Gabriela Fernandes Henriques

Licenciada em Biologia Celular e Molecular

Study of the role of wall teichoic acids in the localization *Staphylococcus aureus* cell wall synthesis protein PBP4

Orientador: Doutora Mariana G. Pinho, Investigador Auxiliar, ITQB AX-UNL

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Resumo

A parede celular de *Staphylococcus aureus* é uma rede extremamente complexa composta maioritariamente por peptidoglicano (PG) com alto teor em pontes interpeptidicas e ácidos teicóicos (TAs), ambos importantes para a manutenção da integridade e viabilidade celular da bactéria. As proteínas de ligação à penicilina (PBP), que catalisam a fase final da biossíntese do PG, são alvos dos antibióticos β –lactâmicos e como tal têm sido um dos principais focos da investigação antibacteriana. *S. aureus* tem quatro PBPs nativas, PBP1 – 4, que estão presentes quer nas estirpes sensíveis á meticilina (MSSA), quer nas resistentes (MRSA). PBP4 cataliza a formação de ligações interpetidicas do peptidoglicano e, como demonstrado recentemente, é essencial para a expressão da resistência aos antibióticos β - lactâmicos em estirpes adquiridas na comunidade (CA-MRSA). Esta proteína, em *S. aureus*, localiza-se no septo celular, localização esta que parece ser espacialmente e temporalmente regulada por um intermediário, ainda não identificado, da biossíntese dos ácidos teicoícos da parede (WTA). Neste sentido, se a síntese dos WTA é comprometida, a PBP4 perde a sua localização septal e surge dispersa por toda a membrana celular. O objetivo deste projeto foi identificar o precursor da síntese dos WTA responsável pelo recrutamento septal da PBP4. Foram construídos mutantes indutíveis de dois genes essenciais para esta via de síntese, o *tarB e tarL,* utilizando a estirpe NCTCPBP4 – YFP (que expressa um derivado fluorescente da PBP4), o que nos permite estudar a localização da PBP4 na presença e ausência da expressão destes genes. Em conclusão, com este trabalho, fomos capazes de mostrar que a ausência destas duas proteínas, TarB e TarL, levam à deslocalização da PBP4, indicando que provavelmente a proteína TarL ou uma proteína ou precursores da síntese WTA dependente de TarL, é responsáveis pelo recrutamento de PBP4.

Palavras-chave: *Staphylococcus aureus*; Parede celular; Resistência aos antibióticos β – lactâmicos; Biossíntese dos ácidos teicoícos; Proteínas de ligação à penicilina; localização de proteínas.

Abstract

The cell wall of *Staphylococcus aureus* is a highly complex network mainly composed of highly cross-linked peptidoglycan (PG) and teichoic acids (TAs), both important for the maintenance of the integrity and viability of bacteria. The penicillin binding proteins (PBPs), which catalyse the final stage of PG biosynthesis, are targets of β-lactam antibiotics and have been a key focus of antibacterial research. *S. aureus* has four native PBPs, PBP1-4 carried by both methicillin-sensitive (MSSA) and –resistant (MRSA) strains. PBP4 is required for the synthesis of the highly cross-linked PG and, as shown in recent studies, is essential for the expression of β-lactam resistance in community-acquired strains (CA-MRSA). This protein has a septal localization that seems to be spatially and temporally regulated by an unknown intermediate of the wall teichoic acids (WTA) biosynthesis pathway. Therefore, if WTA synthesis is compromised, PBP4 becomes dispersed throughout the entire cell membrane. The aim of this project was to identify the WTA precursor responsible for the septal recruitment of PBP4. In order to do so, inducible mutants of *tarB* and *tarL* genes in the background of NCTCPBP4-YFP were constructed allowing for the study of PBP4 localization in the presence and absence of these specific *tar* genes.With this work we were able to show that the absence of TarB or TarL leads to the delocalization of PBP4, indicating that TarL or a protein/WTA precursor whose localization/synthesis is dependent on TarL is responsible for the recruitment of PBP4.

Keywords: *Staphylococcus aureus*; cell wall; β-lactam resistance; wall teichoic acids biosynthesis; penicillin-binding proteins, protein localization

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Introduction

Staphylococcus aureus as an antibiotic resistant pathogen.

The gram-positive cocci *Staphylococcus aureus* is a common commensal organism of the skin and mucosal surfaces, but it is also an important opportunistic pathogen responsible for a wide range of nosocomial and community-acquired infections, such as skin and ocular infections, pneumonia, septicemia, endocarditis and osteomyelitis (Archer, 1998; Diekema *et al.,* 2001). This organism lives as a persistent commensal on 20% of the human population, preferentially on the skin and nasopharynx, and it is intermittently carried by a further 60% of individuals (Edwards *et al.,* 2012; Foster, 2005). Colonization is normally asymptomatic, but clearly increases the risk for subsequent infection, as if the skin barrier or the mucous membranes are breached *S. aureus* can enter into the soft tissues and establish an invasive infection. Colonization also allows the transmission of *S. aureus* by skin-to-skin contact between individuals or contaminated objects (Archer, 1998; Miller and Diep, 2008; Wertheim *et al.,* 2005). The success of *S. aureus* as a virulent pathogen and its ability to cause a large spectrum of infections are due to the expression of several virulence factors, such as surface-attached proteins and secreted enzymes, that allow the adherence to and invasion of human tissues, impart resistance to innate immune defences and act as toxins (Archer, 1998; Edwards *et al.,* 2012; Gordon and Lowy, 2008).

Antibiotic resistance in *S. aureus* is also a serious health-care problem due to its remarkable ability to develop new mechanisms to resist the effects of antimicrobial agents. The introduction of the β-lactam penicillin in the early 1940s, the first effective drug against *S. aureus,* produced in 1928 by the Scottish microbiologist Alexander Fleming, dramatically improved the prognostic of patients with staphylococcal infections (Plord and Sherris, 1974). However, in 1942, as a consequence of the remarkable adaptive efficiency of *S. aureus,* penicillin-resistant staphylococci were recognized, first in hospitals and then in the community. By the late 1960s, more than 80 % of both community- and hospital-acquired staphylococcal isolates were resistant to penicillin (Lowy, 2003; Swoboda *et al.,* 2010; Swoboda *et al.,* 2009; Szweda *et al.,* 2012). The resistance of these strains was conferred by the presence of a plasmid containing the *blaZ* gene that encodes a β-lactamase (called first penicillinase), an extracellular enzyme synthetized when staphylococci are exposed to β-lactam antibiotics. The enzyme functions to hydrolyse the β-lactam ring of penicillin, thus rendering the antibiotic inactive (Lowy, 2003). In the sixties, a semisynthetic β-lactamase-resistant penicillin called methicillin was developed to treat the infections caused by these penicillin-resistant *S. aureus* strains (Barber, 1961; Parker and Hewitt, 1970). However, soon after methicillin therapy in hospitals began, methicillin resistant *Staphylococcus aureus* (MRSA) strains were isolated, initially from patients in a hospital in Colindale, UK. Through the late 1960s and early 1970s, MRSA strains were reported, with increasing frequency, in others countries all over the world, such as Australia, Belgium, Denmark, France, India, Poland, Switzerland and United States of America (Chambers, 1988; Jevons *et al.,* 1963; Lyon and Skurray,

1987; Szweda *et al.*, 2012). Nowadays, MRSA strains are one of the leading causes of nosocomial infections worldwide (Chambers and Deleo, 2009). Recent studies show that in the United States the number of deaths caused by MRSA infections is higher than those related to HIV/AIDS and tuberculosis combined (Boucher and Corey, 2008). Reports from The European Centre of Disease Prevention and Control (ECDC) show that in recent years the percentage of methicillin-resistant *S. aureus* isolates has increased dramatically. For example in Portugal more than 50% of isolates are now resistant to methicillin.

For the first three decades after their appearance, MRSA strains were known only as hospital-acquired pathogens (HA-MRSA). Then, in the early nineties, with an unpredicted epidemiological turn, MRSA strains also began to appear in the community among healthy people, who had no symptoms or risk factors for such infections. These strains, called community-acquired MRSA (CA-MRSA) (Okuma *et al.*, 2002; Rice, 2006; Saravolatz *et al.*, 1982), are less resistant to most antibiotics, other than β-lactams, but exhibit a major virulence potential, and are consequently capable of causing infections in healthy individuals (Szweda *et al.*, 2012). The spread of such a dangerous pathogen to the community is recognized as a disturbing reality and a huge concern in many countries. It also highlights the requirement for an increase in our knowledge about the resistance mechanisms in *S. aureus* to aid in the development of new therapies against these infections.

Cell wall biosynthesis and β-lactam resistance.

The cell wall, the external layer of bacterial cells, is very important for the integrity and viability of bacteria, as it provides physical protection, determines the cell shape and is the principal stress-bearing element, which makes it an ideal target for antibiotics (Scheffers and Pinho, 2005). In Gram-positive bacteria such as *S. aureus* the cell wall is composed of surface proteins, teichoic acids and a thick layer of peptidoglycan (PG). Peptidoglycan, also called murein, is a heteropolymer composed of long glycan chains, made up of alternating β-1,4-linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) subunits, which are cross-linked by flexible peptide bridges to form a strong but flexible structure (Beeby *et al.*, 2013; Scheffers and Pinho, 2005; Schleifer and Kandler, 1972; Szweda *et al.*, 2012). Peptidoglycan is present in almost all bacteria, except in *Mycoplasma* and a few other species that lack detectable cell walls. Attached to the carboxyl group of each MurNAc residue are stem peptides that, unlike glycan chains, have varying composition between different species. In *S. aureus* the stem peptides are composed of the sequentially added L-Alanine (L-Ala), D-Glutamic acid (D-Glu), L-Lysine (L-Lys), D-Alanine (D-Ala), D-Ala amino acids. The interpeptide bridges, created by the addition of five glycine residues to the L-Lys residue, allow for the cross-linking between different layers of PG (Kopp *et al.*, 1996; Schleifer and Kandler, 1972; Vollmer *et al.*, 2008).

Peptidoglycan synthesis is a major target of some of the most successful classes of antibiotics, including the β-lactams such as penicillin or methicillin (Popham, 2013). The biosynthesis of PG can be divided into three different stages, as shown in Figure1.1 (Heijenoort, 1998, 2001). The first stage

involves the cytoplasmic synthesis of the nucleotide sugar-linked precursors UDP-N-acetylmuramyl-pentapeptide (UDP-MurNAc-pentapeptide) and UDP-N-acetylglucosamine (UDP-GlcNAc). In the second stage, which takes place at the inner side of the cytoplasmic membrane, MraY transfers the phospho-MurNAc-pentapeptide moiety of UDP-MurNAc-pentapeptide to the membrane acceptor bactoprenol, generating lipid I [MurNAc-(pentapeptide)-pyrophosphoryl-undecaprenol]. MurG then promotes the β-1,4 linkage between UDP-GlcNAc and lipid I, yielding the final PG precursor, lipid II [GlcNAc-β- (1,4)-MurNAc- (pentapeptide)-pyrophosphoryl-undecaprenol]. Before its translocation to the outer side of the cytoplasmic membrane, the lipid II is modified by a family of peptidyltransferases (FemX, FemA and FemB), which promote the sequential addition of five glycines to the L-Lys residue, creating a pentaglycine bridge peptide for the cross-linking of PG in the cell wall. It has been proposed that the export of the fully modified PG lipid II precursor is catalyzed by a flippase (Roemer *et al.*, 2013; Typas *et al.*, 2012) . The third and final stage of PG biosynthesis, that takes place at the outer surface of the cytoplasmic membrane, consists on the polymerization of the newly synthesized disaccharide–peptide units and its incorporation into the growing PG, by elongation (transglycosylation) and peptide cross-linking (transpeptidation) between glycan strands (Heijenoort, 1998, 2001; Llarrull *et al.*, 2009; Scheffers and Pinho, 2005; Typas *et al.*, 2012; Vollmer *et al.*, 2008). These reactions, which occur mainly at the division septum of *S. aureus*, are catalyzed by the penicillin-binding proteins (PBPs) and monofunctional transglycosylases (Pinho and Errington, 2003). PBPs are membrane-associated proteins, anchored to the cytoplasmic membrane facing the extracellular surface, which can be classified as low-molecular-weight (LMW) and high-molecular-weight (HMW) proteins (Ghuysen, 1991; Goffin and Ghuysen, 1998). LMW PBPs are enzymes that only have a penicillin binding domain, that exhibit a DD- carboxypeptidase leading to the removal of terminal D-aminoacids from the PG muropeptides or transpeptidase activity leading to the formation of the cross-links between the peptides strands of PG. HMW PBPs are enzymes composed of two modules located on the outer side of cytoplasm membrane and an N-terminal anchored to the cytoplasmic membrane. The C-terminal is the penicillin binding domain, with transpetpidasse (TP) activity responsible for the cross-linking of the PG peptides. The N-terminal domain allows, depending on its primary structure and catalytic activity, the classification of HMW PBPs into two major classes: A and B (Ghuysen, 1991; Goffin and Ghuysen, 1998). The N-terminal domain of class A PBPs has a glycosyltransferase activity, catalyzing the elongation of glycan strands. The N-terminal domain of HMW class B PBPs have a non-penicillin-binding domain of unknown function, that has been suggested to have a role in cell morphogenesis (Scheffers and Pinho, 2005).

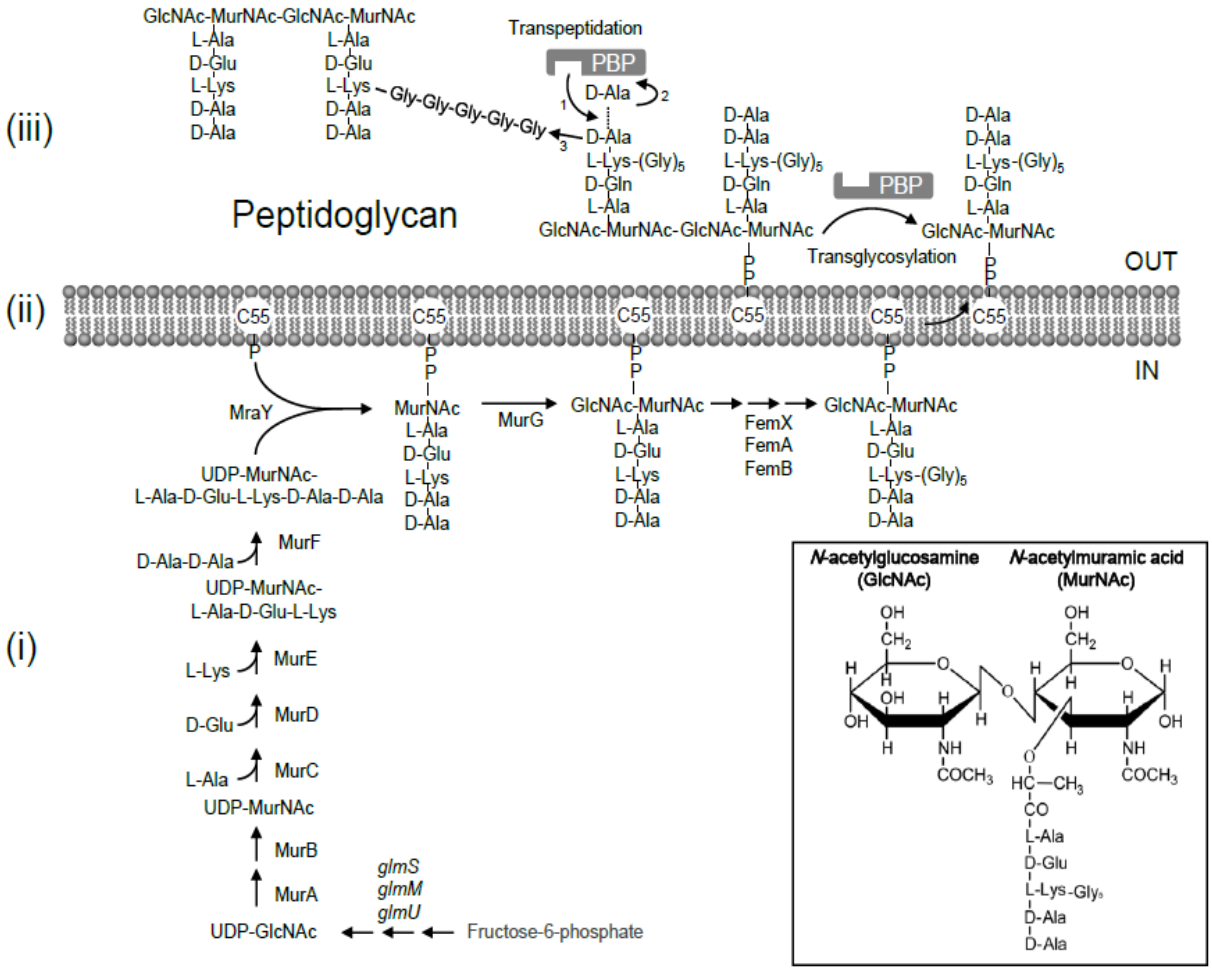


Figure 1.1. Cell wall biosynthesis in *S. aureus*. The image represents the three stages of cell wall synthesis: (i) cytoplasmic synthesis of the UDP-MurNAc-pentapeptide and the UDP-GlcNAc; (ii) inner membrane biosynthesis of the lipid II precursor and (iii) outer membrane polymerization of glycan chains and peptide crosslinking. The chemical structure of a muropeptide and the enzymes which catalyze each biosynthetic step are also represented (reproduced from Pinho (2008)).

Since their discovery as targets of β-lactam antibiotics, PBPs have been a key focus of antibacterial research. β-lactam antibiotics bind irreversibly to the transpeptidase active site of PBPs. Through the formation of an acyl-enzyme complex, they act as pseudosubstrates causing the inhibition of synthesis and cross-linking of PG, resulting in the weakening of the cell wall and leading to eventual cell lysis (Llarrull *et al.*, 2009; Zapun *et al.*, 2008). *S. aureus* have four native PBPs, PBP1-4 carried by both methicillin-sensitive and –resistant strains, to which most β-lactam antibiotics bind (Pereira *et al.*, 2009; Pinho *et al.*, 1998; Zapun *et al.*, 2008). The first three are HMW PBPs, while PBP4, a non-essential protein, is a LMW PBP that has transpeptidase activity performing secondary cross-linking of the PG and therefore leading to the high degree of cross-linking characteristic of the *S. aureus* PG (Leski and Tomasz, 2005; Memmi *et al.*, 2008). Recent studies have also shown that PBP4, is essential for the expression of β-lactam resistance in CA-MRSA (Memmi *et al.*, 2008). MRSA strains encode an

additional PBP, PBP2A, the expression of which is responsible for the resistance of these strains to β-lactam antibiotics. This enzyme is encoded by the *mecA* gene that is situated in the chromosome in a genomic island designated staphylococcal cassette chromosome *mec* (SCC*mec*) (Berger-Bächi *et al.*, 1992; de Lencastre *et al.*, 2007; de Lencastre and Tomasz, 1994; Verghese *et al.*, 2012). The *mecA* gene is not native to *S. aureus*, but was acquired by lateral transfer, possibly from others related organisms, like *Staphylococcus sciuri* or *Staphylococcus fleurettii* (Couto *et al.*, 1996; Crisostomo *et al.*, 2001; de Lencastre *et al.*, 2007). PBP2A has a remarkably low affinity for all β-lactams, and in their presence performs all of the transpeptidase activity, in cooperation with the glycosyltransferase activity of PBP2, ensuring continued cell wall synthesis (Pinho *et al.*, 2001a; Pinho *et al.*, 2001b; Pinho *et al.*, 1997).

Wall teichoic acid biosynthesis and β-lactam resistance.

In addition to peptidoglycan, an important class of cell surface glycopolymers in Gram-positive bacteria are the phosphate rich teichoic acids (TAs). These molecules play a role in a large variety of functions, such as in maintaining the physicochemical properties of the cell surface, cation homeostasis, resistance to antimicrobial peptides and lytic enzymes, acting as phage receptors, in cell division, biofilm formation and host adhesion (Figure 1.2). There are two types of TAs, distinguished by the way they are covalently linked to the surface, the lipo- teichoic acids (LTAs), which are anchored to the cytoplasmic membrane, extending from the cell into the peptidoglycan layer, and the wall teichoic acids (WTAs), which are covalently attached to the peptidoglycan layers and extend beyond them (Figure 1.2). Together, the LTAs and the WTAs, create a negative gradient that goes from the bacterial cell surface until the outer most layers of the PG (Morath *et al.*, 2005; Pasquina *et al.*, 2013; Swoboda *et al.*, 2010; Weidenmaier and Peschel, 2008).

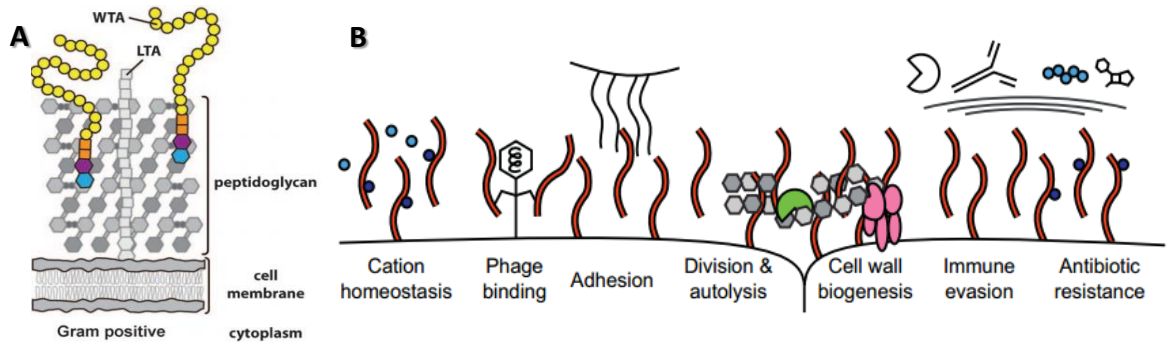


Figure 1.2. Simplified illustration of Gram-positive bacterial cell envelope and the TAs functions. **A)** Representation of the Gram-positive bacterial cell wall. This image does not show proteins, which are also an important element of the cell wall, in order to simplify the scheme. LTA: lipo-teichoic acid; WTA: wall teichoic acid. (Adapted from Swoboda et al. 2010); **B)** Representation of the functions of teichoic acid, which are involved in cell division, charge homeostasis and infection. (Adapted from Pasquina et al. 2013).

It has been shown that the expression of WTAs is critical for the pathogenicity of *S. aureus* strains, so a detailed study of WTA biosynthesis is important for a better understanding of their roles in bacterial physiology and to evaluate their potential as antibacterial targets (Weidenmaier *et al.,* 2005). The chemical structure of WTAs vary among Gram-positive bacteria, but the most common structures are composed of a β-(1,4)-linked N-acetylmannosamine (ManNAc) and N- acetylglucosamine (GlcNAc), attached by a phosphodiester linkage to the C6 hydroxyl of MurNAc residue of PG, followed by two glycerol phosphate units which are linked to a chain of glycerol- or ribitol phosphate repeats (Lazarevic *et al.,* 2002; Sanderson *et al.,* 1962). *S. aureus* WTAs contain polyribitol phosphate (poly-RboP) units with GlcNAc and cationic D-alanine esters substituents at their hydroxyl group (Figure 1.3) (Brown *et al.,* 2010; Weidenmaier and Peschel, 2008)

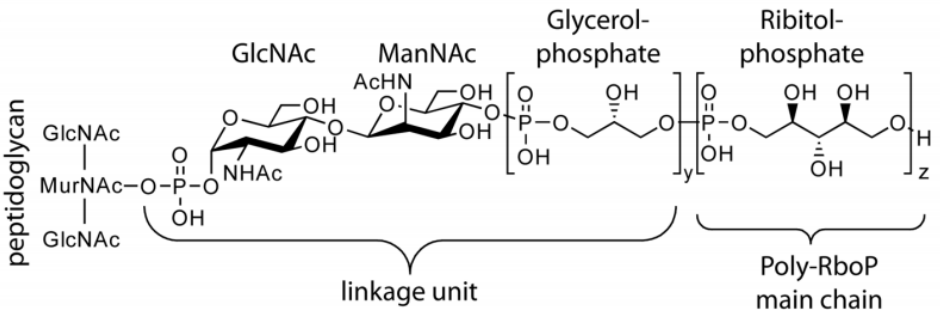


Figure 1.3. Chemical structure of wall teichoic acids (WTAs) in *Staphylococcus aureus*. RboP: ribitol-phosphate; y = 1–2, z = 20–40 (Adapted from Brown et al. 2010).

The biosynthesis of WTAs (shown in Figure 1.4) in *S. aureus* is catalysed by the *tar* genes (for teichoic acid ribitol) whose function has been established based mostly on sequence homology to the *tag* genes (for teichoic acid glycerol) involved in the production of WTAs of the well-studied model organism *Bacillus subtilis* (Lazarevic *et al.,* 2002; Qian *et al.,* 2006). This biosynthesis pathway begins in the cytoplasm, at the wall-membrane interface, with the transfer of GlcNAc-1-P from UDP-GlcNAc to the membrane-anchored undecaprenyl phosphate carrier lipid, an intermediate also used in the PG biosynthesis. This first step is a reversible reaction catalysed by TarO, which is a N-acetylglucosamine-1-phosphate transferase that belongs to the glycosyltransferase family, which also includes the enzyme MraY, required for PG biosynthesis (Anderson *et al.,* 1978; Brown *et al.,* 2008; Soldo *et al.,* 2002). The first irreversible step in WTA biosynthesis is catalysed by an N-acetylmannosaminyl transferase, TarA, that transfers a ManNAc residue from the UDP-ManNAc to the C4 hydroxyl of GlcNAc forming a β-linked disaccharide (Yokoyama *et al.,* 1989; Zhang *et al.,* 2006). Following the formation of the ManNAc(β1-4)GlcNAc disaccharide, the synthesis continues with the addition of two glycerol-3-phosphate units, by TarB and TarF glycerolphosphate transferases (Brown *et al.,* 2008). The glycerol-

3-phosphate derived from CDP-glycerol is a nucleotide-activated precursor of TarD, a cytidylyltransferase (Park *et al.,* 1993). In *S. aureus* the assembly of the WTA main chain (a poly-ribitol-5-P chain), requires a bi-functional poly-ribitol primase/polymerase, TarL, which transfers a single ribitol phosphate residue to the linkage unit and then attaches more than forty ribitol-5-P units to complete the polymer (Brown *et al.,* 2008; Meredith *et al.,* 2008). The ribitol-5-P is derived from CDP-Ribitol, in a reaction performed by the combined action of TarI, a cytidylyltransferase, and TarJ, an alcohol dehydrogenase (Pereira and Brown, 2004). All *S. aureus* strains contain an apparent duplication of the chromosomal region containing the *tarIJL* genes, this second set of genes is designated *tarI'J'K*. The significance of these duplications is still unclear, and it was already shown that the *tarK* gene is highly homologous to the *tarL* gene and consequently their encoded enzymes have similar functions. TarL has a polymerase function that catalyses the formation of a primary TarL-directed WTA polymer (L-WTA) while TarK it's a primase makes a secondary TarK-directed WTA polymer (K-WTA) (Meredith *et al.,* 2008; Pereira *et al.,* 2008; Swoboda *et al.,* 2010). The WTA glycosylation occurs in the cytoplasm, following polymer synthesis, through the addition of α-GlcNAc, by TarM, and β-GlcNAc, by TarS (Brown *et al.,* 2012; Xia *et al.,* 2010). The WTA polymer is then translocated to the external side of the membrane by the ABC transporter complex composed of TarH and TarG. This WTA transporter consists of an ATPase domain, the TarH, which provides the necessary energy to catalyse a conformational change in the transmembrane component, and a transmembrane domain, the TarG, a channel that facilitates the translocation across the membrane (Schirner *et al.,* 2011; Seeger and van Veen, 2009). Once the WTA polymer is outside of the cell, it has to be incorporated into the PG, by a phosphodiester linkage between the polymer and the C6 hydroxyl of the PG MurNAc residue. This reaction is catalysed by unknown proteins, presumably homologous to the TagTUV enzymes (Brown *et al.,* 2013). The D-alanylation of WTAs is another important mechanism, because it allows bacteria modulate their surface charge. This process, which occurs outside the cell, involves the attachment of D-alanine esters to WTAs and is catalysed by four enzymes encoded in the *dltABCD* operon (Kovacs *et al.,* 2006). Although this reaction is not completely understood, it is believed that the DltA, an D-alanyl carrier protein ligase, activates D-alanine as an AMP ester and then, with the help of the membrane-anchored DltD protein, transfers the aminoacyl adenylate to the carrier protein DltC (Heaton and Neuhaus, 1992, 1994). The DltB protein is an uncharacterized transmembrane protein of the membrane-bound-*O*-acetyltransferase (MBOAT) family, that has been suggested to be involved in the translocation of the D-alanine-charged DltC across the cytoplasmic membrane, where D-alanine is then transferred to the WTA backbone (Brown *et al.,* 2013). These final steps of the synthesis pathway are illustrated in Figure 1.5.

WTAs are not essential for *S. aureus* viability, since *tarO* and *tarA* can be deleted and the mutant strains survive (although their growth and virulence are impaired) (D'Elia *et al.,* 2006a). In contrast, the deletion of genes involved in downstream reactions of the WTAs biosynthesis pathway results in a lethal phenotype, indicating that these are conditionally essential genes. The lethal phenotype can be rescued

in a *ΔtarO* or *ΔtarA* background, suggesting that lethality can be due to the accumulation of toxic intermediates in the cell or depletion of cellular undecaprenyl phosphate, an intermediate shared with the PG biosynthesis (D'Elia *et al.*, 2006b; Swoboda *et al.*, 2010).

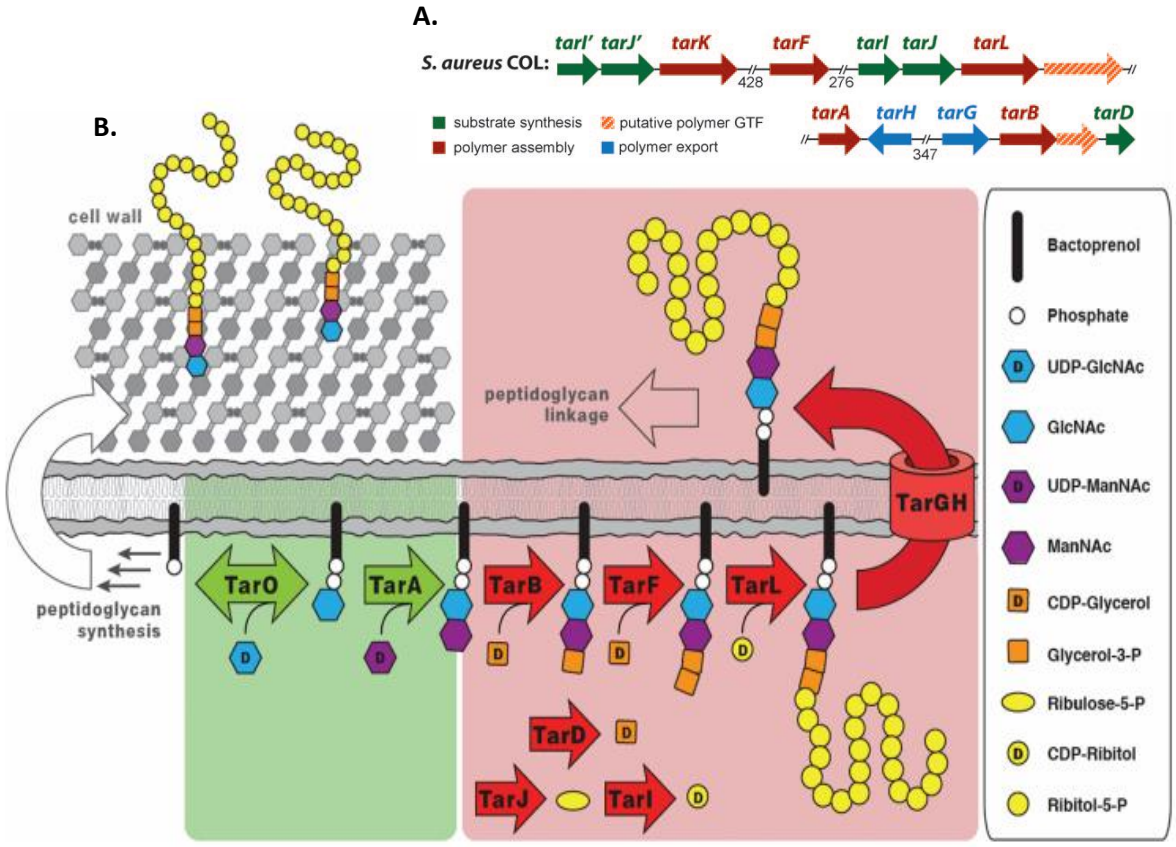


Figure 1.4. Genes and proteins involved in the primary *Staphylococcus aureus* WTA biosynthetic pathway. A) Genetic organization of wall teichoic acid biosynthetic genes in *S. aureus*; tar: teichoic acid ribitol (//: number of nucleic acids between genes if >120 base pairs); **B)** Depiction of the primary *S. aureus* WTA biosynthetic (L-WTA) pathway. After the intracellular production, the poly-ribitol-phosphate polymer is translocated to the outside of the membrane by a two-component ABC transporter, TarGH, and then incorporated into the PG. The green section represents the non-essential WTA pathway enzymes. Conditionally essential enzymes are coloured red, whose deletion is lethal in a wild-type background but permitted in a *ΔtarO* or *ΔtarA* background. Adapted from Swoboda et al. 2009 and Swoboda et al. 2010.

The role of WTA in β-lactam resistance of MRSA strains has remained elusive for a long time. In 1994, Maki *et al* identified the *llm* gene, through transposon insertional inactivation as playing an important role in methicillin resistance of MRSA strains. Although its molecular function was unknown, *llm* mutants had a profoundly restored β-lactam susceptibility in a wide range of MRSA clinical isolates studied (Maki *et al.*, 1994). Recent studies showed, by sequence comparison, that *llm* is the same as *tarO*, the gene encoding the first enzyme in wall teichoic acid (WTA) biosynthesis pathway in *S. aureus* (Campbell *et al.*, 2010).

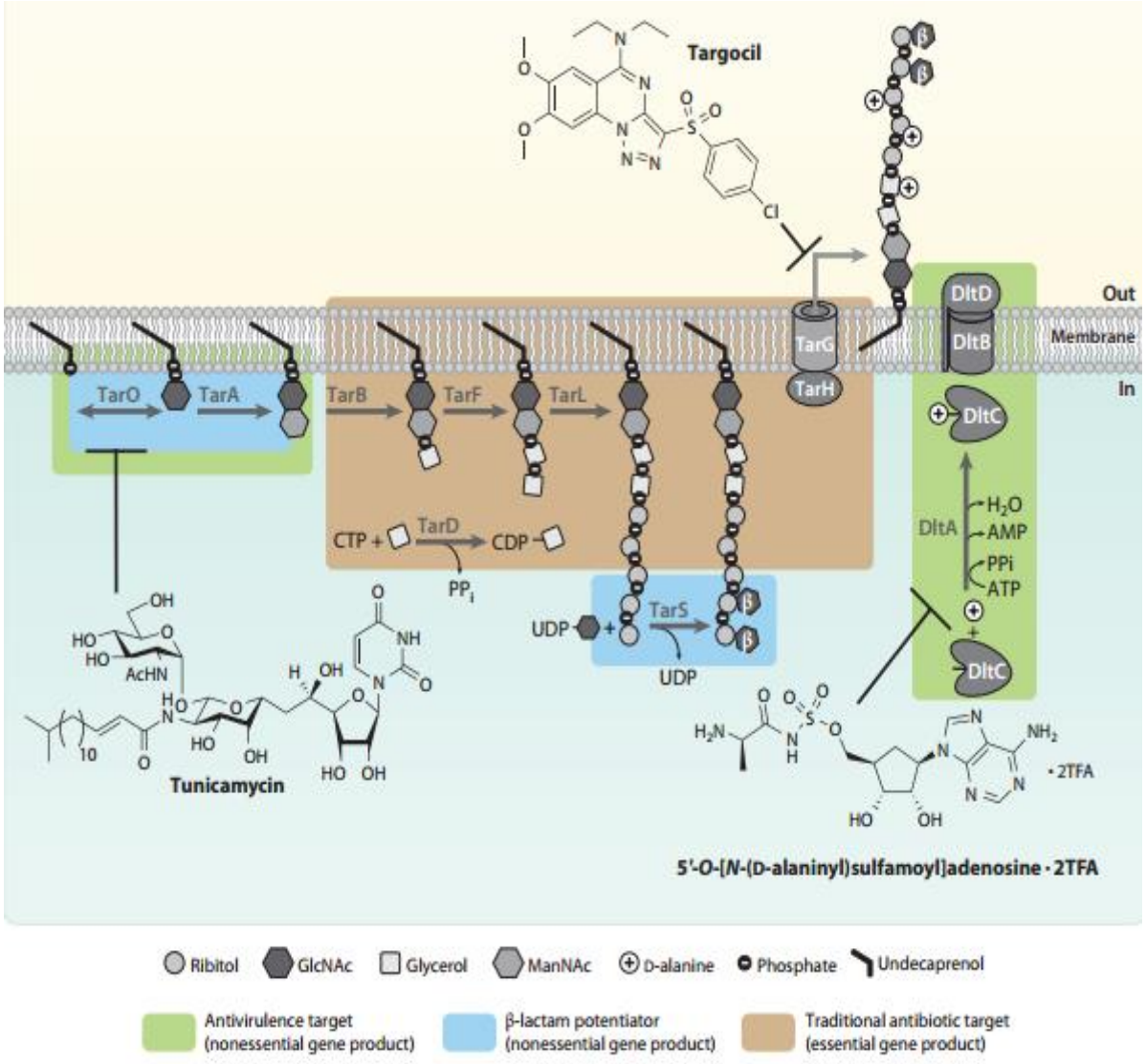


Figure 1.5. *Staphylococcus aureus* WTA biosynthetic pathway, with potential antibiotic targets. The image shows, in boxes with different colours, the three possible types of antibacterial targets in the *S. aureus* WTA pathway: traditional antibiotic targets (Brown), β-lactam potentiators (blue) and antivirulence antimicrobial targets (green). The three chemical structures represented are small molecules known to inhibit the WTA enzymes TarO, TarG, and DltA; GlcNAc: N-acetylglucosamine; ManNAc: N-acetylmannosamine; TFA: trifluoroacetic acid (Brown *et al.*, 2013).

The role of WTA in expression of β-lactam resistance was confirmed with the identification of drugs that targets WTA synthesis and have a synergistic effect with β-lactams. One of these drugs is tunicamycin, a naturally produced inhibitor of a family of enzymes that, in *S. aureus*, includes the TarO and MraY, an essential enzyme involved in PG biosynthesis (Campbell *et al.*, 2010; Campbell *et al.*, 2012). Although tunicamycin inhibits both enzymes, TarO is inhibited at much lower concentrations (Campbell *et al.*, 2010). The use of tunicamycin in conditions that specifically inhibit TarO has shown that the absence of WTAs caused MRSA strains to become more susceptible to β-lactams. Unfortunately, this compound is highly cytotoxic to mammals because it inhibits GPT, an essential phosphotransferase involved in eukaryotic N-linked glycan biosynthesis (Price and Tsvetanova, 2007; Roemer *et al.*, 2013).

A second drug that targets WTA synthesis is targocil, a synthetic small molecule that, through drug resistant mutant isolation, was shown to inhibit TarG, an essential subunit of the WTA ABC transporter (Swoboda *et al.*, 2009; Wang *et al.*, 2013). Resistance to targocil is achieved by loss-of-function mutations in *tarO* or *tarA*, given that in these conditions WTAs become dispensable, and the frequency of resistance (FOR) is high. However, when targocil is used in combination with oxacillin, β-lactam resistance of MRSA strains is impaired and the FOR for targocil mutants is greatly reduced (Campbell *et al.*, 2010; Lee *et al.*, 2010).These findings suggest that WTA inhibitors could work as β-lactam combination agents against MRSA (Roemer *et al.*, 2013; Wang *et al.*, 2013).Given that β-lactams are broad spectrum and safe and the most used class of antibiotics, the study and development of new therapeutic agents that restore β-lactam sensitivity to resistant microorganisms is of great importance (Brown *et al.*, 2013).

The WTA biosynthetic pathway is thus an important target for new antibacterial drugs to treat MRSA infections, given that different Tar enzymes can be considered antivirulence targets, essential targets and β-lactam potentiator targets (Figure 1.5) (Brown *et al.*, 2013). Antivirulence targets do not affect essential genes but disturb the pathogenicity of the cell. The enzymes of the *dlt* operon are an example of such targets, as strains without teichoic acid D-alanine esters are strongly attenuated in animal infection models and yet show minimal growth defects under laboratory growth conditions. In 2005, the 5'-O-[N- (D-alanyl)-sulfamoyl] adenosine molecule, was described as a DltA inhibitor, but remains to be optimized and is likely not specific (Brown *et al.*, 2013; May *et al.*, 2005).

Connection between WTA and PG biosynthesis in *S. aureus*

In 2010, J. Campbell and colleagues, showed that tunicamycin, which blocks the first and non-essential step in the WTA pathway, caused profound morphological defects, even though it did not significantly affect growth rates and had only a modest effect on gene expression (Campbell *et al.*, 2010; Campbell *et al.*, 2012). The morphological defects included aberrations in septal placement, a high frequency of duplicate septa and an inability to separate daughter cells following the completion of new septa. These defects demonstrate that WTAs play a fundamentally important role for properly coordinated cell division and suggest a link between PG and WTA biosynthesis (Campbell *et al.*, 2010).

In 2010 M. Atilano and colleagues discovered that WTAs modulate the degree of PG cross-linking by temporally and spatially regulating the recruitment of PBP4 to the site of cell-wall synthesis, the division septum (Atilano *et al.*, 2010). PBP4, the enzyme responsible for the high degree of PG cross-linking in *S. aureus*, localizes to the septum in wild type strains. However, in *ΔtarO* mutants, in which the level of PG cross-linking was shown to be severely decreased, the PBP4 protein no longer accumulates specifically at the septum, but instead is dispersed over the entire cell membrane. These observations suggested that the septal recruitment of PBP4 was dependent upon the synthesis of WTAs (Atilano *et al.*, 2010). The recruitment of PBP4 was shown not to occur via direct protein-protein interaction with TarO, which reinforces the idea that this recruitment is dependent of the septal synthesis of WTA. A delocalized PBP4 is unable to perform its function, a fact that may be due to the substrate being found only at the septum or to the lateral PG exhibiting a different structure to the septal PG, which may not allow the addition of further cross-links between the glycan strands (Atilano *et al.*, 2010). On the basis of these findings, the authors suggested a model, represented in the figure 1.6, in which the initial cell-wall synthetic machinery is recruited to the division septum in the early stages of its formation. TarO, together with others enzymes involved in WTA biosynthesis, are then recruited to the septum and initiate the WTA synthesis pathway, which functions as a temporal indication that early PG biosynthesis is complete and that PG can be further processed to become highly cross-linked. PBP4 subsequently arrives at the septum, where it catalyzes the last steps of PG synthesis, performing the high cross-linking of the PG mesh.

Importantly, it is likely that recruitment of PBP4 is mediated by an immature form of WTA corresponding to an intermediate of the WTA biosynthesis pathway, which is encountered only at the septum, since the fully synthesized/mature WTAs are present throughout the entire surface of *S. aureus* (Atilano *et al.*, 2010) but this intermediate remains unknown. The objective of this work is to answer to the question *"Which is the WTA precursor responsible for the septal recruitment of PBP4?"* by studying the localization of *S. aureus* PBP4 in presence and absence of specific *tar* genes. This question is of particular importance, not only to gain further insight into a fundamental process of the synthesis of the bacterial cell surface, but also due to the essential role of PBP4 in the expression of β-lactam resistance in CA-MRSA. Understanding how PBP4 localizes is required to fully understand its role in β-lactam resistance.

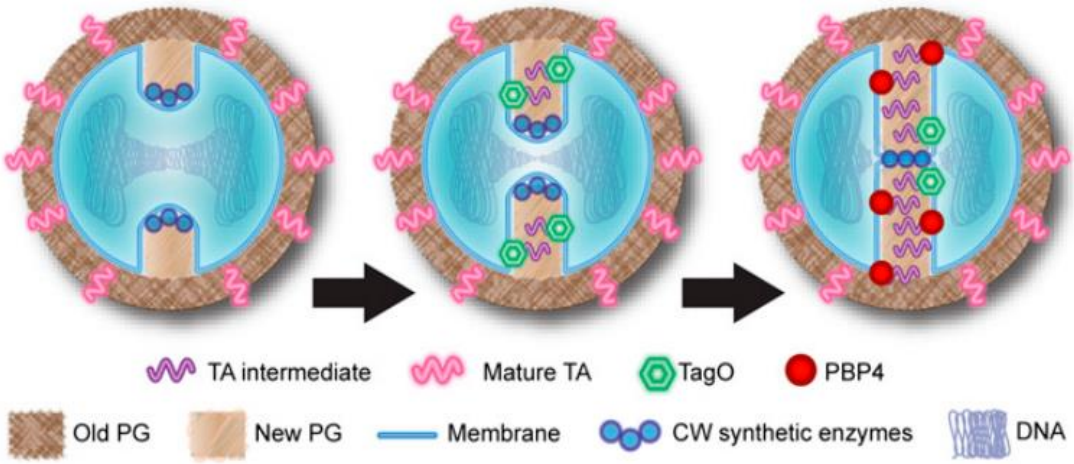


Figure 1.6. Model for the role of teichoic acids synthesis in PBP4 recruitment to the septum. The initial cell-wall synthetic machinery arrives to the division site, leading to the synthesis of new PG, with low levels of crosslinking (Left). TagO, and the remaining enzymes involved in WTA biosynthesis, are recruited to the septum, by an unknown mechanism, and there initiate the synthesis of intermediate molecules in TA biosynthesis (Centre). These intermediates (or another cellular components dependent on TA biosynthesis) function as a temporal and spatial cue for PBP4 recruitment to the division septum, allowing the synthesis of highly cross-linked PG to occur in a regulated manner (Right) (Atilano *et al.*, 2010)

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used and constructed during this study are listed in Tables 2.1 and 2.2. *E. coli* strain Dc10B was grown on Luria-Bertani agar (LA; Difco) or Luria-Bertani broth (LB; Difco) medium, supplemented with ampicillin (100 µg/ml) as required. *S. aureus* strains were grown at 37 ºC, with aeration, in tryptic soy broth medium (TSB; Difco) or in tryptic soy agar (TSA; Difco). The medium was supplemented, when required, with erythromycin 10µg/ml (Ery10; Sigma) and/or chloramphenicol 10 µg/ml (Cm10; Sigma), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside 100 µg/ml (X-Gal; Apollo Scientific) and isopropyl-D-thiogalactopyranoside (IPTG; Apollo Scientic).

General procedures

DNA purification and manipulation. In order to obtain *S. aureus* genomic DNA cells were incubated overnight on TSA plates at 37 ºC. Cells were scraped from confluent growth and re-suspended in 100 µl of 50 mM Ethylenediaminetetraacetic acid (EDTA). Lysostaphin 10 µg/mL (Sigma) and RNase 20 µg/mL (Sigma) were added to degrade the cell wall and RNA respectively, followed by 30 minutes incubation at 37ºC. 400 µL of 50 mM EDTA and 500 µl of Nuclei Lysis Solution (Promega) were added to cells and samples were incubated for 5 minutes at 80 ºC. The samples were then cooled to room temperature before the addition of 200 µl of Protein Precipitation Solution (Promega). Samples were vortexed vigorously then incubated on ice for 10 minutes. DNA was precipitated with isopropanol, washed with 70% ethanol and re-suspended in sterile water. Purified genomic DNA was used as template for the amplification of genes of interest via PCR reactions, using Phusion polymerase (Finnyzymes- Thermo Scientific Molecular Biology), following the manufacturer's instructions.

Plasmid DNA was purified from *E. coli* DC10B using the Wizard SV Plus Miniprep kit (Promega) according to the manufacturers protocols. All DNA digests were performed with fast restriction enzymes acquired from Fermentas- Thermo Scientific Molecular Biology, following the manufacturer's guidelines. DNA ligations were performed following standard molecular biology techniques using T4 DNA ligase (Fermentas). PCR colony screening was performed using GoTaq polymerase (Promega) and all clones were sequenced (Macrogen). All primers used are listed in Table 2.2.

***E. coli* transformation.** *E. coli* competent cells were prepared according to the Rubidium Chloride protocol as previously described (Sambrook 1989). In order to propagate the plasmid DNA of interest, 10 µl of ligated DNA or 1 µl of extracted plasmid DNA, was added to 50 µl of competent cells, incubated on ice for 15 minutes, incubated for 1 minute at 42 ºC, returned to ice for more 5 minutes and rescued

in 1 ml of LB. After 60 minutes incubation at 37 ºC with aeration, the cells were spreaded on LA plates containing ampicillin (100 µg/ml). Positive clones were identified by PCR colony screening. Plasmids were extracted and the insert sequenced.

***S. aureus* transformation.** RN4220 electro-competent cells were prepared as previously described (Kraemer & Iandolo, 1990). For transformation, 0.5µg of purified DNA were mixed with 50µl of RN4220 competent cells, transferred to a 0.2 cm BioRad Gene Pulser cuvette and incubated on ice for 5 minutes. Electroporation of the cells was performed in a gene pulser xcell (Bio-Rad) using the following conditions: 2.5 kV; 25 µF and 100Ω. Immediately after electroporation cells were rescued in 1 ml of TSB and incubated at 30 ºC for 2 hours with aeration, before plating on TSA supplemented with Ery10 (Sigma).

***S. aureus* transduction.** Transductions were performed using phage 80α as previously described (Oshida and Tomasz, 1992). In order to prepare the phage lysates, cells of the donor strain were scraped from plates and re-suspended in 1 ml of TSB containing 5 mM of $CaCl_2$. Serial dilutions of 80α phage to 10^{-7} were made in Phage Buffer ($MgSO_4$ 1mM, $CaCl_2$ 4 mM, Tris-HCl 50 mM pH 7.8, NaCl 5.9 g/L, gelatin 1 g/L). $CaCl_2$ was added to a final concentration of 5 mM to phage top agar (casamino acids 3 g/l, Difco; yeast extract 3 g/L, Difco; sodium chloride 5.9 g/L, Sigma; agar 5 g/L, Difco; pH 7.8) that was kept in the water-bath for 60 minutes at 45 ºC before being mixed with 10 µl of donor strain and 10 µl of each phage dilution. The mixtures were poured onto previously prepared plates of phage bottom agar (the same composition as the phage top agar but containing 15 g/L of agar) containing $CaCl_2$ 5 mM and incubated at 30 ºC overnight. To the plates showing confluent lysis phage buffer was added (3-4 ml) and incubated for 1 hour at 4 ºC, for the phage to be transferred to the phage buffer. The top agar and phage buffer were then collected to a 50 mL centrifuge tube, vortexed to disrupt the phage top agar and incubated at 4 ºC for 1 hour. The tubes were then centrifuged at 3000 rpm for 15 minutes at 4 ºC. The supernatant was recovered and filtered with a 0.45 µm sterile filter.

For transduction the cells of the recipient strain were scraped from confluent growth and re-suspended in 1 ml of TSB containing $CaCl_2$ 5mM. A volume of 100 µl of this cell suspension was mixed with a range of different volumes of phage lysate (0.1 µl, 1 µl, 10 µl, 100 µl) and 100 µl of phage buffer containing $CaCl_2$ 5 mM. A control sample in which no phage lysate was added was also prepared. The samples were incubated for 20 minutes at 30 ºC. The mixtures were then added to the 0.3 GL top agar (casaminoacids 3 g/L; yeast extract 3 g/L; NaCl 5.9 g/L; sodium lactate 60% syrup 3.3 ml/L, Sigma; glycerol 50%, 2 ml/L, Sigma; Tri-sodium citrate 0.5 g/L, Sigma; agar 7,5 g/L; pH 7.8) previously left in the water-bath for 60 minutes at 45 ºC. These samples were poured onto pre-prepared plates (used within an hour of preparation) containing a 10 mL layer of 0.3 GL bottom agar (the same as the 0.3 GL top agar but containing 15 g/L of agar) supplemented with 30 µg/mL of appropriate antibiotic and a 20 mL layer of 0.3 GL bottom agar without antibiotic. The plates were incubated for 48 hours at 30 ºC. When needed, the medium was supplemented with IPTG.

Table 2.1. Bacterial strains used and constructed in this study

Name	Relevant characteristics	Source or reference
E. coli		
DC10B	*E. coli* cloning strain, chromosomal genotype: F^-mcrA Δ (*mrr-hsd*RMS-*mcr*BC) Φ80d*lac*ZΔM15 Δ*lac*X74 *end*A1 *rec*A1 *deo*R Δ (*ara*, *leu*) 7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L λ^-	Lab stock
S. aureus		
RN4220	MSSA strain. Restriction-deficient derivative of *S. aureus* NCTC8325-4, which accepts foreign DNA.	R. Novick
RNpEzrA-CFP	RN4220 with integrated pEzrA-CFP plasmid encoding C-terminal EzrA-CFP fusion; Ery^r	(Pereira *et al.*, 2007)
NCTC8325-4	MSSA strain	R. Novick
NCTCPBP4-YFP	NCTC8325-4 with integrated pMad plasmid encoding a *pbp4-yfp* C-terminal fusion;	Lab stock
NCTC*Δpbp4*	NCTC8325-4 *pbp4* null mutant	Lab stock
NCTC*Δspa::tarB*	NCTC8325-4 *pbp4::pbp4-YFPΔspa::Pspac-tarB-lacI*	This study
NCTC*Δspa::tarL*	NCTC8325-4 *pbp4::pbp4-YFPΔspa::Pspac-tarL-lacI*	This study
NCTC*Δspa::tarBΔtarB*	NCTC8325-4 *pbp4::pbp4-YFPΔspa::Pspac-tarB-lacIΔtarB*	This study
NCTC*Δspa::tarLΔtarL*	NCTC8325-4 *pbp4::pbp4-YFPΔspa::Pspac-tarL-lacIΔtarL*	This study
NCTCΔspa::tarBi	NCTC8325-4 *pbp4::pbp4-YFPΔspa::Pspac-tarB-lacIΔtarB* $lacI^{mC}$; Cm^r	This study
NCTCΔspa::tarLi	NCTC8325-4 *pbp4::pbp4-YFPΔspa::Pspac-tarL-lacIΔtarL* $lacI^{mC}$; Cm^r	This study
NCTCEzrA-CFP	NCTC8325-4 with with integrated pEzrA-CFP plasmid encoding C-terminal EzrA-CFP fusion; Ery^r	Lab stock
NCTCΔspa::tarBi *EzrA-cfp*	NCTC8325-4 *pbp4::pbp4-YFPΔspa::Pspac-tarB-lacIΔtarB* $lacI^{mC}$ *ezrA::ezrA-cfp;* Cm^r; Ery^r	This study
NCTCΔspa::tarLi *EzrA-cfp*	NCTC8325-4 *pbp4::pbp4-YFPΔspa::Pspac-tarL-lacIΔtarL* $lacI^{mC}$ *ezrA::ezrA-cfp*; Cm^r; Ery^R	This study

abbreviations: Ery^r – Erythromycin resistant; Cm^r – Chloramphenicol resistant; $lacI^{mc}$ – cells expressing multiple copies of the *lac*I gene (encoded by pMGPII);

Table 2.2. Plasmids used and constructed in this study

Name	Relevant characteristics	Source or reference
pMAD	*E. coli – S. aureus* shuttle vector with a thermosensitive origin of replication for Gram-positive bacteria; Amp^r; Ery^r; $LacZ^+$	(Arnaud *et al.*, 2004)
pBCB13	pMAD derivative with up- and downstream regions of *spa* gene and *Pspac-lacI* region from pDH88; Amp^r, Ery^r	(Pereira *et al.*, 2010)
pMGPII	Plasmid encoding *lacI* gene; Cm^r	(Pinho *et al.*, 2001)
pEzrA-CFP	Plasmid encoding C-terminal EzrA-CFP fusion; Amp^r Ery^r	(Pereira *et al.*, 2010)
pBCB13*tarB*	pBCB13 derivative containing P*spac-tarB-lacI*	This study
pBCB13*tarL*	pBCB13 derivative containing P*spac-tarL-lacI*	This study
pMAD*tarB*KO	pMAD derivative containing the up-and downstream regions of *tarB*	This study
pMAD*tarL*KO	pMAD derivative containing the up-and downstream regions of *tarL*	This study

abbreviations: Amp^r – Ampicillin resistant; Ery^r – Erythromycin resistant; Cm^r – Chloramphenicol resistant; $lacI^{mc}$ – cells expressing multiple copies of the *lac*I gene (encoded by pMGPII);

Mutant construction

To investigate the localization of *S. aureus* PBP4 in presence and absence of specific *tar genes*, we constructed inducible mutants of these genes in the background of NCTC8325-4 PBP4-YFP. In order to construct an inducible mutant, a full copy of the interest gene was first placed in the *spa* locus under the control of the P*spac* promoter and, subsequently, while in the presence of IPTG, was deleted from its native chromosomal locus. Sequences of the primers used in this study are listed in Table 2.3.

Table 2.3. Primers used in this study

Primer Name	Primer Sequence (5'- 3')*
pSpaTarB3-P1	TACCCGGGACATATTAAGTTGGTG
pSpaTarB-P2	TACTCGAGTCAGTAGAACCACCATC
pTarB-KO-P1	ACGAGAATTCAGTGTGGTTTAATGGAATG
pTarB-KO-P2	GTCACCATCTTATCTATATAAATACACCAACTTAATATG
pTarB-KO-P3	AGTTGGTGTATTTATATAGATAAGATGGTGAC
pTarB-KO-P4	ACTGGATCCGCAGTTTATGGTCATCAATG
pTarB-KO-P5	ATGACGAAACCCCGCTAACC
pTarB-KO-P6	TGTCGTGTGCGTTACTGCTGGGTG
tarBchrom	TCAGAGTGGGTGTTTTGACAC
pSpaTarL-P1	ATTACCCGGGTGAAGCAGACCTGTC
pSpaTarL-P2	ATACTCGAGTACCTCTCCCACTTTGAC
pTarL-KO-P1	ACGAGAATTCAGTTGAATGGAGGAAG

Primer Name	Primer Sequence (5'- 3')*
pTarL-KO-P2	TGACTACTATATAAACCGTTAATTCATCC
pTarL-KO-P3	AGGATGAATTAACGGTTTATATAGTAGTCAAAGTGGGAGAG
pTarL-KO-P4	TCGCA GGATCC TCATGTTGGCTCACAATG
pTarL-KO-P5	TCACCAGAAGGAAGCATTGCACTG
pTarL-KO-P6	ACGCCACATTTCTAGGTTTACCTGG
tarLchrom	AGAAGATGGACAAGCGTCACAACG
pMADI	CTCCTCCGTAACAAATTGAGG
pMADII	CGTCATCTACCTGCCTGGAC
Spa_p1_BamHI	TGAGGATCCCCAGCTTGTTGTTGTCTTC
Spa_p4_NcoI	TGCAGTCCATGGTTGAAAAAGAAAAACATTTATTC
Pspac_p1_pDH88EcoRI	GCTGAATTCTTCTACACAGCCCAGTCCAGAC

* Underlined sequences correspond to restriction sites

Construction of a *tarB* inducible mutant. To clone the *tarB* gene, in the ectopic *spa* locus of *S. aureus* strain NCTCPBP4-YFP, under the control of the IPTG inducible/lacI-repressible P*spac* promoter (Yansura and Henner, 1984), the entire *tarB* gene, including the RBS sequence, was amplified by PCR from NCTC8325-4 genomic DNA using the primers pSpaTarB3-P1 and pSpaTarB-P2. The resulting PCR product was digested with SmaI and XhoI fast restriction enzymes and ligated into pBCB13 plasmid digested with the same enzymes, giving rise to pBCB13 *tarB*. *E. coli* DC10B competent cells were then transformed with this plasmid and after its purification, the insert in pBCB13*tarB* was confirmed by enzymatic digestion and sequencing. The plasmid pBCB13*tarB* was transferred to RN4220 by electroporation (selection with erythromycin) and subsequently transduced to NCTCPBP4-YFP using phage 80α as previously described (Oshida and Tomasz, 1992).

In order to integrate the pBCB13*tarB* plasmid into the chromosome, an erythromycin resistant colony was inoculated into fresh TSB containing Ery10 and incubated at 30 ºC overnight. The overnight culture was diluted 1:1000 into fresh TSB with Ery10, incubated at 30 ºC for 8 hours, then diluted again into the same media and incubated overnight at 43 ºC, a non-permissive temperature that prevents the plasmid replication due to the thermosensitive origin of replication and allows, in presence of erythromycin, the selection of recombinants in which the plasmid had integrated into the chromosome. The overnight culture was serially diluted and 100 µL of each of the 10^{-4}, 10^{-5} and 10^{-6} dilutions were plated on TSA containing Ery10 and X-GAL 100 µg/mL at 43 ºC. Several light blue colonies were chosen and re-streaked in the same conditions. The integration of pBCB13*tarB* plasmid into the chromosome can occur via the upstream or downstream regions of the gene encoded in the plasmid, so the integration by upstream region was confirmed by PCR using primers pMADII and spa_p4_NcoI, while the downstream region was confirmed using primers spa_p1_BamHI and pMADI. Two clones with the plasmid integrated into the chromosome, via the up and downstream regions, were inoculated

in TSB at 30 ºC overnight. The overnight culture was diluted 1:500 in the same conditions, incubated at 30 ºC for 8 hours, serially diluted (10^{-4}, 10^{-5} and 10^{-6}) and then plated on TSA containing X-GAL 100 µg/mL at 43 ºC. White colonies that represent candidates for the loss of the plasmid, were chosen and re-streaked on TSA X-GAL 100 µg/mL and TSA Ery10 X-GAL 100 µg/mL through replica plating. The white and erythromycin sensitive colonies were screened by PCR, to confirm the substitution of the *spa* gene by *tarB* using primers Pspac_p1_pDH88EcoRI and pSpaTarB-P2 and for the wild type phenotype (presence of *spa* gene in *spa* locus) using primers Spa_p1_BamHI and Spa_p4_NocI. The resulting strain, which has two copies of *tarB* gene, one in the native locus and the other in the *spa* locus under the control of *Pspac* promoter was named NCTC*Δspa::tarB*.

Subsequently, to delete *tarB* from its normal locus in the background of strain NCTC*Δspa::tarB*, a PCR fragment containing the upstream and downstream regions of the sequence, approximately 1 Kb each, were amplified from NCTC8325-4 genomic DNA, in two sequential PCR steps. First, the upstream region, that contains the upstream region of *tarB* until the start codon, as amplified using primers pTarB-KO-P1 and pTarB-KO-P2, and the downstream region, containing the downstream region of *tarB* including the 3´end, was amplified using the primers pTarB-KO-P3 and pTarB-KO-P4. These two amplified products were then purified and joined by an overlap PCR reaction, using primers pTarB-KO-P1 and pTarB-KO-P4. The final PCR product was digested with EcoRI and BamHI and cloned into pMAD plasmid, giving rise to pMAD*tarB*KO. The presence of the cloned insert was verified by enzymatic digestion and sequencing. The pMAD*tarB*KO plasmid was electroporated into RN4220 (selection with erythromycin), transduced to NCTC*Δspa::tarB* by phage transduction and subsequently, integrated and excised, as described above. The deletion of the *tarB* gene from the native locus was confirmed by PCR using primers pTarB-KO-P5 and pTarB-KO-P6, resulting in NCTC*Δspa::tarBΔtarB* strain.

The pMGPII plasmid (Pinho *et al.*, 2001), which encodes the *lacI* gene, was also transduced into NCTC*Δspa::tarBΔtarB,* to ensure tight regulation of *tarB* expression. The resultant strain was named NCTCΔspa::tarBi. As a control, we also transduced pEzrA-CFP into this strain, which resulted in NCTCΔspa::tarBi *EzrA-cfp* strain.

Construction of a *tarL* inducible mutant. The construction of this inducible mutant was performed as described above for the construction of *tarB* inducible mutant. The entire *tarL* gene, including the RBS sequence, was amplified by PCR from NCTC8325-4 genomic DNA using the primers pSpaTarL-P1 and pSpaTarL-P2, digested with SmaI and XhoI fast restriction enzymes and cloned into pBCB13 plasmid, giving rise to pBCB13*tarL*. The insert in pBCB13*tarL* was confirmed by enzymatic digestion and sequencing. The plasmid pBCB13*tarL* was electroporated into RN4220 (selection with erythromycin) and subsequently transduced to NCTCPBP4-YFP. The integration and excision of the plasmid into the chromosome was performed as described above, to check the integration by upstream region we made a PCR using primers pMADII and spa_p4_NcoI, while the downstream region was confirmed using

primers spa_p1_BamHI and pMADI. Substitution of the *spa* gene by *tarL* was confirmed by PCR colony screening using primers Pspac_p1_pDH88EcoRI and pSpaTarL-P2 and for the wild type phenotype using primers Spa_p1_BamHI and Spa_p4_NocI. The resulting strain, which has two copies of *tarL* gene, one in the native locus and the other in the ectopic *spa* locus under the control of P*spac* promoter was named NCTC*Δspa::tarL*.

Subsequently, to delete *tarL* from its native locus in the NCTC*Δspa::tarL* background, the pMAD*tarL*KO plasmid was transduced into this strain and, after an integration and excision events, the gene deletion was confirmed by PCR using primers pTarL-KO-P5 and pTarL-KO-P6, resulting in NCTC*Δspa::tarLΔtarL* strain, expressing a single copy of *tarL* from the *spa* locus, under the control of P*spac* promoter.

In order to ensure tight regulation of *tarL* expression the pMGPII plasmid, which expresses the P*spac* repressor *lacI*, was also transduced into NCTC*Δspa::tarLΔtarL* strain, giving rise to a new strain named NCTC*Δspa::tarLi*. As a control, we also transduced pEzrA-CFP into this last strain which resulted in NCTC*Δspa::tarLi EzrA-cfp* strain.

Growth analysis of *S. aureus* strains

The growth of the *S. aureus* strains was analyzed by measuring, at regular intervals, the optical density at 600nm (OD600nm) of the liquids cultures. For that, an overnight culture of parental strain NCTCPBP4-YFP was diluted (1:200) into fresh TSB media and incubated at 37 ºC with aeration, while the inducible mutants were grown overnight, in the same conditions, in TSB medium supplemented with 10 µg/ml of chloramphenicol (Cm10) and 0.5mM of IPTG, then the overnight cultures were harvested, washed three times with fresh TSB and re-inoculated (with a 1:200 dilution) in media with and without IPTG. The inducible mutants were also tested on solid media (TSA) supplemented with chloramphenicol 10 µg/ml (Cm10) with or without 0.5 mM IPTG.

Fluorescence Microscopy

S. aureus strains were grown overnight, in TSB at 37 ºC, with appropriate antibiotic selection and, the next day, were diluted (1:400) in 50 ml of fresh TSB supplemented with 0.5 mM IPTG and grown until OD600nm 0.2. Cultures were then harvested, washed three times with fresh TSB and split into two 25ml cultures of fresh TSB with and without IPTG. To visualize the localization of PBP4 and EzrA, cultures were incubated for at least one hour after the washes, and thereafter at regulated intervals we took the samples to be observed by fluorescence microscopy. For that the samples were centrifuged, re-suspended in 20 µl of 1X Phosphate Buffered Saline (PBS) and 1 µl was placed on a thin film of 1% agarose in 1X PBS. Fluorescence microscopy was performed using a Zeiss Axio Observer.Z1 microscope equipped with a Photometrics CoolSNAP HQ2 camera (Roper Scientific), using Metamorph software (Molecular devices). Analysis of fluorescence images was performed using Metamorph and ImageJ software.

Analysis of the expression of fluorescent proteins in *S. aureus*

In order to confirm whether the *pbp4*-YFP fusion protein was being cleaved in strains NCTCPBP4-YFP, NCTCΔspa::tarBi and NCTCΔspa::tarLi the length of the band relative to YFP was analysed by SDS-PAGE using a Fuji FLA 5100 laser scanner (Fuji Photo Film) to detect the fluorescent protein. For that purpose, the strains were grown overnight in TSB medium supplemented with appropriate antibiotics and 0.5 mM IPTG, when required. To prepare total protein extracts from each strain, the overnight cultures were diluted 1:200 into fresh TSB (supplemented with the same antibiotics) incubated at 37 ºC until an $O.D_{600nm}$ of 0.8**.** Cells were harvested by centrifugation, re-suspended in 1X PBS and disrupted with 250 µl glass beads in a Fast Prep FP120 (Thermo Electro Corporation). The protein extracts were separated from glass beads by centrifugation (4200 x g, 1 minute at 4 ºC)**.** The total protein content of the extracts was quantified by the Bradford method, using bovine serum albumin as a standard (BCA protein assay kit, Pierce) and equal amounts of protein, from each sample, were loaded in a 10% SDS-PAGE gel and separated at 120V. Gel images were acquired on a Fuji FLA 5100 laser scanner (Fuji Photo Film) using 473 nm laser for YFP.

Western blot analysis

To analyze if the *pbp4*-YFP fusion was being cleaved, western blots were performed using a polyclonal anti-PBP4 and anti-GFP antibody. The protein extracts of NCTC8325-4, NCTC*Δpbp4*, NCTCPBP4-YFP, NCTCΔspa::tarBi and NCTCΔspa::tarLi strains and the quantification of total protein content of the extracts were performed as described above. Equal amounts of protein, from each sample, were heated to 100 ºC for 5 minutes, loaded onto a 10% SDS-PAGE gel and separated at 120V. Proteins were then transferred to a Hybond-P Polyvinylidene fluoride (PVDF) membrane (GE Healthcare) using a semidry transfer cell (Bio-Rad) according to standard western blotting techniques (Burnette,W.N., 1980). The membranes were blocked with blocking buffer (PBS, 5% milk, 5% Tween 20), as previously described (Jonhson, D.A. *et al,* 1984), for 1 hour and, after washed three times the membranes with 0.5% of Tween 20 in PBS, were incubated with a polyclonal anti-PBP4 antibody (1/100 dilution in blocking buffer) or an anti-GFP antibody (1/500 dilution in blocking buffer) overnight at 4 ºC. The following day membranes were washed three times with 1 x PBS-T and incubated with secondary antibodies diluted 1/100000 in blocking buffer. The detection was performed using ECL Plus Western blotting detection system from Amersham according to the manufacturers guidelines.

Results

Construction of TarB and TarL inducible mutants

In order to study the localization of *S. aureus* PBP4 in the presence and absence of specific *tar* genes we constructed inducible mutants of these genes in the background of NCTCPBP4-YFP strain (Figure 3.1). For that purpose we replaced the *spa* gene by a full copy of the gene of interest, under the control of IPTG inducible / LacI repressible P*spac* promoter, and subsequently, while in the presence of IPTG, deleted the gene from its native chromosomal locus (Yansura and Henner, 1984).

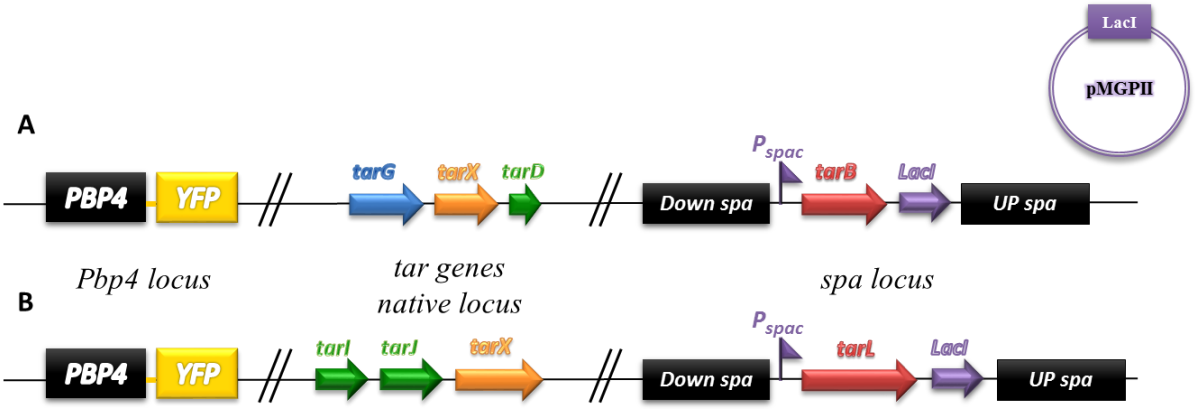


Figure 3.1. Schematic representation of the inducible mutant constructs. A. *S. aureus* strain with inducible *tarB* gene NCTC*Δspa::tarBi*; **B.** *S. aureus* strain with inducible *tarL* gene NCTC*Δspa::tarLi*; The *tarB* and *tarL* genes were cloned at the ectopic *spa* locus, under the control of the P*spac* promoter, and were subsequently deleted from their native loci. The pMGPII plasmid, encoding the LacI repressor protein was transduced into these strains in order to ensure tight regulation from P*spac*.

Most of the *tar genes*, involved in WTA biosynthesis, can not be deleted in a wild type *S. aureus* strain and are encoded within operons, as shown in the figure 1.4.A. Therefore deletion of genes such as *tarB* or *tarL*, can have lethal effects and their placement under the control of an inducible promoter at the wild type locus can have deleterious polar effects on downstream essential genes (Swoboda *et al.*, 2010). These facts were taken in account during the construction of the inducible mutant strains, NCTCΔ*spa::tarBi* and NCTCΔ*spa*::*tarL*i. For the construction of these strains, a copy of the *tarB* or *tarL* gene was placed in the *spa* locus under the control of P*spac*.The *lacI* gene, encoding the repressor protein LacI was also placed at the *spa* locus, to repress the P*spac* promoter. The *tarB* or *tarL* genes were then deleted from their native chromosomal locus. The process for placing the *tar* genes in the *spa* locus is shown in Figure 3.2. A similar process was used for their deletion from the native chromosomal locus, using the pMAD vector containing only the up and downstream regions of the gene of interest. Importantly, deletion of *tar* genes was performed in the presence of IPTG to induce expression of the essential gene from the P*spac* promoter, at the *spa* locus, and thus avoid cell damage or the appearance of suppressor mutations.

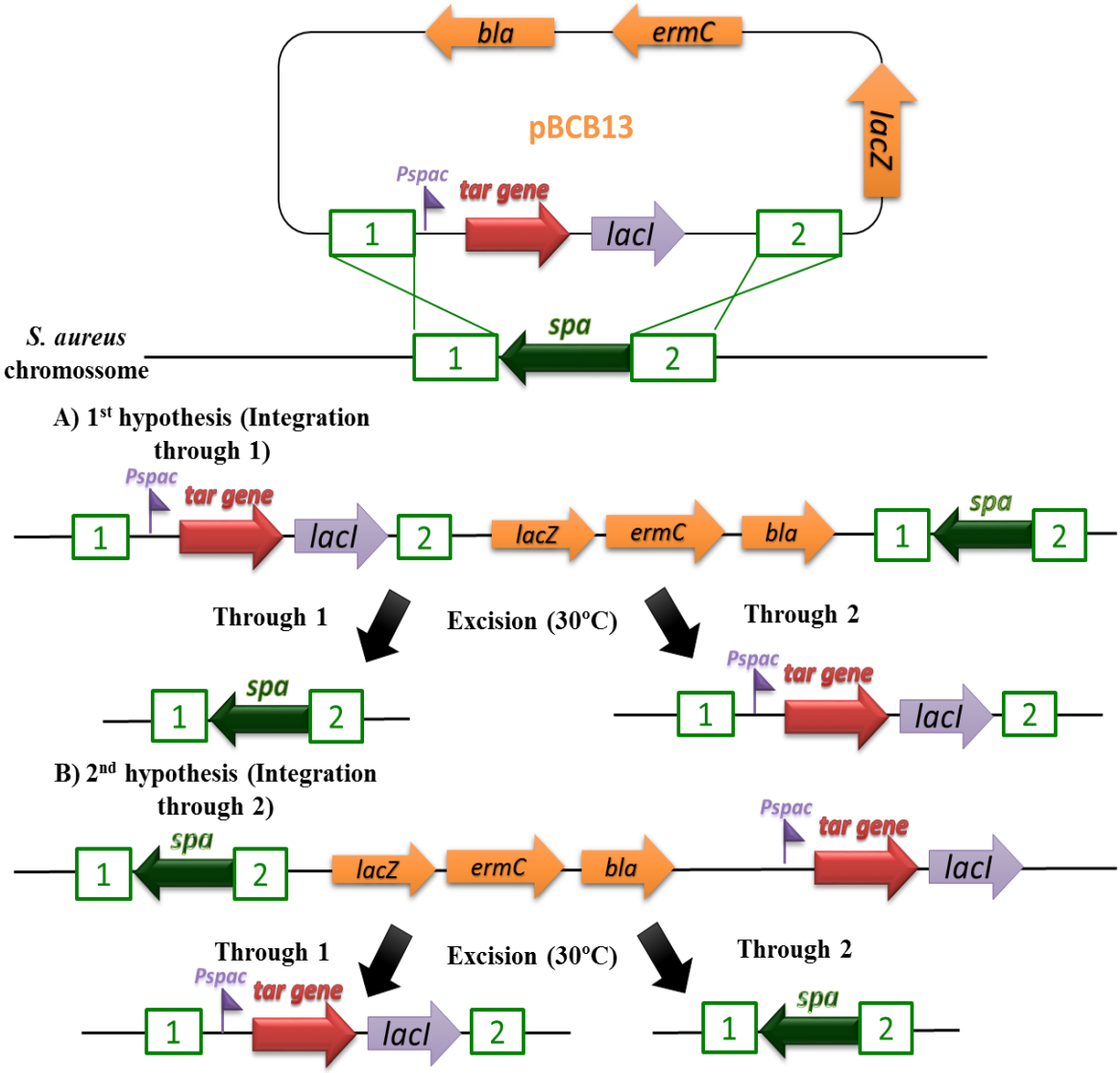


Figure 3.2. Schematic representation of the *spa* gene replacement by an gene of interest. This process, to place *tarB* or *tarL* under the control of P*spac* promoter in the *spa* locus, involves the integration and excision of a plasmid encoding the gene of interest and *lacI* between the up- and downstream regions of the *spa* gene, by homologous recombination, into the parental strain NCTCPBP4-YFP; **A.** Integration through the homologous region 1; **B.** Integration through the homologous region 2.

Although the inducible mutants have a copy of *lacI* in the *spa* locus, we transduced into the mutants the multicopy pMGPII plasmid (Pinho *et al.*, 2001b), which encodes the *lacI* gene, to ensure tight regulation of expression of the *tar* genes from the P*spac* promoter. It has been previously shown that, in *S. aureus*, expression of the *lacI* gene from a multicopy plasmid is required for the tight regulation of genes under the control of the P*spac* promoter (Jana *et al.*, 2000). The resulting strains NCTCΔspa::tarBi and NCTCΔspa::tarLi strains allowed for the study of the localization of PBP4 in the presence and absence of *tarB* and *tarL*, by growing them with and without IPTG, respectively. When the strains were plated on TSA in the presence of IPTG (and therefore in the presence of the *tar* gene) both strains displayed normal growth. In contrast, in the absence of IPTG, and thus the absence of TarB or TarL, cells failed to grow indicating the essentiality of these gene products for viability (Figure 3.3).

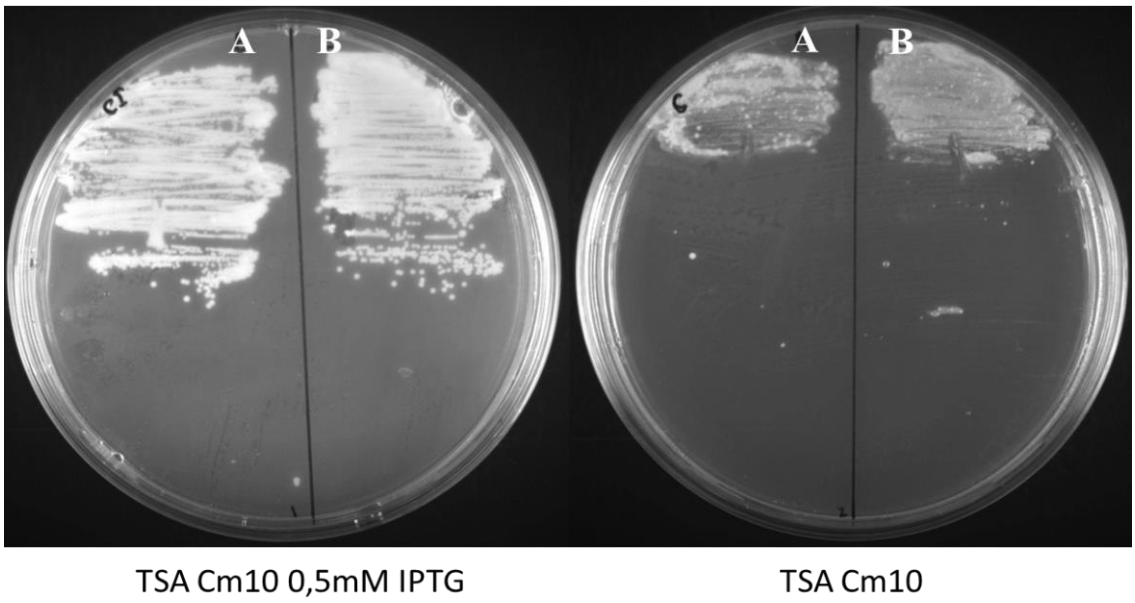


Figure 3.3. Growth of *S. aureus* in the presence or in the absence of TarB and TarL. A) *NCTCΔspa::tarBΔtarBi;* **B)** *NCTCΔspa::tarLΔtarLi;* The strains with *tarB* and *tarL* under control of theIPTG incudible P*spac* promoter were grown overnight at 37ºC on TSA plates with chloramphenicol (10 µg/mL) supplemented (left plate) or not (right plate) with 0.5 mM IPTG.

Growth of the inducible *tar* mutants was also analysed in liquid culture in the presence and absence of IPTG and compared with the parental strain NCTC*PBP4-YFP*, as shown in Figures 3.4 and 3.5. In the absence of IPTG, the NCTC*Δspa::tarBi* and NCTC*Δspa::tarLi* strains grow slower than the parental strain, NCTC*PBP4-YFP*. However in the presence of IPTG, even at low concentrations such 0.1 mM, the growth rates are like the parental strain. These observations show that the ectopic expression of *tarB* or *tarL* from the *spa* locus, in the presence of IPTG, enabled cells to recover the levels of these proteins and grow like the wild-type cells (Figure 3.4 and 3.5). As the results presented in Figures 3.4

and 3.5 show, in order to achieve total suppression of the P*spac* promoter a second dilution of the culture lacking IPTG was necessary.

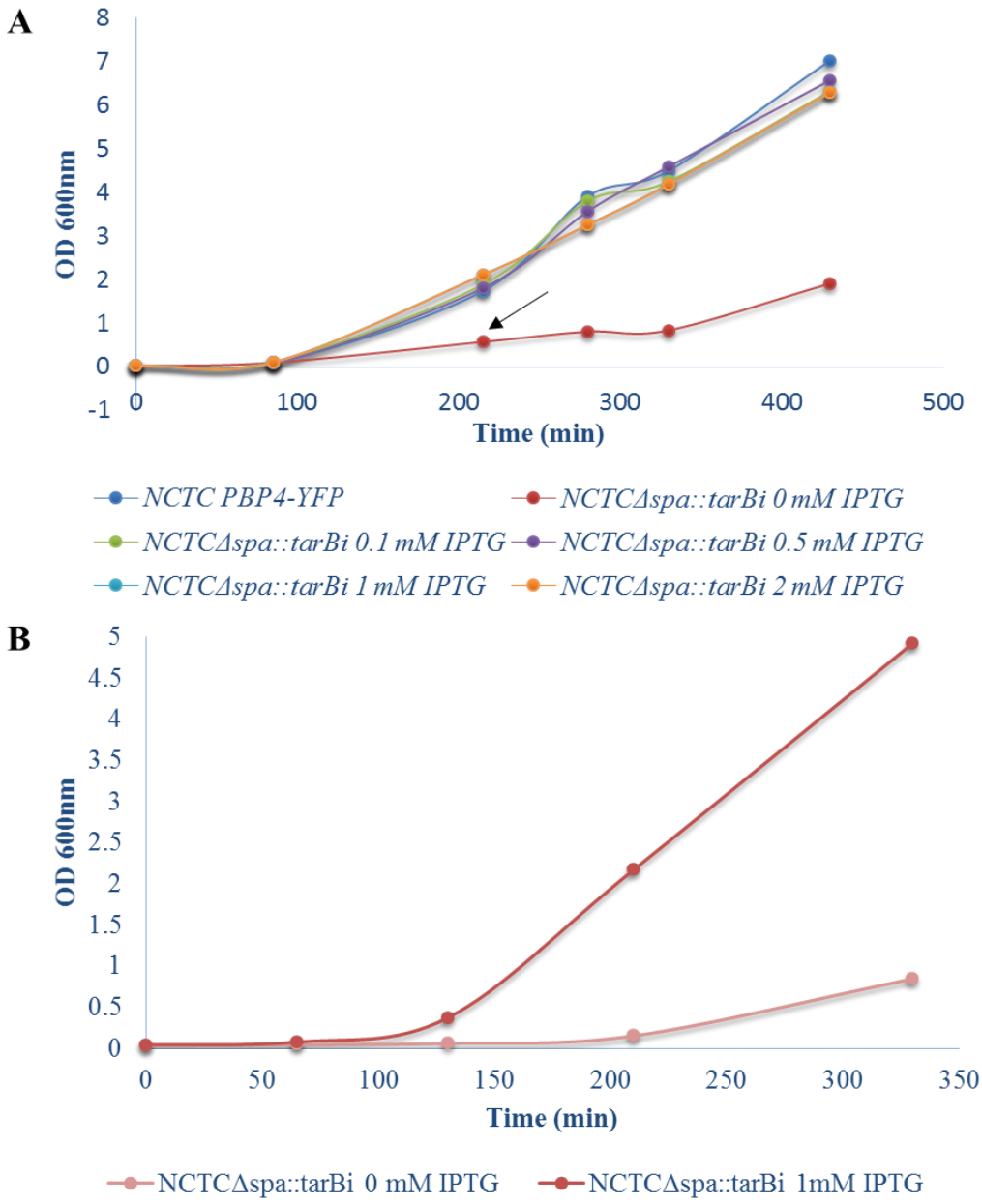


Figure 3.4. **Growth analysis of *NCTCΔspa::tarBi.*** The NCTC*Δspa::tarBi* culture was grown overnight in TSB, Cm 10 µg/mL and 0.5 mM IPTG at 37ºC, washed three times with TSB and re-inoculated in fresh TSB without IPTG or with 0.1, 0.25, 0.5, or 1 mM IPTG. Compared to the parental strain, NCTC*PBP4-YFP*, the *tarB* depletion affects growth of the inducible mutant, which is restored to parental like levels by the addition of IPTG. Panel **A.** shows the growth curves obtained through regular measurements of absorbance at $OD_{600nm.}$ The black arrow indicates the point at which a sample of NCTC*Δspa::tarBi*, without IPTG, was re-inoculated in fresh TSB without and with 1 mM IPTG, resulting in the growth curves shown in panel **B**.

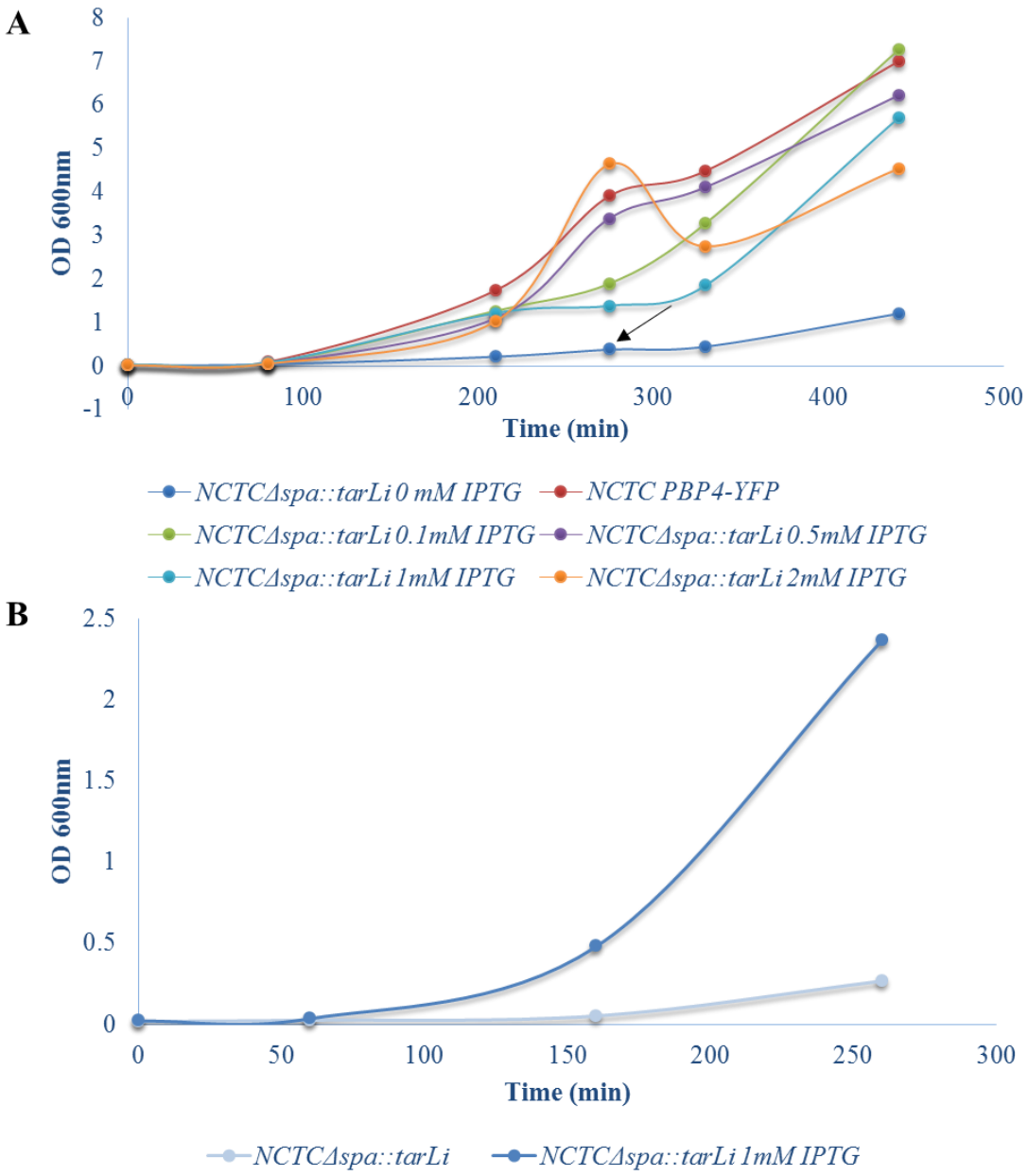


Figure 3.5. Growth analysis of NCTC*Δspa::tarLi*. The NCTC*Δspa::tarLi* culture was grown overnight in TSB, Cm 10 µg/mL and 0.5 mM IPTG at 37ºC, washed three times with TSB and re-inoculated in fresh TSB without IPTG or TSB with 0.1, 0.25, 0.5, or 1 mM IPTG. Compared to the parental strain, NCTC*PBP4-YFP*, the *tarL* depletion affects growth of the inducible mutant, which is restored to parental like levels by the addition of IPTG. Panel **A.** shows the growth curves obtained through regular measurements of absorbance at $OD_{600nm.}$ The black arrow indicates the point at which a sample of NCTC*Δspa::tarLi*, without IPTG, was re-inoculated in fresh TSB without and with 1 mM IPTG, resulting in the growth curves shown in panel **B**.

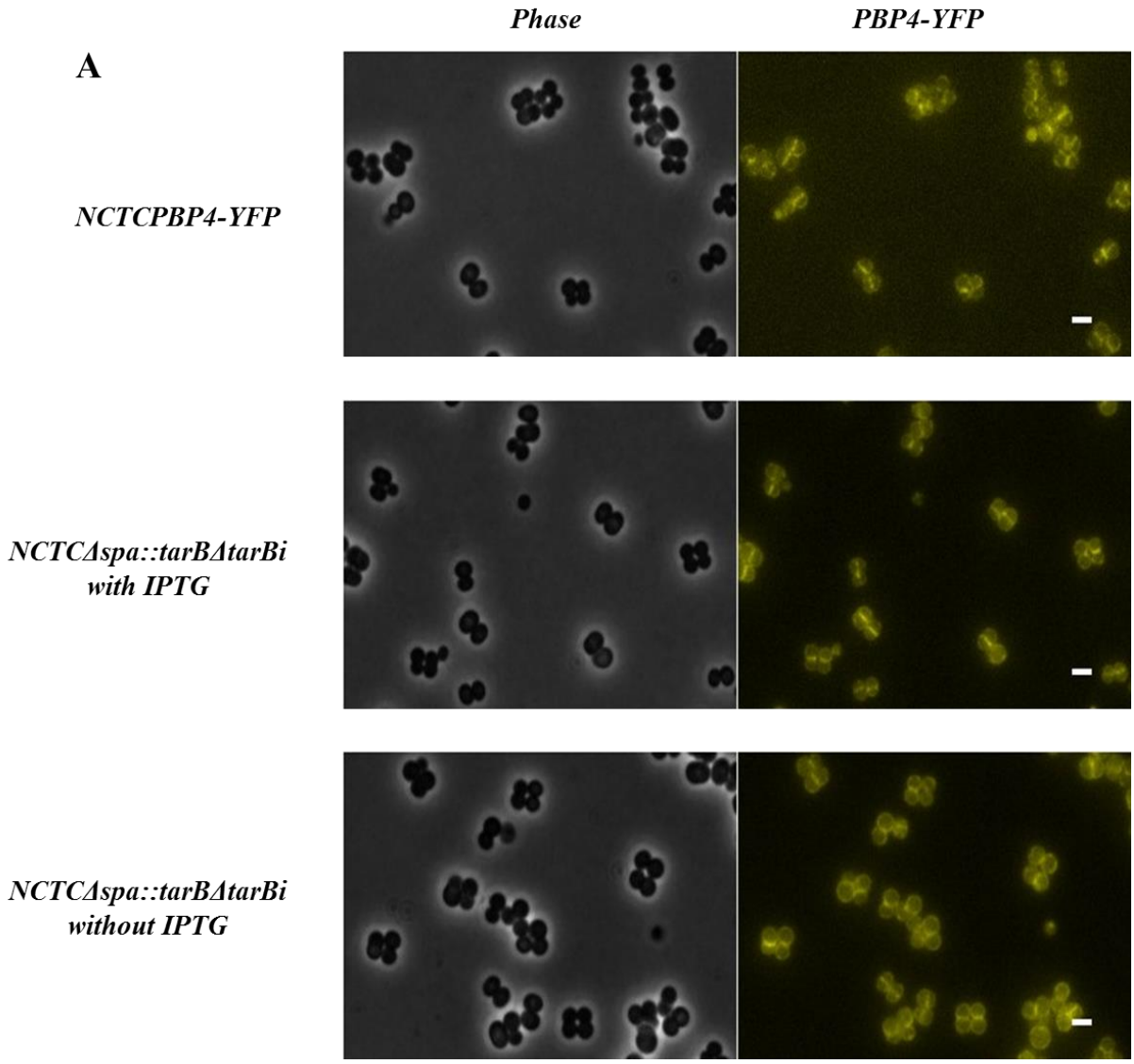
Then we grew the *S. aureus* strains (NCTC*PBP4-YFP,* NCTC*Δspa::tarBi* or NCTC*Δspa::tarLi* without and with several IPTG conditions) in liquid medium the cells tend to form clusters at cell densities corresponding to OD_{600nm} values of 1 or 2. This results in inaccurate OD_{600nm} values and, consequently, in fluctuations in the growth curves, as can be seen in figure 3.5.A. However this phenomenon did not affect our downstream experiments as cultures used for microscopy analysis hadOD_{600nm} values lower than 1.

Deletion of *tarB* or *tarL* leads to delocalization of PBP4.

In 2010 M. Atilano and colleagues discovered that the deletion of the *tarO* gene, the first gene in the WTAs synthesis pathway in *S. aureus*, resulted in the delocalization of PBP4. The recruitment of PBP4 to its normal septal location was shown not to occur via direct interaction between these two proteins, implying that PBP4 is likely recruited by an intermediate in WTA biosynthesis. Using the *tarB* and *tarL* inducible mutants described above, we wanted to test if PBP4 localization was dependent on intermediates that are downstream, in the WTA biosynthesis pathway, the reaction catalyzed by TarB and TarL.

Depletion of TarB causes delocalization of PBP4.

When the PBP4–YFP fusion was expressed in the NCTC parental strain NCTC*PBP4-YFP* and in the *tarB* inducible strain NCTC*Δspa::tarBi* in the presence of IPTG, it localized to the division septum (Figure 3.7) where cell-wall synthesis has been reported to take place in *S. aureus* (Atilano *et al.*, 2010; Pinho and Errington, 2003). However, when the same fusion was expressed in NCTC*Δspa::tarBi* in the absence of IPTG and thus depleted for TarB, PBP4 became delocalized, appearing all around the cellular membrane, with no specific accumulation at the division septum (Figure 3.6). To quantify the delocalization of PBP4 in the absence of the TarB protein, we calculated the ratio of fluorescence measured at the septum versus the fluorescence measured at the "lateral" wall. If the fluorescent protein is specifically accumulated at the division septum (which contains two membranes) then the fluorescence ratio should be higher than two, however if it is delocalized and homogeneously dispersed over the entire cell membrane, the intensity of the fluorescent signal at the septum should be approximately twice the fluorescence at the lateral membrane. When this ratio was calculated for PBP4–YFP in the parental strain NCTCPBP4-YFP we obtained an average value of 3.2±0.98 and a value of 3.3±1.07 for the NCTC*Δspa::tarBi* plus IPTG, a condition that allows the mutant strain to recover and grow like the parental strain. A value of 1.9±0.47 was obtained for the *tarB* inducible mutant NCTC*Δspa::tarBi* grown in the absence of IPTG, indicating that the absence of the TarB protein leads to a loss of the specific accumulation of PBP4 at the septum.


Phase
PBP4-YFP
A
NCTCPBP4-YFP
NCTCΔspa::tarBΔtarBi with IPTG
NCTCΔspa::tarBΔtarBi without IPTG

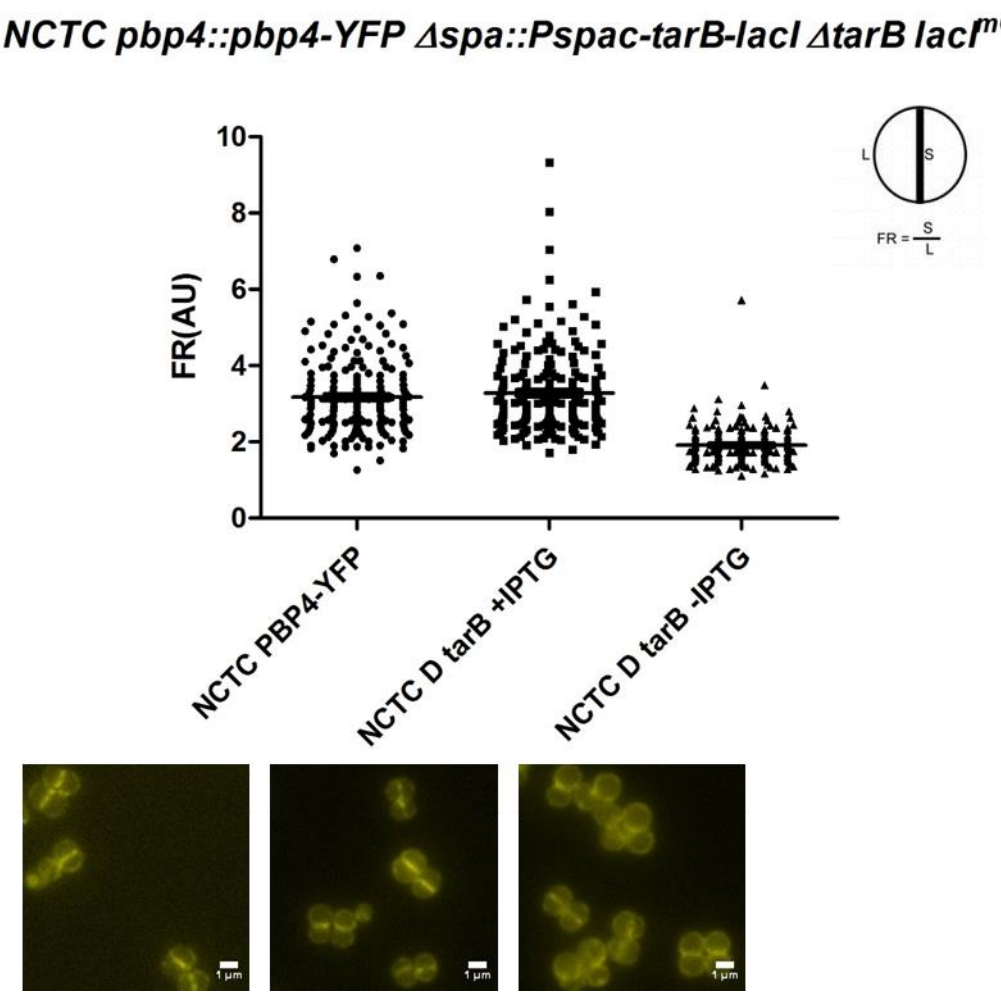
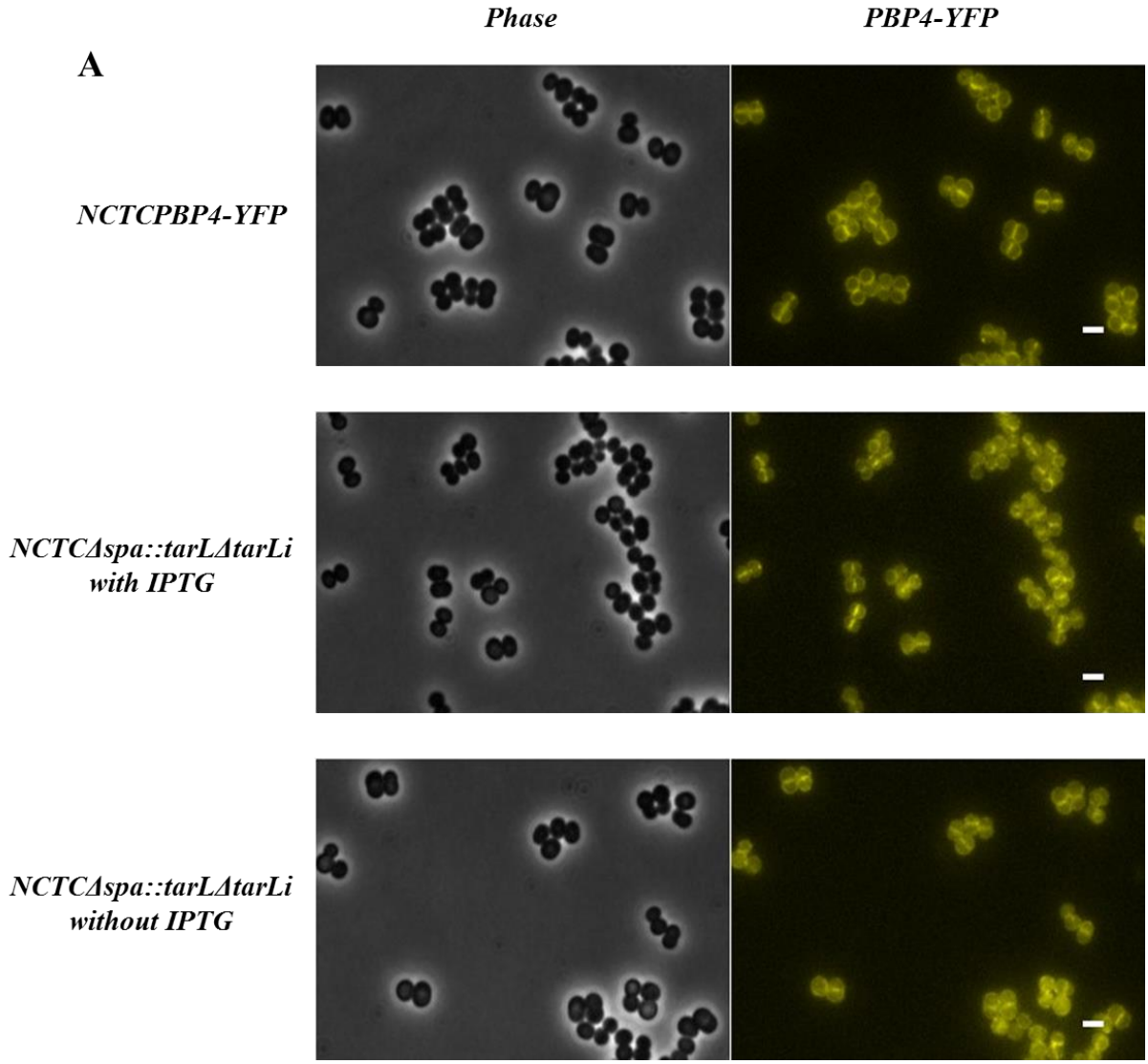


Figure 3.6. Septal localization of PBP4 is lost in absence of TarB in the NCTC*Δspa::tarBi* inducible strain. A) Microscopy images showing cells of NCTC*PBP4-YFP* and NCTC*Δspa::tarBi*, grown in the presence or absence of IPTG. Panels on the left show the phase-contrast image and panels on the right show the localization PBP4-YFP fusion; **B**) Quantification of septum (S) versus lateral membrane (L) fluorescence (fluorescence ratio, FR) of PBP4–YFP in a parental background (NCTCPBP4YFP), in a *tarB* inducible background (NCTC*Δspa::tarBi*) with or without IPTG. Quantification was performed in 200 cells displaying closed septa for each strain/condition. Horizontal lines correspond to average FR values. FR values above 2 indicate septal localization, whereas FR values equal to or under 2 indicate that the protein is distributed over the entire cell surface. $P < 0.001$. All images are false-coloured. Scale bar: 1µm.

Depletion of TarL causes delocalization of PBP4.

When the PBP4–YFP fusion was expressed in the NCTC parental strain, NCTC*PBP4-YFP*, and in the *tarL* inducible strain, NCTC*Δspa::tarLi,* in the presence of IPTG, it localized to the division septum (Figure 3.7) as expected (Atilano *et al.*, 2010; Pinho and Errington, 2003). However, when the same fusion was expressed in the strain NCTC*Δspa::tarLi* in the absence of IPTG (and therefore of TarL), PBP4 is delocalized, appearing all around the cellular membrane with no specific accumulation at the division septum (Figure 3.7). To quantify the delocalization of PBP4 in the absence of the TarL protein, we calculated the fluorescence ratio as descrived above, and obtained an average value of 3.1±0.98 for NCTCPBP4-YFP and of 3.7±1.39 for the NCTC*Δspa::tarLi* plus IPTG, whereas a value of 1.8±0.30 was obtained for the *tarL* inducible mutant NCTC*Δspa::tarLi*. These results indicate that the absence of the TarL protein leads to the delocalization of PBP4 from the septum.



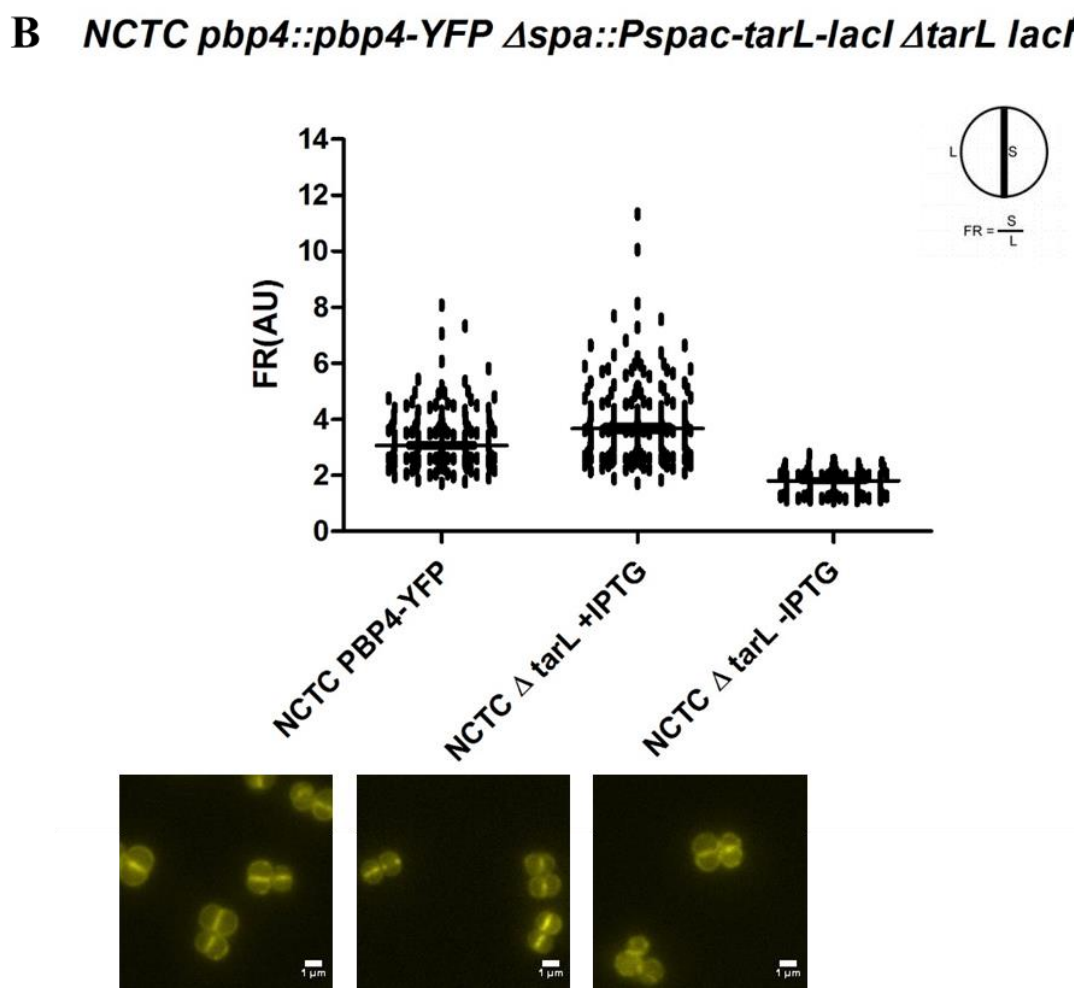


Figure 3.7. Septal localization of PBP4 is lost in absence of TarL in the NCTC*Δspa::tarLi* inducible strain. A) Microscopy images showing cells of NCTC*PBP4-YFP* and NCTC*Δspa::tarLi*, grown in the presence or absence of IPTG. Panels on the left show the phase-contrast image and panels on the right show the localization PBP4-YFP fusion; **B**) Quantification of septum (S) versus lateral membrane (L) fluorescence (fluorescence ratio, FR) of PBP4–YFP in a parental background (NCTCPBP4YFP), in a *tarL* inducible background (NCTC*Δspa::tarLi*) with or without IPTG. Quantification was performed in 200 cells displaying closed septa for each strain/condition. Horizontal lines correspond to average FR values. FR values above 2 indicate septal localization, whereas FR values equal to or under 2 indicate that the protein is distributed over the entire cell surface. $P < 0.001$. All images are false-coloured. Scale bar: 1µm.

Statistical analysis.

Statistical analysis was performed to assess the significance of the differences between PBP4 localization in the parental strain NCTCPBP4-YFP and in the inducible strains NCTC*Δspa::tarBi* and NCTC*Δspa::tarLi* grown in the presence of IPTG, and between these two strains grown in the presence or in the absence of IPTG. For that purpose, we performed the statistical significance tests Kruskal-Wallis and Dunn's Multiple Comparison tests, and calculated the *p*-value, with a confidence level of 0.001. The results obtained for the TarB mutant, presented in Figure 3.6, showed no significant difference between PBP4 localization in the parental strain and in the inducible strain grown in the presence of IPTG, however a significant difference was observed between *tarB* inducible mutant grown in the absence and in the presence of IPTG, indicating that lack of *tarB* causes delocalization of PBP4. Regarding TarL, a significant difference was also noticed between the inducible strain lacking *tarL* and the parental strain, indicating that lack of *tarL* also causes delocalization of PBP4 (Figure 3.7). However, in this case, a difference was also observed between the parental strain NCTCPBP4-YFP and the inducible strain NCTC*Δspa::tarLi* grown with IPTG, which surprisingly has a higher value for PBP4-YFP fluorescence in the septum than the parental strain.

The PBP4-YFP fusion is not cleaved.

In some of the microscopy images showing PBP4-YFP fluorescence many cells show a greater degree of cytoplasmic signal than that previously observed in the NCTCPBP4-YFP strain (Atilano *et al.*, 2010). One possible explanation for this signal would be the cleavage of the PBP4-YFP fusion. In order to address this and to be sure that the PBP4-YFP fusion is not degraded in our mutant strains we analysed the presence of the PBP4-YFP fusion in the NCTC*Δspa::tarBi* and NCTC*Δspa::tarLi* mutants strains by SDS-PAGE followed by imaging in a fluorescent image analyzer and western blotting, using a polyclonal anti-PBP4 antibody, as described in the materials and methods. The cell extracts of the wild type strain NCTC*8325-4*, the parental strain NCTCPBP4-YFP and the null mutant NCTC*Δpbp4* were used as controls. The results, shown in Figure 3.8, show that the PBP4-YFP fusion is not cleaved in the *tar* inducible strains, because only one band is present, corresponding to the molecular weight of this fusion (Figure 3.8 A) instead of one band with lower weight which would result from cleavage of the fusion protein. The western blots (Figure 3.8 B), show the presence of the PBP4 band only in the wild-type strain NCTC*8325-4* as expected. Additionally, one band corresponding to the PBP4-YFP fusion was observed for the NCTC*Δspa::tarBi* and NCTC*Δspa::tarLi* mutants strains.

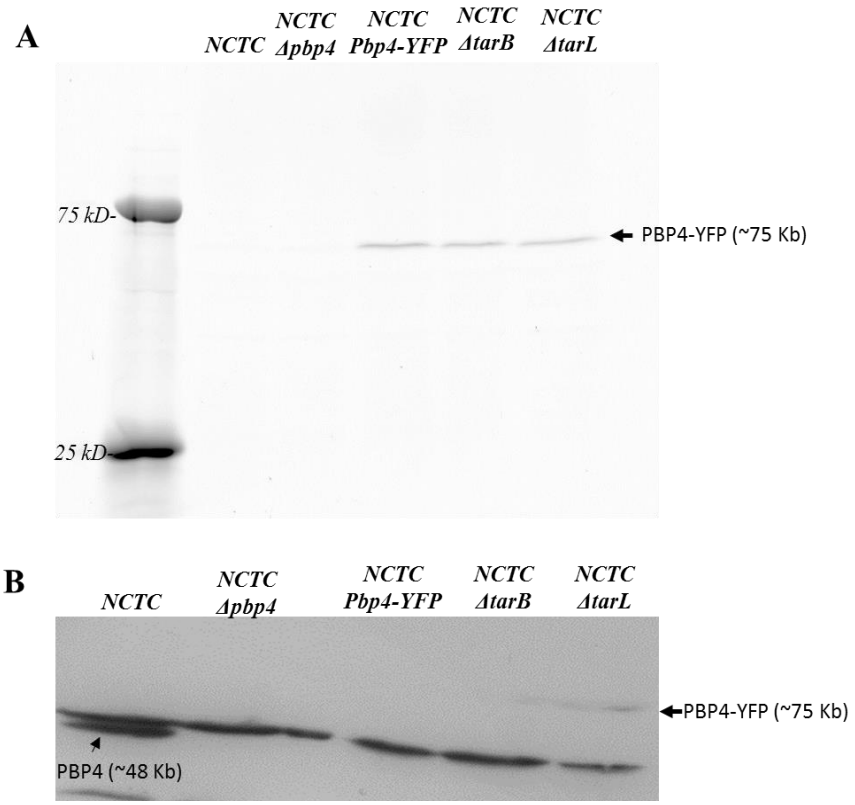


Figure 3.8. The PBP4-YFP fusion is not cleaved in NCTCPBP4-YFP, NCTC*Δspa::tarBi* and NCTC*Δspa::tarLi* strains. A) Analysis of protein bands by fluorescence imaging detected intact PBP4-YFP fusion in strains NCTCPBP4-YFP, NCTC*Δspa::tarBΔtarBi* and NCTC*Δspa::tarLΔtarLi*. **B)** Western blot analysis, using an anti-PBP4 specific antibody, of NCTC*8325-4*, NCTC*Δpbp4,* NCTCPBP4-YFP, NCTC*Δspa::tarBΔtarBi* and NCTC*Δspa::tarLΔtarLi* (from left to right). The band that appear in all strains, between the PBP4 band and PBP4-YFP band, is a nonspecific band, as it appears in the NCTC*Δpbp4* strain lacking PBP4.

Delocalization of PBP4 in the absence of TarL or Tar B is not due to cell death.

Bacterial cell division is a highly regulated process during which cells undergo a series of temporally and spatially controlled events that result in the generation of two identical daughter cells (Adams and Errington, 2009; Jorge *et al.*, 2011). In almost all bacteria, this process begins with the polymerization of a tubulin-like protein, FtsZ, into a ring-like structure located at the future division septum, which serves as a scaffold for the recruitment of other proteins that together form a multi-protein complex called the divisome (Adams and Errington, 2009). One of these proteins is EzrA, a transmembrane protein that acts as a negative regulator of Z-ring assembly, preventing FtsZ assembly at inappropriate locations different from the mid-cell (Jorge *et al.*, 2011). In *S. aureus*, EzrA localizes to the division septum in dividing cells (Jorge *et al.*, 2011).

When cells are unhealthy and begin to die, the divisome is not assembled and consequently there is a loss of septal localization of proteins involved in its formation (Jorge *et al.*, 2011). PBP4 normally has a septal localization, so in order to ensure that its delocalization observed in the mutant strains, NCTC*Δspa::tarBi* and NCTC*Δspa::tarLi*, is due to the absence of the Tar proteins and not due to cell death, we localized EzrA in the same strains. EzrA has previously been shown to delocalize from its normal septal location prior to cell death (Jorge *et al.*, 2011). For that purpose we transduced an integrative plasmid encoding EzrA-CFP to the *tarB* and *tarL* inducible mutants, resulting in the strains NCTCΔspa::tarBi *ezrA-cfp* and NCTCΔspa::tarLi *ezrA-cfp.* These new mutant strains allowed us to quantify the PBP4 and EzrA localization in the same cells and to determine if the delocalization of PBP4 was part of general protein delocalization upon cell death or if it was specifically due to lack of the TarL and TarB proteins. However, these strains displayed a distinct phenotype, even in the presence of IPTG and therefore of the Tar proteins forming clusters (shown in Figure 3.9) not previously seen in the strains lacking the EzrA-*cfp* fusion. This phenomenon limited our ability to correctly quantify the localization of PBP4 as in the cell aggregates it was difficult to measure the PBP4-YFP fluorescence at the septum and "lateral" wall. In contrast to the initial mutant strains, NCTC*Δspa::tarBi* and NCTC*Δspa::tarLi*, these strains have an additional erythromycin resistant marker. We studied the effect of expression of erythromycin resistance on the strains to determine whether it was responsible for the observed phenotype, perhaps interfering with cell growth and causing the formation of cell aggregates. As shown in figure 3.11 the presence or absence of erythromycin (10 ug/mL) had no effect upon the formation of cell aggregates in strains carrying the EzrA-CFP fusion. Given that the control strain, NCTC*EzrA-CFP*, does not show as many aggregates, this phenotype may be caused by the co-expression of the two fusion proteins (PBP4-YFP and EzrA-CFP) in the same cells. We therefore decided to analyse separately the effect of TarB and TarL depletion on ErzA and PBP4 localization, instead of doing the analysis in the same cells, as initially planned.

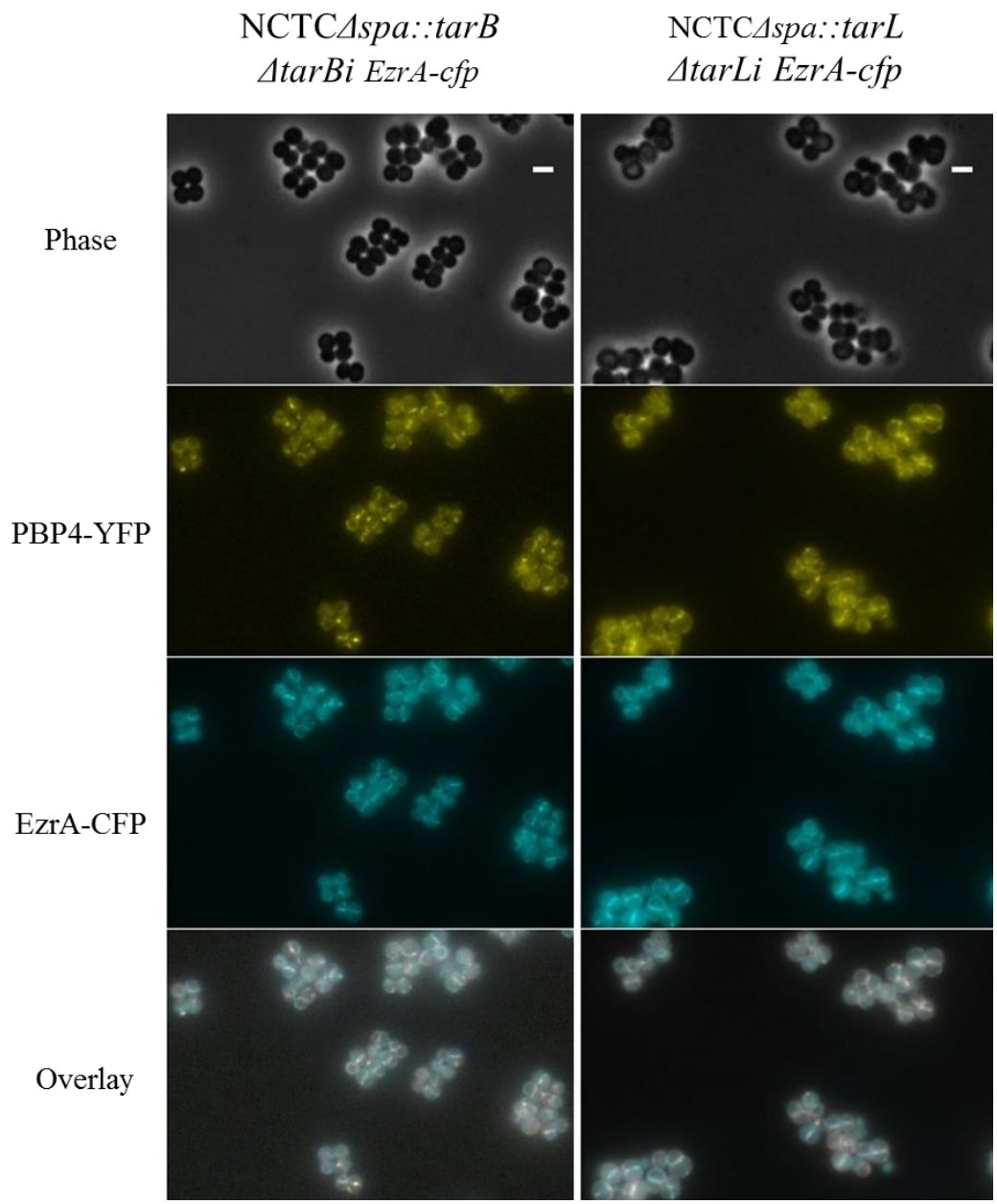


Figure 3.9. Strains NCTC*Δspa::tarBΔtarBi EzrA-cfp and* NCTC*Δspa::tar*L*Δtar*L*i EzrA-cfp* form aggregates. The microscopy images show cell aggregates of NCTC*Δspa::tarBΔtarBi EzrA-cfp* and NCTC*Δspa::tar*L*Δtar*L*i EzrA-cfp*in the presence of IPTG. Panels from top to the bottom show phase-contrast image, PBP4-YFP fusion fluorescence, EzrA-CFP fusion fluorescence and the overlay between PBP4-YFP and EzrA-CFP images. Scale bars 1 µm. All images are false-colored.

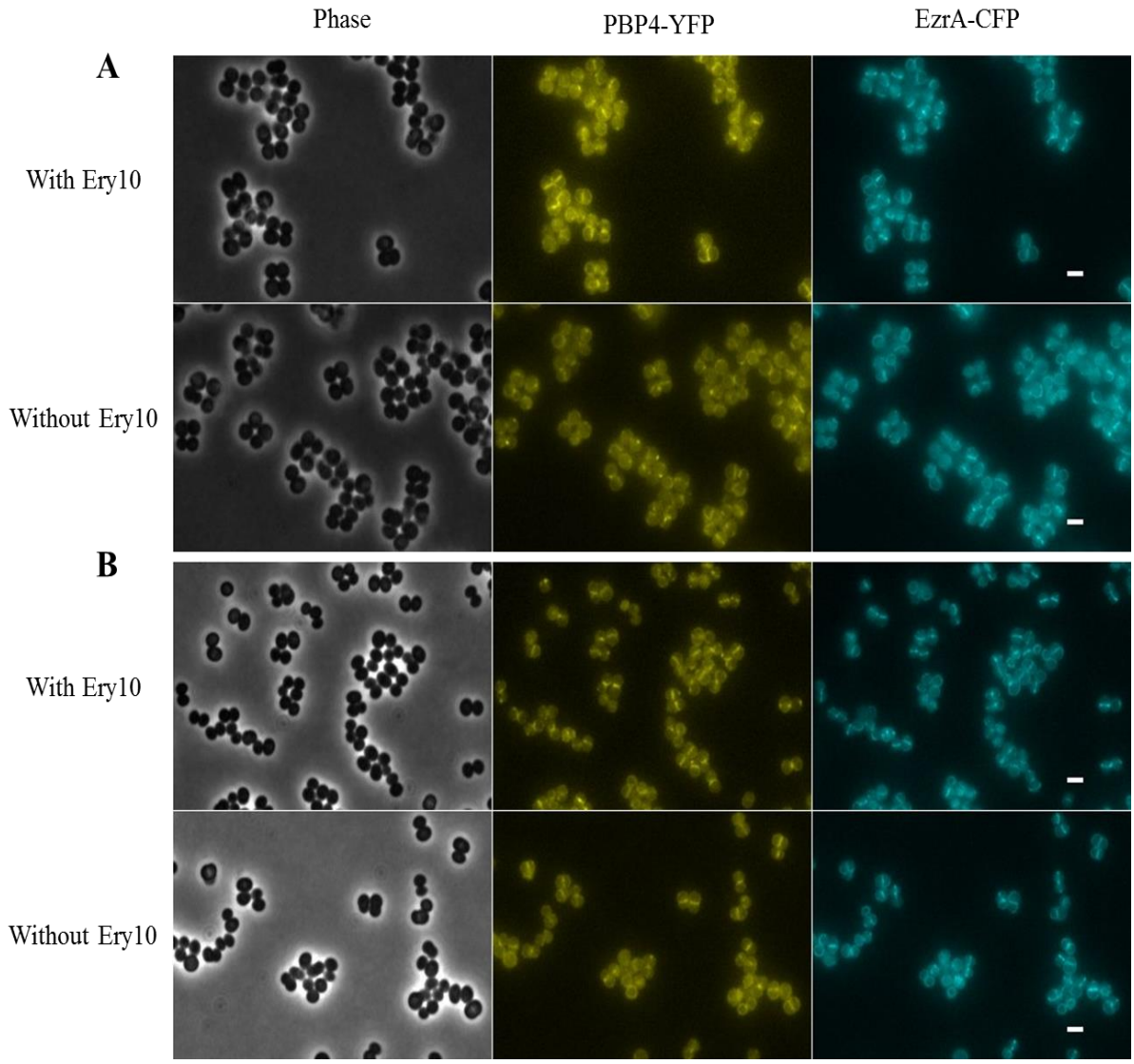


Figure 3.10. The presence of Erythromycin does not affect the formation of cell aggregates in *tarB* and *tar* inducible strains. The microscopy images show cells of NCTC*Δspa::tarBΔtarBi EzrA-cfp* **(A)** and NCTC*Δspa::tar*L*Δtar*L*i EzrA-cfp* **(B)** strains grown in the presence of IPTG and with or without Ery 10. Panels from left to right show phase-contrast image, PBP4-YFP fusion fluorescence, EzrA-CFP fusion fluorescence. Scale bars 1 µm. All images are false-colored.

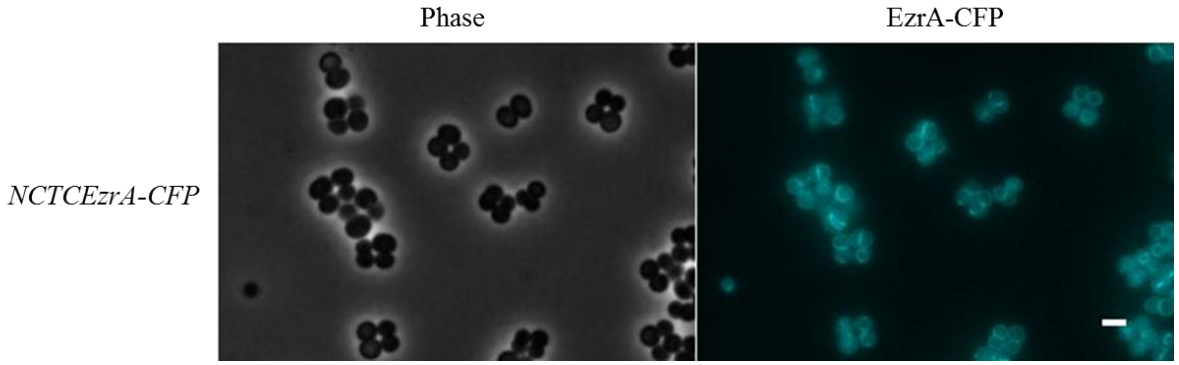
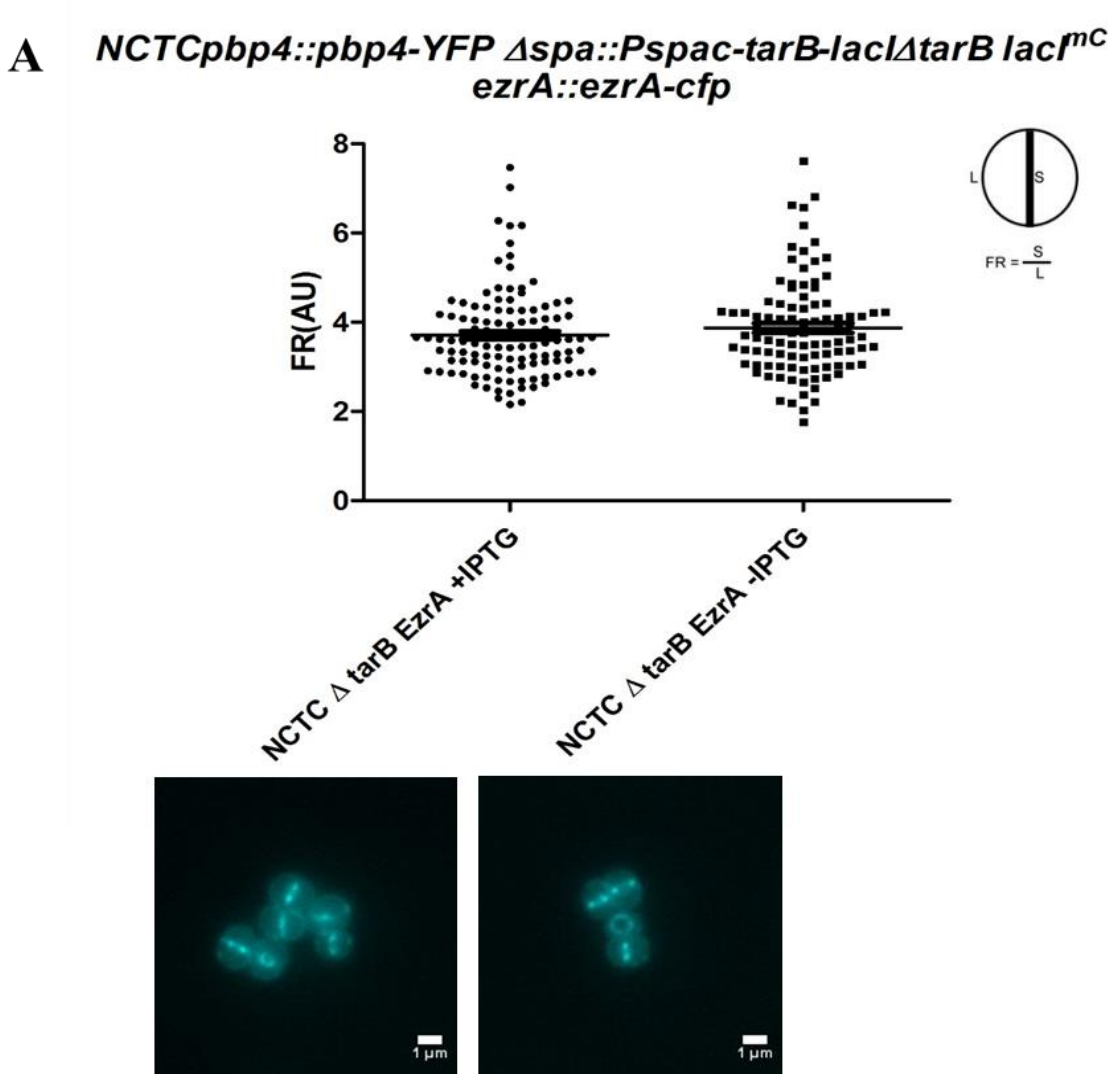


Figure 3.11. Morphology of NCTCEzrA-CFP cells. The microscopy images show cells of NCTCEzrA-CFP which do not form large clusters. Scale bars 1 µm. All images are false-colored.

When the EzrA-CFP fusion was expressed in the NCTC*Δspa::tarBi* and NCTC*Δspa::tarLi* strains, with and without IPTG the protein localized to the division septum (Figure 3.12) as expected (Jorge *et al.*, 2011). However, as stated above, when the PBP4-YFP fusion was expressed in the same background causing the formation of cell clusters we could not accurately quantify the localization of this protein. Therefore we used the strains NCTC*Δspa::tarBi EzrA-cfp* and NCTC*Δspa::tarLi EzrA-cfp* to quantify the localization of EzrA at the same time points at which we analysed PBP4-YFP localization in strains NCTC*Δspa::tarBi* and NCTC*Δspa::tarLi*. In this way we were able to determine whether delocalization of PBP4 occurs before or simultaneously with the delocalization of EzrA, the later of which would indicate that PBP4 could be delocalizing, not specifically due to lack of TarB or TarL, but rather as part of general protein delocalization in cells dying because of the lack of essential Tar proteins. To quantify the localization of EzrA in the presence and absence of the TarB and TarL proteins, we calculated the fluorescence ratio, obtaining average values of 3.7±0.98 and 3.9±1.08 for NCTCΔspa::tarBi *EzrA-cfp* strain in the presence and absence of IPTG, respectively, and average values of 4.2±1.28 and 4.2±1.34 for the NCTCΔspa::tarLi *EzrA-cfp* strain in the same conditions. These results indicate that, in the absence of the TarB or TarL proteins, EzrA remains localized to the septum, indicating that the divisome is correctly assembled and there is no general protein delocalization. Therefore, the observed delocalization of PBP4 was most likely due to lack of TarB and TarL.


A
NCTCpbp4::pbp4-YFP Δspa::Pspac-tarB-lacIΔtarB lacI^mC
ezrA::ezrA-cfp
FR(AU)
8
6
4
2
0
L
S
FR = S/L
NCTC Δ tarB EzrA +IPTG
NCTC Δ tarB EzrA -IPTG
1 µm
1 µm

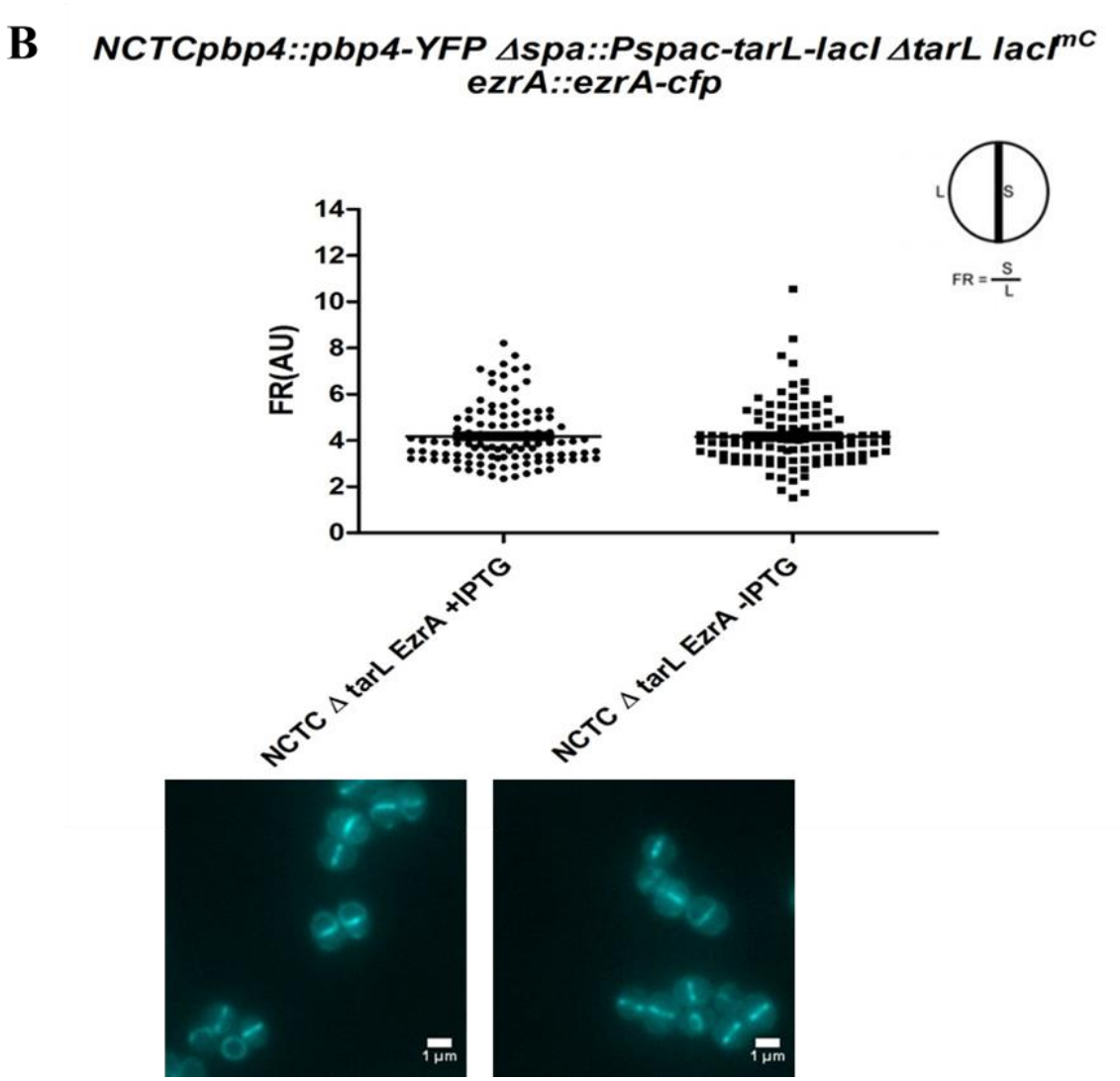


Figure 3.12. Septal localization of EzrA is not lost in the absence of TarB or TarL. Quantification of septum (S) versus lateral membrane (L) fluorescence (fluorescence ratio, FR) of EzrA in **A)** NCTC*Δspa::tarBΔtarBi EzrA-cfp* or **B)** NCTC*Δspa::tarLΔtarLi EzrA-cfp* strains in the presence or absence of IPTG, with the respective microscopy images. Quantification was performed in 100 cells displaying closed septa for each strain. Horizontal lines correspond to average FR values. FR values above 2 indicate a preferential septal localization, whereas FR values equal to or under 2 indicate that a protein is distributed over the entire cell surface. $P< 0.001$. All images are false-colored. Scale bar: 1µm.

Statistical analyses were performed to assess if the differences between the quantifications of EzrA-CFP localization in the strains NCTCΔspa::tarBi *EzrA-cfp* and NCTCΔspa::tarLi *EzrA-cfp*, with and without IPTG were significant. Mann Whitney tests were performed and the *p*-values calculated, with a confidence level of 99.9%. The results obtained for the TarB and TarL mutants (Figures 3.11 and 3.12) showed that a significant difference did not exist indicating that EzrA-Cfp localization remained unchanged in upon depletion of either of the Tar proteins.

Discussion

The cell wall is very important for the integrity and viability of bacteria, as it provides physical protection, determines the cell shape and is the principal stress-bearing element, which makes it an ideal target for antibiotics (Scheffers and Pinho, 2005). In Gram-positive bacteria, such as *S. aureus*, the cell wall is composed of surface proteins, teichoic acids and a thick layer of peptidoglycan (PG), whose synthesis is a major target of some of the most successful classes of antibiotics, including the β-lactams such as penicillin or methicillin (Popham, 2013). The peptidoglycan requires a complex process of synthesis that involves the elongation (transglycosylation) and peptide cross-linking (transpeptidation) of glycan strands, which occurs mainly at the division septum of *S. aureus* and is catalyzed by the penicillin binding proteins (PBPs) (Scheffers and Pinho, 2005). Recent studies have shown that the PBP4 is essential for the expression of β-lactam resistance in CA-MRSA strains (Memmi *et al.*, 2008). This protein has a septal localization and is responsible for the high degree of PG cross-linking notable in *S. aureus*. The localization of PBP4 has recently been shown to be spatially and temporally regulated by an unknown intermediate of the WTA biosynthesis pathway (Atilano *et al.*, 2010). In this work we aimed to identify the WTA precursor responsible for the septal recruitment of PBP4. We constructed inducible mutants of specific *tar* genes in the background of NCTCPBP4-YFP, a strain expressing a fluorescent derivative of PBP4, that allowed us to study its localization in the presence and absence of *tarB* and *tarL* genes.

Deletion of *tarO* and the use of tunicamycin, which blocks the first and non-essential step in the WTA pathway by the inhibiting TarO, have been shown to cause profound morphological defects in *S. aureus*, such as aberrations in septal placement, a high frequency of double septa and an inability to separate daughter cells following the completion of new septa (Atilano *et al.*, 2010; Campbell *et al.*, 2010; Campbell *et al.*, 2012). In both the absence of TarO and in the presence of tunicamycin, the PBP4 protein is dispersed over the entire cell membrane instead of displaying its normal septal localization, indicating that PBP4 is recruited by the TarO protein or the product of a downstream reaction in the WTA biosynthesis pathway (Atilano *et al.*, 2010; Roemer *et al.*, 2013). However, Atilano *et al* have shown that the recruitment of PBP4 does not occur via direct protein–protein interaction with TarO because these two proteins did not interact in a bacterial two-hybrid screening, did not colocalize in 49% of the cells in the early stages of septum synthesis and PBP4 did not retain its septal localization in the presence of an inactive TarO protein properly localized at the septum (Atilano *et al.*, 2010). Therefore it is more likely that PBP4 is recruited by a WTA synthesis intermediate. The use of a second drug, targocil, which inhibits the TarG WTA ABC transporter, demonstrated that the PBP4 is recruited by a precursor of the WTA biosynthesis pathway present inside the membrane. In the presence of this drug PBP4 remains specifically at the division septum which shows that the steps after the WTA translocation to the outside membrane are not involved in the recruitment of PBP4 (Roemer *et al.*, 2013). Based upon

these results we chose to study the *tarB* and *tarL* genes, which catalyze the addition of one glycerol-3-phosphate unit and the addition of a ribitol phosphate residue chain, respectively (Figure 1.4).

In this work, we have shown that in parental strain NCTCPBP4-YFP, PBP4 can be found at the septum of *S. aureus*. However, in the inducible *tar* mutant strains depleted for TarB and TarL (when grown without IPTG), PBP4 no longer accumulates specifically at the division septum, but instead is dispersed over the entire cell membrane (Figures 3.6 and 3.7). There is no statistically significant difference in the PBP4-YFP localization between parental NCTCPBP4-YFP PBP4 strain and the NCTC*Δspa::tarBi* strain plus IPTG, which means the inducible strain behaves as expected in the presence of IPTG, i.e. the expression of *tarB* from the *spa* locus or from its native locus is similar for the purpose of PBP4 localization. However, in the case of NCTC*Δspa::tarLi* there was a significant difference between PBP4 localization in this strain, in the presence of IPTG, and in the parental strain with the former having a higher fluorescence ratio for PBP4 localization, i.e., more PBP4 protein localized at the septum. This observation can be explained by two hypotheses (1) expression of *tarL* in the conditions used to grow the inducible strain, NCTC*Δspa::tarLi*, in the presence of IPTG, could lead to an overexpression of TarL with a consequent increase in the number of precursors that recruit the PBP4 and therefore an increase in the septal signal of the PBP4; (2) by cloning the *tarL* in the *spa* locus, separating it from its operon, we could be changing the regulation network of the teichoic acids synthesis, which could also affect the pathways for cell wall synthesis, including PBP4 production, given that the two pathways have common substrates. Overexpression of PBP4 could therefore be the reason for the increased fluorescence ratio observed in the TarL inducible strain in the presence of IPTG.

We determined PBP4 localization on cells depleted for TarB and TarL for 1 hour and 45 minutes. However, TarB and TarL depletion eventually leads to cell death, which can be accompanied by general protein delocalization. It was therefore important to determine if PBP4 delocalization was part of general protein delocalization in cells approaching death, or if PBP4 was specifically delocalizing in the absence of TarB and TarL, in conditions where other proteins remain properly localized. Given that PBP4 localizes to the septum, we used a divisome protein, EzrA, as a control and determined the effect of TarB and TarL depletion of EzrA septal localization. For that purpose we used a EzrA-CFP fusion as the fluorescence emitted by CFP does not overlap the fluorescence emitted by YFP fused to PBP4, i.e., the emission maxima of the two fluorophores are sufficiently apart to be separated using appropriate filters (Pereira *et al.*, 2010). The strains simultaneously expressing PBP4-YFP and EzrA-CFP, with either *tarB* or *tarL* under the control of the inducible promoter Pspac, NCTCΔspa::tarBi *EzrA-cfp* and NCTCΔspa::tarLi *EzrA-cfp*, should have enabled us to quantify the localization of PBP4 and EzrA in the same cells and confirm if the delocalization of PBP4 is accompanied or not by changes in the localization of EzrA. Unfortunately these strains formed cell clusters which did not permit the correct quantification of PBP4-YFP localization, as we cannot correctly measure the fluorescence at the septum and at the "lateral" wall for calculation of the fluorescence ratio. As an alternative, we quantified EzrA and PBP4 localization in separate strains but under the same TarB/TarL depletion conditions. The

obtained results shown in Figure 3.12 confirm that PBP4 delocalization did not occur as part of general protein delocalization, as EzrA-CFP remained localized at the septum in the absence of TarB/TarL while PBP4-YFP was dispersed throughout the cell membrane.

In this work we were able to determine that in the absence of TarB or TarL, PBP4 loses its normal septal localization and becomes dispersed all around the cell membrane. With these results we can suggest two hypotheses:

(1) The TarL protein itself recruits PBP4 to the division septa. In this case, once the TarL catalyses one of the last steps of the WTA biosynthesis occurring in the inner side of the cytoplasmic membrane (Figure 1.4 and 1.5 in the introduction) and the absence of TarO, the first protein in this synthesis pathway, also leads to PBP4 delocalization, then TarL localization should be dependent on the substrate. Therefore, blocking TarO protein would deplete subsequent intermediates in WTA synthesis, which would in turn affect TarL and PBP4 localization;

(2) PBP4 is recruited by a substrate of the WTA synthetic pathway whose presence is dependent on TarL. In this case, the absence of an earlier protein in the WTA biosynthetic pathway, such as TarO or TarB, would deplete the substrate for TarL, which would therefore be unable to make its product.

In conclusion, these results indicate that the molecule responsible for PBP4 recruitment is probably one involved in the last steps in WTA synthesis pathway at the inner side of the membrane. To prove that hypothesis and identify the intermediate responsible for PBP4 localization, we look for PBP4 interaction partners by a bacterial two-hybrid screening, namely to see if the PBP4 interacts with TarL, TarH and/or TarG. We will also study of the localization of *S. aureus* PBP4 in the absence of TarH and TarG by using *tarH* and *tarG* inducible mutants. Although PBP4 maintains its septal localization in the presence of the antibiotic targocil, which blocks the activity of TarG, we do not know if TarH or TarG physically interact with PBP4 to recruit it to the septa. Uncovering the mode of recruitment of PBP4 is not only important to gain knowledge into the fundamental process of bacterial cell wall synthesis, but also into the essential role of PBP4 in the expression of β-lactam resistance in CA-MRSA strains. For this purpose we have already started to construct the strains to reproduce our studies in the CA-MRSA strain MW2, an understanding of how PBP4 localizes is required to fully understand its role in β-lactam resistance.

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