# Characterization of *Streptococcus pneumoniae* mutants lacking the gene *dacA*, which encodes Penicillin Binding Protein 3 (PBP3)

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# Forord

Oppgaven ble utført ved avdelingen for molekyær mikrobiologi på Universitet for Miljø- og Biovitenskap i perioden august 2011 til mai 2012. Jeg har hatt gleden av å jobbe sammen med hjelpsomme og støttende kolleger, og har lært mye gjennom arbeidet mitt og oppfølgingen fra veiledere.

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Ås, mai 2012
Fredrik Hult Jakobsen

# Sammendrag

Formålet med denne oppgaven var å studere funksjonen til penicillin bindende protein 3 (PBP3), som kodes av genet *dacA* hos *S. pneumoniae*. En pneumokokk-stamme ble fremskaffet av avdelingen for molekylær mikrobiologi ved Universitetet for miljø- og biovitenskap (UMB) ved Ås. Denne stammen ble brukt til å fremstille åtte mutanter, hvorav fem mangler deres opprinnelige *dacA* gen. Ved å utføre analyser og sammenligning av vekst, morfologi, og resistens mot antibiotika hos mutantene og villtypen, kunne vi studere funksjonen til *dacA* i overlevelsesevnen til pneumokokker.

Mutanter som mangler *dacA* viser en svekket vekstrate, sammenlignet med villtypen, og mutanter som mangler enten *pbp2a*, *pbp1a*, eller *pbp1b*. Gjennom vekstekseperimentene ble det observert en grad av samspill mellom *dacA* og *pbp2a*, ettersom mutanter som manglet begge genene viste en relativt høy svekkelse i vekstraten, sammenlignet med andre mutanter som manglet *dacA* og et klasse A HMM PBP-kodende gen.

Differensial Interferens Mikroskopi viser at mutanter som mangler *dacA* har variasjon i form og størrelse av cellene. De har også større grad av kjededannelse, som fører til at cellene danner klaser, heller enn rette kjeder. Tilsettelse av LytA ved begynnelsen av veksten, eller i løpet av den eksponensielle vekstfasen til mutantene uten *dacA* ser ikke ut til å hemme veksten deres, men mutantene som mangler både *dacA* og en klasse A HMM PBP-kodende gen ser ut til å få den eksponensielle veksten inhibert.

Ved antibiotisk sensitivitetstester viser ikke mutanter uten dacA noen særlig sensitivitet for penicillin, men er relativt sårbare mot et annet β-lactam antibiotikum, ceftazidime, så vel som mot bacitricin, vancomycin, erythromycin, novobiocin og tetracycline. Mutanter som mangler både *dacA* og *pbp2a* er svært sårbare mot ceftazidime, bacitricin, vancomycin, erythromycin og tetracycline, selv om mutanter som mangler *pbp2a* alene ikke er særlig sensitive mot noen av disse antibiotikaene. Dette kan indikere et samspill mellom PBP3 og PBP2a i pneumokokkers motstand mot antibiotika.

For å måle aktiviteten til dacA promoteren ble det utført en luminescens reporter test, ved å transformere utgangsstammen med en $P_{dacA}$-*luc* konstruksjon. Det ble også innført *dacA* etter *comX* promotoren, for å regulere uttrykket av PBP3. Mangel på forskjell i målt

luminescens mellom stammer som fikk ekspresjon av *comX* promotoren stimulert med tilsatt ComS, og de som ikke fikk det, kan tyde på at *S. pneumoniae* mangler evnen til å sense fraværet av PBP3. Andre muligheter kan være at $P_{dacA}$-*luc* konstruksjonen er dysfunksjonell, eller at $P_{comX}$-*dacA* kan ha lekket uttrykk.

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# 1 Summary

In this study we wanted to expand our knowledge regarding the role of the Penicillin Binding Protein 3 encoded by the gene *dacA* in *S. pneumoniae*. A pneumococcal strain (RH426) was provided by the department of molecular microbiology at the University of Environmental and Life Science (UMB) in Ås. This strain was used to produce eight mutants, five of which lacked their native *dacA* gene. Through analysis and comparison of the growth, the morphology, and the antibiotic susceptibility of the mutants and wild-type, the functions of *dacA* in pneumococcal survivability were brought up to evaluation.

Mutants deficient in *dacA* showed a lower growth rate, compared with the parent wild type, as well as all of the mutants deficient in either *pbp2a*, *pbp1a*, or *pbp1b*. Through the growth experiments we saw an indication of interplay between *dacA* and *pbp2a*, as mutants deficient in both *dacA* and *pbp2a* showed a relatively large decrease in growth rate compared to other mutants deficient in *dacA* and a class A HMM PBP-encoding gene.

*S. pneumoniae* deficient in *dacA* have an abnormal morphology, and show irregular size. They also displayed an increased cell linkage, with the cells being clustered together, rather than forming a straight chain. Addition of LytA at the start of their growth, or during their exponential growth phase, did not seem to inhibit their growth, although it did decrease the growth rate of double mutants deficient in both *dacA* and a class A HMM PBP-encoding gene.

Antibiotic susceptibility tests were performed, and while *dacA* deficient mutants showed no particular sensitivity to penicillin, they were comparatively sensitive to another β-lactam antibiotic, ceftazidime, as well as to bacitricin, vancomycin, erythromycin, novobiocin and tetracycline. Mutants deficient in both *dacA* and *pbp2a* were severely susceptible to ceftazidime, bacitricin, vancomycin, erythromycin and tetracycline, although single mutants lacking the PBP2a-encoding gene were not particularly sensitive to any of these antibiotics. This could indicate interplay between PBP3 and the PBP2a, with regards to pneumococcal antibiotic resistance.

In order to measure the activity of the *dacA* promoter, we employed a luminescence reporter gene, by inserting a $P_{dacA}$-*luc* construct in the wild type. The $P_{comX}$-*dacA* construct

was also inserted, in order to regulate the expression of PBP3. The lack of any difference in measured luminescence between strains with ComS*-stimulated *comX* expression could indicate that *S. pneumoniae* lacks the ability to sense its lack of PBP3. Other possible explanations could be that the $P_{dacA}$-*luc* construct is dysfunctional, or that *comX* could have leaked some expression.

# 2 Introduction

## 2.1 - *Streptococcus pneumoniae*

*Streptococcus pneumoniae*, also referred to as pneumococcus, is a member of Streptococcus - a genus of Gram-positive cocci or short rods, which exist in pairs or chains. They are non-motile, do not sporulate, and are catalase negative. They are facultative anaerobic chemoorganotrophs that require a complex nourishment and a fermentative metabolism resulting in L(+) lactic acids as a main product of fermentation [1].

### 2.1.1 - General characteristics

*S. pneumoniae* belongs to the oral streptococcal group (mitis phylogenetic group). This group has frequent changes in nomenclature, but currently consists of nine recognized species. On the basis of 16S rDNA sequencing, the most closely related species of *S. pneumoniae* are *Streptococcus oralis* and *Streptococcus mitis*, which share over 99% sequence identity with *S. pneumoniae*. *S. pneumoniae*, can be distinguished from the other two on the basis of three differentiating characteristics: optochin susceptibility, bile solubility, and agglutination with antipneumococcal polysaccharide capsule antibodies [2].

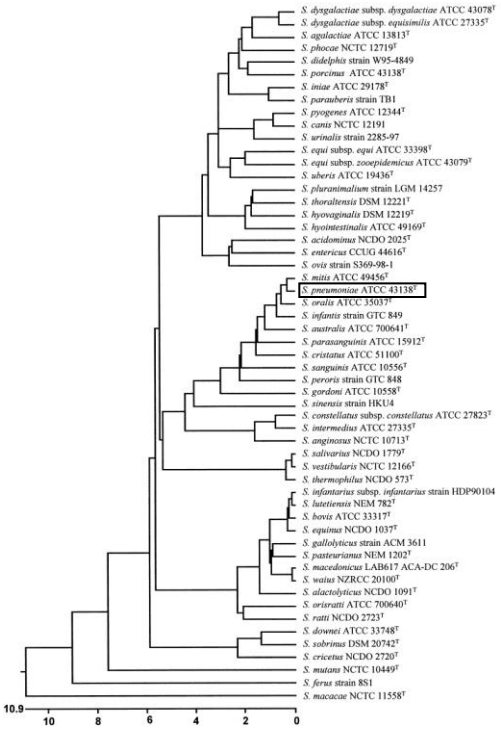



**Figure 2.1.** Phylogenetic tree showing the relationship between bacteria of the Streptococcus genus. *S. pneumoniae* is outlined.

Early experiments performed by Frederick Griffith *et al*. (1928) resulted in the discovery of what we today call the transforming principle. Griffith and co-workers proved that non-virulent or weakly virulent strains of pneumococcus could acquire virulence through transformation [3, 4]. Griffith's experiment involved using different strains of pneumococcus, both virulent and non-virulent, to infect mice. Some isolates of the virulent strains were heat-killed, and lost their ability to harm the mice. However, when injected together with the non-virulent strains, the combination was able to kill the host (Figure 2.2). Griffith also isolated both virulent and non-virulent strains from the host, and concluded that the non-virulent strains had somehow used the heat-killed virulent strains to "transform" themselves into virulent strains. This is known today as the "transforming principle".

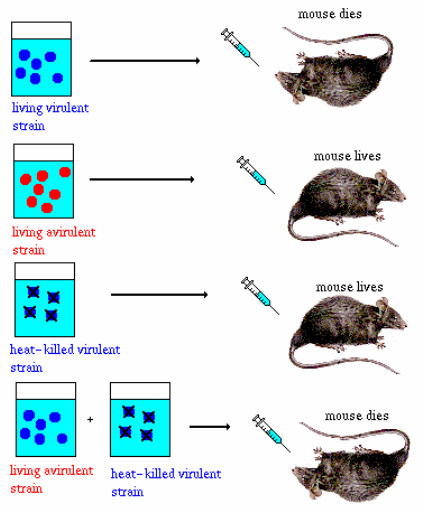



**Figure 2.2.** An illustration of how Griffith's experiment proved the existence of the "transforming principle". The nonvirulent strains become virulent when mixed with heat-killed virulent strains [picture reference: http://www.bios.niu.edu/sims/bios103/GeneConcept/griffith.html].

This kind of transformation could only occur under certain circumstances, as confirmed by Martin Dawson (Dawson and Sia (1931) [5]). Dawson found that transformation did not occur if the bacterial suspensions were heated over, or frozen below, a certain temperature, as this seemed to destroy the transforming principle [3].

Dawson's colleague, Oswald Avery, worked together with Colin MacLeod and Maclyn McCarty to research how the transforming principle worked. Their experiment proved that a nucleic acid of the desoxyribose type (DNA) is the fundamental unit of the transforming principle. Avery and his colleagues also suggested that DNA is the molecule that carries genetic information in organisms [6, 7].

### 2.1.2 - Pathogenicity

*S. pneumoniae* is a common bacterium in humans, normally found in the nasopharynx. Although most individuals with a developed and healthy immune system will never get invasive disease caused by *S. pneumoniae*, it can move beyond the nasopharynx and cause ear infections, pneumonia, meningitis, or bacteraemia. People with weakened immune systems, like children and the elderly, are more vulnerable to diseases like these [8]. In fact, *S. pneumoniae* is the most common cause of community-acquired pneumonia worldwide [9], and causes approximately 100 million infections [10], and at least 1-2 million infant deaths every year worldwide [9]. *S. pneumoniae* has several characteristics that contribute to its pathogenicity during an infection.

The primary virulence factor of *S. pneumoniae* is its capsular polysaccharide layer, and by comparing the differences in polysaccharide composition, *S. pneumoniae* can be divided into more than 90 serotypes [11]. When entering the nasal cavity, *S. pneumoniae* cells encounter mucus secretions of the host. Studies using experimental colonization have revealed that carriage of *S. pneumoniae* induces the production of mucosal and systemic immunoglobulin, which is mainly strain- and type-specific. The pneumococcus can bypass the mucus by expressing a capsule, allowing them to access the epithelial surfaces. Once there, *S. pneumoniae* enters a phase variation, where it switches to expression of a thinner capsule, in order to aid its adherence [12].

Pneumococcal adhesion and binding to the extracellular matrix is performed by pneumococcal adhesion and virulence A (PavA) and enolase. PavA binds to fibronectin, while enolase binds to plasminogen, both being extracellular-matrix components of the host cell [12].

Acute inflammatory responses to pneumococcal infection is ineffective at clearing the carrier state, and is even promoted by pneumolysin-mediated pore formation on epithelial cells, which attracts neutrophiles to the infection site. This suggests that the pneumococcus might have evolved to cause inflammatory responses, and thereby increase the likelihood of transmission through resulting secretions [12].

Pneumococci express the major autolysin LytA, which is an important virulence factor. The highly potent murein hydrolase is a main contributor to the release of the cytolytic toxin pneumolysin during infection. LytA is also responsible for the characteristic autolysis of stationary-phase pneumococci, which is caused by the cleaving of the lactyl-amide bond that links the stem peptides and the glycan strands of the peptidoglycan. *S. pneumoniae* is normally protected from lysis by LytA during exponential growth, but becomes vulnerable during the stationary phase. They are also more sensitive to lysis when their growth or cell wall synthesis is inhibited. Such vulnerability can be achieved through penicillin treatment [13].

It is generally thought that host-mediated killing of *S. pneumoniae* requires serotype-specific binding of antibodies, accompanied by serum proteins which protect against infection, and followed by phagocytosis. Other factors that have been proven to aid in pneumococcal clearance are Toll-like receptor 2 (TLR2), and major histocompatibility complex class II [12].

*S. pneumoniae* can limit the effectiveness of host humoral response on mucosal surfaces with the expression of a secreted zinc metalloprotease that specifically targets human immunoglobulin A1 (IgA1), which constitutes more than 90% of the IgA in the human airways. The IgA1 protease cleaves bound IgA1, producing bacterial surface antigens and preventing inflammation from being initiated through host recognition of the Fc region of the antibody. This ensures that antibody-mediated clearance only occurs after sufficient amounts of other classes and subclasses of specific antibody have been generated [12].

Pathogenic microbial species in the human pharynx compete with each other, as it has been shown that one strains induction of innate immune responses can cause the clearance of another. This implies that while the pneumococcus is resistant to the innate immune responses it stimulates by itself, it can be sensitive to the responses induced by its competitors. Through their mechanisms of natural competence, pneumococcal strains are also in competition with each other [12].

An effective way to reduce mortality from pneumococcal pneumonia is by using vaccines, based on the polysaccharide capsule of the bacteria. This treatment is challenged by the existence of many different serotypes, and to ensure efficient protection it is necessary to include capsule components from a wide variety of serotypes [12].

A heptavalent pneumococcal vaccine (PCV7 vaccine) was licensed in the US and recommended for routine use in infants in 2000. The PCV7 vaccine contained the 7 most common pneumococcal serotypes causing invasive infections in children, and it dramatically reduced the rates of invasive pneumococcal disease, otitis, media and nasal carriage of the serotypes included in the vaccine. While the PCV7 vaccine reduced pneumoccocal disease caused by the included serotypes, infections caused by the serotypes not included prevailed. In 2010, a 13-valent pneumococcal conjugate vaccine (PCV13) was licensed. This vaccine protects against the same seven strains that were in PCV7, but it also has the potential to reduce invasive pneumococcal disease caused by 6 additional strains [http://www.immunizationinfo.org/vaccines/pneumococcal-disease]. Although the PCV13 vaccine covers more serotypes than the PVC7, it still contributes to an increase in the incidences of disease caused by serotypes not included in the vaccine [14].

### 2.1.3 - Natural Competence and Fratricide in *S. pneumoniae*

Pnumococci have the ability to enter a naturally competent state, and take up naked DNA from the environment. This DNA can then be incorporated into their own genome by homologous recombination.

The competent state in *S. pneumoniae* is entered when the concentration of the competence-stimulating peptides (CSP-1) in the extracellular environment reaches a critical level, and transcription of the early and late competence genes are initiated. The concentration of CSP-1 is registered by the cells *comDE*-encoded two-component signal transduction pathway, which comprise a quorum sensing system. ComD monitors the extracellular concentration of CSP-1. When CSP-1 reaches a critical level it triggers a signal cascade through ComD leading to activation of the response regulator ComE, which binds to a conserved direct-repeat motif in the promoter region of about 20 early competence genes. Among these are the alternative sigma factor *comX*, which is required for expression of approximately 80 late competence genes [15].

Fratricide is part of a process where naturally competent pneumococci lyse non-competent cells of closely related bacteria. This step is a product of the late competence gene *cbpD*, which encodes Choline-Binding Protein D (CbpD), the key murein hydrolase responsible for lysis of sensitive target cells [16]. Efficient lysis of the target cells also requires the cell wall

hydrolase LytA and LytC. While CbpD is produced by competent cells exclusively, LytA and LytC can be produced by non-competent cells as well. *comM*, one of the early competence genes, encodes an immunity protein that protects the competent cells against their own lysins CbpD [15].

Studies on the competence of another streptococcal species, *Streptococcus thermophilus*, has revealed a competence stimulating peptide known as ComS. It is likely that intracellular ComS interacts with and activates ComR, a transcriptional regulator, which then binds to an inverted repeat motif upstreams the *comX* gene, and initiates expression of *comX* [17].

Through natural transformation, pneumococcus gain access to a gene pool, which it shares with strains of its own species, and closely related streptococci. Since certain genes are beneficial during a certain kind of stress (e.g. antibiotic resistance genes when the pneumococci are exposed to a specific antibioticum), they grant a higher chance of survival. These genes are then passed on, and will spread quickly among the pneumococci [15].

### 2.1.4 - Antibiotics and resistance of *S. pneumoniae*

*S. pneumoniae* is the most common cause of pneumonia, meningitis, and middle ear infections. Such infections are usually treated with antibiotics, typically penicillins. However, exposing bacteria to an antibiotic that they are vulnerable to also promotes the growth of strains that have the stress-specific advantage of being resistant to this antibiotic. The increasing rate of penicillin resistant strains of pneumococcus have been a worrying trend over the last decades, and is an increasing problem in several countries, especially in Asia [18].

Penicillin is a β-lactam based antibioticum that kills susceptible bacteria by inhibiting transpeptidase enzymes that cross-links cell wall peptidoglycan. The reason for this is that the β-lactam antibiotics have a structural similarity to the natural substrate of transpeptidase enzymes, the D-Ala-D-Ala end of the stem pentapeptide precursors. The enzymes form a long-lived acyl-enzyme with penicillin, that impairs their peptidoglycan cross-linking capability. Because of this, these enzymes are called Penicillin Binding Proteins (PBP) [19].

Streptococci are among the most penicillin-sensitive organisms, but can develop high-level resistance. This mechanism appears to involve complex alterations in target PBPs. The production of β-lactamase (enzyme that breaks down β-lactam based antibiotics) also plays a significant role in the evolution of resistance to β-lactam antibiotics [20].

Pneumococci can gain resistance to β-lactam antibiotics through natural transformation. One way this can happen is through recombinational replacements within and around the capsular biosynthesis (*cps*) locus. The *cps* locus is flanked by the *pbp2x* and *pbp1a* genes, coding for Penicillin Binding Proteins.[21] Thus, recombination of the *pbp2x* and *pbp1a* genes can simultaneously lead to changes in the capsule genes resulting in an altered capsule structure in addition to higher penicillin resistance.

## 2.2 - Cell wall synthesis in bacteria

### 2.2.1 - Role and structure of the bacterial cell wall

The bacterial cell wall is a layer that surrounds the bacterium. Its main function is to protect the bacterium from its own turgor pressure [23]. Bacteria can be categorized as either Gram-positive or Gram-negative, depending on the composition of their cell wall.

The main component of the cell wall is peptidoglycan. The peptidoglycan structure can be defined as linear glycan strands cross-linked by short peptides (Figure 2.3A). The glycan strands consist of N-acetylglucosamine (*GlcNAc*) and N-acetylmuramic acid (*MurNAc*) residues linked by β-1-->4 bonds, and are terminated by a 1,6-anhydroMurNAc residue. The strands are further connected to each other by cross-linking between meso-$A_2$pm- and D-Ala-groups from the MurNAc residues (Figure 2.3A) [24].

The bacterial cell wall of Gram-positive bacteria consists of an inner layer of low density, and an outer layer of higher density peptidoglycan. The outer layer likely has a higher degree of polymeric wall structure. The layers form a sacculus around the cell that protects it from osmotic pressure and lysis, and it is flexible enough to expand and shrink threefold without rupture. The peptidoglycan layer is also riddled with pores of relatively homogenous size.

The composition of the peptidoglycan layer varies greatly between species. Because of its important function in preserving the cell, any degradation of it, or inhibition of its synthesis, will lead to lysis of its cell due to the high turgor pressure inside the bacteria. The peptidoglycan layer contributes to the cell maintaining its normal shape, so in theory, any modification of the layer could lead to a visible difference in the cell's shape [24].

Teichoic acids are found in the cell wall of Gram-positive bacteria, and can be covalently linked to the peptidoglycan layer. Peptidoglycan-associated teichoic acids have a large structural diversity, making it difficult to define what is and what is not a teichoic acid [25]. In *S. pneumoniae*, the covalent linkage region of teichoic acids to the peptidoglycan layer is currently unknown. Pneumococcal teichoic acids have a complex structure consisting of repeating units of ribitol phosphate [26]. The main function of teichoic acids is to work as a docking molecule for pneumococcal surface protein, in particular those containing a Choline binding domain, since choline residues are attached to the teichoic acids. The teichoic and lipoteichoic acids are pneumococcal common antigens, and may have a pathophysiological role [27].

### 2.2.2 - Synthesis of peptidoglycan

The biosynthesis of peptidoglycan proceeds through three steps; the first two are the synthesis of precursor intermediates, which happens in the cytoplasm and cellmembrane. The final step is the polymerization of the newly synthesized disaccharide-peptide units and incorporation into the growing peptidoglycan (Figure 3B). This step is achieved mainly through the action of penicillin-binding proteins (PBPs), which catalyze the reactions responsible for the formation of the glycosidic and peptide bonds of the peptidoglycan. These reactions involve transglycosylation and transpeptidation.

Glycan chains, cross-linked by peptide bridges, are formed through transglycosylation, by connecting the linked amino sugars N-acetylglucosamine and N-acetylmuramic acids. This connection is formed between the reducing end of the MurNAc of one glycan-group and the C-4 carbon of the glucosamine residue of the other glycan-group.

The glycan chains are connected/crosslinked through transpeptidation. The D-Ala-D-Ala bond of one peptide is cleaved by a PBP enzyme, and the peptide-linked D-Ala-terminal is

connected to the last amino acid of the pentaglycine cross bridge. Between these two steps the PBP forms an enzyme-substrate intermediate with the peptide-linked D-Ala-terminal [29].

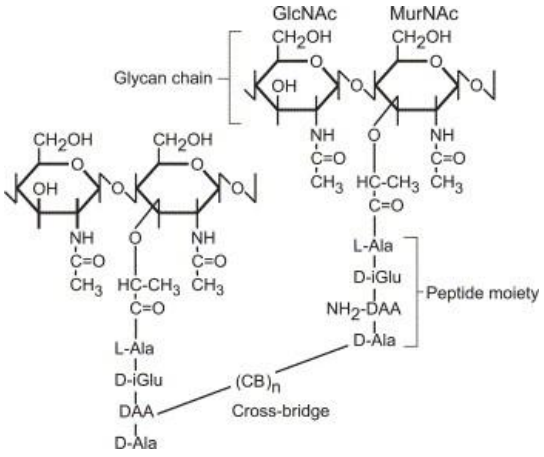



**Figure 2.3A.** A diagram of Gram-positive bacterial peptidoglycan. The residues of GlcNAc and MurNAc are linked together by β-1-->4 bonds, and are cross-linked by short peptides [28].

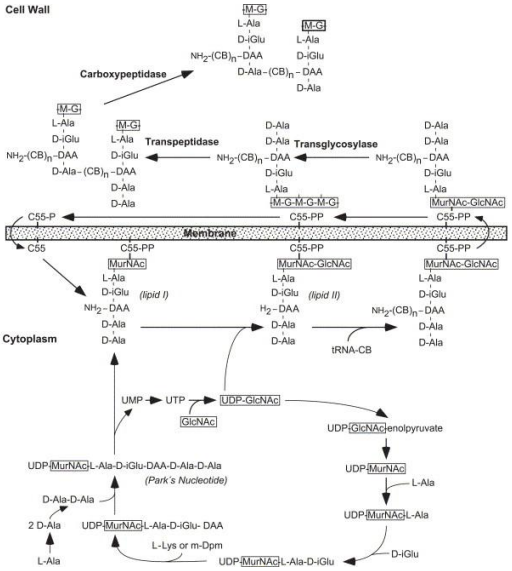



**Figure 2.3B.** Biosynthesis pathway of bacterial cell wall assembly [28].

### 2.2.3 - Penicillin Binding Proteins

PBPs are a family of enzymes associated with the cell division process in bacteria. *S. pneumoniae* carries a set of six PBPs; PBP1a, -1b, -2a, -2b, -2x, and -3. The first five are classified as high-molecular-mass (HMM) PBPs, while PBP3 is a low-molecular-mass (LMM) PBP. Furthermore, PBP1a, -1b, and -2a catalyze both glycosyltransfer and transpeptidation, and are classified as class A HMM PBPs, while PBP2b and -2x only catalyze transpeptidation, and are classified as class B HMM PBPs [30].

The topology of PBPs consists of a cytoplasmic tail, a transmembrane anchor, and two domains joined by a β-rich linker located on the outer surface of the cytoplasmic membrane where peptidoglycan synthesis takes place. Depending on the structure and catalytic activity of their N-terminal domain, PBPs are categorized as either class A or class B. For class A PBPs, the N-terminal domain is responsible for the glycosyltransferase activity, catalyzing the elongation of uncross-linked glycan chains. The role of the N-terminal domain in class B PBPs is believed to play a role in cell morphogenesis by interacting with other proteins involved in

the cell cycle. The C-terminal penicillinbinding domain of both classes has a transpeptidase activity [19].

PBP3 has been shown to act as a D-Ala,D-Ala (D,D) carboxypeptidase. It is hypothesized that since PBP3 is localized throughout the entire bacterial surface except for the future division site, and its activity leaves pentapetides without their COOH-terminal D-alanine, that it guarantees the availability of intact pentapeptidic substrates near the division site. It has been proposed that this could ensure that the FtsZ-ring, which function is to recruit other proteins that produce a new cell wall between dividing cells [http://www.umass.edu/microbio/chime/pipe/ftsz/present/], is located correctly, leading to normal cell division [30].

### 2.2.4 - Cell division in *S. pneumoniae*

Bacterial cell division starts at the cell division site, with the formation of a Z ring, which consists of FtsZ polymers. This is necessary for the recruitment of all other cell division proteins to the cell division site [29].

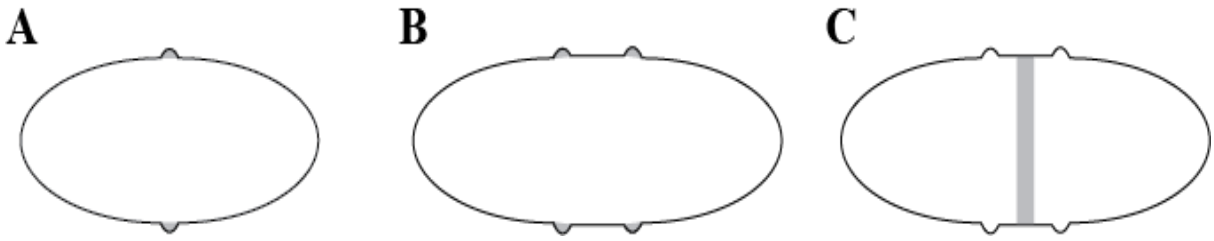



**Figure 2.4.** Cell division in *S. pneumoniae. S.pneumoniae* grow by elongating its cell shape from an equatorial ring surrounding the middle of the cell (indicated by the two bumps) (A). While inserting new cell wall material at this initial ring, another ring is formed, and further cell elongation separates the two rings (B). A division septum is synthesized between the rings (grey), forming the division site of the daughter cells as a result of cell division (C) [29].

A study on localization of PBPs in *S.pneumoniae* was conducted by Morlot and colleagues [31], using immunofluorescence microscopy. PBP1a and -2x both have septal localization and follow FtsZ localization, while PBP2a and -2b follow the localization of the duplicated equatorial rings. PBP1b follows either septal or equatorial localization, varying from cell to cell. Their localization indicates that different PBPs are specialized in peptidoglycan synthesis in either cell division (septal localization) or cell wall synthesis (equatorial localization) [29].

The localization study [31] focused on how localization of HMM PBPs could be changed by the absence of other HMM PBPs. Mutants lacking the PBP2a-encoding gene (*pbp2a*) were

made, and the localization of PBP1a in these mutants was found to have changed to equatorial position in a significant proportion of the cells. The study group hypothesized that PBP2a would relocate to equatorial localization only when PBP1b was positioned at septal localization, so that both cell wall synthesis and cell division could progress. In addition, the study also included localization analysis on two double mutants, deficient in PBP1b and either PBP1a or PBP2a. The remaining class A HMM PBP would then be positioned at either equatorial or septal localization, but never simultaneously.

PBP3 is evenly distributed on both hemispheres and is absent from the future division site. Because it catalyses the removal of the HMM PBP substrate D-Ala-D-Ala, its role has been postulated to ensure that the substrate of HMM PBPs only is present at the division site. When PBP3 is missing from the cell, the substrate is no longer restricted to the division site, and HMM PBPs lose their normal colocalization with FtsZ rings. This implies that localization of HMM PBPs at the middle region of the cell depends on the availability of substrate exclusively at that place [29].

In an article on a PBP3-altered pneumococcal mutant (Schuster *et al*. (1990)) it was noted that LMM PBPs exhibited a different mode of membrane attachment than HMM PBPs. While the HMM PBPs has an N-terminal hydrophobic peptide region that provides membrane binding, the LMM PBPs are anchored to the membrane by their C-terminal amino acids. By producing and cultivating a pneumococcal mutant without the C-terminal producing part of the PBP3-encoding gene present, the research group could observe that most of the PBP3 was present in the growth medium, rather than in the pneumococci. The mutant also had a disturbed morphology, which the research group believed to be an indication of a disturbance in the system which determined the proper initiation sites of new septa, and that this was caused by the absence of cell-associated PBP3. They also noted the possibility that part of the phenotypic effects could be related to some PBP3 activity during its transport through the cell wall layer, or that the C-terminal part of PBP3 serves as an important structural element interacting with other proteins [32].

In order for the peptidoglycan wall to expand, the covalent bonds of the peptidoglycan must be broken so that new material can be inserted. Peptidoglycan hydrolases are therefore essential for cell growth and division. Depending on which specific bond of the

peptidoglycan the hydrolase cleave, they can be classified as muramidases, glucosaminidases, amidases, endopeptidases, and carboxypeptidases [29].

*S. pneumoniae* produces several peptidoglycan hydrolases, but only one (LytB) has hitherto been shown to be involved in cell division. *ΔlytB*-mutants have shown formation of extremely long chains of cells, whereas the mutants lacking the autolysins encoded by *lytA* and *lytC* show no changes in morphology nor growth compared to wild type cells. LytB is required for the final step of cell separation in pneumococci. Another putative cell wall hydrolase called PcsB has also been shown to be essential for *S. pneumoniae* although its activity remains undefined [33].

## 2.3 - Aim for this study

To understand how the bacterial cell wall is synthesized is important for understanding antibiotic action and resistance as well as in identifying targets for new types of antibiotics. In this study we wanted to expand our knowledge on the function of the pneumococcal gene *dacA*, *pbp1a*, *pbp1b* and *pbp2a*encoding PBP3, PBP1a, PBP1b and PBP2a, respectively. By producing *ΔdacA, Δpbp1a*, *Δpbp1b* and *Δpbp2a*-mutants from a strain of *S. pneumoniae R6*, and analyzing the growth rate, morphology and antibiotic resistance of the mutant strains, we aim to observe how well pneumococci survive and respond to antibiotics, with the lack of these genes. In addition, we wanted to explore the interplay between the role of PBP3 and the HMMs PBP1a, PBP1b and PBP2a.

# 3 Materials

## 3.1 - Bacterial strain

The pneumococcal strain RH426 was used in this study. It has a wild type phenotype, and is the original strain for every mutant produced in the study. RH426 has a mutated version of the *rspL*-gene, which makes the strain resistant to streptomycin. This property makes it suitable for genetic manipulations using the Janus cassette (see section 4.4.1). Whenever wild type cells are referred to in this report, it refers to the RH426 strain.

We made several mutants in this study, using either the RH426 or the *ΔdacA*-mutant as the parent strain. The *ΔdacA*::*luc* mutant also contains a $P_{comX}$-*dacA* construct. Templates and primers for the amplification of the *Δpbp2a*::Janus, *Δpbp1a*::Janus and *Δpbp1b*::Janus constructs were provided by Marita Sæther.

| Mutant | Parent |
|---|---|
| *ΔdacA* | RH426 |
| *Δpbp2a* | RH426 |
| *Δpbp1a* | RH426 |
| *Δpbp1b* | RH426 |
| *ΔdacA*::*luc* | RH426 |
| *ΔdacAΔpbp2a* | *ΔdacA* |
| *ΔdacAΔpbp1a* | *ΔdacA* |
| *ΔdacAΔpbp1b* | *ΔdacA* |

## 3.2 - Growth Media

| **C Medium** | |
|---|---|
| Pre C-medium | 150 ml |
| Manganchloride | 150 µl |
| 20 % Glucose | 1,5 ml |
| ADAMS III | 3,75 ml |
| 3 % Glutamine | 110 µl |
| 2 % Sodium pyruvate | 2,25 ml |
| 1,5 M Sucrose | 95 µl |
| 2 mg/ml Uridine/Adenosine | 1,5 ml |
| 8 % Albumin | 1,5 ml |
| 10 % Yeast Extract | 3,75 ml |

| **Todd Hewitt** | |
|---|---|
| Todd Hewitt Broth | 15 g |
| Agar | 7.5 g |
| Water | 500 ml |

## 3.3 - Kits

| Supplier | Kit |
|---|---|
| Macherey-Nagel | Nucleospin® Extract II Kit |

## 3.4 - Enzymes

| Supplier | Enzyme |
|---|---|
| New England Biolabs | Taq-polymerase, Phusion Polymerase |

## 3.5 - Standards

| Supplier | Nucleic acid ladders |
|---|---|
| Invitrogen | 1 kb ladder |

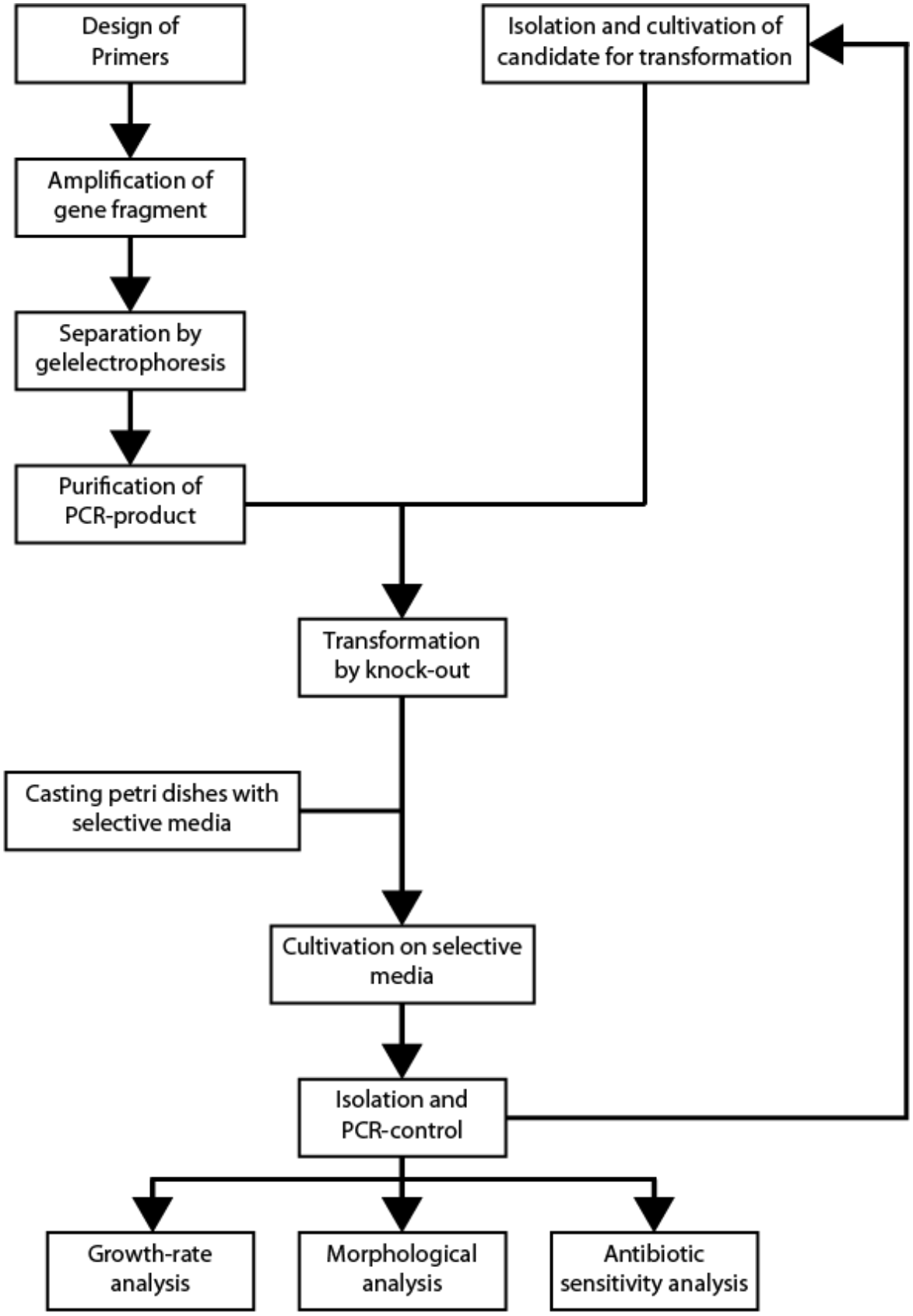
## 3.6 - Chemicals

| Supplier | Chemical |
|---|---|
| BD | Bacto™ Todd Hewitt Broth |
| Fluka | Biotin, Nicotinic acid, Pyridoxine hydrochloride, $FeSO_4.7H_2O$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $MgCl_2.6H_2O$, L-Tryptophan, L-Cysteine hydrochloride monohydrate, L-Glutamine, Glutamine |
| MERCK | MnCl2.4H2O, HCl, Dipotassium hydrogen phosphate, Glucose, Glutaraldehyde, Potassium chloride, Disodium phosphate, Monopotassium phosphate, Glacial acetic acid, Agar |

| | |
|---|---|
| Promega | dATP, dCTP, dGTP, dTTP |
| Riedel-de Haën | Mangan chloride tetrahydrate |
| Sigma | Calcium pantothenate, Thiamine hydrochloride, L-Aspargine.$H_2O$, $CaCl_2$, Adenosine |
| Sigma-Aldrich | Riboflavin, Kanamycin sulfate, Streptomycin sulfate, Sodium pyruvate, Paraformaldehyde, Sodium chloride |
| Sigma Life Science | Choline, Sodium acetate, Uridine, Trisbase, Glycerol |
| VWR | Saccharose, Ethidium bromide, EDTA |

# 4 Methods

Schematic flow-diagram showing the process of transformation and genetic analysis



## 4.1 - Isolation and cultivation of *S. pneumoniae*

Every culture was isolated from frozen stock cultures using a sterile loop, and cultivated in C-medium at 37˚C.

### 4.1.1 - Anaerobic incubation

Since *S. pneumoniae* is a faccultative anaerob bacterium, it grows best in anaerobic conditions. Such conditions were prepared using Oxoid AnaeroGen™ sachets, which rapidly absorbs atmospheric oxygen, while simultaneously producing carbon dioxide. This method does not require any catalyst or the presence of water.

[http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=AN0035&org=53&c=UK&lang=EN]

The plates were put into jars together with either one or two Oxoid AnaeroGen™ sachets, depending on the volume of the jar. The jar was then shut close and stored at 37˚C.

## 4.2 - Design of primers

The web-based genome database at NCBI (http://www.ncbi.nlm.nih.gov/) was used to find the genomic sequence of the gene *dacA* in *S. pneumoniae*, as well as the upstream and downstream sequence of this gene.

The forward primer was copied directly from the sequence, while the reverse primer was reversed and each base were changed to their complementary base. The tail for the forward primer was copied from the upstream sequence of the forward primer, and then reversed. The tail for the reverse primer was copied from the downstream sequence of the reverse primer, and made reverse complementary.

The primers and tails were designed by fulfilling the general rules of primer design. The sequences should be between 18-24 nucleotides, with a GC content sufficient enough to provide an efficient annealing. Ideally the sequences should also end with a G or C, to ensure a strong binding at the start of the elongation site.

## 4.3 - Polymerase Chain Reaction

PCR is a three-step cycling process. First, the double-stranded DNA is denatured to single strands. Then, primers are attached to the ends of the fragments that we wish to amplify. Finally, the primers are extended, and new double-stranded DNA-fragments are made. By repeating these three steps multiple times, the fragment can be copied to a desired amount.

Denaturation of DNA can be accomplished by increasing the temperature to a little under 100°C. The hydrogen bonds between strands are then broken, and the DNA strands are available for synthesis.

Attachment of primers, known as the Annealing step, happens automatically at temperatures between 50 - 60°C, since primers are present in large amounts. They are therefore more likely to anneal to the dissociated strands than the strands are to reanneal to each other. Each primer is complementary to the sequence that they are meant to anneal to.

The primer extension is catalyzed by added DNA polymerase, which adds nucleotides to the 3'-end of the primers. This results in the synthesis of a new DNA strand, which complements the strand that the primer annealed to [34].

### 4.3.1 - Phusion and Taq polymerase

Phusion polymerase is a high fidelity DNA polymerase produced by Finnzymes. It's based on a novel Pyrococcus-like enzyme, but includes a processivity-enhancing domain. According to its producers, phusion polymerase has an error rate of $4.4 \times 10^{-7}$ for every nucleotide polymerized.

Taq polymerase is a stable DNA polymerase with a temperature optimum of 80°C, that has been purified from the extreme thermophile *Thermus aquaticus* [35]. It's relatively cheap compared with Phusion polymerase, but has a much lower replication fidelity. Through a study performed by Tindall and Kunkel [36] Taq polymerase showed a error rate of 1 for each 9000 nucleotides polymerized, which is about two hundred and fifty times higher than what Finnzymes claims that phusion polymerase have.

In order to conserve the valuable phusion polymerase, we use Taq polymerase for PCR control experiments, where perfect replication is not a requirement.

### 4.3.2 - PCR specifics

The PCR reaction mixture is presented in Table 4.1, and the PCR configuration is given in Table 4.2.

**Table 4.1. The Phusion PCR reaction mixture**

| PCR reaction | Volume (µl) |
|---|---|
| Phusion HF-buffer (5X) | 10 |
| dNTP (10 mM) | 1 |
| Forward primer (10 pmol/µl) | 2,5 |
| Reverse primer (10 pmol/µl) | 2,5 |
| Template DNA | 2 (10-100 ng) |
| Phusion polymerase | 0,5 |
| ddH2O to a Total volume | 50 |

**Table 4.2. The PCR temperature cycle**

| Temperature | Time | Cycles |
|---|---|---|
| 94°C | 5 min | 1 |
| 94°C | 10 sek | 25 |
| 54°C | 30 sek | |
| 68°C | x sek* | |
| 68°C | 5 min | 1 |
| 4°C | ∞ | 1 |

*Typically, we add 30 min for every 1.000 bp.

## 4.4 - Overlap Extension PCR

In order to prepare our DNA sequences for transformation of *S. pneumoniae*, they need to contain sequence(s) homologous to the region they are meant to replace. Through overlap extension PCR, we can make nucleotide sequences with an upstream- downstream-sequence identical to the upstream- downstream-sequence of the gene we wish to delete or modify (Figure 4.1). It is a method that combines nucleotide sequences by the use of primers which contain complementary ends of the sequences we wish to fuse together, and by employing PCR, the combined sequences are fused and amplified into single hybrid sequences [37].

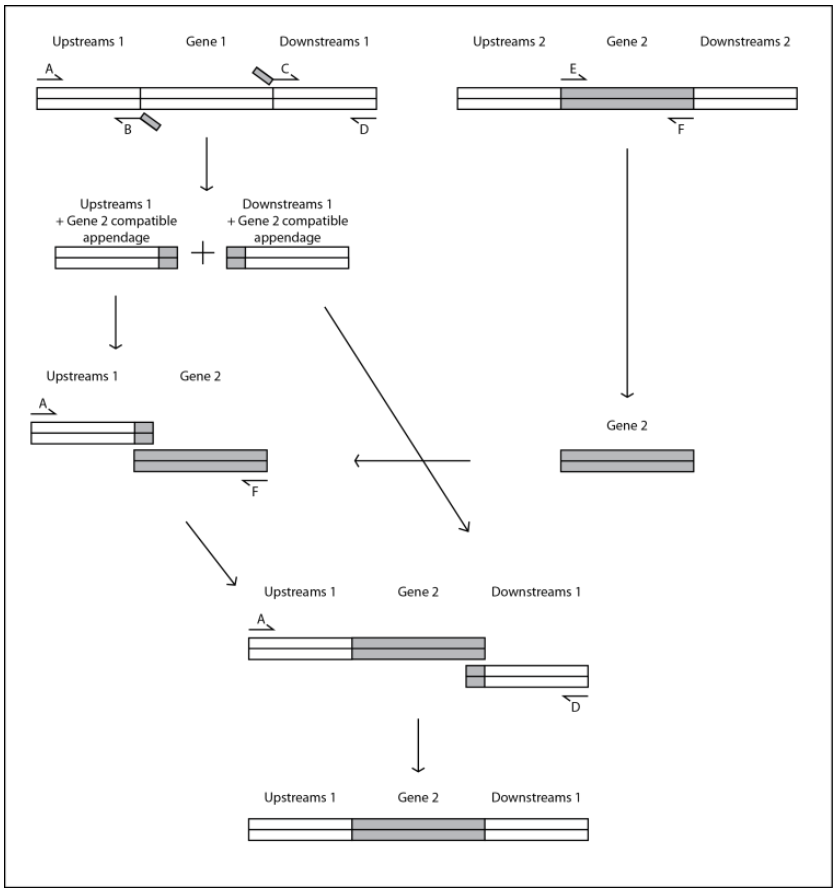



**Figure 4.1.** In order to hybridize the three DNA fragments "Upstreams 1", "Gene 2", and "Downstreams 1", we need to use six different primers, A-F. A, C, and E are forward primers, while B, D, and F are reverse primers for the three fragments. Primer B and C also include a sequence which is complementary to the forward and reverse sequence of "Gene 2". By amplifying "Upstreams 1" and "Downstreams 1" with these primers we gain clones of these sequences with a "Gene 2"-complementary appendage. Amplifying these sequences together with "Gene 2" and forward/reverse primers corresponding to the end of the desired fragment, we can hybridize the three DNA fragments.

### 4.4.1 - The Janus cassette

The Janus cassette is a DNA fragment, which can be incorporated into the genome of pneumococci through natural transformation. For the Janus cassette to function properly in *S. pneumoniae*, the strains need to be streptomycin resistant. The cassette contains both a kanamycin resistance marker and an $rpsL^+$ marker, which gives the mutant dominant streptomycin sensitivity, in spite of the natural streptomycin resistance ($rpsL^-$) of the wild type (RH426). Since pneumococci are naturally sensitive to kanamycin, mutants with the Janus cassette can be selectively cultivated by growth on kanamycin, while the wild-type and

mutants without the Janus cassette can be selectively cultivated by growth on streptomycin [38].

We use the Janus cassette to knock out genes of interest in the pneumococcus genome. Mutants that have their gene replaced by a Janus cassette, can be selectively cultivated on TH-agar containing kanamycin (400 µg/ml). The mutants can then be isolated. If we want to delete another gene from the mutant, we first have to delete the Janus cassette, select the mutants without the Janus cassette by cultivation in streptomycin infused agar, and then use the Janus cassette to knock out the other gene.

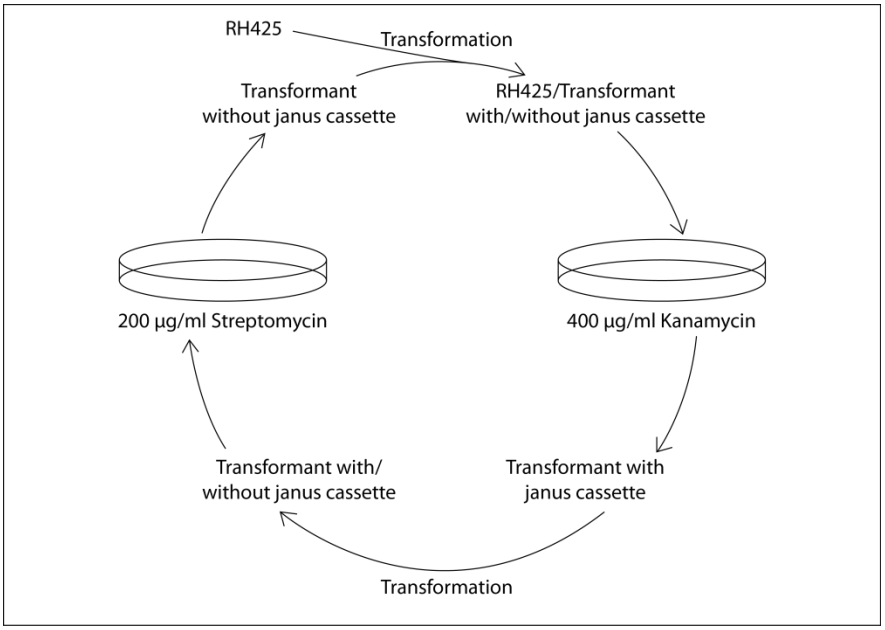



**Figure 4.2.** An illustration showing the procedure of selection for each transformation.

## 4.5 - DNA separation by gel electrophoresis

In order to analyze and purify the amplified DNA fragments, the PCR-product was separated by agarose gel electrophoresis. This technique relies on the negative charge of the phosphate backbone in DNA molecules, and the use of a sieving matrix (a polymer with a conductive medium) to distribute a voltage gradient and create an electromotive force. By placing DNA fragments in wells in the matrix, and applying an electric field, the molecules will migrate in parallel through the matrix in a direction from the cathode towards the positive electrode. Larger molecules will move through the matrix at a slower pace than

smaller molecules, and by comparing the molecules with a "ladder", which contains fragments of predetermined size, we can estimate the size of the DNA fragments in our samples [39]. By applying ethidium bromide (EtBr) to the gel, the DNA fragments can be visualized using ultra violet (UV) light. EtBr is a fluorescent dye that can bind to DNA by intercalation, slip between adjacent base pairs, and cause a stretch of the double helical structure. This binding enhances the fluorescence intensity and lifetime of the DNA.

[http://www.sciencedirect.com/science/article/pii/S0022286006004819, http://arbl.cvmbs.colostate.edu/hbooks/genetics/biotech/gels/agardna.html]

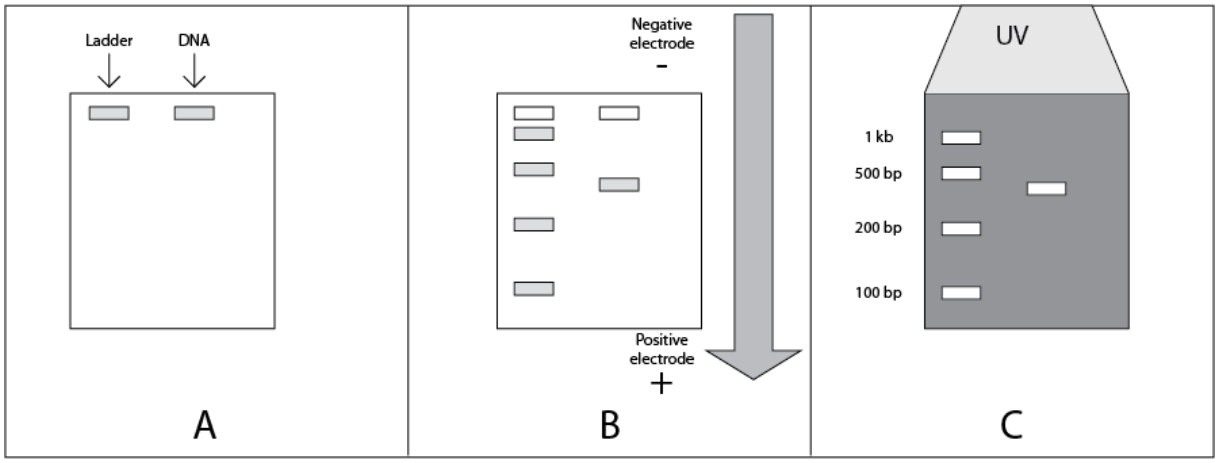



**Figure 4.3.** An illustration of separation by gel electrophoresis. The DNA fragments are applied to a well on a sieving matrix, next to a well for a DNA ladder with fragments of predetermined size (A). Applying an electric field will make the fragments move through the matrix, with a speed depending on the size of the fragment (B). During the electrophoresis the ethidium bromide in the gel has bound to the DNA, and can be visualized by exposure to UV light (C). By comparing the size of the fragments in the ladder with the size of the DNA fragment, we can determine how long the sample fragment is.

### 4.5.1 - Agarose gel electrophoresis separation protocol

1. The gel was made by mixing 0.5 g of agarose with 50 ml 1 X TAE-buffer. Preparation of TAE-buffer is described in table 4.3.
2. This solution was heated in a microwave, cooled to about 60˚C and added with EtBr to a final concentration of 0.5 µg/ml.
3. The solution was then transferred to an electrophoresis vessel, and combs were applied to make wells.

After 10-15 minutes, the agarose gel was ready for electrophoresis.

1. A volume of 10 µl of 1 kb DNA-ladder was used as molecular size marker, and 5 µl of 10x loadingbuffer was added to each 50 µl of sample, which was then applied to the remaining wells.
2. Electrophoresis was performed at 0.6 V/$Cm^2$ for 25 minutes.
3. The separated DNA were visualized in a Gel Doc-1000 (BioRad).

**Table 4.3. Preparation of 50 x TAE-buffer**

242 g Trisbase

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0)

## 4.6 - Purification of PCR-product from agarose gel

In order to isolate and clean the PCR-product, it must be extracted from the agarose gel, and be bound, washed, and eluted. We used the Nucleospin® Extract II Kit to perform this. The kit contains a column with a silica membrane, which DNA binds to in the presence of chaotropic salt. Using the ethanolic buffer NT3, contaminations like salts and soluble macromolecular components can be removed. Since the adsorption of DNA to the silicia membrane is pH dependent, pure DNA can be eluted with an addition of the weakly alkaline buffer NE, under low ionic strength conditions.

[http://www.mbio.ncsu.edu/MB452/unknown/PT3814-1.pdf]

With this method, we can purify the PCR-product with a high yield, while completely removing primers from the PCR reactions.

1. Using a clean scalpel, the DNA fragment was excised from the agarose gel, and transferred to a clean tube.
2. After measuring the gel fragments weight, 200 µl of NT buffer were added for every 100 mg of gel. The tube was then incubated at 50°C until the gel had dissolved completely.
3. A Nucleospin® Extract II Column was placed into a collection tube (2 ml), and the dissolved gel was transferred into the column. The column was then centrifuged at

11,000 x *g* for 1 min. The flow-through was discarded and the column was placed back into the tube.

4. 700 µl of Buffer NT3 was added into the column, which was centrifuged at 11,000 x *g* for 1 min. The flow-through was discarded and the column was placed back into the tube.
5. The column was then centrifuged at 11,000 x *g* for another 2 min, in order to remove the Buffer NT3 completely.
6. The column was then removed from the tube and placed into a new 1.5 ml microcentrifuge tube.
7. Depending on the amount of product, Between 15 and 50 µl of Buffer NE was added into the column, which was stored at room temperature (18°C - 25°C) for 1 min.
8. The column was then centrifuged at 11,000 x *g* for 1 min.

## 4.7 - Casting petri dishes with selective media

Antibiotics were prepared by diluting them with water to a storage concentration. When the antibiotics were used in selective media, they were diluted with Todd Hewitt Agar to a selection concentration. Both of the specific concentrations for each of two antibiotics are given in table 4.4.

**Table 4.4. Concentrations for storage and selection with antibiotics**

| Antibiotic | Stock solution | Selection solution |
|---|---|---|
| Kanamycin (Kan) | 100 mg/ml | 400 µg/ml |
| Streptomycin (Sm) | 100 mg/ml | 200 µg/ml |

Todd Hewitt (TH) Agar was prepared by mixing 500 ml of water with 15 g of TH-media and 7.5 g of agar. A magnet for stirring the solution with antibiotics was added, and the solution was autoclaved at 120˚C for 15 min. The antibiotic was then applied to the right selection solution (Table 4.4) and the solution was stirred on a magnet-stirrer. The agar was then distributed onto petri dishes.

## 4.8 - Transformation of *S. pneumoniae* by homologous recombination

By using the pneumococcus' ability to become naturally competent during exponential growth, we can incorporate the amplified DNA fragment into the pneumococcal genome.

1. The purified DNA fragment was diluted with C-medium to $OD_{550}$ ≈ 0,05, and cultivated at 37˚C for 15 min.
2. 1 ml of culture was then transferred to each of two eppendorf tubes. Each of these were induced with CSP-1 to a concentration of 250 ng/ml.
3. 5 µl of purified DNA was also applied to one of the tubes. Both tubes were incubated at 37˚C for two hours.
4. The cultures were applied to selective TH-media in dosages of either 20 µl or 100 µl.
5. The plates were incubated at 37˚C in anaerobic conditions overnight.

## 4.9 - Isolation and PCR-control

After transformation and overnight incubation, bacteria from the resulting colonies were isolated by gently touching them with sterile toothpicks, which were then dipped in PCR-tubes for PCR-control, and then added to falcon-tubes containing C-medium and the appropriate antibioticum for cultivation. The PCR-reaction mixture follows table 4.5, and the PCR configuration in table 4.2.

**Table 4.5. The PCR reaction mixture**

| PCR reaction | Volume (µl) |
|---|---|
| Taq-buffer (10X) | 5 |
| dNTP (10 mM) | 1 |
| Forward primer (10 pmol/µl) | 2,5 |
| Reverse primer (10 pmol/µl) | 2,5 |
| Template DNA | 2 |
| Taq-polymerase | 0,5 |
| ddH2O to a Total volume | 50 |

After having cultivated the mutants for about 9 hours ($OD_{550}$ = 0.2-0.3), glycerol was added to a final concentration of 15%. The tubes were then stored at -80˚C.

## 4.10 - Growth rate analysis

In order to verify and observe the growth rate of the cell cultures, spectrophotometry was performed. A Novaspec II single beam spectrophotometer was used to take measurements during the cultivation of the cell cultures, while a plate reader was used to periodically measure the growth rate over time. The cell cultures were loaded into cuvettes for the spectrophotometer, and into plate wells for the plate reader.

### 4.10.1 - Spectrophotometry

Spectrophotometry is a way to quantify the cell density in a medium, by measuring the amount of light that is absorbed and refracted by the cells at a specific wavelength [http://www.nist.gov/pml/div685/grp03/spectrophotometry.cfm].

### 4.10.2 - Luminescence reporter gene

A good way to measure the expression of a gene during the growth of bacteria is to replace that gene with a luminescence reporter gene, like the luciferase-encoding gene *luc*.

Luciferase is an enzyme that among other things is used by fireflies to produce light. The biolumiscent reaction turns luciferin and oxygen into oxyluciferin, which is in an electronically excited state. When the oxyluciferin returns to the ground state, it releases a photon of light. This reaction is catalyzed by luciferase [40].

### 4.10.3 - The ComRS system

In a study by Fontaine *et al*. (2010) it is proposed that the addition of the signaling peptide ComS activates ComR, which in turn binds to the ECom box, resulting in activation of the *comX* promoter [41]. Thus, we can promote the transcription of the gene following the *comX* promoter by adding ComS*.

As part of the study, a mutant of the wild type was prepared by replacing the native *dacA* gene with the luciferase-encoding gene. The mutant was also modified by inserting a new *dacA* following the *comX* promoter. By adding ComS*, transcription of *dacA* could then be regulated, and with the induction of luciferin, transcription from the native *dacA*-promoter could be measured.

### 4.10.4 - Growth experiment procedure

To measure the growth of multiple parallels of mutants over time, a FLUOstar OPTIMA plate reader was used. The plate reader was set to measure absorbance at an OD of 550 nm, as well as the luminescence of each well every 10 minutes. The temperature was set to 37°C.

#### *4.10.4.1 - Standard growth experiment*

1. The wild-type and mutants were diluted with C-medium to an $OD_{550}$ ≈ 0,05, cultivated at 37˚C for about an hour, and then diluted to $OD_{550}$ ≈ 0,05 again.
2. The cultures were added onto a plate, with 300 µl of each sample being added to six wells each. This creates three parallels of each culture with two wells in each parallel.
3. One parallel was uninduced, the second parallel was induced with 1,5 µl of LytA from the start, and the final parallel was induced with 1,5 µl of LytA during the exponential growth of the cultures ($OD_{550}$ ≈ 0,3).

#### *4.10.4.2 - Luminescence reporter experiment*

1. During the luminescence reporter tests, the *ΔdacA*::luc-mutant was diluted with C-medium to an $OD_{550}$ ≈ 0,05, cultivated at 37˚C for about an hour, and then diluted to $OD_{492}$ ≈ 0,04.
2. The *ΔdacA*::luc-mutant was added onto a plate, with 300 µl of it being added to two wells, and 280 µl of each sample being added together with 20 µl of luciferin to two other wells. This creates two parallels of the *ΔdacA*::luc-mutant, which contains or lacks the presence of luciferin.
3. In addition, ComS was added to one well of each parallel.

## 4.11 - Morphological analysis

1. The wild-type and mutants were diluted with C-medium to $OD_{550}$ ≈ 0,05 and cultivated to $OD_{550}$ ≈ 0,3.
2. 400 µl of cell fixative solution was added to 1 ml of bacteria solution and placed on ice for one hour. The preparation of cell fixative solution is described in table 4.6.

3. The mixed solution was then sentrifuged at 13200 rpm for 5 minutes, the supernatant was removed, and the precipitate was resuspended with 50 µl of PBS. PBS was made as shown in table 4.7.

**Table 4.6. Preparation of Cell Fixative solution**

| |
|---|
| 2 % (v/v) formaldehyde |
| 0.2 % (v/v) glutaraldehyde |
| 1 X phosphate-buffered saline |

**Table 4.7. Preparation of PBS**

| | |
|---|---|
| NaCl | 8 g |
| KCl | 0.2 g |
| $Na_2HPO_4$ | 1.8 g |
| $KHPO_4$ | 0.3 g |
| $ddH_2O$ | 1 L |

The sample was prepared for microscopy by adding 5 µl of fixed bacteria onto an object plate and by putting a cover plate on top of that. The sample was analyzed by the use of a Differential Interference Contrast microscope at 63 times magnitude with oil added onto the sample.

### 4.11.1 - Differential Interference Contrast Microscopy

Differential Interference Contrast (DIC) Microscopy is a way to achieve contrast in an unstained specimen, by exploiting phase differences between a specimen light ray and a reference ray. The advantage of this method is that an object will appear bright against a dark background, and unlike Phase Contrast Microscopy, it does not produce a diffraction halo.

DIC is based on the principle of interferometry (Figure 4.4), in which a ray of light is split into two rays, one going through the specimen, and the other going through the background. The rays are then recombined at the image plane, and wave interference occurs. By adjusting the phase difference between the rays, the image contrast can be modified.

In DIC, the sample and reference rays are created after the ray has passed through the sample, and has become phase-distorted by it. The ray is then split into two equally phased rays, which are spatially separated by a Wollaston prism. The rays then pass through a

polarizing filter and are vibrationally recombined at the image plane. The contrast is created from the wave interference of the two rays.

[http://microscopy.berkeley.edu/Resources/instruction/DIC.html]

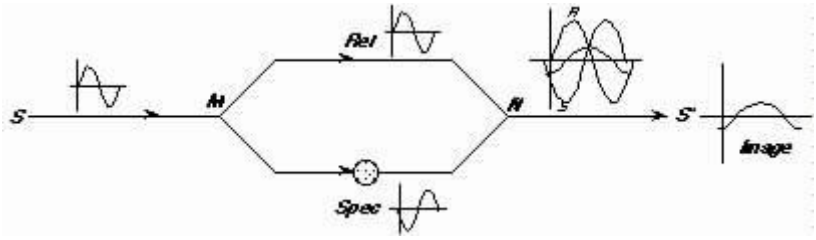



**Figure 4.4.** An illustration of the principle of an interferometer. A ray of light (S) is split into two rays at M, where one ray passes through the specimen (spec), and the other (ref) passes through the background. The rays are recombined at N, where wave interference occurs, resulting in an image ray (S').

## 4.12 - Antibiotic sensitivity analysis

In order to cultivate mutants on petri dishes and expose them to concentrated plates of antibiotics, we prepared TH agar and soft agar. Soft agar is prepared the same way as TH agar (see section 4.6), only with half the amount of TH media. The agar plates were cast the same way as in 4.6, but without antibiotics applied.

1. The wild type and mutants were diluted with C-medium to $OD_{550}$ ≈ 0,05 and cultivated to $OD_{550}$ ≈ 0,3.
2. The soft agar was heated by microwave to a liquid form, and distributed in portions of 5 ml into sterile glass tubes. The number of tubes prepared correlated to the amount of petri dishes. The tubes were incubated at 45˚C, to keep the agar in liquid form.
3. The samples that were grown to $OD_{550}$ ≈ 0,3 were then added into the tubes with soft agar, in portions of 100 µl, stirred by vortexing, and added onto the agar plates.
4. When the plates had solidified, antibiotic discs (BBL™ Sensi-Disc™) were added onto the agar plates.
5. The agar plates were incubated at 37˚C in anaerobic conditions overnight.
6. The antibiotic sensitivity of the mutants was determined by measuring the size of the zone of inhibition surrounding the antibiotic containing disc.

# 5 Results

In this study we wanted to gain further insight into the function of *dacA* in *S. pneumoniae*, and how a Δ*dacA*-mutant would respond to deletions of either *pbp1a*, *pbp1b* or *pbp2a*. Different combinations of knockout mutants of these four genes were created, and their growth rate, morphology and antibiotic resistance were examined. Through these studies we aim to learn more about how pneumococci are affected by the lack of these genes, and what kind of interplay each of the class A PBP-encoding genes have with *dacA*.

## 5.1 - Construction of the Δ*dacA*-mutant

The Janus cassette is a fragment with a size of 1359 bp [42]. The upstream and downstream sequence of the *dacA* fragments are about 1000 bp in size. After amplification of these fragments, we expect them to align on the agarose gel at the same position as the reference fragments of the same size in the DNA ladder.

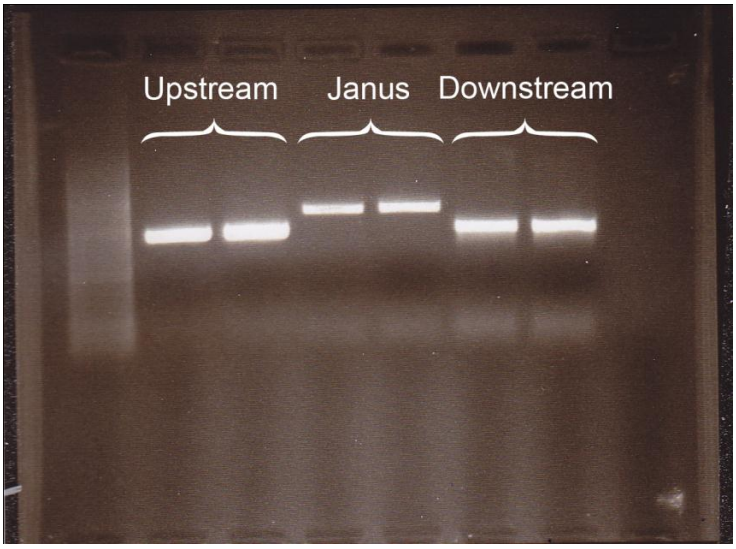



**Figure 5.1.** The agarose gel results from the amplification of the Janus cassette (the two fragments in the middle) and the upstream and downstream fragment of *dacA*. The DNA ladder is unclear on the gel, so we need to run a control PCR to verify the results.

As we can see in the agarose gel (Figure 5.1), the PCR has amplified three distinct fragments. Since the DNA ladder was unclear, we could not verify whether the fragments had the expected size, but the supposed Janus cassette fragment is slightly larger than the other fragments, which correlates with what we know of the sizes of the desired sequences. After

purification and separation in a second agarose gel containing a fresh DNA ladder it was confirmed that these DNA-fragments was of correct sizes.

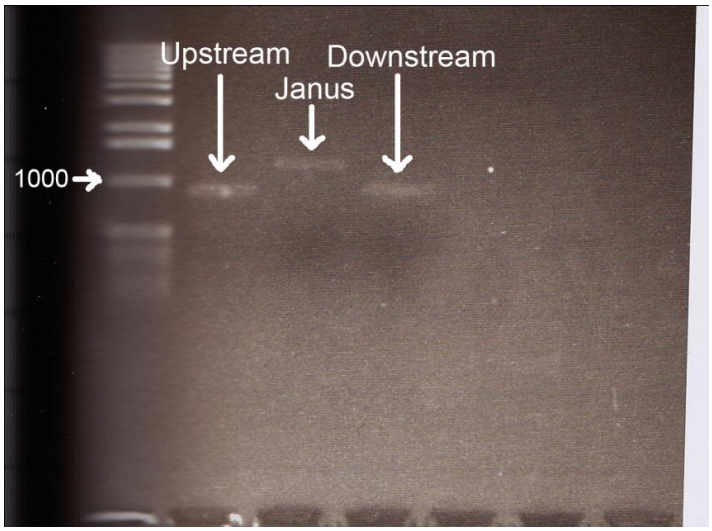



**Figure 5.2.** The control PCR of the purified PCR-product gives clear fragments of approximately right size. Both the upstream and downstream fragment have a size of about 1000 bp, and the Janus cassette is slightly larger than that.

The control PCR was visualized on an agarose gel with a clear DNA ladder, and by comparing our fragments with the ladder, we see that the fragments are approximately the size of the desired sequences (Figure 5.2).

By using overlap extension PCR we aim to produce a sequence consisting of the upstream sequence, the Janus cassette, and the downstream sequence. First, we combine the Janus cassette and either the upstream or downstream sequence. This gives us a clear fragment for the combination of the Janus cassette and the downstream sequence, but not for the Janus cassette and upstream sequence (Figure 5.3).


**Figure 5.3.** The three fragments were fused in pairs using overlap extension PCR. While the fragment composed of the Janus cassette and the downstream sequence was clear, the fragment with the Janus cassette and the upstream sequence was separated.

Another overlap extension PCR was performed to combine the downstream+Janus cassette fragment with the upstream sequence fragment. The agarose gel visualized a series of fragments, and by comparing them with the DNA ladder, we can isolate the correct fragment for purification (Figure 5.4).

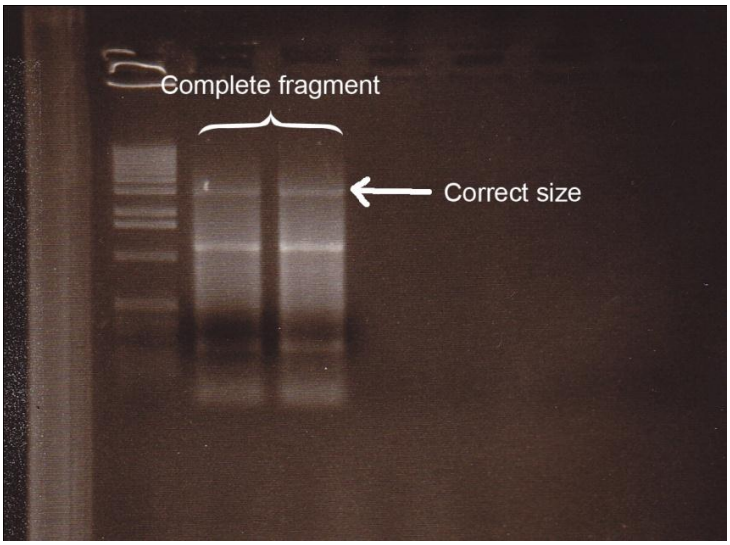



**Figure 5.4.** An overlap extension PCR using both the purified Downstream+Janus cassette fragment and the Upstream fragment has given us a complete fragment with all three sequences. The total length of the desired fragment has a size of approximately 3.300 bp, which we can see as a visible band just above the 3.000 bp band of the ladder.

The DNA-fragment corresponding to the size of upstream-Janus-donwnstream was isolated for the agarose gel. The purified fragment was used to transform *S. pneumoniae*. By using control PCR with the transformants as template, we confirmed that at least two of the isolated transformants had the correct sequence incorporated into their genome resulting in a Δ*dacA* genotype (Figure 5.5).

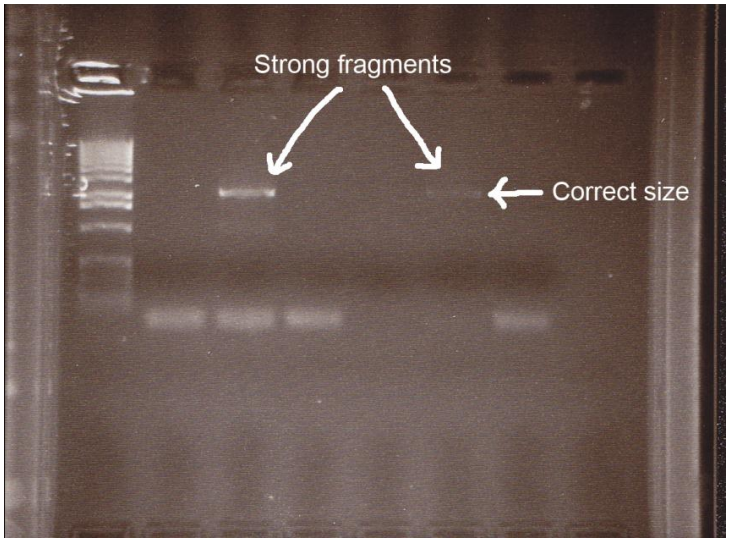



**Figure 5.5.** After transformation of RH426 with the complete fragment, we perform control PCR for the six isolated colonies. Two of them show a clearly visible fragment of about 3.000 bp in length. We keep the two transformants that give these fragments and discard the remaining isolated colonies.

The other mutants were produced and controlled using the same basic procedure as described above.

## 5.2 - Comparison of growth rates

### 5.2.1 - Growth rate measurements of *S. pneumoniae* deficient in PBP-encoding genes

The gene *dacA* encodes PBP3, which is known to cleave off the D-Ala-D-Ala from disaccharidepentapeptides, which are used as substrates for the peptidoglycan synthesis of *S. pneumoniae*. HMM PBPs depend on the energy in the D-Ala-D-Ala bond to synthesize peptidoglycan, and can therefore only use uncleaved disaccharidepentapeptides as substrate for their reactions. We know that this mechanism is hypothesized to coordinate the localization of the HMM PBPs by excluding the availability of uncleaved disaccharidepentapeptides from other places on the cell than near the future division site.

By measuring the growth rate of the mutants and their parent wild type, we aim to determine if the absence of the PBP3-encoding gene or any of the class A HMM PBP-encoding genes have an effect on the ability of the pneumococci to grow. Growth of the wild type and mutants was measured using spectrophotometry, programmed to take measurements at $OD_{550}$ every fifth minute during a period of 18 hours.

First we wanted to examine the growth of the *ΔdacA*-mutant compared to that of the wild type.

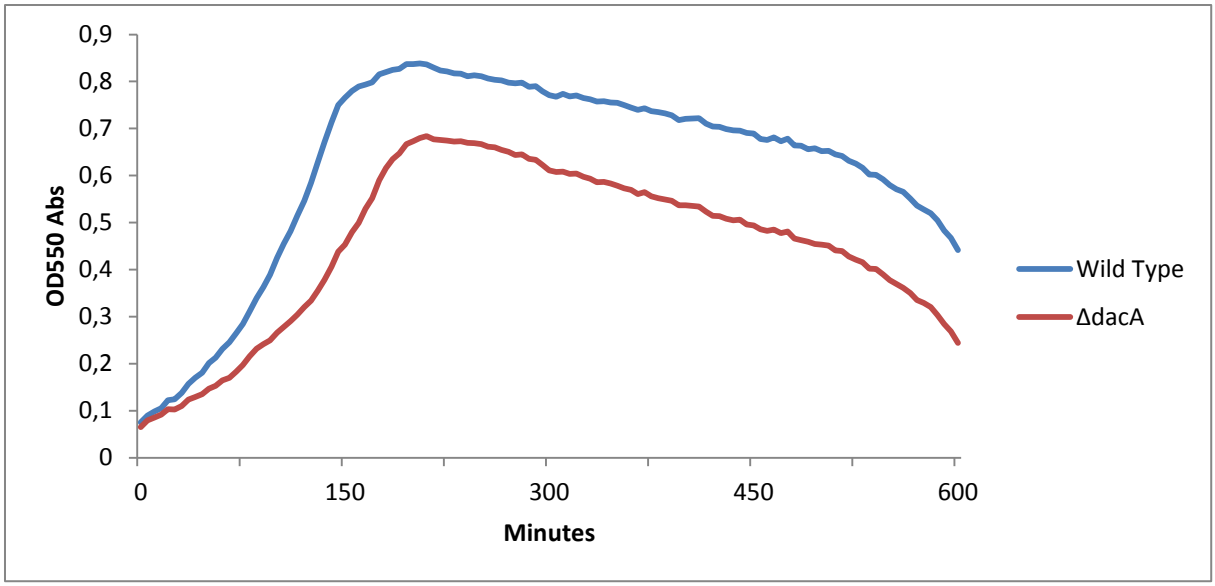



**Figure 5.6.** Growth comparison of the wild type and the Δ*dacA*-mutant. Both samples reach their growth optimum at approximately the same time, around 200 minutes into the experiment, and their cell density steadily declines following this point. The wild type has a higher growth rate and growth optimum than the Δ*dacA*-mutant.

The measurements show that both the wild type and *ΔdacA*-mutant reaches their stationary phase at around 200 minutes of growth (Figure 5.6). They both enter a death phase as the cell density starts to rapidly decline after 550 minutes of growth. During the exponential phase, the wild type displayed a faster growth rate than the *ΔdacA*-mutant, and throughout the rest of the experiment, the *ΔdacA*-mutant had a comparatively lower cell density than the wild type.

Following this experiment, we wanted to compare the growth of the wild type and *ΔdacA*-mutant with that of the three single mutants (*Δpbp2a*, *Δpbp1a*, and *Δpbp1b*), and with that of three double mutants (*ΔdacAΔpbp2a*, *ΔdacAΔpbp1a*, and *ΔdacAΔpbp1b*). Based on the results of these experiments, we will observe if there is any interplay between *dacA* and any of the class A HMM PBP-encoding genes regarding cell growth.

First we compared the growth profile of the wild type, the *ΔdacA*-mutant, and the *Δpbp2a*, *Δpbp1a*, and *Δpbp1b* mutants.

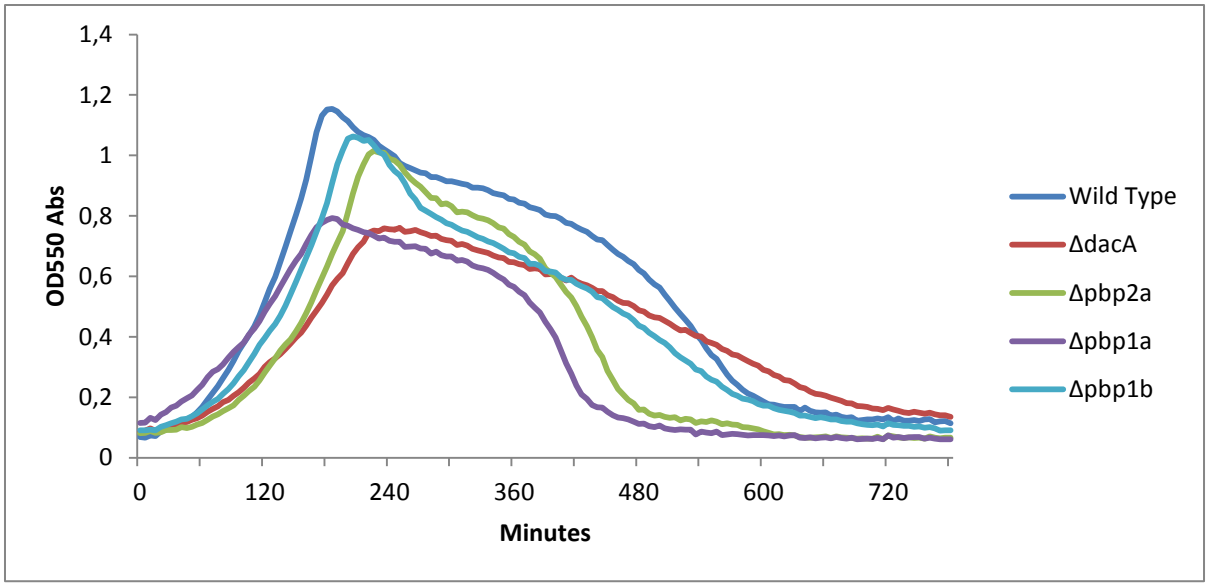



**Figure 5.7.** Comparison of the growth of the wild type and single mutants of Δ*dacA, Δpbp2a,Δpbp1a and Δpbp1b*. The wild type has a highest growth rate and growth optimum, followed by the Δ*pbp1b*-mutant, the Δ*pbp2a*-mutant, the Δ*pbp1a*-mutant, and finally the Δ*dacA*-mutant. The Δ*pbp1a*-mutant has a high cell density at the start of its growth, but is bypassed by other Δ(HMM PBP-encoding gene) mutants after about 180 minutes of growth. All of the Δ(HMM PBP-encoding gene) mutants experience a rapid decline in their cell density at some point following their growth optimum, but the Δ*dacA*-mutant has a steady decline in cell density during the rest of the experiment, following its growth optimum.

The *Δpbp1a*-mutant had a relatively high cell density at the beginning of the experiment (Figure 5.7), compared to the other strains, and this could contribute to a slight misrepresentation of how it grows compared to the other samples. We see that the cell density of the *Δpbp1a*-mutant is bypassed by that of the wild type at around 100 minutes, and by that of the other Δ(HMM PBP-encoding gene) mutants once it enters stationary phase (Figure 5.7). The *Δpbp1a*-mutant seems to have a similar growth curve to that of the *ΔdacA*-mutant, regarding the growth rate, and the maximum cell density.

The wild type and *Δpbp1a*-mutant both reach their stationary phase at around 180 minutes, while the remaining mutants reach stationary phase at between 200 and 240 minutes (Figure 5.5). The wild type, the *Δpbp2a*-mutant, and the *Δpbp1b*-mutant have a drastic decrease in cell density shortly after reaching their growth optimum, and seem to enter a stationary phase at around 260 minutes into the experiment (Figure 5.7). They enter another rapid decrease in cell density at between 400 to 480 minutes into the experiment (Figure 5.7).

Both the *Δpbp2a* and *Δpbp1a*-mutants seem to autolyse (caused by LytA) at around 400 minutes, while the wild type starts to gradually autolyse after around 500 minutes (Figure

5.7). The *ΔdacA*-mutant displayed a slow rate of autolysis from 240 minutes to 720 minutes (Figure 5.7). The *Δpbp1b*-mutant had a somewhat similar "death phase" to that of the *ΔdacA*-mutant, but experiences a rapid decline at between 200 and 260 minutes, and then transitions to a steady decline, which is slightly faster than that of the *ΔdacA*-mutant, lasting from at 260 minutes to at 600 minutes into the experiment (Figure 5.7).

Moving on to the next experiment, we compared the growth of the wild type, the *ΔdacA*-mutant, and the double mutants Δ*dacA*Δ*pbp2a*, Δ*dacA*Δ*pbp1a*, and Δ*dacA*Δ*pbp1b*.

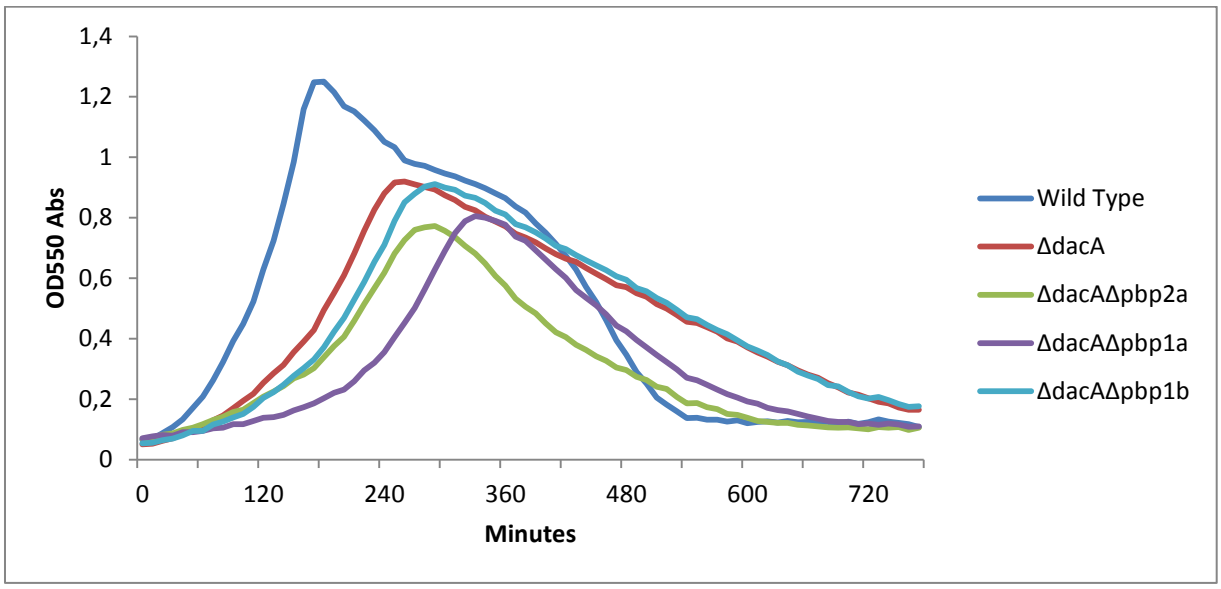



**Figure 5.8.** Growth comparison of the wild type, the Δ*dacA*-mutant*, and* double mutants of Δ*dacA*Δ*pbp2a,* Δ*dacA*Δ*pbp1a and* Δ*dacA*Δpbp1b. The wild type has a highest growth rate and growth optimum, followed by the ΔdacA-mutant, the ΔdacA*pbp1b*-mutant, the ΔdacA*pbp2a*-mutant, and finally the Δ*dacApbp1a*-mutant. Only the wild type has any periods of rapid decline in cell density during the experiment. The mutants all experience a steady decline in cell density following their growth optimum.

The wild type reached maximum cell density after around 180 minutes of growth (Figure 5.8), similar to what was observed in the previous experiment (Figure 5.7). The double mutants all have a lower growth rate than the *ΔdacA*-mutant, and they all have a slower rate of autolysis than the wild type resembling that of the mutant missing only *ΔdacA* from its genome (Figure 5.8). While all of the double mutants have a lower growth rate compared to their *dacA* positive single mutant counterparts, the *ΔdacAΔpbp2a*-mutant seems severely affected by the loss of *dacA*, as it has the lowest growth optimum of the double mutants (Figure 5.8). In comparison, the *Δpbp2a*-mutant had the second to highest growth optimum of the single mutants (Figure 5.7). By comparing the growth rate of the mutants, we see that the deletion of *pbp1b* seems to have lesser negative effect on the cell growth, while deletion

of *dacA* or *pbp1a* seem to have most negative effect on the growth. The deletion of both *dacA* and *pbp1a* (Δ*dacA*Δ*pbp1a)* produced a mutant with even more restrained cell growth (Figure 5.8).

After reaching stationary phase, all the Δ*dacA*-mutants displayed a slower rate of autolysis compared to the wild type and the single mutants lacking class A HMM PBP-encoding genes (Figure 5.7 and Figure 5.8).

### 5.2.2 - Luminescence reporter

After having observed how the lack of *ΔdacA* affected the growth of *S. pneumoniae*, we wanted to examine if the bacterium could sense a deficiency in the amount of PBP3 or its activity present at any time, and would react by activating the *dacA* promoter.

This can be achieved by replacing the native *dacA* gene with the luciferase-encoding gene *luc*. By doing this we can measure the luminescence product of the luciferase activity, which will give an indication of the activity of the *dacA* promoter. In our instrumentation a luciferase measurement that is higher than 1.000 indicates a high degree of luciferase activity, meaning that the promoter would be expressed intensely during the measurement. A Δ*dacA*::*luc* mutant was used in the following experiment.

We also wanted to regulate the availability of PBP3 during the experiment, in order to compare the luciferase expression in mutants that lack any PBP3 expression, and mutants that have PBP3 expressed at the start of the experiment. By using the ComRS gene depletion system (Berg *et al*.), we can activate transcription of the *comX* promoter by adding ComS*. By inserting a the *dacA* gene behind the *comX* promoter we could express different levels of PBP3 by varying the concentration of ComS* in the growth medium. If a low expression of PBP3 was sensed by the cells and it would lead to an induction of the *dacA* promoter we would see this as an increase in the luciferase activity.

During our first experiment with the *ΔdacA*::*luc* mutants we measured the luminescence in transformants with either 0 µM or 2 µM of ComS* added. We also added luciferin to both mutants.

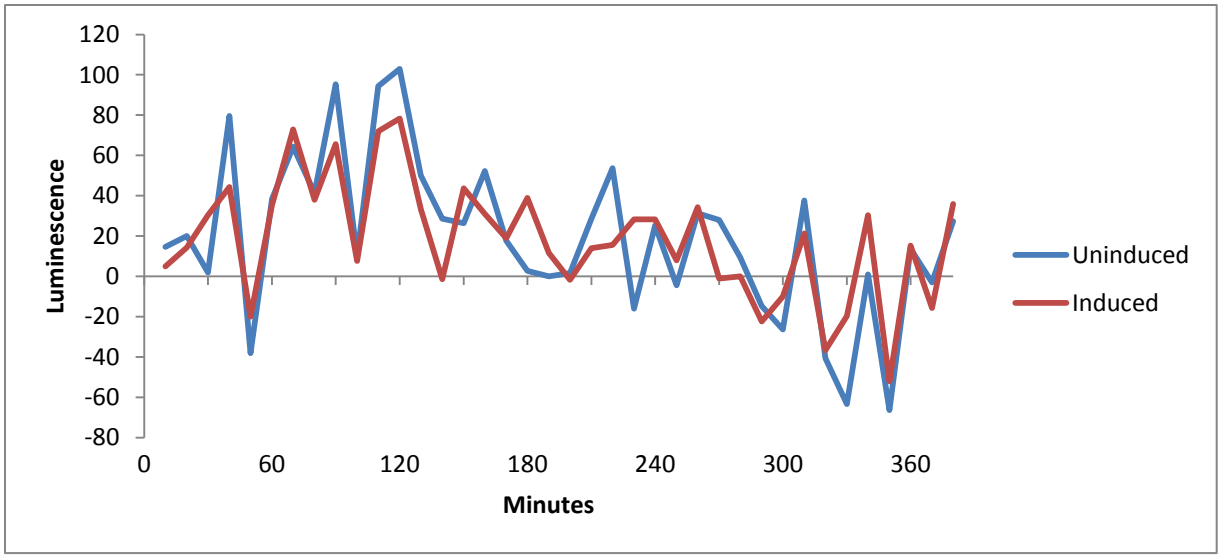



**Figure 5.9.** Comparison of luciferase expression in *ΔdacA*::*luc* mutants. One sample was induced with ComS* from the beginning of the experiment. There does not seem to be any clear difference in the luminescence of the two samples.

We did not observe a luminescence measurement higher than 120 (Figure 5.9), which indicates that the luciferase gene was expressed at a very low level during the experiment. There was no significant difference in the luminescence of the samples based on whether they had ComS* added at the beginning of the experiment or not. Although the measured luminescence varies greatly between each measurement, it seems to decline during the measurements taken after about 140 minutes.

We performed another experiment to study the effect varying expression levels of PBP3 had on the growth of *S. pneumoniae*. The Δ*dacA*::*luc* mutant was grown with increasing amounts of ComS* in the medium ranging from 0-3.2 µM. If a sufficient amount of dacA expressionwas achieved, then perhaps the mutant would reach a growth rate similar to the wild type. ComS* was added to six parallels of the mutant, in concentrations of 3.2 µM, 1.6 µM, 0.8 µM, 0.4 µM, 0.2 µM, or 0 µM.

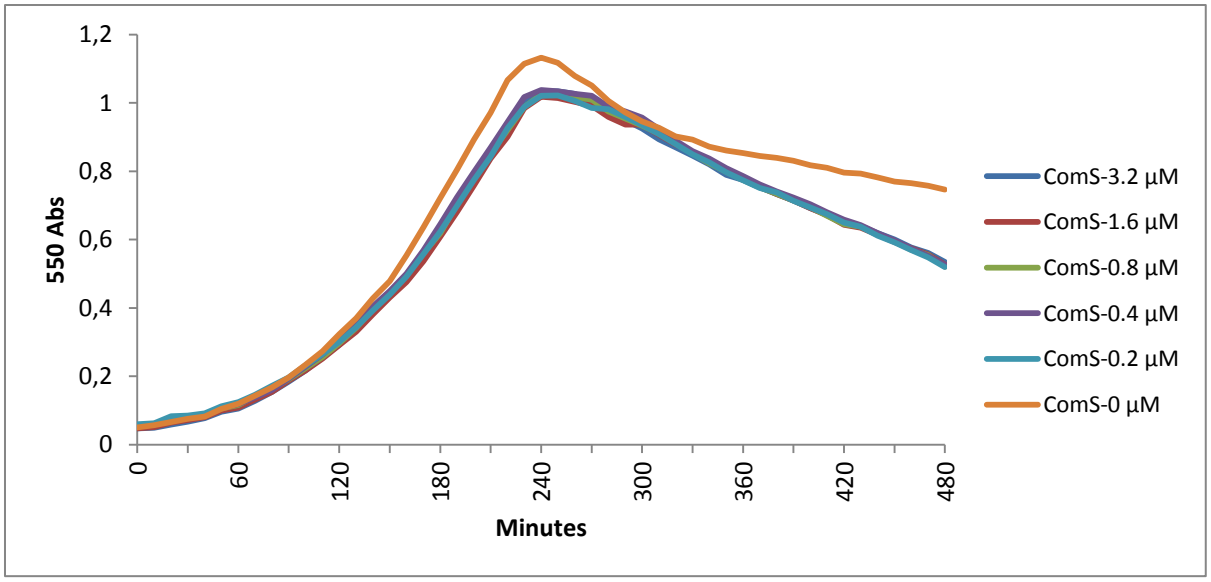



**Figure 5.10.** Comparison of the growth rate in *ΔdacA*::*luc* mutants, with different amounts of ComS added to the samples at the start of the experiment. None of the samples with ComS added had any improvement in the growth rate over the *ΔdacA*-mutant, but the mutant without ComS had a relatively higher growth rate and growth optimum. It also had a decrease in the declining rate of cell density at 300 minutes into the experiment. All samples had a growth optimum at 240 minutes of growth, and all samples with ComS added experienced a steady decline in cell density following their growth optimum.

We did not observe any significant difference in the growth of the samples that had ComS* added, but the sample without ComS* showed a slightly higher growth rate, and a slower rate of autolysis (Figure 5.10). The growth rate of the mutants with ComS* added is very similar to that of the *ΔdacA*-mutant in the double mutant experiment (Figure 5.8), with a growth optimum of approximately $OD_{550}$=1.0 at 240 minutes, and a growth measurement of approximately $OD_{550}$=0.6 at 480 minutes.

## 5.3 - Morphological analysis of *S. pneumoniae* deficient in PBP-encoding genes

The deletion of PBP-encoding genes has consequences for the peptidoglycan synthesis of pneumococci. In addition to inhibiting the cell wall synthesis, the absence of class A HMM PBPs can also affect the efficiency of cell division, as studies on the localization of PBPs have shown that PBP localization can change in order to replace the deficiency of other PBPs [29].

The localization of HMM PBPs is thought to depend on the availability of PBP3, as this enzyme is responsible for ensures higher concentration of the disaccharidepentapeptide

units which are used for peptidoglycan synthesis close to the division site. A deficiency in PBP3 could result in uncoordinated peptidoglycan synthesis, which could give the cells an indefinite morphology.

Through DIC microscopy, we aim to observe the cell shape of the Δ*dacA* mutants, and compare it to that of the wild type. We will also compared the shape of the single mutants with their corresponding double mutants.

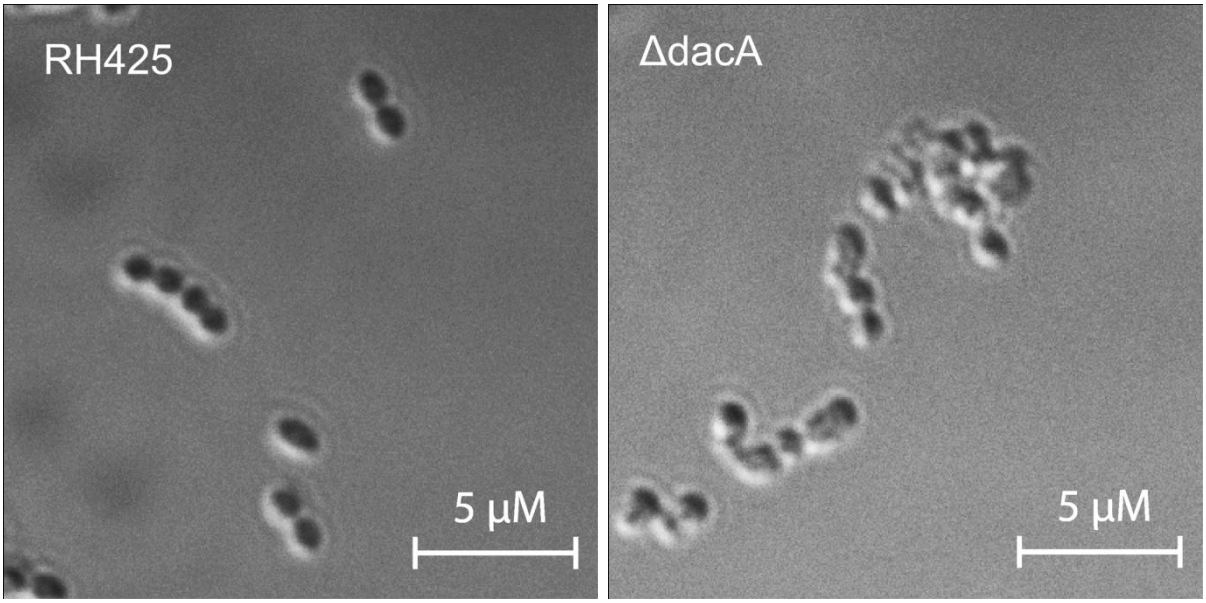



**Figure 5.11.** DIC Microscopic photographs of the wild type (left) and the *ΔdacA*-mutant (right). We can observe a more uniform cell shape in the wild type, as the cells all share the same elliptic morphology. The *ΔdacA*-mutant does not have a definite cell shape, and seems to cluster together, rather than making pairs and chain-like formations.

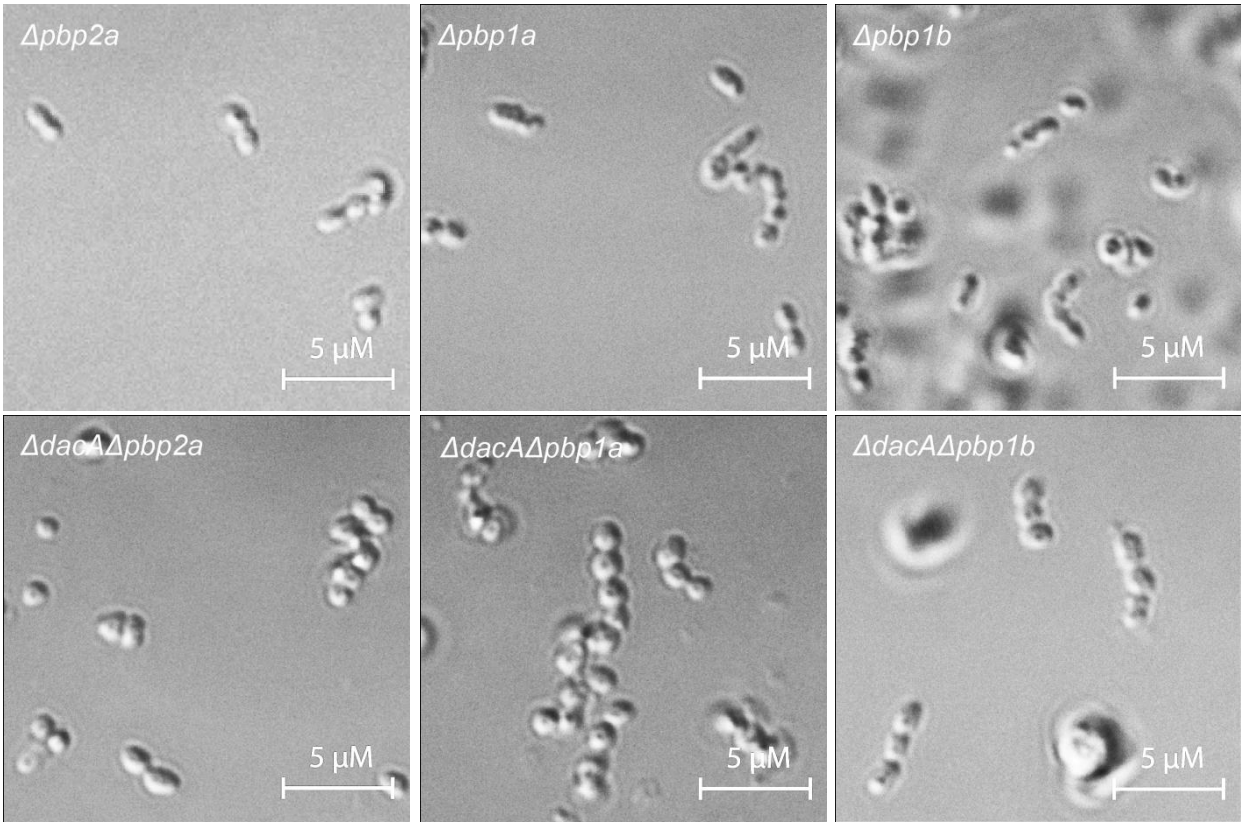



**Figure 5.12.** DIC Microscopic photographs of the *Δpbp2a*, *Δpbp1a*, *Δpbp1b*, *ΔdacAΔpbp2a*, *ΔdacAΔpbp1a*, and *ΔdacAΔpbp1b*-mutants. While the single mutants all show instances of abnormal morphology, they seem to maintain the elliptic shape and an uniform size of the cells to some degree. The *Δpbp1a*-mutant seems to have the least uniform size and shape of the three mutants. Regarding the double mutants, they all seem to share the indefinite morphology and size of the *ΔdacA*-mutant.

The results of the morphology studies (Figure 5.11 and Figure 5.12) show us that mutants deficient in *dacA* have indefinite cell morphology and size. Among the single mutants lacking class A HMM PBP-encoding genes, the *Δpbp1a*-mutant displayed the most abnormal cell shape and indefinite size. We cannot tell if there is a difference in the morphology of the *ΔdacA*-mutant or any of the double mutants, as they all seem to have abnormal cell shape and size.

### 5.3.1 - Sensitivity to exogenous LytA

It is well known that damages to the cell wall of *S. pneumoniae* render the bacterium sensitive to the important virulence factor LytA, while undamaged cell wall is resistant to LytA. Since our Δ(PBP-encoding gene) mutants displayed restrained cell growth we wanted to test whether any of our mutants had acquired LytA sensitivity.

First, we wanted to compare the growth rate of the wild type and Δ*dacA* mutant with and without LytA added to a concentration of 5 µg/ml from the beginning of the experiment. We also wanted to add LytA (5 µg/ml) to a third parallel of samples once they had reach an $OD_{550}$ of 0.3 (Figure 5.13).

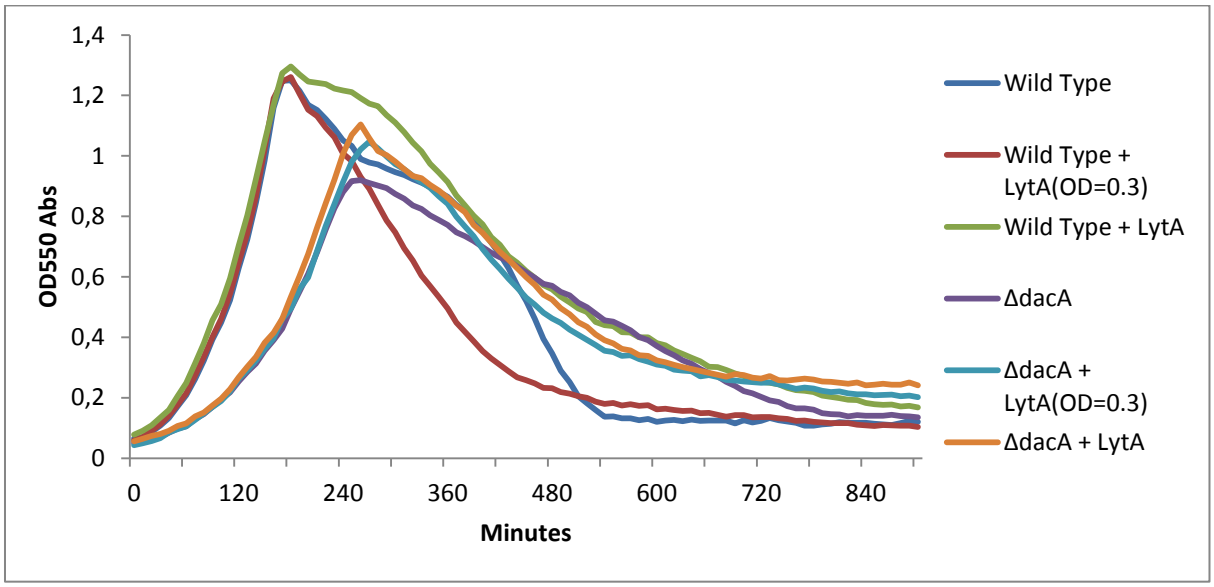



**Figure 5.13.** Comparison of the growth of the wild type and the *ΔdacA*-mutant. One parallel of each sample had LytA added to a final concentration of 5 µg/ml at the beginning of the experiment, while two parallel cultures had LytA added to the same concentration when their growth reached an $OD_{550}$ of 0.3.The *ΔdacA* mutant was not significantly affected by the addition of exogenous LytA, demonstrating that the lack of PBP3 did not make the pneumococci susceptible to LytA.

Addition of LytA (5 µg/ml) both from the start of the experiment or at $OD_{550}$=0.3 did not seem to have any lytic effect on the *ΔdacA* mutant during its growth phase. Application of LytA at $OD_{550}$ = 0.3 created a more drastic cell lysis in the wild type after entering stationary phase, and in the Δ*dacA* mutant it had more or less the same effect as if applied from the start.

The sensitivity to LytA was also tested in a growth experiment including the single mutants.

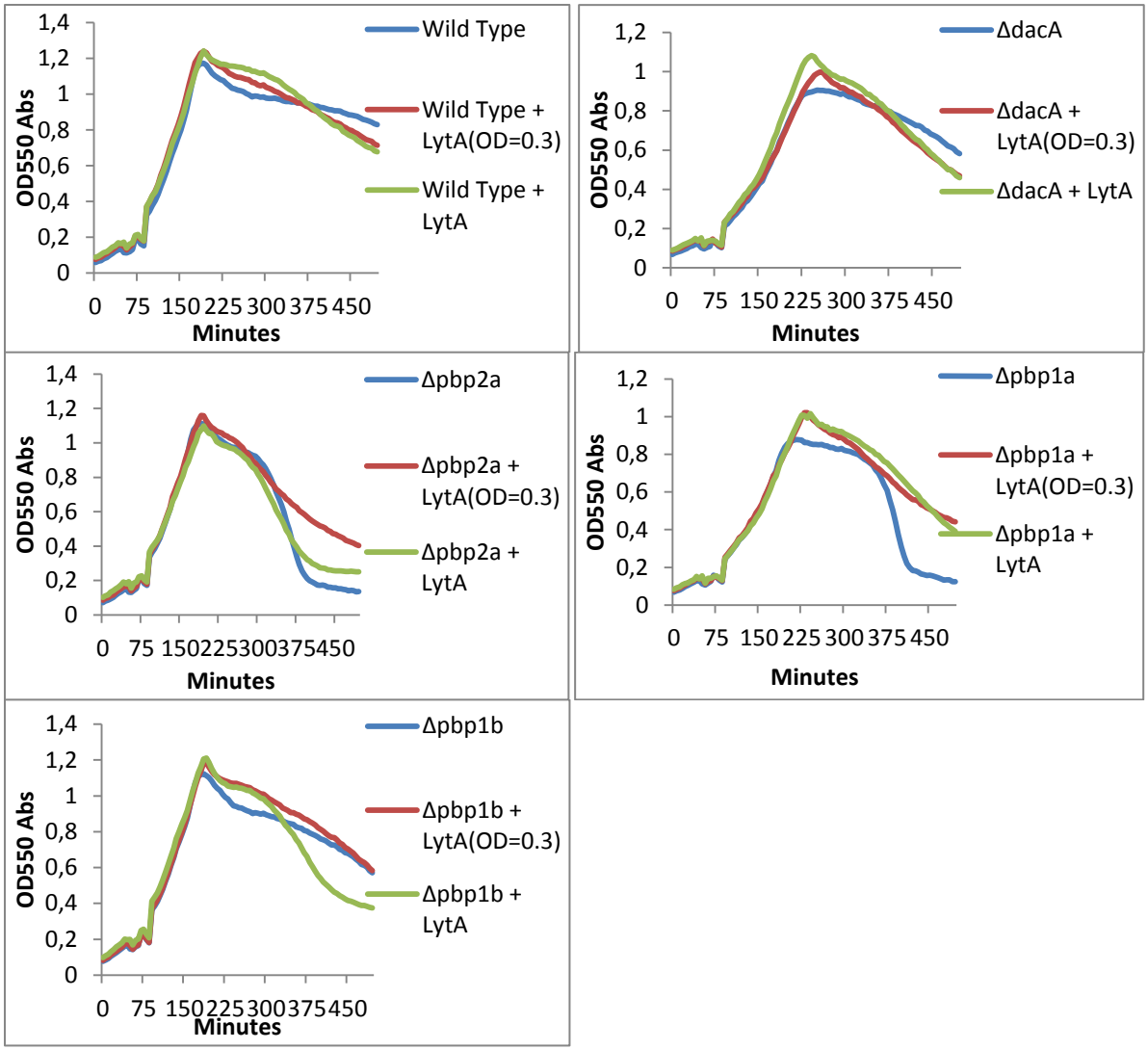



**Figure 5.14.** Comparison of the growth of the wild type, the Δ*dacA* mutant, and all three of the single mutants. One parallel of each sample had LytA added to a concentration of 5 µg/ml at the beginning of the experiment, while another parallel had LytA added to the same concentration when its samples reached an $OD_{550}$ of 0.3. Adding LytA seems to increase the maxiumu cell density of each sample, as well as lower the rate of autolysis during stationary phase. The LytA had a stronger influence autolysis of the Δ*pbp2a* mutant when it was added during the exponential growth ($OD_{550}$=0.3).

The results show that none of the mutants were sensitive to exogenous LytA during exponential growth. Adding LytA to the single mutants did not seem to affect their growth rate, but it did decrease the rate of the cell death for the *Δpbp2a*-mutant and the *Δpbp1a*-mutant. The *Δpbp2a*-mutant had a less declining death phase when LytA had been added $OD_{550}$=0.3, rather than from the beginning of the experiment. The opposite of this is observed in all the other samples (Figure 5.14).

We also wanted to compare the double mutants in an experiment, using the same method.

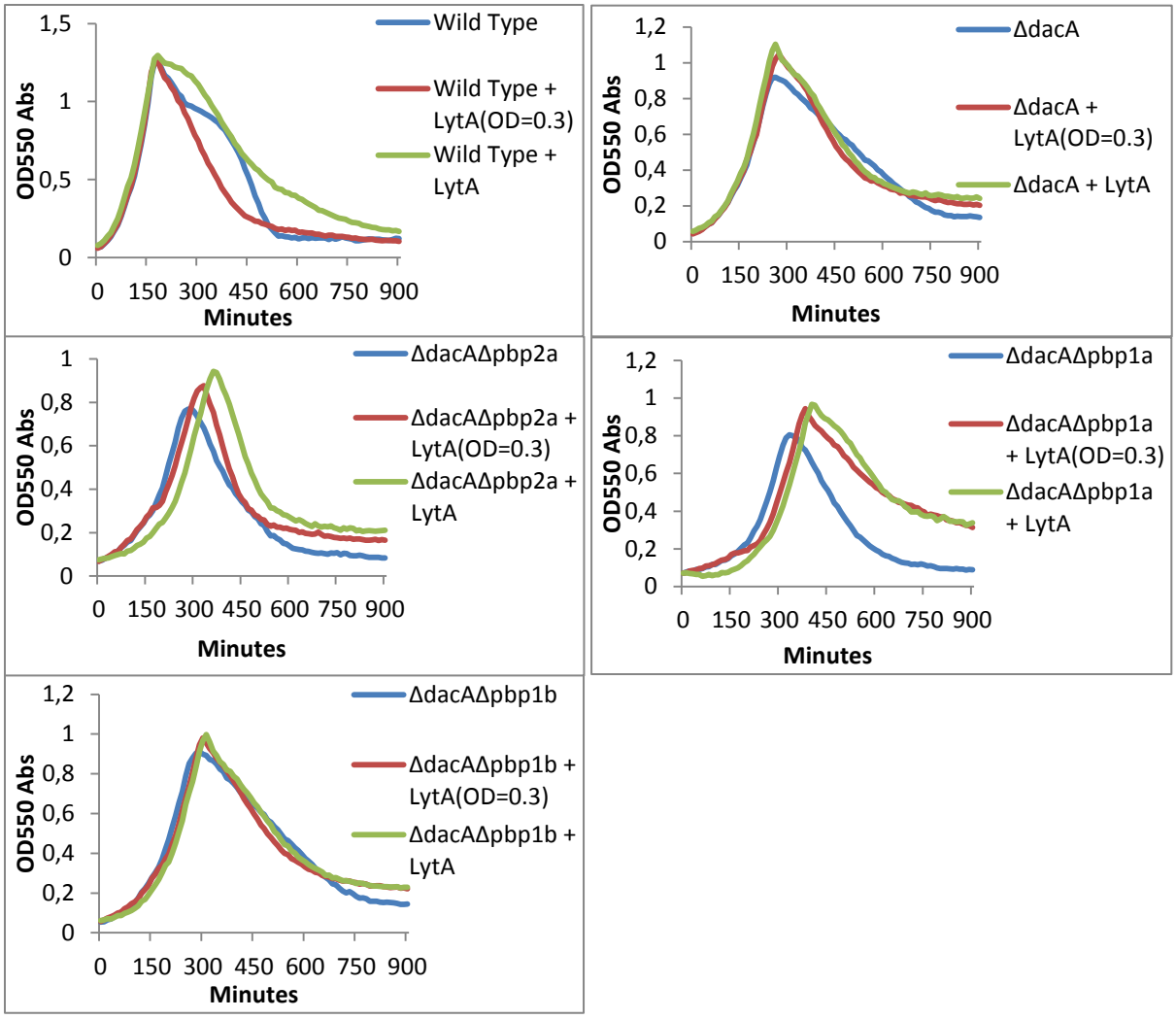



**Figure 5.15.** Comparison of the growth of the wild type, the *ΔdacA*-mutant, and all three of the double mutants. One parallel of each sample had LytA added to a concentration of 5 µg/ml at the beginning of the experiment, while another parallel had LytA added to the same concentration when its samples reached an $OD_{550}$ of 0.3. Adding LytA seems to decrease the growth optimum of the double mutants, but it also increases their growth optimum slightly. Their death rate seems unaffected.

The earlier LytA was added to the *ΔdacAΔpbp2a*-mutant and the *ΔdacAΔpbp1a*-mutant, the more their growth rate seems to decrease, and the higher their growth optimum became. The autolysis of each sample seems unaffected by the addition of LytA. The *ΔdacAΔpbp1b*-mutant seems unaffected by when LytA was added, but the addition seemed to lower the growth rate and increase the growth optimum (Figure 5.15).

Through these experiments, we have observed that pneumococci that do not express PBP1a, PBP2a, PBP1b or PBP3, still have resistance to LytA-mediated autolysis during exponential growth. Pneumococci that lack the ability to express both PBP3 and a class A HMM PBP,

show sensitivity to exogenous LytA, as these mutants had a decreased growth rate when exposed to extracellular LytA.

## 5.4 - Antibiotic sensitivity results of *S. pneumoniae* deficient in PBP-encoding genes

Since β-lactam antibiotics specifically target and inhibit the function of PBPs, we wanted to test if mutants lacking the genes that express PBPs have a change in the susceptibility to this kind of antibiotics. We also wanted to test the mutant susceptibility to antibiotics that inhibit peptidoglycan synthesis without targeting PBPs, and antibiotics that target other cell functions not directly related to peptidoglycan synthesis.

The antibiotics employed in the sensitivity test experiment includes Ceftazidime and Penicillin, which are both β-lactam antibiotics, Bacitricin and Vancomycin, which are related to peptidoglycan synthesis, but not PBPs directly, and Erythromycin, Novobiocin and Tetracycline, which are not directly related to peptidoglycan synthesis.

The sensitivity of the mutants to each antibioticum was tested by cultivating the mutants anaerobically on TH-agar with antibiotic diffusion discs added. The mutants will not cultivate close to antibiotics that they are susceptible to, and the greater the susceptibility, the further away from the antibiotics they can grow. This creates a zone of inhibition, which gives an indication on how sensitive the mutant is to the antibioticum in the disc.

The results from the antibiotic sensitivity testing are displayed in table 5.1-5.3. Each table shows the zone of inhibition in mm (left) and the percentage-based increase of susceptibility in mutants from that of the wild type (right).

**Table 5.1. Test results of the wild type and its mutant's sensitivity to β-lactam antibiotics.**

| | Ceftazidime | Penicillin | | Ceftazidime | Penicillin |
|---|---|---|---|---|---|
| Wild Type | 9,7 | 15,0 | Wild Type | 0 % | 0 % |
| *ΔdacA* | 14,0 | 16,5 | *ΔdacA* | 45 % | 10 % |
| *Δpbp2a* | 10,0 | 18,0 | *Δpbp2a* | 3 % | 20 % |
| *Δpbp1a* | 11,7 | 16,7 | *Δpbp1a* | 21 % | 11 % |
| *Δpbp1b* | 9,3 | 15,7 | *Δpbp1b* | -3 % | 4 % |
| *ΔdacAΔpbp2a* | 16,0 | 19,0 | *ΔdacAΔpbp2a* | 66 % | 27 % |
| *ΔdacAΔpbp1a* | 15,0 | 17,5 | *ΔdacAΔpbp1a* | 55 % | 17 % |
| *ΔdacAΔpbp1b* | 14,0 | 19,3 | *ΔdacAΔpbp1b* | 45 % | 29 % |

The *ΔdacA*-mutant displayed a high susceptibility to ceftazidime, but does not deviate significantly from the other single mutants regarding penicillin sensitivity. While the *Δpbp2a*-mutant is fairly resistant to ceftazidime, the *ΔdacAΔpbp2a*-mutant has the highest ceftazidime sensitivity. Similarly, the *Δpbp1b*-mutant has the lowest sensitivity to penicillin, while the *ΔdacAΔpbp1b*-mutant has the highest penicillin sensitivity (Table 5.1).

**Table 5.2. Test results of the wild type and its mutant's sensitivity to antibiotics related to peptidoglycan synthesis, but not directly to PBPs.**

| | Bacitracin | Vancomycin | | Bacitracin | Vancomycin |
|---|---|---|---|---|---|
| Wild Type | 6,0 | 6,0 | Wild Type | 0 % | 0 % |
| *ΔdacA* | 10,0 | 8,3 | *ΔdacA* | 67 % | 39 % |
| *Δpbp2a* | 7,0 | 6,3 | *Δpbp2a* | 17 % | 6 % |
| *Δpbp1a* | 7,5 | 6,7 | *Δpbp1a* | 25 % | 11 % |
| *Δpbp1b* | 7,0 | 6,7 | *Δpbp1b* | 17 % | 11 % |
| *ΔdacAΔpbp2a* | 12,0 | 8,7 | *ΔdacAΔpbp2a* | 100 % | 44 % |
| *ΔdacAΔpbp1a* | 10,7 | 8,3 | *ΔdacAΔpbp1a* | 78 % | 39 % |
| *ΔdacAΔpbp1b* | 9,5 | 6,3 | *ΔdacAΔpbp1b* | 58 % | 6 % |

The *ΔdacA*-mutant displays a high susceptibility to both bacitracin and vancomycin. The *ΔdacAΔpbp2a*-mutant displays the highest susceptibility to both antibiotics, while the *Δpbp2a*-mutant has the lowest of all the mutants. The *ΔdacAΔpbp1b*-mutant has a relatively low sensitivity compared to its parent strain, *ΔdacA*, as well as the single mutant *Δpbp1b* (Table 5.2).

**Table 5.3. Test results of the wild type and its mutant's sensitivity to other antibiotics.**

| | Erythromycin | Novobiocin | Tetracycline | | Erythromycin | Novobiocin | Tetracycline |
|---|---|---|---|---|---|---|---|
| Wild Type | 3,0 | 6,7 | 10,0 | Wild Type | 0 % | 0 % | 0 % |
| *ΔdacA* | 10,5 | 10,0 | 13,0 | *ΔdacA* | 250 % | 50 % | 30 % |
| *Δpbp2a* | 3,0 | 8,0 | 10,0 | *Δpbp2a* | 0 % | 20 % | 0 % |
| *Δpbp1a* | 2,3 | 8,7 | 10,3 | *Δpbp1a* | -22 % | 30 % | 3 % |
| *Δpbp1b* | 3,0 | 8,3 | 10,3 | *Δpbp1b* | 0 % | 25 % | 3 % |
| *ΔdacAΔpbp2a* | 13,0 | 10,5 | 16,0 | *ΔdacAΔpbp2a* | 333 % | 58 % | 60 % |
| *ΔdacAΔpbp1a* | 10,0 | 10,7 | 13,7 | *ΔdacAΔpbp1a* | 233 % | 60 % | 37 % |
| *ΔdacAΔpbp1b* | 9,5 | 8,7 | 12,7 | *ΔdacAΔpbp1b* | 217 % | 30 % | 27 % |

The *ΔdacA*-mutant has a high susceptibility to erythromycin, novobiocin and tetracycline, particularly erythromycin. The *ΔdacAΔpbp2a*-mutant displays a high susceptibility to all three of the antibiotics, while the *Δpbp2a*-mutant does not have any notable sensitivity to them. Surprisingly, the *ΔdacAΔpbp1b*-mutant had a lower susceptibility to novobiocin and vancomycin than its parent strain, the *ΔdacA*-mutant (Table 5.3).

# 6 Discussion

## Main summary

The objective of this study was to analyze the survivability of *S. pneumoniae* mutant that lack the *dacA* gene, and compare it with the parent wild type and mutants that lack a class A HMM PBP-encoding gene. We also wanted to analyze mutants that lack both *dacA* and a class A HMM PBP-encoding gene, to observe if there is any interplay between the gene-products.

The results of the study have shown that *dacA* is essential for the growth, the cell morphology, and the antibiotic resistance of *S. pneumoniae*. With the exception of the penicillin resistance test, the lack of *dacA* proved more critical to *S. pneumoniae* in every test, than the lack of any class A HMM PBP-encoding gene.

A possible interpretation of the necessity of *dacA* for normal survivability, compared to the class A HMM PBP-encoding genes, could be that PBP3 has a very specialized function in the peptidoglycan synthesis, which is to coordinate the localization of the disaccharidepentapeptides that are used as peptidoglycan substrate. While this function is not essential for the survivability, as the deletion of *dacA* has not proven to be lethal, it is still important for the efficiency of the peptidoglycan synthesis, as *ΔdacA*-mutants display a severely inhibited growth rate.

While the class A HMM PBPs are fundamental for the peptidoglycan synthesis, and each of them has specialized localization and role in the process, we know from other studies [31] that class A HMM PBPs can change their localization in order to make up for each other's absence. Thus, a class A HMM PBP-encoding gene deletion is not necessarily a devastating loss to *S. pneumoniae*, as the *dacA* deletion has proven to be.

The LytA experiments showed that the absence of PBP3 did not trigger LytA sensitivity during the exponential growth of *S. pneumoniae*. The same results were obtained for *S. pneumoniae* lacking PBP1a, PBP1b or PBP2a. Since *S. pneumoniae* is protected from the activity of LytA during exponential growth, unless its growth is inhibited by nutrient

depletion, or by specific arrest of the cell wall synthesis, we can hypothesize that an absence of more PBPs is required to trigger LytA sensitivity.

The Δ*dacA* mutants have problems carrying out their cell wall synthesis, and are therefore more susceptible to cell lysis caused by LytA during their exponential phase. This would explain why addition of LytA causes them to have a decreased growth rate when they also lack a class A HMM PBP-encoding gene.

The *ΔdacA*-mutants have problems carrying out their cell wall synthesis, and are therefore more susceptible to cell lysis caused by LytA during their exponential phase. This would explain why addition of LytA causes them to have a decreased growth rate when they also lack a class A HMM PBP-encoding gene.

In the test where the activity of the *dacA* promoter was measured through a luminescence reporter, we observed no significant difference in the measured luminescence of the strains that were added with ComS* and those who were not. This would indicate that *dacA* is expressed at a normal rate even in the absence of PBP3, and that *S. pneumoniae* is unable to sense the absence of PBP3.

On the other hand, it is possible that the *comX* promoter that we inserted *dacA* gene behind "leaked" some expression of *dacA*, which gave *S. pneumoniae* a sufficient amount of PBP3 to carry out peptidoglycan synthesis as normal. If so, induction of ectopic expression of *dacA* from $P_{comX}$ using ComS* would not affect the activity of $P_{dacA}$ since the cells already have sufficient levels of PBP3. Another possibility is that the *luc* gene we inserted had a mutation which prevented it from expressing a functional luciferase gene. Sequencing the $P_{dacA}$-*luc* region of the mutant would help us to determine if the mutant had a functional *luc* gene.

Induction of the *comX* promoter with the addition of ComS* proved to decrease the growth rate of the Δ*dacA*::*luc* mutant. It is possible that an over-expression of PBP3 could be damaging for the cell, and that the *comX* promoter "leaks" expression on its own, which is sufficient to maintain the cell without wasting resources. In such a scenario, the induction of the *comX* promoter would be unnecessary, and the addition of ComS* only serves to inhibit the growth of the cell.

The morphological analysis shows that Δ*dacA*-mutants have a far less uniform cell shape and size compared to *dacA* positive pneumococci. It is hypothesized that the function of PBP3 is to coordinate the localization of peptidoglycan synthesis substrate [30], and thus indirectly coordinate the localization of HMM PBPs to the equatorial rings and future division site. By doing this, it ensures a focused cell growth process, with the goal of dividing into viable cells as efficiently as possible. Since mutants lacking *dacA* has displayed abnormal cell morphology, it stands to reason that the peptidoglycan synthesis in these mutants has no defined localization on the cells, which leads to the formation of new peptidoglycan at various places of the cell, while wasting resources and postponing the cell division, or performing division that produces non-viable cells. Our results support the hypothesis described above, however further evidence is required to prove that this is the case. Nevertheless, we show that PBP3 is very important for normal cell morphology in *S. pneumoniae*.

Antibiotic sensitivity testing show that *ΔdacA*-mutants does not have a greater susceptibility over the Δ(class A HMM PBP-encoding gene) mutants to penicillin. However, it is far more vulnerable to the other β-lactam antibiotic, ceftazidime, as well as the five other antibiotics tested for, including bacitracin, vancomycin, erythromycin, novobiocin and tetracycline. We also observed some possible interplay between *dacA* and *pbp2a* as the *Δpbp2a*-mutants were the least susceptible mutant to nearly all of the antibiotics tested for, while the *ΔdacAΔpbp2a*-mutant was the most susceptible mutant to nearly all of the antibiotics tested for.

The increase in the antibiotic susceptibility could reflect the mutants inhibited ability to grow, but it could also be that the lack of PBP3-mediated PBP coordination could make the PBPs more exposed to the β-lactam antibiotics.

## 6.1 - Growth rate and LytA sensitivity of *S. pneumoniae* deficient in PBP3 and class A HMM PBPs

The results of the growth experiments (Figure 5.7) show that the decreased growth rate of *ΔdacA*-mutants exceeded the loss of growth in any of the Δ(class A HMM PBP)-encoding

gene mutants. The coordination of peptidoglycan substrate seems vital for an efficient cell wall synthesis and effective cell growth. While the class A HMM PBPs have the ability to relocalize in order to make up for the deficiency of each other, the function of PBP3 is likely irreplaceable, and it is therefore probable that the cell has no way to effectively coordinate the peptidoglycan synthesis without it, leading to a severely inhibited growth rate.

The lack of any rapid decline in the cell density of the *ΔdacA*-mutant, and to some degree the *Δpbp1b*-mutant (following the spontaneous instance of lysis shortly after the stationary phase, 260 minutes into the experiment), indicates that these mutants are more resistant to autolysis than the wild type. The *Δpbp2a*-mutant and *Δpbp1a*-mutant experiences autolysis earlier than the wild type, which could indicate that these mutants are more sensitive to autolysis, compared to the wild type. Since we know that the lack of PBP3 disturbs the cell morphology of *S. pneumoniae*, and creates a cluster-linkage between the cells, as we have observed in our morphological analysis (Figure 5.11), a possible assumption could be that LytA has problems interacting with the peptidoglycan layer of pneumococcal cells when they are in this condition. However, this does not explain why *Δpbp1b*-mutants, which had a fairly normal morphology, did not experience autolysis.

The double mutants all display a similar growth pattern to that of the *ΔdacA*-mutant, with a slower growth than the wild type and a gradual autolysis in the stationary phase. The *ΔdacAΔpbp2a*-mutant shows that there could be some interplay between *dacA* and *pbp2a*, as the *Δpbp2a*-mutant displays a greater decrease in its growth rate when *dacA* is also deleted, compared to that of the other class A HMM PBP-encoding genes.

LytA is a cell wall hydrolase, which in addition to causing cell lysis in pneumococci, also aid their virulence. However, LytA can only lyse pneumoccocal cells that have either reached their stationary phase of growth, or have their growth inhibited during their exponential phase [12].

We wanted to test the mutants for their susceptibility to extracellular LytA. The results from our experiments (Figure 5.13, Figure 5.14 and Figure 5.15) show that adding LytA at either the beginning of the experiment, or during the exponential growth phase, did not mediate lysis of *S. pneumoniae*, regardless of its lack of *dacA*, *pbp2a*, *pbp1a* or *pbp1b*. While it did inhibit the growth rate of mutants lacking both *dacA* and a class A HMM PBP-encoding gene

somewhat, we can assume that *S. pneumoniae* is not sensitive to autolysis mediated by extracellular LytA during exponential growth, regardless of its deficiency in PBP3.

## 6.2 - Morphology of *S. pneumoniae* deficient in PBP3 and class A HMM PBPs

Analysis of the cell morphology of *ΔdacA*-mutants (Figure 5.11) showed irregularity in the shape and size of the cells. Although there were some degree of variation in the shape of the Δ(class A HMM PBP-encoding gene) mutants, their size was fairly consistent. A possible interpretation of this is that since the *ΔdacA*-mutants are unable to focus peptidoglycan synthesis to occur within the equatorial rings, the variation in how the cells grow and are divided is higher than in the wild type and Δ(class A HMM PBP-encoding gene) mutants.

There was a notable increase in the cell linkage in all of the mutants, which could be caused by the slowdown in peptidoglycan synthesis, as the case was with the Δ*lytB*-mutants in another experiment [33]. Since the function of PBP1a is to process the cell division, and we observe a high degree of cell linkage in the *Δpbp1a* and *ΔdacAΔpbp1a*-mutants (Figure 5.12), it is probable that the specific inhibition of cell division could be the main cause of increased cell linkage. Conversely, the function of PBP2a is synthesis of the cell wall, and we see little cell linkage in the *Δpbp2a* and *ΔdacAΔpbp2a*-mutant, compared to the other mutants (Figure 5.12).

In all the *ΔdacA*-mutants, there was also some degree of cells clustering together (Figure 5.11 and Figure 5.12), rather than making chain like formations, like the cells of the wild type and Δ(class A HMM PBP-encoding gene) mutants. Since coordination of the localization of HMM PBPs is important for the cell wall synthesis and cell division to occur normally, we could hypothesize that the cell division of *ΔdacA*-mutants occurs at irregular sites, rather than just between the equatorial rings. On the other hand, we also need to consider the effect of other cell division factors, like the FtsZ ring, before we can draw a conclusion on the cause of the cell clustering.

In general, we can assume that the removal of any PBP-encoding gene leads to a disturbance in the peptidoglycan synthesis procedure, resulting in abnormal cell morphology. The *Δpbp1b*-mutants seem to have the least disturbed of the mutants in terms of morphology,

size and linkage (Figure 5.12), which is appropriate, considering that they had the least inhibited growth rate of the mutants (Figure 5.7 and Figure 5.8). We know little about the function of PBP1b, other than that it seems to have either equatorial or septal localization [29], but we can assume that it is less necessary for the survivability of *S. pneumoniae* than PBP3 and the other class A HMM PBPs. As studies performed by Morlot *et al*. (2003) has shown us, PBP1b can function as a "backup" for peptidoglycan synthesis at either equatorial or septal localization, in the absence of PBP2a or PBP1a, respectively [31].

## 6.3 - Antibiotic sensitivity in *S. pneumoniae* deficient in *dacA* and class A HMM PBP-encoding genes

We used two types of β-lactam antibiotic, penicillin and ceftazidime, as well as two antibiotics related to peptidoglycan synthesis, bacitracin and vancomycin, and three other antibiotics related to protein synthesis, erythromycin, novobiocin and tetracycline.

### 6.3.1 - β-lactam antibiotics

β-lactam antibiotics target bacterial PBPs, and inhibit the peptidoglycan synthesis[43]. Ceftazidime and Penicillin are both β-lactam antibiotics.

The results in table 5.1 shows that the penicillin sensitivity in the wild type is relatively unaffected by the deletion of PBP-encoding genes. We see a small increase in sensitivity for the *Δpbp2a*- and *ΔdacAΔpbp2a*-mutants, and the *ΔdacAΔpbp1b*-mutants are significantly more vulnerable to penicillin than either the *ΔdacA*- or the *Δpbp1b*-mutants, suggesting that the two genes may be important to penicillin resistance, but that they perform redundant functions in this area.

In the case of ceftazidime, deletion of *pbp2a* or *pbp1b* seem to have little influence on the sensitivity to this antibiotic, but the large increase in the zone of inhibition shown in *ΔdacAΔpbp2a*-mutants indicates that there could be a case of interplay between PBP3 and PBP2a, in regards to how they affect the pneumococcal resistance to ceftazidime. If, for instance, PBP3 performs a similar function as PBP2a in the resistance against this antibioticum, then the loss of PBP2a is not critical to the pneumococcal sensitivity to

ceftazidime, but the deficiency in both PBP2a and PBP3 is. There could therefore be a possible redundancy in the effect PBP2a and PBP3 have in connection with ceftazidime sensitivity. Nonetheless, all the *ΔdacA*-mutants have shown a significant effect on ceftazidime resistance by themselves.

### 6.3.2 - Antibiotics related to peptidoglycan synthesis

Bacitracin and vancomycin both inhibit the peptidoglycan synthesis process, by preventing the recycling of undecaprenyl pyrophosphate[44], and by complexing with the D-alanyl-D-alanine moieties [45], respectively.

The bacitracin sensitivity increase in *ΔdacA*-mutants looks strikingly similar to the results from the ceftazidime sensitivity tests. It could be postulated that both the effect of PBPs, and the recycling of undecaprenyl pyrophosphate, have a similarly important effect on the sensitivity to these antibiotics, and that the difference in zone of inhibition is merely a result of how healthy the cells are due to deficiency in PBP-encoding genes.

All of the *ΔdacA*-mutants were more sensitive to vancomycin than the wild type and the mutants, *Δpbp2a*, *Δpbp1a* and *Δpbp1b*. The only exception was the *ΔdacAΔpbp1b*-mutant, which had a lower sensitivity than its parent strain. Although the parallel results for the *ΔdacApbp1b*-mutants were fairly identical, the parallel results for the Δ*dac*-mutants were not, and one result of the former showed a zone of inhibition of 6 mm. The variance could be a result of suboptimal cultivation conditions, a poor preparation of the agar plate, or simply a misreading of the results.

Nevertheless, it cannot be ignored that both the *Δpbp1b*- and *ΔdacAΔpbp1b*-mutants had a relatively low sensitivity to vancomycin. Since the absence of PBP1b leads to its function being replaced by the other two class HMM PBPs, this could indicate that PBP1b is comparatively more sensitive to the effects of vancomycin than PBP2a and PBP1a. Another possible interpretation could be that the relatively low vancomycin sensitivity of the *ΔdacAΔpbp1b*-mutant reflects the low decrease of its growth rate compared to that of the parent Δ*dac*-mutant.

### 6.3.3 - Antibiotics related to protein synthesis

Erythromycin binds the entrance of the exit tunnel for nascent peptides, at the large ribosomal subunit, thus blocking the synthesis of peptides longer than eight amino acids [46]. Novobiocin interferes with an ATP-requiring step in the transcription of RNA polymerase III [47]. Tetracycline prevent the addition of new amino acids to the growing polypeptide during protein synthesis, by binding to the ribosome [48].

Deletion of *pbp1a* or *pbp1b* seem to have little effect on the sensitivity to both erythromycin and tetracycline. The same can be said for *pbp2a*, but when *pbp2a* is removed in a *dacA*-mutant, we observe a greater increase in the sensitivity to either antibiotic. This could be yet another example of redundant antibiotic resistance effect, as indicated earlier with ceftazidime.

### 6.3.4 - An overview of what the results tell us of *S. pneumoniaes* susceptibility to antibiotics

The presence of the *dacA*-gene seems to be critical to either the general health of the pneumococci, or the specific pneumococcal sensitivity to antibiotics, because the *ΔdacA*-mutants had a higher zone of inhibition than the other PBP-encoding gene-mutants had to every antibiotic, except for penicillin. A possible explanation for the increased sensitivity to the other β-lactam antibiotic, ceftazidime, could be that PBP3 coordinates the localization of other PBPs, and the absence of this coordination could make the PBPs more exposed to the β-lactam antibiotic.

Although the peptidoglycan-related antibiotic tests showed fairly similar results, it is hard to derive any conclusive information on whether PBP2a, PBP1a, PBP1b or PBP3 have any effect on the sensitivity to such antibiotics themselves. A more probable reasoning would be that the lack of these genes cause the pneumococci to synthesize peptidoglycan at a much slower pace, that it makes them more vulnerable to stress in general, and not necessarily more specifically susceptible to either bacitrin or vancomycin.

The results for sensitivity to ceftazidime, bacitracin, erythromycin and tetracycline indicate that *dacA* and *pbp2a* have a redundant effect in the resistance against these antibiotics, and that the removal of both genes causes a much greater sensitivity than the removal of either

of them, or *dacA* and one of the other PBP-encoding genes together. This is interesting, as *pbp2a*-mutants have a stronger resistance to antibiotics in general, with the exception of penicillin.

## 6.4 - Activity of the *dacA* promoter

The measured luminescence of the *ΔdacA::luc* mutants seems to increase to about 120 minutes into the experiment, after which it begins to decrease. This correlates somewhat with a typical growth curve of the *ΔdacA* mutant, where the cell density would stop increasing at around 240 minutes.

The lack of any significant difference in the luminescence measurement of *ΔdacA::luc* mutants, regardless of the addition of ComS*, suggests that pneumococci can not sense a lack of PBP3, but it could also be caused by an error in the PCR of the inserted *luc* gene. For instance, a mutation could have caused a stop codon or a frame-shift to appear in the middle of the *luc* gene, leading to incomplete translation, and thus a lack of functional luciferase expression. The $P_{dacA}$-*luc* construct that was transformed into *S. pneumoniae* needs to be sequenced before any conclusion can be drawn from this experiment.

The induction of *dacA* expression in *S. pneumoniae* using the ComRS system resulted in a small reduction in the growth rate. A possible explanation is that the ectopically expressed *dacA* gene, following the *comX* promoter, was expressed regardless of addition of ComS*, leading to an expression of PBP3 at a healthy rate. Induction of *dacA* with ComS* could then lead to an over-expression of *dacA*, and production of toxic amounts of PBP3. Of course, if the transformant had a normal expression of *dacA*, it would not have the same growth rate as the *ΔdacA*-mutants. The validity of this hypothesis is therefore debatable.

## 6.5 - Conclusions

Compared with the wild type and the Δ(class A HMM PBP)-encoding gene mutants, the *ΔdacA*-mutants have more dramatic morphological abnormalities and a slower growth rate.

In addition, the *ΔdacA*-mutants displayed a higher susceptibility to several different types of antibiotics such as ceftazidime, bacitracin, vancomycin, erythromycin and tetracycline.

We also observed a possible interplay between *dacA* and *pbp2a*, as the deletion of both these genes seem to make pneumococci highly susceptible to antibiotics that *Δpbp2a*-mutants were not sensitive to, including ceftazidime, bacitracin, erythromycin and tetracycline. The *ΔdacAΔpbp2a*-mutants also had growth rate which seemed to be lower than what the deletion of *dacA* from other mutants would indicate.

*S. pneumoniae* are not sensitive to autolysis mediated by intracellular or extracellular LytA during its exponential growth, regardless of the absence of PBP3. *S. pneumoniae* deficient in PBP3 also displays an increased resistance to LytA following its stationary phase.

# 7 Future work

Since none of the transformations conducted in this experiment proved to be lethal, it could be possible to produce mutants lacking *dacA* and two class HMM PBP-encoding genes. As the localization study performed by Morlot *et al*. (2003) shows, it is possible to produce viable mutants lacking both *pbp1b* and either *pbp1a* or *pbp2a* [31]. Continuing the work to produce mutants with more deficiency in PBP-encoding genes should eventually give mutants that are fatally vulnerable to LytA-mediated lysis during exponential growth, as our study on double mutants has shown (Figure 5.10).

None of the mutants were sequenced, and doing this would help the credibility of the results. Particularly, sequencing of the $P_{dacA}$-*luc* construct used to produce the *ΔdacA*::*luc* mutant could help us understand why there was no significant luminescence measurement during the luminescence reporter experiment.

The antibiotic sensitivity test produced some interesting results, especially considering the difference in effect the two β-lactam antibiotics had on the mutants. While penicillin had an overall stronger effect on the mutants lacking any PBP-encoding gene, ceftazidime was clearly more effective against the *ΔdacA*-mutants. More testing on β-lactam antibiotics could give a wider overview on how these antibiotics affect each PBP specifically, or how the localization of PBPs affect their vulnerability to β-lactam antibiotics.

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