cross-linking, similar to that seen with NCTCΔ*pbpD*. The fluorescent derivative of PGRP-SA protein was not able to the surface of NCTCΔ*pbpD* bacteria that produces teichoic acids at their surface. Exponential phase cells of RN4220 (a laboratory strain that is *agr* defective), RNΔ*tagO* and RNΔ*dltABCD* were also incubated with the protein. The RNΔ*dltABCD* is a mutant strain whose WTAs lacks D-alanine residues. The fluorescent derivative of PGRP-SA protein was not able to the surface of RNΔ*dltABCD* bacteria that produces teichoic acids with no D-alanines at their surface. Grey panels are phase-contrast images of bacterial cells (white scale bar represents 1 μm); black panels mCherry-PGRP-SA binding. Images also show that mcherry-PGRP-SA bound strongly to tagO null mutants constructed in both NCTC8325-4 (*agr* positive) and RN4220 (*agr* negative) strains. (D) Estimated survival curves for wild type flies infected with *S. aureus agr*

positive (NCTC8325-4 and NCTCΔ*tagO*) and negative strains (RN4220 and RNΔ*tagO*). Flies were infected with ~100 bacterial cells and fly survival was assessed every 24 hours over 3 days. *S. aureus* RN4220 strain with *agr* negative phenotype is not affected in the ability to kill drosophila flies.

Survival curves for a particular fly background when infected with either NCTC8325-4 or NCTCΔ*pbpD* were statistically inseparable, except for those obtained for the wild type background, where flies succumbed more to infection with NCTCΔ*pbpD* (Figure 8; 62% and 38% survival at 72 hours post-infection, respectively).

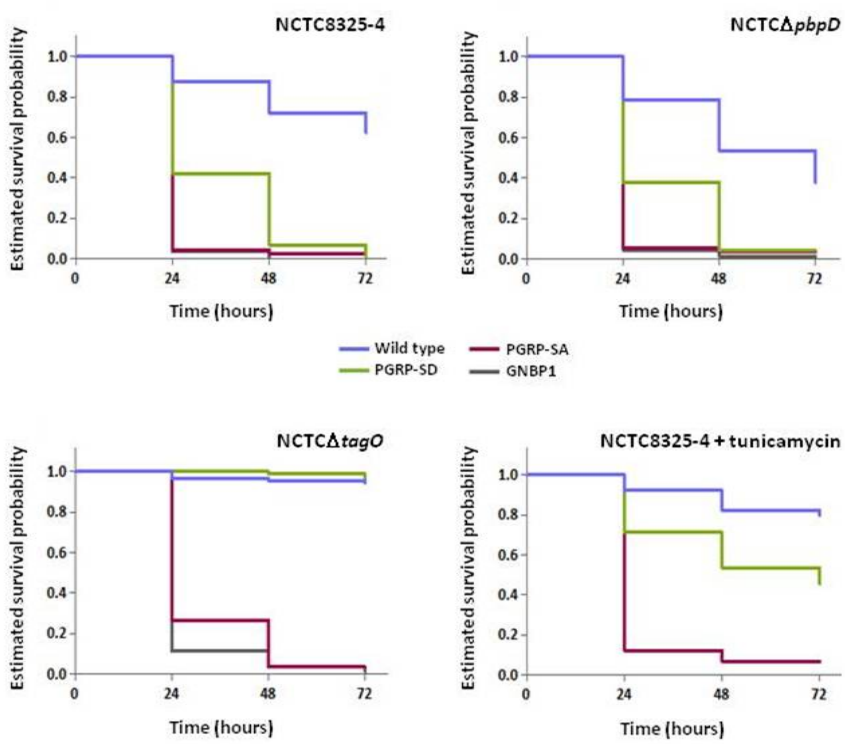


Figure 8. PGRP-SA and not PGRP-SD is required to control infection by *S. aureus* mutant lacking WTAs. Flies assayed for survival were injected concurrently with those for determining CFUs (Figure 5). The survival of infected

flies (n = 90) was monitored at 24-hour intervals for three days, and estimates of survival plotted (for clarity, 95% confidence intervals have been omitted). For each fly background – except wild type – survival curves were statistically inseparable for flies infected with NCTC8325-4 or NCTCΔ*pbpD* (log-rank test, $P > 0.05$). PGRP-SD, PGRP-SA and GNBP1 mutant flies succumbed strongly to infection by 72 hours, whereas wild type survived up to ~60%. Infection with NCTCΔ*tagO* originates different results. While wild type and PGRP-SD mutant flies were barely susceptible to infection with NCTCΔ*tagO*, PGRP-SA and GNBP1 flies succumbed strongly. A similar survival trend was seen when flies were infected with tunicamycin-treated NCTC8325-4 (GNBP1 mutant flies were not infected for this experiment).

Nearly all PGRP-SA and GNBP1 mutant flies infected with parental *S. aureus* bacteria died by 24 hours, whereas ~40% of PGRP-SD mutant flies survived beyond this time point, succumbing to infection around 48 hours (~5% of flies surviving). In contrast, ~95% of wild type and PGRP-SD mutant flies survived the NCTCΔ*tagO* infection up to 72 hours (furthermore, taking CFUs at this time-point revealed that NCTCΔ*tagO* had been eliminated from these flies, 0 CFUs per fly). The majority of PGPR-SA and GNBP1 flies had succumbed to infection by 48 hours (3% of flies surviving). A similar trend in survival outcome was observed with the parental strain NCTC8325-4 after treatment with tunicamycin (Figure 8). These data confirmed that WTAs were indeed required to counter host immunity, because without them, infection could be controlled in a PGRP-SA/GNBP1 dependent manner. Differences in CFUs were apparent 6 hours post-infection suggests that recognition, and reduction of propagation or killing of bacteria, occurs rapidly following infection.

Interestingly, these results also showed that a requirement for PGRP-SD was by-passed when WTAs are removed and PGRP-SA has far greater access to PGN.

To further demonstrate the necessity for WTAs to protect PGN from host recognition, we monitored survival of flies infected with the aforementioned TagO point mutations (Figure 4B and Figure 9). In these experiments, we wanted to rule out unknown causes that may occur due to the absence of the TagO protein *per se*, and also, lessen adverse effects that may occur due to a complete lack of WTAs. The survival trend for flies infected with RNΔ*tagO* pMAD, that lacks *tagO* and carries an empty pMAD plasmid vector (vector control), was similar to that for NCTCΔ*tagO*: the PGRP-SA mutant succumbed rapidly, whereas the PGRP-SD mutant and wild type flies generally survived, their curves being statistically inseparable (Figure 9). The injection of the complemented strain (RNΔ*tagO* p*tagO*) resulted in survival outcomes that were characteristic of NCTC8325-4, with PGRP-SD mutant and wild type flies succumbing to the infection, with their curves being statistically separated (Figure 9). Notably, wild type and PGRP-SD mutant flies infected with RNΔ*tagO* p*tagO*^{G152A} (which produces ~24% WTAs relative to RNΔ*tagO* p*tagO* but produces similar levels of the TagO protein) [24] survived to intermediary levels (Figure 9).

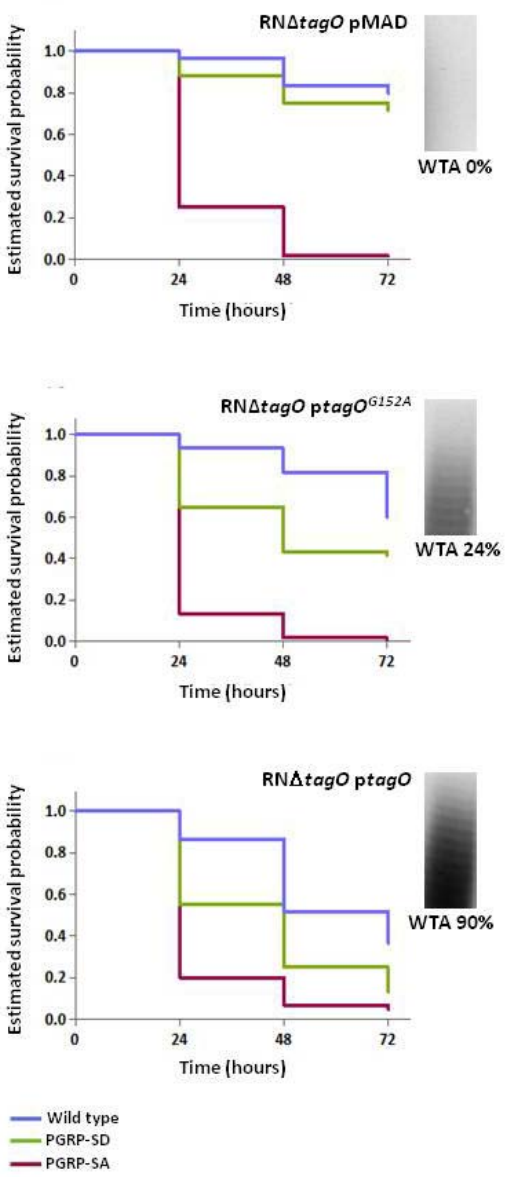


Fig 9. The levels of WTAs modulate the requirement for PRRs. Survival of infected flies (n = 60) was monitored at 24 hours intervals for three days, and estimates of survival constructed from the raw data. Flies were infected with *S. aureus* mutants that produce different levels of WTAs (percentage of WTAs

produced by each strain was quantified as the signal intensity of bands of WTA in the native gels, and it was normalized against the corresponding value for the wild type – considered as 100%): RNΔ*tagO* pMAD lacks WTA; RNΔ*tagO* p*tagO* produces 90% WTAs relative to the parental RN4220; and RNΔ*tagO* p*tagO*G152A produces 24% WTAs relative to the parental RN4220 strain. Wild type flies succumb successively to infection as the levels of WTAs increase (log-rank test, $P < 0.05$), likewise for the PGRP-SD mutant. In addition, survival of wild type and PGRP-SD mutant flies increasingly separates for each of the bacterial mutants: wild type versus PGRP-SD, $P = 0.2452$ (log-rank test, RNΔ*tagO* pMAD); $P = 0.0053$ (RNΔ*tagO* p*tagO*G152A); $P = 0.0001$ (RNΔ*tagO* p*tagO*). For all infections, PGRP-SA mutant flies succumb equally to infection (log-rank test, $P > 0.05$).

Overall, survival of wild type flies decreased as WTAs levels increased (with a concomitant decrease in PGRP-SA binding, Figure 4B), and likewise for the PGRP-SD mutant; with the difference between wild type and PGRP-SD mutant survival successively increasing. In contrast, survival of PGRP-SA mutant flies was independent of WTAs levels, with flies succumbing strongly for all infections in a statistically inseparable manner (Figure 9). These data confirmed that it was indeed *in vivo* protection of PGN by WTAs against the consequences of PGRP-SA binding, and furthermore, suggested that a requirement for PGRP-SD gradually became redundant as WTA levels decreased.

Wall teichoic acids and not D-alanylation of WTAs are the responsible for decreased PGRP-SA binding to PGN

It has been reported previously that D-alanylation of WTAs is also required for the pathogenicity of *S. aureus* [11]; D-alanylation is a process

that incorporates D-alanine residues into the glycerol- / ribitol-phosphate backbone of WTA, thereby reducing the negative charge of the polymer [43]. We examined therefore, whether a *S.* aureus mutant that lacks the D-alanylation pathway (RNΔ*dltABCD*) bound mCherry-PGRP-SA equivalently to RNΔ*tagO*. Binding of, mCherry-PGRP-SA to RNΔ*dltABCD* was similar to the binding to the wild type bacteria (Figure 7C). This prompted us to assess how RNΔ*dltABCD* affected survival of the wild type, PGRP-SD and PGRP-SA mutant flies. In contrast to RNΔ*tagO*, PGRP-SA mutant flies did not succumb strongly to RNΔ*dltABCD* infection, with 83% surviving at 72 hours post-infection (Fig 10).

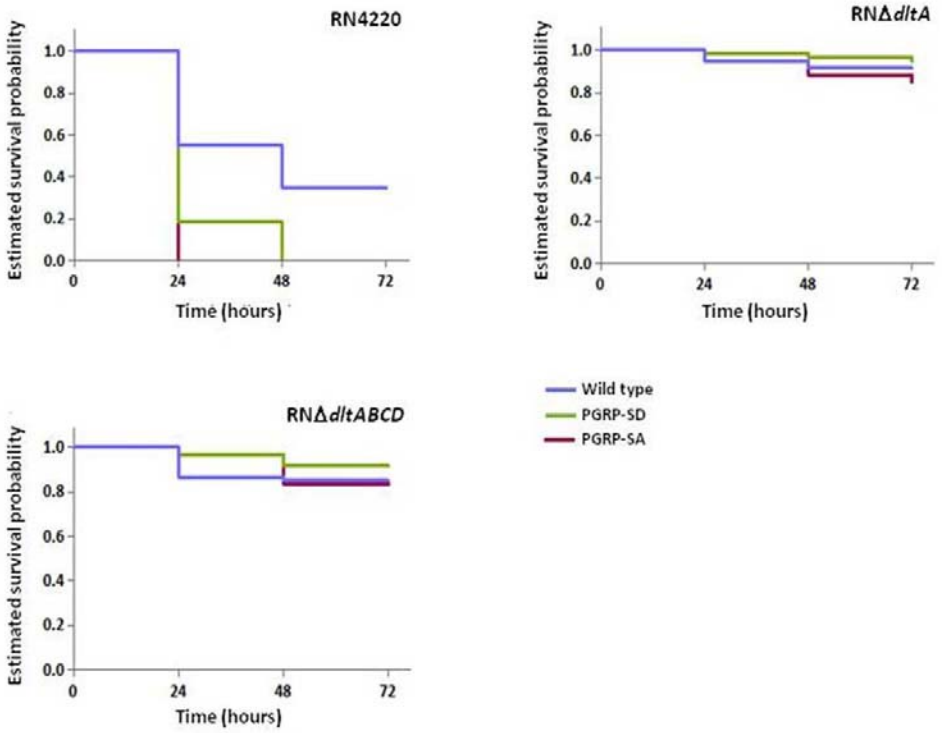


Fig 10. PGRP-SA mutant flies survive infection by *S. aureus* strains defective in the D-alanylation of WTAs. The *dltABCD* operon encode proteins involved in the D-alanylation of WTAs. Deletion of *dltA*, or of the *dltABCD* operon, result in bacteria that produce D-Alanine free WTAs. With all backgrounds, more than 80% of flies survived infection by RNΔ*dltABCD* or RNΔ*dltA*; all curves being statistically inseparable (log-rank, $P > 0.05$). Survival outcomes with the parental RN4220 strain are similar to those seen with NCTC8325-4 parental strain.

Furthermore, survival was statistically inseparable for the different fly backgrounds (Figure 10). These data demonstrated that D-alanylation is not necessary for WTAs to limit the access of PGRP-SA, that neither PGRP-SD nor PGRP-SA were required to control the RNΔ*dltABCD* infection and consequently, the reduced killing effect of RNΔ*dltABCD* had nothing to do with recognition.

DISCUSSION

The results shown here indicate that in respect to Gram-positive bacteria, where the cell wall is not concealed by outer membrane (e.g. *staphylococci*), pathogen recognition, via recognition of PGN, is tightly linked to host survival. Our studies bring forward the notion that one of the strategies used by pathogens to reduce recognition is to restrict accessibility to inflammatory non-self components of the cell wall. Specifically, the results here show that presence of WTAs in a range of Gram-positive bacteria impaired PGRP-SA binding. The use of tunicamycin to abolish WTAs synthesis dramatically improved receptor recognition of bacteria as well as host survival of flies infected with

antibiotic treated *S. aureus*. Genetically deleting a major component of the WTAs synthesis (TagO) in *S. aureus* also increased PGRP-SA binding leading to increased host survival. It should also be noted that, rPGRP-SA was capable of binding *in vitro* significantly better to WTAs-free PGN than to WTAs-linked PGN(CW) that were purified from wild type *S. aureus* bacteria, treated with trypsin to remove any attached surface proteins and adjusted to the same concentration of PGN (Figure 3). This observation confirmed the results obtained with live bacteria and allowed us to eliminate the hypothesis that deletion of *tagO* gene may influence the amount of proteins present at the cell surface and that this change in protein levels was influencing the binding of PGRP-SA. Effectively during the course of this work we have removed WTAs from PGN by treatment with antibiotic, by deletion of the *tagO* gene and finally we have chemically removed them from PGN. In all the cases binding of PGRP-SA to PGN has increased.

S. aureus produces WTAs composed of about 40 ribitol phosphate-repeating units modified with N-acetylglucosamine (GlcNAc) and D-alanine [7]. The latter modification is mediated by the D-Alanine ligase DltA and partially neutralizes the negative charge of the cell surface thus reducing attraction of cationic AMPs [43]. *S. aureus* Δ*dltA* mutants are more susceptible to killing by cationic AMPs and neutrophils *in vitro* and have markedly reduced virulence in several animal infection models including Drosophila [11, 44]. In one of these studies [11], Tabuchi and colleagues showed that *S. aureus* producing WTAs without D-alanylation were impaired in their ability to kill *Drosophila*. Surprisingly, they reported that *S. aureus* Δ*dltA* mutant was more impaired in the ability to

kill flies than an independently generated *tagO* mutant [11]; the latter according to the authors had the same pathogenicity as wild type *S. aureus* [11], contrary to our findings. There is a crucial point to be made in reference to this however, which is at the heart of our experimental design and gives physiological relevance to our results. We propose that WTAs are important to reduce *S. aureus* recognition by the host and thus help the pathogen increase its numbers inside the fly. The host uses PGRP-SA to control bacterial numbers and the more PGRP-SA binds to the cell wall (see Fig 4B) the more the bacterial load is controlled (as seen by comparing CFUs between wild type NCTC8325-4 *S. aureus* and NCTCΔ*tagO* in Fig 8). In PGRP-SA mutants the control mechanism is absent and NCTCΔ*tagO* was able to proliferate and kill the host (Fig 8). We were able to observe this because we started from a low bacterial load (10^2 cells/initial infection/fly) and followed the progress of pathogen load inside the host. Tabuchi *et al* injected 10^4-10^6 cells per fly for all bacterial strains used [11]. In our hands this concentration overwhelmed the host from the beginning and it is not surprising that these authors were unable to resolve statistical differences in host survival.

In order to rule out possible pleiotropic effects produced by the inactivation of the *tagO* due to the insertion of non-replicative plasmids or reversion of the mutation by elimination of the plasmid from the chromosome, we have specifically deleted the *tagO* gene in a manner that left no resistance marker in the bacterial chromosome and thus minimized possible alterations on the transcription of neighbouring genes. Finally, in order to increase the confidence of our results, we have complemented the *tagO* null mutant with plasmids that allowed the expression of a partially

active TagO protein, ($TagO^{G152A}$), and have statistically analyzed the estimated host survival probability curves obtained. Finally we should emphasize that deletion of the *tagO* gene in NCTC8325-4 strain (an *agr* positive strain) and in RN4220 (an *agr* negative strain) resulted in similar outcomes (Figure 7A, D): reduced pathogenicity in the Drosophila infection model and production of a bacterial cell surface that was better recognized by mCherry-PGRP-SA.

In parallel experiments we have also generated a *S. aureus* Δ*dltA* deletion mutant (this study) as well as a deletion of the Δ*dltA* operon (Δ*dltABCD*) [24] and found that both were indeed less pathogenic than wild type *S. aureus* (Figure 10), similar to what was previously reported [11]. However, this reduced pathogenicity was also observed in PGRP-SA and PGRP-SD single mutant flies (in contrast to Δ*tagO*). This indicated that the non-pathogenicity of Δ*dltA* was not linked to recognition by PGRP-SA or PGRP-SD.

We propose that increased "visibility" of PGN to PGRP-SA when WTAs were removed, dramatically improved survival of the host. However, alternative interpretations of our results may exist. In the following section we will attempt to challenge and rule them out:

1. We have recently reported that removal of WTAs has an impact on PGN cross-linking and consequently on the susceptibility to host lysozyme [24]. The possibility that the increased host survival may be the result of decreased pathogen resistance to the lysozyme constitutively expressed in the fly (due to the reduced PGN cross-linking in the *S. aureus tagO* null mutant) rather than removal of a physical entity (WTAs), which blocked access to PGN, was ruled out as follows. We generated an *S.*

aureus mutant unable to produce high-level PGN cross-linking but capable of producing regular levels of WTAs, by deleting the *pbpD* gene [24]. The *pbpD* gene encodes to PBP4 which is responsible for the final stages of PGN maturation and results in highly cross-linked PGN. As shown in Figure 4, bacteria that produce PGN with a low level of cross-linking, but normal levels of WTAs, were able to kill wild type flies similarly to the parental *S. aureus* strain. In addition, similar amounts of mCherry-PGRP-SA bound to the surface of both wild type bacteria and NCTCΔ*pbpD* (Figure 7C). These results indicate that in S. aureus Δ*tagO* strain increased recognition by mCherry-PGRP-SA and the inability to kill flies is due to the absence of the WTA and not due to alterations in PGN cross-linking.

2. The hypothesis that the absence of WTAs could turn *S. aureus* bacteria more susceptible to enzymes present in the haemolymph of *Drosophila*, such as lysozyme-like enzymes, which would make the bacteria unable to kill flies, was also considered and ruled out. In accordance with previous reports [44] we have verified that the *S. aureus tagO* null mutant is as resistant to lysozyme as the parental strain. The *tagO* null mutant only becomes susceptible to lysozyme when an additional mutation in the *oat* gene, encoding a protein responsible for PGN O-acetylation, is introduced (data not shown). Most importantly, injection of *S. aureus tagO* null mutant into PGRP-SA mutant flies was lethal, indicating that the *S. aureus tagO* null mutant bacteria were able to multiply in the haemolymph of flies if undeterred by PGRP-SA.

3. The possibility that PGRP-SA is responsible for directly killing bacteria lacking WTAs was also ruled-out as PGRP-SA is non-lytic [45].

There was no alteration in the growth rate of *S. aureus tagO* null mutant when grown in the presence of recombinant PGRP-SA (data not shown). At the present moment, we cannot exclude that a protein present in the haemolymph is capable of mediating killing of *S. aureus tagO* in complex with PGRP-SA.

4. The possibility that the absence of WTAs could turn *S. aureus* bacteria more susceptible to AMPs (produced as a consequence of the recognition of an invading pathogen) was also tested (Figure 11). Injection of *S. aureus tagO* null mutant into mutant flies affected in the ability to produce AMPS, such as *Dif*^{1}*-key*^{1}, *spz*^{rm7} and *spz*^{1} was not lethal to the flies, indicating that the *S. aureus tagO* null mutant bacteria were being eliminated in a way that was dependent on recognition by PGRP-SA but not dependent upon activation of the production of AMPs.

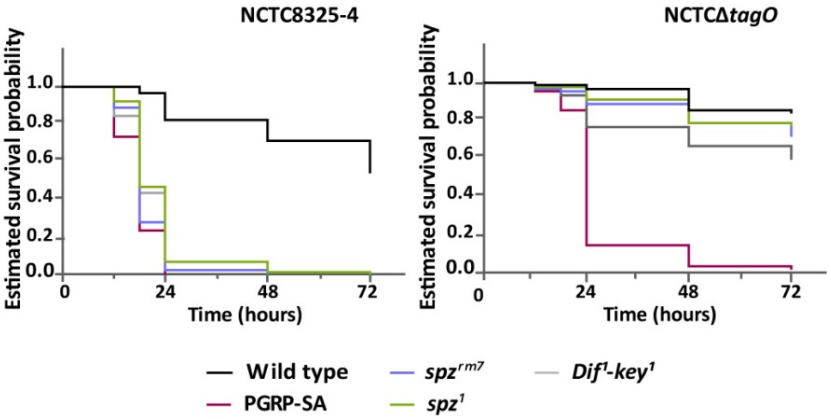


Figure 11. Flies severely compromised in AMP production are able to survive upon infection with *S. aureus* lacking WTAs. To assess the contribution of AMPs with regards to determining how flies survive infection with NCTC8325-4 or NCTCΔ*tagO*, flies compromised in their ability to produce AMPs (PGRP-SA, Dif-

key, spz^{1} and spz^{rm7}) were infected (~ 100 cells per fly) and survival recorded every 24 hours over 3 days. For each fly background – except wild type – survival curves were statistically inseparable for flies infected with NCTC8325-4 (log-rank test, $P > 0.05$). Flies affected in the production of AMPs succumbed strongly to infection with wild type bacteria NCTC8325-4 by 72 hours, whereas wild type flies survived up to ~55%. When infected with NCTCΔ*tagO*, survival curves for each fly background were statistically different from the PGRP-SA mutant flies (log-rank test, $P > 0.05$). PGRP-SA mutant flies succumbed to infection, whereas the rest of the mutants containing functional PGRP-SA but affected in the ability to produced AMPs survived up to more than 60 % by 72 hours.

At the moment we are unable to identify how Drosophila flies are killing invading *S. aureus tagO* null mutant bacteria. It is possible that bacteria, upon recognition by PGRP-SA, are more easily phagocytised or that, as *in Tenebrio molitor* [46], PGRP-SA binding recruits the local melanization cascade, triggering such a response.

Our results underline an important aspect of pathogen recognition by the host, which remains relatively unexplored. Namely, how does the host recognition machinery respond to changes in the surface of bacteria? Here we manipulated the amount of WTAs on the cell surface of *S. aureus*. Previously, two host PGRPs, PGRP-SA and PGRP-SD were found to be involved in recognition of wild type *S. aureus* [14, 15]. We found here that when WTA were genetically removed, the requirement for PGRP-SD was abolished. Flies deficient for PGRP-SD had estimated survival probabilities comparable to wild type flies following infection by *S. aureus ΔtagO* or $\Delta tagOptagO^{D87/D88A}$. When a small amount of WTAs was left on the surface through the residual activity of the *S. aureus* $\Delta tagOptagO^{G152A}$

then PGRP-SD mutants were less able to survive infection. However this sensitivity was not as pronounced as when infected with *S. aureus ΔtagOptagO*, the strain with reconstituted wild type levels of WTAs. Previous studies have established that PGRP-SD does not bind Gram-positive Lys-type PGN [14, 47]. However, in its presence, PGRP-SA was able to bind substantially better to cell wall from *S. aureus* and *S. saprophyticus* [14]. Our results, combined with the latter observation, support a role for PGRP-SD in neutralizing the effect of WTAs obstructing access to PGN. The alternate hypothesis that PGRP-SD may directly recognize WTAs, and is therefore not necessary when flies are infected with bacteria that lack teichoic acids, will be further investigate in the immediate future.

The role of teichoic acids in concealing PGN at the surface of Gram-positive bacteria may be also effective in preventing recognition by innate immune sensors of other organisms. It is now established that insect PGRPs have mammalian homologues and mice and humans express four genes encoding members of this family [45]. Our results correlate with data, which attributed a significantly reduced virulence of *tagO* mutants in cotton rat nasal colonisation model [48] as well as a mouse endophthalmitis model [49] and suggest a mechanism for how this may happen: absence of WTAs may render PGN at the bacteria surface more exposed to the host immune system.

BIBLIOGRAPHY

1. Foster, T.J., *Immune evasion by staphylococci.* Nat Rev Microbiol, 2005. **3**(12): p. 948-58.

2. Chaput, C. and I.G. Boneca, *Peptidoglycan detection by mammals and flies.* Microbes Infect, 2007. **9**(5): p. 637-47.

3. Vollmer, W., D. Blanot, and M.A. de Pedro, *Peptidoglycan structure and architecture.* FEMS Microbiol Rev, 2008. **32**(2): p. 149-67.

4. Schleifer, K.H. and O. Kandler, *Peptidoglycan types of bacterial cell walls and their taxonomic implications.* Bacteriol Rev, 1972. **36**(4): p. 407-77.

5. Scott, J.R. and T.C. Barnett, *Surface proteins of gram-positive bacteria and how they get there.* Annu Rev Microbiol, 2006. **60**: p. 397-423.

6. Kadioglu, A., J.N. Weiser, J.C. Paton, and P.W. Andrew, *The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease.* Nat Rev Microbiol, 2008. **6**(4): p. 288-301.

7. Weidenmaier, C. and A. Peschel, *Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions.* Nat Rev Microbiol, 2008. **6**(4): p. 276-87.

8. Humann, J. and L.L. Lenz, *Bacterial peptidoglycan degrading enzymes and their impact on host muropeptide detection.* J Innate Immun, 2009. **1**(2): p. 88-97.

9. Vollmer, W., *Structural variation in the glycan strands of bacterial peptidoglycan.* FEMS Microbiol Rev, 2008. **32**(2): p. 287-306.

10. Boneca, I.G., O. Dussurget, D. Cabanes, M.A. Nahori, S. Sousa, M. Lecuit, E. Psylinakis, V. Bouriotis, J.P. Hugot, M. Giovannini, A. Coyle, J. Bertin, A. Namane, J.C. Rousselle, N. Cayet, M.C. Prevost, V. Balloy, M. Chignard, D.J. Philpott, P. Cossart, and S.E. Girardin, *A critical role for peptidoglycan N-deacetylation in Listeria evasion from the host innate immune system.* Proc Natl Acad Sci U S A, 2007. **104**(3): p. 997-1002.

11. Tabuchi, Y., A. Shiratsuchi, K. Kurokawa, J.H. Gong, K. Sekimizu, B.L. Lee, and Y. Nakanishi, *Inhibitory role for D-alanylation of wall teichoic acid in activation of insect Toll pathway by peptidoglycan of Staphylococcus aureus.* J Immunol, 2010. **185**(4): p. 2424-31.

12. Kurokawa, K., J.H. Gong, K.H. Ryu, L. Zheng, J.H. Chae, M.S. Kim, and B.L. Lee, *Biochemical characterization of evasion from peptidoglycan recognition by Staphylococcus aureus D-alanylated wall teichoic acid in insect innate immunity.* Dev Comp Immunol, 2011. **35**(8): p. 835-9.

13. Filipe, S.R., A. Tomasz, and P. Ligoxygakis, *Requirements of peptidoglycan structure that allow detection by the Drosophila Toll pathway.* EMBO Rep, 2005. **6**(4): p. 327-33.

14. Wang, L., R.J. Gilbert, M.L. Atilano, S.R. Filipe, N.J. Gay, and P. Ligoxygakis, *Peptidoglycan recognition protein-SD provides versatility of receptor formation in Drosophila immunity.* Proc Natl Acad Sci U S A, 2008. **105**(33): p. 11881-6.

15. Bischoff, V., C. Vignal, I.G. Boneca, T. Michel, J.A. Hoffmann, and J. Royet, *Function of the drosophila pattern-recognition receptor PGRP-SD in the detection of Gram-positive bacteria.* Nat Immunol, 2004. **5**(11): p. 1175-80.

16. Michel, T., J.M. Reichhart, J.A. Hoffmann, and J. Royet, *Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein.* Nature, 2001. **414**(6865): p. 756-9.

17. Gobert, V., M. Gottar, A.A. Matskevich, S. Rutschmann, J. Royet, M. Belvin, J.A. Hoffmann, and D. Ferrandon, *Dual activation of the Drosophila toll pathway by two pattern recognition receptors.* Science, 2003. **302**(5653): p. 2126-30.

18. Lemaitre, B. and J. Hoffmann, *The Host Defense of Drosophila melanogaster.* Annu Rev Immunol, 2007. **25**: p. 697-743.

19. Ferrandon, D., A.C. Jung, M. Criqui, B. Lemaitre, S. Uttenweiler-Joseph, L. Michaut, J. Reichhart, and J.A. Hoffmann, *A drosomycin-GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway.* Embo J, 1998. **17**(5): p. 1217-27.

20. Lemaitre, B., E. Nicolas, L. Michaut, J.M. Reichhart, and J.A. Hoffmann, *The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults.* Cell, 1996. **86**(6): p. 973-83.

21. Lindsley, D.L. and G.G. Zimm, *The Genome of Drosophila melanogaster*1992, San Diego: Academic Press.

22. Rutschmann, S., A. Kilinc, and D. Ferrandon, *Cutting edge: the toll pathway is required for resistance to gram-positive bacterial infections in Drosophila.* J Immunol, 2002. **168**(4): p. 1542-6.

23. Vergara-Irigaray, M., T. Maira-Litran, N. Merino, G.B. Pier, J.R. Penades, and I. Lasa, *Wall teichoic acids are dispensable for anchoring the PNAG exopolysaccharide to the Staphylococcus aureus cell surface.* Microbiology, 2008. **154**(Pt 3): p. 865-77.

24. Atilano, M.L., P.M. Pereira, J. Yates, P. Reed, H. Veiga, M.G. Pinho, and S.R. Filipe, *Teichoic acids are temporal and spatial regulators of peptidoglycan cross-linking in Staphylococcus aureus.* Proc Natl Acad Sci U S A, 2010. **107**(44): p. 18991-6.

25. Wieser, M., E.B. Denner, P. Kampfer, P. Schumann, B. Tindall, U. Steiner, D. Vybiral, W. Lubitz, A.M. Maszenan, B.K. Patel, R.J. Seviour, C. Radax, and H.J. Busse, *Emended descriptions of the genus Micrococcus, Micrococcus luteus (Cohn 1872) and Micrococcus lylae (Kloos et al. 1974).* Int J Syst Evol Microbiol, 2002. **52**(Pt 2): p. 629-37.

26. Jacob, A.E. and S.J. Hobbs, *Conjugal transfer of plasmid-borne multiple antibiotic resistance in Streptococcus faecalis var. zymogenes.* J Bacteriol, 1974. **117**(2): p. 360-72.

27. Henriques, A.O., P. Glaser, P.J. Piggot, and C.P. Moran, Jr., *Control of cell shape and elongation by the rodA gene in Bacillus subtilis.* Mol Microbiol, 1998. **28**(2): p. 235-47.

28. Glittenberg, M.T., S. Silas, D.M. MacCallum, N.A. Gow, and P. Ligoxygakis, *Wild-type Drosophila melanogaster as an alternative model system for investigating the pathogenicity of Candida albicans.* Dis Model Mech. **4**(4): p. 504-14.

29. Abramoff, M., P. Magalhaes, and S. Ram, *Image processing with ImageJ.* Biophoton Int, 2004. **11**: p. 36-42.

30. Campbell, J., A.K. Singh, J.P. Santa Maria, Jr., Y. Kim, S. Brown, J.G. Swoboda, E. Mylonakis, B.J. Wilkinson, and S. Walker, *Synthetic lethal compound combinations reveal a fundamental connection between wall teichoic acid and peptidoglycan biosyntheses in Staphylococcus aureus.* ACS Chem Biol, 2011. **6**(1): p. 106-16.

31. Wang, L., A.N. Weber, M.L. Atilano, S.R. Filipe, N.J. Gay, and P. Ligoxygakis, *Sensing of Gram-positive bacteria in Drosophila: GNBP1 is needed to process and present peptidoglycan to PGRP-SA.* Embo J, 2006. **25**(20): p. 5005-14.

32. Kashyap, D.R., M. Wang, L.H. Liu, G.J. Boons, D. Gupta, and R. Dziarski, *Peptidoglycan recognition proteins kill bacteria by activating protein-sensing two-component systems.* Nat Med, 2011. **17**(6): p. 676-83.

33. Schlag, M., R. Biswas, B. Krismer, T. Kohler, S. Zoll, W. Yu, H. Schwarz, A. Peschel, and F. Gotz, *Role of staphylococcal wall teichoic acid in targeting the major autolysin Atl.* Mol Microbiol, 2010. **75**(4): p. 864-73.

34. Grundling, A., D.M. Missiakas, and O. Schneewind, *Staphylococcus aureus mutants with increased lysostaphin resistance.* J Bacteriol, 2006. **188**(17): p. 6286-97.

35. Steen, A., G. Buist, K.J. Leenhouts, M. El Khattabi, F. Grijpstra, A.L. Zomer, G. Venema, O.P. Kuipers, and J. Kok, *Cell wall attachment of a widely distributed peptidoglycan binding domain is hindered by cell wall constituents.* J Biol Chem, 2003. **278**(26): p. 23874-81.

36. Davison, A.L. and J. Baddiley, *The Distribution of Teichoic Acids in Staphylococci.* J Gen Microbiol, 1963. **32**: p. 271-6.

37. Swoboda, J.G., J. Campbell, T.C. Meredith, and S. Walker, *Wall teichoic acid function, biosynthesis, and inhibition.* Chembiochem, 2010. **11**(1): p. 35-45.

38. Wang, Y., J. Huebner, A.O. Tzianabos, G. Martirosian, D.L. Kasper, and G.B. Pier, *Structure of an antigenic teichoic acid shared by clinical isolates of Enterococcus faecalis and vancomycin-resistant Enterococcus faecium.* Carbohydr Res, 1999. **316**(1-4): p. 155-60.

39. Salton, M.R.J., *The bacterial cell envelope - a historical perspective*, in *Bacterial Cell Wall*, J.-M. Ghuysen and R. Hakenbeck, Editors. 1994, Elsevier. p. 1-22.

40. Brown, S., Y.H. Zhang, and S. Walker, *A revised pathway proposed for Staphylococcus aureus wall teichoic acid biosynthesis based on in vitro reconstitution of the intracellular steps.* Chem Biol, 2008. **15**(1): p. 12-21.

41. Archer, G.L., *Staphylococcus aureus: a well-armed pathogen.* Clin Infect Dis, 1998. **26**(5): p. 1179-81.

42. Galac, M.R. and B.P. Lazzaro, *Comparative pathology of bacteria in the genus Providencia to a natural host, Drosophila melanogaster.* Microbes Infect, 2011. **13**(7): p. 673-83.

43. Peschel, A., M. Otto, R.W. Jack, H. Kalbacher, G. Jung, and F. Gotz, *Inactivation of the dlt operon in Staphylococcus aureus confers sensitivity to defensins, protegrins, and other antimicrobial peptides.* J Biol Chem, 1999. **274**(13): p. 8405-10.

44. Bera, A., R. Biswas, S. Herbert, E. Kulauzovic, C. Weidenmaier, A. Peschel, and F. Gotz, *Influence of wall teichoic acid on lysozyme resistance in Staphylococcus aureus.* J Bacteriol, 2007. **189**(1): p. 280-3.

45. Dziarski, R. and D. Gupta, *The peptidoglycan recognition proteins (PGRPs).* Genome Biol, 2006. **7**(8): p. 232.

46. Park, J.W., B.R. Je, S. Piao, S. Inamura, Y. Fujimoto, K. Fukase, S. Kusumoto, K. Soderhall, N.C. Ha, and B.L. Lee, *A synthetic peptidoglycan fragment as a competitive inhibitor of the melanization cascade.* J Biol Chem, 2006. **281**(12): p. 7747-55.

47. Leone, P., V. Bischoff, C. Kellenberger, C. Hetru, J. Royet, and A. Roussel, *Crystal structure of Drosophila PGRP-SD suggests binding to DAP-type but not lysine-type peptidoglycan.* Mol Immunol, 2008. **45**(9): p. 2521-30.

48. Weidenmaier, C., J.F. Kokai-Kun, E. Kulauzovic, T. Kohler, G. Thumm, H. Stoll, F. Gotz, and A. Peschel, *Differential roles of sortase-anchored surface proteins and wall teichoic acid in Staphylococcus aureus nasal colonization.* Int J Med Microbiol, 2008. **298**(5-6): p. 505-13.

49. Suzuki, T., J. Campbell, J.G. Swoboda, S. Walker, and M.S. Gilmore, *Role of wall teichoic acids in Staphylococcus aureus endophthalmitis.* Invest Ophthalmol Vis Sci, 2011. **52**(6): p. 3187-92.

CHAPTER V

Concluding remarks and future perspectives

Over the past few decades, several studies have highlighted the importance of WTAs in the physiology and pathogenicity of Gram-positive bacteria. However their specific roles have been difficult to identify because these polymers affect several cell envelope properties. In this thesis I have revealed two additional aspects of WTA function in *S. aureus* bacteria: 1) the synthesis of WTAs regulates the level of PGN cross-linking by localizing PBP4 to the division septum, and 2) WTAs can reduce accessibility of the pattern recognition receptor PGRP-SA to the PGN and allow bacteria to proliferate in the host.

Role of WTAs in PGN synthesis

Recent studies in *B. subtilis* have provided evidence that WTAs play an important role in PGN synthesis. The disruption of *tagO* and *tagA* genes in this rod-shaped bacterium, resulted in the formation of coccoid cells [1, 2]. This indicates that WTAs are somehow required for the correct assembly of the cell wall synthetic machinery in order to maintain correct cell morphology. In addition, fluorescence analysis revealed that WTA biosynthetic enzymes in *B. subtilis* are localized at division sites and along the lateral walls of the bacterial cells [3]. The WTA synthesizing enzymes localized in a helical pattern similar to that displayed by fluorescently labeled probes capable of binding to the nascent PGN [4]. WTA synthesizing enzymes have also been shown to interact with MreB, MreC and MreD, which are proteins proposed to work as scaffolds for the PGN biosynthetic enzymes such as the PBPs [5]. However, until now there has been no further evidence suggesting a coordinated biosynthesis of the two

major cell wall components. WTA synthesis, similarly to PGN synthesis, starts with formation of nucleotide sugars in the cytoplasm, proceeds to the sugar phosphate transference to the same lipid carrier undecanoprenyl phosphate, followed by translocation to the external side of the membrane and linkage to the PGN macromolecule.

In this work we have uncovered new evidence for the link between PGN and WTA biosynthesis in *S. aureus*. We have shown that the TagO protein, involved in WTA biosynthesis, is localized at the division septum together with other proteins involved in PGN synthesis, and its activity is essential to keep PBP4 at this site. These findings suggest that WTAs regulate the level of PGN cross-linking through the recruitment of PBP4 to the division septa. Although recruitment of PBP4 to the septum is dependent upon the synthesis of WTAs, the precise WTA intermediates that are involved remain unknown. PBP4 localization analysis in conditional mutants in the WTA biosynthetic machinery, that lead to the accumulation of different WTA intermediates, might clarify the WTA intermediate structures involved in this process. If the WTA intermediates involved could be identified through this approach, questions regarding the mechanism by which the protein is localized and regulated would still remain. For example, does PBP4 interact directly with the WTAs or through other mediator proteins (Figure 1)? The enzymes involved in cell wall biosynthesis and degradation are localized outside of the bacterial cell membrane and their regulation is crucial to the maintenance of cell integrity. It has long been proposed that these enzymes might interact with cell wall components, such as other surface proteins, in order to be regulated [6, 7]. Recent studies in *E. coli* suggest that outer-membrane

lipoproteins LpoA and LpoB interact with PBP1A and PBP1B respectively, which are situated between the inner membrane and the PGN layer regulating their activity [8, 9]. Since *S. aureus* lacks an outer membrane, one possibility is that these bacteria use WTAs instead of outer-membrane lipoproteins as PBP4 accessory molecules to regulate its activity (Figure 1A). Membrane–anchored WTAs at the septum might be inserted through pores in the PGN layers and therefore act as a scaffold for the PBP4 protein.

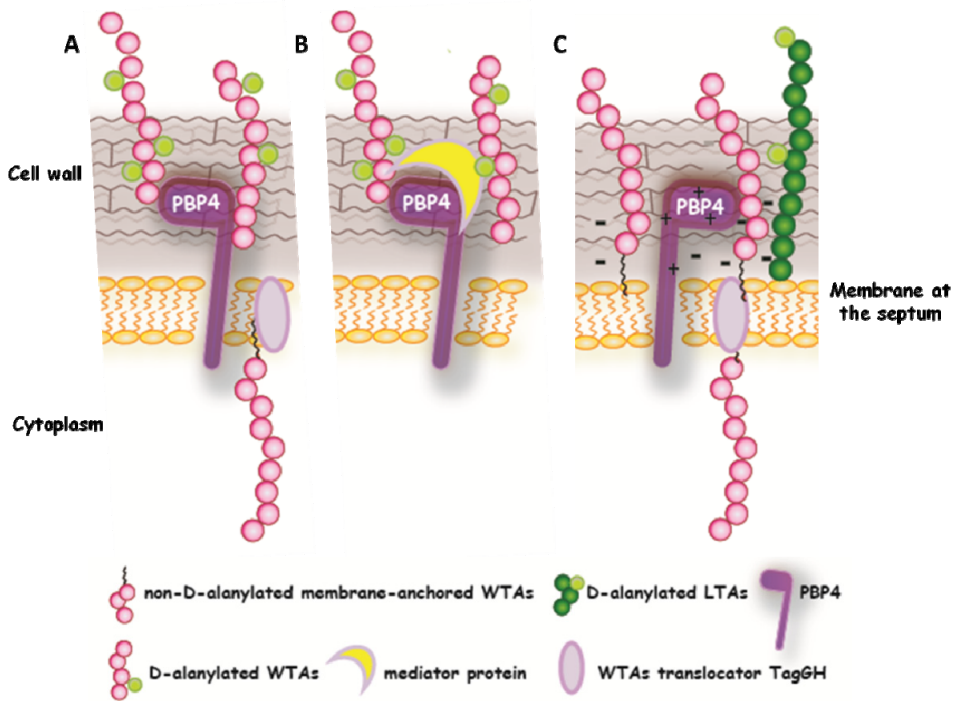


Figure 1. Models for PBP4 localization. Shown are schematic representations of putative mechanisms for PBP4 localization at the division septum. (A) PBP4 directly interacts with WTAs intermediates present at the septum. (B) PBP4 interacts indirectly with WTAs present at the septum through a mediator protein.

(C) PBP4 PBP4 localizes at the septum via electrostatic interactions with the highly negative charged TAs present in that region.

Binding to WTAs through a specific domain, would allow PBP4 to reach its PGN substrate and perform the transpeptidation reaction. PBP4 possess a DUF1958 domain that is also present in other D, D-carboxypeptidases from Gram-positive bacteria containing WTAs but not in the other PBPs. The biological function of this domain is not yet known but could be involved in binding to WTAs. Thus, it would be interesting to study the effect of point mutations within this uncharacterized DUF1958 domain upon PBP4 localization.

WTAs and LTAs are polyanionic cell wall components that highly contribute to negative charge of the bacterial cell envelope [10]. An equally attractive possibility is that PBP4 localization could be dependent upon a charge gradient at the septum (Figure 1C). The highly negative charge gradient produced by the non-D-alanylated membrane-anchored teichoic acids at the septum could serve as a key determinant for protein localization as some proteins, such as PBP4, which contain cationic domains in their amino acid sequence. Interestingly, [11] has recently revealed that FmtA, a putative PBP involved in PGN biosynthesis, binds to WTAs via electrostatic interactions. The proposed negative charge produced at the septum by WTAs is also in agreement with studies which have proposed the binding of cationic autolysins to anionic teichoic acids as a control mechanism that regulates autolysins [12, 13]. Moreover, the delocalization of Atl observed by Schalag and colleagues in the absence of WTAs [14] further supports of this model.

Although these models are possible on a theoretical basis, experimental support for them is still lacking. Pull down assays with purified PBP4 protein and WTAs should allow us to elucidate the existence or not of electrostatic interactions or direct binding between PBP4 and WTAs glycopolymers. Moreover, limited trypsin digestion follow by Maldi-TOF analysis might help to determine the protein regions involved in interactions with WTAs.

It is important to highlight that although WTAs are thought to be distributed uniformly over the entire lateral cell wall, there is not yet any consensus about their existence or distribution at the septum. The use of specific compounds to target the WTAs or the use of microscopy techniques such as fluorescence, atomic force and electron microscopy might clarify their distribution in the cell and provide new insights into the mechanisms by which they localize proteins.

In summary, the role of WTA synthesis on the localization of PBP4 [15] together with the recent reports of Atl [14] and FmtA [11] localization dependent on WTA, clearly supports the importance of WTAs in the regulation of PGN synthesis and degradation/autolysis. Furthermore, the discovery that WTAs regulate PGN cross-linking, reinforces the idea that bacteria coordinate both the PGN and WTA synthesizing machineries, in order to assemble the complex cell wall envelope that can protect the bacteria from the host immune system.

WTAs reduce accessibility of the Drosophila PGRP-SA to the PGN

The PGN from *S. aureus*, a clinical relevant bacterial human pathogen, is highly modified. PGN modifications have been associated with the ability of bacteria to survive or proliferate within the host, by enabling them to counter particular aspects of host immunity [16-20] For example, it has been shown that WTAs contribute to the protection of bacteria from detrimental host factors such as lysozyme [21], and are also required for adherence to host tissues [22], which in turn, elicits virulence.

In this work we revealed a new role in virulence of the WTAs and have proposed a model for their role in bacteria/host interaction (Figure 2). According to this model, WTAs prevent the pattern recognition receptor PGRP-SA from binding to its PGN substrate, which may reduce immune detection of PGN within the host. In addition, they also control the level of secondary cross-linking of the PGN, which turns it more susceptible to host lysozyme [15]. The former function is fundamental for the bacteria to survive within a host, whereas the latter is dispensable in this regard. Although the increased binding of PGRP-SA to bacteria lacking WTAs results from the combined effect of the absence of two factors, WTAs and a high degree of cross-linking, it would be interesting to evaluate only the effect of the first in PGRP-SA accessibility to PGN of live cells.

Another important observation is the ability of PGRP-SA to bind specific sites of bacteria that produce WTAs (usually, division septa). One possible explanation for this observation could be that the putative intermediate forms of WTAs, present at the division septum, [14, 15], are

not as efficient preventing the PGRP-SA binding to PGN as the putative mature form of WTAs.

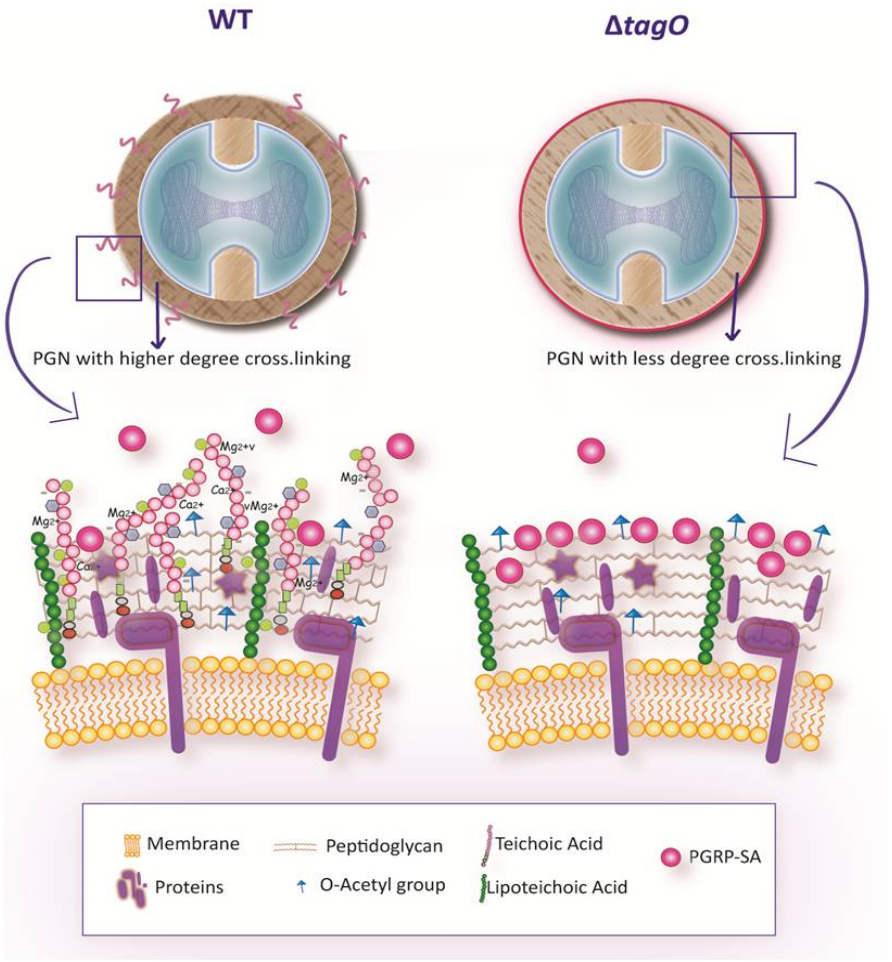


Figure 2. Model for the role of WTAs in immune recognition of *S. aureus*. Recognition of bacterial cells by PGRP-SA is limited in cells containing WTAs in their PGN. Absence of WTAs and decreased PGN cross-linking leads to better PGN detection due to an increase in the number of PGRP-SA molecules binding to the bacterial cells.

An alternative explanation might be the increase autolytic activity that is expect to occur at the septum, the site better recognized by PGRP-

SA [23]. Therefore the PGN at these regions would have a different composition/structure, due to the action of the autolysins during the separation process, allowing the PGRP-SA to better access its MurNAc-pentapetide substrate. In agreement with this, Kashyap and colleagues [24] revealed a similar binding of mammalian PGRPs to the splitting septa, as was observed with PGRP-SA, showing that these PGRPs co-localized, at the same cell separation sites, with the peptidoglycan hydrolases LytE and LytF in *B. subtilis.* Consistent with the model proposed here, the Atl enzyme was shown to delocalize in *S. aureus* Δ*tagO* [14].

Our *in vivo* data indicated that increased binding of Drosophila PGRP-SA to *S. aureus* lacking WTAs reduced bacterial growth within the host. However, the consequences of this binding and the exact role of PGRP-SA in this phenomenon remain unclear. Recognition of bacterial PGN by PGRPs in insects activates a broad inflammatory response characterized by the production of AMPs, the recruitment of phagocytes, and activation of the prophenoloxidase pathway [25-27]. Although PGRP-SA has some sequence homology to bacteriophage T7 lysozyme, an amidase able to hydrolyze the MurNAc linkage to the stem peptide, PGRP-SA has no amidase activity and according to our results is not bactericidal (*in vitro* data; data not shown) unlike the human PGRPs, recently described by Kashyap *et al* [24]. Even though the reduced bacterial load does not seem to be dependent upon antimicrobial peptide production, PGRP-SA might interact with, activate or increase the activity of another antibacterial system such as the prophenoloxidase cascade.

The exact function of PGRP-SD in PGN recognition remains unclear. Although PGRP-SD have a weak binding affinity for Lys-type PGN [28], both PGRP-SA and PGRP-SD are thought to be involved in *S. aureus* recognition by the Drosophila immune system [29] . However, PGRP-SD seems not to be required for recognition of *S. aureus* lacking WTAs. Our results suggest that PGRP-SD might be needed for recognition of bacteria containing WTAs in their PGN. The purification of a fluorescent derivative of PGRP-SD protein and analysis of the protein binding to the surface of bacteria may help to better elucidate the role of PGRP-SD in bacterial recognition and the Drosophila immune response.

In summary, the work described in this thesis reveals new insights about how bacteria have evolved a sophisticated and complex cell wall that allows them to survive in hostile environments. In particular, it describes how WTAs contribute to the cell wall architecture and to help in the establishment of a successful host infection.

BIBLIOGRAPHY

1. D'Elia, M.A., M.P. Pereira, Y.S. Chung, W. Zhao, A. Chau, T.J. Kenney, M.C. Sulavik, T.A. Black, and E.D. Brown, *Lesions in teichoic acid biosynthesis in Staphylococcus aureus lead to a lethal gain of function in the otherwise dispensable pathway.* J Bacteriol, 2006. **188**(12): p. 4183-9.

2. D'Elia, M.A., J.A. Henderson, T.J. Beveridge, D.E. Heinrichs, and E.D. Brown, *The N-acetylmannosamine transferase catalyzes the first committed step of teichoic acid assembly in Bacillus subtilis and Staphylococcus aureus.* J Bacteriol, 2009. **191**(12): p. 4030-4.

3. Formstone, A., R. Carballido-Lopez, P. Noirot, J. Errington, and D.J. Scheffers, *Localization and interactions of teichoic acid synthetic enzymes in Bacillus subtilis.* J Bacteriol, 2008. **190**(5): p. 1812-21.

4. Daniel, R.A. and J. Errington, *Control of cell morphogenesis in bacteria: two distinct ways to make a rod-shaped cell.* Cell, 2003. **113**(6): p. 767-76.

5. Kawai, Y., J. Marles-Wright, R.M. Cleverley, R. Emmins, S. Ishikawa, M. Kuwano, N. Heinz, N.K. Bui, C.N. Hoyland, N. Ogasawara, R.J. Lewis, W. Vollmer, R.A. Daniel, and J. Errington, *A widespread family of bacterial cell wall assembly proteins.* EMBO J, 2011. **30**(24): p. 4931-41.

6. Goffin, C. and J.M. Ghuysen, *Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs.* Microbiol Mol Biol Rev, 1998. **62**(4): p. 1079-93.

7. Rice, K.C. and K.W. Bayles, *Molecular control of bacterial death and lysis.* Microbiol Mol Biol Rev, 2008. **72**(1): p. 85-109, table of contents.

8. Typas, A., M. Banzhaf, B. van den Berg van Saparoea, J. Verheul, J. Biboy, R.J. Nichols, M. Zietek, K. Beilharz, K. Kannenberg, M. von Rechenberg, E. Breukink, T. den Blaauwen, C.A. Gross, and W. Vollmer, *Regulation of peptidoglycan synthesis by outer-membrane proteins.* Cell, 2010. **143**(7): p. 1097-109.

9. Paradis-Bleau, C., M. Markovski, T. Uehara, T.J. Lupoli, S. Walker, D.E. Kahne, and T.G. Bernhardt, *Lipoprotein cofactors located in the outer membrane activate bacterial cell wall polymerases.* Cell, 2010. **143**(7): p. 1110-20.

10. Neuhaus, F.C. and J. Baddiley, *A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria.* Microbiol Mol Biol Rev, 2003. **67**(4): p. 686-723.

11. A, Q., *Investigation of the function and role of FMTA in the Staphylococcus aureus response to antibiotic stress*, in *Biology*2011, York university: Toronto.

12. Peschel, A., C. Vuong, M. Otto, and F. Gotz, *The D-alanine residues of Staphylococcus aureus teichoic acids alter the susceptibility to vancomycin and the activity of autolytic enzymes.* Antimicrob Agents Chemother, 2000. **44**(10): p. 2845-7.

13. Fedtke, I., D. Mader, T. Kohler, H. Moll, G. Nicholson, R. Biswas, K. Henseler, F. Gotz, U. Zahringer, and A. Peschel, *A Staphylococcus aureus ypfP mutant with strongly reduced lipoteichoic acid (LTA) content: LTA governs bacterial surface properties and autolysin activity.* Mol Microbiol, 2007. **65**(4): p. 1078-91.

14. Schlag, M., R. Biswas, B. Krismer, T. Kohler, S. Zoll, W. Yu, H. Schwarz, A. Peschel, and F. Gotz, *Role of staphylococcal wall teichoic acid in targeting the major autolysin Atl.* Mol Microbiol, 2010. **75**(4): p. 864-73.

15. Atilano, M.L., P.M. Pereira, J. Yates, P. Reed, H. Veiga, M.G. Pinho, and S.R. Filipe, *Teichoic acids are temporal and spatial regulators of peptidoglycan cross-*

linking in Staphylococcus aureus. Proc Natl Acad Sci U S A, 2010. **107**(44): p. 18991-6.

16. Humann, J. and L.L. Lenz, *Bacterial peptidoglycan degrading enzymes and their impact on host muropeptide detection.* J Innate Immun, 2009. **1**(2): p. 88-97.

17. Kraus, D. and A. Peschel, *Staphylococcus aureus evasion of innate antimicrobial defense.* Future Microbiol, 2008. **3**(4): p. 437-51.

18. Bishop, J.L., E.C. Boyle, and B.B. Finlay, *Deception point: peptidoglycan modification as a means of immune evasion.* Proc Natl Acad Sci U S A, 2007. **104**(3): p. 691-2.

19. Gotz, F., *Staphylococci in colonization and disease: prospective targets for drugs and vaccines.* Curr Opin Microbiol, 2004. **7**(5): p. 477-87.

20. Koprivnjak, T. and A. Peschel, *Bacterial resistance mechanisms against host defense peptides.* Cell Mol Life Sci, 2011. **68**(13): p. 2243-54.

21. Bera, A., R. Biswas, S. Herbert, E. Kulauzovic, C. Weidenmaier, A. Peschel, and F. Gotz, *Influence of wall teichoic acid on lysozyme resistance in Staphylococcus aureus.* J Bacteriol, 2007. **189**(1): p. 280-3.

22. Weidenmaier, C., J.F. Kokai-Kun, S.A. Kristian, T. Chanturiya, H. Kalbacher, M. Gross, G. Nicholson, B. Neumeister, J.J. Mond, and A. Peschel, *Role of teichoic acids in Staphylococcus aureus nasal colonization, a major risk factor in nosocomial infections.* Nat Med, 2004. **10**(3): p. 243-5.

23. Touhami, A., M.H. Jericho, and T.J. Beveridge, *Atomic force microscopy of cell growth and division in Staphylococcus aureus.* J Bacteriol, 2004. **186**(11): p. 3286-95.

24. Kashyap, D.R., M. Wang, L.H. Liu, G.J. Boons, D. Gupta, and R. Dziarski, *Peptidoglycan recognition proteins kill bacteria by activating protein-sensing two-component systems.* Nat Med, 2011. **17**(6): p. 676-83.

25. Royet, J. and R. Dziarski, *Peptidoglycan recognition proteins: pleiotropic sensors and effectors of antimicrobial defences.* Nat Rev Microbiol, 2007. **5**(4): p. 264-77.

26. Park, J.W., C.H. Kim, J.H. Kim, B.R. Je, K.B. Roh, S.J. Kim, H.H. Lee, J.H. Ryu, J.H. Lim, B.H. Oh, W.J. Lee, N.C. Ha, and B.L. Lee, *Clustering of peptidoglycan recognition protein-SA is required for sensing lysine-type peptidoglycan in insects.* Proc Natl Acad Sci U S A, 2007. **104**(16): p. 6602-7.

27. Garver, L.S., J. Wu, and L.P. Wu, *The peptidoglycan recognition protein PGRP-SC1a is essential for Toll signaling and phagocytosis of Staphylococcus aureus in Drosophila.* Proc Natl Acad Sci U S A, 2006. **103**(3): p. 660-5.

28. Wang, L., R.J. Gilbert, M.L. Atilano, S.R. Filipe, N.J. Gay, and P. Ligoxygakis, *Peptidoglycan recognition protein-SD provides versatility of receptor formation in Drosophila immunity.* Proc Natl Acad Sci U S A, 2008. **105**(33): p. 11881-6.

29. Bischoff, V., C. Vignal, I.G. Boneca, T. Michel, J.A. Hoffmann, and J. Royet, *Function of the drosophila pattern-recognition receptor PGRP-SD in the detection of Gram-positive bacteria.* Nat Immunol, 2004. **5**(11): p. 1175-80.