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# Molecular characterisation of *tagO*-deficient mutants of *Staphylococcus aureus*

**A thesis submitted for the degree of Doctor in Philosophy**

**by**

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## Summary

Wall teichoic acid (WTA) is a major component of the Gram-positive bacterial cell wall. Despite not being essential, it plays important roles in cell growth, division, morphology and virulence.

The enzyme TagO initiates the assembly of WTA. Here, isogenic *tagO* mutants of methicillin sensitive and methicillin resistant strains of *S. aureus* were generated. All mutants failed to produce WTA. RNA-seq was carried out to investigate transcriptional changes in a *tagO* mutant of strain Newman. The lack of *tagO* caused the up-regulation of 107 genes and the down-regulation of 85 genes compared to the wild-type. Genes from the cell wall stress stimulon and genes responsible for capsule production (*cap* operon) were up-regulated. While genes involved in virulence and colonisation, such as the *agr* system (the main accessory regulatory system that controls virulence factors) *spa* and *clfB* were down-regulated. These changes in gene expression were confirmed using qRT-PCR.

The expression of some of the proteins encoded by differentially expressed genes was measured by Western immunoblot and flow cytometry.

The cell wall anchored proteins Spa and ClfB were present at lower levels in the *tagO* mutant compared to the wild-type. As a consequence, the mutants adhered poorly to the ClfB ligand loricrin and the Spa ligand immunoglobulin G. Gene expression of ClfA and the extracellular proteins alpha toxin and V8 protease was not altered, but protein levels were drastically reduced. As a consequence the *tagO* mutant had an impaired capacity to bind to the ClfA ligand fibrinogen and to cause alpha toxin-mediated hemolysis. The wild-type phenotypes were restored by complementation with a plasmid expressing *tagO*.

TarH is the protein responsible for the translocation of the final WTA polymer to the cell surface, during WTA synthesis. A Newman *tarH* mutant was isolated to investigate the possible effects of the WTA intermediates in the regulation and expression of Spa. This preliminary studied showed that intermediates of the WTA pathway can be involved in the regulation of Spa and possibly other genes.

Lipoteichoic acid is another important polymer of the cell envelope. Despite sharing some functions with WTA, their structures and synthesis are different. LAC

LTA-deficient mutants were used to investigate the expression and functional activity of the cell wall anchored proteins Spa, ClfB and ClfA and the secreted protein Hla. The results showed that the absence of LTA causes an even more drastic effect on the surface proteins than WTA, suggesting that LTA might also have a role in the surface display of these proteins.

## Publications

**Molecular characterisation of *tagO*-deficient mutants of *Staphylococcus aureus.*** *Joana Carvalho, Carsten Kröger, Timothy J. Foster, Joan A. Geoghegan.* Manuscript in preparation.

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## Key to abbreviations

| | |
|---|---|
| Agr | Accessory gene regulator |
| ATc | Anhydrotetracycline |
| CA-MRSA | Community acquired methicillin resistant *S. aureus* |
| CWA | Cell wall anchored |
| CWSS | Cell wall stress stimulon |
| Fg | Fibrinogen |
| HA-MRSA | Hospital acquired methicillin resistant *S. aureus* |
| Ig | Immunoglobulin |
| kDa | Kilodaltons |
| LTA | Lipoteichoic acid |
| PCR | Polymerase chain reaction |
| PG | Peptidoglycan |
| PVDF | Polyvinyldene fluoride |
| RboP | Polyribitol |
| SLIC | Sequence ligation independent cloning |
| Tag | Teichoic acid glycerol |
| Tar | Teichoic acid ribitol |
| TCA | Trichloroacetic acid |
| TCS | Two component system |
| WTA | Wall teichoic acid |
| ECM | Extracellular matrix |
| FITC | Fluorescein isothiocyanate |
| X-Gal | β-galactosidase |
| MSCRAMMS | Microbial surface components recognising adhesive matrix molecules |

# Chapter 1

# Introduction

## 1.1 Biology of the Staphylococci

Bacteria of the genus *Staphylococcus* are Gram-positive, approximately 1μm in diameter, that form grape-like structures. The genus *Staphylococcus* is classified in the family *Micrococcaceae* (Stackebrandt, 1979). Staphylococci belongs to the *Bacillus-Lactobacillus-Streptococcus* cluster that is defined as Gram-positive bacteria with a low G + C content (33-40%) (Ludwig *et al.*, 1981).

### 1.1.1 Defining properties of *Staphylococcus aureus*

*Staphylococcus aureus* (*S. aureus*) is a Gram-positive facultative anaerobic, non-motile, non-spore-forming, catalase-positive coccus. *S. aureus* forms glistening, smooth, entire, raised and translucent colonies with a golden pigment. All species of *staphylococcus* grow in the presence of bile salts and are extremely halotolerant, growing at up to 3.5 M NaCl. *S. aureus* can be differentiated from other species by its ability to ferment mannitol and to produce extracellular thermostable DNase and coagulase.

Coagulases are proteins that bind to fibrinogen and also activate prothrombin to initiate the host blood coagulation pathway. The presence or absence of these proteins has been used to divide *Staphylococcus* species into two different groups, coagulase-positive and coagulase-negative. *S. aureus* is coagulase-positive and so coagulase expression is often associated with virulence. The most common *S. aureus* infections are superficial skin lesions such as impetigo, but it can also be the cause of severe invasive infections such as bacteraemia. *S. aureus* can be responsible for serious diseases such as osteomyelitis, pneumonia and is the leading cause of infective endocarditis (Lowy, 1998). Invasive disease is by far the most severe.

However the greatest burden of morbidity is associated with skin and soft tissue infections which are extremely common and often recurrent.

Coagulase-negative staphylococci are less frequent pathogens of humans. However, species like *S. epidermidis, S. lugdunensis* or *S. haemolyticus* can cause severe infections in humans. The conversion of coagulase-negative staphylococci from skin commensals to human pathogens is often directly related to the use of indwelling medical devices (Rogers *et al.*, 2009).

*S. aureus* is regarded as a transient resident of healthy skin, except for community acquired MRSA (CA-MRSA). However, it can persist in the moist squamous epithelium of the nares. Asymptomatic carriage of *S. aureus* is widespread in the population. It persistently colonizes the nose of 20% of the human population while the remaining 80% are intermittent carriers. Humans are often infected by the strain that they carry. Carriers who become infected, have a reduced risk of mortality compared to non-carriers (Wertheim *et al.*, 2004).

### 1.1.2. Colonisation and disease

Humans are frequently exposed and recurrently colonized by *S. aureus*. The primary colonisation site is the anterior nares, followed by skin, throat, perineum, vagina and gastrointestinal tract (Mertz *et al.*, 2007, Bourgeois-Nicolaos *et al.*, 2010, Acton *et al.*, 2009). The nasal site is often the source of inoculation of other sites. The higher the bacterial burden in the nares, the more likely an individual is to be colonized in other parts of the body and the colonization to be persistent (Nouwen *et al.*, 2004, Mermel *et al.*, 2011).

*S. aureus* is exposed to a large number of antimicrobial host factors during colonization and infection. However, *S. aureus* possesses a plethora of resistance mechanisms that contribute to the ability to evade host cutaneous defences and survive during colonization (Foster, 2005, Grenfell *et al.*, 2004). Host defense mechanisms and *S. aureus* factors essential to evade those mechanisms are the subject of co-evolution, resulting in major variations between different hosts and bacterial strains (Grenfell *et al.*, 2004, Woolhouse *et al.*, 2002).

#### 1.1.2.1 Determinants of nasal carriage

The nasal cavity is the major reservoir of *S. aureus*, but the reasons underlying the predisposition and tropism for the nares are still unclear. Some studies showed that *S. aureus* colonizes mostly the anterior part of the nares by adhering to desquamated cells (squames) on the epithelial surface of the nasal vestibule. Five surface proteins of *S. aureus* promote adhesion to squamous cells *in vitro*. Clumping factor B (ClfB) has the ability to bind to fibrinogen (Ní Eidhin *et al.*, 1998), but also

binds to loricrin (Mulcahy *et al.*, 2012) and cytokeratin 10 (Walsh *et al.*, 2004), two major components on the squamous epithelium. The properties of ClfB are covered in detail in section 1.2.5.2.1.3. The nasal mucosa is an iron-deficient environment that stimulates the expression of the iron-regulated surface determinant (Isd) family of surface proteins. These proteins are involved in iron acquisition and survival on skin and the mucosa (Skaar & Schneewind, 2004). IsdA is also involved in promoting bacterial adhesion to squames (Clarke *et al.*, 2006). Additionally SdrC, SdrD and SasG also promote adhesion to squamous epithelial cells but their ligands are not known (Corrigan & Foster, 2009, Corrigan *et al.*, 2007). However, there is very clear evidence that *S. aureus* also interacts with living ciliated cells in deeper areas of the nasal cavity. Wall teichoic acid (WTA), a surface-exposed polyanionic polymer of the cell wall plays a key role in *S. aureus* nasal colonisation (Weidenmaier *et al.*, 2004). A WTA-deficient strain adheres less efficiently than its parental strain to human nasal epithelial cells, and loses the ability to colonize the nares of cotton rat (Weidenmaier *et al.*, 2004). However, the conclusions from this study have to be evaluated carefully. The changes on the bacterial cell surface caused by the absence of WTA in the WTA-deficient mutant, such as the display of cell wall anchored proteins, were not studied. A deficient display of these proteins could be responsible for the reduced colonization of the WTA-deficient mutant.

SREC-1 is the WTA receptor in nasal epithelial cells (Baur *et al.*, 2014). In binding assays with immobilized WTA, SREC-1 bound WTA in a dose-dependent manner. This binding could be inhibited by the addition of a SREC-1 antibody.

Thus, *S. aureus* nasal colonization is a multifactorial process. Cell wall proteins, such as ClfB, seem to be more important in long-term colonization, whereas WTA is important for the initial stages of colonization (Baur *et al.*, 2014).

### 1.1.3 Methicillin resistant *S. aureus*

Shortly after the introduction of penicillin in the 1940s, the first penicillinase-producing *S. aureus* strains were described. This led to the development of penicillinase-resistant drugs, such as methicillin and oxacillin. The first methicillin-resistant *S. aureus* (MRSA) strain was reported in the United Kingdom one year after of

the introduction of this drug (Jevons, 1961). MRSA strains have a modified penicillin-binding protein (PBP2a) encoded by *mecA*, which confers resistance to all β-lactam antibiotics. The *mecA* gene is located on complex mobile genetic elements, known as *SCCmec* (staphylococcal chromosomal cassette). *SSCmec* includes recombinase genes, regulatory elements and additional genes encoding resistance to other antibiotics, such as macrolides (Ito *et al.*, 2001, Oliveira *et al.*, 2000). Hospital acquired MRSA (HA-MRSA) strains account for more than 50% of all *S. aureus* isolates causing disease in hospitalized patients and those with chronic diseases, in the United States (Maree *et al.*, 2007). HA-MRSA expresses high levels of resistance to oxacillin, are resistant to multiple antibiotics and has sacrificed high virulence for high β-lactams resistance. They typically cause wound infection and bacteraemia (McCarthy *et al.*, 2015).

The emergence of community-associated MRSA (CA-MRSA) strains was reported in the 1990s. CA-MRSA strains express lower levels of resistance to oxacillin, are resistant to fewer antibiotics and are highly virulent. They typically cause severe skin and soft tissue infections in the community (Watkins *et al.*, 2012).

## 1.2. The cell wall of *S. aureus*

### 1.2.1. Peptidoglycan

One of the major differences between Gram-positive and Gram-negative bacteria is the outer membrane of the latter. In Gram-negative organisms, this outer membrane protects the cell from the environment, filtering out toxic molecules and retains (in the periplasm) important proteins required for cell wall growth and degradation. It also serves as a scaffold to which proteins and polysaccharides that interacts with other cells and with the environment are attached (DiRienzo *et al.*, 1978). The cell wall of Gram-positive bacteria lacks an outer membrane and a periplasm but the layer of peptidoglycan is very thick (Vollmer *et al.*, 2008). The peptidoglycan (PG) stabilizes the cell membrane and provides sites to which other molecules can be attached. The *S. aureus* peptidoglycan (PG) is composed of glycan chains made from the amino sugars N-acetylglucosamine and N-acetylmuramic acid. Stem pentapeptides (L-Ala-D-iso-Gln-L-Lys-D-Ala-D-Ala) are attached to the carboxyl group of each N- acetylmuramic acid.

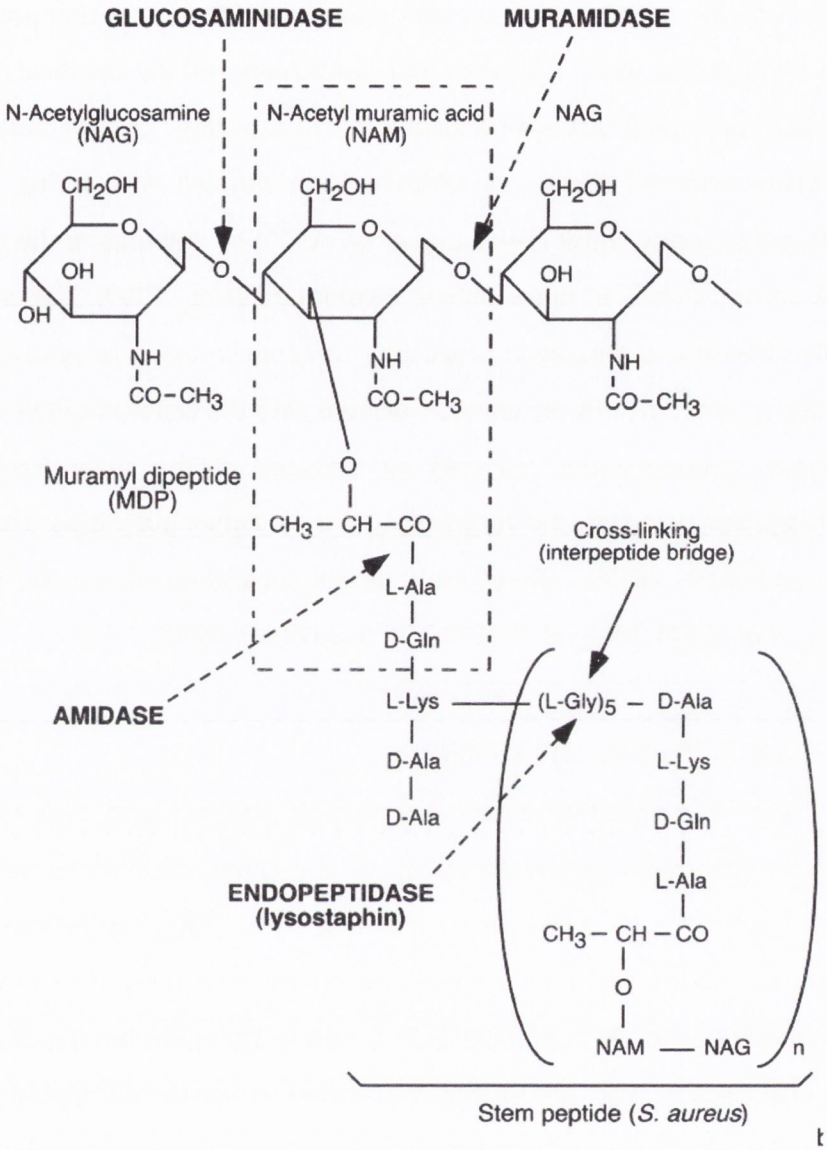



**FIG 1.1** Structure of peptidoglycan of *S. aureus*. The peptidoglycan of Gram-negative bacteria and Gram-positive bacilli is indicated on the right. Digestion by different enzymes (glucosaminidase, muramidase, amidase, and endopeptidase) is shown by dotted arrows. MDP structure is indicated in the dotted square. NAG, *N*-acetylglucosamine; NAM, *N*-acetylmuramic acid; m-DAP, *m*-diaminopimelic acid. Extracted from (Fournier & Philpott, 2005).

Pentaglycine bridges connect the lysine from one stem peptide to the penultimate D-alanine of the neighbour stem peptide (FIG 1.1) (Dmitriev *et al.*, 2004). A major function of the cell wall is to prevent overexpansion by creating pressure when the water enters the cell. Enzymes that participate in PG synthesis are targets of antibiotics such as β-lactams (penicillin, cephalosporins), glycopeptides (vancomycin), and fosfomycin. PG fragments released from the cell wall during infection act as mediators of septic shock (Veldkamp *et al.*, 1997) by stimulating the toll-like receptor TLR-2 on the surface of macrophages (Yoshimura *et al.*, 1999). The cell wall of Gram-positive bacteria undergoes a process of turnover in order for growth to occur. This function is performed by enzymes associated with the cell wall called autolysins. Some autolysins hydrolyse the cell wall by cleavage of the sugar backbone, allowing peptidoglycan turnover and recycling (FIG 1.1). Other autolysins exhibit amidase or glucosaminidase activity. They promote cell separation during the division process (Wong *et al.*, 1974, Mani *et al.*, 1994, Yamada *et al.*, 1996).

### 1.2.2. Wall teichoic acids

The Gram-positive bacterial envelope also contains two major phosphate polymers that have similar functions to the outer membrane, such as providing stability to the plasma membrane, mediating extracellular interactions, and along with the peptidoglycan, acting as scaffolds for enzymes required for cell wall growth and degradation (Swoboda *et al.*, 2010). In *S. aureus* there are two types of teichoic acids (TA), wall teichoic acid (WTA) that is attached to the peptidoglycan and lipotheicoic acid (LTA), which are anchored to the plasma membrane via a glycolipid (FIG 1.2). Together, these polymers create a negative charge that extends from the bacterial cell surface beyond the outermost layers of PG (Neuhaus & Baddiley, 2003, Weidenmaier & Peschel, 2008).

#### 1.2.2.1 Wall teichoic acid structure and chemical composition

Wall teichoic acid is an anionic glycopolymer containing 40 to 60 polyol repeats (Kojima *et al.*, 1985) that is covalently attached to PG via a phosphodiester bond to the C6 hydroxyl of N-acetyl muramic acid (MurNAc) (FIG 1.2).

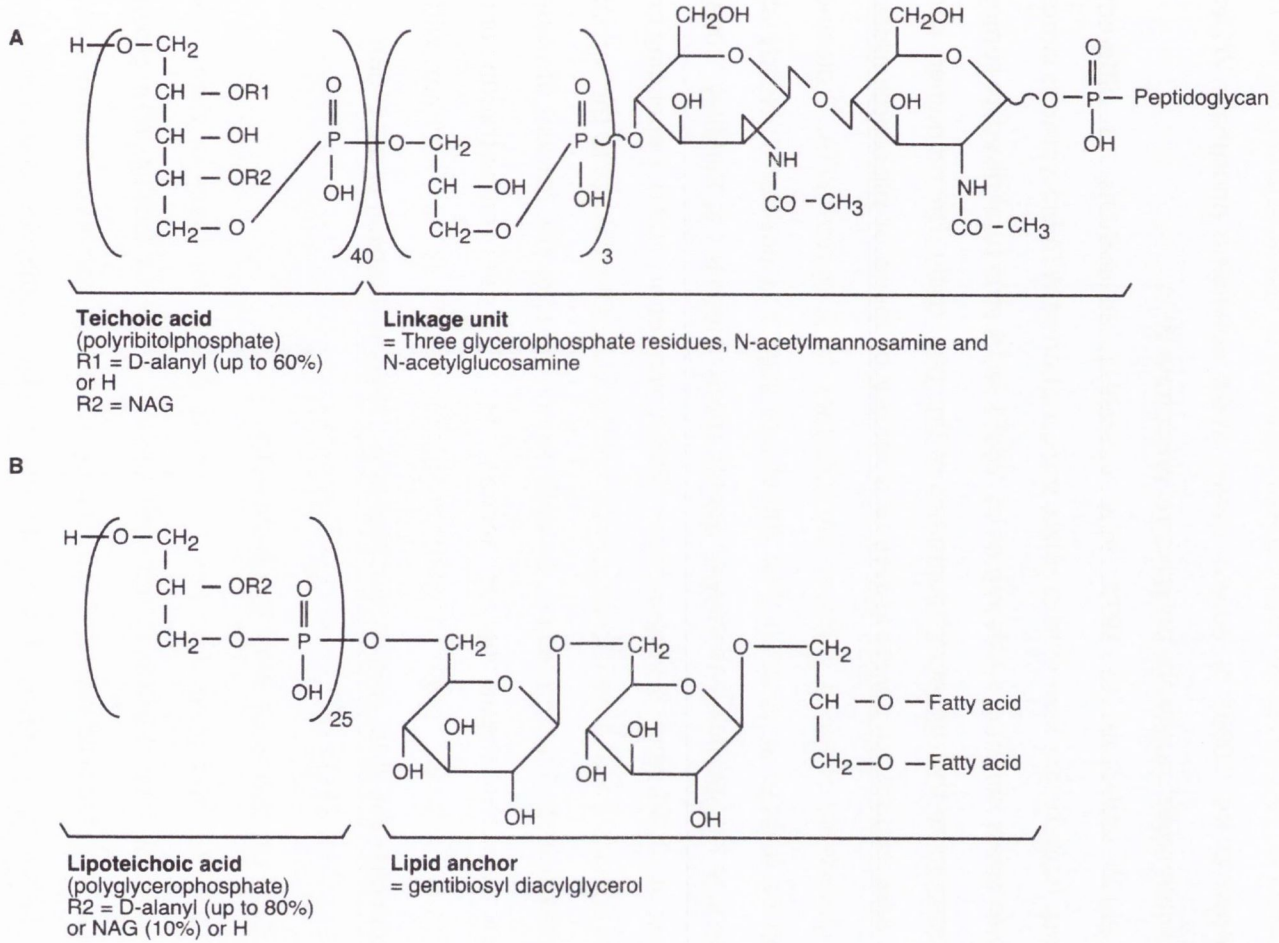



**FIG. 1. 2** Chemical structure of teichoic acids . A) Wall teichoic acid with poly-ribitol phosphate chain; B) Lipoteichoic acid with poly-glycerol phosphate chains. R, R1, R2 indicate potential substituent groups on the polyol chains (e.g., D-Ala, Glc, Gal, GlcNAc). Extracted from (Fournier & Philpott, 2005).

The chemical structure of WTA varies among different bacterial species (Neuhaus & Baddiley, 2003). The most common structure is composed of a ManNAc(β1→4)GlcNAc dissacharide with one or three glycerol phosphates attached to the C4 hydroxyl of the ManNAc residue (linkage unit) followed by a much longer chain of glycerol or ribitol repeats (main chain) (Neuhaus & Baddiley, 2003, Kojima *et al.*, 1985, Araki & Ito, 1989). *S. aureus* strains make polyribitol phosphate WTAs, whereas *S. epidermidis* usually has polyglycerol phosphate WTA.

Structural variations to WTA may represent adaptations to different environments. Bacteria are known to regulate transcription of WTA biosynthesis genes in response to stress conditions (Minnig *et al.*, 2005). WTA structural diversity comes from modifications to the repeating monomers of the main chain. The monomers can be tailored with cationic D-alanine esters or a variety of mono or oligosaccharides, commonly glucose or N-acetyl glucosamine (GlcNAc). In *S. aureus* WTA, D-alanine residues can be located at position 2 of ribitol and sugars at position 4. Nearly all ribitol-phosphate repeats have O-GlcNAc modifications (Neuhaus & Baddiley, 2003, Vinogradov *et al.*, 2006, Jenni & Berger-Bächi, 1998, Collins *et al.*, 2002). Depending on the bacterial species the anomeric configuration of the glycosidic linkage to the repeat unit can be exclusively β, exclusively α, or a mixture of the two. The content of these residues are variable, depending on factors like pH, salt concentration and temperature. In contrast, sugar modifications are less likely to fluctuate with environmental changes (Endl *et al.*, 1983, Endl *et al.*, 1984, Nathenson *et al.*, 1966).

### 1.2.2.2 Functions of wall teichoic acid

Wall teichoic acid plays a variety of roles in which, for many of them, the underlying molecular mechanism is not well known. *S. aureus* lacking WTA grows slower compared to the wild type and cells clump, due to the inability to complete cell separation following division. These strains exhibit numerous morphological abnormalities, including a thicker peptidoglycan layer, increased cell size, and defects in the number of septa and their position, showing a role for WTA in cellular morphology and division (Campbell *et al.*, 2011, Cole *et al.*, 1970, D'Elia *et al.*, 2006, Park *et al.*, 1974, Schlag *et al.*, 2010).

These phenotypes indicate that WTA is necessary for proper localization, assembly and/or activation of cell wall elongation and division machinery. Consistent with this is the fact that the PG and WTA biosynthetic machinery have functional interactions (Campbell *et al.*, 2011). PG cross-linking enzymes PBP4 and FmtA lose their septal localization when WTA is missing which causes a decrease in PG cross-linking (Atilano *et al.*, 2010, Qamar & Golemi-Kotra, 2012). In MRSA strains, the absence of WTA sensitized the bacterial cells to β-lactams, potentially due to the disruption of the PG cross-linking enzyme PBP2a (Farha *et al.*, 2013). Recent studies found that the absence of WTA β-GlcNAc modifications led to susceptibility to β-lactams antibiotics, which may be due to WTA β-GlcNAc moieties acting as scaffolds for proteins involved in resistance. Accordingly, WTA was shown to interact with PBP2a, the oxacillin resistant transpeptidase found in MRSA strains (Brown *et al.*, 2012, Farha *et al.*, 2013, Maki *et al.*, 1994, Suzuki *et al.*, 2011, Wecke J, 1997, Peschel *et al.*, 1999).

WTA can also regulate autolytic activity. Strains lacking WTA have altered endogenous autolysin expression profiles and rates of increased autolysis (Schlag *et al.*, 2010, Biswas *et al.*, 2012, Maki *et al.*, 1994, Wecke J, 1997). WTA plays a crucial role in targeting the Atl autolysin since a mutant strain lacking WTA is unable to target autolysins to the septum. Instead they are evenly distributed around the cell (Schlag *et al.*, 2010).

Wall teichoic acid is also associated with the regulation of ion homeostasis due to its ability to bind to extracellular metal cations. WTA has a high affinity for metals, and its biosynthesis is upregulated under metal limitation. This ion binding capacity is thought to minimize repulsion between nearby phosphate groups, affecting polymer structure and therefore cell wall integrity (Kern *et al.*, 2010, Wickham *et al.*, 2009). WTA also protects bacteria against host defences and antibiotics. WTA-deficient cells are more susceptible to human antibacterial fatty acid, presumably because these molecules penetrate the mutant cells more easily and bind to the cell membrane more efficiently, where they can cause damage (Kohler *et al.*, 2009).

D-alanylation of WTA protects cells against host defence mechanisms (Kristian *et al.*, 2003, Kristian *et al.*, 2005). Bacterial cells with WTA lacking D-alanine have an increased susceptibility to neutrophil killing (Collins *et al.*, 2002) and to lysostaphin

and lysozyme (Peschel *et al.*, 2000). The absence of D-alanine increases the overall cell surface negative charge, attracting cationic molecules and thus increasing the susceptibility to cationic antimicrobial peptides (Peschel *et al.*, 1999).

Wall teichoic acid also has a role in biofilm formation. WTA-deficient mutants showed reduced adherence to artificial surfaces such as glass and polystyrene (Gross *et al.*, 2001) and were impaired in their ability to form biofilms. WTA has an important role both in the primary attachment and accumulation phases of the *S. epidermidis* biofilm associated with an increased cell surface hydrophobicity, and reduced polysaccharide intercellular adhesin (PIA) expression (Holland *et al.*, 2011).

Finally, as mention before WTA is involved in adhesion to host tissue. WTA-deficient mutants are impaired in their ability to adhere to nasal epithelial cells and unable to colonize the nares of cotton rats (Weidenmaier *et al.*, 2004). Later, WTA was also described to be essential for colonization of endothelial tissues from kidney and spleen (Weidenmaier *et al.*, 2005). However, WTA-deficient mutants have cell surface and growth defects when compared to the wild type. Therefore the cells are less fit and more prone to immune clearance by the host. More recently, the amount of WTA on the cell surface of a CA-MRSA strain was associated with increased skin abscess formation (Wanner *et al.*, 2017).

### 1.2.2.3 Biosynthesis of wall teichoic acid

Wall teichoic acid is sequentially synthesized by a series of teichoic acid ribitol (Tar) enzymes, which have the same biological functions as the enzymes teichoic acid glycerol (Tag). The biosynthesis occurs on an undecaprenyl – phosphate lipid carrier that anchors the nascent polymer to the inner face of the cytoplasmic membrane (FIG1.3). The TagO, TagA and TagB proteins are responsible for completing synthesis of the linkage unit. TagO catalyzes the first synthetic step by transferring GlcNAc-1-P from UDP-GlcNAc to the membrane-anchored carrier lipid, an intermediate shared with PG synthesis (Rubinchik *et al.*, 2011, Soldo *et al.*, 2002) (FIG 1.3). The next step is catalysed by TagA and consists in the transfer of ManNAC from UDP-ManNAC to the C4 hydroxyl of ManNAC from UDP-ManNAC to the C4 hydroxyl of GlcNAc to form the

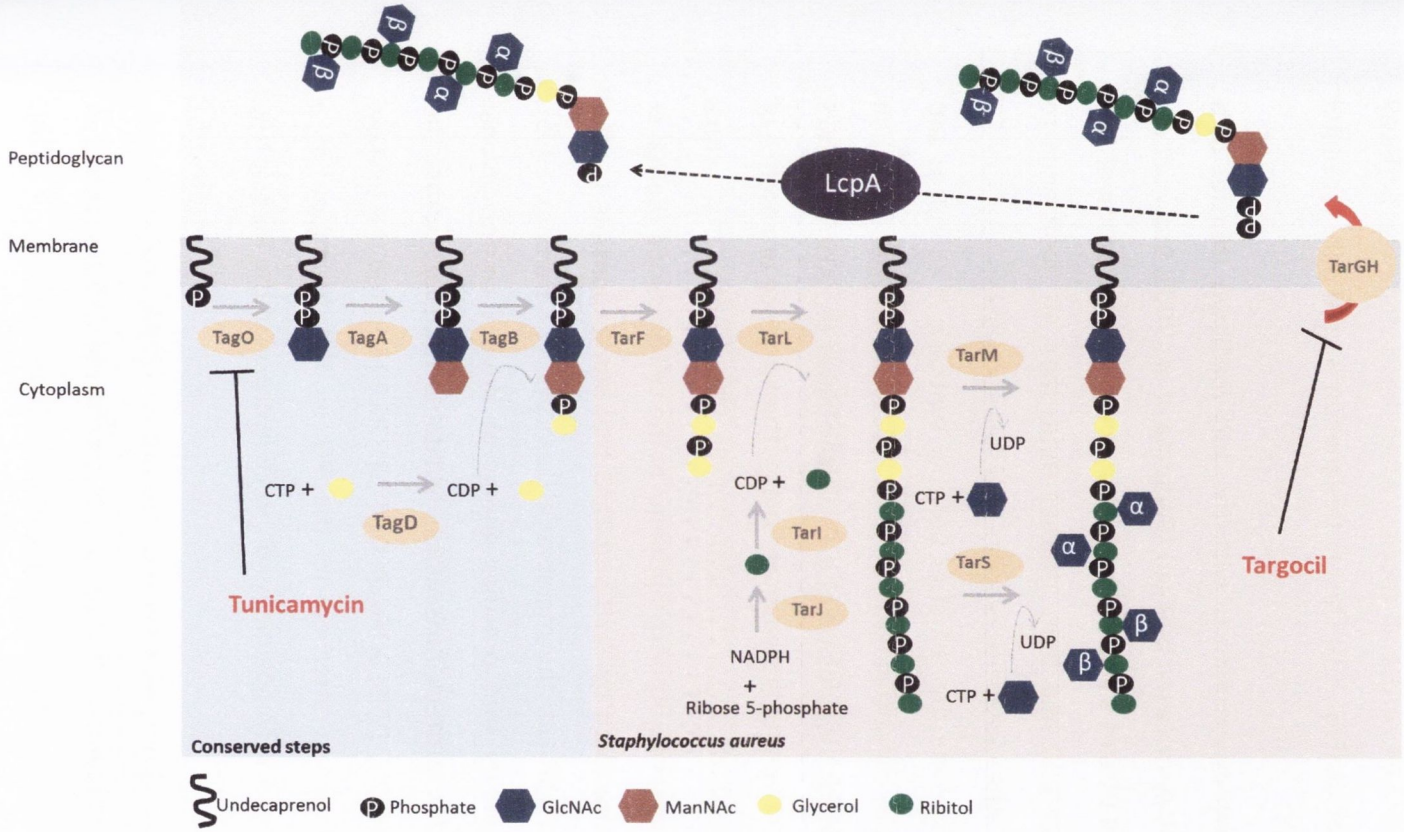



**FIG 1.3** Schematic representation of the WTA biosynthetic pathway. In different organisms the WTA biosynthesis initial steps (involving TagO, TagA and TagB) are conserved (in blue), but then the pathway becomes specific of each organism (light rose). The final polymer is translocated to the cell surface by TarGH and attached to the peptidoglycan by LcpA. Tunicamycin and targocil are antibiotics that block TagO and TagG activity, respectively. Abbreviations: GlcNAc – N-acetylglucosamine; ManNAc – acetylmannosamine.

ManNAc(β1→4)GlcNAc disaccharide (D'Elia *et al.*, 2009, Ginsberg *et al.*, 2006, Zhang *et al.*, 2006). Then, TagB, a glycerophosphotransferase transfers a GroP unit from CDP-glycerol to the C4 position of ManNAc (Bhavsar *et al.*, 2005, Ginsberg *et al.*, 2006). The steps carried out by TagO, TagA and TagB enzymes (necessary for the assembly of the linkage unit) are highly conserved across all species. After these steps the pathway diverges among different species (FIG. 1.3). The tag designation is used here for ribitol WTA synthesis.

Polyribitol (RboP) synthesis continues with the TarF-mediated transfer of a single unit from CDP-glycerol, synthesized by TagD (Badurina *et al.*, 2003, Brown *et al.*, 2008). The enzyme TarL (that utilizes a substrate made by the combined action of TarI and TarJ) primes the linkage unit and then attaches more than 40 RboP units to complete the polymer (FIG 1.3).

Glycosylation of WTA occurs inside the cell after polymer synthesis is complete and prior to its translocation to the cell surface (Allison *et al.*, 2011, Brown *et al.*, 2012). In *S. aureus*, TarM is responsible for the addition of α-*O*-GlcNAc (Xia *et al.*, 2010) and TarS for the modifications with β-*O*GlcNAc (Brown *et al.*, 2012). The presence or absence of modifications as well as the nature of the glycosidic linkage affects polymer structure and also influences interactions with other components in the cell envelope (Bernal *et al.*, 2009). The final step is mediated by TarH and TarG that are responsible for the translocation of the polymer through the membrane (Lazarevic & Karamata, 1995). TarH contains an ATPase-domain that provides energy to drive a conformational change in the transmembrane protein TarG which facilitates transport across the membrane (Lazarevic & Karamata, 1995, Schirner K 2011). Once exported the teichoic acid polymer becomes covalently attached to the PG by the formation of a phosphodiester linkage between WTA polymer and the C6 hydroxyl of the PG MurNAc unit (Sewell & Brown, 2014).

*S. aureus* encodes 3 LCP genes, which encode the proteins LcpA, LcpB and LcpC. LcpC is responsible for the attachment of capsular polysaccharide CP5 (described in detail in section 1.2.4) to the peptidoglycan (Chan *et al.*, 2014).

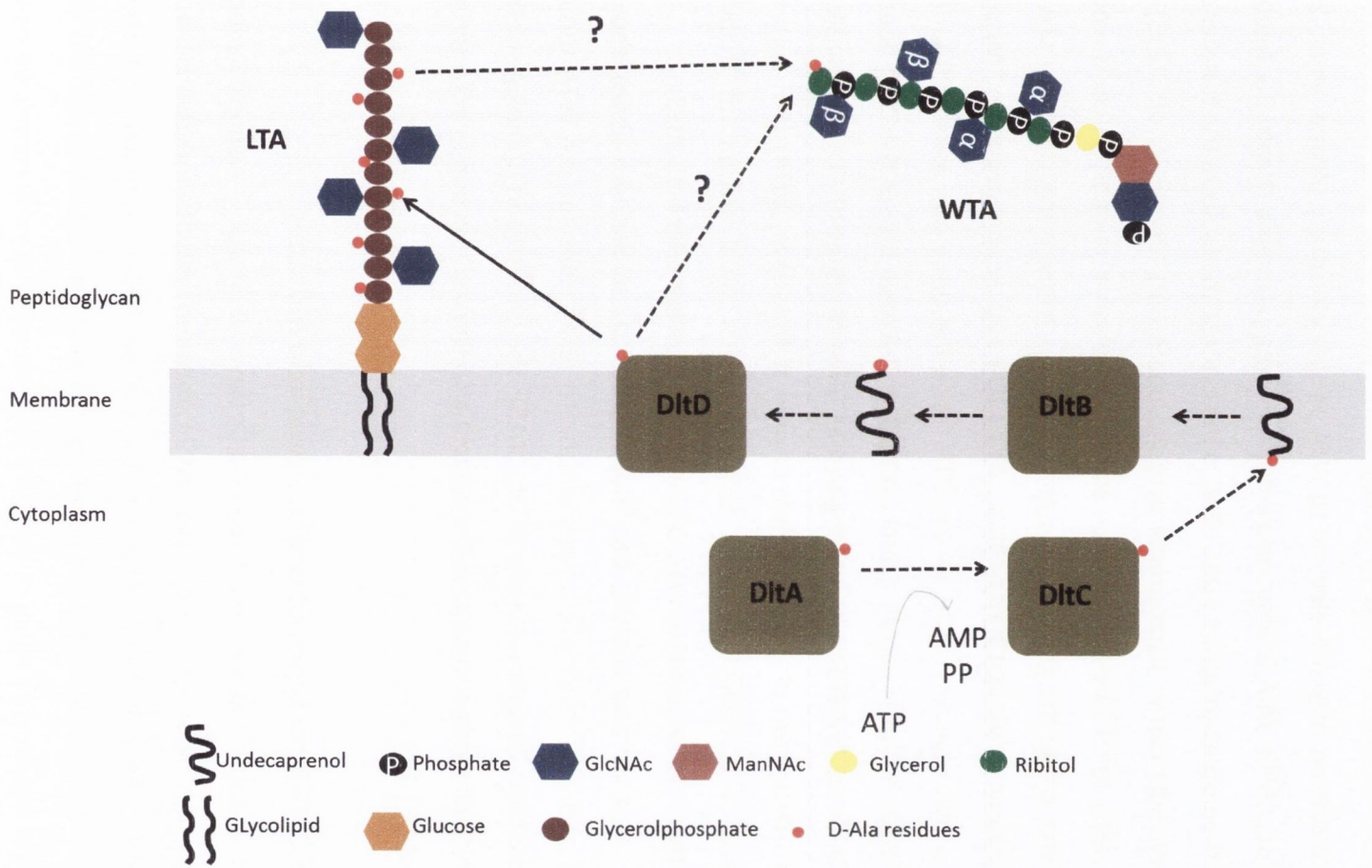



**FIG 1.4** Schematic representation of D-alanylation of teichoic acids. Abbreviations: GlcNAc – N-acetylglucosamine; ManNAc – acetylmannosamine. Based in (Reichmann *et al.*, 2013)

*lcpA* and *lcpB* mutants displayed a great reduction in WTA anchoring, suggesting to have a role in the WTA anchoring to peptidoglycan (Chan *et al.*, 2014, Chan *et al.*, 2013).

D-alanylation of the polymer is an important mechanism by which bacterial cells modulate their surface charge (Collins *et al.*, 2002). Four enzymes, encoded by the *dltABCD* operon, attach D-alanines to WTA on the cell surface (Kristian *et al.*, 2003, Perego *et al.*, 1995). DltA is responsible for activating D-alanine (Heaton & Neuhaus, 1992) and then transferring it to DltC. DltC in the cytoplasm results in the transfer of D-Ala onto the lipid carrier bactoprenol pyrophosphate (PP) (Perego *et al.*, 1995) (FIG 1.4). DltB flips the D-Ala-PP complex across the plasma membrane. It is unclear whether DltD assists the DltB flippase or contributes to D-Ala transfer onto LTA and WTA (Reichmann *et al.*, 2013, May *et al.*, 2005) (FIG 1.4). Schnneewind and Missiakas (2014) proposed a second model where DltD facing the cytoplasm assists DltA for the loading of D-Ala onto the carrier protein DltC. The D-Alanylation of the WTA does not required catalysis. Therefore, it is thought that D-Ala residues of LTA are transferred onto WTA (Reichmann *et al.*, 2013, Schneewind & Missiakas, 2014) (FIG 1.4). WTA isolated from a LTA-deficient strain contains only a small amount of D-alanine, indicating that LTA has a role in WTA D-alanylation (Reichmann *et al.*, 2013).

In *Bacillus subtilis*, the PhoP regulon comprises two genes encoding hydrolyses called teichoicase enzymes which are responsible for the WTA turnover during phosphate starvation (Myers *et al.*, 2016). The widespread homologs of these enzymes in *Staphylococcaceae* families suggest a conserved function in WTA turnover (Myers *et al.*, 2016).

### 1.2.2.4 Phenotypes of wall teichoic acid mutants

Null mutants that are defective in TagO, the protein that is responsible for the first step of the WTA synthesis, have mildly impaired growth and defects in cell division, biofilm formation and adherence to nasal epithelium. A mutant defective in TagA is also viable with similar phenotype to the *tagO* mutant. However the genes encoding the late-step proteins of the WTA biosynthetic pathway cannot be deleted unless *tarO* or *tarA* is deleted first (D'Elia *et al.*, 2006). The proteins encoded by these

genes are required for viability in a strain background containing an otherwise functional WTA biosynthetic pathway, but are not required in a WTA-null background. Two explanations have been considered. One explanation attributes toxicity to the accumulation of toxic intermediates when the later stages of the pathway are disrupted (D'Elia *et al.*, 2006). Alternatively, bactoprenol carrier lipid also required for PG biosynthesis is depleted whereas blockage at the early-stages (TagO and TagA) is not (Watkinson *et al.*, 1971)

WTA, but not LTA, serves as a receptor for staphylococcal phage infection (Xia *et al.*, 2011).

### 1.2.2.5 Wall teichoic acid pathway inhibitors

The important role of WTA in bacterial physiology and pathogenesis suggests that WTA synthesis enzymes are good candidates as targets for novel antibiotics. The antibiotic tunicamycin inhibits TagO (FIG 1.3) at very low concentrations (Campbell *et al.*, 2011) and at higher concentrations also inhibits MraY (UDP-MurNAc-pentapeptide transferase) that is essential for PG biosynthesis (Price & Tsvetanova, 2007). This antibiotic has been used to investigate the effects of inhibiting WTA synthesis in bacteria but cannot be used to treat infection because it inhibits GPT, an essential phosphotransferase involved in eukaryotic N-linked glycan biosynthesis (Price & Tsvetanova, 2007).

Targocil is a late-stage WTA pathway inhibitor that blocks TarG (FIG 1.3). It was discovered by screening a *tagO* mutant and its parental strain with a library of compounds to identify those that selectively killed the wild-type strain. This compound inhibited TarG. Importantly, targocil is synergistic with β-lactams, in restoring sensitivity to β-lactams in MRSA (Wang *et al.*, 2013).

## 1.2.3. Lipoteichoic acids

Lipoteichoic acid (LTA) is a zwitterionic polymer found in the cell membrane of many Gram-positive bacteria. LTA consists of a polyglycerol-phosphate (PGP) chain attached to the membrane via a glycolipid anchor (FIG 1.2 B).

LTA synthesis begins in the cytoplasm with the production of the glycolipid anchor. The LTA anchor consists of a disaccharide linked to diacylglycerol (Fischer, 1988) that is produced by a glycosyltransferase. Following synthesis in the cytoplasm, the glycolipids are transported to the outer face of the cytoplasmic membrane where they are used as the LTA anchor. LtaA is a membrane transporter protein from the major facilitator family that is encoded in an operon with *ypfP* that is involved in this process (Gründling & Schneewind, 2007a). Then, the protein LtaS polymerizes the PGP chain by adding GroP units to the distal end of the chain, from the head group of the membrane lipid phosphatidylglycerol (Fischer, 1990, Taron *et al.*, 1983, Koch *et al.*, 1984). LtaS is predicted to be a membrane protein with a large extracellular enzymatic domain responsible for LTA synthesis (Gründling & Schneewind, 2007b, Lu *et al.*, 2009). The structure of LTA is shown in FIG 1.2 B, however biosynthesis is described in detail in Chapter 5.

*S. aureus* LTA-null mutants lacking *ypfP* produce LTA bound to DAG lacking a glycolipid anchor. The cells are enlarged and misshapen (Kiriukhin *et al.*, 2001). Deletion of the *ltaS* gene results in an increased cell size and an aberrant placement of division septa. Bacteria can only grow in the presence of high sucrose or NaCl concentrations. The phenotype is even more obvious in rod-shaped bacteria, where the lack of LTA causes filamentation (Gründling & Schneewind, 2007b, Oku *et al.*, 2009).

### 1.2.4. Capsular polysaccharides

The majority of *S. aureus* strains produce capsular polysaccharides (CP) of which eleven distinct serotypes are recognised. Strains belonging to the serotypes 1 and 2 produce a macrocapsule whereas strains producing CP of serotypes 3-11 are microencapsulated. The vast majority of *S. aureus* produce CP5 and CP8. These capsular polysaccharides are uronic acid polymers, and consist of trisaccharide repeats of N-acetylmannosaminuronic acid and two N -acetylfucosamine. Despite their chemical similarity CP5 and CP8 are immunologically different due to variations in linkages and substitutions of the sugar residues (O'Riordan & Lee, 2004). Expression of CP5 and CP8 *in vitro* is very sensitive to various environmental signals. Bacterial

growth conditions can influence capsule production. The *cap5* and *cap8* gene cluster each contains 16 co-transcribed genes. Several global regulators have been shown to mediate the production of the capsule, including *agr, sarA, arl* and *mgrA*, as well as others (Luong *et al.*, 2002, Pöhlmann-Dietze *et al.*, 2000, Gupta R. K., 2013, Luong & Lee, 2006). Little capsular polysaccharide is produced in the logarithmic phase of growth and maximal capsule production occurs during the post-exponential growth phase, which is consistent with Agr mediated control (Dassy & Fournier, 1996).

Notably, strains of the USA300 lineage (such as LAC) do not produce capsule due to mutations in the *cap* promoter and in the *capD* and *capE* genes (Boyle-Vavra S 2015).

The production of CP contributes to virulence. The capsule enhances virulence by impeding phagocytosis which promotes persistence in the bloodstream of infected hosts (O'Riordan & Lee, 2004). However, mice inoculated with CP5 expressing *S. aureus* had a higher frequency of arthritis than mice inoculated with the isogenic non-encapsulated mutant (Nilsson *et al.*, 1997).

A detailed description of the genes involved in the capsule biosynthesis is presented in Chapter 3 (see 3.2.3.3).

### 1.2.5 Cell wall-associated surface proteins

The diversity of infections caused by *S. aureus* is a reflection of its ability to produce an array of virulence factors that participate in the pathogenesis of infection, allowing the cells to adhere to surfaces/tissues, evade the immune system and cause harmful toxic effects to the host. These factors are numerous and include surface-associated adhesins, capsular polyssacharide, exoenzymes and exotoxins. Cell wall anchored (CWA) proteins in *S. aureus* have many features in common. An N-terminal signal sequence of about 40 amino acids targets the protein to the secretory pathway (Sec). The AXA motif at the end of the sequence is cleaved by the membrane signal peptidases SpsB during translocation across the cytoplasmic membrane (Cregg *et al.*, 1996, Mazmanian *et al.*, 2001). These proteins also have a wall-spanning region

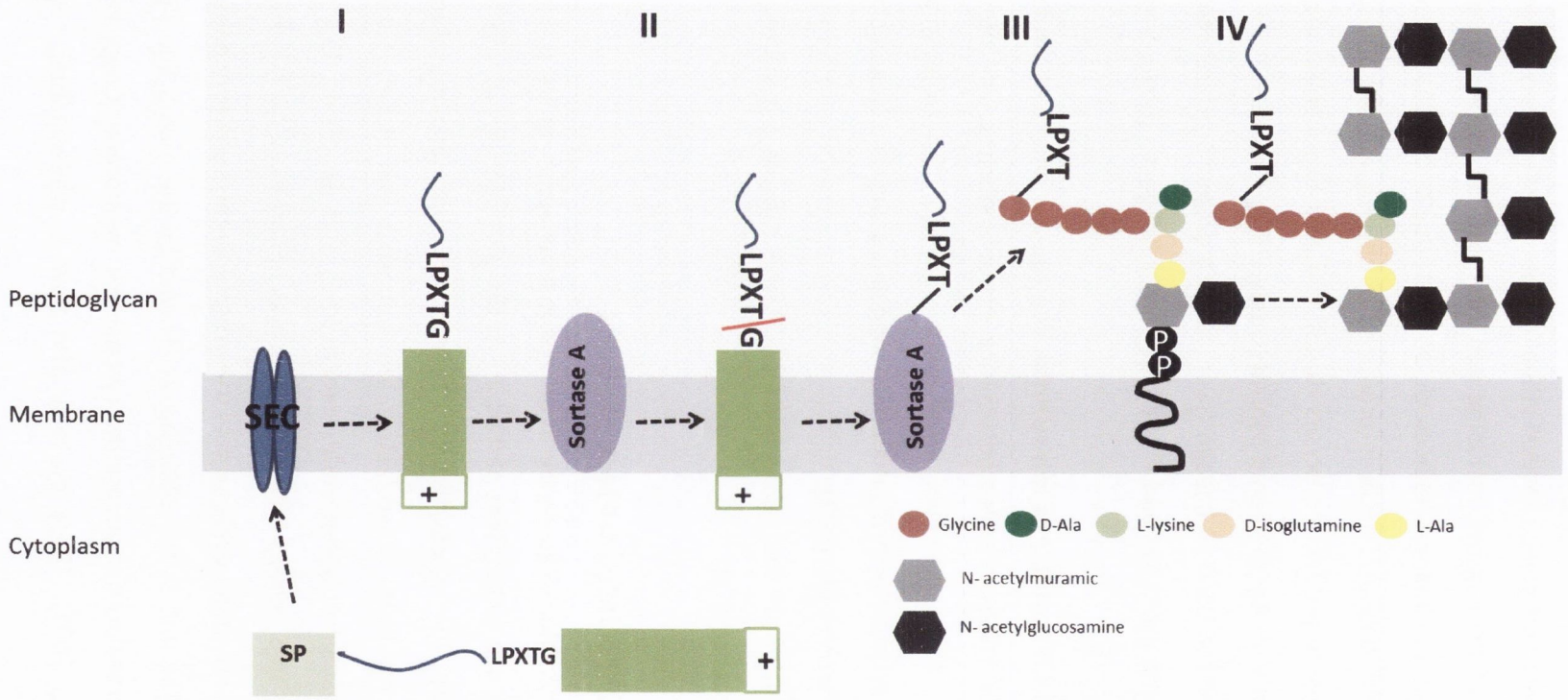



**FIG 1.5** Schematic representation of the anchoring of CWA proteins in *S. aureus*. (I) Precursor proteins with an N-terminal signal peptide (SP) are initiated into the secretory (Sec) pathway and the signal peptide is removed. The C-terminal sorting signal retains polypeptides within the secretory pathway. (II) Sortase cleaves between the threonine and the glycine of the LPXTG motif, resulting in the formation of a thioester enzyme intermediate. (III) The amino group of the cell wall cross bridges within lipid II (pentaglycine) cleaves the thioester bond between SrtA and its substrate, thereby forming an amide bond between the protein C-terminal and lipid II (IV) Lipid-linked surface protein is first incorporated into the cell wall via the transglycosylation reaction. The murein pentapeptide subunit with attached surface protein is then cross-linked to other cell wall peptides, generating the mature murein tetrapeptide (not shown).

(hydrophobic membrane-spanning domain) and a positively charged cytoplasmic tail at the C-terminus. Surface proteins contain a motif that is responsible for the targeted attachment to the cell, whereby these proteins become covalently attached to the nascent pentaglycine cross-bridges of peptidoglycan.

Adherence is mediated by a family of surface adhesins named MSCRAMMs (microbial surface components recognising adhesive matrix molecules) that are covalently anchored to the peptidoglycan via specific signals at the C-terminus (FIG 1.5). CWA proteins recognize host proteins, such as those from the extracellular matrix (ECM), and blood components, such as fibrinogen. Expression of CWA proteins promotes adhesion to damaged tissue, to the surfaces of host cells and to implanted medical devices (Foster *et al.*, 2014, Geoghegan & Foster, 2015).

Secreted factors, such as exoenzymes, proteases and cytolytic toxins facilitate tissue destruction and spreading. *S. aureus* also causes toxin-mediated disease such as toxic shock syndrome (due in part to the production of toxic shock toxin-1 and enterotoxins), severe necrotising pneumonia (due to the production of Panton-Valentine leucocidin), scalded skin syndrome (caused by the epidermolytic toxins) and food poisoning (caused by ingestion of enterotoxins) (Geoghegan & Foster, 2015, Foster *et al.*, 2014).

### 1.2.5.1. Sortase-mediated anchoring of cell-wall associated proteins

*S. aureus* surface proteins are covalently anchored to the cell wall peptidoglycan by a process called sorting that requires a C-terminal sorting signal with an LPXTG motif (FIG 1.5). Surface proteins are first synthesized in the bacterial cytoplasm and are then transported across the cytoplasmic membrane. Sortase A (SrtA), a membrane-anchored transpeptidase, cleaves the C-terminal sorting signal (Mazmanian *et al.*, 2000, Mazmanian *et al.*, 2001) between the threonine and glycine residues of the LPXTG motif and covalently attaches the threonine to terminal glycine in the pentaglycine of the nascent PG subunits of lipid II (Ton-That *et al.*, 1997, Navarre *et al.*, 1998). SrtA-null mutants lack surface proteins and consequently are

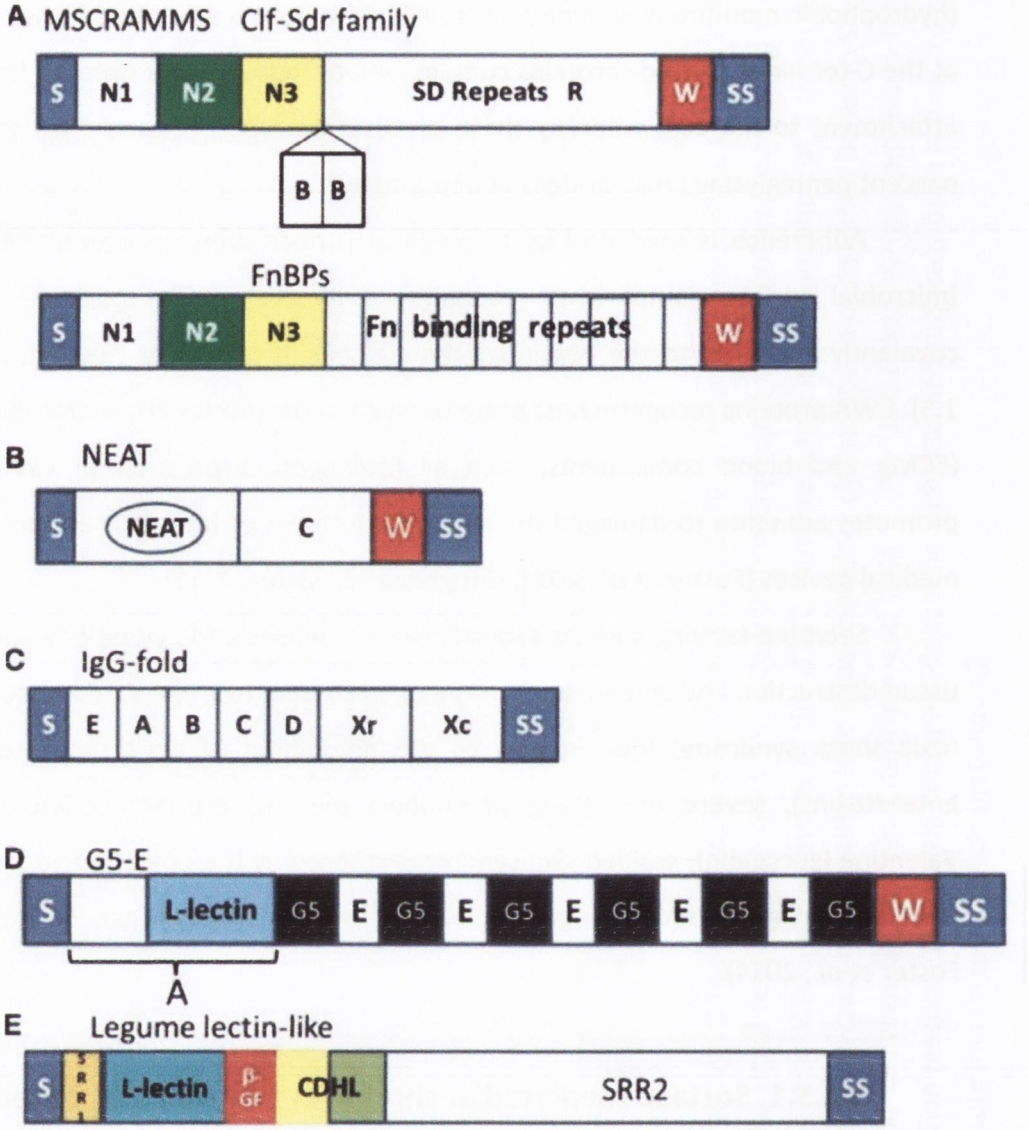



**FIG. 1.6** Schematic representation of the common motif organisation of the *S. aureus* CWA proteins. An N-terminal signal sequence directs the pre-protein for secretion via the Sec machinery. The C-terminus is composed of the cell wall-spanning region, or Xc region in the case of the three-helical bundle family, and the sorting signal, which facilitates covalent anchoring of CWA proteins to peptidoglycan. CWA proteins are characterised based on shared structural and functional motifs. The MSCRAMMs (A) are the largest family of CWA proteins and possesses an N-terminal A domain which contains two consecutive IgG-fold like domains (green and yellow boxes). The domain structures of the neat motif family (B), three-helical bundle family (C) G5-E repeat family and (E) legume-lectin family are also shown. S – signal sequence; W and Xc-wall-spanning region; SS-sorting signal; Xr – repeat containing region;. Adapted from (Foster *et al.*, 2014) and (Speziale *et al.*, 2014).

attenuated in animal models of septic arthritis and endocarditis (Jonsson *et al.*, 2002, Jonsson *et al.*, 2003, Weiss *et al.*, 2004).

Cell wall anchored proteins can be classified into structural and functional groups such as MSCRAMMS, NEAT motif family, Three-helical bundle family, G5-E repeat family and Legume lectin like proteins (FIG 1.6). They are all structurally similar possessing a signal sequence and a sorting signal. They also share a wall spanning region with exception of the three-helical bundle family.

The NEAT family (Isd proteins) binds either haemoglobin or haem and are only expressed under iron limiting conditions, such as in serum within host infection (Mazmanian *et al.*, 2002).

The G5-E repeat family comprises SasG which promotes biofilm accumulation and bacterial adhesion to desquamated epithelial cells (Corrigan *et al.*, 2009, Roche *et al.*, 2004).

The Legume-lectin domain family is composed by SraP which plays a role in infective endocarditis due to its affinity for human platelets (Siboo *et al.*, 2005), promotes bacterial adhesion and invasion of host epithelial cells by binding to N-acetyl neuraminic acid on mammalian cells (Speziale *et al.*, 2014, Yang *et al.*, 2014).

This chapter describes with more emphasis the MSCRAMM and the three helical bundle families of cell wall proteins.

### 1.2.5.2 The MSCRAMM family

#### 1.2.5.2.1. The Clf/Sdr subfamily of MSCRAMMS

##### 1.2.5.2.1.1 ClfA

Fibrinogen (Fg) is a 340 kDa glycoprotein and is the major clotting factor in the blood plasma with an important role in blood coagulation and thrombosis (Aleman *et al.*, 2014). *S. aureus* can clump in the presence of Fg (McDevitt *et al.*, 1997, Rivera *et al.*, 2007). and can promote bacterial adhesion to immobilized Fg and to fibrin blood clots. This was attributed primarily to clumping factor A (ClfA) (McDevitt *et al.*, 1997). However, *S. aureus* expresses other surface located Fg-binding proteins, including clumping factor B (ClfB), which is related to ClfA in terms of sequence and structure (Ní Eidhin *et al.*, 1998). The N—terminal 520 residue A domain of ClfA is composed of

three subdomains, N1, N2 and N3. The N2N3 subdomains of ClfA have been crystalized and this has allowed identification of a hydrophobic trench between N2 and N3 that forms the fibrinogen binding site employed by the binding mechanism Dock, Lock and Latch (Deivanayagam *et al.*, 2002) (see section 1.2.5.2.1.2). The A domain is followed by region R consisting of serine-aspartate (SD) dipeptide repeats (McDevitt *et al.*, 1994) (FIG1.6). Region R of ClfA acts as a stalk that allows the projection of the A domain away from the cell surface (Hatford *et al.*, 1997)

ClfA binds to the C-terminus of the γ-chain of fibrinogen at the same site as the human platelet integrin GpIIb/IIIa (McDevitt *et al.*, 1997, Medved *et al.*, 1997). ClfA can stimulate platelet activation by forming a fibrinogen bridge between bacterium and the platelet receptor GPIIb/IIIa (Loughman *et al.*, 2005). Thus, ClfA contributes to the development of infective endocarditis (Moreillon & Que, 2004, Sullam *et al.*, 1996). ClfA is also anti-phagocytic and protects *S. aureus* by phagocytosis from murine macrophages and human neutrophils in vitro (Palmqvist *et al.*, 2004, Higgins *et al.*, 2006). ClfA –defective mutants caused significantly less severe arthritis than the isogenic wild-type strain or a complemented mutant (Josefsson *et al.*, 2001).

#### 1.2.5.2.1.2 Dock, Lock and Latch mechanism

The "dock, lock and latch" mechanism mediates ligand binding by staphylococcal MSCRAMMs. Crystal structures of the ligand region of SdrG as an apo-protien and in complex with a synthetic fibrinogen peptide revealed the structural changes that occur during "dock, lock and latch" (Ponnuraj *et al.*, 2003). SdrG in its apo form, contains an exposed hydrophobic trench between subdomains N2 and N3. Ligand docking within the trench induces a conformational change. The subdomain N3 extension (the latching peptide), is redirected to cover the ligand, locking it in place. Finally, a short segment of the N3 extension inserts into a trench located in the surface of subdomain N2 by β-strand complementation (FIG 1.7).

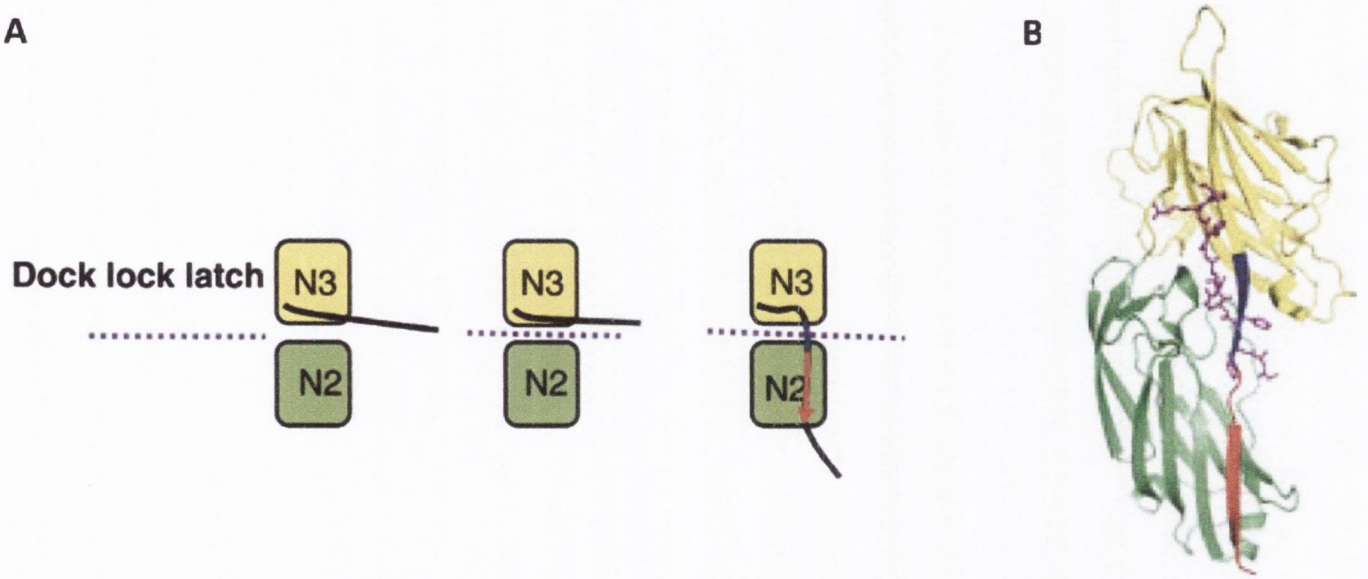



**FIG 1.7 A)** Schematic representation of the dock lock and latch mechanism; **B)** Ribbon diagram of the bacterial protein in complex with its ligand. MSCRAMMs such as ClfA and ClfB bind their ligands by the 'dock, lock, and latch' mechanism. The red arrow indicate the new β-strands that are formed when the complex is stabilized. In MSCRAMMs, the latching peptide, an extension of the N3 subdomain (yellow), binds to a β-strand in N2 subdomain (green). The purple dashed line represents the peptide ligand. Adapted from (Geoghegan & Foster, 2015).

#### 1.2.5.2.1.3 ClfB

Clumping factor B (ClfB) has a very similar structure to ClfA, sharing 26% sequence identity in the A domain and 93% amino acid identity in the SD-repeat region (Ní Eidhin *et al.*, 1998) (FIG 1.6). The ligand binding A region of ClfB can bind to fibrinogen, cytokeratin 10 and loricrin (Ní Eidhin *et al.*, 1998, O'Brien *et al.*, 2002a, Mulcahy *et al.*, 2012). Similarly to ClfA, ligand binding by ClfB occurs by the "dock, lock and latch" mechanism. Unlike ClfA which binds the fibrinogen Y-chain, ClfB binds the α-chain (Walsh *et al.*, 2008). The binding domain in fibrinogen has been localized to glycine and serine-rich repeat 5 of the flexible connector region of the αC-domain (Walsh *et al.*, 2008).

Cytokeratin 10 is expressed on the surface of desquamated nasal epithelial cells and ClfB promotes adherence to these cells in vitro (O'Brien *et al.*, 2002b). However, loricrin represents the major target ligand for ClfB during *S. aureus* colonisation. Loricrin comprises three separate GS-rich omega loops regions and ClfB was found to bind most strongly to region 2 (Mulcahy *et al.*, 2012).

ClfB plays an important role in nasal colonization by *S. aureus*. *clfB* mutants were defective in colonization of the nares in a mouse colonisation model and in human volunteers (Wertheim *et al.*, 2008, Schaffer *et al.*, 2006, Mulcahy *et al.*, 2012). Systemic administration of an anti-ClfB monoclonal antibody protected against colonization in mice. ClfB has a crucial role in promoting adherence of clinically relevant strains of *S. aureus* to corneocytes of patients with atopic dermatitis (Fleury O., not published).

#### 1.2.5.2.1.4 Sdr family

Sdr proteins have a structural organisation that is similar to ClfA and ClfB, with the exception of additional B repeats located between region A and region R (FIG 1.6). An important characteristic of the B region is the presence of a consensus EF-hand loop for high affinity $Ca^{2+}$ binding. The structural integrity of this region depends upon this cation and when it is removed the structure collapses. SdrC and SdrD promote adhesion of bacteria to desquamated epithelial cells (Schaffer *et al.*, 2006). SdrD binds

to desmoglein 1 from human squamous cells isolated from the nose (Askarian *et al.*, 2016), while the ligand for SdrC is unknown.

#### 1.2.5.2.2. The Fibronectin binding protein subfamily of MSCRAMMS

Fibronectin is a dimeric glycoprotein that is present in plasma. It constitutes a significant part of the extracellular matrix conditioning layer that coats many implanted medical devices such as heart valves and catheters. *S. aureus* expresses two closely related fibronectin-binding proteins A and B (FnBPA and FnBPB), encoded by the closely linked genes *fnbA* and *fnbB*, respectively (Signäs *et al.*, 1989). The majority of *S. aureus* contains both genes although some lack the *fnbB* gene (Greene *et al.*, 1995). FnBPs are subjected to proteolytic degradation by V8 protease (McGavin *et al.*, 1997) in the stationary phase of growth.

The N-terminal A domains of FnBPs are predicted to fold into 3 subdomains like ClfA and mediate binding to fibrinogen and elastin while repeated domains of the C-terminus bind to fibronectin (FIG 1.6).

Adherence of *S. aureus* to mammalian cells is mediated by a fibronectin bridge between FnBP and the $\alpha_5\beta_1$ integrin (Dziewanowska *et al.*, 1999, Sinha *et al.*, 1999, Peacock *et al.*, 1999, Fowler *et al.*, 2000). This is crucial for internalisation of *S. aureus* by endothelial cells and consequent bacterial dissemination from the bloodstream to other parts of the body.

#### 1.2.5.2.3 Collagen binding protein subfamily of MSCRAMMS

The collagen binding protein (Cna) possesses an N-terminal A region that composed of three subdomains, similar to the other subclasses MSCRAMMS (FIG 1.6). However, the ligand binding by Cna is mediated by the N1 and N2 subdomains rather than the N2 and N3 subdomains (Zong *et al.*, 2005). The A region of Cna is separated from the wall spanning region and sorting signal region by a variable number of $B_{cna}$ repeated domains that differ in sequence from the $B_{sdr}$ domains (Deivanayagam *et al.*, 2000). Cna does not possess a flexible stalk region linking the B domains and the sorting signal.

#### 1.2.5.2.4 Three helical bundle family

Protein A (Spa) is a CWA protein that is expressed on the surface of over 95% of *S. aureus* strains (Forsgren & Nordström, 1974). At the N-terminus Spa contains four or five homologous 56 - 62 amino acid ligand-binding repeat domains designated the E, D, A, B and C domains, followed by a short polymorphic repeat region of variable length (Xr) and a conserved region (Xc) (Uhlén *et al.*, 1984, Moks *et al.*, 1986) (FIG 1.6). The ligand-binding repeat domains consist of single separately folded three-helical bundles that can bind to several different ligands. Spa is encoded by the *spa* gene which is repressed by the regulatory loci *agr* and *sarA* in stationary phase (Cheung *et al.*, 1997).

Protein A is a cell wall-anchored (CWA) protein although a significant fraction of it is present in the culture supernatant due to a sub-optimal sorting sequence (O'Halloran *et al.*, 2015). Spa recognizes the Fc domain of IgG with high affinity (Cedergren *et al.*, 1993); (Gouda *et al.*, 1992) which blocks C1q (and FCγ receptor) binding sites. Spa can also bind to the variable region of the Fab heavy chain of Vh3 class of antibodies (IgM molecules) (Hartleib *et al.*, 2000). Other ligands for Spa are von Willebrand factor (Hartleib *et al.*, 2000) and tumor necrosis factor – α receptor 1 (TNFR-1) (Gómez *et al.*, 2004).

Spa is a virulence factor in subcutaneous infection, sepsis and septic arthritis and pneumonia murine models (Patel *et al.*, 1987, Palmqvist *et al.*, 2002, Gómez *et al.*, 2004) and a potent inhibitor of phagocytosis in vitro (Gemmell, 1991). The binding of the Fc region of IgG to Spa results in the bacterium becoming coated with incorrectly oriented IgG molecules. These IgG molecules are not recognised by the Fcγ receptors of neutrophils, therefore not triggering complement fixation by the classical pathway avoiding opsono-phagocytosis (Gemmell, 1991). Binding to IgM on B-cells causes their activation, proliferation and consequent apoptotic destruction (Goodyear & Silverman, 2003), conferring on Spa immunosuppressive properties.

#### 1.2.5.2.5 Other IgG binding proteins

*S. aureus* expresses a second Ig-binding protein named Sbi (second binding protein of immunoglobulin) (Williams, 1963; Zhang *et al.*, 1999). This protein is encoded by the *sbi* gene and is transcriptionally regulated by RNAIII and SprD (Chabelskaya *et al.*, 2014). Sbi is non-covalently associated with lipoteichoic acid in the cell envelope (Smith *et al.*, 2012). It comprises four three-helical bundles, two of which have sequence similarity to the IgG-binding domains of Spa (Burman *et al.*, 2008). Sbi is released into the culture supernatant because it is not covalently attached to the cell wall peptidoglycan (Smith *et al.*, 2012). The other two domains are independently folded and bind to complement factor C3 (Upadhyay *et al.*, 2008). When released the protein forms a platform that triggers complement fixation resulting in futile consumption of C3 away from the cell surface. Thus Sbi has an important role in the ability of *S. aureus* to avoid phagocytosis in human blood by reducing the uptake of bacteria by human neutrophils in the presence of serum opsonins (Smith *et al.*, 2012).

## 1.3. Extracellular factors

### 1.3.1 Cytolytic pore-forming toxins

*S. aureus* produces several toxins that damage the membranes of host cells (leucotoxins). A large number of secreted proteins are believed to be involved in virulence mediating adhesion to the host tissue, evasion of host defence mechanisms, tissue damage and invasion. The archetypal toxin that forms β-barrel in the cytoplasmic membrane of host cells is alpha-toxin (α-toxin).

#### 1.3.1.1 Alpha-toxin (α-toxin)

Alpha-toxin is secreted as a monomeric water-soluble protein of 33KDa. When binding to the membrane the monomer oligomerises to form a heptameric ring that constitutes a membrane pore. The formation of such pores disrupts the integrity of the cytoplasmic membrane causing cell lysis.

Alpha-toxin is an important virulence factor in different animal infection models, such as mastitis (Bramley *et al.*, 1989), subcutaneous abscesses (Patel *et al.*, 1987) and keratitis (O'Callaghan *et al.*, 1997). The toxin is expressed in the post-

exponential phase of growth and is encoded by the *hla* gene which is under a very complex transcriptional regulation by Agr, SarA and SarA homologues.

At high concentration pore formation is receptor-independent. However, at low concentrations pore formation is dependent on the interaction with the ADAM10 receptor, a disintegrin and metalloproteinase receptor (Wilke & Bubeck Wardenburg, 2010). Another study showed that the complex alpha-toxin-ADAM10 results in the cleavage of E-cadherin in epithelial cells and consequently the loss of epithelial barrier function (Inoshima *et al.*, 2011).

### 1.3.1.2 Beta-toxin (β-toxin)

Beta-toxin (β-toxin) is a $Mg^{2+}$-dependent toxin secreted by *S. aureus* in the post-exponential phase of growth with the capacity to cause "hot-cold" lysis of erythrocytes (Bohach G. and Foster, 2000). B-toxin is a sphingomyelinase C, which degrades the membrane lipid sphingomyelin (Doery *et al.*, 1963). It has the capacity to lyse cells with significant amounts of sphingomyelin such as monocytes and granulocytes (Otto, 2014). Beta-toxin contributes to pathogenesis in a murine mastitis model (Bramley *et al.*, 1989), plays a role in corneal infections (O'Callaghan *et al.*, 1997) and damages human keratinocytes (Katayama *et al.*, 2013).

### 1.3.1.3 Bi-component toxins

The bi-component leucotoxins comprise two subunits that are secreted separately and assemble together within the host cell membrane forming a pore (Jayasinghe & Bayley, 2005). There are five types of bi-component toxins, Panton-Valentine leukocidin (PVL), leukocidin E/D, leukocidin A/B, γ-hemolysins (Hlg A/B and Hlg C/B) (Otto, 2014). These toxins recognise specific receptor molecules on the surface of the host cells which explains their individual cell and host specificity.

PVL and the leukocidins AB and ED only lyse leukocytes whereas γ-toxin lyses both erythrocytes and leukocytes (Alonzo & Torres, 2014).

### 1.3.2 Phenol soluble modulins

Phenol soluble modulins (PSMs) are encoded by the core genome. They are short, amphipathic, α-helical peptides (Wang *et al.*, 2007). PSMs are divided into two classes according to peptide length. The α-type peptides (PSM α1-PSM α4) are 20-25 amino acids long and the β-type peptides (PSM β1 and PSM β2) are ~40 amino acids long (Peschel & Otto, 2013). The α-type PSMs lyse a variety of cells including monocytes, erythrocytes and osteoblasts (Cheung *et al.*, 2012, Rasigade *et al.*, 2013, Wang *et al.*, 2007). Furthermore, the α-type peptide was identified as a key virulence factor in a murine bacteraemia model and a skin infection model whereas no role for β-PSMs could be demonstrated (Wang *et al.*, 2007). In addition to this, PSMs have pro-inflammatory capacities. Peptides are recognised by formyl peptide receptor 2 found on neutrophils leading to neutrophil chemotaxis and activation (Kretschmer *et al.*, 2010, Wang *et al.*, 2007).

The delta – toxin (δ-toxin) is part of the PSM family. This toxin is produced by nearly all *S. aureus* isolates. It is a 26aa heat stable cytolytic peptide toxin encoded by the *hld* gene which is located near the 5´end of the Agr RNAIII transcript (see section 3.2.3.4.4.; (Fitton *et al.*, 1980). This toxin has been implicated in the degranulation of mast cells in the allergic skin disease atopic dermatitis (Nakamura *et al.*, 2013).

### 1.3.3 Secreted proteins

#### 1.3.3.1 Coagulases

The production of coagulase is often used for the identification of *S. aureus* strains. Coagulases, Coa and vWbp are secreted to the extracellular medium. Coagulases bind to prothrombin to form a complex named staphylothrombin, which activates the conversion of fibrinogen to fibrin clots. The transcription of Coa is activated in exponential and is repressed in the stationary phase by RNAIII (Chevalier *et al.*, 2010).

*S. aureus* also secretes von Willebrand factor (vWbp). This protein promotes a similar reaction as coagulase. The formation of fibrin (the product of coagulase activity) enables this pathogen to disseminate as thromboembolic lesions and resist opsonophagocytosis (McAdow *et al.*, 2012).

### 1.3.3.2 Extracellular complement and fibrinogen-binding proteins

The extracellular fibrinogen-binding (Efb) and the extracellular complement-binding (Ecb) proteins modulate the alternative pathway convertase by directly binding to the C3 molecule (Jongerius *et al.*, 2010). In a murine pneumonia infection model Efb and Ecb together promoted bacterial survival and blocked neutrophil influx into the lung (Jongerius *et al.*, 2012).

Efb binds to fibrinogen, creating a shield surrounding the bacteria which potently blocks phagocytic uptake of the pathogen. This mechanism and the presence of capsular polysaccharide can work in concert to enhance the resistance of *S. aureus* against phagocytosis (Kuipers *et al.*, 2016).

### 1.3.3.3 Proteases

*S. aureus* produces several different secreted proteases, the metalloprotease aureolysin, the serine protease V8 also known as SspA, two cysteine proteases, staphopain A and staphopain B and six serine-like proteases that are SspA homologues (SplABCDEF) (Reed *et al.*, 2001, Shaw *et al.*, 2004).

Proteases are multifunctional proteins that are required for growth in peptide-rich environments, serum, in the presence of antimicrobial peptides and in human blood and for resisting phagocytosis by human leukocytes (Kolar *et al.*, 2013). These proteins can inhibit the formation of protein dependent biofilm (Loughran *et al.*, 2014) and in promote virulence in murine infection models (Kolar *et al.*, 2013, Zielinska *et al.*, 2012).

Aureolysin can cleave ClfB (McAleese *et al.*, 2001). This enables the bacteria to regulate the abundance of an important virulence factor depending on the specific niche encountered within the host (Lindsay & Foster, 1999). Aureolysin can also modulate the stability of the secreted proteins α-toxin and phenol-soluble modulins (Cassat *et al.*, 2013); (Gonzalez *et al.*, 2012, Zielinska *et al.*, 2011). V8 is enzymatically processed into its mature form by the mature form of Aureolysin (Drapeau, 1978, Lindsay & Foster, 1999), and the cysteine protease SspB, which is encoded within the same operon as the *sspA* gene, is enzymatically processed by mature V8 (Chan & Foster, 1998, Rice *et al.*, 2001). The extracellular protease V8 is responsible for

removing fibronectin-binding proteins from the staphylococcal cell surface (McGavin *et al.*, 1997).

#### 1.3.3.4 Superantigens

Superantigens are potent activators of the immune system. These proteins bind directly to MHC class II molecules on the surface of antigen-presenting cells resulting in hyperactivation of B cells (Krakauer *et al.*, 2016). Secreted superantigen-like protein SSL7 binds to IgA and blocks recognition by the neutrophil receptor FcαRI impeding phagocytosis (Wines *et al.*, 2006). SSL10 binds to human IgG1, but not to immunoglobulins of lower vertebrates. This interaction inhibits IgG1binding to Fcγ receptors on the surface of neutrophils thereby interfering with the phagocytic uptake of IgG1-opsonized bacteria *in vitro* (Itoh *et al.*, 2013, Patel *et al.*, 2010).

### 1.4. Transcriptional regulation in *S. aureus*

In *S. aureus*, transcriptional regulation is governed by sigma factors, two – component signal transduction systems (TCS), metabolite-responsive regulatory proteins, RNA-binding proteins and regulatory RNAs (Ibarra *et al.*, 2013). The presence of some of the factors can vary among strains as a result of genome rearrangements and evolution by the acquisition of mobile genetic elements (Messina *et al.*, 2016, Ibarra *et al.*, 2013).

### 1.4.1 RNA regulation in *S. aureus*

In bacteria, mRNA levels are a function of both RNA synthesis and degradation. Modulation of mRNA turnover is an important means of regulating gene expression. *S. aureus* can alter its mRNA degradation properties in response to growth phase (Roberts *et al.*, 2006), and extracellular environment stresses (Anderson *et al.*, 2006, Anderson *et al.*, 2010), including antibiotic exposure (Blickwede *et al.*, 2005b, Blickwede *et al.*, 2005a). Additionally, changes in mRNA turnover have been associated with increased antibiotic resistance (Anderson *et al.*, 2006), adherence to epithelial cells (Blickwede *et al.*, 2005a) and pathogenesis (Huntzinger *et al.*, 2005). There are three functional groups in the mRNA turnover machinery: regulatory or small RNAs (sRNA), RNA-binding proteins and ribonucleases (RNases).

In *S. aureus* small RNAs (sRNA) are key players in gene regulation. sRNAs are limited in size (50-300nt) and accomplish a variety of regulatory functions, acting at the level of transcription, translation or RNA degradation. The majority of these RNA molecules regulate pathways that sense and transfer intracellularly various external signals, and adapt the cell population in response to environmental change (Cech & Steitz, 2014, Storz *et al.*, 2011, Fineran & Charpentier, 2012). There are approximately 160 sRNA in the Staphylococcal Regulatory RNA database. However, their functions have not been extensively investigated. Some sRNAs are known to regulate virulence factors (Bordeau *et al.*, 2016, Fechter *et al.*, 2014).

The most studied mechanism of action of sRNAs is direct RNA-RNA interactions. These interactions consist of two classes: cis-acting RNA and trans-acting RNA. Cis-acting RNA is located within the transcriptional unit of the gene itself, while trans-acting RNAs are located in a different region and are only complementary to small portions of their target (Morrison & Dunman, 2011, Fechter *et al.*, 2014).

RNA-binding proteins have an important role in mRNA turnover by directly interacting with target transcripts and affecting their degradation. As described earlier, *S. aureus* mRNA turnover is modulated by certain stresses and it is likely that RNA-binding proteins are among trans-acting factors that modulate the organism´s RNA degradation. However, the identity of such proteins in *S. aureus* is limited.

Ribonucleases coordinate mRNA degradation. They degrade the RNA-RNA duplexes formed between the regulatory RNA (e.g. RNAIII; see section 1.4.2.2) and the target mRNA transcript, resulting in a tight regulation of virulence factors expression by immediate cessation of the mRNA translation (Morrison & Dunman, 2011).

### 1.4.2 Regulation of virulence factors

Genes involved in virulence are regulated in a highly coordinated manner according to the biological needs of the organism. However, regulation genes and pathways are different in different *S. aureus* strains.

Staphylococci have a major quorum sensing system, called the accessory gene regulator (Agr) (FIG 1.8), which is the central regulatory element governing the expression of virulence factors.

### 1.4.2.1 The Accessory gene regulation system

The *agr* locus is expressed from two divergent promoters P2 and P3. The P2 promoter is responsible for activating the *agrBDCA* operon. *agrA* and *agrC* encode a classical two-component system and *agrB* and *agrD* encode two proteins that are responsible for the synthesis of a peptide that contains a 5 amino acid thiolactone ring called the auto-inducing peptide (AIP). Upon reaching a threshold concentration the AIP binds and activates AgrC by triggering phosphorylation of the conserved cytoplasmic histidine residue (Lina *et al.*, 1998). AgrC activates the response regulator AgrA through phosphorylation. The activated AgrA up-regulates its own promoter, P2 (Novick & Geisinger, 2008, Mayville *et al.*, 1999); (Novick *et al.*, 1995) and P3 which controls transcription of the regulatory RNAIII.

### 1.4.2.2 RNAIII

RNAIII is a 514 nucleotide RNA and is the intracellular effector of the *agr* regulon. It also encodes a 26 aminoacid peptide, delta-hemolysin or Hld, which has no regulatory function (Novick *et al.*, 1993). Expression of RNA III reaches its maximum in the late exponential and stationary phase of growth. RNAIII not only represses early virulence factors such as CWA proteins and activates post- exponentially expressed extracellular toxins but also regulates several two-component system (ArlRS, SaeRS) as well as global regulators such as Rot (Dunman *et al.*, 2001, Cassat *et al.*, 2006, Jones *et al.*, 2008). The regulation of Rot by RNAIII results in the indirect transcriptional regulation of many genes (FIG 1.9) The 5´end RNAIII positively regulates *hla* translation by activating the *hla* ribosomal binding site (Novick *et al.*, 1993); (Morfeldt *et al.*, 1995). The 3´end RNAIII represses the synthesis of virulence factors such as coagulase, protein A and Rot (FIG 1.9). After transcription, RNAIII anneals to target mRNA and the RNA-RNA complexes formed represses the initiation of translation and triggers mRNA rapid degradation by the ribonuclease RNase III. .

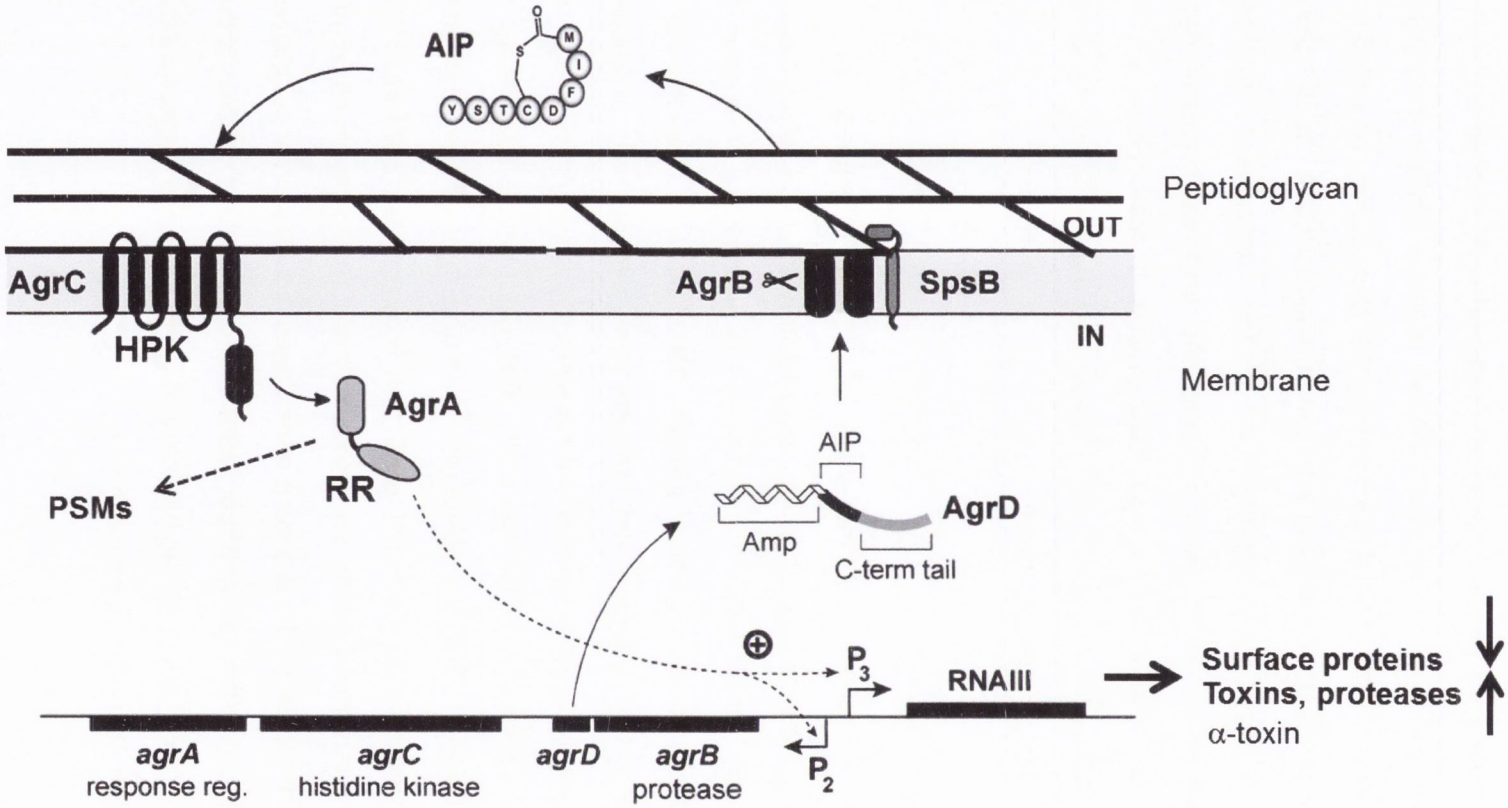



**FIG. 1.8** Schematic representation of the Agr pathway. The pathway consists of the auto-activation of RNAII and RNAIII transcription by their respective P2 and P3 promoters. During the exponential growth phase, the AgrD is secreted into the extracellular environment by AgrB, resulting in the mature autoinducing peptide (AIP). The *agr* locus is activated when the AIP concentration reaches a threshold that is directly linked to specific cell density. This type of regulation, whereby the bacterial population responds to cell density, defines quorum sensing. In the case of alpha-toxin RNAIII activates *hla* gene expression at the translational level. The binding of RNAIII to the *hla* results in a conformational change that allows translation to occur. RNAIII represses the expression of protein A and other CWA proteins by triggering mRNA degradation (Thoendel & Horswill, 2009).

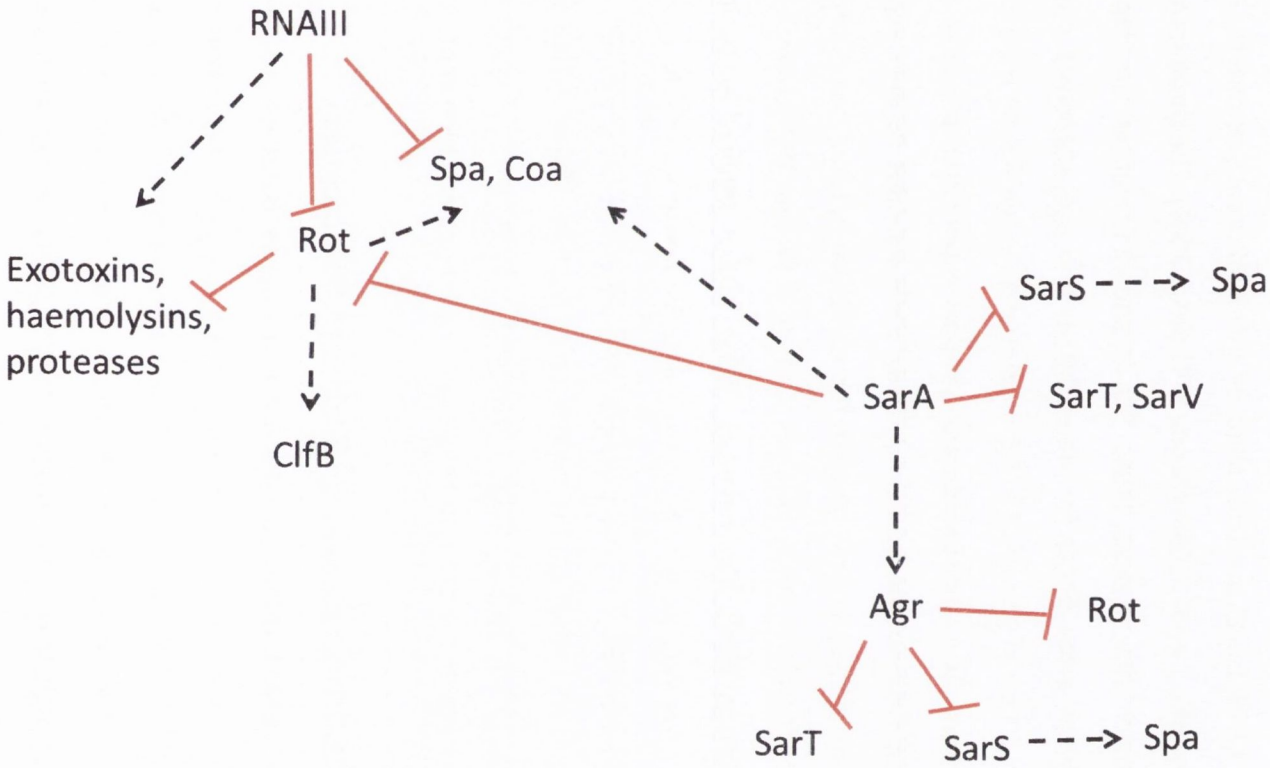



**FIG 1.9** Genetic regulatory network involving RNAIII and SarA in *Staphylococcus aureus*. Arrows indicate activation (black). Blocked arrows indicates repression (red).

### 1.4.2.3 Accessory regulator SarA

The *sarA* locus was initially identified as a Tn 917 transposon insertion into a region of the chromosome distinct from the *agr* locus that decreased the expression of several exoprotein genes belonging to the *agr* regulon (Cheung *et al.*, 1992). The *sarA* operon consists of three overlapping transcripts. The transcripts, sarP1 (*sarA*), sarP2 (*sarB*) and sarP3 (*sarC*) are controlled by three different promoters, P1, P2 and P3, respectively (Bayer *et al.*, 1996, Manna & Cheung, 2001). The three transcripts encode the SarA protein (Bayer *et al.*, 1996). The P1 and P2 promoters are dependent on the primary sigma factor (SigA) for transcription and are expressed during the early exponential phase of growth. The P3 promoter is recognised by the alternative sigma factor B (SigB) and is induced during the post-exponential phase of growth during environmental stress (Manna *et al.*, 1998). SarA activates its own expression and is down-regulated by SarR. SarA binds to the P2-P3 promoter region of the *agr* locus and up-regulates the transcription of both (Cheung & Projan, 1994, Heinrichs *et al.*, 1996, Chien & Cheung, 1998); (Rechtin *et al.*, 1999). SarA is required for optimal expression of RNAIII. However, SarA can also regulate virulence gene expression in an Agr-independent manner in certain strains, such as transcription of *spa* (FIG 1.9). This protein regulates negatively the expression of many proteases (Chan & Foster, 1998, Chien *et al.*, 1999). SarA binds as a homodimer structure to a conserved A/T – rich recognition motif in the promoter regions of target genes (Chien *et al.*, 1999).

#### 1.4.2.3.1 Accessory regulator SarA homologues

Besides SarA, there are nine SarA homologues. Based on size, the SarA protein family members are classified into three major groups (i) single domain proteins such as SarA, SarR, SarT, *S. aureus* expresses two sigma factors, (i) the primary sigma factor SigA required for the expression of housekeeping genes and (ii) a stationary phase sigma factor SigB that is homologous to the stress responsive transcription factor expressed by *B. subtilis* (Kullik *et al.*, 1998; Deora *et al.*, 1997). SigB is involved in the regulation of gene expression upon changes in the environment such as starvation, heat shock and osmotic shock (Wu *et al.*, 1996).

**Table 1.1** – List of the SarA protein family

| Protein | Putative function | Growth phase | Reference |
|---|---|---|---|
| **SarS** | An activator of protein A synthesis and *hla* | early exponential phase | (Tegmark *et al.*, 2000) (Cheung *et al.*, 2001) |
| **SarR** | represses expression of SarA by binding to the P1-P2 promoters during the | late exponential and stationary growth phases | (Cheung *et al.*, 2004) |
| **Rot** | Activates transcription of *spa, sdrC, sdrD and clfB* | transcribed throughout the growth cycle | (Saïd-Salim *et al.*, 2003) (Xue *et al.*, 2011) |
| **MgrA** | A regulator of autolysis and *agr* (also called Rat or NorR) | Exponential phase | (Truong-Bolduc *et al.*, 2003) |
| **SarT** | An activator of *sarS* and a repressor of alpha hemolysin synthesis | Exponential phase | (Cheung *et al.*, 2004) |
| **SarV** | A regulator of autolysis repressed by SarA and MgrA | Not known | (Manna & Cheung, 2006) |
| **SarU** | A positive regulator of *agr* | Exponential phase | (Cheung *et al.*, 2004) |
| **SarX** | Negative regulator of agr and repressed by MgrA | Exponential phase | (Manna & Cheung, 2006) |
| **SarZ** | Positive regulator of hla | Exponential phase | (Kaito *et al.*, 2006) |
| **SarY** | unknown | unknown | - |

### 1.4.2.4. Sigma factor B

The RNA polymerase core enzyme is composed of four subunits. The association of the core enzyme with a sigma factor forms a holoenzyme that is able to recognise specific promoters and initiate transcription. *S. aureus* expresses two sigma factors, (i) the primary sigma factor SigA required for the expression of housekeeping genes and (ii) a stationary phase sigma factor SigB that is homologous to the stress responsive transcription factor expressed by *B. subtilis* (Kullik *et al.*, 1998; Deora *et al.*, 1997). SigB is involved in the regulation of gene expression upon changes in the environment such as starvation, heat shock and osmotic shock (Wu *et al.*, 1996).

The *sigB* gene is present in an operon along with three other genes, *rsbU, rsbV* and *rsbW*. The first two genes are involved in regulating SigB expression (Palma & Cheung, 2001). RsbU is activated by SigA and is required for SigB activation (Kullik *et al.*, 1998). RsbW acts as an anti-SigB that binds to SigB and RsbV is an anti-anti-SigB that competes with SigB for the binding with RsbW. At least 30 of the *S. aureus* genes, most of them involved in stress responses are controlled by SigB (Gertz *et al.*, 2000). SigB is involved in bacterial aggregation by modulating the expression of the clumping factor and other adhesins. Additionally, SigB is also responsible for the protection of the bacteria against UV radiation by controlling the expression of staphyloxanthin (Giachino *et al.*, 2001).

## 1.4.3 Two component signal transduction systems

A two-component system (TCS) is a signal transduction mechanism by which bacteria respond to their environmental. Typically, a TCS consists of a sensor histidine kinase (HK) and a response regulator (RR). The sensor is a membrane protein consisting of an autokinase domain and a signal binding domain. The RR is a cytoplasmic protein with DNA binding activity comprising an N-terminal regulatory domain and a C-terminal effector domain. After the sensor protein detects the outside signal and auto-phosphorylates the histidine residue, the phosphate group is transferred to the RR N-terminal domain. The RR protein then binds to its target promoters activating transcription (Hoch, 2000, Stock *et al.*, 2000, West & Stock, 2001).

In *S. aureus* 16 TCS have been described, with an additional system present in the staphylococcal cassette chromosome *mec* of MRSA that regulates expression of methicillin resistance (Kuroda *et al.*, 2001). These TCS are involved in sensing environmental stimuli such as nutrient concentration, cell density, pH, ionic strength and membrane stresses. This helps *S. aureus* to adapt to the diverse microenvironments it encounters during its life cycle.

Several TCS are major regulators of virulence factor gene expression such as AgrCA (described before), SaeRS, ArlRS, PhoPR and VicRS (Giraudo *et al.*, 1999, Novick & Jiang, 2003, Haag & Bagnoli, 2016), antibiotic resistance (Gardete *et al.*, 2006, Meehl *et al.*, 2007, Hiron *et al.*, 2011), cell wall metabolism (Martin *et al.*, 1999, Fournier & Hooper, 2000, Brunskill & Bayles, 1996), bacterial respiration (Pragman *et al.*, 2004, Fedtke *et al.*, 2002, Kamps *et al.*, 2004, Sun *et al.*, 2012) and nutrient sensing (Stauff *et al.*, 2007).

A review of the TCS relevant to this work follows.

### 1.4.3.1. The SaeRS system

*S. aureus* exoproteins expression (*sae)* locus encodes a TCS crucial for expression of many exoproteins. This operon is composed of four different genes *saeR, saeS, saeP* and *saeQ*.

Two classes of target genes have been identified. Class I genes require high levels of phosphorylated SaeR for activation (*coa*, *sae* P1 promoter, *fnbA* and *eap*) and class II need lower basal phosphorylation levels of *saeR* (*hla* and *hlb*) (Mainiero *et al.*, 2010). Rot and SigB repress expression of the *sae* P1 promoter, thus indirectly repressing *hla* expression (Li & Cheung, 2008, Goerke *et al.*, 2005).

The *sae* system is influenced by other global regulatory mechanism such as Agr and the ferric iron uptake regulator (Fur) (Johnson *et al.*, 2011). Sae activates a variety of genes involved in immune evasion and adhesion to host molecules such as *seb, efb, eap, lukF, lukM, hlgACB, chp, scn, sspA, fnbA* and *fnbB* (Rogasch *et al.*, 2006, Harraghy *et al.*, 2005, Rooijakkers *et al.*, 2006, Kuroda *et al.*, 2007). Moreover, Sae acts in conjunction with Rot to activate expression of Staphylococcus superantigen-like (SSL)

proteins while *ssl* gene expression is repressed by *agr* (Benson *et al.*, 2012, Pantrangi *et al.*, 2010).

### 1.4.3.2 The ArlRS system

The autolysis-related locus (ArlRS) TCS is involved in the regulation of the DNA supercoiling response to high osmotic pressure (Fournier & Klier, 2004). However, it is not known which environmental signal activates *arlRS*. An *arlS* mutant had increased autolysis as a result of increased peptidoglycan hydrolase activity (Fournier & Hooper, 2000). The Arl system regulates the agglutination mechanism during exposure to human plasma or fibrinogen (Walker *et al.*, 2013).

Microarray analysis showed that ArlR positively regulates the expression of the LytRS TCS involved in autolysis and *lrgA/lrgB* (encoding two holin-like proteins involved in murein hydrolase transport and inhibition (Liang *et al.*, 2005).

Capsule biosynthesis is affected indirectly by the ArlRS system. This system activates the transcription of the transcriptional regulator *mgrA*, which activates capsule biosynthesis (Luong & Lee, 2006).

### 1.4.3.3. The WalKR system

WalKR (also called VicRS or YycFG) is the only essential TCS in *S. aureus*. This TCS is involved in diverse cellular processes, including cell wall metabolism, virulence regulation, biofilm production, oxidative stress resistance and resistance to antibiotics. WalKR regulates all these different cellular processes via direct or indirect regulation of autolysins, the *saeRS* two component system, haemolysins and other key virulence factors (Delauné *et al.*, 2012, Dubrac *et al.*, 2007, Dubrac *et al.*, 2008, Martin *et al.*, 1999). A transcriptome analysis was used to determine the genes comprising the WalKR system regulon. The analysis showed positive regulation of virulence genes in host matrix interactions (*efb, emp, fnbA* and *fnbB*), cytolysis (*hlgACB, hla* and *hlb*) and innate immune defense evasion (*scn, chp* and *sbi*), through activation of the SaeRS TCS (Delauné *et al.*, 2012).

### 1.4.3.4 The PhoPR system

The PhoPR TCS is responsible for the phosphate starvation response and cell wall homeostasis under phosphate deprivation in *Bacillus subtillis* (Hulett, 1996, Hulett, 2002). The function of this system in *S. aureus* is not known.

In *Bacillus subtilis*, this system also controls the teichoic acid/teichuronic acid transition that occurs in response to phosphate limitation. PhoPR represses the *tagABC* operon and activates the operon encoding the teichuronic acid synthesis enzymes (Bisicchia *et al.*, 2010).

### 1.4.3.5 The VraRS system

The vancomycin resistance associated (VraRS) two component signal transduction system. This system is induced by exposure of *S. aureus* to cell-wall affecting antibiotics such as glycolipids and β-lactams. The activation of this system triggers the activation of transcription of several different genes (Kuroda *et al.*, 2003). This system is described in detail in Chapter 3.

## 1.5. Aims and Rationale for this study

Wall teichoic acid was described to be a factor that is required for host colonization (Weidenmaier, 2004). It was reported that WTA is essential for nasal colonization by *S. aureus* in a cotton rat model. Furthermore, a WTA deficient mutant had a decreased capacity to establish infection in a rabbit endocarditis model (Weidenmaier *et al.*, 2005). The authors did not consider the possible effect of the absence of WTA on the disposition of the cell wall proteins or the loss of fitness. This data has to be analysed carefully since the authors used a strain that had previously been subjected to chemical mutagenesis and might have defects in other genes (Iordanescu & Surdeanu, 1976). Secondly, the method employed to isolate the *tagO* mutation used the pBT2 vector with a thermosensitive origin of replication that required cells to be grown at 42ºC. This causes temperature stress that can stimulate the acquisition of secondary mutations (Redder & Linder, 2012). A *tagO* mutant in *S. aureus* strain Newman was generated using a similar method (Gründling & Schneewind, 2006). None of these mutant strains was tested for the absence of other

mutations (eg. by whole genome sequencing and/or gene restoration). Both mutant strains were utilised, by these and other authors, in several studies analysing the importance of WTA in colonization/pathogenesis of *S. aureus* (Weidenmaier *et al.*, 2005, Weidenmaier *et al.*, 2010). Later, the same strain was used to show that WTA binds to SREC-I receptors which mediates the *S. aureus* adhesion to cells from the inner nasal cavity (Baur *et al.*, 2014), and more recently, to show that the amount of WTA on the cell surface of MRSA strains is associated with increase abscess formation in a skin abscess model (Wanner *et al.*, 2017).

Considerable evidence that CWA surface proteins are important for nasal colonisation and adhesion to squames arose when a mutant of Newman defective in ClfB, SdrC, SdrD and IsdA (CWA proteins relevant to adhesion) showed no adherence to human desquamated nasal epithelial cells. This impled that WTA is of limited importance in binding to squames (Corrigan *et al.*, 2009).

Lack of WTA on the cell surface causes pleiotropic effects on the cell. These mutants have severe morphological and growth defects, and in forming biofilm (Peschel *et al.*, 1999, Holland *et al.*, 2011, Gross *et al.*, 2001, Kohler *et al.*, 2009, Schlag *et al.*, 2010, Campbell *et al.*, 2011).

Thus WTA could be of importance for *S. aureus* to colonise the nares, but *in vivo* studies with *tagO* mutants cannot be used to support this hypothesis.

Here WTA-deficient mutants were isolated by allelic exchange using a temperature sensitive vector that imposed less stress than the one used previously. Furthermore several independent mutants in four different strains were compared. Complementation analysis was also performed (Chapter 3).

The complete transcriptional profile of a *tagO* mutant of strain Newman was compared to the wild type and a number of genes were found to be up-regulated and down-regulated. The changes in transcription of several genes that are important for host colonization and bacterial virulence were also examined by RT-PCR (Chapter 3). The expression of important cell wall anchored proteins and secreted proteins were then measured by Western immunoblotting and functional analysis (Chapter 4).

TarH is responsible for the last step of the WTA biosynthetic pathway, the translocation of the WTA polymer to the cell surface, where it becomes attached to the peptidoglycan. Another objective of this study was to isolate a *tarH*-null mutant to

investigate if the intermediates of the WTA pathway that accumulate in the *tarH* mutant have a role in the regulation of other genes/pathways (Chapter 4).

Lipoteichoic acid is a major component of the cell envelope. Preliminary studies were performed to determine if loss of LTA led to similar changes in CWA and secreted proteins expression as in the WTA mutants (Chapter 5).

# Chapter 2

# Materials and Methods

## 2.1 Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 2.1. *E. coli* strains were grown with shaking (200 rpm) in Luria (L) broth or on L agar (Difco) at 37ºC. *S. aureus* cultures were grown on tryptic soy agar (TSA; Oxoid) or in tryptic soy broth (TSB; Oxoid) at 37°C. Cultures were supplemented with ampicillin (Amp; 100 μg/ml), chloramphenicol (Cm; 10 μg/ml) or anhydrotetracycline (ATc) at 1 μg/mL, as required. Unless otherwise stated, all reagents were obtained from Sigma.

## 2.2 Plasmids

All plasmids used in this study are listed in Table 2.1

## 2.3 DNA Manipulation

DNA manipulations were performed using standard methods (Sambrook, 2001). Restriction endonucleases were purchased from New England Biolabs and Roche and were used according to manufacturers'instructions. DNA ligase was purchased from Roche. DNA sequencing was carried out by GATC-Biotech. Sequence and ligase-independent cloning (SLIC) was carried out as described by (Li & Elledge, 2012).

### 2.3.1 Preparation of plasmid and genomic DNA

Plasmid DNA was isolated using the Wizard® Plus SV Miniprep kit (Promega) or Qiagen® Plasmid Plus Midi Kit. DNA purification was carried out using the Wizard® SV Gel and PCR Clean-up System (Promega) or the High Pure PCR Product Purification Kit (Roche). Genomic DNA was prepared using Bacterial Genomic DNA purification kit (Edge BioSystems). Plasmid and genomic DNA extracts from *S. aureus* required the addition of lysostaphin (200 μg/ml) of to digest cell wall peptidoglycan.

**Table 2.1:** Strains and plasmids used in this study. ATc, anhydrotetracycline; $Cm^r$, chloramphenicol resistant; $Ap^r$ , ampicillin resistant; $Tc^r$ , tetracycline resistant; $Em^S$, erythromycin sensitive; $Kan^r$, kanamycin resistant

| *S. aureus* | Relevant features | Reference |
|---|---|---|
| Newman | CC8, MSSA, genome sequenced | (DUTHIE ES, 1952) |
| Newman *spa* | *spa*::$Ka^r$ | (Higgins *et al.*, 2006) |
| Newman *clfAclfB* | Δ*clfA*; *clfB*::$Tc^r$ | (Ní Eidhin *et al.*, 1998) |
| Newman *srtA* | *srtA*::$Em^r$ | (Mazmanian *et al.*, 2000) |
| Newman *agr* | *agr::*Tn551; $Em^r$ | (Dassy *et al.*, 1993) |
| Newman *tagO* | Δ*tagO* | This study |
| Newman *tagO* comp. | Δ*tagO* complemented with pALC2073::*tagO*; $Cm^r$ | This study |
| LAC $Em^s$ | Erythromycin-sensitive derivative of CC8 CA-MRSA strain LAC | (Boles *et al.*, 2010) |
| LAC $Em^s$ *spa* | *spa::*$Ka^r$ | (O'Halloran *et al.*, 2015) |
| LAC $Em^s$ *clfA* | *clfA::* $Tc^r$ | J. Geoghegan; TCD collection |
| LAC $Em^s$ *clfB* | Δ*clfB* | L. Hays; TCD collection |
| LAC $Em^s$ *srtA* | *srtA*::$Tc^r$ | L. Hays; TCD collection |
| LAC $Em^s$ *tagO* | Δ*tagO* | This study |
| LAC $Em^s$ *tagO* comp. | Δ*tagO* complemented (pALC2073::*tagO*); $Cm^r$ | This study |
| SH1000 | CC8, MSSA, genome sequenced | (Horsburgh *et al.*, 2002) |
| SH1000 *spa* | *spa*::$Tc^r$ | J. Geoghegan; TCD collection |
| SH1000 *clfAclfBfnbAfnbB* | *clfA*::Tn917$Em^r$ *clfB*::$Tc^r$ *fnbA*::$Tc^r$ *fnbB*::$Em^r$ | (O'Neill *et al.*, 2007) |
| SH1000 *tagO* | Δ*tagO* | This study |
| SH1000 *tagO* comp. | Δ*tagO* complemented (pALC2073::tagO); $Cm^r$ | This study |
| BH1CC | CC8, HA-MRSA, genome sequenced | (O'Neill *et al.*, 2007) |
| BH1CC *spa* | *spa*::$Ka^r$ | J. Geoghegan; TCD collection |
| BH1CC *tagO* | Δ*tagO* | This study |
| BH1CC *tagO* comp. | Δ*tagO* complemented (pALC2073::*tagO*); $Cm^r$ | This study |
| LAC $Em^s$ *ltaS gdpP* 2426 | *Δlta::erm;* with a rearrangement in the promotor region of *gdpP* | (Corrigan *et al.*, 2011) |
| LAC $Em^s$ *ltaS gdpP* 2434 | (*Δlta::erm* suppressor, with a *gdpP* mutation | (Corrigan *et al.*, 2011) |
| LAC $Em^s$ *gdpP* | *ΔgdpP* | (Corrigan *et al.*, 2011) |
| Newman *tagO tarH* | *tarH* frameshift mutation in Newman *tagO* background | This study |
| Newman *tagO tarH* pRMC2 | Carrying pRMC2 plasmid; $Cm^r$ | This study |
| Newman *tagO tarH* pRMC2::*tagO* | Carrying pRMC2::*tagO*; $Cm^r$ | This study |

| *E. coli* | Relevant features | Reference |
|---|---|---|
| SA08B | DC10BΩ$P_{help}$-hsdMS (CC8-2) (SAUSA300_1751) of NRS384 integrated between *atpI* and *gidB* | (Monk *et al.*, 2015) |
| DC10B | Δ*dcm* in the DH10B background; Dam methylation only | (Monk & Foster, 2012) |
| **Plasmids** | **Relevant features** | **Reference** |
| pIMAY-Z | Temperature sensitive vector for allelic exchange; carries *lacZ*; $Cm^r$ | (Monk *et al.*, 2015) |
| pIMAY-Z::*tagO* | pIMAY-Z with cassette for isolating *tagO* mutation; $Cm^r$ | This study |
| pALC2073 | Multicopy shuttle vector; Atc inducible; $Cm^r$ | (Bateman *et al.*, 2001) |
| pALC2073*tagO* | pALC2073::*tagO;* $Cm^r$ | This study |
| pRMC2 | Anhydrotetracycline-inducible expression vector. $Amp^r$, $Cm^r$. | (Corrigan & Foster, 2009) |
| pIMAY-Z::*tarH* FS | pIMAY-Z::*tarH* with a 2 nucleotides insertion; $Cm^r$ | This study |
| pRMC2::*tagO* | pRMC2::*tagO;* $Cm^r$ | D. O'Halloran; TCD collection |
| TOPO | Plasmid for the direct insertion of blunt-end PCR products; carries *ccd*B gene fused to the C-terminus of the LacZα fragment; $Kan^r$ | Thermo Fischer |
| TOPO::tarH FS | TOPO::*tarH* with a 2 nucleotide insertion; $Kan^r$ | This study |

**Table 2.2**: List of all the primers used in this study.

| Primers | Sequence 5´- 3´ |
|---|---|
| pIMAY fw | CCTCACTAAAGGGAACAAAAGCTGGGTACC |
| pIMAY rv | CGACTCACTATAGGGCGAATTGGAGCT C |
| tagO A | CCTCACTAAAGGGAACAAAAGCTGGGTACCACGATAAGGGATAGGGTAG |
| tagO B | GGGTGGTACAGTGATTCTC |
| tagO C | GGGTGGTACAGTGATTCTCCGTAAGTCATCTCATAAAGAGGAATAG |
| tagO D | CGAATTAAACCGCCACCACC |
| OUT fw | CTGTGGCAGATAATGTATAGACC |
| OUT rv | CGACACACGCATTATGAACATCG |
| ALC fw | CCGGGAGCTCCGATGAAGGTGAATAAATGGTTAC |
| ALC rv | CCGGGAATTCCAGCTATGCTTTCATTCCCTATTCC |
| spa fw | CCTGGTGATACAGTAAATGACATTGC |
| spa rv | CCAAATACAGTTGTACCGATG |
| clfB fw | GCCATCATTGCACCAAATAAAGTTGC |
| clfB rv | CCGATTCAAGAGTTACACCACC |
| gyr fw | TTATGGTGCTGGACAGATACA |
| gyr rv | CACCGTGAAGACCGCCAGATA |
| ori fw | CACGATGCGAGCAATCAAATTTC |
| ori rv | GTGCATAAGTGGATAACTTTTCC |
| RNAIII fw | GAAGGAGTGATTTCAATGGCACAAG |
| RNAIII rv | GAAAGTAATTAATTATTCATCTTATTTTTTAGTGAATTTG |
| hla fw | GGTAGTCATCACGAACTCGTTCG |
| hla rv | GCTCTATGAAAGCAGCAGATAACTTC |
| ebpS fw | GGCCACCACCTTGACGTTGTTG |
| ebpS rv | CAAAAGAGGATCAAGACAAAGCG |
| clfA fw | CGATTGGCGTGGCTTCAGTGC |
| clfA rv | CGATGTTTTAGTATCACTCACG |
| sigB fw | GGCGAAAGAGTCGAAATCAGC |
| sigB rv | GCACCTATTAAACCAACCATACC |
| atpA fw | GACGAATGCTGTACAAATAACGG |
| atpA rv | CATCGTCTAAAGGTCTTAACGC |
| walR fw | CGATGTGTACTGTGCATACGATG |
| walR rv | CGACGTAAGTTCGCTTTCACACG |
| saeR fw | CGTGGATGATGAACAAGACATTG |
| saeR rv | CATCTGCACCTAAAGTTAAGGCG |
| phoR fw | GCTGGTTATGAAGTTGTTGTCGC |
| phoR rv | GTCATCGGCACCTAATTCTAAACC |
| arlR fw | GGACACAGAGTATGATGGACAAG |
| arlR rv | CTTAACTATATAATCGTCTGCACC |
| tagO fw | GGGTGGTACAGTGATTCTC |
| tagO rv | GGCAGCGGCAATTTGACCAGCC |
| OUT tarH fw | GGTATTGAGTTGATGGATGAATG |
| OUT tarH rv | GGTACACCATGAATTGGTGC |
| pIMAY MCS fw | TACATGTCAAGAATAAACTGCCAAAGC |
| pIMAY MCS rv | AATACCTGTGACGGAAGATCACTTCG |
| tarH FS fw | CGTATGAAAGATCTGCGCTCATTCCC |
| tarH FS rv | GGGAATGAGCGCAGATCTTTCATACG |
| M13 fw | GTAAAACGACGGCCAG |
| M13 rv | CAGGAAACAGCTATGAC |
| pRMC2 MCS fw | ATTCAGGCTGCGCAAC |
| pRMC2 MCS rv | TTGTTGACATTATATCATTG |

### 2.3.2 Polymerase chain reaction

Polymerase chain reaction (PCR) amplification was carried out in a DNA thermal cycler (PYKO). Reactions were typically carried out in 50 µL volumes using Phusion™ Hot Start DNA polymerase in Phusion HF Buffer (Thermo Fischer), Phire Hot Start DNA polymerase in Phire buffer or Velocity DNA polymerase in Hifi Buffer (Bioline). Plasmid DNA (10 ng) or genomic DNA (150 ng) were used as templates for PCR. Primers and dNTPs (Bioline) were used at a final concentration of 0.2 mM and 200 µM, respectively. Primers were purchased from Integrated DNA Technologies and are listed in the Table 2.2. When using Phusion the initial denaturation was carried out at 98 ºC (30 sec) followed by 30-35 cycles of denaturation (10 sec) at 98 ºC, 20 sec annealing (temperature dependent on primer used) and extension at 72ºC. When amplifying plasmid DNA, a 15 sec extension time per kb DNA was used. For genomic DNA a longer extension time was used (30 sec per Kb). A final extension step was carried out at 72ºC for 5 min. PCR products were purified using Wizard SV gel and PCR clean-up system (Promega) or High Pure PCR product Purification Kit (Roche).

Velocity reaction starts with an initial denaturation of 98 ºC (2 min) followed by 30-35 cycles of denaturation (30 sec) at 98 ºC, 30 sec. annealing (temperature dependent on primer used) and elongation at 72 ºC for 15 s/kb being amplified. This was followed by incubation at 72ºC for 10 min.

### 2.3.3 Agarose electrophoresis

Gels containing 1% agarose Tris-acetate-EDTA (TAE) buffer were cast in mini trays (Life technologies). DNA samples in loading buffer were added to wells along with DNA size markers (Bioline). Electophoresis of samples was routinely performed at 90 V. Gels were stained with ethidium bromide for 10 min., washed and viewed under UV light. Gels images were collected using an Alpha imager.

## 2.4 Transformation

### 2.4.1 Preparation and electroporation of *E. coli*

Electrocompetent cells were prepared by inoculating L-broth (400 mL in a 2 L baffled flask) with 4 mL overnight culture of *E. coli*. The culture was incubated at 37 ºC

with shaking (200 rpm) until and $OD_{600}$ of 0.6 was reached. The culture was then chilled on ice for 15 min. Cells were harvested at 8, 000 x g for 10 min. at 4 ºC and resuspended in 400 mL sterile ice-cold distilled water. Cells were harvested again at 8, 000 x g for 10 min. at 4 ºC and the pellet was resuspended in 5 mL 10 % (v/v) glycerol. Aliquots (100 µl) were stored at -80 ºC. Cells were thawed for 5 min. on ice before adding up to 10 ng plasmid in a volume of 5 µL. Cells were then left on ice for 1 min. before being added to prechilled 0.1 cm electroporation cuvettes (Biorad) and were then pulsed at 1.8 kV/cm, 200 Ω and 25 uF. Cells were immediately resuspended in LB and were incubated for 1 h at 37ºC with shaking (200 rpm). Recovered cells were plated onto L-agar containing the appropriate antibiotic. Transformants were screened by PCR.

### 2.4.2 Preparation and electroporation of *S. aureus*

Plasmids isolated from *E. coli* SA08B or DC10B were introduced into *S. aureus* by electroporation. Electrocompetent cells were prepared by diluting an overnight culture of *S. aureus* to an $OD_{600}$ of 0.5 mL in 50 mL pre-warmed BHI, and the culture was incubated at 37 ºC for 30 min. Cells were cooled in an ice-water slurry for 10 min. Cells were harvested at 3,900 x g for 10 min. at 4 ºC. The supernatant was discarded and the pellet was resuspended in 50 mL sterile filtered ice-cold water. Cells were harvested again and the pellet was then washed in decreasing volumes of sterile ice-cold 10 % (v/v) glycerol (5 mL, 1 mL and 210 µL). Aliquots (50 µL) were stored at – 80 ºC.

Aliquots were thawed for 5 min on ice and 5 min. at room temperature. Cells were harvested at 5, 000 x g for 1 min. and were re-suspended in sterile 10% (v/v) glycerol / 500 mM sucrose (50 µL). Up to 5 µg of plasmid was added to the cells in a volume of 5 µL, this was mixed and loaded in a 0.1 cm electroporation cuvette (Biorad). The cells were pulsed at 21 kV/cm, 100 Ω and 25 uF. Following electroporation cells were immediately resuspended in sterile BHI/500 mM sucrose (1 mL) and were incubated for 1.5 h at 37 ºC with shaking (200 rpm). Cells were then plated out on TSA containing the appropriate antibiotic. Transformants were screened by PCR.

### 2.4.3 Screening of Transformants

Colonies intended for screening were toothpicked onto the appropriate agar and also resuspended in 40 µL of TE buffer (1 mM EDTA / 10 mM Tris – HCL, pH 7.8). The suspension was then boiled at 100 ºC for 10 min. and centrifuged at 6, 000 rpm for 10 min. Supernatant (2 µL) was used as a template for PCR using Phire polymerase.

## 2.5 Strain and plasmid construction

### 2.5.1 Plasmid construction

The pALC2073 plasmid is a shuttle vector that contains the *tetA* operator/promoter which is induced by anhydrotetracycline, which inactivates the TetR (Corrigan & Foster, 2009). For complementation studies, the full length *tagO* gene, including the ribosome binding site sequence, was cloned into pALC2073, using the enzymes EcoRI and SacI and their specific restriction sites in the vector multiple cloning site. The construct was transformed into *E. coli*. The plasmid was extracted and the correct size of the insert was confirmed by PCR using primers for the insert up and downstream outside regions (ALC fw and rv primers, table 2.2) and by DNA sequencing. It was then transformed into *S. aureus tagO* mutants. All primers are listed in the Table 2.2, in the appendices.

### 2.5.2 Sequence and ligase-independent cloning to generate *tagO* deletion cassette

For sequence and ligase-independent cloning (SLIC) (Li & Elledge, 2012) both vector and insert were amplified by PCR. Primers for amplification of insert were designed with 5´extensions, typically 24 bp in length that possessed sequence homology to the ends of the amplified vector (Table 2.2). Primers were designed to amplify 500 bp of DNA located upstream and downstream of *tagO* to delete the entire gene (*tagO* A, B, C and D primers). Primers B and C were design with a complementary sequence to allow the A/B and C/D fragments to reanneal, which is achieved by joining the two different A/B and C/D products. The religated fragment was then amplified with the A and D primers, resulting in a 1000 bp fragment. Both vector and insert DNA (1 µg) were incubated with T4 polymerase (3 U; NEB) in a final reaction volume of 40

µL containing NEB buffer 2, molecular grade BSA (NEB), Dithiothreitol (DTT, 5 mM) and urea (200 mM). The reaction mix was incubated at 23 ºC for 20 min. and stopped by the addition of EDTA (25 mM), followed by further incubation at 75 ºC for 20 min. 5 µL each of vector and insert with single stranded ends were mixed and allowed to anneal. This reaction was incubated at 65 ºC for 10 min. followed by a slow decrease in temperature from 65 – 25 ºC with 1 min. hold for each degree. The entire reaction was used for transformation into *E. coli* SA08B.

### 2.5.3 Allelic exchange mutagenesis of *tagO*

Plasmid pIMAY-Z::*tagO* was isolated from *E. coli* SA08B and introduced into *S. aureus* by electroporation as described in section 2.4.2. The presence of the *lacZ* gene in the pIMAY-Z plasmid helped to identify colonies with the *tagO* mutation (white colonies) against blue colonies (still with integrated plasmid).Transformants were selected for on TSA incorporating chloramphenicol at 28ºC, the temperature at which pIMAY-Z replicates. Colonies were screened with primers that anneal externally to the multiple cloning site (MCS primers) of pIMAY-Z. Positive clones were incubated on TSA plates containing Cm at 37°C (not permissive for pIMAY-Z replication) to select for single crossover integration into the chromosome. Larger colonies were screened by PCR with the pIMAY MCS primers (Table 2.2) for loss of replicating pIMAY-Z. Colonies than did not carry replicating plasmid were analysed for the side of integration. A band of approximately 1 kb was amplified in both clones, showing that the integration occurred on the upstream side. These two clones were incubated at 28°C to stimulate pIMAY-Z replication in the presence of anhydrotetracycline (1 µg/ml) and x-gal. The plasmid contains an anhydrotetracycline inducible antisense-*secY* gene. Bacteria containing the replicating plasmid will have a growth defect due to the essential gene *secY* being knocked down, and mutant colonies will be white. Additionally plates supplemented with 7. 5% NaCl and 40% sucrose (separately) were used. These compounds were chosen since they were required for an LTA mutant to grow (Corrigan *et al.*, 2009, border-box *et al.*, 2010, Corrigan *et al.*, 2011). Several white small and large colonies were restreaked (at 37ºC and 28ºC) for single colonies in the same media used in the last step. No advantage was seen with the different media

supplements, so both white large and small colonies from the one grown at 37ºC (in TSA with ATc and X-gal) were selected and PCR was carried out using primers for the 600 bp region up and downstream of the AB and CD sequences. A 1.3 kb fragment was amplified in 5 of the colonies screened indicating successfully deletion of *tagO*. These colonies were also plated on TSA containing Cm to confirm loss of the plasmid, and sensitive colonies were chosen. The PCR fragment resulting from deletion was sent for DNA sequencing to confirm the fidelity of the *tagO* deletion. The same procedure was used to isolate *tagO* mutants in Newman *Δspa*, LAC, SH1000 and BH1CC strains. Additionally a second independent mutant was isolated for all the four strains. The *tagO* mutant strains were complemented with pALC2073::*tagO*.

### 2.5.4. Frameshift mutation of *tarH*

Using the primers OUT *tarH* fw and OUT *tarH* rv (Table 2.2) the *tarH* gene was amplified plus 529 bp downstream and 450 bp upstream the gene. A 1.8 kb fragment was amplified and cloned into TOPO vector (Thermo Fischer) by direct insertion of the blunt-end insert into the vector. This reaction was transformed into electrocompetent *E. coli* TOP10 cells and transformants were selected in on L agar with kanamycin and screened by colony PCR using primers M13 fw and M13 rv. The plasmid was extracted and confirmed by DNA sequencing. The TOPO::*tarH* plasmid was amplified using primers for the insertion of 2 nucleotides (CT) and the insertion of the restriction site for the BglII enzyme (AGATCT) into the *tarH* fragment, creating the TOPO::*tarH* FS plasmid. TOPO::tarH FS plasmid was treated with DpnI and transformed into chemically competent *E.coli* XL1 Blue. Transformants were selected in L agar with kanamycin and were screened by colony PCR and M13 fw and rv primers, the plasmid was extracted and sent for sequencing.

The vector TOPO::*tarH* FS was then double digested with the restriction enzymes EcoRI and NotI, the *tarH* FS insert was gel extracted and cloned into pIMAY-Z between the same restriction sites by a ligation reaction. The pIMAY-Z::*tarH* FS vector was then transformed into *E. coli* DC10B and transformants were screened with pIMAY MCS primers (primers that anneal externally to the multiple cloning site). This plasmid was then extracted, sequenced and transformed into *S. aureus* Newman *tagO* mutant

at 28ºC, the temperature at which pIMAY-Z replicates. Two positive clones were incubated on TSA plates containing Cm at 37°C (not permissive for pIMAY-Z replication) to select for single crossover integration into the chromosome. Larger colonies were screened by PCR with the MCS primers for loss of replicating pIMAY-Z. Negative and positive controls of Newman with replicating pIMAY-Z were included. Every colony screened showed no amplification, possibly indicating integration. Two of these were further analysed for integration. Using the OUT primers the 1.8 kb was (now inserted into the genome) was amplified by PCR and digested with the restriction enzyme BglII. One colony was positive for integration (presence of 2 fragments after digestion) and was used for excision. This clone was incubated at 28°C to stimulate pIMAY-Z replication in the presence of anhydrotetracycline (1 µg/ml) and several big colonies were restreaked (at 37ºC and 28ºC) for single colonies in the same media used in the last step (as explained in section 2.5.1). Colonies grown at 37ºC were selected for genomic extraction. A PCR with OUT primers was carried out for the amplification of a 1.8 kb fragment. Four of the fragments were then digested with the restriction enzyme BglII and one was successfully digested, showing 2 bands in the agarose gel after digestion. This suggests that the *tarH* gene with the 2 nucleotide insertion was successfully inserted into the genome. This was also confirmed by sequencing. This colony was also plated on TSA containing Cm to confirm loss of the plasmid.

To restore the *tagO* mutation the double mutant *tagO tarH* was transformed with pRMC2::*tagO* or pRMC2 (O´Halloran, not published) and selected on TSA with chloramphenicol and tunicamycin. The transformants were screened using a colony PCR and pRMC2 MCS fw and rv primers (that anneal outside of the MCS region of the pRMC2).

## 2.6 RNA-seq

### 2.6.1 Total RNA extraction

Exponential growth phase cultures (4 mL) were mixed with ice cold 5% (v/v) phenol pH 4.3, 95% (v/v) ethanol (1, 6 mL). This incubated for 30 min. to stabilise the RNA and prevent degradation, centrifuged and the pellets were resuspended with the

residual liquid and transferred to a 1, 5 mL eppendorf tube. The samples were spun down at maximum speed and the remaining liquid was discarded. The pellet was then dissolved in 1 mL TRIzol on ice and transferred to phase-lock tubes (Phase Lock Gel Heavy 2 ml 5Prime from VWR). 400 µL of $CHCl_3$ were added and the mixture was agitated for 10 sec., incubated for 2 minutes at room temperature and centrifuged for 15 min. The supernatant was then transfered to a new tube and 450 µL of isopropanol was added and incubated for 30 min. at room temperature. The samples were then centrifuged for 30 min. at full speed, the supernatant was removed and the pellet washed with ethanol. After a 10 min. centrifugation the pellet was left to air-dry. The pellet was then resuspended with 25 µL of RNA-free water and dissolved for 5 min. with incubation at 65ºC, vortexing every 2-3 min.

### 2.6.2 cDNA library preparation for RNA-seq

Strand-specific cDNA library preparation and high throughput cDNA sequencing (RNA-seq) of Newman wild type and Newman *tagO* was performed on DNase I digested total RNA by Vertis Biotecnologie AG (Freising, Germany). RNA was depleted for ribosomal RNA (using the Ribo-Zero kit), fragmented by sonication and poly (A) – tails were added to each fragment by poly (A) polymerase. The 5´end of each fragment was de-phosphorylated using tobacco acid pyrophosphatase (TAP). An RNA adaptor, containing a 6-10 nucleotide bar-code was ligated to the 5´end of each fragment to allow for identification of each RNA fragment after sequencing. First strand cDNA synthesis was performed using oligo priming and Moloney murine leukemia virus reverse transcriptase (M-MLV RT). The resulting cDNA was amplified by PCR to approximately 20 - 30 ng/µL using a high fidelity DNA polymerase. cDNA was purified using the Agencourt AMPure XP kit (Beckman Coulter Genomics) and analysed by capillary electrophoresis. The strand specific cDNA libraries were generated using the ScriptSeq kit. The library preparation method also involves the tagging of fragmented RNA with adaptor molecules for directional sequencing.

### 2.6.3 RNA sequencing

The sequencing of the cDNA was performed by Vertis Biotechnologie AG on an Illumina HiSeq 2000 platform. cDNA samples were immobilised on a flow cell surface and amplified by bridge amplification to create clusters of identical copies of single template molecules. Illumina sequencing uses "sequencing by synthesis" (SBS) technology, involving the incorporation of dNTPs reversibly labelled with fluorescent terminators. The labels serve as terminators for polymerase so each dNTP fluoresces and is imaged before being enzymatically cleaved to allow the addition of the next nucleotide. This technique allows for the identification of the individual nucleotide that is incorporated into the nucleic acid chain during each sequence cycle.

## 2.7 Subcellular fractionation

Cultures of *S. aureus* were harvested, the cell free supernatant was precipitated with trichloroacetic acid (TCA) and the cells washed in phosphate-buffered saline (PBS), and resuspended to an $OD_{600}$ of 10 in lysis buffer (50 mM Tris-HCl, 20 mM $MgCl_2$, pH 7.5) supplemented with raffinose (30% w/v) and complete protease inhibitors (40 µl/mL; Roche). To isolate cell wall associated proteins, exponential or stationary phase cultures were harvested, washed twice in PBS and resuspended at an $OD_{600}$ of 10 in 250 µL lysis buffer (50 mM Tris-HCl, 20 mM $MgCl_2$, pH 7.5) supplemented with 30 % (w/v) raffinose and complete EDTA-free protease inhibitors (40 µL/mL, from a 10x stock, Roche). Cell wall associated proteins were solubilized by incubation with lysostaphin (200 µg/mL; Ambi, NY) for 8 min at 37°C. Protoplasts were removed by centrifugation at 16,000 x *g* for 5 min, and the supernatant containing solubilized cell wall proteins was aspirated and boiled for 10 min to use in SDS-PAGE or stored at – 20ºC. The filtered cell-free supernatants were incubated with 100 % TCA, at 4ºC for 10 minutes, and after centrifuging at 4000 rpm for 10 min the precipitated proteins were washed with ice-cold acetone and resuspended in final sample buffer. The protoplasts were disrupted by several freeze-thaw cycles and the different compartments of the cell (membrane and cytoplasm) were separated by centrifugation at 18,000 x *g* for 1 h. The samples containing these two fractions were aspirated and boiled for 10 min in final sample buffer.

## 2.8 Protein electrophoresis and Western Immunoblot

### 2.8.1 SDS-PAGE

Electrophoresis was carried out loading 20 µL of the diluted protein onto acrylamide gels (4.5% stacking and 10% separating gel) and separated by SDS-PAGE at 120 V. Proteins were then electroblotted onto PVDF membranes (Roche) for Western Immunoblotting.

### 2.8.2 Western immunoblotting

Non-specific binding to membranes was inhibited by incubation for 1 h in 10% (w/v) skimmed milk powder (Marvel) in TS buffer (10 mM Tris-HCL, 0.9% (w/v) NaCl, pH 7.4). Blots were then probed with the appropriate primary antibody for 1h and washed three times with agitation for 10 min in TS buffer. The primary antibody was detected mostly using a horseradish peroxidase (HRP)-conjugated secondary antibody or protein A. All antibodies are listed in Table 2.3. Reactive bands were developed using the LumiGLO reagent and peroxidase detection system (Cell signalling technology). Blots were visualized using ImageQuant TL software (GE). This software was also used for measuring the bands intensity by densitometry.

Proteins were separated on 10% or 12.5% (wt/vol) polyacrylamide gels, transferred onto polyvinylidene difluoride membranes (Roche), and blocked in 10% (wt/vol) skimmed milk proteins. Blots were probed with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (1:2,000; Dako) to detect SpA. Alternatively, blots were probed with rabbit anti - ClfA (Geoghegan *et al.*, 2010)( 1 : 2000), anti-ClfB (1:1,000) (Ní Eidhin *et al.*, 1998), anti – alpha toxin (1:200) (Dajcs *et al.*, 2002), anti-V8 serum (1:250) (a gift from Martin McGavin), anti-SrtA (1:3000) (Abcam), anti-EbpS (Downer *et al.*, 2002), anti-ATPase(1:5000) (a gift from P. Owen) and bound antibodies were detected using HRP-conjugated goat anti-rabbit IgG or HRP-conjugated protein A (1:500).

**Table 2.3**: List of all the antibodies used in this study.

| Antibody | Working dilution | Reference |
|---|---|---|
| HRP-conjugated rabbit anti-mouse IgG | 1:2000 / 1:500 | DAKO |
| Rabbit anti-V8 serum | 1:250 | M. McGavin |
| FITC-labelled rabbit anti-mouse IgG | 1:3000 | DAKO |
| Polyclonal rabbit anti-ClfA | 1 : 2000 | (Geoghegan *et al.*, 2010) |
| Polyclonal rabbit anti-ClfB | 1:1,000 | (Ní Eidhin *et al.*, 1998) |
| Polyclonal rabbit anti - α-toxin | 1:200 | (Dajcs *et al.*, 2002) |
| Polyclonal rabbit anti-SrtA | 1:3000 | Abcam |
| Polyclonal rabbit anti-EbpS | 1:1500 | (Downer *et al.*, 2002) |
| Rabbit anti-*Micrococcus luteus* $F_1/F_0$ ATPase serum | 1:5000 | P. Owen |
| HRP-conjugated goat anti-rabbit IgG | 1:500 | DAKO |
| HRP-conjugated protein A | 1:500 | DAKO |

Reactive bands were visualized using LumiGLO reagent and a peroxide detection system (Cell Signaling Technology). Cell wall and membrane proteins Western immunoblots of Newman and LAC were also analysed by densitometry, using serial dilutions of the samples.

## 2.9 Detection of surface displayed SpA by flow cytometry

*S. aureus* cells were washed once with phosphate-buffered saline (PBS) and once in a solution of bovine serum albumin (BSA, 0.1%, w/v) and then adjusted to an OD600 of 0.3 in PBS. Bacteria were incubated with an equal volume of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (1:800 or 1:3,200; Dako) for 30 min. Unbound antibody was removed by one wash in PBS. Bacteria were resuspended in formaldehyde (2%, vol/vol), and bound IgG was detected using flow cytometry. Bacteria were gated on the basis of forward and side scatter. The fluorescence intensity of 20,000 bacteria was analyzed and the median fluorescence was calculated.

## 2.10 Adhesion of bacteria to recombinant ligands

The microtiter plates (Nunc) were incubated for 24 hours at 4ºC with serial dilutions of the ligand (fibrinogen, loricrin and fibronectin). The wells were washed three times in PBS and blocked with filtered 5% (w/v) BSA for 2 h at 37ºC. Bacterial cells were grown to stationary phase for fibrinogen adhesion or to exponential phase for loricrin. The cell cultures were spun down, washed and resuspended in PBS at an OD of 10 and were incubated (100 µL) in the wells with the ligand for 2 hours at 37ºC in agitation (400 rpm), fixed with formaldehyde (25% v/v, 100 µL per well) for 20 min. and stained with crystal violet (0.5% v/v, 100 µL per well) for 1 min. After several PBS washes the crystal violet was solubilized with acetic acid (5% v/v, 100 µL per well) and incubated for 5 min. at room temperature with shaking. The absorbance was measured at 570 nm in an ELISA plate reader (Labsystems).

## 2.11 Total RNA extraction and Real time qPCR

Exponential or stationary phase cultures (10 mL) were mixed with 23 mL of guanidine thiocyanate buffer (5 M guanidine thiocyanate, 0.5 % N-lauryl sarcosine, 0.1

M β-ME, 0.5 % Tween-80 and 10 mM Tris pH 7.5) and centrifuged at 7000 rpm for 10 min most of the supernatant was removed, the pellet resuspended with the remaining buffer and the samples were transferred to a 1.5 mL eppendorf and spun down again for 3 min at 17, 000 x *g* to remove the supernatant. The pellet was resuspended in 1 mL of RNApro (FastRNA® Pro Blue kit) and transferred to the lysing matrix b tubes (silica beads) and incubated on ice for 5min. These tubes were then loaded into the FastPrep®24 and the cells were lysed using setting 6 three times for 45 sec. with 2 min incubation on ice in between. The tubes were centrifuged for 5 min at 12,000 x *g* at 4ºC and the upper phase was transferred to a new eppendorf and incubated at room temperature. Nucleic acids were separated by adding 300 µL of chloroform, vortex for 10 min, incubate at room temperature for 5 min and centrifuged at 12, 000 x g for 5 min at 4ºC. The upper phase was transferred to a new eppendorf and the step with chloroform was repeated to ensure full separation. The nucleic acids were then precipitated adding 500 µL of pure ethanol and incubated for 30 min. at -20ºC. The samples were then centrifuged at 12, 000 rpm for 15 min at 4ºC, the supernatant was removed and the pellet was washed with 500 µL ice cold 100% ethanol and then 75% ethanol (made up in DEPC water). The samples were centrifuged at 12, 000 x *g* for 5 min. at 4ºC and the ethanol was removed. This step was repeated to ensure a complete ethanol removal. The pellet was air dried for 5 min. at room temperature, resuspended in 100 µL of RNA secure (Invitrogen) and incubated at 60ºC for 10 min.

The samples were then submitted to DNase treatment to eliminate any DNA contaminants and purified using the kit RNeasy RNA cleanup from Qiagen. Purified RNA was stored at -20ºC in SUPERase – in RNase inhibitor (Ambion). RNA purity was confirmed by running the samples on an agarose gel and by measuring the ratio A260/A280, which should be around 2. To prepare the standards, genomic DNA was extracted from the wild type Newman strain and diluted to 1:100 followed by 5 serial 10 - fold dilutions. The extracted RNA was then converted to cDNA using reverse transcriptase (High capacity cDNA reverse transcription kit from Thermo Fisher) under the following conditions: 25ºC for 10 min, 37ºC for 120 min and 85ºC for 5 min. Two reactions were prepared, one with specific primers for the gene concerned and another with primers specific for the housekeeping gene *gyrB.* A control without reverse transcriptase was included. The *gyrB* gene is constitutively expressed in *S.*

*aureus* and was used as an internal standard. The primers used in this experiment were designed to amplify a fragment of around 200 bp with an annealing temperature of 60ºC. The cDNA samples and controls were used as template for a PCR, to check primers efficiency and absence of DNA contamination. The cDNA samples were diluted to 1/100, to make sure that the readings were within the standards. SYBER® Green PCR Master Mix (AmpliTaq Gold® DNA Polymerase) was added to 2 µL of the samples and the standards and 0, 5 µL of the respective primers. The reaction was carried out in a 96-well plate, after a brief spin to eliminate any bubbles. The RT-PCR was performed in a StepOne™ System (Applied Biosystems) under the following conditions: 94ºC for 2 min followed by 40 cycles of 94ºC for 15 sec, 55ºC for 60 sec and 72ºC for 60 sec. Prior to performing the quantitative analysis of the data, the efficiency of the reaction (Eff) was determined through the standard graphics slope line. The formula Eff $=(10^{-1/slope})-1$ was used. Data sets with an efficiency of less than 0. 98 were discarded.

For the gene copy number experiment, the genomic DNA was extracted and treated with RNAse. The Newman wild type genomic DNA was diluted to 1:100 followed by 6 serial 10-fold dilutions. The $10^{-2} - 10^{-6}$ dilutions were used as standards. Newman wild type and *tagO* mutant genomic DNA $10^{-4}$ dilution was used to run the PCR, using the same conditions described above. The *oriC* gene (one copy in the chromosome) is constitutively expressed and was used as an internal control. A control without reverse transcriptase was included.

To calculate the fold change in expression between the Newman wild type and Newman *tagO*, cycle thresholds (Ct) values were used. The comparative Ct method is also known as the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), where ΔΔCt= ΔCt sample - ΔCt control. The ΔCt sample is the Ct value for any sample normalized to the internal control and ΔCt control is the Ct value for the control also normalized to the internal control. For the ΔΔCt to be valid, the amplification efficiencies of the sample and control must be similar.

## 2.12 Alpha hemolysin detection

Cells grown to the stationary phase were harvested and the supernatants filtered. Wells of approximately 2 cm diameter were cut in sheep blood agar and 100 uL of the supernatants were added to the wells and incubated at room temperature for 24 to 48 hours after which hemolysis was visualized.

## 2.13 WTA extraction and detection

WTA was isolated from cells from a 20 mL culture of *S. aureus* grown to stationary phase in TSB at 37°C. Cells were collected by centrifugation at 2,000 x *g* for 10 min, washed once with 30 mL of buffer 1 (50 mM 2-(*N*-morpholino)ethanesulfonic acid or MES, pH 6.5), and resuspended in 30 ml of buffer 2 (4% (w/v) sodium dodecyl sulfate or SDS, 50 mM MES; pH 6.5). Samples were placed in a boiling water bath for 1 h, and then collected by centrifugation at 10,000 x *g* for 10 min. The pellet was resuspended in 500 µL of buffer 2, transferred to a 2 mL microcentrifuge tube, and spun down at 14,000 x *g* for 10 min. Next, the pellet was washed once with 500 µL buffer 2, once with 500 µL of buffer 3 (2% NaCl, 50 mM MES, pH 6.5), and finally with 500 µL of buffer 1. After the last wash, samples were treated with 500 µL of proteinase K solution (20 mM Tris-HCl, pH 8.0, 0.5% (w/v) SDS, and 20 µg of proteinase K in 1 mL) and incubated at 50°C for 4 h. Following digestion, samples were washed once with 1mL of buffer 3 and then at least three times with 1 mL of distilled water to remove SDS. Samples were thoroughly resuspended in 1 mL of 0.1 M NaOH and shaken at room temperature for 16 h to hydrolyze WTA. Insoluble cell wall debris was removed by centrifugation at 14,000 x *g* for 10 min and the supernatant containing the hydrolyzed WTA was analyzed by polyacrylamide gel electrophoresis. Samples were diluted 1:3 in loading buffer (50% glycerol in running buffer with a trace of bromophenol blue) and 10 µL was loaded in a 20% acrylamide gel (the stacking gel contained 3% of acrylamide) and run at 4°C for 18 h using constant current (80 mA) and a Tris-Tricine running buffer (0.1 M Tris base, 0.1 M Tricine, pH 8.2). The gel was stained using an Alcian blue - silver staining protocol (Krueger & Schwartz, 1987). The gel was incubated for 30 min with Alcian blue and washed several times with water until complete destaining. The gel was then treated for 7 min in a solution of 3.4 mM

potassium dichromate with 3.2 mM citric acid (sensitization solution), washed again three times with water and incubated in 12 mM of silver nitrate for 25 min with agitation. The silver nitrate solution was removed and the gel was washed in water. A solution of 0.28M sodium carbonate with 6 mM formaldehyde (developing solution) was added to the gel and removed when the gel was turning brown and it was possible to see the bands. The gel was immediately flooded with 0.1 M acetic acid solution to stop the reaction.

### 2.14 Microscopy

Stationary phase cultures (5 µL) of the *tagO* mutants and their respective parental strains were spread onto a slide and air dried. Once dried, 100% ethanol was added to the slide ensuring the dried culture was immersed. Using a forceps the ethanol was burned off and the slide was let to cool. A cover-slip was added on top of preparation to visualize under the phase contrast microscope.

### 2.15 Growth curves in the presence of tunicamycin or anhydrotetracycline

Tunicamycin stocks were made and diluted in 95% ethanol. Overnight cultures of Newman wild type and *tagO* mutant were diluted 1/200 in fresh TSB and different concentrations of tunicamycin were added to the Newman wild type cultures (0. 4, 2, 4 and 20 µg/mL). The flasks were then incubated at 37ºC (with shaking) and the optical density (600nm) of each time point was recorded after resuspension.

Anhydrotetracycline (ATc;) 1 mg/mL stock was made in ethanol. Overnight cultures of Newman wild type, Newman *tagO* pALC2073::*tagO*, Newman *tagO tarH* pRMC2 and Newman *tagO tarH* pRMC2::*tagO* were diluted 1/200 in fresh TSB and 800 ng/mL of ATc were added to the different cultures when they reached an $OD_{600nm}$ of 0.2. The culture flasks were incubated at 37ºC (with shaking) and the optical density of each time point was recorded after resuspension, using a spectrophotometer.

## 2.16 Densitometry analysis

Densitometry analysis was performed using ImageQuant TL imaging software (GE Corporation)

## 2.17 Statistical analysis

Statistical analysis was performed using Prism Graphpad 5 software. P values were calculated using Student´s t-test.

# Chapter 3

## Global analysis of the transcriptional profile of a *tagO* mutant of *Staphylococcus aureus*

## 3.1 Introduction

The idea to investigate the importance of wall teichoic acid (WTA) in the elaboration of cell-wall anchored and secreted proteins stemmed from the publication by Weidenmaier *et al*, (2004) which implicated WTA as an adhesin in the colonization of the nares of cotton rats by promoting binding to nasal epithelial cells. Conclusions taken from studying nasal colonization in a cotton rat model were likely to be compromised because of WTA-defective mutants having altered cell division and slower growth than the wild type. Because of the pleiotropic nature of the effects of WTA mutants it was also important to study the consequences of WTA loss on the transcriptome.

One of the criticisms of early work with WTA-defective mutants was that the host strain SA113 had been subjected to chemical mutagenesis prior to isolating a WTA mutant by allelic exchange with the likely consequence of the presence of mutations in other genes (Iordanescu & Surdeanu, 1976).

Secondly, the method used to isolate the *tagO* mutants employed a plasmid vector with a thermosensitive replication (Rep) system that necessitated growth at temperatures of 42-43ºC (Redder & Linder, 2012) which can stimulate the acquisition of mutations in global regulatory genes such as *saeRS* and *agr* (Sun *et al.*, 2010, Monk *et al.*, 2017). The *tagO* mutants were not tested for the absence of secondary mutations by whole genome sequencing or by complementation analysis.

Therefore, in this study, manipulation methods were employed to isolate a *tagO* deletion mutant by allelic exchange using the vector pIMAY-Z (Monk & Foster, 2012). The major advantage of the pIMAY vector is that the *rep* mutation causes the plasmid to cease replication at 37ºC, the optimum growth temperature of *S. aureus*, so bacteria are not subjected to temperature stress during mutagenesis. Secondly, the same mutation was isolated in several different strains (two MSSA and two MRSA) and the phenotypes compared.

Thirdly, note was taken of the need for a compensatory mutation in order to establish growth of mutants defective in lipotheicoic acid (Corrigan *et al.*, 2011). Therefore, the initial mutant selection procedure incorporated high concentrations of NaCl or sucrose to compensate for possible osmotic stress.

Fourthly, complementation analysis was performed using the plasmid vector pALC2073 (Bateman *et al.*, 2001). Several different types of complementation system are available such as non-replicating plasmids that integrate at phage attachment sites allowing complementation with a single gene copy, while multi-copy vectors allow constitutive or inducible expression of the complementing gene (e.g. pRMC2 and pALC2073) (Bateman *et al.*, 2001, Corrigan & Foster, 2009). An alternative approach is to perform gene restoration by allelic exchange to reverse the mutation (Monk & Foster, 2012).

This chapter describes the isolation and validation of *tagO* deletion mutations in several strains of *S. aureus* by allelic exchange.

In order to examine the effect of loss of WTA on the behaviour of the cells, transcriptional analysis was performed using RNA-seq. This enables the level of mRNA of each gene in the chromosome to be measured in the wild type and in the *tagO* mutant grown under identical conditions.

Total mRNA is isolated and genomic DNA and ribosomal RNA removed. The RNA fragments were coupled to specific primers to generate cDNA. High throughput sequencing of the cDNA fragments allowed accurate quantification of transcripts present in the sample. The DNA sequences resulting from the transcripts were mapped to the location of their corresponding gene in the chromosome to provide reproducible and comprehensive gene expression datasets (Kröger *et al.*, 2012, Kröger *et al.*, 2013). Finally in order to validate the RNA-seq analysis the transcript level of several important genes was analysed by quantitative reverse transcriptase PCR (qRT-PCR).

## 3.2 Results and Discussion

### 3.2.1 Isolation of the *tagO* mutant

In order to construct a *tagO* deletion mutantion in strain Newman, the temperature-sensitive plasmid pIMAY-Z (Monk *et al.*, 2015) was used (FIG. 3.1 A). This plasmid contains a multiple cloning site (MCS) for conventional ligase-dependent cloning.It also carries the *lacZ* gene that expresses β-galactosidase which cleaves the chromogenic substrate X-gal to a blue chromophore. This enables cells lacking the

plasmid to be readily detected as colourless colonies on an agar plate. pIMAY-Z also contains a segment of the essential gene *secY* in the antisense orientation. The *secY* antisense is only expressed in the presence of anhydrotetracycline which inactivates the TetR repressor that normally prevents transcription. This plasmid has the p15A replication functions that allow the plasmid to replicate autonomously at 37ºC in *E. coli* and the *rep* system from the *Lactococcus* plasmid pVE6007with a thermosensitive mutation in the *repA* gene. The *repA* mutant is defective in replication at 37ºC but functions normally at 28ºC. Finally, pIMAY-Z possesses a strongly expressed *cat* gene which confers chloramphenicol resistance both in *E. coli* and in *S. aureus*.

For the construction of pIMAY-Z::*tagO* sequence and ligase independent cloning (SLIC) was used. The plasmid vector was amplified and linearized by inverse PCR using pIMAY fw and rv primers (FIG. 3.1 A). In order to construct a *tagO* deletion cassette 645 bp of DNA located upstream of *tagO* incorporating the first 159 nucleotides of *tagO* gene was amplified by PCR using primers A and B. 517 bp of DNA located downstream the *tagO* gene, including the last 27 nucleotides of the *tagO* gene was amplified using primers C and D (FIG 3.1 B). The sequence of primer B was complementary to the 5´extension included on primer C. Primers A and D contained 5´extensions

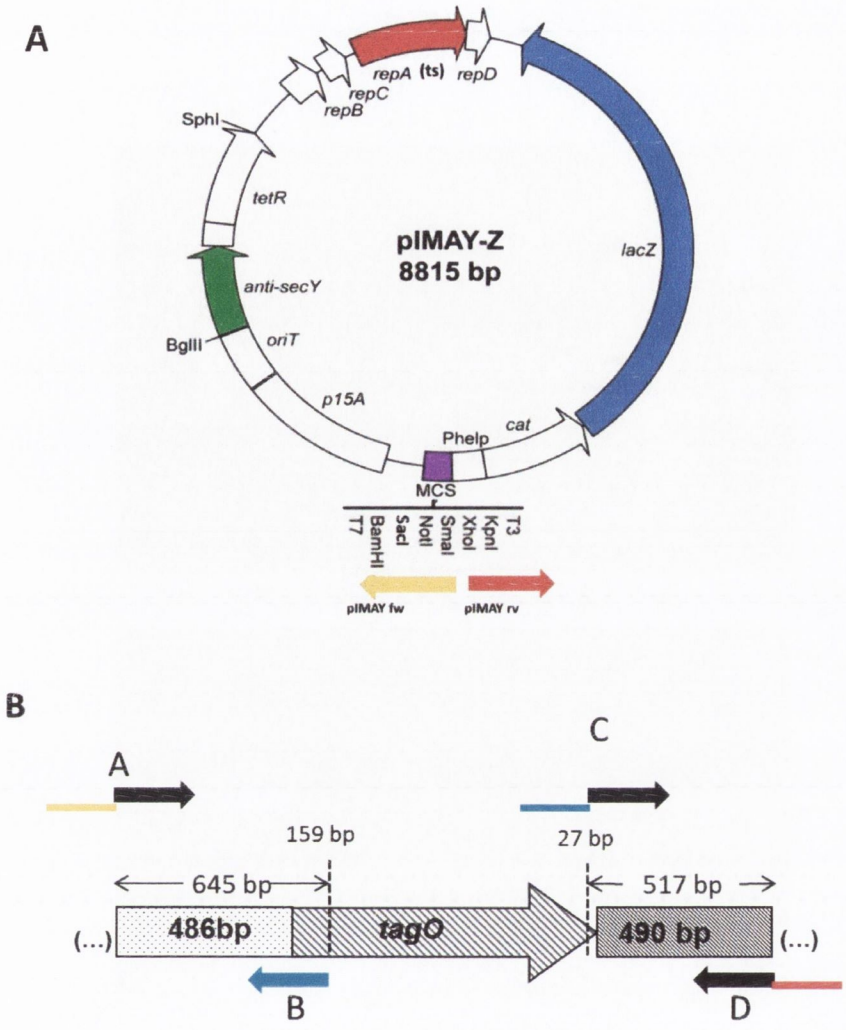



**FIG. 3.1** pIMAY-Z map and sequence and ligation independent cloning (SLIC). **A)** Schematic representation of plasmid pIMAY-Z::*tagO*. pIMAY-Z possesses the temperature-sensitive *repA* (ts) (red arrow) replicon from the plasmid pVE6007. It also encodes a *secY* antisense RNA fragment (anti-*secY*-green arrow) under the control of the *Pxyl/tetO* promoter. This vector possesses the *lacZ* gene (blue arrow), which encodes β-galactosidase and also possess a chloramphenicol resistance gene (*cat*; expressed both in *E. coli* and *S. aureus*). The position of the multiple cloning site (MCS) is indicated. The entire pIMAY-Z plasmid was amplified by inverse PCR using primers pIMAY fw and pIMAY rv (yellow and red arrows, respectively). These primers are complementary to the overhangs of the primers A and D (yellow and red lines, respectively). **B)** The up-stream (645 bp) and down-stream (517 bp) fragments were amplified from genomic DNA isolated from Newman strain using primers A/B and C/D, respectively. The primer B is complementary to the C primer overhang (blue line). AB and CD fragments were then joined by complementarity (between B and C) by PCR using primers A and D, to create the AD insert.

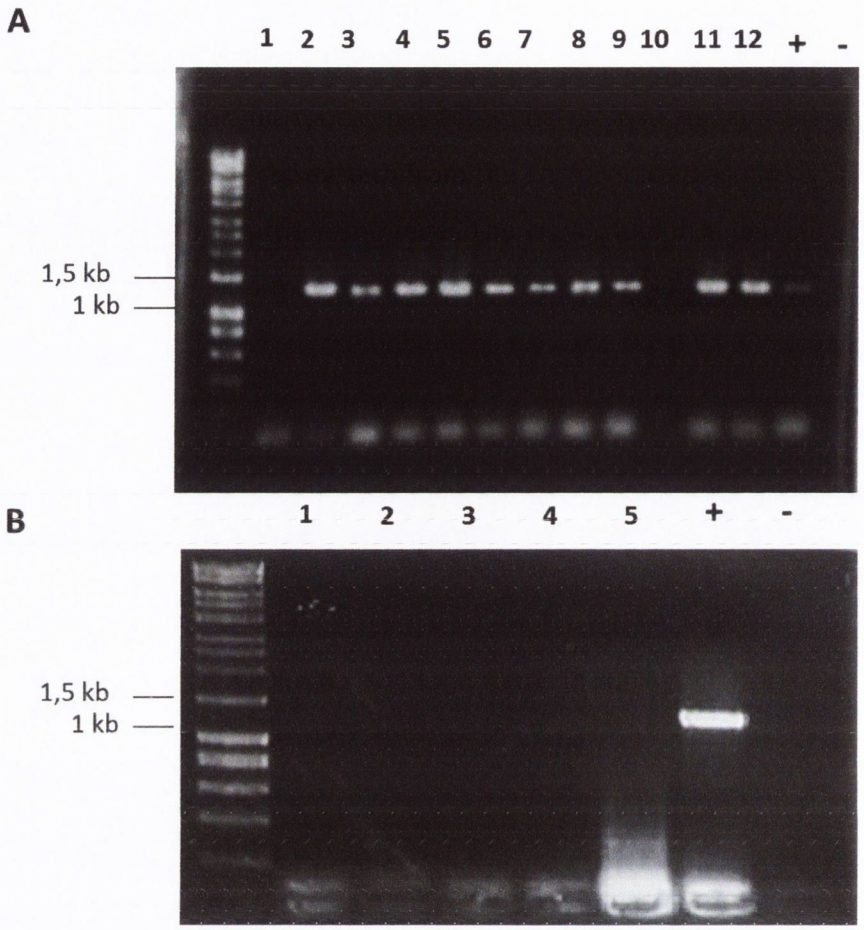



**FIG. 3.2** pIMAY-Z::*tagO* integration. **A)** The plasmid pIMAY-Z::*tagO* was transformed into *E. coli* SA08B and after 24 hours of growth the plasmid was extracted and moved into Newman at 28°C in the presence of chloramphenicol. Twelve colonies were screened for the presence of replicating plasmid by colony PCR and with primers pIMAY MCS (anneal approximately 100bp up - and downstream of the multiple cloning site). A positive colony (containing replicating pIMAY with insert) yielded a band of approx. 1.2 kb (e.g. colonies 2 and 3). **B)** After the integration step five colonies were tested for the presence of replicating plasmid. Integration of the pIMAY-Z::*tagO* into the Newman chromosome was selected by growth at 37ºC in the presence of chloramphenicol. After 24h colonies were once again screened using the MCS primers. A successful integration result was the absence of a band, e.g. colonies 1 and 2. The presence of a 1.2kb band indicates the retention of replicating plasmid. In both cases a positive control (+) of *S. aureus* containing replicating pIMAY, and a negative control (-) of *S. aureus* without plasmid, were included.

complementary to the ends of the linearised pIMAY-Z vector. The plasmid vector and the A/B and C/D fragments were denatured and allowed to anneal via the complementary sequences, to form pIMAY-Z::*tagO*. The pIMAY-Z::*tagO* plasmid was transformed into *E.coli* SA08B (Monk *et al.*, 2015). The *E. coli* SA08B strain has a defect in cytosine methylation of plasmid DNA that allows molecules to avoid being degraded by the type IV restriction system SauUS1, which is the primary defense of *S. aureus* against incoming foreign DNA. This strain expresses the type I restriction system and modification proteins of the *S. aureus* clonal complex 8 (HsdMS (CC8-2)) from a cassette that is integrated into the chromosome (Monk *et al.*, 2015). This facilitates efficient transformation of CC8 strains including the laboratory strains Newman and SH1000 as well as MRSA strains, BH1CC and LAC.

All four *S. aureus* strains were transformed with pIMAY-Z::*tagO*. Transformants were selected at 28ºC on agar containing chloramphenicol. Colonies were screened for the presence of extrachromosomal plasmid DNA by PCR using pIMAY MCS Fw and Rv primers. These primers will only amplify circular plasmid DNA and will generate a fragment of around 1.3 kb which encompasses the *tagO* cassette along with approximately 200 bp of flanking plasmid DNA.

FIG 3.2 A shows the results from 12 putative transformants of strain Newman along with PCR controls. All except transformants 1 and 10 have the 1.3 kb band that is indicative of extrachromosomal pIMAY-Z::*tagO* plasmid.

In order to select for cells where the plasmid had integrated into the chromosome, clones carrying pIMAY-Z::*tagO* were grown at 37ºC. Five colonies were tested for loss of extrachromosomal plasmid DNA by colony PCR employing the MCS fw and rv primers. In this case the primers are facing outwards from the integrated plasmid which results from a single cross-over recombination event between upstream or downstream *tagO* gene homology (FIG 3.3 A). FIG 3.2 B shows a gel with the lack of amplification except in the case of the positive control. Integration event can occur either upstream or downstream of the *tagO* gene (FIG 3.3 A).

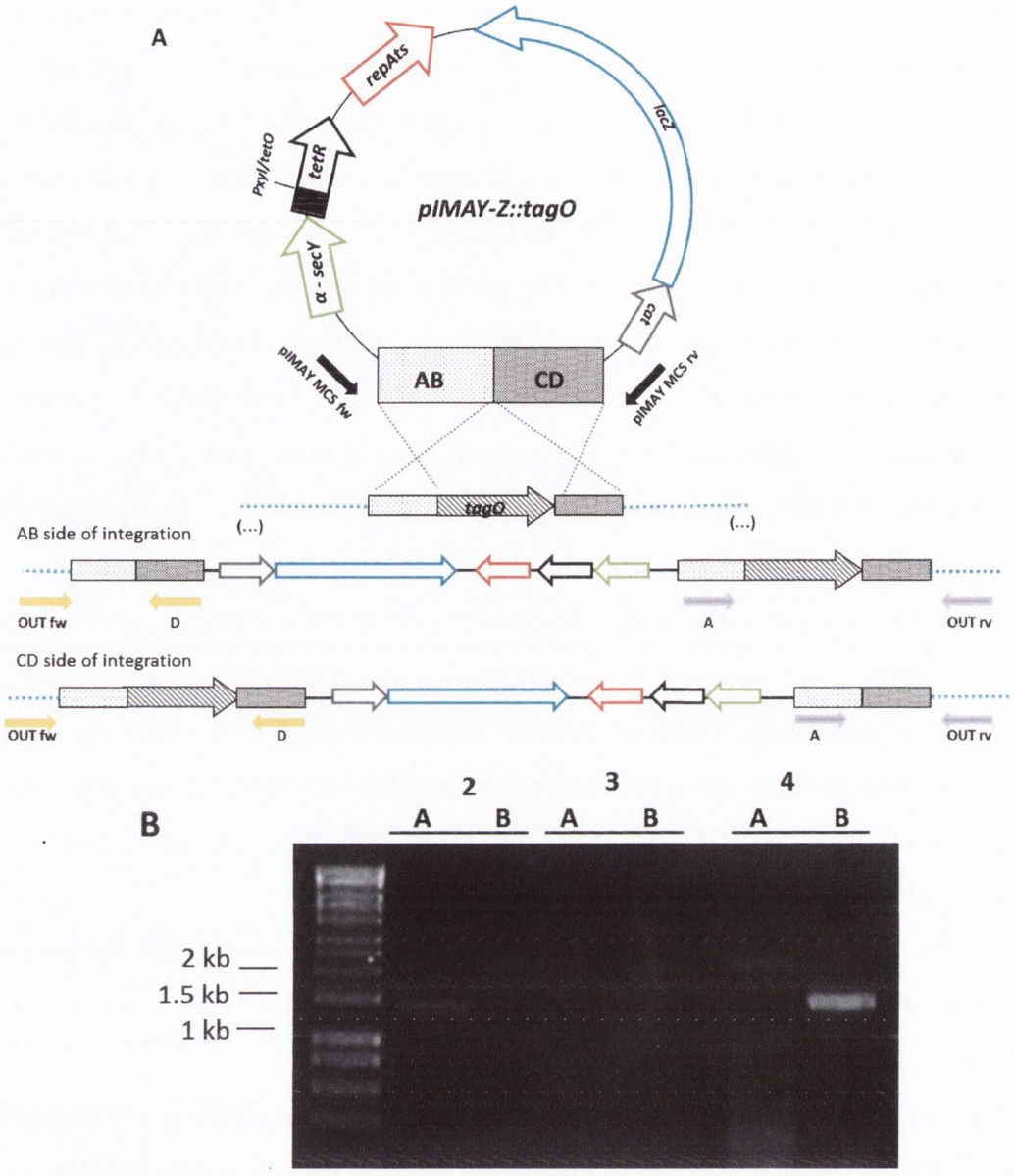



**FIG. 3.3** pIMAY-Z::*tagO* side of integration **A)** Schematic representation of pIMAY-Z::*tagO* integration into the *S. aureus* Newman chromosome. pIMAY-Z::*tagO* can integrate Via homologous recombination into the Newman chromosome either upstream or downstream of the *tagO* gene. **B)** To determine the side of plasmid integration colonies were screened by PCR using a combination of primers. Three colonies (2, 3 and 4) were screened using a combination of primers. Combination A (orange) was OUT fw/D primer and B (purple) was A primer/OUT rv. A positive integrant on the AB side of integration (colony 2) yielded a band of 1.2kb with primers from the combination A and 2 kb with primers from combination B. An integrant on the CD side of integration (colony 4) had a band of 2 kb with primers from the combination A and 1.2kb band with primers from the combination B. Size markers are indicated (kb).

In order to determine the side of plasmid integration, PCR was performed with a combination of primers internal to the AD fragment (DNA that flanks *tagO*, either upstream and downstream) and OUT primers that anneal in the chromosome DNA up- and downstream the AD fragment (A - OUT fw + primer D and B - primer A + OUT rv; table 2.2). A colony with an integrant on the AB side yielded a band of around 1.2 kb with primers combination A and a band of approximately 2 kb with combination B (FIG. 3.3 B colony 2). A colony with an integrant on the CD side yielded a band of approximately 2 kb with primers combination A and a band of around 1.2 kb with combination B (FIG. 3.3 B colony 4).

Colonies with integrated plasmid were grown on agar containing anhydrotetracyclin (ATc, 1 μg/ml) at 28ºC. ATc induces the expression of the anti-*secY* antisense RNA that inhibits the expression of *secY* gene which is essential for growth of bacteria carrying pIMAY. X- gal was also added to the plates to screen for white colonies (without replicating plasmid) against blue (with replicating plasmid). The *lacZ* gene encodes β-galactosidase (enzyme that degrades X-gal forming a blue pigment) which is active in colonies with the plasmid.

Additionally, plates supplemented with 7.5% NaCl and 40% sucrose (separately) were used. These compounds were chosen since they were required for an LTA mutant to grow (Corrigan *et al.*, 2009). However, it was found that these supplements were not necessary for colonies to grow at 37ºC on TSA.

Excision can occur at one of two sites by a single cross-over (i) exact reversal of the integration event to regenerate the wild type *tagO* gene or (ii) on the opposite side of the integration event and generate *tagO* deletion (FIG 3.4 A). Excision was assessed by PCR using primers to the chromosome region flanking the *tagO* gene (OUT fw and rv) and colonies that grew in the presence of ATc and were sensitive to chloramphenicol. Wild type bacteria yielded a product of 2171 bp whereas mutants yielded a product of 1142 bp (FIG. 3.4 B, colony 5). Newman wild type genomic DNA was included as a positive control.

The plasmid pALC2073 (FIG. 3.5 A) was used for *tagO* complementation. The plasmid vector pALC2073 is a multicopy shuttle vector derived from the pC194 plasmid vector that allows expression of genes from the TetR-regulated promoter which can be induced by tetracycline (Bateman *et al.*, 2001). The repression is leaky so that it is not

necessary to add an inducer for the cloned gene to be expressed at a moderate level. The plasmid pALC2073 contains a multiple cloning site (MCS) for conventional ligase-dependent cloning along with penicillin and chloramphenicol resistance genes for selection in *E. coli* and *S. aureus,* respectively. This plasmid contains the tetracycline inducible *xyl/tetO* promoter and the *tetR* gene (encoding the TetR repressor).

The *tagO* gene, including the ribosomal binding site was amplified by PCR using primers ALC fw and ALC rv and cloned into pALC2073 by a ligation reaction between the restriction sites EcoRI and SacI. This placed the gene in the correct orientation for expression (FIG. 3.5 A). Transformants were validated by PCR, using the primers pRMC2 MCS fw and rv that anneal outside the MCS region of the plasmid and by sequencing the PCR product. FIG. 3.5 B shows an agarose gel with the screening of 17 Newman *tagO* positive transformants with a band of around 1.3 kb. The plasmid was isolated from *E.coli* SA08B and transformed into the *tagO* mutants of Newman, SH1000, BH1CC and LAC.

To determine if the *tagO* mutants contained WTA SDS-PAGE and silver staining were performed after extracting the WTA from the cell wall. The WTA appears as a smear in the wild type and complemented mutant, but is completely absent in the *tagO* mutants (FIG. 3.6). This shows the lack of WTA in the mutants and its restoration in the complemented mutant.

To assess the morphology of the *tagO* mutant cells were visualized under a phase contrast microscope and compared to the wild type. The *tagO* mutant formed big clumps of cells presumably due to the lack of daughter cell separation. The wild type phenotype was restored by the presence of the *tagO* gene in the complemented strain (FIG. 3.7).

In order to determine if growth of the *tagO* mutant in liquid medium was compromised, wild type Newman and the *tagO* mutant were inoculated into TSB and growth curves were performed (FIG 3.8)

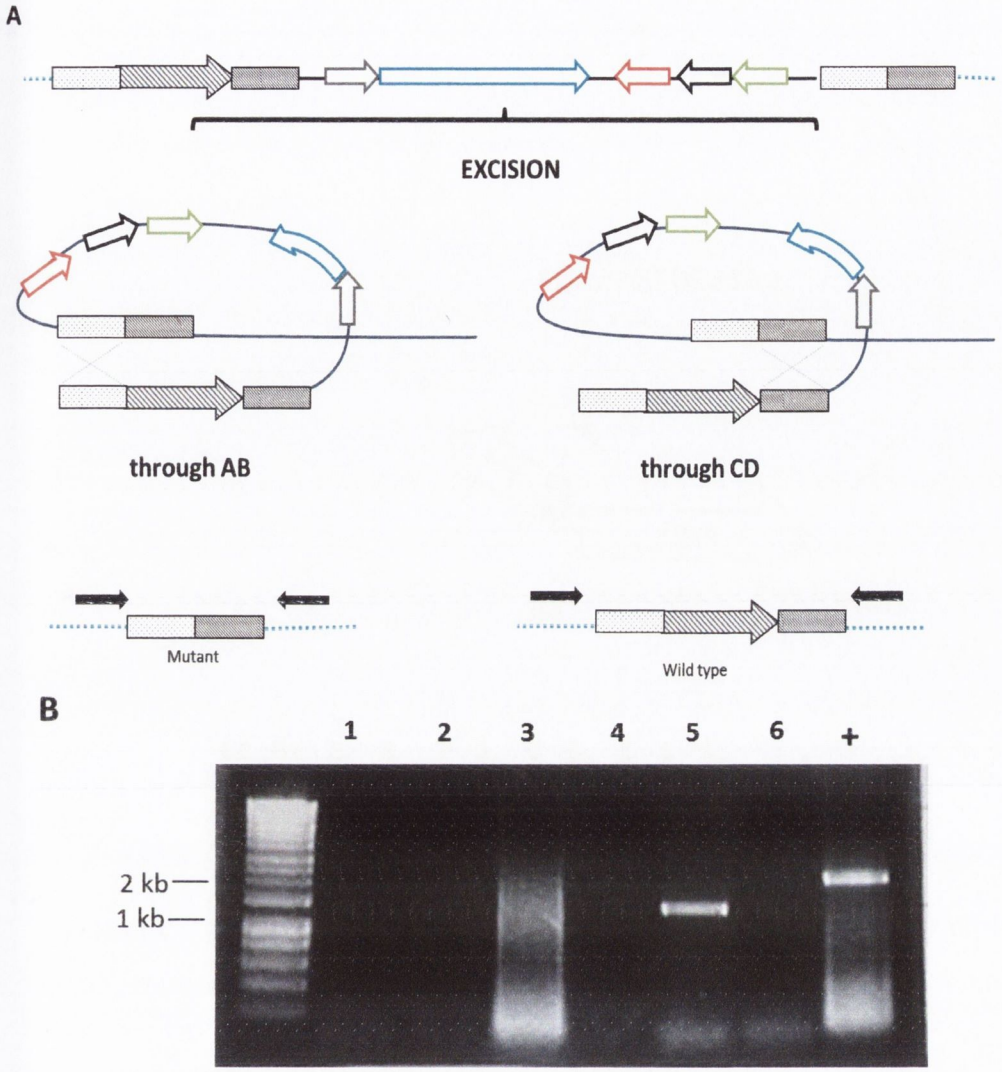



**FIG. 3.4** Excision of pIMAY-Z::*tagO* from the Newman chromosome. **A)** Colonies harbouring integrated pIMAY-Z::*tagO* were replated on agar containing anhydrotetracycline which induces the expression of the α-*secY* antisense RNA. Following this one of two events may occur, either plasmid excision occurs leaving behind the wild type *tagO* gene, or it can result in the co-excision of the wildtype *tagO* gene leaving behind *tagO* deletion fragment comprised of the upstream and downstream regions, creating a clean *tagO* deletion. **B)** The outcome of this excision event was determined by PCR using primers that anneal up-and downstream of the and fragments (OUT fw and rv primers; black arrows). The wild-type gene will generate a product of approximately 2 kb whereas colonies possessing the *tagO* deletion fragment will generate a band of approximately 1.2 kb, e.g. colony 5. Newman was included as a positive control (+) to indicate presence of the wild type *tagO* gene. Size markers are indicated (kb).

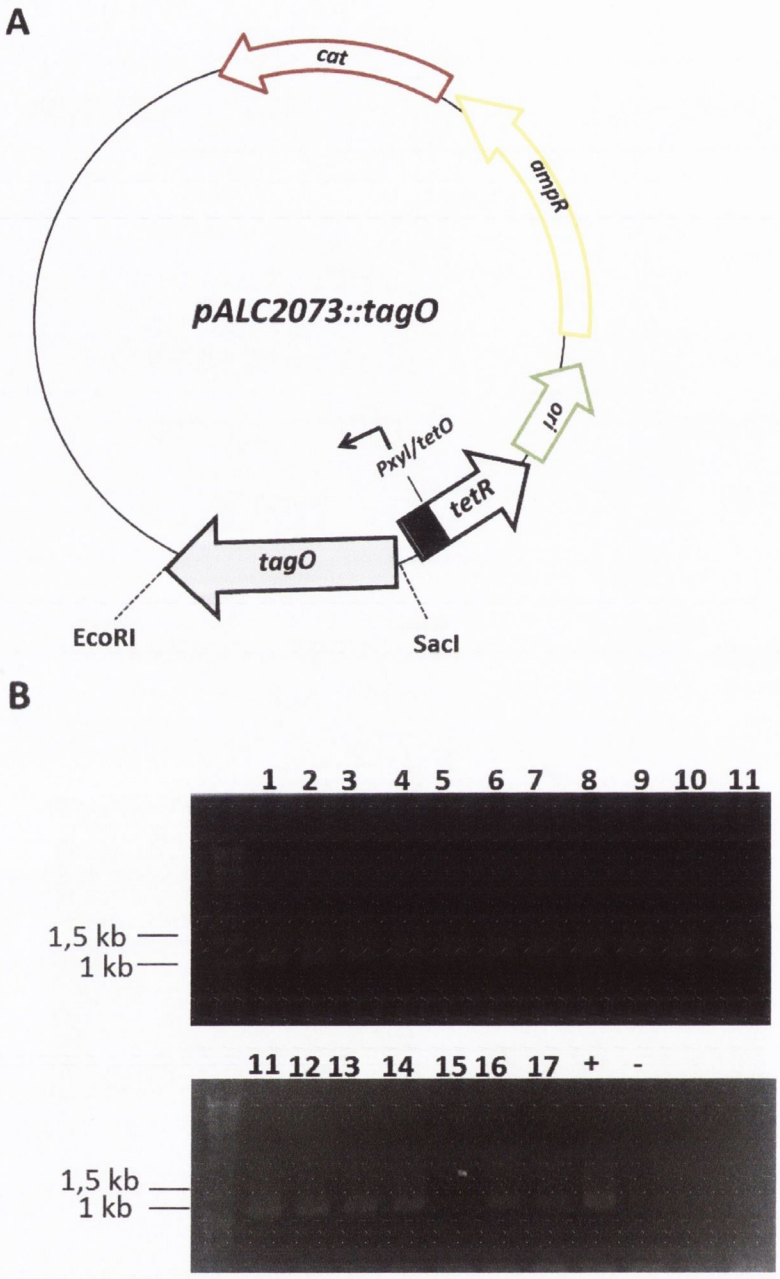



**FIG. 3.5** pALC2073 map and cloning of the *tagO* gene. **A)** Schematic representation of pALC2073. pALC2073 is a multicopy shuttle vector derived from the pC194 plasmid vector that allows expression of genes from the TetR-regulated promoter. pALC2073 contains penicillin (amp; yellow arrow) and chloramphenicol (cat; red arrow) resistance genes and tetracycline inducible *xyl/tetO* promoter and the *tetR* gene (encoding TetR repressor; black arrow). Plasmid map drawn using SnapGene software. **B)** Seventeen colonies were screened by colony PCR using the primers ALC MCS fw and rv. A positive colony yielded a product of approx. 1.3 kb (e.g. 1-11). Size markers indicated (kb). *E.coli* pALC2073::*tagO* was included as a positive control (+) to indicate presence of the wild type *tagO* gene and *E.coli* pALC2073 was included as a negative control (-).

The rate of growth in exponential phase was reduced in the *tagO* mutant (50 min.) compared to the wild type (25 min.). Also, the final growth yield was lower (an $OD_{600nm}$ of 7.63 in the wild type and 4.98 in the *tagO* mutant).

### 3.2.2 Transcriptome analysis

In order to study the Newman *tagO* mutant transcriptome total RNA was extracted from cells of *S. aureus* strain Newman wild type and the *tagO* mutant from the exponential phase of growth (FIG. 3.9). The cDNA libraries used for RNA-seq in this study were generated from total RNA by Vertis Biotechnologie AG, Freising, Germany. RNA-seq was performed using an Illumina Hiseq 2000 platform. Total RNA was fragmented by sonication and poly-A tails were added to each fragment by poly (A) polymerase (FIG 3.9). The 5´end of each fragment was de-phosphorylated and short bar-code sequences were attached to the 5´ends of the RNA fragments to allow for identification of each RNA fragment after sequencing. cDNA synthesis was performed using oligo priming and Moloney murine leukemia virus reverse transcriptase and the strand specific cDNA libraries were generated using the ScriptSeq kit. cDNA samples were amplified by bridge amplification to create clusters of identical copies of single template molecules. Illumina sequencing uses "sequencing by synthesis" technology, involving the incorporation of dNTPs reversibly labelled with fluorescent terminators. The labels serve as terminators for the polymerase so each dNTP fluoresces and is imaged before being enzymatically cleaved to allow the addition of the next nucleotide. This technique allows identification of the individual nucleotide that is incorporated into the nucleic acid chain during each sequence cycle. Analysis of the sequencing data was limited to the sequencing reads that mapped to a single location on the chromosome (uniquely mapped reads) (FIG. 3.9). The development of RNA-seq analysis pipelines (Sivasankaran, 2014) and the use of transcript per million (TPM) values to quantify gene expression permitted information to be managed from these large datasets.

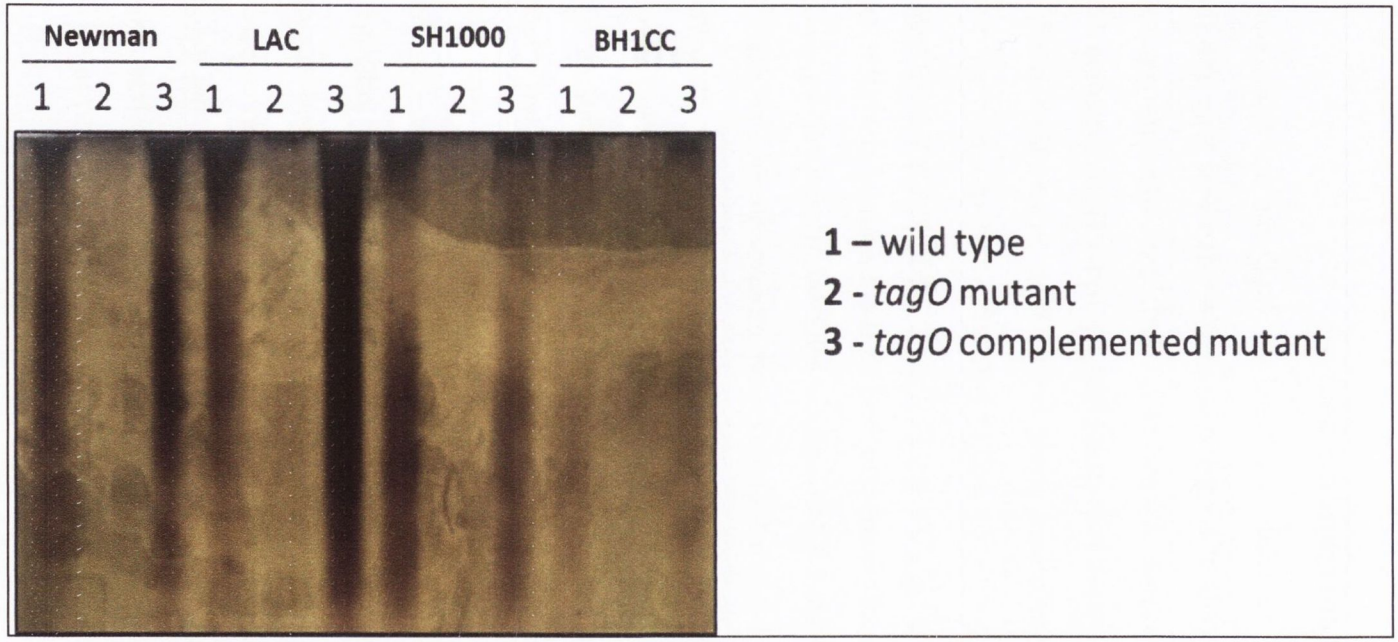



**FIG. 3.6** Extraction and detection of WTA. WTA was isolated from cells in the stationary phase of growth by treatment with SDS in a boiling water bath for 1h to detach WTA from the cell surface. After a centrifugation step the supernatants were separated and treated with proteinase K to eliminate any remaining proteins. WTA was hydrolized by treatment with NaOH and subjected to SDS Page. The gels were stained with a Alcian blue-silver staining method. The figure is representative of N=2 data.

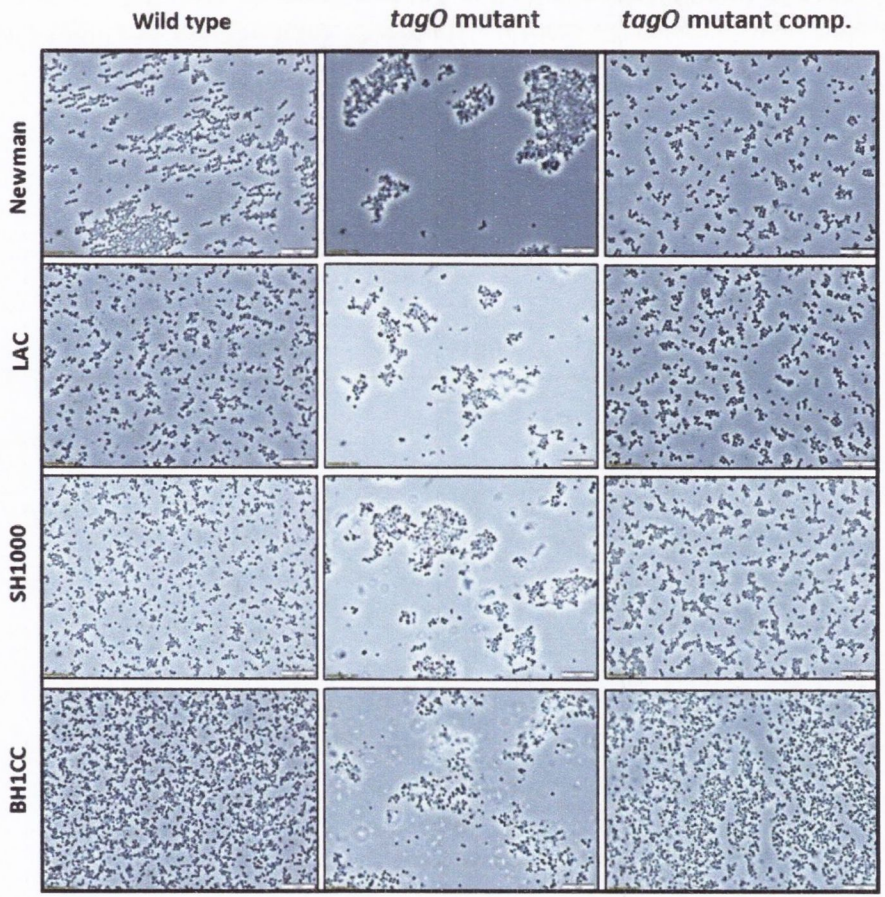



**FIG. 3.7** Morphology of the *tagO* mutants**.** Phase contrast microscopy using ethanol-fixed cells from stationary phase cultures. Broth cultures (5µL) were spread into slides and air dried. The dried culture was fixed with ethanol and heat and observed under the microscope;

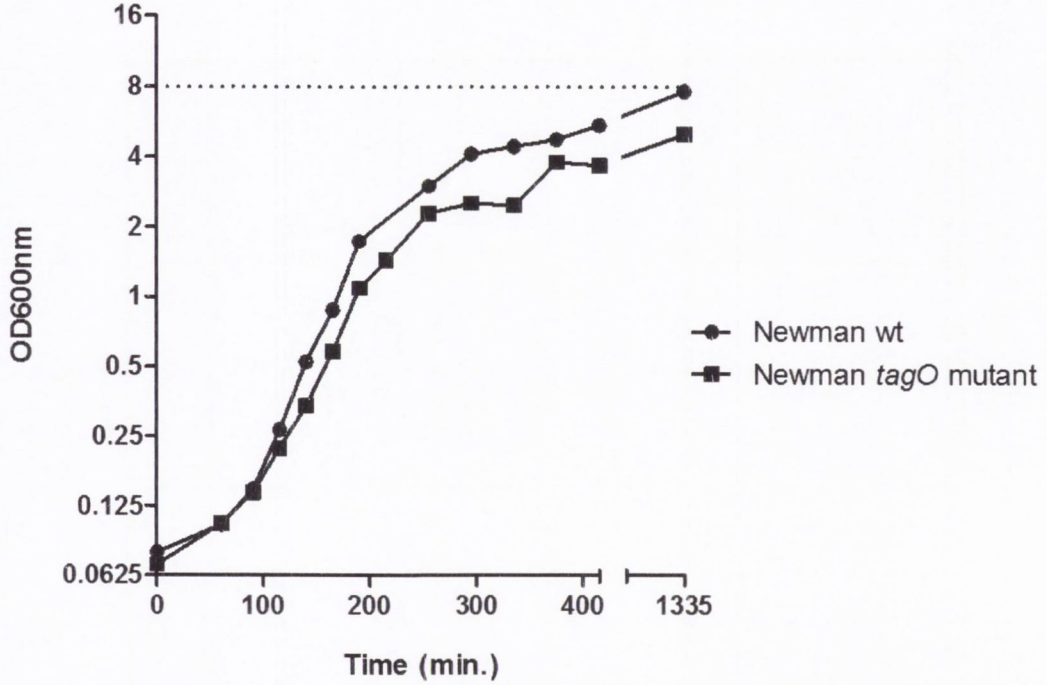



**FIG. 3.8** Growth curve of the Newman wild type and *tagO* mutant. Overnight cultures of Newman wild type and *tagO* mutant were diluted 1/200 in fresh TSB. The flasks were incubated at 37ºC (with shaking) and the optical density (600nm) of each time point was recorded. This is representative of N=3 data.

The sequencing data obtained in this study was acquired from 3 independent RNA samples sequenced in the same sequencing run. The reproducibility of the RNA-seq transcriptomic data set was confirmed using correlative analysis of the biological replicates from samples sequenced in the same sequencing run and in the Illumina flow cells (Kröger *et al.*, 2013).

The gene expression TPM values show a high level of correlation (Pearson correlation coefficient: 0.9997) between biological replicates of wild type and *tagO* (FIG. 3.10) which demonstrates the reproducibility of the RNA extraction, cDNA library preparation and RNA-seq methods.

The replicates of the Newman wild-type and *tagO* mutant mRNA reads were mapped to the *Staphylococcus aureus* Newman genome sequence that was used as a reference. An average of 22,140,664 reads were obtained for the wild type with an overall alignment rate of 95.34%, and an average of 21,331,991 reads were obtained for the *tagO* mutant with an overall alignment rate of 97.57%.

Coverage files were created, by in-house perl scripts, for viewing the data using the Integrated genome browser (IGB) program and the fold differences in transcript per million (TPM) values were calculated. The gene annotation was performed using and the BLAST tool available from GenBank. A total of 163 genes were altered (a change in expression less than 2-fold was not considered relevant, therefore the threshold was set at 2-fold difference), 85 were downregulated and 107 were upregulated (Table 3.1 and 3.2). The function of the proteins encoded by the altered genes was analysed further.

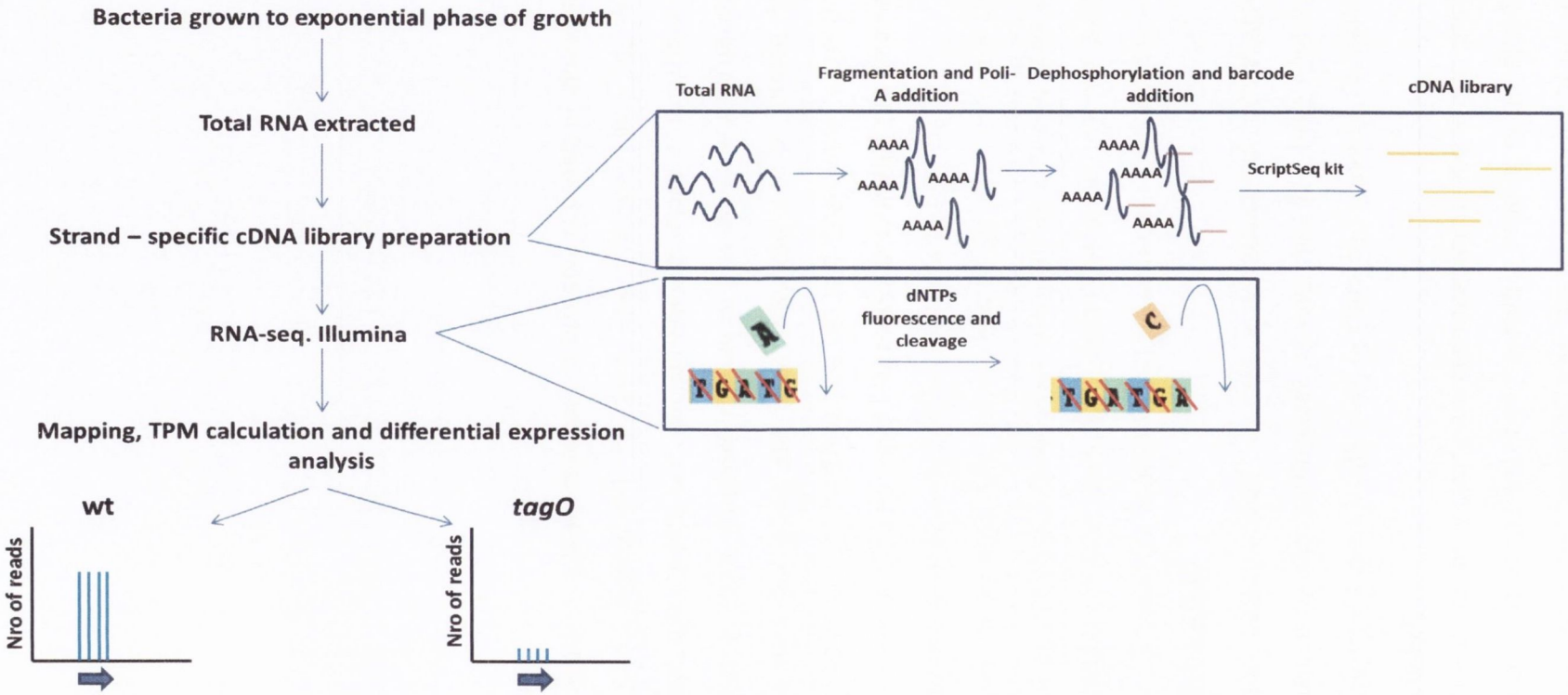



**FIG. 3.9** Schematic representation of the RNA-seq method. Bacteria was grownto exponential phase of growth and cells were harvested for RNA extraction. The cDNA libraries and RNA-seq were performed from total RNA by Vertis Biotechnologie AG. RNA was fragmented by sonication and poly-A tails were added to each fragment. The 5´end of each fragment was de-phosphorylated and short bar-code sequences were attached to the 5´ends of the RNA fragments. cDNA synthesis was performed using specific primers and Moloney murine leukemia virus reverse transcriptase. The strand specific cDNA libraries were generated using the ScriptSeq kit. RNA-seq was performed using an Illumina Hiseq 2000 platform. dNTPs were reversibly labelled with fluorescent terminators for the polymerase. The fluorescence of each dNTP is recorded before being enzymatically cleaved to allow the addition of the next nucleotide. Each sequencing read was mapped to a single location on the chromosome. TPM (transcripts per million) values were calculated and differential expression was assessed

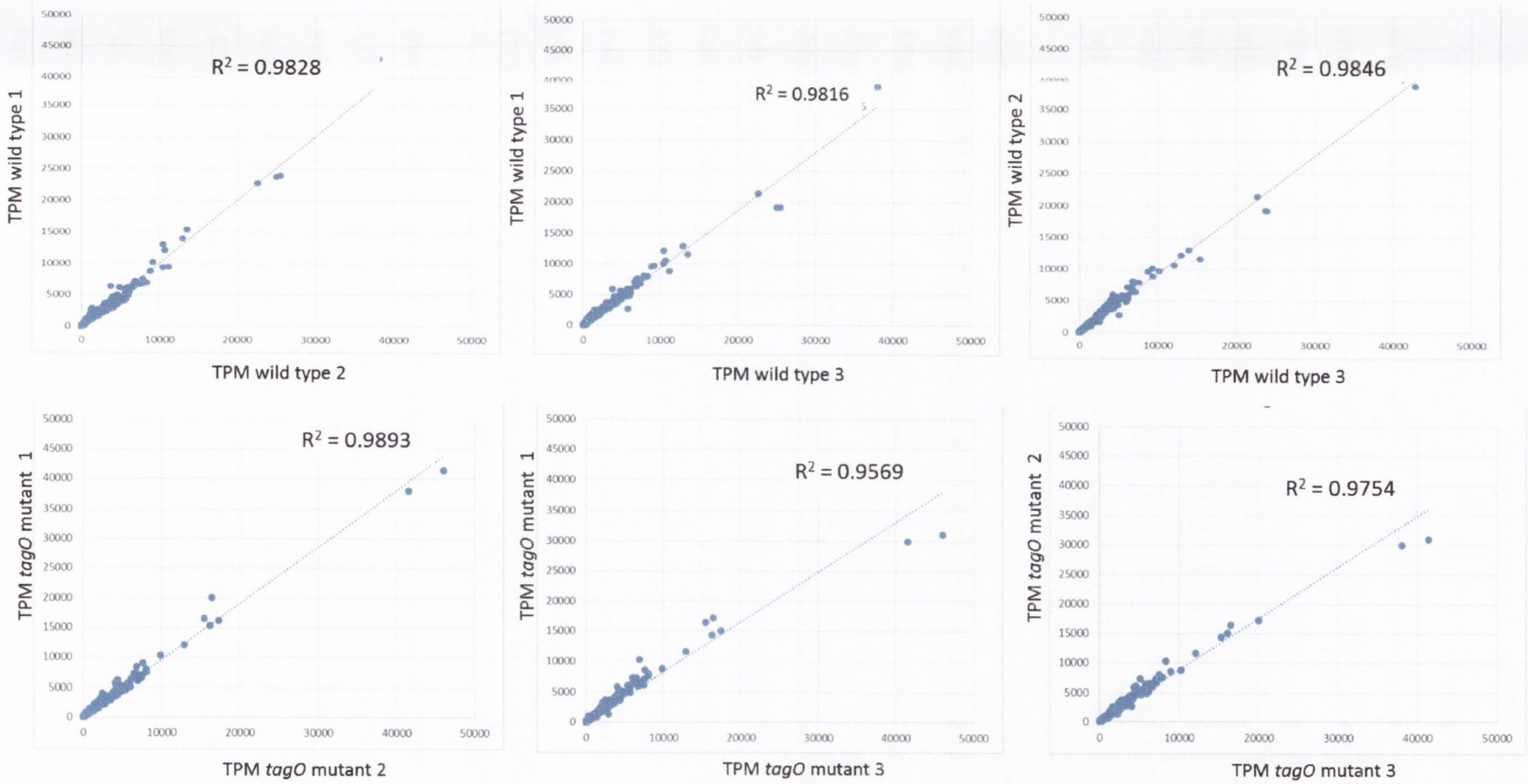



**FIG. 3.10** RNA-seq **r**eproducibility graphs of the technical replicates between the different biological replicates in the Newman wild type strain. Transcript per million (TPM) values of each biological replicates of the wild type strain (wt 1, 2 and 3) and the *tagO* mutant (*tagO* 1, 2 and 3) were ploted and correlation coefficient was calculated. The correlation coefficient is indicated ($R^2$).

### 3.2.3 Differentially expressed genes

#### 3.2.3.1 Overview

The RNA-seq data showed that there is complete absence of *tagO* transcript in the *tagO* mutant strain corresponding to DNA that was deleted (FIG 3.11). The deletion of tagO gene removed all but the 159 nucleotides at the 5′end and 27 nucleotides at the 3′end of the sequence. FIG 3.11 shows transcriptional activity at the 5′end including the untranslated promoter region, the first 159 nucleotides and the last 27 nucleotides of the gene. The gene upstream *tagO* encodes a hypothetical protein and its transcription did not change in the *tagO* mutant. In addition the unknown gene downstream of *tagO* was unchanged.

Genes important for cell wall processes/pathways were investigated, including genes involved in LTA biosynthesis (*ypfP, ltaA, ltaS* and *dagK*), genes involved in WTA and LTA D-alanylation (*dltA, dltB, dltC* and *dltD*), genes involved in attachment of WTA to the cell wall such as *lcpA* and *lcpC* and peptidoglycan biosynthesis genes (*murA, murB, murC, murD, murE, murF, mraY, murG, fmhB, femA, femB, ftsW, pbp4, pbpA, pbpB* and *pbpC*) (FIG 3.12). The expression of these genes was not changed with the exception of LCP proteins A and C (2.26 and 6.81-fold induced, respectively; essential for capsule and WTA attachement), DagK (2.37-fold induced; involved in LTA production) (FIG. 3.12) and the peptidoglycan synthesis protein MurZ (described further in the cell wall stress stimulon section). This shows that the majority of the genes responsible for the cell wall biosynthesis and composition are not affected by the loss of WTA.

Table 3.1 shows that among the 107 up-regulated genes, two were massively increased, *vraX* (128-fold increase; FIG. 3. 13) and *cwrA* (9.1-fold increase; FIG. 3. 15). Of the up-regulated genes, some were *vraR* and *vraS* encoding the two component system VraRS, known to regulate up to 46 different genes including the genes encoding the cell wall biosynthesis enzymes Pbp2 (1.92-fold increased), MurZ (3.83-fold increased) and SgtB (3.98-fold increase) comprising the cell wall stimulon (Kuroda *et al.*, 2003, McAleese *et al.*, 2006). VraR was 3.69-fold increased and VraS was 3.45-fold increased (FIG. 3. 14). Other up-regulated genes were genes encoding proteins involved in peptidoglycan turnover, such as the autolysins *atl* (2.57–fold increase) and

*sle1* (2.4-fold increase) essential for the cell-daughter separation during division (Biswas *et al.*, 2006) (Table 3.1). Genes encoding proteins involved in protein fate were also up-regulated such a the PrsA (7.08-fold increase; FIG. 3.17) involved in the extracellular folding of several secreted proteins (Alonzo *et al.*, 2011, Heikkinen *et al.*, 2009, Vitikainen *et al.*, 2004), *et al* 2004) and the chaperone ClpB (3.21–fold increase) important for the recovery of the cell from heat-induced damage (Trey Matthews 2007) (Table 3.1). The genes encoding the proteases HtrA (FIG. 3.18) and SpsB (Table 3.1) that are important for cleavage of secreted and periplasmic proteins (Bruton *et al.*, 2003, Rigoulay *et al.*, 2005) were also up-regulated (3.04 and 5.96-fold increases, respectively). The gene encoding the protein FmtA, that is involved in expression of methicillin resistance in MRSA (Qamar & Golemi-Kotra, 2012) and more recently (Rahman *et al.*, 2016) described as a D-amino esterase that hydrolyses the ester bond between D-Ala and the backbone of teichoic acids, was also up-regulated (3.48-fold; FIG. 3.19).

Several genes encoding proteins involved in metabolic processes were also up-regulated (Table 3.1). The most represented set of affected metabolic genes were those involved in urea catabolism (*ureG*, *ureA*, *ureE*, *ureD* and *ureF*). The gene encoding the highly antigenic protein called staphylococcal secretory antigen A (SsaA) involved in biofilm-associated infections (Lang *et al.*, 2000) was also up-regulated (7.58-fold increased) (Table 3.1).

Another major class of up-regulated genes are those encoding the proteins responsible for capsular biosynthesis (the *cap* operon; *capK, capO, capH, capJ, capA, capM, capN, capI* and *capL*; FIG. 3.21) important for virulence and pathogenesis (O'Riordan & Lee, 2004). The down-regulated genes are mainly genes involved in virulence such as the transcriptional regulators SarS (5-fold decrease; FIG. 3.23) and Rot (3-fold decrease; FIG.3.24), that are involved in the expression of protein A (Spa; 100-fold decrease; FIG. 3.23) and clumping factor B (ClfB; 9-fold decrease; FIG. 3.25), respectively.

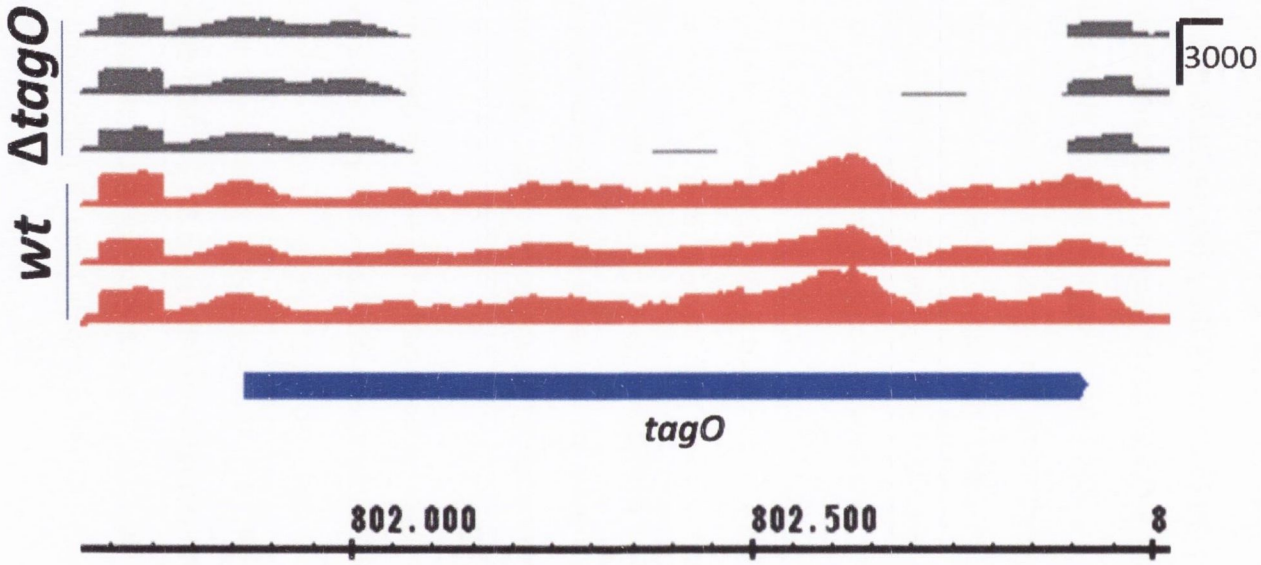



**FIG. 3.11** Integrated genome browser (IGB) visualization of the *tagO* gene. The colour of each track represents the sequencing reads that map to that locus and are directly proportional to the level of expression of that locus. In the annotation tracks, blue arrows define the protein-encoding gene. The arrow indicates the direction of transcription. The black line defines the genome coordinates. IGB Y axis scale value of the peaks indicated.

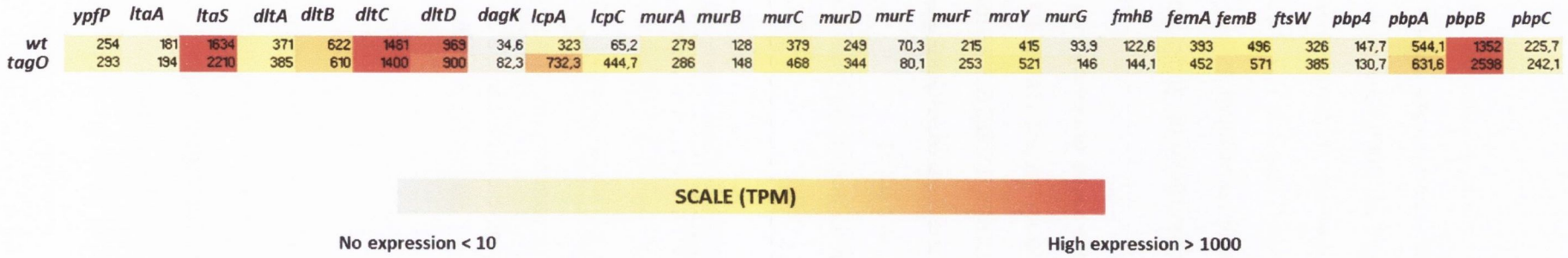



**FIG. 3.12** Heatmap of the expression of genes related to the cell wall. Absolute TPM values, calculated using by RNA-seq data, of each cell wall gene of Newman wild type and *tagO* mutant were used. Genes related in the synthesis of LTA, such as *ypfP, ltaA, ltaS*, *dagK* and genes associated with alanine modifications of the teichoic acids such as *dltA, dltB, dltC* and *dltD* were used. Genes encoding the proteins LcpA and LcpC are responsible for the attachment of WTA and capsular polysaccharides to the peptidoglycan, respectively. Genes involved in the peptidoglycan assembly were also used such as *murA, murB, murC, murD, murE, murF,mraY, murG, fmhB, femA, femB, ftsW, pbp4, pbp1, pbp2* and *pbp3*. These genes were under the threshold of 2-fold difference in expression, except for *dagK, lcpA* and *lcpB*. Genes in grey have a low expression (less than 10 TPM) and genes in red have a high expression (more than 1000 TPM).

Spa is a CWA protein important for immune evasion and ClfB is responsible for the attachment of *S. aureus* to the skin corneocyte ligand loricrin and therefore colonisation of human nares (Corrigan *et al.*, 2009, Gemmell, 1991). The *agr* system is essential for virulence in *S. aureus* and an *agr* mutant has an impaired pathogenesis. The entire *agr* operon is down-regulated (Table 3.2; FIG. 3.26) in the *tagO* mutant, as well as the *agr* effector molecule RNAIII (annotated as *hld;* 2.94-fold decrease; FIG. 3.26), that is responsible for the expression of genes involved in pathogenesis including alpha-toxin (Hla).

Other down-regulated genes are those encoding extracellular factors such as superantigen-like proteins (encoded by *ssl* genes) (Table 3.2) and the enterotoxin A (encoded by *sea*) which is 2.56-fold decreased. *clpC* is 3.22-fold decrease and encodes a protein which is a ATPase with functions in physiology, metabolism, and late-stationary phase survival (Chatterjee *et al.*, 2009).

In the next section, the expressed flanking genes of the gene of interest, in the *tagO* mutant are shown in the figures.

### 3.2.3.2 Cell wall stress stimulon

The genes *vraX*, *vraR*, *vraS*, *vraT*, *vraU*, *cwrA*, *prsA*, *htrA*, *fmtA*, *murZ*, *sgtB* and *pgk* are part of the cell wall stress stimulon (McAleese *et al.*, 2006) and can be induced by cell wall targeting antibiotics such as vancomycin. This suggests that this group of genes are being induced by the cell wall damage caused by the lack of WTA in the *tagO* mutant.

#### 3.2.3.2.1 VraX

*vraX* is the most up-regulated gene in the Newman *tagO* mutant, being increased 128-fold (FIG. 3.13). FIG. 3.13 shows a very high gene expression at the gene 5′end in the wild type. No phenotypic differences were detected in a *vraX* deletion mutant of SH1000 (Helena Rosado 2012). When compared to the SH1000 parental strain no differences were detected in growth rate, viability or susceptibility to antibiotics such as vancomycin, oxacillin, erythromycin and mitomycin.

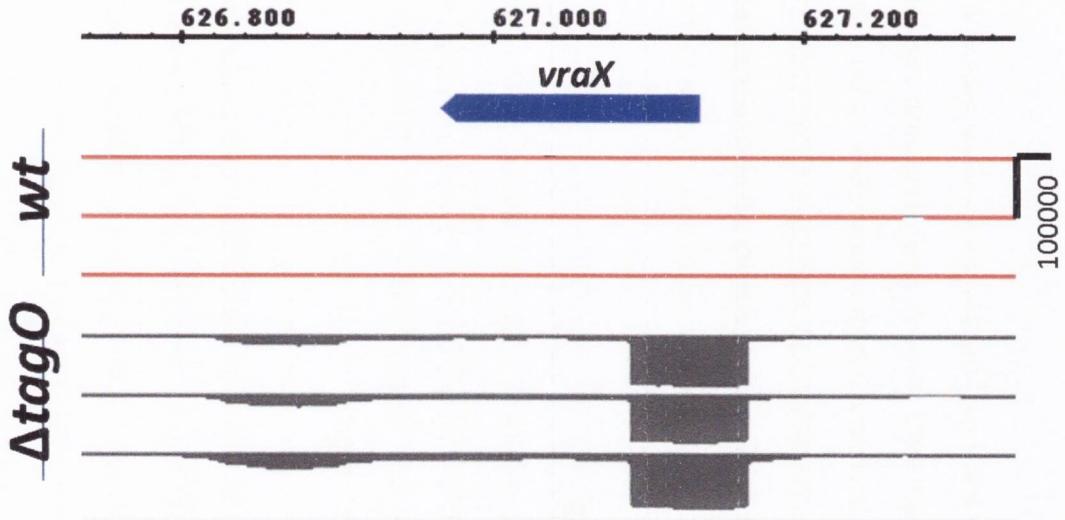



**FIG. 3.13** Integrated genome browser (IGB) visualization of the *vraX* gene. The colour of each track represents the sequencing reads that map to that locus and are directly proportional to the level of expression of that locus. In the annotation tracks, blue arrows define the protein-encoding gene. The arrow indicates the direction of transcription. The black line defines the genome coordinates. IGB Y axis scale value of the peaks indicated.

Additionally, no differences were detected with respect to pigment production and alpha-toxin activity. Thus the function of VraX is unclear.

However, protein-ligand docking stimulation showed that VraX might bind to the β-lactam imipenem close to a serine residue in a conserved motif found in all VraX homologues (Helena Rosado 2012). This small peptide also harbours a putative phosphorylation site (Scherl *et al.*, 2006) that could be involved in regulatory processes.

*vraX* is under the control of the two component system VraRS, which is also up-regulated in the Newman *tagO* mutant. *vraR* (response regulator) and *vraS* (sensor histidine kinase) are located on a 2.8 kb operon (FIG. 3. 14), together with two other genes of unknown function, *vraU* and *vraT* (also called *yvqF*) (Yin *et al.*, 2006). This operon is flanked by the downstream gene *map1*, also designated as *eap* (extracellular adherence protein) which has similar levels of transcription to the wild type strain.

#### 3.2.3.2.2 VraRS

The VraRS two component system is induced by cell wall synthesis inhibitors activating about 46 unlinked genes in the *vra* regulon. Several of the genes from the *vra* regulon encode cell wall biosynthesis enzymes (Kuroda *et al.*, 2003, Yin *et al.*, 2006) that can presumably repair the cell wall damage.

VraS is an integral membrane-sensing histidine kinase and responds to cell wall stress caused by antibiotics by autophosporylation and subsequent phosphorylation of VraR. VraR then binds to its own promoter and to the genes in the *vra* regulon to activate or repress their transcription. The specific molecular signal that activates *vraS* and *vraR* is still not known. VraT is a putative membrane protein that is a homologue of the cell wall response protein LiaF in *Bacillus subtilis* (Mascher, 2006, Jordan *et al.*, 2006). It is not clear the mechanism by which *vra* affects methicillin resistance. Alternatively, VraT or VraU could be responsible for the attenuation of methicillin resistance seen in the *vraRS* mutant (Boyle-Vavra *et al.*, 2006).

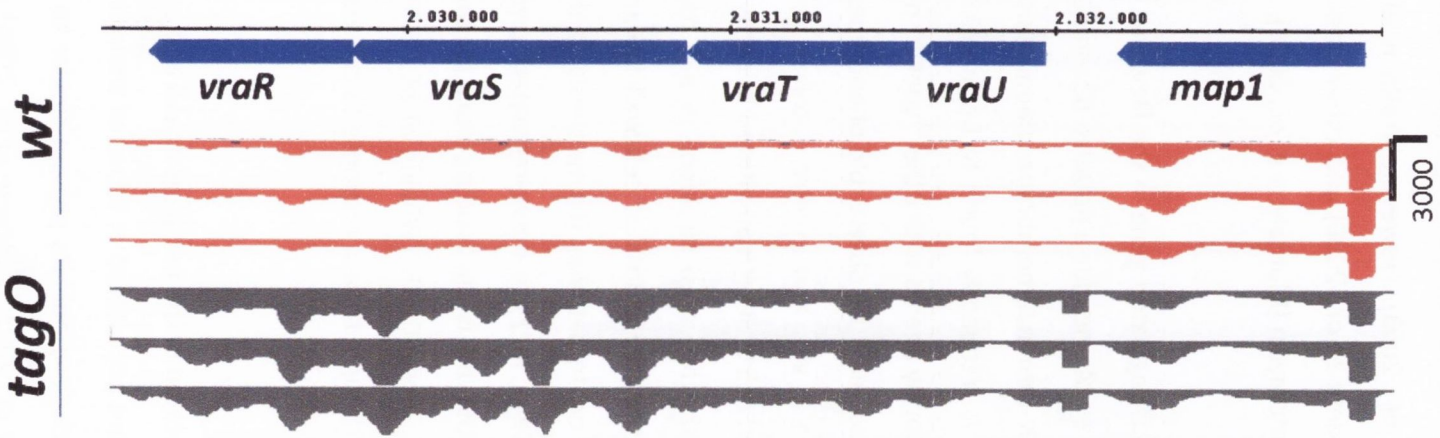



**FIG. 3.14** Integrated genome browser (IGB) visualization of the *vra* operon. The colour of each track represents the sequencing reads that map to that locus and are directly proportional to the level of expression of that locus. In the annotation tracks, blue arrows define the protein-encoding gene. All arrows indicate the direction of transcription. The black line defines the genome coordinates. IGB Y axis scale value of the peaks indicated.

The post-translational changes of the extracellular cysteine protease (SpeB) in *Streptococcus* are dependent on appropriate amounts of PrsA (Olsen *etal.*,2010).

#### 3.2.3.2.6 HtrA

The *htrA1* gene is located upstream of an unknown gene and downstream the gene *tyrS* which encodes a tyrosyl-tRNA synthetase. Both genes are expressed at the same levels in the wild type and the *tagO* mutant (FIG. 3.18). The transcription of *htrA1* was increased 3.04-fold in the *tagO* mutant when compared to the wild type.

*S. aureus* encodes two putative HtrA – like proteases encoded by *htrA1* and *htrA2*. It has been shown that HtrA proteases are involved in stress resistance and survival in *Streptococcus pyogenes* (Jones *et al.*, 2001).

Both *htrA1* and *htrA2* genes were expressed in a *Lactococcus lactis htrA* mutant using a conditional expression system and HtrA1 was shown to be important for protection against thermal stress having a just a weak protease activity while HtrA2 displayed no phenotype (Rigoulay *et al.*, 2004). The roles of HtrA-like proteins differ between different strains. In *S. aureus* RN6390 strain HtrA1 is needed for puromycin (which generates truncated proteins during biosynthesis; (Goldberg, 1972) stress resistance, suggesting a role for this protein in eliminating truncated proteins (Rigoulay *et al.*, 2005). Both HtrA1 and HtrA2 have a role in virulence of strain RN6390 in a rat endocarditis model which is probably due to their role in the expression of secreted virulence factors such as haemolysins. However, in *S. aureus* COL strain both HtrA1 and HtrA2 are needed for growth at high temperatures but they do not contribute to virulence in the endocarditis model (Rigoulay *et al.*, 2005), showing different phenotypes in different hosts.

#### 3.2.3.2.7 FmtA

The *fmtA* gene is well isolated from any other gene. It is induced 3.48-fold in the Newman *tagO* mutant (FIG. 3.19). FmtA was first reported to be a factor in the expression of methicillin resistance of MRSA strains (Komatsuzawa *et al.*, 1997).

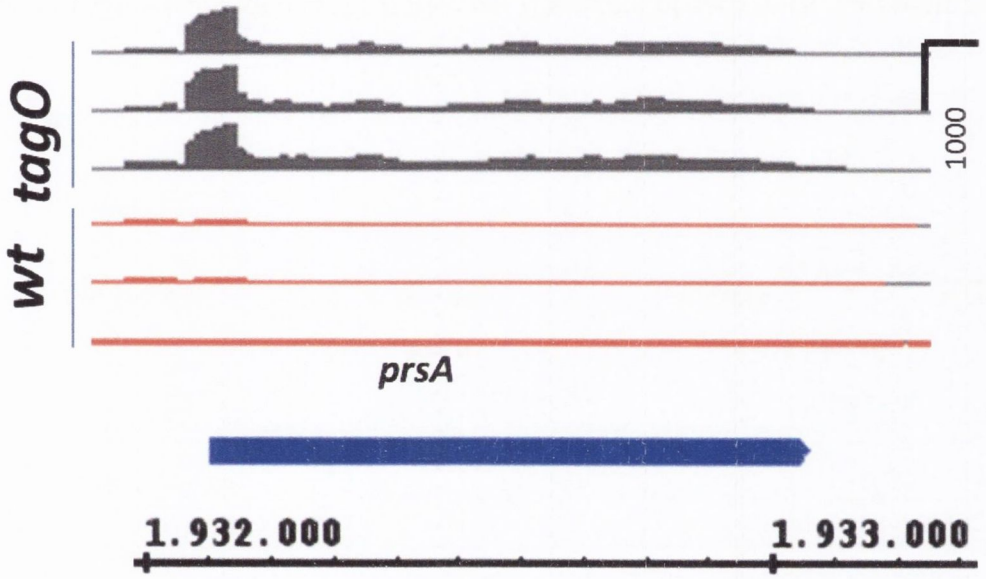



**FIG. 3.17** Integrated genome browser (IGB) visualization of the *prsA* gene. The colour of each track represents the sequencing reads that map to that locus and are directly proportional to the level of expression of that locus. In the annotation tracks, blue arrows define the protein-encoding gene. The arrow indicates the direction of transcription. The black line defines the genome coordinates. IGB Y axis scale value of the peaks indicated.

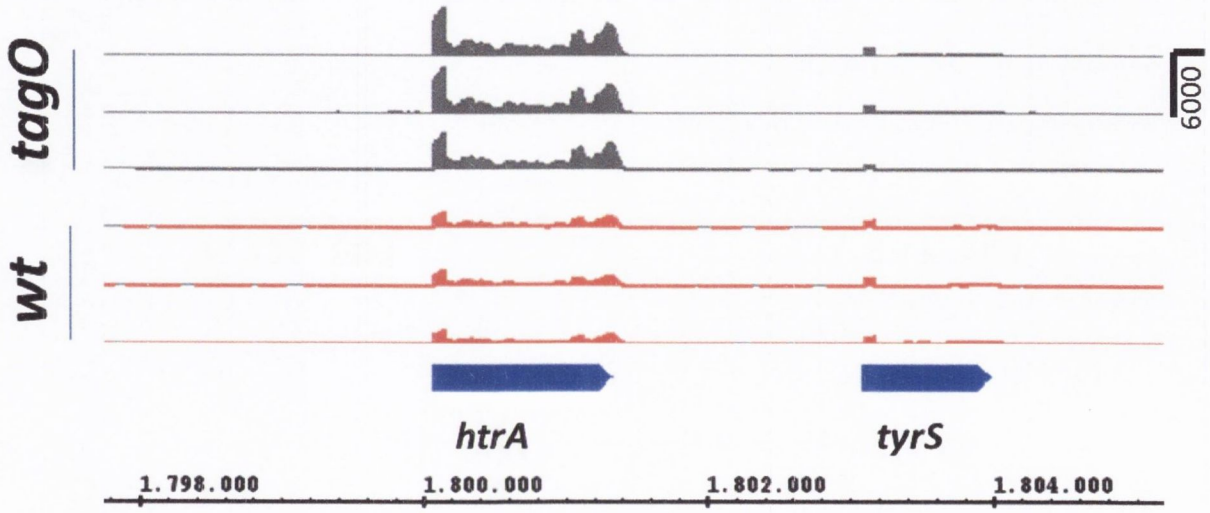



**FIG. 3.18** Integrated genome browser (IGB) visualization of the *htrA* gene. The colour of each track represents the sequencing reads that map to that locus and are directly proportional to the level of expression of that locus. In the annotation tracks, blue arrows define the protein-encoding gene. The arrow indicates the direction of transcription. The black line defines the genome coordinates. IGB Y axis scale value of the peaks indicated

Furthermore, FmtA was also shown to be involved in the stress response caused by cell wall – active agents, since its expression increases in the presence of cell wall inhibitors or when cell wall biosynthesis genes are deleted (Bernal *et al.*, 2010, McAleese *et al.*, 2006), (McCallum *et al.*, 2006, Utaida *et al.*, 2003).

FmtA protein harbours two of three conserved motifs, SXXK and SND, found in the serine active site of PBPs and β-lactamases. The third conserved motif has not been identified in FmtA. It has been described that FmtA binds covalently to β-lactams via the SXXK motif, and also with peptidoglycan (Fan *et al.*, 2007).

A more recent study found that FmtA acts as an esterase by removing D-Ala groups from WTA and LTA. Deletion of *fmtA* resulted in a 7-fold increase in the D-Ala content of WTA, suggesting that FmtA might modulate D-alanylation of WTA in *S. aureus* (Rahman et al., 2016). In another study, FmtA was exogenously added to *S. aureus* cells which induced autolysis, suggesting that binding of FmtA to WTA might affect the activity of the major autolysin AtlA by modulating the charge of WTA. However a direct interaction between FmtA and AtlA cannot be ruled out (Qamar & Golemi-Kotra, 2012). Therefore FmtA seems to be involved both in cell wall biosynthesis and autolysis depending on the presence or absence of cell wall stress.

#### 3.2.3.2.8 MurZ

The *murZ* gene is 2.83-fold increased in the *tagO* mutant. This gene is flanked by the downstream gene NWMN_2027 that is 1.79-fold up-regulated in the *tagO* mutant and the gene NWMN_2029 that is expressed at the same level as in the wild type (FIG 3.20) The gene *murA* encodes the enzyme responsible for the first step of peptidoglycan biosynthesis (H.J. Rogers, 1980). Gram positive bacteria with a low GC content, also encodes the *murZ* gene. In *S. aureus* this enzyme compensates for the lack of MurA in the synthesis of peptidoglycan since it catalyses the same reaction (Blake *et al.*, 2009). However, the role of *murZ* is not well understood..

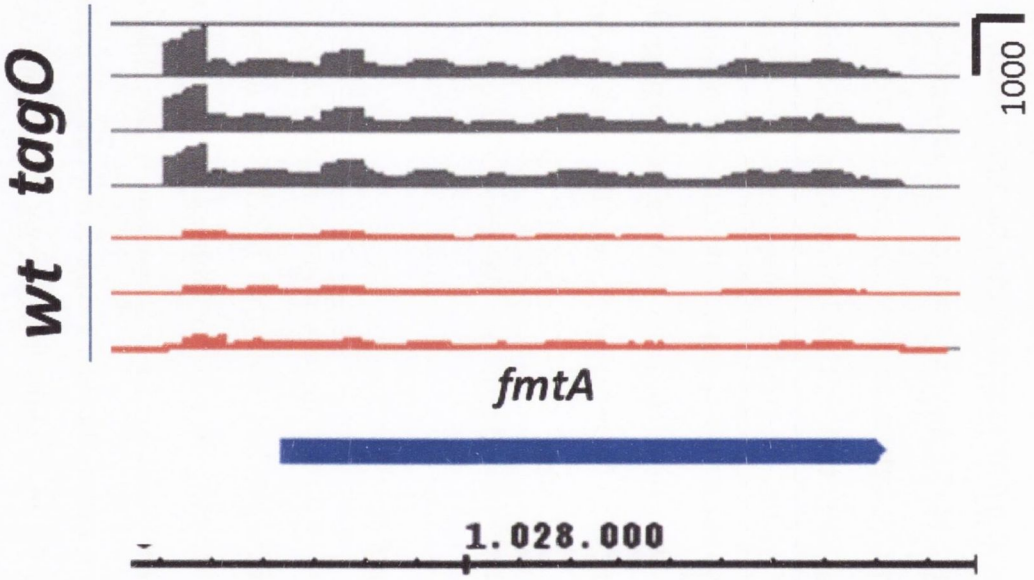



**FIG. 3.19** Integrated genome browser (IGB) visualization of the *fmtA* gene. The colour of each track represents the sequencing reads that map to that locus and are directly proportional to the level of expression of that locus. In the annotation tracks, blue arrows define the protein-encoding gene. The arrow indicates the direction of transcription. The black line defines the genome coordinates. IGB Y axis scale value of the peaks indicated

The inactivation of *murA* or *murZ* is not lethal in *S. pneumonia* suggesting that either enzyme can sustain viability (Du *et al.*, 2000).

### 3.2.3.3 Capsule biosynthetic genes

Capsule genes are located in an operon containing 16 closely linked genes transcribed in one orientation, comprising a region of approximately 17.5 kb. This operon is flanked by the *adhE* gene (downstream) and the *aldA* gene (upstream) (FIG. 3.21). Neither *adhE* nor *aldA* are transcriptionally altered in the *tagO* mutant. The *cap5* and *cap8* gene clusters share a high level of similarity since 12 out of 16 genes are nearly identical (Sau *et al.*, 1997). The type-specific genes are the ones located in the central part of the operon (*capH*, *capI*, *capJ* and *capK*) and show little homology between the two clusters (Wann *et al.*, 1999). *cap5* genes are regulated positively by the Agr system (Dassy *et al.*, 1993, Pöhlmann-Dietze *et al.*, 2000). Little capsular polysaccharide is produced in the logarithmic phase of growth and maximal capsule production occurs during the post-exponential growth phase, which is consistent with the *agr* system that regulates virulence factor in a growth dependent manner (Dassy & Fournier, 1996).

The gene products for which functions have been experimentally determined are CapE, CapP, CapO, CapF, CapG and CapH. The remaining gene products have predicted roles based in homology and bioinformatics, except CapC whose function is unkown. CapH belongs to the O-acetyltransferase family and is responsible for adding O-acetyl groups on N-acetylmannosaminuronic acid residues (Bhasin *et al.*, 1998). CapP and CapO share a high level of homology with epimerase and dehydrogenase enzymes, respectively. These enzymes are responsible for the biosynthesis of UDP-N-acetylmannosaminuronic acid (UDP-ManNAcA) from the precursor UDP-N-acetylglucosamine (UDP-GlcNAc) (Sau *et al.*, 1997). CapE is a multifunctional enzyme that catalyzes the 4, 6 –dehydration and 3, 5 – epimerization of UDP-GlcNAc (Kneidinger *et al.*, 2003). Possible functions of CapB, CapD and CapN have been described based on sequence homology (O'Riordan & Lee, 2004).

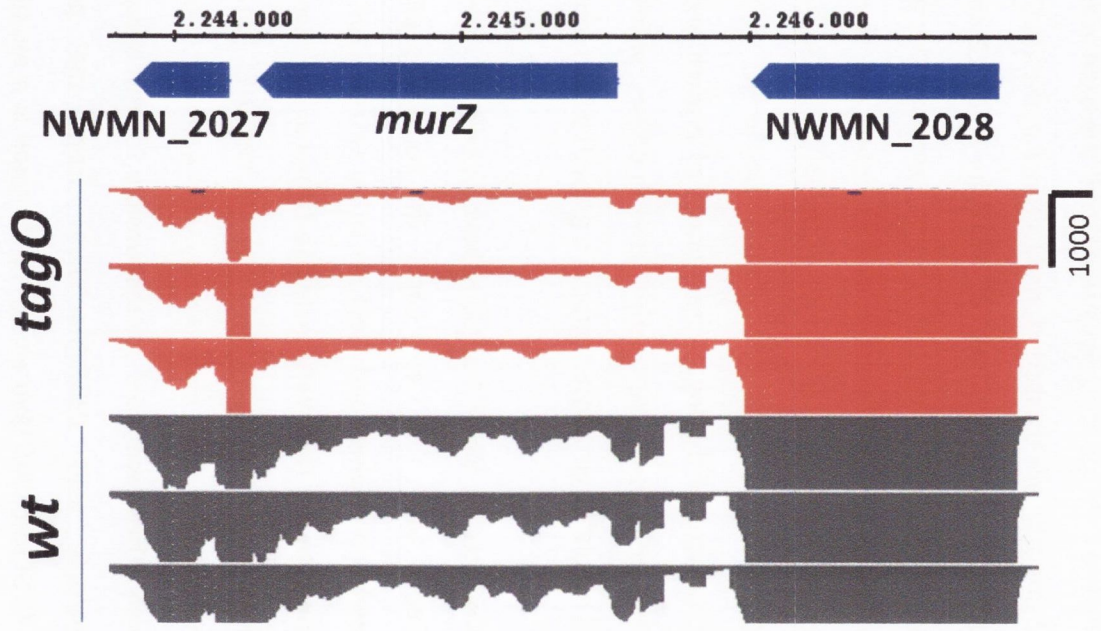



**FIG. 3.20** Integrated genome browser (IGB) visualization of the *murZ* gene. The colour of each track represents the sequencing reads that map to that locus and are directly proportional to the level of expression of that locus. In the annotation tracks, blue arrows define the protein-encoding gene. The arrow indicates the direction of transcription. The black line defines the genome coordinates. IGB Y axis scale value of the peaks indicated

However, there is no experimental evidence supporting their actual roles. CapB has been described has being a regulator of polysaccharide chain length and CapD and CapN are likely involved in the synthesis of UDP-D-FucNAc (O'Riordan & Lee, 2004). The function of these genes and other *cap* genes, such as *capA*, *capI*, *capJ*, *capK*, *capL* and *capM*, involved with putative transferase, polymerase and flippase activities are still unconfirmed.

These capsular polysaccharides are uronic acid polymers, which consist of trisaccharide repeats of N-acetylmannosaminuronic acid and two N –acetylfucosamine (O'Riordan & Lee, 2004). Expression of CP5 in vitro is very sensitive to various environmental signals. Bacterial growth conditions, such as growth medium, have been shown to influence capsule production.

The production of CP is associated with virulence. Macrocapsules are important anti-phagocytic virulence factors but the role of microcapsules is not clear. However, mice inoculated with CP5 expressing *S. aureus* had a higher frequency of arthritis than mice inoculated with the isogenic non-encapsulated mutant (Nilsson *et al.*, 1997).

The 16 genes of the *cap* operon in the Newman *tagO* mutant are up-regulated (Table 3.1), suggesting that there is a higher production of capsular polysaccharides in the absence of WTA.

### 3.2.3.4 Down-regulated genes

The down-regulated genes are mainly those encoding proteins involved in transcriptional regulation, virulence and some metabolic processes. The most highly down-regulated gene is the one encoding the CWA protein Spa (100-fold reduction). As stated in Chapter 1 section 1.2.5.4.1., Spa is virulence factor that is important for *S. aureus* pathogenesis and immune evasion. The *spa* gene is activated by *sarS* which encodes a 250-residue DNA binding protein (Tegmark *et al.*, 2000, Cheung *et al.*, 2004) and is located immediately upstream the *spa* gene (FIG. 3.22).

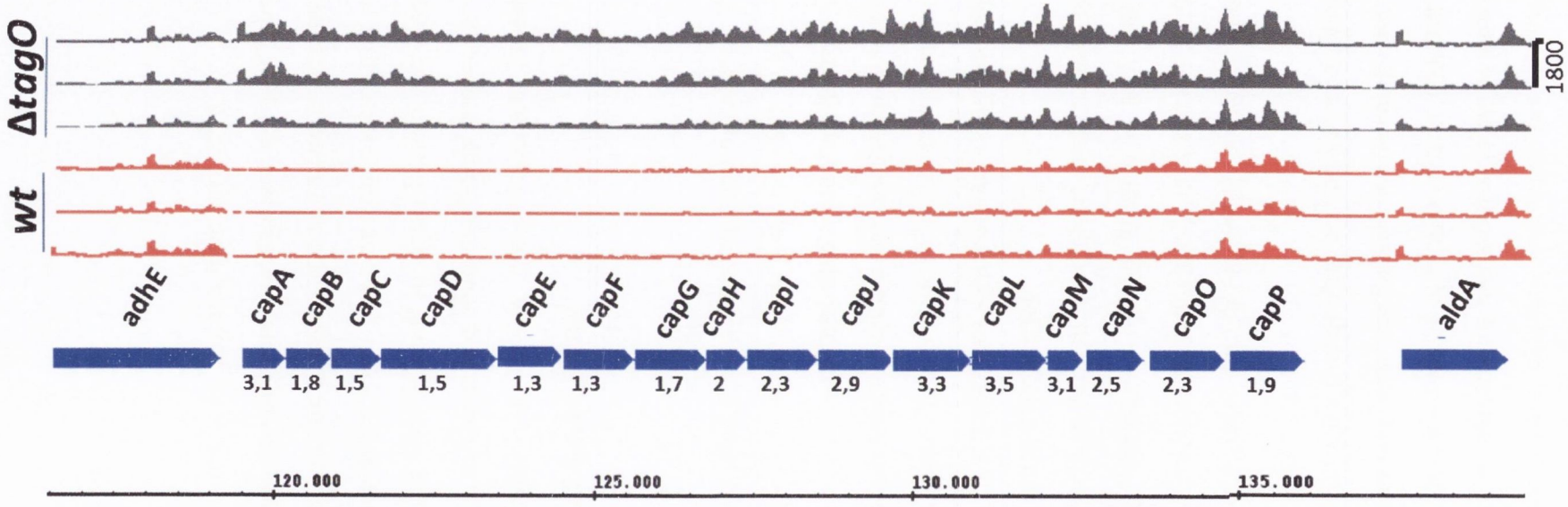



**FIG. 3.21** Integrated genome browser (IGB) visualization of the *cap* operon. The colour of each track represents the sequencing reads that map to that locus and are directly proportional to the level of expression of that locus. In the annotation tracks, blue arrows define the protein-encoding gene. All arrows indicate the direction of transcription. The black line defines the genome coordinates. IGB Y axis scale value of the peaks indicated. The fold-increase in expression of each gene is indicated.

#### 3.2.3.4.1 SarS and Spa

The *sarS* gene is also down-regulated (5-fold reduced) which is most likely the cause of the reduced transcription of *spa*. The *spa* and *sarS* gene are located upstream of a gene encoding a hypothetical protein that is not expressed under the conditions tested and downstream the *sir* operon, encoding a system for the acquisition of iron. The *sir* operon is composed of genes *sirA*, *sirB* and *sirC* encoding a siderophore transport system (Heinrichs *et al.*, 1999). The *sirC* gene is up-regulated (2.57-fold increase) in the *tagO* mutant. *sirC* is predicted to encode a transmembrane protein through which the iron-siderophore complex is transported across the membrane (Heinrichs *et al.*, 1999) under iron-restricted conditions. Additional transcription studies would have to be performed in iron-limiting conditions to investigate relevance of the increased expression of *sirC* in the *tagO* mutant. The SarS protein is a member of the Sar family of transcriptional regulators. SarS is a DNA binding protein and, thus, may up-regulate *spa* expression by directly binding to its promoter (Schmidt *et al.*, 2003).

#### 3.2.3.4.2 Rot

Another member of the Sar family of transcriptional regulators is Rot (repressor of toxins), which has been shown to activate *sarS*, and subsequently *spa* expression (Saïd-Salim *et al.*, 2003). *rot* is located downstream of an unknown gene and upstream the ORF NWMN_1652 encoding a hypothetical protein. There was no change no change in expression of either in the *tagO* mutant (FIG. 3. 23). The gene *rot* is down-regulated in the *tagO* mutant (3-fold reduction). Other factors positively regulated by Rot include cell surface adhesins such as ClfB (Ní Eidhin *et al.*, 1998) and SdrC (Josefsson *et al.*, 1998).

#### 3.2.3.4.3 ClfB

The *clfB* gene is also down-regulated (3.2 – fold reduction) in the *tagO* mutant (FIG. 3.24). As described in the section 1.2.5.2.2 in Chapter 1 ClfB is an important CWA protein for adhesion and nasal colonisation of *S. aureus*. *clfB* is located downstream the *arc* operon and the *aur* gene (encoding aureolysin)

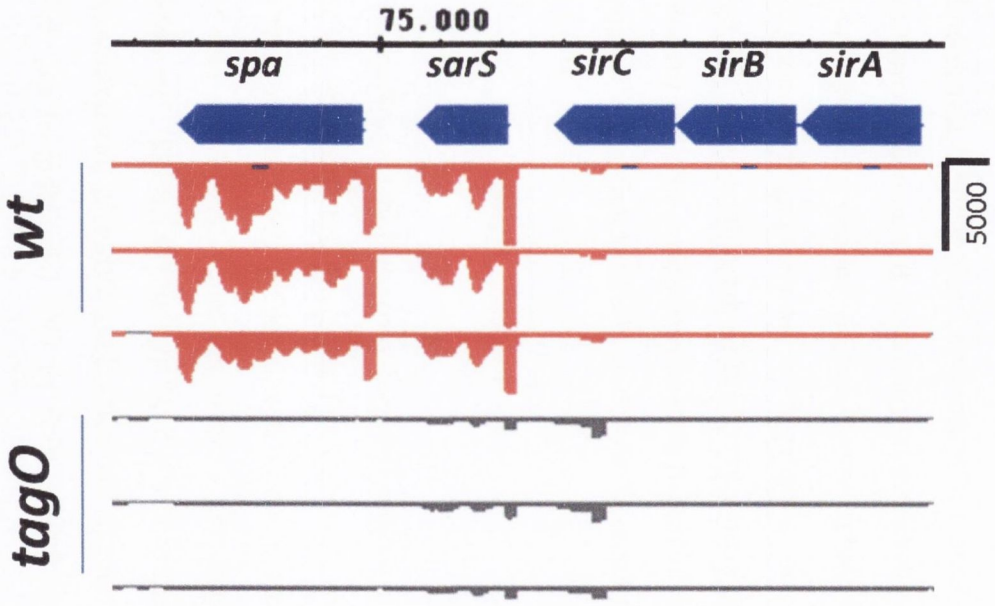



**FIG. 3.22** Integrated genome browser (IGB) visualization of the *spa* and *sarS* gene. The colour of each track represents the sequencing reads that map to that locus and are directly proportional to the level of expression of that locus. In the annotation tracks, blue arrows define the protein-encoding gene. The arrow indicates the direction of transcription. The black line defines the genome coordinates. IGB Y axis scale value of the peaks indicated

and upstream the *estA* gene. The genes flanking *clfB* are not expressed under the conditions tested (FIG. 3. 24). Rot can activate the transcription of *clfB* during the early-exponential phase, through direct binding of Rot to the *clfB* promoter (Xue *et al.*, 2011). The reduced transcription of ClfB may be caused by the reduction in rot transcription.

#### 3.2.3.4.4 Agr

The *agr* operon is flanked upstream and downstream by genes encoding hypothetical proteins that were not expressed in the conditions tested (FIG. 3.25). The 4 genes comprising the *agr* operon *agrB*, *agrD*, *agrC* and *agrA* are down-regulated in the Newman *tagO* mutant (Table 3.2) which may explain the down-regulation of genes important for the virulence of *S. aureus* Newman. The *agr* operon (as described in section 1.3.2. in Chapter 1) is the main regulatory system responsible for the expression of virulence factors in *S. aureus*.

This operon is also responsible for the activation of RNAIII which is the effector molecule of the *agr* system. RNAIII is an anti-sense RNA induced by the *agr* system. It is responsible for the switch between the expression of surface proteins and secreted toxins (Novick *et al.*, 1993). This RNA also has a small ORF near the 5´end that encodes for the delta-hemolysin (FIG. 3.25), while the noncoding regions have regulatory functions (Novick & Jiang, 2003). RNA III is an anti-sense small RNA that is transcribed from the DNA strand opposite to the *agr* operon (FIG. 3.25) and has multiple regulatory functions (Novick & Geisinger, 2008). The RNAIII 5´domain activates translation of alpha-hemolysin by interacting with the *hla* mRNA (Morfeldt *et al.*, 1995) and its 3´domain represses the translation of *rot* and virulence factors such as *coa* and *lytM* (Boisset *et al.*, 2007, Chevalier *et al.*, 2010, Liu *et al.*, 2011).

Delta-toxin is a member of the phenol-soluble modulin (PSM) peptide family and a key contributor to infection by *S. aureus*. Others PSM peptides such as PSMα which are also down-regulated in the *tagO* mutant (Table 3.2), are under the direct regulation of AgrAC (Queck *et al.*, 2008). PSM peptides are strongly associated with the capacity of MRSA strains to lyse human neutrophils and other cell types, to promote skin infection and sepsis (Wang *et al.*, 2007). PSMs also promote

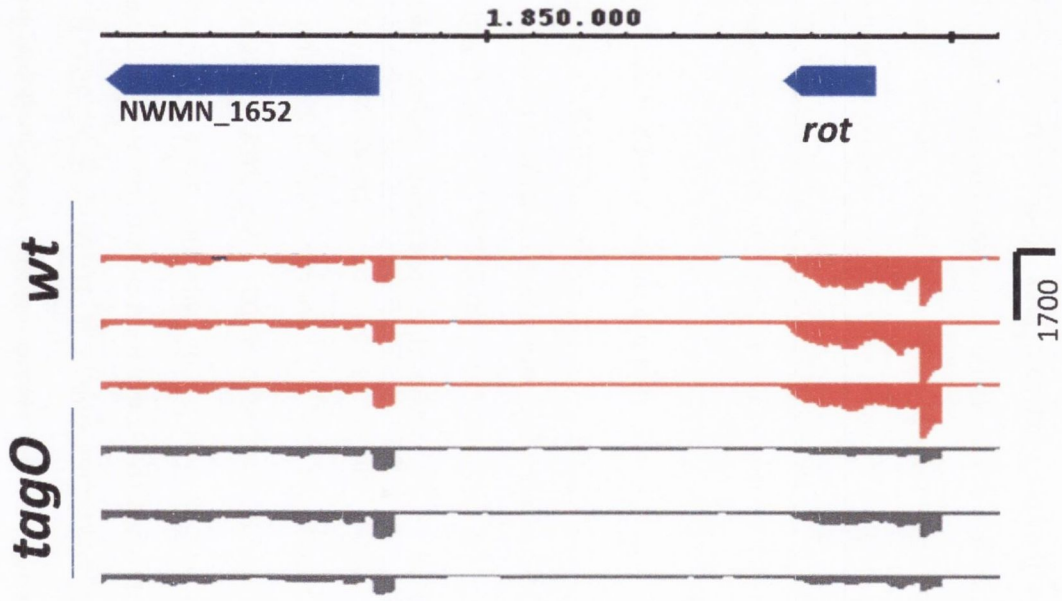



**FIG. 3.23** Integrated genome browser (IGB) visualization of the *rot* gene. The colour of each track represents the sequencing reads that map to that locus and are directly proportional to the level of expression of that locus. In the annotation tracks, blue arrows define the protein-encoding gene. The arrow indicates the direction of transcription. The black line defines the genome coordinates. IGB Y axis scale value of the peaks indicated.

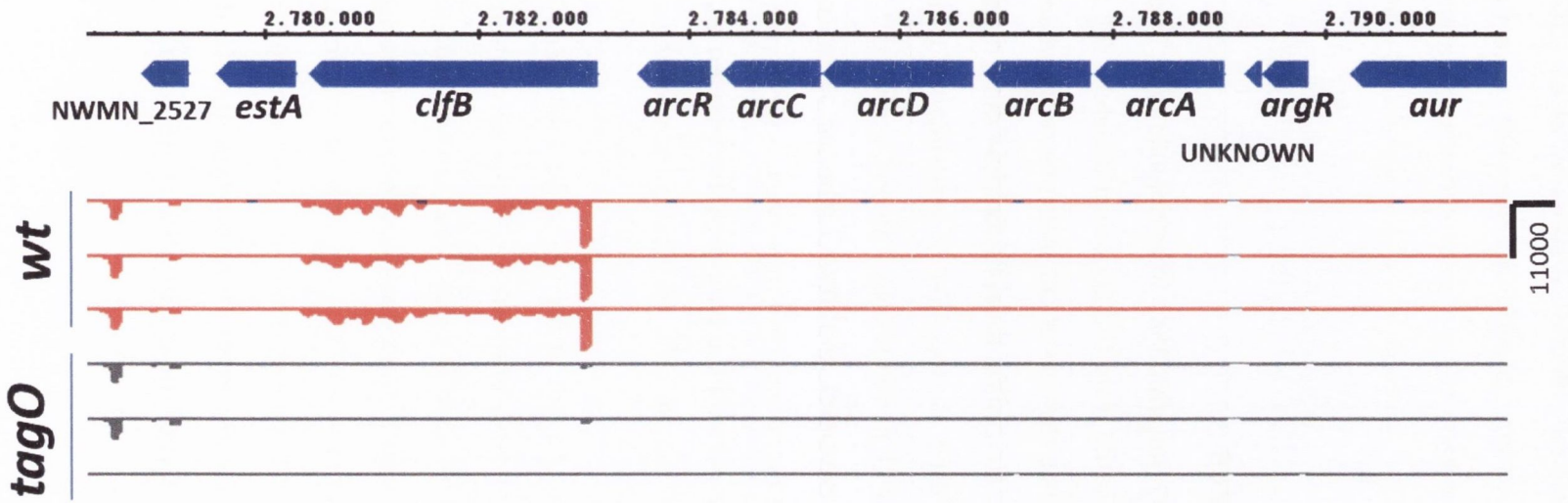



**FIG. 3.24** Integrated genome browser (IGB) visualization of the *clfB* gene. The colour of each track represents the sequencing reads that map to that locus and are directly proportional to the level of expression of that locus. In the annotation tracks, blue arrows define the protein-encoding gene. The arrow indicates the direction of transcription. The black line defines the genome coordinates. IGB Y axis scale value of the peaks indicated.

inflammatory responses, biofilm structuring, detachment and the systemic dissemination of biofilm-associated infection (Periasamy *et al.*, 2012, Wang *et al.*, 2011). The delta-toxin is a 26aa long peptide that does not contain a signal peptide (Chatterjee *et al.*, 2013). Delta-toxin triggers mast cell degranulation and likely has an important contribution to the development of the skin disease, atopic dermatitis (Nakamura *et al.*, 2013).

### 3.2.4 Quantitative PCR

The growth of the *tagO* mutant differs from the wild type, in particular due to the formation of clumps originated from the abnormal daughter cell separation after cell division which may influence the use of OD measurements when preparing cells for isolating RNA and proteins. To confirm that precisely the same number of wild type and mutant cells was being used for the RT-PCR experiments, gene copy number experiments were performed with genomic DNA. The *oriC* was used as internal control (only one copy in the chromosome) and DNA extracted from cells during RNA extraction, the gene concerned was amplified (one copy in the chromosome), and no differences between the wild type and the mutant were detected indicating that the same number of wild type and mutant cells were being used for the experiment (FIG. 3.26).

In order to confirm the changes in the transcript levels seen in the RNA-seq, real – time (RT) PCR was performed. With this study the correlation between these two methods was determined for specific genes. Newman wild type and *tagO* mutant total RNA was extracted from cells in the exponential phase of growth. The RNA was converted to cDNA and measured by RT-PCR using specific primers (Chapter 2, Table 3.2). The slope of the standard curve was measured to determine the efficiency and efficiencies of less than 0.98 were discarded. Using the cycle thresholds (ct) values the fold-differences were calculated using the comparative ct method.

The *tagO* gene transcripts were measured in the Newman wild type, *tagO* and *tagO* complemented strains (FIG. 3.27). No transcripts were detected in the *tagO* mutant. However in the *tagO* complemented strain, the level of *tagO* transcripts was higher than the wild-type (although not fully induced) which is likely to be a consequence of the gene being expressed from a high copy number plasmid in the complemented strains, although the value was not statistically significant.

Relative (FIG.3.28) and absolute expression (FIG.3.29) showed that the level of transcripts for *spa* was reduced, with a 10-fold reduction in the *tagO* mutant when compared to the wild type. *clfB* transcripts were also reduced (although this was not statistically significant), with a 6-fold reduction in the *tagO* mutant when compared to the wild type. This method was also used to measure the transcript level of *hld* (RNAIII), genes encoding the membrane proteins EbpS and AtpA, the extracellular protein Hla (alpha-toxin) and the two-component system response regulators VicR (WalR), PhoP, ArlR and SaeR (FIG. 3.28 and 3.29). The transcript levels of the response regulators of the different two component systems were determined by RT-PCR to confirm the RNA-seq results. The two component systems that were chosen to be studied by RT-PCR are involved in important pathways in *S. aureus*, including the expression of virulence factors (as described in Chapter 1). An altered transcription of these proteins would most likely originate changes in the transcription of virulence factors. The levels of *hld* mRNA were significantly reduced, with a 100-fold difference. However, no changes were detected in the transcripts of the other proteins with exception of PhoP and ArlR. The transcript levels of the surface protein ClfA and the transcriptional regulator SigB were also measured, this time with RNA from cells in the stationary phase of growth, and no significant changes were seen when compared to control sample (FIG. 3.30). These results suggested that the RNA-seq results correlate with the RT-PCR performed for the different genes.

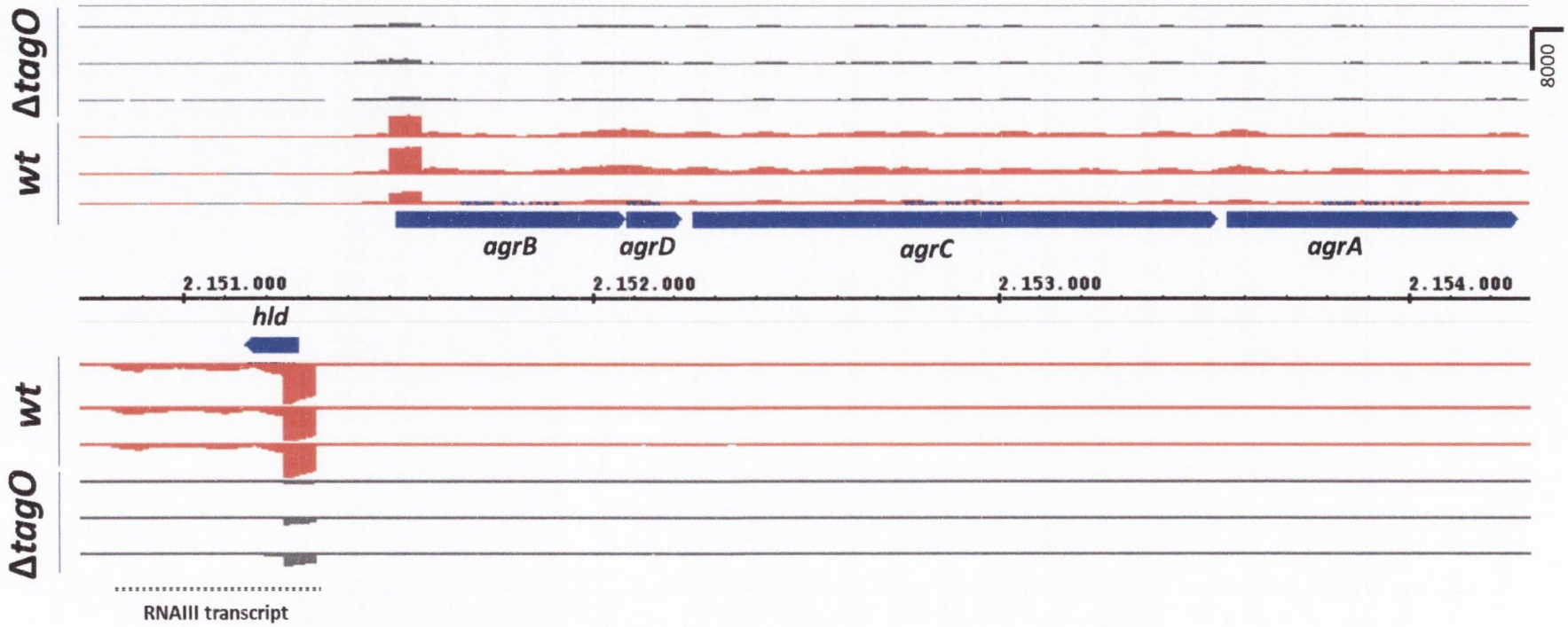



**FIG. 3.25** Integrated genome browser (IGB) visualization of *agr* operon. RNAIII extent of the transcript is indicated. The colour of each track represents the sequencing reads that map to that locus and are directly proportional to the level of expression of that locus. In the annotation tracks, blue arrows define the protein-encoding gene. All arrows indicate the direction of transcription. The black line defines the genome coordinates. IGB Y axis scale value of the peaks indicated.

**Table 3.1:** List of the up-regulated genes in the Newman *tagO* mutant determined by RNA-seq. *SAZ172_1070 is unannotated on Newman genome, located between NWNM_1811 and NWNM_1812.

| Putative Function | GENE | Fold increase | Locus ID |
|---|---|---|---|
| **REGULATORY FUNCTION** | | | |
| **Two component system** | | | |
| response regulator; control of the cell wall peptidoglycan biosynthesis | ***vraR*** | 3.69 | NWMN_1822 |
| sensor protein; activates VraR by phosphorylation | ***vraS*** | 3.45 | NWMN_1823 |
| **Transcription regulation** | | | |
| conserved hypothetical protein from the vra operon | ***vraT*** | 3.56 | NWMN_1824 |
| conserved hypothetical protein from the vra operon | ***vraU*** | 3.45 | NWMN_1825 |
| molecular chaperone that facilitates folding of outer membrane porins | ***surA*** | 2.28 | NWMN_1252 |
| HTH cro/C1-type DNA-binding | ***NA*** | 3.82 | SAZ172_1070* |
| involved in the response to aldehyde stress | ***adhR*** | 2.52 | NWMN_2105 |
| transcription antiterminator | ***glcT*** | 2.21 | NWMN_1270 |
| transcriptional regulator Cro/CI family protein | ***NA*** | 2.18 | NWMN_2606 |
| **CELL ENVELOPE** | | | |
| **Capsule biosynthesis proteins** | | | |
| capsular polysaccharide biosynthesis protein | ***capK*** | 3.35 | NWMN_0105 |
| acting on the CH-OH group of donors, NAD or NADP as acceptor | ***capO*** | 2.35 | NWMN_0109 |
| capsular polysaccharide synthesis enzyme Cap5H; O-acetyl transferase | ***capH*** | 2 | NWMN_0102 |
| required for the biosynthesis of type 1 capsular polysaccharide. | ***capJ*** | 2.96 | NWMN_0104 |
| sugar efflux transmembrane transporter activity | ***capA*** | 3.15 | NWMN_0095 |
| transferase activity, transferring glycosyl groups | ***capM*** | 3.12 | NWMN_0107 |
| Catalytic activity; coenzyme | ***capN*** | 2.52 | NWMN_0108 |
| coenzyme binding; lyase activity; racemase and epimerase activity | ***capI*** | 2.32 | NWMN_0103 |
| oxidoreductase involved in capsule synthesis | ***capL*** | 3.56 | NWMN_0106 |
| attachment of capsular polysaccharides to the peptidoglycan | ***lcpC*** | 6.81 | NWMN_2211 |
| **Peptidoglycan** | | | |
| dephosphorylation of undecaprenyl diphosphate. Resistance to bacitracin. | ***bacA*** | 2.14 | NWMN_0652 |
| Cell wall formation. Adds enolpyruvyl to UDP-N-acetylglucosamine | ***murZ*** | 2.83 | NWMN_2028 |
| M23 family peptidase | ***NA*** | 3.89 | NWMN_RS01180 |
| Penicillin-binding protein | ***sgtB*** | 3.98 | NWMN_1766 |
| metallo β-lactamase fold exonuclease | ***yycJ*** | 2.07 | NWMN_1601 |
| Peptidoglycan catabolic process | ***atl*** | 2.57 | NWMN_0922 |
| Murein DD-endopeptidase MepM and murein hydrolase activator NlpD | ***lytM*** | 2.95 | NWMN_0150 |
| Hydrolase involved in the splitting of the septum during cell division | ***sle1*** | 2.4 | NWMN_0634 |
| putative exported protein; cell wall damage protection | ***cwrA*** | 93.1 | NWMN_2456 |
| **Teichoic acid synthesis** | | | |
| involved in WTA attachment to peptidoglycan | ***lcpA*** | 2.26 | NWMN_1274 |
| phosphorylation of diacylglycerol (DAG) into phosphatidic acid | ***dagK*** | 2.37 | NWMN_0695 |

| | | | |
|---|---|---|---|
| **Cell wall anchored proteins** | | | |
| cell surface-associated protein causing human platelet aggregation. | ***sdrE*** | 2.67 | NWMN_0525 |
| **PROTEIN FATE** | | | |
| **Post-tranlational modification, protein turnover and chaperones** | | | |
| post-translocational extracellular folding of several secreted proteins. | ***prsA*** | 7.08 | NWMN_1733 |
| involved in the recovery of the cell from heat-induced damage | ***clpB*** | 3.21 | NWMN_0845 |
| **Proteases** | | | |
| stress resistance and bacterial survival | ***htrA1*** | 3.04 | NWMN_1621 |
| essential for cell viability; Cleavage of secreted and periplasmic proteins | ***spsB*** | 5.96 | NWMN_0836 |
| **Transport activity** | | | |
| iron ABC transporter permease | ***sirC*** | 2.57 | NWMN_0057 |
| oligopeptide transport ATP-binding protein | ***oppD*** | 3.44 | NWMN_0858 |
| part of a transport system that may be involved in oligopeptide uptake. | ***oppD2*** | 2.45 | NWMN_1290 |
| part of a transport system that may be involved in oligopeptide uptake. | ***oppC2*** | 3.11 | NWMN_0173 |
| Macrolide MFS transporter | ***NA*** | 2.1 | NWMN_2081 |
| ferrichrome ABC transporter | ***fecB*** | 2.67 | NWMN_2078 |
| ABC-type dipeptide oligopeptide nickel transport system permease component | ***nikB*** | 3.19 | NWMN_1293 |
| ammonium transmembrane transporter activity | ***amt*** | 3.29 | NWMN_1950 |
| urea transmembrane transporter activity | ***yut*** | 2.43 | NWMN_2187 |
| Iron-dicitrate ABC transporter | ***fecD*** | 2.02 | NWMN_2076 |
| **ANTIOBIOTIC RESISTANCE** | | | |
| putative sequester of β-lactam agents before they gain access to their target in the cell envelope | ***vraX*** | 128 | NWMN_0542 |
| affects the methicillin resistance level and autolysis as well as the cell wall structure | ***fmtA*** | 3.48 | NWMN_0926 |
| **AMINOACID TRANSPORT AND METABOLISM** | | | |
| cystathionine beta-lyase activity | ***mccB*** | 2.43 | NWMN_0425 |
| Sulfurtransferase | ***ybfQ*** | 2.26 | NWMN_2587 |
| cysteine biosynthetic process from serine | ***cysM*** | 2.35 | NWMN_0424 |
| L-proline glycine betaine binding ABC transporter protein ProX | ***proX*** | 2.08 | NWMN_2345 |
| amino-acid biosynthesis | ***thrA*** | 2.06 | NWMN_1239 |
| **METABOLIC PROCESSES** | | | |
| **Oxireductases** | | | |
| glyoxylate bypass, Tricarboxylic acid cycle | ***citZ*** | 3.52 | NWMN_1588 |
| catalyzes the reduction of glutamyl-tRNA to glutamate 1-semialdehyde | ***hemA*** | 4.42 | NWMN_2607 |
| response to arsenic-containing substance | ***arsC*** | 3.01 | NWMN_1666 |

| | | |
|---|---|---|
| organic hydroperoxide resistance protein ***ohrB*** | 2.2 | NWMN_0729 |
| mannitol metabolic process ***mtlD*** | 2.25 | NWMN_2060 |
| glycine betaine biosynthetic process ***betB*** | 2.23 | NWMN_2510 |
| glycerol catabolic process ***glpD*** | 2.33 | NWMN_1209 |
| thioredoxin; Electron transport, Transport ***NA*** | 2.99 | NWMN_RS10720 |
| **Hydrolase** | | |
| hydrolase activity, acting on glycosyl bonds ***sceD*** | 17.2 | NWMN_1999 |
| calcium binding ***drp35*** | 2.26 | NWMN_2586 |
| **Alkaline phosphatase** | | |
| O-mannosyltransferase ***ydjZ*** | 2.48 | NWMN_0834 |
| **Kinase** | | |
| Glycolysis ***pgk*** | 3.63 | NWMN_2331 |
| guanosine tetraphosphate metabolic process ***relA*** | 2.06 | NWMN_1536 |
| phosphoenolpyruvate-dependent sugar phosphotransferase system ***ptsG*** | 2 | NWMN_0133 |
| phosphoenolpyruvate-dependent sugar phosphotransferase system ***mtlF*** | 2.47 | NWMN_2059 |
| **Ligase** | | |
| glutamine biosynthetic process ***glnA*** | 2.12 | NWMN_1217 |
| **Urea catabolic process** | | |
| facilitates the functional incorporation of the urease nickel metallocenter ***ureG*** | 2.05 | NWMN_2193 |
| urea catabolic process ***ureA*** | 2.62 | NWMN_0525 |
| involved in urease metallocenter assembly. Binds nickel ***ureE*** | 3.01 | NWMN_2191 |
| required for maturation of urease ***ureD*** | 2.17 | NWMN_2194 |
| required for maturation of urease ***ureF*** | 2.51 | NWMN_2192 |
| **PATHOGENESIS** | | |
| **Extracellular factors** | | |
| immunogenic protein ***ssaA*** | 7.58 | NWMN_2195 |
| truncated MHC class II analog protein ***NA*** | 3.97 | NWMN_2109 |
| **RNA PROCESSING** | | |
| methyltransferase ***rumA*** | 3.27 | NWMN_1835 |
| **DNA REPAIR** | | |
| DNA polymerase involved in untargeted mutagenesis ***umuC*** | 2.06 | NWMN_1276 |
| equivalent to the polymerase V from gram-negative bacteria ***NA*** | 2.21 | NWMN_1860 |
| **HYPOTHETICAL PROTEINS** | | |
| **Function** | **Fold increase** | **Locus ID** |
| Unknown function | 9.77 | NWMN_1552 |
| Unknown function | 5.63 | NWMN_RS08700 |
| Unknown function | 5.3 | NWMN_0931 |
| Unknown function | 4.77 | NWMN_2223 |
| Unknown function | 4.65 | NWMN_0402 |
| phage anti-repressor protein - Transcription | 3.83 | NWMN_0995 |

| | | |
|---|---|---|
| xenobiotic response family of transcription regulators - Transcription | 3.37 | NWMN_RS05585 |
| Unknown function | 3.33 | NWMN_RS10385 |
| Unknown function | 2.93 | NWMN_1834 |
| Unknown function | 2.8 | NWMN_RS07475 |
| Unknown function | 2.73 | NWMN_0996 |
| Unknown function | 2.72 | NWMN_0120 |
| Alkylhydroperoxidase family enzyme - Metabolic processes | 2.68 | NWMN_2371 |
| Unknown function | 2.66 | NWMN_1196 |
| Unknown function | 2.62 | NWMN_RS05300 |
| Peptidase propeptide and YPEB domain - Protein fate, transport and binding | 2.61 | NWMN_0364 |
| aldo-keto reductase - Metabolic processes | 2.49 | NWMN_2104 |
| Domain of unknown function with cystatin-like fold - Lipoprotein | 2.34 | NWMN_0245 |
| XRE family transcriptional regulator - Transcription | 2.33 | NWMN_RS06845 |
| amino acid transporter - Protein fate, transport and binding | 2.29 | NWMN_2349 |
| Oxireductase - Metabolic processes | 2.28 | NWMN_2229 |
| Unknown function | 2.2 | NWMN_RS13315 |
| Cell envelope | 2.16 | NWMN_0923 |
| Unknown function | 2.07 | NWMN_RS12990 |
| Unknown function | 2.06 | NWMN_RS12695 |
| Unknown function | 2.03 | NWMN_0767 |
| Oxireductase - Metabolic processes | 2.03 | NWMN_2048 |
| hydrolase - Metabolic processes | 2.02 | NWMN_2480 |

**Table 3.2:** List of the down-regulated genes in the Newman *tagO* mutant determined by RNA-seq.

| Putative function | | Fold decrease | Locus ID |
|---|---|---|---|
| **REGULATORY FUNCTION** | **GENE** | | |
| **Transcription regulation** | | | |
| cold shock protein | ***cspB*** | 2.7 | NWMN_260 |
| cold shock protein | ***cspC*** | 2.5 | NWMN_0761 |
| atl autolysin putative transcriptional regulator | ***NA*** | 2.5 | NWMN_0921 |
| teicoplanin resistance operon transcriptional regulator | ***tcaR*** | 2.32 | NWMN_2258 |
| protein-N(PI)-phosphohistidine-sugar phosphotransferase activity | ***pfoR*** | 2.9 | NWMN_0252 |
| global regulator of several genes involved in virulence | ***Rot*** | 3.0 | NWMN_1655 |
| Two component system phosphorelay sensor kinase activity | ***agrC*** | 2.70 | NWMN_1945 |
| response regulator AgrA | ***agrA*** | 2.32 | NWMN_1946 |
| activator of the gene encoding protein A (spa) | ***sarS*** | 5 | NWMN_0056 |
| **CELL ENVELOPE** | | | |
| **Wall teichoic acid** | | | |
| first step of wall teichoic acid biosynthesis | ***tagO*** | 7.1 | NWMN_0716 |
| Glycosyl transferase | ***tarM*** | 10 | NWMN_0906 |
| **PROTEIN FATE** | | | |
| **Transport activity** | | | |
| formate/nitrite transporter | ***nirC*** | 5.5 | NWMN_0247 |
| lactate transmembrane transporter activity | ***lldP*** | 7.69 | NWMN_2268 |
| ABC transporter involved in phosphate import | ***ydhJ*** | 14.28 | NWMN_2414 |
| sodium/glutamate symport carrier protein | ***gltS*** | 2.7 | NWMN_2246 |
| **Proteases** | | | |
| protease involved in resistance to antimicrobial peptides in *B. subtilis* | ***prsS*** | 4.34 | NWMN_0172 |
| **Chaperones** | | | |
| ATP-dependent Hsp100/Clp chaperone involved in protein quality control | ***clpC*** | 3.22 | NWMN_2448 |
| **ANTIBIOTIC RESISTANCE** | | | |
| multidrug efflux pump; resistance against diverse quinolones | ***norB*** | 5.88 | NWMN_1346 |
| **AMINOACID TRANSPORT AND METABOLISM** | | | |
| arginine biosynthesis; argininosuccinate synthase | ***argG*** | 2.08 | NWMN_0832 |

| | | | |
|---|---|---|---|
| anaerobic formation of alpha-ketobutyrate and ammonia from threonine | ***tdcB*** | 4.54 | NWMN_1348 |
| constituent of the peptidoglycan layer | ***ald*** | 2.85 | NWMN_1349 |
| branched-chain amino acid transport system II carrier protein | ***brnQ1*** | 2 | NWMN_0248 |
| basic amino acid/polyamine antiporter | ***frlA*** | 7.69 | NWMN_1347 |
| **METABOLIC PROCESSES** | | | |
| **Oxireductases** | | | |
| phosphodiesterase activity | ***azoR2*** | 2.63 | NWMN_0149 |
| D-lactate dehydrogenase activity | ***ddh*** | 2.70 | NWMN_2422 |
| oxireductase; NAD(P)H-quinone oxidoreductase subunit 5 | ***ndhF*** | 2 | NWMN_0418 |
| alcohol degydrogenase; zinc-dependent alcohol dehydrogenase | ***adh1*** | 2.17 | NWMN_0577 |
| flavohemoprotein; nitric oxide dehydrogenase | ***yhb1*** | 4.54 | NWMN_0175 |
| superoxide dismutase [Mn/Fe] 2 | ***sodM*** | 2.32 | NWMN_0077 |
| L-lactate dehydrogenase activity | ***ldh1*** | 25 | NWMN_0176 |
| non-heme ferritin; intracellular sequestering of iron ion | ***ftnA*** | 2.27 | NWMN_1831 |
| **Hydrolase** | | | |
| formaldehyde catabolic process | ***geh*** | 4.76 | NWMN_0262 |
| formate acetyltransferase | ***pflA*** | 5 | NWMN_0163 |
| formate acetyltransferase | ***pflB*** | 6.6 | NWMN_0162 |
| nitrate assimilation, Transport | ***narK*** | 7.69 | NWMN_2288 |
| **Transferases** | | | |
| glucose-specific transporter protein | ***glcA*** | 2.17 | NWMN_0133 |
| **PURINE METABOLISM** | | | |
| purine transporter | ***pbuG*** | 2 | NWMN_2155 |
| Purine metabolism | ***purC*** | 2 | NWMN_0935 |
| **PATHOGENESIS** | | | |
| quorum sensing system signal molecule, the autoinducing peptide (AIP). | ***agrB*** | 3.44 | NWMN_1943 |
| autoinducing peptide of the agr pathway | ***agrD*** | 2.63 | NWMN_1944 |
| promotion of bacterial attachment to human fibrinogen | ***clfB*** | 9 | NWMN_2529 |
| immunoglobulin G binding protein A | ***spa*** | 100 | NWMN_0055 |
| **Extracellular factors** | | | |
| exfoliative toxin that binds to the skin protein profilaggrin | ***eta*** | 2.56 | NWMN_1082 |
| extracellular fibrinogen binding protein | ***vWbp*** | 6.25 | NWMN_0759 |
| conversion of fibrinogen to fibrin | ***coa*** | 2.85 | NWMN_0166 |
| fibrinogen-binding protein precursor | ***efb*** | 2.27 | NWMN_1069 |
| staphylococcal complement inhibitor | ***scn*** | 2.5 | NWMN_1876 |
| binds to human plasma haptoglobin-hemoglobin complexes | ***isdH*** | 5.88 | NWMN_0401 |
| virulence factor, important for the establishment of infection in the host | ***esxA*** | 5.26 | NWMN_2163 |
| binds directly to host FPRL1 inhibiting leukocytes | ***flr*** | 7.69 | NWMN_1067 |

| | | |
|---|---|---|
| inhibits the response of human neutrophils and monocytes to complement ***chp*** | 3.7 | NWMN_1877 |
| superantigen-like protein 11 ***ssl11nm*** | 5.26 | NWMN_0400 |
| superantigen-like protein 8 ***ssl8nm*** | 2.22 | NWMN_0395 |
| ***ssl7nm*** Inhibits complement activation and IgA action | 16.6 | NWMN_0394 |
| staphylococcal enterotoxin-like toxin ***ssl1nm*** | 3 | NWMN_0388 |
| phenol-soluble modulin alpha ***PSM α2*** | 2.7 | NWMN_2618 |
| phenol-soluble modulin alpha ***PSMα3*** | 2.56 | NWMN_2617 |
| phenol-soluble modulin alpha ***PSMα4*** | 2.63 | NWMN_2616 |
| lyses erythrocytes and many other mammalian cells ***hld*** | 2.94 | NWMN_2624 |
| enterotoxin type A precursor ***sea*** | 5 | NWMN_1883 |
| **HOLIN-LIKE PROTEINS** | | |
| putative holin-like toxin ***NA*** | 3.56 | NWMN_RS05040 |
| holin-like protein ***cidA*** | 2.5 | NWMN_2440 |
| holin-like protein ***lrgA*** | 3.8 | NWMN_0196 |
| anti-holin ***lrgB*** | 3.33 | NWMN_0197 |
| **DNA METABOLISM** | | |
| **DNA degradation** | | |
| excalibur extracellular calcium-binding domain protein ***NA*** | 4.34 | NWMN_RS09470 |
| **Nucleotidases** | | |
| 5'-nucleotidase family protein ***sasH*** | 2.94 | NWMN_0022 |
| 5'-nucleotidase, lipoprotein e(P4) family protein ***NA*** | 3 | NWMN_0249 |
| **Restriction enzymes** | | |
| subunit R is required for both nuclease and ATPase activities ***hsdR*** | 3.22 | NWMN_0139 |
| **HYPOTHETICAL PROTEINS** | | |
| **Function** | **Fold decrease** | **Accession nro** |
| unknown function | 2.17 | NWMN_0766 |
| unknown function | 2.22 | NWMN_RS13415 |
| unknown function | 2.27 | NWMN_0904 |
| unknown function | 2.27 | NWMN_1074 |
| unknown function | 2.32 | NWMN_1874 |
| unknown function | 2.38 | NWMN_RS02075 |
| unknown function | 2.38 | NWMN_2490 |
| unknown function | 2.43 | NWMN_RS06085 |
| unknown function | 2.43 | NWMN_0078 |
| unknown function | 2.43 | NWMN_2304 |
| unknown function | 2.32 | NWMN_RS06860 |
| XRE family transcriptional regulator | 2.08 | NWMN_1220 |
| Oxyreductase, nitroreductase family - metabolic processes | 2.7 | NWMN_1941 |
| Immunoprotective lipoprotein, antigen MPT63- Pathogenesis | 4 | NWMN_1689 |
| XRE family transcriptional regulator- Transcription | 9 | NWMN_2377 |
| ABC-transport - Protein fate, transport and binding | 11.1 | NWMN_2413 |

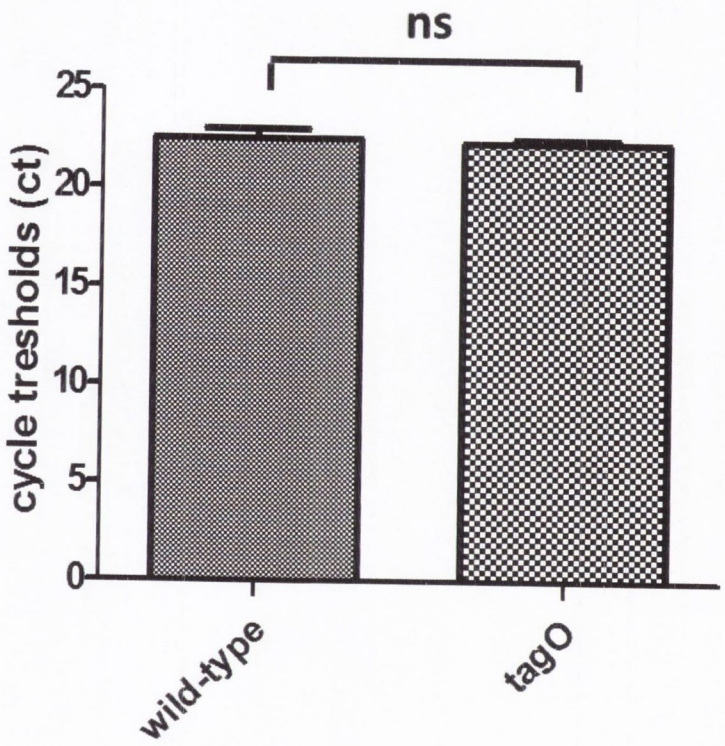



**FIG 3.26** Bar graph representing the Ct values of the *oriC* gene measured by RT-PCR in the Newman wild type strain and the *tagO* mutant. Genomic DNA of cells in the exponential phase of growth were used for this experiment. Statistical analysis was performed using an unpaired t-test. ns = not significant P>0.05

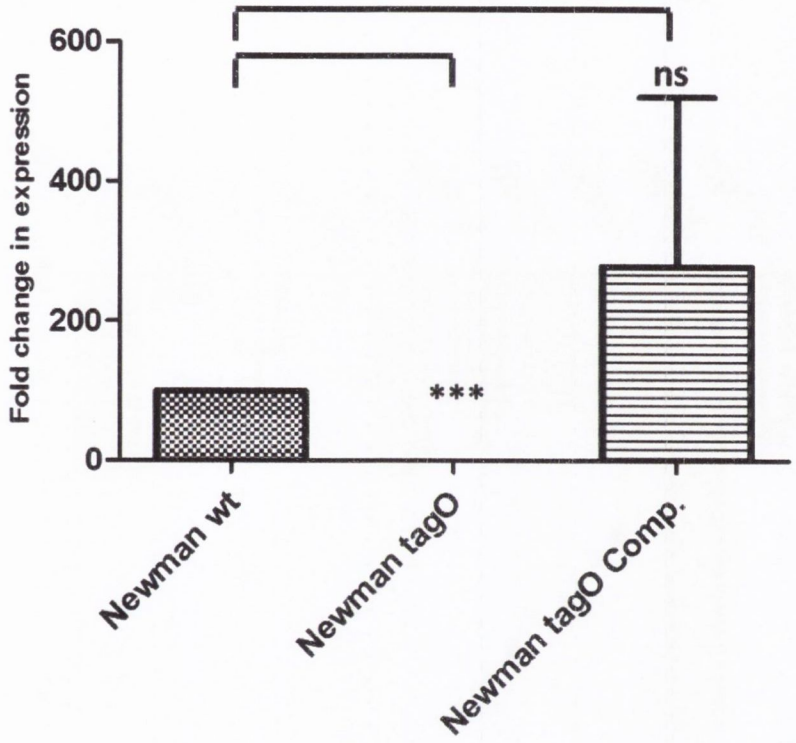



**FIG. 3.27** Bar graph representing the fold change in *tagO* transcripts between the Newman wild type strain, the *tagO* mutant and the *tagO* complemented strain (Newman *tagO* pALC2073::*tagO*). Cells in the exponential phase of growth were used for this experiment. The relative expression ratio, of N=2 experiment, was calculated by assessing the fold change in expression using cycle thresholds (Ct). Statistical analysis was performed using an unpaired t-test. Statistically significant result is ***P<0.001. ns = not significant P>0.05

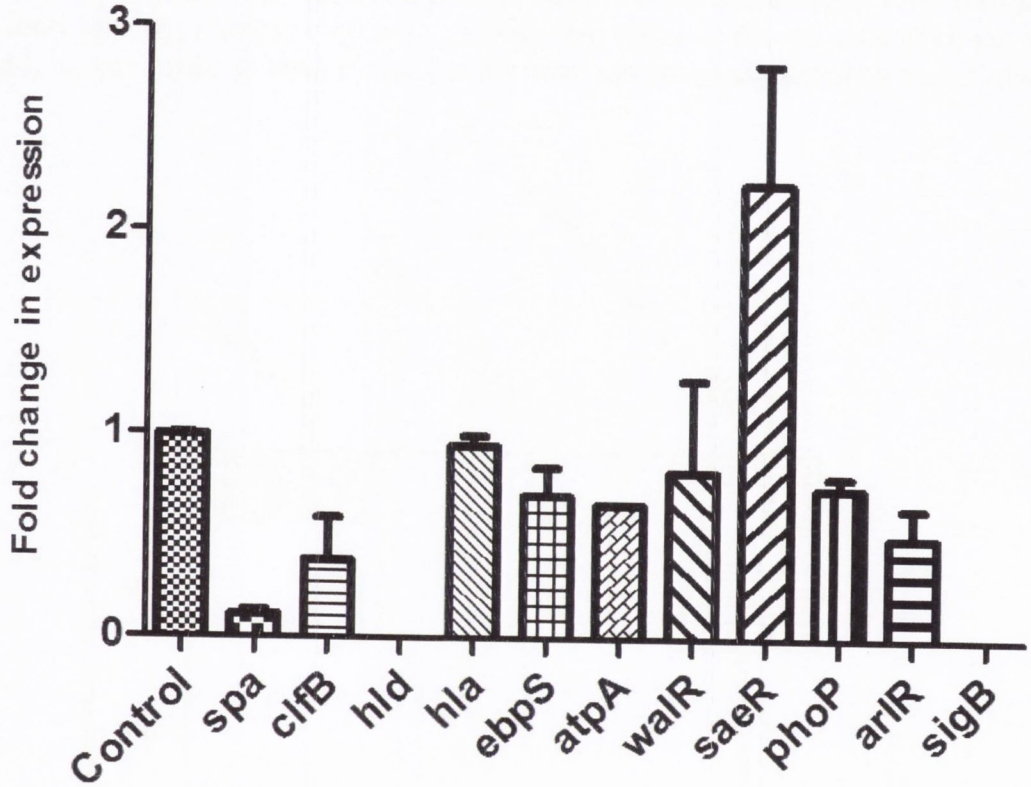



**FIG. 3.28** Bar graph representing the fold change in transcripts between the Newman wild type strain and the *tagO* mutant. Cells in the exponential phase of growth were used for this experiment. The relative expression ratio, of N=2 experiment, was calculated by assessing the fold change in expression using cycle thresholds (Ct). The data presented here is representative of 2 biological replicates.

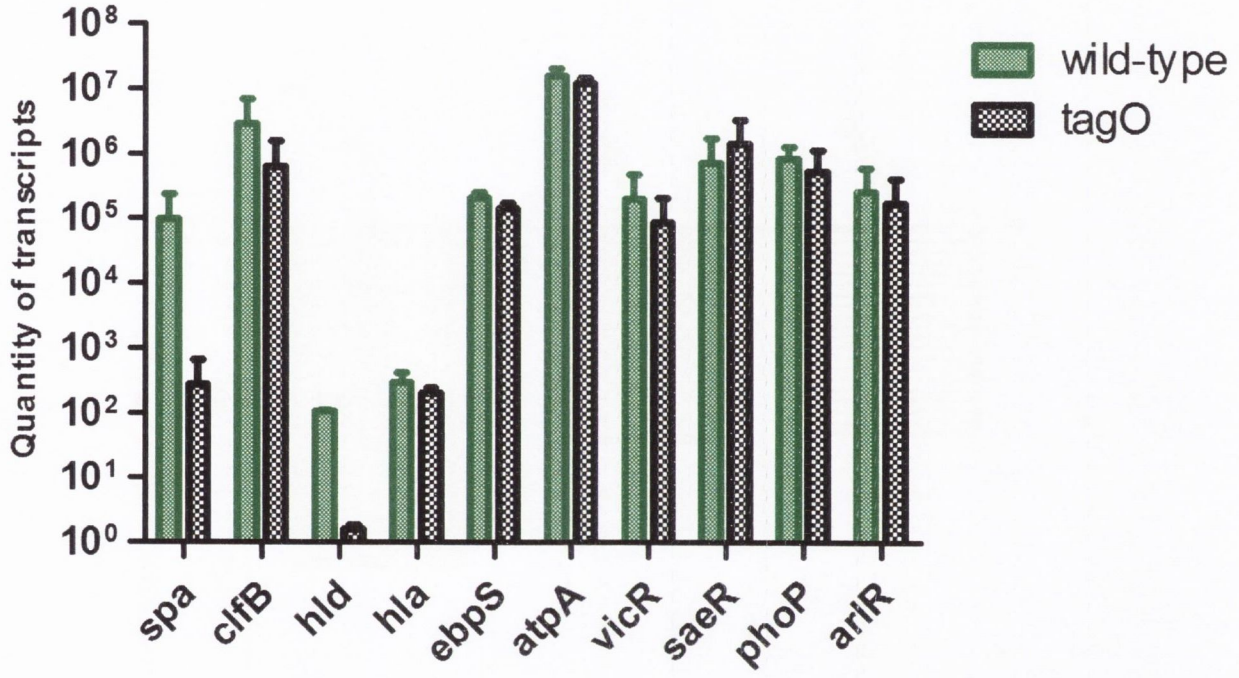



**FIG. 3.29** Bar graph representing the absolute expression of the genes detected by RT-PCR in the Newman wild type strain and the *tagO* mutant. Cells in the exponential phase of growth were used for this experiment. Statistical analysis was performed using an unpaired t-test. The data presented here is representative of 2 biological replicates.

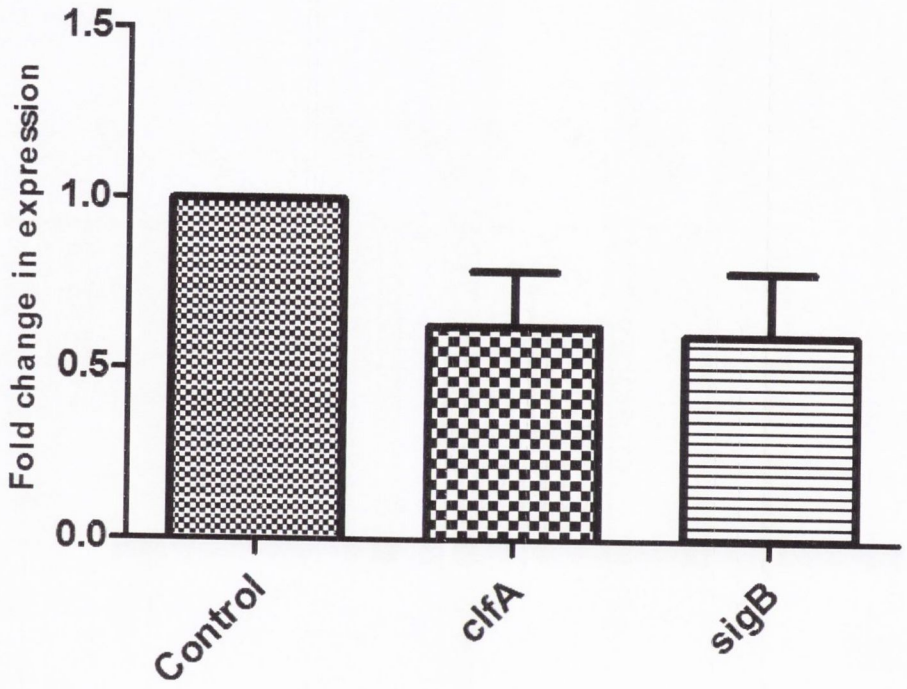



**FIG. 3.30** Bar graph representing the fold change in transcripts between the Newman wild type strain and the *tagO* mutant. Cells in the stationary phase of growth were used for this experiment. The relative expression ratio, of N=2 experiment, was calculated by assessing the fold change in expression using cycle thresholds (Ct). The data presented here is representative of 2 biological replicates.

## 3.3 Summary

Wall teichoic acid was described as a factor that is required for nasal host colonization by *S. aureus* in a cotton rat model (Weidenmaier *et al.*, 2004). Today, it is known that the lack of WTA exerts pleiotropic effects on *S. aureus* cells which would compromise the use of a WTA deficient mutant in animal studies. In order to investigate if these effects are a consequence of altered transcription of important genes, such as those encoding CWA proteins and other virulence factors and genes involved in cell division and cell wall biosynthesis, the whole cell transcriptome was analysed by RNA-seq. The transcriptional profile of Newman wild type and Newman *tagO* was compared and the differently expressed genes were assessed. The *tagO* mutant was constructed by allelic exchange using the temperature sensitive vector pIMAY-Z::*tagO*. Corrigan (2011) showed that LTA mutants can only be isolated under osmotically stabilizing conditions. With WTA mutants no growth advantage was seen in using supplemented media (with sucrose or NaCl), suggesting that these osmoprotectants are not necessary for WTA mutant cells to grow. The wild-type phenotype was successfully restored by complementing the *tagO* mutant with pALC2073::*tagO*, which indicated that all phenotypes tested associated with the WTA mutants were due to the specific mutation and not secondary mutations.

Total RNA of 3 different cultures of Newman wild type and *tagO* was extracted and subjected to rRNA depletion and DNase I treatment. These samples were used to create a cDNA library using a reverse transcriptase and then sequenced.

The sequencing data obtained in this study was acquired by running 3 biological replicates of the Newman wild type and *tagO* mutant strains and the reproducibility of the results was quantified in order to compare gene expression across all strains. The gene expression values between the biological replicates show a high level of correlation demonstrating the reproducibility of the RNA extraction, cDNA library preparation and RNA-seq methods.

Importantly, the absence of the *tagO* gene transcript was shown (FIG. 3.11) in the *tagO* mutant, showing that the *tagO* gene is not being transcribed. None of the genes involved in peptidoglycan synthesis and LTA synthesis were altered (FIG. 3. 12),

showing that the changes in transcription in the WTA-deficient mutant are solely due to the absence of WTA and not due to any other event on the cell surface.

Bacterial cell wall synthesis is a major target for antibiotics. Cell wall active agents such as β-lactams and vancomycin are bactericidal since they target the peptidoglycan synthesis. *S. aureus* rarely acquired high level resistance to vancomycin encoded by the van genes in *Enterococcus*. However, strains that are less sensitive to vancomycin have arisen from a combination of mutations that cause the VISA phenotype (vancomycin intermediate *S. aureus*) (Liu & Chambers, 2003). More recently, it has become clear that bacteria orchestrate a complex program of gene and protein expression and repression following exposure to cell wall active antibiotics. The transcriptomes of several *S. aureus* strains treated with different antibiotics were combined and a set of 17 up-regulated and 2 down-regulated genes were found to be common to all four studies (Kuroda *et al.*, 2003, McAleese *et al.*, 2006, McCallum *et al.*, 2006, Utaida *et al.*, 2003). This group of genes have been termed cell wall stress stimulon (FIG. 3.31) which appears to be a specific response to inhibition of peptidoglycan biosynthesis. It is composed by the up-regulated *genes sgtB, murZ fmt, vraT, tcaA, htrA, prsA, vraS, cwrA, pgk, vraU*, NWMN_1834, NWMN_2211, NWMN_2332, NWMN_2405, and downregulated genes, *atl* and NWMN_0429.

In this study 11 of the 17 genes from the cell wall stimulon were found to be up-regulated in the Newman *tagO* mutant (FIG 3.31). As described in the results section the genes *sgtB, pgk, murZ, fmt, vraT, htrA, prsA, vraS, vraR, vraU* and *cwrA* were all found to be up-regulated. Other genes induced by cell wall active antibiotics were also up-regulated such as *bacA* and *lytM*.

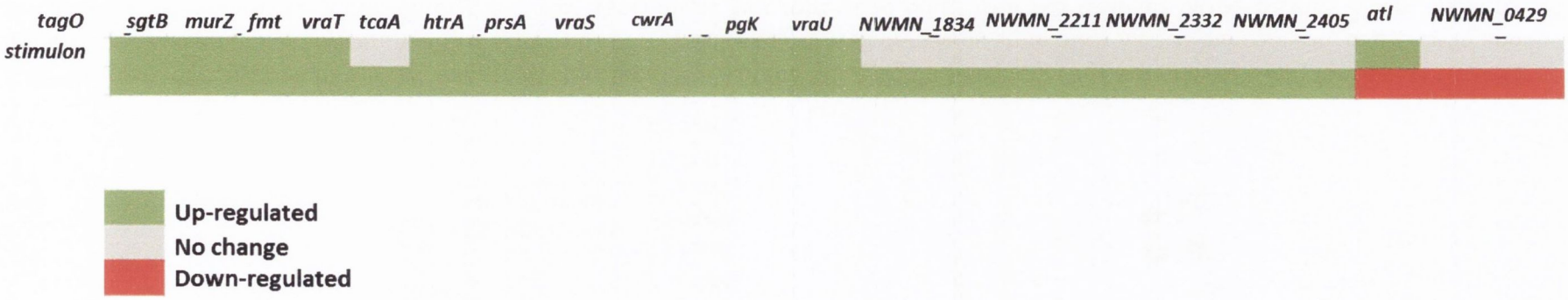



**FIG. 3.31** Comparison of the cell wall stress stimulon genes established by McAleese (2006) with results obtained in this study. In the McAleese study a *S. aureus* strain was treated with vancomycin and the expression of genes was determined by the GeneChips (Affymetrix) technology.

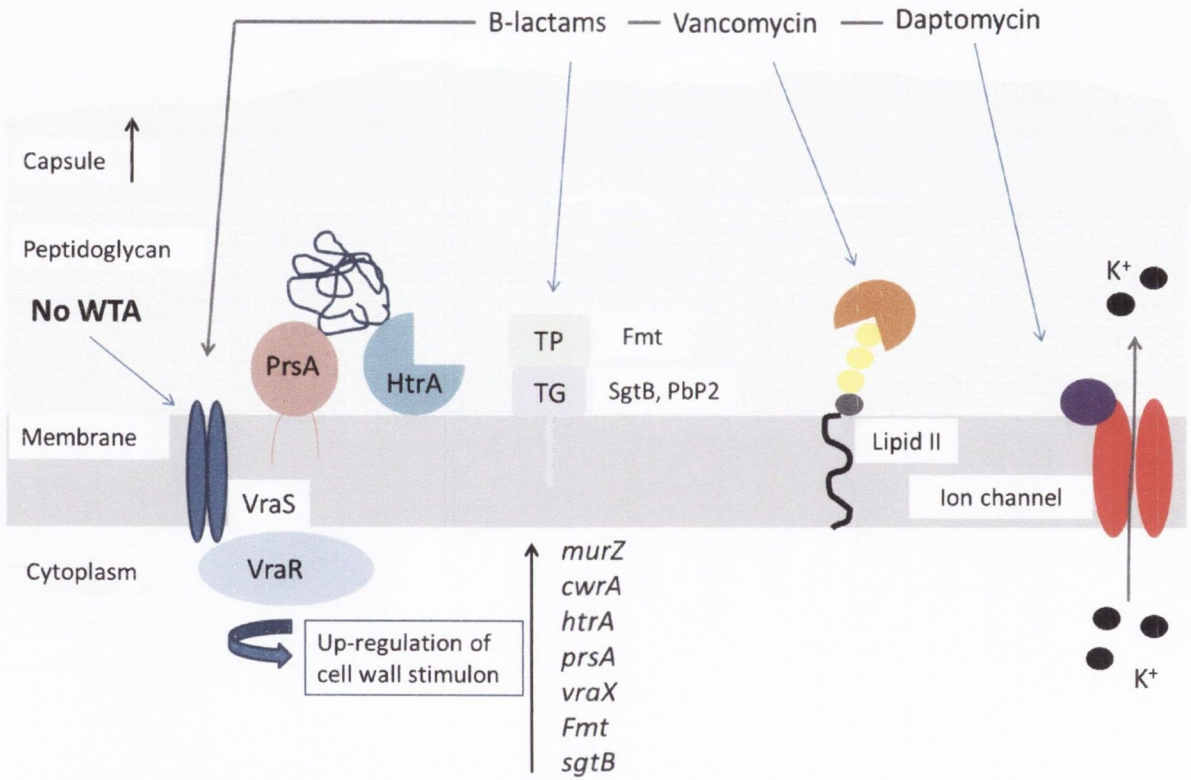



**FIG. 3.32** Summary of activation of the cell wall stress stimulon (CWSS) in the MSSA strain Newman. Only enzymes relevant for this study were mentioned. The two component system VraRS is activated upon eposure to cell-wall damaging antibiotics such as Vancomycin, daptomycin and β-lactams, which originates the activation of the CWSS. Vancomycin recognizes and binds to D-Ala residues in the peptide chain inhibiting peptidoglycan cross-linking. Daptomycin binds to the membrane causing depolarization and the formation of ion channels. The leakage of ions inhibits DNA and protein synthesis. β-lactams bind to penicillin binding proteins (PBPs) inhibiting the peptidoglycan cross-linking. The CWSS comprises genes encoding PrsA and HtrA, that accumulation in the membrane to overcome the presence of damaged and misfolded proteins. Fmt and SgtB are also part of the CWSS and are essential for peptidoglycan synthesis. The absence of WTA in the Newman *tagO* mutant also activates the CWSS originating a similar stress response. Two black arrows represents up-regulation. TG-tranglycosylases; TP-transpeptidases.

*bacA* encodes a bacitracin resistance protein that is a putative undecrapenol kinase homolog and is potentially associated with increasing undecrapenol phosphate in the cell which may be a response to an increase in the acceptor of lipid I product (reaction catalysed by MraY enzyme). The transcription of the *lytM* gene is 2.95-fold induced in the *tagO* mutant. This gene has shown to be elevated 2-3 fold in *S. aureus* strains with increased vancomycin (Mongodin *et al.*, 2003). LytM is a secreted glycyl-glycine endopeptidase of *S. aureus* that is responsible for the release of Spa from the bacterial envelope by cutting the pentaglycyl cross-bridge of Spa cell wall anchor structure (O'Halloran *et al.*, 2015, Becker *et al.*, 2014).

The protease *htrA* and the chaperone *prsA* are both up-regulated in the *tagO* mutant, which may suggest that damaged and misfolded proteins accumulate upon exposure to cell-wall active antibiotics. It has been speculated (Pechous *et al*, 2004) that the deficient assembly of cell surface proteins in the cell wall promotes their accumulation in the cytoplasmic membrane and cytoplasm, inducing chaperone and protease genes.

The *atl* gene is up-regulated in the *tagO* mutant, while it is commonly down-regulated in the presence of cell wall active antibiotics. This gene encodes for the major autolysin Atl which is involved in peptidoglycan turnover and is up-regulated in the *tagO* mutant. Schlag *et al* (2010) showed that in a *S. aureus* wild-type strain Atl was localized at the cross-wall region, while in a *tagO* mutant (lacking WTA) this enzyme was evenly distributed on the cell surface, which explains the increased fragility and autolysis of the mutant.

The VraRS two-component system is responsible for the induction of the cell wall stress stimulon, with the exception of the *htrA* gene. This two component system regulates the important steps in cell wall synthesis such as murein monomer precursor synthesis (*murZ*) and peptidoglycan polymerization (*fmt*, *pbp2* and *sgtB*). In agreement with this a VraRS deletion mutant acquires an increased susceptibility to cell wall targeting agents (Kuroda *et al.*, 2003). The transglycosylase SgtB and the transpeptidase Fmt are essential for peptidoglycan synthesis. The up-regulation of these two proteins in the *tagO* mutant can be a mechanism of the cell to overcome the abnormal synthesis of the peptidoglycan due to the absence of WTA.

The VraRS system is also responsible for the induction of *vraX* (128-fold increased expression in the *tagO* mutant; FIG. 3.14), which encodes a small polypeptide (55 amino acids) that is also induced in vancomycin intermediate resistance (McAleese *et al.*, 2006) during experimental *S. aureus* infections and (Scherl *et al.*, 2006) exposure to vancomycin and daptomycin (Utaida *et al.*, 2003, McAleese *et al.*, 2006, Muthaiyan *et al.*, 2008). The function of VraX is not known. However this small peptide harbours a phosphorylation site that could be involved in regulatory processes (Scherl *et al.*, 2006).

The second most increased expression in the *tagO* mutant was of the gene *cwrA* (93-fold increase; FIG. 3.15). Overexpression of *cwrA* is believed to be a response to cell wall damage, since *cwrA* is up-regulated when any of the genes in the mevalonate pathway, responsible for biosynthesis of the isoprenoid precursor isopentenyl pyrophosphate (IPP), is downregulated (Balibar *et al.*, 2009). IPP is the building block for the undecaprenyl lipid II, essential for the synthesis of peptidoglycan (Wilding *et al.*, 2000).

Cell wall targeting antibiotics, such as vancomycin, daptomycin and β-lactams, which inhibit the normal formation of peptidoglycan, activate the two component system VraRS. This system activates the transcription of the cell wall stress stimulon (FIG 3.32). A similar stress response happens when in the absence of WTA in the *S. aureus* Newman strains (FIG 3.32), most likely due to the abnormal peptidoglycan synthesis.

*S. aureus* strains genome harbours three genes encoding proteins carrying the LCP domain, LcpA, LcpB and LcpC. These proteins catalyse the final step in WTA synthesis (FIG 1.3), the linkage of WTA to peptidoglycan (Kawai *et al.*, 2011). The deletion of *lcpA* and *lcpB* is responsible for the largest reduction in WTA anchoring, while *lcpC* mutation causes only a small reduction (Chan *et al.*, 2013). In another study, *lcpC* (which is 6.81 – fold increased in the *tagO* mutant of this study), was shown to encode the key LCP protein for capsule polysaccharide attachment to peptidoglycan (Chan *et al.*, 2014). These proteins are up-regulated upon exposure to cell-wall active antibiotics and cell wall damage (McAleese *et al.*, 2006, Utaida *et al.*, 2003Dengler *et al.*, 2011). As described in the results section, the capsule genes (*cap* operon) are up-regulated in the *tagO* mutant. LcpC up-regulation correlates with the increase of

capsule production and the need to attach the increased amount of capsule polymers to the peptidoglycan. An increased synthesis of capsule was observed previously when the WTA synthesis was inhibited (Chan *et al.*, 2014). That study suggested that both wall polymers occupy the same C6 hydroxyl of N-acetylmuramic acid or adjacent sites on peptidoglycan and that this occupancy by one polymer results in the attachment of the other. It is also known that capsule genes are induced in VISA strains such as Mu50 and that cell wall increased thickness is a trait present in strains with increased vancomycin resistance (Katayama *et al.*, 2016).

The transcriptional regulation of genes encoding capsule synthesis enzymes is down-regulated when in the presence of targocil (FIG. 3.33), an antibiotic that blocks TarH (the ABC transporter that translocate WTA to the cell surface where it becomes attached to the peptidoglycan) (Campbell *et al.*, 2011). These findings might suggest that WTA intracellular intermediates have a role regulating transcription of capsule genes. WTA intracellular intermediates were shown recently to have a role in transcription of genes that regulate teichuronic acid synthesis in *Bacillus subtilis* (Botella *et al.*, 2014). *S. aureus* does not have teichuronic acid and *Bacillus subtilis* does not express a capsule, which leads to the hypothesis that capsule, performs a similar function to teichuronic acid to compensate for the loss of WTA. This was shown not to be essential because SH1000 is defective in capsule expression due to a mutation in a *cap* gene however is still viable when *tagO* is deleted.

The up-regulation of the cell wall stress stimulon in the *tagO* mutant, followed by the loss of WTA activates different genes and pathways responsible for helping the cell overcome the cell wall damage caused by the lack of WTA. The up-regulation of the capsule genes in the *tagO* mutant that most likely originates an increased production of capsule compensates for the loss of WTA from the cell surface.

The down-regulated genes in the *tagO* mutant are mostly involved in virulence, such as the transcriptional regulators Rot and SarS which activate genes such as those encoding the CWA proteins ClfB and Spa. The two component system composed by *agrA* and *agrC*, the other *agr* genes and the *agr* effector molecule RNAIII are also down-regulated in the *tagO* mutant, resulting in a down-regulation of genes encoding superantigen-like proteins, hemolysins (*hld*) and other extracellular factors like proteases and enterotoxins (*sea*). Several genes encoding superantigen-like proteins

are down-regulated such as such as *ssl11*, *ssl8*, *ssl7* and *ssl1*. These proteins are structurally similar to superantigens but do not stimulate T cells (Kuroda *et al.*, 2001, Williams *et al.*, 2000). Superantigen-like proteins are found in all *S. aureus* strains and have different roles in immune evasion, such as ssl7 which binds to IgA, blocking its action and binds to complement C5 also inhibiting its action (Langley *et al.*, 2005).

Several genes encoding proteins involved in metabolic processes are also down-regulated. *pflA* and *pflB* are two of the most down-regulated. These enzymes catalyse the conversion of pyruvate to formate in anaerobic conditions, thereby producing acetyl coenzyme A, which is important for energy supply (Leibig *et al.*, 2011). The gene encoding the NarK protein, which is involved in the nitrate and nitrite transport (Fast *et al.*, 1996), is also highly down-regulated.

These non-essential genes and pathways that are down-regulated in the *tagO* mutant are likely to be a result of the cell energy management activated by the severe stress in the cell wall, caused by the absence of WTA. One can hypothesise that this saved energy is necessary to activate the mechanisms crucial for survival, such as the cell wall stimulon.

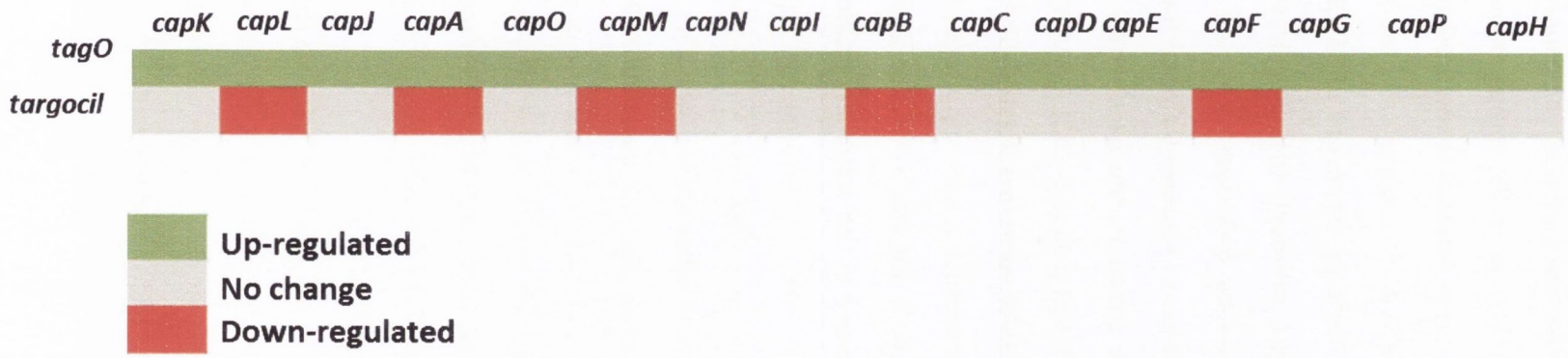



**FIG. 3.33** Comparison of the *cap* genes up- and down-regulated in the Newman *tagO* mutant (this study) and the *S. aureus* strain from the Campbell et al 2011 study. In the Campbell study the protein TarH in the *S. aureus* strain was blocked using the antibiotic targocil, and the expression of genes was investigated by Microarray analysis.

Quantitative PCR was performed in order to confirm the RNA-seq results and correlate the data obtained by the two methods. The expression of the *tagO* gene in the Newman wild type, *tagO* mutant and in the complemented strain was assessed by RT-PCR and, no *tagO* transcripts were detected in the *tagO* mutant. However, a higher number of transcripts were detected in the complemented strain, when compared to the wild type strain (FIG. 3.27). The leaky expression of pALC2073 multicopy plasmid is still sufficient for overexpression, although the data presented is not statistically significant because of the high SD in an N=2 experiment.

In order to have a set of genes representative of the different functions that proteins can have in *S. aureus* grown to the exponential phase of growth, genes encoding proteins from the cell wall (*spa* and *clfB*), membrane (*ebpS* and *aptA*) and from the extracellular medium (*hla*) were investigated. The expression of the genes encoding the response regulator protein of four important two component systems in *S. aureus (saeR, vicR, arlR* and *phoP*) was also measured, as well as the transcriptional regulator *sigB*. The genes expressed in the stationary phase of growth *clfA* and *sigB* were also tested, whereas the RNA-seq was performed with exponential phase cells. RT-PCR revealed reduced levels of transcripts for *spa* and *clfB* (10 and 6-fold reductions, respectively). Consistent with RNA-seq, no alteration was detected for *hla*. The levels of RNAIII transcripts were also measured by RT-PCR and found to be 100-fold reduced (FIG. 3.28). Transcription of the membrane proteins EbpS and AtpA and the extracellular protein Hla was not altered in the WTA-mutant which is consistent with the RNA-seq data. *saeR, vicR, arlR, phoP* and *sigB* transcripts were not found to be altered in the RNA-seq and confirmed by RT-PCR, as well as the *clfA* and *sigB* genes expressed in the stationary phase (FIG. 3.30).

The variation between RNA-seq and RT-PCR results shown in FIG. 3.34 can have several explanations, such as bias introduced by different normalisation methods or PCR primer efficiency. In general, the fold change differences in the genes in study between RNA-seq and RT-PCR showed the same trend.

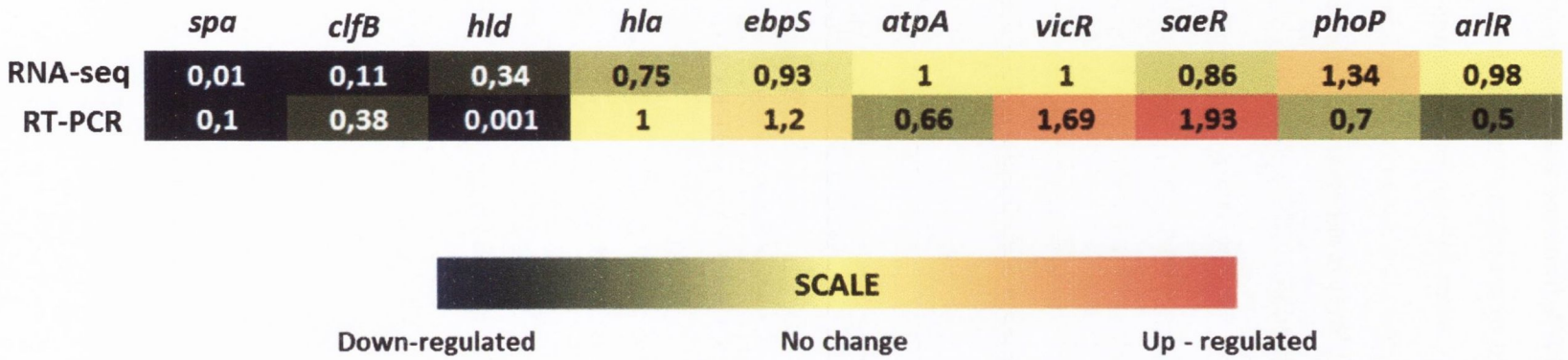



**FIG. 3.34** Comparison of mRNA detected by RNA-seq and RT-PCR. The values presented are the fold change in expression calculated using RNA-seq and RT-PCR data. For the RT-PCR experiments the relative expression ratio, of N=2 experiment, was calculated by assessing the fold change in expression using cycle thresholds (Ct). The threshold of the fold differences was 2. The colour red represents up-regulation (2- fold increase).

This chapter showed that the deletion of the *tagO* gene in *S. aureus* Newman and consequent absence of WTA from the cell surface originates the activation of transcription of genes involved in processes that will help the cell to overcome and compensate the loss of WTA. Concomitantly, the cell shuts down the transcription of several non-essential genes, such as the virulence genes Spa and ClfB, as a result of cell energy management that most likely is necessary to active the mechanisms crucial for survival such as the cell wall stimulon.

# Chapter 4

## Elaboration of surface and secreted proteins in the *S. aureus tagO* mutants

## 4.1 Introduction

Mutants of *S. aureus* that lack the ability to synthesize wall teichoic acid as a result of disruption of the *tagO* gene have been used by others to examine the role of WTA in nasal colonisation and virulence (Weidenmaier *et al,* 2004). It is difficult to draw any meaningful conclusions from these studies because *tagO* mutants that lack WTA are less fit than the wild type due to growth defects. Furthermore, RNA-seq results presented in Chapter 3 revealed gross changes in transcription of many genes.

Of particular interest was the greatly reduced transcription of two genes that encode important CWA proteins. ClfB is a crucial factor for colonisation of the anterior nares by virtue of its ability to bind the squamous cell envelope protein envelope Loricrin. Protein A is an important virulence factor because of its ability to help bacteria evade the host immune responses. In addition the global regulator of virulence factor expression Agr was expressed at a reduced level.

In this chapter expression of the CWA proteins ClfB and Spa was measured by western immunoblotting and by functional tests. CWA proteins were solubilized by dissolving cell wall peptidoglycan with the endopeptidase lysostaphin in the presence of the osmotic stabilizer raffinose. The protoplasts remained intact and only proteins present in the cell wall were released.

The functional activity of Spa and ClfB was measured with intact cells so that only protein exposed on the cell wall surface was measured. Spa was measured by flow cytometry after labelling cells with FITC-conjugated IgG which binds via the Fc region to the repeated binding domains of the bacterial protein.

It should be borne in mind that *S. aureus* expresses a second binding protein called Sbi. This has the potential to complicate studies to detect Spa by Western immunoblotting and flow cytometry. Sbi binds non-covalently to lipoteichoic acid and does not fractionate with CWA proteins following protoplast formation. It is present in the culture supernatant of cells from the stationary phase of growth but little is present in exponential phase. It is expressed at relatively low levels compared to Spa and contributes only to a small extent to IgG binding by intact cells.

For ClfB the ability of bacteria to adhere to immobilized loricrin was measured. ClfB can also bind to fibrinogen and cytokeratin 10, but it is the only known adhesin for loricrin.

In contrast to ClfB which is expressed in the exponential phase of growth, ClfA is expressed predominantly in stationary phase. The RNA-seq analysis was only performed with exponentially growing bacteria so expression of ClfA was measured with cells from stationary phase. Functional activity was measured by performing adhesion assays with immobilized fibrinogen. Under these growth conditions ClfA is the only CWA protein expressed by strain Newman and LAC that can bind to fibrinogen.

In addition, the expression of the two secreted protein alpha-toxin (Hla) and V8 protease was measured by Western immunoblotting, and in the case of Hla, by a functional test.

The membrane proteins EbpS, AtpA and SrtA were studied as controls for the cell fractionation process and because the RNA-seq did not reveal any reduction of transcription of their genes. EbpS is an integral membrane protein with an N-terminus exposed in the extracellular milieu that contains the elastin binding-site (Downer *et al.*, 2002). The ATP synthase subunit alpha (AtpA) is an essential *S. aureus* protein, responsible for the production of ATP from ADP in the presence of a proton gradient across the membrane. The alpha chain is a regulatory subunit. The membrane protein SrtA is the protein responsible for the anchoring of CWA proteins to the cell wall of *S. aureus.* Any reduction in Sortase A would likely be reflected in a reduced level of CWA proteins on the cell surface.

To confirm that the reduction in Spa expression was due to the loss of TagO enzymatic activity the activity of TagO in wild-type cells was inhibited by the antibiotic tunicamycin.

Finally, preliminary experiments to investigate the effect of the build-up of intracellular WTA intermediates on CWA proteins expression were performed. TarH is involved in exporting the WTA precursor across the cytoplasmic membrane. Because a *tarH* mutation alone is lethal, the mutation was constructed in a *tagO* mutant (to prevent precursor accumulation and/or lipid II accumulation) and expression of *tagO*

was inhibited from an inducible plasmid vector. Expression of protein A expression was measured by flow cytometry.

## 4.2. Results

### 4.2.1 Elaboration of protein A (Spa) on the surface of *tagO* mutants of S. *aureus*

Protein A is a cell wall-anchored (CWA) protein although a significant fraction of it is present in the culture supernatant partially due to a sub optimal sorting sequence (O'Halloran *et al.*, 2015). Since the level of Spa mRNA was reduced in the *tagO* mutant the level of this CWA protein was examined, in cell extracts by Western immunoblotting and on intact cells by flow cytometry (see section 3.2.2.1.4).

Firstly, cells from the exponential phase of growth of strains Newman, LAC, SH1000 and BH1CC were harvested and CWA proteins were released by lysostaphin in the presence of the osmotic stabilizer raffinose. The solubilized CWA proteins were subjected to SDS-PAGE and Spa detected with HRP-conjugated IgG which binds to the ligand binding repeats of Spa by the Fc region.

In each case the wild type strain was compared to the *spa* mutant, the *tagO* mutant and the complemented *tagO* mutant. The *spa* mutant was used as a negative control strain, bearing in mind that the cells also express a second IgG binding protein called Sbi which normally does not appear in the cell wall fraction because it is attached to the membrane via lipoteichoic acid (LTA) (Smith *et al.*, 2012) (FIG 4.1). In Newman (FIG 4.2 A), SH1000 (FIG 4.2 C) and BH1CC (FIG 4.2 D) no Spa was detected in the cell wall fraction while in the case of LAC some reactive material was detected (FIG 4.2 B). Densitometry analysis of the blots was performed on three independent samples of Newman and LAC cells and for the Newman *tagO* mutant the residual level of Spa was found to be <1% and for the LAC *tagO* was found to be reduced by 65% (FIG 4.2). Spa expression was restored by introducing the wild type *tagO* gene on a complementing plasmid (FIG 4.2).

Having shown that Spa was absent or substantially reduced in the *tagO* mutant it was important to confirm this, using functional tests with intact cells. The level of IgG-binding protein expressed on the surface of Newman and LAC cells was measured

by flow cytometry. Cells grown to exponential phase were incubated with FITC-conjugated IgG and the fluorescence measured. Sbi does not contribute significantly to the fluorescence because it is much less expressed than Spa and Sbi only possesses two IgG binding sites whereas Spa has five (O'Halloran *et al.*, 2015).

For the Newman *tagO* mutant no IgG binding was detected whereas in LAC it was greatly reduced (by approximately 95%; FIG 4.3). A *spa* null mutant was included in both cases and no Spa was detected in this mutant. Complementation of the *tagO* mutants restored the level of Spa expression (FIG 4.3).

Because Spa was undetectable in the solubilized cell wall fraction or on the surface of Newman *tagO* it remained a possibility that the protein was mislocalized in the mutant. Therefore it was important to perform subcellular fractionation to detect IgG-reactive material by Western immunoblotting.

The reduced levels of Spa in the *tagO* mutant were likely due to the reduced level of *spa* expression (FIG 3.23). Additionally, it was possible that Spa was mislocalized during secretion/sorting due to the lack of WTA. To study this Newman, Newman *spa* and Newman *tagO* were grown to exponential phase and fractionation experiments were performed. Sedimentation of cells yielded the culture supernatants which were tested unconcentrated or in the case of the wild type diluted 1 in 10 to account for the fact that *spa* expression was reduced 100-fold in the *tagO* mutant (Chapter 3; Table 3.2). The strongly reactive band in each of the samples that migrated slightly farther than Spa (detectable in wild type sample) is Sbi (FIG 4.4 B and D). Sbi is released into the supernatant fraction and is non-covalently associated with LTA in the membrane (Smith *et al.*, 2012). No Spa protein was detected in the culture supernatant of the *tagO* mutant.

No reactive protein of the size of Spa was detected in the cell wall, membrane or cytoplasm fractions of the *tagO* mutant. In contrast, Spa was detected in the cell wall, membrane and cytoplasm of Newman wild type.

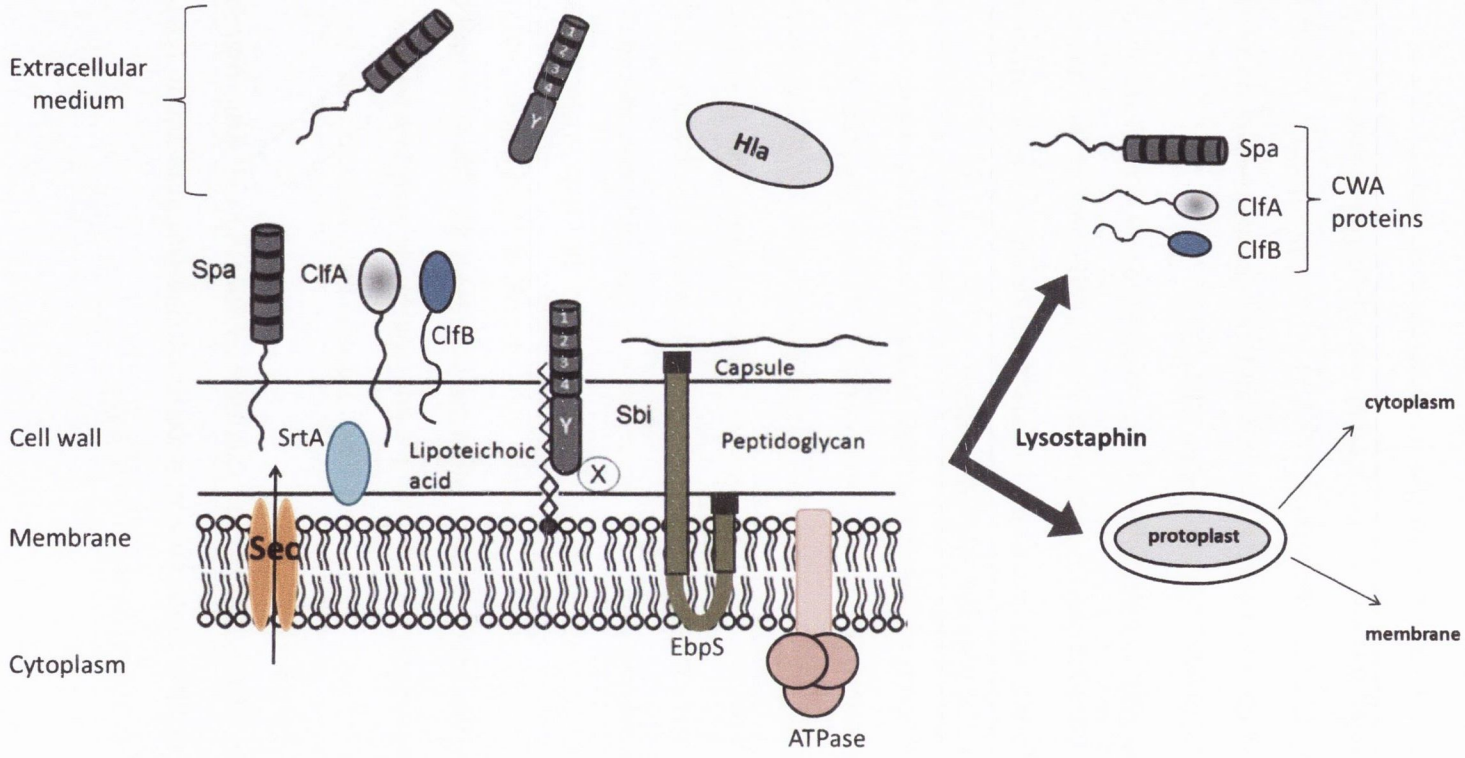



**FIG 4.1**. Schematic representation of the proteins studied in this chapter present in the extracellular medium (culture supernatant), cell wall, membrane and cytoplasm. The cell wall anchored proteins were solubilized by treating the cells with lysostaphin which is an enzyme that cleaves the cross linking of the peptidoglycan pentaglycine bridges liberating the cell wall proteins and creating stable protoplasts. The protoplasts were lysed to
create a membrane and a cytoplasmic fraction.

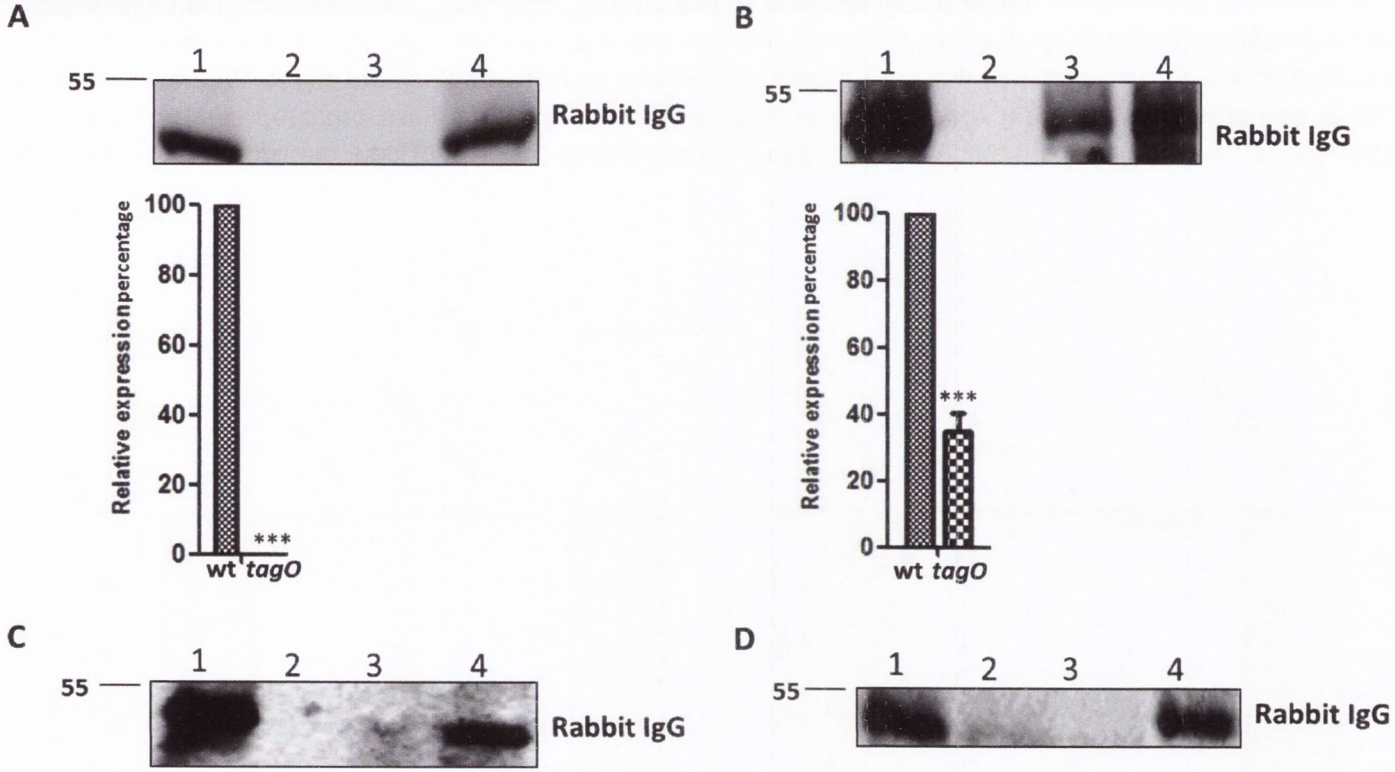



**FIG. 4.2** Western immunoblots to detect protein A in A) Newman, B) LAC, C) SH1000 and D) BH1CC strains. Cell wall anchored proteins were solubilized by treating cells grown to exponential phase with lysostaphin. The cell wall extracts were subjected to SDS-PAGE and Western immunoblotting using HRP-labelled IgG to detect Spa. 1- wild type; 2- *spa* mutant; 3-*tagO* mutant; 4-complemented *tagO* mutant. Molecular weight is indicated. Densitometry of Newman and LAC Western immunoblots was also performed to compare the wild type and the *tagO* mutant. The intensity of the bands of three independent Western immunoblotting was measured by densitometry and statistical analysis was performed using an unpaired t-test. Statistically significant result is ***P<0.001.

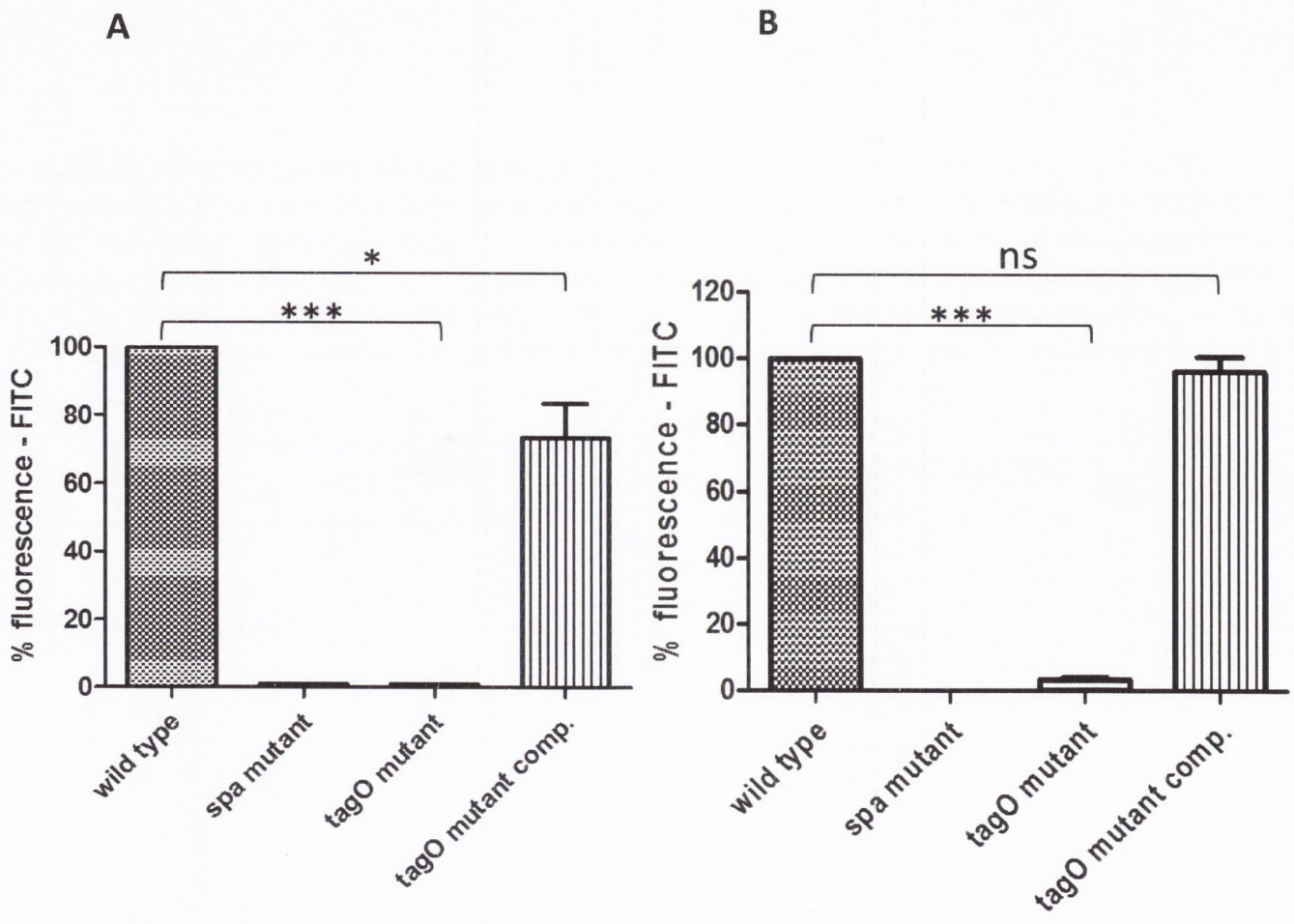



**FIG. 4.3** Flow cytometry to detect protein A on the surface of A) Newman and B) LAC, including the *tagO* complemented mutant (*tagO* mutant comp.). Surface located SpA was detected using FITC-labelled rabbit IgG and fluorescence intensity was measured using flow cytometry. Values are plotted as a percentage of the median fluorescence intensity measured for the wild-type strain. Error bars represent the standard error of three independent experiments. The *spa* mutant strain was included as a negative control. Statistical analysis was performed using an unpaired t-test. Statistically significant result is ***P<0.001, *P<0.05 and ns =not significant >0.05.

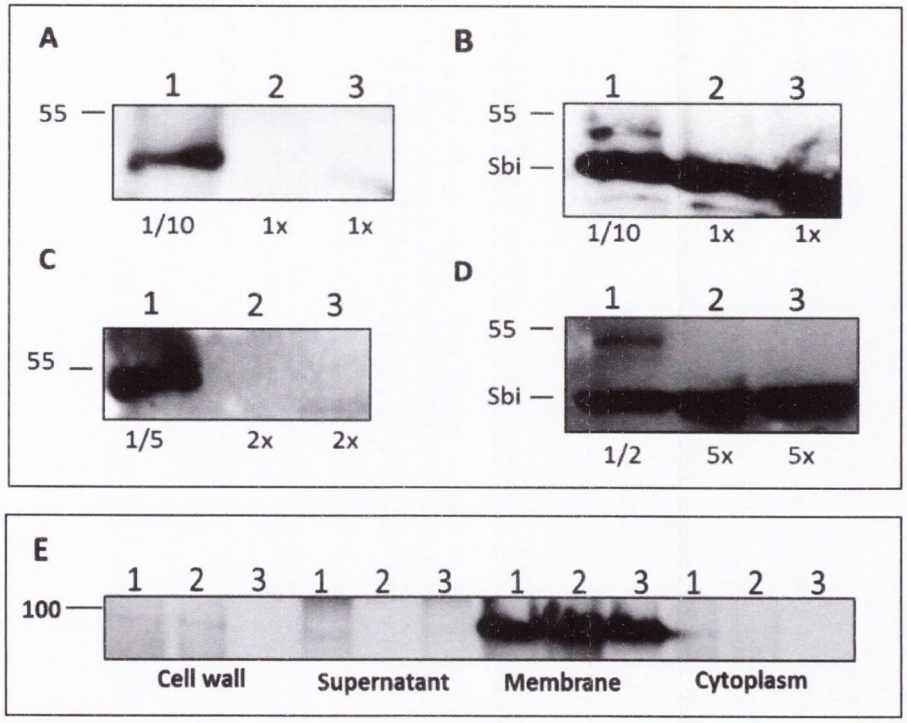



**FIG. 4.4** Subcellular fractionation and Western immunoblotting for SpA detection in Newman. A) Cell Wall, B) Supernatant, C) Cytoplasm and D) Membrane E) detection of EbpS; 1- wild type, 2- *spa* mutant, 3- *tagO* mutant. A and B wild type samples are diluted 1/10; C wild type sample is diluted 1/5 in comparison with samples 2 and 3; D wild type is diluted 1/2 in comparison with samples 2 and 3. Protein A was detected using rabbit IgG-HRP. A *spa* mutant was used as a negative control. The integrity of the fractionation process was tested using anti-EbpS IgG followed by HRP-conjugated protein A. Blots are representative of three biological replicas

The wild type, membrane and cytoplasmic fractions have some detectable Spa (FIG 4.4 B and C), possibly due to Spa being transiently associated with the membrane during secretion/sorting and being present in the cytoplasm following translation and prior to secretion. It is interesting to note that Sbi was not detected in the cytoplasm of Newman (FIG 4.4 C). It is present in the membrane fraction of the wild type, the *tagO* mutant and the *spa* mutant due to its association with LTA. Also, the level of Sbi was not reduced in the *tagO* mutant culture supernatant or in the membrane fractions in agreement with the RNA-seq results showing that the levels of Sbi transcript was not altered in the *tagO* mutant.

Probing for an integral membrane protein EbpS with a specific rabbit antibody served as a control for the integrity of the fractionation process and the purity of the fractions (FIG 4.4 E). EbpS was only detected in the membrane fraction and not in the cytoplasm, culture supernatant or cell wall fractions.

### 4.2.2. Elaboration of ClfB on the surface of the *tagO* mutants

Clumping factor B (ClfB) is a CWA protein that is covalently attached to the peptidoglycan and extends into the extracellular environment (FIG 4.1). ClfB transcript levels in the *tagO* mutant were found to be reduced (see section 3.2.2.1.4). In order to investigate if the reduction in mRNA resulted in reduced levels of the protein, cells from the exponential phase of growth were harvested and treated with lysostaphin to solubilize cell wall proteins. The solubilized CWA proteins were subjected to SDS-PAGE and the released ClfB detected with anti-ClfB IgG followed by HRP-conjugated protein A.

The wild type was compared to the *clfB* mutant, the *tagO* mutant and the complemented *tagO* mutant. Densitometric analysis of the blots was performed on three independent samples of Newman, LAC wild type and their corresponding *tagO* mutants.

In Newman, LAC and SH1000 a reduced level of ClfB protein was detected in the cell wall fraction of the *tagO* mutant (FIG 4.5). In the case of Newman the *tagO* band intensity was reduced to approximately 30% (+/- 17.3%) of wild type levels and in the case of LAC *tagO* was approximately. 50% (+/- 14.7%) reduced (FIG 4.5 A and B).

No ClfB was detected in the BH1CC *tagO* mutant (FIG 4.5 D) while in the SH1000 *tagO* mutant the expression was reduced (FIG 4.5 C). For both Newman and LAC *tagO* mutants the ClfB protein was restored by introducing the wild type *tagO* gene on a complementing plasmid. Having shown that ClfB was substantially reduced in the *tagO* mutant it was important to confirm this by functional tests with intact cells.

In order to correlate the decreased expression of ClfB with the capacity of the mutant to adhere to its ligands, assays to measure adhesion to loricrin were performed. Surface protein expression varies during the growth cycle. ClfB is predominantly expressed in the exponential phase and it is known to be the only factor responsible for adhesion to loricrin. Cells from the exponential phase of growth from Newman and LAC strains were harvested and incubated in a 96-well plate coated with decreasing concentrations of loricrin. Bound cells were fixed and stained with crystal violet. Cells were resuspended and the $OD_{570nm}$ was recorded.

In each the wild type was compared to a mutant deficient in ClfB, the *tagO* mutant and the complemented *tagO* mutant. The level of binding to loricrin was greatly reduced in the *tagO* mutant and was restored to wild type levels by introducing the wild type *tagO* gene on a complementing plasmid (FIG 4.6). ClfB attachment to loricrin was dose-dependent in the wild type strains and in the complemented Newman and LAC *tagO* mutants (FIG 4.6 A and B). Both *tagO* mutants had an impaired binding capacity which is similar to the *clfB* mutant strains that were employed as negative controls (FIG 4.6 A and B).

Thus despite some ClfB being detectable in the cell wall fractions by densitometry of Western blots this must be insufficient to promote bacterial adhesion to loricrin.

### 4.2.3 Elaboration of ClfA on the surface of the *tagO* mutants

The CWA protein clumping factor A (ClfA) is covalently attached to the peptidoglycan and is elaborated on the cell surface (FIG 4.1). ClfA transcripts in the *tagO* mutant were found to be at the same levels as the wild type strain in the RNA-seq results, using cells from the exponential phase of growth. However, ClfA is mostly expressed in the stationary phase of growth. The RT-PCR data (with cells in late stationary phase; FIG 3.30) did not show a significant difference

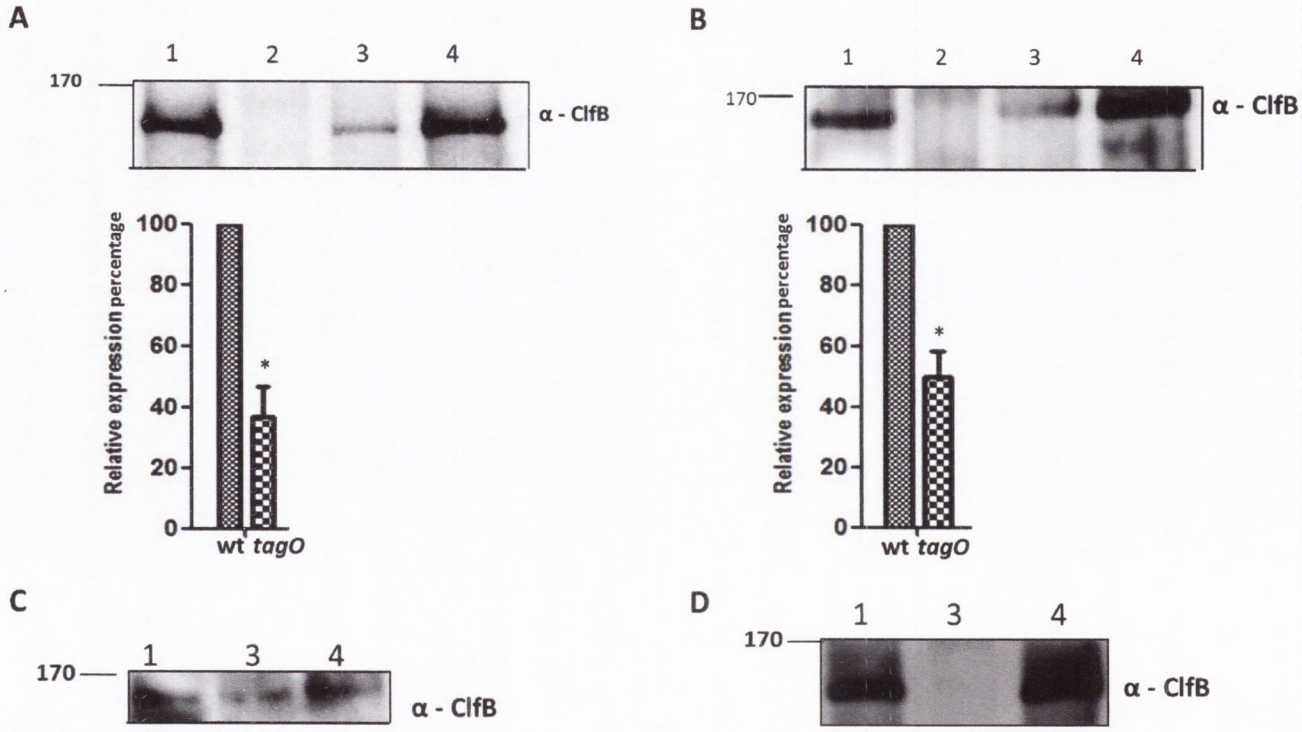



**FIG. 4.5** Western immunoblots to detect ClfB in A) Newman, B) LAC, C) SH1000 and D) BH1CC strains. Cell wall anchored proteins were solubilized by treating cells grown to exponential phase with lysostaphin. The cell wall extracts were subjected to SDS-PAGE and Western immunoblotting using anti-ClfB IgG followed by HRP-labelled protein A to detect ClfB. 1- wild type; 2- *clfB* mutant strain; 3-*tagO* mutant; 4-complemented *tagO* mutant. Molecular weight is indicated. Densitometry of Newman and LAC Western immunoblots was also performed. The intensity of the bands of three independent Western immunoblots of the respective protein was measured by densitometry and statistical analysis was performed using an unpaired t-test. Statistically significant result is *P<0.05.

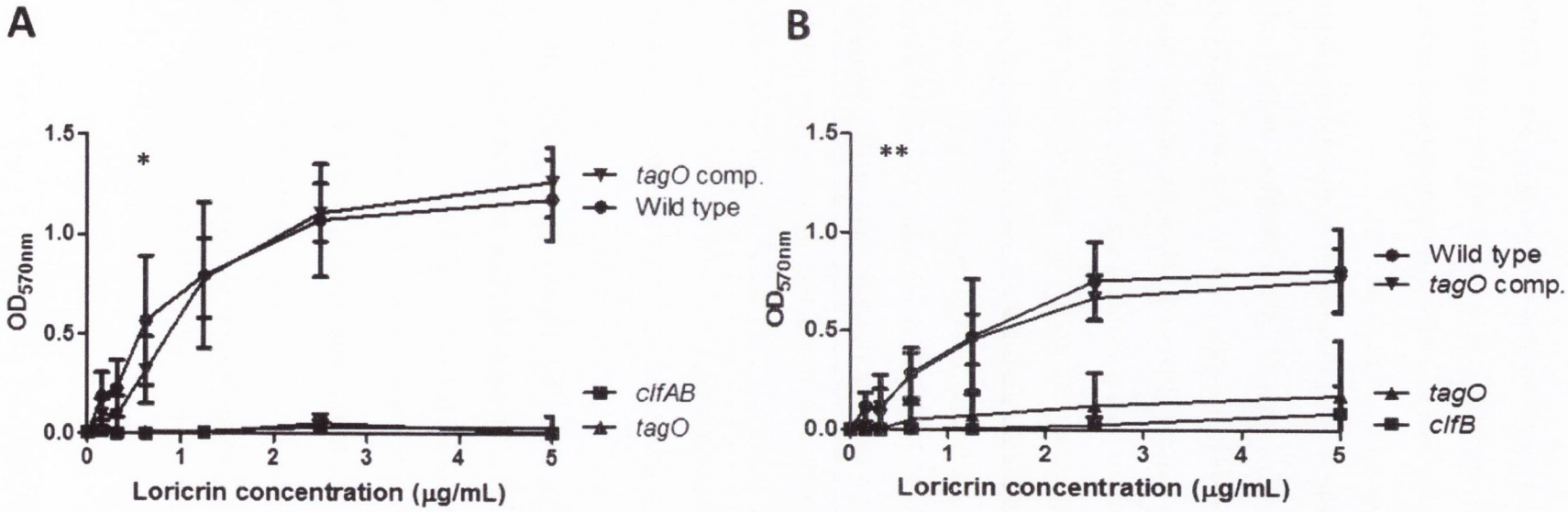



**FIG 4.6** Dose-dependent adhesion assays to detect ClfB activity in A) Newman and B) LAC strains. Cells from the exponential phase of growth were harvested and incubated in wells pre-coated with doubling dilutions of loricrin (starting at 5 µg/ml). Attached cells were fixed and stained with crystal violet. The *clfB* mutants were present as negative control. The $OD_{570nm}$ of three independent assays was recorded and statistical analysis was performed on a wild type and *tagO* mutant single point from the linear range of the graph was using an unpaired t-test. Statistically significant result is *P<0.05 and **P<0.01.

in *clfA* mRNA between the Newman *tagO* mutant and wild type.

Cell wall extracts of Newman and LAC strains were prepared by treating stationary phase cells with lysostaphin and subjecting the proteins to SDS-PAGE and Western immunoblotting. The released ClfA was detected with anti-ClfA IgG followed by a HRP-conjugated protein A (chapter2, Table 2.3).

In each strain the wild type was compared to the *clfA* mutant, the *tagO* mutant and the complemented *tagO* mutant. Densitometric analysis of the blots was performed on three independent samples of wild type and *tagO* mutant of Newman and LAC. In Newman *tagO* mutant 50% (+/-3.5%) of the protein was detected (FIG 4.7 A) and in the LAC *tagO* mutant 50% (+/-20.5%) was detected (FIG 4.7 B). In each case the level of ClfA expression was restored to wild type levels by introducing the wild type *tagO* gene on a complementing plasmid.

Having shown that ClfA was substantially reduced in the *tagO* mutant it was important to confirm this by functional tests with intact cells. Assays to measure adhesion to fibrinogen were performed. Cells from the stationary phase of growth of Newman and LAC strains were harvested and incubated in wells pre-coated with different concentrations of fibrinogen. Bound cells were stained with crystal violet and the $OD_{570nm}$ measurements were recorded.

For each strain the wild type was compared to the *clfA* mutant, the *tagO* mutant and the complemented *tagO* mutant. In both mutants the level of adherence was restored to wild type levels by introducing the wild type *tagO* gene on a complementing plasmid (FIG 4.8). Some binding was still found in the Newman and LAC *tagO* mutants which correlates with the Western immunoblots, however the differences were not statistically significant (FIG 4.8 A and B).

### 4.2.4 Detection of membrane proteins in the *tagO* mutants

The integral membrane proteins EbpS, SrtA and AtpA (FIG 4.9) were detected by SDS-PAGE and Western immunoblotting of membrane fractions. The mRNA levels of these proteins were found not to be altered in the *tagO* mutant.

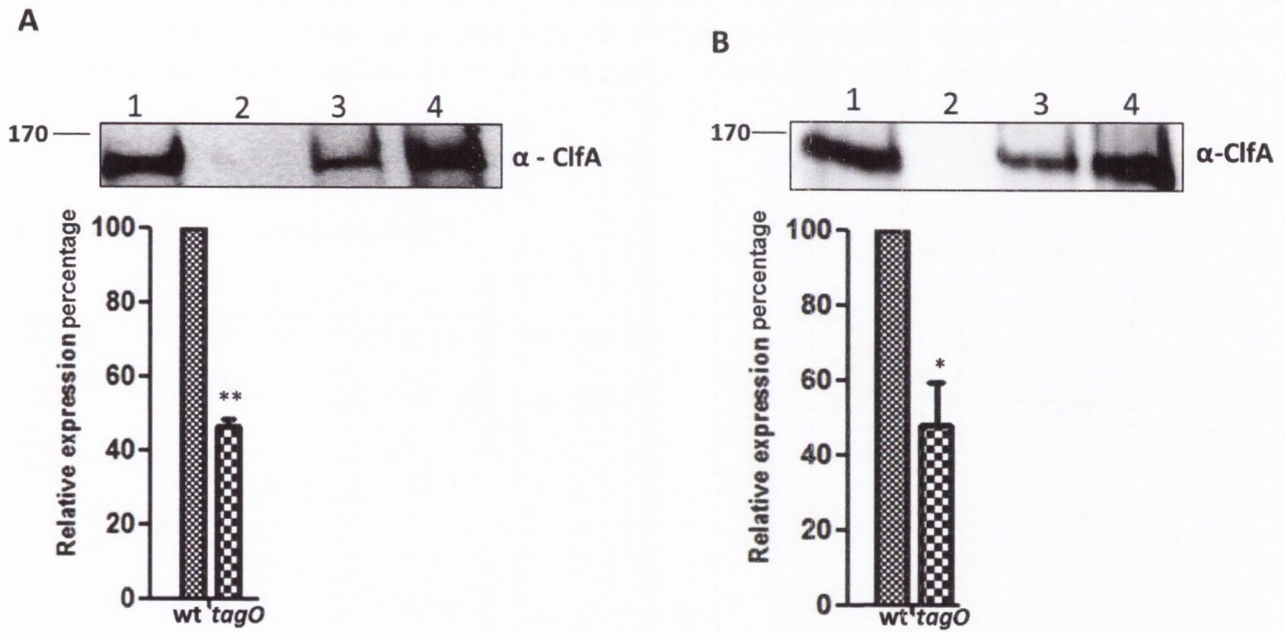



**FIG. 4.7** Western immunoblots to detect ClfA in A) Newman and B) LAC strains. Cell wall anchored proteins were solubilized by treating cells grown to stationary phase with lysostaphin. The cell wall extracts were subjected to SDS-PAGE and Western immunoblotting using anti-ClfA IgG followed by HRP-conjugated protein A to detect ClfA. 1- wild type; 2- *clfA* mutant; 3-*tagO* mutant; 4-complemented *tagO* mutant. Molecular weight is indicated. Densitometry of Newman and LAC Western immunoblots of wild type and *tagO* mutant strains was also performed. The intensity of the bands of three independent Western immunoblots was measured by densitometry and statistical analysis was performed using an unpaired t-test. Statistically significant result is *P<0.05 and **P<0.01.

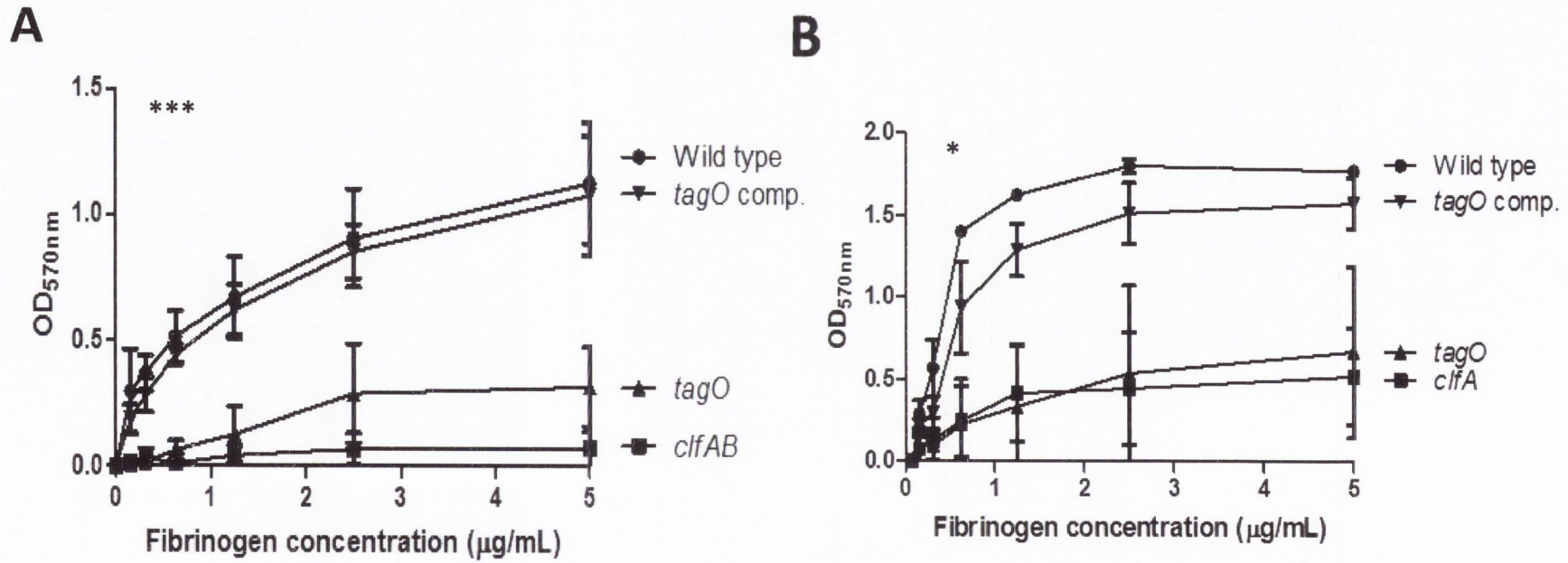



**FIG 4.8** Dose-dependent adhesion assays to detect the activity of ClfA in A) Newman and B) LAC strains. Cells from the exponential phase of growth were harvested and incubated in wells pre-coated with decreasing concentrations of fibrinogen (starting at 5μg/ml). Attached cells were fixed and stained with crystal violet. The *clfA* mutants were present as negative control. The $OD_{570nm}$ of three independent assays was recorded and statistical analysis was performed on a wild type and *tagO* mutant single point from the linear range of the graph was using an unpaired t-test. Statistically significant result is *P<0.05 and ***P<0.001.

The membrane protein SrtA is the protein responsible for the anchoring of CWA proteins to the cell wall of *S. aureus.* A reduction in expression of this protein could lead to reduced levels of sortase anchored proteins in the cell wall (such as Spa, ClfA and ClfB). It was important to investigate the expression of this protein in the Newman *tagO* mutant (FIG 4.9 A).

In order to measure the level of each protein, membrane extracts were prepared by lysing the stable protoplasts after treatment with lysostaphin. The lysate was subjected to centrifugation and the membrane and cytoplasm fractions were separated (FIG 4.1). The membrane proteins were separated by SDS-PAGE and detected with specific antibodies followed by HRP-conjugated protein A. The Newman wild type was compared to the *tagO* mutant and the complemented *tagO* mutant. Densitometric analysis of the blots was performed on three independent samples. The apparent small difference in expression of these proteins between Newman and LAC wild type and *tagO* mutant were not found to be statistically significant (FIG 4.9).

The level of protein expression was similar for the wild type and the *tagO* mutant where the wild type *tagO* gene was present on a complementing plasmid (FIG 4.9).

### 4.2.5. Detection of extracellular proteins in the *tagO* mutants

Alpha-toxin (Hla) and the V8 protease are secreted proteins that are present in the supernatant of *S. aureus* strains. Both Hla and V8 mRNA levels were not altered in the RNA-seq experiments.

In order to investigate if mRNA levels correlate with the expression of the protein, cells from the exponential phase of growth of strains Newman and LAC were spun down and the supernatants were precipitated with TCA. The precipitated proteins were resuspended in a smaller volume and subjected to SDS-PAGE. Secreted Hla was detected using an anti-Hla IgG followed by HRP-conjugated protein A and V8 protein detected with an anti-V8 IgG followed by HRP-conjugated goat anti-rabbit IgG. In the case of Hla, Newman and LAC wild type strains were compared to an *agr* mutant (lacks Hla production), the *tagO* mutant and the complemented *tagO* mutant. In the case of V8, Newman and LAC wild type were compared to the *tagO* mutant and the

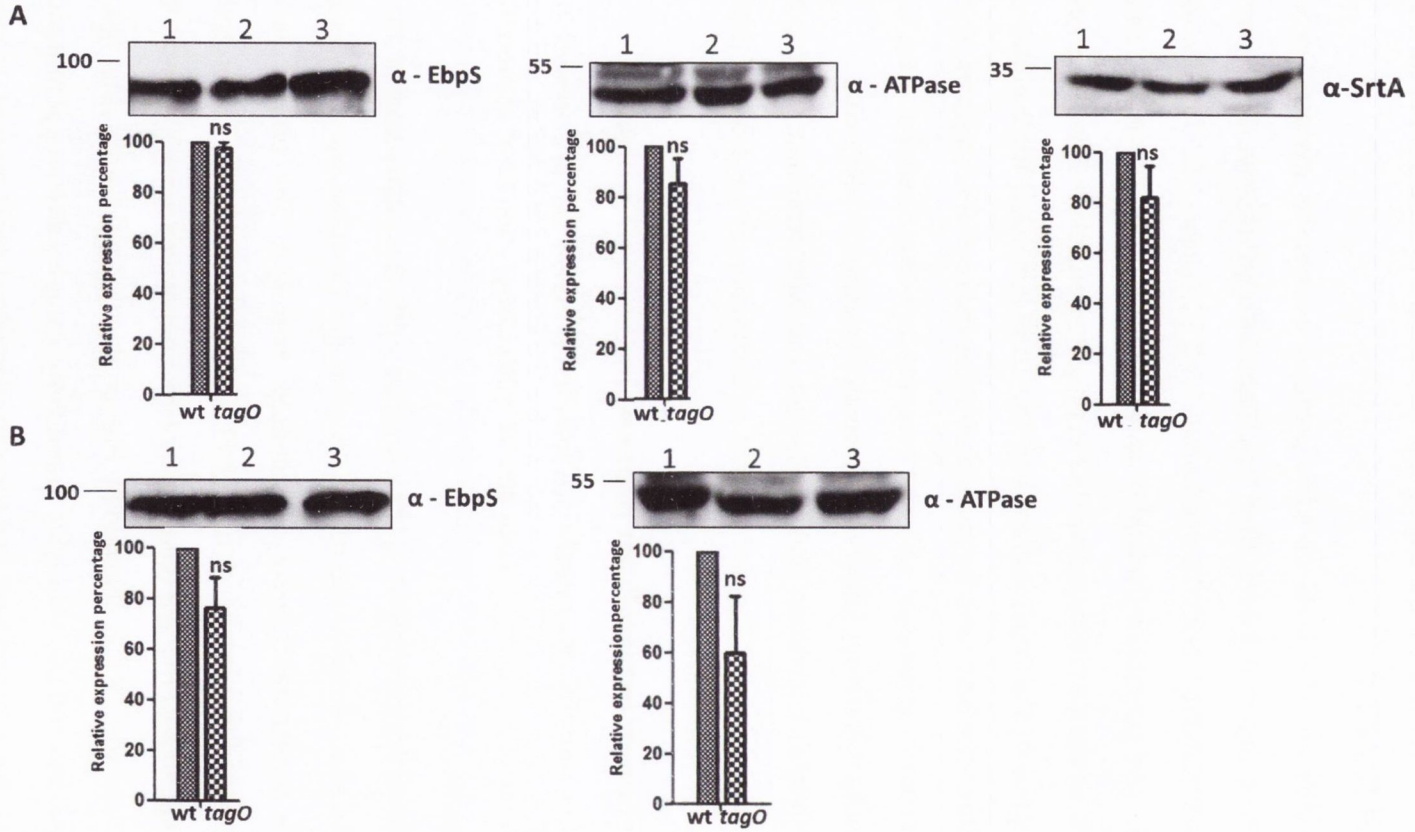



**FIG. 4.9** Western immunoblots to detect membrane proteins in A) Newman and B) LAC strains. Cells from exponential phase cultures were harvested and treated with lysostaphin to create stable protoplasts. Protoplasts were lysed and the cell content separated by centrifugation. Membrane fractions were solubilized and subjected to SDS-PAGE and Western immunoblotting using anti-EbpS, anti-ATPase or anti-SrtA antibody, followed by HRP-labelled protein A. 1- wild type; 2-*tagO* mutant; 3-complemented *tagO* mutant. Molecular weight is indicated. Densitometry of Newman and LAC wild type and *tagO* mutant strains Western immunoblots was also performed. The intensity of the bands of three independent Western immunoblots was measured by densitometry and statistical analysis was performed using an unpaired t-test. ns =not significant >0.05.

complemented *tagO* mutant. No reactive Hla was found in the Newman *tagO* strain while a reduced level of V8 protein was found in supernatant (FIG 4.10 A), whereas in LAC some Hla and V8 reactive proteins were found in the *tagO* mutant supernatant (FIG 4.10 B). This was not measured quantitatively. In both cases the level of Hla and V8 expression was restored to wild type levels by introducing the wild type *tagO* gene on a complementing plasmid (FIG 4.10). In order to determine if reduced expression levels of Hla in culture supernatants of the *tagO* mutant correlated with reduced protein activity haemolytic assays were performed. Hla is an extracellular cytolytic pore-forming toxin that disrupts the integrity of the cytoplasmic membrane causing cell lysis. This protein is expressed in Newman and LAC at levels below the limits of detection during the exponential phase of growth, therefore cells from the stationary phase of growth were used to perform this test. The lysis of red blood cells was tested by pipetting culture supernatants into wells in sheep blood agar. Haemolysis was not detected in the wells corresponding to the Newman *tagO* mutant (FIG 4.11 A.) or the LAC *tagO* mutant (FIG. 4.11 B), showing that there is no detectable α-toxin being produced by the mutant strains. In both cases the level of Hla activity was restored to wild type levels by introducing the wild type *tagO* gene on a complementary plasmid

### 4.2.6 The effect of tunicamycin on WTA synthesis and protein A expression

To confirm that the massive reduction of Spa in the cell wall of the Newman *tagO* mutant was due to loss of enzymatic activity of TagO, tunicamycin was used to inhibit the TagO enzyme and the levels of surface exposed Spa were measured by flow cytometry. Tunicamycin is a natural product inhibitor of a family of enzymes that transfer hexose-1-phosphates from nucleotide – sugar donors to membrane embedded lipid phosphates to form lipid-pp-hexose products In *S. aureus*, TagO and MraY (essential for peptidoglycan biosynthesis) have this enzymatic activity. It has been reported that TagO activity is more susceptible to tunicamycin inhibition than MraY. Thus it is possible to specifically inhibit TagO and not MraY with tunicamycin

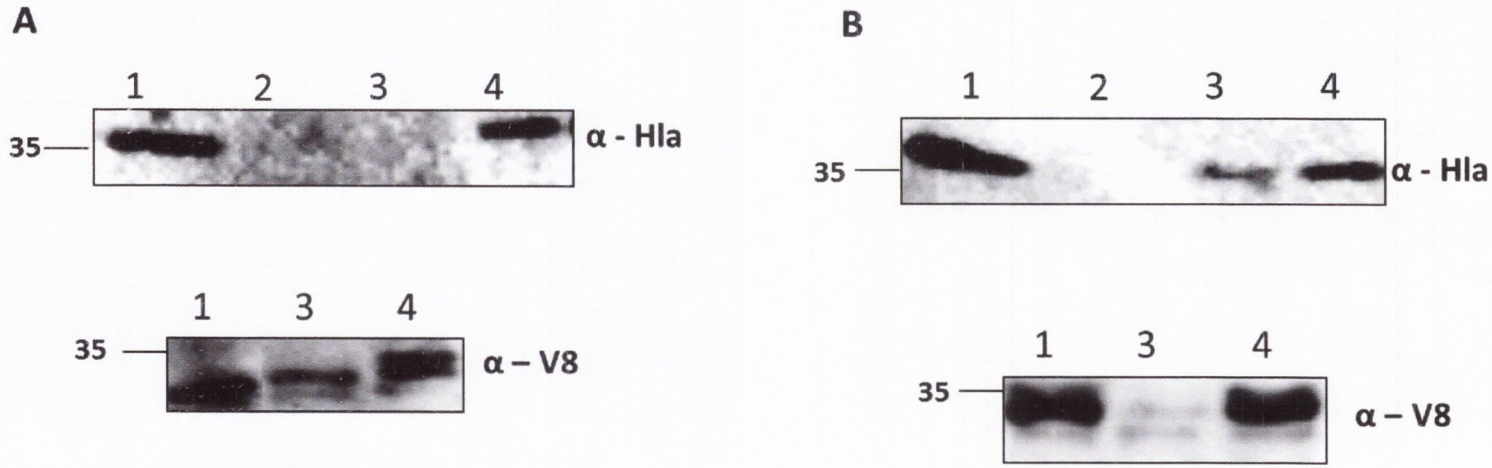



**FIG. 4.10** Western Immunoblot to detect extracellular proteins Hla and V8 protease tested in A) Newman and B) LAC strains by Western immunoblot. The culture supernatant of cells grown to exponential phase was separated and the proteins were precipitated with TCA. Supernatant proteins were solubilized and subjected to SDS-PAGE and Western immunoblotting using anti-Hla and anti-V8 serum antibodies, respectively, followed by HRP-conjugated protein A and HRP-conjugated goat anti-rabbit IgG, respectively. 1- wild type; 2- *agr*-mutant; 3-*tagO* mutant; 4-complemented *tagO* mutant. Molecular weight is indicated. Blots are representative of three biological replicates.

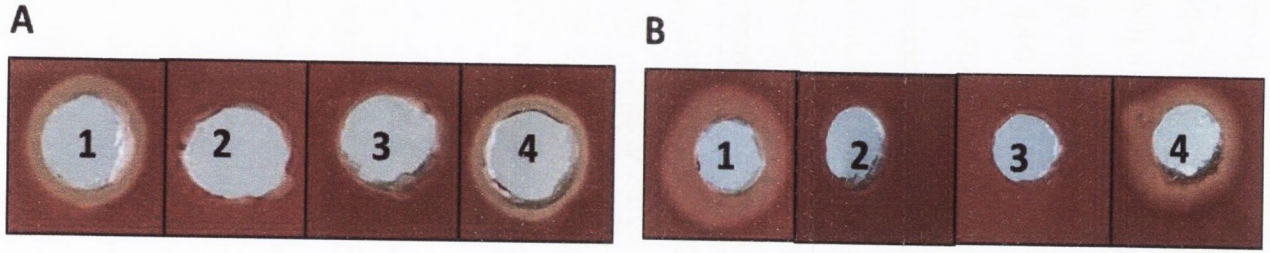



**FIG. 4.11** Haemolysis test to detect the activity of α-toxin in A) Newman and B) LAC. Supernatant of cells grown to stationary phase were collected and extracellular proteins were resuspended in a smaller volume. 100µL was added to wells made in the agar. 1- wild type, 2- *agr*- mutant, 3- *tagO* mutant and 4- *tagO* mutant complemented. Representative of three biological replicas.

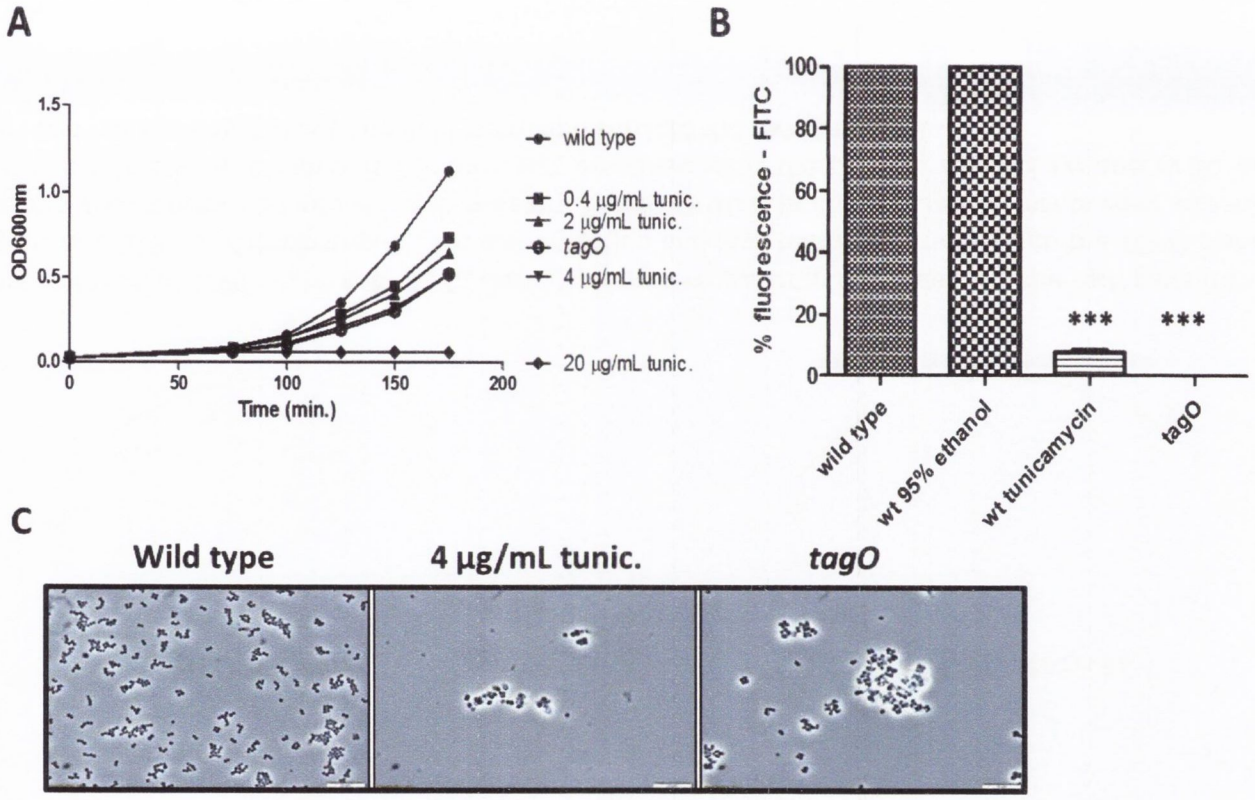
concentrations of between 2-10 μg/mL (Campbell *et al.*, 2011). Firstly, it was important to determine the correct amount of tunicamycin to add to the cultures. Growth curves of Newman wild type in the presence of different concentrations of tunicamycin were performed (FIG. 4.12 A). Newman wild type grown with 20 μg/mL of tunicamycin was employed as a negative control, since this is the minimum concentration of tunicamycin that inhibits MraY. At this concentration no growth occurred (FIG 4.12 A). A control with only the tunicamycin vehicle solution (95% ethanol) was used to show that this does not affect growth (FIG 4.12 A).

The concentration of tunicamycin that reduced the growth of Newman wild type to that of the *tagO* mutant was found to be 4 μg/mL (FIG. 4.12 A).

Phase contrast microscopy was employed to study Newman wild type cells grown with and without tunicamycin (4 μg/mL) and the *tagO* mutant cells (FIG 4.12 C). Newman wild type cells grown with 4 μg/mL of tunicamycin not only have a defect in growth to the same extent as the *tagO* mutant but also have a very similar clumping phenotype (FIG 4.12 C). Newman cells grown to the exponential phase in the presence of tunicamycin were harvested and labelled with FITC-conjugated IgG and the fluorescence measured by flow cytometry. In Newman grown in tunicamycin Spa expression was reduced by approximately 90% (FIG 4.12 B) when compared to the Newman wild type strain. This shows that the inhibition of TagO enzymatic activity is sufficient to reduce Spa expression in *S. aureus.*

### 4.2.7 Isolation of the double mutant Newman *tagO tarH*

Chapters 3 and 4 showed that a *tagO* deletion causes major changes in growth, morphology, gene transcription and protein expression. However it is not known if these changes are caused by the lack of WTA on the cell surface or due to the lack of intracellular WTA intermediates. The publication of Botella *et al* (2014) showed that PhoPR two component system monitors WTA metabolism in *Bacillus subtiliis* by sensing the level of a WTA intermediate. PhoR activity is inhibited by this intermediate when WTA is high in cells




**4.12** WTA synthesis inhibition using tunicamycin. **A)** Growth curve of Newman wild type. Starter cultures grown in antibiotic-free broth were diluted into fresh broth containing tunicamycin (0. 4, 2, 4 and 20 µg/mL). Tunicamycin at 20 µg/mL was used as a negative control. **B)** Surface located Spa was detected using FITC-labelled rabbit IgG and fluorescence intensity was measured using flow cytometry. Values are plotted as a percentage of the median fluorescence intensity measured for the wild-type strain. Error bars represent the standard error of three independent experiments. The wild type grown in the medium supplemented with just 95% ethanol (tunicamycin vehicle) was used as a control. Statistical analysis was performed using an unpaired t-test. Statistically significant result is ***P<0.001**. C)** Phase contrast microscopy using ethanol-fixed cells from stationary phase cultures of Newman wild type, *tagO* mutant and wild type grown in the presence of 4 µg/mL and *tagO* mutant.

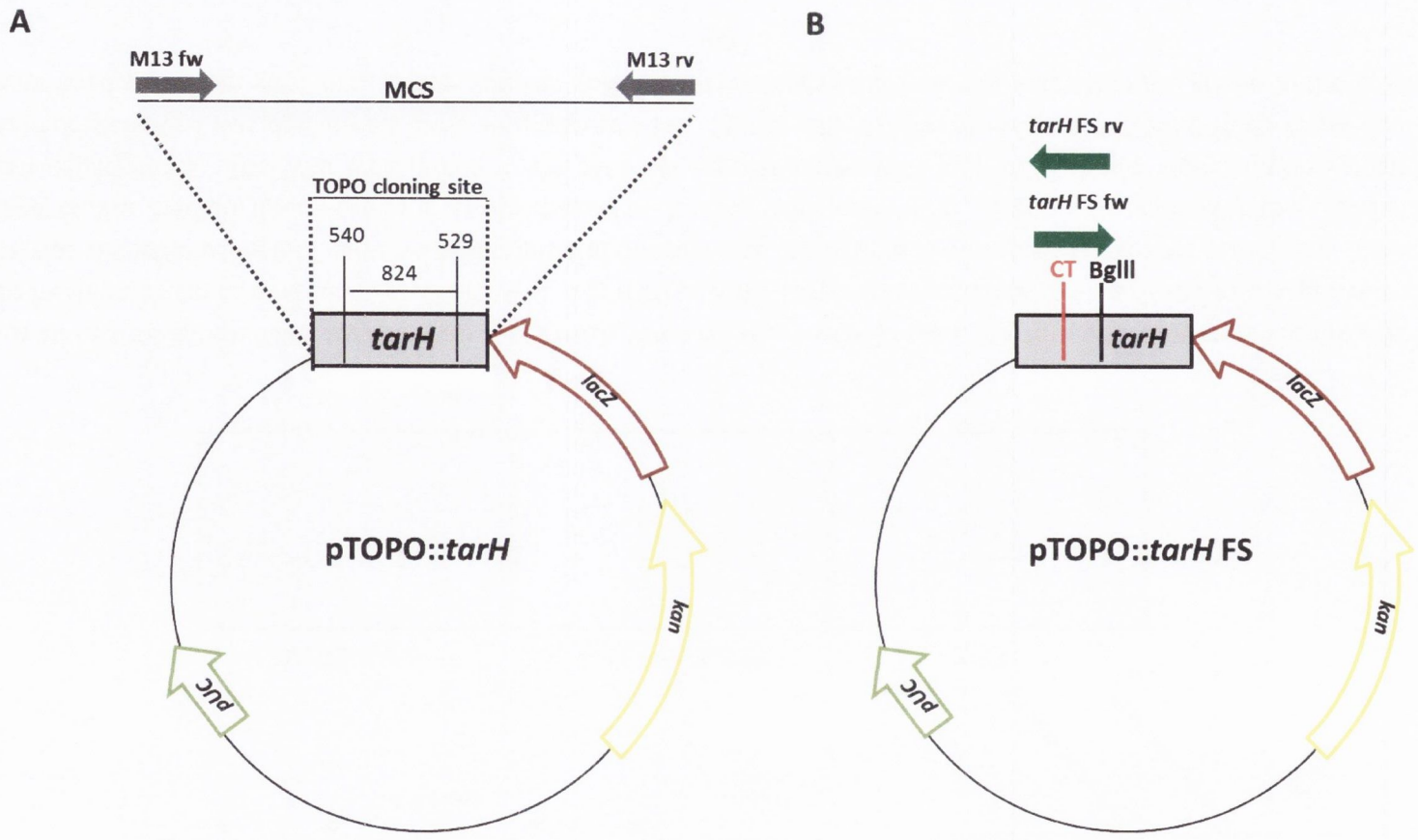



**FIG 4.13** Schematic representation of TOPO::*tarH* and TOPO::*tarH* FS plasmid vector from Invitrogen**. A)** the *tarH* gene (824 bp), 540 bp of DNA upstream of the tarH gene and 529 bp of downstream DNA was amplified and inserted by blunt-ends into the TOPO cloning site of the TOPO plasmid vector. This plasmid was transformed into electrocompetent *E. coli* TOP10 cells and the transformants were screened with M13 fw and rv primers **B)** after being extracted the plasmid TOPO::*tarH* was amplified with the primers *tarH* FS fw and rv to produce the plasmid TOPO::*tarH* FS and insert two nucleotides (CT) and the BglII restriction site into the *tarH* sequence.

growing under phosphate-replete conditions. It is possible that *S. aureus* WTA intermediates also activates or represses sensing of the transcription of certain genes.

Therefore, in this study, genetic manipulation was employed to isolate a *tarH* mutant. TarH encodes the ABC transporter that translocates the final WTA polymer to the cell surface, where it becomes attached to peptidoglycan (FIG 1.3). The mutation was isolated in a Newman *tagO* background because disrupting *tarH* is lethal in a wild-type background (see Chapter 1 section 1.2.2.5). However, the *tarH* mutation is tolerable in a Δ*tagO* background due to the absence of these intermediates.

The wild type *tagO* gene was cloned into the inducible expression vector pRMC2. This plasmid was then introduced into the Newman *tarH* mutant and the effects of turning on *tagO* gene expression monitored. In order to study the possible effects of the presence of WTA intermediates, surface-exposed protein A was detected by flow cytometry in the Newman *tarH* mutants as a measure of *spa* gene expression.

In order to construct a *tarH* mutant in a Newman *tagO* background, site-directed mutagenesis was performed using the plasmid PCR-Blunt II-TOPO (pTOPO; FIG 4.13). The pTOPO plasmid was used for the ligase-independent direct insertion of a blunt-end PCR product into a plasmid vector. This plasmid contains a multiple cloning site (MCS) for the cloning of blunt-end PCR products, a kanamycin resistance cassette for selection in *E. coli*, and the pUC origin of replication that allows the plasmid to replicate autonomously.

The plasmid vector pTOPO is supplied linearized with *Vaccinia* virus DNA topoisomerase I covalently bound to the 3′end of each DNA strand. The *tarH* gene (824 bp) plus a fragment of the upstream sequence (540 bp) and a fragment of the downstream sequence (529 bp) was amplified from Newman genome using *tarH* OUT fw (annealing 540 bp upstream of the tarH gene) and rv (annealing 529 bp downstream of the *tarH* gene) primers. The linearized plasmid was incubated with the blunt-ended *tarH* PCR product and the DNA was transformed into electrocompetent *E. coli* TOP10 cells. Transformants were selected in medium with kanamycin, screened by a colony PCR using primers M13 fw and rv (primers that anneal

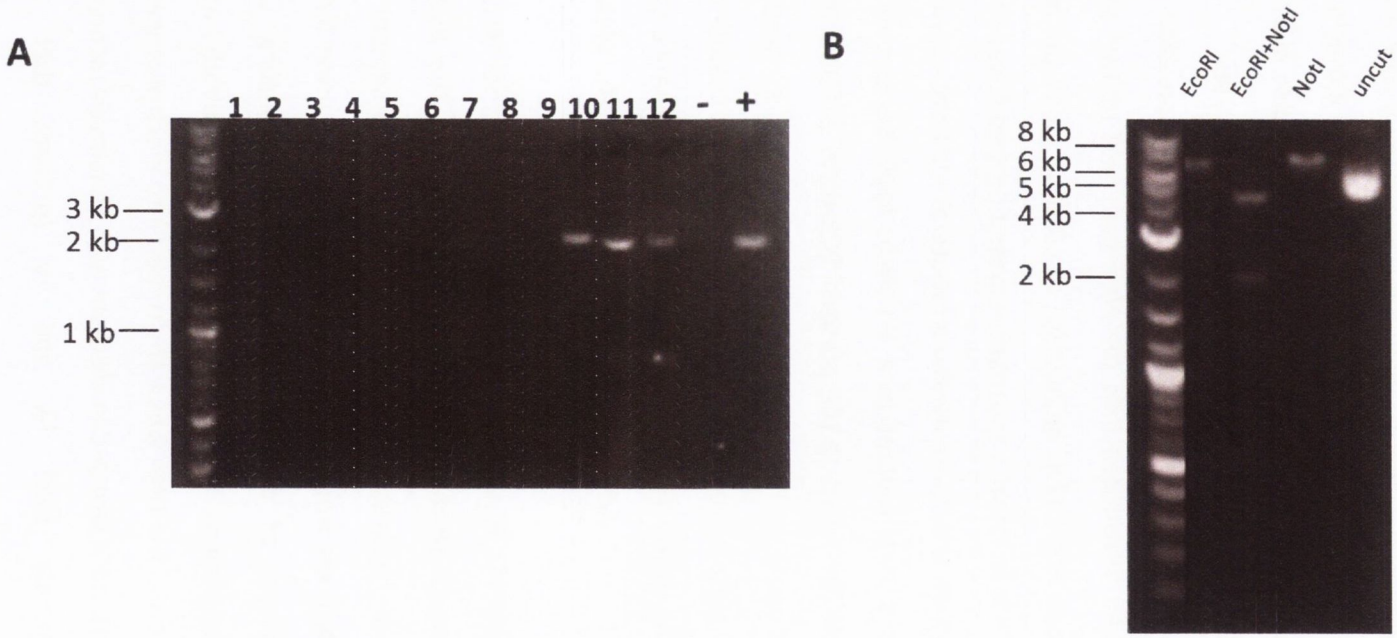



**FIG. 4.14** Manipulation of *tarH* FS **A)** Screening of *E. coli* XL1 Blue transformants with pTOPO::*tarH* FS by colony PCR. A positive colony (containing TOPO with insert) yielded a band of approx. 2 kb (e.g. colony 12). A positive control (+) of *E. coli* containing TOPO::*tarH*, and a negative control (-) of *E.coli* without plasmid, were included. Size markers indicated (kb). **B)** Cleavage of pTOPO::tarH FS with the restriction enzymes EcoRI and NotI. The single cut and uncut plasmid were included as controls. Size markers are indicated (kb)

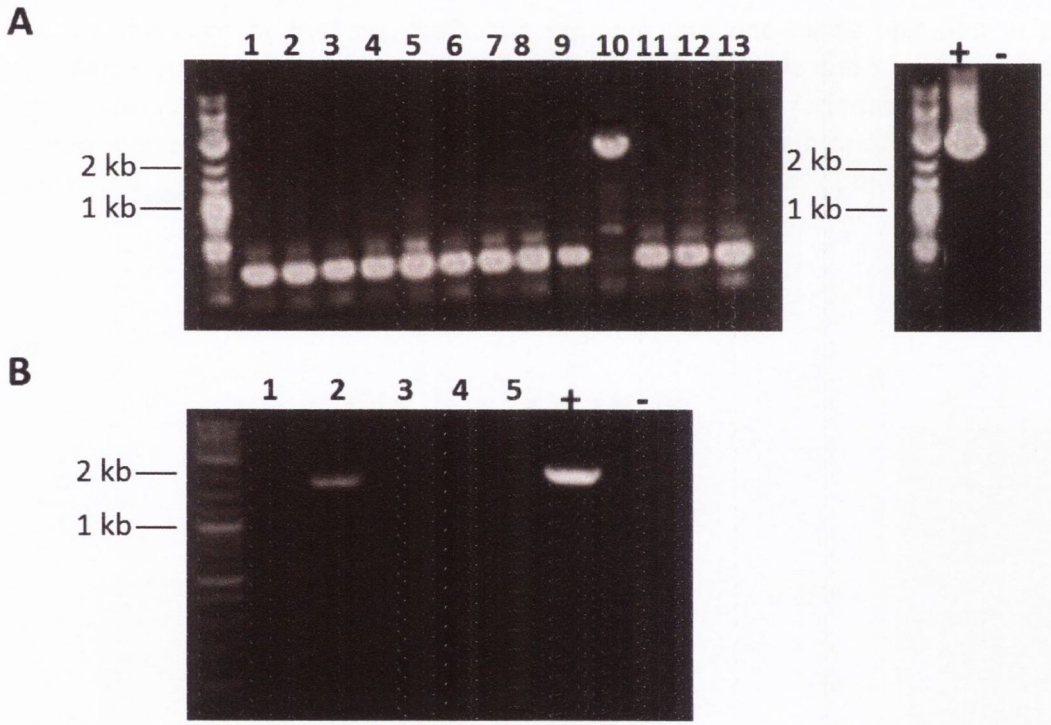



**FIG. 4.15** *S. aureus* tarH mutant **A)** Screening of *E. coli* DC10B transformants with pIMAY-Z::*tarH* FS by colony PCR. A positive colony (containing TOPO with insert) yielded a band of approx. 2 kb (e.g. colonie 10). **B)** Screening of Newman *tagO* pIMAY-Z::*tarH* FS transformants by colony PCR. Only one colony (2) showed to have a band of the correct size (2 kb). For both a positive control (+) of *E. coli* containing TOPO::*tarH*, and a negative control (-) of *E.coli* without plasmid, were included. Size markers are indicated.

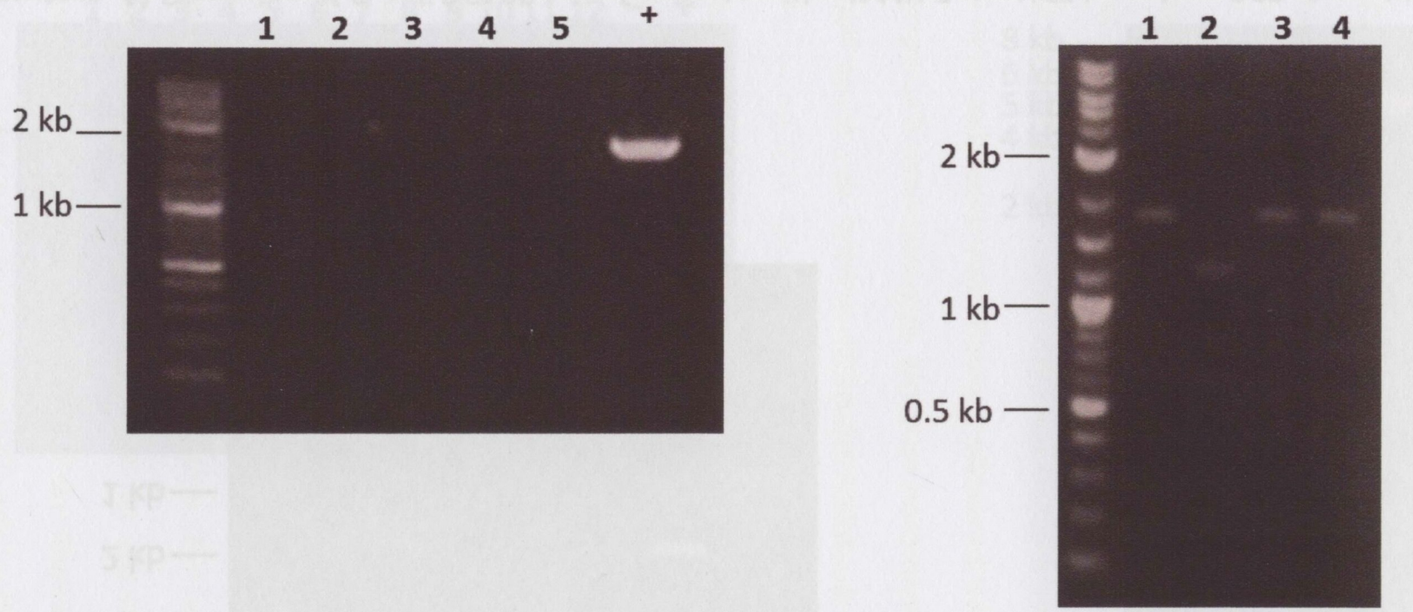



**FIG. 4.16.** Integration and excision of pIMAY-Z::*tarH* FS from Newman *tagO* **A)** Screening of pIMAY-Z::*tarH* FS integrants in Newman *tagO* by colony PCR. Absence of a band indicates successful integration.e.g. colonies 1-5. The presence of a 2 kb band indicates the retention of replicating plasmid. A positive control (+) of *S. aureus* containing replicating pIMAY was included. **B)** Screening of pIMAY-Z::*tarH* FS excision in Newman *tagO* by restriction digest with the restriction enzyme BglII. Two bands of 648 bp and 1.2 kb were produced after digestion of the PCR product with BglII revealing the presence of the *tarH* fragment with the two nucleotide insertion in the Newman *tagO* chromosome.

to the upstream and downstream region of the pTOPO MCS; Chapter 2Table 2.2) and the PCR product was sent for sequencing. The pTOPO::*tarH* (FIG 4.13 A) plasmid was amplified using the primers *tarH* FS fw and rv (Chapter 2, Table 2.2), for the insertion of 2 nucleotides (CT) between nucleotides 105 and 106, to generate a frameshift mutation while simultaneously creating a BglII restriction site (AGATCT) creating pTOPO::*tarH* FS (FIG 4.13 B). The plasmid was transformed into *E.coli* XL1 Blue. Transformants were selected on kanamycin and were screened by colony PCR and M13 fw and rv primers.

FIG 4.14 A shows the results from 12 transformants along with PCR controls. Two transformants (11 and 12), with a band of approximately 2 kb indicative of pTOPO::*tarH* FS plasmid, were selected for DNA extraction and sent for sequencing. Only one plasmid was found to have the 2 nucleotide insertion in the *tarH* plasmid insert. This pTOPO::*tarH* FS plasmid was double digested with the restriction enzymes EcoRI and NotI and cloned into pIMAY-Z between the same restriction sites by a ligation reaction.

FIG 4.14 B shows the double digestion of pTOPO::*tarH* FS with EcoRI and NotI restriction enzymes, with a fragment of 4 kb that corresponds to the plasmid and another fragment of 2 kb corresponds to the *tarH* FS insert. Single digestions and uncut plasmid were included controls.

The pIMAY-Z::*tarH* FS vector was then transformed into *E. coli* DC10B. *E. coli* DC10B is a Dcm (methylase)-deficient mutant that has been engineered to be used to transfer *E. coli* derived non-cytosine plasmid DNA directly into *S. aureus* with high efficiency (Monk & Foster, 2012).

FIG 4.15 A A shows the PCR screening of 13 *E. coli* DC10B colonies along with controls. One transformant has the 2kb band indicative of extrachromosomal pIMAY-Z::*tarH* FS plasmid. This plasmid was extracted, confirmed by sequencing and transformed into Newman *tagO*.

Newman *tagO* pIMAY-Z::*tarH* FS transformants were selected at 28ºC on agar containing chloramphenicol. Colonies were screened for the presence of extrachromosomal DNA by PCR using pIMAY MCS Fw and Rv primers. These primers will only amplify plasmid DNA and will generate a fragment of approximately 2 kb which encompasses the *tarH* cassette along with 540 bp upstream and 529 bp

downstream of the *tarH* gene and approximately 300 bp of flanking plasmid DNA. FIG 4.15 B shows the screening of 5 colonies for the presence of pIMAY-Z::*tarH* FS. One colony showed a 2 kb band indicative of the presence of extrachromosomal pIMAY-Z::*tarH* FS.

The procedure for allelic exchange is the same as in Chapter 3 (section 3.2.1) with the exception of the detection of the mutant.

One plasmid carrying transformant was examined for temperature sensitivity by plating a broth culture grown at 28ºC in chloramphenicol onto agar plates containing chloramphenicol in duplicate for 24h at 28ºC and 37ºC.

FIG 4.16 A shows a gel with the lack of amplification in 5 colonies except in the positive control. The colony with integrated plasmid was then selected for excision by growing them in TSA with anhydrotetracyclin (ATc, 1 µg/ml) at 28ºC. Genomic DNA was extracted from 4 colonies grown at 37ºC. A PCR with OUT primers was carried out for the amplification of a 2 kb fragment.

FIG 4.16 B shows the digestion of four of the fragments with the restriction enzyme BglII. One was successfully digested (colony 2), showing two bands of 1.3 kb and 0.7 kb. This suggests that the *tarH* gene with the 2 nucleotide insertion was successfully inserted into the genome. This was also confirmed by sequencing. This colony was also plated on TSA containing chloramphenicol to confirm loss of the plasmid.

The double mutant *tagO tarH* was transformed with either pRMC2 or pRMC2::*tagO* (O´Halloran, not published) and selected in TSA with chloramphenicol and tunicamycin. The transformants were screened using colony PCR and pRMC2 MCS fw and rv primers (that anneal outside of the MCS region of the pRMC2) The vector pRMC2 is a derivative of pALC2073 that expresses the TetR repressor at a higher level than pALC2073.

In the first attempts of transforming the Newman *tagO tarH* double mutant with this plasmid no transformant colonies grow on TSA with chloramphenicol.The reason for this was likely to be due to leaky expression of *tagO* even without induction.

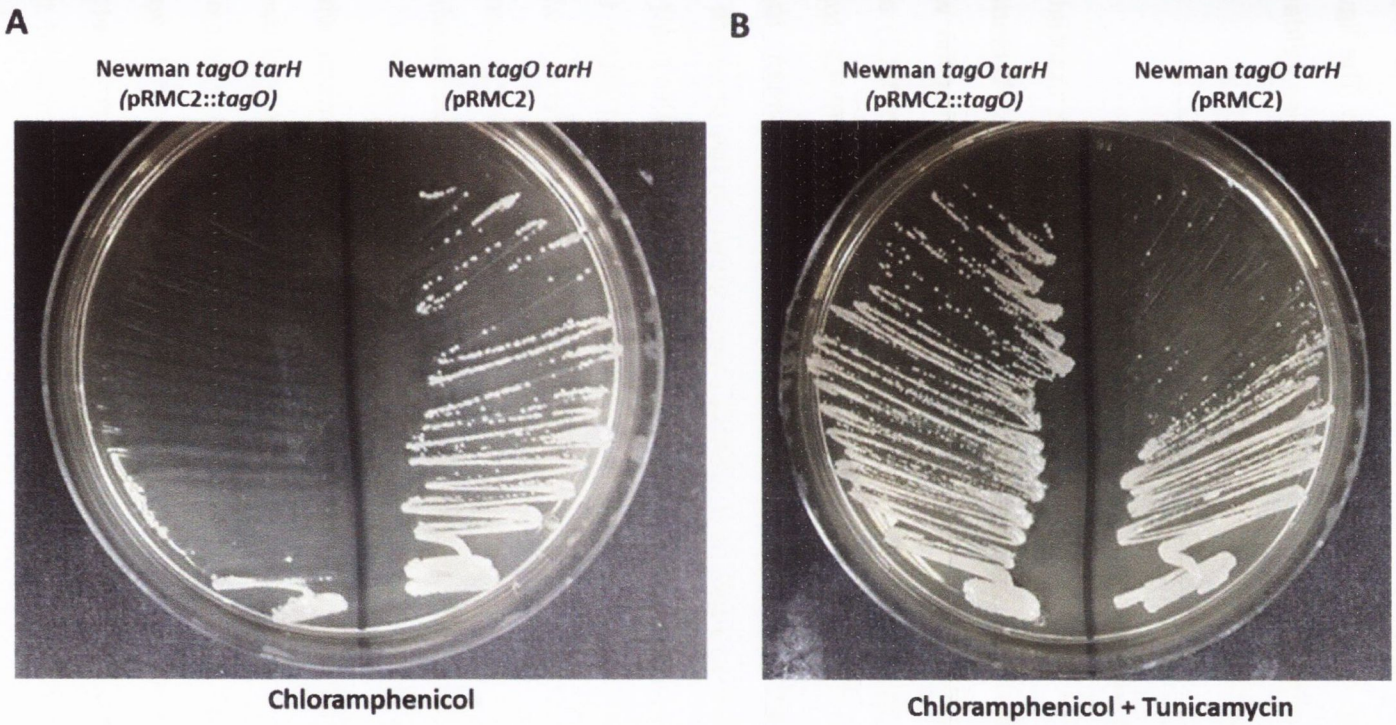



**FIG. 4.17.** Growth of Newman *tagO tarH (*pRMC2::*tagO)* in the presence of tunicamycin. **A)** Newman *tagO tarH (*pRMC2::*tagO)* and Newman (pRMC2) grown on TSA with chloramphenicol. **B)** Newman *tagO tarH (*pRMC2::*tagO)* and Newman (pRMC2) grown in TSA with chloramphenicol and tunicamycin.

of pRMC2. To overcome this, colonies of transformants were selected on TSA with chloramphenicol and tunicamycin. Tunicamycin is an antibiotic that blocks the activity of the TagO enzyme.

FIG 4.17 shows agar plates with restreaked Newman *tagO tarH* (pRMC2::*tagO)* growing in the presence of chloramphenicol with or without tunicamycin. The figure shows that the strain can only grow in the presence of tunicamycin. This also validates the *tarH* mutation in a Newman *tagO* background.

### 4.2.8 Expression of protein A in *tarH* mutants

In order to investigate if the accumulation of WTA intermediates is associated with interfering with expression of surface-exposed Spa on intact cells, the IgG binding protein was measured by flow cytometry. Newman wild type Spa expression was compared with Newman *tagO* (pALC2073::*tagO),* Newman *tagO tarH* (pRMC2) and Newman *tagO tarH* (pRMC2::*tagO).* The expression of *tagO* by the pRMC2::*tagO* plasmid was induced with 800 ng/mL of ATc. The strains Newman *tagO* (pALC2073::*tagO)* and Newman *tagO tarH* (pRMC2) were grown in the presence or absence of ATc and used as controls. A *spa* null mutant was used as a negative control.

Firstly, it was important to confirm that 800 ng/mL ATc did not affect the growth of the strains Strains were grown overnight in TSB supplemented with chloramphenicol and in the case of Newman *tagO tarH* (pRMC2::*tagO)* in the presence of tunicamycin. Strains were grown to the exponential phase and ATc was added when cells reached an $OD_{600nm}$ of 0.2.

The wild type and *tagO* (pALC2073::*tagO)* strains showed similar growth, while Newman *tagO* (pALC2073::*tagO)* grown in the presence of 800 ng/mL ATc showed slower growth. Newman *tagO tarH* (pRMC2) showed slower growth, characteristic of a *tagO* mutant growing in the absence of ATc. Strain Newman *tagO tarH* (pRMC2::*tagO)* showed a slower growth when compared to the wild type strain but still grew better than Newman *tagO tarH* (pRMC2), presumably due to the complementation of the *tagO* mutation. The growth of this strain was not completely restored possibly

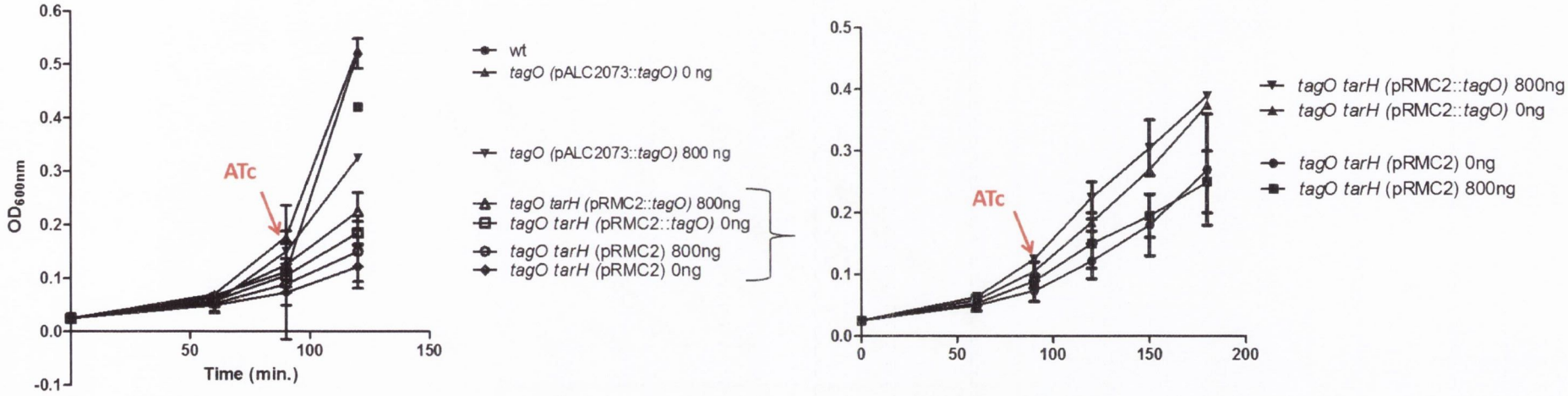



**FIG 4.18.** Growth of Newman strains in the presence of ATc (800 ng/mL) and its absence. Starter cultures grown in the presence of tunicamycin were diluted in fresh broth to an $OD_{600nm}$ of 0.025. ATc was added once the cultures had reached an $OD_{600nm}$ of 0.2. Newman *tagO* (pALC2073::*tagO)* and Newman *tagO tarH (*pRMC2) were used as controls. Each data point represents the mean OD value from three independent experiments.

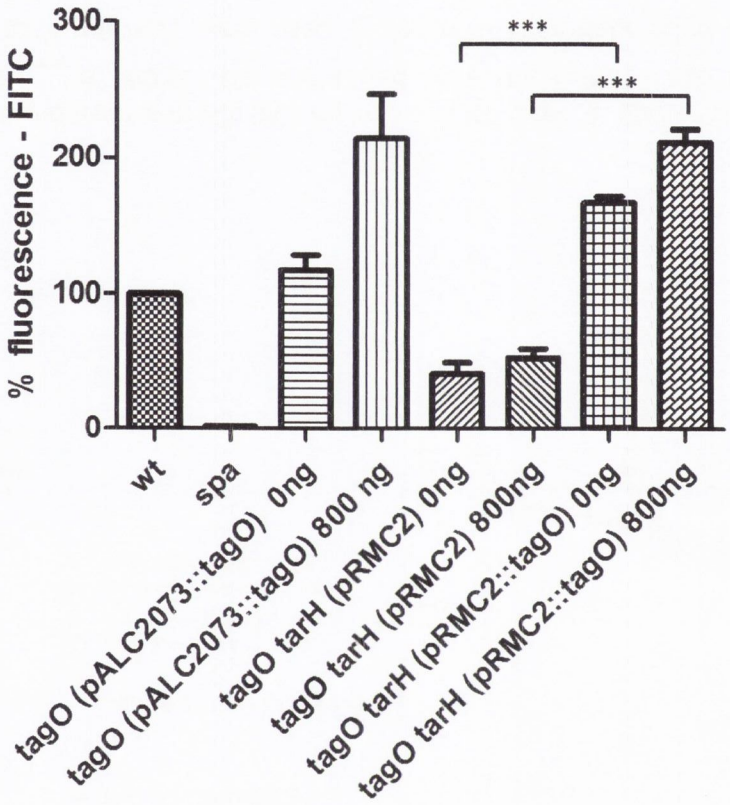



**FIG 4.19** Surface located Spa was detected using FITC-labelled rabbit IgG and fluorescence intensity was measured using flow cytometry. Values were plotted as a percentage of the median fluorescence intensity measured for the wild-type strain. Error bars represent the standard error of three independent experiments. The *spa* mutant strain was included as a negative control. Statistical analysis was performed using an unpaired t-test. Statistically significant result is ***P<0.00

due to toxic effects of WTA intermediates because of leaky *tagO* expression even in the absence of ATc. As shown in FIG 4.18, 1 hour after the addition of ATc strain Newman *tagO tarH* (pRMC2::*tagO)* reached an $OD_{600nm}$ of approximately 0.3, showing that the strain was actively growing even in the presence of 800 ng/mL of ATc.

Cells were harvested and labelled with FITC-conjugated IgG and fluorescence was measured by flow cytometry. Newman *tagO (*pALC2073::*tagO)* expressed Spa at slightly higher levels than the wild type (described before in Chapter 4) and at an even higher rate when the plasmid was induced with 800 ng/mL of ATc (FIG 4.19). Strain Newman *tagO tarH* (pRMC2) showed low levels of fluorescence while Newman *tagO tarH* (pRMC2::*tagO)* showed fluorescence at an even higher level than the wild type strain (even with no ATc induction of the plasmid) (FIG 4.19). These data suggest that expression of *tagO* in a *tarH*-deficient background restores production of the Spa protein.

This result suggests that the production of WTA biosynthetic pathway intermediates may allow the activation of *spa* transcription and consequent expression of this protein on the cell surface.

## 4.3 Discussion

The data presented in this chapter were obtained from investigations into the expression of important cell wall anchored (CWA) proteins, membrane proteins and secreted proteins.

Transcription of the genes encoding the cell anchored proteins Spa and ClfB were reduced in the *tagO* mutant. Expression of a protein can be regulated at both transcriptional and translational levels and so it is important to correlate mRNA and protein levels (Vogel & Marcotte, 2012).

In order to investigate if the transcription levels of the genes encoding the CWA proteins Spa and ClfB correlated with the level of protein expression and protein activity in the *tagO* mutants, Western immunoblot and functional assays were performed. Spa was found in the cell wall and supernatant of Newman wild type as well as in the membrane and cytoplasm possibly due to Spa being transiently associated with the membrane during secretion/sorting and being present in the cytoplasm following translation and prior to secretion. This could be a consequence of a reduced sorting efficiency causing a blockage. In the Western blots of LAC the level of Spa in the *tagO* mutant was reduced to 75% (+/- 9.5%) of the wild type. The reason for this could be because LAC produces Spa in higher levels when compared to Newman.

To test if the IgG binding capacity of the surfaced exposed protein A was reduced in the *tagO* mutants, flow cytometry was used. This method does not depend on the efficiency of cell wall digestion and the proteins are not subjected to protease activity during protoplast formation. The reason for the discrepancy between LAC protein A Western immunoblot and flow cytometry is not known. However, densitometry is not a very accurate method since the background interferes with the intensity of the bands, and as these are very different methods that measure different things, they are not directly comparable. Flow cytometry is a more informative experiment that measures functional protein on the cell surface.

To test the binding capacity of surface exposed ClfB, adhesion assays to loricrin, were performed. Reduced levels of binding were found in the *tagO* mutant of Newman and LAC strains.

These results showed that both proteins are reduced in Newman *tagO*, LAC *tagO*, BH1CC *tagO* and SH1000 *tagO* mutants. Moreover, the activity of both proteins is reduced in the Newman *tagO* mutant and LAC *tagO* mutant.

These results suggest that Newman *tagO* and LAC *tagO* most likely have an impaired capacity to bind IgG and escape the host immune system, due to the reduced levels of Spa and an impaired capacity to bind to the squamous epithelium in the nares of rodents or humans, due to the reduced expression and activity of ClfB. This suggests that data generated by Weidenmaier (2004) should be carefully interpreted. The reduced levels of the CWA proteins Spa and ClfB in the SH1000 and BH1CC *tagO* mutant strains show that the phenotypic changes caused by the lack of WTA occurs in both MSSA and MRSA strains.

The observation that *clfB* and *spa* transcription are reduced in the *tagO* mutant is most likely due to the reduction of their transcriptional regulators *rot* and *sarS*, respectively, as described in Chapter 3.

*S. aureus* expresses another IgG binding protein, Sbi. Sbi is located both extracellularly in the culture supernatant and is non-covalently attached to the cell envelope by binding to LTA and possibly a second membrane associated factor (Smith *et al.*, 2012). This protein was found to be in the supernatant of Newman wild type and the *tagO* mutant and in the membrane fraction due to its association to LTA. Sbi levels were not reduced in the *tagO* mutant. This is in agreement with the level of *sbi* gene expression being similar in the wild type and *tagO* strains.

The expression and activity of ClfA was also investigated by Western Immunoblotting and adhesion assays. ClfA was reduced by about 50% from the cell wall of Newman and LAC *tagO*. No fractionation studies were performed for ClfA because the sorting of this protein is very efficient and it is all located in the cell wall (McCormack *et al.*, 2014). However, no differences were seen in the transcript levels of *clfA* in stationary phase cells of the Newman *tagO* mutant, while ClfA protein expression was reduced. Since transcription of the ClfB gene was dramatically reduced in the *tagO* mutant it is likely that the loss of ClfB from the cell wall is due to reduce expression and not due to mislocalisation. Fractionation studies would be necessary to study this further. It is possible that in stationary phase *tagO* mutant cells the protein is mislocalised, or is subjected to increased degradation. Another explanation could be

that the *clfA* transcript is already degraded in a *tagO* mutant in the late stationary phase culture and so the protein is less expressed in the cell wall. To test the binding capacity of surface exposed ClfA, adhesion assays to fibrinogen were performed. Reduced levels of adherence were found in the *tagO* mutant of Newman and LAC strains. Our results suggest that the Newman and LAC *tagO* mutants have an impaired capacity to bind to fibrinogen and consequently are likely to be less capable of infecting a host due to the reduced expression of ClfA.

The mRNA levels of the membrane proteins EbpS and AtpA were not altered and no differences in expression of these proteins were found in Newman and LAC *tagO* mutant blots. Transmembrane F0/F1 ATPases, such as AtpA are essential proteins (Ferrándiz MJ et al, 2002), suggesting that only accessory genes, regulated by the *agr* and other regulators of virulence factors are being affected.

The expression of SrtA in the Newman *tagO* mutant was also tested by Western immunoblotting in order to investigate if this protein has a different expression pattern in the mutant that could alter the CWA proteins sorting to the cell wall. No differences were detected in SrtA expression in the Newman *tagO* mutant which correlated with the RNA-seq results for this protein.

The expression of the secreted proteins Hla and V8 protease were tested by Western immunoblotting using culture supernatants of Newman and LAC strains grown to exponential phase. Both Hla and V8 were drastically reduced from the extracellular medium of Newman *tagO* and LAC *tagO*. Hla transcription was not altered in the Newman *tagO* mutant (Chapter 3). However this protein was not detected in a supernatant from Newman *tagO* and was extremely reduced in the LAC *tagO* mutant supernatant. The explanation for this apparent discrepancy is that while the *hla* gene is transcribed at the same levels in the *tagO* mutant and the wild type (activated by SaeR/S the transcription of *hla* is undiminished), the lack of Agr RNAIII in the *tagO* mutant means that translation of the *hla* mRNA cannot occur. RNAIII binds to the long 5´UTR of the *hla* mRNA causing a conformational change that frees the ribosome binding site allowing translation to start (Morrison & Dunman *et al*, 2011). The data from the RNA-seq showed that RNAIII transcripts are ca 3-fold reduced which could cause a reduction in Hla translation.

In the exponential phase of growth Hla is expressed at levels below the limits of detection in a sheep blood agar assay with unconcentrated material. The haemolytic activity of Hla was tested by incubating culture supernatants from cultures grown to the stationary phase of growth in blood agar, which showed no haemolytic activity in the Newman and LAC *tagO* mutants.

Hla was described before as being implicated in bloodstream infection and in establishing organ colonization (see section 1.2.5.2). The steadily increasing expression of Hla from nasal colonisation to bacteraemia to heart lesions in a mouse infection model suggests a role of this protein in the transition from commensal to pathogen (Jenkins *et al.*, 2015). The fact that this protein is reduced in Newman and LAC *tagO* mutants, suggests that the absence of Hla can be related to the incapacity of the mutants to cause infection.

The TagO enzyme activity was inhibited during growth by the antibiotic tunicamycin in Newman wild type cells in order to block WTA synthesis and measure the expression of surface exposed Spa. A tunicamycin concentration causing the same growth defects as in the Newman *tagO* mutant and the same clumping phenotype was used. Newman wild type cells were grown to exponential phase in the presence of 4 µg/ml tunicamycin and then labelled with FITC and the fluorescence measured by flow cytometry. Spa was drastically reduced from the cell wall of Newman wild type grown in the presence of tunicamycin, suggesting that this effect in is solely due to the absence of WTA synthesis and not due to any other change in the cell.

A *S. aureus* WTA deficient strain has severe morphological defects due to effects on peptidoglycan and consequently cell division. These strains induce stress responses to improve their fitness. We hypothesise that the cell shuts down several non-essential accessory pathways and genes at a cost of loss in virulence.

This work shows that the reduced capacity of the mutant to adhere to host ligands, bind to IgG and produce alpha-toxin is not merely due to the absence of WTA (as stated before by Weidenmaier et al, 2004) but to the reduced expression of CWA and secreted proteins.

The most down-regulated gene in the *tagO* mutant was *spa* (100-fold reduced) which resulted in a complete absence of protein on the cell surface.

The study of Botella et al (2014) showed that WTA intermediates can have a role in transcription. PhoR activity is inhibited by a WTA intermediate when WTA is high in cells growing under phosphate-replete conditions.

Inhibition of TarH with the antibiotic targocil caused down-regulation of their genes in the capsule biosynthetic operon (Campbell et al, 2011). Presumably WTA intermediates accumulate intracellularly when TarH is inhibited. Inactivation of either TagO or TarH will prevent WTA from reaching the cell surface. Inhibition of TarH has the opposite effect to disrupting TagO activity. However, while inhibition of TarH reduced expression of the capsule synthesis genes, inactivation of *tagO* increased their expression. Therefore it is possible that WTA intermediates play a role in down-regulating the expression of capsule genes. It would be interesting to study genes from the *cap* operon by RT-PCR using this strain, to investigate if WTA intermediates also interfere with the transcription of genes responsible for capsule synthesis in *S. aureus.*

We hypothesised that the transcription of *spa* could be affected by the absence of WTA intermediates and not necessarily by the absence of WTA from the cell surface. To study this, a *tarH* mutant was isolated in a Newman *tagO* background and the mutant was transformed with pRMC2::*tagO*. The expression of *tagO* initiated the synthesis of WTA intermediates with no export of the polymer due to inactivity of the TarH enzyme. The effects of the synthesised WTA intermediates on Spa expression were studied by flow cytometry to detect the protein on the surface of *S. aureus.*

The growth of the strains in the presence of ATc was assessed and the growth curves performed (FIG 4.18) showed that strains were actively growing even in the presence of 800 ng/mL of ATc. The cells were harvested and surface exposed Spa was measured by flow cytometry (FIG 4.19). The flow cytometry results showed that there is a significant increase of Spa expression in strain Newman *tagO tarH* (pRMC2::*tagO)* (regardless of the induction or pRMC2 with ATc), which is synthesizing WTA intermediates, when compared to the strain Newman *tagO tarH* (pRMC2) which is not.

This suggests that the intermediates of the WTA biosynthesis pathway are interfering with *spa* transcription, possibly through *sarS* activation. More studies would have to be performed with the Newman *tagO tarH* double mutant complemented with pRMC2::*tagO*, such as RT-PCR, to investigate which other genes the WTA intermediates interfere with

# Chapter 5

## Elaboration of surface proteins in a *S. aureus* LAC *ltaS* mutant

### 5.1 Introduction

The major part of this thesis concerned the properties of *tagO* null mutants of *S. aureus* defective in production of wall teichoic acid. The bacterium expresses another anionic polymer composed of polyglycerol phosphate (instead of polyribitol phosphate) and linked to the cytoplasmic membrane, lipoteichoic acid (LTA). It is linked to the membrane via a glycolipid anchor which consists of a disaccharide linked to diacylglycerol (DAG). Following synthesis in the cytoplasm, the glycolipids are transported to the outer membrane by the enzyme LtaA (FIG 5.1), where they are used as the LTA anchor. Then, the protein LtaS polymerizes the polyglycerolphosphate (PG) chain by adding glycerolphospate units to the distal end of the chain, from the head group of the membrane lipid phosphatidylglycerol (FIG 5.1), as described in section 1.2.3 in Chapter 1. In addition many of the glycerol moieties are modified with D-alanine substitutions. According to Reichmann (2013) D-alanylation is controlled by the Dlt locus. The combined activity of DltA and DltC in the cytoplasm results in the transfer of D-Ala groups onto the lipid carrier bactoprenol pyrophosphate. Then, DltB, which is a transmembrane protein translocates D-Ala-bactoprenol pyrophosphate to the plasma membrane. However, according to Schneewind & Missiakas (2014) the D-alanylation of WTA do not require catalyses and therefore it is likely that the D-Ala residues are transferred from the LTA final polymer to WTA.

LTA is likely not a surface exposed polymer (Reichmann & Gründling, 2011), being localized in the periplasmic region.

The precise function of LTA is not fully understood but like WTA it is involved in correct localization of peptidoglycan hydrolases (Yamada *et al.*, 1996), cell surface charge and in bacterium-host cell interaction, such as adhesion, phagocytosis and the induction of inflammatory cytokines (Ginsburg, 2002, Morath *et al.*, 2002, Weidenmaier & Peschel, 2008) and interacts with the cell division machinery (Reichmann *et al.*, 2014).

Chapter 3 showed that *tagO* null mutants could be isolated by allelic exchange, most likely without the necessity of secondary mutations. In contrast, mutants defective in LTA (*ltaS* deletion mutants) could only be propagated on normal laboratory medium at 37ºC in the presence of the osmoprotectants 7.5% NaCl or 40% sucrose (Corrigan *et al*, 2011). When the *ltaS* mutant strains were plated on TSA

without the addition of sucrose or salt a small number of colonies were obtained. Whole genome sequencing was performed on these mutants and compensatory mutations were found in the *gdpP* gene that allowed the bacteria to grow normally in the absence of LTA (Corrigan *et al,* 2011). The GdpP protein has phosphodiesterase activity responsible for the complete conversion of the cyclic dinucleotide c-di-AMP to 5'-pApA. Disruption of *gdpP* leads to an increase in intracellular c-di-AMP levels, presumably leading to effects on activity and/or expression of proteins that compensates for defects caused by the absence of LTA (Corrigan *et al*, 2011). The growth of the LAC *ltaS gdpP* supressor mutant closely resembled the parental wild type strain (Corrigan *et al*, 2011). The cells have near wild type size and a considerable improvement in the division site placement accuracy, although misplacement of septa still occurs in some cells (Corrigan *et al*, 2011).

The data in Chapters 3 and 4 revealed that a *tagO* mutant of strain Newman suffered dysregulation of a number of genes including the cell wall stress stimulon regulators. Genes encoding CWA proteins and secreted proteins were reduced. In this chapter, mutants of the CA-MRSA strain LAC containing a deletion of the *ltaS* gene and compensatory mutations in *gdpP* were compared to the parental strain as well as a strain containing a loss of function mutation in *gdpP*. Time did not allow a comprehensive analysis of transcripts and protein expression but a limited comparison of protein expression and phenotype was completed in order to determine if the consequences of LTA deficiency are similar to those of the WTA mutant.

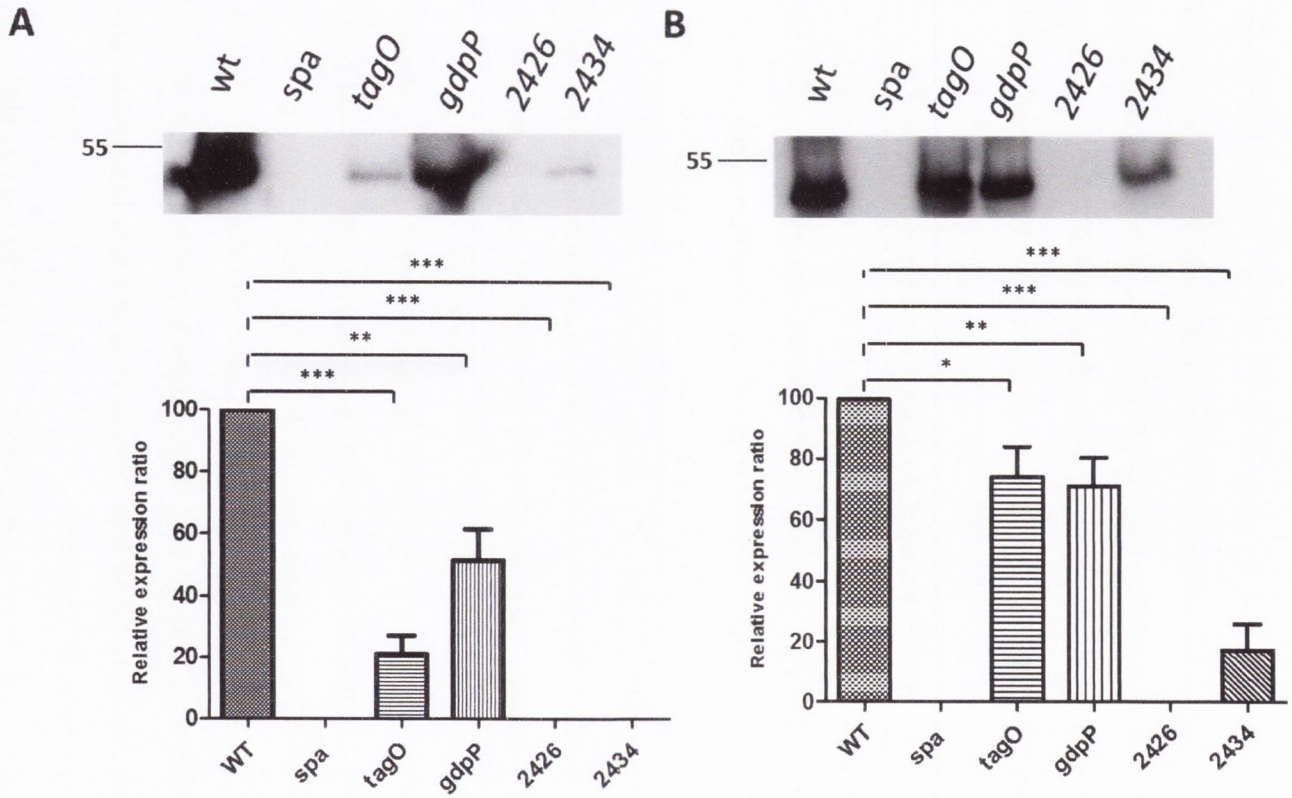



**FIG. 5.2** Western immunoblots to detect protein A in LAC LTA deficient mutants **A)** in the exponential phase of growth and **B)** the stationary phase of growth. Cell wall anchored proteins were solubilized by treating cells grown to both growth phases with lysostaphin. The cell wall extracts were subjected to SDS-PAGE and Western immunoblotting using HRP-labelled IgG to detect Spa. The *spa* mutant was included as a negative control. Molecular weight is indicated. Densitometry of LAC Western immunoblots was also performed to compare the wild type, the *gdpP* mutant and *ltaS gdpP* double mutants. The intensity of the bands of three independent Western immunoblotting was measured by densitometry and statistical analysis was performed using an unpaired t-test. Statistically significant result is *P<0.05., **P<0.01 and ***P<0.001.

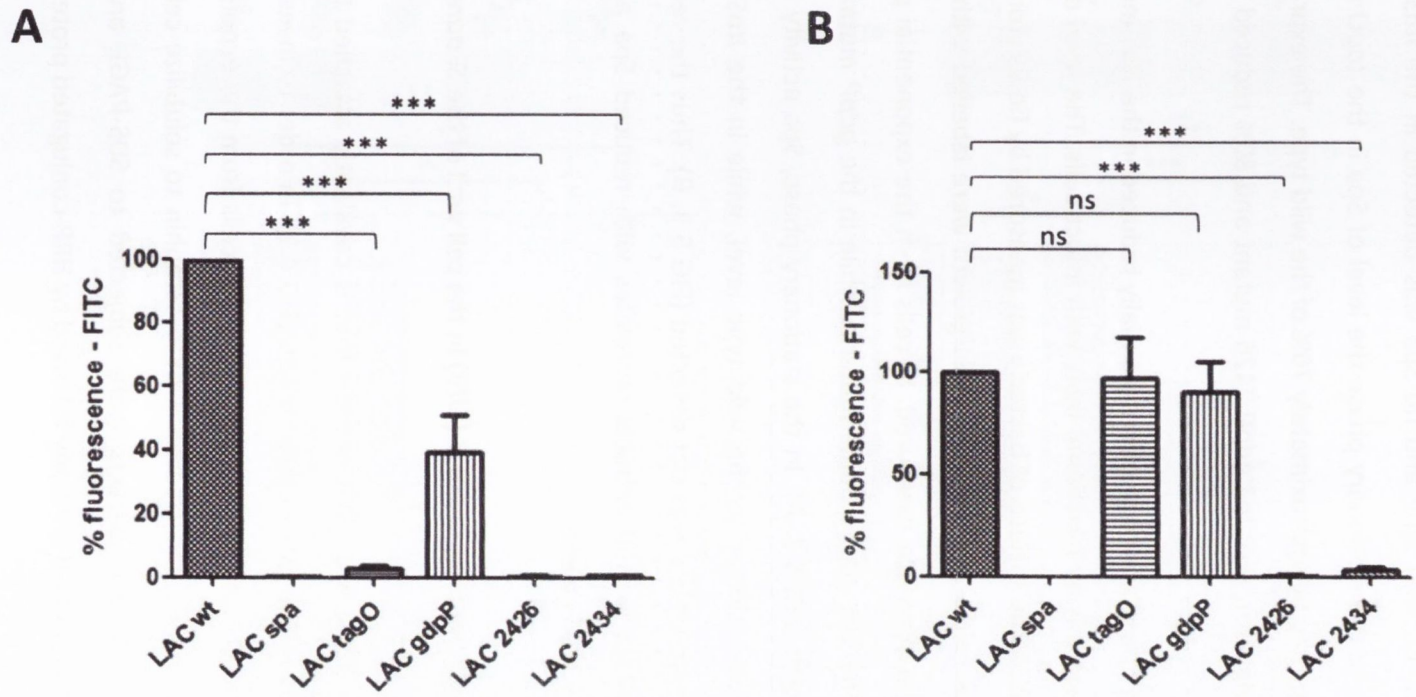



**FIG. 5.3** Flow cytometry to detect protein A on the surface of **A)** LAC strains grown to the exponential phase of growth and **B)** LAC strains grown to the stationary phase of growth. Surface located SpA was detected using FITC-labelled rabbit IgG and fluorescence intensity was measured using flow cytometry. Values are plotted as a percentage of the median fluorescence intensity measured for the wild-type strain. Error bars represent the standard error of three independent experiments. The *spa* mutant strain was included as a negative control. Statistical analysis was performed using an unpaired t-test. Statistically significant result is ***P<0.001 and ns = not significant.

Densitometry analysis of the blots was performed on three independent samples. In the exponential phase the level of Spa in the *tagO* mutant was found to be approximately 20 % of the wild type but in the *gdpP* mutant expression was found to be approximately 50% of the wild type and no Spa was detected in the *ltaS gdpP* double mutants (FIG 5.2). In the stationary phase the level of Spa in the *tagO* and in the *gdpP* mutants was found to be approximately 70% of the wild type. The expression of Spa remained undetectable in the *ltaS gdpP* 2426 mutant and 80% reduced in the *ltaS gdpP* 2434 mutant.

Having shown that Spa was absent or substantially reduced in the *ltaS* mutants it was important to confirm this by functional tests with intact cells. The level of IgG-binding protein expressed on the surface of bacteria was measured by flow cytometry. Cells from the exponential and stationary phases of growth were labelled with FITC-conjugated IgG and the fluorescence measured. In cells from the exponential phase, no Spa was detected in the *ltaS gdpP* double mutants while in the *gdpP* mutant Spa activity was reduced by 60% (FIG 5.3. A). In the stationary phase, Spa activity in the *tagO* and *gdpP* mutants was similar to the wild type level, while in the *ltaS gdpP* mutants 2426 and 2434 Spa activity was not detected (FIG 5.3. B). Thus the reduced expression of Spa detectable in wall extracts correlates with reduced Spa activity detected on whole cells by fluorescence.

### 5.2.2 Elaboration of clumping factor B (ClfB) in the cell wall of the *S. aureus* LAC *ltaS* mutant

Clumping factor B (ClfB) is a CWA protein that is covalently attached to the peptidoglycan and extends to the extracellular milieu (FIG 4.1). In order to investigate if ClfB expression was also affected by the absence of LTA, cells from the exponential phase of growth were harvested and treated with lysostaphin to solubilize cell wall proteins. The solubilized cell wall extracts were subjected to SDS-PAGE and the released ClfB detected with anti-ClfB antibody followed by HRP-conjugated protein A.

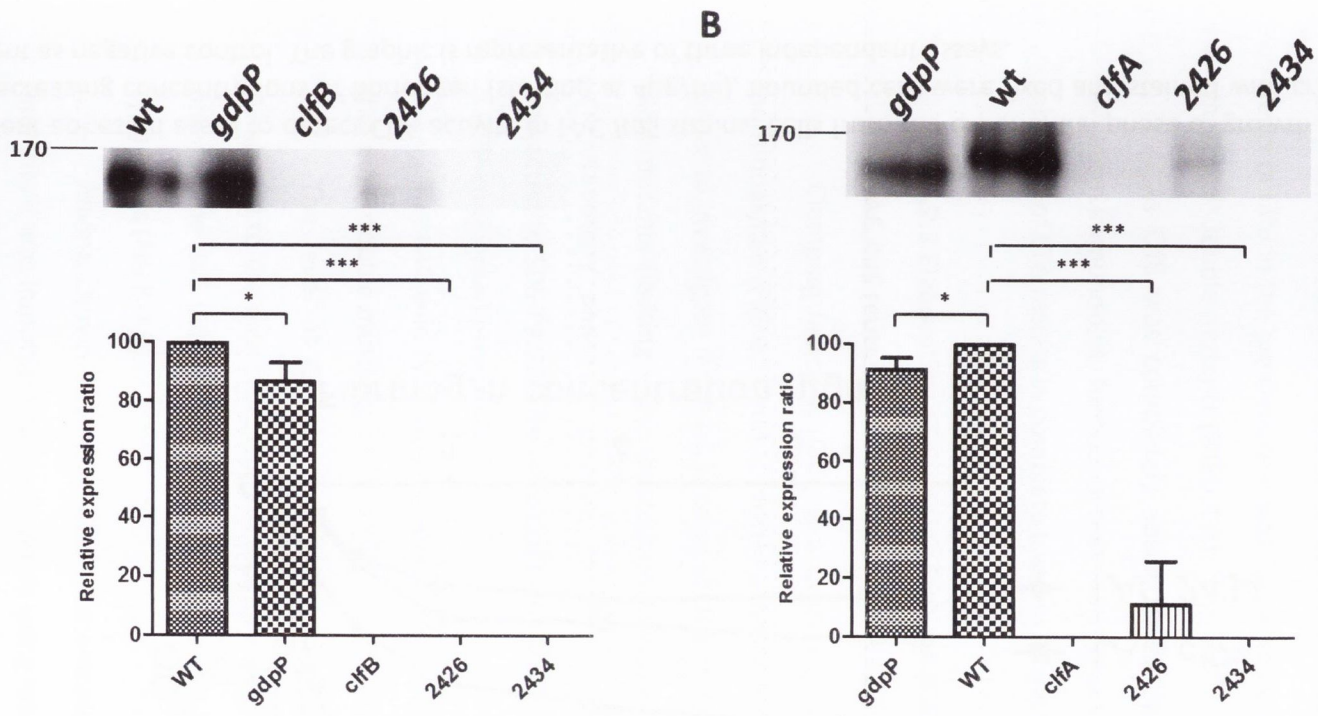



**FIG. 5.4** Western immunoblots to detect **A)** ClfB and **B)** ClfA in LAC strains. Cell wall anchored proteins were solubilized by treating cells grown to exponential phase with lysostaphin. The cell wall extracts were subjected to SDS-PAGE and Western immunoblot using anti-ClfB and anti-ClfA antibody, respectively, followed by HRP-labelled protein A. *clfB* and *clfA* mutant strains were included as negative controls. Molecular weight is indicated. Densitometry of LAC Western immunoblots was also performed to compare the wild type, the *gdpP* mutant and *ltaS gdpP* double mutants. The intensity of the bands of three independent Western immunoblotting was measured by densitometry and statistical analysis was performed using an unpaired t-test. Statistically significant result is *P<0.05 and ***P<0.001.

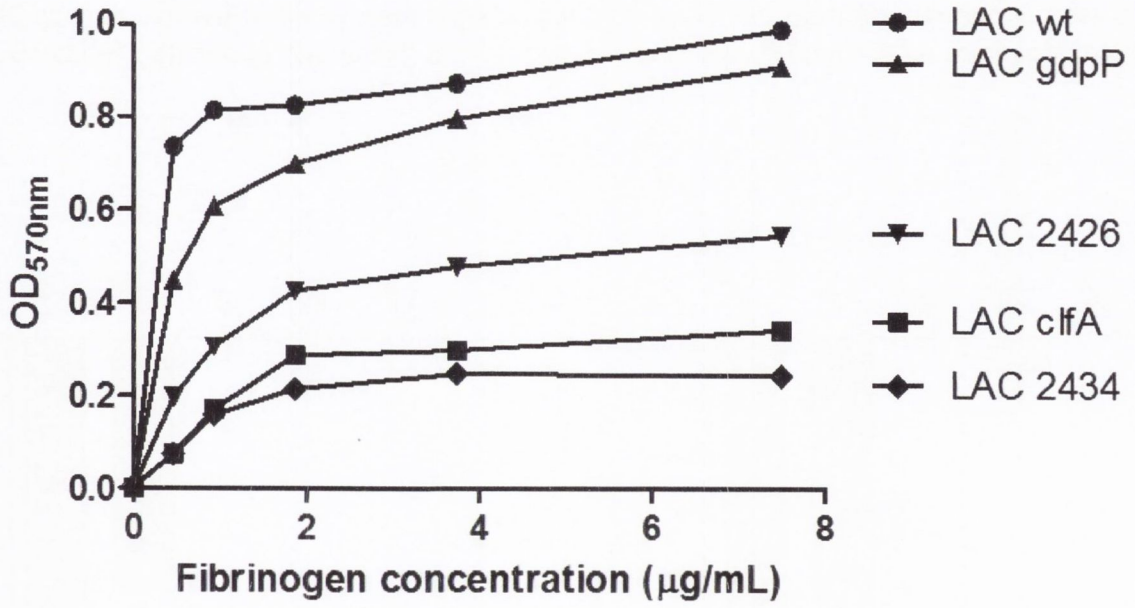



**FIG. 5.5** Dose-dependent adhesion assay to detect ClfA activity in LAC *ltaS* strains. Cells from the exponential phase of growth were harvested and incubated with decreasing concentrations of fibrinogen (starting at 4μg/ml), bounded cells were fixed and stained with crystal violet. The *clfA* mutant was present as negative control. The graphic is representative of three independent assays.

The wild type was compared to the LAC *clfB* mutant, the *gdpP* mutant and the *ltaS gdpP* double mutants. ClfB was detected in the wild type and in the *gdpP* mutant, and no detectable protein was found in the *ltaS gdpP* double mutants (FIG 5.4 A). Densitometry analysis of the blots was performed on three independent samples. The level of ClfB in the *gdpP* mutant was found to be 90 % and no ClfB was detected in the *ltaS gdpP* double mutants (FIG 5.4 A).

As ClfB was completely absent from the *ltaS gdpP* double mutants in the Western immunoblot, functional tests with intact cells were not performed. Thus the absence of LTA results in complete loss of ClfB on the surface of LAC *ltaS* mutants.

### 5.2.3 Elaboration of clumping factor A (ClfA) in the cell wall of the *S. aureus* LAC *ltaS* mutant

Clumping factor A (ClfA) is a CWA protein that is covalently attached to the peptidoglycan (FIG 4.1) and is mostly expressed in the stationary phase of growth. In order to investigate if the expression of this protein was altered in the cell wall of LAC *ltaS* mutants (lacking LTA), Western immunoblotting was performed with cells from the stationary phase of growth. These cells were treated with lysostaphin and subjected to SDS-PAGE. The released ClfA was detected with anti-ClfA IgG followed by a HRP-conjugated protein A.

The LAC wild type was compared to the *clfA* mutant, the *gdpP* mutant and the *ltaS gdpP* double mutants.

The level of ClfA in the *gdpP* mutant was found to be slightly reduced compared to the wild type. No ClfA was detected in the *ltaS gdpP* 2426 double mutant and for the *ltaS gdpP* 2434 double mutant. ClfA levels were reduced by 10% of wild type levels (FIG 5.4 B).

Having shown that ClfA was substantially reduced in the *ltaS gdpP* double mutants it was important to confirm this using functional tests with intact cells. Assays to measure adhesion to fibrinogen were performed. Cells from the stationary phase of growth were harvested and incubated in wells coated with decreasing concentrations of fibrinogen (starting at 4 μg /mL). Bound cells were stained with crystal violet, resuspended and the $OD_{570nm}$ were recorded.

The adhesion of the *gdpP* mutant to fibrinogen was slightly reduced when compared to the wild type while the binding of the *ltaS gdpP* 2434 double mutant was reduced to the same level as the *clfA* mutant (FIG 5.5). The *ltaS gdpP* 2426 double mutant showed some binding which correlates with the low amount of ClfA detected in the Western immunoblot. Thus the reduced expression of ClfA detectable in wall extracts of the LAC *ltaS gdpP* correlates substantially with reduced ClfA-mediated adhesion to fibrinogen.

### 5.2.4 Detection of the secreted alpha-toxin in the *S. aureus* LAC *ltaS* mutant culture supernatant

Alpha-toxin (Hla) is a secreted protein present in the supernatant of *S. aureus* strains. In order to investigate the activity of this protein in the LAC *ltaS gdpP* mutants, haemolytic assays were performed. Hla is an extracellular cytolytic pore-forming toxin that disrupts the integrity of the cytoplasmic membrane causing cell lysis. The lysis of red blood cells was tested by pipetting culture supernatants from cells in the stationary phase of growth into wells in sheep blood agar. Zones of haemolysis were detected in the supernatants taken from the wild type strain and the *gdpP*, but not the *ltaS gdpP* mutants 2426 and 2434. This suggested that the *ltaS*-deficient mutants produced far less alpha-toxin than the LTA-producing strains (FIG 5.6).

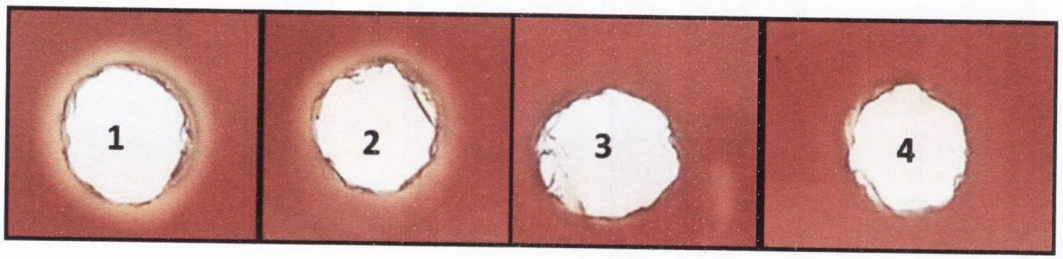



**FIG. 5.6** Haemolysis test to detect the activity of α-toxin in LAC *ltaS* strains. Supernatant of cells grown to stationary phase were collected, filtered and added to wells made in the agar. 1- wild type, 2- *gdpP* mutant, 3- *ltaS gdpP* 2426 mutant and 4- *ltaS gdpP* 2434 mutant complemented. Representative of two biological replicates.

### 5.3 Discussion

LTA is an anionic polymer present in the cell envelope of *S. aureus,* involved in important functions similar to the WTA functions. The absence of LTA from the cell envelope causes a variety of different phenotypes, including changes in morphology and a loss in virulence. *S. aureus* WTA mutants are also known for their inability to colonize and establish infection in the host, while having profound morphological defects. Moreover, LTA has been assumed to serve as a scaffold for the presentation of adhesins and surface proteins that initiate infectious processes (Neuhaus & Baddiley, 2003). LTA is also responsible for the attachment of Sbi, the second *S. aureus* IgG binding protein important for immune evasion (see section 1.2.5.2.6), to the membrane (Smith *et al.*, 2012).

We hypothesised that the lack of LTA from the cell wall of *S. aureus* would cause similar changes in the expression of CWA proteins as a WTA defect. This chapter studied the changes in the levels of CWA proteins Spa, ClfB and ClfA. These proteins were found to be drastically reduced in the LAC *ltaS* mutants. Additionally, the ligand binding activity of the Spa and ClfA proteins was reduced as a consequence of the reduced level of each protein tested.

As stated in Chapter 4, defects resulting in a reduction of proteins such as Spa, ClfB, ClfA and Hla, causes a loss in virulence and colonisation potential. The LAC *ltaS* mutants are incapable of binding to IgG, due to the reduced Spa expression and of binding to host ligands such as loricrin (in the squames of nares) and fibrinogen (in the host blood) due to a loss of ClfB and ClfA, respectively.

In the Newman *tagO* mutant *spa* mRNA was reduced due to a reduced transcription of *sarS*, the transcriptional regulator that directly activates Spa transcription. Moreover, the reduced expression of ClfB was likely due to the reduction of *rot* which is the transcriptional activator of *clfB*. More studies would have to be performed with the LAC *ltaS* mutants, such as RT-PCR or RNA-seq, to investigate if the changes in the levels of CWA proteins are due to changes at the level of transcription, as in the Newman *tagO* mutant.

The expression of Spa in cells from the stationary phase of growth was found to be substantially reduced in the *ltaS gdpP* 2434 mutant and was completely absent from the surface of the same mutant grown to exponential phase (FIG 5.2 B). This is

most likely due to the accumulation of the protein during the growth cycle, becoming detectable in the stationary phase while in the exponential phase of growth it was not being expressed in detectable amounts. The small difference in the levels of Spa in the 2426 and 2434 mutants in stationary phase could be due to the nature of the *gdpP* mutations in the different mutants, which might result in some *gdpP* expression in the 2434 (stop codon insertion) mutant. While the *gdpP* compensatory mutation is necessary for the *ltaS* mutant strains to grow, the *gdpP* mutation alone affects the levels of Spa.

The *gdpP* mutation alone apparently does not affect ClfB or ClfA as much as Spa, since both proteins are expressed in the *gdpP* mutant at approximately 90% of wild type. However, in the stationary phase of growth the 2426 (promoter rearrangement) mutant expresses ClfA approximately 10% of wild type (FIG 5.4 B).

The overall patterns of *S. aureus* SA113 wild type and *ypfP* mutant CWA proteins was investigated previously, using lysostaphin solubilized CWA proteins in an SDS-PAGE (Fedtke *et al.*, 2007). The YpfP enzyme is responsible for the lipid anchor assembly, the first step of the LTA synthesis pathway. Only minor changes were detected in the CWA proteins overall patterns and no changes were observed in the major proteins. The analysis of the CWA proteins in an SDS-PAGE is not as accurate as using specific antibodies to detect the proteins of interested in a Western immunoblot. Moreover, the strain used in Fedtke (2007) study was a SA113 *ypfP* mutant, that still presented a low amount of LTA (13% of the wild type) while this study used a LAC *ltaS* mutant strain (no LTA). The expression of Sbi was not investigated in the LAC *ltaS* mutants. However the expression of Sbi would most likely be dramatically reduced from the cell envelope given that this protein is non-covalently associated with LTA.

The LtaS enzyme is responsible for the polymerization of the LTA chain (last step of the biosynthetic pathway), which is also necessary for the addition of D-alanine. These findings suggest that either the complete loss of LTA, LtaS enzyme activity or D-alanylation patterns are associated with the reduced expression of the CWA proteins Spa, ClfB and ClfA and the secreted protein Hla in the *S. aureus* LAC strain. Since D-alanine modification of WTA is proposed to be at least partially-

dependent on D-alanylated LTA it is possible that D-alanylation of WTA is reduced in LtaS-deficient mutants which may impact on WTA function.

# Chapter 6

# Discussion

## 6. 1 Discussion

*S. aureus* wall teichoic acid (WTA) is an important surface polymer that provides adittional stability to the plasma membrane, mediates extracellular interactions and act as scaffold for enzymes required for cell wall growth and degradation (Swoboda *et al.*, 2010). Despite not being an essential polymer, the lack of WTA causes morphological abnormalities and a loss in fitness.

The *tagO* gene encodes the first enzyme in the WTA biosynthesis pathway. This study, investigated other changes that occur in *tagO*-deficient mutants of *S. aureus* cells, by studying changes in transcription and expression of proteins.

The enzymes comprising the WTA biosynthesis pathway are considered excellent targets for the development of new antibiotic drugs (Wang *et al.*, 2013, Price & Tsvetanova, 2007). Moreover, a recent study showed that the amount of WTA on the cell surface is directly related to an increased capacity of MRSA strains to cause skin abscesses (Wanner *et al.*, 2017). Significant progress has been made towards the discovery of new antibiotics targeting teichoic acids (Pasquina *et al.*, 2013), however many potential targets remain underexplored.

The transcriptome of the *tagO* mutant was studied using RNA-seq. The major changes in transcription that occurred in the *tagO* mutant were the up-regulation of genes related to the cell wall stress and capsule synthesis and the down-regulation of genes involved in colonisation and virulence. The cell wall stress stimulon is activated when the cells are exposed to cell wall damaging agents such as vancomycin and β-lactams (Dengler *et al.*, 2011). This suggests that the damage that occurs in the cell envelope in the absence of WTA might be similar to that caused by these antibiotic agents. Moreover, *tagO* (encodes the enzyme responsible for the first step in the synthesis of WTA) was found to be mutated in strains that acquired the mutations responsible for the vancomycin-intermediate resistance phenotype (Matsuo *et al.*, 2013). This might suggest a closer association of the gene *tagO* to the vancomycin resistance phenotype. To investigate this, more studies would have to be performed such as determining the effects of *tagO* presence/absence in a vancomycin intermediate- *S. aureus* (VISA) strain.

Moreover, the polysaccharide capsule synthesis genes were also up-regulated in the WTA-deficient mutant. Capsule synthesis is also increased in vancomycin resistant strains of *S. aureus* (Howden *et al.*, 2010). We hypothesise that the increased transcription of capsule genes is associated with the increased expression of capsule in the cell wall which might be a compensatory mechanism activated by the cell to overcome the absence of WTA from the cell surface. However, *tagO* deletion mutants could be isolated in the capsule deficient strains SH1000 and LAC indicating that *S. aureus* can survive in the absence of both WTA and capsule. More studies are necessary to investigate if there is another compensatory mechanism that compensates for the absence of WTA and capsule from the cell surface of *S. aureus.*

To activate the cell wall stress stimulon and the capsule synthesis genes, the cell needs a considerable amount of energy. The expression of virulence factors becomes secondary when the cell is managing the energy necessary to express genes essential for survival. Therefore, in the WTA-deficient mutant genes related to virulence, such as genes encoding CWA and secreted proteins are down-regulated.

The fact that a WTA-deficient mutant has an impaired transcription and expression of virulence factors such as Spa, ClfB and Hla essential for immune evasion, nasal colonisation and lysis of host cells, suggests that results originated from studies that used WTA-deficient mutants need to be carefully interpreted. Weidenmaier (2004) characterised WTA has being an important factor for nasal colonisation, without considering the changes in transcription/expression of other important factors in the WTA-deficient mutant used. More recently, the same WTA-deficient mutant was used to determine the importance of WTA in the capacity of MRSA strains to cause skin abscesses (Wanner *et al*, 2017).

Authors using defective mutants, especially in major components of the cell, such as WTA, should consider studying that the mutation might alter the expression of other genes and that the effects of the mutation need to be assessed fully before carrying out any other experiments with the mutant.

This study shows that a WTA-deficient mutant has an impaired ability to adhere to loricrin (present in the squamous epithelium of nares) and fibrinogen (present in the blood and host tissue) due to the lack of ClfB and ClfA, respectively and not due to the lack of WTA itself. Furthermore, the mutant lacks the capacity to bind to IgG, due

to the absence of surface bound Spa, which is important to host immune evasion, which might suggest that the mutant would be more easily killed by the host immune cells. The WTA-mutant also does not express Hla (alpha-toxin) which is another important virulence factor in *S. aureus* . More studies would have to be performed to investigate the virulence-related proteins mainly expressed in the stationary phase.

To determine if the absence of another important cell envelope polymer LTA would cause the same changes in expression of CWA and secreted proteins, LTA-deficient mutants of strain LAC were studied. An LTA deficient mutant of strain Newman has not yet been isolated. The results showed that the reduction in expression of Spa, ClfB and ClfA is even more drastic in LTA-deficient mutants of LAC than in the WTA-deficient mutant. This suggests that the stress imposed to the cell by the lack of LTA is even more severe that the one caused by the absence of WTA. This correlates with the fact that LTA is essential for growth at 37ºC and that for an LTA-deficient strain to survive at 37ºC the cells need to acquire a compensatory mutation in the *gdpP* gene. The study of the transcriptome of this mutant would reveal the transcriptional changes occurring in this strain.

In order to investigate if the intermediates of the WTA biosynthesis pathway could have a role in the changes in Spa expression seen in the WTA-deficient mutant, a *tarH* mutant was isolated.

The gene *tagO* was then expressed in the *tarH* mutant to allow for the WTA intermediates to accumulate, with no export of the final polymer. This mutant was studied and the levels of surface-located Spa were measured. The results showed that there is an increase in Spa expression when the WTA intermediates are present compared to when they are not being produced. This suggests that WTA intermediates could be responsible for the regulation of Spa expression. More expression and transcriptional studies would reveal all the genes that WTA intermediates interfere with. These results show a more broad range of functions that could be attributed to WTA.

The study of Campbell et al (2011) showed that inhibition of TarH with the antibiotic targocil caused the down-regulation of the capsule genes, while this study shows that the entire capsule operon is up-regulated when *tagO* is deleted. This might suggest that WTA intermediates can also have a role in regulating the genes

responsible for the capsule production. *Bacillus subtilis* produces a glycerol phosphate WTA, however it does not possess a polysaccharide capsule, it instead synthesises teichuronic acid under phosphate-limited conditions (Ellwood & Tempest, 1969)(Ellwood et al, 1969). This process is regulated by the two component system PhoPR and a WTA intermediate (Botella et al, 2014). More studies need to be performed to investigate if WTA intermediates have a role in regulating capsule production in *S. aureus*, such as measure the transcription of *cap* genes in the Newman *tagO tarH* (pRMC2::*tagO*) strain.

In summary, results from this study found that the absence of WTA in MSSA and MRSA strains of *S. aureus* causes pleiotropic effects. A WTA-deficient mutant enters in an energy saving mode by down-regulating virulence factors and activating a variety of genes called the cell wall stress regulon, essential for survival. This mutant also has an impaired display of CWA proteins such as Spa, ClfB and ClfA and the secreted protein Hla, showing that a WTA mutant is not adequate to study processes where these proteins are involved, such as adherence. The abnormal display of these proteins was even more drastic in a LAC LTA mutant, suggesting that this mutant might undergo the same changes seen in the WTA- deficient mutants. Moreover, the WTA synthesis intermediates have an apparent role in regulating the expression of Spa. Furthermore, here we report additional evidences that support the potential of teichoic acids as targets for the development of new antibiotic agents, and propose that targeting WTA biosynthesis could be a strategy to an anti-virulence therapy. The significant loss of virulence seen in WTA-deficient strains indicates that these strains would not cause infection in the host and that they would be more vulnerable to immune clearance.

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