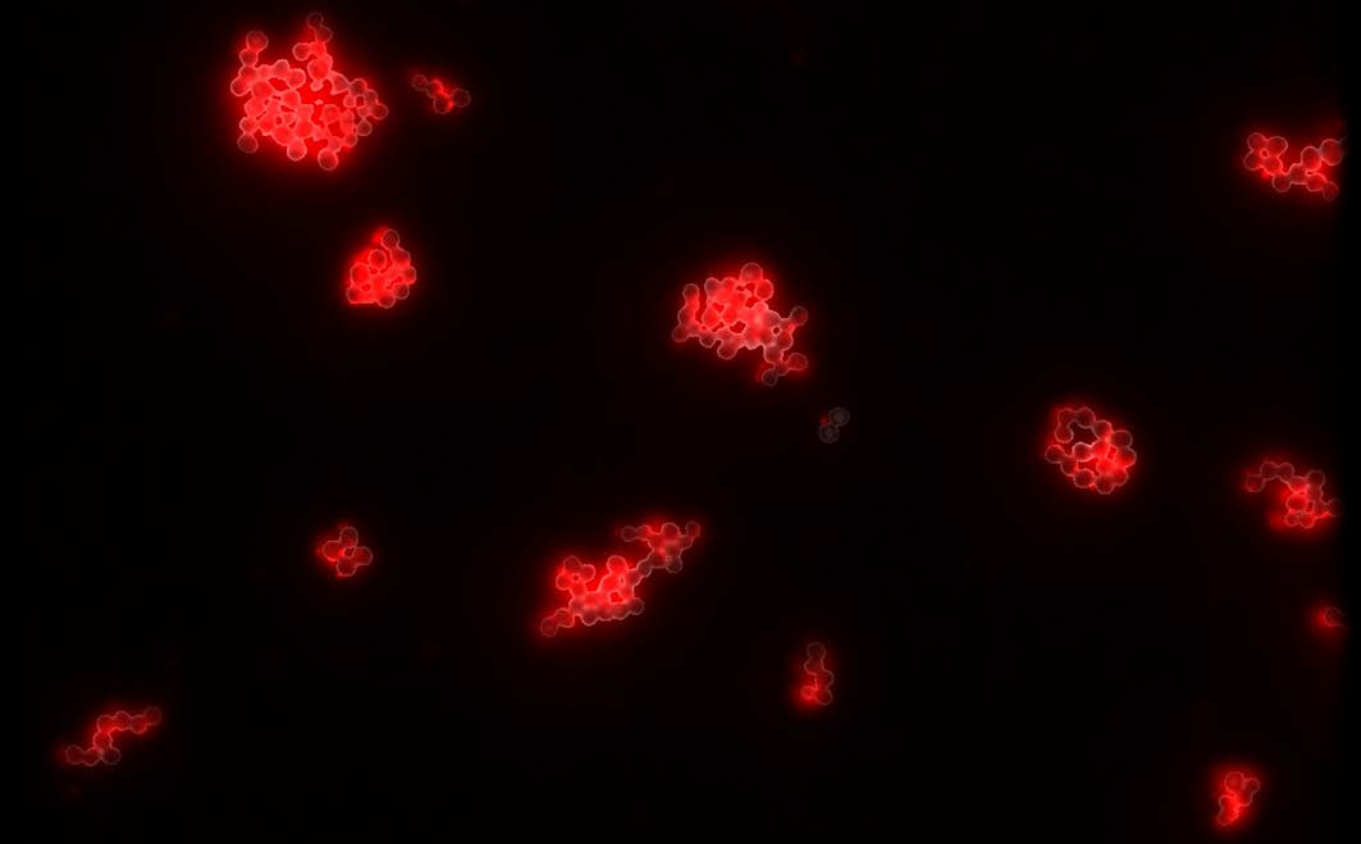
Synthesis and host recognition of *Staphylococcus aureus* peptidoglycan

Gonçalo Neff Valadares Gomes Covas



Dissertation presented to obtain the Ph.D degree in Molecular Biosciences

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
December, 2019

UNIVERSIDADE NOVA DE LISBOA

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COVER IMAGE

mCherry_PGRP-SA binding to *S. aureus* JE2 Δ*atl*Δ*sagB* mutant cells.

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Publications in International Peer-reviewed Journals:

- Queda, F., **Covas, G.**, Silva, T., Santos, C. A., Bronze, M. R., Cañada, F. J., Corvo M. C., Filipe, S. R., Marques, M. M. B. (2019). A top-down chemo-enzymatic approach towards *N*-acetylglucosamine-*N*-acetylmuramic oligosaccharides: Chitosan as a reliable template. Carbohydrate Polymers, 224, 115133.

- Monteiro, J. M., **Covas, G**., Rausch, D., Filipe, S. R., Schneider, T., Sahl, H.-G., & Pinho, M. G. (2019). The pentaglycine bridges of *Staphylococcus aureus* peptidoglycan are essential for cell integrity. Scientific Reports, 9(1), 5010.

- Szymczak P, Filipe SR, **Covas G**, Vogensen FK, Neves AR, Janzen T (2018). Cell wall glycans mediate recognition of the dairy bacterium *Streptococcus thermophilus* by bacteriophages. *Appl Environ Microbiol., AEM.01847-18*;

- **Covas, G**; Vaz, F; Henriques, G; Pinho, MG; Filipe, SR. (2016). Analysis of Cell Wall Teichoic Acids in *Staphylococcus aureus*. *Methods in Molecular Biology*, 1440, 201-13;

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Contents

Abbreviations and acronyms

amiDAP	Amidated *meso*-diaminopimelic acid
AMP	Antimicrobial Peptides
Amp	Ampicillin
anhMur*NAc*	1,6-anhydro-*N*-acetylmuramic acid
CA-MRSA	Community Acquired MRSA
CDFI	2-(2-Chlorophenyl)-3-[1-(2,3-dimethylbenzyl)piperidin-4-yl]-5-Fluoro-1H-indole
CFU	Colony Forming Units
CHAP	Cysteine, Histidine-dependent Amidohydrolases/Peptidases
CPS	Capsule
CW	Cell Wall
DAP	*Meso*-diaminopimelic acid
D-Fuc*NAc*	*D*-*N*-Acetyl Fucosamine
D-Man*NAcA*	*D*-*N*-Acetyl Mannosaminuronic Acid
DNase	Deoxyribonuclease
EDTA	Ethylenediamine tetraacetic acid
EM	Electronic Microscopy
Ery	Erythromycin
Glc*NAc*	*N*-Acetyl Glucosamine
GNBP1	Gram negative binding protein 1
GNBP3	Gram negative binding protein 3
GroP	Glycerol-3-Phosphate
HPAEC-PAD	High Performance Anion Exchange Chromatography Coupled with Pulsed Amperometric Detection
IM	Inner Membrane

IPTG	Isopropyl *β-D*-1-thiogalactopyranoside
Kan	Kanamycin
LA	Luria-Bertani Agar
LB	Luria-Bertani
L-Fuc*N*	*L-N*-Acetyl Fucosamine
LPS	Lipopolysaccharides
LTA	Lipoteichoic Acids
Lys	*L*-Lysine
MAMP	Microbe Associated Molecular Pattern
Man*NAc*	*N*-Acetyl Mannosamine
MATE	Multidrug and Toxic Extrusion
MIC	Minimal Inhibitory Concentration
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSSA	Methicillin Susceptible *Staphylococcus aureus*
MTSES	Sodium (2-sulfonatoethyl) methanethiosulfonate
Mur*NAc*	*N*-Acetyl Muramic acid
N-acetyl-glucosaminidases	Endo-*N*-acetyl-*β-D*-glucosaminidases
N-acetyl-muramidases	*N*-Acetyl-*β-D*-muramidases
NBD	7-Nitro-2,1,3-benzoxadiazol-4-yl
Neo	Neomycin
NFκB	Nuclear Factor Kappa B
O.D.	Optical Density
OM	Outer Membrane
Oxa	Oxacillin
PAMP	Pathogen Associated Molecular Pattern
PBP	Penicillin Binding Proteins
PBS	Phosphate Buffered Saline
PG	Peptidoglycan

PRR	Pattern Recognition Receptor
RboP	Ribitol-5-Phosphate
RNase	Ribonuclease
ROI	Region of interest
RP-HPLC	Reverse Phase High-Performance Liquid Chromatography
RP-UHPLC	Reverse Phase Ultra High-Performance Liquid Chromatography
SDS	Sodium Dodecyl Sulphate
SEDS	Shape, Elongation, Division and Sporulation
TA	Teichoic Acid
TG	Transglycosylation
TMR	Tetramethylrhodamine Cadaverine
TP	Transpeptidation
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UDP-Glc*NAc*	Uridine Diphosphate-Glc*NAc*
UDP-Mur*NAc*	Uridine Diphosphate-Mur*NAc*
Van	Vancomycin
WTA	Wall Teichoic Acids
X-Gal	5-bromo-4-chloro-3-indolyl *β-D*-galactopyranoside

Abstract

Bacteria are able to establish relationships with other organisms, which may be temporary, as in a bacterial infection, or long-term, as in symbiosis. From the interplay between different organisms emerged the concept of "identity", i.e. the need to distinguish "self" from "non-self". Multicellular organisms have developed immune systems responsible for the detection of "non-self" organisms that lead to the activation of appropriate responses to maintain the organism in a state of homeostasis. In opposition, bacteria have developed mechanisms (virulence factors) to avoid their identification as harmful, in order to assure their survival within the host.

Host detection of the presence of a microorganism relies on the recognition of conserved microbial structures from invading microorganisms, also known as Microbe Associated Molecular Patterns (MAMP), by host Pattern Recognition receptors (PRR). Bacterial peptidoglycan (PG), a major component of the bacterial cell wall, the outmost layer of bacteria, is considered a bona-fide MAMP. PG can be efficiently recognized by different PRR that are produced in a plethora of hosts that range from dipteran, such as *Drosophila melanogaster,* to vertebrate animals and plants.

D. melanogaster has proven to be a good invertebrate model organism to study human pathways since its organs and signalling pathways for development, metabolism and innate immunity share evolutionary conserved elements with vertebrates. Furthermore, the mechanisms used in different model organisms to detect invading microorganisms show that the molecular mechanisms of innate immunity have a degree of conservation between plants, invertebrates and vertebrates, including humans, and that bacterial virulence factors and immune evasion strategies of different bacterial pathogens are frequently conserved. Moreover, since *D. melanogaster* is naturally infected with bacteria, fungi and viruses and, can be experimentally infected with human

pathogens, such as the clinically relevant Gram-positive *Staphylococcus aureus*, it constitutes a good "tool" to study host-pathogen interactions.

In this work we used *S. aureus* as a bacterial model to I) unveil how *D. melanogaster* Peptidoglycan Recognition Proteins (PGRP) discriminate bacteria based on the variation of the PG concealment within the assembled cell wall; II) reveal bacterial mechanisms that can impair PG recognition by host receptors; III) enquire about the role of a specific membrane protein in the translocation of PG precursors that are used to produce the PG macromolecule.

The current model of *D. melanogaster* innate immunity proposes that these invertebrate organisms induce a tailored humoral response to the infecting bacteria. This model defends that bacteria with Lysine (Lys) type PG (frequently found in Gram-positive bacteria) are recognized by PGRP-SA, which induces the Toll pathway, and that bacteria with diaminopimelic acid (DAP) type PG (found in Gram-negative bacteria and in some Gram-positive *bacilli*) are recognized by PGRP-LC or PGRP-LE, which induce the Imd pathway. We propose that the existing model of *Drosophila* humoral immune response may oversimplify the mechanisms used by flies to discriminate different bacteria. The current model reduces the variability found in the composition, organization and shape of different bacteria to whether a cell produces a PG with a Lys or a DAP residue on the third position of the stem peptide.

Using *S. aureus* and *Bacillus subtilis* as model organisms for bacteria that produce a surface harbouring a Lys or amidated DAP (amiDAP) PG, respectively, we showed that in a buffer that mimics the composition of *D. melanogaster* haemolymph (HemoBuffer) PGRP-SA does not have a binding preference for either Lys type or amiDAP type PG in *vitro.* Moreover, we have observed that both PGRP-SA or PGRP-LC can bind exposed PG present at the surface of either *S. aureus* or *B. subtilis* bacteria that lack wall teichoic acids (WTA). We have reported that *Drosophila* immune responses to *S. aureus* and *B. subtilis* mutants that lack WTA, and therefore have a highly exposed PG, are mediated by both PGRP-SA and PGRP-LC. These observations do not support the

previous model of bacteria discrimination by PGRPs based on the presence of a Lys/amiDAP residue and suggest that accessibility to PG may play an important role in the discrimination between different classes of bacterial pathogens. We put forward an alternative model that proposes a cross-play between the two pathways of *Drosophila* innate immunity in mounting an effective humoral response to an evading bacterium.

Previous studies have unveiled that the Methicillin-Susceptible *S. aureus* (MSSA) NCTC 8325-4 strain can prevent recognition of PG at its surface by host innate immune receptors by coating it with WTA and/or by trimming the excessive/exposed PG through the activity of the secreted Atl, an autolytic enzyme with *N*-acetyl-glucosaminidase and *N*-Acetylmuramyl-*L*-alanine amidase activities. Moreover, while these mechanisms were identified as virulence factors for a *S. aureus* MSSA NCTC 8325-4 strain in a *D. melanogaster* infection model, Atl seemed to play a redundant role in the concealment of PG in other Methicillin-Resistant *S. aureus* (MRSA) strains and to be redundant for virulence in a murine infection model with *S. aureus* CA-MRSA USA300 LAC strain.

In this work, we have found that the MRSA *S. aureus* JE2 strain presents a cell surface with a PG that is less exposed to host immune PG receptors, such as of *Drosophila* PGRPs, than NCTC 8325-4 strain. We observed a bias towards a lower binding of PGRP-SA to the cell surface of JE2 when compared with NCTC 8325-4, and found that it is correlated with the ability of JE2 to produce the PG hydrolase SagB in higher amounts or with higher activity than NCTC8325-4. Deletion of *sagB* in the JE2 genetic background produced a cell surface that is better recognized by PGRP-SA. In accordance with the hypothesis that SagB is redundant with Atl in the JE2 strain, we found that a NCTC 8325-4 *atl* deletion mutant and a JE2 *atl sagB* deletion mutant strains produce cell surfaces that are equally recognizable by PGRP-SA.

We have extended our studies of the role of SagB in the metabolism of the bacterial cell surface and determined that deletion of SagB in *S. aureus* results in cells producing a PG with an altered architecture/composition. We observed that cells lacking SagB produce a PG with lower amounts of cross-linked muropeptides and with longer

glycan chains. It is therefore possible that SagB reduces PGRP binding to *S. aureus* cell surface by the altering PG composition or by trimming the exposed PG, in a manner similar to that previously proposed for Atl.

The process used by bacteria to transport the PG precursors to the subcellular locations where there is a need to produce a robust and concealed PG molecule is of extreme importance. Although the PG biosynthesis pathway has been extensively studied in the recent years, there is still an intense debate regarding which protein(s) are responsible for flipping the lipid linked precursor(s) to the outer leaflet of the cytoplasmatic membrane. The flipping of PG linked precursors takes place at the membrane, which is underneath the PG and WTA surface layers and is efficiently concealed from outside recognition. In this work we observed that inhibition of MurJ activity results in a reduction of precursor incorporation into nascent PG, with accumulation of lipid linked precursors most likely at the inner leaflet of the cytoplasmatic membrane. These observations support the hypothesis that flipping of lipid-linked PG precursors in *S. aureus* is dependent on MurJ.

Overall, this thesis has tackled three major steps that occur at the cell surface of bacteria propagating within an infected host (PG assembly, maturation and concealment), increasing our understanding of the bacteria-host interaction.

Resumo

As bactérias são capazes de interagir com outros organismos, quer de uma forma transitória, como é o caso de infecções bacterianas, quer de forma duradoura, como as observadas quando estabelecem relações simbióticas. A existência destas interações estabeleceu a necessidade de os organismos criarem um sistema capaz de diferenciar o que pertencia à sua entidade de outras entidades (estranhas). Esta pressão evolutiva poderá ter conduzido ao desenvolvimento do sistema imune no organismo hospedeiro que é responsável pela monitorização da presença de entidades estranhas e pelo espoletar de uma resposta imune que promova o retorno à homeostase. Em contrapartida, as bactérias necessitaram de desenvolver mecanismos que asseguram a sua sobrevivência dentro dos hospedeiros, conhecidos como fatores de virulência.

A detecção de um microorganismo invasor passa pelo reconhecimento de estruturas moleculares, que se assumem conservadas nos microrganismos e que são denominadas por MAMP ("*Microbe Associated Molecular Patterns*"), através de recetores do hospedeiro (intitulados PRR ou "*Pattern Recognition Receptors*"). O peptidoglicano (PG), um polímero presente na parede celular da maior parte das bactérias, apresenta uma estrutura que é capaz de ser reconhecida por recetores de organismos hospedeiros tão variados como animais vertebrados, plantas e organismos dípteros (entre eles a mosca do vinagre ou *Drosophila melanogaster*).

A mosca do vinagre apresenta-se como um bom organismo modelo para estudar vias metabólicas humanas dado os seus órgãos serem funcionalmente semelhantes aos encontrados nos organismos vertebrados, e as suas vias metabólicas serem reguladas por mecanismos conservados e também encontrados nos organismos vertebrados. Deste modo, os mecanismos de virulência que os microorganismos empregam por forma a garantir a sua sobrevivência num organismo infetado também se encontram frequentemente conservados. Sendo assim, *D. melanogaster* apresenta-se como um

bom organismo modelo para estudar as interações entre um hospedeiro e um organismo patogénico dado que é possível a sua infeção com patógenos humanos, para além dos microrganismos que naturalmente conseguem infetar *D. melanosgater* tais como bactérias, fungos e vírus.

Neste trabalho tivemos como objetivo elucidar como as PGRPs ("*Peptidoglycan Recognition Proteins"*), receptores de *D. melanogaster* que são capazes de reconhecer o PG, discriminam as bactérias com base na variação da sua parede celular e, quais os mecanismos que as bactérias podem empregar para prevenir o seu reconhecimento.

O modelo actual da resposta imune inata de *D. melanogaster* defende que este organismo constrói uma resposta imune dependente do microrganismo invasor. Este modelo advoga que a via metabólica *Toll* é ativada após o reconhecimento, pela PGRP-SA, de bactérias com um PG que tenha uma lisina (Lys) na sua composição, como é o caso da maior parte das espécies Gram-positivas. Por outro lado, a via metabólica *Imd* é ativada quando bactérias Gram-negativas ou bacilos Gram-positivos, que apresentam um PG que inclua um resíduo de ácido diaminopimélico (DAP), são reconhecidas pela PGRP-LC ou PGRP-LE. Neste trabalho propomos que este modelo simplifica demasiado os mecanismos de discriminação das bactérias por parte das PGRPs visto reduzir a variabilidade da parede celular das bactérias, que se pode observar em diferentes parâmetros como a composição, a organização e a forma, a um pequeno detalhe, como a capacidade de uma bactéria produzir um PG com uma lisina ou um resíduo de ácido diaminopimélico.

Utilizando as bactérias *Staphylococcus aureus* e *Bacillus subtilis* como organismos modelo, dado que produzem, respetivamente, uma parede celular com um PG do tipo Lys ou DAP, demonstrámos que a PGRP-SA não apresenta uma preferência para PG do tipo Lys ou DAP se os ensaios de análise *in vitro* forem executados num tampão que mimetiza a composição da hemolinfa de *D. melanogaster*. Também demonstrámos que quer a PGRP-SA quer a PGRP-LC se conseguem ligar ao PG existente na superfície das bactérias *S. aureus* e *B. subtilis*, se este estiver exposto pela remoção

dos *wall teichoic acids* (WTA). Em colaboração com o Professor Petros Ligoxygakis da Universidade de Oxford, reportámos que a resposta imune de *D. melanogaster* a uma infecção por estirpes de *S. aureus* e *B. subtilis* sem WTA é dependente de ambas as PGRPs. Estas observações, que contradizem o modelo atual da resposta imune inata de *D. melanogaster*, sugerem que a acessibilidade do PG à superfície de uma bactéria é um fator importante na discriminação de bactérias patogénicas e desafia-nos a ponderar a hipótese de haver uma comunicação entre as vias metabólicas *Toll* e *Imd*.

A identificação das estratégias que as bactérias empregam para lidarem com os sistemas de deteção e defesa do organismo infetado foi também considerada nesta tese. Foi reportado anteriormente que a estirpe *S. aureus* MSSA NCTC 8325-4 consegue prevenir o reconhecimento do PG na sua parede celular através da produção de WTA e/ou através da degradação pela enzima Atl do PG exposto/excessivo. Embora estes componentes possam ser considerados como fatores de virulência num modelo de infeção de *D. melanogaster*, a enzima Atl foi, entretanto, reportada como um factor de virulência redundante na estirpe *S. aureus* CA-MRSA USA300 LAC JE2 quando esta foi usada num modelo de infeção em ratinho.

Neste trabalho demonstrámos que a estirpe *S. aureus* CA-MRSA USA300 LAC JE2 apresenta uma superfície em que o PG está menos exposto a uma PRR quando comparada com a superfície da estirpe *S. aureus* MSSA NCTC 8325-4. Observou-se uma menor capacidade da PGRP-SA em se ligar à superfície da estirpe JE2 quando comparada com a estirpe NCTC 8325-5, que parece estar correlacionada com a maior expressão, ou a uma maior atividade enzimática, da enzima hidrolítica do PG SagB na estirpe JE2. Em concordância, também se observou que uma estirpe JE2 mutante com a deleção do gene *sagB* apresenta uma superfície celular que é melhor reconhecida pela PGRP-SA e, que uma estirpe JE2 com a deleção dos genes *atl* e *sagB* produz uma superfície celular que é reconhecida pela PGRP-SA do mesmo modo que a superfície da estirpe mutante NCTC 8325-4 com a deleção do gene *atl*. Concluímos que a enzima SagB poderá ser responsável pela redundância do Atl como factor de virulência na estirpe JE2.

O papel da enzima SagB na composição da superfície celular de *S. aureus* também foi analisado nesta tese. A deleção do gene que codifica a enzima SagB resultou em células que produzem um PG com uma composição/arquitectura alterada. As bactérias *S. aureus* que não expressam SagB, produzem um PG empobrecido em muropéptidos multiméricos, resultantes do "cross-linking" de muropeptidos presentes em diferentes cadeias, e enriquecido em cadeias de glicanos longas. Estas alterações na composição do PG produzido pelas bactérias *S. aureus* que não expressam SagB, poderão não justificar uma maior deteção pela PGRP-SA. É possível que o SagB influencie a ligação da PGRP-SA à superfície bacteriana através da degradação do PG excessivo/exposto, de forma similar ao proposto para o Atl.

Na parte final desta tese foram também analisados os processos que as bactérias *S. aureus* usam para produzir o seu PG. Embora a via metabólica de síntese do PG tenha sido intensamente estudada nos últimos anos ainda existe um intenso debate sobre qual a(s) enzima(s) responsável pela translocação dos percursores lipídicos através da membrana citoplasmática. Este processo deverá decorrer de forma a proteger o acesso dos percussores ao reconhecimento exterior e a manter estas moléculas disponíveis para as enzimas que polimerizam o PG. Neste trabalho observámos que a inibição da atividade do MurJ resulta numa redução da incorporação de precursores no PG nascente. Concomitantemente, também se observou uma acumulação de precursores lipídicos na membrana citoplasmática, provavelmente na folha interior desta. Estas observações suportam que a translocação de precursores lipídicos em *S. aureus* é dependente da presença do Murj.

Chapter I

General Introduction

Bacteria and their interaction with other organisms

Bacteria are among the first live forms known to exist on earth (Donoghue and Antcliffe 2010). As any organism, to assure the survival of their species, bacteria adapt to the surrounding environment to multiply and propagate in different niches. As such, bacteria are nowadays found to be present on most of earth habitats, being able to survive under conditions considered inhospitable to other domains (Rothschild and Mancinelli 2001). Bacteria are also able to establish relationships with other organisms that belong to the same species, which permit them to live as multicellular structures, with organisms that belong to other species or that belong to the different life domains. These biological relationships may be temporary, as those observed in an episode of a common bacterial infection or of a long-term nature, known as symbiosis and, can range from mutualism, benefiting both symbionts, to parasitism, where one symbiont (the parasite) benefits from the other (the host) while causing harm (Paracer, Surindar; Ahmadjian 2000).

From this interplay between different organisms rouse the concept of "identity", i.e. the need to distinguish "self" from "non-self" and the need to discriminate "non-self" entities as beneficial or harmful. As such, multicellular organisms have developed immune systems responsible for both the detection of "non-self" organisms and for the activation of appropriate responses that maintain the organism in a state of homeostasis (Sansonetti 2006). In opposition, bacteria developed mechanisms to avoid their identification (as harmful) in order to assure their survival within hosts (Hornef et al. 2002; Veldkamp and van Strijp 2009; Foster 2005; Graves, Kobayashi, and DeLeo 2010; Actor 2014).

The responses built by a given immune system can be divided into those put forward by the innate immune system and those triggered by the acquired/adaptive immune system. While the acquired/adaptive immune system seems to be restricted to vertebrates, the innate immune system seems to be

ubiquitous in the *Eukarya* Domain and forms the first line of defence in an organism (Actor 2014).

Detection of invading microorganisms relies on constitutively expressed evolutionary conserved receptors known as Pattern-Recognition Receptors (PRR) that perceive conserved microbial structures, specific to microbes but absent in the host. These conserved microbial structures constitute Pathogen Associated Molecular Patterns (PAMP), also known as Microbe Associated Molecular Patterns (MAMP). Since these microbial structures are not unique to pathogenic microorganisms, the latter designation (MAMP) is favoured (Mackey and McFall 2006; Janeway and Medzhitov 2002; Sansonetti 2006).

The study of the mechanisms used in different model organisms to detect invading microorganisms show that the molecular mechanisms of innate immunity have a degree of conservation between plants, invertebrates and vertebrates, including humans (Ronald and Beutler 2010; Ausubel 2005) and that bacterial virulence factors and immune evasion strategies are frequently conserved (Hornef et al. 2002; Veldkamp and van Strijp 2009; Foster 2005).

Bacterial Cell wall

The bacterial cell wall (CW), as the outmost layer of bacteria, is a major target of host PRR, and consequently paramount for immunological studies. This bacterial component has been extensively studied as the bacteria cell wall is simultaneously responsible for the bacterial shape, resistance to turgor pressure (up to 5 atm or 50 atm in Gram-Positive and Gram-negative bacteria, respectively) and paramount for survival of most bacteria (Young 2003).

Historically, bacteria have been divided according to the outcome of Gram staining into two major groups: Gram-positive bacteria, which retain crystal violet dye, and Gram-negative that are not labelled with the crystal violet staining

procedure. The differential staining of this method, first used to detect *Streptococcus pneumoniae* in lung tissue autopsies, is due to the physicochemical and structural differences in the cell surface of the bacteria [Figure 1] (Beveridge 2001; Popescu and Doyle 1996).

While *Escherichia coli* is the main model organism for Gram-negative bacteria and the composition of its CW is used as a reference for this group, there are different bacteria that are frequently used as model organisms for Gram-positive bacteria. *Staphylococcus aureus* CW is frequently used as a reference for this group. Figure 1 shows a schematic comparison of the CW structure of both groups of bacteria.

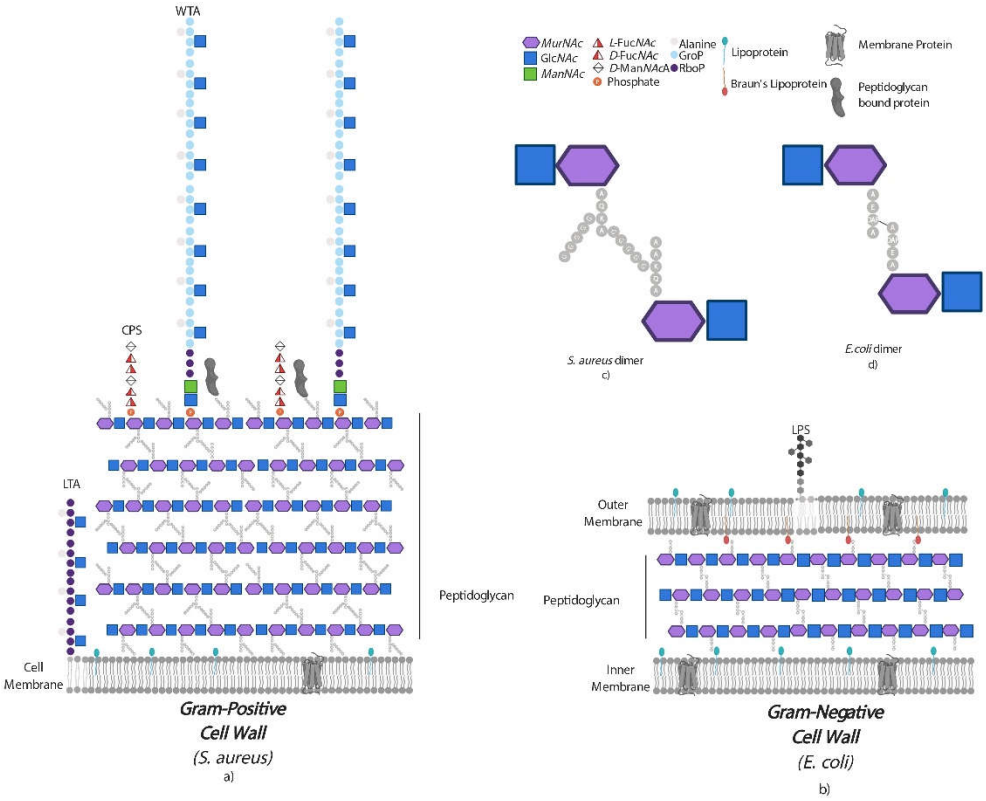

Figure 1 – Schematic comparison of the structure of Gram-positive and Gram-negative cell walls. The structure of *S. aureus* is depicted as an example of Gram-positive bacteria [a)] while the structure from *E. coli* is depicted as an example from Gram-negative bacteria [b)]. While Gram-negative CW is composed of an outer membrane and an inner membrane that confines the periplasm region and surrounds a thin peptidoglycan layer, Gram-positive CW is characterized by a thick peptidoglycan layer that is associated with Wall Teichoic Acids (WTA) and Lipoteichoic acids (LTA). Some bacteria can be further coated by a covalently linked and sugar-rich external layer: the capsular polysaccharide or Capsule (CPS) in Gram-positive bacteria and the lipopolysaccharide (LPS) in Gram-negative bacteria. The cell wall structure of the two groups of bacteria also differ on peptidoglycan architecture. While most Gram-positive bacteria typically have a *L*-lysine as the third amino acid on the stem peptide[c)], Gram-negative bacteria usually have *meso*-diaminopimelic acid [d)]. Furthermore, *S. aureus* is characterized by having a penta-glycine bridge connecting the two stem peptides while in *E. coli* and *B. subtilis* two stem peptides from different glycan chains are connected directly.

Gram-negative cell wall

The Gram-negative CW [Figure 1 b)] is composed of an outer membrane (OM) and an inner membrane (IM) that confine the periplasm region, which

harbours a thin peptidoglycan (PG) layer (Osborn et al. 1972; Silhavy, Kahne, and Walker 2010; Miura and Mizushima 1968). The OM is an asymmetric lipid bilayer, with the inner leaflet mainly composed of phospholipids while the outer leaflet is enriched in proteins, glycolipids and lipopolysaccharides (LPS) (Kamio and Nikaido 1976; Arora et al. 2000; Cowan et al. 1992; Hwang et al. 2002; Miyadai et al. 2004; Nikaido 2003). The OM is linked to the PG through a lipoprotein called Lpp, murein lipoprotein or Braun's lipoprotein (V. Braun 1975). The LPS molecules that are attached to the outer leaflet of the OM are composed of lipid A, a core polysaccharide that connects lipid A to the variable *O*-polysaccharide or *O*-antigen, which is responsible for the antigenic specificity (Raetz and Whitfield 2002). The periplasm is densely packed with proteins (Heppel 1967) involved in CW biosynthesis, chemotaxis and transport of sugars and amino acids (Michael Ehrmann 2006).

Gram-positive cell wall

Gram-positive CW [Figure 1 a)] is characterized by a thick PG layer that accounts for between 30% and 70% of the total weight of the CW (Schleifer and Kandler 1972). It is thought that this thick (30-100 nm) PG layer forms a protection layer similar to the Gram-negative OM (Waldemar Vollmer, Blanot, and De Pedro 2008). Gram-positive CW is further characterized by the presence of teichoic acids (TA), either connected to the PG, known as Wall Teichoic Acids (WTA), or connected to the membrane, known as Lipoteichoic acids (LTA) (Neuhaus and Baddiley 2003). Neither WTA nor LTA are essential for survival on lab conditions. However, it is not possible to delete both WTA and LTA since they are synthetically lethal and seem vital for CW architecture and integrity (Oku et al. 2009; Morath, von Aulock, and Hartung 2005).

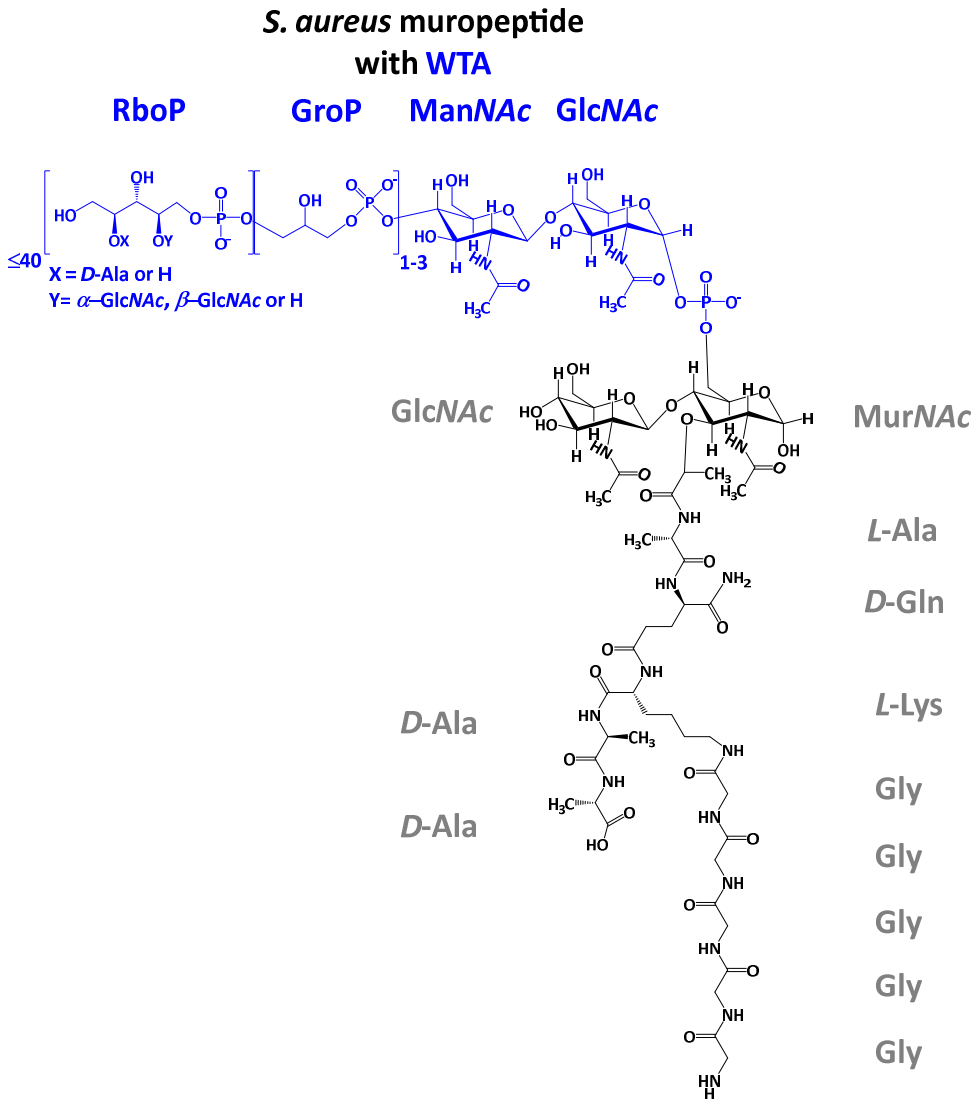

Figure 2 - Structure of a *S. aureus* muropeptide with WTA polymer attached. In *S. aureus* WTA are a polymer of RboP linked to PG by a linker containing GroP, Man*NAc* and Glc*NAc*.

WTA polymers have a linkage unit composed of an *β*-(1,4) - linked disaccharide of *N*-Acetyl mannosamine (Man*NAc*) and *N*-Acetyl glucosamine (Glc*NAc*) connected through the non-reducing end (Man*NAc*) to a polymer of 1 to 3 units of glycerol-3-phosphate (GroP). The other saccharide of the linkage unit, the Glc*NAc* (at the reducing end), is linked through a phosphodiester linkage to the

C6 hydroxyl group of *N*-Acetyl muramic acid (Mur*NAc*) residues of the PG. In *S. aureus* and in some strains of *B. subtilis*, the last GroP of the linkage unit is connected to a polymer of variable size (up to 40 units) of ribitol-5-phosphate (RboP) while in most *B. subtilis spp.* the last GroP of the linkage unit is connected to a polymer of variable size of GroP [Figure 2] (Brown, Santa Maria, and Walker 2013; Swoboda *et al*. 2010; Endl *et al*. 1983). It is estimated that on average *S. aureus* and *B. subtilis* CW has one WTA polymer for every nine Glc*NAc*-Mur*NAc* disaccharides (Kojima, Araki, and Ito 1985).

While *S. aureus* WTA can be further modified by addition of Glc*NAc* to the C4 hydroxyl group of RboP residues on the WTA polymer, *B. subtilis spp.* tailor the C4 hydroxyl group of RboP on the WTA polymer through the addition of glucose residues. *S. aureus* and *B. subtilis spp.* that produce a WTA with a RboP polymer can further modify RboP by *D*-alanylation of the C2 hydroxyl group [Figure 2]. Strains of *B. subtilis spp.* that produce WTA with a GroP polymer modify their WTA polymer by either *D*-alanylation or through the addition of glucose to GroP C2 hydroxyl group (Brown, Santa Maria, and Walker 2013; Swoboda et al. 2010). The modifications introduced in the WTA molecules are able to tune their charge and structure and are relevant for cation homeostasis (Marquis, Mayzel, and Carstensen 1976; Heptinstall, Archibald, and Baddiley 1970). WTA modification is also important to the bacterial resistance to host antimicrobial peptides, to resistance to lytic enzymes and to the promotion of bacteria immune evasion (Neuhaus and Baddiley 2003; Peschel et al. 1999; Collins et al. 2002; Peschel et al. 2000; Gerlach et al. 2018; Carvalho et al. 2015; Bera et al. 2007; Gautam et al. 2015; Lee et al. 2015; Kurokawa et al. 2011).

WTA are proposed to extend from the PG layer, perpendicularly in respect to the surface of bacteria, coating and masking the PG [Figure 1] (Neuhaus and Baddiley 2003; Magda L. Atilano et al. 2011; Gautam et al. 2015; Doyle et al. 1975;

Birdsell, Doyle, and Morgenstern 1975; Arizono, Umeda, and Amako 1991; Umeda et al. 1992).

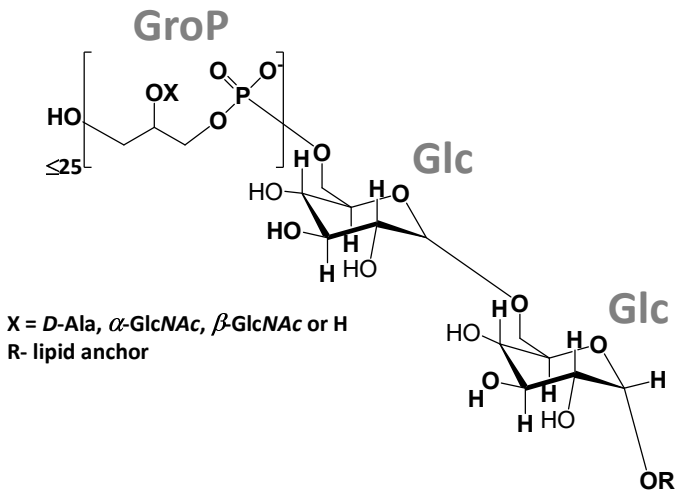

Figure 3 - Structure of *S. aureus* Lipoteichoic acid. In *S. aureus* LTA are polymers of GroP linked by a diglucosyl disaccharide to a lipid anchor.

LTA polymers are structures of a variable number of GroP residues linked to a glycolipid anchor that is present in the bacterial membrane. The C6 hydroxyl group of a diglucosyl disaccharide connects the GroP polymer to a diacylglycerol lipid. Similarly to WTA, LTA can be modified by reactions of *D*-alanylation or through the addition of Glc*NAc* residues to the C2 hydroxyl groups of the GroP backbone. LTA molecules are usually shorter (up to 25 GroP units) than WTA [Figure 3]. Although the exact function of LTA is still unknown (Percy and Gründling 2014) it has been reported that variations of LTA composition are important to regulation of autolysis (Perea Vélez et al. 2007; Steen et al. 2005), to cation homeostasis (Archibald, Baddiley, and Heptinstall 1973), to adhesion to host cells and invasion (Armstrong et al. 1959; Abachin et al. 2002), to biofilm formation (Fabretti et al. 2006) and to resistance to AMP (Peschel et al. 1999; Abachin et al. 2002). Furthermore, LTA have been reported as a virulence factor in *S. aureus* and *Listeria monocytogenes* (Collins et al. 2002; Abachin et al. 2002). Similarly to WTA,

it is estimated that one in every nine lipids in the outer leaflet has a LTA (Percy and Gründling 2014; Neuhaus and Baddiley 2003; Morath, von Aulock, and Hartung 2005; Reichmann et al. 2014; Reichmann, Cassona, and Gründling 2013; Fischer 1994; Heaton and Neuhaus 1992; Iwasaki et al. 1989; Mancuso and Chiu 1982).

The CW produced by Gram-positive bacteria may also be decorated by surface proteins that can be inserted on the cellular membrane (membrane proteins), covalently bonded to the PG or associated with the PG and TA. Proteins covalently bonded to PG are produced with a LPXTG sorting motif at the C-terminal, which is then cleaved by an enzyme named sortase between the threonine and the glycine. The resulting carboxyl group of the threonine is linked by the sortase to the amine group of a glycine residue of the peptide bridge that is present in the *S. aureus* PG (DeDent et al. 2008; Dramsi et al. 2008; Fischetti, Pancholi, and Schneewind 1990; Scott and Barnett 2006; Marraffini, Dedent, and Schneewind 2006; Sjöquist et al. 1972). Surface proteins have different roles and can be involved in iron-uptake [siderophores] (Volkmar Braun and Hantke 2011; Schalk 2013; Mazmanian et al. 2003; Skaar et al. 2004), host colonization [adhesins] and pathogenicity [virulence factors] (Clarke and Foster 2006; Weidenmaier et al. 2004, 2005, 2008).

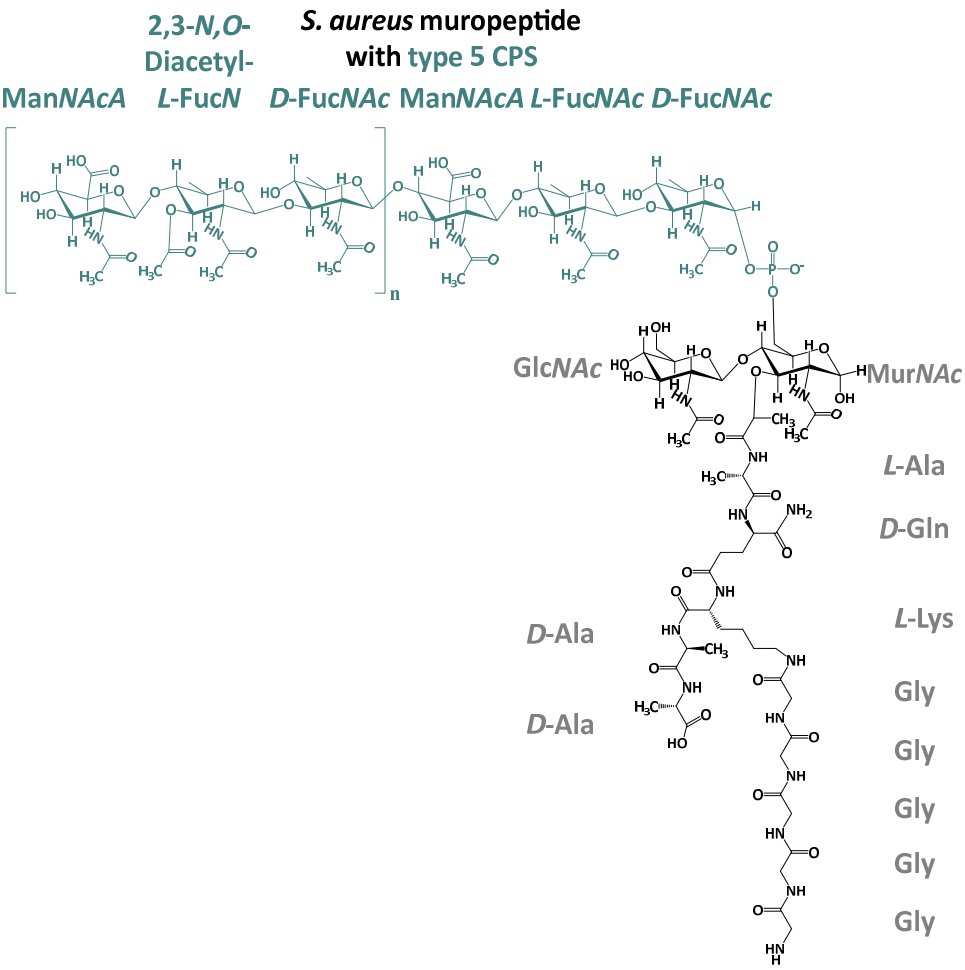

Figure 4 - Structure of a *S. aureus* muropeptide with type 5 CPS polymer attached. In *S. aureus* a type 5 capsule is a polymer of a repeating unit of Man*NAcA*, 2,3-*N,O*-Diacetyl-Fuc*N*, *D*-Fuc*NAc* linked by a Man*NAcA*, *L*-Fuc*NAc*, *D*-Fuc*NAc* trisaccharide to the PG.

Gram-positive bacteria can be further coated by extracellular polysaccharides, the capsule (CPS) (Gilbert 1931). The structure of these glycopolymers has a high degree of variation within the same bacterial species. They are involved in bacterial strategies of evasion to the host immune system and are frequently considered to be a virulence factor (Nilsson et al. 1997; Thakker et al. 1998; Portolés et al. 2001). It has been shown that *S. pneumoniae* can switch their capsule serotype *in vivo* (Venkateswaran, Stanton, and Austrian 1983).

Although *S. aureus* has eleven different serotypes, currently, due to loss of prototype strains and antisera, it is only possible to serotype capsule types 1, 2, 5 and 8. Staphylococcal strains that belong to serotypes 5 and 8, also described as microencapsulated in opposition to serotypes 1 and 2, represent 25% and 50 %, respectively, of all *S. aureus* isolated strains (Nilsson et al. 1997; Karakawa et al. 1985; Sompolinsky et al. 1985). It is thought that CPS, like WTA, is attached by its reducing end monosaccharide to the C6 hydroxyl group of Mur*NAc* of PG [Figure 4].

Serotypes 5 and 8 are structurally very similar. They are polymers of a repeating trisaccharide of, from reducing end to non-reducing end, *D*-*N*-Acetyl Fucosamine (*D*-Fuc*NAc*), *L*-*N*-Acetyl Fucosamine (*L*-Fuc*NAc*) and *D*-*N*-Acetyl Mannosaminuronic acid (*D*-Man*NAcA*). They differ in the type of glycosidic bond between the monosaccharide's residues and in the *O*-Acetylation of *L*-Fuc*NAc*. Type 5 capsule has a [(-4) Man*NAcA* (1-4) *L*-Fuc*NAc* (1-3) *D*-Fuc*NAc* (1-)] with *O*-acetylation of the C2 hydroxyl of *L*-Fuc*NAc* repeating unit [Figure 4], while Type 8 capsule has a [(-3) Man*NAcA* (1-3) *L*-Fuc*NAc* (1-3) *D*-Fuc*NAc* (1-)] repeating unit (O'Riordan and Lee 2004; Rausch et al. 2019; Jones 2005).

Peptidoglycan composition, architecture and variation

As the name suggests, peptidoglycan (PG) is composed of a peptide moiety and a glycan chain. This macromolecule, also known as murein[1] is unique to bacteria and present in most bacteria. PG glycan chains are a linear alternating copolymer of *β*-(1,4) - linked Glc*NAc* and Mur*NAc* residues. The Mur*NAc* C3 lactyl group has an amide bond to the peptide stem. Since the PG stem peptides have in their composition diamino acids, they can "branch out" and connect to

[1] From the Latin *murus*, meaning wall.

neighbouring peptide stems, thus cross-linking adjacent glycan chains (Schleifer and Kandler 1972).

The glycan chains present in the PG, can be further modified by reactions of *N*-deacetylation of either Mur*NAc* or Glc*NAc* residues, or reactions of *O*-acetylation of Mur*NAc* in both Gram-positive and Gram-negative bacteria [Figure 5 b) and c) respectively] (Waldemar Vollmer 2008; Abrams 1958; Araki et al. 1971; Atrih et al. 1999; Bera et al. 2005, 2006; Boneca et al. 2007; Crisóstomo et al. 2006; Logardt and Neujahr 1975; Snowden et al. 1989; W Vollmer and Tomasz 2000). In Gram-negative bacteria, and in some Gram-positive *Bacilli*, the terminal Mur*NAc* is substituted by a 1,6-anhydro-*N*-acetylmuramic acid (anhMur*NAc*) that is the result of lytic transglycosylases activity [Figure 5 d)]. The activity of these enzymes explains the absence of a reducing end in the glycan chains of the PG macromolecule produced by Gram-negative bacteria, while most Gram-positive bacteria typically have a reducing end (Waldemar Vollmer 2008; Glauner, Holtje, and Schwarz 1988; Harz, Burgdorf, and Höltje 1990; Höltje et al. 1975; Quintela, Caparrós, and de Pedro 1995). In *Bacilli* spores, half of the Mur*NAc* residues are modified to muramic lactam [Figure 5 e)] (Warth and Strominger 1969, 1972; Popham et al. 1996; Gilmore et al. 2004; Waldemar Vollmer 2008).

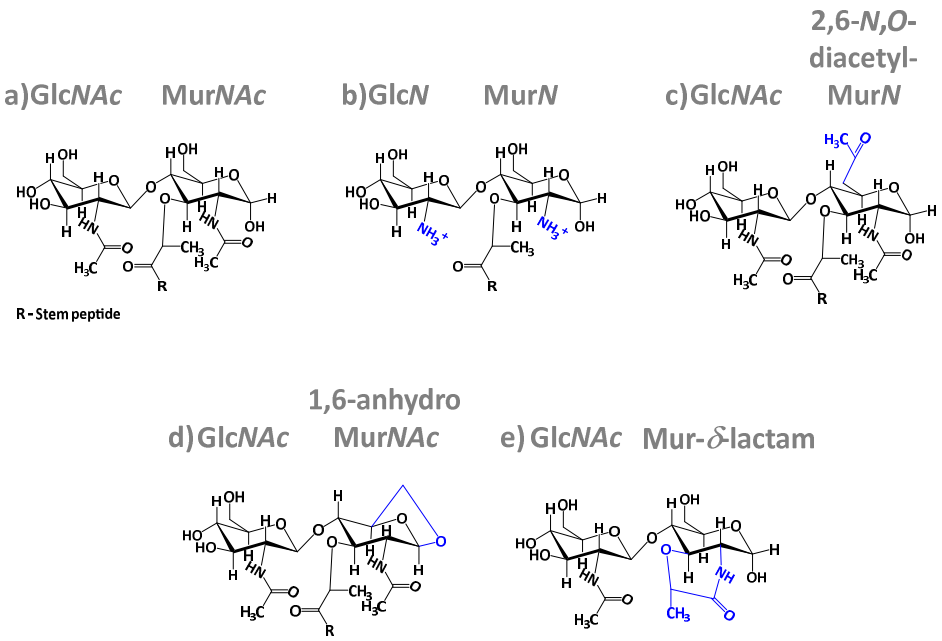

Figure 5 - The glycan chain of PG can be modified by b) *N*-deacetylation of either Mur*NAc* and Glc*NAc*, c) *O*-acetylation of Mur*NAc* or by conversion of Mur*NAc* to d) 1,6-anhydroMur*NAc* by lytic transglycosylases or to e) Mur-*δ*-lactam.

The length of the PG glycan chains varies between bacteria. The glycan chains present in mature PG of *S. aureus* and of *E. coli* have an average length of 6 disaccharides (Boneca et al. 2000) and of 33 disaccharides (Harz, Burgdorf, and Höltje 1990), respectively. The glycan chains found in the PG of *B. subtilis* can have up to 5 µm of length and are estimated to be built of 5000 disaccharides (Hayhurst et al. 2008).

The composition of stem peptides in the PG macromolecule is very similar between Gram-positive and Gram-negative species. The unbranched stem peptides are typically pentapeptides of alternating *L* and *D* amino acids: usually *L*-alanine, *D*-glutamic acid, a diamino acid and two terminal *D*-alanines. In Gram-negative bacteria, and also in Gram-positive *Bacilli* and in *Listeria* spp., the diamino acid in the third position is a *meso*-diaminopimelic (DAP). On the other hand, most

Gram-positive bacteria contains the diamino acid *L*-lysine (Lys) in the third position [Figure 6 a), b) and c)] (Schleifer and Kandler 1972).

Variations in the composition of the PG stem peptides can be due to either the insertion of alternative amino acids during biosynthesis or due to posterior chemical modifications of the amino acids. Most modifications, such as those inserted through reactions of amidation, hydroxylation, acetylation and attachment of proteins affect the second or third amino acid of the stem peptide (Waldemar Vollmer, Blanot, and De Pedro 2008). Gram-positive bacteria, such as *S. aureus*, can further modify their PG through the amidation of *D*-Glutamic acid to *D*-Glutamine [Figure 6 a)](Figueiredo et al. 2012; Münch et al. 2012).

Although *B. subtilis* and *E. coli* PG composition is very similar, *B. subtilis* PG is amidated on DAP (amiDAP) while *E. coli* PG is not [Figure 6 b) vs c)]. This modification may be responsible for differential recognition of both PGs by host immune receptors (Q. Wang et al. 2016; Leone et al. 2008).

The type of cross-link between two PG stem peptides shows higher variability than the main peptide stem chain and form the criteria for the tripartite (see I to III below) PG architecture classification system established by Schleifer and Kandler.

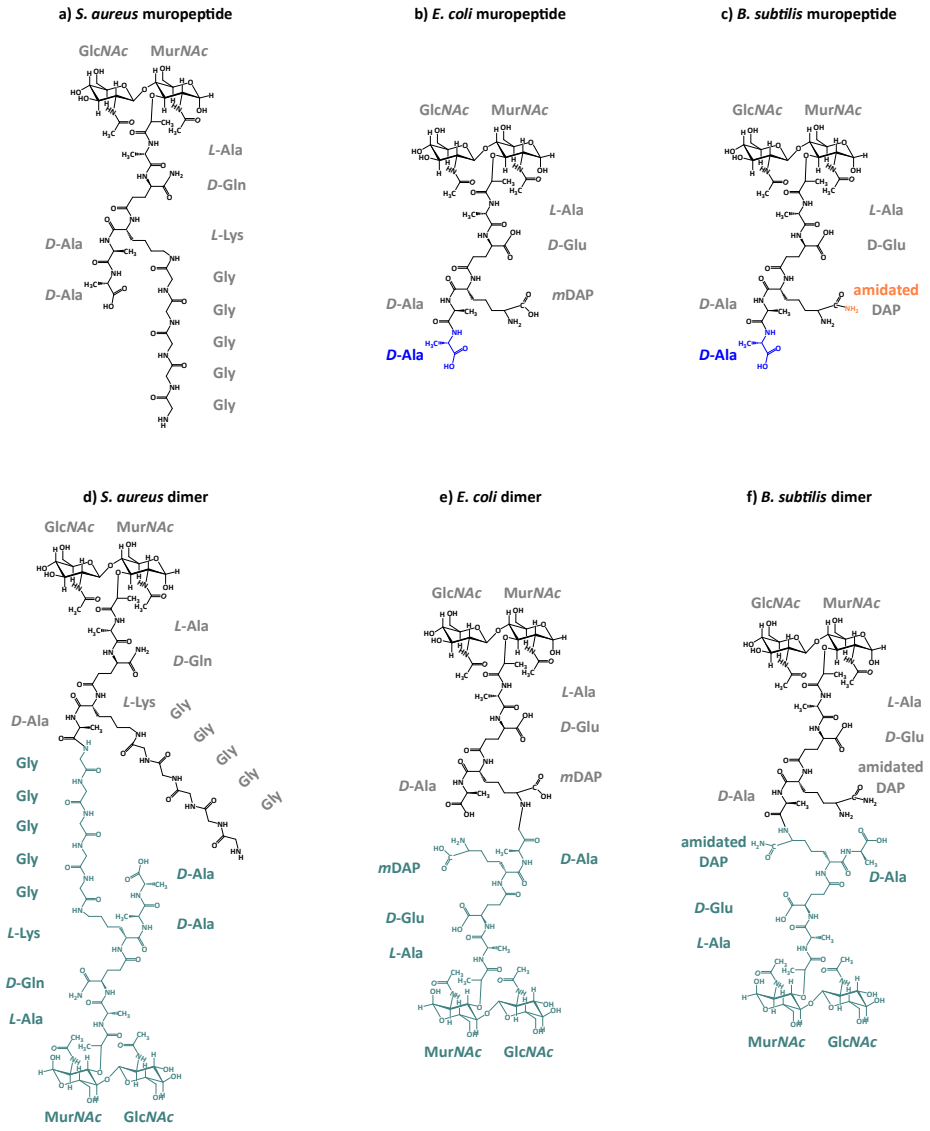

Figure 6 - Muropeptide structure in different bacteria. a) The PG stem peptide produced in Gram-positive bacteria *S. aureus* is composed of *L*-Ala, *D*-Gln, *L*-Lys, *D*-Ala, *D*-Ala with a pentaglycine bridge attached to the *L*-Lys. On the other hand, c) Gram-positive bacteria *B. subtilis* and b) Gram-negative bacteria *E. coli* produce PG stem peptides that have a DAP residue in the third position. The DAP residue in *B. subtilis* can be amidated (amiDAP, in orange). Please note that *D,D*-carboxypeptidases can cleave the terminal *D*-ala in mature PG from *E. coli* and *B. subtilis* (depicted in blue). Since PG stem peptides have diamino acids, they can branch out and cross-link to PG stem peptides in adjacent glycan strands. The different types of cross-link are the basis of the classification system established by Schleifer and Kandler. While stem peptides are crosslinked in e) *E. coli* and f) *B. subtilis* through a direct 3-4 connection (type A1), in d) *S. aureus* there is 3-4 cross-link with a monocarboxylic acid bridge, i.e. a pentaglycine bridge (type A3).

Briefly, the PG produced by different bacteria are classified in a hierarchically system based on:

I) the position of the amino acids involved in the cross-link between the two stem peptides. This allows the classification of the PG with a capital Latin letter. A PG with a cross-link between the 3rd and 4th amino acids of two peptide stems (3-4 connection) belongs to group A. On the other hand, a PG with a cross-link between the 2nd and 4th amino acids (2-4 connection) belongs to group B;

II) the type of cross-link that links two different PG stem peptides. This allows the classification of the PG with an Arabic number. e.g. *E. coli* and *B. subtilis* with a direct (3-4) cross-link is an A1 type PG [Figure 6 e) and f)] while *S. aureus* with a (3-4) cross-link by a interpeptide bridge of monocarboxylic acids, i.e. glycines, is an A3 type PG [Figure 6 d)] ;

III) the identity of the amino acid in the 3rd position of stem peptide and that is involved in the PG crosslinking. This allows the classification of the PG with a Greek letter (Schleifer and Kandler 1972).

Interestingly, some organisms besides having *D,D*-transpeptidases (enzymes that catalyse the (3-4) cross-linking of adjacent stem peptides, see below) also have *L,D*-transpeptidases. The latter enzymes (*L,D*-transpeptidases) use the carboxyl of the amino acid on the 3rd position of the stem peptide as donor for the transpeptidation, in opposition to the 4th amino acid for *D,D*-transpeptidases. This results in a (3-3) cross-link instead of a (3-4) cross-link [Figure 7]. This different cross-link architecture, whose synthesis may be activated due to environmental signals perceived by bacteria, seems to neither essential nor

universal. Its relative abundance in different bacteria seems to increase with PG maturation (stationary phase) and β-lactams resistance (Mainardi et al. 2000; Wietzerbin et al. 1974; Cava et al. 2011; Glauner and Höltje 1990).

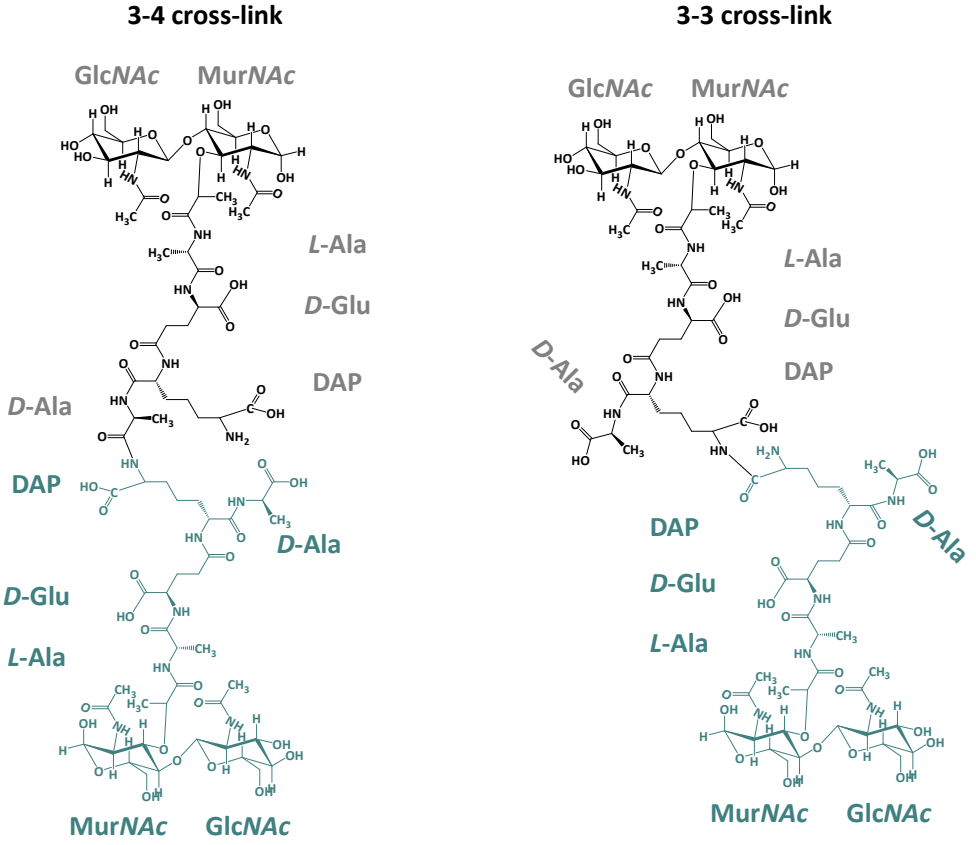

Figure 7 - Comparison muropeptides dimers with a 3-4 direct cross-link and alternative direct 3-3 cross-link present in some organisms due to the action of *L,D*-transpeptidases.

There is a high degree of variability in the percentage of cross-link observed in the bacterial PG, i.e. number of cross-links per disaccharide unit (muropeptide) (Snowden and Perkins 1990). This percentage varies within the bacteria of the same species in a way that is dependent on growth conditions and on the stage where bacteria have been harvested as shown by the observation that the cross-link degree seems to increase in stationary phase [mature PG] (Pisabarro, de Pedro, and Vázquez 1985; Glauner, Holtje, and Schwarz 1988; Blasco, Pisabarro,

and de Pedro 1988). Also different bacterial species have remarkably different typical cross-linking levels, e.g. *B. subtilis* and *E. coli* are characterized by a PG with low cross-link, around 40-50% in exponential phase, while *S. aureus* has PG with high cross-link levels, from 70% to 80% (Snowden and Perkins 1990; Jonge et al. 1992; Glauner, Holtje, and Schwarz 1988; Hayhurst et al. 2008) . It should be highlighted that the free peptide bridge (not connected to other PG stem peptides) can also serve to attach proteins covalently bonded to PG (see above).

Since the disaccharide units in the glycan chain rotate in a right-handed helical structure consecutive peptide stems in a glycan chain are oriented in different directions (Labischinski et al. 1979). In *S. aureus* every stem peptide has an angle close to 90ºC to the prior stem peptide in the glycan chain, meaning that every 4 disaccharides in a glycan chain have a stem peptide orientated in the same direction (Labischinski et al. 1985). Also, the glycan chains are densely packed in an ordered structure with the stem peptides parallel to each other (S. J. Kim, Chang, and Singh 2015). Kim et. al proposed that the size of the peptide bridge can dictate the structure of the glycan chains. PG with long bridges, e.g. *S. aureus* with 5 glycines, have a parallel peptide stem structure that enables high levels of cross-link (theoretically up to 100%). In opposition, PG of *S. pneumoniae* with intermediate size bridges (a dipeptide) have stem peptides with a perpendicular orientation and *E. coli* with short bridges (direct link) has stem peptides with an antiparallel orientation that allows theoretically up to 70% and 50% of cross-link, respectively (S. J. Kim, Chang, and Singh 2015).

As already mentioned, bacteria have PG with different thickness i.e. Gram-positive bacteria have a thicker PG layer than Gram-negative bacteria. Most of the *S. aureus* and *B. subtilis* cell wall envelope, around 33 nm, is occupied by the PG-WTA-LTA structure, while in *E. coli* PG ranges only from 3 nm to 6 nm in thickness. Interestingly, studies of Gram-positive CW by atomic force microscopy and cryogenic electron microscopy showed that the CW has a bipartite structural

organization, with an outer wall zone of higher density and an inner wall zone of lower density. In *S. aureus* and *B. subtilis* the inner wall zone can have a thickness of 16 and 22 nm, respectively (Valério R F Matias and Beveridge 2005; Valerio R F Matias and Beveridge 2006). Matias and Beveridge further shown that the division septa of *S. aureus* has a low density zone, similar to the inner wall zone, separating what will be the future outer wall zones (V. Matias and Beveridge 2007).

Although PG structure acts as a protection layer, it is though that it can be permeable to uncharged globular proteins smaller than 20 kDa, since it has been experimentally demonstrated that PG can have pores with an average radius of 2.06 nm and 2.12 nm in *E. coli* and *B. subtilis*, respectively (Demchick and Koch 1996; Hughes, Thurman, and Stokes 1975).

Peptidoglycan biosynthesis

PG synthesis is conserved among Gram-positive and Gram-negative bacteria and occurs in three stages that are carried in three different locations: 1) the synthesis of a PG precursor in the cytoplasm; 2) the synthesis of lipid linked precursors at the inner leaflet of the cytoplasmatic membrane with flipping to the outer leaflet; 3) the incorporation of the precursor on nascent PG in the CW [Figure 8] (Scheffers and Pinho 2005).

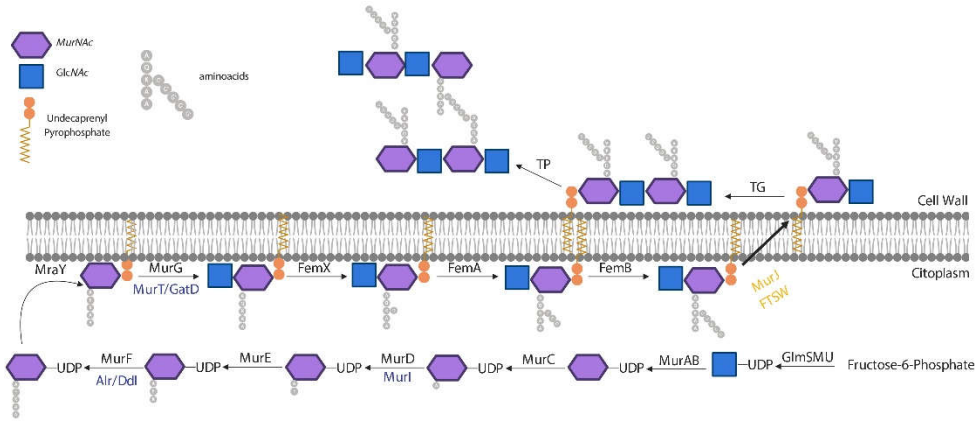

Figure 8 – Schematic representation of three stages of PG biosynthesis in *S. aureus*: 1) synthesis of a PG precursor in the cytoplasm; 2) synthesis of lipid linked precursors at the inner leaflet of the cytoplasmatic membrane with flipping to the outer leaflet; 3) incorporation of the precursor on nascent PG in the CW. Enzymes in blue synthesize substrates added on the depicted reaction: MurI and Alr racemases convert *L*-Glu to *D*-Glu and *L*-Ala to *D*-Ala, respectively, while Ddl ligases form the *D*-Ala-*D*-Ala dipeptide. There is debate regarding which enzyme is responsible for Lipid II flipping, with FtsW and MurJ (in yellow) being the two candidates.

Peptidoglycan synthesis – Cytoplasmatic steps

PG synthesis starts with the conversion of fructose-6-phosphate into uridine diphosphate-Glc*NAc* (UDP-Glc*NAc)* by the GlmSMU enzymes (Badet, Vermoote, and Le Goffic 1988; Badet-Denisot and Badet 1992; Gehring et al. 1996; Jolly et al. 1999; Mengin-Lecreulx and van Heijenoort 1994, 1996). Part of the UDP-Glc*NAc* pool is converted to uridine diphosphate-Mur*NAc* (UDP-Mur*NAc*) by MurAB enzymes (Benson et al. 1993; Benson, Walsh, and Massey 1997; Du et al. 2000). The *D*-amino acids present in the PG are produced by the MurI and Alr racemases, which convert *L*-Glu to *D*-Glu and *L*-Ala and *D*-Ala, respectively (Doublet, van Heijenoort, and Mengin-Lecreulx 1994; Doublet et al. 1993; Scaletti, Luckner, and Krause 2012). The aminoacids *L*-Ala, *D*-Glu and *L*-Lys or DAP are added sequentially by the MurCDE enzymes, respectively, to the lactoyl group of UDP-Mur*NAc* (Kouidmi, Levesque, and Paradis-Bleau 2014; Ruane et al. 2013; Bertrand

et al. 1999; Emanuele et al. 1996). In *B. subtilis* DAP is further amidated by AsnB (Dajkovic et al. 2017). The *D*-Ala-*D*-Ala dipeptide present at the carboxyl end of the PG stem peptide is first formed by the Ddl ligases (Zawadzke, Bugg, and Walsh 1991) and then incorporated into the UDP-Mur*NAc*-tripeptide by MurF (M. S. Anderson et al. 1996; al-Bar et al. 1992), forming a UDP-Mur*NAc*-pentapeptide known as Park nucleotide.

Peptidoglycan synthesis – Lipid linked precursors synthesis and flipping

The Park nucleotide is connected to bactoprenol (undecaprenyl pyrophosphate), in the inner leaflet of the cytoplasmatic membrane by MraY with the release of UDP. The resulting Mur*NAc*-(pentapeptide)-pyrophosphoryl-undecaprenol is known as lipid I (Al-Dabbagh et al. 2008; Chung et al. 2013; Bouhss et al. 2004). MurG converts lipid I into lipid II [Glc*NAc*-β-(1,4)-Mur*NAc*-(pentapeptide)-pyrophosphoryl-undecaprenol] by adding the Glc*NAc* of UDP-Glc*Nac* to lipid I with release of UDP (J. S. Anderson et al. 1965; van Heijenoort 2007; Mengin-Lecreulx et al. 1991).

In *S. aureus* FemX adds the first glycine of the cross bridge, to the amine group of the lysine in the PG stem peptide, forming a lipid II-Gly_1 molecule. Afterwards, the cross bridge is extended by FemA, which adds the second and the third glycines forming lipid II-Gly_3, and by FemB, which adds the fourth and the fifth glycines forming lipid II-Gly_5 (Schneider et al. 2004; Rohrer et al. 1999; Ehlert, Schröder, and Labischinski 1997). The GatD/MurT complex is responsible for the amidation of *D*-Glu to *D*-Gln. Since the GatD/MurT complex was shown to amidate all 5 lipid precursors *in vitro*, suggesting that GatD/MurT may lack a preference for a specific PG lipid precursor, it is unknown which substrate is preferentially used *in vivo* (Figueiredo et al. 2012; Münch et al. 2012).

The resulting product lipid II-Gly_5 (and possibly the intermediary products lipid II-Gly_3 and lipid II-Gly_1 (Monteiro et al. 2019)) is flipped from the inner leaflet to the outer leaflet of the cytoplasmatic membrane. There is a debate regarding which enzyme is responsible for this step, with FtsW and MurJ being the two strongest candidates (Mohammadi et al. 2011; Ruiz 2008).

Peptidoglycan synthesis – precursor incorporation into nascent PG

Precursor incorporation into nascent PG is achieved by reactions of transglycosylation (TG), to extend glycan chains, and reactions of transpeptidation (TP), to connect adjacent glycan chains through their stem peptides. TG connects the lipid II-Gly_5 Glc*Nac* C4 to the Mur*NAc* C1 of the nascent PG with release of the undecaprenyl pyrophosphate. Reactions of TG in *S. aureus* can be carried out by the monofunctional transglycosylases, MGT and SgtA, by the transglycosylase domain of PBP2 and, as recently shown, by FtsW (Sauvage et al. 2008; Barrett et al. 2005; Heaslet et al. 2009; Taguchi et al. 2019; Terrak and Nguyen-Distèche 2006; Reed et al. 2011). After the incorporation of the PG precursor into the PG macromolecule, the undecaprenyl pyrophosphate is subsequentially dephosphorylated, to remove one phosphate group, and recycled for another cycle of Lipid precursor synthesis. Reactions of TP in *S. aureus* are achieved by cleaving the amide bond between the two terminal *D*-Ala and connecting the carboxyl of the *D*-Ala, in the fourth position of the stem peptide, to the amine group of the glycine present in another PG stem peptide. This reaction, carried by the transpeptidase domain of penicilin binding proteins (PBPs), results in the mature cross-linked PG being deficient of terminal *D*-Ala (Sauvage et al. 2008).

Peptidoglycan Hydrolysis

Peptidoglycan can also be modified and degraded by specific hydrolytic enzymes, the peptidoglycan hydrolases. If these enzymes are synthetized and act upon the same cell (species) and their enzymatic action can result in cell lysis, they are called autolysins. Since autolysins are important for cell wall turnover and daughter cell separation (Layec, Decaris, and Leblond-Bourget 2008; Waldemar Vollmer et al. 2008), peptidoglycan synthesis and autolysins activity are thought to be tightly regulated and coordinated (Antignac, Sieradzki, and Tomasz 2007).

Peptidoglycan hydrolases activities can be divided into two major groups: the enzymes that cleave the glycosidic bonds that are present in the glycan chains, known as glycosydases and, the enzymes that cleave amide bonds, known as amidases (Waldemar Vollmer et al. 2008; Ghuysen, Tipper, and Strominger 1966; Frirdich and Gaynor 2013).

Glycosydases can show hydrolysis specificity, cleaving after a specific monosaccharide residue. Endo-*N*-acetyl-β-*D*-glucosaminidases (*N*-acetyl-glucosaminidases) cleave after a Glc*NAc* residue, generating a Glc*NAc* reducing end while *N*-acetyl-β-*D*-muramidases (*N*-acetyl-muramidases) cleave after a Mur*NAc* residue. *N*-acetyl-muramidases activity can be further divided into lysozyme like activity, creating a Mur*NAc* reducing end or lytic transglycosylases that cleave the β 1,4-glycosidic bond between Mur*NAc* and Glc*NAc* with the concomitant formation of a 1,6-anhydro ring at the Mur*NAc* residue (Waldemar Vollmer et al. 2008; Ghuysen, Tipper, and Strominger 1966; Frirdich and Gaynor 2013).

Amidases can cleave the amide bond between the lactyl residue of Mur*NAc* and the first amino acid of the peptide stem, known as *N*-Acetylmuramyl-*L*-alanine amidases, or between two amino acids of the peptide stem, acting as peptidases. The peptidases can be classified as carboxypeptidases if they remove a terminal amino acid or endopeptidases if they cleave within the peptide stem.

Endopeptidases and carboxypeptidases can be further classified as *DD*, *DL* or *LD* based on the chirality of α- carbon of the amino acids involved [Figure 9] (Waldemar Vollmer et al. 2008; Ghuysen, Tipper, and Strominger 1966; Frirdich and Gaynor 2013).

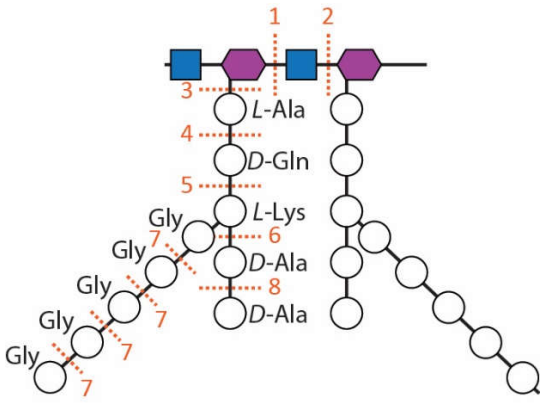

Figure 9 – Schematic representation of *S. aureus* PG and specificity of cleavage sites of peptidoglycan hydrolases. Note that virtually all bonds present in the peptidoglycan can be cleaved by a peptidoglycan hydrolase. The cleavage sites are depicted with an orange dashed line for 1) *N*-acetyl-muramidases, 2) *N*-acetyl-glucosaminidases, 3) *N*-Acetylmuramyl-*L*-alanine amidases, 4) *LD* endopeptidase, 5) *DL* endopeptidase, 6) *LD* Carboxypeptidase, 7) endopeptidase, 8) *DD* carboxypeptidase.

Peptidoglycan hydrolases, both those encoded by bacteria and phage genomes, have a modular structure. They usually contain hydrolytic domains, which are responsible for their enzymatic activity(ies) and may have one or more localization domains, which target the hydrolytic enzyme to a specific cellular localization (García et al. 1988; López et al. 1992).

Several catalytic domains have been described in bacterial encoded peptidoglycan hydrolases (Layec, Decaris, and Leblond-Bourget 2008; Waldemar Vollmer et al. 2008; Chapot-Chartier 2010). The identity of the catalytic domains present in each enzyme may allow the prediction of the hydrolase enzymatic activity, as described in Table 1. Table 2 describes some of the localization domains found in bacterial encoded peptidoglycan hydrolases (Layec, Decaris, and Leblond-Bourget 2008; Waldemar Vollmer et al. 2008; Chapot-Chartier 2010).

Table 1 – Hydrolytic domains commonly found in bacteria peptidoglycan hydrolases and their predicted hydrolytic activity.

Domain Name	PFAM entry	Predicted activity
Glyco_hydro_25	PF01183	Muramidase (lysozyme -like)
Glucosaminidase	PF01832	*N*-acetyl-Glucosaminidase
Glyco_hydro_18	PF00704	Glycosidase
Transglycosylase-like	PF06737	Lytic transglycosylase
SLT	PF01464	Lytic transglycosylase
Amidase_2	PF01510	*N*-acetylmuramyl-*L*-alanine amidase
Amidase_3	PF01520	*N*-acetylmuramyl-*L*-alanine amidase
Amidase_5	PF05382	*N*-acetylmuramyl-*L*-alanine amidase
Cysteine, **H**istidine-dependent **a**midohydrolases/**p**eptidases (**CHAP)**	PF05257	Amidase or peptidase
NLPC_P60	PF00877	Peptidase
Pepdidase_M23	PF01551	Gly-Gly endopeptidase
Peptidase_S66	PF02016	*LD*-carboxypeptidase
VanY	PF02557	*D*-alanyl-*D*-alanine carboxypeptidase
Peptidase_S11	PF00768	*D*-alanyl-*D*-alanine carboxypeptidase
Peptidase_S13	PF02113	*D*-Ala-*D*-Ala carboxypeptidase
Hydrolase_2	PF07486	Unknown

Table 2 – Cell wall binding domains commonly found on peptidoglycan hydrolases.

Domain	PFAM entry
LysM	PF01476
CW_binding_1 (choline binding domain)	PF01473
SH3_5	PF08460
GW	PF13457
SPOR	PF05036
CW_binding_2	PF04122
PG_binding_1	PF01471

Although virtually all known peptidoglycan bonds can be degraded by autolysins (see Figure 9), a single bacterium may not have all known activities. Furthermore, due to bacteria codifying autolysins with redundant activity, the characterization of autolysins activities and their physiological role has proven to be a difficult challenge [Figure 10] (Waldemar Vollmer et al. 2008; Smith, Blackman, and Foster 2000, 1996; Firczuk and Bochtler 2007).

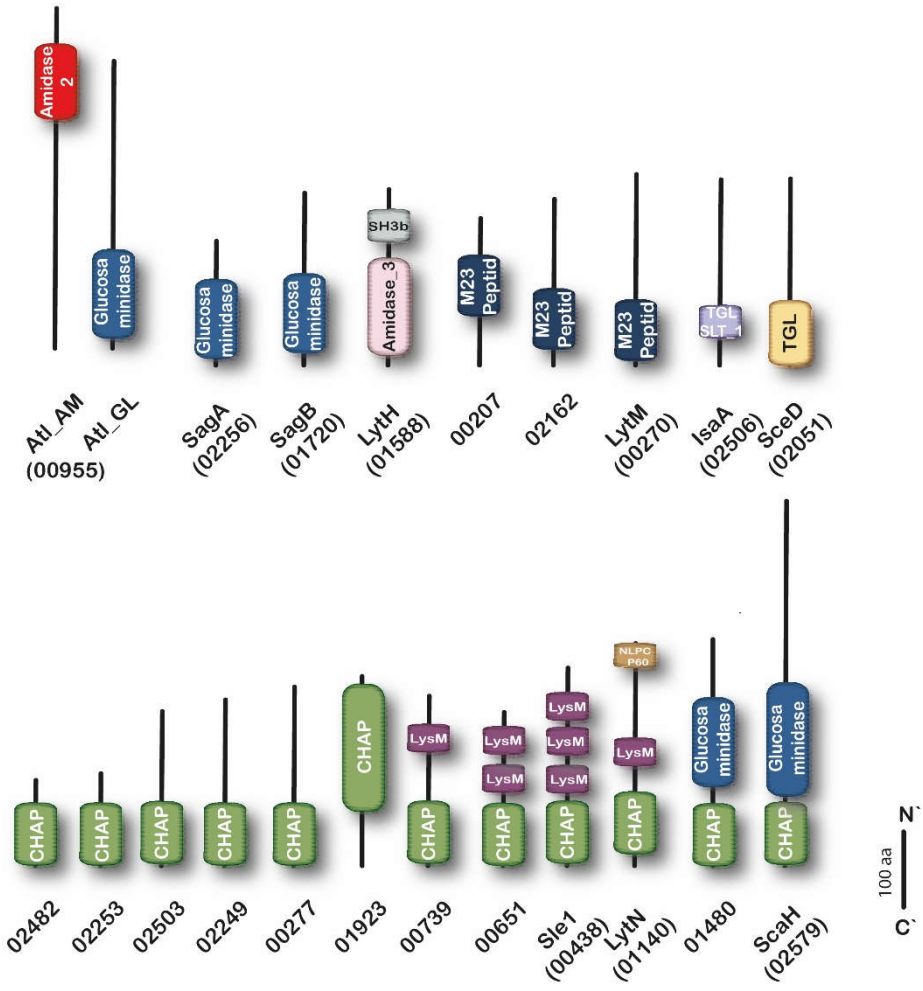

Figure 10 – Schematic representation of the known and putative PG hydrolases encoded by *S. aureus* genome that portrays the modular organization of the catalytic and localization domains. Proteins are identified by name and/or of their gene locus in the genome of *S. aureus* USA300_FPR3757. Atl is depicted after being processed to two lytic enzymes with *N*-acetyl-glucosaminidase and *N*-acetyl-muramyl-*L*-alanine activities.

Activity of PG hydrolases can be regulated either at transcription level or at post-translational level. Transcriptional regulation of genes that encode for PG hydrolytic enzymes is typically achieved by two component systems. In *S. aureus*, the LytSR system together with the *lrgAB* operon was shown to regulate PG

hydrolases activity using an holin-antiholin system similar to bacteriophages (Brunskill and Bayles 1996; Groicher et al. 2000; Rice et al. 2003). PG hydrolases activity can be further regulated by the *arlRS* two component system that may be a positive regulator of the LytSR-*lrgAB* system (Fournier and Hooper 2000; Liang et al. 2005). The *S. aureus* WalKR two component system, homologous to the YycFG system in *B. subtilis*, can control PG hydrolases activity and biofilm formation. The WalKR system was shown to control the activity of both Atl and LytM in *S. aureus* (Dubrac et al. 2007).

Regulation of PG hydrolases at the post-translational level can be achieved by proteolysis. An inactive form of the enzyme can be activated by proteolysis or an active form of the enzyme can be inactivated by proteolysis, e.g. in *B. subtilis* extracellular proteases degrade PG hydrolases involved in cell wall turnover (Jolliffe, Doyle, and Streips 1980). Peptidoglycan hydrolases can also be regulated by interaction with a modifier protein that modulates activity or by interaction with a particular cell wall component, e.g. CwbA modulates the activity of *B. subtilis* CwlB amidase (Kuroda and Sekiguchi 1992) and LytA from *S. pneumoniae* is activated by interaction with choline present in pneumococcal WTA (Tomasz and Westphal 1971). Chemical modification of the substrate, such as those inserted by reactions of *O*-acetylation, de-*N*-acetylation of the glycan or *D*-alanylation of the WTA, can modulate peptidoglycan hydrolases activity (Veiga et al. 2007; Waldemar Vollmer 2008; Peschel et al. 2000). Peptidoglycan hydrolases can also be regulated by controlling their cytoplasmatic export and localization with a signal peptide or localization domains (see above) or by blocking access to the substrate by steric hindrance by other cell wall components, such as WTA or LTA (Steen et al. 2003).

PG hydrolases are involved in autolysis, in remodelling of the PG sacculus to determine the bacterial cell shape, in the assembly of cell surface organelles, in sporulation and in the germination of spores, in resuscitation of dormant cells, in biofilm formation and in the bacterial lysis that ensure the survival of bacteria in

species competition (Waldemar Vollmer, Blanot, and De Pedro 2008; Chapot-Chartier 2010; Kajimura et al. 2005; Stapleton et al. 2007; Sugai et al. 1995; Waldemar Vollmer et al. 2008; Frirdich and Gaynor 2013). Furthermore, PG turnover products released due to autolysins activity can be detected by host receptors (Goodell and Schwarz 1985; Girardin et al. 2003; C.-I. Chang et al. 2006).

Peptidoglycan recognition by host innate immune system

Peptidoglycan a major component of the bacterial CW and a bona-fide MAMP, can be efficiently recognized by several PRR that are produced in a plethora of hosts that range from dipteran, such as *Drosophila melanogaster,* to vertebrate animals and plants (Buckley and Rast 2015; Bruno Lemaitre and Hoffmann 2007; Girardin et al. 2003; Mesnage et al. 2014). These PRR include the Toll-Like Receptor proteins (TLR), the Nucleotide binding Oligomerization Domain proteins (NOD), the Lysin-Motif domain proteins (LysM) and the Peptidoglycan Recognition Proteins (PGRP).

The molecular mechanisms of innate immunity available in different organisms, including humans, show, as already mentioned, a degree of conservation (Neyen et al. 2014; Ausubel 2005; Ronald and Beutler 2010). Since bacterial virulence factors, and the strategies used by different microorganisms to evade immune detection, are also conserved among bacteria, we can study these mechanisms in model organisms (Foster 2005; Veldkamp and van Strijp 2009; Hornef et al. 2002; Vallet-Gely, Lemaitre, and Boccard 2008). Furthermore, it has been shown that virulence factors for disease in one host also contribute to the outcome of a bacterial infection in non-natural hosts (Sifri et al. 2003; Glittenberg et al. 2011; Panayidou, Ioannidou, and Apidianakis 2014; Kounatidis and Ligoxygakis 2012).

D. melanogaster has proven to be a good invertebrate model organism to study human pathways (Bruno Lemaitre and Hoffmann 2007; Padmanabha and Baker 2014) since its organs and signalling pathways for development, metabolism and innate immunity share evolutionary conserved elements to vertebrates' (Buchon, Silverman, and Cherry 2014). Furthermore, several research groups have a well-established set of genetic tools that facilitate studying the aforementioned molecular processes (Venken and Bellen 2014). Additionally, since *D. melanogaster* are naturally infected with bacteria, fungi and viruses, and can be experimentally infected with human pathogens, they present them-selves as a good "tool" to study host-pathogen interactions (Magda L. Atilano et al. 2011; Bera et al. 2007; Magda L. Atilano et al. 2014; Panayidou, Ioannidou, and Apidianakis 2014; Kounatidis and Ligoxygakis 2012; Neyen et al. 2014; Vallet-Gely, Lemaitre, and Boccard 2008).

Drosophila melanogaster innate immune response

Drosophila, being dipteran organisms, only have innate immune defences to fight a bacterial infection as they lack an adaptive immune system. Innate immunity associated processes can be divided between immediate cellular responses and inducible humoral responses. Passive mechanisms that act as physical barrier *i.e.* the integument (the outer surface of *Drosophila*), and the midgut peritrophic membrane in *Drosophila* are also considered to be part of the innate immune system (Ashida and Brey 1995; Hegedus et al. 2009).

Cellular immune responses rely on haemocytes. In *Drosophila* different type of haemocytes are responsible for several defence responses: plasmatocytes comprise about 90% the total number of haemocytes and are responsible for pathogen phagocytosis or formation of nodules that physically contain a large bacterial load; lamellocytes, which are only present in the larval developmental

stage, promote encapsulation of parasitic organisms; crystal cells promote reactions of melanisation that help on the formation of capsules or nodules that contain parasites or groups of pathogens, respectively (Parsons and Foley 2016; Crozatier and Meister 2007; Stanley, Miller, and Tunaz 2009; Bruno Lemaitre and Hoffmann 2007).

On the other hand, humoral immune responses involve the inducible production of antimicrobial peptides (AMP) in the fat-body, the insect equivalent of the liver, and production of reactive oxygen species (ROS) (Imler and Bulet 2005). Since the humoral immune response is induced upon detection of an infection, it is believed that cellular responses act as the first layer of defence (Stanley, Miller, and Tunaz 2009; Haine et al. 2008).

AMP can be grouped into three families according to their antimicrobial activities: 1) defensins, which are active against Gram-positive bacteria; 2) cecropins, drosocin, attacins and diptericin that are active against Gram-negative bacteria; 3) drosomycin and metchnikowin that are active against fungi (Imler and Bulet 2005).

AMP production is induced after recognition of a MAMP by a PRR and involve signalling pathways that converge on the differential activation of nuclear factor kappa B (NF-κB) transcription factors (Bruno Lemaitre and Hoffmann 2007; Ganz 2003; Hultmark et al. 1980, 1983; Steiner et al. 1981). Two signalling pathways have been identified in *Drosophila* [Figure 11], the Toll pathway and the Imd (immunodeficiency) pathway, that are differentially activated mainly due to the recognition of PG by evolutionary conserved PGRPs (Bruno Lemaitre and Hoffmann 2007; Valanne, Wang, and Rämet 2011; Kleino and Silverman 2014; Myllymäki, Valanne, and Rämet 2014).

Drosophila PGRPs are germline encoded receptors responsible for the recognition of PG. These innate immune receptors bind PG through their PGRP domains, which are type 2 amidase domains that are structurally similar to

bacteriophage T7 lysozyme and bacterial type 2 amidase [see Table 1] (Royet, Gupta, and Dziarski 2011). *Drosophila* genome encodes 19 different PGRPs that can be divided into groups based on their length or hydrolytic activity (Dziarski et al. 2006; Kurata 2014).

Short PGRPs, denoted as PGRP-S, for short, usually harbour a conserved C-terminal PGRP domain and a variable N-terminal signal peptide (Royet, Gupta, and Dziarski 2011). Long PGRPs can have one or more PGRP domains in the C-terminal domain and a variable N-terminal sequence unique for a given PGRP that lacks homology to other PGRPs or proteins (Dziarski et al. 2006; Charroux et al. 2009).

PGRPs with an enzymatically active PGRP domain have *N*-acetylmuramyl-*L*-alanine amidase activity and harbour a conserved Zn^{2+} binding site (M.-S. Kim, Byun, and Oh 2003; Mellroth, Karlsson, and Steiner 2003). The available crystal structures of PGRPs have revealed the existence of a PG binding groove, whose net charge has been described to be responsible for the discrimination between PG of different composition (Reiser, Teyton, and Wilson 2004; Basbous et al. 2011; Leone et al. 2008; Charroux et al. 2009; M.-S. Kim, Byun, and Oh 2003; C. I. Chang et al. 2004).

The Toll pathway, initially identified has a developmental pathway, is described as being responsible for the immune response to Gram-positive bacteria or fungi infection (Bruno Lemaitre and Hoffmann 2007; Valanne, Wang, and Rämet 2011; Lindsay and Wasserman 2014) while the Imd pathway is described as being responsible for the immune response to Gram-negative bacteria [Figure 11] (Kleino and Silverman 2014; Myllymäki, Valanne, and Rämet 2014).

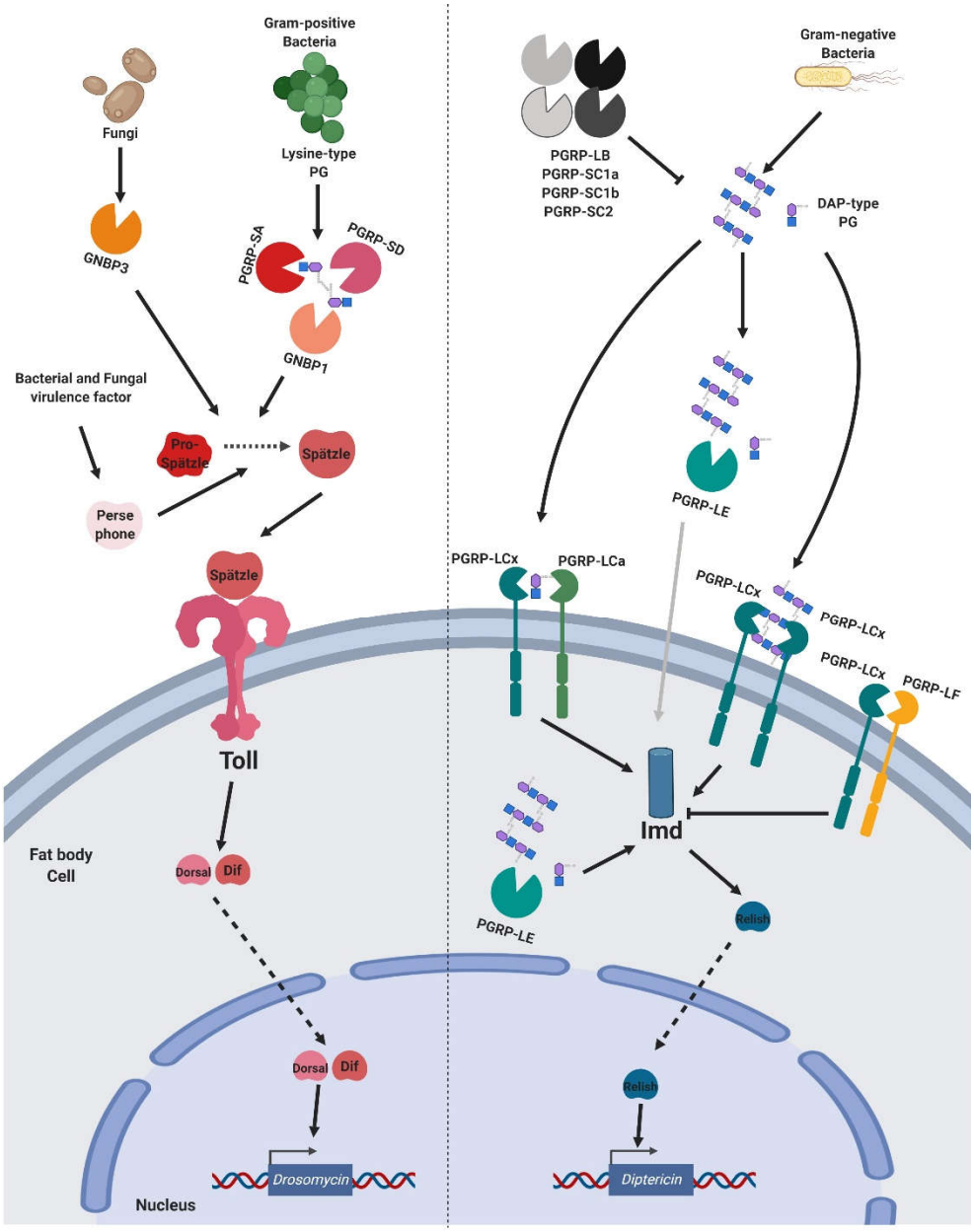

Figure 11 – Model of the *Drosophila melanogaster* Toll and Imd pathways that control the expression of different AMPs upon recognition of a MAMP by PGRPs. The Toll pathway is described as being responsible for induction of an innate immune response to Gram-positive bacteria after recognition of Lys-type PG by the secreted PGRP-SA or to invading fungi after recognition of *β*-1,3-glucan by GNBP3. Alternatively, Persephone can be proteolytic degraded into an active form by bacterial and fungal virulence factors. These three events converge on the activation of Spätzle, by proteolysis of proSpätzle. Spätzle will bind to membrane Toll receptors on the Fat Body cells that will activate a

signalling pathway that trigger the activation of Dorsal and Dif NF-κB transcription factors that will promote the production of Drosomycin among other AMPs. The Imd pathway is described as being responsible for the innate immune response to Gram-negative bacteria after recognition of DAP type PG by PGRP-LC isoforms or by PGRP-LE. Upon binding to PG, PGRP-LC or PGRP-LE will dimerize and bind to Imd protein. This event will initiate a signalling pathway that triggers the activation of the Relish NF-κB transcription factor that will promote production of Diptericin among other AMPs. A homodimer of the PGRP-LC_x isoform is thought to bind to polymeric DAP type PG while the PGRP-LC_x/PGRP-LC_a heterodimer is thought to recognized monomeric DAP type PG. Since PGRP-LF can bind to PGRP-LC_x but not to PG it is thought to be an inhibitor of the Imd pathway. PGRP-LB, PGRP-SC1a, PGRP-SC1b and PGRP-SC2 amidase activity cleaves DAP type PG to a form that cannot be recognized by PGRP-LC or PGRP-LE proteins.

Upon recognition of Lys type PG by PGRP-SA (Michel et al. 2001) or recognition of β-1,3-glucan from invading fungi by the Gram-negative binding protein 3 (GNBP3) (Mishima et al. 2009), serine proteases cascades trigger the proteolysis of proSpätzle to produce the activated form of Spätzle (Shia et al. 2009). Active Spätzle is the ligand of *Drosophila* Toll receptors, contrary to mammal Toll receptors (Coscia et al. 2011), and Spätzle binding to toll receptors on the cell membrane initiates a signalling pathway that activates the Dorsal and Dif NF-κB transcription factors. These events can regulate the induction of an immune response, such as the production of Drosomycin and other AMPs (Valanne, Wang, and Rämet 2011; Lindsay and Wasserman 2014; Bruno Lemaitre and Hoffmann 2007). Gram-negative binding protein 1 (GNBP1) and possible PGRP-SD are thought to cooperate with PGRP-SA in the detection of Lys-Type PG (L. Wang et al. 2008, 2006), although more recently it was reported that PGRP-SD can recognize DAP type PG and activate the Imd pathway (Iatsenko et al. 2016). Moreover, persephone can be proteolytically cleaved to an active form directly by bacterial and fungal virulence factors. Active Persephone will promote toll pathway activation by proteolytically activating Spätzle (Bruno Lemaitre and Hoffmann 2007; B Lemaitre et al. 1996).

The Imd pathway is activated when PGRP-LC, or PGRP-LE, bind DAP type PG (Kleino and Silverman 2014; Myllymäki, Valanne, and Rämet 2014; Kaneko and Silverman 2005; Kaneko et al. 2006). Binding of PG is thought to promote

dimerization of the PGRP-LC or PGRP-LE that, in turn, facilitate the binding to Imd protein (Choe, Lee, and Anderson 2005). This initiates a signalling pathway that will promote activation of Relish NF-κB transcription factor (Choe et al. 2002) and production of Diptericin among other AMPs. A homodimer of the PGRP-LC_x isoform is thought to bind to polymeric DAP type PG while the PGRP-LC_x/PGRP-LC_a heterodimer is thought to recognized monomeric DAP type PG (Kaneko et al. 2004; Mellroth et al. 2005). Contrary to PGRP-LC isoforms that are transmembrane proteins, PGRP-LE is either intracellular or secreted to the haemolymph, similarly to PGRP-SA and PGRP-SD that are extracellular (Bruno Lemaitre and Hoffmann 2007). PGRP-LE can recognize both monomeric and polymeric PG. Extracellular PGRP-LE upon binding to PG will activate the Imd pathway in a PGRP-LC dependent manner, while intracellular PGRP-LE interacts directly with the Imd protein (Yano et al. 2008). Since PGRP-LF can bind to PGRP-LC_x but not to PG it is thought to prevent formation of the PGRP-LC_x homodimer or the PGRP-LC_x/PGRP-LC_a heterodimer and, therefore, be an inhibitor of the Imd pathway (Basbous et al. 2011). PGRP-LB, PGRP-SC1a, PGRP-SC1b and PGRP-SC2 amidase activity cleaves DAP type PG to a form that cannot be recognized by PGRP-LC or PGRP-LE proteins. These proteins are therefore thought to be responsible for the inactivation of the Imd pathway to homeostasis/non-infection levels (Mellroth and Steiner 2006; M.-S. Kim, Byun, and Oh 2003; Mellroth, Karlsson, and Steiner 2003; Bischoff et al. 2006).

In this study we focused on assessing how bacterial PG is recognized by host PRR, more specifically, *Drosophila melanogaster* PGRPs. To this end we explored the hypothesis that different *S. aureus* strains can present alternative/additional mechanism of modifying their PG to evade recognition. We also assessed how *Drosophila* PGRPs can discriminate PG with different compositions. Finally, since PG is simultaneous paramount for bacterial survival

and a bona fide MAMP we explored the mechanism of lipid-linked precursors flipping in *S. aureus*.

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Chapter II

Accessibility of Peptidoglycan is Important for Recognition of Gram-Positive Bacteria

Contributions to this chapter:

Gonçalo Covas performed all experiments.

Publications in this chapter:

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Abstract

The current model of *Drosophila melanogaster* innate immunity proposes that *Drosophila* induces a tailored response to the bacterial strain causing an infection. This differential immune response is thought to rely on the ability of peptidoglycan recognition proteins (PGRPs) to discriminate the invading bacteria and to induce the differential expression of NF-κB transcriptional factors, which culminates in the expression of suitable antimicrobial peptides (AMPs). The current model defends that while bacteria with Lys type peptidoglycan ([PG], Gram-positive bacteria) are recognized by PGRP-SA, which leads to the induction of the Toll pathway, bacteria with DAP type PG (Gram-negative bacteria and some Gram-positive *bacilli*) are recognized by PGRP-LC, or PGRP-LE, which culminates in the activation of the Imd pathway.

In this study, we propose that this model may oversimplify the *Drosophila* immune response, as it states that bacterial discrimination is only dependent on the variability of the third amino acid present in the peptidoglycan stem peptides. We further report that accessibility of peptidoglycan also plays an important role. We observed that both PGRP-SA and PGRP-LC can bind to PG purified from bacteria, with either Lys type or amidated DAP type peptidoglycan (*Staphylococcus aureus* and *Bacillus subtilis*, respectively), when the assay is carried out using a synthetic buffer akin to *Drosophila*'s haemolymph (HemoBuffer). We observed that PGRP-LC has a preference to bind amidated DAP type PG, and that PGRP-SA seems to bind either Lys type or amidated DAP type PG with a similar preference.

Remarkably, both PGRP-SA and PGRP-LC can bind to the surface of *S. aureus* or a *B. subtilis* mutant bacteria that are unable to produce TagO. This observation highlights the role of Wall Teichoic Acids (WTA) in blocking access of different PG receptors, such as PGRP-SA and PGRP-LC, to PG of different composition.

Introduction

Drosophila melanogaster relies solely on an innate immunity response to maintain homeostasis during their interaction with invading bacteria. Innate immunity can be divided into passive mechanisms that act as physical barrier, such as the integument and the midgut peritrophic membrane, a set of immediate cellular responses and several inducible humoral responses (Ashida and Brey 1995; Hegedus et al. 2009).

The cellular responses that are built immediately after a bacterial infection rely on specialized haemocytes that are responsible for pathogen phagocytosis, the encapsulation of the pathogen, the nodulation of different host cells around the microorganism and the melanisation of the surrounding tissues (Parsons and Foley 2016; Crozatier and Meister 2007; Stanley, Miller, and Tunaz 2009; Lemaitre and Hoffmann 2007). On the other hand, the different humoral immune responses put forward in an innate immune response involve an inducible production of antimicrobial peptides (AMP) in the fat-body (Imler and Bulet 2005). AMP can be grouped into three families according to their antimicrobial activities and are differentially induced as part of a differential immune response that can discriminate the infecting microorganism. *Drosophila* immune response relies on the ability of PRR, such as Peptidoglycan Recognition Proteins (PGRPs) or Gram-negative Binding Proteins (GNBPs), to recognize and discriminate PG of different composition in order to tune the innate immunity response (Lemaitre and Hoffmann 2007; Panayidou, Ioannidou, and Apidianakis 2014; Neyen et al. 2014; Dziarski et al. 2006; Julien Royet, Gupta, and Dziarski 2011; Kurata 2014). The *Drosophila* innate immune responses can be divided into two groups defined by the pathways that are differentially activated, under the control of NF-κB

transcription factors, upon recognition of a microbial infection, the Toll and Imd Pathways (see Figure 11 of Chapter 1 for summary).

The Toll pathway is described as being responsible for the activation of an innate immune response to infections caused by Gram-positive bacteria or fungi, upon the recognition of Lys type bacterial PG, by PGRP-SA (Michel et al. 2001), or the recognition of β-1,3-glucan from invading fungi, by the Gram-negative binding protein 3 (GNBP3) (Mishima et al. 2009). These events can trigger a serine proteases-dependent cascade of reactions that promote the hydrolysis of proSpätzle protein [inactive form] to Spätzle [active form] (Shia et al. 2009). Spätzle can then bind to *Drosophila'*s Toll receptors and initiate a signalling pathway that activates the Dorsal and Dif NF-κB transcription factors. This activation regulates the induction of an immune response, such as the production of Drosomycin and other AMPs (Valanne, Wang, and Rämet 2011; Lindsay and Wasserman 2014; Lemaitre and Hoffmann 2007; Ferrandon et al. 2007). Gram-negative binding protein 1 (GNBP1) and PGRP-SD are thought to cooperate with PGRP-SA in the detection of Lys-Type PG (L. Wang et al. 2008, 2006), although, controversially, more recently it was reported that PGRP-SD recognizes DAP type PG and activates the Imd pathway (Iatsenko et al. 2016). On additional note, different bacterial and fungal virulence factors can proteolytically activate Persephone that promotes the hydrolysis of proSpätzle to Spätzle.

The Imd pathway is described as being responsible for the *Drosophila* immune response to Gram-negative bacteria (Lemaitre and Hoffmann 2007; Valanne, Wang, and Rämet 2011; Lindsay and Wasserman 2014). This pathway is activated when PGRP-LC, or PGRP-LE bind DAP type PG (Kleino and Silverman 2014; Myllymäki, Valanne, and Rämet 2014; Kaneko and Silverman 2005; Kaneko et al. 2006), which is thought to promote dimerization of PGRP-LC or PGRP-LE. On one hand, a homodimer of the PGRP-LC_x isoform binds to polymeric DAP type PG while the PGRP-LC_x/PGRP-LC_a heterodimer can recognize monomeric DAP type PG

(Kaneko et al. 2004; Mellroth et al. 2005). On the other hand, PGRP-LE can recognize both monomeric and polymeric PG. Dimerization of PGRPs facilitates their association to the Imd protein (Choe, Lee, and Anderson 2005), a process that activates the Imd signalling pathway and promotes activation of Relish NF-κB transcription factor (Choe et al. 2002) and the consequent production of Diptericin and other AMPs. Interestingly, while extracellular PGRP-LE binding to PG activates the Imd pathway in a PGRP-LC dependent manner, the intracellular PGRP-LE can interact directly with the Imd protein in the activation of an innate immune response (Yano et al. 2008).

In summary, the literature defends a paradigm that has PGRP-SA as the main responsible for triggering an immune response against Gram-positive bacteria, that produce a Lys-type PG, while has PGRP-LC as the main responsible for triggering an immune response against Gram-negative bacteria, that are surrounded by a DAP-type PG. This oversimplified model to describe bacteria discrimination by *Drosophila*'s immune system, fails to explain conflicting data:

- It reduces all discrimination of bacterial variability to the third amino acid residue of the stem peptide present in the bacterial PG;
- It defines a role for only three PG receptors (PGRP-SA, PGRP-LC or PGRP-LE) but doesn't define a role for the 13 different PGRPs encoded in the *Drosophila* genome (J. Royet, Sciences, and Royet 2004; Kurata 2014).
- It doesn't fully explain the recognition of certain Gram-positive bacteria, such as *B. megaterium* and *B. subtilis*, that produce PG with a DAP or an amidated DAP (amiDAP), respectively, in the stem peptides of their PG. Of notice is the observation, made by Stenbak and colleagues, that amiDAP PG induces a lower expression of diptericin than DAP PG (Stenbak et al. 2004).

- The observation, made by Chang and colleagues, that recombinant PGRP-SA binds to both Lys type PG (*M. luteus* and *E. faecalis*) and non-amidated DAP type PG (*E. coli* and *P. aeruginosa*) but not to amiDAP type PG (*B. subtilis* and *B. thuringiensis*) in 20 mM Tris-HCl pH 7.8, 300 mM NaCl Buffer (C. I. Chang et al. 2004).
- The report, made by Mellroth and colleagues, that in a buffer with a particular composition (20 mM Hepes, pH 7.2, 150 mM NaCl), it was possible to observe a strong binding of recombinant PGRP-LC to polymeric PG harvested from bacteria with Lys type PG (such as *M. luteus, S. aureus* and *L. casei*); to PG produced by bacteria with DAP type PG (such as *B. megaterium, E. coli,* and *L. plantarum*) and to PG isolated from bacteria that produced a Ornithine-type PG (such as *L . fermentum*) (Mellroth et al. 2005).
- The observation, made also by Mellroth and colleagues, that in the same buffer (20 mM Hepes, pH 7.2, 150 mM NaCl) recombinant PGRP-SA can also show strong binding to polymeric PG harvested from both bacteria with Lys type PG (such as *M. luteus, S. aureus* and *L. casei)* and from bacteria with DAP type PG (such as *E. coli,* and *L. plantarum*) (Mellroth et al. 2005). By contrast, Mellroth and colleagues observed weak binding of recombinant PGRP-SA to *B. megaterium* (DAP type PG) and *L. fermentum* (Ornithine type PG) in 20 mM Hepes, pH 7.2, 150 mM NaCl (Mellroth et al. 2005).
- The interesting description, carried out by Leone and colleagues, that in a buffer of different composition (20 mM Tris-HCl pH 7.8, 300 mM NaCl), recombinant PGRP-SD, but not recombinant PGRP-SA, can bind amiDAP type PG from *B. subtilis* (Leone et al. 2008).

- The report that, in *Tenebrio molitor*, polymeric DAP type PG from *B. subtilis* (amiDAP) or *E. coli* (DAP) is bonded by PGRP-SA to produce a successful activation of Toll pathway (Yu et al. 2010).

As different reports have showed that PGRP-SA and PGRP-LC can bind both Lys-type and DAP-type PG (C. I. Chang et al. 2004; Mellroth et al. 2005), and previous studies by our group showed that in *S. aureus*, WTA can impair recognition of PG at the bacterial cell surface (Atilano et al. 2011), we decided to test the hypothesis that accessibility of PG plays an important role in the recognition/evasion of both Lys or DAP type bacteria. To test this hypothesis, we assessed the ability of different fluorescent PGRP derivatives to bind the surface of model Gram-positive bacterial organisms. We used *S. aureus* and *B. subtilis* strains that produce a Lys-type or an amiDAP-type PG, respectively, covered by WTA or lacking these glycopolymers. We further assessed if the buffer used in assays for determining PGRP binding preferences can have a significant influence on the result, and therefore, may be a factor of variability that is responsible for the conflicting observations in the literature. To test this, we compared PGRP-SA and PGRP-LC binding preferences to PG of different composition in a buffer that has an ionic composition and pH similar to that found in the haemolymph of *D. melanogaster* flies (named HemoBuffer).

Results

Wall Teichoic Acids prevent PGRP-SA or PGRP-LC binding to *S. aureus* and *B. subtilis* cells

In order to test the hypothesis that differential activation of an immune response in *Drosophila* is not restricted to the discrimination of the amino acid in the third position of the PG stem peptide and that it may be influenced by PG

accessibility, which could be determined by a particular CW architecture or composition, we quantified and compared the binding of mCherry_PGRP-SA and mCherry_PGRP-LC to *S. aureus* NCTC 8325-4 and *B. subtilis* EB6 wt strains with and without WTA at their surface due to the absence of TagO protein. To mimic, as closely as possible, the conditions where the *Drosophila* PG receptor binds the surface of a bacteria *in vivo* we performed the binding assays in HemoBuffer, a buffer optimized to have an ionic composition and pH as close as possible to the known composition of *Drosophila* haemolymph (see materials and methods for composition; van der Meer and Jaffe 1983; Croghan and Lockwood 1960).

We observed a weak binding ability of mCherry_PGRP-SA to both *S. aureus* and *B. subtilis* wt cells [Figure 1 and Figure 2, respectively]. Similarly, mCherry_PGRP-LC seemed to lack the ability to bind to both *S. aureus* and *B. subtilis* wt cells [Figure 1 and Figure 2, respectively]. Interestingly, the intensity of the fluorescent signals, derived from mCherry_PGRP-SA or from mCherry_PGRP-LC, that were associated with either *S. aureus* cells or *B. subtilis* cells were not statistically distinguishable [Supplementary Table 1]. These observations suggest that, in the conditions that were used, both *S. aureus* and *B. subtilis* cells have a cell surface where PG is similarly concealed. Moreover, the ability of PG to be hidden to either PGRP was independent of its composition (Lys- or amiDAP-type).

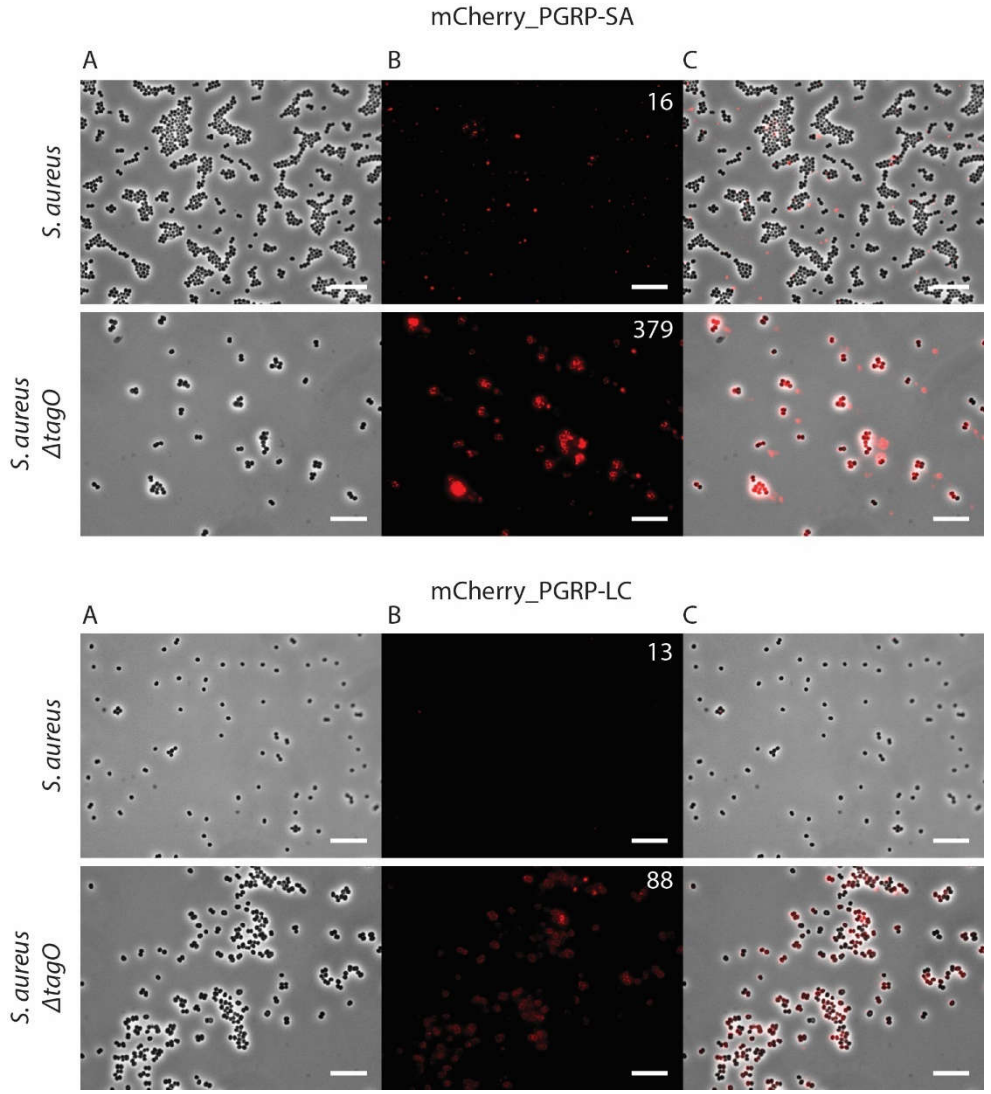


Figure 1 – mCherry_PGRP-SA and mCherry_PGRP-LC binding to *S. aureus* wt and *tagO* deletion mutants. Deletion of *tagO* results in an increased binding of either mCherry_PGRP-SA or mCherry_PGRP-LC to *S. aureus* with a Lys-Type PG. Panel A) shows a representative image acquired by Phase contrast microscopy, panel B) presents the mCherry_PGRP-SA or mCherry_PGRP-LC fluorescent signal acquired with a 10 ms acquisition time and panel C) shows an overlay of the two images. White scale bar represents a line of 10 μm. Median value of the binding intensity is shown on the upper right corner of panel B. See Supplementary Figure 1 for quantification data.

However, a completely different result was observed when mutants lacking WTA were used. We were able to observe that mCherry_PGRP-SA and mCherry_PGRP-LC showed an increase binding ability to the surface of the *S.*

aureus tagO deletion mutant, or to the surface of a *B. subtilis* deletion mutant that lacks TagO, when compared with their parental strains [Figure 1 and Figure 2, respectively]. The binding intensities of mCherry_PGRP-SA to either a *S. aureus* or a *B. subtilis* deletion mutant lacking TagO were statistically distinguishable from the binding intensities to the respective parental strains [Supplementary Table 1]. It should be highlighted that despite the binding intensity of mCherry_PGRP-LC to a *S. aureus* deletion mutant lacking TagO is not statistically distinguishable from the binding to the parental strain, when microscopy analysis was carried out at 10 ms [Supplementary Table 1], using a longer exposure time (500 ms) we were able to observe binding intensities that are statistically distinguishable (data not shown). In contrast, the mChery_PGRP-LC binding intensities to a *B. subtilis* mutant lacking TagO and to the parental strain were statistically distinguishable at a 10 ms exposure time [Supplementary Table 1]. These observations support the hypothesis that WTA can prevent recognition of PG by either PGRP, independently of the PG composition (Lys or amiDAP-type). Interestingly, both recombinant PGRPs showed a higher binding intensity to the *B. subtilis* deletion mutant lacking TagO than to the *S. aureus* deletion mutant lacking TagO, although PGRP-SA being described has having a binding preference to Lys type PG.

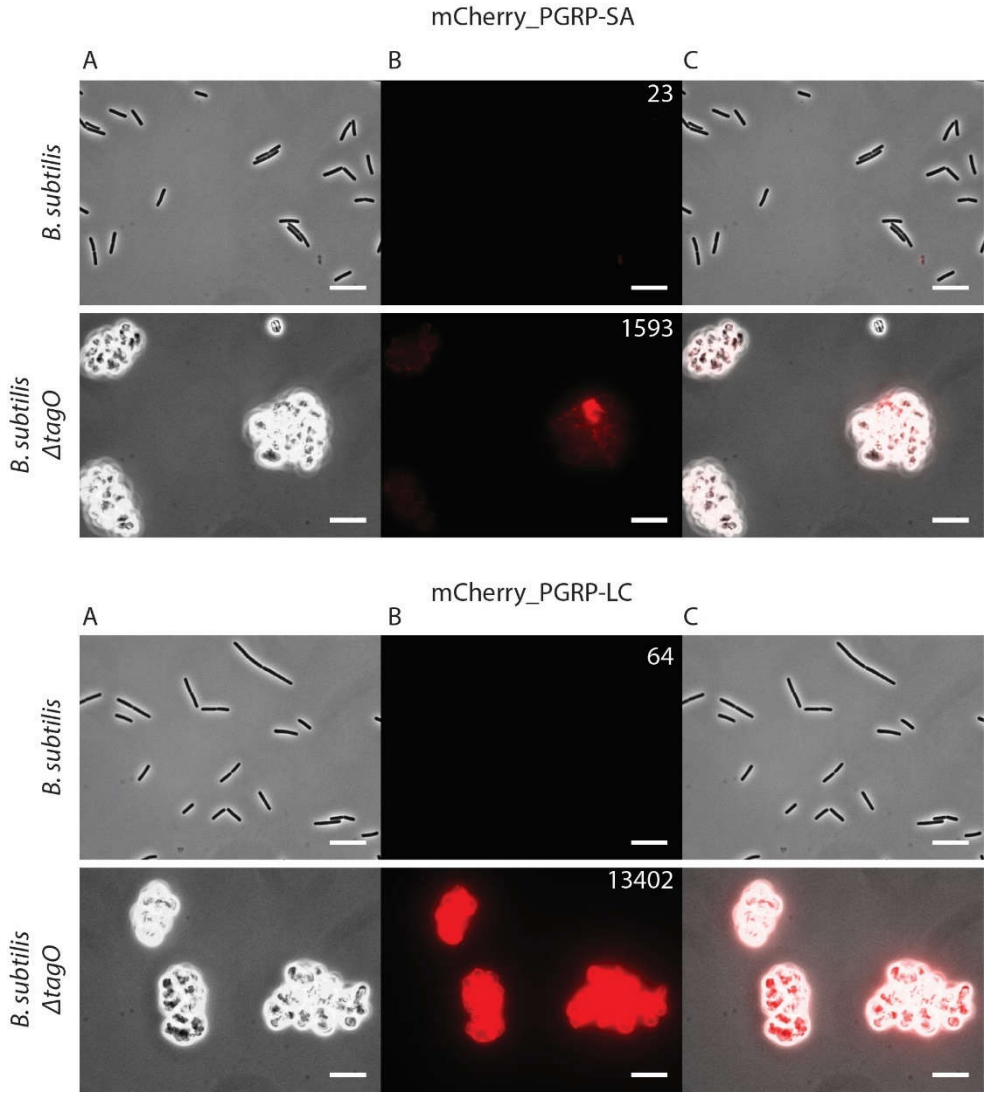


Figure 2 – mCherry_PGRP-SA and mCherry_PGRP-LC binding to *B. subtilis* wt and *tagO* deletion mutants. Deletion of *tagO* results in an increased binding of either mCherry_PGRP-SA or mCherry_PGRP-LC to *B. subtilis* with an amiDAP type PG. Panel A) presents an image acquired by Phase contrast microscopy, panel B) shows the fluorescent mCherry_PGRP-SA or mCherry_PGRP-LC signal observed with a 10 ms acquisition time and C) is an overlay of the two images. White scale bar represents a line of 10 µm. Median value of the binding intensity is shown on the upper right corner of the panel B. See Supplementary Figure 1 for quantification data.

To discard the hypothesis that the mCherry fluorescent tag (common to both recombinant proteins) was influencing the binding of the different proteins to PG we quantified the fluorescent signal present at the surface of bacteria that

have been exposed to a mCherry_PGRP-SA recombinant protein that should be unable to bind PG. This fluorescent PGRP construct has two mutations (C48Y and C54Y) that disrupt the highly conserved disulfide bridge considered to be essential for a correct and functional PGRP domain fold (Michel et al. 2001; C. I. Chang et al. 2004). The role of these residues for the activation of an innate immune response is highlighted by the observation that *Drosophila* semmelweis flies, that have a non-functional PGRP-SA also have a C48Y mutation (Michel et al. 2001). Despite mCherry_PGRP-SA_C48YC54Y showing a higher binding intensity to a *S. aureus* deletion mutant lacking TagO than to the parental strain the variation observed is negligible when compared to the functional mCherry_PGRP-SA [Figure 3]. As seen in Figure 4, a mCherry_PGRP-SA_C48YC54Y recombinant protein did not show a stronger binding intensity to a *B. subtilis* deletion mutant lacking TagO than to the parental strain. These observations allowed us to state that the PG binding domain determines the ability of the fluorescent PGRP receptors to bind the surface of bacteria and that the mCherry fluorescent domain does not have a role in this activity. Quantification of microscopy images showing the binding of mCherry_PGRP-SA or mCherry_PGRP-SA_C48YC54Y to *S. aureus* and *B. subtilis* wt cells and mutants lacking TagO are shown in Supplementary Figure 2.

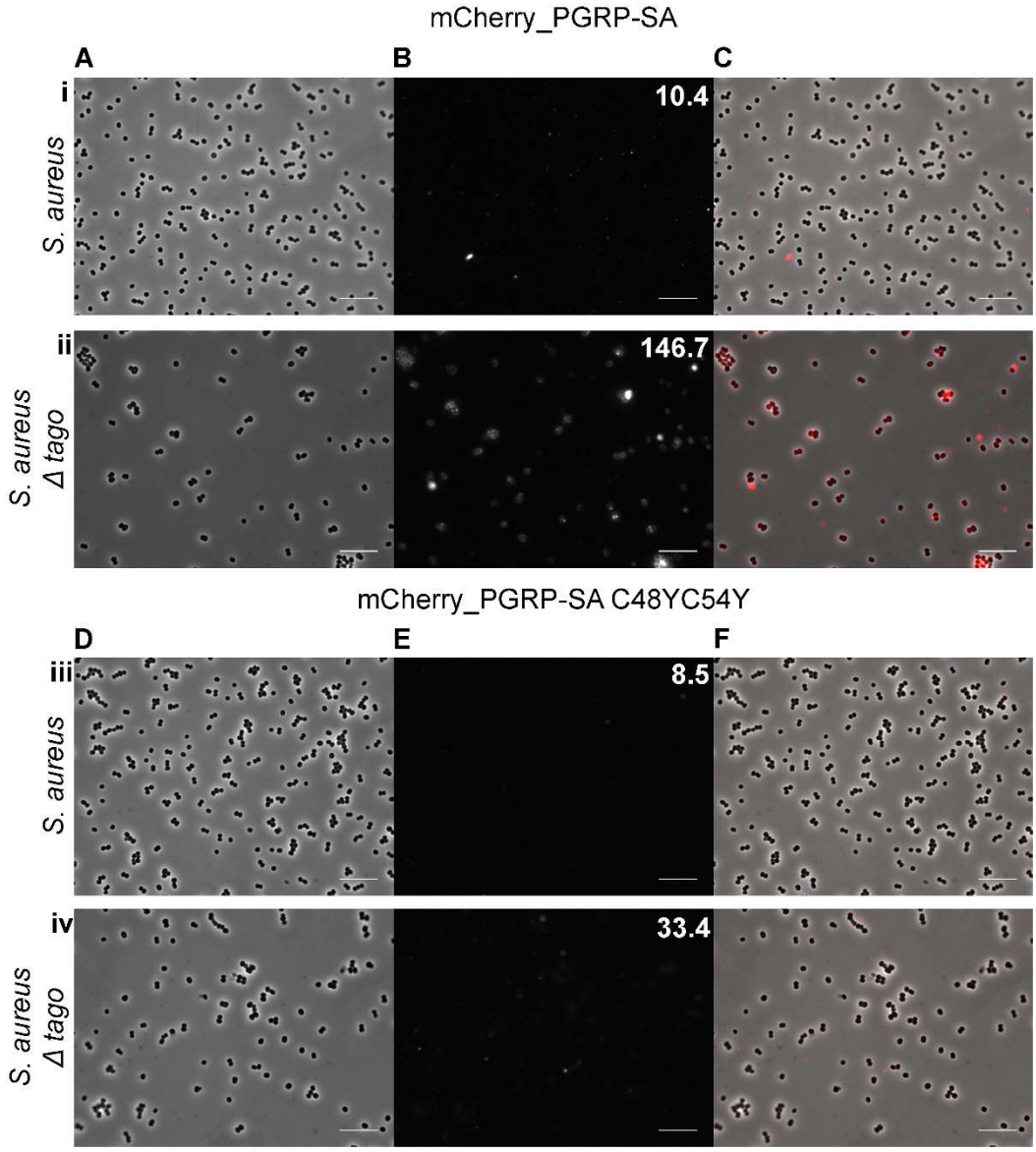


Figure 3 – Comparison of binding of a functional mCherry_PGRP-SA recombinant protein and a non-functional mCherry_PGRP-SA recombinant protein (With a C48Y and C54Y double mutation) to a *S. aureus* deletion mutant lacking TagO and the parental strain show that increased binding upon deletion of *tagO* is not due to the mCherry fluorescent tag. Panel A) represents images acquired by Phase contrast microscopy, B) show the mCherry_PGRP-SA fluorescent signal detectable with a 10 ms acquisition time and C) is an overlay of the two images. White scale bar is 10 µm. Median value of the binding intensity is shown on the upper right corner of the mCherry_PGRP-SA image. See Supplementary Figure 2 for quantification data.

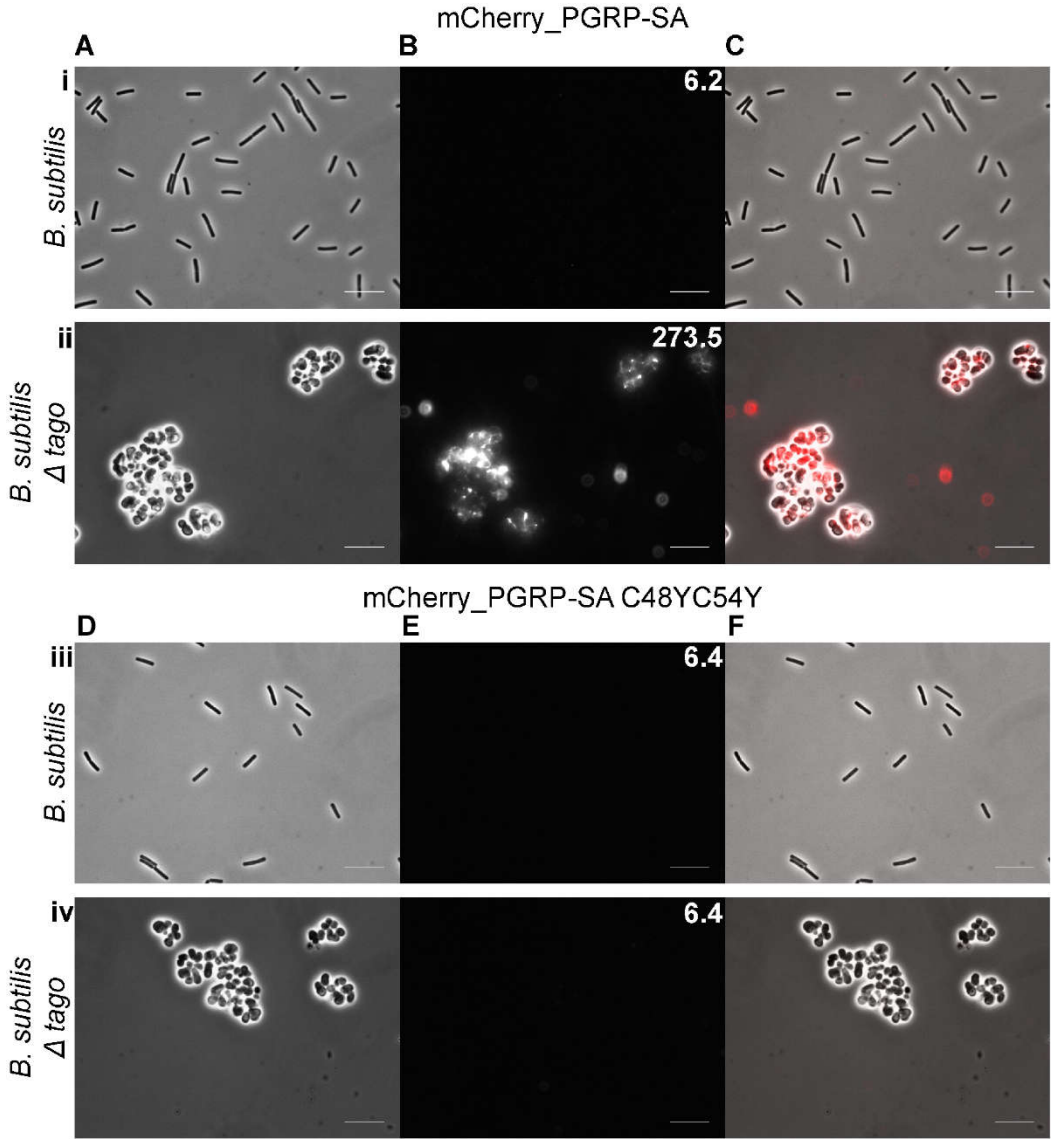


Figure 4 - Comparison of binding of a functional mCherry_PGRP-SA recombinant protein and a non-functional mCherry_PGRP-SA recombinant protein (With a C48Y and C54Y double mutation) to a *B. subtilis* deletion mutant lacking TagO and the parental strain show that increased binding upon deletion of *tagO* is not due to the mCherry fluorescent tag. Panel A) presents a representative image acquired by Phase contrast microscopy, B) shows the mCherry_PGRP-SA fluorescent signal that was detected with a 10 ms acquisition time and C) is an overlay of the two images. White scale bar is 10 µm. Median value of the binding intensity is shown on the upper right corner of the mCherry_PGRP-SA image. See Supplementary Figure 2 for quantification data.

PGRP-SA and PGRP-LC bind both Lys and DAP PG in HemoBuffer

To test the hypothesis that the discrepancy observed on the binding preferences of PGRPs on previously reported studies may be due to assays performed in buffers that are not representative of conditions found in haemolymph of flies, we assessed the binding preferences of PGRP-SA and PGRP-LC to Lys type and amiDAP type PG by co-precipitation assays on three different buffers: 1) 20 mM Tris-HCl 300 mM NaCl pH 7.8 (as used in Chang et al. 2004; and Leone et al. 2008); 2) Phosphate Buffered Saline (PBS) at pH 6.0; and 3) HemoBuffer. In order to be able to compare binding of PGRPs to PGs that have been purified from bacteria with different compositions, we quantified the number of moles of muramic acid per weight in each PG sample. In this way we were confident that the same amount of PG, in number of muramic acid residues, was added to the different co-precipitation assays.

As seen in Figure 5 panel A, while recombinant mCherry_PGRP-SA showed a weak binding to the *B. subtilis* amiDAP-type PG when the co-precipitation assay was performed in 20 mM Tris-HCl 300 mM NaCl pH 7.8. In contrast, when the co-precipitation assay was performed in HemoBuffer, we observed a stronger binding of recombinant mCherry_PGRP-SA to amiDAP. It was previously reported by Chang and Leone that PGPR-SA could not bind amiDAP PG (C. I. Chang et al. 2004; Leone et al. 2008). Considering that these studies were performed in 20 mM Tris-HCl 300 mM NaCl pH 7.8 and that we observed an increased binding of PGRP-SA to amiDAP PG in HemoBuffer, it is possible that the buffer choice is responsible for the conflicting observations in the literature. Similarly, the amount of mCherry_PGRP-LC that co-precipitated with *B. subtilis* PG was increased in HemoBuffer. Furthermore, it is clear that PGRP-LC has a stronger binding than PGRP-SA to amiDAP PG [Figure 5 panel A]. This is in accordance with (ami)DAP type PG being preferentially recognized by PGRP-LC.

Using a PG from *S. aureus* we found that the binding (amount of co-precipitated) of either PGRP-SA or PGRP-LC was higher when the assay was performed with either PBS or HemoBuffer when compared with 20 mM Tris-HCl 300 mM NaCl pH 7.8 [Figure 5 panel B]. Furthermore, in a co-precipitation assay with *S. aureus* PG and *B. subtilis* PG performed in HemoBuffer the amount of co-precipitated PGRP-SA seems to be comparable [Figure 5 panel C]. This contradicts the current model of *Drosophila* innate immunity.

It has been reported that WTA influence the level of PG crosslinking in *S. aureus* (Atilano et al. 2010). These alterations could explain why PG receptors were able to bind to the surface of *tagO* mutant *S. aureus* and *B. subtilis* bacteria. In order to test this possibility, we compared the ability of each PG receptor to bind PG produced by bacteria unable to produce WTA and their parental strains. We observed that a PG purified from either a wt strain or a deletion mutant strain lacking *tagO* showed no difference on the amount of co-precipitated PGRP-SA or PGRP-LC independently of being Lys type (*S. aureus*) or amiDAP type (*B. subtilis*) [Figure 5 panel C]. This supports the hypothesis that the binding differences observed by microscopy when comparing a deletion mutant strain lacking TagO with the parental strain are due to a more exposed PG and not due to alterations of the PG composition [Figure 1 and Figure 2].

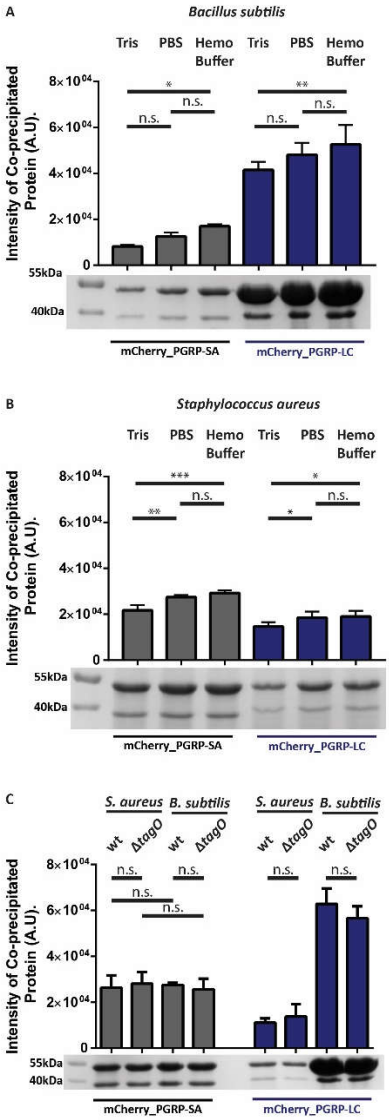


Figure 5 – Panel A) and B) PGRP:PG co-precipitation assays of PGRP-SA and PGRP-LC to A) PG purified from *S. aureus* bacteria (a Lys-type PG) and to B) PG harvested from *B. subtilis* bacteria (an amiDAP-type PG) in 20 mM Tris-HCl 300 mM NaCl pH 7.8, PBS at pH 6.0 and in HemoBuffer show that: 1) PGRP-SA is capable of binding to amiDAP type PG in HemoBuffer; 2) PGRP-LC is able of binding to Lys type PG. Panel C) PGRP:PG co-precipitation assays of PGRP-SA and PGRP-LC to PG purified from a deletion mutant lacking TagO or from the parental strain. The results obtained through this analysis show a similar binding intensity, supporting that the binding differences seen in a wt and single deletion mutant without TagO are not due to changes of the PG composition.

Since the observation that PG purified from *S. aureus* (Lys type) or *B. subtilis* co-precipitates a comparable amount of PGRP-SA is not in accordance with the current model of *Drosophila* innate immunity discriminating bacteria, which is based on the composition of the PG stem peptides, we decided to further assess the binding of PGRP-SA and PGRP-LC to Lys and amiDAP type PG. To this end, we performed co-precipitation assays that were carried out with a fixed amount of protein (PGRP) and variable levels of PG. In this way, we were able to titrate the amount of PG to span a range that covers a saturated and weak binding signal in the stained protein gels.

Using the data obtained through the determination of the amount of PG receptor found co-precipitated with the PG, and assuming that each PGRP only has one binding site, i.e. it can only perform one binding event at a time to PG (Swaminathan et al. 2006), we plotted the one site specific binding regression curve seen in Figure 6. Since this curve is based on the equation {S + E ⇌ [SE] → E + P} we can calculate the theoretical maximum binding (B_{max}), and the K_d value for each PGRP:PG pair [Table 1]. The K_d is defined as the concentration at which the binding of the PG receptor is half of the maximum theoretical binding (B_{max}) and, is a good measure of the affinity of a substrate to a protein (ligand to a receptor).

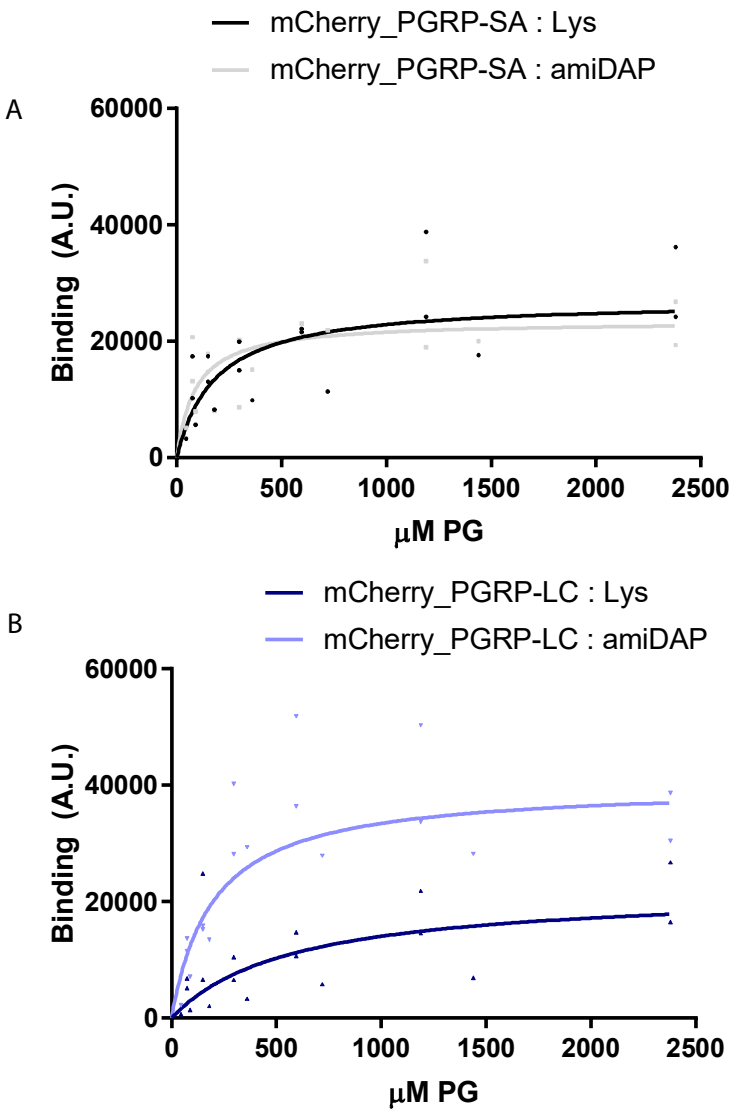


Figure 6– One site specific binding regression curves for titration of PG in PGRP:PG co-precipitation assays for A) mCherry_PGRP-SA and B) mCherry-PGRP-LC. While mCherry_PGRP-SA has a similar curve for Lys and amiDAP type PG, mCherry_PGRP-LC has a higher maximum binding for amiDAP type PG.

As seen in Figure 6 panel A, while mCherry_PGRP-SA has a similar one site specific binding regression curve to PG purified from either *S. aureus* or *B. subtilis*, mCherry_PGRP-LC shows one site specific binding regression curves with a different profile for each PG type [Figure 6 panel B].

We observed that mCherry_PGRP-SA has a similar B_{max} value for PG purified from either *S. aureus* (Lys type) or *B. subtilis* (amiDAP type), which supports the hypothesis of PGRP-SA not having a preference for Lys or (ami)DAP type PG [Table 1]. By contrast, mCherry_PGRP-LC showed a higher B_{max} value for amiDAP type PG than Lys type, supporting the hypothesis that PGRP-LC has a preference for DAP type [Table 1]. Nevertheless, mCherry_PGRP-LC is capable of binding Lys type PG.

The assays carried out with mCherry_PGRP-LC, clearly showed a lower K_d for an amiDAP type PG than for a Lys type PG [Table 1], reinforcing the preference of PGRP-LC for DAP type. Surprisingly mCherry_PGRP-SA also showed a lower K_d for an amiDAP type PG than for a Lys type PG [Table 1]. This observation in not in accordance with the hypothesis that PGRP-SA has a preference for Lys type PG over DAP type PG. Interestingly, mCherry_PGRP-SA binding to either PG types showed a lower K_d than mCherry_PGRP-LC, hinting the possibility that PGRP-SA is capable to effectively bind PG (Lys or amiDAP type) at low concentration better than PGRP-LC.

Table 1– mCherry_PGRP-SA and mCherry_PGRP-LC K_d and B_{max} values to a Lys type and DAP type PG, obtained by applying a one site specific binding regression curve to the titration of PG in PGRP:PG co-precipitation assays.

mCherry_PGRP-SA				**mCherry_PGRP-LC**			
Lys type		amiDAP type		Lys type		amiDAP type	
K_d (μM)	B_{max} (A.u.)	K_d (μM)	B_{max} (A.u.)	K_d (μM)	B_{max} (A.u.)	K_d (μM)	B_{max} (A.u.)
182.4	$2.7x10^4$	88.94	$2.3x10^4$	575.9	$2.2x10^4$	198.3	$4.0x10^4$

Discussion

The current model of *Drosophila* melanogaster innate immune system asserts that the differential activation of an immune response to different bacteria is mainly achieved by discrimination of the amino acid in the third position of the stem peptide. Therefore, the current model proposes that bacteria with Lys type PG, found in most Gram-positive bacteria, are recognized by PGRP-SA while bacteria with DAP type PG, produced in most Gram-negative bacteria and in some Gram-positive *Bacilli*, are recognized by PGRP-LC or PGRP-LE (Julien Royet and Dziarski 2007).

However, despite the current model being a good framework at predicting how different bacteria will trigger an innate immune response in *Drosophila* flies, it has some shortcomings. First, it may be an oversimplification of *Drosophila*'s immune system, since it reduces recognition of bacteria by the immune response of *Drosophila* to mainly three PGRPs (PGRP-SA, PGRP-LC or PGRP-LE) when *Drosophila*'s genome encodes for at least 13 different PGRPs (J. Royet, Sciences, and Royet 2004; Kurata 2014). Moreover, it reduces the variability found in the CW of different bacteria to the identity of the amino acid in the third position of the PG stem peptide. Secondly, while PGRP-SA is described as being responsible for triggering the immune response to infection by Lys type bacteria, it was reported that it can also bind non amidated DAP type PG (C. I. Chang et al. 2004; Mellroth et al. 2005; Leone et al. 2008). Interestingly, PGRP-SA was reported to be not able to bind amiDAP (Leone et al. 2008; C. I. Chang et al. 2004). Similarly, while PGRP-LC is described as being responsible for triggering an immune response to DAP type PG, it was reported that it can bind to Lys type or Ornithine type PG (Mellroth et al. 2005). Furthermore, it was reported in *Tenebrio molitor* that polymeric DAP type PG from *B. subtilis* (amiDAP) or *E. coli* (DAP) was bonded by PGRP-SA with a successful activation of Toll pathway (Yu et al. 2010).

Therefore, there is a discrepancy between the capabilities of PGRP-SA and PGRP-LC to bind different PGs and the predictions/statements supported by the current model.

The conflicting observations in the ability of a given PGRP to bind different PGs may derive from the fact that most studies of PGRPs binding preferences were performed with buffers that do not mimic haemolymph composition (the media in which most PGRP-bacteria interactions occur), e.g. 20 mM Hepes, pH 7.2, 150 mM NaCl or 20 mM Tris-HCl pH 7.8, 300 mM NaCl. Moreover, the different assays used PG purified from different bacteria, whose concentrations were dependent on the weight of the purified PG sample. This may have led to a significant variation of PG concentration used in each assay due to: 1) variability of the PG architecture/composition from bacterial strain to bacterial strain; 2) the presence of attached polymers such as WTA or capsular polysaccharides that may vary between different bacteria and that removal from purified PG is dependent on the purification procedure used.

Since it was previously reported that both PGRP-SA and PGRP-LC can bind to either Lys type or DAP type PG and considering that our group has previously showed that WTA can impair recognition of *S. aureus* by PGRP-SA, possibly by exerting steric hindrance (Atilano et al. 2011), we hypothesized that differential activation of an immune response in *Drosophila* is not restricted to the discrimination of the amino acid in the third position of the stem peptide but is also influenced by PG accessibility due to different CW architectures/compositions. To test this hypothesis, we used *S. aureus* and *B. subtilis* strains that produce a PG with Lys or amiDAP, respectively, while having a similar Gram-positive CW architecture, i.e., presence of WTA and absence of an outer membrane. To mimic as closely as possible the *in vivo* binding conditions we performed the binding assays in HemoBuffer, a buffer optimized to have an ionic composition and pH as similar as possible to the known composition of *Drosophila* haemolymph.

When fluorescent PG receptors were added to cells that have been harvested from a liquid culture, we observed a weak binding of mCherry_PGRP-SA or mCherry_PGRP-LC to either *S. aureus* or *B. subtilis* wt cells [Figure 1 and Figure 2, respectively]. Furthermore, quantification of the binding intensities showed that binding of PGRP-SA to either *S. aureus* or *B. subtilis* wt cells was not statistically distinguishable [Supplementary Table 1]. Similarly, quantification of the binding intensities showed that binding of PGRP-LC to either *S. aureus* or *B. subtilis* wt cells was not statistically distinguishable [Supplementary Table 1]. These observations suggest that in HemoBuffer *S. aureus* and *B. subtilis* cells (Lys type PG and amiDAP type PG, respectively) have a cell surface with a similarly concealed PG from either PGRP-SA or PGRP-LC.

However, when *S. aureus* or a *B. subtilis* mutant bacteria that lacked TagO and consequently lacked WTA were used, we observed an increased binding of both mCherry_PGRP-SA or mCherry_PGRP-LC when compared with the parental strain [Figure 1 and Figure 2]. Despite the binding intensity of mCherry_PGRP-LC to a *S. aureus* deletion mutant lacking TagO not being statistically distinguishable from the binding to the parental strain at this acquisition time (10 ms) the binding intensities of mCherry_PGRP-LC to a *B. subtilis* mutant lacking TagO and to the parental strain were statistically distinguishable [Supplementary Table 1]. Similarly, the binding intensities of mCherry_PGRP-SA to either a *S. aureus* or a *B. subtilis* deletion mutant lacking TagO were statistically distinguishable from the binding intensities to the respective parental strain [Supplementary Table 1]. These observations support the hypothesis that WTA impair recognition of both Lys type or (ami)DAP type PG, and that steric hindrance is not restricted to *S. aureus* strains. Also, contrary to Leone et al. 2008; and Chang et al. 2004, we observed binding of PGRP-SA to amiDAP PG. Furthermore, the observation of a higher binding of both mCherry_PGRP-SA or mCherry_PGRP-LC to *B. subtilis* single deletion mutants lacking TagO when compared to *S. aureus* single deletion mutants lacking TagO

contradicts the current model of discrimination of PG by *Drosophila*'s PGRPs, that proposes that PGRP-SA as having a preference to Lys type PG.

To discard the hypothesis that our observations were being influenced by the mCherry tag that is common to both recombinant proteins we quantified the binding of non-functional recombinant mCherry_PGRP-SA that is unable to bind PG. mCherry_PGRP-SA C48YC54Y has two mutations that disrupt the highly conserved disulfide bridge essential to a correct PGRP domain fold (Michel et al. 2001; C. I. Chang et al. 2004). This mutation is similar to the mutation observed in *Drosophila* semmelweis flies, that have a non-functional PGRP-SA due to a C48Y mutation (Michel et al. 2001). The additional C54Y mutation was inserted to prevent non-specific disulfide bridges. mCherry_PGRP-SA_C48YC54Y recombinant protein does not show a higher binding intensity to a *B. subtilis* deletion mutant lacking TagO than to the parental strain [Figure 4] and despite having a slightly higher binding intensity to a *S. aureus* deletion mutant lacking TagO than to the parental strain the variation observed is negligible when compared to the functional mCherry_PGRP-SA [Figure 3 and Supplementary Figure 2]. Therefore, it is unlikely that our observations are the result of the presence of a mCherry tag on our recombinant proteins.

To test the hypotheses that the discrepancy between our observation of PGRP-SA binding to amiDAP PG and the reported studies of Leone et al. 2008; and Chang et al. 2004 and that the discrepancies between the current model of PG discrimination by *Drosophila* PGRPs and the binding possibilities of PGRP-SA and PGRP-LC are due to studies performed in buffers that are not representative of haemolymph, we assessed the binding preferences of PGRP-SA and PGRP-LC to Lys type and amiDAP type PG by co-precipitation assays on three different buffers: 20 mM Tris-HCl 300 mM NaCl pH 7.8, as used in Chang et al. 2004; and Leone et al. 2008, Phosphate Buffered Saline (PBS) at pH 6.0 and HemoBuffer. To circumvent a possible bias in the PG concentrations due to possible architecture/composition

differences in the PG of *S. aureus* and *B. subtilis* we quantified the PG monosaccharides concentration and adjusted the amount of loaded PG by molar concentration of muramic acid and not by weight. This allow us to theoretically have the same amount of epitopes on both PGs and also allows us to assess the purity of the PG samples, since a poor PG preparation with leftover WTA would result in the observation of other monosaccharides than muramic acid and glucosamine.

While we observed a weak binding of PGRP-SA to amiDAP PG by co-precipitation assay in 20 mM Tris-HCl 300 mM NaCl pH 7.8, using HemoBuffer we observed a strong binding of recombinant PGRP-SA to amiDAP [Figure 5 A], similarly to what was observed to a *B. subtilis* single deletion mutant lacking tagO [Figure 2]. This observation favours the hypothesis that part of the discrepancy observed in the literature can be due to assays performed with a buffer not representative of the Haemolymph and/or due to poor adjustment of the PG samples. Similarly to PGRP-SA, the amount of co-precipitated mcherry_PGRP-LC was higher in an assay with HemoBuffer than in an assay with 20 mM Tris-HCl 300 mM NaCl pH 7.8.

PGRP-SA and PGRP-LC showed an increase in the amount of co-precipitation with Lys type PG in PBS or HemoBuffer when compared with 20 mM Tris-HCl 300 mM NaCl pH 7.8 [Figure 5 panel B], further highlighting the importance of choosing a buffer that mimics the Hemolymph to perform binding assays. A co-precipitation of amiDAP type PG in HemoBuffer with mCherry_PGRP-SA or mCherry_PGRP-LC resulted in a higher co-precipitation of mCherry_PGRP-LC [Figure 5 panel A], which is in accordance with the current model. Since we observed that a co-precipitation of either mCherry_PGRP-SA or mCherry_PGRP-LC with PG from a wt strain or a deletion mutant lacking TagO did not result in a co-precipitation of a distinguishable different amount of protein [Figure 5 panel C] we consider that it is unlikely that an increased binding to cells of a deletion mutant

lacking TagO by microscopy [Figure 1 and Figure 2] is due to a difference in composition/architecture of the PG and favour the hypothesis that the observed binding is due to a more exposed PG in both bacterial strains.

Since our observation of PGRP-SA showing no clear binding preference to either pure Lys type or amiDAP PG is in conflict with the current model of discrimination of PG by *Drosophila*'s PGRPs and to rule out the hypothesis of this observation being an artefact of the tested conditions, more particularly, the ratio of substrate (PG) available to ligand (PGRP) used in this assay we performed co-precipitation assays were we titrated the amount of PG added to the reaction while maintaining the amount of PGRP used stable. PG concentration was increased in two-fold steps in a range that covered both a weak binding signal and a saturated signal. Based on the published structures of PGRP-SA and PGRP-LC we assumed that both PGRPs only have on binding site (Reiser, Teyton, and Wilson 2004; C. I. Chang et al. 2004; C.-I. Chang et al. 2006; Lim et al. 2006). Furthermore, Swaminathan observed a 1:1 ratio of human PGRP-I to muropeptide in microcalorimetry studies (Swaminathan et al. 2006).

The one site specific binding regression curve of the titration data for both proteins is shown in Figure 6. From the one site specific binding regression curve we can calculate two parameters that describe the tested ligand-substrate pair: the theoretical maximum binding (B_{max}) and the K_d value. The K_d is defined as the concentration at which the binding is half of the maximum theoretical binding (B_{max}) and is a good measure of the affinity of a substrate to a protein.

It was reported that PGRP-LC dimerizes upon binding to PG and that PGRP-LC dimerization facilitates association with the IMD protein (Kaneko et al. 2004; Mellroth et al. 2005; Choe, Lee, and Anderson 2005). It was also reported that PGRP-SA from *Tenebrio molitor* activates downstream signalling upon clustering to polymeric Lys type PG (Park et al. 2007). Furthermore, it was reported that monomeric muropeptides of Lys type PG inhibit an immune response dependent

of PGRP-SA (Filipe, Tomasz, and Ligoxygakis 2005) and that muramidase treated PG is unable to induce either Toll or Imd activation (Filipe, Tomasz, and Ligoxygakis 2005; Leulier et al. 2003). Also, it was reported that PGRP-LB, PGRP-SB1, PGRP-SB2, PGRP-SC1 and PGRP-SC2 amidase activity cleaves DAP type PG into smaller fragment that are not immunostimulatory (Mellroth and Steiner 2006; Kim, Byun, and Oh 2003; Mellroth, Karlsson, and Steiner 2003; Bischoff et al. 2006). All these data suggest that activation of *Drosophila* immune responses require polymeric PG to promote PGRP dimerization/clustering. From this model one can argue that for the same amount (molar concentration) of PG, a PG with a higher amount of total binding (B_{max}) produces more opportunities for PGRP dimerization/clustering and, therefore, is more immunostimulatory. In other words, one can argue that there is a correlation between PGRP binding intensity to PG (B_{max}) and the efficiency of immune response induction.

The observation of a similar B_{max} value of mCherry_PGRP-SA to a PG purified from either *S. aureus* (Lys type) or *B. subtilis* (amiDAP type) supports that PGRP-SA does not have a preference between Lys type or amiDAP type PG [Table 1]. By contrast, mCherry_PGRP-LC shows a higher B_{max} value to an amiDAP type PG than to a Lys type PG. This supports PGRP-LC having a binding preference for a DAP type PG [Table 1]. Controversially, while mCherry_PGRP-LC showed a lower K_d for a amiDAP type PG than for a Lys type PG [Table 1], reinforcing the preference of PGRP-LC for DAP type, mCherry_PGRP-SA also showed a lower K_d for a amiDAP type PG than for a Lys type PG [Table 1], contradicting the hypothesis that PGRP-SA does not have a preference for Lys or DAP type PG. Also, mCherry_PGRP-SA showed a lower K_d than mCherry_PGRP-LC to either Lys type or amiDAP type PG. Since K_d is defined as the concentration at which the binding is half of the maximum theoretical binding, one can argue that when we compare this value between two PGRP-PG pairs we have a “read-out” of how many of the available epitopes are occupied at a given concentration below saturation. By other words, a lower K_d

indicates that more epitopes are occupied at a given concentration, therefore one can argue that K_d indicates how efficiently a PGRP can bind to a PG type at a given concentration. This means that PGRP-SA having a lower K_d values for either Lys type or amiDAP type PG than PGRP-LC suggests that PGRP-SA is capable of efficiently binding PG (Lys or amiDAP type) at lower concentration better than PGRP-LC, and therefore may be better at binding PG that is more concealed than PGRP-LC.

Therefore, it is feasible that at a PG concentration below saturation PGRP-SA is capable of occupying a similar or higher number of epitopes (higher binding) and therefore reach a threshold need for dimerization/clustering and, therefore, initiate an immune response more efficiently than PGRP-LC, even if PGRP-LC has higher number of maximum binding sites (B_{max}) at saturation (see Supplementary Figure 3).

In sum, the co-precipitation titration data shows that mCherry_PGRP-SA has a similar curve for both Lys type and amiDAP type PG [Figure 6 A], contradicting the current model that states that PGRP-SA has a preference for Lys type PG. Previous observations in *Tenebrio molitor* showed that PGRP-SA could bind DAP type and amiDAP type PG (*E.* coli and *B. subtilis*, respectively*)* with a successful activation of the Toll pathway (Yu et al. 2010). This supports PGRP-SA having a promiscuous binding to PG, similarly to our observation of PGRP-SA not having a preference for DAP type or Lys type PG.

By contrast, the one site specific binding regression curve of mCherry_PGRP-LC [Figure 6 B] shows that mCherry_PGRP-LC binds amiDAP type PG better than Lys type PG, supporting the current model that states that PGRP-LC has a preference for DAP type PG. Nevertheless, mCherry_PGRP-LC is still capable of binding Lys type PG [Figure 6 B]. Stenbak and colleagues produced an *E. coli* mutant strain that can incorporate lysine into the PG and showed that an anhydromuramic acid muropeptide with lysine purified from this strain can induce

diptericin expression, albeit with a two fold less expression than a anhydromuramic acid muropeptide with DAP (Stenbak et al. 2004). Therefore, it is feasible that a PG with a lysine residue can induce the Imd pathway, presumibly in a PGRP-LC dependent way. Interestingly, the same authors also showed that a (non-anhydro) muramic acid muropeptide induces diptericin expression 2.5 fold less than a anhydromuramic acid muropeptide (Stenbak et al. 2004). Nevertheless, these observations highlight that the amino acid in the third position of PG the stem peptide is not the sole responsible for recognition.

Structural data comparison between PGRP-SA and PGRP-LC suggest that the presence of a positively charged arginine (Arg 413 in PGRP-LC) favours binding of DAP type PG to the PGRP binding groove (C.-I. Chang et al. 2006). Chang et. al. further suggested that presence of a lysine from a monomeric PG ligand would destabilize the interaction. In the PGRP-SA binding groove we can find a neutral threonine instead of an arginine (C.-I. Chang et al. 2006; C. I. Chang et al. 2004; Reiser, Teyton, and Wilson 2004). Therefore, the observation that PGRP-SA can efficiently bind both Lys type and DAP type PG is not in conflict with PGRP-SA structural data. Also, the presence of a positively charged arginine in PGRP-LC is suggested to favour binding of DAP type PG and we observed a preference of PGRP-LC for (ami)DAP type PG. However, we observed that PGRP-LC can also bind, although less efficiently, Lys type PG. Chang et. al. also noted that the ε-amino group of Lys in polymeric PG, or PG with a peptide bridge, the case of *S. aureus*, will not retain a basic charge (C.-I. Chang et al. 2006). Therefore, it is feasible that PGRP-LC can bind polymeric Lys type PG, while maintaining a preference for DAP type PG. Furthermore, since, as previously stated, both PGRP-SA and PGRP-LC dimerize/cluster in order to efficiently trigger an immune response, and monomeric or muramidase treated PG are not immunostimulatory, it may be possible that PGRP-LC can trigger an immune response upon binding to polymeric Lys type PG.

Our lab has reported, together with the data presented in this chapter, that while *PGRP-SA*^{seml} flies [flies lacking a functional PGRP-SA (Michel et al. 2001)] are more susceptible to infection with *S. aureus* than *PGRP-LC*$^{\Delta E12}$ flies [flies lacking PGRP-LC (Gottar et al. 2002)], *PGRP-SA*^{seml}*; PGRP-LC*$^{\Delta E12}$ double mutant flies are more susceptible than *PGRP-SA*^{seml} single mutant flies to an infection with *S. aureus*. This suggests that PGRP-LC also contributes to induction of an immune response to an infection with *S. aureus* cells (see Vaz et al. 2019-Figure 5). Furthermore, infection with *S. aureus* single deletion mutant lacking TagO showed a higher contribution of PGRP-LC to the immune response than with a *S. aureus* wt strain (Vaz et al. 2019). The same study also showed that both PGRP-SA and PGRP-LC contributed to survival upon infection with *B. subtilis* cells (see Vaz et al. 2019-Figure 5). This highlights that WTA can impair recognition of PG by PGRPs and that PG accessibility also plays a role in immune recognition.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2 and 3, respectively. *Escherichia coli* and *B. subtilis* strains were grown at 30ºC in Luria-Bertani (LB, Alfa Aesar) or Luria-Bertani agar (LA, Alfa Aesar) medium; *S. aureus* strains were grown at 30ºC in Tryptic Soy broth (TSB, Difco) or in Tryptic Soy agar (TSA, Difco) medium. When needed, the medium was supplemented with ampicillin (Amp, 100 µg/mL for *E. coli* strains, Apollo Scientific) or with Isopropyl *β-D*-1-thiogalactopyranoside (IPTG 1mM, Apollo Scientific).

Table 2 – Strains used this study

Strain	Description	Source or Reference
S. aureus NCTC 8325-4	MSSA strain	
S. aureus NCTC 8325-4 Δ*tagO*	Obtained using NCTC 8325-4 as parental strain for *tagO* deletion	Atilano et al. 2010
B. subtilis EB6	*B. subtilis* wt strain	Elia et al. 2006
B. subtilis EB1451	*B. subtilis EB6 tagO::Erm*^{r};	Elia et al. 2006

Table 3 – Plasmids used in this study

Plasmid	Description	Source or Reference
pET21α-mCherry_PGRP-SA	pET21a vector codifying T7-mCherry_PGRP-SA-Histag used for protein purification of mCherry_PGRP-SA in *E. coli* BL21(DE3); Amp^R	Magda L. Atilano et al. 2011
pET21α-mCherry_PGRP-LC	pET21a vector codifying T7-mCherry_PGRP-LC-Histag used for protein purification of mCherry_PGRP-LC in *E. coli* BL21(DE3); Amp^R	This study
pET21α-PGRP-SA	pET21a vector codifying T7-PGRP-SA-Histag used for protein purification of PGRP-SA in *E. coli* BL21(DE3); Amp^R	Magda L. Atilano et al. 2011
pET21α-PGRP-LC	pET21a vector codifying T7-PGRP-LC-Histag used for protein purification of mCherry_PGRP-SA in *E. coli* BL21(DE3); Amp^R	This study
pET21α-mCherry_PGRP-SA C48YC54Y	pET21a vector codifying T7-mCherry_PGRP-SA-Histag used for protein purification of inactive mCherry_PGRP-SA in *E. coli* BL21(DE3); Amp^R	Vaz et al. 2019

Table 4 – Primers used in this study.

Primer	Sequence 5'-3'
PGRP-LC Fw	GGGAAGGGAATTCAACCAAACGGACTTGGATG
PGRP-LC Rv	TGCGGCCGCAAGCTTTTAGTGATGGTGATGGTGATGGATTTCGTGTGACCAGTGCG

Construction of plasmids for recombinant PGRP-LC proteins

The PGRP-LC$_x$ PGRP domain (966 bp to 1500 bp of the codifying sequence) was amplified from plasmid IP15793 (*Drosophila* Genomics Resource Centre) with primers PGRP-LC Fw and PGRP-LC Rv [Table 4]. The amplified fragment was cloned into the pET21α-mCherry_PGRP-SA and pET21α-PGRP-SA vectors that had been digested with EcoRI and EagI restriction sites to create the pET21α-mCherry_PGRP-LC and pET21α-PGRP-LC vectors respectively.

Construction of plasmid for recombinant mCherry PGRP-SA C48YC54Y protein

The 48th and 54th cysteines of pET21α-mCherry_PGRP-SA were mutated to a tyrosine by site-directed mutagenesis PCR with appropriate primers followed by DpnI digestion at 37ºC.

Cell wall and peptidoglycan purification

S. aureus and *B. subtilis* CW and PG preparation were obtained as described in Jonge et al., 1992. Briefly, an overnight culture of the strain of interest was back diluted to an OD_{600nm} of ≅ 0.001 and incubated at appropriate conditions until an OD_{600nm} between 0.5 and 0.9 was reached. A high dilution of the overnight culture together with a growth not surpassing the exponential phase is used to maximize the amount of cells actively growing. Upon reaching the desired OD_{600nm}

the culture was quickly chilled by incubation in an ice/ethanol bath before collecting the cells by centrifugation at 13.000 x *g* for 5 min at 4ºC. The pellet was resuspended in ice cold Milli-Q water and added drop by drop to boiling sodium dodecyl sulfate (SDS, Sigma-Aldrich) to a final concentration of 4% (w/v) SDS. The resulting solution was boiled for 30 minutes to promote cellular membrane disruption and inactivation of metabolic enzymes that may modify/degrade the CW/PG (autolysins). SDS was washed-off by repetitive centrifugation of the sample in fresh warm Milli-Q water until no SDS could be detected by the Hayashi method (Hayashi 1975). The SDS-free pellets were broken by performing 10 cycles of 45 seconds at 6 RPM in a FastPrep-24 5G (MP biomedicals) homogenizer with acid washed glass beads (Sigma-Aldrich, ≤106 μm) as abrasive. The samples were clarified of DNA, RNA and contaminant proteins by treatment with DNase (10 μg/mL, Sigma-Aldrich) and RNase (50 μg/mL, Sigma-Aldrich), in 50 mM Tris-HCl pH 7.5 20 mM $MgSO_4$, and with Trypsin (100 μL/mL, Sigma-Aldrich) in 50 mM Tris-HCl pH 7.5 20 mM $MgSO_4$ 10 mM $CaCl_2$. These hydrolytic enzymes were inactivated by boiling in 1% (w/v) SDS and the clarified sample was washed twice with Milli-Q water, once with 8M LiCl (VWR), once with 100 mM Ethylenediamine tetraacetic acid (EDTA, VWR) pH 7.0 and once with acetone (Sigma-Aldrich) to remove SDS, ionically bonded material and endotoxins. The resulting sample, purified CW, was lyophilized and quantified by weight or monosaccharide content (see below).

Pure PG samples were obtained by removing the WTA by incubating CW in Hydrofluoric Acid (HF, Sigma-Aldrich 48% (w/v)) at 4ºC for 48 hours. HF was washed off by repetitive centrifugation with cold 100 mM Tris-HCl pH 7.0. The resulting sample was lyophilized and quantified by weight or monosaccharide content (see below).

Peptidoglycan quantification by monosaccharide content

The monosaccharide composition of the pure PG samples was measured to assess sample purity and quantify the molecular concentration of PG by Mur*NAc* content, as described in Covas, Vaz, Henriques, Pinho, & Filipe, 2016. 0.2 mg of PG were hydrolysed to the constitutive monosaccharides by incubation in 3M HCl (Carl Roth) for 2 hours at 95ºC without agitation. Reaction was stopped by removing HCl by lyophilizing the sample in a SpeedVac (LabConco) at room temperature. Any residual HCl was diluted by resuspending the sample in 1 mL of Milli-Q water and removed by a second lyophilization of the sample. The released monosaccharides were separated by high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) using a Thermo-Scientific Dionex CarboPac PA10 4 mm x 250 mm column with a Thermo-Scientific Dionex AminoTrap Column, 4 x 50 mm as a pre-column in a Thermo-Scientific Dionex ICS5000. The monosaccharides were resolved in a 20-minute isocratic step at 18 mM NaOH followed by a 5-minute linear gradient from 0 mM $NaCH_3COO$ to 200 mM $NaCH_3COO$ in 18 mM NaOH. A 5-minute isocratic step at 200 mM $NaCH_3COO$, 18 mM NaOH was followed by a 5-minute linear gradient to 800 mM $NaCH_3COO$ and a 5-minute isocratic step at the same conditions to elute any remaining monosaccharides. All sample resolution steps were performed at 1 mL/min at 30ºC using a PAD waveform adapted from Engel & Händel, 2011 and gold disposable electrodes (Thermo-Scientific). All buffers were made from ionic chromatography grade NaOH (VWR) and $NaCH_3COO$ (Fluka) in Milli-Q water with $\geq$ 18 MΩ resistivity and kept under a nitrogen (Air Liquide) blanket to minimize carbonate formation in the eluents by dissolved CO2. A Thermo-Scientific Dionex BorateTrap Inline Trap Column was used between the pump and the injection loop to capture any residual borate from the eluents. The sample content of Glc*Nac*, Mur*Nac*, Glucosamine, Muramic acid and Ribitol was calculated using a calibration curve of standards

(Sigma-Aldrich) of known concentration. All quantifications were performed with 3 independent hydrolysis. Note that a complete hydrolysis of a pure PG sample will only show Glucosamine and Muramic acid content. A detectable amount of Ribitol may indicate a WTA contamination while a detectable amount of Glc*NAc* and a Mur*NAc* may indicate a partial hydrolysis of the PG sample, since Glc*NAc* and a Mur*NAc* are de-*N*-acetylated under the used hydrolysis conditions.

Purification of muropeptides and analysis by RP-HPLC

Non-reduced muropeptides were obtained by digesting pure PG samples with mutanolysin (0.2 mg/mL, from *Streptomyces globisporus* ATCC 21553, Sigma-Aldrich) in 12.5 mM $NaPO_4$ pH 5.5 buffer overnight at 37ºC and agitation. Muropeptides were reduced by incubation with fresh $NaBH_4$ (3.125 mg/mL) in 0.25 M Borate pH 9.0 buffer for 2 hours at room temperature. The reduction reaction was stopped by lowering the pH to ≤ 2.0 with H_3PO_4.

Reduced muropeptides were chromatographically separated by Reversed Phase High-Performance Liquid Chromatography (RP-HPLC) using a Thermo-Scientific ODS Hypersil C18 250 mm x 4.6 mm, 5 µm particle size HPLC column coupled with a Thermo-Scientific ODS Hypersil C18 5 µm 4mm x 4.6 mm guard column in a Shimadzu Prominence HPLC. Reduced muropeptides were resolved in a linear gradient from 5 % (v/v) methanol to 30% (v/v) methanol in 100 mM $NaPO_4$ pH 2.0 buffer over 155 minutes followed by a washing step at 30% (v/v) methanol over 5 minutes at 1 mL/min and 52 ºC. Elution of muropeptides was recorded by monitoring Abs_{205nm}. Muropeptide species were quantified with the peak area as percentage of total chromatogram area with Shimadzu LCSolution v1.21 SP1 software. Data shown is representative of 3 replicates.

Recombinant PGRP protein purification

Recombinant PGRPs were purified as described in Magda L. Atilano et al. 2011. Briefly, pET21α plasmid carrying the codifying sequence for the protein of interest was transformed into *E. coli* BL21(DE3) using Ampicillin as selection marker. An overnight culture was back diluted to an $OD_{600nm} \cong 0.05$ in fresh LB with Amp (100 µg/mL) and incubated at 25ºC with agitation until an $OD_{600nm} \cong 0.5$ was achieved. At this point IPTG was added to a final 1mM concentration and the culture was incubated overnight at 25ºC with agitation.

The cells were collected by centrifugation at 13.000 x $\mathcal{g}$ for 5 min at 4ºC, washed once with equilibration buffer [50 mM Na_2PO_4 pH 7.4, 300 mM NaCl] by centrifugation at 13.000 x $\mathcal{g}$ for 5 min at 4ºC and lysed by performing two runs on a French Press at 1000 psi. The lysate was centrifuged at 16.000 x $\mathcal{g}$ for 20 min at 4ºC, the pellet (with inclusion bodies) was resuspended in Resuspension Solution [50 mM Na_2PO_4 pH 7.4, 500 mM NaCl, 8M Urea] and incubated at 4ºC with agitation until sample was homogeneous. At this point the sample was added to an equal volume of Dilution Solution [50 mM Na_2PO_4 pH 7.4, 500 mM NaCl] in order to dilute Urea to a 4M final concentration.

After clarifying the sample of any insoluble debris by repetitive centrifugation at 16.000 x $\mathcal{g}$ for 20 min at 4ºC the sample was incubated with TALON Metal Affinity Resin (Clontech) at 4ºC with agitation for 30 minutes. The resin was recovered by spin-down centrifugation and washed with Wash Solution [50 mM Na_2PO_4 pH 7.4, 300 mM NaCl, 4M Urea], Wash Solution with 5 mM and Wash Solution 10 mM of Imidazole (VWR), sequentially , before eluting the protein with Elution Solution [50 mM Na_2PO_4 pH 7.4, 300 mM NaCl, 150 mM Imidazole] in a gravity flow column. Elution Solution was exchanged with Phosphate-buffered saline [PBS-pH 6.0; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4

adjusted to pH 6.0 with HCl] by overnight dialysis with a 10 kDa cut-off Dialysis tubing (SnakeSkin, Pierce). Purified proteins were aliquoted and stored at -20ºC.

HemoBuffer optimization

HemoBuffer was optimized to mimic the ionic composition and pH of *Drosophila* hemolymph [Table 5]. However, we were unable to create a buffer with identical composition to the *Drosophila* hemolymph, due to salt precipitation, possibly due to formation of complex salts. We found that precipitation occurred after addition of either $CaCl_2$ and/or $MgCl_2$ and titrated the maximum concentration of both Calcium and Magnesium that would result in a soluble solution. However, since HemoBuffer has a tendency to form insoluble salts (2-3 days of shelf-life) we advise to always use freshly prepared buffer using the salts described in Table 6.

Table 5 - Reported ionic composition of *Drosophila* hemolymph and HemoBuffer ionic composition.

Ion/ Concentration (mM)	Croghan and Lockwood 1960	van der Meer and Jaffe 1983	HemoBuffer pH 6.0
K+	**25**	**24**	**25**
Na+	**106**	**87**	**58.6**
Mg2+	**14.4**	**26**	**14.4**
Ca2+	**7.2**	**10.6**	**2**
Cl-	**58**	**-**	**57.8**
PO42-	**39**	**-**	**39**
SO4 2-	**7.3**	**-**	**7.3**

Table 6 - Suggested salts to prepare HemoBuffer (final concentration)

Salt	mM
$Na_2SO_4.10H_2O$	7.3
$NaH_2PO_4.H_2O$	34
$Na_2HPO_4.7H_2O$	5
$CaCl_2.2H_2O$	2
$MgCl_2.6H_2O^*$	14.4
KCl	25

Quantifying PGRP binding to cells by fluorescent microscopy

Binding of mCherry_PGRP-SA or mCherry_PGRP-LC (purified in-house, see above) to *S. aureus* and *B. subtilis* strains cells *in vivo* was assessed by Wide-field fluorescence microscopy. Briefly, cells were collected at mid exponential growth ($OD_{600nm} \cong 0.5$) by centrifugation at 13.000 x g for 5 min at room temperature, washed once with HemoBuffer and incubated with either mCherry_PGRP-SA or mCherry_PGRP-LC at 0.3 mg/mL in HemoBuffer for 5 minutes at room temperature with agitation. After incubation the cells were washed twice with fresh HemoBuffer by centrifugation and applied in 1.2% (w/v) agarose in HemoBuffer patch prior to observation in a Zeiss Axio Observer Z1 microscope with a Plan-Apochromat 100×/1.4 oil Ph3 objective. Images were acquired using a Retiga R1 CCD camera (QImaging) using Metamorph 7.5 software (Molecular Devices) with a 500-millisecond exposure time and a Texas-red filter.

Quantification of the recombinant PGRPs binding to the cell surface was carried with Fiji image software (Schindelin et al. 2012). Briefly, a threshold was applied to the phase image to obtain a mask that harbours the cells. Since recombinant PGRPs bind the cells extracellularly this mask was slightly dilated in order to not truncate the binding signal. The mask of close neighbouring cells was separated by applying a watershed algorithm and resulting masks were converted

to regions of interest (ROIs). This list of ROIs (each harbouring an individual cell) was used to measure the fluorescence signal intensity after subtracting the background fluorescence to the fluorescence image. The average signal intensity of each ROI was used as a measure of the binding intensity. Statistical analysis of the data was performed using the Uncorrected Fisher LSD test. Data is plotted as a Tukey box and whiskers graph with boxes showing the 25th, median, and 75th percentiles; Whiskers are draw at 1.5 times the interquartile range from either the 25th or the 75th percentile; Data points outside the 1.5 times interquartile range are considered an outlier and shown as a point in the graph.

Quantifying PGRP binding to purified PG by pull down assay

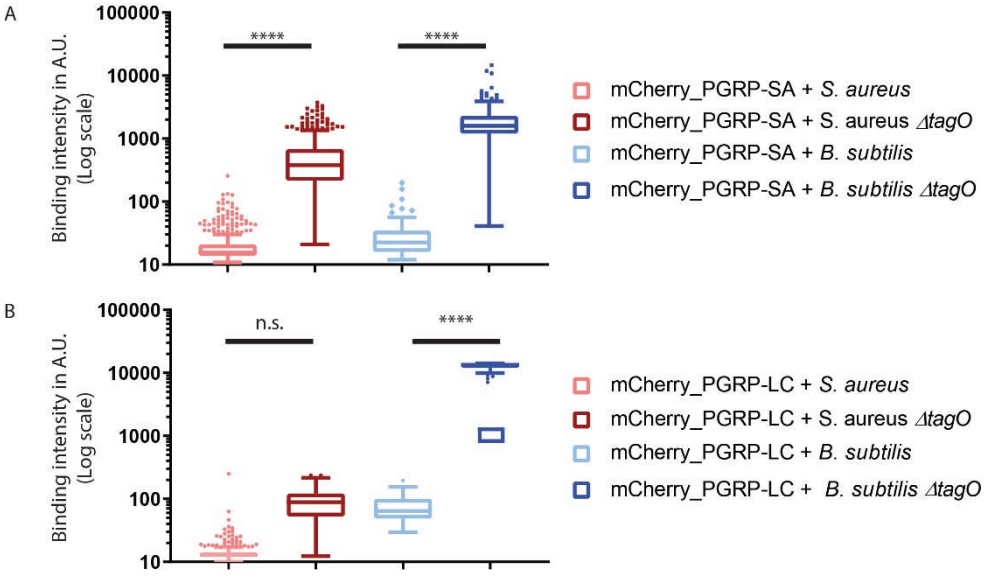
Binding of recombinant PGRPs to pure PG was performed by incubating a PG sample containing 0.038 μmol of Mur*NAc* (see Peptidoglycan quantification by monosaccharide content above) with recombinant PGRPs at 0.3 mg/mL in either 20 mM Tris-HCl 300 mM NaCl pH 7.8, PBS at pH 6.0 or HemoBuffer for 30 minutes at 25ºC with agitation. The pellet containing the PG:bonded recombinant PGRP co-precipitate was washed twice with fresh same buffer by centrifugation before resuspension in SDS-PAGE loading buffer and incubation in boiling water for 5 minutes.

After electrophoretic separation of the resulting samples, the gels were stained with BlueSafe (NZYTech) and the intensity of the recombinant PGRP proteins bands used as a measure of the binding intensity. Gels were digitalized in a Bio-Rad Gel Doc EZ system with Image Lab software. Intensity of the protein bands was measured using the Image Lab software (v6.0 build 25, Bio-Rad) and statistical analysis was performed from 3 independent co-precipitation reactions using an Uncorrected Fisher's LSD.

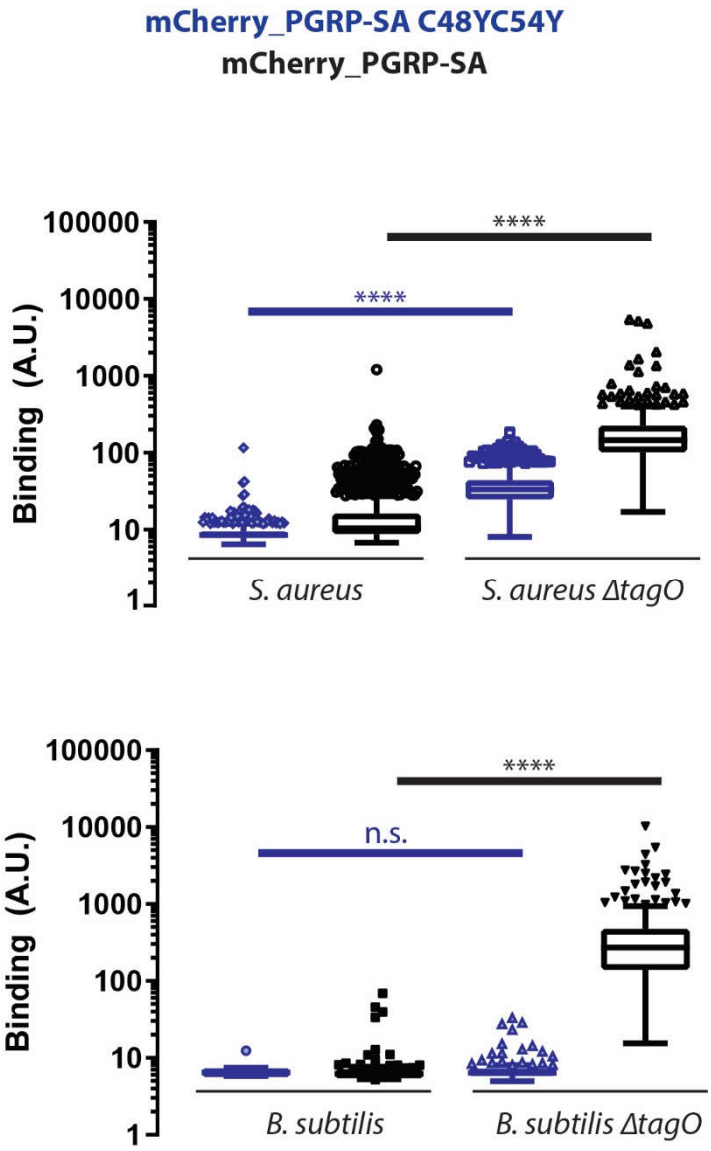
Supplementary Data

Supplementary Table 1 – Summary of statistical analysis of comparisons of the binding of mCherry_PGRP-SA or mCherry_PGRP-LC to *S. aureus* and *B. subtilis* wt strains or single deletion mutants lacking TagO.

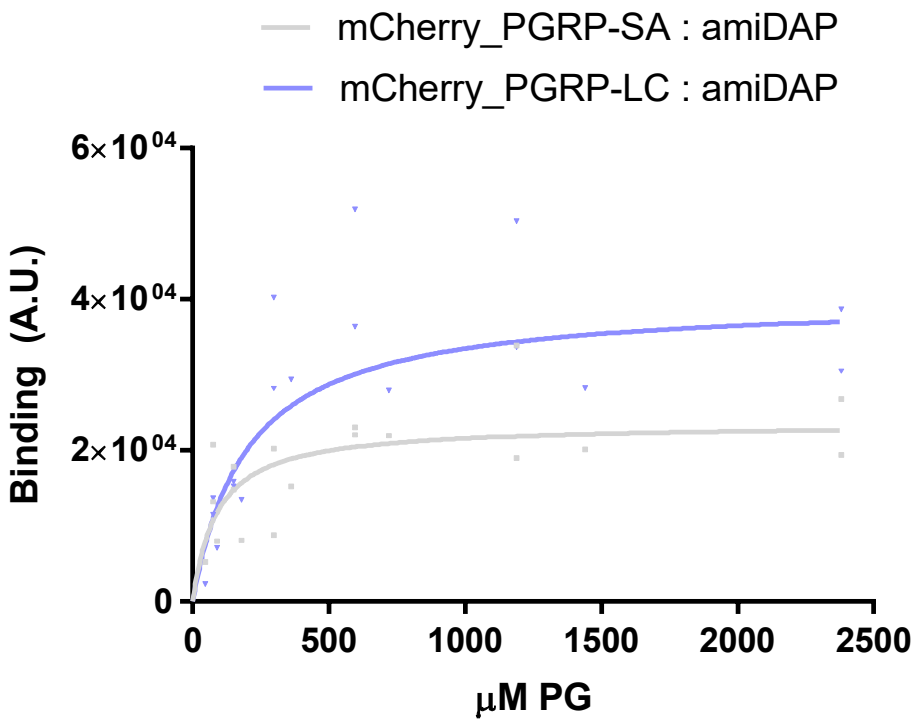
Uncorrected Fisher's LSD	Mean Diff.	95% CI of diff.	Significant?	Summary	Individual P Value
PGRP-LC + *B. subtilis* vs. PGRP-LC + *B. subtilis Δtago*	-12690	-12873 to -12507	Yes	****	< 0.0001
PGRP-LC + *B. subtilis* vs. PGRP-LC + *S. aureus*	59.91	-92.49 to 212.3	No	ns	0.4408
PGRP-LC + *B. subtilis* vs. PGRP-LC + *S. aureus Δtago*	-16.07	-171.0 to 138.9	No	ns	0.8389
PGRP-LC + *B. subtilis* vs. PGRP-SA + *B. subtilis*	40.07	-181.2 to 261.4	No	ns	0.7225
PGRP-LC + *B. subtilis* vs. PGRP-SA + *B. subtilis Δtago*	-2058	-2235 to -1881	Yes	****	< 0.0001
PGRP-LC + *B. subtilis* vs. PGRP-SA + *S. aureus*	51.89	-99.80 to 203.6	No	ns	0.5024
PGRP-LC + *B. subtilis* vs. PGRP-SA + *S. aureus Δtago*	-480.4	-630.7 to -330.1	Yes	****	< 0.0001
PGRP-LC + *B. subtilis Δtago* vs. PGRP-LC + *S. aureus*	12750	12614 to 12887	Yes	****	< 0.0001
PGRP-LC + *B. subtilis Δtago* vs. PGRP-LC + *S. aureus Δtago*	12674	12535 to 12814	Yes	****	< 0.0001
PGRP-LC + *B. subtilis Δtago* vs. PGRP-SA + *B. subtilis*	12730	12520 to 12941	Yes	****	< 0.0001
PGRP-LC + *B. subtilis Δtago* vs. PGRP-SA + *B. subtilis Δtago*	10633	10469 to 10796	Yes	****	< 0.0001
PGRP-LC + *B. subtilis Δtago* vs. PGRP-SA + *S. aureus*	12742	12607 to 12878	Yes	****	< 0.0001
PGRP-LC + *B. subtilis Δtago* vs. PGRP-SA + *S. aureus Δtago*	12210	12076 to 12344	Yes	****	< 0.0001
PGRP-LC + *S. aureus* vs. PGRP-LC + *S. aureus Δtago*	-75.98	-171.6 to 19.62	No	ns	0.1192
PGRP-LC + *S. aureus* vs. PGRP-SA + *B. subtilis*	-19.84	-204.5 to 164.9	No	ns	0.8332
PGRP-LC + *S. aureus* vs. PGRP-SA + *B. subtilis Δtago*	-2118	-2246 to -1989	Yes	****	< 0.0001
PGRP-LC + *S. aureus* vs. PGRP-SA + *S. aureus*	-8.02	-98.25 to 82.22	No	ns	0.8617
PGRP-LC + *S. aureus* vs. PGRP-SA + *S. aureus Δtago*	-540.3	-628.2 to -452.5	Yes	****	< 0.0001
PGRP-LC + *S. aureus Δtago* vs. PGRP-SA + *B. subtilis*	56.14	-130.6 to 242.9	No	ns	0.5556
PGRP-LC + *S. aureus Δtago* vs. PGRP-SA + *B. subtilis Δtago*	-2042	-2173 to -1910	Yes	****	< 0.0001
PGRP-LC + *S. aureus Δtago* vs. PGRP-SA + *S. aureus*	67.96	-26.49 to 162.4	No	ns	0.1584
PGRP-LC + *S. aureus Δtago* vs. PGRP-SA + *S. aureus Δtago*	-464.4	-556.6 to -372.2	Yes	****	< 0.0001
PGRP-SA + *B. subtilis* vs. PGRP-SA + *B. subtilis Δtago*	-2098	-2303 to -1892	Yes	****	< 0.0001
PGRP-SA + *B. subtilis* vs. PGRP-SA + *S. aureus*	11.82	-172.3 to 195.9	No	ns	0.8998
PGRP-SA + *B. subtilis* vs. PGRP-SA + *S. aureus Δtago*	-520.5	-703.5 to -337.5	Yes	****	< 0.0001
PGRP-SA + *B. subtilis Δtago* vs. PGRP-SA + *S. aureus*	2110	1982 to 2237	Yes	****	< 0.0001
PGRP-SA + *B. subtilis Δtago* vs. PGRP-SA + *S. aureus Δtago*	1577	1451 to 1703	Yes	****	< 0.0001
PGRP-SA + *S. aureus* vs. PGRP-SA + *S. aureus Δtago*	-532.3	-619.0 to -445.7	Yes	****	< 0.0001



Supplementary Figure 1– Quantification of A) mCherry_PGRP-SA or B) mCherry_PGRP-LC binding to *S. aureus* and *B. subtilis* wt strains or deletion mutants lacking TagO. For representative images of the data used in this quantification see Figure 1 and Figure 2. Quantification results are showed as a Tukey graph.



Supplementary Figure 2 – Quantification of mCherry_PGRP-SA or non-functional mCherry_PGRP-SA C48YC54Y to *S. aureus* and *B. subtilis wt* cells or deletion mutants lacking TagO. For representative images of the data used in this quantification see Figure 3 and Figure 4. Quantification results are showed as a Tukey graph.



Supplementary Figure 3 - One site specific binding regression curves for titration of amiDAP PG in PGRP:PG co-precipitation assays with mCherry_PGRP-SA and mCherry_PGRP-LC.

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Recognition Mechanism for Bacterial Polymeric Meso-Diaminopimelic Acid-Type Peptidoglycan in Insects." *The Journal of Biological Chemistry* 285 (43): 32937–45. https://doi.org/10.1074/jbc.M110.144014.

Chapter III

SagB contributes to peptidoglycan concealment at the surface of the CA-MRSA *Staphylococcus aureus* JE2 strain

Contributions to this chapter:

Gonçalo Covas designed and performed all the experiments and constructed bacterial mutant strains;

Publications in this chapter:

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

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Abstract

Staphylococcus aureus MSSA NCTC 8325-4 bacteria can prevent host innate immune receptors from recognizing the peptidoglycan (PG) at their surface by coating it with wall teichoic acids (WTA) and/or trimming the excessive/exposed PG with Atl, an autolytic enzyme with *N*-acetyl-glucosaminidase and *N*-acetylmuramyl-*L*-alanine amidase activities (Magda L. Atilano et al. 2011; Magda L. Atilano et al. 2014). However, due to the possible redundant activity of *S. aureus* codified PG hydrolases and due to genetic variation of the staphylococcal accessory genome it is possible that more virulent *S. aureus* strains may have alternative mechanisms to prevent PG recognition or that the mechanisms unveiled for the MSSA NCTC 8325-4 strain are not as relevant in other strains. In accordance with these hypotheses, it has been observed that in a CA-MRSA USA300 LAC derivative JE2 strain, Atl was redundant for virulence when tested in a murine device-related infection model (McCarthy et al. 2016).

In this study we observed that single deletion mutants lacking Atl or Sle1, or both Atl and Sle1 PG hydrolases, which had been constructed in NCTC 8325-4 and JE2 strains, presented a different ability to be bonded by mCherry_PGRP-SA, a fluorescent derivative of the *Drosophila melanogaster* PGRP-SA innate immunity receptor. Mutants constructed in the JE2 strain were less prone to recognition by the PG receptor, which supports the hypothesis that there are additional factor(s) in the JE2 strain capable of preventing PG recognition by host receptors. Since, we also observed a similar bias in JE2 *tagO* mutants that are unable to produce wall teichoic acids (WTA), it is unlikely that the differential binding of the PG receptor to the surface of bacteria of the two genetic backgrounds is due to modifications of the structure, the composition or the amount of these glycopolymers.

Surprisingly, we observed an additional band in autolytic extracts harvested from JE2 strain. The presence of this band was dependent on the expression of *Stahylococcus aureus* glucosaminidase B (SagB). In order to confirm

the role of SagB in the concealment of PG at the surface of JE2 bacteria, we constructed a *sagB* deletion mutant. We observed that the inability to produce SagB results in major alterations of PG architecture. Deletion mutants lacking SagB produce a PG with a lower degree of cross-linking between stem peptides and with longer glycan chains. The role of SagB in the concealment of PG at the bacterial cell surface was confirmed as *sagB* deletion mutants also presented an increased ability to be bonded by mCherry_PGRP-SA. The observation that absence of SagB and Atl have a synergistic effect in the increase of mCherry_PGRP-SA binding suggests that the two enzymes may have some degree of redundancy in JE2. Both enzymes may prevent PG recognition and suggests that the lower binding intensity observed in the JE2 background is partly due to a higher activity of SagB. Unfortunately, due to lack of a enzymatic active recombinant SagB protein, we could not positively assess *in vitro* if SagB influences the concealment of PG to mCherry_PGRP-SA through trimming of the exposed PG, similarly to Atl, or whether the presence of SagB alters the PG composition/architecture to produce a macromolecule that is not easily recognized by PG innate immune receptors.

Introduction

Peptidoglycan is a major component of most bacteria cell surface and it is a bona-fide MAMP that can be recognized by different PRR such as the *Drosophila* PGRP proteins (Lemaitre and Hoffmann 2007; Panayidou, Ioannidou, and Apidianakis 2014; Neyen et al. 2014). *Drosophila* PGRPs are germline encoded receptors that can bind PG through their PGRP domains (Royet, Gupta, and Dziarski 2011; Kurata 2014; Dziarski et al. 2006) and activate an innate immune response. The *Drosophila* innate immune system can be divided into two pathways that are differentially activated upon recognition of a microbial infection. The Toll pathway is described as being responsible for the immune response to Gram-positive

bacteria or fungi infection while the Imd pathway is described as being responsible for the immune response to Gram-negative bacteria, see Figure 11 of Chapter 1 for summary (Lemaitre and Hoffmann 2007; Valanne, Wang, and Rämet 2011; Lindsay and Wasserman 2014).

The Toll pathway is activated upon recognition of Lys type PG by PGRP-SA (Michel et al. 2001), or recognition of β-1,3-glucan from invading fungi by the Gram-negative binding protein 3 (GNBP3) (Mishima et al. 2009), and results in the activation of several immune responses, such as the production of Drosomycin among other AMPs (Ferrandon et al. 2007; Lindsay and Wasserman 2014; Valanne, Wang, and Rämet 2011).

However, modification of the PG can influence its recognition/binding by PG targeting proteins. It has been shown that de-*O*-acetylated PG (Bera et al. 2007), or PG with a lower degree of cross-link between different stem peptides (Magda L Atilano et al. 2010), are more susceptible to lysozyme. Therefore, when studying how accessible PG is, we must also consider modifications of the PG organization, either coarse modifications that alter significantly the architecture of the CW that surrounds bacteria (e.g. such as deletion of *tagO* or *atl*, see below) or fine PG modifications that alter some of the PG residues with "local" effects.

Our lab has previously established that binding of PGRP-SA to *S. aureus* cells is hindered when PG is coated with WTA. We have observed that removal of WTA by either deleting *tagO*, the gene that encodes the enzyme responsible for the first committed step of WTA synthesis, or by chemically inhibiting WTA synthesis, through the propagation of bacteria in the presence of a sub-MIC concentration of tunicamycin to block TagO activity but not MraY activity [PG synthesis, (Campbell et al. 2011)] will result in a cell surface that is better recognized by PGRP-SA both *in vitro* and *in vivo*. Moreover, an increased exposure of PG results in bacteria that are less virulent in a *Drosophila* infection model, see Figure 1 (Magda L. Atilano et al. 2011). This seems not to be specific to the

Drosophila model. Misawa has recently shown that a *S. aureus* Newman deletion mutant that lacks TagO, fails to colonize the nose and the gastrointestinal track in a mouse model of persistent *S. aureus* gastrointestinal colonization (Misawa et al. 2015).

The role of WTA in the virulence of *S. aureus* has been the subject of intense research. It has been shown that WTA can impair binding of antibodies to epitopes that have been exogenously added to the CW via sortase A/LPXTG motif (Gautam et al. 2015). WTA can impair the successful *S. aureus* phagocytosis by macrophages (Shiratsuchi et al. 2010). Furthermore, it was shown that a *S. aureus* mutant harbouring a modified WTA that is depleted of *D*-alanines (a mutant lacking *dlt* operon) is more susceptible to autolysis, to AMPs, to lysozyme, to lysostaphin, to vancomycin (Peschel et al. 1999, 2000; Collins et al. 2002; Herbert et al. 2007) and, is a better target for host innate immune receptors (Kurokawa et al. 2011; Tabuchi et al. 2010) and phagocytosis by macrophages (Shiratsuchi et al. 2010).

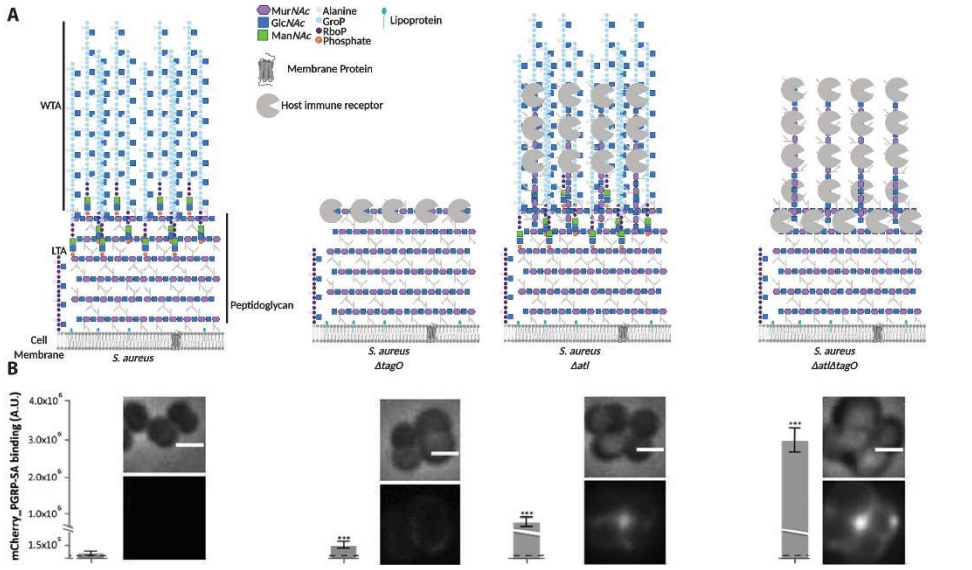


Figure 1 – A) Schematic representation of the effect of deletion of *atl* and *tagO* may exert on the cell surface architecture of *S. aureus* mutants and, consequentially, on the binding of mCherry_PGRP-SA to the cell surface. B) mCherry_PGRP-SA binding to *S. aureus* NCTC 8325-4 and single and double mutants that are unable to produce Atl, and/or TagO, show that absence of WTA or Atl results in an increased binding of PGRP-SA to the surface of *S. aureus* cells. Furthermore, it is shown that deletion of *atl* and *tagO* has a synergistic effect (Adapted from Magda L. Atilano et al. 2014).

Our research group has also reported that Atl, the major autolysin of *S. aureus*, can impair recognition of *S. aureus* NCTC 8325-4 cells both *in vitro* and *in vivo*. Deletion of *atl* gene resulted in bacterial cells with a cell surface that is better recognized by PGRP-SA and in bacteria that present a decreased virulence in the *Drosophila* infection model (Magda L. Atilano et al. 2014). The *N*-acetyl-glucosaminidase and *N*-acetylmuramyl-*L*-alanine amidase enzymatic activities associated with Atl and the observation that *atl* mutants show a thicker and rougher cell wall when observed by electron microscopy (EM) suggest that Atl is responsible for trimming the peptidoglycan that would protrude through the outermost WTA layer and would, therefore, be exposed if Atl was absent [Figure 1] (Magda L. Atilano et al. 2014; Foster 1995; Takahashi et al. 2002). It would be expected that the effect of Atl in the concealment of bacteria would be important

in different infected hosts. However, it was recently shown that absence of Atl is redundant for the virulence of a CA-MRSA USA300 LAC derivative JE2 strain in a murine device-related infection model (McCarthy et al. 2016). A JE2 *atl* deletion mutant was capable of forming biofilms and resulted in a number of CFU and weight loss on infected animals similar to an infection with the wt strain. This led the authors to conclude that, in this model, *S. aureus* bacteria that lack Atl are as virulent as bacteria of the parental strain (McCarthy et al. 2016).

Deletion of *tagO* and *atl* has a synergistic effect further indicating an alternative and complimentary mechanism of peptidoglycan occlusion [Figure 1] (Magda L. Atilano et al. 2014). However, it was also shown that WTA and Atl activities can be corelated. It was shown that Atl localizes to the mid septa in a WTA dependent manner. It was shown that Atl is blocked from binding to the staphylococcal cell surface due to presence of WTA, but was able to bind to the mid septa, probably due to a lower WTA content or an immature WTA being present (Schlag et al. 2010).

Furthermore, it was shown that *S. aureus* cells treated with sub-inhibitory concentrations of Targocil I or Targocil II, which are able to inhibit TarG and TarH activities respectively, have a lower autolytic activity. Since this effect was not observed in a mutant that is unable to produce WTA precursors, due to the deletion of *tagO*, and as it is accepted that TarG and TarH enzymes are responsible for the translocation of WTA precursors across the cell membrane, the authors hypothesized that WTA precursors were sequestering autolysins in the cytoplasm and preventing these enzymes from finding the PG substrate. This hypothesis was supported by the observation that cells treated with a sub-inhibitory concentrations of Targocil I or Targocil II have increased Atl activity, as seen by zymography analysis, that is associated with the membrane fraction (Tiwari et al. 2018).

In accordance with an important role of WTA in the determination of the PG composition, the host research group has also shown that deletion of *tagO* impairs the activity of PBP4, a low molecular weight PBP that is involved in the maturation of the staphylococcal PG, which results in cells that produce a PG with a low degree of crosslinking. These bacteria, which are enclosed within a PG macromolecule with lower cross-link degree, are not better targeted by PGRP-SA *in vivo* (Magda L Atilano et al. 2010).

The balance between the activity of enzymes involved with PG synthesis and the activity of enzymes that degrade PG at the cell surface has to be controlled in order to prevent loss of viability of bacteria that may result from the inhibition of the separation of cells that have been divided or from the activation of a premature lysis. There is growing evidence of a coregulation between CW synthesis enzymes and autolytic activity. Antignac and colleagues have shown that cells treated with a sub-inhibitory concentration of β-lactam antibiotics, or with a lower PBP2 activity, and therefore, a PG with a lower cross-link degree, have also a low activity of PG hydrolases. The authors have suggested that this reduced activity of autolysins may be an adaptation to prevent uncontrolled autolysis (Antignac, Sieradzki, and Tomasz 2007).

As described in the Chapter I, the genome of a *S. aureus* JE2 strain encodes for over 20 known or putative PG hydrolases and due to enzymes with redundant activity, characterization of PG hydrolases activities and their physiological role has proven to be a difficult challenge (Vollmer et al. 2008; Smith, Blackman, and Foster 2000, 1996; Firczuk and Bochtler 2007). We have put forward the hypothesis, supported by the putative redundancy in the activity of the PG hydrolases and the significant genetic variation of the staphylococcal accessory genome, that those *S. aureus* strains known to be more virulent/pathogenic may have alternative and/or additional strategies to evade recognition by host immune receptors beside the production of WTA or the expression of Atl. In this chapter we assess the

hypothesis of the CA-MRSA USA300 LAC derivative JE2 having alternative and/or additional strategies of evading recognition by host immune receptors to the previous strategies uncovered in the NCTC 8325-4 strain.

Results

An unknown factor enhances PG concealment in CA-MRSA JE2 strain

To determine the role of factors previously described as being involved in PG concealment in the MSSA NCTC 8325-4 strain, i.e. coating the PG with WTA or trimming excess/exposed PG by Atl (Magda L. Atilano et al. 2014; Magda L. Atilano et al. 2011), in the concealment of PG at the surface of CA-MRSA JE2 bacteria, and to assess if there are any additional factors present in the CA-MRSA JE2 strain involved in PG concealment that were not previously identified, we constructed single and double deletion mutants of *atl* and *sle1* genes in the JE2 background. To determine how accessible is the PG on their surface, we quantified and compared the binding of mCherry_PGRP-SA, a fluorescent derivative of the *Drosophila* PG receptor. The intensity of the fluorescence signal present at the surface of the different mutants that have been constructed in both the NCTC 8325-4 and JE2 backgrounds was determined by epifluorescence microscopy. We observed that mutant strains that have been constructed in the NCTC 8325-4 genetic background showed a significant bias toward a higher binding intensity than those constructed in the JE2 genetic background [Figure 2]. We also observed that this bias toward a higher binding in the NCTC 8325-4 genetic background in the parental NCTC 8325-4 and JE2 strains, albeit to a lower extent. See Supplementary Figure 1 for microscopy images with a larger field of view.

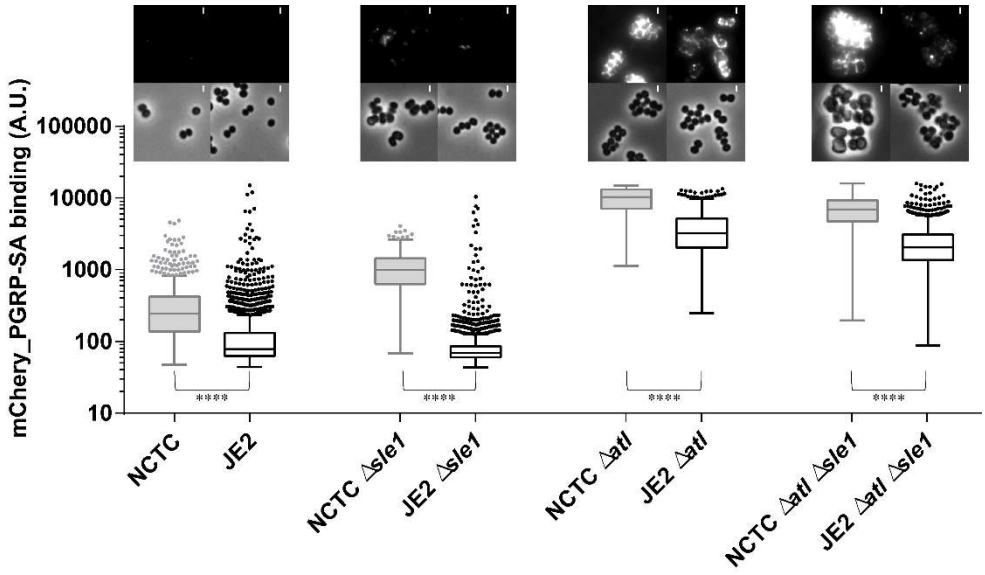


Figure 2 – Comparison of mCherry_PGRP-SA binding to *S. aureus* NCTC 8325-4 and *S. aureus* JE2 mutant strains with deletions of *atl* and/or *sle1* genes show a bias for an increased binding on the MSSA NCTC 8325-4 background. Bottom microscopy panels correspond to images obtained by phase contrast microscopy while top panels show mCherry_PGRP-SA bonded to the surface of bacteria by fluorescence microscopy. White scale bar corresponds to 1 µm. See Supplementary Figure 1 for microscopy images with a larger field of view. Quantification of the intensity of the fluorescent signals observed at the surface of at least 500 bacteria are showed as a Tukey graph (see material and methods).

These observations allowed us to conclude that, together with Atl and Sle1 PG hydrolases, there is an additional factor(s) involved in PG concealment on the JE2 background strain. In the NCTC *sle1* deletion mutant it was possible to detect mCherry_PGRP-SA binding to the cell surface exposed at the mid-section of dividing cells. Interestingly, this was not observed in the JE2 *sle1* deletion mutant [Figure 2].

S. aureus JE2 bacteria have increased activity of a peptidoglycan hydrolase

A lower binding of mCherry_PGRP-SA to the cell surface of a bacteria can result from several factors that include an altered CW composition or an alteration

of the activity, or expression levels, of enzymes that act upon the CW, such as PG hydrolases.

When we compared the zymography profile obtained with crude autolytic extracts from NCTC 8325-4 and JE2 strains, we observed an additional band of unknown identity on the JE2 sample [Figure 3, arrow]. This band is neither dependent on the activity of Atl or Sle1, since it is still observed in single and double deletion mutants lacking *atl and* sle1 genes [Figure 3, arrow]. The presence of the hydrolytic band was dependent on the expression of SagB enzyme, since it is not detected in *sagB* deletion mutants [Figure 3]. As SagB (*Staphylococcus aureus* glucosaminidase B) is identified as a transmembrane *N*-acetyl-glucosaminidase (Wheeler et al. 2015; Chan et al. 2016), the hydrolytic band observed in the zymogram may be the direct result of the SagB hydrolytic activity. However, we were not able to confirm that the observed hydrolytic band is the direct result of SagB hydrolytic activity since attempts to purify a recombinant SagB protein only resulted in enzymes that lack hydrolytic activity *in vitro* (data not shown)

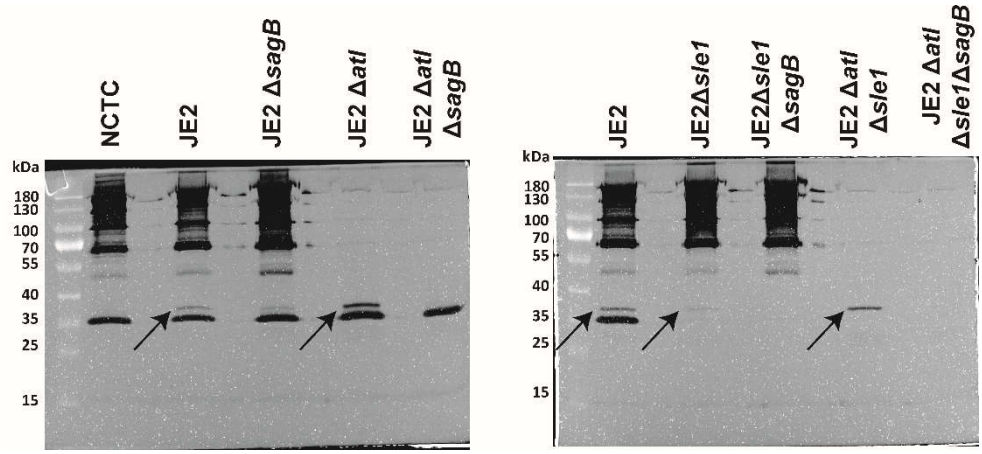


Figure 3 – Gel zymography analysis of the crude autolytic extracts of NCTC and JE2 wt strains show the presence of an additional hydrolytic band in strains derived from JE2 (arrow). This hydrolytic band is not dependent on the presence of either Atl or Sle1 since it is still observed in deletion mutants lacking the genes codifying these proteins. Since a deletion mutant lacking SagB does not show the new hydrolytic band it is possible that this band is the direct result from SagB hydrolytic activity.

SagB is involved in the concealment of PG from host immune receptors

We have observed that strains derived from JE2 show a bias toward a lower ability to be bonded by mCherry_PGRP-SA when compared with NCTC 8325-4 strains. We also observed that in these JE2 strains, there is a PG hydrolase with a higher activity or produced at higher levels, that is dependent on the presence of SagB. However, it was unclear if SagB is responsible for the lower binding of mCherry_PGRP-SA to JE2 strains and if its PG concealment mechanism is independent of previously observed factors such as WTA and Atl.

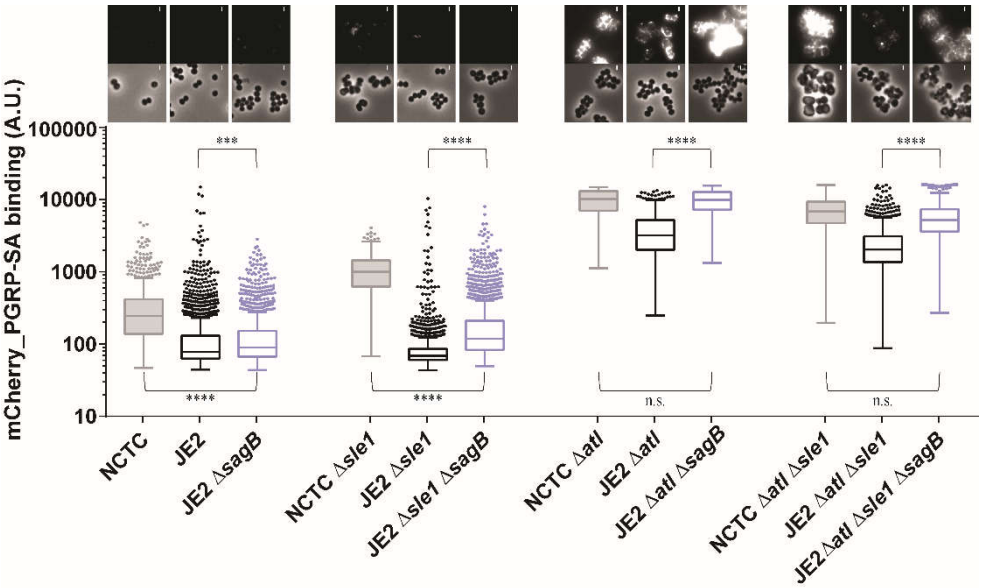


Figure 4 - Comparison of mCherry_PGRP-SA binding to JE2 deletion mutant strains lacking SagB. Mutant strains with deletion of *sagB* show an increased binding when compared with the respective parental strains. Interestingly, *sagB* deletion in a background that already lacks Atl has a higher effect in the binding intensity of mCherry_PGRP-SA to the cell surface. Bottom microscopy panels correspond to images obtained by phase contrast microscopy while the top panels show mCherry_PGRP-SA binding detected by fluorescence microscopy. White scale bar corresponds to 1 µm. See Supplementary Figure 2 for microscopy images with a larger field of view. Quantification of the intensity of the fluorescent signals observed at the surface of at least 500 bacteria are showed as a Tukey graph (see material and methods).

In order to determine how SagB was able to contribute to the concealment of PG at the surface of JE2 bacteria, we quantified the fluorescent signal from mCherry_PGRP-SA bonded to the surface of different *S. aureus* deletion mutant strains that were unable to produce SagB and/or the two Atl and Sle1 PG hydrolases [Figure 4]. JE2 deletion mutant strains that lack SagB showed a higher binding of mCherry_PGRP-SA to their surface than their parental strains. However, this effect was stronger in deletion mutants that lacked Atl, or both Atl and Sle1, than in the parental strains or in mutants that lacked the Sle1 PG hydrolase. This observation suggests that the mechanism of PG concealment dependent on SagB may be similar to Atl and may have some degree of redundancy with Atl. It is worth noting that binding of mCherry_PGRP-SA to the surface of a JE2 double deletion mutant that lacks SagB and Atl is very similar to that observed in the NCTC 8325-4 *atl* deletion mutant. The same was observed for a JE2 triple deletion mutant lacking SagB, Atl and Sle1 when compared with a NCTC double deletion mutant lacking Atl and Sle1. See Supplementary Figure 2 for microscopy images with a larger field of view.

SagB is involved in the maturation of PG produced by *S. aureus*

Since SagB has been reported to have an *N*-acetyl-glucosaminidase activity (Wheeler et al. 2015; Chan et al. 2016), it is possible that a higher activity of this enzyme could modify the PG produced by *S. aureus* and convert it in a substrate that would not be easily recognized by host PG receptors such as PGRPs. Therefore, to assess if s*agB* deletion influences the architecture of the *S. aureus* PG, we purified the cell envelope produced by *S. aureus* JE2 mutant strains lacking SagB and analysed the cross-link degree between different stem peptides and the size distribution of the glycan chains that are present in the PG.

We observed that strains that do not express SagB produce a PG with reduced level of cross-link. As shown in Figure 5, the PG macromolecule of these strains have increased levels of low cross-linked muropeptides, which elute at

retention times lower than 17 minutes (monomers to tetramers), and reduced levels of highly cross-linked muropeptides, that elute at retention times higher than 17 minutes (pentamers and higher level of cross-link). See Supplementary Figure 3 for original PG muropeptide profiles of each strain and Supplementary Table 1 for quantification of the muropeptide species variation.

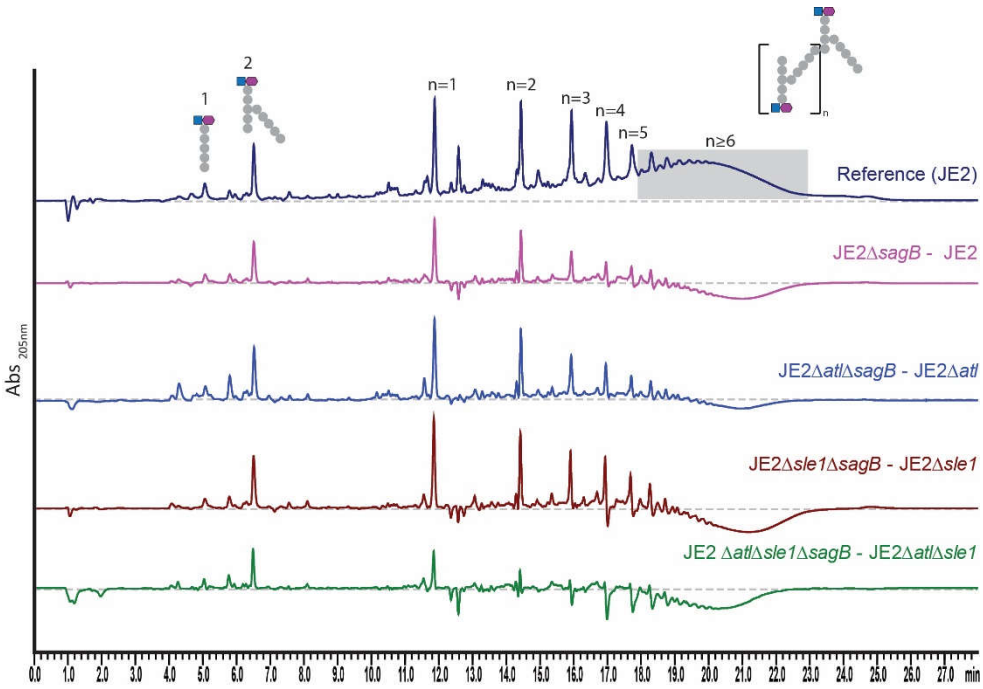


Figure 5 – Highly cross-linked muropeptides are less abundant in the peptidoglycan of *S. aureus* strains that lack SagB. Results are shown as the subtraction of the HPLC profiles, which result from the analysis of mutanolysin-digested PG, of parental strain profile to the mutant profile. The dashed line represents no difference between the parental and mutant strains. See Supplementary Figure 3 for original PG muropeptide profiles of each strain and Supplementary Table 1 for quantification of peak variation. Deletion of *sagB* resulted in an increase of low cross-linked muropeptide species (retention time $\leq$ 17 min) with a simultaneous decrease of highly cross-linked species (retention time $\geq$ 17 min).

We also observed that the PG produced by mutant strains that lack SagB have an altered size distribution of the glycan chains. As shown in Figure 6, deletion of *sagB* results in a major loss of all glycan chains species that can be chromatographically separated using these separation conditions (elute at retention times lower than 14 min), with a simultaneous increase of the peak that

corresponds to unresolved high molecular weight glycan chains (the major peak that elutes at approximately 15 minutes, see Supplementary Figure 4 for graph with an unedited "YY" scale at 15 minutes). The alterations exerted on the PG by deletion of *sagB* result in an elution profile that does not resemble the typical "hedge-hog" profile of *S. aureus* glycan chains described by Boneca and colleagues (Boneca et al. 2000). For quantification of peak variations see Supplementary Table 2.

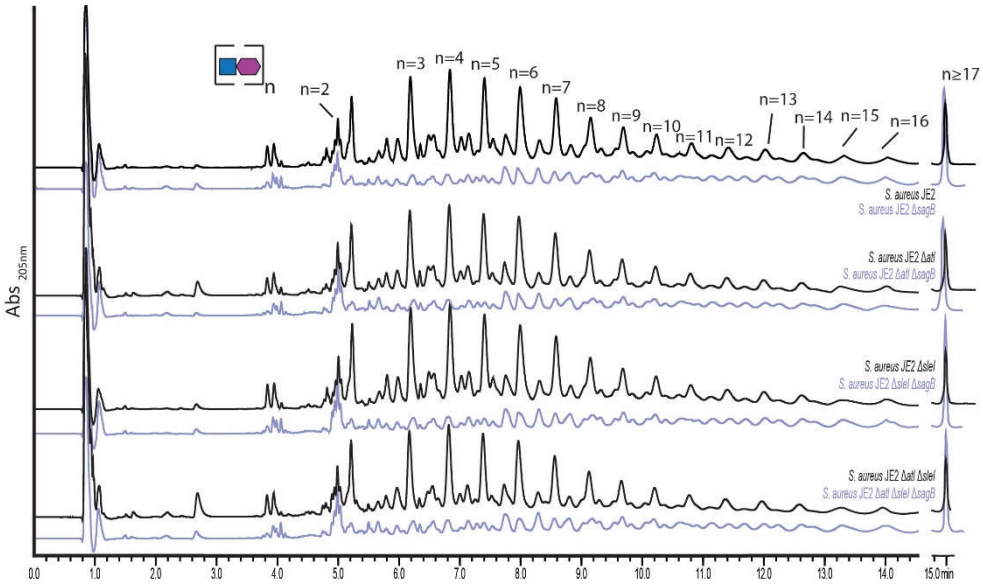


Figure 6 – PG glycans profile of *S. aureus* JE2 mutant strains lacking SagB. Deletion of *sagB* results in a major loss of glycan species that elute at retention times earlier than 14 minutes with a simultaneous increase of glycan species that cannot be resolved and that elute at 15 minutes. JE2 mutant strains lacking SagB produce a PG with a glycan profile that does not resemble the typical hedge-hog profile of *S. aureus* glycan chains. Please note that the "YY" axis scale is discontinued at 14.5 minutes (indicated with a break on the XX axis). For a graph with an unedited "YY" axis see Supplementary Figure 4. For quantification of peak variations see Supplementary Table 2.

Deletion of *sagB* alters *S. aureus* generation time

Since *sagB* deletion alters the architecture of PG produced by *S. aureus* bacteria, and as it has been shown that PG architecture can vary between cell cultures collected in exponential or stationary growth phase (Pisabarro, de Pedro, and Vázquez 1985; Glauner, Holtje, and Schwarz 1988; Blasco, Pisabarro, and de

Pedro 1988), we hypothesized that deletion of *sagB* could interfere with the propagation of *S. aureus*, namely on the growth rate or the onset of the stationary growth phase. These events could be a result of a delayed cell splitting or possible deleterious effects that absence of a PG hydrolase may exert in growing bacteria.

As shown in Figure 7, deletion of *sagB* alone or in a mutant lacking Sle1 led to an increase of the generation time of the mutants when compared with the parental strains. However, deletion of *sagB* in a mutant lacking Atl or lacking both Atl and Sle1 did not result in a significant increase of the generation time.

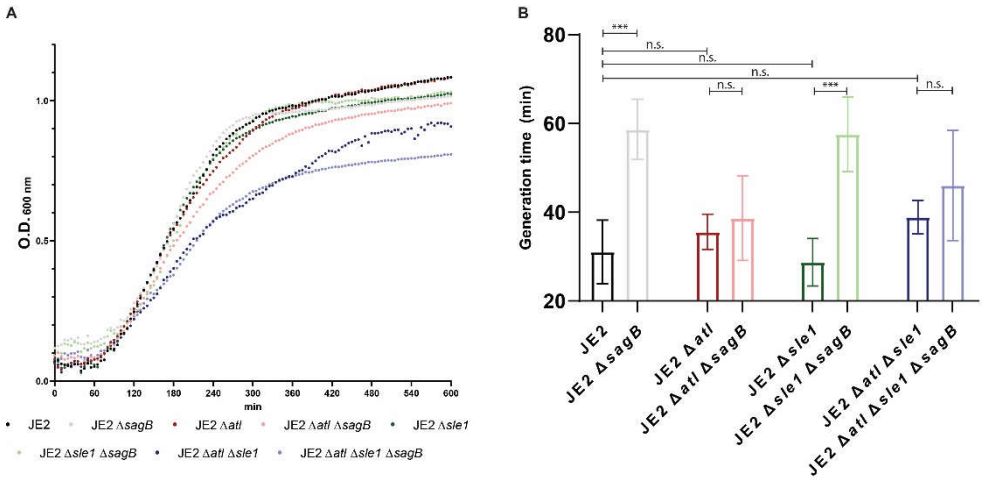


Figure 7 – A) Comparison of growth curves of JE2 mutant strains lacking SagB with parental strains. Data is shown as the average of 5 independent growth curves. Although deletion of *sagB* alone or in a mutant lacking Sle1 influences the generation time of the mutant strain when compared with the parental strain, this effect is not enough to significantly alter the growth profile of the SagB mutant strain when compared with the its parental strain. B) Average generation time of JE2 mutants lacking SagB and respective parental strains.

We also observed that the growth curve of the single *sagB* deletion mutant was similar to the JE2 parental strain and that the transition to stationary phase occurred at similar optical densities. The same was observed when we compared the JE2 double deletion mutant lacking Sle1 and SagB with a JE2 single deletion mutant lacking Sle1 or when we compared the JE2 triple deletion mutant lacking Atl, Sle1 and SagB with a JE2 double deletion mutant lacking Atl and Sle1. When we compared the JE2 double deletion mutant that lacks Atl and SagB with the JE2

single deletion mutant that lacks Atl, we were able to see a small difference on the optical density at which the growth culture curve transitions from exponential to stationary phase.

However, these variations are not enough to alter the growth phase at which the PGRP binding and PG architecture experiments were performed, when comparing the parental and mutant strains and consequently, it is unlikely that the differences observed are due to a comparison of cultures with PG in different maturation states.

SagB may not alter the monosaccharide composition of PG

Since SagB is described as being a *N*-acetyl-glucosaminidase, its hydrolytic activity may lead to an asymmetric release of monosaccharides from the glycan chains and alter the PG Mur*NAc* : Glc*NAc* ratio. It is possible that such PG composition modification could be responsible for a reduced ability to be recognized by host PRR, such as PGRPs.

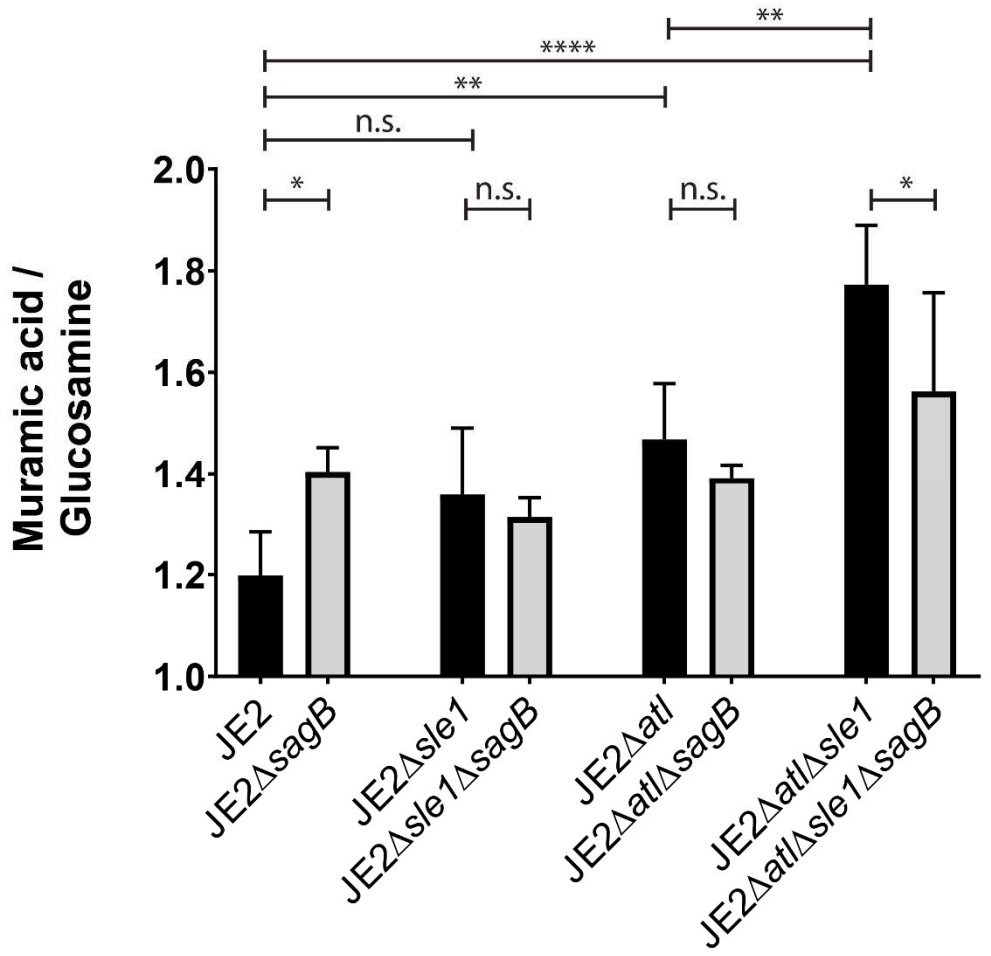


Figure 8 – Determination of the Muramic acid: Glucosamine ratio present in the PG from different *S. aureus* JE2 mutants that lack SagB. Results are shown as the average and standard deviation of 3 independent quantifications. Statistical analysis of data performed using Uncorrected Fisher's LSD. Please note that the Mur*NAc* and Glc*NAc* are de-*N*-acetylated during the hydrolysis of PG to monosaccharides.

We observed that absence of different PG hydrolases results in the increase of the Mur*NAc* : Glc*NAc* ratio, which suggests that the PG that is not trimmed due to the absence of Atl and Sle1 PG hydrolases is enriched in muramic acid residues. Despite the observation that the JE2 deletion mutant lacking SagB has a PG with a slightly higher muramic acid content when compared with the parental strain, we did not observe a significant alteration of the proportion of

muramic acids when SagB was deleted in combination with other PG hydrolases. If we compare the Muramic acid: Glucosamine ratios of JE2 double deletion mutants lacking Atl and SagB or lacking Sle1 and SagB with their respective parental strains, there is no clear effect of SagB deletion [Figure 8]. Thus, it seems unlikely that SagB activity modifies PG in a way that alters the monosaccharide composition of the PG significantly. Any modifications of the PG due to SagB activity may be architectural and not compositional in nature.

Remarkably, a single deletion mutant lacking Atl also has a PG with increased muramic acid content when compared with the wt strain, similarly to what was observed to a mutant lacking SagB. However, deletion of *sagB* in a mutant already lacking Atl did not result in a significant alteration of the Muramic acid: Glucosamine ratio, indicating that the possible effect of SagB and Atl on the PG monosacharide composition is not additive [Figure 8].

Interestingly, deletion of *sle1* alone does not seem to significantly alter the PG monosaccharide composition relatively to the parental strain, but deletion of *sle1* in a mutant lacking Atl results in a further increase of the PG muramic acid content [Figure 8].

It should be highlighted that the JE2 triple deletion mutant lacking Atl, Sle1 and SagB showed a slight decrease on the muramic acid (please note that Mur*NAc* and Glc*NAc* are de-*N*-acetylated during the hydrolysis of PG to monosaccharides) content when compared with the parental strain [Figure 8].

SagB may modify PG to a form that is a worst target for host PRR

We have established that SagB is involved in the concealment of PG from host innate immune receptors and that SagB activity is needed to produce a PG with physiological glycan chain sizes and cross-link level. We also observed that SagB, as other PG hydrolases, may alter PG monosaccharide composition. If SagB modifies PG to form a substrate less recognizable by host innate immune

receptors, it would be expected that PG purified from a mutant lacking SagB would show increased binding of mCherry_PGRP-SA.

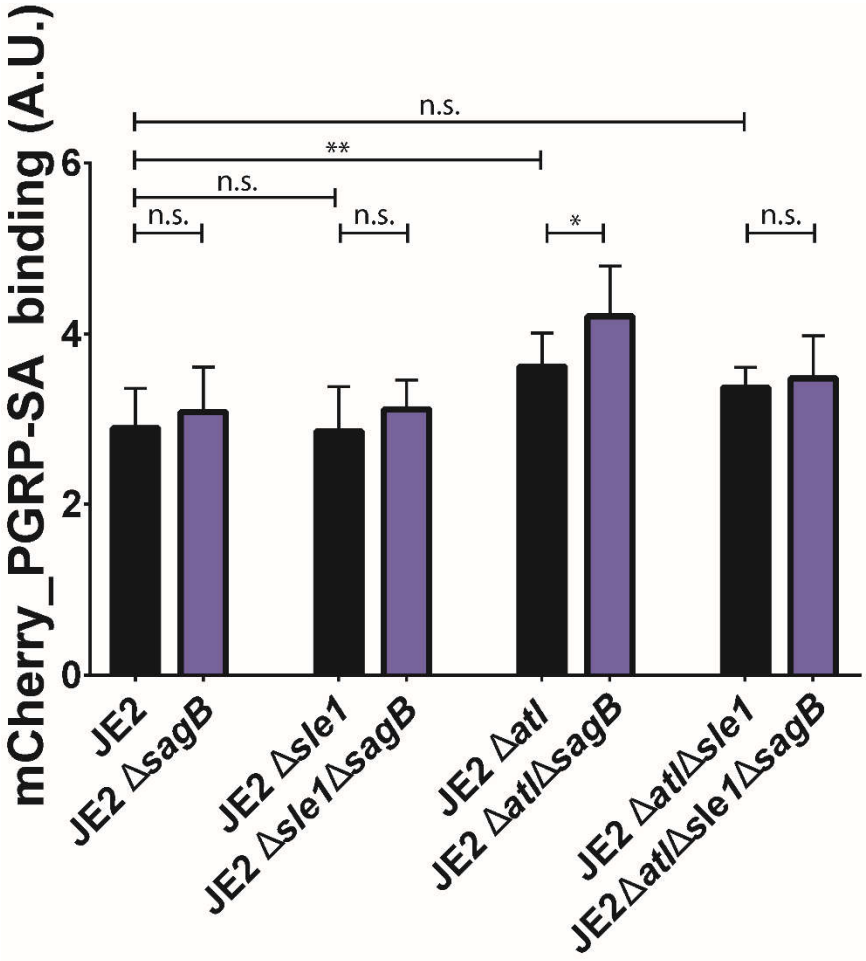


Figure 9 – mCherry_PGRP-SA binding to PG purified from *S. aureus* JE2 mutants lacking different PG hydrolases and SagB. Only a JE2 mutant lacking Atl produces a PG that is better recognized by mCherry_PGRP-SA. Deletion of *sle1* or *sagB* alone does not seem to modify the PG composition in a way that alters mCherry_PGRP-SA binding. Interestingly, similarly to what was observed by quantifying the mCherry_PGRP-SA binding by microscopy, a double deletion mutant without SagB and Atl produces a PG that is better recognized by mCherry_PGRP-SA than a single deletion mutant lacking just Atl. However, contrary to what was observed by microscopy, a triple deletion mutant that lacks Atl, Sle1 and SagB does not produce a PG that is better recognized by mCherry_PGRP-SA than the PG produced by a double deletion mutant lacking just Atl and Sle1. Data shown as average and standard deviation of 5 independent replicates.

However, when we compared the mCherry_PGRP-SA binding to PG purified from a JE2 *sagB* deletion mutant with the binding to PG purified from JE2

parental strain by co-precipitation, we could not observe a statistical significant increase in the amount of mCherry_PGRP-SA co-precipitated [Figure 9]. The same was also observed when we compared the binding to PG from a mutant that lacked both SagB and Sle1 with the binding to PG from the *sle1* mutant or when we compared the binding to PG from the triple deletion mutant, which lacked Atl, Sle1 and SagB, with the binding to the PG purified from the double deletion mutant that lacked both Atl and Sle1 [Figure 9].

We were only able to observe an altered ability to be recognized by mCherry_PGRP-SA in the PG purified from the *atl* deletion mutant which was more prominent in the PG harvested from the double deletion mutant, which lacked both Atl and SagB [Figure 9]. Although a bigger effect of *sagB* deletion on a mutant lacking Atl is not surprising, since a similar result was observed when we determined the ability of mCherry_PGRP-SA to bind the surface of bacteria from different mutants [Figure 4]. However, the absence of an increased binding to PG from a triple deletion mutant lacking Atl, Sle1 and SagB relatively to the parental strain is unexpected. Therefore, although SagB is needed to maintain a physiological PG architecture, it seems that SagB influence on mCherry_PGRP-SA binding may be mainly due to trimming the exposed PG, similarly to Atl. However, we cannot discard the hypothesis that quantification of mCherry_PGRP-SA binding to pure PG by pull down assay is not sensitive enough to resolve the differences of affinity to a PG from a mutant lacking SagB.

SagB deletion influences β-lactam antibiotic resistance

Since it has been shown a correlation between the activity of PG hydrolytic enzymes and the activity of enzymes involved with synthesis of PG (Antignac, Sieradzki, and Tomasz 2007), we hypothesized that a reduced activity of SagB, a peptidoglycan hydrolase with a major impact on the PG architecture, could influence PG synthesis and, consequently, influence antibiotic susceptibility. To this end we determined the minimum inhibitory concentration (MIC) that impairs

growth of *S. aureus* JE2 deletion mutants lacking SagB with Oxacillin, Vancomycin, Kanamycin and Neomycin. Oxacillin is a β-lactam antibiotic that inhibits PG synthesis by binding covalently to PBPs, which will be able to block transpeptidation. Vancomycin is able to bind to the terminal *D*-ala-*D*-ala dipeptide and is, therefore, also capable of blocking transpeptidation. Kanamycin and Neomycin can prevent protein synthesis by binding to the 30S subunit of the ribosome, which will be able to block translation of new proteins.

Table 1 – Minimum inhibitory concentration of Oxacillin (Oxa), Vancomycin (Van), Kanamycin (Kan) and Neomycin (Neo) for *S. aureus* deletion mutants lacking SagB.

Strain | MIC µg/mL	Oxa	Van	Kan	Neo
JE2	> 16	1.25	25	26
JE2 Δ*sagB*	2	1.25	25	26
JE2 Δ*atl*	> 16	1.25	12.5	13
JE2 Δ*atl*Δ*sagB*	1	2.5	25	13
JE2 Δ*sle1*	1	1.25	25	13
JE2 Δ*sle1*Δ*sagB*	< 0.5	2.5	50	26
JE2 Δ*atl*Δ*sle1*	4	2.5	25	13
JE2 Δ*atl*Δ*sle1*Δ*sagB*	< 0.5	2.5	25	13

As we can see in Table 1, deletion of *sagB* had no major effect on the susceptibility to Kanamycin or Neomycin, suggesting that SagB has no effect on protein synthesis. However, deletion of *sagB* significantly increased the susceptibility to Oxacillin, seen as a major decrease of MIC for a deletion mutant lacking SagB. Interestingly, SagB does not seem to have an effect on the susceptibility to vancomycin, another antibiotic that also targets PG synthesis. One possible explanation for this difference may be that Oxacillin interacts directly with the PBPs responsible for PG transpeptidation while Vancomycin interacts with the substrate of PBPs such as PG precursors.

Discussion

It was already shown by our lab that MSSA *S. aureus* NCTC 8325-4 strain can impair recognition of PG at the cell surface by coating it with WTA or by trimming the excessive PG that is exposed in an Atl dependent manner [Figure 1]. Moreover, both mechanisms were found to have a synergistic effect and to influence the virulence of *S. aureus* in a *Drosophila* infection model. However, due to the genetic variability of *S. aureus* strains it is possible that additional and/or alternative mechanisms of evading recognition by host immune receptors are used by more virulent/pathogenic strains. Furthermore, since Atl was recently reported to be redundant to virulence in a murine device-related infection model (McCarthy et al. 2016) it is also possible that the evasion mechanisms unveiled for MSSA *S. aureus* NCTC 8325-4 strain are not relevant in more virulent/pathogenic strains.

To address this issue, we decided to assess if absence of Atl in the CA-MRSA USA300 LAC derivative JE2 strain would also result in a bacterial cell surface with a more exposed PG. We observed that a JE2 deletion mutant lacking Atl, similarly to what was observed for a NCTC 8325-4 deletion mutant lacking Atl, showed a higher binding of mCherry_PGRP-SA by fluorescent microscopy when compared to the parental strain [Figure 2]. However, the mCherry_PGRP-SA binding intensity to the JE2 deletion mutant lacking Atl was lower than the binding intensity to the NCTC 8325-4 deletion mutant lacking Atl. Since a *sle1* single deletion mutant and an *atl sle1* double deletion mutant in the JE2 genetic background also showed a lower binding intensity when compared to identical mutants constructed in the NCTC 8325-4 background, we hypothesized that the JE2 strain had an additional factor(s) that could influence recognition by host innate receptors. Interestingly, we also observed a lower binding of mCherry_PGRP-SA to the JE2 parental strain when compared with the NCTC 8325-4 parental strain, albeit to a less extent [Figure 2].

Since preliminary data showed that mCherry_PGRP-SA binding to a *tagO* single deletion mutant in the JE2 background was lower than the binding to a *tagO*

single deletion mutant in the NCTC 8325-4 background it was unlikely that the bias toward a lower binding in the JE2 background was due to a CW with an altered content of WTA or due to the production of WTA harbouring specific modifications [Supplementary Figure 5].

A zymography analysis of the crude autolytic extracts of the JE2 and NCTC 8325-4 strains [Figure 3] showed the presence of an additional PG hydrolytic band in the JE2 autolytic extracts that was not present in NCTC 8325-4. The migration of this band was compatible with the presence of a protein that had a molecular weight of approximately 37 kDa. Since we used *S. aureus* NCTC 8325-4 inactive cells as a substrate for the zymography analysis, it is possible that the activity of this PG hydrolytic enzyme can result in autolysis, and therefore, can be an unidentified autolysin.

The hydrolytic activity of this PG hydrolase was not dependent on the activity of Atl or Sle1, since single deletion mutants lacking either Atl or Sle1 or a double deletion mutant lacking both Atl and Sle1 still showed the hydrolytic band of the unidentified PG hydrolase [Figure 3]. However, the activity of the PG hydrolytic enzyme was dependent on the presence of SagB, since a single deletion mutant of *sagB*, a double deletion mutant lacking *sagB* in combination with either *atl* or *sle1* or a triple deletion mutant lacking *atl, sle1* and *sagB* did not show the new hydrolytic band [Figure 3]. Since SagB is described as a type II transmembrane protein with *N*-acetyl-glucosaminidase activity (Wheeler et al. 2015; Chan et al. 2016) it is possible that the hydrolytic band is the direct result of PG hydrolysis by SagB. However, since our attempts in purifying a recombinant SagB protein with hydrolytic activity failed, we could not confirm this hypothesis.

Deletion of SagB resulted in an increased ability of the cell surface of *S. aureus* JE2 mutants to be bonded by mCherry_PGRP-SA [Figure 4]. Interestingly, this effect was stronger when we deleted both *sagB* and *atl*. This suggests that SagB mechanism of PG concealment has some degree of redundancy with Atl.

Since both enzymes are described as having *N*-acetyl-glucosaminidase activities it is possible that a higher activity of SagB on the JE2 background can partially compensate for absence of Atl. The observation that a double deletion JE2 mutant lacking both Atl and SagB has an identical binding intensity to a single deletion NCTC 8325-4 mutant lacking Atl supports the hypothesis of a higher activity of SagB on the JE2 background compensating the absence or a reduced activity of Atl. This compensation mechanism can be responsible for the bias toward a lower binding intensity on the JE2 background when comparing single deletion mutants for the same gene between the two backgrounds. Remarkably, a *sle1 sagB* double deletion mutant on the JE2 background did not have a binding intensity similar to a single *sle1* mutant on the NCTC 8325-4 background. This may indicate the presence of an additional mechanism of PG concealment present on the JE2 background that is still not identified.

To address if SagB influences the accessibility of PG to host innate receptors by altering the architecture/composition of the PG available at the surface of *S. aureus* bacteria, we purified and analysed the muropeptide and glycan composition of PGs produced by different *S. aureus* mutants that lacked SagB and other PG hydrolases. We observed that deletion of SagB drastically alters PG architecture. Mutants lacking SagB produce a PG with a lower cross-link degree [Figure 5] and made of longer glycan chains [Figure 6]. An increase of the glycan chains size upon *sagB* deletion was also observed by Wheeler et al. and Chan et al. (Wheeler et al. 2015; Chan et al. 2016).

Since SagB is described as having *N*-acetyl-glucosaminidase activity it is more likely that it affects the glycan chains size directly and the cross-link degree in an indirect way. This is supported by the observation the the mutant lacking SagB produced a PG with a glycan profile that is greatly dissimilar to the typical hedge-hog glycan profile previously reported for *S. aureus* (Boneca et al. 2000). Unfortunately, since we were not able to purify an enzymatically active

recombinant SagB protein we could not assess if adding rSagB exogenously would modify the glycan chains profile of a SagB mutant to a profile more similar to wt profile and, consequently, impair mCherry_PGRP-SA binding. If that was the case, we would be able to propose a direct correlation between glycan chain sizes and mCherry_PGRP-SA binding intensity.

Contrary to us, two different groups reported the purification of a recombinant SagB with hydrolytic activity. Wheeler and colleagues were able to show the activity of SagB by zymography against purified CW from vegetative *B. subtilis* at pH 5.0 (Wheeler et al. 2015). They also showed that although both SagB and the glucosaminidase domain of Atl can cleave *B. subtilis* purified glycans chains *in vitro*, the two recombinant proteins produced glycan chains with a different size distribution. Interestingly, the glucosaminidase domain of Atl was able to cleave *B. subtilis* purified glycans to chains of smaller size than the glycan chains produced by hydrolysis with recombinant SagB (Wheeler et al. 2015). Similarly, Chan and colleagues managed to show that glycan chains purified from a *S. aureus* mutant lacking SagB were digested differently by purified SagB and the glucosaminidase domain of Atl. When purified PG was digested with the recombinant glucosaminidase domain of Atl, they were able to observed the production of mainly disaccharides. However, when the same sample was digested with a recombinant SagB, authors observed the production of glycan chains whose size distribution, and consequently the observed HPLC chromatogram, was very similar to that present in the PG from the parental (wt) *S. aureus* strain (Chan et al. 2016).

Despite the experimental evidence that SagB can cleave glycan chains into shorter fragments, none of the groups proved experimentally that SagB has *N*-acetyl-glucosaminidase activity. They deduced that SagB has *N*-acetyl-glucosaminidase activity based on protein homology and the presence of a glucosaminidase domain (PF01832, see Table I from Chapter I). As mentioned in Chapter I, both *N*-acetyl-muramidases and *N*-acetyl-glucosaminidases are

glycosydases, that is they are able to cleave the β-(1,4) linkage between Glc*NAc* and Mur*NAc* subunits of the PG glycan chains. However, *N*-acetyl-muramidases cleave after a Mur*NAc* residue, producing an Mur*NAc* reducing end while *N*-acetyl-glucosaminidases cleave after a Glc*NAc* producing a Glc*NAc* reducing end.

It is possible to prove experimentally whether an enzyme can act as an *N*-acetyl-glucosaminidase or as an *N*-acetyl-muramidase. Mihelič and colleagues showed experimentally that staphylococcal SagA (also named as AtlE or LytD) and the glucosaminidase domain of Atl have *N*-acetyl-glucosaminidase activities by mass spectrometry analysis of the products that result from digestion of a Glc*NAc*-β-(1,4)-Mur*NAc*-β-(1,4)-Glc*NAc*-β-(1,4)-reduced Mur*NAc* (Mihelič et al. 2017). Furthermore, the authors have shown that both recombinant proteins do not cleave a hexasaccharide of Glc*NAc*, contrary to lysozyme that has *N*-acetyl-muramidase activity. These authors have also shown that SagA has a heart-shaped globular fold composed of two separate (left and right) domains that form two lobes connected at the "bottom" in a core region. Using a bioinformatics approach to search for similar structures, the authors found that SagA has a structure similar to other putative glucosaminidases but also to lysozyme enzymes such as the human lysozyme (PDB entry: liwt) and the lysozyme from Atlantic cod (PDB entry 3gxr). The observation that SagA may be structurally similar to the mentioned lysozymes (although they have lower similarity in the amino acid sequence), led the authors to question the mechanism responsible for the differential activity of *N*-acetyl-muramidases and *N*-acetyl-glucosaminidases. The authors found that while *N*-acetyl-glucosaminidases have structural features that accept the lactate moieties of Mur*NAc* on the left lobe, *N*-acetyl-muramidases exclude them from the left lobe. However, since *N*-acetyl-muramidases do not require interaction of the lactate moieties of Mur*NAc* with the right lobe, they can accommodate and cleave polymers of just Glc*NAc* (Mihelič et al. 2017).

The glycan chains profile of *S. aureus* has a peak distribution that resemble a "hedgehog" with the major peaks being polymers of a Glc*NAc*-β-(1,4)-Mur*NAc* with Mur*NAc* in the reducing end and smaller satellite peaks that correspond to structures with uneven number of Glc*NA*c to Mur*NAc* residues (Boneca et al. 2000). This led Boneca and colleagues to postulate that the satellite peaks are the result of glycan chains digestion with an unknown enzyme with *N*-acetyl-glucosaminidase activity.

The observation carried out by Mihelič and colleagues that *N*-acetyl-glucosaminidases and *N-acetyl*-muramidases have a very similar structure, support the hypothesis that SagB is a muramidase, whose activity converts long glycan chains into smaller fragments and produce the "hedgehog" distribution observed in the parental strain. To date, to the best of our knowledge, no enzyme with *N*-acetyl-muramidase activity was found to be encoded by *S. aureus* genome. Since a mutant lacking SagB is characterized by the loss of the major peaks of glycan chain profile with a Mur*NAc* reducing end, one can hypothesize that SagB is the enzyme that is cleaving longer glycan chains to the physiological size by *N*-acetyl-muramidase activity. However, since we were unable to produce an enzymatic active recombinant SagB enzyme we could not test this hypothesis. In accordance with our proposal, Chan and colleagues reported that SagB is required for producing a PG with glycan chain of physiological size (Chan et al. 2016).

Since we observed that *sagB* deletion from the *S. aureus* genome results in bacteria that produce a PG with an altered architecture, and as it was previously shown that the PG architecture/composition can change with PG maturation (Pisabarro, de Pedro, and Vázquez 1985; Glauner, Holtje, and Schwarz 1988; Blasco, Pisabarro, and de Pedro 1988), i.e. switch from exponential growth to stationary phase, we asked if deletion of *sagB* could also result in an early entry in stationary phase, which could influence how PG was perceived at the bacterial cell surface by a PG receptor. As seen in Figure 7, deletion of *sagB* alone or in a deletion

mutant lacking Sle1 did result in an increase of the generation time when compared with the parental strains. Curiously, deletion of *sagB* in a deletion mutant lacking Atl or a deletion mutant lacking both Atl and Sle1 did not result in a significant increase of the generation time [Figure 7]. The observation of a milder phenotype when *sagB* is deleted in a mutant that also lacks Atl, further reinforces a possible interplay between SagB and Atl. Furthermore, although it was previously reported that deletion of *atl* and/or *sle1* affects cell separation (Kajimura et al. 2005; Monteiro et al. 2015; Takahashi et al. 2002; Oshida et al. 1995), in this study deletion of either *atl* and/or *sle1* did not altered the generation time of a culture significantly [Figure 7]. The growth curves of the deletion mutants lacking SagB and the parental strain are always similar apart from the double deletion mutant lacking Atl SagB when compared with the single deletion mutant lacking just Atl. Therefore, it is unlikely that the PG architecture modifications observed are due to comparison of cells in different growth stages.

The mechanism used by SagB to cleave a glycan chain is unknown as it may present an exo-glycosasydase or endo-glycosydase activity. Depending on the mode of cleavage, SagB may cleave the glycan chain asymmetrically, i.e. it may release muropeptides without a 1:1 ratio of Mur*NAc*:Glc*NAc*. If this would be the case, it would be possible that mutant strains that lack SagB may produce a PG enriched in Glc*NAc* or Mur*NAc* residues. Although a single deletion mutant of *sagB* resulted on an increase of Muramic acid content, double deletion mutants of *sagB* and *atl* or *sle1* did not (Figure 8, Please note that Mur*NAc* and Glc*NAc* are de-*N*-acetylated during the hydrolysis of PG to monosaccharides). Furthermore, a triple deletion mutant of *sagB*, *atl* and *sle1* had a decrease of the Muramic acid content when compared with the parental strain (*atl, sle1* double deletion mutant). So, it is unclear if SagB activity modifies the PG Mur*NAc*:Glc*NAc* ratio.

Remarkably, we also observed that the absence of different PG hydrolases seems to result in the production of a PG with an altered Mur*NAc*:Glc*NAc* ratio.

The *atl* single deletion mutant showed an increased muramic acid content when compared with the parental strain. The same was also observed with the *atl sle1* double deletion mutant, which showed an increased muramic acid content when compared with the parental strain (*atl* single deletion mutant). However, a *sle1* single deletion mutant did not changed the PG Mur*NAc*:Glc*NAc* when compared with the parental strain (wt). Since *S. aureus* encodes at least 20 putative and known PG hydrolases, and their activities can have some degree of redundancy, it may be difficult to appreciate the consequences of loss of activity of a PG hydrolase by directly analysing the PG purified from a deletion mutant. An analysis of the products that result of an incubation of the PG hydrolase with a pure PG sample with a previously known structure/profile may be a more informative approach.

Since SagB deletion resulted in a major alteration of the PG architecture it is possible that the increased mCherry_PGRP-SA binding observed in JE2 strains that lacked SagB activity was due to a PG not processed by SagB being a better substrate for mCherry_PGRP-SA or being present in higher amounts. To test the first hypothesis, we quantified the binding intensity of mCherry_PGRP-SA to PG purified from JE2 mutants lacking SagB. As seen in Figure 9, a PG purified from single deletion mutant lacking Atl has a higher binding of mCherry_PGRP-SA than the parental JE2 strain. This in accordance with what was observed for mCherry_PGRP-SA binding to whole cells [Figure 4]. Moreover, the *atl sagB* double JE2 mutant produces a PG that shows higher binding than the parental strain (*atl* single deletion mutant). This observation is in accordance with what was seen for the mCherry_PGRP-SA binding to whole cells [Figure 9 and Figure 4, respectively] and supports the hypothesis that SagB may modify the *S. aureus* PG to form a substrate that is not well recognized by mCherry_PGRP-SA. However, contrary to what was observed for mCherry_PGRP-SA binding to whole cells, a *sagB* single deletion mutant does not have a higher binding than the parental JE2 strain. The same was also observed with the *sle1 sagB* double deletion mutant or the *atl sle1*

sagB triple deletion mutant as they were equally recognized by mCherry_PGRP-SA as their parental strains (*sle1* single deletion mutant and *atl sle1* double deletion mutant respectively). This contradiction can be the result of a purified PG sample lacking any structural or compositional variation that is important for mCherry_PGRP-SA binding and is lost during the purification, i.e. treatment with HF to remove WTA may remove other PG modifications such as *O*-acetylation (Magda L. Atilano et al. 2011). Alternatively, a PG:mCherry_PGRP-SA co-precipitation assay may lack the sensitivity to discriminate the binding variation between a PG purified from a mutant lacking SagB and the respective parental strain. This means that while it may still be possible that SagB can modify PG composition/architecture to a form that is a worse substrate to host receptors, at this point we cannot discard the hypothesis that the binding differences seen between a mutant lacking SagB and the parental strain are due to SagB trimming the exposed PG at the CW, similarly to Atl. The observation that the phenotype of *sagB* deletion is stronger in a mutant that also lacks Atl highlights some degree of redundancy between Atl and SagB. It also favours the hypothesis that SagB influences the ability of mCherry_PGRP-SA to bind the bacterial cell surface by trimming exposed PG. However, the two hypotheses are not mutually exclusive, i.e. it is possible that the *atl sagB* double deletion mutant has more exposed PG, similarly to the *atl* single deletion mutant, and that the exposed PG is a better substrate to host receptors due to the PG lacking the modifications that result from SagB activity.

The mechanisms that determine the composition of the bacterial cell surface may be redundant and complex. It has been previously proposed that there is a correlation between the activity of PG hydrolytic enzymes and the activity of PG synthesis enzymes (Antignac, Sieradzki, and Tomasz 2007). A lower activity of PG synthesis enzymes due to either sub-MIC concentrations of β-lactam antibiotics or to the deletion of *pbp2*, which encodes a PBP with both transpeptidase and

transglycosylase activities, results in a PG with a reduced cross-link degree and in autolytic extracts with reduced PG hydrolase activities. It was also proposed that this correlation may prevent uncontrolled autolysis (Antignac, Sieradzki, and Tomasz 2007). Since *sagB* deletion results in major alterations of the PG architecture, namely longer glycan chains that produce an atypical size distribution profile and a reduced degree of cross-link between stem PG peptides, it may be possible that a mutant lacking SagB could influence the activity of PG synthesis enzymes and, consequently, interfere with the ability of bacteria to resist or tolerate antibiotics that target the synthesis of their cell surface.

To assess this hypothesis, we determined the MIC of Oxacillin, Vancomycin, Kanamycin and Neomycin in mutants lacking SagB. Oxacillin is a β-lactam antibiotic that inhibits PG synthesis by irreversibly binding to PBPs and blocking transpeptidation. Vancomycin is able to bind to the terminal *D*-ala-*D*-ala dipeptide and is, therefore, an antibiotic that also blocks PG transpeptidation. Kanamycin and Neomycin are antibiotics that do not target the synthesis of the cell wall. They both block protein synthesis by binding to the 30S subunit of the ribosome. As we can see in Table 1, the MIC of a mutant strain lacking SagB to Kanamycin and Neomycin was very similar to the MIC of the parental strain. Some small variations were observed that can be attributed partially to the expected variation for a 96 well microdilution assay. This suggests that SagB deletion has no effect on protein synthesis. However, deletion of *sagB* significantly reduced the MIC of Oxacillin but not the MIC of Vancomycin. Both antibiotics block PG synthesis by interfering with transpeptidation, but one possible explanation for the discrepancy of MIC variation may be due to the mechanism of action of each antibiotic. While Oxacillin interacts with PBPs, Vancomycin interacts with PG. It is feasible to imagine that a PG with longer glycan chains and a lower cross-link degree has the same amount, if not a higher amount, of terminal *D*-ala-*D*-ala dipeptides, and therefore, have a marginal effect on the MIC of Vancomycin or

slightly increase it (compare the MIC of an *atl sagB* double deletion mutant and *sle1 sagB* double deletion mutant with the respective parental strains).

On the other hand, since SagB is a glycosydase it is likely that it affects the glycan chain size directly and the level of PG cross-link indirectly. Therefore, one possible explanation for the observed lower level of PG cross-link is a lower PBP activity in the SagB mutant. It is feasible to imagine that a lower PBP activity would result in a lower MIC for Oxacillin. Chan and colleagues also observed a reduction of the MIC of Oxacillin and an increase of the MIC of Vancomycin upon deletion of *sagB* (Chan et al. 2016). Remarkably, these authors also observed an increased resistance to lysostaphin induced autolysis in *atl* or *sagB* single deletion mutants and, a synergistic effect in the *atl sagB* double deletion mutant. They proposed that a PG with longer glycan chains is less liable to lysis by hydrolysis of the pentaglycine bridges (Chan et al. 2016). However, we should highlight that we and others did not observe a significant alteration of the glycan chain size and PG cross-link in the *atl* single deletion mutant (Takahashi et al. 2002).

Interestingly, deletion of Atl and SagB seem to have phenotypic overlap, suggesting that SagB and Atl may have some redundancy. Deletion of Atl results in a rougher CW, as seen by electron microscopy (Takahashi et al. 2002; Foster 1995) and alters the CW non-covalently bonded proteins (Takahashi et al. 2002). Similarly, SagB results in a rougher CW surface by EM and alters protein export across the CW (Chan et al. 2016). This observation reinforces the idea that SagB influence on accessibility of PG to host PRR is exerted mainly by a mechanism similar to that of Atl, trimming the exposed/excessive PG.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 2 and Table 3, respectively. *Escherichia coli* strains were grown at 37ºC in Luria-Bertani (LB, Alfa Aesar) or Luria-Bertani agar (LA, Alfa Aesar) medium; *S. aureus* strains were grown

at 37ºC in Tryptic Soy broth (TSB, Difco) or in Tryptic Soy agar (TSA, Difco) medium. When needed, the medium was supplemented with: antibiotics ampicillin (Amp, 100 μg/mL for *E. coli* strains, Apollo Scientific), Erythromycin (10 μg/mL for *S. aureus* strains, Apollo Scientific); with 5-bromo-4-chloro-3-indolyl *β-D*-galactopyranoside (X-Gal, 100 μg/mL, Apollo Scientific) and with Isopropyl *β-D*-1-thiogalactopyranoside (IPTG 1mM, Apollo Scientific).

Table 2 – Strains used this study

Strain	Description	Source or Reference
***S. aureus* NCTC 8325-4**	MSSA strain	
S. aureus* NCTC 8325-4Δ*atl	Obtained using NCTC 8325-4 as parental strain for *atl* deletion	Atilano et al. 2014
S. aureus* NCTC 8325-4Δ*sle1	Obtained using NCTC 8325-4 as parental strain for *sle1* deletion	F. B. da C. Vaz 2016
S. aureus* NCTC 8325-4Δ*atl*Δ*sle1	Obtained using NCTC 8325-4Δ*atl* as parental strain for *sle1* deletion	F. B. da C. Vaz 2016
***S. aureus* JE2**	MRSA strain	Fey et al. 2013
S. aureus* JE2Δ*atl	Obtained using JE2 as parental strain for *atl* deletion	This study
S. aureus* JE2Δ*sle1	Obtained using JE2 as parental strain for *sle1* deletion	This study
S. aureus* JE2Δ*sagB	Obtained using JE2 as parental strain for *sagB* deletion	This study
S. aureus* JE2Δ*atl*Δ*sle1	Obtained using JE2Δ*atl* as parental strain for *sle1* deletion	This study
S. aureus* JE2Δ*atl*Δ*sagB	Obtained using JE2Δ*atl* as parental strain for *sagB* deletion	This study
S. aureus* JE2Δ*sle1*Δ*sagB	Obtained using JE2Δ*sle1* as parental strain for *sagB* deletion	This study
S. aureus* JE2Δ*atl*Δ*sle1*Δ*sagB	Obtained using JE2Δ*atl*Δ*sle1* as parental strain for *sagB* deletion	This study

Table 3 – Plasmids used in this study

Plasmid	Description	Source or Reference
pET21α-mCherry_PGRP-SA	pET21a vector codifying T7-mCherry_PGRP-SA-Histag used for protein purification of mCherry_PGRP-SA in *E. coli* BL21(DE3); Amp^R	Magda L. Atilano et al. 2011
pMAD	*E. coli – S. aureus* shuttle vector with thermosensitive origin of replication; Amp^R, Ery^R; lacZ	Arnaud, Chastanet, and Débarbouillé 2004
pMAD_Δ*atl*	pMad with *atl* upstream and downstream regions for constructing null mutant; Amp^R, Ery^R; lacZ	This study
pMAD_Δ*sle1*	pMad with *sle1* upstream and downstream regions for constructing null mutant; Amp^R, Ery^R; lacZ	This study
pMAD_Δ*sagB*	pMad with *sagB* upstream and downstream regions for constructing null mutant; Amp^R, Ery^R; lacZ	This study

S. aureus mutant strains construction

S. aureus null mutants were constructed using the thermosensitive plasmid pMAD (Arnaud, Chastanet, and Débarbouillé 2004). Fragments containing the upstream and downstream region of the gene of interest were PCR amplified from JE2 genomic DNA using the primers P1 and P2 for the upstream fragment and

P3 and P4 primers for the downstream fragment (listed for each gene of interest in Table 4). The upstream and downstream fragments were joined by overlap PCR using P1 and P4 primers and the resulting fragments were digested with *Nco*I *and Bam*HI restriction enzymes (FastDigest, ThermoFisher Scientific) and cloned into pMAD vector using T4 DNA ligase (ThermoFisher Scientific) using the vendor recommended protocols.

The resulting pMAD plasmids (listed in Table 3) were transformed into *E. coli* DC10B and, after confirming the plasmid sequence by Sanger sequencing, the plasmids were electroporated into *S. aureus* RN4220 strain at 30ºC using Erythromycin and X-gal for colony selection. The pMAD plasmids were transduced into the *S. aureus* JE2 strain of interest using phage 80α (see Table 2 for details about parental strains used in mutant construction). This was followed by a selection of colonies where an integration and excision of the plasmid resulted in a deletion of the gene of interest from the chromosome, as described in Arnaud, Chastanet, and Débarbouillé 2004. Deletion of gene of interest was confirmed by PCR with primers P5 and P6 (listed for each gene of interest in Table 4).

Table 4 – Primers used in this study.

Primer	Sequence 5'-3'
SagB_P1	atgcacccatggatgcgaatgttatcggttctg
SagB_P2	ggtgtggatatgtaatttgataagctacgagttg
SagB_P3	tatcaaattacatatccacacctcttaggtc
SagB_P4	gtgcatggatccaaagattgcttgcttgaggg
SagB_P5	gggcgcaccttggaatatag
SagB_P6	tatatgaagatgttagggtgtttcaacatc
Sle1_P1	atgcgccatggccgaataaagcattaaatgatggtg
Sle1_P2	cgtaagactttacactttaaaatcctcctcttgc
Sle1_P3	ggattttaaagtgtaaagtcttacgtatataaatatataatgaattcc
Sle1_P4	tgcatggatcccattatttgtcatgtttaccacc
Sle1_P5	gacactaaggcttgggagaagg
Sle1_P6	ggccacacattgcctgccatcc
Atl_P1	atgcacCCATGGagttgcagcagctttagaag
Atl_P2	catgttgcttacattctatttattactcctaac
Atl_P3	aaatagaatgtaagcaacatgaacataggatc
Atl_P4	ctgGGATCCatttttcgcgaaataaccag
Atl_P5	gggtatatgatgaaaatggc
Atl_P6	ggtactatcaaacgttttg

Cell wall and peptidoglycan purification

S. aureus CW and PG preparation were obtained as described in Jonge et al., 1992. Briefly, an overnight culture of the strain of interest was back diluted to an OD_{600nm} of ≅ 0.001 and incubated at appropriate conditions until an OD_{600nm} between 0.5 and 0.9 was reached. A high dilution of the overnight culture, together with growth conditions that do not surpass the exponential phase, were used to maximize the amount of cells actively growing. Upon reaching the desired OD_{600nm},

the culture was quickly chilled by incubation in an ice/ethanol bath before collecting the cells by centrifugation at 13.000 x *g* for 5 min at 4ºC. The pellet was resuspended in ice cold Milli-Q water and added drop by drop to boiling sodium dodecyl sulfate (SDS, Sigma-Aldrich) to a final concentration of 4% (w/v) SDS. The resulting solution was boiled for 30 minutes to promote cellular membrane disruption and inactivation of metabolic enzymes that may modify/degrade the CW/PG (autolysins). SDS was washed-off by repetitive centrifugation of the sample in fresh warm Milli-Q water until no SDS could be detected by the Hayashi method (Hayashi 1975). The SDS-free pellets were broken by performing 10 cycles of 45 seconds at 6 RPM in a FastPrep-24 5G (MP biomedicals) homogenizer with acid washed glass beads (Sigma-Aldrich, ≤106 μm) as abrasive. The samples were clarified of DNA, RNA and contaminant proteins by treatment with RNase (50 μg/mL, Sigma-Aldrich) and DNase (10 μg/mL, Sigma-Aldrich) in 50 mM Tris-HCl pH 7.5 20 mM $MgSO_4$ and Trypsin (100 μL/mL, Sigma-Aldrich) in 50 mM Tris-HCl pH 7.5 20 mM $MgSO_4$ 10 mM $CaCl_2$. The hydrolytic enzymes were inactivated by boiling in 1% (w/v) SDS and the clarified sample was washed twice with Milli-Q water, once with 8M LiCl (VWR), once with 100 mM Ethylenediamine tetraacetic acid (EDTA, VWR) pH 7.0 and once with acetone (Sigma-Aldrich) to remove SDS, ionically bonded material and endotoxins. The resulting sample, purified CW, was lyophilized and quantified by weight or monosaccharide content (see below).

Pure PG samples were obtained by removing the WTA by incubating CW in pure Hydrofluoric Acid (HF, Sigma-Aldrich 48% (w/v)) at 4ºC for 48 hours. HF was washed off by repetitive centrifugation with fresh cold 100 mM Tris-HCl pH 7.0. The resulting sample was lyophilized and quantified by weight or monosaccharide content (see below).

Peptidoglycan quantification by monosaccharide content

The monosaccharide composition of the pure PG samples was measured to assess sample purity and quantify the molecular concentration of PG by Mur*NAc*

content, as described in Covas, Vaz, Henriques, Pinho, & Filipe, 2016. Samples of PG (0.2 mg) were hydrolysed to the constitutive monosaccharides by incubation in 3M HCl (Carl Roth) for 2 hours at 95ºC without agitation. Reaction was stopped by removing HCl by lyophilizing the sample in a SpeedVac (LabConco) at room temperature. Any residual HCl was diluted by resuspending the sample in 1 mL of Milli-Q water and removed by lyophilizing the sample. The monosaccharides were separated by high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) using a Thermo-Scientific Dionex CarboPac PA10 4 mm x 250 mm column with a Thermo-Scientific Dionex AminoTrap Column, 4 x 50 mm as a pre-column in a Thermo-Scientific Dionex ICS5000. The monosaccharides were resolved in a 20-minute isocratic step at 18 mM NaOH followed by a 5-minute linear gradient from 0 mM $NaCH_3COO$ to 200 mM $NaCH_3COO$ in 18 mM NaOH. A 5-minute isocratic step at 200 mM $NaCH_3COO$, 18 mM NaOH was followed by a 5-minute linear gradient to 800 mM $NaCH_3COO$ and a 5-minute isocratic step at the same conditions to elute any remaining monosaccharides. All sample resolution steps were performed at 1 mL/min at 30ºC using a PAD waveform adapted from Engel & Händel, 2011 and gold disposable electrodes (Thermo-Scientific). All buffers were made from ionic chromatography grade NaOH (VWR) and $NaCH_3COO$ (Fluka) in Milli-Q water with $\geq$ 18 MΩ resistivity and kept under a nitrogen (Air Liquide) blanket to minimize carbonate formation in the eluents by dissolved CO2. A Thermo-Scientific Dionex BorateTrap Inline Trap Column was used between the pump and the injection loop to capture any residual borate from the eluents. The sample content of Glc*Nac*, Mur*Nac*, Glucosamine, Muramic acid and Ribitol was calculated using a calibration curve of standards (Sigma-Aldrich) of known concentration. All quantifications were performed with 3 independent hydrolysis. Note that a complete hydrolysis of a pure PG sample will only show Glucosamine and Muramic acid content since Glc*NAc* and a Mur*NAc* residues present in the PG are de-*N*-acetylated under the hydrolysis conditions that

are used. A detectable amount of Ribitol may indicate a WTA contamination while a detectable amount of Glc*NAc* and a Mur*NAc* may indicate a partial hydrolysis of the PG sample.

Purification of muropeptides and analysis by UHPLC

Non-reduced muropeptides were obtained by digesting pure PG samples with mutanolysin (0.2 mg/mL, from *Streptomyces globisporus* ATCC 21553, Sigma-Aldrich) in 12.5 mM $NaPO_4$ pH 5.5 buffer overnight at 37ºC and agitation. Muropeptides were reduced by incubation with fresh $NaBH_4$ (3.125 mg/mL) in 0.25 M Borate pH 9.0 buffer for 2 hours at room temperature. The reduction reaction was stopped by lowering the pH to ≤ 2.0 with H_3PO_4.

Reduced muropeptides were chromatographically separated by Reversed Phase Ultra-High-Performance Liquid Chromatography (RP-UHPLC) using a Waters Acquity CSH C18 1.7µm 2.1 mm x 100 mm UHPLC column coupled with a Waters VGuard Acquity CSH C18 1.7µm 2.1mm x 5 mm guard column in a Shimazu Nexera-i LC-2040C 3D UHPLC. Reduced muropeptides were resolved in a linear gradient from 5 % (v/v) methanol to 24% (v/v) methanol in 100 mM $NaPO_4$ pH 2.0 buffer over 21.5 minutes followed by a washing step at 24% (v/v) methanol over 0.7 minutes at 0.3 mL/min and 52 ºC. Elution of muropeptides was recorded by monitoring Abs_{205nm}. Muropeptide species were quantified by determining the area of each peak as percentage of total chromatogram area with Shimadzu LabSolutions v5.92 software. Data shown is the average of 3 replicates.

Purification of glycans and analysis by UHPLC

Purified glycans were obtained by first digesting pure PG with Lysostaphin (100 µg/mL, from *Staphylococcus simulans*, Sigma-Aldrich) in 25 mM $NaPO_4$ buffer pH 7.0. at 37ºC with agitation. After heat inactivation of Lysostaphin, the sample was incubated in a sonic bath for 5 min to facilitate sample resuspension and release from the pellet matrix and digested with LytA (50 µg/mL, from *Streptococcus pneumoniae*, purified in-house) in 12.5 mM $NaPO_4$ buffer pH 7.0. at

37ºC with agitation. Pre-treatment with Lysostaphin increases the amount of material recovered after digestion with LytA (Boneca et al. 2000). After heat inactivating LytA the sample was clarified of any residual contaminant by centrifugation and acidified to a pH $\leq$ 2.0 with H_3PO_4. At this pH the neutral glycans were separated from the charged peptides using a MonoS 5/50Gl column from GE Healthcare in a Shimazu Nexera-i LC-2040C 3D UHPLC. The neutral glycans eluted within a 10-minute isocratic step at 10 mM $NaPO_4$ buffer pH 2.0 at 0.5 mL/min and 40ºC. The charged peptides were eluted in a 10-minute linear step to 10 mM $NaPO_4$ buffer pH 2.0 100 mM NaCl also at 0.5 mL/min and 40ºC. Elution of sample was recorded by monitoring the $Abs_{205\ nm}$.

Purified glycans were reduced by incubation with fresh $NaBH_4$ (3.125 mg/mL) in 0.25 M Borate pH 9.0 buffer for 2 hours at room temperature without agitation. The reduction reaction was stopped by lowering the pH to ≤ 2.0 with H_3PO_4. Reduced glycans were chromatographically separated by Reversed Phase Ultra-High-Performance Liquid Chromatography (RP-UHPLC) using a Waters Acquity CSH C18 1.7µm 2.1x100mm UHPLC column coupled with a Waters VGuard Acquity CSH C18 1.7µm 2.1x5mm guard column in a Shimazu Nexera-i LC-2040C 3D UHPLC. Reduced glycans were resolved in a convex gradient (-4 curve of Shimadzu LabSolutions v5.92 software) from 0 % (v/v) acetonitrile to 10.5% (v/v) acetonitrile in 100 mM $NaPO_4$ pH 2.0 buffer over 12.5 minutes followed by a washing step at 30% (v/v) acetonitrile over 4.3 minutes at 0.3 mL/min and 30 ºC. Elution of muropeptides was recorded by monitoring Abs_{205nm}. Glycan species were quantified by determining the area of each peak as a percentage of total chromatogram area with Shimadzu LabSolutions v5.92 software. Data shown is average of 3 replicates.

Crude autolytic extracts activity quantification by zymography

Crude autolytic extracts were prepared as described in Vaz & Filipe, 2015. Briefly, an overnight culture of the strain of interest was back diluted to a to an

OD_{600nm} of ≅ 0.05 and incubated at appropriate conditions until an OD_{600nm} between 0.5 and 0.9 was reached. Upon reaching the desired OD_{600nm} the culture was quickly chilled by incubation in an ice/ethanol bath before collecting the cells by centrifugation at 13.000 x *g* for 5 min at 4ºC. The pellet was washed once with 50 mM Tris-HCl pH 7.5 150 mM NaCl before incubation in 4% (w/v) SDS for 30 minutes at 25ºC with agitation. The crude autolytic extracts were obtained as the supernatant after a centrifugation at 30.000 x *g* for 15 min at 25ºC. Crude autolytic extracts were used freshly prepared and the concentration was standardized by adjusting the OD_{600nm} of the cultures prior to the extraction procedure.

Crude autolytic extracts were analysed by zymography using heat inactivated cells of *S. aureus* at 2 mg/mL as a substrate in a PAGE [10 % (w/v) total acrylamide; 2.7% cross-linker, Bio-Rad]. The PAGE gels were prepared without any SDS added to the acrylamide. The sample loading buffer had SDS but not had any reduction agent, i.e. *β*-mercaptoetanol or dithiothreitol. *Inactivated S. aureus* cells were obtained by growing *S. aureus* NCTC8325-4 cells as stated for the crude autolytic extracts. Upon reaching the desired OD_{600nm} the culture was quickly chilled by incubation in an ice/ethanol bath before collecting the cells by centrifugation at 13.000 x *g* for 5 min at 4ºC. The pellet was washed once with ice-cold Milli-Q water and autoclaved for 15 min at 121 ºC. After electrophoretic resolution of the samples, the gels were washed off of running buffer and any residual denaturing agents, such as SDS, by 3 incubations in fresh Milli-Q water for 15 minutes and incubated in renaturing buffer [50 mM Tris-HCl pH 7.5 0.1% (v/v) Triton X-100 10 mM $CaCl_2$, 10 mM $MgCl_2$] overnight at 37ºC with gentle agitation in order to allow for the renaturation and enzymatic activity of the peptidoglycan hydrolases to occur. The undigested substrate was stained with a 0.1% (w/v) Methylene blue (Sigma-Aldrich) in 0.01% (w/v) KOH solution for increased contrast with the transparent halos that result from enzymatic digestion of the substrate.

The gels were digitalized in a GE HealthCare ImageScannerIII with LabScan 6.0 software.

mCherry_PGRP-SA protein purification

mCherry_PGRP-SA was purified as previously described in Magda L. Atilano et al. 2011. Briefly, pET21α-mCherry_PGRP-SA was transformed into *E. coli* BL21(DE3) using ampicillin (Amp) as selection marker. An overnight culture was back diluted to an $OD_{600nm} \cong 0.05$ in fresh LB with Amp (100 µg/mL) and incubated at 25ºC with agitation until an $OD_{600nm} \cong 0.5$ was achieved. At this point IPTG was added to a final 1mM concentration and the culture was incubated overnight at 25ºC with agitation.

The cells were collected by centrifugation at 13.000 x g for 5 min at 4ºC, washed once with equilibration buffer [50 mM Na_2PO_4 pH 7.4, 300 mM NaCl] by centrifugation at 13.000 x g for 5 min at 4ºC and lysed by performing two runs in a French Press at 1000 psi. The lysate was centrifuged at 16.000 x g for 20 min at 4ºC, the pellet (with inclusion bodies) was resuspended in Resuspension Solution [50 mM Na_2PO_4 pH 7.4, 500 mM NaCl, 8M Urea] and incubated at 4ºC with agitation until sample was homogeneous. At this point the sample was added to an equal volume of Dilution Solution [50 mM Na_2PO_4 pH 7.4, 500 mM NaCl] in order to dilute Urea to a 4M final concentration.

After clarifying the sample of any insoluble debris by repetitive centrifugation at 16.000 x g for 20 min at 4ºC the sample was incubated with TALON Metal Affinity Resin (Clontech) at 4ºC with agitation for 30 minutes. The resin was recovered by spin-down centrifugation and washed with Wash Solution [50 mM Na_2PO_4 pH 7.4, 300 mM NaCl, 4M Urea], Wash Solution with 5 mM and Wash Solution 10 mM of Imidazole (VWR), sequentially , before eluting the protein with Elution Solution [50 mM Na_2PO_4 pH 7.4, 300 mM NaCl, 150 mM Imidazole] in a gravity flow column. Elution Solution was exchanged with Phosphate Buffered Saline [PBS-pH 6.0; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4

adjusted to pH 6.0 with HCl] by overnight dialysis with a 10 kDa cut-off Dialysis tubing (SnakeSkin, Pierce). Purified mCherry_PGRP-SA was aliquoted and stored at -20ºC.

Quantifying PGRP binding to cells by fluorescent microscopy

Binding of mCherry_PGRP-SA (purified in-house, see above) to *S. aureus* strains cells *in vivo* was assessed by Wide-field fluorescence microscopy. Briefly, cells were collected at mid exponential growth ($OD_{600nm} \cong 0.5$) by centrifugation at 13.000 x g for 5 min at room temperature, washed once with PBS-pH 6.0 and incubated with mCherry_PGRP-SA at 0.3 mg/mL in PBS-pH 6.0 buffer for 5 minutes at room temperature with agitation. After incubation the cells were washed twice with fresh PBS-pH 6.0 buffer by centrifugation and applied in 1.2% (w/v) agarose in PBS-pH 6.0 buffer patch prior to observation in a Zeiss Axio Observer Z1 microscope with a Plan-Apochromat 100×/1.4 oil Ph3 objective. Images were acquired using a Retiga R1 CCD camera (QImaging) using Metamorph 7.5 software (Molecular Devices) with a 500-millisecond exposure time and a Texas-Red filter.

Quantification of the mCherry_PGRP-SA binding to the cell surface was carried with Fiji image software (Schindelin et al. 2012). Briefly, a threshold was applied to the phase image to obtain a mask that harbours the cells. Since mCherry_PGRP-SA binds the cells extracellularly this mask was slightly dilated in order to not truncate the binding signal. The mask of close neighbouring cells was separated by applying a watershed algorithm and resulting masks were converted to regions of interest (ROIs). This list of ROIs (each harbouring an individual cell) was used to measure the fluorescence signal intensity after subtracting the background fluorescence to the fluorescence image. The average signal intensity of each ROI was used as a measure of the binding intensity. Statistical analysis of the data was performed using the non-parametric Uncorrected Dunn's test since quantification data did not show a normal/gaussian distribution. Data is plotted as a Tukey box and whiskers graph with boxes showing the 25th, median, and 75th

percentiles; Whiskers are draw at 1.5 times the interquartile range from either the 25th or the 75th percentile; Data points outside the 1.5 times interquartile range are considered an outlier and shown as a point in the graph.

Quantifying PGRP binding to purified PG by pull down assay

Binding of mCherry_PGRP-SA to pure PG was performed by incubating a PG sample containing 0.038 μmol of Mur*NAc* (see Peptidoglycan quantification by monosaccharide content above) with mCherry_PGRP-SA at 0.3 mg/mL in PBS-pH 6.0 for 30 minutes at 25ºC with agitation. The pellet containing the PG : bonded mCherry_PGRP-SA co-precipitate was washed twice with fresh PBS-pH 6.0 buffer by centrifugation before resuspension in SDS-PAGE loading buffer and incubation in boiling water for 5 minutes.

After electrophoretic separation of the resulting samples, the gels were stained with BlueSafe (NZYTech) and the intensity of the mCherry_PGRP-SA protein bands used as a measure of the binding intensity. Gels were digitalized in a Bio-Rad Gel Doc EZ system with Image Lab software. Intensity of the protein bands was measured using the Image Lab software (v6.0 build 25, Bio-Rad) and statistical analysis was performed from 5 independent co-precipitation reactions using an Uncorrected Fisher's LSD.

Growth curves of *S. aureus* cultures

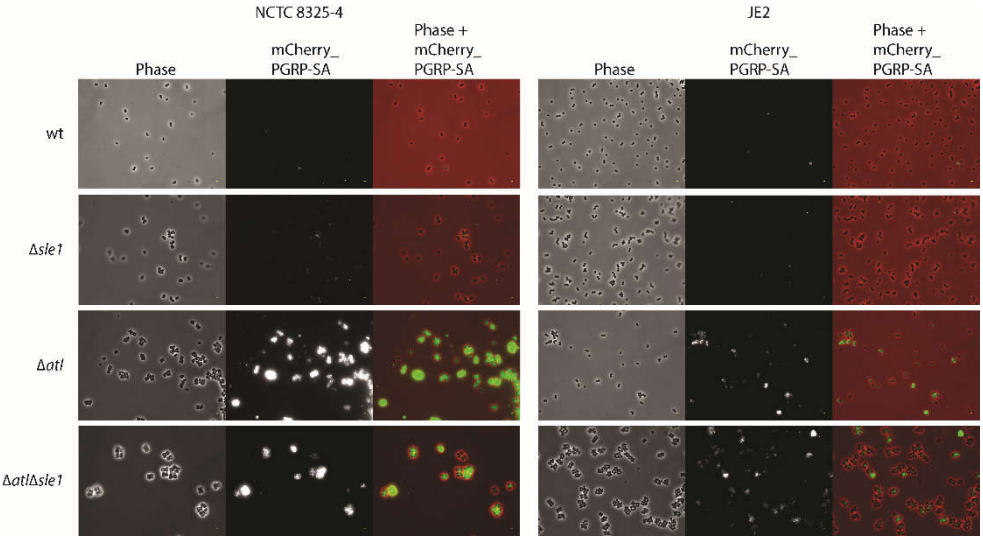
Overnight cultures of the strains of interest were back diluted to an $O.D._{600\ nm}$ of ≅ 0.05 in fresh TSB and incubated at 37ºC with shaking. Growth of all strains were monitored for 24 hours, with a reading of the $O.D._{600\ nm}$ taken every 15 minutes, in a BioScreen C Analyser (Oy Growth Curves Ab). Growth curves were obtained from 5 independent biological replicates.

Minimum inhibitory concentration assay by microdilution method

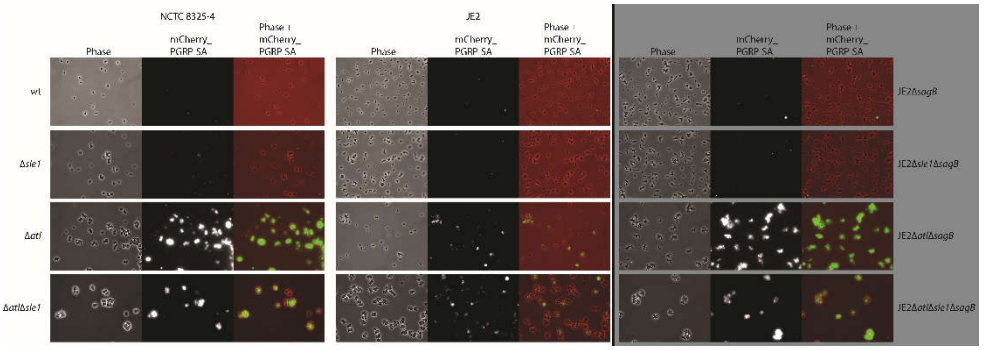
MICs were determined by microdilution in sterile 96-well plates with medium containing a series of two-fold dilutions of each compound. Cultures of *S. aureus* strains were added at a final density of ≅5×10^5 CFU/mL to each well. Each

plate had wells reserved for sterility control (no cells added) and cell viability (no compound added). Plates were incubated at 37 °C. MIC was determined as the lowest concentration that inhibited growth after 48h of incubation. All assays were done in triplicate.

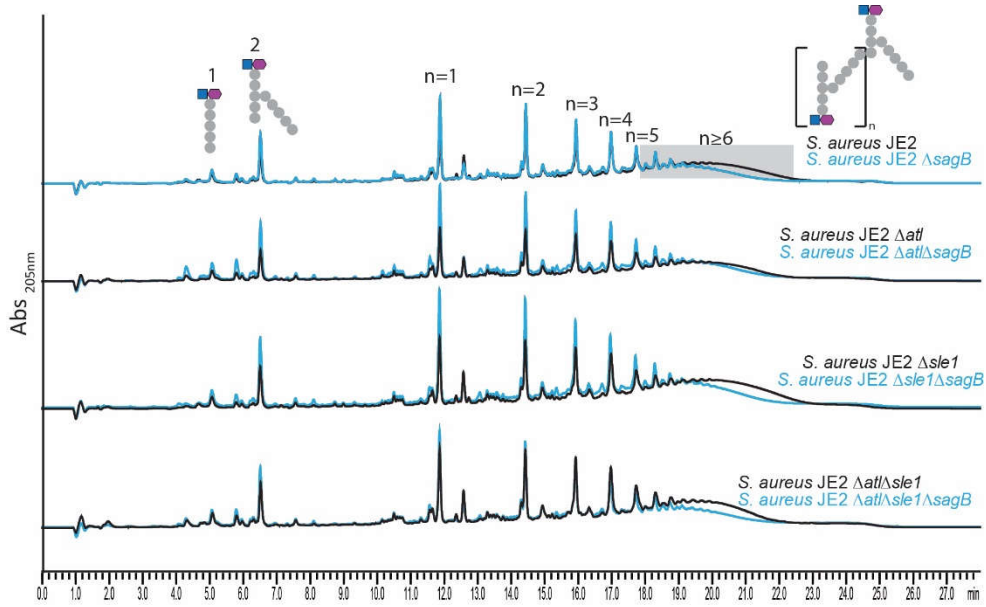
Supplementary Data



Supplementary Figure 1 – Comparison of mCherry_PGRP-SA binding to *S. aureus* NCTC 8325-4 and *S. aureus* JE2 mutant strains with a single deletion of either *atl* or *sle1* genes or both *atl* and *sle1* genes show a bias for an increased binding on the MSSA NCTC 8325-4 background. White scale bar corresponds to 1 µm.



Supplementary Figure 2 - Comparison of mCherry_PGRP-SA binding to JE2 strains lacking SagB. Mutant strains with deletion of *sagB* show an increased binding when compared with the parental strain. Interestingly, *sagB* deletion in a background that already lacks Atl has a higher effect in the binding intensity of mCherry_PGRP-SA to the cell surface. White scale bar corresponds to 1 µm.

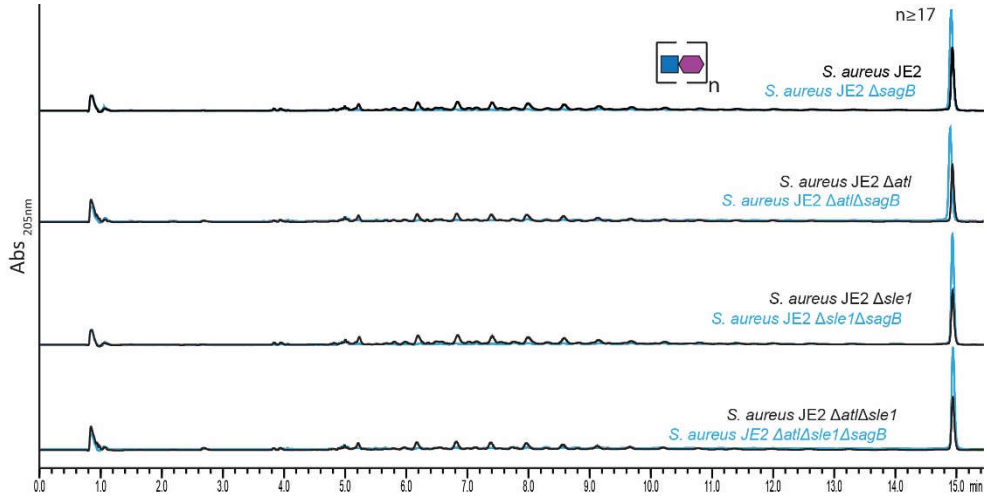


Supplementary Figure 3 - PG muropeptides profile of *S. aureus* JE2 mutant strains lacking SagB. Deletion of *sagB* results in an increase of low cross-linked muropeptide species (retention time ≤ 17 min) with a simultaneous decrease of highly cross-linked species (retention time ≥ 17 min).

Supplementary Table 1 - Change of PG muropeptide species as percentage of the total chromatogram area for JE2 mutants lacking SagB. For peak identification see the chromatography profile in Figure 5 or Supplementary Figure 3. Deletion of *sagB* results in an increase of low cross-linked muropeptide species with a simultaneous decrease of highly cross-linked species. Data shown is average of 3 replicates.

peak id	JE2 Δ*sle1* - JE2	JE2 Δ*atl* - JE2	JE2 Δ*atl*Δ*sle1* - JE2	JE2 Δ*sagB* - JE2	JE2 Δ*sle1*Δ*sagB* - JE2 Δ*sle1*	JE2 Δ*atl*Δ*sagB* - JE2 Δ*atl*	JE2 Δ*atl*Δ*sle1*Δ*sagB* - JE2 Δ*atl*Δ*sle1*
1	-0.065	0.331	0.637	2.949	3.053	2.164	3.191
2	-0.218	0.434	0.735	2.211	2.069	1.343	1.561
n=1	-0.129	-0.107	0.163	-0.191	0.073	-0.028	-0.241
n=2	-0.256	0.556	0.866	2.019	2.285	0.641	0.433
n=3	-0.490	-0.112	0.034	1.087	1.667	0.392	0.723
n=4	-0.277	0.120	0.448	0.635	0.660	0.006	-0.240
n=5	-0.361	0.125	0.240	0.402	0.633	-0.197	-0.239
n≥6	3.294	-2.958	-5.812	-13.931	-15.526	-11.919	-10.769

% variation	legend
≥2	⇑
0.5 to 2	▲
-0.5 to 0.5	–
-2 to -0.5	▼
≤-2	⇓

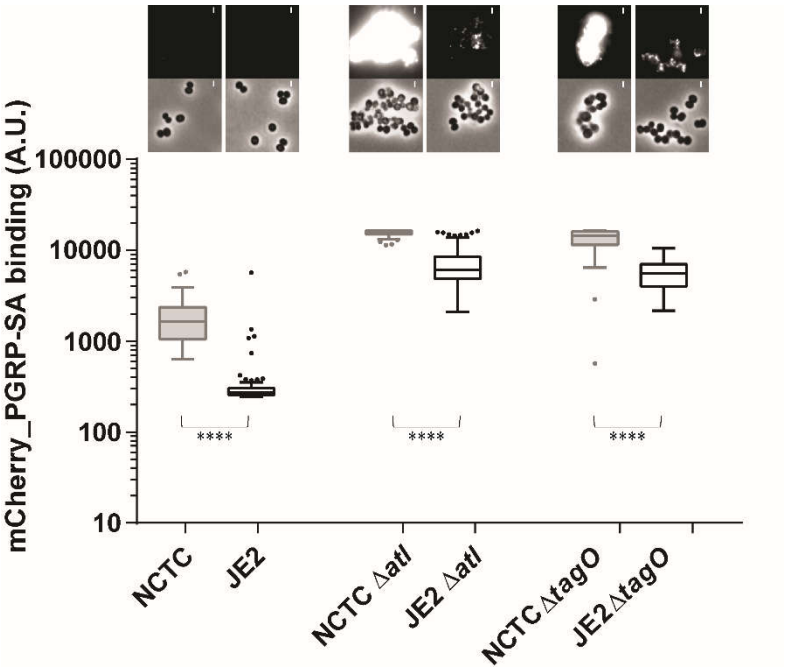


Supplementary Figure 4 – PG glycans profile of *S. aureus* JE2 mutant strains lacking SagB with a YY scale that allows visual appreciation of the variation of the peak that elutes at 14.5 minutes. For quantification of peak variations see Supplementary Table 2.

Supplementary Table 2 - Change of PG glycan species as percentage of the total chromatogram area for JE2 mutants lacking SagB. For peak identification see the chromatography profile in Figure 6. JE2 mutants with a single or double deletion of *atl* or *sle1* shown small variation of glycan species. In opposition, deletion of sagB causes a major variation of glycan species, with a major increase of peak 17 and concomitant decrease of peaks 3 to 11. Data shown is average of 3 replicates.

n=	JE2Δ*sle1* -JE2	JE2 Δ*atl* -JE2	JE2 Δ*atl*Δ*sle1* - JE2	JE2 Δ*sagB* -JE2	JE2 Δ*sle1*Δ*sagB* - JE2 Δ*sle1*	JE2 Δ*atl*Δ*sagB* - JE2 Δ*atl*	JE2 Δ*atl*Δ*sle1*Δ*sagB* - JE2 Δ*atl*Δ*sle1*
2	0.1	0.4	0.6	0.1	0.5	0.3	-0.1
3	0.4	0.1	0.2	-2.7	-3.0	-2.7	-2.8
4	0.3	0.0	0.2	-3.1	-3.3	-3.2	-3.2
5	0.2	0.0	-0.3	-3.6	-3.7	-3.6	-3.2
6	0.2	0.3	0.2	-2.7	-2.9	-3.0	-2.9
7	0.2	0.0	0.0	-2.4	-2.5	-2.4	-2.3
8	0.1	0.1	0.1	-2.4	-2.4	-2.3	-2.3
9	0.4	0.3	0.4	-1.8	-2.2	-2.1	-2.1
10	0.0	0.0	0.1	-0.7	-0.5	-0.6	-0.6
11	-0.1	0.1	0.1	-1.6	-1.4	-1.2	-1.4
12	-0.1	0.1	0.1	-0.3	-0.1	-0.3	-0.4
13	-0.1	0.1	0.4	0.0	0.2	0.0	-0.4
14	-0.1	0.1	-0.1	-0.6	-0.4	-0.6	-0.5
15	-0.1	0.1	0.0	1.1	1.4	1.1	1.1
16	-0.1	0.1	0.0	1.3	1.6	1.2	1.2
17	-2.6	0.2	-1.9	23.7	23.2	19.9	20.0

% variation	legend
≥2	
0.5 to 2	
-0.5 to 0.5	
-2 to -0.5	
≤-2	



Supplementary Figure 5 - Comparison of mCherry_PGRP-SA binding to *S. aureus* NCTC 8325-4 and *S. aureus* JE2 mutant strains with a single deletion of either *atl* or *tagO* genes show a bias for an increased binding on the MSSA NCTC 8325-4 background. Bottom microscopy panel corresponds to images obtained by phase contrast while the top panel shows mCherry_PGRP-SA binding detected by fluorescence microscopy. White scale bar corresponds to 1 μm- Quantification data is represented as a Tukey Graph from at least ≥30 cells.

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Chapter IV

Lipid II translocation across the membrane in *Staphylococcus aureus* is dependent on the presence of MurJ

Contributions to this chapter:

Gonçalo Covas performed all experiment except construction of a MurJ inducible strain

Andreia Tavares performed all experiments

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Abstract

Peptidoglycan (PG) is a major component of the bacterial cell wall (CW) and its biosynthesis pathway is conserved among Gram-positive or Gram-negative bacteria. PG biosynthesis starts with precursors at the cytoplasm and terminates with integration of precursors into nascent PG in the periplasm/extracellular space that surrounds bacteria. Although this pathway has been extensively studied there is still debate regarding which protein(s) are responsible for flipping the lipid linked precursor to the outer leaflet of the cytoplasmatic membrane. Several reports favour MurJ as the bona-fide flippase, despite experimental and biochemical evidence that support the involvement of FtsW or AmJ in lipid linked precursors flipping across the cytoplasmatic membrane. In this study we show that MurJ depletion arrests *Staphylococcus aureus* growth with a simultaneous increase of cell size that is not accompanied by cell elongation. Additionally, inhibiting MurJ activity results in a reduction of precursor incorporation into nascent PG and in the accumulation of lipid linked precursors in the cytoplasmatic membrane, most likely at its inner leaflet. Our observations suggest that MurJ is necessary for lipid linked precursors flipping in *S. aureus*.

Introduction

PG biosynthesis is conserved among Gram-positive and Gram-negative bacteria and occurs in three stages that are carried in different subcellular locations: 1) synthesis of a PG precursor in the cytoplasm; 2) attachment and modification of a lipid linked precursor at the inner leaflet of the cytoplasmatic membrane, which is followed by its flipping to the outer leaflet; 3) incorporation of the translocated PG precursor into the nascent PG that is part of the bacterial cell surface (see Figure 8 of Chapter I). Despite the extensive studies performed on

this essential bacterial pathway, there is still active debate regarding which protein(s) are responsible for flipping the lipid linked precursor to the outer leaflet of the cytoplasmatic membrane.

In vitro assays performed with *Escherichia coli* vesicles using lipid II labelled with 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) observed that Lipid II flipping is not spontaneous and requires the active transport of the PG precursor (van Dam et al. 2007). It was initially believed that FtsW, or its paralog RodA, which are members of the shape, elongation, division and sporulation (SEDS) protein family, were responsible for the flipping of the lipid-linked PG precursor (Höltje 1998; Matsuhashi 1994). However, a bioinformatic study suggested that the transporter of the lipid-linked precursor, could also be a protein of the mouse virulence factor family (MVF) and, member of the multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) exporter superfamily (Ruiz 2008). This study identified predicted inner membrane proteins that are present in *E. coli* and in several endosymbiotic Gram-negative bacteria that contain PG synthesis machinery and intersected this list with the genomes of bacteria that produce or not produce PG. A protein, initially identified as MviN and then renamed MurJ, was the only candidate membrane protein that was present in all bacteria that produce PG. MurJ was proposed to be the lipid II flippase (Ruiz 2008). Interestingly, the same study argued that FtsW was unlikely to be the flippase as it was present in the genome of bacteria that do not produce PG (Ruiz 2008). Furthermore, since attempts to delete *murJ* gene failed, it was proposed that *murJ* is essential due to being indispensable for PG synthesis (Ruiz 2008; Inoue et al. 2008; Meeske et al. 2015; Huber et al. 2009; Rudnick et al. 2001). Additionally, using an L-arabinose conditional mutant for *murJ*, Ruiz found that MurJ depletion in *E. coli* resulted in accumulation of Lipid Iinked and cytoplasmatic PG precursors, which reinforce the role of MurJ as the flippase of Lipid II (Ruiz 2008).

Nevertheless, Henrichfreise and colleagues observed the presence of lipid II biosynthesis pathway in PG-less bacteria, which counter argues against the premise used in the study by Ruiz (Henrichfreise et al. 2009).

On a different note, *in vitro* studies performed by Mohammadi and colleagues with *E. coli* vesicles showed that FtsW could flip Lipid II across the membrane (Mohammadi et al. 2011). Briefly, the authors used vancomycin and Lipid II derivatives, that were respectively labelled with tetramethylrhodamine cadaverine (TMR) and NBD, that were able to act as FRET pairs. With these tools, authors showed that over-expression of FtsW resulted in an increased TMR fluorescence with simultaneous NBD quenching. Since vancomycin, which cannot cross the cytoplasmatic membrane, binds Lipid II, these results indicated that upon over-expression of FtsW there was an increased flipping of Lipid II to the outer leaflet (Mohammadi et al. 2011). Moreover, it has also been reported that deletion of the four *murJ* homologs in *Bacillus subtilis* is not lethal nor did the bacteria mutant cells exhibit any morphological defects (Fay and Dworkin 2009). The authors have therefore proposed that MurJ is not the bona fide flippase and that another unidentified protein may compensate for its absence (Fay and Dworkin 2009).

More recently, Ruiz and colleagues have demonstrated MurJ flippase activity *in vivo* in *E. coli* cells. Briefly, authors used *E. coli* cells expressing a mutated version of MurJ protein, which can be inactivated by treatment with sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES), and colicin M (ColM), a polypeptide toxin that cleaves Lipid II into bactoprenol and 1-pyrophosphate-Mur*NAc*-Glc*NAc*-pentapeptide that is then released into the periplasm of bacteria. With these tools, the authors observed that blocking mutated MurJ activity, through the incubation of cells with MTSES, prevented cleavage of Lipid II by ColM. This was not observed when they used cells with mutated MurJ not treated with MTSES or wild-type cells. In these later cases, authors observed a ColM activity in

the periplasm (Sham et al. 2014). The authors further showed that depletion of FtsW did not prevent ColM activity and that, therefore, depletion of FtsW does not impair significantly the flipping of lipid linked precursors across the bacterial membrane.

However, a recent study by Meeske and colleagues found another protein that seem to have a Lipid II flipping activity (Meeske et al. 2015). These authors observed that a *B. subtilis* strain with a deletion of the four *murJ* homologs and six other MOP exporter superfamily proteins can still grow at a rate similar to a wild type strain. Then, by performing a synthetic lethality screening, these authors identified *amj* (renamed from *ydaH*) as an alternative flippase (Meeske et al. 2015). Meeske and colleagues observed that *amj* cannot be deleted in a mutant lacking *murJ* and that *amj* is upregulated in a *murJ* deletion mutant. It was also observed that expression of *amj* or *murJ* from *B. subtilis* could rescue a *murJ* mutant in *E. coli* and that these two proteins could flip Lipid II in an *E. coli in vivo* assay with ColM similar to the study performed by Ruiz and colleagues (Meeske et al. 2015).

Biochemical and structural analysis of the different proteins may help resolve the candidate that most likely has a major role in the flipping of lipid II, or other bactoprenol-linked PG precursors across the bacterial membrane. FtsW, MurJ and Amj are membrane proteins with 12, 14 and 6 transmembrane domains, respectively (Lara and Ayala 2002; Butler et al. 2013; Meeske et al. 2015). FtsW is a member of the SEDS family that includes other proteins such as the FtsW paralog RodA. Members of this SEDS family were shown recently to have a PG transglycosylase activity, which suggests that FtsW may have a role in incorporation of the precursor into nascent PG in the cell surface rather than flipping lipid linked precursors (Meeske et al. 2016; Emami et al. 2017; Taguchi et al. 2019). On the other hand, MurJ is a member of the MOP exporter superfamily and an early computational modelling of MurJ revealed a structure with a solvent exposed “V”-shaped cavity (Butler et al. 2013). It is possible that this predicted

structure may present some bias as all crystal structures available for proteins of the MOP exporter superfamily at the date of the study were of the Multidrug and Toxic Extrusion (MATE) class. Nevertheless, the proposed structure, with a solvent exposed "V"-shaped cavity, has been validated with PhoA-LacZα reporter fusions and with substituted cysteine accessibility methods (Butler et al. 2013). It is interesting that the "V"-shaped cavity of MATE proteins has been shown to alternate its conformation to "open" to the inner or outer side of the cytoplasmic membrane, binding and releasing cargo, respectively (Lu, Radchenko, et al. 2013; Lu, Symersky, et al. 2013; Tanaka et al. 2013). A recent study has also identified a "V"-shaped cavity in MurJ from *E. coli* and *Thermosipho africanus* with an inward-facing conformation (Kuk, Mashalidis, and Lee 2017) and a crystallography and sequence co-evolution study has showed that MurJ from *E. coli* can adopt an inward-open conformation and an outward-open conformation, supporting a rocker-switch model for Lipid II flipping (Zheng et al. 2018). More recently, it was reported an *in vivo* structure-guided cysteine crosslinking and proteolysis-coupled gel analysis of the conformational changes of MurJ in *E. coli* cells, which showed that MurJ can adopt both inward-facing and outward-facing conformations and, that absence of membrane potential favours the outward-facing state (Kumar et al. 2019). Since Amj does not have sequence similarity to either MOP or ABC-transporter families and is predicted to only have 6 transmembrane domains, against the 12 and 14 for FtsW and MurJ, respectively, it was suggested that Amj oligomerizes to form a channel for flipping Lipid II (Meeske et al. 2015).

In overall, the identity of protein(s) responsible for Lipid II flipping remains controversial. This debate has been further ignited by conflicting reports of *murJ* essentiality. While *murJ* seems to be essential in *E. coli*, in *B. subtilis* this doesn´t seem to be the case due to the redundant role of *amj*. Additionally, it is still not clear if FtsW can also flip Lipid II, either as the bona-fide flippase or as a redundancy mechanism. Interestingly, biochemical assays carried out in *E. coli* showed that

MurJ can form a complex with Lipid II with a higher affinity than FtsW (Bolla et al. 2018). The same study argues that considering the expected ratio of MurJ:FtsW:Lipid II (1.2:1:45) in bacterial cells, it is likely that MurJ binds all available lipid II (Bolla et al. 2018).

Nevertheless, the literature seems to currently favour MurJ as the bona-fide flippase. MurJ is essential in *S. aureus* and its inhibition leads to accumulation of PG biosynthesis precursors and to a defective cell wall (Huber et al. 2009; Mott et al. 2008). MurJ was recently shown to localize to the division septum and to be responsible for directing PG synthesis to midcell (Monteiro et al. 2018).

In this study, we present experimental evidence that clarified the identity of the flippase of lipid II PG precursor. We show that absence of MurJ leads to the accumulation of lipid linked precursors, with a simultaneous decrease of PG precursors incorporation into nascent PG and cell enlargement in *S. aureus*. Our data demonstrate that *in vivo* MurJ has a significant role in Lipid II flipping in *S. aureus*.

Results

MurJ depletion in *S. aureus* alters cell morphology and arrests cell growth

Since MurJ has been described as being essential in *E. coli* and that its deletion was reported to lead to significant alterations in cell morphology (Ruiz 2008; Inoue et al. 2008; Meeske et al. 2015; Huber et al. 2009; Rudnick et al. 2001), we decided to assess the effects of MurJ depletion in *S. aureus*. As MurJ was previously reported being essential in *S. aureus* (Huber et al. 2009; Mott et al. 2008), we constructed a conditional mutant, COLpMurJi, where the expression of MurJ was under the control of the IPTG-inducible P*spac* promoter in the native locus.

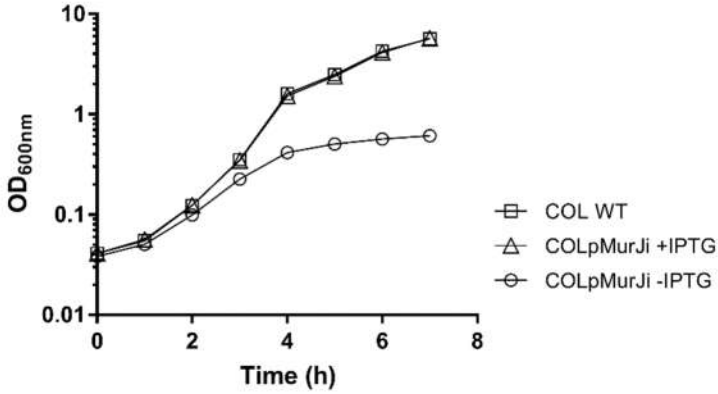

Figure 1 – Growth curve of *S. aureus* COL and its *murJ* conditional mutant in the presence or absence of inducer shows that under MurJ depletion cells arrest growth. Data is shown as mean of three independent experiments.

As seen in Figure 1, when the *murJ* conditional mutant was grown in the presence of inducer, we observed a growth curve that was similar to the wild type strain. On the other hand, in the absence of inducer, the conditional mutant seized growth prematurely. MurJ depletion also resulted in the enlargement of cells of approximately 30% of the normal cell volume without sustained cell elongation (cell asymmetry or formation of oblong cells) during the 3 cell cycle phases [Figure 2].

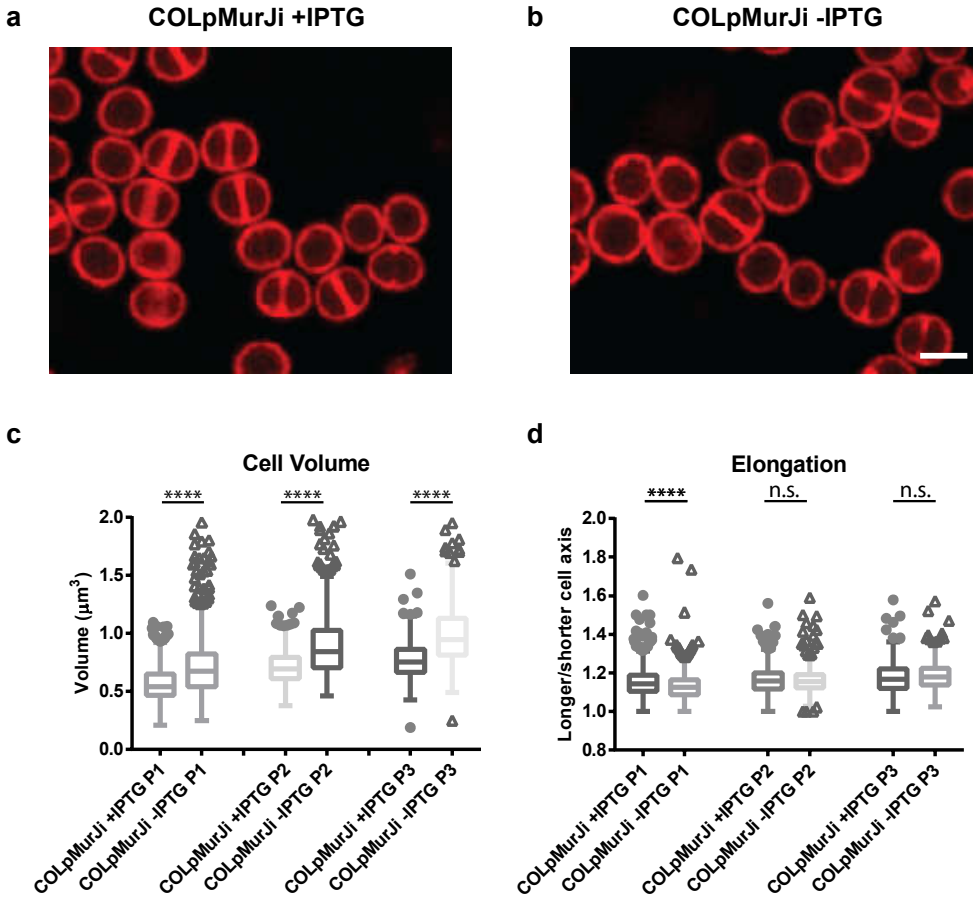

Figure 2 – Absence of MurJ results in the enlargement of *S. aureus* cells. Representative Structured Illumination Microscopy (SIM) images of *murJ* conditional mutant cells that were incubated with (a) and without (b) inducer and stained with membrane dye Nile Red are shown in top panels. Scale bar is 1 µm. Quantification of *murJ* conditional mutant cell volume (c) and (d) cell elongation measured for different *S. aureus* cells during each cell cycle show that depletion of MurJ results in cell enlargement without elongation. Data is represented as a Tukey graph of ≥300 cells.

MurJ depletion results in reduced incorporation of PG precursors and accumulation of lipid-linked precursors

To test the hypothesis that the observed reduced cell growth and increased cell volume upon MurJ depletion were due to the expected blockage of the PG synthesis, we assessed the incorporation of PG precursors into nascent PG

by measuring the incorporation of the fluorescent *D*-amino acid NADA (Kuru et al. 2012, 2015). As seen in Figure 3, depletion of MurJ results in a reduced NADA incorporation when compared with the cells grown in the presence of inducer. Despite the overall reduction of NADA incorporation into the bacterial cell surface, in both the septum and the peripheral cell wall, we were able to observe in these cells, a robust decrease of the fluorescence ratio (FR) determined between the division septum and the cell periphery. This highlights a higher reduction of septal incorporation of NADA, where most of the synthesis of the new PG takes place, than that observed into the peripheral cell wall.

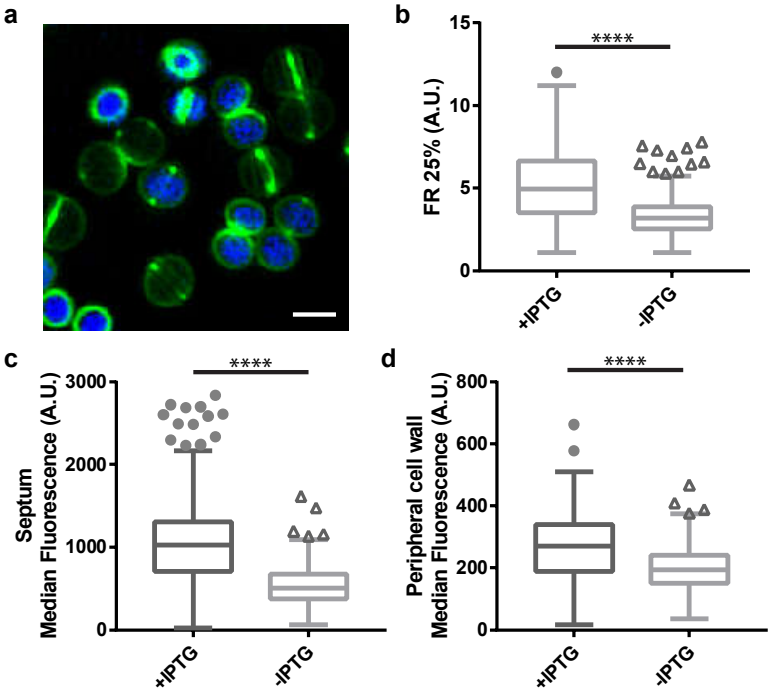

Figure 3 – Absence of MurJ results in a robust decrease of PG precursors into the recently synthesized PG. A) Representative SIM image of NADA incorporation in a *murJ* conditional mutant grown in presence of inducer (identified by having their chromosome stained with Hoechst 33342 DNA label) or absence of inducer (identified by lacking any blue stain in the cytoplasm). Scale bar is 1 µm. Quantification of the B) Fluorescence ratio (FR) between the NADA-dependent fluorescence intensity measured in C) the septal and D) peripheral cell wall suggest that upon MurJ depletion there is a decrease of NADA incorporation more robust in the division septum that in the peripheral cell surface. Data is represented as a Tukey graph of ≥200 cells.

In order to assess that absence of MurJ impairs the flipping of lipid linked precursors, and consequently, their incorporation into the PG macromolecule, we purified and analysed the lipid linked precursors produced in *S. aureus murJ* mutant cells in the presence and absence of inducer. We observed that depletion of MurJ resulted in the accumulation of lipid linked PG precursors [Figure 4 and Table 1] whose glycopeptide moiety, released after an acidic hydrolysis, seem to have a elution time similar to the elution time to the peak 1 and peak 5 of muropeptide samples, peak A and D, respectively (Jonge et al. 1992). Moreover, we also observed accumulation of a peak with an elution time of 3.6 minutes (Peak B) that had a mass compatible with a non-amidated Mur*NAc*-[*L*-Ala-*D*-Glu-*L*-Lys-*D*-Ala-*D*-Ala] glycopeptide and that the peak with a retention time of 5.6 minutes (peak D) has a mass compatible with a non-amidated Glc*NAc*-β(1-4)-Mur*NAc*-[*L*-Ala-*D*-Glu-*L*-Lys(Gly5)-*D*-Ala-*D*-Ala] glycopeptide. Absence of significant accumulation of lipid linked PG precursors on a COL wild type strain, propagated with and without the inducer, suggests that the peaks observed on the *murJ* conditional mutant are not an artefact of the extraction methods. The observation that upon MurJ depletion we have a reduced precursor incorporation into nascent PG and a simultaneous accumulation of lipid linked PG precursors suggest that MurJ is involved in flipping of lipid II.

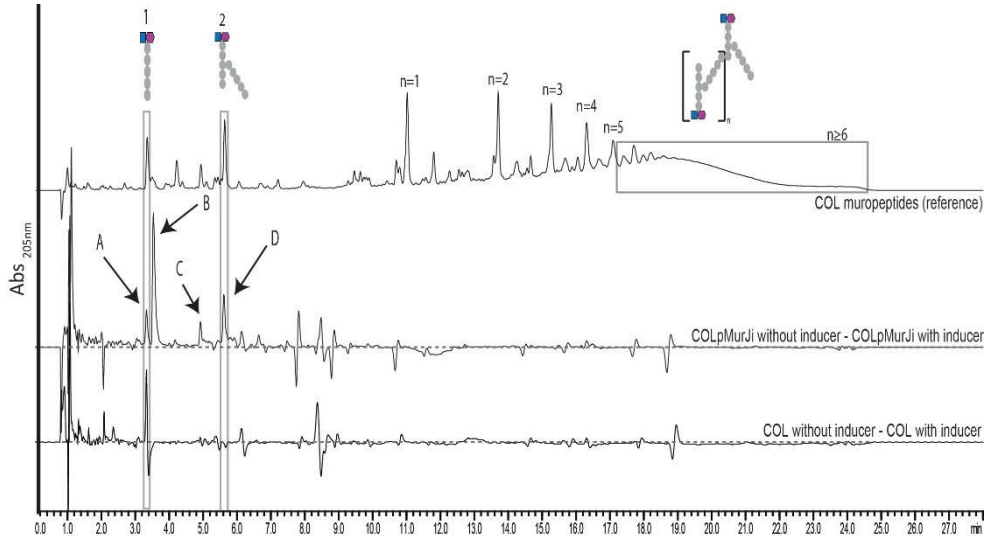

Figure 4 – Comparison of lipid linked PG precursors extracted from a COL *murJ* conditional mutant grown in presence of inducer or absence of inducer show accumulation of 4 peaks upon MurJ depletion suggest that MurJ is involved in flipping of lipid II precursors. Absence of significant peak accumulation in a COL wild type strain suggests that the peaks observed in the *murJ* conditional mutant are not an artefact of the extraction methods. Results are shown as the subtraction of induced sample profile to the depleted sample profile. The dashed line represents no difference between the two profiles and a positive peak represents a higher amount of the analyte on the MurJ depleted sample. Muropeptides of COL PG are shown as a reference. See Table 1 for peak identification. Data shown is representative of three independent extractions.

Table 1 – Identification of Lipid linked PG precursors peaks that are accumulated upon depletion of MurJ. See Figure 4 for Chromatogram.

Peak ID	Retention time /minutes	Retention time similar to muropeptide peak	Measured mass by MS analysis /Da	Predicted structure
A	3.3	1	-	Glc*NAc* β(1-4) Mur*NAc*-[*L*-Ala-*D*-Gln-*L*-Lys-*D*-Ala-*D*-Ala]
B	3.6	-	765.38	Mur*NAc*-[*L*-Ala-*D*-Glu-*L*-Lys-*D*-Ala-*D*-Ala]
C	4.9	-	-	-
D	5.6	5	1252.80	Glc*NAc* β(1-4) Mur*NAc*- [*L*-Ala-*D*-Glu-*L*-Lys (Gly5) -*D*-Ala-*D*-Ala]

MurJ inhibition reduces lipid linked PG precursors in the outer leaflet of the cytoplasmatic membrane

We observed that MurJ depletion in *S. aureus* bacteria results in a reduced precursor incorporation into nascent PG and a simultaneous accumulation of lipid linked PG precursors. However, if MurJ is responsible for flipping lipid linked precursors across the staphylococcal membrane, we should observe an accumulation of lipid linked PG precursors in the inner leaflet of the cytoplasmatic

membrane and not at the outer leaflet. To test the role of MurJ in flipping lipid linked precursors, we decided to use a antibacterial polypeptide, named colicin M (ColM), which was reported to be able to cleave lipid II into bactoprenol and 1-pyrophospho-Mur*NAc*(pentapeptide)-Glc*NAc* that is released into the *E. coli* periplasm (Sham et al. 2014).

Despite the observation that *E. coli* cells incubated with ColM displayed a growth inhibition halo in a disk diffusion assay, and a lower cell viability seen as a lower value of CFU counts and cell lysis by microscopy [Figure 5], *S. aureus* cells seems to be insensitive to ColM activity [Figure 5]. The same was also observed when we used a *S. aureus* strain that has an altered cell surface and a more exposed PG (*S. aureus tagO* null mutants or wild type cells grown with tunicamycin- Atilano et al. 2011) or with a less cross-linked PG (*S. aureus* Δ*pbpD* – Memmi et al. 2008).

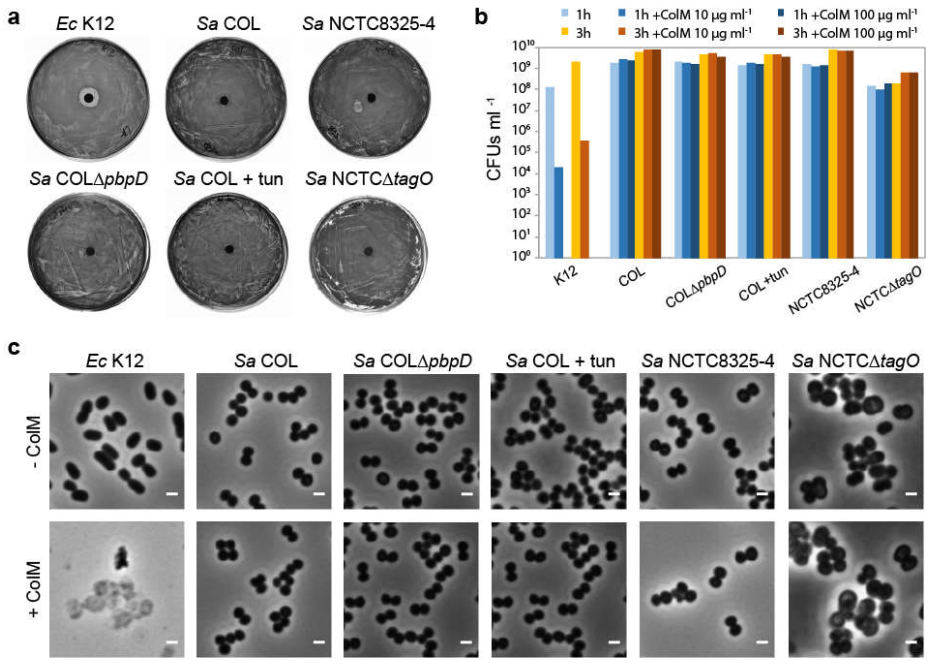

Figure 5 – *S. aureus* cells are insensitive to ColM activity. *E. coli* K12, *S. aureus* COL, *S. aureus* COL Δ*pbpD* (lower cross-linked PG), *S. aureus* NCTC 8325-4 and *S. aureus* without WTA (*S. aureus* 8325-4 Δ*tagO* and with 0.4 µg/mL tunicamycin) cells sensitivity to ColM was assessed by A) Disk diffusion assays with 50 µg of ColM impregnated into the disk; B) measuring Colony Forming Units (CFUs) per mL after 1h and 3h incubation with 10 µg/mL and 100 µg/mL of ColM and c) cell integrity by phase contrast microscopy.

Since vancomycin binds to lipid II and is unable to cross the cytoplasmic membrane (Mohammadi et al. 2011; Bolla et al. 2018), we used fluorescently labelled vancomycin (Van-FL) as an alternative tool to assess if accumulation of the lipid linked precursors occurs mainly in the outer or inner leaflet of the cytoplasmatic membrane. We believed that this approach would be successful as 1) vancomycin binds the terminal *D*-Ala-*D*-Ala amino acids of the peptidoglycan peptide moiety (Bolla et al. 2018), 2) it has been reported that *S. aureus* cells grown in media with an excessive amount of *D*-serine can incorporate *D*-serine, instead of *D*-Ala, into the terminal *D*-Ala-*D*-Ala (Jonge et al. 2002; Pereira et al. 2007) and, 3) *D*-Ala-*D*-Ser is a poor substrate to vancomycin. We expected that we would be able to use Van-FL labelling to assess incorporation of *D*-Ala-*D*-Ala or *D*-Ala-*D*-Ser

upon MurJ inhibition by CDFI [2-(2-Chlorophenyl)-3-[1-(2,3-dime- thylbenzyl)piperidin-4-yl]-5-fluoro-1H-in- dole] (Huber et al. 2009).

As seen in Figure 6, if *S. aureus* cells were grown in 0.125 M *D*-serine and then resuspended in fresh TSB, we were able to observe an increase of the binding intensity of Van-FL to the division septum, that is where the majority incorporation of new PG precursors with *D*-Ala-*D*-Ala is expected to take place. If the same procedure was repeated with cell that were resuspended in fresh TSB medium with an excess of 0.125 M *D*-serine, there was a low binding intensity of Van-FL to the surface of bacteria as a consequence of the continuing incorporation of *D*-Ala-*D*-Ser. Interestingly, cells that were incubated in fresh TSB medium but in the presence of molecules capable of blocking the MurJ activity [TSB with 8μg/mL CDFI, 2 fold the MIC (Huber et al. 2009)], presented a cell surface where we observed a low binding of Van-FL despite the absence of an excessive concentration of *D*-ser. This observation supports the model that blocking MurJ prevents precursor incorporation into nascent PG. Furthermore, we observed a decrease of the fluorescence ratio (FR) between the septum and the periphery upon MurJ inhibition, similarly to what was observed for NADA incorporation, that highlights a higher reduction of septal incorporation than peripheral cell wall incorporation.

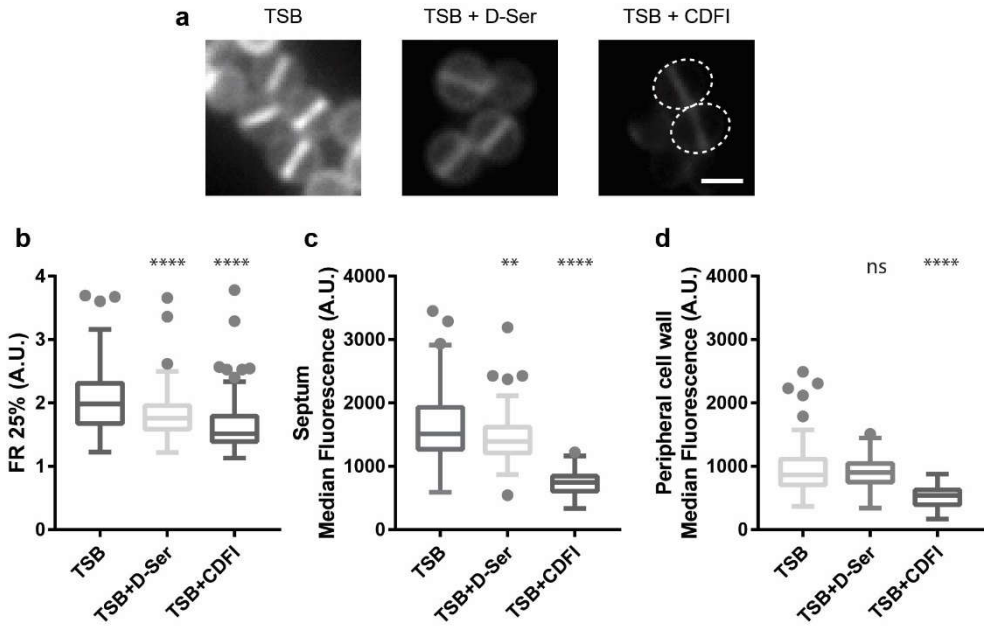

Figure 6 – Inhibition of MurJ by incubation of *S. aureus* bacteria in TSB with 8 μg/mL CDFI prevents incorporation of precursors into nascent PG. A) Epifluorescence imaging of Van-FL binding to COL cells grown in TSB with 0.125 M *D*-serine and incubated for 30 minutes with fresh TSB, TSB with 0.125 M *D*-serine or TSB with 8 μg/mL. Scale bar is 1 μm. In the lower panels, we are able to observe B) fluorescence ratio (FR) between the septal (C) and peripheral cell wall (D) fluorescence intensities that are a consequence of the binding of fluorescent vancomycin to the bacterial surface. Data is represented as a Tukey graph of ≥80 cells and is representative of an experiment performed in triplicate.

To reinforce the observations made with cells that have a high incorporation of *D*-Ser containing precursors into PG due to the excess of this aminoacid in the surrounding medium, we decided to use the unnatural dipeptide *D*-Ala-*D*-Pra (Sarkar et al. 2016). This unnatural dipeptide has an alkyne group that is able to covalently bind azide containing compounds in a copper-catalysed click-reaction (Sarkar et al. 2016) and, by using a permeable and an impermeable fluorescent probe containing azide groups, we should be able to ascertain if MurJ inhibition leads to accumulation of lipid linked precursors in the inner or the outer leaflet of the cytoplasmatic membrane.

We could observe binding of the impermeable probe (AF 488-Azide), a compound capable of binding to PGN stem peptides containing *D*-Ala-*D*-Pra, to

cells with a functional MurJ. In accordance with the role of MurJ flipping the lipid linked precursors across the membrane, inhibition of MurJ activity by incubation in TSB with 8 µg/mL CDFI resulted in a loss of fluorescence [Figure 7]. These observations strongly support the hypothesis that inhibition of MurJ results in an accumulation of lipid-linked precursors in the inner leaflet of the cytoplasmatic membrane and not in the outer leaflet. However, we observed the same results with a permeable probe (5-TAMRA Azide), precluding us from directly observe an accumulation of lipid linked precursors in the inner leaflet of the cytoplasmatic membrane (data not shown).

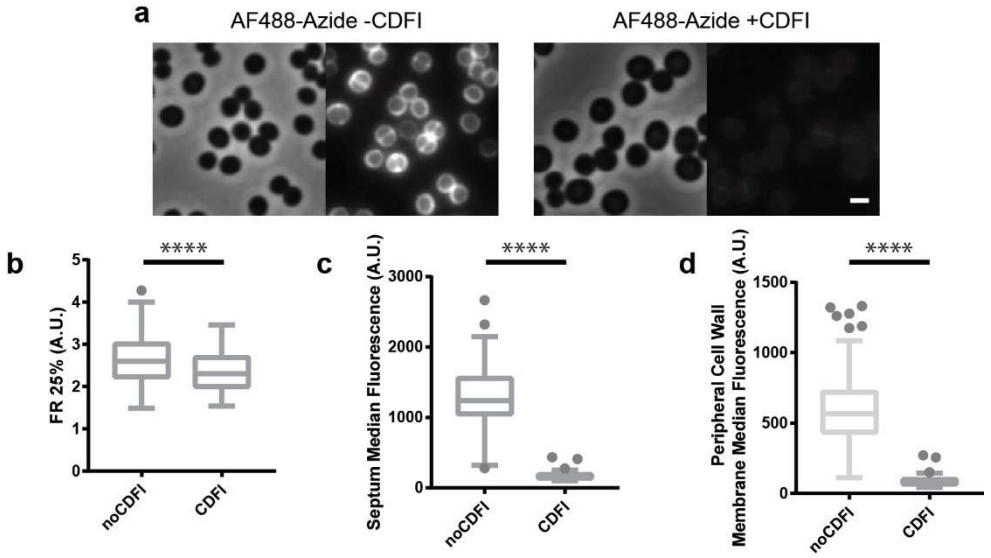

Figure 7 – MurJ inhibition by 8 µg/mL CDFI results in a reduction of lipid-linked precursors at the outer leaflet of the cytoplasmatic membrane. A) Phase contrast (left panel) and epifluorescence (right panel) representative images of COL cells incubated for 30 min with *D*-Ala-*D*-Pra unnatural dipeptide with a functional MurJ (without CDFI – left panel) or with a inhibited MurJ (with 8 µg/mL CDFI – right panel). These cells were further labelled with AF488-Azide impermeable fluorescent dye through a copper-catalysed click-reaction. Scale bar is 1 µm. Also shown in bottom panels are the B) fluorescence ratio (FR) between the sepal (C) and peripheral cell wall (D) fluorescence intensities. Data is represented as a Tukey graph of ≥100 cells and is representative of an experiment performed in triplicate.

Discussion

Although PG biosynthesis is conserved among Gram-positive and Gram-negative bacteria and has been extensively studied, there is still debate regarding which protein(s) are responsible for flipping of lipid linked PG precursors to the outer leaflet of the cytoplasmatic membrane. In overall, the literature currently available seems to favour MurJ as the bona-fide flippase and while in *E. coli murJ* seems to be essential, *amj* seems to provide a redundancy mechanism in *B. subtilis*. Additionally, it is still not clear if FtsW can also flip Lipid linked PG percursors, either as the bona-fide flippase or as a redundancy mechanism. In this study we assess if MurJ is the bona-fide flippase in *S. aureus*.

Similarly to what was reported in *E. coli* (Ruiz 2008; Inoue et al. 2008; Meeske et al. 2015; Huber et al. 2009; Rudnick et al. 2001), MurJ was reported to be essential in *S. aureus* (Huber et al. 2009; Mott et al. 2008). As such, to assess the effects of MurJ depletion in *S. aureus* we had to construct a conditional mutant with *murJ* expression under the control of the IPTG-inducible P*spac* promoter in the native locus (COLpMurJi).

MurJ depletion resulted in a premature arrest of cell growth [Figure 1] and in cells with approximately 30 % higher volume without measurable sustained elongation [Figure 2] in all 3 cell cycle phases. This observation is in accordance with previous reports that inhibiting lipid linked precursors flipping leads to cell morphology defects. However, contrary to previous reports in *E. coli* or *B. subtilis* we did not observed cell asymmetry [elongantion] (Sham et al. 2014; Huber et al. 2009; Mott et al. 2008; Ruiz 2008; Inoue et al. 2008; Meeske et al. 2015). This observation may be explained with the reduced level of elongation observed in *S. aureus* cell cycle. We were also able to observe that compromise of MurJ activity resulted in a reduced incorporation of PG precursors into nascent PG. This was successfully observed either through the reduction of NADA incorporation into the

bacterial cell surface [Figure 3] upon MurJ depletion or through the reduction of the fluorescence intensity of Van-FL bonded to the cell surface after MurJ activity inhibition by CDFI [Figure 6]. This is in accordance with previous reports that MurJ blockage in *E. coli* and *B. subtilis* results in a reduction of PG synthesis (Sham et al. 2014; Ruiz 2008; Inoue et al. 2008; Meeske et al. 2015). Interestingly, we observed a higher reduction of precursor incorporation at the septum, where most of the polymerization of the PG may take place, than at the peripheral cell wall [Figure 3 and Figure 6].

Additionally, we observed that depletion of MurJ also results in the accumulation of lipid linked PG precursors [Figure 4 and Table 1] with a retention time and/or molecular weight compatible with a Glc*NAc*-β(1-4)-Mur*NAc*-[*L*-Ala-*D*-Glu-*L*-Lys-*D*-Ala-*D*-Ala] (peak A), Mur*NAc*-[*L*-Ala-*D*-Glu-*L*-Lys-*D*-Ala-*D*-Ala] (peak B) or Glc*NAc*-β(1-4)-Mur*NAc*-[*L*-Ala-*D*-Glu-*L*-Lys(Gly5)-*D*-Ala-*D*-Ala] (peak D) glycopeptides. Interestingly, the two most accumulated molecules (Peak B and Peak D) correspond to lipid linked precursors that are non-amidated, suggesting that MurJ depletion leads to accumulation of precursors that were not subjected to GatD/MurT activity and may suggest that the amidation of the lipid linked PG precursors may take place in the outer leaflet of the bacterial membrane or that requires the presence of the MurJ enzyme. Additionally, we observed that peak B (Mur*NAc*-[*L*-Ala-*D*-Glu-*L*-Lys-*D*-Ala-*D*-Ala]) corresponding to the structure of a non-amidated Lipid I molecule was the lipid linked precursor with higher accumulation upon MurJ depletion. It is currently unclear if the observation of a higher accumulation of Lipid I than a Lipid II precursor reflects a role for MurJ in the activation of MurG, which converts Lipid I into Lipid II or an artefact that could have been originated from the extraction method. It is possible that MurJ depletion also leads to accumulation of cytoplasmatic precursors, and a lipid linked precursor preparation contaminated with cytoplasmatic precursors would be enriched in the structure predicted for peak B, since it is also compatible with the structure of the

park nucleotide with the UDP removed. Nevertheless, our results are in accordance with previous reports that have shown accumulation of PG precursors in *E. coli* upon inhibition of the MurJ activity (Sham et al. 2014; Ruiz 2008; Inoue et al. 2008; Meeske et al. 2015).

The reduced precursor incorporation into nascent PG, with a simultaneous accumulation of lipid linked PG precursors, suggests that MurJ is involved in flipping of PG lipid linked precursors. In accordance, we observed that inhibition of MurJ by CDFI resulted in a lower binding intensity of Van-FL. Considering that vancomycin cannot cross the cytoplasmatic membrane [Figure 6], it is likely that the lipid linked PG precursors are not accumulating at the outer leaflet of the cytoplasmatic membrane. However, this assay does not allow to assess if there was an accumulation of lipid linked PG precursors at the inner leaflet of the cytoplasmatic membrane directly. In order to clarify this question, an assay using the unnatural dipeptide *D*-Ala-*D*-Pra that can covalently bind azide groups and an impermeable fluorescent probe (AF 488-Azide) were used. The observed results reinforced the observation that inhibition of MurJ by CDFI resulted into a lower amount of lipid linked PG precursors on the outer cytoplasmatic membrane leaflet [Figure 7], however, since we were unable to label the inner lipid linked PG precursors with a permeable fluorescent probe (5-TAMRA Azide, data not shown), we could not directly observe an accumulation of lipid linked PG precursors at the inner leaflet of the cytoplasmatic membrane.

In overall , MurJ is necessary for maintaining a physiological cell size and growth of *S. aureus* bacteria and blocking its activity results in the reduction of precursor incorporation into nascent PG with the simultaneous accumulation of lipid linked PG precursors, which are located, most likely, on the inner leaflet of the cytoplasmatic membrane. Therefore, the data obtained in this study supports the hypothesis of MurJ being involved in lipid linked precursors flipping.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 2 and Table 3, respectively. *E. coli* strains were grown at 37ºC in Luria-Bertani (LB, Alfa Aesar) or Luria-Bertani agar (LA, Alfa Aesar) medium. *S. aureus strains* were grown at 37ºC in Tryptic Soy broth (TSB, Difco) or in Tryptic Soy agar (TSA, Difco) medium. When needed the medium was supplemented with ampicillin (Amp, 100 µg/mL, Apollo Scientific), erythromycin (Ery, 10 µg/mL, Apollo Scientific), Kanamycin (Kan, 25 µg/mL, Apollo Scientific), CDFI (8 µg/mL, gift from Merck), *D*-Serine (0.125 M, Sigma-Aldrich) or with Isopropyl *β-D*-1-thiogalactopyranoside (IPTG, 0.5 mM, Apollo Scientific).

Construction and propagation of a *S. aureus* strain with an inducible expression of MurJ

A DNA fragment containing the sequence that encodes the ribosome-binding site and the initial 636 bp of *S. aureus murJ* gene from COL strain was amplified by PCR using primers listed in Table 4. The resulting fragment was digested with *Hind*III/*Bam*HI restriction enzymes and cloned into pMUTIN4, generating plasmid pMutMurJi, which was electroporated into RN4220 strain. Transduction of this plasmid, using phage 80α, into COL generated strain COLpMurJi, which has the native *murJ* gene under the control of IPTG-inducible P*spac* promoter and a truncated *murJ* gene under the control of the native promoter.

COLpMurJi overnight cultures grown in the presence of 0.5 mM IPTG were back diluted to an OD_{600nm} of 0.1 and grown until an OD_{600nm} of approximately 1 in the presence of 0.5 mM IPTG. Cells were washed three times with fresh TSB, back

diluted to $OD_{600nm} \cong 0.05$ and grown in the presence or absence of 0.5 mM IPTG for 3h.

Table 2 – Strains used this study

Strain	Description	Source or Reference
E. coli K12	*E. coli* wt strain	
S. aureus COL	HA-MRSA strain	Gill et al. 2005
S. aureus COLpMurJi	COL with *murJ* gene under P*spac* control in the chromosome	This study
S. aureus COLΔ*pbpD*	COL *pbpD* (PBP4) null mutant	Memmi et al. 2008
S. aureus NCTC 8325-4	MSSA strain	
S. aureus NCTC 8325-4 Δ*tagO*	Obtained using NCTC 8325-4 as parental strain for *tagO* deletion	Atilano et al. 2010

Table 3 – Plasmids used in this study

Plasmid	Description	Source or Reference
pMutMurJi	*S. aureus* integrative vector including the IPTG-inducible P*spac* promoter and *lacI* gene; Amp^R; Ery^R	This study
pMLD23	pET2430 encoding His6-ColM. Kan^R	Barreteau et al. 2010

Table 4 – Primers used in this study.

Primer	Sequence 5'-3'
1804P1HindIII	cgcaagcttacatgagatagggagattcg
1804P2BamHI	ataggatccaataatcgcaccaactgctg

Bacterial growth curves

Overnight cultures of COLpMurJi grown with 0.5 mM IPTG were washed three times with fresh TSB and back diluted to OD_{600nm} of 0.02 in 20 mL TSB with or without 0.5 mM IPTG. OD_{600nm} measurements were taken every hour in an Amersham Biosciences Ultrospec Pro 2100 spectrophotometer. Growth curve was performed in triplicate.

Recombinant ColM protein purification

ColM purification was carried as described in Sham et al. 2014. Briefly, pMLD237 plasmid carrying the codifying sequence for the Histag-ColM protein was transformed into *E. coli* BL21(DE3) using kanamycin as selection marker. An overnight culture was back diluted to an $OD_{600nm} \cong 0.05$ in fresh LB with Kan (25 µg/mL) and incubated at 30ºC with agitation until an $OD_{600nm} \cong 0.5$ was achieved. At this point IPTG was added to a final 0.5mM concentration and the culture was incubated for 3 hours at 30ºC with agitation.

The cells were collected by centrifugation at 13.000 x g for 5 min at 4ºC, washed once and resuspended in buffer A [20 mM Tris-HCl pH 7.4, 500 mM NaCl] containing protease inhibitor cocktail III (EMD Millipore) and lysed by performing two runs on a French Press at 15000 psi. The lysate was clarified by repetitive centrifugation at 16.000 x g for 20 min at 4ºC and the supernatant was incubated with Ni-NTA agarose (Qiagen) at 4ºC with agitation for 60 minutes.

The resin was recovered by spin-down centrifugation, loaded into a gravity flow column (BioRad) and washed twice with 4 mL of buffer A, before eluting the protein with 2 mL of buffer B [20 mM Tris-HCl pH 7.4, 500 mM NaCl, 300 mM Imidazole]. Elution Solution was exchanged with Phosphate-buffered saline with 10% (v/v) glycerol [PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4] by overnight dialysis with a 10 kDa cut-off Dialysis tubing (SnakeSkin, Pierce). Purified proteins were aliquoted and stored at -20ºC.

ColM Antibacterial Activity Test assays

Disc Diffusion Assay: overnight cultures of *E. coli* and *S. aureus* were back diluted to an $OD_{600nm} \cong 0.05$ in fresh LB or TSB, respectively, and incubated at 37ºC with aeration until $OD_{600nm} \cong 0.6$. At this point, cells from 1 mL aliquots were collected by centrifugation at 16.000 x *g* for 5 min at room temperature, resuspended in fresh media and plated onto LA (*E. coli* strains), TSA or TSA with 0.4 µg/mL tunicamycin (*S. aureus* strains). Sterile 6mm-diameter paper discs (Quilaban) were placed on the agar surface and were loaded with 50 µg of purified ColM protein. A control with sterile water was also performed. Growth inhibition was observed after 24h of incubation at 37°C.

Colony Forming Units (CFUs) determination and cell imaging: overnight cultures of *E. coli* and *S. aureus* were back diluted to an $OD_{600nm} \cong 0.05$ in fresh LB or TSB, respectively, and incubated at 37ºC with aeration until $OD_{600nm} \cong 0.6$. At this point the cultures were divided and ColM was added to a final concentration of 0 µg/mL, 10 µg/mL or 100 µg/mL. The cultures were incubated at 37ºC with aeration for 1 hour and 3 hours, after which a 5 µL aliquot was applied in a 1.2% (w/v) agarose in PBS patch prior to observation by phase contrast microscopy. In addition, a 100 µL aliquot from a 10^{-5} dilution or a 10^{-6} dilution (*S. aureus* strains) or a 10^{-2} dilution, 10^{-5} dilution or 10^{-6} dilution (*E. coli* strains) were plated on TSA or

TSA with tunicamycin 0.4 µg/mL plates (*S. aureus* strains) or LA plates (*E. coli* strains). Values of CFUs/mL were determined after 24h. Images were acquired in a Zeiss Axio Observer Z1 microscope with a Plan-Apochromat 100×/1.4 oil Ph3 objective and a Retiga R1 CCD camera (QImaging) using Metamorph 7.5 software (Molecular Devices).

Purification of muropeptides and analysis by RP-HPLC

S. aureus CW and PG preparation were obtained as described in Jonge et al., 1992. Briefly, an overnight culture of the strain of interest was back diluted to an OD_{600nm} of ≅ 0.001 and incubated at appropriate conditions until an OD_{600nm} between 0.5 and 0.9 was reached. A high dilution of the overnight culture together with a growth not surpassing the exponential phase is used to maximize the amount of cells actively growing. Upon reaching the desired OD_{600nm} the culture was quickly chilled by incubation in an ice/ethanol bath before collecting the cells by centrifugation at 13.000 x g for 5 min at 4ºC. The pellet was resuspended in ice cold Milli-Q water and added drop by drop to boiling sodium dodecyl sulfate (SDS, Sigma-Aldrich) to a final concentration of 4% (w/v) SDS. The resulting solution was boiled for 30 minutes to promote cellular membrane disruption and inactivation of metabolic enzymes that may modify/degrade the CW/PG (autolysins). SDS was washed-off by repetitive centrifugation of the sample in fresh warm Milli-Q water until no SDS could be detected by the Hayashi method (Hayashi 1975). Cells present in SDS-free bacterial pellets were disintegrated by exposing them to 10 cycles of 45 seconds at 6 RPM in a FastPrep-24 5G (MP biomedicals) homogenizer with acid washed glass beads (Sigma-Aldrich, ≤106 µm) as abrasive. Samples were clarified of DNA, RNA and contaminant proteins by treatment with RNase (50 µg/mL, Sigma-Aldrich) and DNase (10 µg/mL, Sigma-Aldrich) in 50 mM Tris-HCl pH 7.5 20 mM $MgSO_4$ and Trypsin (100 µL/mL, Sigma-Aldrich) in 50 mM Tris-HCl pH

7.5 20 mM $MgSO_4$ 10 mM $CaCl_2$. The hydrolytic enzymes were inactivated by boiling in 1% (w/v) SDS and the clarified sample was washed twice with Milli-Q water, once with 8M LiCl (VWR), once with 100 mM Ethylenediamine tetraacetic acid (EDTA, VWR) pH 7.0, once with acetone (Sigma-Aldrich) and then twice with Milli-Q water to remove SDS, ionically bonded material and endotoxins. The resulting sample, purified CW, was lyophilized and quantified by weight or monosaccharide content (see below).

Pure PG samples were obtained by removing the WTA by incubating CW in a solution of Hydrofluoric Acid (HF, Sigma-Aldrich 48% (w/v)) at 4ºC for 48 hours. HF was washed off by repetitive centrifugation with cold 100 mM Tris-HCl pH 7.0. The resulting sample was lyophilized and quantified by weight or monosaccharide content (see below).

Non-reduced muropeptides were obtained by digesting pure PG samples with mutanolysin (0.2 mg/mL, from *Streptomyces globisporus* ATCC 21553, Sigma-Aldrich) in 12.5 mM $NaPO_4$ pH 5.5 buffer overnight at 37ºC and agitation. Muropeptides were reduced by incubation with fresh $NaBH_4$ (3.125 mg/mL) in 0.25 M Borate pH 9.0 buffer for 2 hours at room temperature. The reduction reaction was stopped by lowering the pH to ≤ 2.0 with H_3PO_4.

Reduced muropeptides were chromatographically separated by Reversed Phase Ultra-High-Performance Liquid Chromatography (RP-UHPLC) using a Waters Acquity CSH C18 1.7µm 2.1 mm x 100 mm UHPLC column coupled with a Waters VGuard Acquity CSH C18 1.7µm 2.1mm x 5 mm guard column in a Shimazu Nexera-i LC-2040C 3D UHPLC. Reduced muropeptides were resolved in a linear gradient from 5 % (v/v) methanol to 24% (v/v) methanol in 100 mM $NaPO_4$ pH 2.0 buffer over 21.5 minutes followed by a washing step at 24% (v/v) methanol over 0.7 minutes at 0.3 mL/min and 52 ºC. Elution of muropeptides was recorded by monitoring Abs_{205nm}. Muropeptide species were quantified with the peak area as

percentage of total chromatogram area with Shimadzu LabSolutions v5.92 software. Data shown is average of 3 replicates.

Extraction of lipid linked PGN precursors and UHPLC analysis

COLpMurJi was grown as described above. COL overnight cultures were back diluted in fresh TSB to an OD_{600nm} of ≅ 0.05 and incubated at 37ºC with aeration until an OD_{600nm} of 0.6-0.8 was achieved. Lipid linked PG precursors were purified as previously described in Qiao et al. 2014. Briefly, the cells were harvested by centrifugation at 16.000 x g for 5 min at room temperature, resuspended in a 1:1:2 mixture of PBS:$CHCl_3$:MeOH and broken in a FastPrep-24 5G (MP biomedicals) homogenizer with acid washed glass beads (Sigma-Aldrich, ≤106 µm) as abrasive for 3 sets of 2 cycles of 45 seconds at 4.5 RPM with a 5-minute interval on ice between sets. Cell debris were removed by centrifugation at 4000 x g for 10 min at 4ºC. The resulting sample was added to a mixture of 2 mL of $CHCl_3$ and 1.5 mL PBS and vortexed for 10 minutes to allow partition. To ease phase separation the samples were centrifuged for 10 minutes at 4000 x g at room temperature. The interface and organic phase fractions (containing the lipid linked PG precursors) were collected and evaporated to dryness in a speedvac at 30ºC.

The lipid linked PG precursors extracts were resuspended in 200 µL of Milli-Q H_2O and homogenized by performing one cycle of 45 seconds at 4.5 RPM in a FastPrep-24™ 5G Homogeniser. Samples were hydrolysed to a muropeptide-like structure by incubation in 0.1 M HCl for 15 minutes at 95ºC without shaking. This step hydrolyses the pyrophosphate bond that connects bactoprenol to the glycopeptide moiety. The resulting samples were reduced by incubation with fresh $NaBH_4$ (3.125 mg/mL) in 0.25 M Borate pH 9.0 buffer for 2 hours at room temperature. The reduction reaction was stopped by lowering the pH to ≤ 2.0 with H_3PO_4.

The resulting samples were chromatographically separated by RP-UHPLC and compared to the profile of *S. aureus* COL muropeptides using the method described for muropeptide analysis above.

Alternatively, the samples were chromatographically separated by RP-HPLC using two Merck Chromolith Performance RP8 endcapped 4.6 mm x 100 mm monolithic columns in tandem in a Shimazu Nexera-i LC-2040C 3D UHPLC for manual peak collection before MS-analysis. Reduced muropeptides were resolved in a linear gradient from 0 % (v/v) acetonitrile to 15 % (v/v) acetonitrile in 0.01 % (v/v) trifluoroacetic acid over 58 minutes followed by a washing step at 50% (v/v) acetonitrile over 6 minutes at 1 mL/min and 30 ºC. Elution of muropeptides was recorded by monitoring Abs_{205nm}. The samples of interest were lyophilized and resuspended in Milli-Q water before UHPLC-MS analysis in a MicroLC-MS Eksigent LC4500 coupled to tripleTOF 6600.

S. aureus imaging by fluorescence microscopy

Cell size measurements: COLpMurJi strain was grown as described above and a 1 mL aliquot was labelled with Nile Red membrane dye (5 µg/mL in TSB, Invitrogen) for 5 minutes at 37ºC. After washing the sample with PBS by centrifugation at 16.000 x *g* at room temperature a 5 µL aliquot was applied in a 1.2% (w/v) agarose in PBS patch prior to observation by Structured Illumination Microscopy (SIM) performed on an Elyra PS.1 microscope (Zeiss) with a Plan-Apochromat 63x/1.4 oil DIC M27 objective. SIM images were acquired using five grid rotations, with 34 µm grating period for the 561 nm laser (100 mW), 28 µm period for 488 nm laser (100 mW) and 23 µm period for 405 nm laser (50 mW) and captured using a Pco.edge 5.5 camera. Images were reconstructed using ZEN software (black edition, 2012, version 8.1.0.484) based on a structured illumination algorithm (Heintzmann, R. & Cremer 1999).

Peptidoglycan incorporation localization was performed by evaluating fluorescent *D*-amino acid NADA incorporation (Kuru et al. 2012, 2015): COLpMurJi was grown as described above and a 1 mL aliquot was labelled with 250 µM of NADA for 5 minutes at 37ºC. To allow comparison of absolute fluorescence of incorporated NADA in two different samples in the same microscopy image, one of the samples was previously labeled with DNA dye Hoechst 33342 (1 µg/mL, Invitrogen). After washing the samples with PBS by centrifugation at 16.000 x g at room temperature, the samples with and without Hoechst labelling were mixed in a 1:1 ratio and a 5 µL aliquot was applied in a 1.2% (w/v) agarose in PBS patch prior to observation by SIM (see above) or epifluorescence microscopy in a Zeiss Axio Observer Z1 microscope with a Plan-Apochromat 100×/1.4 oil Ph3 objective. Images were acquired using a Retiga R1 CCD camera (QImaging) using Metamorph 7.5 software (Molecular Devices).

Labelling recently flipped lipid II: *S. aureus* COL culture grown overnight in TSB with an excess of *D*-serine (0.125 M) were back diluted to OD_{600nm} of ≅ 0.1 in TSB with 0.125 M of *D*-serine and incubated at 37ºC with aeration until OD_{600nm} of ≅ 0.5 was achieved. The cells in 1 mL aliquots were collected by centrifugation at 16.000 x g at room temperature and resuspended in 1mL of TSB, 1 mL of 0.125 M *D*-serine in TSB or 1 mL of 8 µg/mL CDFI in TSB. The samples were incubated at 37ºC with aeration for 30 minutes, labelled for 5 min with 0.8 µg/mL of an equal volume mixture of vancomycin (Sigma) and BODIPY FL conjugate of vancomycin (Van-FL, Molecular Probes) and a 5 µL aliquot was applied in a 1.2% (w/v) agarose in PBS patch prior to observation by epifluorescence microscopy (see above).

Copper-catalysed click-reaction: An overnight cultures of *S. aureus* COL was back diluted to an OD_{600nm} ≅ 0.05 in fresh TSB and incubated at 37ºC with aeration until OD_{600nm} ≅ 1.2. At this point the culture was split in two and incubated 30 minutes at 37ºC with aeration in 4 mM of the unnatural *D*-Ala-*D*-Pra dipeptide 2 (Sarkar et al. 2016) in TSB with or without 8 µg/mL of CDFI. Aliquots of 1 mL were

washed 3 times with PBS and incubated in 0.2 mL click-reaction solution [1 mM $CuSO_4.5_H2O$, 250 µM Tris(3-hydroxypropyltriazolylmethyl) amine (THPTA), 1.2 mM Ascorbic Acid; SigmaAldrich] with 100 µM of Alexa Fluor 488-azide (Jena Bioscience) probe for 15 min at 37ºC with agitation. The samples were washed with PBS and a 5 µL aliquot was applied in a 1.2% (w/v) agarose in PBS patch prior to observation by epifluorescence microscopy (see above). The aliquots from the culture with 8 µg/mL of CDFI were washed with PBS and incubated in a click-reaction solution with 8 µg/mL of CDFI.

Microscopy Image Analysis: Cell volume and elongation, septum median fluorescence and peripheral cell wall median fluorescence were measured using our in-house software, eHooke, as previously described in Monteiro et al. 2018. Fluorescence ratio (FR) of septal to membrane signal was measured by eHooke taking in consideration only the 25% brightest pixels. Data is plotted as a Tukey box and whiskers graph with boxes showing the 25^{th}, median, and 75^{th} percentiles; Whiskers are draw at 1.5 times the interquartile range from either the 25^{th} or the 75^{th} percentile; Data points outside the 1.5 times interquartile range are considered an outlier and shown as a point in the graph.

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Chapter V

General Discussion and Conclusions

The interplay between pathogens and the immune system is a complex subject that has been extensively studied. While the host employs several mechanisms that aim at detecting invading organisms, such as pathogens, and at triggering a response that restore homeostasis, pathogens can employ virulence factors that will promote their survival within the host. Part of this interplay relies in the detection of conserved microbial structures, known as Microbe Associated Molecular Patterns (MAMP), from invading microorganisms by host Pattern Recognition receptors (PRR) (Mackey and McFall 2006; Janeway and Medzhitov 2002; Sansonetti 2006). In this work we used *Drosophila melanogaster* Peptidoglycan Recognition Proteins (PGRP) as probes to unveil bacterial mechanisms that can impair peptidoglycan (PG) recognition.

Accessibility of Peptidoglycan is Important for Recognition of Gram-positive Bacteria

The current model of *Drosophila melanogaster* innate immunity proposes that *Drosophila* flies induce a tailored humoral response to the bacteria that are infecting these invertebrate organisms. It further proposes that while bacteria with Lysine (Lys) type PG (frequently found in Gram-positive bacteria) are recognized by PGRP-SA, which will induce the Toll pathway, bacteria with diaminopimelic acid (DAP) type PG (found in Gram-negative bacteria and in some Gram-positive *bacilli*) are recognized by PGRP-LC or PGRP-LE, which will induce the Imd pathway (Lemaitre and Hoffmann 2007) [See Figure 11 of Chapter I]. However, several biochemical studies have reported that both PGRP-SA and PGRP-LC binding is not restricted to the PG of a particular type (Lys type or DAP type PG, respectively) (Chang et al. 2004; Mellroth et al. 2005). This divergence between the established model based in the bacterial (PG) discrimination by *Drosophila* PGRPs and observed binding abilities of PGRPs, led us to question which factor(s) are involved in PG recognition by different PGRPs that will:

I) influence the PGRPs ability to bind PG at the surface of a bacterial cell;

II) what are the factors involved in evolving a binding event to a successful trigger of a signalling pathway;

III) which bacterial factors modulate binding of PGRPs to the PGN at the bacterial cell surface.

In this work we propose that the current model of *Drosophila* humoral immune response may oversimplify the mechanisms used to discriminate different bacteria. The current model reduces the variability found in the composition, organization and shape of different bacteria to the PG chemotype, i.e., it reduces the discrimination to whether a cell produces a PG with a Lys or a DAP residue on the third position of the stem peptide. Bacterial cell wall architecture and composition shows a plethora of variations other than PG chemotype (Vollmer 2008; Vollmer, Blanot, and De Pedro 2008; Schleifer and Kandler 1972). Since it has been reported that WTA can prevent binding of PGRP-SA to the PG at the surface of *Staphylococcus aureus* cells (Magda L. Atilano et al. 2011) and prevent antibody binding to epitopes within the cell wall of *S. aureus* (Gautam et al. 2015) we tested the hypothesis that accessibility of peptidoglycan is important for the recognition of bacteria, regardless of PG chemotype. To this end, we used *S. aureus* and *Bacillus subtilis* as model organisms for bacteria with a surface harbouring a Lys or amidated DAP (amiDAP) PG, respectively. Since both organisms are Gram-positive bacteria, they produce a CW with a similar architecture, e.g. not concealed by an outer membrane, permitting us to limit the influence of other factor(s) when comparing the binding of PGRPs to the cell surface produced by these organisms. We carried out co-precipitation assays in a buffer that was designed to mimic the known composition of *Drosophila* haemolymph (HemoBuffer) to show that PGRP-SA does not have a binding preference for either Lys type or AmiDAP type PG in *vitro,* an observation that contradicts the current model of bacteria discrimination by PGRPs. Furthermore, using fluorescence microscopy, we also observed that fluorescent derivatives of either PGRP-SA or PGRP-LC can bind to the surface of either *S. aureus* or *B. subtilis* bacteria that lack WTA when resuspended in HemoBuffer. This is in accordance with previously reported

biochemical studies that have shown binding of either PGRP-SA or PGRP-LC to purified Lys type or DAP type PG (Chang et al. 2004; Mellroth et al. 2005). These observations reinforce the possibility that both PGRP-SA and PGRP-LC can bind to the surface of bacteria with either PG chemotype and, reinforces the importance of questioning about which factor(s) influence the PGRPs ability to bind PG at the surface of a bacterial cell and what are the factor(s) involved in evolving a binding event to a successful trigger of an immune pathway.

In collaboration with Prof. Petros Ligoxygakis from the University of Oxford, we have reported that *Drosophila* immune response to *S. aureus* and *B. subtilis* strains lacking WTA is mediated by both PGRP-SA and PGRP-LC (Vaz et al. 2019). These results indicate that accessibility to PG is important for recognition of Gram-positive bacteria and that accessibility may play an important role in the discrimination between classes of bacterial pathogens. Altogether, these observations challenge the current dichotomy present in the model of bacteria discrimination by *Drosophila* PGRPs to be re-evaluated to assess possible cross-play between the two pathways in mounting an effective humoral response to an evading bacterium.

SagB contributes to a bacterial cell surface with a PG as a worse (less exposed) substrate to host immune receptors

Previous studies have highlighted that *S. aureus* MSSA NCTC 8325-4 strain can prevent recognition of PG at its surfasce by host innate immune receptors by coating it with wall teichoic acids (WTA) and/or by trimming the excessive/exposed PG through the activity of the secreted Atl, an autolytic enzyme with *N*-acetyl-glucosaminidase and *N*-Acetylmuramyl-*L*-alanine amidase activities. Furthermore, NCTC 8325-4 mutants that have these mechanisms impaired showed a reduced virulence in a *Drosophila* infection model (Magda L. Atilano et al. 2011; Magda L Atilano et al. 2014). The role of these

mechanisms in other infection models is a subject of current research. McCarthy and colleagues have shown that in a CA-MRSA USA300 LAC derivative JE2 strain Atl was redundant for virulence in a murine device-related infection model (McCarthy et al. 2016). This observation rises the hypothesis that more clinically relevant *S. aureus* strains, or those that express resistance to beta-lactams due to the presence of an extra enzyme capable of synthesize PG in the presence of these antibiotics, may have alternative mechanisms to prevent recognition of their PG and/or that the mechanisms unveiled in a particular strain may not be as relevant in a different strain. The genetic variation of the staphylococcal accessory genome, which includes the set of genes not presence in all strains, further expands the possibility that different strains may employ a specific set of mechanisms to evade PG recognition.

In this work, we concluded that *S. aureus* JE2 strain presents a cell surface with a PG that is a worse (less exposed) substrate to fluorescent derivatives of host immune PG receptors, such as of *Drosophila* PGRPs, than the *S. aureus* NCTC 8325-4 strain. This was observed as a bias toward a lower binding intensity of mCherry_PGRP-SA to the cell surface of the JE2 strain when compared to a NCTC 8325-4 strain. Since a JE2 *tagO* deletion mutant (without WTA) also showed a lower binding than the NCTC 8325-4 *tagO* deletion mutant, it was unlikely that the bias towards a lower binding observed in the JE2 strain was due to a significantly difference in the amount or composition of the WTA produced by the JE2 strain.

We then observed that the JE2 strain seems to produce a peptidoglycan hydrolase in higher amounts, or with an higher activity, than the NCTC8325-4 strain. This peptidoglycan hydrolase activity was shown to be dependent on the presence of SagB. The role of SagB in the mechanism that produces a cell surface with a less recognizable PG was highlighted by the observation that a deletion of *sagB* in the JE2 genetic background produced a cell surface that is better recognized by mCherry_PGRP-SA. The observation that deletion of *sagB* in the JE2 *atl* deletion mutant produces a much stronger phenotype suggests that both Atl and SagB contribute to the production of a

cell surface with a less recognizable PG. Moreover, the importance of SagB in the JE2 strain is highlighted by the observation that the NCTC 8325-4 *atl* deletion mutant and the JE2 *atl sagB* deletion mutant produce a cell surface that is equally recognizable by mCherry_PGRP-SA. It is therefore possible that SagB is responsible for the Atl redundancy as a virulence factor in the JE2 strain previously described in a murine device-related infection model (McCarthy et al. 2016). Although we did not test if a strain that is unable to produce SagB in the presence or absence of Atl is less virulent in the *Drosophila* or murine infection models, it would be interesting to test this hypothesis to reinforce the hypotheses that SagB is a staphylococcal virulence factor, and it may have redundancy with Atl *in vivo*.

Despite the observation that lack of SagB in *S. aureus* results in the production of PG with an altered architecture/composition, we could not assess if these alterations, which resulted in the production of a PG with lower amounts of cross-linked muropeptides and with glycan chains of increased size, produce a better substrate to host immune receptors. It would be interesting to test this hypothesis. Since previous studies have shown that a *S. aureus* NCTC 8325-4 strain with deletion of *pbp4*, and therefore, a lower cross-linked PG do not produce a cell wall that is better recognizable by PGRP-SA (Magda L. Atilano et al. 2011), it is conceivable the hypothesis that SagB influences PGRP-SA binding to PG through the modulation of the PG glycan chain size. It is also possible that SagB reduces PGRP binding to *S. aureus* cells surface by trimming the exposed PG, in a similar manner to that previously proposed for Atl. The observation that a deletion mutant lacking *sagB* produces a rougher CW, as seen by electron microscopy (Chan et al. 2016) similarly to the phenotype observed for a mutant lacking Atl (Takahashi et al. 2002; Foster 1995) reinforces the hypothesis that SagB may trim the exposed/excessive PG. It should be highlighted that it is currently unknown whether SagB and Atl target the same PG at the cell surface.

On a different note, we should also take in consideration that the primary role of SagB cannot be reduced to its contribution to the concealment of PG at the cell surface

produced by *S. aureus* bacteria. This PG hydrolase may be part of a complex structure that coordinates PG synthesis with the PG degradation, which is of vital importance to the normal propagation of bacteria. Since SagB is needed to produce a PG with a physiological architecture it would be interesting to assess its activity and localization during the cell cycle and, probably more interestingly, if SagB interacts with the PG synthesis machinery. It is of interest the results acquired through the analysis of the PG glycan chains produced in *S. aureus* mutants that lack SagB. It has been reported that *S. aureus* glycan chains have a characteristic "hedge-hog" chromatographic profile, with the major peaks being polymers of a Glc*NAc*-β-1-4-Mur*NAc* disaccharide (Boneca et al. 2000). We observed that strains lacking *sagB* in their chromosome produce longer glycan chains that fail to resemble the profile observed in the parental strain and show a clear dominance by long polymers of a Glc*NAc*-β-1-4-Mur*NAc* disaccharide.

If SagB is responsible for cleaving glycan chains to their physiological size, as proposed by both Chan and colleagues and Wheeler and colleagues (Chan et al. 2016; Wheeler et al. 2015), to produce glycan chains with a Mur*NAc* residue in the reducing end it is expected that either SagB needs to have *N-acetyl*-muramidase activity, or that the products of SagB are further processed by a *N-acetyl*-muramidase. This is not in accordance with the proposed glucosaminidase activity for SagB (Wheeler et al. 2015; Chan et al. 2016). Therefore, considering that Mihelič et al has observed that enzymes with *N*-acetyl-glucosaminidase and *N-acetyl*-muramidase activity have a very similar structure (Mihelič et al. 2017), it would be interesting to assess if SagB (*Staphylococcus aureus* glucosaminidase B) was misnamed and have *N-acetyl*-muramidase activity. This finding would be particularly interesting, since to date, to the better of our knowledge no enzyme with *N-acetyl*-muramidase activity has been identified in *S. aureus*. Unfortunately, this was not possible during the work associated with this thesis since we were unable of producing a recombinant SagB protein with enzymatic activity. We could neither assess if the hydrolytic band observed in zymography assays is the direct result

of SagB enzymatic activity nor that SagB has *N*-acetyl-glucosaminidase or *N-acetyl*-muramidase activity.

Flipping of lipid-linked PG precursors is dependent on MurJ

Although PG composition and architecture can vary, as discussed above, its biosynthesis pathway is conserved among Gram-positive and Gram-negative bacteria. Even though the PG biosynthesis pathway has been extensively studied there is still debate regarding which protein(s) are responsible for flipping the lipid linked precursor(s) to the outer leaflet of the cytoplasmatic membrane (Mohammadi et al. 2011; Ruiz 2008). The flipping of the PG precursor is an event of great importance in the cell cycle of a bacterial organism. It takes place at a specific sub-cellular localization that is efficiently concealed from outside recognition (Pereira et al. 2007).

Most reports recently published seem to favour MurJ as the bona-fide flippase, however there is evidence supporting that FtsW or AmJ can also be involved in the flipping of lipid linked precursors across the cytoplasmatic membrane (Meeske et al. 2015). In this work we observed that MurJ depletion arrests *S. aureus* growth with a simultaneous increase of cell size without cell elongation. Additionally, inhibition of the MurJ activity results in a reduction of precursor incorporation into nascent PG with accumulation of lipid linked precursors most likely at the inner leaflet of the cytoplasmatic membrane. Unfortunately, we were unable to directly observe accumulation of lipid-linked PG precursors at the inner leaflet of the cytoplasmatic membrane but remarkably, preliminary data with FtsW depletion showed no accumulation of lipid-linked PG precursors (data not shown), therefore, suggesting that MurJ is necessary for flipping lipid linked precursors in *S. aureus*.

Analysis of the two most accumulated peaks by MS revealed that the accumulated lipid-linked precursors had a mass compatible with a Mur*NAc*-[*L*-Ala-*D*-Glu-*L*-Lys-*D*-Ala-*D*-Ala] structure, a lipid I precursor, and a Glc*NAcβ*-(1-4)-Mur*NAc*-[*L*-Ala-*D*-

Glu-*L*-Lys(Gly5)-*D*-Ala-*D*-Ala structure, a lipid II precursor. Interestingly both these structures harbour a non-amidated glutamic acid, suggesting that the accumulated lipid linked precursors had not been processed by the MurT/GatD complex (Figueiredo et al. 2012; Münch et al. 2012). It would be noteworthy to assess if this observation is the result of a saturated MurT/GatD system or that a functional flipping mechanism is needed to have a functional MurT/GatD system. Moreover, since *S. aureus* incorporates a pentaglycine cross bridge via the FemXAB system in a step-wise manner (Schneider et al. 2004; Rohrer et al. 1999; Ehlert, Schröder, and Labischinski 1997), it would also be interesting to assess if flipping of the lipid II-Gly_1 and lipid II-Gly_3 intermediate forms is possible.

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