Sara Filipa dos Santos Pinheiro

Bachelor degree in Biochemistry

Development of a *Streptococcus pneumoniae* system to control exposure of immature peptidoglycan

Dissertation to obtain the Master degree in
Molecular Genetics and Biomedicine

Supervisor: Doutor Sérgio R. Filipe, Professor Auxiliar, FCT NOVA

Jury:

President: Doutor José Paulo Nunes de Sousa Sampaio, Professor Associado com Agregação, FCT NOVA;

Examiner: Doutor Mário Nuno Ramos d'Almeida Ramirez, Professor Associado com Agregação da Faculdade de Medicina, Universidade de Lisboa.

November 2020

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Abstract

Streptococcus pneumoniae is a Gram-positive bacterial pathogen capable of causing from mild infections to more severe conditions such as pneumonia and meningitis. Pneumococcal virulence is dependent on the production of different virulence factors, such as particular peptidoglycan-associated surface proteins or the production of a capsular polysaccharide structure surrounding the bacteria. Peptidoglycan, a major cell wall component, offers structural stability to the cell and is dynamically tailored by hydrolases, such as LytA, during physiological mechanisms like cell elongation, division and septation. Capsule production, which is strictly regulated, requires expression of several genes present in the *cps* locus, including genes with a regulatory role such as *wzd* and *wze.* The interaction between proteins Wzd and Wze, in the presence of ATP, is thought to play a central role in the regulation of capsule synthesis.

The aim of this dissertation was to develop a *S. pneumoniae* system capable of controlling the exposure of immature peptidoglycan to enzymes present in the surrounding medium. To achieve this goal, pneumococcal strains were constructed where the *Escherichia coli* LacI repressor (encoded by the *lacI* gene) is constitutively expressed and the capsule regulatory genes pneumococcal *wzd* and *wze* are placed under the control of an IPTG-inducible promoter (Plac). This was performed in the background of *S. pneumoniae wzd* and *wze* deletion mutants, where immature peptidoglycan is exposed at the surface of the septal region due to absence of capsule at the division septum. One of the constructed strains, 6314Δ*wze-lacI*-Plac-*wze*, was analyzed by immunofluorescence microscopy to assess if septal capsule expression was restored at the division septum upon expression of IPTG-inducible *wze*. This analysis showed that the constructed mutant, in the presence of IPTG, presented a seemingly intermediate phenotype between absence of capsule in the division septum and full encapsulation.

Keywords: *Streptococcus pneumoniae***,** LytA**,** IPTG-inducible promoter**,** immature peptidoglycan, immunofluorescence

Resumo

Streptococcus pneumoniae é uma bactéria patogénica Gram-positiva, responsável por diferentes tipos de infeções, desde infeções ligeiras a doenças bastante graves como pneumonia e meningite. A virulência pneumocócica é dependente da produção de diferentes fatores de virulência que incluem proteínas na superfície da bactéria que se associam ao peptidoglicano ou a estrutura capsular polissacarídica que envolve a maior parte dos isolados clínicos desta bactéria. O peptidoglicano, um dos componentes mais relevantes da parece celular, proporciona estabilidade estrutural à célula e é dinamicamente moldado por hidrolases, como LytA, durante diferentes mecanismos fisiológicos como o elongamento da bactéria, a sua divisão celular e a separação das células-filhas. A produção de cápsula, sujeita a uma regulação bastante controlada, requer a expressão de vários genes presentes no locus *cps*, incluindo os genes *wzd* e *wze*. Atualmente, considera-se que a interação entre as proteínas Wzd e Wze, na presença de ATP, tem um papel bastante relevante na regulação da síntese de cápsula.

O objetivo desta dissertação foi o desenvolvimento de um sistema em *S. pneumoniae* que permitisse controlar a exposição de peptidoglicano imaturo a enzimas que estejam presentes no meio circundante. Para tal, foram construídas estirpes pneumocócicas que expressam o repressor LacI (codificado pelo gene *lacI*) de *Escherichia coli* de um modo constitutivo e que têm os genes que codificam as proteínas reguladoras da síntese de cápsula, Wzd e Wze, sob controlo de um promotor indutível por IPTG (Plac). Esta construção foi realizada utilizando mutantes de *S. pneumoniae* de deleção dos genes *wzd* e *wze*, uma vez que nestes organismos o peptidoglicano imaturo está continuamente exposto na zona septal, à superfície da bactéria, devido à ausência de cápsula no septo de divisão celular. Uma das estirpes construídas, 6314Δ*wze-lacI*-Plac-*wze*, foi analisada por imunofluorescência, de modo a determinar se a indução de *wze* por IPTG leva à produção de cápsula no septo. Com estes ensaios de microscopia foi determinado que o mutante construído, na presença do indutor IPTG, apresentava um fenótipo aparentemente intermédio entre ausência de cápsula no septo e total encapsulamento da bactéria.

Palavras-chave: *Streptococcus pneumoniae***,** LytA**,** promotor indutível por IPTG**,** peptidoglicano imaturo, imunofluorescência

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Abbreviations

% - Percentage

% (m/v) – Mass/volume percentage

% (v/v) – Volume/volume percentage

AATGal - 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose

ATP - Adenosine triphosphate

BBB – Blood-brain barrier

bp – Base pairs

BSA – Bovine serum albumin

CBPs – Choline-binding proteins

CPS – Capsular polysaccharide

CSF – Cerebrospinal fluid

CSP – Competence-stimulating peptide

GalNAc - *N*-acetyl-D-galactosamine

gDNA – Genomic DNA

Gent – Gentamycin

GFP – Green fluorescent protein

GlcNAc - *N*-acetylglucosamine

HGT – Horizontal gene transfer

HPLC - High performance liquid chromatography

IF – Immunofluorescence

IMAC - Immobilized metal affinity chromatography

IPTG - Isopropyl β-D-1-thiogalactopyranoside

Kan – Kanamycin

LA - Luria-Bertani agar

LB - Luria-Bertani

LTAs – Lipoteichoic acids

MurNAc – *N*-acetylmuramic acid

NF-κB - Nuclear factor-κB

PAFr - Platelet-activating factor receptor

PAMPs – Pathogen-associated molecular patterns

PBPs - Penicillin-binding proteins

PBS - Phosphate-buffered saline

PCho - Phosphorylcholine

PCR – Polymerase chain reaction

PCV-7 - 7-valent pneumococcal conjugate vaccine

pDNA – Plasmid DNA

PGN – Peptidoglycan

Ply - Pneumolysin

rpm - Revolutions per minute

SNP – Single nucleotide polymorphism

Spec – Spectinomycin

TAE - Tris-acetate-EDTA

TLR - Toll-like receptor

TSA – Tryptic soy agar

UP – Undecaprenyl phosphate

URT – Upper respiratory tract

WT – Wild-type

WTAs – Wall teichoic acids

Chapter 1 – Introduction and objectives

1.1. *Streptococcus pneumoniae*: an overview

Streptococcus pneumoniae, commonly referred to as pneumococcus, is a Gram-positive bacterial pathogen responsible for the annual death of 1.6 million people accordingly to a WHO estimate in 2005 (https://www.who.int/ith/diseases/pneumococcal/en/ last access 12.10.2020).

Although an opportunistic pathogen, *S. pneumoniae* bacteria colonize the human upper respiratory tract (URT), especially in young children, by establishing a commensal relationship with the carrier (Weiser, Ferreira, & Paton, 2018).

However, when in certain conditions that are associated with increased virulence or invasiveness of particular strains, pneumococcus is responsible for a range of diseases, from mild mucosal infections, such as otitis media and sinusitis, to more complicated conditions, such as pneumonia, bacteremia and meningitis (Weiser et al., 2018).

The ability of pneumococcus to cause such range of disease outcomes is due to the production of different virulence factors, namely a capsular polysaccharide (CPS) structure and particular choline-binding proteins (CBPs). The production of these factors contributes to host colonization at a cellular level and their importance will be discussed in later sections (Mitchell & Mitchell, 2010).

1.1.1. Transmission and colonization

S. pneumoniae inter-host transmission begins necessarily with nasopharyngeal colonization of a carrier followed by aerosol dispersion from person to person. As colonization of URT epithelial cells occurs, bacteria develop mechanisms to endure host defenses long enough so that they are able to spread and invade other locations of the host, which will consequently cause disease (Weiser et al., 2018).

For colonization, *S. pneumoniae* bacteria need to undertake innate and adaptive immune responses, such as mucous entrapment in which action of antimicrobial peptides and immunoglobulins are involved. Therefore, this first step of the pneumococcal life cycle is dependent on several molecular mechanisms that are promoted by many surface proteins, adhesins and enzymes, such as bacteria uptake into the host organism, adherence to the nasopharyngeal epithelium and biofilm production (Henriques-Normark & Tuomanen, 2013; Weiser et al., 2018).

As bacteria escape these mucous defense mechanisms, migration to the lower respiratory tract begins and invasive pneumonia can be initiated as a response to pneumococcus adherence and replication in the alveolar epithelium (Henriques-Normark & Tuomanen, 2013).

At this stage of the pneumococcal life cycle, different pathogen-associated molecular patterns (PAMPs), such as peptidoglycan (PGN), pneumolysin (Ply) and pneumococcal DNA are recognized by the innate immune system, flagging the presence of bacteria within the infected host. The recognition of these PAMP molecules triggers an inflammatory response due to the stimulation of different host pathways, such as the Toll-like receptors (TLRs) signaling pathway, which ultimately results in the activation of nuclear factor-κB (NF-κB) production. Consequently, the activation of NF-κB production induces severe inflammation, as it promotes cytokine release and phagocyte recruitment and activation, which contributes further to the damage of lung tissues and impairment of the functions of this organ (Henriques-Normark & Tuomanen, 2013).

Similarly to other respiratory pathogens, pneumococcal disease progression, represented in Figure 1.1, requires bacterial dissemination across different cellular barriers, such as the lung epithelium, which may result in bacteremia. This barrier crossing is possible due to the presence of phosphorylcholine (PCho) at the surface of *S. pneumoniae* which allows the interaction of bacteria with the host platelet-activating factor receptor (PAFr). Bacterial PCho mimics the bioactivity of this moiety on the ligand (PAF chemokine) that binds to PAFr. Therefore, as pneumococcal PCho interacts with PAFr through its natural ligand, bacteria is taken up in vacuoles allowing their crossing of epithelial cells and bloodstream entering (Radin et al., 2005; Thornton, Durick-Eder, & Tuomanen, 2010).

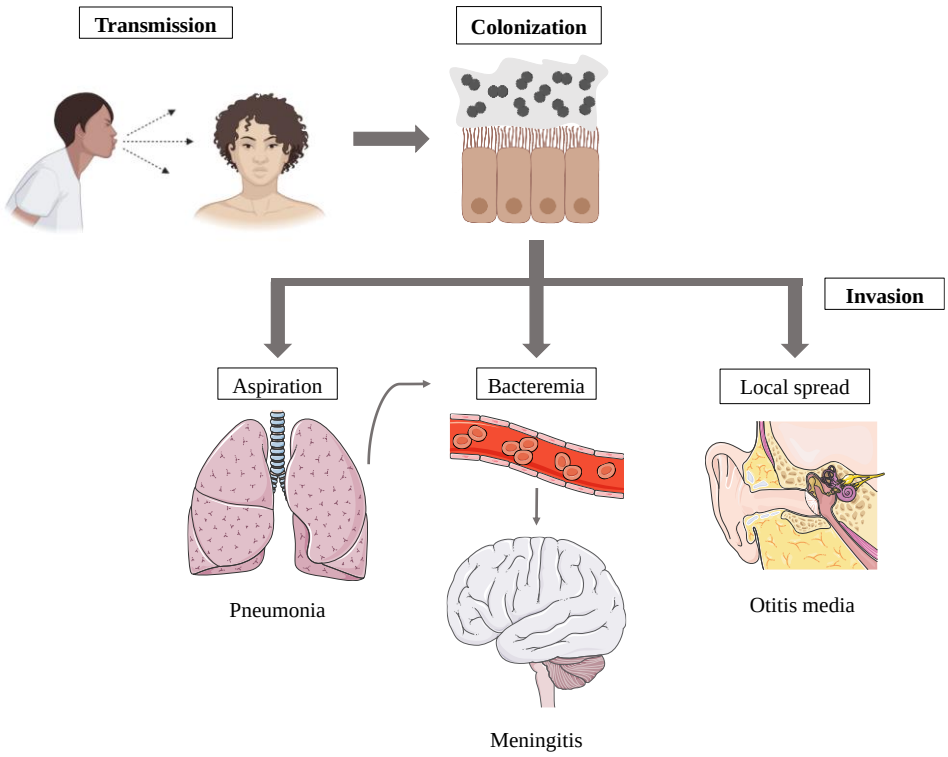

Figure 1.1 - Life cycle of *Streptococcus pneumoniae*. Pneumococcal life cycle begins with URT colonization of a carrier which can lead to transmission to other individuals and/or progression to invasive disease. Pathogenesis includes mild infections such as otitis media, when bacteria dissemination happens locally, and serious conditions such as pneumonia and meningitis, resulting from aspiration to the lower respiratory tract and bacteremia, respectively. Adapted from Weiser et al., 2018.

As bacteria enters the bloodstream, pneumococcal CPS plays a vital role in the inhibition of complement deposition, which is part of the innate host defenses, allowing a high level of bacteremia that often precedes meningeal invasion (Coureuil, Lécuyer, Bourdoulous, & Nassif, 2017; Henriques-Normark & Tuomanen, 2013).

Alike lung epithelium crossing, meningitis occurs when *S. pneumoniae* bacteria adhere and bridge the endothelial cells of another host cellular barrier, the blood-brain barrier (BBB), through binding of bacterial components to several host receptors, including the previously mentioned PAFr. In the cerebrospinal fluid (CSF), bacteria multiply uncontrollably due to the absence of innate defenses, causing neuronal toxicity that ultimately leads to brain damage. Thus, bacterial meningeal invasion, a condition with high morbidity and mortality, represents the further extent of pneumococcal pathogenesis. It also highlights the importance of understanding the details of the infectious process in order to prevent or improve the outcome of pneumococcal infection (Coureuil et al., 2017; Henriques-Normark & Tuomanen, 2013; Iovino, Seinen, Henriques-Normark, & van Dijl, 2016).

1.1.2. Antibiotic resistance and pneumococcal vaccine

For a long period of time, standardized treatment of pneumococcal infections included antibiotic usage, specifically penicillin administration. Soon after, antimicrobial-resistant pneumococcal clones started to arise with penicillin reduced susceptibility being reported as early as the mid-1960s, only 20 years past mass production of this β-lactam antibiotic. Before long, particular pneumococcal strains, which have become resistant to most antibiotics and allowed the emergence of multidrug-resistant clonal types, have disseminated globally (Henriques-Normark & Tuomanen, 2013; Kim, McGee, Tomczyk, & Beall, 2016).

Such occurrences rely on the capability of *S. pneumoniae* to evolve and survive through selection pressure induced by antibiotic misuse, vaccine stress and its natural competence for transformation (Reinert, 2009).

1.1.2.1. Natural competence for transformation

S. pneumoniae competence for natural genetic transformation, a form of horizontal gene transfer (HGT), in which exogenous DNA is incorporated into the genome by homologous recombination, seems to be crucial, and possibly a prerequisite, for antibiotic resistance development in specific clonal types or serotypes (Reinert, 2009; Straume, Stamsås, & Håvarstein, 2014).

The development of competence depends on a quorum-sensing-like system consisting of five proteins: an ABC-transporter, ComAB, and a three-party regulatory system, ComCDE. All these proteins play a role in integrating internal and external signals, like the competence-stimulating peptide (CSP) encoded by *comC*. CSP is a precursor peptide that, after maturation, is

secreted by the ABC-transporter and thus, accumulates extracellularly under competence-permissive conditions. After CSP accumulation, which is detected by the receptor ComD, the competent state is accomplished with the help of ComE, a response regulator protein capable of modulating the expression of different pneumococcal genes. Consequently, released DNA from lysed cells can be taken up by competent cells and integrated into the genome before the competent state is shut-off, as shown in Figure 1.2 (Straume et al., 2014).

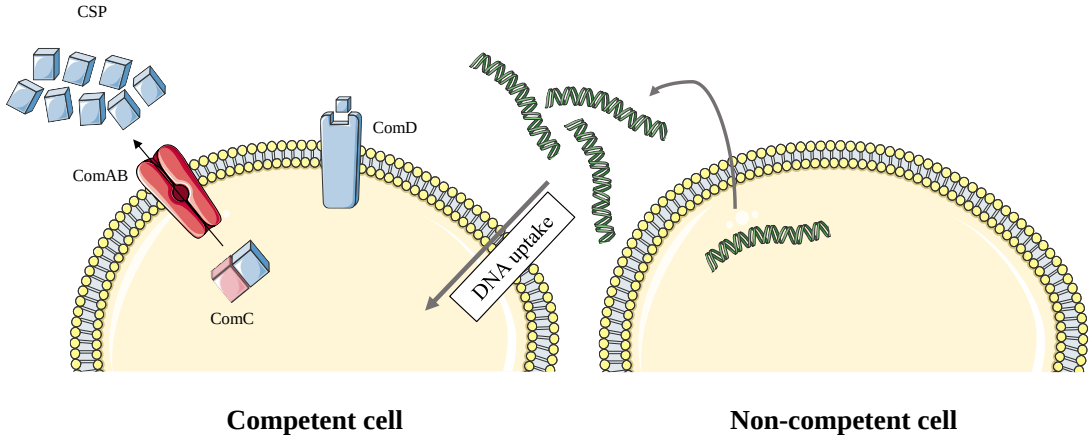

Figure 1.2 – Representation of the pneumococcal natural genetic transformation mechanism. *S. pneumoniae* is naturally competent in taking up exogenous DNA and integrating it in its genome. The regulation of this mechanism is mainly dependent of four proteins (ComA, ComB, ComD and ComE) and an external signal, CSP, encoded by *comC*, which accumulates extracellularly to induce competence. After a chain of events caused by CSP recognition, DNA from lysed cells can be taken up by competent pneumococcal cells. Adapted from Straume et al.,2014.

Pneumococcus shares this capability of natural competence for transformation with around 40 different species of bacteria which contributes to its genetic versatility and ultimately, to antibiotic resistance, including multidrug-resistance (Reinert, 2009).

1.1.2.2. Vaccine selection pressure

In the early 2000s, a 7-valent pneumococcal conjugate vaccine (PCV-7) emerged in the US and became a part of the childhood vaccination program. This vaccine consisted in 7 different capsular polysaccharides produced by 7 specific pneumococcal serotypes that were predominantly responsible for invasive disease in the country (Henriques-Normark & Tuomanen, 2013).

As the administration of PCV-7 also targeted the majority of β-lactam resistant clonal types, a noticeable reduction in invasive disease and carriage of vaccine serotypes was observed, as expected, in the younger population as well as in the elderly (Straume et al., 2014).

Nevertheless, since the implementation of vaccination programs, pneumococcus evolved due to vaccine stress and colonization serotype distribution was altered. Non-vaccine clonal types expanded and certain serotypes, which were not covered by PCV-7, started to, increasingly, cause

invasive disease. The same situation occurred after the development of more recent vaccines that include 10 and 13 different serotypes. Efforts to reach a higher variety of serotypes targeted by different vaccines were not made, since pneumococcus large number of different serotypes would make it economically infeasible (Henriques-Normark & Tuomanen, 2013; Reinert, 2009; Straume et al., 2014).

This minority selection and vaccine circumvention may be caused by capsular switch events promoted by pneumococcal natural competence for transformation through recombination and highlights once more the capacity of *S. pneumoniae* to evolve through different types of pressure (Reinert, 2009).

1.1.2.3. Novel therapeutic targets

The observed selection of particular pneumococcal clinical isolates caused by vaccination programs, highlights the urgent need for novel therapeutic targets that are common to all serotypes like alternative vaccines based on virulence contributing proteins. If such vaccine target would be able to produce immunological memory and be highly immunogenic, the cost of vaccination for pneumococcal disease would be more affordable and all serotypes would be covered in an economically feasible way (Kadioglu, Weiser, Paton, & Andrew, 2008).

A few candidates for a novel therapeutic target have been identified that include pneumolysin and choline-binding proteins like PspA and CbpA, involved in inhibition of complement deposition and lung epithelium crossing, respectively (Kadioglu et al., 2008).

Human trials data have shown that Ply-based vaccines were successful in reporting immunogenicity and protection especially when non-toxic derivatives of the protein were used. Moreover, one of the most promising alternative vaccine has been the one that targets Ply, PspA and CbpA simultaneously (Kadioglu et al., 2008; Nishimoto, Rosch, & Tuomanen, 2020).

1.2. Pneumococcal cell wall

The pneumococcal cell wall is a complex and dynamic structure that encloses bacteria offering protection against the extracellular surroundings. The cell wall structurally supports the bacterial cell, playing a role in keeping its shape through growth and division processes. In addition, individual components of this structure contribute to the pathogenesis of these Gram-positive bacteria through interaction with the host defenses (Rajagopal & Walker, 2017; Vollmer, Massidda, & Tomasz, 2019).

Commonly to all Gram-positive bacteria, pneumococcal cell wall is composed of a thick layer of peptidoglycan to which teichoic acids can be covalently linked. Proteins can also be present either covalently linked at cell surface or in close contact with the cell wall (Rajagopal & Walker, 2017; Vollmer et al., 2019).

The understanding of the cell wall composition, the mechanisms involved in its biosynthesis and regulation, is of major importance to find new targets for the development of novel anti-infective strategies, especially considering that *S. pneumoniae* is currently resistant to the most commonly used antibiotics as mentioned in previous sections (Rajagopal & Walker, 2017).

1.2.1. Peptidoglycan assembly

Peptidoglycan, a heteropolymer identified as one of the main cell wall components, is constituted by long glycan chains cross-linked by short stems of peptides. The glycan chains are composed of two alternating glucose byproducts, GlcNAc (*N*-acetylglucosamine) and MurNAc (*N*-acetylmuramic acid) residues (Engholm, Kilian, Goodsell, Andersen, & Kjærgaard, 2017).

One of the initial steps of PGN biosynthesis is the cytosolic assembly of the peptide stem linked exclusively to the MurNAc residues of the glycan chain. Different Mur ligases are involved in this process by adding the amino acids L-alanine, D-glutamate, L-lysine and a D-alanine dipeptide to form a MurNAc-pentapeptide (Engholm et al., 2017).

The MurNAc-pentapeptide is then transferred to a membrane-associated acceptor, undecaprenyl phosphate (UP), that will facilitate hydrophobic cell membrane crossing. After UP-MurNAc-pentapeptide complex (or lipid I) is created, another transferase adds a GlcNAc sugar residue to the complex. This cytosolic glycosylation step leads to the creation of UP-MurNAc-pentapeptide-GlcNAc complex, also called lipid II, as represented in Figure 1.3 (Engholm et al., 2017).

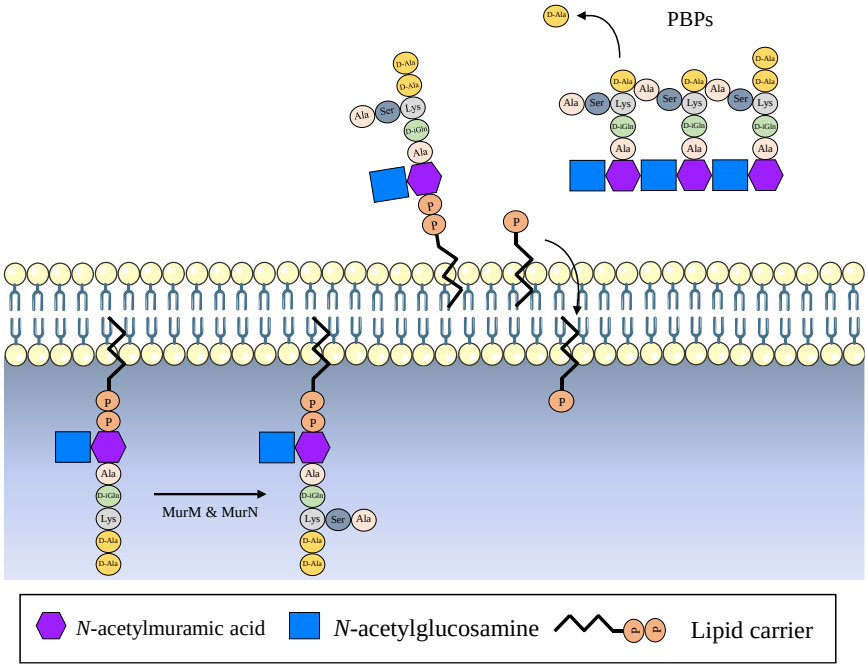

Figure 1.3 – Representation of the peptidoglycan assembly mechanism. Lipid II is a substrate for ligases MurM and MurN responsible for cell wall branching. After lipid II translocation, the precursor is attached to the preexisting cell wall by glycosyltransferases and the MurNAc-pentapeptide is trimmed. The last step in PGN assembly is the recycling of the lipid carrier. Adapted from Vollmer et al, 2019.

Additionally, a strain-variable branch of L-amino acids is added to the L-lysine residue of the peptide stem by the MurM and MurN ligases. This branch is involved in the indirect PGN crosslinking but is also involved in the expression of β-lactam antibiotic resistance (Vollmer et al., 2019).

After lipid II is translocated across the cell membrane, immature PGN undergoes a maturation process involving polymerization and crosslinking, which can be direct or indirect, mainly by penicillin-binding proteins (PBPs) (Engholm et al., 2017).

PBPs exhibit three different catalytic functions: they act as glycosyltransferases to polymerize immature PGN to the preexisting cell wall, as transpeptidases to crosslink the glycan chains and as carboxypeptidases to trim the MurNAc-pentapeptide. The MurNAc-pentapeptide is hydrolyzed by the carboxypeptidase domain of PBPs at the D-alanine residue terminal to produce a tetrapeptide (Rajagopal & Walker, 2017; Vollmer et al., 2019).

The final step in PGN biosynthesis is the recycling of the membrane-associated UP as it is required as an acceptor for other cell wall components, like teichoic acid precursors, and exists in limited quantities in bacterial membranes (Rajagopal & Walker, 2017).

1.2.2. Teichoic acids synthesis

As mentioned in section 1.2., teichoic acids are the second main component of the cell wall of Gram-positive bacteria. Teichoic acids can be categorized into wall teichoic acids (WTAs) or lipoteichoic acids (LTAs), if they are covalently attached to PGN or anchored at the cell membrane, respectively (Denapaite & Hakenbeck, 2012).

Unlike other Gram-positive bacteria, pneumococcal teichoic acids are atypical not only in their repeating unit composition but also in the structural similarity between its two classes, which might suggest a common biosynthetic pathway (Denapaite & Hakenbeck, 2012).

Its complex repeating unit consists of a pseudo-pentasaccharide structure that includes the rare amino sugar 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose (AATGal), D-glucose, ribitol 5-phosphate and two *N*-acetyl-D-galactosamine (GalNAc) residues. In addition and depending on the pneumococcal strain, one or two phosphocholine residues can be added to the GalNAc residues of the repeating unit through a phosphodiester bond. The teichoic acids chain can contain between 4 to 8 repeating units that are attached to each other by an α-1,4 glycosidic linkage between the AATGal and the first GalNAc residue of the posterior pentasaccharide structure (Vollmer et al., 2019).

Although, the two classes of teichoic acids seem to have a common biosynthetic pathway, very few enzymes have been biochemically studied and the biological processes were deduced with the use of bioinformatics. In sum, the pathway consists in the cytoplasmic assembly of the repeating unit linked to a carrier lipid and in the addition of phosphocholine residues to the GalNAc subunits. In the final steps, after the cell membrane translocation and maturation steps,

teichoic acids are then transferred to either peptidoglycan (WTAs) or membrane glycolipids (LTAs) (Vollmer et al., 2019).

1.3. Peptidoglycan hydrolases

Peptidoglycan hydrolases are a diverse group of enzymes with the ability to cleave different bonds in the heteropolymer, whose main function is to degrade the bacterial backbone of this cell wall component and/or its respective soluble fragments. Some of these enzymes can promote cell lysis and are designated autolysins (Jedrzejas, 2001; Vollmer, Joris, Charlier, & Foster, 2008).

According to their cleavage site, PGN hydrolases can be categorized as amidases or peptidases whether they hydrolyze the amide bond between the MurNAc residue of the glycan chain and the N-terminal L-alanine or the amide bond between two amino acids present in the stem peptide (Vollmer et al., 2008).

Furthermore, PGN hydrolases seem to participate under strict regulation in physiological mechanisms in which cell wall tailoring is necessary, such as cell elongation, septation and daughter cells separation, through cell wall expansion (Uehara & Bernhardt, 2011).

1.3.1. LytA, a pneumococcal autolysin

One of the most characterized bacterial autolysins is the pneumococcal *N*-acetylmuramoyl-L-alanine LytA amidase. As mentioned before, LytA as an amidase cleaves the lactyl-amide bond between the MurNAc residue and the L-alanine amino acid that links the glycan chains and stem peptides, as shown in panel A of Figure 1.4 (Mellroth et al., 2012).

LytA structural organization, which is represented in panel B of Figure 1.4, consists of two distinct domains: the C-terminal choline binding domain responsible for its attachment to the cell wall, specifically to teichoic acids with the PCho residues, and the N-terminal catalytic domain responsible for its amidase function (Jedrzejas, 2001; Mellroth et al., 2012).

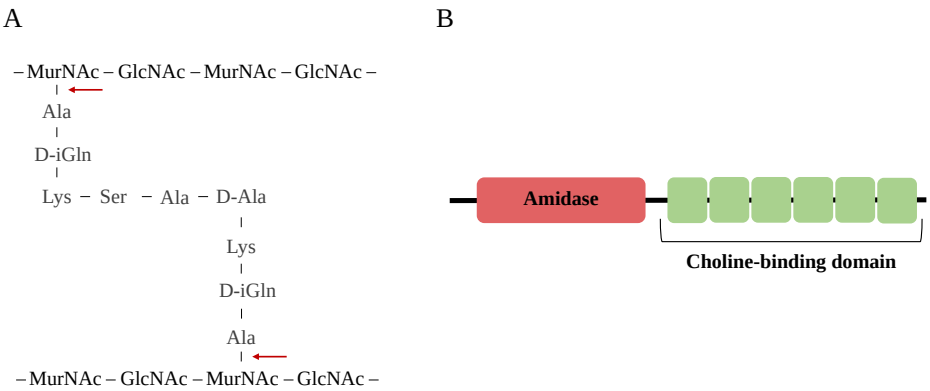

Figure 1.4 – LytA amidase cleavage site and structural organization. In panel A, the PGN cleavage site of LytA is illustrated with an arrow between the MurNAc residue and the L-alanine amino acid residue. In panel B, a structural representation of LytA is presented with an amidase domain and a choline-binding domain. Adapted from Mellroth et al., 2012.

As mentioned in section 1.1, choline-binding proteins, such as LytA, are one of the main virulence factors produced by *S. pneumoniae* that promotes bacterial pathogenesis. Although its role is still unclear, mutations in *lytA* showed reduced virulence in murine models suggesting the importance of this enzyme for the bacteria to cause invasive disease (Jedrzejas, 2001; Kadioglu et al., 2008).

The main hypothesis for the role of LytA in pneumococcal virulence is the release of inflammatory cell wall components triggered by its amidase activity and the release of other virulence factors after cell lysis, such as pneumolysin (Jedrzejas, 2001; Mellroth et al., 2012).

Nevertheless, the basic mechanisms regarding LytA regulation are equally unclear and need further investigation, including what activates its lytic function, especially in such a predictable and consistent timeframe and what makes the cell wall its target (Mellroth et al., 2012).

1.4. CPS, a capsular polysaccharide structure

The outermost layer of the pneumococcus surface is a capsular polysaccharide structure that surrounds pneumococcal bacteria. It has a thickness of around 200 to 400 nm and it is considered the most important virulence factor as it is the first pneumococcal component in contact with the host (Engholm et al., 2017; Kadioglu et al., 2008).

CPS is involved in different mechanisms capable of influencing the pathogenesis of pneumococcal bacteria. Due to its highly negative charge, this structure likely physically repels phagocytes and provides protection against these host cells that are involved in host innate immune responses. Consequently, this protection prevents phagocytic cell receptors from recognizing certain bacterial antigens, since their localization is much deeper in the pneumococcal cell (Kadioglu et al., 2008; Li, Weinberger, Thompson, Trzcin, & Lipsitch, 2013).

Pneumococcal virulence is not promoted by a static capsule but rather by a dynamic structure that adjusts its thickness and density according to the stage of infection. For instance, in early colonization stages, capsule is down-regulated to permit pneumococcal adhesins to be exposed and, consequently, adhesion of bacteria to epithelial cells of the nasopharynx to occur. On the contrary, later in the infection as bacteria enter the bloodstream, CPS is up-regulated to promote virulence. Its complete absence has been associated with mild infections, such as conjunctivitis (Engholm et al., 2017; Kadioglu et al., 2008).

1.4.1. The *cps* locus

As established in the previous section, CPS presence is essential for invasive disease to occur and its maximum expression is necessary for the systemic aspects of pneumococcal infection. Thus, regulation of the capsule production in a transcriptional, translational and post-translational manner is vital for pneumococcal virulence (Kadioglu et al., 2008).

The basis of this regulation relies on the chromosomal *cps* locus, which is conserved amongst most of the original serotypes of *S. pneumoniae* sequenced in 2006 by the Sanger Institute (Geno et al., 2015).

The *cps* locus is almost exclusively located in the *dexB*/*aliA* region of the pneumococcal chromosome. Its first four genes *wzg*, *wzh*, *wzd* and *wze*, which are common to most serotypes with the exception of two (serotypes 3 and 37), regulate and play an important role in the capsule expression (Geno et al., 2015; Kadioglu et al., 2008).

The remaining locus is composed of central glycosyltransferases encoding genes that are serotype specific and are involved in the cytoplasmic assembly of the oligosaccharide repeating units of the CPS structure. Additionally, it includes genes that encode for the capsule repeating unit flippase, a polymerase that links the repeating units, additional enzymes involved in the synthesis of activated sugars precursors and other regulatory conserved genes (Kadioglu et al., 2008).

1.4.1.1. CPS biosynthesis

Depending on the serotype, capsule synthesis can occur either in a synthase-dependent or polymerase-dependent mechanism. The synthase-dependent mechanism occurs exclusively in the serotypes mentioned above that lack the first four regulatory conserved genes of the *cps* locus. In this case, only a single enzyme is responsible for synthesis initiation and its role is to, continuously, add sugar residues, such as glucose and glucuronic acid, to a lipid acceptor until the structure is extended to an octosaccharide. At this point, the same enzyme translocates the chain to the outside of the cell and extension continues as long as its precursors are available (Geno et al., 2015).

The polymerase-dependent synthesis mechanism represented in Figure 1.5 is common to all other serotypes. It begins with a glycosyltransferase that adds a glucose-1-phosphate residue to an UP intracellular acceptor. The assembly of the repeating unit is continued by the subsequent glycosyltransferases, encoded in the *cps* locus, that sequentially add sugars to the chain. Before flippase Wzx translocates the repeating unit to the outside of the cell, some of the sugars in the chain can suffer modifications by acetyltransferases. After export to the extracellular space, the polymerase Wzy links the different polysaccharide units (Geno et al., 2015; Kadioglu et al., 2008).

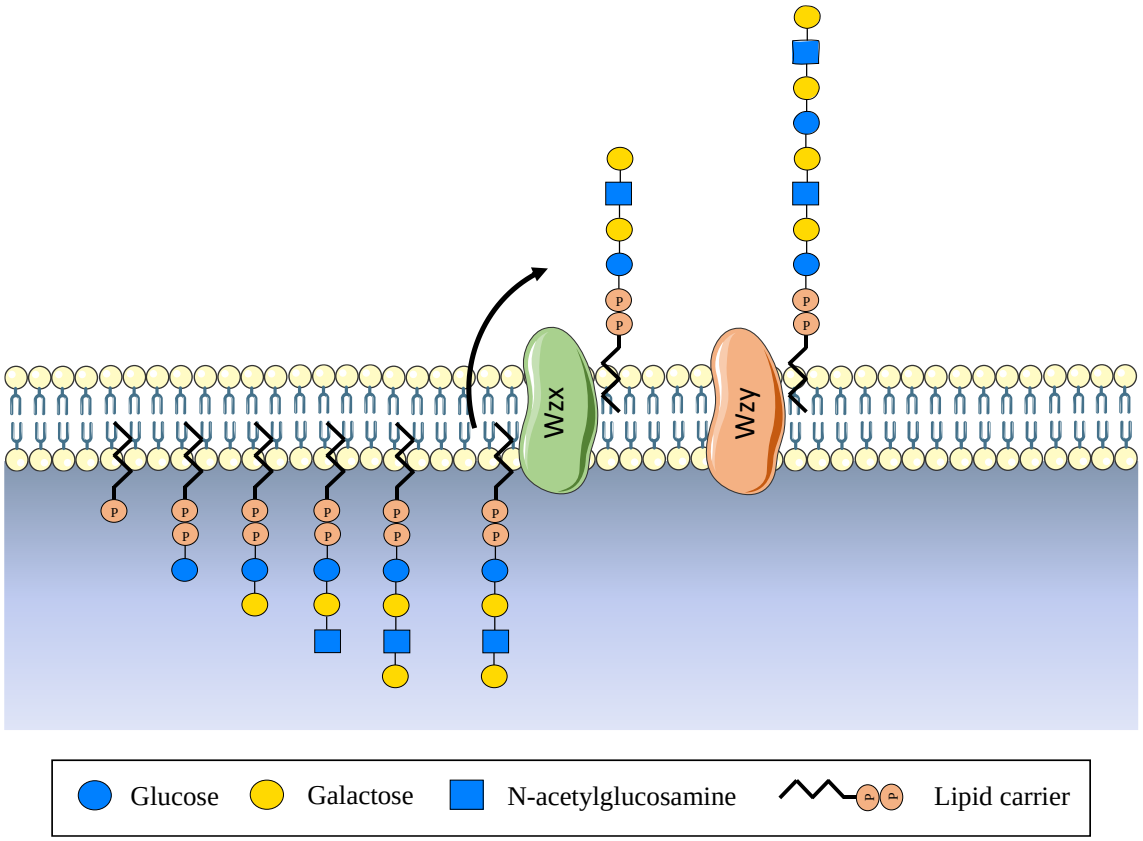

Figure 1.5 – Representation of the polymerase-dependent capsule synthesis mechanism. Glycosyltransferases are involved in the cytoplasmic assembly of the capsular repeating unit before its translocation to the extracellular space by Wzx, a flippase. After export, the polymerase Wzy links different repeating units to form the polysaccharide structure, the outermost layer of the pneumococcal cell. Adapted from Bentley et al., 2006 and Geno et al., 2015.

1.4.1.2. Regulatory genes *wzd* and *wze*

Out of the four conserved regulatory genes of the *cps* locus, *wzd* and *wze* (commonly referred to as *cpsC* and *cpsD*, respectively) are especially relevant to the subject of this dissertation.

Proteins encoded by these two genes, which are co-transcribed, act as a two-party system involved in the regulation of the kinase activity of the Wzd/Wze complex, with Wze taking the catalytic role and Wzd acting as an external domain that regulates its partner. Wzd protein is located at the cell membrane and is associated with the polysaccharide co-polymerase enzyme family whereas Wze is an autophosphorylating bacterial tyrosine kinase located at the cytoplasm (Henriques, Rodrigues, Carido, Ferreira, & Filipe, 2011; Kadioglu et al., 2008; Whittall, Morona, & Standish, 2015).

The interaction between Wzd and Wze proteins promotes capsule expression and the absence of either one has deleterious consequences on CPS production. The tyrosine kinase Wze, similarly to other members of this bacterial enzyme family, is composed of a Walker A and Walker B motifs, that specifically bind adenosine triphosphate (ATP), and a C-terminal end that

is characteristically rich in tyrosine residues. The interaction between Wzd and Wze, in the presence of ATP, induces the autophosphorylation of the tyrosine residues of Wze, which consequently regulates capsule expression (Henriques et al., 2011; Whittall et al., 2015).

1.4.2. The rise of a multitude of serotypes

As early as late 19th century, the existence of different groups of *S. pneumoniae* was described due to the serologic heterogeneity found in sera from convalescent patients. Later on, in early 20th century, through the use of serotype-specific antisera, more than 30 serotypes were described, which corresponded to most pneumococcal clinical isolates (Geno et al., 2015).

Along the years, the need for other forms of serotyping was imperative and many procedures emerged to discriminate the different pneumococcal clinical isolates, which include the Quellung reaction that later became the *status quo* of capsular typing and allowed the mass discovery of serotypes (Geno et al., 2015).

Nowadays, a clear distinction has been made between serotypes and serogroups. Serotypes are defined as strains that produce a chemical and immunologically distinct capsular structure and serogroups are the association of different serotypes that share serologic properties. Until recently, 100 different serotypes have been reported divided in almost 50 serogroups (Ganaie et al., 2020; Geno et al., 2015).

This multitude of serotypes is not surprising. It has been reported that the *cps* locus can form up to 2000 distinct coding sequences, as revealed by systematic genetic sequencing. This locus has also been classified as an evolutionary hotspot with potential to generate novel serotypes due to elevated rates of substitution and recombination. Comparison between different serotypes has shown that acquisition and loss of genetic material in this locus, specifically near the *wzd*/*wze* genes, supports the huge diversity of serotypes (Mostowy et al., 2017).

1.4.2.1. Serotype 14

Although most CPS structures are anionic, which contributes to the anti-phagocytic role as described in section 1.4., some serotypes can have neutral capsules, such as the capsular serotype 14, the capsular type most relevant to the subject of this dissertation (Geno et al., 2015).

Capsular biosynthesis for this serotype occurs in a Wzy-dependent mechanism and the gene organization of the *cps* locus as well as the putative functions for each gene product is represented in Figure 1.6 (Geno et al., 2015).

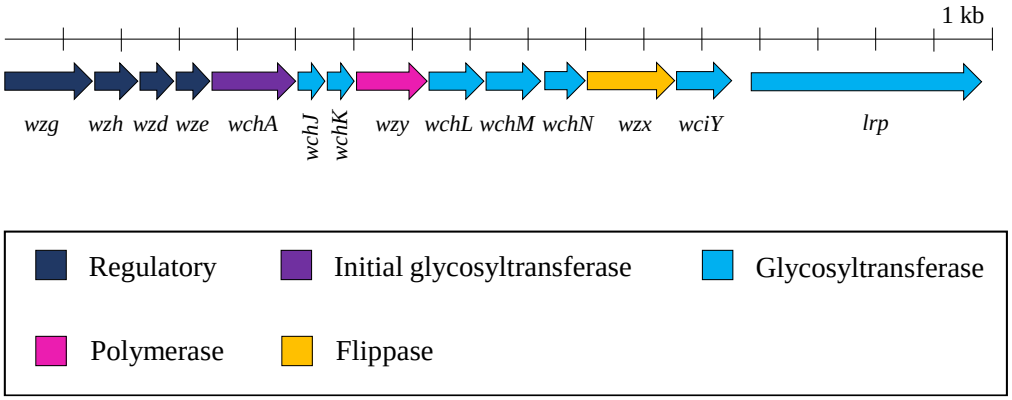

Figure 1.6 – Representation of serotype 14 *cps* gene cluster. The different illustrated genes play various roles in capsular biosynthesis accordingly to their putative functions. This representation was based on *S. pneumoniae* strain 34359 serotype 14 (GenBank accession no. CR931662.1). Adapted from Bentley et al.,2006 and Geno et al.,2015.

Serotype 14 is one of the several capsular types covered in the current pneumococcal conjugate vaccination program because, in the pre-vaccination era, it was highly responsible for invasive disease in clinical cases often causing the worst outcomes, such as necrotizing pneumonia. As serotype 14 pneumococcal isolates were commonly associated with antimicrobial resistance to chemicals, like penicillin and erythromycin, it urged the need for vaccination coverage. Since covered by vaccination, serotype 14 has been overthrown as the main responsible for invasive disease by non-vaccine covered serotypes (Ding et al., 2009).

1.5. Group previous work

The Bacterial Cell Surfaces and Pathogenesis group is interested in the process of capsular synthesis and the biological role of proteins Wzd and Wze.

Recently, some of the results obtained by Henriques et al., 2011 showed that both Wzd and Wze co-localize at the division septa of live and dividing pneumococcal cells when co-expressed. In the absence of the partner protein, Wzd localizes scattered across the cell membrane and Wze is dispersed through the cytoplasm.

Knock-down mutants of these genes were also constructed to clarify their biologic roles. Capsular synthesis seemed impaired in these null mutants due to the results obtained in Quellung reaction experiments of decreased agglutination in the presence of serum against serotype 14 capsular polysaccharide. However, contrary to previous reports indicating a complete lack of capsule (Morona, Miller, Morona, & Paton, 2004; Morona, Paton, Miller, & Morona, 2000), authors also showed that these mutants produce CPS at different subcellular sites relative to what was observed in the parental strain, as represented in Figure 1.7.

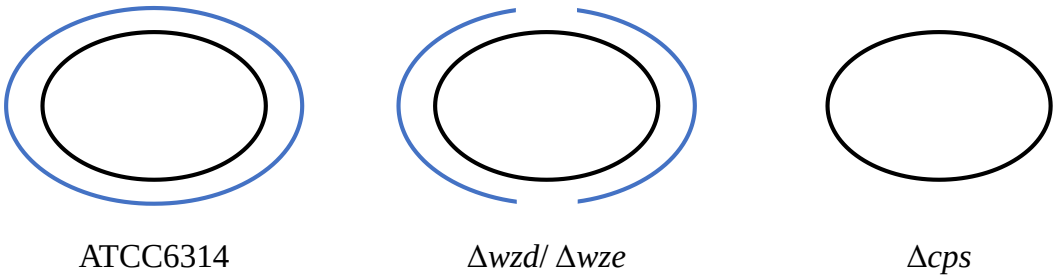

Figure 1.7 – Representation of different pneumococcal phenotypes. Knocked-down mutants of regulatory genes *wzd* and *wze* showed absence of capsule in the division septum of pneumococcal cells, whilst covering the rest of the mature cell wall. These phenotypes were observed by Henriques et al., 2011.

In the *wzd* or *wze* null mutants, capsule was produced in lower quantities than in the parental strain (ATCC6314) as it was absent specifically from the division septum but present in the mature cell wall.

Based on these results, a model was proposed reporting Wzd and Wze as spatial regulators in the capsular synthesis process. In this model, Wze interacts with Wzd, in an ATP-binding dependent manner, and the complex migrates to the septum. In the septum, the complex either activates CPS export by the synthetic machinery or themselves act as exporters ensuring the concealment of newly synthesized cell wall in encapsulated strains, such as ATCC6314.

1.6. Objectives of this dissertation

The work performed in this dissertation aims to develop a *Streptococcus pneumoniae* system capable of controlling the exposure of immature peptidoglycan to enzymes present in the surrounding medium, which will allow further analysis of this cell wall component and, therefore, contributing to the understanding of its composition and regulation mechanisms.

To achieve this goal, several objectives were accomplished and can be summarized as:

- Selection of the appropriate genetic background, confirmation and viability assessment of *S. pneumoniae* ATCC6314 derived mutants. This subject is discussed in Chapter 2.

- Construction of *S. pneumoniae* strains with constitutive expression of the *lac* repressor (*lacI*) in the genetic background Δ*wzd* and Δ*wze*. This subject is discussed in Chapter 3.

- Construction of *S. pneumoniae* strains with capsule regulatory genes *wzd* and *wze* under the control of an inducible promoter (Plac) in the genetic background 6314Δ*wzd-lacI* and 6314Δ*wze-lacI*. This subject is discussed in Chapter 4.

- Expression and purification of autolysin LytA-GFP for susceptibility assessment of the constructed strains to confirm the successful construction of a pneumococcal system to expose immature peptidoglycan. This subject is discussed in Chapter 5.

Chapter 2 – *S. pneumoniae* ATCC6314 and derived mutants confirmation

2.1. Introductory remarks

In this chapter, the *S. pneumoniae* strains used in this study, previously constructed in the laboratory, were confirmed by polymerase chain reaction (PCR) for mutations and tested by immunofluorescence (IF) to assess their viability. Detailed information about the strains is presented in Table 2.1., including their original designation and the adopted designation for each one throughout this dissertation.

Table 2.1 - *S. pneumoniae* strains, previously constructed in the host laboratory, used in this study.

***S. pneumoniae* strains**			
Original designation	Designation in this thesis	Characteristics	Source
ATCC6314	WT	Encapsulated serotype 14 strain	American Type Culture Collection
BCSMC001	Δ*cps*	ATC6314Δ*cps**	(Henriques et al., 2011)
BCSMH001	Δ*wzd*	ATCC6314Δ*wzd*	(Henriques et al., 2011)
BCSMH002	Δ*wze*	ATCC6314Δ*wze*	(Henriques et al., 2011)

* Δ*cps* mutant lacks the whole *cps* operon

Mutants Δ*wzd* and Δ*wze* are null mutants for the regulatory genes *wzd* and *wze*, respectively, and their main phenotypic feature is the lack of capsule in the division septum, which should expose immature PGN at that particular sub-cellular site. Therefore, these mutants are the ideal model to develop a *S. pneumoniae* system to control exposure of the immature cell wall component, concealed at the bacterial surface.

2.2. Materials and methods

2.2.1. Bacterial growth conditions and genomic DNA extraction

S. pneumoniae strains were grown in semi-synthetic C+Y pH 8 liquid medium, whose detailed composition is described in Supplementary Information 1, in a water bath at 37 °C

without aeration or in tryptic soy agar (TSA) plates supplemented with 5 % (v/v) defibrinated sheep blood (Thermo Fisher Scientific) at 37 ºC and in a 5 % CO_2 atmosphere.

When bacterial liquid cultures reached the stationary phase, they were diluted 1:50 in sterile water. Afterwards, genomic DNA (gDNA) was extracted by boiling the cells for 2 minutes and cooling them on ice immediately after. These gDNA samples were stored at -20 ºC until future usage.

2.2.2. PCR and agarose gel analysis

Bacterial gDNA was used for PCR confirmation of the different strains. Wild-type (WT) and Δ*wzd* mutant strain were confirmed using primers 1 and 2. On the other hand, all strains were confirmed using primers 1 and 3. All primers mentioned throughout this dissertation are described in Supplementary Table 1. PCR fragments were amplified using DreamTaq DNA polymerase (Thermo Fisher Scientific), an annealing temperature of 56 ºC, and an extension time of 1:30 minutes.

After amplification, PCR fragments were analyzed by agarose gel electrophoresis. The gel was prepared with 1 % (m/v) agarose in Tris-acetate-EDTA (TAE) buffer and 2 µL of GreenSafe Premium (NZYTech) stain. Amplification samples from the different strains were run at 100 V alongside the GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific).

2.2.3. Serotype 14 antiserum purification for immunofluorescence

As previously mentioned, the viability of the different strains of *S. pneumoniae* was assessed via IF. The capsular polysaccharide structure was detected *in vivo* at the surface of pneumococcal cells with the use of a serotype 14 specific antiserum (SSI Diagnostica).

This antiserum required previous purification against the unencapsulated mutant Δ*cps* due to the presence of antibodies that, in previous studies, were responsible for non-specific capsule detection. The purification process started with the inoculation of 5 mL C+Y pH 8 with Δ*cps* bacteria to grow overnight at 37 ºC. The following day, 3 mL of the pre-inoculum were used to inoculate 300 mL C+Y pH 8. After reaching early exponential phase, at an optical density at 600 nm (OD_{600nm}) of 0.3, the bacterial culture of the unencapsulated strain was heat-killed at 56 ºC for 45 minutes.

Bacterial culture was then centrifuged at 4 ºC and 4000 *g* for 15 minutes, the supernatant was discarded and the cells were washed twice with Phosphate-buffered saline (PBS) solution. The resultant cell pellet was resuspended in 300 µL of Anti-CPS14 serum, previously diluted in PBS in a 1/100 ratio, and incubated at 4 ºC, overnight, with gentle agitation.

In the following day, cells were centrifuged for 20 minutes at 4 ºC and 10000 *g*, and the resultant supernatant, which contained the purified serotype 14 specific antiserum, filtered (0.2 µm pore, Millipore) and stored at 4 ºC.

2.2.4. Immunofluorescence assays

CPS detection of live pneumococcal cells through IF began with the inoculation of 5 mL C+Y pH 8 with cells from each strain presented in Table 2.1. to grow overnight at 37 °C. The following day, 200 µL of each pre-inoculum were used to inoculate 5 mL C+Y pH 8 medium. After reaching an OD_{600nm} of 0.3-0.4, 1 mL aliquots of each culture were centrifuged at room temperature and 13000 *g* for 3 minutes.

Pneumococcal cells were then incubated on ice with 50 µL of purified capsule-specific serotype 14 antibodies for 5 minutes, centrifuged and washed twice with 200 µL C+Y pH 8 at 37 °C. The cell pellet was resuspended and incubated on ice with 100 µL of 4 µg/mL Goat anti-Rabbit Alexa Fluor 488 (Invitrogen) secondary antibody for 5 minutes.

Cells were then washed twice with C+Y, resuspended in 50 µL of the liquid medium and 2-3 µL of this suspension were loaded on microscopy slides that were covered by a thin layer of PreC + 1 % (m/v) agarose. The composition of PreC, one of the components of C+Y liquid medium, is described in Supplementary Information 1.

IF images were acquired using a Zeiss Axio Observer. Z1 microscope equipped with a Photometrics CoolSNAP HQ2 camera with a 500 msec exposure. Images were analyzed, adjusted and cropped using Image J software (Abràmoff, Magalhães, & Ram, 2004).

2.3. Results and discussion

2.3.1. PCR confirmation of ATCC6314 and derived mutants

The different strains were confirmed by PCR amplification using primers 1, 2 and 3. In the capsule gene cluster, primers 1 and 2 hybridize with the upstream and downstream regions of the *wzd* gene, respectively, whereas primer 3 hybridizes with the downstream region of the *wze* gene, as represented in Figure 2.1.

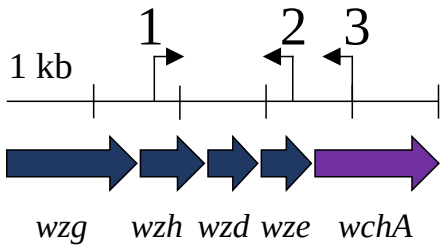

Figure 2.1 - Representation of the regions hybridized by primers 1, 2 and 3 on the genome of *S. pneumoniae* ATCC6314. Primers 1 and 2 bind to the upstream and downstream regions that flank the *wzd* gene. Primer 3 binds to the downstream flanking region of the *wze* gene.

The results of the agarose gel electrophoresis are presented in Figure 2.2. Their analysis confirmed the predicted amplification product for each one of the tested strains and the purity of the samples.

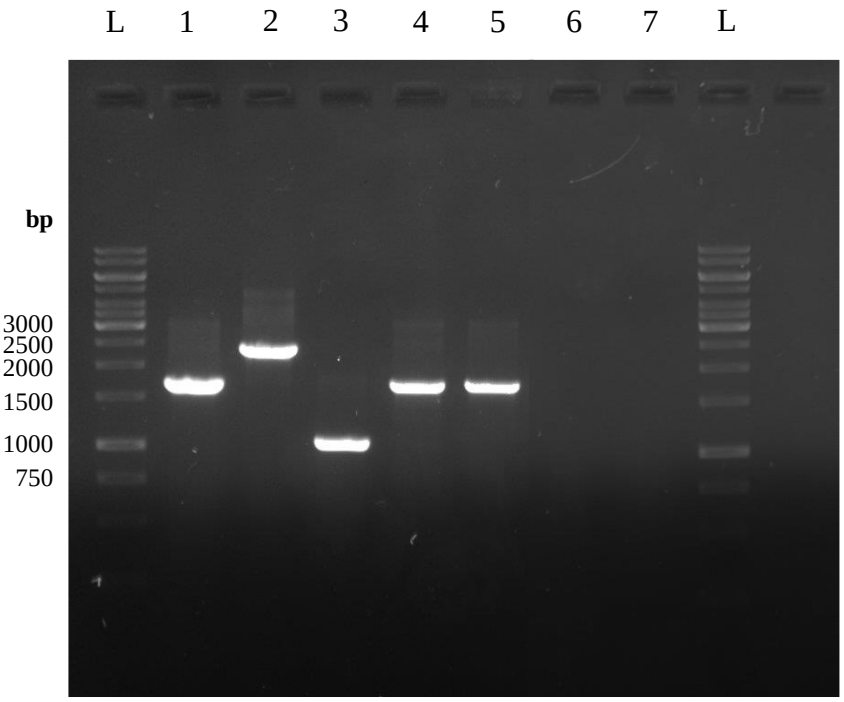

Figure 2.2 – Agarose gel electrophoresis results of *S. pneumoniae* strains. Electrophoresis run was fixed at 100 V and GeneRuler 1 kb DNA Ladder was used for sizing of the amplification products which corresponds to lane L. 1 – ATCC6314 amplification product using primers 1 and 2; 2 - ATCC6314 amplification product using primers 1 and 3; 3 – Δ*wzd* amplification product using primers 1 and 2; 4 – Δ*wzd* amplification product using primers 1 and 3; 5 – Δ*wze* amplification using primers 1 and 3; 6 – Δ*cps* amplification product using primers 1 and 2; 7 – Δ*cps* amplification product using primers 1 and 3.

The amplification products obtained for ATCC6314 strain with both primer pairs demonstrated the presence of intact *wzd* and *wze* genes as expected. Amplification product length with primers 1 and 2 was around 1700 base pairs (bp) (lane 1, Figure 2.2) and with primers 1 and 3 around 2400 bp (lane 2, figure 2.2), as the latter primer hybridizes downstream of *wze* gene. As these genes are intact, it can be assumed that so is the rest of the *cps* operon and that the capsular structure is present, which can be corroborated with the IF assays.

As for the Δ*wzd* mutant, amplification products length coincided with the expected results for a *wzd* null mutant. Primers 1 and 2 originated a 1015 bp fragment (lane 3, Figure 2.2), as primer 2 hybridizes downstream of the non-existent *wzd* gene in this mutant, and primers 1 and 3 originated a 1700 bp fragment (lane 4, Figure 2.2). Amplification with primers 1 and 3 originated a 1715 bp fragment (lane 5, Figure 2.2) in the *wze* null mutant, as expected.

Lastly, the Δ*cps* mutant showed no amplification products (lanes 6 and 7, Figure 2.2) which is explained by the absence of the whole *cps* operon and therefore, the absence of capsular structure.

2.3.2. Immunofluorescence assays

IF images of live pneumococcal cells of the ATCC6314 strain and derived mutants are presented in Figure 2.3.

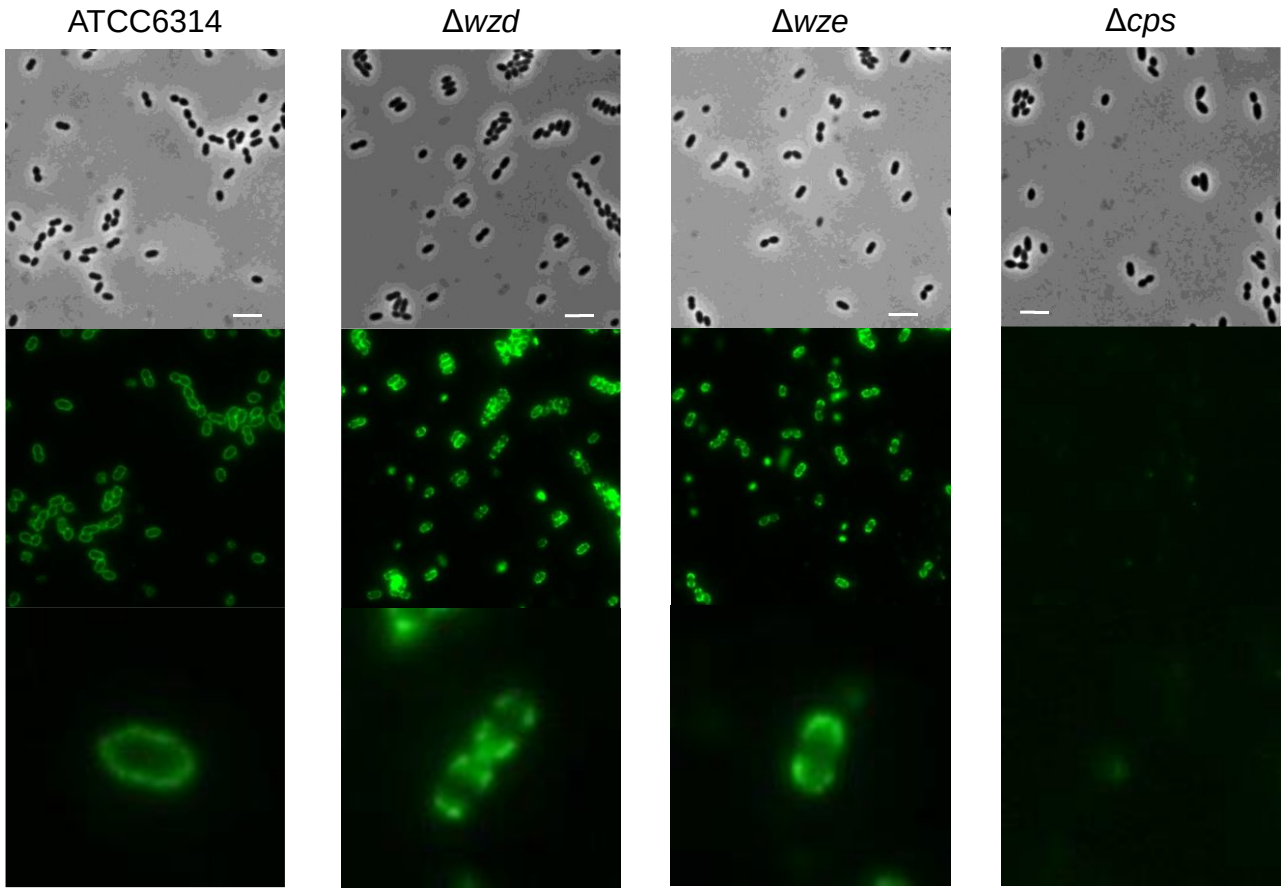

Figure 2.3 – Immunofluorescence of the different *S. pneumoniae* strains. Capsular detection of ATCC6314 and derived mutants Δ*wzd*, Δ*wze* and Δ*cps* was achieved through IF assays using a serotype 14 capsule specific antiserum and secondary antibody Alexa 488. The first panel in all strains corresponds to brightfield images, the second to fluorescence images and the third to the ampliation of an area of the image with a single and representative bacteria cell. Scale bar, 2 µm.

In the WT strain, an intact capsular structure was detected by IF microscopy, confirming the integrity of the *cps* operon responsible for CPS production and regulation.

Regarding Δ*wzd* and Δ*wze* mutants, a different capsular production pattern from the parental strain was observed with the polysaccharide being absent from the division septum but present in the rest of the cell.

The *cps* operon null mutant had no capsular *in vivo* detection confirming the operon deletion.

The serotype 14 antiserum purification process against this strain was required due to previous non-specific detection results in the unencapsulated mutant. The purification allowed enrichment of serotype 14 capsule specific antibodies and therefore, Δ*cps* mutant acts as a negative control for this process due to its absence of capsule and IF images confirms its effectiveness as no detection was observed.

The IF results obtained and described here for each strain confirmed what has been previously observed by Henriques *et al.*, 2011 and corroborated the statements made in the previous section about the purity and viability of the different strains.

Chapter 3 – Construction of *S. pneumoniae* strains with the *lac* repressor (*lacI*)

3.1. Introductory remarks

In this chapter, *S. pneumoniae* Δ*wzd* and Δ*wze* strains, whose viability was assessed in Chapter 2, were altered so that they are able to express the *Escherichia coli* LacI repressor under the control of a constitutive promoter. An integrative plasmid, pPEPY-PF6-lacI, which is capable of being replicated in *E. coli* and that carries the repressor gene flanked by upstream and downstream sequences with high homology with a large number of <pneumococcal strains, was used. Information in Table 3.1 summarizes some of its features and antibiotic selection in different bacterial organisms.

Table 3.1 – Information on pPEPY-PF6-lacI, an integrative plasmid carrying *lacI*, used in this study.

		Antibiotic selection	
Name	Features	*E. coli*	*S. pneumoniae*
pPEPY-PF6-lacI	Constitutive PF6 promoter and *lac* repressor (LacI)	Kan 50 µg/mL	Gent 150 µg/mL *

Kan – Kanamycin; Gent – Gentamycin

* Determined in this thesis

3.2. Materials and methods

3.2.1. PCR amplification and purification of *lacI*

The *lacI* gene present in plasmid pPEPY-PF6-lacI (a gift from Jan-Willem Veening; Addgene plasmid # 85589 ; http://n2t.net/addgene:85589 ; RRID:Addgene_85589) was amplified from an isolated *E. coli* colony through PCR using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific), primers 4 and 5, an annealing temperature of 55 °C and an extension time of 1:30 minutes.

The amplified PCR fragment was analyzed through electrophoresis in a 1 % (m/v) agarose gel prepared and run as described in section 2.2.2.

Then, the amplification product was purified accordingly to the NZYGelpure protocol provided by NZYTech©. First, the volume of the reaction mixture was transferred to a 1.5 mL

microcentrifuge tube to which five volumes of Binding Buffer were added, followed by inversion of the tube for mixing. The volume of the mixture was then added to a NZYTech spin column followed by a 30 seconds to a minute centrifugation at 12000 *g*.

After discarding the flow-through, 600 µL of the Wash Buffer were added to the column and the tube centrifuged for 30 seconds to a minute. Once more the flow-through was discarded and the tube was centrifuged for 1 minute to dry the NZYTech spin membrane of residual ethanol.

At last, 50 µL of the Elution Buffer were added to the center of the NZYTech spin column, previously placed into a clean 1.5 mL microcentrifuge tube and after a 1 minute incubation at room temperature, the tube was centrifuged for 1 minute for DNA elution. After elution, purified DNA was quantified (NanoDrop) and stored at -20 ºC.

3.2.2. Preparation of *S. pneumoniae* competent cells

Pneumococcal competent cells of all strains listed in Table 2.1., except Δ*cps* mutant, were prepared. For that, 5 mL C+Y pH 8 were inoculated with the different strains to grow overnight at 37 °C.

In the following day, 100 µL of the overnight culture were used to inoculate 5 mL of fresh C+Y pH 8 medium. After reaching an OD_{600nm} of 0.4-0.5, mid-exponential phase, the different cell cultures were placed on ice and aliquoted with glycerol at a final concentration of 15 % (v/v).

At last, competent cells were frozen in dry ice and stored immediately at -80 °C until further usage.

3.2.3. Antibiotic selection determination

Before transforming Δ*wzd* and Δ*wze* mutants with the DNA fragment that encoded the LacI repressor, all pneumococcal strains presented in Table 2.1, as well as the unencapsulated R36A strain, were plated in TSA supplemented with 5 % (v/v) defibrinated sheep blood and gentamycin (Gent) in increasing concentrations (40, 80, 100, 120, 150, and 200 µg/mL), in order to determine the adequate antibiotic concentration for the selection of transformants.

3.2.4. Transformation of Δ*wzd* and Δ*wze* and colony screening

The transformation of pneumococcal competent cells was achieved based on the protocol described by (Pozzi et al., 1996). Transformation process began with the addition of the following listed components to 100 µL of competent cells:

a. 200 µL of 1.6 % (m/v) bovine serum albumin (BSA);
b. 200 µL of 0.1 % (m/v) $CaCl_{2;}$
c. 40 µL of 12.5 µg/mL competence stimulating peptide-1 (CSP-1);

d. DNA to a final concentration of 1 µg/mL. Δ*wzd* and Δ*wze* mutants were transformed with the amplified DNA fragment described in section 3.2.1.
e. C+Y pH 8 liquid medium for a final reaction volume of 2 mL.

After an incubation period of 150 minutes in a water bath at 37 °C, cells were seeded in TSA supplemented with 5 % (v/v) defibrinated sheep blood and 150 µg/mL Gent to grow overnight at 37 °C and in a 5 % CO_2 atmosphere.

In the next day, several colonies were picked, plated onto new TSA supplemented with antibiotic and incubated overnight as before. These isolated colonies were screened for the presence of *lacI* by PCR with primers 4 and 5 and DreamTaq DNA polymerase (Thermo Fisher Scientific) at an annealing temperature of 55 °C and extension time of 2:10 minutes.

Positive *lacI* colonies were inoculated in C+Y pH 8 and 150 µg/mL Gent and grown in the water bath at 37 °C until stationary phase. Cell cultures were stored in aliquots with glycerol at a final concentration of 10 % (v/v).

3.3. Results and discussion

3.3.1. Amplification of *lacI* for transformation

Plasmid pPEPY-PF6-lacI contains two regions homologous with the non-essential *prsA* locus in the genome of *S. pneumoniae* D39 strain, which have high similarity with several other pneumococcal strains, including ATCC6314 (Liu et al., 2017). These upstream and downstream regions flank the *lacI* gene and a gentamycin resistance marker as seen on Figure 3.1.

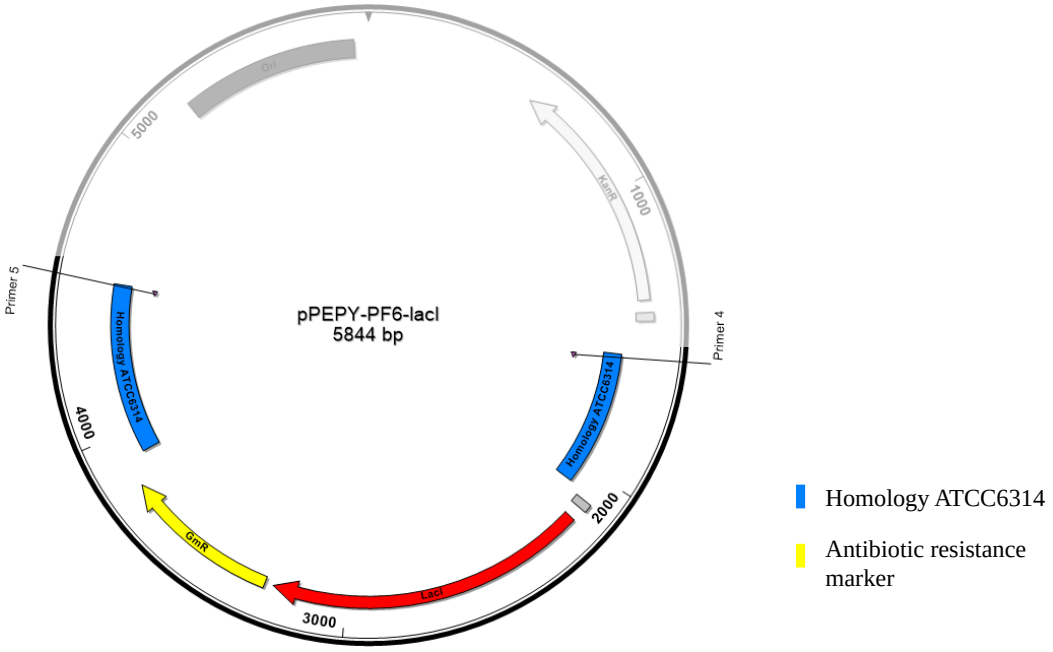

Figure 3.1 – Representation of integrative plasmid pPEPY-PF6-lacI. This plasmid includes two regions homologous with the genome of *S. pneumoniae* ATCC6314 strain, the LacI repressor (red arrow) and two antibiotic resistance markers, gentamycin (GmR) for *S. pneumoniae* and kanamycin (KanR) for *E. coli*.

Here, a DNA fragment that includes the *lacI* gene was amplified from the pPEPY-PF6-lacI plasmid with primers 4 and 5 that hybridize at the 5´ end of the upstream and 3´ end of the downstream homologous regions, respectively as illustrated in Figure 3.1. Therefore, transforming ATCC6314 derived mutants, Δ*wzd* and Δ*wze*, with this DNA fragment will result in transformants that can be selected by their gentamycin resistance and that have integrated the *lacI* gene in the genome at that specific locus by homologous recombination.

In pPEPY-PF6-lacI, expression of the *lacI* gene is driven by the constitutive promoter PF6, resulting in the constant production of the repressor after integration into the genome of a pneumococcal strain, in this case Δ*wzd* and Δ*wze* strains.

This constitutive expression of LacI triggers a very slow growth rate in *E. coli* cells. In the absence of its natural inducer 1,6 – allolactose or analogues, the repressor binds to a specific DNA sequence of the operator present in the *lac* operon. After binding, LacI induces a repression in the transcription of the downstream genes of the operon, including gene *lacZ* encoding the β-galactosidase enzyme. This explains the decreased growth rate considering that enzymatic degradation of β-galactosidase substrates promotes cell growth (Rondon & Wilson, 2019; Swerdlow & Schaaper, 2015).

For this reason, the repressor was amplified directly from *E. coli* cells containing the plasmid as, due to poor growth, isolation of plasmid DNA was not possible.

The results of the agarose gel electrophoresis relative to *lacI* amplification are presented in Figure 3.2.

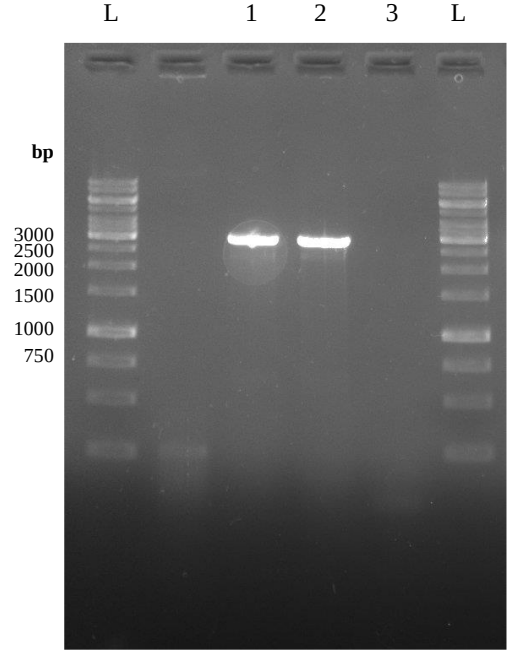

Figure 3.2 – Agarose gel electrophoresis results of *lacI* amplification. Electrophoresis run was fixed at 100 V and GeneRuler 1 kb DNA Ladder was used for sizing of the amplification products which corresponds to lane L. 1, 2 – Amplification product containing *lacI* using primers 4 and 5; 3 – Amplification negative control.

The *lacI* gene amplification using primers 4 and 5 resulted in a 2947 bp product, as expected. The samples appear pure, even though amplification was done with isolated *E. coli* colonies instead of purified plasmid DNA. The amplification product was then purified for later use in the transformation of pneumococcus.

3.3.2. Determination of the antibiotic concentration for transformants selection

Colony growth was observed for *S. pneumoniae* ATCC6314 and derived mutants when plated in the concentration of 40 µg/mL gentamycin, which is recommended for pneumococcal cells carrying the plasmid pPEPY-PF6-lacI (Liu et al., 2017). Therefore, these strains, as well as the unencapsulated strain R36A, were plated in increasing concentrations of gentamycin to determine the antibiotic concentration that would specifically select pneumococcal cells that successfully integrated the repressor in their genome.

The results obtained showed that Δ*wzd* and Δ*wze* mutants have higher gentamycin susceptibility as their growth was inhibited by lower concentrations (100 µg/mL) of this antibiotic. Additionally, 150 µg/mL of gentamycin was sufficient for minimal or no growth of all pneumococcal strains tested hence the optimal concentration for transformants selection.

3.3.3. Transformation of Δ*wzd* and Δ*wze* with the DNA fragment containing LacI

Besides extracellular DNA in saturating concentration and competent cells, the transformation reaction included BSA and Ca^{2+} because they seem to play a role in increasing the transformation frequency, as demonstrated before in other naturally competent organisms, such as the human pathogen *Acinetobacter baumannii* (Traglia, Quinn, Schramm, Soler-bistue, & Ramirez, 2016).

Although, ATCC6314 and derived mutants are naturally competent and therefore, CSP producers, this stimulating peptide was added to the mixture to ensure that all cells in the culture sample were in a permissive competent state (after CSP detection by its receptor ComD).

After transformation of the Δ*wzd* and Δ*wze* mutants with the amplification product containing the *lacI* gene, 20 isolated colonies from each strain were screened by PCR with primers 4 and 5 for the integration of the repressor in the genome. Agarose gel electrophoresis results of the colony screening are presented in Figure 3.3.

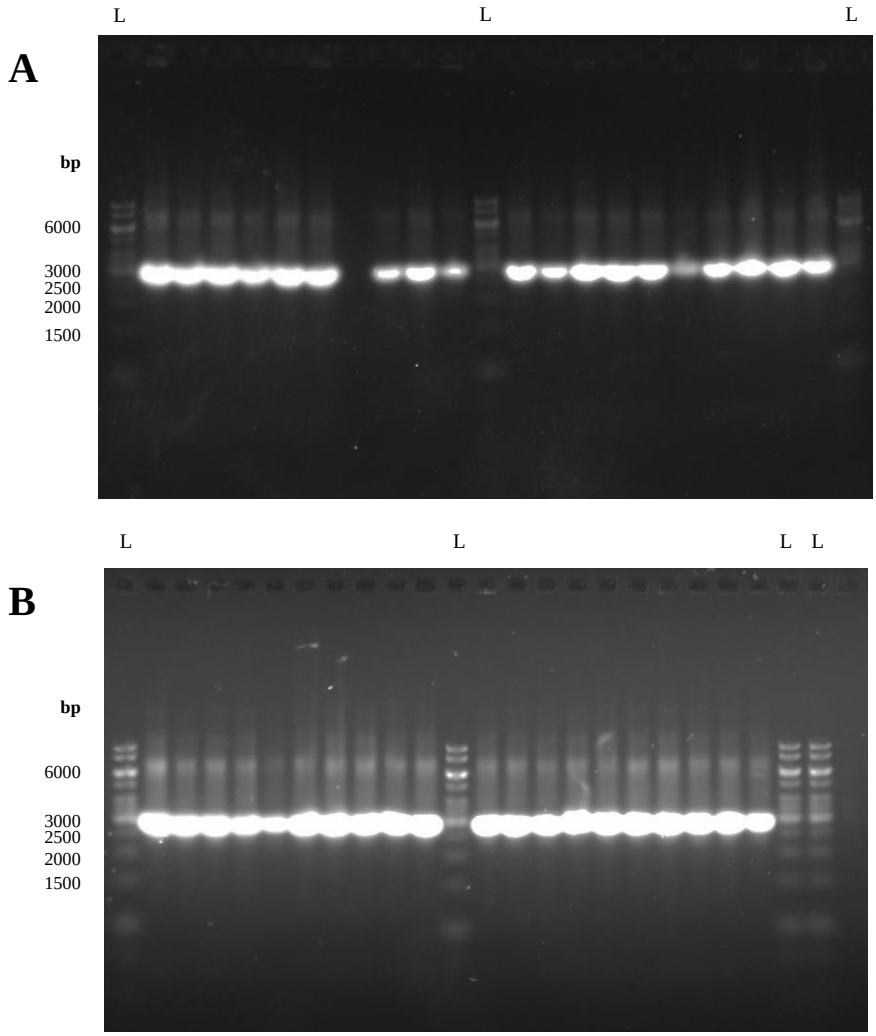

Figure 3.3 - Agarose gel electrophoresis results of *lacI* positive colony screening. Electrophoresis run was fixed at 100 V and GeneRuler 1 kb DNA Ladder was used for sizing of the amplification products which corresponds to lanes L. In panel **A** are the colony screening results for the Δ*wzd* mutant. In panel **B** are the colony screening results for the Δ*wze* mutant. Primers 4 and 5 were used for the screening of positive *lacI* colonies in both strains.

According to the agarose gel electrophoresis results, nearly all colonies screened were positive for the presence of the *lacI* gene in both genetic backgrounds Δ*wzd* and Δ*wze* due to the amplification of the 2947 bp *lacI* containing fragment (shown before in section 3.3.1.). These results show a good transformation efficiency and effectiveness of both protocols for preparation of pneumococcal competent cells and subsequent transformation.

In contrast to the growth impairment observed in *E. coli* cells, the *lacI* positive pneumococcal colonies grew normally in C+Y pH 8 liquid medium and 150 µg/mL gentamycin. In pneumococcus, genes involved in lactose degradation are organized differently with the existence of a *lac* gene cluster divided in two operons, I and II. Previous reports have shown that

LacR acts as a repressor of the *lac* operon I in the absence of lactose in *S. pneumoniae*, similarly to LacI in *E. coli* (Afzal, Shafeeq, & Kuipers, 2014).

As LacR and LacI are two different proteins with no reported homology, their DNA binding motifs might differ explaining why the constitutive presence of LacI in *S. pneumoniae* strains does not impact the growth rate as it does in *E. coli* cells. In sum, although pneumococcus can utilize lactose, its uptake and metabolism seems to be independent of the exogenous LacI.

Chapter 4 - Construction of *S. pneumoniae* strains with *wzd* and *wze* genes under the control of an inducible promoter

4.1. Introductory remarks

In this chapter, *S. pneumoniae* strains were constructed with the expression of the capsule regulatory *wzd* and *wze* genes under the control of an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible promoter, the Plac promoter. These strains were constructed in the genetic background of the strains constructed in the previous chapter, 6314Δ*wzd-lacI* and 6314Δ*wze-lacI*, which will should permit the development of a system to control exposure of immature PGN.

To do so, an integrative plasmid, which is capable of replicating in *E. coli* and that carries this specific promotor flanked by DNA with homology with a large number of pneumococcal strains, was used. Information in Table 4.1 summarizes some of its features and antibiotic selection in different bacterial organisms.

Table 4.1 – Information on pPEPZ-Plac, an integrative plasmid carrying the Plac promoter, used in this study.

		Antibiotic selection	
Name	Features	*E. coli*	*S. pneumoniae*
pPEPZ-Plac	IPTG-inducible Plac pomoter	Spec 50 µg/mL	Spec 200 µg/mL

Spec – Spectinomycin

4.2. Materials and methods

4.2.1. *S. pneumoniae* genomic DNA extraction

Pneumococcal gDNA was extracted accordingly to the NZY Microbial gDNA Isolation kit protocol provided by NZYTech© that allows a small scale highly pure gDNA preparation based on mechanical disruption to achieve cell lysis instead of enzymatic procedures.

For the extraction of pneumococcal gDNA, ATCC6314 cells were grown overnight at 37 °C in 5 mL C+Y pH 8 liquid medium. In the following day, cells were harvested by centrifugation, the supernatant discarded and the cells resuspended in 100 µL of Elution Buffer

NME. Afterwards, the cell suspension was transferred into a NZYSpin Microbial Bead Tube and 40 µL of Buffer NML and 10 µL of Proteinase K were added to the mixture.

The Bead tube with the cell suspension was then placed on a swing mill in three cycles of one minute each. After mechanical disruption of the Gram-positive bacteria cells, the cell suspension was centrifuged for 30 seconds at 11000 *g*.

The resultant cell pellet was resuspended in 600 µL of Buffer NML and this suspension was mixed in the vortex for 3 seconds before it was centrifuged again for 30 seconds at 11000 *g*. The supernatant was transferred to a NZYSpin Microbial Column, which was also centrifuged for 30 seconds at 11000 *g*. After centrifugation, the column was placed in a new collection tube.

The column silica membrane was then washed with 500 µL of Buffer NMW1 followed by 500 µL of Buffer NMW2. After each centrifugation, the flow through was rejected. The column was centrifuged one more time for the silica membrane to dry and to remove the residual wash buffers.

For DNA elution, 100 µL of NME Buffer was added directly to the column silica membrane, previously placed into a clean 1.5 mL microcentrifuge, and after a 1 minute incubation period at room temperature, the tube was centrifuged for 30 seconds at 11000 *g*.

Purified DNA was quantified (NanoDrop) and then stored at -20 ºC.

4.2.2. PCR amplification and purification of *wzd* and *wze*

Fragments of DNA encoding the capsule regulatory genes *wzd* and *wze* were amplified from the gDNA of *S. pneumoniae* ATCC6314 by PCR using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) with primers 6 and 7 and primers 8 and 9, respectively. An annealing temperature of 57 °C and extension time of 30 seconds were used in the PCR process. The primers used were designed to create amplicons with an upstream BamHI and a downstream XhoI restriction sites.

PCR fragments were analyzed through electrophoresis in a 1 % (m/v) agarose gel, prepared and run as described in section 2.2.2. and directly purified accordingly to the NZYGelpure protocol provided by NZYTech© described in the previous chapter.

4.2.3. Enzymatic digestion of pPEPZ-Plac, *wzd* and *wze* and ligation reaction

The integration plasmid pPEPZ-Plac (a gift from Jan-Willem Veening; Addgene plasmid # 122635; http://n2t.net/addgene:122635; RRID:Addgene_122635) and *wzd* and *wze* amplicons were digested with FastDigest BamHI and FastDigest XhoI (Thermo Fisher Scientific). For DNA restriction, the following reaction mixture was prepared:

a. 1 µL of each FastDigest enzyme;

b. 2 µL of 10X FastDigest buffer;
c. 300 ng or 500 ng of DNA;
d. water for a final reaction volume of 20 µL.

The reaction mixture was mixed gently and incubated at 37 °C in a heat block (Eppendorf) for 15 minutes. Lastly, enzymes were inactivated by incubating the reaction mixture at 80 °C for 5 minutes.

Both *wzd* and *wze* digested amplicons were cloned into pPEPZ-Plac separately. The ligation was achieved with the preparation of the following reaction mixture:

a. 1 U of T4 DNA Ligase (Thermo Fisher Scientific) enzyme;
b. 2 µL of 10X T4 DNA Ligase buffer;
c. 65 ng of linear plasmid DNA;
d. a 3:1 molar ratio of insert DNA over plasmid DNA;
e. water for a final reaction volume of 20 µL.

The ligation reaction mixture was incubated for 1 hour at 22 °C to maximize transformation efficiency. The resultant plasmids pPEPZ-Plac-*wzd* and pPEPZ-Plac-*wze* were used to transform competent *E. coli* DH5α for plasmid propagation.

4.2.4. Preparation of *E. coli* DH5α competent cells

E. coli DH5α competent cells preparation started with the inoculation of 5 mL Luria-Bertani (LB) medium with DH5α cells to grow overnight at 37 °C and 180 revolutions per minute (rpm) (Innova 40, New Brunswick). In the following day, 200 µL of the pre-inoculum were used to inoculate 100 mL LB medium incubated at 37 °C and 180 rpm until the OD_{600nm} reached 0.35-0.45.

Then, the cell culture was kept on ice for 15 minutes and centrifuged at 3000 *g* for 5 minutes at 4 °C. The pellet was kept and resuspended in 15 mL of RF1 solution. The resuspension was kept on ice for 15 minutes and centrifuged at 3000 *g* for 5 minutes at 4 °C. After discarding the supernatant, the cell pellet was resuspended in 4 mL of RF2 solution and kept on ice for 15 minutes. The composition of solutions RF1 and RF2 are described in Supplementary Information 2.

At last, 800 µL of 50 % (v/v) glycerol were added to the cells. The competent *E. coli* cells were aliquoted and stored at -80 °C.

4.2.5. Transformation of competent *E. coli* DH5α cells, colony screening and plasmid purification

To transform competent *E. coli* DH5α cells, each ligation reaction containing pPEPZ-Plac-*wzd* or pPEPZ-Plac-*wze* (total volume of 20 µL) was added to 100 µL of competent cells previously defrosted on ice. Then, the cells were incubated on ice for 10 minutes, followed by 1 minute at 42 ºC and 5 minutes on ice.

Subsequently, 900 µL LB medium were added to the culture followed by an incubation at 37 ºC with agitation for 1 hour.

After incubation, 100 µL of the cell culture were plated in Luria-Bertani agar (LA) supplemented with 50 µg/mL of spectinomycin (Spec) and the rest centrifuged. After discarding the supernatant, the pellet was resuspended in 200 µL LB medium and 100 µL were plated in the same conditions as before.

In the next day, several colonies were picked, plated onto new LA plates supplemented with antibiotic and incubated overnight at 37 ºC. These isolated colonies were screened for the presence of capsule regulatory genes *wzd* and *wze* by PCR with primer pairs 6/7 and 8/9, respectively, DreamTaq DNA polymerase (Thermo Fisher Scientific), an annealing temperature of 57 °C and extension time of 1 minute.

PCR fragments were analyzed through electrophoresis in a 1 % (m/v) agarose gel, prepared and run as described in section 2.2.2.

Then, a positive colony was picked to inoculate 5 mL LB medium supplemented with 50 µg/mL Spec to grow overnight at 37 °C with vigorous shaking (180 rpm).

The next day, highly pure plasmid DNA was prepared accordingly to the NZY Miniprep protocol provided by NZYTech©. Plasmid purification began with cell harvesting by centrifugation for 30 seconds at 12000 *g*. The resultant cell pellet was resuspended with 250 µL of Buffer A1 by vigorous vortexing, followed by the addition of 250 µL of Buffer A2 (maximum incubation period of 4 minutes) and 300 µL of Buffer A3. The suspension was then mixed exclusively by inversion of the reaction tube.

After cell lysis, the culture was centrifuged for 10 minutes at room temperature and the supernatant loaded onto a NZYTech spin column, which was centrifuged for 1 minute. Following flow through rejection, 500 µL of Buffer AY and 600 µL of Buffer A4 were added to the column, each followed by a 1 minute centrifugation and the respective supernatant discarded. The column was centrifuged one more time for the silica membrane to dry and to remove the residual wash buffers.

At last, 50 µL of Buffer AE were added directly to the column silica membrane, previously placed into a clean 1.5 mL microcentrifuge. After a 1 minute incubation period at room temperature, the tube was centrifuged for 1 minute for plasmid DNA elution.

Purified DNA was quantified (NanoDrop) and stored at -20 ºC.

4.2.6. PCR amplification of Plac-*wzd*, Plac-*wze* and Plac products

The products Plac-*wzd* and Plac-*wze* were amplified from the purified plasmids pPEPZ-Plac-*wzd* and pPEPZ-Plac-*wze*, respectively, through PCR using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) with primers 10 and 11 (listed in Supplementary Table 1) at an annealing temperature of 55 °C and extension time of 2:00 minutes. Empty pPEPZ-Plac plasmid was also used as a control.

PCR fragments were analyzed through electrophoresis in a 1 % (m/v) agarose gel prepared and run as described in section 2.2.2. and purified accordingly to the NZYGelpure protocol provided by NZYTech© described in section 3.2.1.

4.2.7. Preparation of pneumococcal 6314Δ*wzd-lacI* and 6314Δ*wze-lacI* competent cells, transformation and colony screening

The preparation of *S. pneumoniae* 6314Δ*wzd-lacI* and 6314Δ*wze-lacI* competent cells, from mutant strains constructed in the previous chapter, and their transformation with respective Plac-*wzd*, Plac-*wze* and Plac amplification products were performed as described in sections 3.2.2. and 3.2.4., except that transformants were plated in TSA supplemented with 5 % (v/v) defibrinated sheep blood, 150 µg/mL Gent and 200 µg/mL Spec.

Several colonies were screened by PCR reaction with primers 10 and 11 and DreamTaq DNA polymerase (Thermo Fisher Scientific) at an annealing temperature of 55 °C and extension time of 3:00 minutes. Positive Plac-*wzd*, Plac-*wze* and Plac colonies were inoculated in C+Y pH 8 supplemented with 150 µg/mL Gent and 200 µg/mL Spec and grown in the water bath at 37 °C until stationary phase. Cell cultures were stored in aliquots with glycerol at a final concentration of 10 % (v/v).

4.2.8. Immunofluorescence assays of *S. pneumoniae* 6314Δ*wze-lacI*-Plac-*wze* and 6314Δ*wze-lacI*-Plac strains

Prior to the immunofluorescence assays, strains 6314Δ*wze-lacI*-Plac-*wze* and 6314Δ*wze-lacI*-Plac were grown in C+Y pH 8 supplemented with 150 µg/mL Gent, 200 µg/mL Spec and 1mM IPTG for 4 generations.

CPS detection in live pneumococcal cells by IF as well as serotype 14 antiserum purification necessary in these assays were achieved as described in sections 2.2.3. and 2.2.4.

IF images were acquired using a Zeiss Axio Observer. Z1 microscope equipped with a Photometrics CoolSNAP HQ2 camera with a 250 msec exposure. After acquisition, images were analyzed, adjusted and cropped using Image J software (Abràmoff et al., 2004).

4.3. Results and discussion

4.3.1. PCR amplification of *wzd* and *wze*

DNA fragments that include the capsule regulatory genes *wzd* and *wze* were amplified through PCR from the genomic DNA of ATCC6314 pneumococcal strain with upstream BamHI and downstream XhoI restriction sites for posterior cloning into the pPEPZ-Plac integration plasmid. The results of the agarose gel electrophoresis are presented in Figure 4.1. Their analysis confirmed the predicted amplification products for both genes.

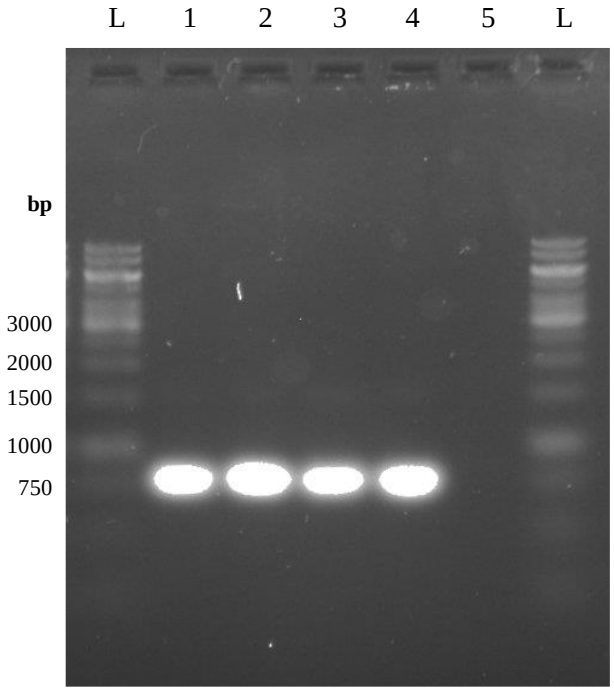

Figure 4.1 - Agarose gel electrophoresis results of *wzd* and *wze* amplification. Electrophoresis run was fixed at 100 V and GeneRuler 1 kb DNA Ladder was used for sizing of the amplification products which corresponds to lane L. 1, 2 – *wze* amplification product using primers 8 and 9; 3, 4 - *wzd* amplification product using primers 6 and 7; 5 – Amplification negative control.

The *wzd* amplification product using primers 6 and 7 had a size similar to the expected 775 bp. The same was observed with the *wze* amplification product using primers 8 and 9, whose size was similar to the expected 791 bp. All samples were apparently pure, confirming a

successful genomic DNA extraction. Therefore, these amplification products were purified before enzymatically digested.

4.3.2. Construction of pPEPZ-Plac-*wzd* and pPEPZ-Plac-*wze*

Integration plasmid pPEPZ-Plac and *wzd* and *wze* genes were enzymatically digested with BamHI and XhoI prior to cloning. The digestion products of pPEPZ-Plac were analyzed by agarose gel electrophoresis (Figure 4.2).

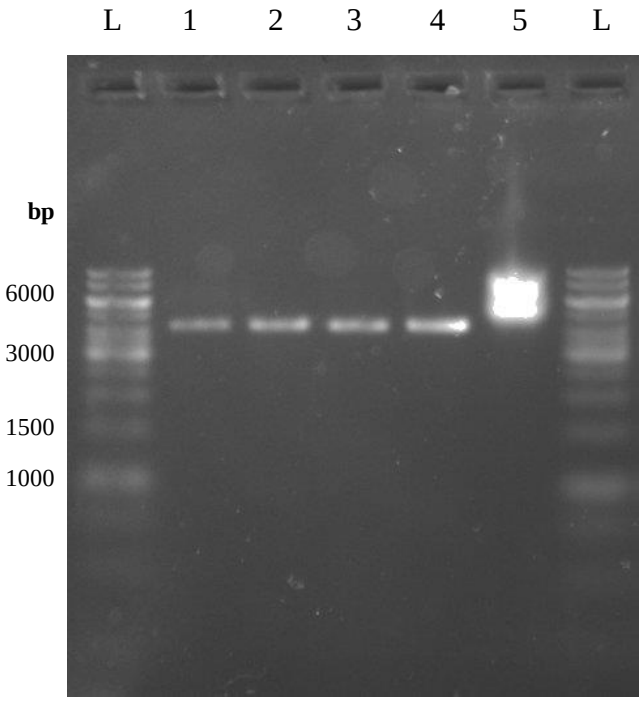

Figure 4.2 - Agarose gel electrophoresis results of pPEPZ-Plac digestion products. Electrophoresis run was fixed at 100 V and GeneRuler 1 kb DNA Ladder was used for sizing of the amplification products which corresponds to lane L. 1 – pPEPZ-Plac single digestion with XhoI; 2 - pPEPZ-Plac single digestion with BamHI; 3 - pPEPZ-Plac double digestion with XhoI and BamHI (300 ng pDNA); 4 - pPEPZ-Plac double digestion with XhoI and BamHI (500 ng pDNA); 5 – Undigested pPEPZ-Plac (300 ng pDNA).

The size of the products obtained after single digestion with each of the enzymes or after a double digestion with both enzymes corresponded to 4314 bp, the expected size of the linear plasmid.

The integrative plasmid pPEPZ-Plac that was used as the backbone for cloning the capsule regulatory genes *wzd* and *wze* under the control of the Plac promoter is represented in panel A of Figure 4.3.

The BamHI/XhoI digested genes *wzd* and *wze* were each cloned into BamHI/XhoI digested pPEPZ-Plac resulting in plasmid constructs pPEPZ-Plac-*wzd* and pPEPZ-Plac-*wze*, respectively, represented in panel B of Figure 4.3.

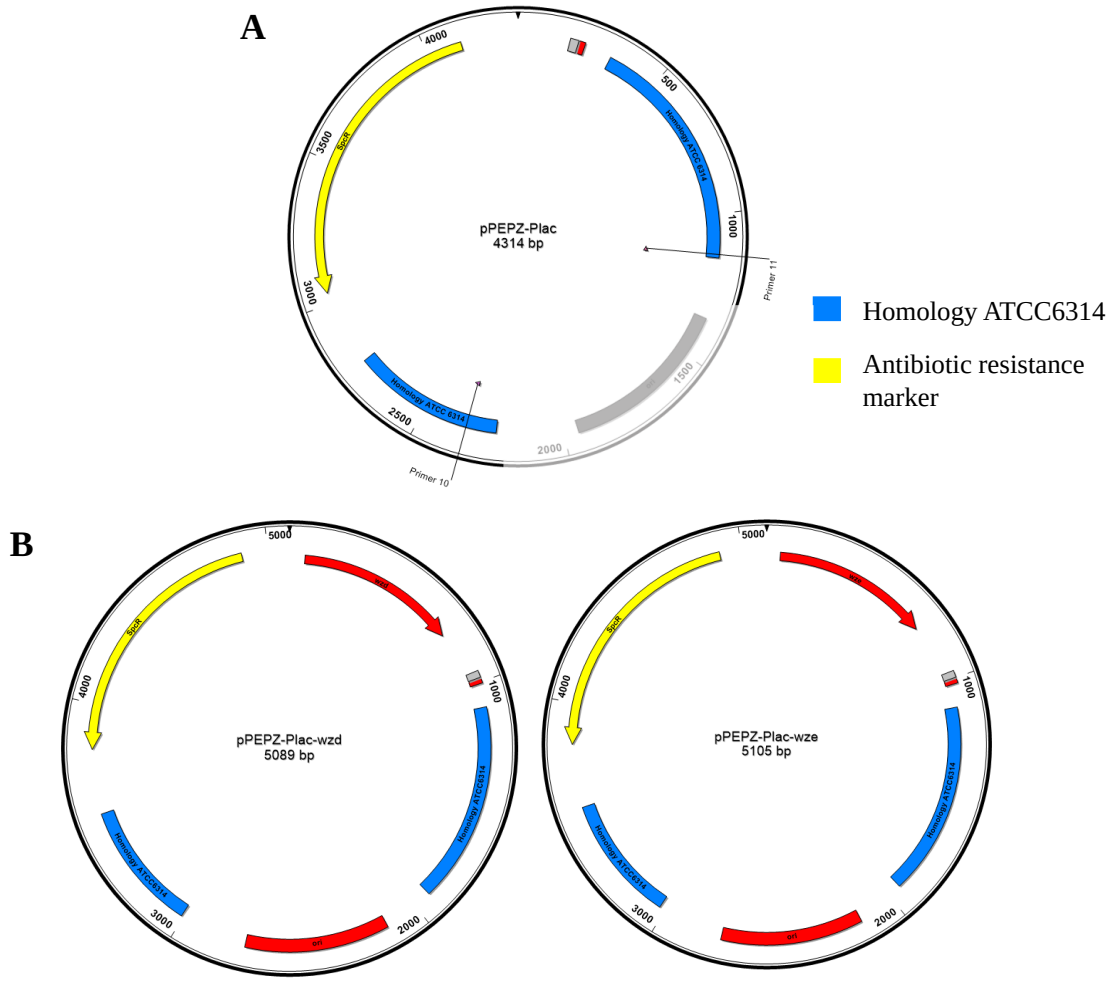

Figure 4.3 – Representation of integrative plasmid pPEPZ-Plac and plasmid constructs pPEPZ-Plac-*wzd* and pPEPZ-Plac-*wze*. In panel **A**, pPEPZ-Plac includes two regions homologous with the genome of *S. pneumoniae* ATCC6314 strain and a spectinomycin (SpcR) resistance marker for *S. pneumoniae* and *E. coli*. This plasmid was used as the backbone for cloning the capsule regulatory genes *wzd* and *wze* under the control of the Plac promoter originating constructs pPEPZ-Plac-*wzd* and pPEPZ-Plac-*wze* that are represented in panel **B**.

Therefore, in the resultant plasmids both genes were placed under the control of the IPTG-inducible promoter Plac present in the original plasmid pPEPZ-Plac. After cloning, both constructs were propagated in *E. coli*.

4.3.3. Transformation of *E. coli* with pPEPZ-Plac-*wzd* and pPEPZ-Plac-*wze*

E. coli DH5α competent cells were transformed with the constructed pPEPZ-Plac-*wzd* and pPEPZ-Plac-*wze* for plasmid propagation. This strain of *E. coli* has, characteristically, high transformation efficiency due to several mutations affecting recombination systems and inactivating endonucleases that could degrade the inserted construct (Woodcock et al., 1989).

After selection with Spec, several colonies were screened by PCR with primer pairs 6/7 and 8/9 previously used for *wzd* and *wze* amplification, respectively, to confirm the presence of the regulatory genes in the constructs. The results of the agarose gel electrophoresis are presented in Figure 4.4.

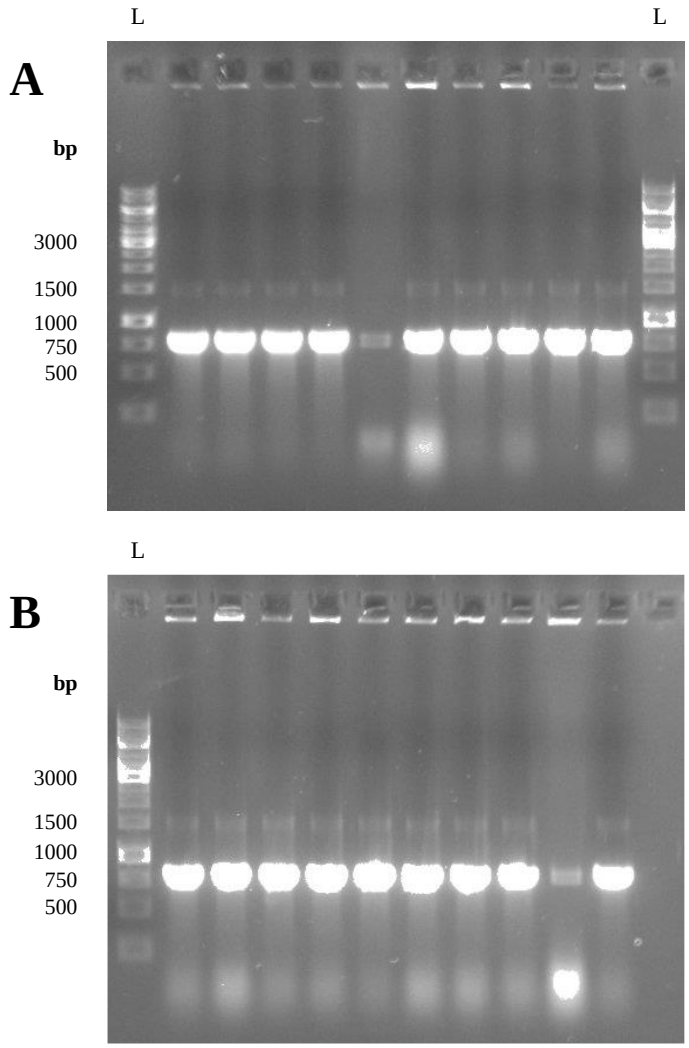

Figure 4.4 - Agarose gel electrophoresis results of *wzd* and *wze* positive colony screening. Electrophoresis run was fixed at 100 V and GeneRuler 1 kb DNA Ladder was used for sizing of the amplification products which corresponds to lanes L. In panel **A** are the colony screening results for the transformation of *E. coli* with pPEPZ-Plac-*wzd.* In panel **B** are the colony screening results for the the transformation of *E. coli* with pPEPZ-Plac-*wze.* Primer pairs 6/7 and 8/9 were used for the screening of positive *wzd* and *wze* colonies, respectively.

These results demonstrate the effectiveness of the ligation process and plasmid construction. The fact that 90% of the screened colonies were positive supports the previously described high transformation efficiency of the *E. coli* strain used for plasmid propagation.

Positive colonies from each construct transformation were then grown in liquid medium for posterior DNA purification.

4.3.4. PCR amplification of Plac-*wzd*, Plac-*wze* and Plac DNA fragments

For pneumococcal transformation, a DNA fragment containing the regulatory genes *wzd* or *wze* under the control of an inducible promotor and the resistance marker for spectinomycin was amplified from both plasmid constructs pPEPZ-Plac-*wzd* and pPEPZ-Plac-*wze* with primers 10 and 11. The same was done for the empty plasmid pPEPZ-Plac. As represented in panel A of Figure 4.3, these primers hybridize, respectively, at the 5' end of the upstream and at the 3' end of the downstream homologous regions with *S. pneumoniae* ATCC6314 genome. The presence of these two regions of homology in the amplified DNA fragment will allow homologous recombination to proceed by a double crossover event that replaces the target sequences in the pneumococcal genome by the Plac-*wzd*, Plac-*wze* or Plac DNA fragments that have been transported into the competent pneumococcal bacteria.

The results of the agarose gel electrophoresis correspondent to the amplification of these fragments are present in panel A of Figure 4.5.

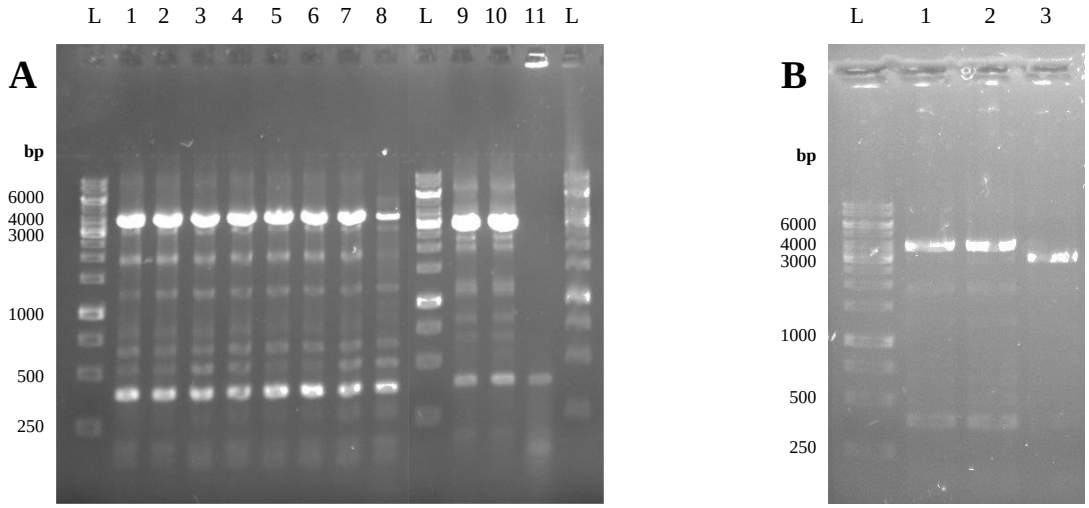

Figure 4.5 - Agarose gel electrophoresis results of fragments Plac-*wzd*, Plac-*wze* and Plac amplification. Electrophoresis run was fixed at 100 V and GeneRuler 1 kb DNA Ladder was used for sizing of the amplification products which corresponds to lanes L. In panel **A** are the amplification products of pPEPZ-Plac-*wzd* (lanes 1-4), pPEPZ-Plac-*wze* (lanes 5-8) and empty pPEPZ-Plac (lanes 9-11) using primers 10 and 11. In panel **B** is a representation of the amplification products of pPEPZ-Plac-*wzd* (lane 1), pPEPZ-Plac-*wze* (lane 2) and empty pPEPZ-Plac (lane 3) using primers 10 and 11 after purification.

A prominent band was present in almost every lane that corresponded to the expected product Plac-*wzd* with 3890 bp or Plac-*wze* with 3906 bp long. The amplification product for the pPEPZ-Plac with no insert, used as a control, was 3115 bp long.

However, the results demonstrate the low purity level of the samples. After several attempts of PCR optimization, including changing the different reagents and parameters in the PCR run (such as temperature and duration of the cycle steps), the smaller bands still appeared in the

agarose gel electrophoresis analysis. Therefore, these bands could be attributed to non-specific amplification products present in much lower concentration that the target band.

Indeed, after purification of the amplification products, all extra bands, except the band of interest, seemed to be in negligible concentrations probably not sufficient to interfere with the transformation process, as shown in the electrophoresis results presented in panel B of Figure 4.5.

4.3.5. Transformation of pneumococcal 6314Δ*wzd-lacI* and 6314Δ*wze-lacI* with Plac-*wzd*, Plac-*wze* and Plac DNA fragments

The homologous upstream and downstream regions for recombination in plasmid pPEPZ-Plac permit the insertion of the DNA fragments in a different integration locus of the ATCC6314 genome than those homologous regions present in plasmid pPEPY-PF6-lacI as represented in Figure 4.6.

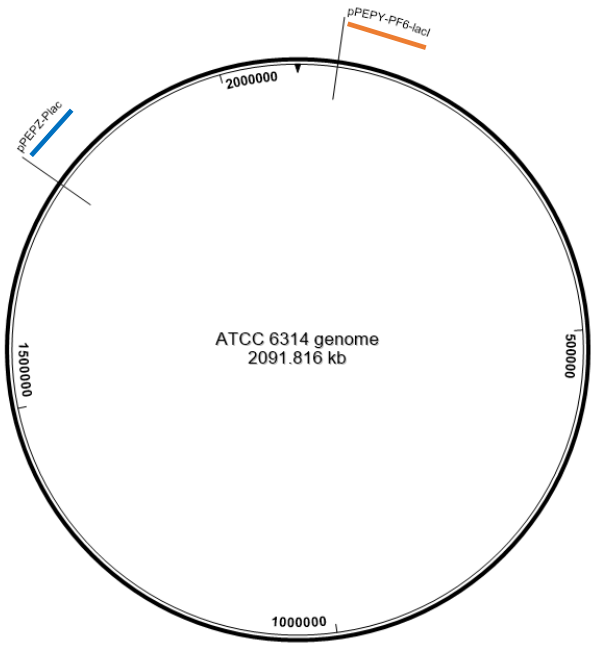

Figure 4.6 – Representation of integration loci for pPEPY-PF6-lacI and pPEPZ-Plac in *S. pneumoniae* ATCC6314 genome.

Considering the different integration sites in the bacterial chromosome of both plasmids, pneumococcal transformation with the amplified products Plac-*wzd*, Plac-*wze* and Plac will enable the construction of strains with the capsule regulatory genes *wzd* and *wze* under the control of the inducible promoter Plac in the genetic background of the mutant strains already with the repressor LacI (constructed in the previous chapter).

After transformation of the 6314Δ*wzd-lacI* and 6314Δ*wze-lacI* mutant strains with the Plac-*wzd*, Plac-*wze* and Plac fragments, four isolated colonies of each strain from two independent *lacI* transformants were screened by PCR with primers 10 and 11 to confirm the integration of the capsule regulatory genes and inducible promoter in the genome. The results of the agarose gel electrophoresis are shown in Figure 4.7.

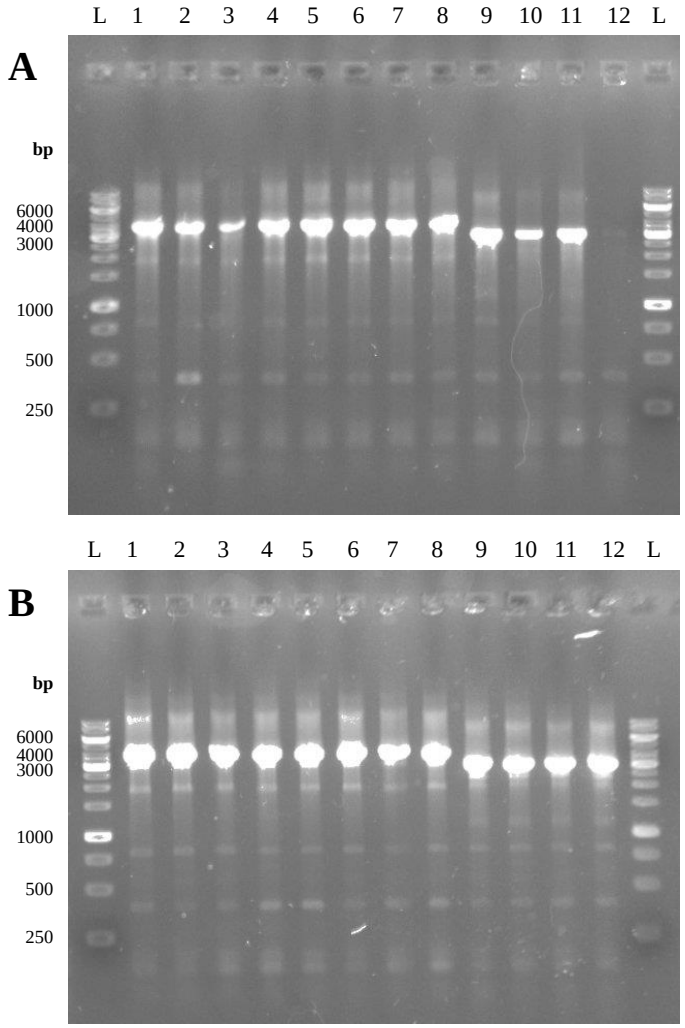

Figure 4.7 - Agarose gel electrophoresis results of Plac-*wzd*, Plac-*wze* and Plac positive colony screening. Electrophoresis run was fixed at 100 V and GeneRuler 1 kb DNA Ladder was used for sizing of the amplification products which corresponds to lanes L. In panel **A** are the colony screening results for the transformation of two independent 6314Δ*wzd-lacI* colonies (lanes 1-4 and lanes 5-8) with Plac-*wzd* and transformation of 6314Δ*wzd-lacI* with the fragment carrying only the Plac promoter (lanes 9-12). In panel **B** are the colony screening results for the transformation of two independent 6314Δ*wze-lacI* colonies (lanes 1-4 and lanes 5-8) with Plac-*wze* and transformation of 6314Δ*wze-lacI* with the fragment carrying only the Plac promoter (lanes 9-12). Primers 10 and 11 were used for the screening of positive Plac-*wzd*, Plac-*wze* and Plac after each each transformation.

The results of the colony screening confirmed the integration of the Plac-*wzd*, Plac-*wze* and Plac fragments in 6314Δ*wzd-lacI* and 6314Δ*wze-lacI*, respectively. However, some non-specific

amplification products were still present suggesting that primers 10 and 11 might hybridize with several parts of the sequence of the amplification products. It is also possible that they amplify other regions of the pneumococcal genome.

Therefore, the colony screening of some of the selected mutants was repeated with primers 12 and 13, whose sequence is presented in Supplementary Table 1, that were designed so that they hybridize with regions in the ATCC6314 genome that are approximately 450 bp upstream and downstream of the 5' and 3' end of the homology region used for integration of the amplification products, respectively.

The results of the agarose gel electrophoresis for this amplification are shown in Figure 4.8.

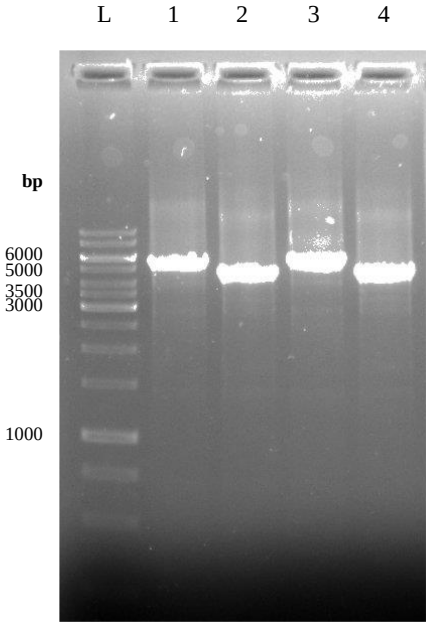

Figure 4.8 - Agarose gel electrophoresis of fragments Plac-*wzd*, Plac-*wze* and Plac amplification. Electrophoresis run was fixed at 100 V and GeneRuler 1 kb DNA Ladder was used for sizing of the amplification product which corresponds to lanes L. 1 – Plac-*wzd* amplification in 6314Δ*wzd-lacI*-Plac-*wzd*; 2 - Plac amplification in 6314Δ*wzd-lacI*-Plac; 3 - Plac-*wze* amplification in 6314Δ*wze-lacI*-Plac-*wze*; 4 - Plac amplification in 6314Δ*wze-lacI*-Plac

The results show the expected band for the amplicons with primers 12 and 13 and no extra bands corresponding to non-specific products, confirming the successful construction of the mutant strains with the insertion of the Plac-*wzd*, Plac-*wze* and Plac fragments specifically in the target sequence of the pneumococcal genome.

At this point in this thesis, there are 4 main strains constructed that are described in Table 4.2.

Table 4.2 – Pneumococcal strains constructed throughout this thesis and respective features.

Strain name	Features	Antibiotic selection
6314Δ*wzd-lacI*-Plac-*wzd*	*wzd* only expressed in the presence of IPTG	Gent 150 µg/mL and Spec 200 µg/mL
6314Δ*wzd-lacI*-Plac	*wzd* expression is not observed either in the presence or absence of IPTG	
6314Δ*wze-lacI*-Plac-*wze*	*wze* only expressed in the presence of IPTG	
6314Δ*wze-lacI*-Plac	*wze* expression is absent in the presence or absence of IPTG	

All these strains have both the LacI repressor and the inducible Plac promoter integrated into their chromosomes. The repressor LacI is controlled by a constitutive PF6 promoter, hence it is constantly repressing the Plac promoter integrated in a different region of the pneumococcal genome.

However, in the presence of IPTG (an analogue of the natural LacI inducer 1,6-allolactose), the repressor affinity for the operator in the *lac* operon decreases drastically due to a conformational shift upon binding of the chemical signal. This affinity decrease will allow the transcription of genes downstream of Plac such as *wzd* and *wze* in the strains 6314Δ*wzd-lacI*-Plac-*wzd* and 6314Δ*wze-lacI*-Plac-*wze* respectively, as the physical block of RNA polymerase ceased to exist (Rondon & Wilson, 2019).

4.3.6. Immunofluorescence assays of 6314Δ*wze-lacI*-Plac-*wze* and 6314Δ*wze-lacI*-Plac strains

In the presence of IPTG, genes downstream of the Plac promoter are transcribed. This means that in strain 6314Δ*wze-lacI*-Plac-*wze*, endogenous *wze* expression starts to occur. In contrast, in strain 6314Δ*wze-lacI*-Plac, endogenous Wze is absent because this gene is deleted in the original mutant and the Plac promoter is not controlling its expression.

Images of immunofluorescence of live pneumococcal cells from the different strains are presented in Figure 4.9

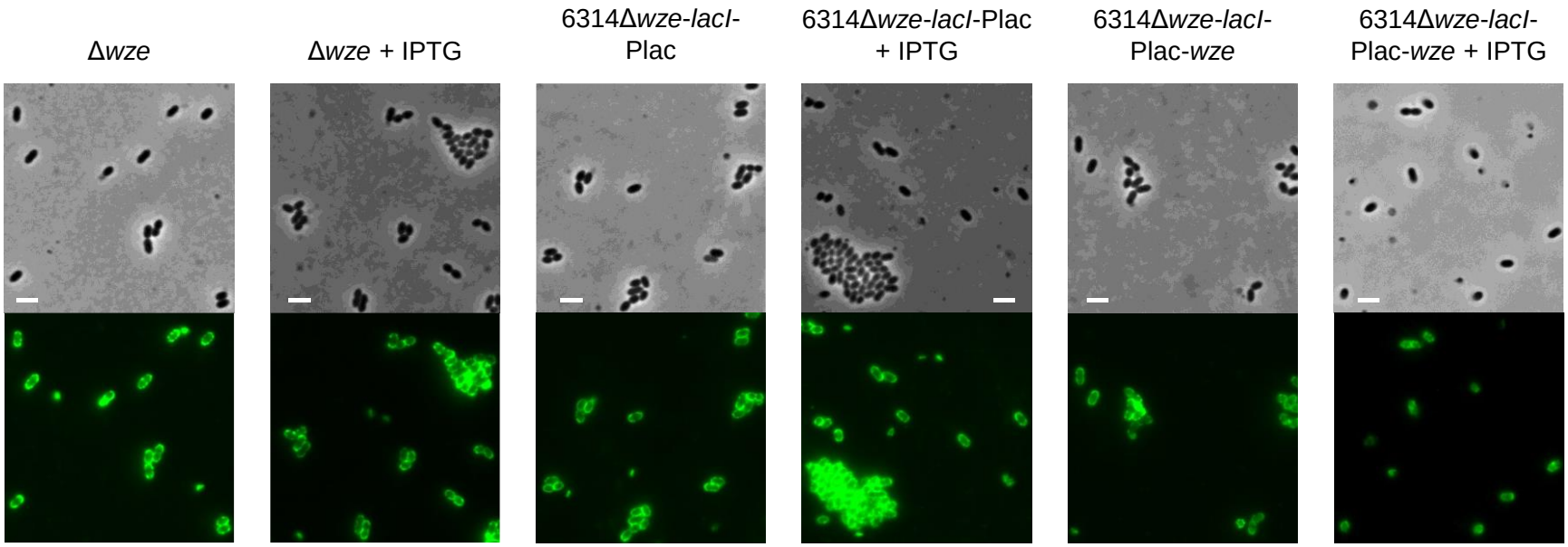

Figure 4.9 - Immunofluorescence of the different *S. pneumoniae* strains. Capsular detection of ATCC6314 derived mutant Δ*wze* and constructed strains 6314Δ*wze-lacI*-Plac and 6314Δ*wze-lacI*-Plac-*wze* in the absence and presence of IPTG was achieved through IF assays using a serotype 14 capsule specific antibody and secondary antibody Alexa 488. The first panel in all strains corresponds to brightfield images and the second to fluorescence images. Scale bar, 2 µm.

The results of the immunofluorescence assays showed that there is no apparent difference in the phenotypes between the 6314Δ*wze-lacI*-Plac strain and the original mutant Δ*wze* in the presence or absence of the inducer, as expected. The absence of the capsular polysaccharide from the division septum, when *wze* has been deleted, is in accordance with what was described by Henriques et al., 2011. As there is no *wze* expression in the four different conditions studied here, there is no interaction between this protein and the other regulatory protein, Wzd, and consequently, the capsule is not produced in the septal region of the pneumococcal cell.

In the absence of IPTG, the phenotype of the strain 6314Δ*wze-lacI*-Plac-*wze* is similar to those described above as there is no induction and LacI is still bound to the operator of the *lac* operon physically blocking the action of the RNA polymerase.

The phenotype of the strain 6314Δ*wze-lacI*-Plac-*wze* grown in the presence of IPTG hypothetically should be similar to the phenotype of the wild-type ATCC6314 strain, considering that *wze* should be expressed and thus, the respective protein would be available to interact with Wzd allowing septal capsule synthesis. However, IF results showed a seemingly intermediate phenotype between absence of capsule in the septum and full encapsulation of the cell.

Importantly, the cells also seem smaller and a large quantity of dead cells were detected by IF in this strain in contrast to the others, which might indicate the existence of a stress factor.

Several methods can be performed to ensure that *wze* expression under the Plac promoter is restored, in the presence of IPTG, and full capsule synthesis is occurring, such as incubation of pneumococcal cells with LytA. *S. pneumoniae* strains will have different susceptibilities to autolysin LytA according to distinct capsular expression levels. CPS has been reported to inhibit access of hydrolases, such as LytA, to peptidoglycan, their cell wall target. Therefore, ATCC6314 and IPTG-induced 6314Δ*wze-lacI*-Plac-*wze*, provided that the pneumococcal system was successfully constructed, should be less susceptible to the lytic activity of LytA than the mutants Δ*wzd* and Δ*wze*, due to reduced PGN exposure (Normark & Normark, 2002).

Alternatively, results of the Quellung reaction for strain 6314Δ*wze-lacI*-Plac-*wze*, in the presence of IPTG, could also be compared with those obtained for ATCC6314 and Δ*wze* strains, since agglutination ability after exposure to a serotype specific serum is reduced if capsular expression is impaired (Henriques et al., 2011).

However, in case the pneumococcal system is not working and *wze* expression under the control of the Plac promoter is not restored in the presence of IPTG, the core mutant Δ*wze* was further analyzed and studied. As shown in Figure 4.10, the *wze* null mutant exhibited colonies with different phenotypes when plated in TSA supplemented with defibrinated sheep blood and observed with a stereo microscope.

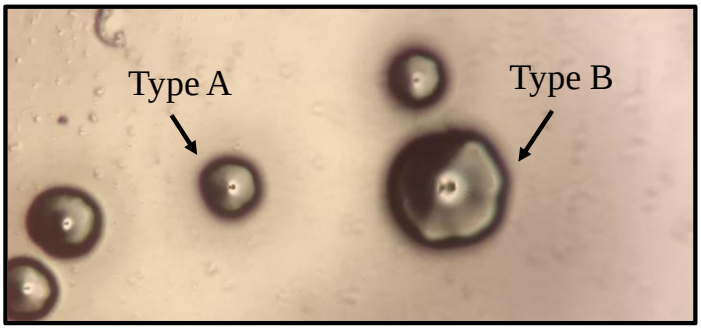

Figure 4.10 – Colony morphology heterogeneity observed in original Δ*wze* mutant. *wze* null mutant showed a mixture of colony phenotypes when observed in a stereo microscope. The two phenotypes could be categorized in type A, smaller colonies, and type B, denser and larger colonies.

This phenotype heterogeneity included a small sized colony, type A, and a denser and larger colony, type B, and prompt the isolation of each phenotype and confirmation through both PCR, whose results of the agarose gel electrophoresis analysis are presented in Figure 4.11, and IF assays, as performed in Chapter 2.

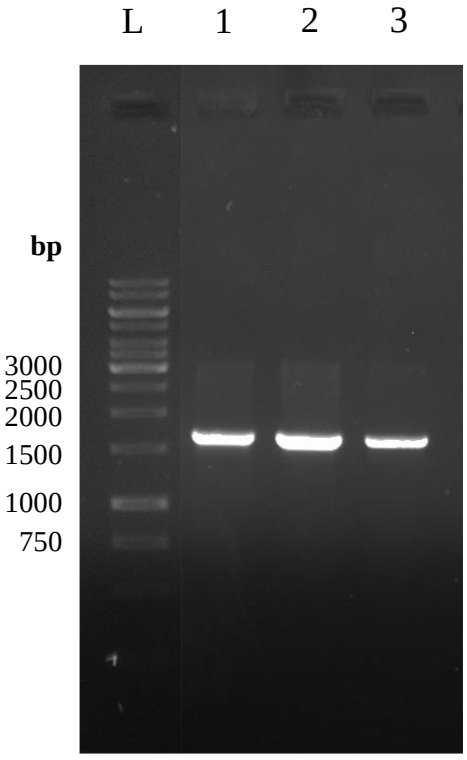

Figure 4.11 - Agarose gel electrophoresis results of *S. pneumoniae* strains. Electrophoresis run was fixed at 100 V and GeneRuler 1 kb DNA Ladder was used for sizing of the amplification products which corresponds to lane L. 1 – Δ*wze* amplification product using primers 1 and 3; 2 - Δ*wze* type A amplification product using primers 1 and 3; 3 - 2 - Δ*wze* type B amplification product using primers 1 and 3.

The results of the agarose gel electrophoresis showed that the three samples, the *wze* null mutant, which is a mixture of two different phenotypes, and both A and B phenotypes, exhibit consistent results for a mutant lacking the *wze* gene. Amplification products of the three samples with primers 1 and 3 originated a 1715 bp fragment (lanes 1, 2 and 3; Figure 4.11), as expected.

Images of immunofluorescence of live pneumococcal cells from the *wze* null mutant and isolated phenotypes are present in Figure 4.12.

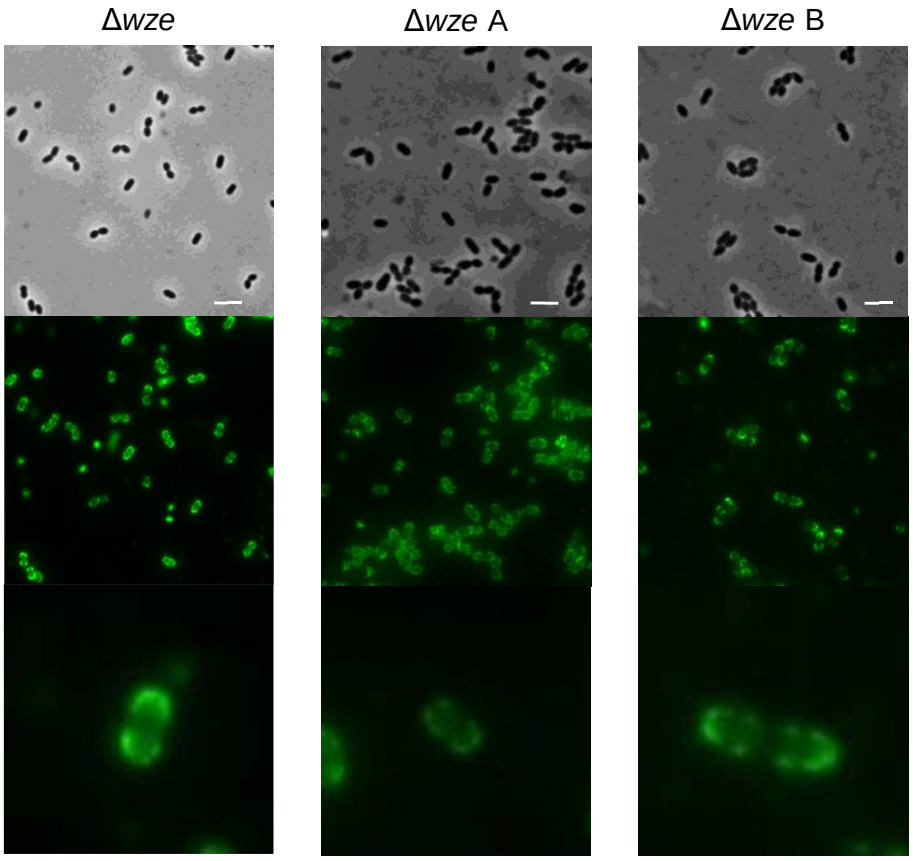

Figure 4.12 – Immunofluorescence of the different *S. pneumoniae* strains. Capsular detection of ATCC6314 derived mutant Δ*wze* and different isolated phenotypes, Δ*wze* A and B, was achieved through IF assays using a serotype 14 capsule specific antibody and secondary antibody Alexa 488. The first panel in all strains corresponds to brightfield images, the second to fluorescence images and the third to the ampliation of an area of the image with a single and representative bacteria cell. Scale bar, 2 µm.

IF assays showed that lack of capsule in the division septum is conserved between phenotypes but seems more prominent in phenotype B.

The phenotype heterogeneity might be related to single nucleotide polymorphisms (SNPs), possibly located outside the *cps* locus since colonies maintain the same features as the original mutant regarding capsule production but seem to differ in size.

These findings and IF results might suggest that phenotype B of the Δ*wze* mutant could be a more appropriate background for the construction of the pneumococcal system to expose immature peptidoglycan to enzymes present in the surround medium. Complementation and full encapsulation after *wze* expression under the Plac promoter is restored in the presence of IPTG could be easier to observe in these colonies than in the original Δ*wze* strain.

Chapter 5 – LytA-GFP expression and purification

5.1. Introductory remarks

In this chapter, pneumococcal autolysin LytA-GFP was expressed and purified with the intent of incubating this amidase with the pneumococcal strain 6314Δ*wze-lacI*-Plac-*wze*. This would allow the assessment of its autolysin susceptibility and posterior comparison with the susceptibility obtained for the strains ATCC6314 and derived mutant Δ*wze.*

Therefore, autolysin incubation could be a process to confirm if the pneumococcal system to expose immature peptidoglycan is well established and working and to release PGN fragments of the immature PGN that, in the future, could be analyzed by high performance liquid chromatography (HPLC).

5.2. Materials and methods

5.2.1. Preparation of *E. coli* BL21 (DE3) competent cells and transformation with pET21a_lytA_GFP

E. coli BL21 (DE3) competent cells were prepared as described previously in section 4.2.4. and transformation was achieved accordingly to the protocol described in section 4.2.5.,¸except that plasmid DNA (pDNA) (pET21a_lytA_GFP*) was previously propagated in *E. coli* DH5α cells and purified accordingly to the NZY Miniprep protocol provided by NZYTech©. Additionally, *E. coli* BL21 (DE3) cells were transformed with 100-200 ng of pDNA and transformants selected in LA medium supplemented with 100 µg/mL ampicillin.

* Construction of pET21a_lytA_GFP was done by A. R. Narciso.

5.2.2. Expression of LytA-GFP and lactose induction

After transformant selection with ampicillin, one colony was pre-inoculated in 50 mL LB medium supplemented with 100 µg/mL ampicillin at 37 °C and 170 rpm (Innova 40, New Brunswick) for around 5 hours.

After this period, 12.5 mL of the pre-inoculum was added to 500 mL LB medium supplemented with 100 µg/mL ampicillin and 30 mM lactose to induce protein expression. The inoculum was incubated overnight at 37 °C and 170 rpm (Innova 40, New Brunswick).

5.2.3. Cell harvest and lysis

E. coli BL21 (DE3) cells expressing LytA-GFP were harvested by centrifugation at 4 ºC and 8000 rpm for 9 minutes (JA-10 rotor, Beckman Coulter Avanti J-26S XPI) and washed

once with 30 mL of Equilibration Buffer. The composition of all buffers used in cell harvesting and protein purification is described in Supplementary Information 3.

In 50 mL falcons, cells were pelleted again by centrifugation at 4 ºC and maximum speed for 8 minutes. Cell pellet was resuspended, once more, in 30 mL of Equilibration Buffer and cell lysis was carried out by French Press, twice each sample, at 1000 psi.

The lysate was centrifuged at 4 ºC and maximum speed for 15 minutes. This process was repeated until no pellet was observed after each centrifugation.

5.2.4. LytA-GFP purification and SDS-PAGE analysis

After cell lysis, LytA-GFP was purified from other soluble proteins by immobilized metal affinity chromatography (IMAC) using a Co^{2+} charged TALON® resin (Clontech Laboratories, Inc.).

The first step of the purification process consisted in resin equilibration by centrifuging 6 mL of slurry resin at 4 ºC and 7000 rpm for 3 minutes with the removal of the supernatant (ethanol), yielding 3 mL of TALON® resin.

Afterwards, the resin was washed with 15 mL of Wash Buffer and centrifuged once more to remove as much alcohol as possible.

Subsequently, the supernatant of the lysate acquired in section 5.2.3. was added to the resin for binding and incubated on ice for 1 hour on a platform shaker.

After this incubation, the mixture was centrifuged and the supernatant was removed. Then, the resin was loaded onto a gravity-flow column, settled with a small centrifugation and washed three times with 5 mL of Wash Buffer. Every buffer added to the resin should drain no further than the top of the resin bed to stop air bubbles from entering the resin.

Afterwards, the resin was washed two times with 5 mL of 5 mM Imidazole Wash Buffer, 5 mL of 10mM Imidazole Wash Buffer and 3 mL of Elution Buffer. At last, the purified eluted protein was dialysed (SnakeSkin™ Dialysis Tubing, 10K MWCO, 22 mm, Thermo Fisher Scientific) overnight against 5 L PBS pH 6.0.

Different aliquots collected during protein expression and purification were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). The recipe followed for the preparation of a 4 % acrylamide stacking gel and a 10 % acrylamide resolving gel is present in Supplementary Table 2.

The different samples were prepared by mixing 40 µL with 10 µL of Laemmli buffer, whose detailed composition is described in Supplementary Information 4, and boiling the mixture at 100 ºC for 5 minutes. Preparation of pellet samples required 50 µL of sample buffer for resuspension of the cell pellet. After denaturing, samples were delivered to each well alongside PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific). The voltage parameter for the electrophoresis run was set to 100 V.

Protein concentration was determined by the absorbance at 280 nm measured in the NanoDrop.

5.3. Results and discussion

5.3.1. LytA-GFP purification and SDS-PAGE analysis

E. coli BL21 (DE3) has been widely used for recombinant protein expression because its main characteristic is the presence of a λDE3 phage harboring the T7 RNA polymerase gene (Studier & Moffatt, 1986). This polymerase is highly specific for its own T7 promoters and therefore, originates high levels of transcription of the genes under their control, such as the fusion sequence *lytA-GFP* in the construct pET21a_lytA_GFP.

As mentioned before, several aliquots of the different steps of protein expression and purification were collected and analyzed by SDS-PAGE. As shown in Figure 5.1, a prominent band with the expected molecular weight of around 67 kDa representative of LytA-GFP is present in several samples analyzed.

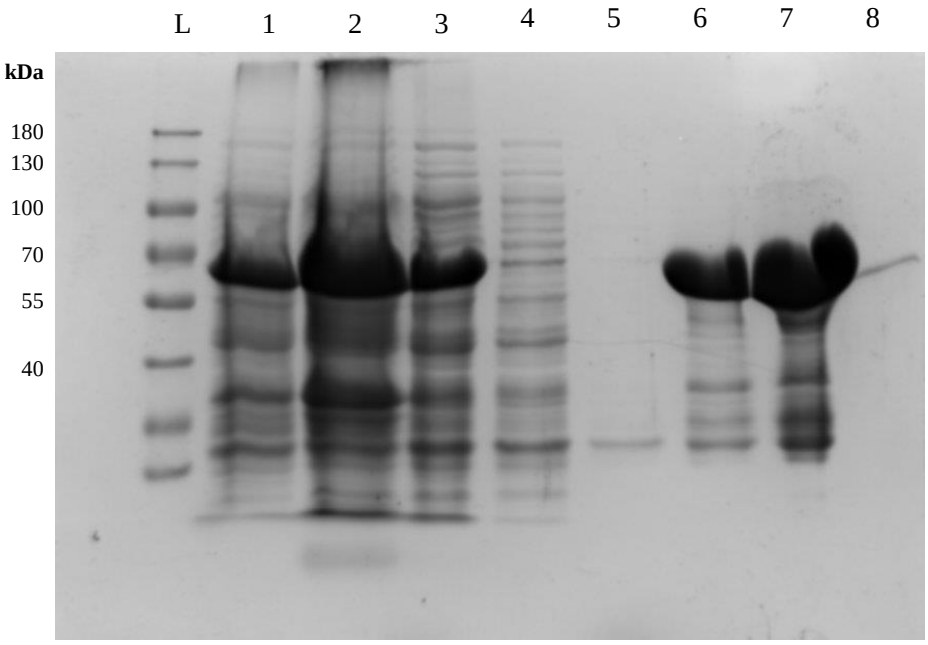

Figure 5.1 - Analysis of samples from different steps of LytA-GFP expression and purification by SDS-PAGE. Electrophoresis run was fixed at 100 V and PageRuler™ Prestained Protein Ladder was used for determination of the molecular weight of the proteins which corresponds to lane L. 1 – Cell pellet after overnight lactose induced expression; 2 – Cell pellet after cell lysis by French Press; 3 – Supernatant after cell lysis by French Press; 4 – 1st resin wash with Wash Buffer; 5 – 3rd resin wash with Wash Buffer; 6 – LytA-GFP elution during 1st resin wash with 5 mM Imidazole Wash Buffer; 7 - LytA-GFP elution during 2nd resin wash with 5 mM Imidazole Wash Buffer; 8 – Resin wash with 10 mM Imidazole Wash Buffer.

The presence of the 67 kDa band in the cell pellet after overnight induction (lane 1, Figure 5.1) shows that the use of lactose instead of IPTG as an inducer, is also effective in promoting protein expression via recognition by T7 RNA polymerase of the T7 promoter present in the construct pET21a_lytA_GFP.

LytA-GFP is also present in the cell pellet after cell lysis (lane 2, Figure 5.1) indicating that the French Press method was not effective in breaking all the cells expressing the protein, which might be resolved by passing the cell culture through the apparatus several times. Alternatively, it could indicate that most of the protein might be in an insoluble form as the band in this fraction seems larger than the band in the supernatant after cell lysis (lane 3, Figure 5.1), suggesting a higher protein comcentration.

Moreover, when the resin was first washed with the Wash Buffer, most of the proteins with no affinity for the cationic Co^{2+} charged resin were eluted, while LytA-GFP remained bound to the resin as the band of 67 kDa was absent (lanes 4 and 5, Figure 5.1).

The fact that LytA-GFP was eluted during both resin washes with 5 mM Imidazole Wash Buffer (lanes 6 and 7, Figure 5.1) suggests that the protein is not strongly bound to the resin, which may be due to the conformational inaccessibility of the protein anionic groups to the Co^{2+} ions. In this case, a low concentration of imidazole is sufficient to elute the protein as this compound replaces LytA-GFP binding site in the resin due to its higher affinity to cobalt. In addition, there was no protein elution when the imidazole concentration was increased to 10 mM (lane 8, Figure 5.1), indicating that all protein was eluted in the previous washes.

The estimated concentration of the purified LytA-GFP based on an absorbance at 280 nm of 8.752 was 4.4 mg/mL, which should be sufficient for future susceptibility assessment experiments with 6314Δ*wze-lacI*-Plac-*wze* and other constructed strains.

Chapter 6 – Conclusions and future work

The work accomplished in this dissertation was an attempt at developing a pneumococcal system that would enable controlled exposure of immature peptidoglycan concealed at the surface of the cell.

The Δ*wzd* and Δ*wze* genetic background was chosen as appropriate to achieve this goal due to the immature peptidoglycan exposed at the division septum in these mutants. As an attempt to reverse this phenotypic feature, strains were constructed with repressor *lacI* and capsule regulatory genes *wzd* and *wze* expression controlled by an IPTG-inducible promoter (Plac). Hypothetically, if these strains were grown in an IPTG-rich medium, the *wzd*/*wze* expression under the Plac promoter control would be restored and capsule synthesis would occur all around the cell. Therefore, immature peptidoglycan should be accessible by depletion of the inducer and posteriorly analyzed, in different stages of the cell life cycle.

The IF results for the strain 6314Δ*wze-lacI*-Plac-*wze* showed an intermediate capsular phenotype that did not confirm the successful development of this system, which highlights the need to do it with other methodologies in the future as well as study other colonies from this strain, especially considering two independent 6314Δ*wze-lacI* colonies were used for the second transformation, and the other constructed strain, 6314Δ*wzd-lacI*-Plac-*wzd.* Moreover, constructing this system in the isolated Δ*wze* B should be considered, as capsular absence in the division septum seems more noticeable than in the original Δ*wze* mutant.

One of the possible methodologies to further study this system would be autolysin LytA-GFP incubation with pneumococcal cells. For this purpose, LytA-GFP was expressed and purified effectively and was isolated in sufficient concentration for incubation and susceptibility assessment of the different strains. Additionally, a reporter gene, such as the one encoding the green fluorescent protein (GFP), under the control of Plac and in the presence of IPTG, could also act as a control for the system.

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Supplementary material

Supplementary information 1 - C + Y pH 8 liquid medium composition and respective component solutions.

C + Y (pH 8) liquid medium

400 mL of PreC **(1)**

15 mL of 1 M KPO_4 buffer pH 8

13 mL of Supplement **(2)**

10 mL of 1 mg/mL glutamine

10 mL Adams III **(3)**

5 mL of 2 % pyruvate

10 mL of 5 % yeast extract

(1) PreC

2.42 g of anhydrous sodium acetate

10 g of casamino acids

0.01 g of L-Tryptophan

0.1 g of L-Cysteine·HCl

Add dH_2O to 2000 mL and adjust to pH 7.4 - 7.6.

Then autoclave and store at room temperature.

(2) Supplement

30 mL of 3 in 1 salts **(4)**

60 mL of 20 % glucose

3 mL of 50 % sucrose

60 mL of 2 mg/mL adenosine

60 mL of 2 mg/mL uridine

Filter with a 0.22 µm sterile filter and store at 4 °C.

(3) Adams III

0.8 g of asparagine

0.08 g of choline

0.64 mL of 1 % $CaCl_2$

64 mL of Adams I **(5)**

16 mL of Adams II **(6)**

Add dH_2O to 400 mL and filter with a 0.22 µm sterile filter.

Then store in the dark at 4 °C.

(4) 3 in 1 salts

10 g of $MgCl_2·6H_2O$

0.05 g of anhydrous $CaCl_2$

0.02 mL of 0.1 M $MnSO_4·4H_2O$

Add dH_2O to 100 mL and autoclave.

Then store at room temperature.

(5) Adams I

0.03 g of nicotinic acid

0.035 g of pyridoxine

0.12 g of Ca-pantothenate

0.032 g of thiamine-HCl

0.014 g of riboflavin

0.06 mL of 0.5 mg/mL biotin

Add dH_2O to 200 mL and filter with a 0.22 µm sterile filter.

Then store in the dark at 4 °C.

(6) Adams II

0.05 g of $FeSO_4·7H_2O$

0.05 g of $CuSO_4·5H_2O$

0.05 g of $ZnSO_4·7H_2O$

0.02 g of $MnCl_2·4H_2O$

1 mL of concentrated HCl

Add dH_2O to 100 mL and autoclave.

Then store at room temperature.

Supplementary Table 1 – Primers used in this dissertation.

Primer name	Sequence (5' to 3')
1	CGCGGATCCGGCGCAGAGATATACTATAC
2	CCGGAATTCGTGATAATTACCGCATCAATAAC
3	CCGGAATTCCTGCTACCAGTTTCCATGAAAG
4	CATCATCGGTAAGGTTGAAGGC
5	CTACAAACACATCGCTCCTG
6	CGCGGATCCGCGAAAGAACTTTTTGTAGATAATCC
7	CCGCTCGAGGTCGGCATTCCTCTCTCCTATTTC
8	CGCGGATCCGCAGATGACACTTTTGGGAGTTG
9	CCGCTCGAGCTGCCAGAAAAATTTCCAATCC
10	GTTGGGAGCTGATGGTGAAG
11	GTCAATAAAAAGGCAATCCACG
12	GCTGCGCTTTCTCATCTTTCTGAC
13	CGTACATTCAGACGGCTCTTATCC

Supplementary information 2 – Composition of solutions RF1 and RF2 used in the preparation of *E. coli* competent cells.

RF1 solution

1.2 g of RbCl

0.99 g of $MnCl_2 \cdot 4H_2O$

0.15 g of $CaCl_2 \cdot 2H_2O$

15 g of Glycerol

3 mL of 1M KAc pH 7.5

Add dH_2O to 100 mL and adjust to pH 5.8.

Then filter with a 0.22 µm sterile filter and store at 4 °C.

RF2 solution

0.12 g of RbCl

0.83 g of $CaCl_2 \cdot 2H_2O$

15 g of Glycerol

2 mL of 0.5 M MOPS pH 6.8

Add dH_2O to 100 mL and adjust to pH 6.8.

Then filter with a 0.22 µm sterile filter and store at 4 °C.

Supplementary information 3 – Composition of buffers used in cell harvesting and protein purification.

Equilibration/Wash buffer

50 mM Na_2PO_4 pH 7.4

300 mM NaCl

5 mM Imidazole Wash buffer

50 mM Na_2PO_4 pH 7.4

300 mM NaCl

5 mM Imiddazole

10 mM Imidazole Wash buffer

50 mM Na_2PO_4 pH 7.4

300 mM NaCl

10 mM Imiddazole

Elution buffer

50 mM Na_2PO_4 pH 7.4

300 mM NaCl

150 mM Imiddazole

Supplementary Table 2 - Recipe for preparation of a 4 % acrylamide stacking gel and a 10 % acrylamide resolving gel.

	Stacking gel	**Resolving gel**
	4 %	10%
30 % Acrylamide/Bis	0.4 mL	3.3 mL
0.5 M TrisHCl pH 6.8	0.756 mL	-
1.5 M TrisHCl pH 8.8	-	2.5 mL
10 % SDS	30 µL	100 µL
ddH_2O	1.8 mL	4.05 mL
TEMED	3 µL	5 µL
10 % APS	15 µL	50 µL
Total volume	3 mL	10 mL

Supplementary information 4 – Composition of Laemmli buffer used in sample preparation for SDS-PAGE.

60mM Tris-HCl pH 6.8
2 % SDS
10 % Glycerol
5% β-mercaptoethanol
0.01 % Bromophenol blue