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60 credits

The immunity protein ComM; effects of overexpression in *Streptococcus pneumoniae*

Immunitetsproteinet ComM; innvirkning på *Streptococcus pneumoniae* ved overekspresjon

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Vegard Osa Lie
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Abbreviations

CbpD	Choline-Binding Protein D
StkP	Serine/Threonine kinase phosphorylase
ComS*	Competence stimulating peptide in *S. thermophilus*
CSP-1	Competence stimulating peptide in *S. pneumoniae*
dsDNA	Double stranded DNA
ssDNA	Single stranded DNA
EDTA	Ethylene Diamine Tetra acetic Acid
$Km^{S/R}$	Kanamycin sensitive/resistant
OD	Optical Density
PCR	Polymerase Chain Reaction
PVDF	Polyvinlyidenflourid membrane
$Sm^{S/R}$	Streptomycin sensitive/resistant
TAE-buffer	Tris-acetate-EDTA buffer
TBS/T	Tris-buffered saline/Tween 20
UV	Ultra Violet
WT	Wild Type

Sammendrag

Streptococcus pneumoniae er en opportunistisk Gram-positiv bakterie som koloniserer det øvre respiratoriske systemet hos dyr og mennesker og kan være ansvarlige for mulig dødelig sykdommer som lungebetennelse, bakteriemi, hjernehinnebetennelse og endokarditt. Pneumokokker er kompetent for naturlig transformasjon, som tillater dem å fort tilpasse seg til eventuelle miljøforandringer. Denne egenskapen gjør at de kan erverve nye fenotyper, som for eksempel antibiotikaresistens, ved å inkorporer fremmed DNA via homolog rekombinasjon. Denne evnen er strengt regulert via et peptid-feromon kalt CSP-1, som virker via en «qourum sensing» mekanisme. Under kompetanse uttrykker bakterien et murein hydrolase-protein kalt CbpD som lager kutt i celleveggen til nært beslektete streptokokker. Dette proteinet resulterer i lysis og frigjøring av DNA som kan tas opp av de kompetente pneumokokkene. Denne mekanisme kalles fratricide «Brodermord». Siden CbpD gjør skade på celleveggen er det nødvendig for de kompetente cellene å beskytte seg selv mot sitt egenproduserte CbpD. Dette blir gjort via et kompetanseinduserte immunitets protein kalt ComM. Dette proteinet blir produsert under kompetanse, men i et tidligere stadium enn CbpD og som gir tid for cellene til å beskytte seg selv. ComM er forventet å være et membranintegrert protein med immunitet rettet mot CbpD, men mekanismen for dette er fortsatt ukjent. Målet med denne oppgaven var å få en bedre forståelse av immunitetsproteinet ComM ved ektopisk utrykk ved bruk av ComRS-system, i tillegg til å prøve å lokalisere ComM i cellen. I dette arbeidet ble det utført et vekstforsøk av ComM-utrykkende stammer ved bruk av ComRS systemet. Dette eksperimentet viste en morfologisk forandring i celle morfologi og n viss økt resistens mot LytA hos de immune cellene, tyder på at ComM er ansvarlig for en modifikasjon av celleveggen. Dette ledet til en undersøkelse av stempeptidene i peptioglykan laget til immune celler ved hjelp av revers HPLC analyse. Disse undersøkelsene viste ingen forandring. Dette kan tyde på at modifikasjonene er lokalisert et annet sted. Videre ble det utført zymografi forsøk som viste at cellevegg fra immune (ComM+) celler var mer resistente mot renset CbpD i forhold til cellevegg fra sensitive (ComM-) pneumokokker. Dette støtter tanken at ComM er ansvarlig for en forandring i celleveggen som beskytter mot CbpD. Dette viste at CHAP domene et katalytisk inaktivt uten de cellevegg bindende domener (SH3b domenene og et kolin bindende domene) Den andre delen av dette arbeidet var å prøve å lokalisere ComM hos immune celler ved hjelp av fusjon-proteinet sfGFP-ComM. Siden CbpD har vist seg å angripe det septale området hos ikke-kompetente pneumokokker, er det god grunn til å tenke at dette er lokasjonen til ComM. Lokalisasjonen studiene i dette arbeidet ledet ikke til noen signifikante resultater

Abstract

Streptococcus pneumoniae is an opportunistic Gram-positive bacterium that resides in the upper respiratory tracts of humans and can be responsible for fatal diseases such as pneumonia, bacteraemia, meningitis, and endocarditis. Pneumococci are capable of natural transformation, allowing them to quickly adapt to environmental change. This makes pneumococcal infections challenging to treat as they can acquire new phenotypes by incorporating foreign DNA by homologous recombination. This state of competence is induced by a peptide pheromone called CSP-1, which is sensed by a quorum sensing mechanism. During competence, the bacterium secretes a murein hydrolase called CbpD which targets closely related streptococci resulting in lysis and release of DNA. This predatory mechanism is called fratricide, and CbpD is regarded as a fratricine. To avoid self-lysis, the competent cells needs to protect themselves against their own fratricine. This is accomplished by expressing of the immunity protein called ComM. This protein is also produced during competence, albeit at an earlier stage then CbpD, thus giving the cell time to protect itself. ComM is predicted to be an integral membrane protein with immunity focused against CbpD. The mechanism for this is still unknown. The aim of this study was to obtain a deeper understanding of how the immunity protein ComM works through ectopic expression using the ComRS-system while also attempting to determine the location of ComM in the cell. In this work a growth experiment was conducted by ectopically expressing ComM using the ComRS system. This experiment showed morphological changes of the cell shape and an increased resistance towards LytA from the immune cells, which leads to the assumption that ComM is responsible for modification(s) to the cell envelope. To test this hypothesis, reverse phase HPLC analysis of the stem-peptides of immune cells was conducted. The results showed no detectable difference in the stem peptide composition, which could mean that the modification is located elsewhere. Furthermore, zymography experiments showed that cell walls derived from immune (ComM+) cells were more resistant against purified CbpD compared to cell wall derived from sensitive (ComM-) pneumococci. This supports the notion that ComM somehow induces changes in the cell envelope that provide protection against CbpD. This showed that the CHAP domain is catalytically inactive without its cell wall binding domains (two SH3b domains and a choline binding domain). Also part of this work was an attempt to localize ComM in immune cells with the use of the fusion protein sfGFP-ComM. Since CbpD has been shown to target the septal region of target cells it has been speculated that ComM is located in the septum. This localization studies in this work, however, did not lead to any conclusive data.

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1 Introduction

1.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae is a commensal bacterium mostly found in the mucus layer of the upper respiratory tract of humans. It is a Gram-positive bacterium among the low G-C (34-46%) bacteria in the phylum *Firmicutes* (Garrity, Bell et al. 2004). The cells of *S. pneumonia* are ovoid in shape and mostly grow in pairs, but can also form shorter chains. They are aerotolerant and lack an electron transport chain, making them chemoorganotrophic. Therefore, *S. pneumoniae* requires a complex growth medium containing amino acids, carbohydrates, purines, pyrimidines, salts and vitamins. They obtain energy by fermenting sugars where lactic acids is the primary end product of their fermentative metabolism (Hardie and Whiley 1997).

Table 1.1.1 Taxonomic classification of *S. pneumoniae*

Taxon	**Name**
Domain	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Lactobacilliales
Family	Streptococcacea
Genus	*Streptococcus*
Species	*S. pneumoniae*

The *Streptococcus* genus has been divided into six phylogenetic sub-groups based on of 16S rDNA sequencing analysis. The groups are: pyogenic, anginosus, mitis, salivarius, bovis and mutans. Figure 1.1 shows the phylogenetic tree of the different groups, where *S. pneumoniae* can be found in the mitis group along with 11 other species including *S. mitis* and *S. oralis.* The figure only shows six species but this has been updated to 12 in the gene bank database (Kilian, Poulsen et al. 2008).

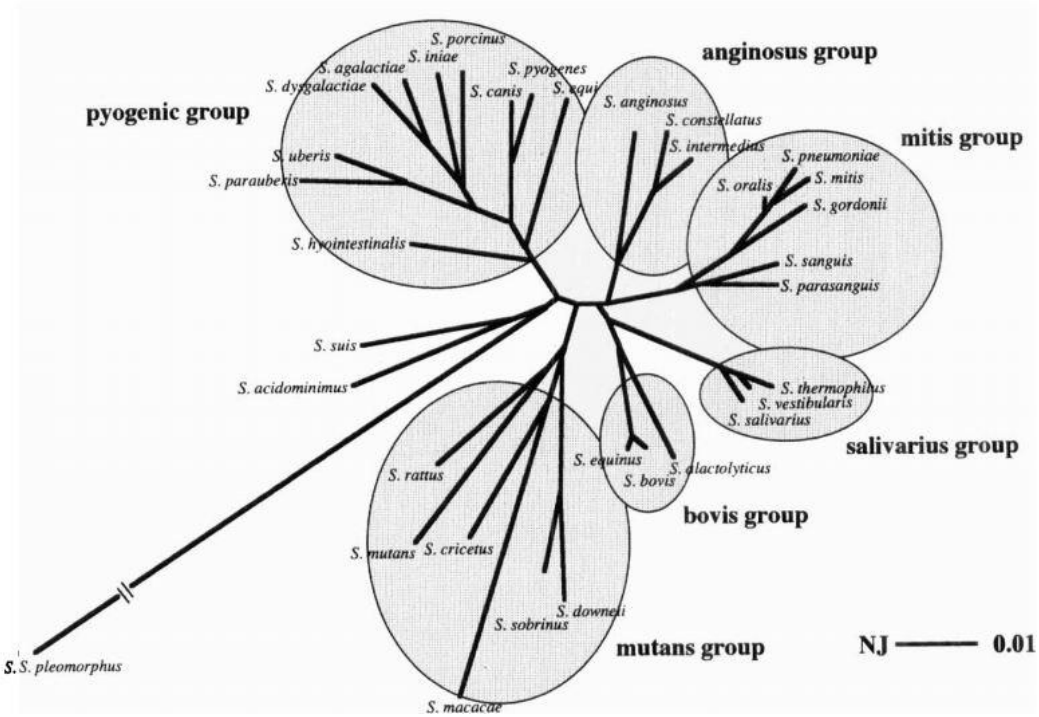


Figure1.1 **Phylogenetic tree of the Streptococcus genus**. The groups are divided based on 16S rDNA sequencing analysis where *S. pneumoniae* is placed in the mitis group. The figure is taken from (Kawamura, Hou et al. 1995).

S. pneumoniae is an opportunistic bacterium which has its primary reservoir in the naso-pharynx of humans and other animals. In most cases the bacterium will act as a passing commensal bacterium, however *S. pneumoniae* is also the cause of serious diseases like bacterial pneumonia, bacteraemia, meningitis, arthritis and endocarditis. Though these diseases are most common among groups with already compromised immune systems like in young children, elderly and individuals with underlying diseases such as AIDS or other immune compromising afflictions. What makes *S. pneumoniae* a human pathogen is the virulence factors, which include: a polysaccharide capsule, pneumolysin (Ply), autolysin (primarily LytA) and IgA1- proteases (Hoskins, Alborn et al. 2001). The most important virulence factor is considered to be the polysaccharide capsule, and for this reason the strain used in this work, *S. pneumoniae* R6, does not have the ability to produce this capsule making them avirulent. With the use of autolysins such as LytA, which degrade the peptidoglycan layer of the bacterial cell wall, the *S. pneumoniae* commits suicide releasing the pneumolysins in the cytoplasm of the cell. One of the modes of action of the pneumolysins is to bind to the cholesterol in the cell membrane of the host cell, resulting in the formation of large pores in the membrane. This will lead to lysis of the host's cells (Jedrzejas 2001).

S. pneumoniae is a major cause of fatalities, especially among children and in developing countries, where it has been shown to be responsible for more than 1.2 million deaths of children

annually (Kadioglu, Weiser et al. 2008). Pneumococcal infections are treated with antibiotics, where penicillins are the first in line choice. However, since *S. pneumoniae* is naturally competent, the spread of antibiotic resistance genes among pneumococcal isolates occurs at a concerning high rate and it is starting to pose serious medical concern. Penicillins are in the β-lactam family of antibiotics, which interfere with the cell wall synthesis machinery of bacteria by inhibiting the function of penicillin binding proteins (PBPs). PBPs are responsible for synthesizing the major constituent of the bacterial cell wall called peptidoglycan by performing transglycosylation and transpeptidation reactions. By structural resemblance with the natural substrate of the PBP's, the β-lactams bind and occupy their active transpeptidase site, leading to stalled peptidoglycan synthesis. In penicillin resistant strains, the PBPs have been mutated to have a lower affinity for the antibiotics without losing its original function.

In the case of vaccinations, the polysaccharide capsule is the target for the PCV7 vaccine which was introduced in the US in the year 2000. This vaccine consisted of the polysaccharides of seven of the more than 92 capsule types (Serotypes) (Golubchik, Brueggemann et al. 2012). This vaccine showed great success as the rate of pneumococcal infections in vaccinated children age 0-2 years decreased by 69%. As stated before, the transformation of modified PBPs might also involve a capsule change (Serotype shift), and this poses a challenge for vaccinations. Since the serotype covered by the vaccine may transform to a new serotype, the immune system of the host may not recognise the bacteria. Because of this, it is important to gain a better understanding of natural transformation in *S. pneumoniae* in order to find new targets for antibiotics and vaccines.

1.2 Horizontal gene transfer

Horizontal gene transfer is the transfer of genetic material between two organisms through other channels then reproduction. The acquisition of genetic material from one organism to another can lead to new genotypes and even phenotypes. There are three different ways of acquiring DNA: conjugation, transduction and transformation. Conjugation is the transfer of DNA via direct cell-to-cell contact. Transduction is mediated by a bacteriophage and transformation is the active acquisition of naked DNA from the environment. Transformation is different from the other two in that the transfer is initiated by the receiving cell itself (Johnsborg, Eldholm et al. 2007).

1.2.1 Conjugation

Conjugation, as stated earlier, is the transfer of genetic material through direct cell-to-cell contact. This is done by two bacteria cells, where one is the donor and the other the acceptor of either a plasmid or a piece of chromosome. The link between the two cells is done through a pilus, called a conjugation bridge. The ability to be a donor is determined by the conjugation plasmid referred to as a fertility plasmid. The fertility plasmid contains a cluster of transfer genes, responsible for the expression of the conjugation bridge and for synthesis and transfer of DNA. The transfer begins with the pilus binding to the cell membrane of the recipient cell, and a single strand of the fertility plasmid DNA is transferred in a 5' to 3' direction. Once inside the recipient cell the single stranded DNA is converted to a double-stranded fertility plasmid, and the missing strand in the donor cell is replaced, resulting in both cells harbouring the fertility plasmid (Holloway 1993).

1.2.2 Transduction

Transduction is the transfer of genomic material though a bacteriophage as a vector. As the bacteriophage infects a bacterium and starts to replicate during their lytic growth cycle, they might pick up and replicate a fragment of the bacterial genome instead of viral DNA. When the bacteriophages are then released after lysis of the host, the bacteriophages with bacterial genome can infect other bacteria where the new genes can be transferred to the recipient where it can become part of the genome (Holloway 1993).

1.2.3 Natural Transformation

Transformation is the uptake and incorporation of exogenous DNA from the environment. As cells undergo lysis their cytoplasmic content is released, including genomic material. This genomic material can then be incorporated by recipient bacterial cells, provided they are competent. Competence refers to the ability to take up extracellular DNA and become transformed. Competence is induced at different stages for different groups of bacteria. For example, the *Acinetobacter, Chlorobium, Deinococcus, Neisseria* and *Synechoccus* have been shown to exhibit competence all throughout their exponential growth phase, while some express their competence genes constitutively, like *Neissera gonorrhoeae* and *N. meningitidis.* On the other hand, *S. pneumoniae* are only competent for a short period of time. In case of, *N. gonorrhoeae* DNA uptake is dependent on a 10-basepair long sequence which is recognized and is necessary for uptake and recombination (Hamilton and Dillard 2006). For *S. pneumoniae*

this is not the case, where DNA uptake is not sequence specific. During competence several genes are expressed called *com*-genes, and which code proteins responsible for binding, uptake, processing and recombination of the exogenous DNA. Competence is usually induced during stressful conditions for the bacteria, as in high cell density, shortage of nutrients or even sub-lethal levels of antibiotics (Johnsborg, Eldholm et al. 2007). This gives the competent cells the advantage to survive changes in the environment, and establishes a selection pressure for competent bacteria.

Natural transformation in *Streptococcus* is primarily found in the mitis group, though examples of natural transformation have also been found in the anginosus group: *S. anginosus, S. constellatus, S. intermedius*, and there is even one example in the salivarius group, namely *S. thermophilus.* Late competence genes, which are expressed during the later stages of competence in *S. pneumoniae* and some of which are essential for the binding, uptake and recombination of extracellular DNA, seem to be present in the entire *Streptococcus* family. The reason that transformation has yet to be seen in a majority of the species of the streptococcus family, may be because the correct requirements for competence are unknown or difficult to realize in the laboratory (Johnsborg, Eldholm et al. 2007).

1.3 Natural Competence in *S. pneumoniae.*

As stated earlier, competence in bacteria refer to their ability to take up naked DNA from their environment or neighbouring cells and incorporating it into their own genome. This capability presents a huge advantage for the pneumococcus, as well as being a major driving force in the evolution of this bacterium. The ability to take up genes from the environment makes it possible for *S. pneumoniae* to rapidly adapt to a changing environment. These adaptations might be the acquisition of antibiotic resistances, capsular switching or even new virulence factors, opening new ways for the bacterium to survive. Of course, acquiring resourceful genes is not always the case, as the genes might also do nothing, or might even be harmful to the cells. But as these either do not survive or do not give advantages to the cells, there is no selection pressure to keep these genes.

In 1928 Frederick Griffith conducted an experiment (Griffith 1928), now called Griffith's experiment, while working on creating a vaccine against pneumonia. The experiment consisted of two *S. pneumoniae* strains which he injected into mice. The two strains were type III-S

(Smooth) and type II-R (Rough). The smooth strain was protected from the immune system of the host by a polysaccharide capsule (virulent strain) while the rough strain was not (non-virulent strain). Individually, the virulent strain resulted in mouse death, while the mice infected with the non-virulent strain survived. The great discovery of Griffith was that when he co-infected heat inactivated virulent (smooth) strain with the non-virulent (rough) the mouse died (Griffith 1928). This experiment showed that the non-virulent strain had been transformed to a virulent strain by integrating the capsule of the dead virulent strain. Griffith at the time did not know that what he had discovered was the uptake of DNA, which was later discovered by Avery, McLeod and McCarty in 1944 (Avery, Macleod et al. 1944).

1.3.1 Regulation of competence in *S. pneumoniae*

Entrance of the competent state in *S. pneumoniae* is regulated by the gene products of the competence induced operon *comCDE* (Håvarstein, Gaustad et al. 1996). ComD and ComE constitute a two component system where ComD is a membrane histidine kinase and ComE its cognate response regulator. ComC encodes a peptide pheromone called CSP (Competence-Stimulating Peptide), which acts as a quorum sensing signal that is monitored by ComD (Håvarstein, Coomaraswamy et al. 1995). CSP is produced with a leader-sequence (pre-CSP) that is cleaved off by the ComAB transporter during export out of the cells. Outside the cells, mature CSP binds to the receptor domain of ComD which then autophosphorylates its cytoplasmic kinase domain. This phosphorylated group is transferred to the response regular ComE (Johnsborg et al. 2007). ComE recognizes a set of tandem repeats in the promoters of the so called early competence genes. When ComE is phosphorylated it will activate transcription of these early genes, which include about 20 genes, of which seven are required for developing competence: *comAB, comCDE, comX* and *comW.* Also among these early genes is *comM* which is a fratricide immunity gene which protects the cell against the fratricine CbpD (Choline binding protein D) produced among the late genes. Since ComE induces its own production in addition to ComC and ComD, this regulatory circuit functions as an autocatalytic loop. Competence is induced when the level of extracellular CSP reaches a critically high level as a result of growth or environmental stress, resulting in more phosphorylated ComE and which eventually triggers the autocatalytic loop. The gene *comX1* codes for the alternative sigma factor ComX, which promotes the RNA-polymerase to initiate transcription of the late competence genes (Lee and Morrison 1999). Expressed among the late genes are products that are responsible for the uptake and recombination of the extracellular DNA. In addition, the

competent cells produce and secrete the fratricine called CbpD, whose function is to lyse neighbouring non-competent cells to release their DNA content. The competent cells can then take up DNA from the lysed cells. CbpD, being a late competence product, ensures that the early competence protein ComM (CbpD immunity protein) is produced first to avoid CbpD mediated suicide.

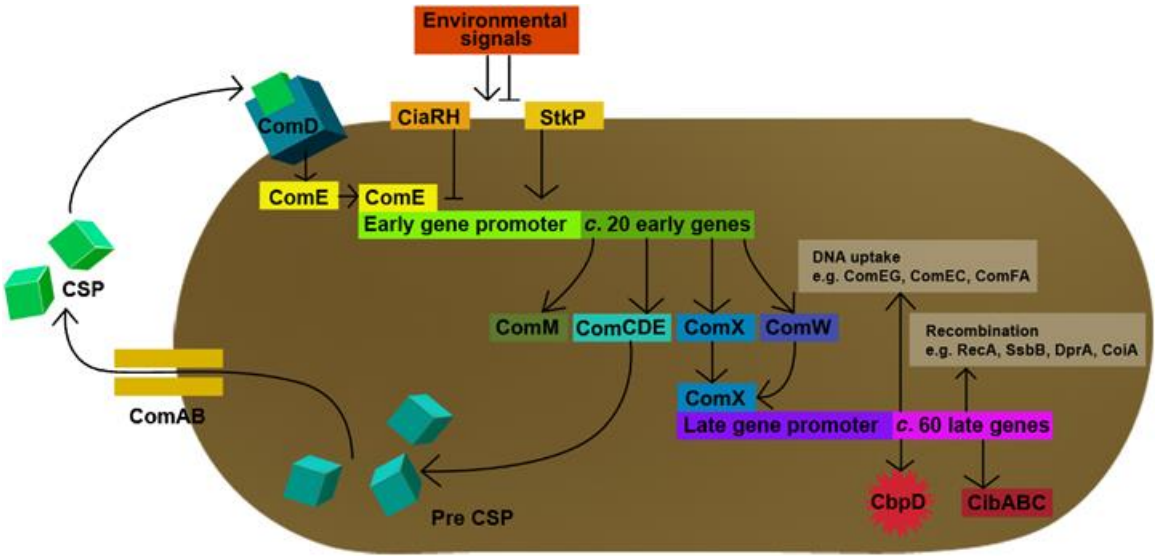


Figure 1.2 **Regulation of competence in *S. pneumoniae*.** The figure shows the two component regulation and expression of competence in *S. pneumoniae*. The CSP is transported out of the cell using the ABC-transporter ComAB. As the extracellular concentration of CSP increases, the ComD receptor is activated and in turn activates ComE, which initiates the transcription of the early genes. ComW protects ComX from degradation which initiates the transcription of the late genes. Figure from (Johnsborg and Håvarstein 2009).

As the immunity protein ComM is only expressed during competence, the *S. pneumoniae* will only be able to lyse non-competent cells of its own kind, or closely related species that are not induced to competence by the CSP variant (different pherotype) (Johnsborg and Håvarstein 2009). The cell exits the competent state by expression of the late competence gene *dprA.* The protein DprA has a dual function during competence. It functions as a RecA loader, where RecA is a DNA recombination protein. DprA promotes the loading of RecA onto the single stranded DNA that has been transported into the cytoplasm. This stimulates the ssDNA-dependent ATPase activity of RecA, which triggers formation of paranemic and plectonemic joints between homologous sequences (Mortier-Barrière, Velten et al. 2007). DprA's second function is to bind phosphorylated ComE, thereby quenching its ability to promote transcription of the early competence genes.

1.4 Fratricides, a competence induced mechanism to acquire homologous DNA

Fratricide is the act of killing closely related species, which can result in the release of nutrients or in some cases the release of homologous DNA. By targeting closely related bacteria there is an almost guarantee to acquire homologous DNA with the purpose of recombination. In the case of *S. pneumoniae* fratricide is a competence induced mechanism, where during competence the cells will produce the murein hydrolase CbpD which is a late gene product. CbpD targets non-competent cells and attacks the cell wall, releasing the DNA. CbpD has been shown to be the main component of pneumococcal fratricide, but fully effective lysis requires several murein hydrolases such as the enzymes LytA and LytC. (Johnsborg, Eldholm et al. 2007).

LytA, LytC and CbpD all contain choline binding domains consisting of a series of repeating choline binding sequences, which can bind to the choline in the wall teichoic acid (WTA) and the lipoteichoic acid (LTA) of the cell wall, allowing their active sites to reach their substrate (Kausmally, Johnsborg et al. 2005). Because of these choline binding domains, the targets for the fratricides are limited to bacteria with choline in their cell wall. Choline is rarely found in the cell wall of other bacteria than streptococci. Bacteria without choline in their cell wall will not undergo lysis by these fratricides (Johnsborg, Eldholm et al. 2008). This enhances the probability for accessing only homologous DNA.

LytA is an amidase which is an autolysin and lyses the *S. pneumoniae* cells during the stationary phase. This is achieved by cleaving the amide binding between the N-acetylmuramine acid and the first amino acid (L-alanine) in the stem peptide chain. LytA is localised internally in the cell and is released from cells undergoing spontaneous lysis in the stationary phase. When LytA is released into the environment it is free to bind and degrade the cell wall of other cells. Though LytA is expressed constitutively, the expression is increased roughly 6 times during competence. As LytC is also expressed constitutively during the exponential growth phase without affecting the growth of healthy cells, it is likely that both of these autolysins require a trigger to active them. As CbpD is capable of killing cells on its own, but the effect is strongly amplified by the presence of LytA and LytC, it is reasonable to assume that CbpD has some responsibility for the activation of these cell wall hydrolases (Eldholm, Johnsborg et al. 2009). This is most likely achieved by CbpD creating the initial damages to the cell wall of non-competent cells, allowing for the release of LytA and LytC, while also assisting the secreted LytC from the attacking cell, as shown in the figure below (Figure 1.3).

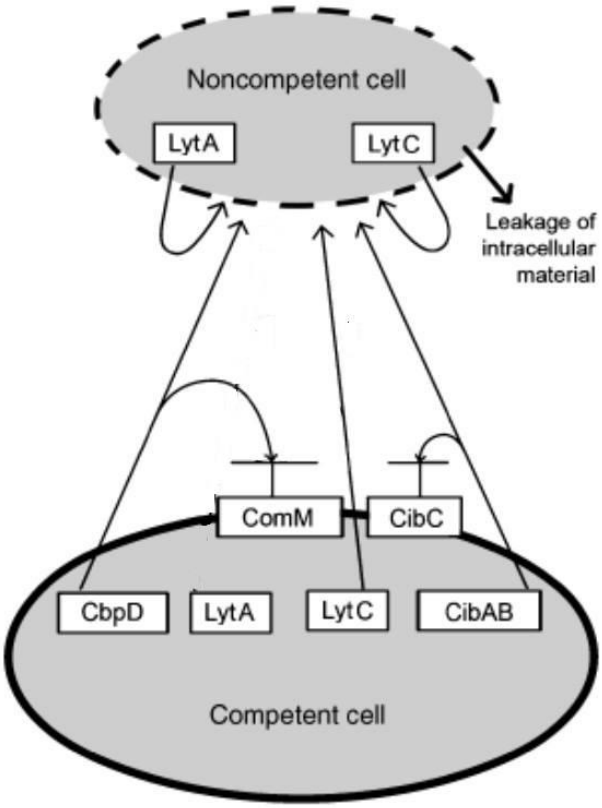


Figure 1.3 **Fratricide in *S. pneumoniae*.** Figure demonstrating that the autolysins LytA and LytC are contributed from both the attacking cell and the target cell. Also shown is the immunity protein ComM protecting the competent cell against autolysis. CbpD assists the LytA and LytC in compromising the target cell wall and releasing the autolysins. Figure modified from (Johnsborg and Håvarstein 2009).

For the uptake of extracellular DNA, a study performed by Laurenceau et al (Laurenceau, Péhau-Arnaudet et al. 2013) showed that during competence *S. pneumoniae* produce a pilus of about 2-3 µm in length that binds double stranded DNA. Only single stranded DNA is transported into the cytoplasm. The endonuclease EndA in the cytoplasmic membrane breaks down the non-transforming DNA strand while the complementary strand is transported into the cytoplasm in a 3' to 5' direction (Straume, Stamsås et al. 2014) (Laurenceau, Péhau-Arnaudet et al. 2013). Once the ssDNA is internalized, and provided that the strand is sufficiently homologous to any regions in the genome of the transforming cells, it will undergo recombination. Among the late genes there are five gene products involved in this process. They are: RecA, DprA, SsbB, CoiA and RadA. SsbB stands for single-stranded DNA-binding protein B and is responsible for protecting the ssDNA fragments from degradation and also preparing them for recombination. This is done by the ssDNA being coated in SsbB and thus protecting it from endogenous nucleases.

There have been speculations as to whether the uptake of DNA might be a means of acquiring nutrients and not only for recombination. The fact that ssDNA is taken into the cytoplasm and is coated and protected against degradation, seems to disprove this point. The fact that *S. pneumoniae* only target closely related bacteria during competence suggests that the interest for the DNA lies in recombination, as these closely related neighbouring cells have a higher chance of yielding homologous DNA. If the DNA were to be used for nutrients, this process would limit the cells potential by targeting only a small group. On the other hand, DNA that is not going to be used for recombination can instead be used as nutrients (Johnsborg, Eldholm et al. 2007).

1.4.1 CbpD, a competence induced cell wall hydrolase

CbpD consists of 4 domains. One, the N-terminal cysteine, histidine dependent amidohydrolase/peptidase (CHAP), which is the enzymatically active domain. The function of this CHAP domain is most likely to cleave peptide bonds within the stem peptides of the peptidoglycan chain as it shows homology to the corresponding domain of other cell wall hydrolases that have this function (Eldholm, Johnsborg et al. 2009). Then two src-homology 3b (SH3b) domains, which functions in recognizing and binding to the peptidoglycan (Eldholm, Johnsborg et al. 2010). At the C-terminal end is the CBD (Choline Binding Domain), consisting of 4 repeating choline binding sequences, which direct the CbpD to the septal region of the *S. pneumoniae* cells, and electron microscopy analysis has shown that cells attacked by CbpD rupture at the septal region (Eldholm, Johnsborg et al. 2010).

CbpD targets non-competent cells to release homologous DNA to be acquired by competent cells. CbpD has been shown to increase the proficiency of DNA uptake 1,000-fold compared to CbpD-deficient cells. This was demonstrated with a mixed culture of competent attacker cells and non-competent target cells. The CbpD-proficient cells were much more efficient in capturing an antibiotic resistance marker from non-competent cells than CbpD-deficient cells (Johnsborg, Eldholm et al. 2008)

As mention earlier, the effects of CbpD is strongly enhanced by the presence of LytA and LytC. CbpD is likely to activate LytC, either directly or indirectly, as LytC at high concentrations have been shown to be harmless against non-competent cells in the exponential growth phase (Eldholm, Johnsborg et al. 2009). The synergistic effect of CbpD and LytA is most likely from the initial damage from CbpD to the cell wall, resulting in the disruption of the cell and release of internal LytA.

1.5 Structure and synthesis of the *S. pneumoniae* cell wall

S. pneumoniae along with other Gram-positive bacteria, have a thick cell wall which allows the cells to resist the internal turgor pressure while also maintaining their overall shape. The cell wall is comprised of several layers of glycan chains which are cross linked by stempeptides forming a net-like structure called peptidoglycan. The glycan chain consists of alternating β-1,4 linked N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) with stem peptides crosslinking two NAMs of different glycan chains. The stem peptide consists of a chain of a variable number, usually four to five of amino acids. The amino acids are L-alanine, D-glutamine, L-lysine and D-alanine. Covalently bound to these peptidoglycans are wall teichoic acids (WTAs) and capsular polysaccharides (Denapaite, Bruckner et al. 2012). WTA and peptidoglycan are the major components of the *S. pneumoniae* cell wall (Bui, Eberhardt et al. 2012). *S. pneumoniae* also have lipoteichoic acids (LTAs) which are bound to the cytoplasmic membrane of the cell, but these are far less abundant than the WTAs.

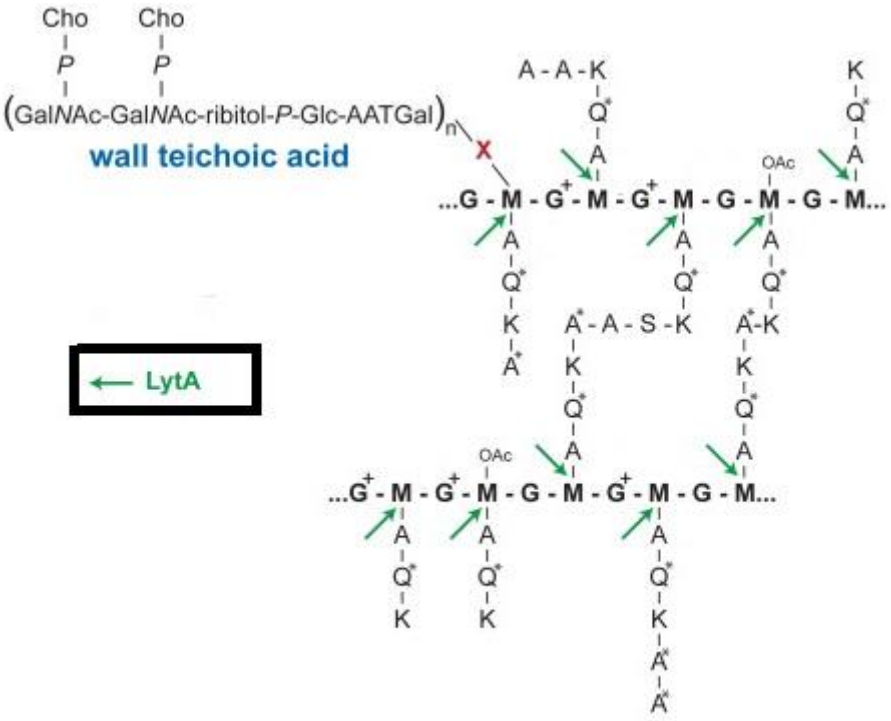


Figure 1.4 Cell wall components. Schematic overview of the cell wall structure in *S. pneumoniae*, also showing the cleavage sites for LytA. G and M represent N-acetylglucosamine and N-acetylmuramic acid, respectively. The amino acids are represented by their single letter code. Figure modified from (Bui, Eberhardt et al. 2012)

As shown in the figure above, the wall teichoic acid is comprised of 2-acetoamido-4-amino-2,4,6-trideoxygalacose (AATGal), a glucose molecule (Glc), Ribitol-5-phospate (Ribitol-p) and two units of N-Acetyl-galactosamine (Gal*N*Ac). These two units of Gal*N*Ac contain phosphocholine through ester-linkage. Choline is rarely found in other bacteria and *S. pneumoniae* is the only bacterial species known where choline is required for growth (Bui, Eberhardt et al. 2012).

Penicillin binding proteins (PBPs) were first named after the ability to bind penicillin. They are responsible for synthesising the peptidoglycan layer. PBPs can be categorized into 2 groups: Low Molecular Weight (LMW) and High Molecular Weight (HMW), where the HMW PBPs are responsible for the polymerization of the glycan strand through glycosyltransferase activities, and the formation of peptide cross linkage through transpeptidase activities (Morlot, Zapun et al. 2003). The LMW PBPs are responsible for D,D-carboxypeptidase activity that degrade the substrate of the HMW PBPs (Scheffers and Pinho 2005). In *S. pneumoniae* there are six PBPs: PBP1a, PBP1b, PBP2b, PBP2x, PBP3 and DacB, where PBP3 and DacB are of the LMW PBPs while the others are HMW PBPs.

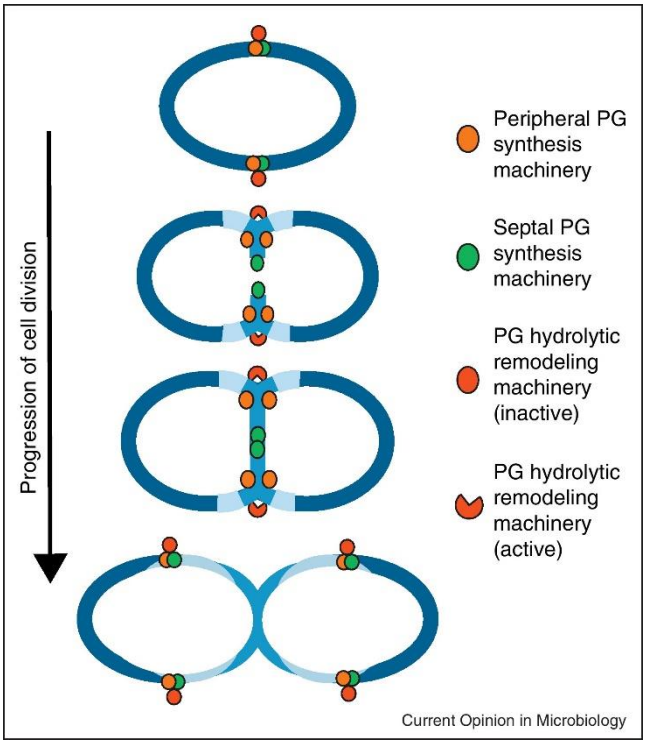


Figure 1.5 **Model showing the peptidoglycan synthesis in *S. peunomiae***. The cell division starts at the equatorial line of the cell, synthesising peptidoglycan in the peripheral and septal directions. Once the new septal peptidoglycan layers meet, cell wall is synthesised in the peripheral direction, resulting in division of the cell. The red, green and orange oval shapes represent the PBP's. Figure taken from (Sham, Tsui et al. 2012)

The biosynthesis of peptidoglycan begins in the cytoplasm of the cells, where the precursor UDP-N-acetulmuramyl is synthesised into a UDP-N-acetulmuramyl-pentapeptide through the stepwise addition of amino acids. The UDP-N-acetylmuramyl-pentapeptide is then positioned near the membrane where it is linked to a transport lipid forming Lipid I. The addition of UDP-N-acetylglucosamine forms Lipid II. Lipid II is the precursor to the peptidoglycan and is transported through the membrane; this is suggested to be performed by a flippase protein (Bouhss, Trunkfield et al. 2008). Outside of the cell Lipid II is synthesised into peptidoglycan by the transpeptidation and transglycosylation by the penicillin binding proteins (Pinho, Kjos et al. 2013). For ovoid formed cells, such as *S. pneumoniae*, cell wall synthesis occurs in two directions, the peripheral and the septal. This has led to the model that two different peptidoglycan synthesis machineries are required, as shown in figure 1.4. Whether these machineries operate as two distinct complexes or form a single large complex, is still unknown (Sham, Tsui et al. 2012). An immunofluorescence microscopy experiment conducted by (Morlot, Zapun et al. 2003) showed the localization of the PBPs in *S. pneumoniae*, where PBP1a and PBP2x were shown to be located in the septal part of cell division, while PBP1a and PBP2b were shown to be located in the peripheral part. This would suggest that the septal and peripheral peptidoglycan synthesis machinery operates as two complexes, located at their respective sites during cell division.

1.6 The immunity protein ComM

As competent cells themselves are targets for their own fratricine, it is imperative that they manage to protect themselves so they do not commit suicide during the competent state. This is done by producing immunity proteins. During competence in *S. pneumoniae*, it is ComM that grants immunity against CbpD (Håvarstein, Martin et al. 2006). As mentioned earlier the *comM* gene is an early competence gene, while *cbpD* is a late competence gene. ComM can therefore render the competent pneumococci immune against CbpD before they start to produce this fratricine during the later phase of competence. By deletion experiments it has been shown that ComM is the only protein required for full protection against CbpD (Eldholm, Johnsborg et al. 2010). Moreover, it has also been shown that ComM is not involved in the uptake, processing or recombination of extracellular DNA (Knutsen, Ween et al. 2004).

Figure 1.6 Figure showing the genetic organization of ComM in *S. pneumoniae* R6. ComM is transcribed from the ComE dependent promoter which is triggered by competence. *Spr1761*, *spr1760* and *lytR* are transcribed constitutively during normal growth conditions by the extended -10 promoter, but is upregulated during competence. Figure taken from (Johnsborg and Håvarstein 2009)

The *comM* gene is a part of the *comM*-operon which consists of four *comM* genes as shown in figure 1.6. *spr1761, spr1760* and *lytR* are constitutively expressed, but during competence the ComE dependent *comM* promoter is activated. This also results in an up-regulation of the *spr1761, spr1760* and *lytR* genes. The genetic organization of *comM* is shown in Figure 1.6.

ComM is an integral transmembrane protein consisting of 206 amino acids predicted to form six or seven membrane crossing alpha helixes (Stamsås, Håvarstein et al. 2013), with both N- and C- Terminal positioned into cytoplasm. A predicted topology of ComM is shown in figure 1.7 using the topological prediction programme TMHMM (Krogh, Larsson et al. 2001). The actual three dimensional structure of ComM is still unknown, as it is generally difficult to purify and crystallize membrane proteins.

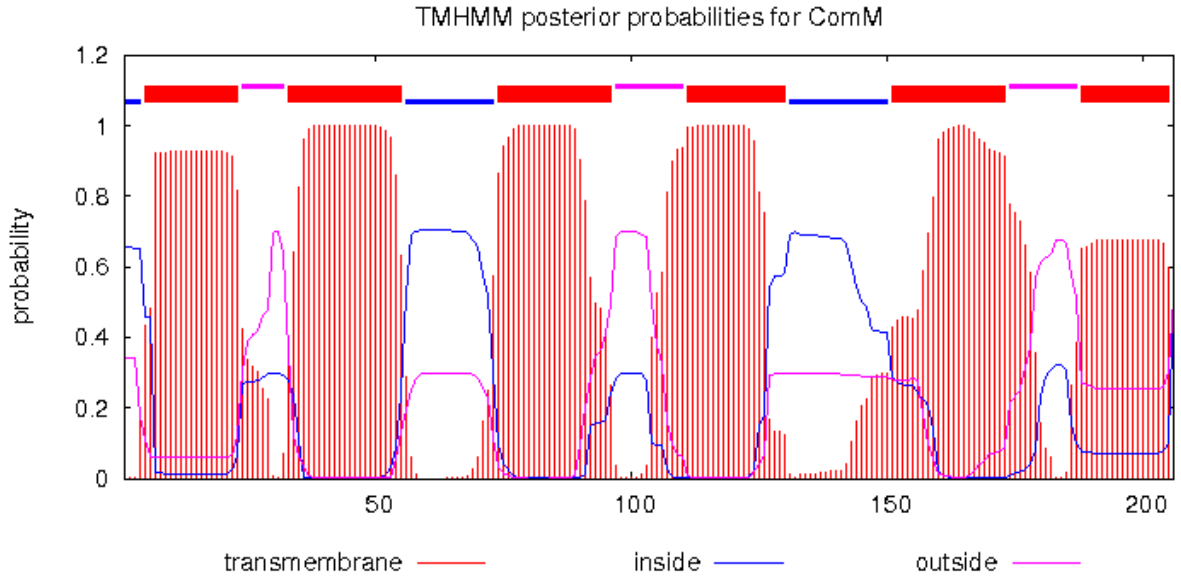


Figure 1.7 **Topology prediction of ComM.** Figure was created using the TMHMM, a prediction program based on a hidden Markov model. Red colour means the residues are in a transmembrane segment, while blue is inside the cytoplasm and purple is outside. Showing six transmembrane helixes with both N-terminal and C-terminal inside of the cytoplasm. (Krogh, Larsson et al. 2001) http://www.cbs.dtu.dk/services/TMHMM/.

Homology searches do not give any clues to what activity ComM might have. However, ComM has been shown to have some similarities to a group of membrane proteins called CAAX amino-

terminal protease (C= Cysteine, A= aliphatic amino acids, X= any residue). This protease is defined by 3 conserved motifs. One, comprised of two glutamate residues next to each other, and an arginine residue divided by three arbitrary(X) amino acids (EEXXXR). Two, the second motif is comprised of one phenylalanine and any three amino acids followed by a histidine (FXXXH). The third motif is a conserved histidine. These CAAX proteases can be found in eukaryotes where they cleave off the AAX motif when the cysteine has been prenylated (Pei and Grishin 2001). As ComM shares some similarities with this family of proteins, it has been proposed to have a proteolytic activity. This is supported by the fact that ComM contains conserved amino acids such as a histidine, which are frequently found in the active sites of enzymes (Kjos, Snipen et al. 2010, Rodriguez, Callahan et al. 2011).

A

B

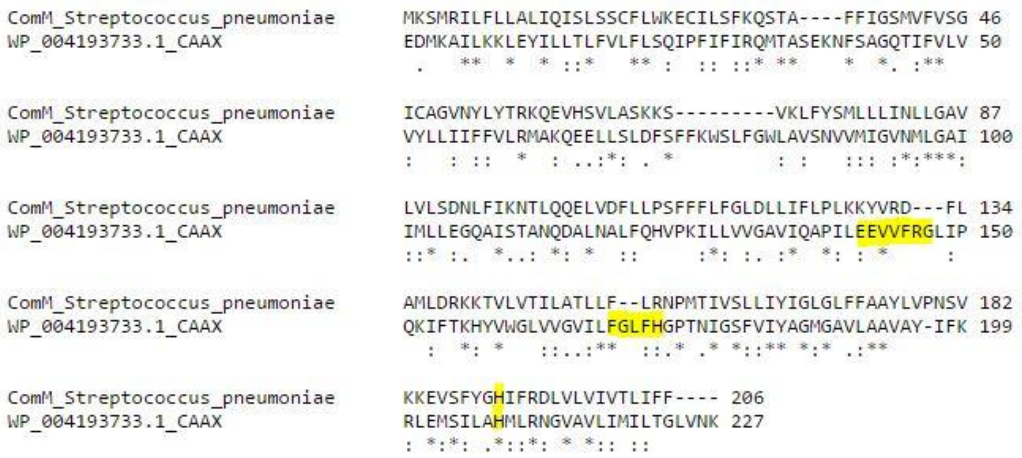


Figure 1.8 **Homology between ComM and the CAAX proteases.** A multiple alignment of the CAAX prenyl protease family (A) demonstrating the conserved motifs, identical residues are marked with red. An alignment of ComM from *S. pneumoniae* and a CAAX protease from *S. sanguinis*.(B) The alignment shows the conserved histidine in both ComM and the CAAX protease (Marked with yellow).(*) annotates identical residues, (:) annotates conserved substitutions, and (.) annotates semi-conserved substitutions. Figure (A) is taken from NCBI's conserved domain database (Marchler-Bauer, Derbyshire et al. 2015). Figure (B) was created using ClustalW (Larkin, Blackshields et al. 2007).

An alignment was done between a protein of the CAAX protease family from *S. sanguinis* and ComM from *S. pneumoniae*, demonstrating the conserved histidine marked on both sequences in figure 1.8 (B).

ComM protects the cells from the activity of CbpD. How it provides immunity is still unknown. However, since ComM is fully embedded in the membrane with no extracellular loops it is unlikely that ComM and Cbpd get in direct contact. It is more likely that ComM either modifies CbpD's substrate or is involved in a mechanism that inhibits the catalytic activity of CbpD (Straume et al 2014). If the activity of ComM is targeted at the peptidoglycan, it is possible that ComM modifies the cell wall of the bacteria in such a way that CbpD loses affinity to its substrate. This will lower the efficiency of CbpD, granting immunity. CbpD has been shown to target the division zone (septum) and the poles of non-competent cells (Eldholm, Johnsborg et al. 2010). It is therefore tempting to assume that ComM is located at the septum of dividing cells, where it might modify the new peptidoglycan layer being synthesized in such a way that the CHAP domain of CbpD loses affinity towards its substrate.

1.7 The ComRS gene expression/depletion system

To study the effects of overexpression and depletion of genes, a gene expression/depletion system was constructed by Berg et al 2011(Berg, Biørnstad et al. 2011). Initially it was constructed to study essential genes in *S. pneumoniae*, but proved an excellent tool to study other non-essential genes as well. This system is called the ComRS system and is based on the competence regulating system of *Streptococcus thermophilus.* Here the Ami transporter transports the inducer peptide ComS* into the cell, where it interacts and activates the response regulator ComR. Once activated ComR will bind to promoter containing a so called ECom box and activate transcription. The *comR* gene and the ECom box-containing P_{comX} promoter of *S. thermophilus* were introduced in a neutral site in the *S. pneumoniae* genome. By adding specific amounts of synthetic ComS* to pneumococcal strains harbouring the ComRS system, the expression of genes cloned downstream of P_{comX} can be fine-tuned (Figure 1.8). The Ami transporter is not necessary in *S. pneumoniae*, as they have an oligopeptide permease which allows ComS* to be transported through the membrane.

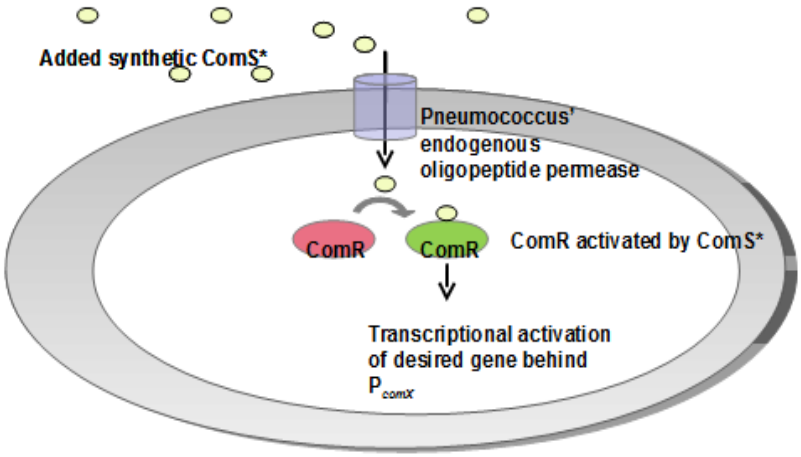


Figure 1.10 **The ComRS system.** A simplified figure demonstrating the ComRS system introduced into *S. pneumoniae*. The added ComS* is acquired from the environment using an oligopeptide permease. The ComS* then activates ComR, which in turn activates the transcription of the gene located downstream of the P_{comX} promoter. (Figure published with permission from Dr. Kari Helen Berg)

As this system is not related to the competence regulatory system of *S. pneumoniae*, and since the ComRS system does not have any homologous components in *S. pneumoniae*, it does not disturb the natural physiology or other functions in the cell. This makes it an ideal candidate for studying essential genes, while also being an excellent tool for controlling the expression of ComM without having to induce the cell cultures for competence.

With the insertion of the desired gene downstream of P_{comX} and the deletion of the native gene, one can manipulate the expression of the gene with the addition of ComS* to the medium. Hence, by controlling the concentration of ComS* in the growth medium, one can also control the expression of the desired gene and study its effects. In this study the ComRS system has been used to express different levels of ComM in *S. pneumoniae*.

1.8 Objectives of the present work

In view of the previous description and discussion of the state of knowledge, the objective of the current work has been two-fold:

- Obtain a deeper understanding of how the immunity protein ComM works towards endowing *S. pneumoniae* with immunity against the CbpD, and, secondly, to
- Attempt to determine the location of ComM in the cell, using the established technique of a Green Fluorescent Protein(GFP) attached to ComM

The following two chapters, Methodology and Results, provide details of the methodology and specific results in this endeavour.

2 Materials

2.1 Bacterial strains

Table 2.1.1 Strains and plasmids. Table showing the strains and plasmids used in this thesis including a description of their relevant genotype.

Strain/Plasmids	Genotype	Reference
RH1	*Δegb::spc, EryR, SpcR*	(Johnsborg et al. 2008)
RH14	RH1 but *ΔLytA :: kan, Ery^R spc^R Kan^r*	Eldholm et al 2009
RH420	*PcomR* inserted between *amiR* and *treF*. *PcomX* inserted downstream of *cpsO*. *Janus* inserted between *cpsO* and *cpsN*, *EryR*, *KanR*	(Eldholm, Johnsborg et al. 2009)
RH609	Ami R1::comM, SmR	Stip. S.C. Sandanger
RH610	Ami R2::ComM, SmR	Stip. S.C. Sandanger
RH611	Ami R3::ComM, SmR	Stip. S.C. Sandanger
RH612	Ami R4::ComM, SmR	Stip. S.C. Sandanger
SPH131	SPH130, but *Δluc::janus, KanR*	(Berg et al. 2011)
E. coli:		
E. coli BL21 (DE3)pLysS	*F$^-$ ompT*, *hsdSB* (r_B^- m_B^-), *gal*, *dcm* (DE3), pLysS (CmR), CmR	Invitrogen
ds28	*E.coli* with LytA in pRSET	(Berg, Stamsas et al. 2013)

Table 2.1.2 *Streptococcus pneumoniae* mutants constructed in this work. Table showing the mutants constructed in this thesis including a description of their relevant genotype.

Strain name	Genotype
V.L 5	ds 109 but *ΔLytA*
V.L 10	ds 109 but *ΔRseP::Janus*
V.L 11	DH5α but contains the plasmid *pRSET-6xHis-CHAP*

V.L 12	DH5α but contains the plasmid *pRSET-CHiC-CHAP*
V.L 13	BL21 but contains the plasmid *pRSET-6xHis-CHAP*
V.L 14	BL21 but contains the plasmid *pRSET-CHiC-CHAP*
V.L 15	SPH131 but *sfGFP-comM::Janus*
V.L 16	V.L 15 but *wtcomM::Janus*

2.2 Peptides

Table 2.2.1: Peptide pheromones used in this thesis.

Pheromone	Amino acid sequence (N→C)	Stock solution
CSP-1	H_2N-EMRLSKFFRDFILQRKK-COOH	100µg/ml
ComS*	H_2N-LPYFAGCL-COOH	500µM

2.3 Primers

Table 2.3.1: Nucleotide sequence of the primers used in this thesis.

Primer name	Oligo nucleotide sequence (5'→3')	Description	Reference
VE17	TGTATCTATCGGCAGTGTGAT	Forward primer for LytA	Unpublished
VE20	TCAACCATCCTATACAGTGAA	Reverse primer for LytA	Unpublished
KHB138	**ATTGGGAAGAGTTACATATTAGAA** ACTAAAAGAAAATGAGCGTAACAAT G	Reverse primer for ComM	Berg et al. 2011

KHB31	ATAACAAATCCAGTAGCTTTGG	Forward primer for *cpsO*	Berg et al. 2011
KHB36	TGAACCTCCAATAATAAATATAAAT	Reverse primer for *cpsO*	Berg et al. 2011
KHB33	TTTCTAATATGTAACTCTTCCCAAT	Forward primer for *cpsN*	Berg et al. 2011
KHB34	CATCGGAACCTATACTCTTTTAG	Reverse primer for *cpsN*	Berg et al. 2011
Ds196	TACGTCTAGATTAAGAAGGAGATATACATATGCATCATCATCATCATCATGAGAACCTGTACTTCCAAGGTAGAGGAAATGGATCGATTG	Forward primer for *6xHis-CHAP*	This study
Ds3	TACGAAGCTTCTAAGCTGAGGATTGACTATTCC	Reverse primer for *CHAP*	Unpublished
Ds197	GAGAACCTGTACTTCCAAGGTAGAGGAAATGGATCGATTG	Forward primer for *CHiC-CHAP*	This study
VE168	AATACGACTCACTATAGGGAGA	Forward primer for pRSET	Unpublished
Ds58	ACCTTGGAAGTACAGGTTCTC	Reverse primer for pRSET	Unpublished
TH009	ACGTTTGAGCAATTTCCTTCC	Forward primer for RseP	Unpublished
TH021	ATGAAATCAATGAGAATCTTATTTTTG	Forward primer for ComM	Unpublished
TH022	CTAAAAGAAAATGAGCGTAACAATG	Revers primer for ComM	Unpublished
TH024	CATTGTTACGCTCATTTTCTTTTAGAGAAAGCCTGTTTTTTATGGATG	Forward primer downstream of ComM	Unpublished

ds222	ATGAAACATCTTACCGGTTCTA	Forward primer for sfGFP	This study
ds223	tagaaccggtaagatgtttcatCTTCTCTCTCCCTTCCTACC	Revers primer upstream ComM, linked to ds222	This study
Gro200	ACTCTGAGTCCGTCTCGCT	Forward primer 1kb upstream of spr 1761	Unpublished
VE109	GACCGAACTTACCTTGAATGGA	Forward primer 1kb upstream of ComM	Unpublished
VE112	TGCCCCACGCTCTTGG	Reverse primer 1kb downstream of ComM	Unpublished

2.4 Molecular weight standards, enzymes and nucleotides

Table 2.4.1: Molecular weight standards, enzymes and nucleotides. Table showing the molecular weight standards, enzymes and nucleotides used in this thesis

Name	**Stock solution**	**Product number**	**Manufacturer**
1kb DNA ladder	50ng/µl in loading buffer and dH_2O	N3232	New England BioLabs® Inc.
dATP, dCTP, dGTP and dTTP	100mM	-	-
Phustion™ High-Fidelity DNA Polymerase	2.0 U/µL	M0530	New England BioLabs® Inc.
Taq DNA Polymerase	5.0 U/µL	M0273	New England BioLabs® Inc.
SYTOX® Green Nucleic Acid Stain	5 mM	S7020	Invitrogen

2.5 Antibiotics

Table 2.5.1: Antibiotics. Table showing the antibiotics used in this thesis, along with the stock solution.

Antibiotic	Stock solution	Product number	Supplier
Kanamycin	100mg/ml	K4000	Sigma-Aldrich
Streptomycin	100mg/ml	S6501	Sigma-Aldrich
Spectinomycin	50mg/ml	-	Sigma-Aldrich
Ampicillin	100mg/ml	A-9518	Sigma-Aldrich

2.6 Kits

Table 2.6.1: Kits. Table showing the kits used in this thesis

Name	Use	Product number	Manufacturer
Nucleospin ® Extract II	DNA purification from agarose gel	740609	Machnery-Nagel
NucleoSpin® Plasmid	Plasmid purification from agarose gel		Machnery-Nagel

2.7 Chemicals

Table 2.7.1: Chemicals. An overview of chemicals used in this thesis along with chemical formula, provider and product number.

Name	Formula	Product number	Provider
2-Mercaptoethanol	$C_{22}H_6OS$	M6250	Sigma Aldrich
Acrylamide 4x (40%) 37,7:1	C_3H_5NO	B1AC41	Saveen Werner
Active Coal	C	1.02182.1000	Merck
Adenosine	$C_{10}H_{13}N_5O_4$	A9251	Sigma
Agarose		1.01614.1000	Invitrogen
Ammonium persulphate	$(NH_4)_2S_2O_8$	A3678	Sigma

Bacto Agar		1.01614.1000	Merck
Bacto ™ Todd Hewitt Broth		249240	BD Diagnostic Systems
Bacto ™Casitone		225930	BD Diagnostic Systems
Biotin	$C_{10}H_{16}N_2O_3S$	19606	Sigma-Aldrich
Bovine Albumin Serum		A7906	Sigma
Bromophenol Blue 1%	$C_{19}H_9Br_4O_5SNa$	B-5525	Sigma Aldrich
Calcium Chloride dehydrate	CaCL	21075	Fluka
Calcium pantothenate	$C_{18}H_{32}CaN_2O_{10}$	C8731	Sigma Aldrich
Choline Chloride	$C_5H_{14}NO.Cl$	C1879	Sigma Aldrich
Cobbersuphate pentahydrate	$CuO_4S.5H_2O$	61240	Fluka
di-Potassium hydrogen phosphate	KH_2PO_4	1.05104.1000	Merck
di-Sodium hydrogen phosphate	Na_2HPO_4	1.06580.1000	Merck
EDTA	$C_{10}H_{16}N_2Na_2O_8H_2O$	20 296.360	VWR
Ethidium bromide	$C_{12}H_{20}BrN_3$	E1510	Sigma Aldrich
Glucose	$C_6H_{12}O_6$	101176K	VWR
Glutaraldehyde, 25% solution	$C_5H_8O_2$	1.06009.2511	Merck
Glycerol	$C_3H_5(OH)_3$	49781	Sigma
Iron sulphate Heptahydrate	$FeO_4S.7H_2O$	44970	Fluka
L-Asparagine monohydrate	$C_4H_8N_2O_3.H_2O$	A8381	Sigma Aldrich
L-Cysteine Hydrochloride Monohydrate	$C_3H_7NO_2S.HCl.H_2O$	30130	Fluka
L-Tryptophan	$C_{11}H_{12}NaO_2$	93660	Fluka
Magensium Chloride Hexahydrate	$ClMg.6H_2O$	63072	Fluka
Mangan(II) Chloride Tetrahydrate	$MnCl_2.4H_2O$	31422	Riedel-deHaën

Methanol	CH_2OH	1.06009.2500	Merck
Nicotine Acid	$C_6H_5NO_2$	72309	Fluka
Paraformaldehyde, powder 95%	$(CH_2O)_n$	15.812-7	Sigma Aldrich
Poly-L-Lysine hydrobromide	L-Lys-(L-Lys)$_n$-L-Lys.xHBr	P1274	Sigma Aldrich
Potassium Chloride	KCl	1.04094.1000	Merck
Pyridoxin hydrochloride	$C_8H_{11}NO_3HCl$	95180	Fluka
Riboflavin	$C_{17}H_2ON_4O_6$	R-7649	Sigma Aldrich
SDS, Sodium dodecyl sulphate	$NaC_{12}H_{25}SO_4$	UN1325	AppliChem
Skim Milk Powder		1.15363.05000	Merck
Sodium acetate	$C_2H_3NaO_3$	27650.292	Sigma Aldrich
Sodium Chloride	NaCl	1.06404.1000	Merck
Sodium hydroxide	NaOH	1.06469.1000	Merck
Sucrose	$C_{12}H_{22}O_{11}$	102754C	BHD
Sulphuric acid	H_2SO_4	-	-
TEMED (N,N,N',N'-tetramethylethylenediamine)	$C_6H_{16}N_2$	T9281	Sigma
Thiamine Hydrochloride	$C_{12}H_{17}ClN_4OS.HCl$	67038	Sigma Aldrich
Triton® X-100	$C_{14}H_{22}O(C_2H_4O)n$	X100	Sigma Aldrich
Trizma® base	$NH_2C(CH_2OH)_3$	T1503	Sigma
Tween 20	$C_{58}H_{114}O_{26}$	170-6531	BioRad
Uridin	$C_9H_{12}N_2O_6$	U6381	Sigma
Yeast Extract granulated		1.03753.0500	Merck
Zink Sulphate Heptahydrate	$O_4SZn.7H_2O$	96500	Fluka

2.8 Equipment and instruments.

Table 2.8.1: Equipment and instruments. Table showing the equipment and instrument used in this thesis.

Equipment/instrument	Model	Manufacturer
1.5 ml disposable cuvettes	759015	Brand
Anaerobic incubation bags	AnaeroGen™	Oxoid
Autoclave	cv-el 12L / 18L	Certoclav
Avanti centrifuge	J-26 XP	Beckman Coulter®
Benchtop homogonizer	Fast prep 24	MP™
Centrifuge I	Multi centrifuge 3 S-R	Heraeus
Centrifuge II	5430R	Ennendorf
Container for anaerobic incubation		Oxoid
Electrophoresis box for agarose gel electrophoresis	Mini-Sub Cell® GT	BioRad
Filter (0.2 um)		Sarstedt
Gel documentation system	c400	Azure systems
Gel documentation system	GelDoc	Bio Rad
Gel pump	GP100	Savant
Glass beads, acid washed	Sigma	G4649
Image printer	P91	Mitsubishi
Incubator		Termaks
Incubator	Multitron standard	Infors HT
Magnetic stirrer	MR 3001 K	Heidolph
Micro plate reader	Synergy H1 Hybrid Reader	BioTek

Equipment/instrument	Model	Manufacturer
Micro plate reader	FLUOstar OPTIMA	BMG LABTECH
Microtiter plates, 96 wells	3604	Corning
Multi channel pipette	Finnpipette, 30-300 ul	Thermo Scientific
pH-meter	PHM210	MeterLab®
Pipettes	Finnpipettes (0.5-10ul, 10-100ul, 50-200ul, 100-1000ul, 1-5 ml)	Thermo Scientific
Power supply for electrophoresis	Power pac 1000	Bio-rad
Power supply for electrophoresis	Power pac 300	Bio-rad
Refrigirated condensation trap		Savant
Scale, 0.0001g-200g	CP124S	Sartorious
Spectrophotometer	Novaspec® II	Pharmacia Biotech
SpeedVac concentrator	SVC-100H	Savant
Syringes for sterile filtration	Div volumes	BD Plastipak™
Table centrifuge	5424	Eppendorf
Thermal cycler (PCR)	2720 Thermal Cycler	Applied Biosystems
Water bath	MB	Julabo
Water bath	19	Julabo
Water bath	Tw20	julabo
Water bath (maximum 100C)	D 3006	GLF

2.9 Recipe for SDS-PAGE gels

Table 2.9.1: Ingredients for two separation gels. Table showing the amount of ingredients needed for two separation gels of different concentrations

Reagents	**10%**	**12%**	**15%**
dH_2O	4.78 ml	4.3 ml	3.55 ml
1,5 M Tris HCl, pH 8.8	2.5 ml	2.5 ml	2.5 ml
10% (w/v) SDS	0.1 ml	0.1 ml	0.1 ml
40% Acrylamide + 0.8% Bis-acrylamide	2.5 ml	3 ml	3.75 ml
10% APS	0.1 ml	0.1 ml	0.1 ml
TEMED	0,005 ml	0.005 ml	0.005 ml

Table 2.9.2: Ingredients for two stacking gels. Table showing the amount of ingredients needed for 2 stacking gels of 4%.

Reagents	**4%**
dH20	3.15 ml
1 M Tris-HCl , pH 6.8	1.25 ml
10% (w/v) SDS	0.05 ml
40% Acrylamide + 0.8% Bis-Acrylamide	0.5 ml
10% APS	0.05 ml
TEMED	0.005 ml

2.10 Growth Mediums

2.10.1 **Pre-C-Medium**

For 4 Litres:

0.045g L-cysteine HCl

8g Sodium Acetate

20g Bacto™ Casitone

0.024g L-tryptophan

34g di-Potassiumhydrogenphosphate (K_2HPO_4)

Sterile water is added to a final volume of 4 litres, and dispensed to 150 ml per bottle and autoclaved at 121°C.

2.10.2 **C-Medium**

For 150ml of pre-C-Medium

0.4 mM Mangan(II) Chloride Tetrahydrate	150µl
20% (w/v) Glucose	1.5 ml
ADAMS III	3.75 ml
3% (w/v) Glutamine	110µl
2% (w/v) Sodium Pyruvate	2.25ml
1.5M Sucrose	95µl
2mg/ml Uridine-Adenosine	1.5ml
8% (w/v) Albumine	1.5 ml
10% (w/v) Yeast extract	3.75 ml

*Stored at 4°C for 24 hours.

2.10.3 **Todd Hewitt Agar**

15g Todd Hewitt Broth

7.5g Agar

Sterile water added to a final volume of 500ml, and autoclaved for 15 minutes, moulded in plates and stored in plastic bag at 4°C.

2.10.4 **Luria Bertani (LB)**

10g Tryptone

5g Yeast extract

10g NaCl

Sterile water added to a final volume of 1 litre, and autoclaved for 15 minutes. Stored at 4^{o}C

2.11 Solutions and buffers

2.11.1 **Solutions for C-Medium**

ADAMS I

0.15ml of 0.5mg/ml Biotin

75mg Nicotinic Acid

87.5mg Pyridoxine Hydrochloride

30mg Potassium Panthothenate

80mg Thiamine Hydrochloride

35mg Riboflavin

Sterile water added to a final volume of 500ml and pH was adjusted to 7.0. Stored at 4^{o}C

ADAMS II 10X

500mg Iron sulphate Heptahydrate

500mg Copper sulphate Pentahydrate

500mg Zink sulphate Heptahydrate

200mg Manganese (II)-Chloride Tetrahydrate

10ml Hydrochloric acid

Sterile water added to a final volume of 100ml, stored at 4^{o}C.

ADAMS III

128ml ADAMS I

3.2ml ADAMS II 10X

1.6g Asparagine monohydrate

0.160g Choline Chloride

0.4g Potassium Chloride dehydrate

16g Magnesium Chloride Hexahydrate

Sterile water added to a finale volume of 800ml, and pH adjusted to 7.6, sterile filtrated and stored at 4^{o}C.

2.11.2 Solutions for agarose gel electrophoresis

50X TAE Buffer

242g Tris Base

57.1 ml Acetic Acid

100ml of 0.5 M EDTA (pH 8.0)

Sterile water added to a final volume of 1 litre. Stored at room temperature.

10x Loading buffer

2 ml 1% Bromophenol blue (0.14% v/v)

5 ml 50% Glycerol (16.7% v/v)

8 ml dH_2O

Stored at 4 ^{o}C.

2.11.3 Solutions for SDS-PAGE

10% Ammonium Persulphate

0.02g APS

200µl dH_2O

Dissolved by vortexing and prepared right before use.

0.5M Tris-HCl, pH 6.8

30.28g Tris Base

400 ml dH_2O.

pH adjusted to 6.8 with HCl, and sterile water added to a finale volume of 500ml.

1.5 M Tris-HCl, pH 8.8

90.83g Tris base

400 ml dH_2O

pH adjusted to 8.8 with HCl, and sterile water added to a finale volume of 500ml.

10% SDS

50 ml 20% SDS adjusted to a total volume of 100ml with sterile water. Stored at room temperature.

10x Tris-Glycine Running buffer.

30g Tris Base (0.25M)

144g Glycine (1,92M)

50 ml 20% SDS (1% v/v)

Final volume adjusted to 1 litre with sterile water. Stored at room temperature.

2x SDS Sample Buffer

1 ml 20% SDS (4% v/v)

1.176 ml 85% Glycerol (20% v/v)

1.250 ml 0.5 M Tris-HCl pH 6.8 (0.125M)

0.1 ml β- Mercaptoethanol (2% v/v)

0.01 % Bromophenole Blue

Final volume adjusted to 5 ml with sterile water. Stored at 4 ^oC.

2.11.4 Solutions for zymography

Refolding buffer

20mM Tris-HCl pH 7.6
50mM NaCl
20mM MgCl
0.5% Triton X-100

2.11.5 Solutions for Coomassie-staining of SDS-Gels

Coomassie staining solution

For 500ml
0.5g Coomassie Brilliant Blue
200ml Methanol
20ml Acetic acid
250 ml dH_2O

Destaining solution

50ml Acetic acid
50ml Methanol
400ml dH_2O

2.11.6 Solutions for Western Blot

0.1 M TBS

12.114 g Tris-base (0.1 M)
pH adjusted to 7.5 and final volume was adjusted to 1 litre using sterile water.
The addition of Tween-20 to a final concentration of 1% yields TBST.

10x TBS

30.1 g Tris Base
87.7 g NaCl
pH adjusted to 7.6 and final volume adjusted to 1 litre using sterile water.
The addition of Tween-20 to a final concentration of 0.05% yields TBST

Blocking solution

3% Dry skim milk in TBST (w/v)

Towbin transfer buffer

3.03 g Tris-base (25mM)

14.4 g Glycine (192mM)

100 ml Methanol (10% v/v)

Final volume adjusted to 1 litre using sterile water. Store at 4 ^{o}C.

3 Methodology

3.1 Growth and storage of *S. pneumoniae*

S. pneumoniae was grown either in liquid C-medium, made fresh the same day, or on Todd Hewitt agar plates. When grown on TH-agar plates AnaeroGen™ bags were used. (Oxoid 2002) The Todd Hewitt plates were placed in an air tight container and an AnaeroGen™ bag was placed with the plates. The bags will reduce the oxygen level in the container to less than 1% in about 30 minutes. The container was then placed in an incubator set at 37°C.

Start cultures were prepared by adding 50% glycerol to a final concentration of 15% when the cultures reached an OD_{550} of around 0.2-0.3. The freezing stocks were prepared by taking 1 ml of cell culture and 15% glycerol. The frozen stocks and start cultures were stored at -80°C until needed. When using the freezing stock, only a small amount of ice scraped from the top was used, and the stock was never thawed.

3.2 Growth experiments using Synergy H1 Microplate reader

To study the growth curves of the different *S. pneumoniae* strains under different conditions, the Synergy H1 plate reader was used. The machine reads the optical density for each selected well of the 96-well plate for a set period of time. This way the doubling time and the exponential curves can be investigated. In this study the plate reader was used to look at the effects of ComM on a *LytA* mutant (V.L 5) and ds109 with the *comM* gene behind the P_{comX} promoter. A dilution series of the inducer ComS* was used to express ComM ectopically at different levels.

The instrument was also used to examine if the V.L 16 (*ΔJanus::sfGFP-comM, ΔwtcomM::Janus)* strain still displayed immunity when induced for competence and for expression of sfGFP-comM. For this experiment SYTOX® was used to show the level of cell lysis during the growth experiment. SYTOX® fluoresces upon binding to the nucleic acids. As it cannot cross the cell membrane of living cells it can be used to monitor cell lysis in real time. When SYTOX® binds to nucleic acids it can be exited by light of 428 nm and emits a bright green fluorescence light which can be detected by reading the emission peak at 532nm.

3.2.1 Protocol for the induction of ds109 and V.L 5

1. The cells were inoculated and grown in liquid C-medium, starting at an OD_{550} of 0.025, and incubated at 37°C in a water bath for about 25 minutes.
2. While the cultures were incubating in the water bath, the 96-well plate was prepared. This was done first by adding 50 µl of C-medium in all of the appropriate wells, 100µl in the first two wells of the two dilution series. For the dilution of ComS*, 2.5µl of ComS* from a 500µM Stock was added to the first well of the dilution series, 50µl was then taken from the first well and mixed with the next well, and so on, resulting in a 2-fold dilution series of ComS*.
3. Once the bacteria cultures reached an OD_{550}-0.05, 250 µl of the cell culture was transferred to each of the appropriate wells. A blank only containing C medium was also included. RH1 and RH14 were also used as controls.
4. The plate was then put in the Synergy H1 machine, and set to 20 hours with readings at 492 nm to measure cell growth every 5 minutes.

3.2.2 Protocol for immunity test of V.L16

1. The cells were grown to an OD_{550} of 0.04 in liquid C-medium, and 280µL was transferred to each of the appropriate wells. 20µl of a 1/1000 dilution of SYTOX® was added to each well, including the blank.
2. The plate was placed inside the Synergy H1 machine and set to read OD_{492} and to detect fluorescence at 523nm when exiting SYTOX® at 428 nm.
3. Once the OD_{492} reached 0.2, the cells were induced with a final concentration of 250ng/ml of CSP-1.

3.3 Polymerase Chain Reaction (PCR)

PCR stands for Polymerase Chain Reaction, and is a method used for specific amplification of a DNA sequence. The PCR methodology allows a single copy of DNA segment to be amplified by several magnitudes following an exponential curve. These DNA segments can then further be used for diagnostic purposes, such as sequencing, or they can be used in an overlap extension of DNA fragments and transformation of bacteria. A PCR must contain a template, a set of primers (reverse and forward), buffer and a polymerase. The reaction depends on temperature, and a thermocycler is most commonly used. The thermocycler controls the temperature in the PCR-tubes, and cycles through the different temperatures at the correct time. What temperatures

and for how long the temperatures should be kept is based on the size of the DNA fragment, primers and which polymerase used. The first step in the PCR reaction is to increase the temperature to 94-98 ^{o}C, this is to denature the double stranded template DNA into single stranded DNA facilitating annealing of the primers to their complementary sequence on the ssDNA. The temperature of the primer hybridisation step of the thermoccler was set to be around the melting temperature (T_m) for the primers used. In this study the primer was designed to have a T_m of 58-60 ^{o}C based on the estimation that G & C = 4 ^{o}C, and A & T = 2 ^{o}C. The primers also need to be complementary to a sequence on the DNA strand at each end of the fragment you want to amplify. A forward and a reverse primer are required. The 3' free OH-group of the primers are the starting points of the elongation of the desired fragment, which starts synthetizing in step 4, elongation. The temperature in the elongation step is determined by the optimal temperature of the polymerase. In this study the polymerases used were primarily Phusion®DNA Polymerase and Taq-DNA Polymerase which have a temperature optimum of 68 ^{o}C and 72 ^{o}C, respectively. In the elongation step, the polymerase uses the available dNTP to synthesize the new DNA strand. The length of the desired fragment and the speed of the polymerase, decides the time required for this step. Phusion® works at a speed of around 30 seconds per kilobase for genomic DNA as template, and 15 seconds per kilobase for PCR-products as templates. Taq Polymerase uses about twice as long time per kilobase. These steps are repeated 25-30 times, and theoretically the number of target sequences doubles each cycle following an exponential curve.

Table 3.4.1. For a reaction with the Phusion®DNA Polymerase, which is used for amplifying DNA-fragments for transformation purposes and in the construction of new DNA-fragments as Phusion polymerase is a more precise but more expensive polymerase.

Volume	*Reagents*	*Final concentration/ -Volume*
X µl	Mili-Q H_2O	50 ul
10 µl	5x Phusion® HF-buffer	1x
1 µl	dNTP (10mM)	0,2mM
2.5 µl	Primer A (10pmol/µl stock)	0,5mM
2.5 µl	Primer B (10pmol/µl stock)	0,5 mM
X µl*	Template-DNA	-
0.5 µl	Phusion®DNA Polymerase (2 U/µl)	0,02 U/µl

* Generally 2µl of cell-culture, or 1 µl of each PCR-fragment.

Step	*Temperatures and time*
1. Initial denaturation	94ºC, 5 minutes (10 minutes for whole cells as template)
2. Denaturation	94ºC, 30 seconds
3. Primer hybridisation	50-60ºC*, 30 seconds
4. Elongation	72ºC, X seconds
5. Go to step 2	25-35 cycles**
6. Final extension	68ºC, 5 minutes
7. Hold	4ºC, ∞

* Depending on the melting temperature of your primer

** 25 cycles most common for PCR amplification.

Table 3.4.2. The reaction mixture when using Taq-DNA Polymerase, which was primarily used for screening of transformants.

Volume	*Reagents*	*Final concentration/ -Volume*
X µl	Mili-Q H_2O	20 ul
2 µl	Thermo-pol reactionbuffer	1x
0.5 µl	dNTP (10mM)	0,2mM
0.5 µl	Primer A (10pmol/µl stock)	0,5mM
0.5 µl	Primer B (10pmol/µl stock)	0,5 mM
X µl*	Template-DNA	-
0.5 µl	Taq DNA Polymerase (2 U/µl)	0,02 U/µl

* Generally 2µl of cell-culture, or 1 µl of each PCR-fragment.

Step	*Temperatures and time*
1. Initial denaturation	94ºC, 5 minutes (10 minutes for whole cells)
2. Denaturation	94ºC, 30 seconds
3. Primer hybridisation	50-60ºC*, 30 seconds
4. Elongation	68ºC, X seconds
5. Go to step 2	25-35 cycles**
6. Final extension	72ºC, 5 minutes
7. Hold	4ºC, ∞

***** Depending on the melting temperature of your primer

** 30 cycles common for screening.

3.3.1 **Overlap extension-PCR**

To create gene knockout cassettes and to introduce point mutations or insertions/deletions in the genome of *S.pneumoniae,* a procedure called overlap extension-PCR was employed. The principle of this procedure is to fuse two or more PCR-fragments together to form a new fragment (Higuchi, Krummel et al. 1988). To fuse two DNA fragments together they need to have complementary ends. This is accomplished by designing a reverse primer with a 5' tail which is complementary to the 5' tail of the forward primer of the PCR product it is being fused with (Figure 3.1). When these two PCR-products are used as templates in the third reaction their complementary 3' ends will anneal to each other and work as a starting point for the DNA polymerase which will close the gap resulting in a fused PCR product. Then using primers at the opposite ends of each fragment, the entire new fragment can be amplified, and can be further used in knock-outs or insertions. In this work overlap extension PCR has been used to construct the fusion gene *sfGFP-comM* and fusing the CHiC-tag encoding sequence with the CHAP encoding sequence of *cbpD*.

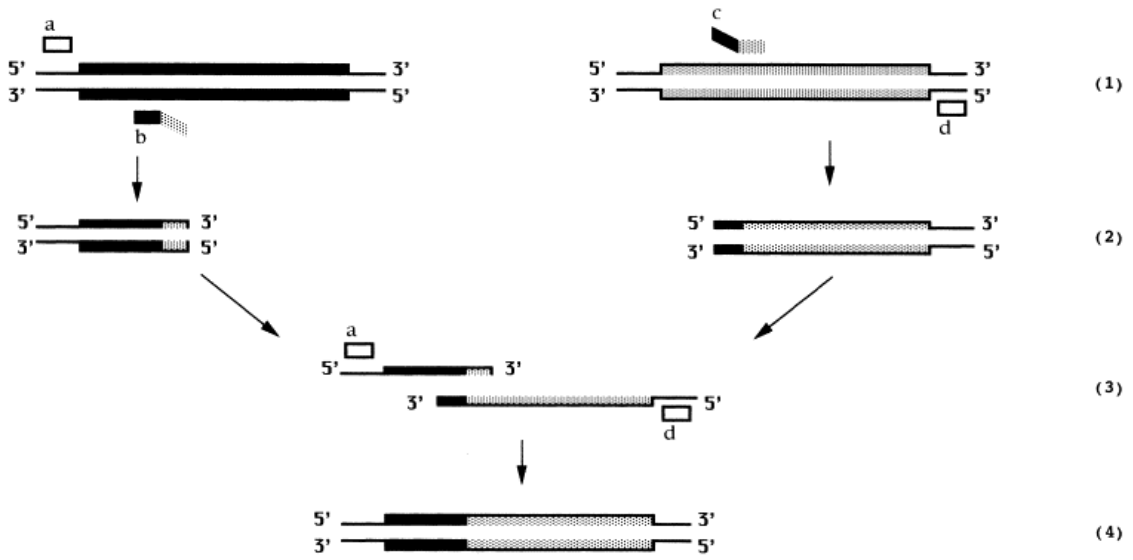


Figure 3.1 **Overlap extension PCR**. A schematic overview of the splicing and annealing of two genes to yield a new construct. a,b,c,d annotate the primers used, going in a 5'-3' direction. The construct from primer a-b and c-d are spliced together with the use of their overlapping ends to form the final product. Figure taken from (Warrens, Jones et al. 1997).

3.4 Agarose Gel Electrophoresis

To separate DNA fragments by size a method called agarose gel electrophoresis is used. The agarose gel is submerged in TAE buffer and connected to two electrodes which creates an electric current through the buffer and gel from the negative pole, cathode, to the positive pole, anode. Since DNA is negatively charged because of the phosphate groups, the DNA will migrate from the cathode to the anode. The agarose works as a mesh like network, which allows smaller DNA fragments to migrate faster through the agarose gel than larger DNA fragments. The lower the concentration of agarose the bigger the pores will be, making it easier for larger DNA molecules to travel through the gel. The DNA fragments are separated by size since the larger molecules use longer time going through the pores than smaller molecules do. To visualize the DNA, PeqGreen was added to the heat soluble agarose before casting the gel. PeqGreen binds DNA and fluoresces only upon DNA binding when exposed to UV-light. A loading buffer is added to the sample solution containing bromophenol blue and glycerol. The colouring additive bromophenol blue makes it easier to apply the sample to the wells and it migrates with the samples during electrophoresis. In addition, it migrates faster than DNA, so the electrophoresis can be stopped before the DNA migrates out of the gel. The glycerol gives the solution a higher density than the TAE buffer so that the sample sinks to the bottom of the well.

To be able to tell the size of the separated DNA fragments are, a molecular weight standard is used. It contains DNA-fragments of known sizes. In this study 1kb DNA Ladder (Invitrogen) was used.

3.4.1 Protocol for agarose gel electrophoresis

1. For 50 ml of TAE buffer, 0.5 g of agarose is added resulting in a 1% (w/v). This solution is heated to the boiling point in a microwave oven to solubilize the agarose.
2. Once the agarose was completely dissolved, the solution was cooled down to about 60^{o}C and the PeqGreen was added, 2 µl resulting in a final concentration of 0.5µg/ml in 50 ml of solution.
3. Agarose solution was then poured into a casting form, and well combs of desired size were placed in the gel.
4. Once the gel had set it was placed in the electrophoresis bath and completely submerged in 1x TAE buffer.
5. The electrophoresis was performed by using 0,6V/cm^2 for 20-30 minutes. The Gel Doc-1000 (Bio Rad) was then used to visualize the DNA using UV-light.

3.5 Extraction of DNA from agarose gel.

To extract the DNA from the gel, a kit called Nucleo Spin® Extract from Macherey-Nagel was adopted. The procedure involves cutting a DNA band of correct size from the agarose gel and placing it in the binding buffer NT. The gel is solved in the binding buffer which contains chaotropic salts causing the DNA to be more hydrophobic and more easily bind to the silica membrane of the spin column. A wash buffer, NT3, containing ethanol is then used to clean the column of unwanted contaminations and salts. To remove the DNA from the membrane an elution buffer is used, NE, containing Tris-HCl, and the DNA is stored at -20^{o}C.

3.5.1 Protocol for DNA extraction

1. The desired DNA fragment was cut out from the agarose gel using a sterile scalpel blade and transferred to an Eppendorf tube.
2. For every 100mg of agarose gel, 200µL of the NT binding buffer was added. The solution was incubated at 50 degrees Celsius until the agarose gel had dissolved.
3. The solution was transferred to a NucleoSpin® 2ml spin column and centrifuged at 11,000g for 30 seconds.

4. The membrane in the column was washed using 700µL NT3 wash buffer by centrifugation at 11,000g for 30 seconds. The membrane of the spin column was then dried by centrifugation for 1 minute at 11,000 g for 1 minute.
5. The spin column was then placed in a sterile 1.5 ml Eppendorf tube and 30µL (Or more or less depending of the strength of the band) NE elution buffer. The solution was allowed to incubate at room temperature for 1 minute and subsequently centrifuged at 11,000g for 1 minute.
6. The purified DNA was stored at -20 degree Celsius.

3.6 Selection with the use of the Janus-cassette

The Janus-cassette is a *kan-rpsL*$^+$ DNA-cassette constructed by Sung et al (2001) which can be used for negative selection of *S. pneumoniae* that are naturally streptomycin resistant (*rpsL*$^-$*)* (Sung, Li et al. 2001). This is possible because the cassette contains a kanamycin resistance marker and a counter selectable *rpsL*$^+$ marker, meaning that the cassette will make streptomycin resistant *S. pneumoniae* strains sensitive to streptomycin. This way you can select for your transformation by using TH-agar plates containing kanamycin (400µg/ml) when replacing a gene with the Janus-cassette. In fact, the cassette will confer a kanamycin resistance for successful transformations. TH-agar plates containing streptomycin (200µg/ml) can be used when removing the Janus. This is because the gene in the Janus cassette, *rpsL*$^+$, is no longer present in the bacterium. In fact, the streptomycin resistance is a recessive quality and is no longer repressed by the dominant streptomycin sensitivity of the Janus-cassette. This method can then be used to replace a specific gene with a gene of your choice via a two-step transformation. First by a Janus-cassette with flanking regions to the target gene is inserted via homologous recombination, and then it can be selected on plates containing kanamycin. For the second transformation the transforming DNA contains e.g a gene of interest with flanking sequences corresponding to the flanking regions of the Janus to be removed. When the original phenotype of being streptomycin resistant is restored, agar plates containing streptomycin can be used for the final selection.

3.7 Transformation of *Streptococcus pneumoniae*

1. The bacterial culture is grown in C-medium at 37^{o}C to an OD_{550} of around 0,05-0.1 before the cells are induced to competence by adding CSP-1 (250 ng/ml) and 0.5-1

µg/ml of the transforming DNA. A negative control is also used, where CSP-1 was added but no DNA. The cultures were the incubated for 2 hours in a water bath at 37°C.

2. After 2 hours 20µL and 100µL of the cultures were plated out on TH-agar plates containing the appropriate with antibiotics. The plates where incubated anaerobically over night at 37°C.
3. The next day the colonies were picked with a toothpick and grown in C-medium containing antibiotics, and frozen when they were in exponential growth. PCR and sequencing was used to confirm the success of the transformation.

3.8 Purification of peptidoglycan

To analyse the differences in cell wall composition of pneumococcal strains producing the immunity protein ComM along with the native composition, one needs a pure product of the cell wall that can be analysed by HPLC. The isolation of the cell wall is achieved by boiling the samples in SDS. This will solubilize the cell membrane and most of the proteins. The cell wall can then be isolated by a series of centrifugations in NaCl and water before the cell wall is mechanically broken, using Acid-Washed glass beads and the cell wall samples were finally incubated with DNase, RNase and proteases to remove any unwanted DNA, RNA and proteins.

3.8.1 Protocol for peptidoglycan purification

1. Cells of strain ds109, one control (ComM-) and one induced with ComS* (ComM+), were grown in a 1 litre container of C-medium to an OD_{550} of 0.3 – 0.5. The strain ds109 was induced at an OD~0.15 with 0.2 µM ComS*. The cells were harvested by centrifugation at 7,000 g for 5 minutes.
2. The cell pellet was then resuspended in 40 ml of 50mM Tris/HCl pH 7.4 and was added drop-wise to 120 ml of 5% boiling SDS with stirring. Once the entirety of the cell pellet had been added to the SDS, boiling was continued for 15 minutes and then cooled to room temperature.
3. The samples were then centrifuged and the cell wall material collected at 12,000 g for 10 minutes at room temperature and the pellet is washed free of SDS by resuspending and centrifugation twice with 20 ml of 1 M NaCl, and then 4 additional times, or as many as needed, with 20 ml of water.
4. The pellet is then resuspended in 2 ml of water and is mechanically broken using acid-washed glass beads. This was done using the FastPrep machine (MP BioMedicals) using

8 pulses at max speed (6.5 m/s) for 20 seconds each. Between each pulse the samples were allowed to be cooled on ice for about 30 seconds. The samples were then placed on the work bench and the glass beads was allowed to sink to the bottom. The supernatant containing fragmented cell wall material was then collected and the volume was adjusted to 25ml, and unbroken cells and larger cell debris were removed by centrifugation at 2,000 g for 5 minutes.

5. The supernatant was collected and the cell wall was collected by centrifugation at 25,000 g for 15 minutes at room temperature.
6. The pellet was then resuspended in 2 ml of 100mM Tris/HCl pH 7.4 containing 20mM $MgSO_4$, DNase (10µg/ml) and RNase (50µg/ml). The samples were then incubated at 37^{o}C for 2 hours with stirring. After the 2 hours 10mM $CaCl_2$ and 100µg/ml trypsin was added, and the samples were incubated at 37^{o}C overnight.
7. The next day SDS was added from an 8% stock to a final concentration of 1% and the samples were incubated at 80^{o}C for 15 minutes before the volume was adjusted to 20 ml with water and centrifuged at 25,000 g for 30 minutes at room temperature.
8. The pellet was then resuspended in 10 ml of 8 M LiCl and incubated for 15 minutes at 37^{o}C, before being sedimented as before.
9. The pellet was resuspended in 10 ml of 100mM EDTA pH 7.0 and again incubated at 37^{o}C, before being sedimented as before.
10. The pellet was then washed with 20 ml of water, then with 20 ml of acetone and again with 20 ml of water. The peptidoglycan was collected by centrifuging and resuspending at 13,000 g for 30 minutes. After the last wash the pellet was resuspended in about 1 ml of water and transferred to an Eppendorf tube and vacuum centrifuged to dry the cell wall sample for weighing. The amount of dry cell wall was determined and then resuspended in water to yield a concentration of 50mg/ml. The peptidoglycan samples were stored at -20^{o}C.

3.9 Preparation of stem peptides for HPLC analysis

The cell wall samples were digested using LytA amidase which breaks the N-acetylmuramoyl-L-alanine bonds in the peptidoglycan layer, resulting in the stem peptides being released. The composition of these stem peptides can then be analysed with HPLC (High-Performance Liquid Chromatography). The reverse HPLC analysis is a technique used to separate the components in a sample mixture to identify and quantify each component. The gradual increase in eluent

from the stationary phase will allow components to be released according to their hydrophobicity. As each component is released, their absorbance can be measured in the column.

3.9.1 Protocol for release of stem peptides from pneumococcal peptidoglycan

1. An amount of 2mg peptidoglycan was suspended in 100µl of 100mM phosphate buffer, pH 7.0, containing 2.5 µg LytA amidase.
2. The reaction mixture was stirred well by tapping the Eppendorf tube, and then incubated at 37^{o}C overnight.
3. The next day the sample was heated to 95^{o}C in a water bath for about 20 minutes, this was done to precipitate the LytA.
4. The sample was then centrifuged at 20,000 g for 10 minutes, and the supernatant was transferred to new Eppendorf tubes and 100µl of phosphate buffer pH 7.0 was added.
5. The sample was again centrifuged at 20,000 g for 10 minutes and the supernatants were again collected and transferred to a new Eppendorf tube.
6. The pH was adjusted to pH~ 3 by adding approximately 2µl of 20% ortho phosphoric acid, the pH of which was checked by adding 1µl of the sample onto a piece of pH paper
7. Two buffers were prepared for the reverse HPLC. The mobile phase, consisting of 0.05% Tri-fluoric acid and dH_2O to a final volume of 500ml. The second buffer, the eluent, consisting of 15% acetonitrile in 0.05% Tri-fluoric acid.
8. The column was first equilibrated using the mobile phase buffer, set at 0.5 ml/minute for 30 minutes. A volume of 30µl of the sample was then loaded on the column and the HPLC instrument was set for 206nm absorbance, and with a flow rate of 0.5 ml/min.
9. The program then proceeded with a 5 minute wash, 100 min of gradual elution from 0% to 15% acetonitrile, and finally 15 minutes of regeneration with the mobile phase.

3.10 DEAE-cellulose affinity chromatography.

To purify the CHAP domain from the CbpD protein, an *E. coli* (V.L 14) containing the pRSET plasmid with CHiC-CHAP sequence inserted was grown and induced for the expression of CHiC-CHAP using IPTG. The protein was then purified from the lysed cells using DEAE-cellulose affinity chromatography. The plasmid was transformed into DH5α and then isolated and transformed into the expression strain BL21. The column chromatography depends on the inherent ability of the CHiC tag (choline-binding histidine combination tag) to bind choline residues in the teichoic and lipoteichoic acids in the cell wall. This means it will also bind very

strongly to the DEAE-celluose in the DEAE-cellulose column as it is very structurally similar to choline. The CHiC tag consists of an N-terminal 6xHis-tag, a choline binding domain followed by a proteolytic site specific for the tobacco etch virus (TEV) endopeptidase, and was construced by Gro et al 2013 (Stamsas, Havarstein et al. 2013). This method was also used to purify LytA from the *E. coli* strain ds28, with LytA in the pRSET plasmid. The procedure is the same only in this case LytA contains a choline binding domain, so there is no need for a CHiC-tag.

3.10.1 Protocol for DEAE-cellulose chromatography

1. Volumes of 600 millilitres LB medium, containing ampicillin at a concentration of 100µg/ml, was used to grow the V.L14 strain (BL21 cells containing pRSET – ChiC-CHAP) and ds28. The culture was grown to an OD_{600} of 0.3 before it was induced with 0.1mM IPTG and placed at 20^{o}C with shaking overnight.
2. The next day the cells were harvested by centrifugation at 5000g for 5 minutes, the supernatant was discarded and the pellet was resuspended in 10mM Tris-HCl, pH 7.4 containing 100mM NaCl.
3. The cells were then mechanically lysed by shaking in the presence of acid-washed glass beads, using the fast prep 24 instrument for 6 pulses of 20 seconds at max speed of 6.5m/s. Between bursts the cells were put on ice to cool for about 1 minute.
4. Unbroken cells and insoluble cell debris were removed by centrifugation at 20,000 g for 10 minutes.
5. The column was prepared by resuspending approximately 4ml of DEAE-cellulose powder in 10mM tris-HCl pH 7.4 containing 100mM NaCl which was mixed well before settling in the column. After settling, a piece of Whatman filter was placed on top. The column was washed once with 10ml of 10mM tris-HCl pH 7.4 containing 100mM NaCl before the soluble protein fraction was passed through the column at approximately 2ml/min allowing CHiC-CHAP to bind DEAE.
6. The column was washed a total of 4 times using 15ml of 10mM Tris-HCl pH 7.4 containing 1.5 M NaCl to remove other proteins bound electrostatically to the positively charged DEAE-cellulose.
7. After the washing steps the CHiC-CHAP was eluted from the DEAE-cellulose by adding 0.14M Choline to the 10mM Tris-HCl pH 7.4 containing 1.5 M NaCl. The choline binding domain of the CHiC-tag will bind to the excess of choline in the buffer

instead of the DEAE-cellulose. The choline binding domain of LytA will also bind more strongly to the Choline.

8. The CHiC-CHAP was eluted in 2ml fractions and examined by SDS-PAGE.

3.11 SDS-PAGE

SDS-PAGE (Sodium DodecylSulphate PolyAcrylamide Gel Electrophoresis) is a method used to separate different proteins by size. This is done by the SDS binding to the proteins in the samples linearizing the protein and giving it a negatively charged surface. Larger proteins will bind more SDS and therefore be more negatively charged. The negative charge to protein size ratio is therefore constant, making it possible to separate the SDS coated proteins by size using electrophoresis. The proteins travel through the gel at a speed that correlates with the size of the proteins and the pore size of the gel itself. The pore size is determined by the acrylamide concentration. Before the samples can be applied onto the gel, the proteins need to be denatured. This is achieved by heating the samples at 95^{o}C in a sample buffer containing Tris-HCl pH 6.8, Bromophenol blue, glycerol, β- mercaptoethanol and SDS. The SDS and β- mercaptoethanol denature the proteins; the β- mercaptoethanol by disrupting the disulphide bonds between cysteine residues both intra- and inter molecularly. The SDS is a detergent and disrupts the three dimensional structure of the protein. The Bromophenol Blue is used to follow the samples migrated through the gel, as it travels with the proteins. The electrophoresis is then stopped when the blue colour is migrating out of the gel, while the glycerol makes the samples easier to apply in the wells by giving the samples higher density than water. SDS-PAGE was executed as described by Laemmli (1970) (Laemmli 1970).

3.11.1 Protocol for the preparation of the SDS- polyacrylamide gel

1. The separation gel and stacking gel solutions were mixed as described in section 2.10, and not adding the TEMED before you are ready to apply the mixture between the glass plates, as this will start the polymerisation process.
2. The glass plates were mounted in the casting frame and placed on the casting rack and then tested with water to make sure there were no leaks.
3. 3.2 ml of the separation gel was applied in-between the two glass plates, water was added on top to make sure the separation gel became straight.
4. After about 20-30 minutes, when the gel had polymerised, the stacking gel mixture was added to the rim, and the well-forming comb was inserted. The gels were allowed to

polymerise for another 20-30 minutes.

5. The gels were placed in the gel chamber, and running buffer was added between the two gels to the rim and around the mount until it covered the required amount. The well-forming combs were then removed. The samples were prepared and applied to the gel along with a molecular weight standard. The samples were separated by using 200V for 30-40 minutes or until the bromophenol blue could be seen migrating out of the gel.

To visualize the proteins in the gel, the gel was placed in a container with a Coomassie Brilliant Blue solution and gently shaken for about 1 hour. The $-SO_3^-$ anions in the Coomassie staining solutions form electrostatic interactions with the protonated amino groups in the proteins, meaning when the solution is washed off with the distaining solution, bands of protein in the gel will be coloured. Destaining was performed until the gel was sufficiently destained.

3.12 Zymography

A zymogram is an SDS-PAGE only with whole cell cultures moulded with the acrylamide. In this case cells from the strain ds109, expressing ComM or not, were used. The zymogram is used to detect enzyme activity, in this case to see what effect long term production of ComM has on the cells immunity to CbpD and to see if the CHAP domain of the CbpD protein has any effect in itself. Using ds109, where the *comM* gene is placed behind the P_{comX} promoter, we can induce the cells to produce the protein ComM without inducing for competence. The loaded samples will be inactive during the SDS-PAGE because of the denaturing effects of SDS, but will be reactivated again using the refolding buffer, resulting in the lytic proteins forming clearing zones in the gel (Leclerc and Asselin 1989). The refolding buffer contains Triton X-100 which will remove the SDS and the proteins will return to their three dimensional shape.

3.12.1 Protocol for zymography

1. Volumes of 300 ml of bacterial culture were grown for each gel, one induced with 0.05µM ComS* to drive ComM expression and one control which was not induced. The cells were grown at 37°C to an OD_{550} of about 0.3.
2. The cells were harvested by centrifugation at 5000g for 5 minutes and then the pellet was resupended in 1.25 ml of 1.5M Tris-HCl, pH 8.8 and heat treated at 95°C for 10 minutes.
3. The cells were then moulded into the 15% separation gel, and the 4% stacking gel was

constructed like previously described.

4. The electrophoresis was performed at 100V for 10 minutes for the samples to migrate into the stacking gel. The voltage was then increased to 200V when the samples reached the separation gel. The electrophoresis was stopped once the blue colouring additive was seen migrating out of the gel.
5. The zymograms were then first washed 3x30 minutes with water at room temperature with gentle shaking. Then refolding buffer was added and the gels were shaken gently at room temperature until the clearing zones were sufficiently visible.

3.13 Western Blotting

Western blotting is a procedure to detect specific proteins from a mixture of several proteins and was first described by (Towbin, Staehelin et al. 1979), in this case proteins with threonines that have been phosphorylated during competence in RH1 (wt) and RH420 (Δ*comM*). This was done by using a primary antibody from rabbit, which will bind to the proteins having phosphorylated threonine residues. This in turn can be detected using a secondary anti-rabbit antibody conjugated to a detector, in this case from goat, conjugated to Horse Radish Peroxidase. When adding the substrate for the conjugated Horseradish Peroxidase, H_2O_2 and luminol, a detectable light signal is created. The proteins are first separated using SDS-PAGE before they are transferred to a PVDF membrane using the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell. The gel and membrane are sandwiched between Whatman filters soaked in transfer buffer. The gel is placed on top of the membrane since the cathode is on the top of the transfer cell. During the electrophoresis any negatively charged molecules will migrate from the negatively charged cathode towards the positively charged anode, causing the SDS-coated proteins in the gel to move and bind in the membrane. Once the proteins have migrated to the membrane, a blocking solution made from skim milk powder is used to avoid unspecific binding of antibodies. This is avoided with the blocking solution which will block the unoccupied binding surfaces on the membrane, removing background noise.

3.13.1 Protocol for western blotting

1. The PVDF membrane and six Whatman filter papers were cut to fit the gel. The membrane was handled with gloves.
2. The PVDF membrane was activated by soaking it in 100% methanol for about 30 seconds. The filters, gel and membrane were then soaked in cold transfer buffer for a

few minutes.

3. Three filter papers where placed on the bottom plate of the transfer cell, making sure there was no air between the papers and the plate since this will disturb the protein transfer. The membrane was then gently placed on the filter papers and the rest of the filter papers were placed on top.
4. The gel was blotted for 1 hour at constant voltage of 15.
5. The membrane was then transferred to a tub containing 10 ml of 5% skim milk in TBST (50 mM Tris and 150 mM NaCl, pH 7.6, 0.05% Tween-20) and gently shaken at room temperature for 1 hour before being placed at 4^{o}C overnight.

3.13.2 Protocol for immunoblotting

1. The membrane was first incubated with the primary antibody diluted 1:1000 in TBST and incubated for 1 hour.
2. After one hour the membrane was washed three times with TBST before the secondary antibody (1:4000 in TBST) was added and incubated for 1 hour.
3. The membrane was again washed 3x10 minutes with TBST, and substrate from the Supersignal® West Pico kit was added. After about 5 minutes the membrane was placed in the Gel Doc (Azur C400 from AH diagnostics) and the light signal from the horseradish peroxidase was captured.

3.14 Remazol® Brilliant Blue release assay

To study the efficiency of mutanolysin hydrolysis on the cell wall of ds109, expressing ComM or not, the previously purified cell wall material was labelled with Remazol® Brilliant Blue which will bind to the peptidoglycan. Once the cell wall is broken by the use of mutanolysin the dye will leak into the supernatant which can be measured. Thus the efficiency of mutanolysin can be measured by measuring the amount of Remazol® Brilliant Blue in the supernatant.

3.14.1 Protocol for Remazol® Brilliant Blue release assay

1. Volumes of 50µl of cell wall material (50mg/ml) was centrifuged and resuspended in 500µl of 0.02M Remazol Brilliant Blue and 0.25M NaOH, the samples were then incubated at 37^{o}C overnight.

2. The next day the samples were centrifuged at 12, 000 g for 5 minutes and washed repeatedly until the supernatant became clear.
3. Once the supernatant was clear, 10µl was transferred to a set of 4 new tubes with 3 parallels and resuspended in 90µl sterile H_2O and 15µl of 4000U Mutanolysin was added.
4. The samples were incubated at 37^{o}C for 10, 20, 30 and 40 minutes. Then they were centrifuged at 12000g for one minute and the supernatant was collected and the absorbance was measured at 592nm.

3.15 Fluorescent microscopy

Fluorescent microscopy was used in this work to observe the location of ComM with the use of the fluorescent fusion protein sfGFP-ComM. This is possible by sfGFP emitting energy when radiated with light with a wavelength of 488nm. When radiated at this wavelength the GFP is excited and emits light which can then be photographed though a microscope objective. The cell cultures were first induced with the desired concentration of ComS* and 4µL was placed on glass slides to be examined in the Zeiss Microscope.

3.15.1 Protocol for fluorescent microscopy

1. The strain V.L 16 (*sfGFP-ComM*) was incubated at 37 degrees Celsius to an OD_{550} of about 0.2
2. Once the cells reached the appropriate optical density, they were induced with the desired ComS* concentration. Ranging from 0.2µM to 0.025µM. The cells were then incubated further for 15 minutes.
3. 4µL was then transferred from the cell cultures to sterile microscope slides, and a cover glass was placed on top.
4. The samples were then viewed in the Zeiss LSM microscope, using a 488nm wavelength to excite the sfGFP-ComM.

4 Results

4.1 The effect of ComM on auolysis in *S. pneumoniae*

When *S. pneumoniae* are induced for competence they start producing the fratricine CbpD to kill nearby cells that are closely related. Consequently, the competent cells will have to protect themselves from fratricines of their own and other competent cells. Among the genes that are upregulated early in the competence stages is *comM,* codes for ComM, which works as an immunity protein protecting the cell against the cell wall hydrolase CbpD. The mechanism behind the ComM induced immunity against CbpD, is still unknown. ComM is an integral membrane protein, while CbpD targets the cell wall of target cells from the outside. This makes it unlikely that ComM protects the cells by direct interaction with CbpD. The substrate of CbpD is the peptidoglycan layer, and it is reasonable to think that ComM could be responsible for inducing some modifications, or several modifications, to the peptidoglycan or to other parts of the cell envelope, preventing CbpD from damaging the cell wall. This notion is supported by unpublished data from our laboratory. The transient expression of ComM during competence constitute a challenge when studying its effects on the cells. For this reason we chose to take advantage of the ComRS system to be able to express ComM continuously at different levels during growth, in order to see if prolonged expression of ComM could give a better understanding of how the protein works.

This experiment was conducted in two parts. First we wanted to examine the growth of cells overexpressing ComM during the entirety of the growth period. We reasoned that if ComM somehow alters the normal structure of the pneumococcal cell wall, this could also affect cell growth. The second part was to see whether the activity of LytA (the major pnumococcal autolysin) was in any way influenced by the presence of ComM, as LytA cleaves the same stem peptide substrate as CbpD is predicted to do. To test this the *S. pneumoniae* strains ds109 and V.L 5 (both expressing *comM* with the ComRS system) were grown in the presence of a dilution serious of ComS*. The V.L 5 strain has deleted the *lytA* gene in its genome, and was used as a LytA negative control. Any difference in growth or lytic activity by LytA in ComM expressing

cells was monitored by measuring the optical density (OD) throughout growth and into the stationary phase. (Figures 4.1 and 4.2)

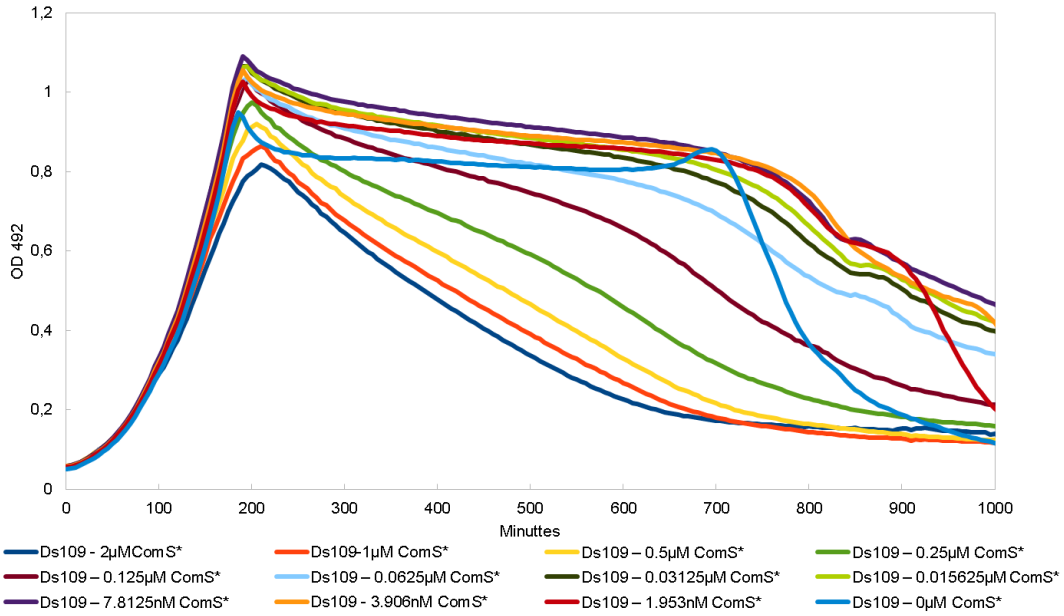


Figure 4.1 **Growth curves for ds109.** The figure shows the dilution series of ComS*, resulting in overexpression of ComM in the highest dilutions. Readings were done each 5 minutes for 16 hours and 40 minutes. 0µM ComS* shows a standard growth curve for *S.pneumoniae* while the over expression of ComM causes stress to the cells, and they never reach the same optical density as the control.

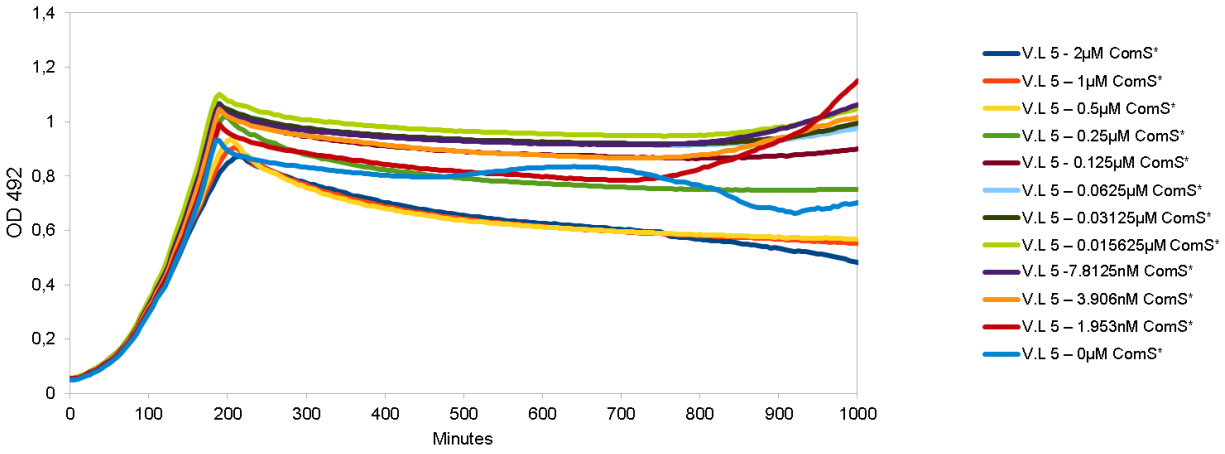
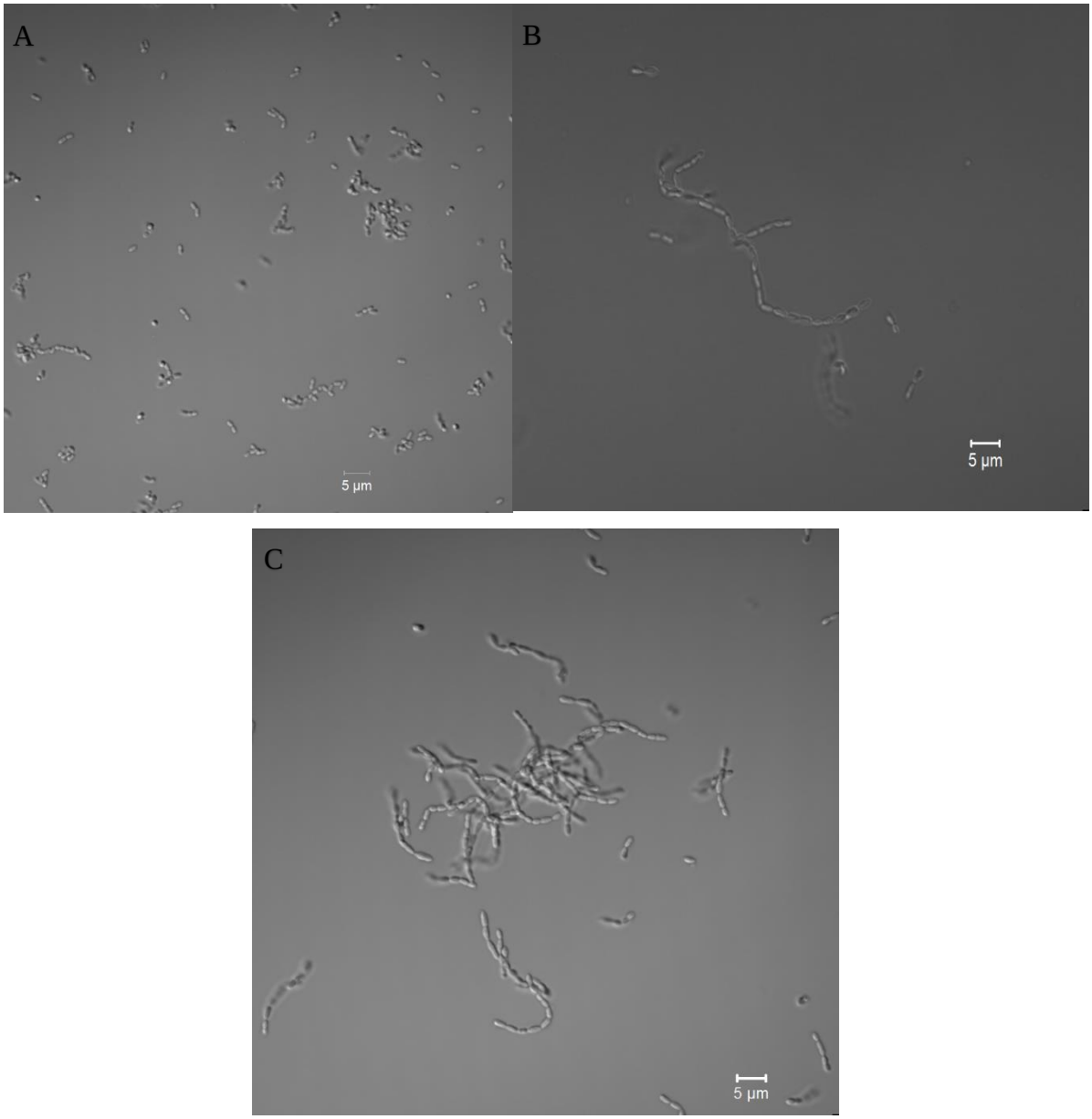


Figure 4-2 **Growth curves for the LytA mutant V.L 5**. These curves demonstrate a dilution series of ComS* on strains without LytA, demonstrating the extended stationary phase that is characteristic of LytA mutants. Higher concentrations of ComM does not reach as high optical density as the control.

During the stationary phase of *S. pneumoniae* some cells spontaneously undergo lysis, and as LytA is expressed constitutively, it will be released from the dead cells once their cell wall is destroyed during lysis. The release of LytA will then target living cells and result in the lysis of these cells. Eventually the cumulative LytA in the medium will result in the lysis of all the cells.

This can be seen in Figure 4.1, especially for 0µM ComS* where after about 700 minutes the cells lyse. In figure 4.2 the bacteria do not have LytA, so no lysis is observed and we see the characteristic extended stationary phase. In Figure 4.1 there can be seen a difference in lysis already at 0.015nM ComS*, where the stationary phase is not particularly extended but the decay rate is clearly affected. With only small amounts of ComM, LytA ceases to be as effective as with no ComM. The reason for this might relate to some modification of the cell wall which affects the efficiency of LytA. The growth experiment showed that the highest levels of ComM expression resulted in a slight reduction in growth rate, and that the cells reach stationary phase at a lower density compared to cells not expressing ComM. This makes sense since ComM has been known to cause stress to the cell (Unpublished data). Interestingly, the LytA positive cells expressing highest levels of ComM started to lyse immediately after reaching stationary phase. However, the rate of lysis seems to be slower than normal autolysis of the control cells taking place further into the stationary phase. For the cells expressing lower levels of ComM autolysis occurred later in the stationary phase, but still with a slower rate than ComM negative cells. One thing that can be noted is that even though the cells do not reach a proper stationary phase, they still do not decay as rapidly as the non-immune cells, supporting the hypothesis that ComM could function by inducing changes to the cell envelope. However, this needs to be verified experimentally.

To demonstrate the stress that higher levels of ComM induced in *S. pneumoniae*, a microscope experiment was conducted on ds109 with different concentrations of ComS* to see that this stress looks like on the cells.

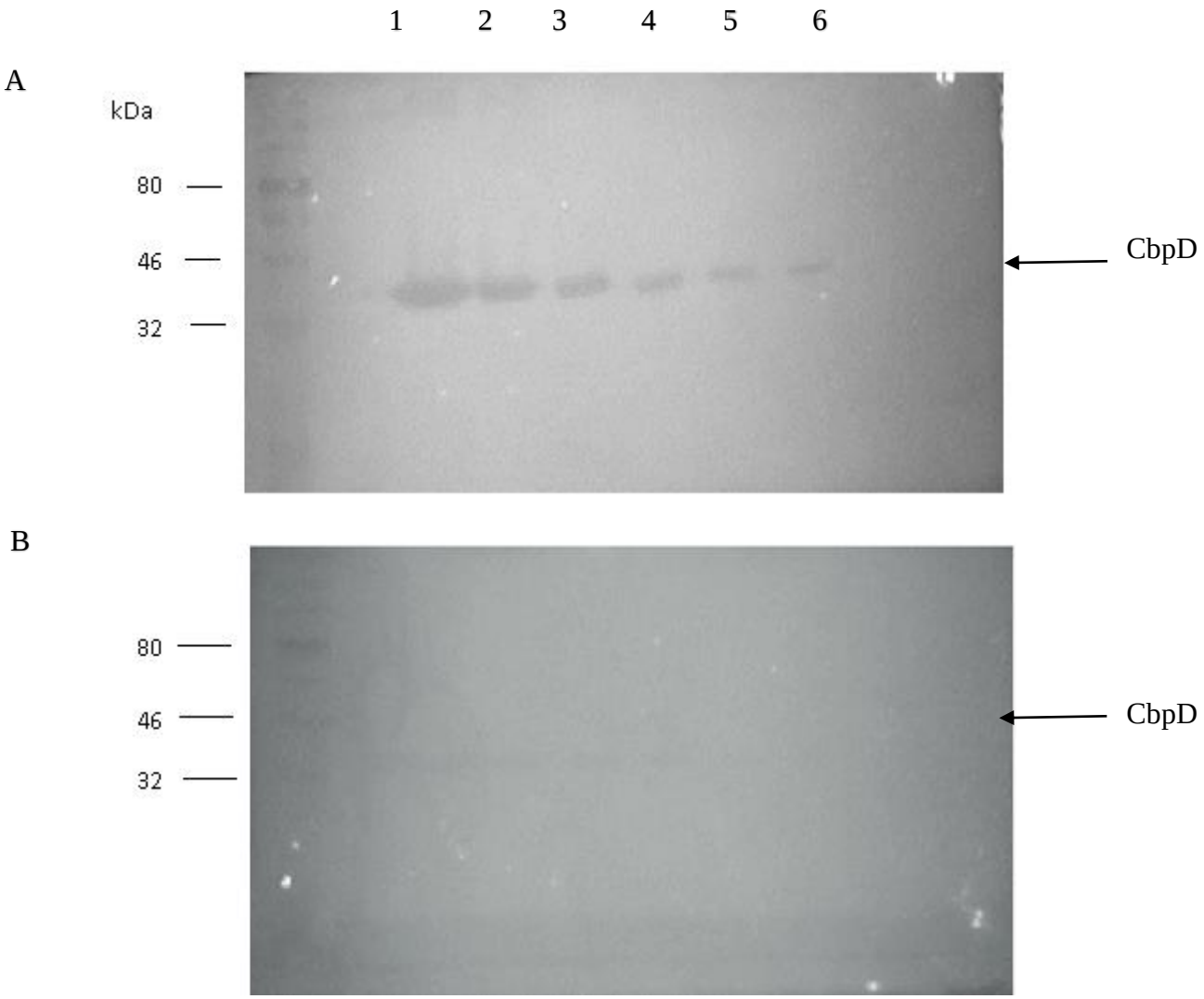


Figur 4.3. Microscope pictures of ds109. (A) – Uninduced. (B) – 0.05µM ComS* and (C) – 1µM ComS*. Demonstrating the chain formation and shows signs of elongated cells.

The microscopy of ds109 with 1µM ComS* demonstrated that the cells grew in long chains, and also showed signs of changed morphology in the form of elongated cells. This elongation is that as clear at lower levels of ComM (Figure 4.3- B) but the chain formation is still evident. As a control, ds109 uninduced (Figure 4.3- A) shows normal *S. pneumoniae* cell growth.

4.2 Hydrolytic activity of CbpD on immune cells

The hydrolytic activity of CbpD on pneumococcal cell wall can be assayed by doing zymography. Previous experiences with CpbD in Prof. Håvarstein's laboratory have shown that the life time of purified CbpD in solution is very short. However, in zymogram assays the activity of CbpD is prolonged, thus this assay is suitable to monitor its hydrolytic activity. In brief, zymography is based on an SDS-PAGE gel that contains whole cells or cell wall material as substrate for the cell wall hydrolases. The enzymes are separated in the gel by conventional SDS-PAGE and refolded in the gel by incubation in a refolding buffer. Any hydrolytic activity can be seen as a clearing zone in the opaque gel. To study the effects of long term production of ComM in regards to gained immunity towards the cell wall hydrolase CbpD, whole cells of the strain V.L 5 was used as substrate. V.L 5 growing in C-medium containing 0.05µM ComS* (previously found to provide optimal ComM expression for immunity) was used as immune cells, while cells grown without ComS* was used as CbpD sensitive substrate. This strain does not express LytA which would lyse all the cell material in the gel. Any clearing zones are therefore from the activity of CbpD (previously isolated in the laboratory) loaded onto the gel. This experiment will show whether ComM expressing cells that are heat inactivated and treated with SDS still show immunity to CbpD.



Figur 4. 4 **Zymograph analysis of CbpD on V.L 5, induced (B) and not induced (A) for the expression of ComM.** A zymogaph analysis of V.L 5 with a 1/2x dilution series of CbpD (1-6) showing the lytic zones created after 1 hour and 30 minutes of incubation in refolding buffer. This was the time that weak bands started to appear in the induced (B) zymogram, while the lytic zones in the uninduced (A) appeared much earlier.

Both these pictures were taken 1 hour and 30 minutes after being incubated in refolding buffer, which allows the CbpD to refold into its active three dimensional shape. While the first signs of clearing zone in the control first appeared after about 30 minutes, weak bands in the zymogram with induced V.L 5 cells did not appear until 1 hour and 30 minutes, as can be seen in Figure 4.4. This experiment demonstrated that ComM gives immunity to the competent cells from even high concentrations of CbpD, and extended production of ComM from an early stage does not diminish the effect of the immunity protein. Further exposure to refolding buffer did not have any effect on the lytic zone of the induced zymogram. It also shows that immunity is retained in the cells even after ComM was removed from the cells by SDS, demonstrating that

it is not ComM directly that gives immunity. The resistance against CbpD is rather an effect of a physiological change that ComM has induced on the cells during growth.

If the resistance against CbpD is the result of a physiological change, it could mean that immunity is related to the choline binding domain or the SH3 domains. To test this, the CHAP domain of CbpD was isolated to see if the CHAP domain was still catalytically active on immune and sensitive cells. The protein CbpD was also tested on the zymograms as a control.

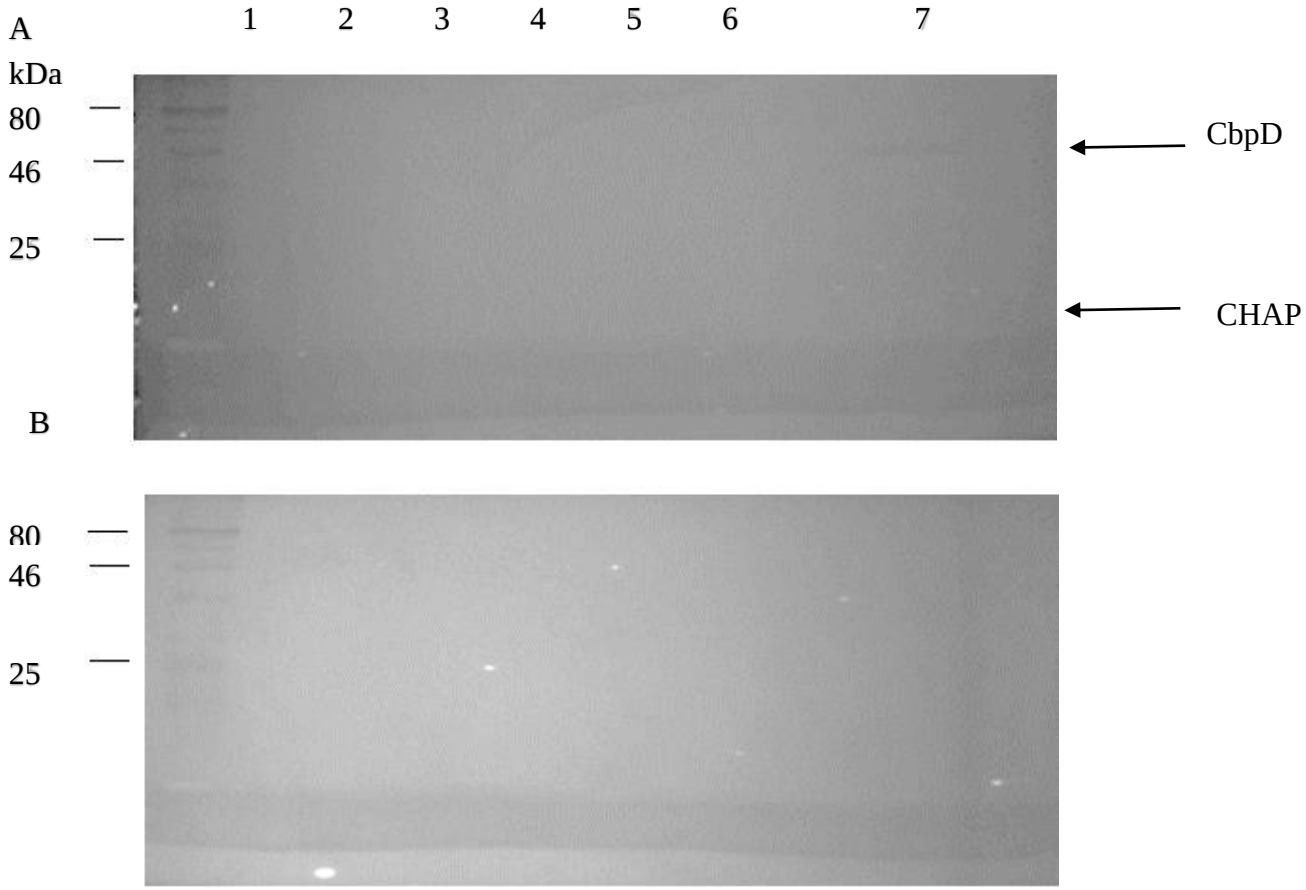


Figure 4. 5 **Zymograph analysis of CHAP domain on V.L 5.** A second zymograph analysis was performed using V.L 5 and a 1/2x dilution series of the purified CHAP domain (1-6) and CbpD (7) on an induced (B) and uninduced (A) 15% SDS-page zymogram. The lytic zone for CbpD is marked, and the expected location of the lytic zone of the CHAP domain is also marked. B was induced using 0.05µM ComS* and the pictures were taken after refolding for 34 minutes. No lytic zones for CHAP domain was observed.

After 34 minutes, the first signs of lytic zone appeared in the zymogram containing no ComM, but only for the control sample of 15µL of CbpD, while the dilution series of the CHAP domain did not show any signs of lytic zones. Even after 24 hours, there were still no lytic zones for the CHAP domain in either zymogram, but a clearing zone did appear in the induced zymogram

for the CbpD, but this was much weaker than for the non-induced. It also appeared several hours later for the cell culture without ComM.

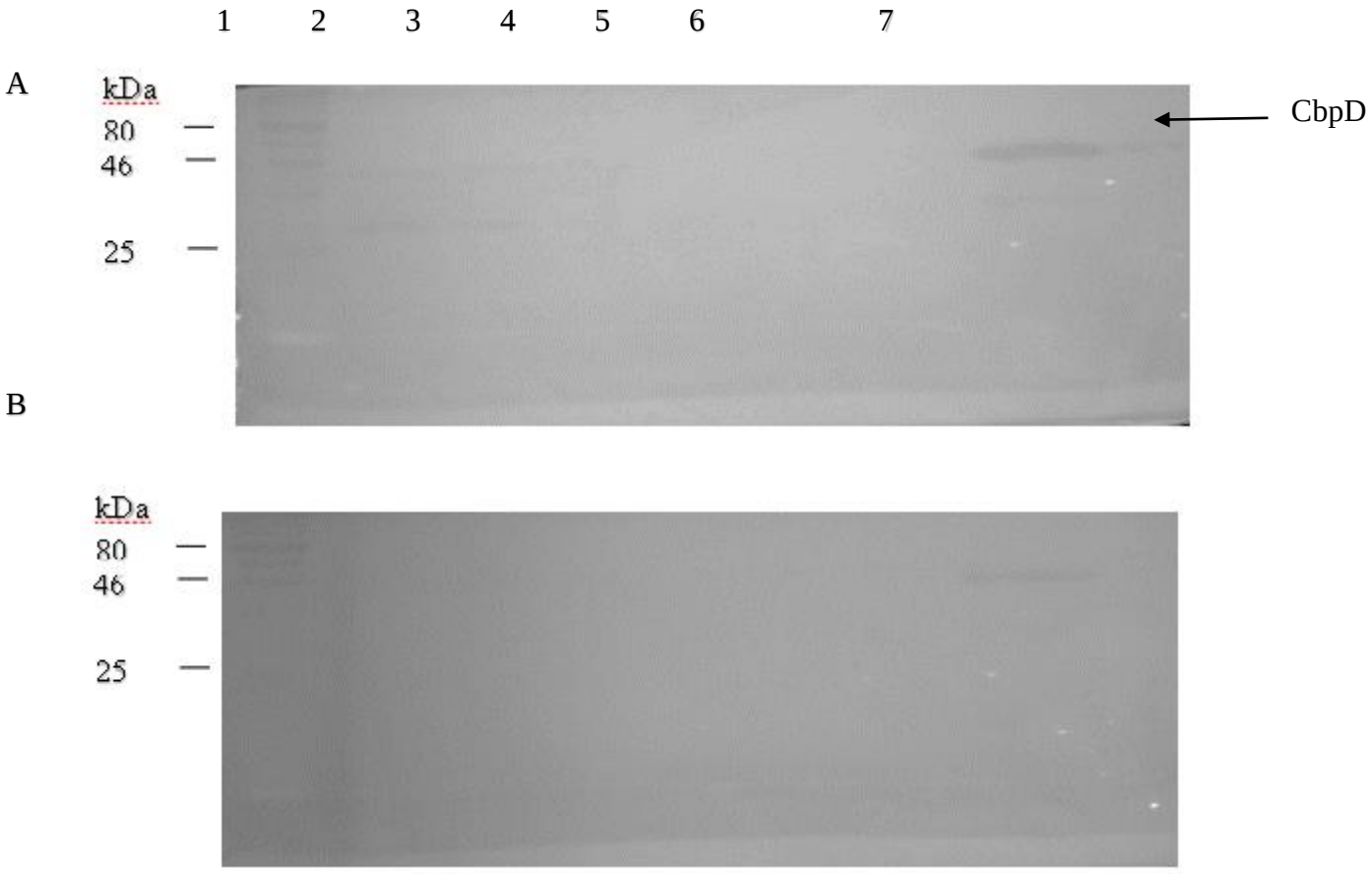


Figure 4.6 **Zymograph analysis of CHAP domain on V.L 5 after 24 hours.** After 24 hours a weak lytic zone appeared on the induced zymogram (B), though much weaker and later than the uninduced one. Still no bands for the CHAP domain.

This experiment showed that the catalytic domain of CbpD is not active without its choline binding domain and its SH3b domains. Whether this is because the active site simply cannot find and bind the substrate without the CBD and SH3b domains, is unknown. There might also be a conformational change in the catalytic site that occurs during choline binding and which allows the protein to bind its substrate.

4.3 Possible ComM influence on the DivIVA phosphorylation

DivIVA is a protein involved in the cell division of *S. pneumoniae* and other Gram-positive bacteria. The proteins are most frequently studied in the bacteria *Basillus subtilis*, and they work together with a complex consisting of two proteins, MinC and MinD. This complex inhibits cell division at the poles of the cell. This strongly indicates that DivIVA has a predominant role in the cell morphology and cell division (Nováková, Bezoušková et al. 2010). DivIVA is activated

by the Serine/threonine-protein kinase StkP, which phosphorylates serines and threonines. The phosphorylated threonines can be detected using immunoblotting. Because of this, it is of interest to see whether ComM influences the phosphorylation of this protein involved in cell division. An immunoblotting experiment was conducted using a primary antibody that binds to the phosphorylated amino acids threonines. The samples used were RH1 and RH420 which were induced for competence with CSP-1 and incubated for 5, 10, 15 and 30 minutes, respectively. This experiment was performed to see if there was any differences in the induced RH1 and the ComM mutant RH420, which could point to ComM being responsible for activating a serine/threonine specific kinase in the competence stage.

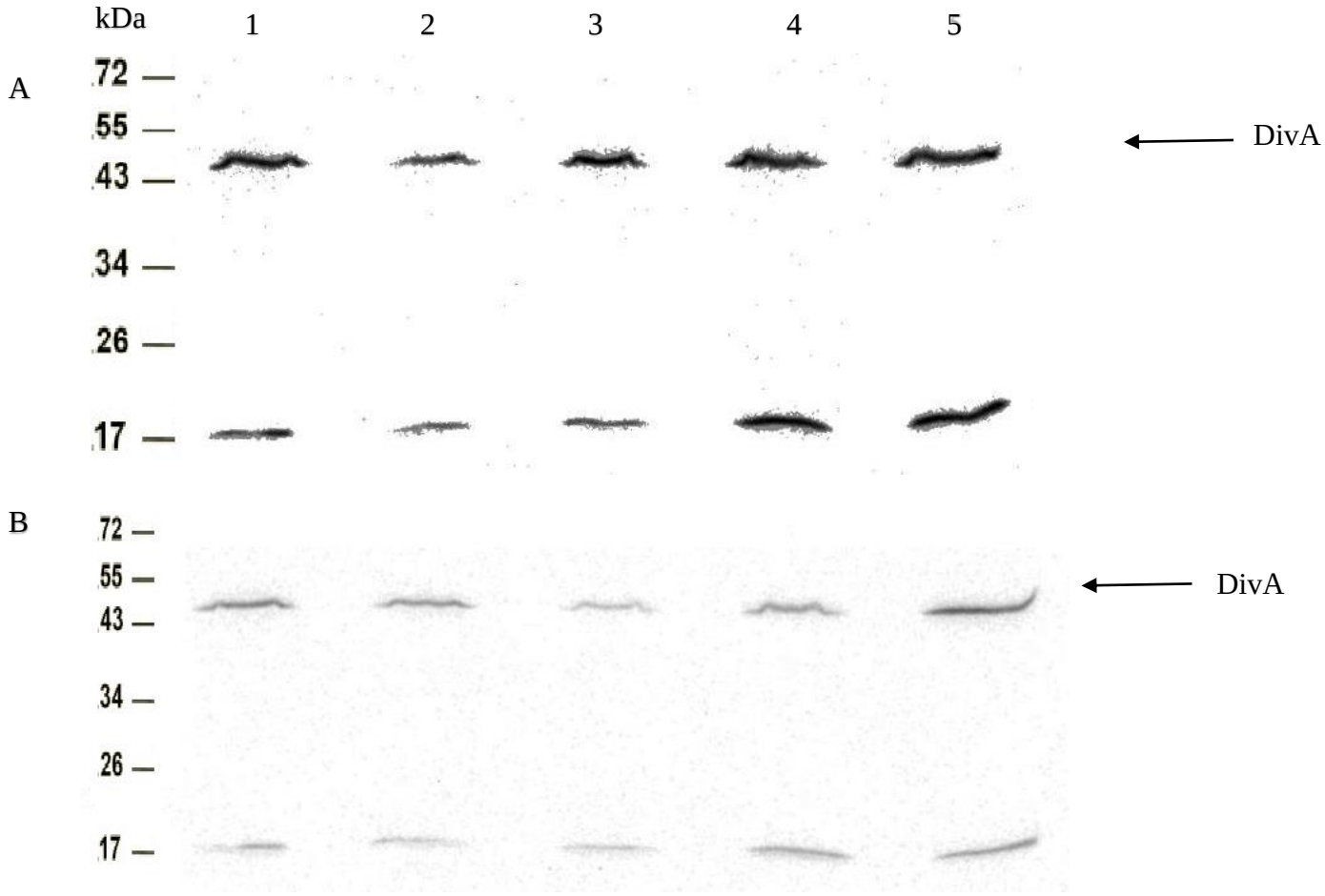


Figure 4. 7. **Western blot of RH1 (A) and RH420 (*ΔComM*) (B)** A western blot using primary antibodies that bind to the phosphorylated amino acid threonine. Both cell cultures were induced with CSP-1 and incubated at different time intervals. (1)- uninduced, (2)-5 minutes,(3) – 10 minutes, (4) – 15 minutes and (5) – 30 minutes. The two bands show the two proteins Div4A and an unknown protein, which show no differences in phosphorylation during competence.

Any phosphorylated protein gives off a bright fluorescence when exposed to UV light, and the two proteins DivIVA and an unknown protein are clearly shown in Figure 4.6, but no other bands can be seen. There is also no difference in degree of phosphorylation between the samples

when taking into account the OD_{550} differences from 5 to 30 minutes of induction. These results show that competence does not affect the phosphorylation of these proteins, and that ComM being expressed does not affect the level of phosphorylation.

4.4 Purification of LytA.

To examine whether ComM render competent cells immune by alteration of the stem peptide composition in the cell wall, HPLC was used to compare the stem peptides from immune cells with those of sensitive cells. In order to analyse the stem peptide composition in the cell walls the amidase LytA was used to release stem peptides from the peptidoglycan. Therefore, LytA first needed to be purified from an *E.coli* strain (ds28) containing the *LytA* plasmid (pRSET A). The strain ds28 will then express LytA when induced with IPTG as this strain includes a T7 polymerase cloned into the genome which is needed to express genes (*LytA*) placed behind the IPTG induced promotor *T7lac*. The culture was grown in LB-medium and induced with IPTG at an OD_{550} of 0.3. The strain expressed LytA for four hours before being harvested at 8,000g and lysed. The LytA from the cells were purified using the DEAE-cellulose affinity chromatography described in Methodology. LytA was eluted from the column in 2 ml fractions which were analysed in a 12% SDS-PAGE (Figure 4.8).

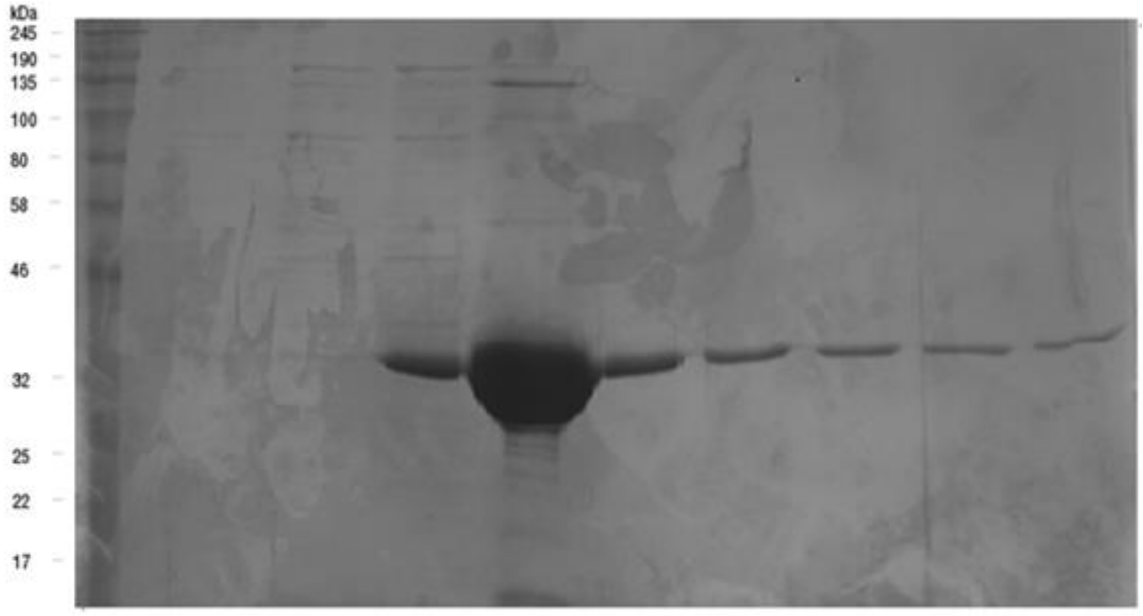


Figure 4 .8. **SDS-PAGE for the 9 fractions from the column chromatography.** From each of the fractions 40µL were taken and mixed with 40µL SDS sample buffer and applied onto the gel. All wells were used. Fraction 4 clearly had more protein than the other fractions. LytA has a mass of about 36kDa.

Fraction four contained most LytA with approximately 90% purity. The concentration of LytA in this fraction was determined based on its absorption at 280nm and the percent extinction

coefficient of LytA ($\varepsilon_{percent}$ = 3.156). The purified LytA sample gave an absorption of 11.69 at 280nm when taking into account the 100x dilution. Since $A_{280}/\varepsilon_{percent}$ = mg/ml, the concentration of LytA was estimated to be 3.7mg/ml. The purified LytA was aliquoted and stored at -80 degrees Celsius until further use.

4.5 Stem peptide composition of peptidoglycan from sensitive and immune cells.

It would seem that ComM leads to modifications in the pneumococcal cell envelope that leads to immunity against CbpD. We wanted to examine if this could be modifications of the stem peptides or their composition. To study the differences in the stem peptide composition between cells with and without ComM expressed, a reverse phase HPLC analysis was performed on previously purified cell wall from ds109, induced with ComS* (ComM+) and not induced (ComM-), respectively. Once the cell peptidoglycan was purified it was treated with LytA (10µg/ml) for 24 hours. LytA cleaves the peptides that crosslink the two sugar molecules N-acetylglucosamine and N-acetylmuramic acid leaving only various peptides of variable amino acid length. These peptides can now be analysed using reverse phase HPLC, which will separate the different peptides based on their hydrophobicity using a gradual increase in elution. The HPLC analysis should then be able to show if ComM is responsible for any changes in the peptides in the bacterial cell wall, changes that would contribute to the cells' immunity towards CbpD.

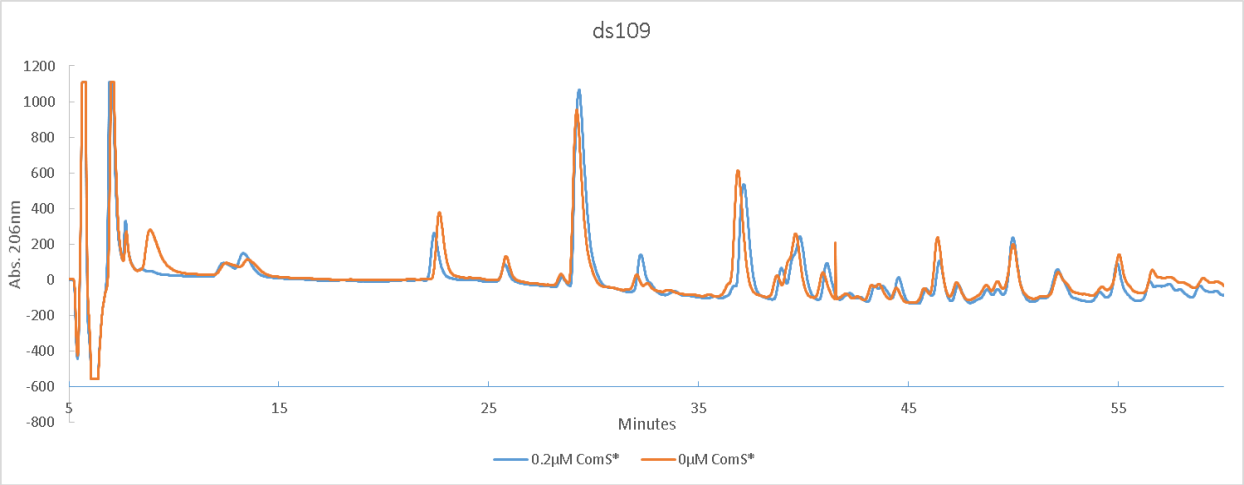


Figure 4. 9. **HPLC analysis of the stem peptides of immune and non-immune cells.** Graph showing the HPLC analysis of ds109, induced with 0.2µM ComS* and uninduced, respectively. Each peak indicates a peptide chain, while the magnitude determines the amount of peptide. Apart from the one peak around 10 minutes, there are no differences that can be noticed in the cell wall composition of the two samples.

The HPLC analysis did not show any significant differences in stem peptide composition between the two samples. The magnitude of the peaks determine the amount of peptide, and the later a peak is eluted the more hydrophobic the peptides are, which indirectly reflects the size of the peptides. As shown in Figure 4.9 all stem peptides eluted at the same time and with more or less similar accounts between the two samples, indicating that ComM is not responsible for any major changes in the peptides composition in the peptidoglycan layer of *S. pneumoniae.*

4.6 Mutanolysin susceptibility of immune cell wall material

Since the activity of LytA and CbpD, the hydrolytic activity of which is targeting the stem peptides, were affected when immune cell walls served as substrate, we wanted to examine whether this also was the case for peptidoglycan hydrolases targeting other parts of the peptidoglycan than LytA and CbpD. Mutanolysin, which breaks the 1-4β glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid in the peptidoglycan, was chosen for this purpose. A so-called release assay using Remazol Brilliant Blue® was conducted on the purified cell wall materials from immune and non-immune cells, respectively, using mutanolysin. The release assay was done by incubating the peptidoglycan samples in Remazol Brilliant Blue® and NaOH over night at 37°C. The samples were then centrifuged and resuspended in dH_2O until the supernatant was clear, then the samples were treated with 40

Units mutanolysin for different time intervals to study the differences in hydrolytic efficiency of the two samples. Remazol Brilliant Blue® binds to the peptidoglycan and is released when the cell wall is broken down by the mutanolysin, resulting in dye leaking into the supernatant and which can be detected by measuring the absorbance of the supernatant. Any significant differences in mutanolysin activity between immune and non-immune peptidoglycan would indicate that cells expressing ComM somehow have altered their cell wall compared to the non-immune control cells.

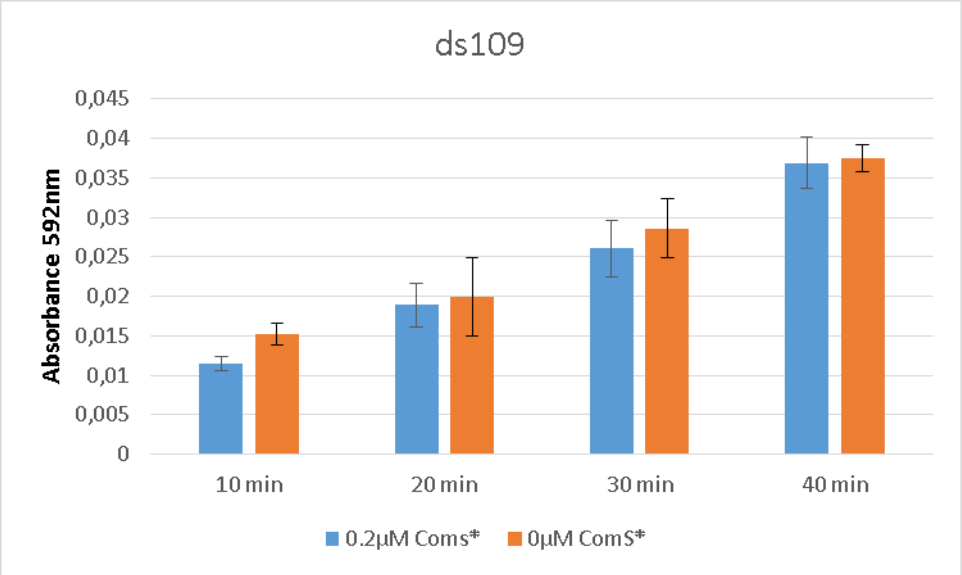


Figure 4. 14. **Release assay of ds109 cell wall.** Cell wall material from ds109 induced (Blue) and uninduced (Orange) were tested using a Remazol Brilliant Blue® release assay. The absorbance at 592nm was measured after treating the cell wall material with mutanolysin for different time periods. A slight difference during the first 10 minutes can be seen, but eventually evens out. The experiment was conducted 3 times, these are the averages with standard deviation calculated.

As can be seen in the figure above the difference between the two samples are not very significant, but there is some slight difference in the first 10 minutes. It would seem that mutanolysin has a slightly smaller efficiency in the first 10 minutes, so perhaps ComM slightly delays the reaction, but this is difficult to assert by this experiment alone as the result is not very significant.

4.7 Localization of ComM in *S. pneumoniae*

4.7.1 Design and immunity of Superfold GFP-ComM

Considering that CpbD specifically targets the septal region of pneumococcal cells, it is reasonable to assume that ComM is localized in this area, where the new cell wall is synthesised. So if ComM is responsible for modifications of the newly synthesised cell wall it should be located close to where the new peptidoglycan is synthesised. To try to figure out where ComM is located in the cell a protein called GFP, Green Fluorescent Protein, was used. This protein was first isolated from jellyfish called *Aequorea victoria*, and has since been used extensively as a fluorescent reporter. In this thesis a modified version of GFP called superfold GFP was used. This is an enhanced version of the protein in regards to proper folding. The *sfGFP* gene was fused to *comM* using overlap extension PCR resulting in a fusion gene that would produce ComM having sfGFP at its N-terminus. The *sfGFP-comM* gene was placed behind the *PcomX* promoter of SPH131. ComS* could now be used to control the amount of sfGFP-ComM produced, which can be seen in the microscope by exposing the cells to a laser with a wavelength of 488 nm. Before the cells were studied in the microscope, they were first tested to see if ComM was still active while coupled with sfGFP. This was done using a micro well plate reader by letting different amounts of ComS* drive sfGFP-ComM expression and then inducing the cells to competence using CSP-1 at an OD_{492} = 0.2. The cells used had the native ComM first knocked out with the use of the Janus Cassette so that the native ComM did not interfere with the immunity of sfGFP-ComM.

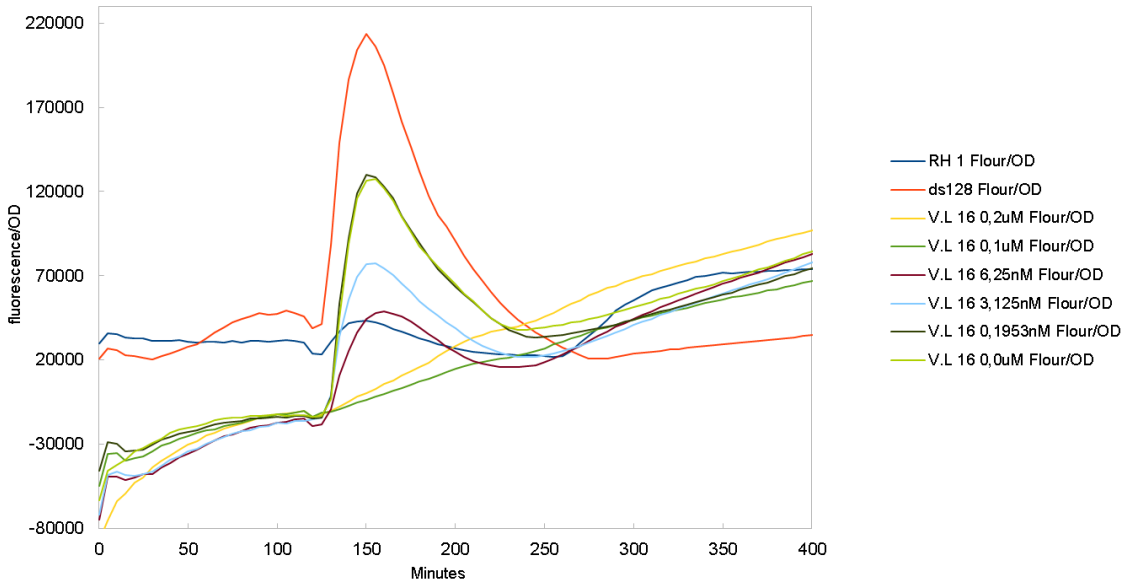


Figure 4. 10. **Relative fluorescence units of V.L 16 demonstrating immunity from sfGFP-ComM.** A dilution series of ComS* was used to express sfGFP-ComM at OD_{492}-0.05, and the cultures were then induced with CSP-1 once they reached and OD_{492} = 0.2. RH1 and ds128 were used as controls. As the graph shows the higher concentrations show immunity, and the lowest still showed signs of immunity.

As seen in the figure above (Fig.4.7), the cells were immune to a point in between 0.1µM and 6.25nM ComS* , showing that sfGFP-ComM was still active. The ds128 (RH420) was used as a *ΔComM* mutant which is lysed by CbpD giving a high peak at two and a half hours, while RH1 and the two first dilutions of V.L 16 are immune and show no peaks at this time. The peak is the emission from the SYTOX® used to illustrate cell death as it will bind to DNA released from lysed cells. Once it had been established that the activity of ComM had not been compromised by the addition of sfGFP, the cells were ready to be studied under the microscope.

4.7.2 **Microscopy of sfGFP-ComM**

As sfGFP-ComM was functionally active its localization would probably be similar as the native protein. Fluorescence microscopy was therefore used to detect the cellular localization of sfGFP-ComM. For examination by microscopy the cell cultures of V.L 16, where first grown to an OD_{550} of about 0.2 before being induced with ComS* concentrations. Different concentrations of ComS* were used to find the optimum expression level of sfGFP-ComM that would give a clear signal in the microscope. The cells were also allowed to express sfGFP-

ComM for time periods ranging from 5 minutes up to 30 minutes. Once the cultures were induced with the desired concentration of ComS*, 4µL was placed on glass slides and examined.

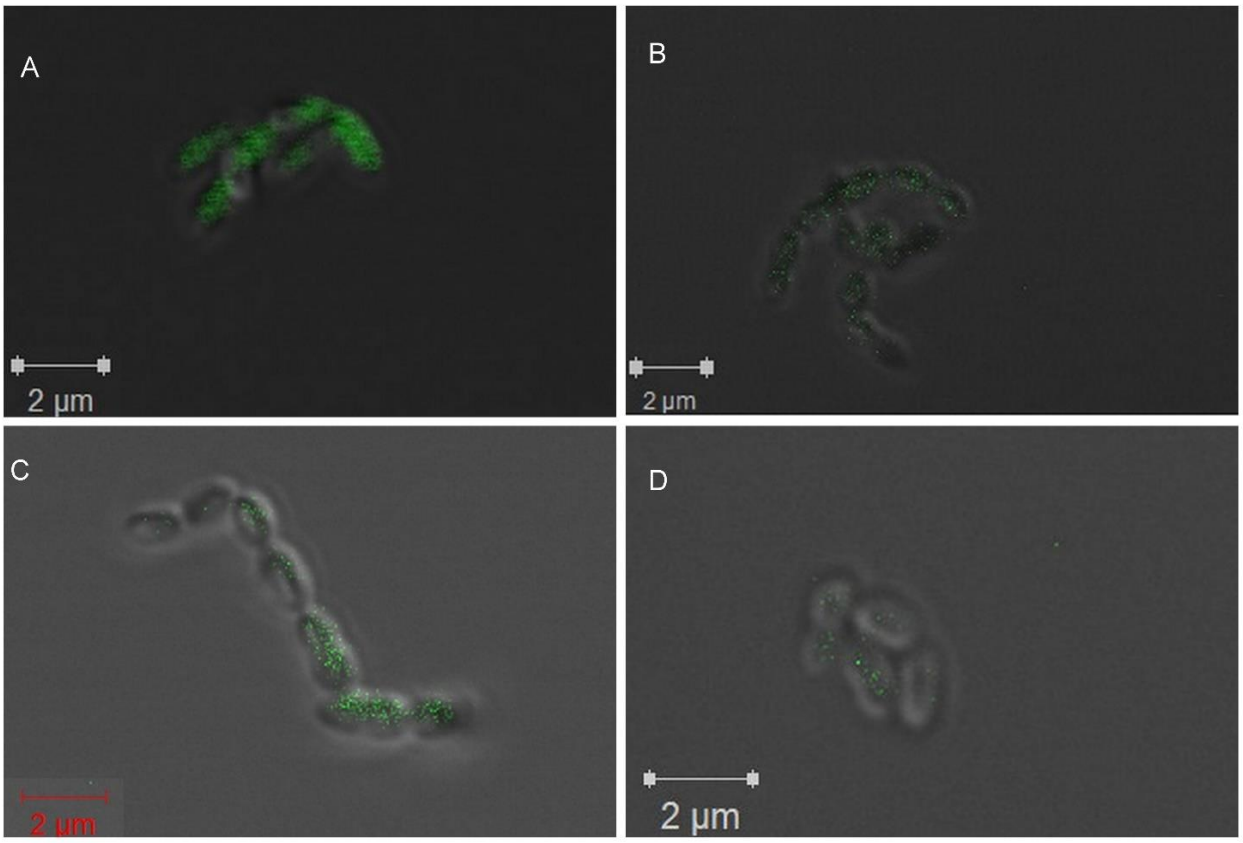


Figure 4.11. V.L 16 induced with ComS*. A-D was taken after 15 minutes, the four pictures demonstrate different ComS* concentrations. A – 0.2µM ComS*, B – 0.1µM ComS*, C – 0.05µM ComS* and D – 0.025µM ComS*. The pictures were taken with the Zeiss fluorescence microscope. These pictures show the random spread of signal throughout the cell, even at low concentrations.

The first two pictures A and B in Figure 4.11 were both taken after 15 minutes of induction with 0.2 µM and 0.1µM ComS*, respectively, while the two others, C and D, were induced with 0.05µM and 0.025µM ComS*. These concentration were chosen to potentially achieve a small amount of signal from the GFP-ComM, because as higher concentrations result in an abundance of fluorescent proteins, while lower concentrations showed little to no signal.

In the pictures A, B and C an increased amount of green signal is observed and also that the cells form long chains which is common for cells that overexpress ComM, as they appear to have problems of separation during cell division. Any lower concentrations than 0.025µM resulted in a signal too weak to be observed. Fifteen minutes seemed to be the optimal induction

time, as any shorter resulted in little signal, while any longer resulted in the stress from ComM to become more apparent without the addition of more signal.

Another observation that the pictures do not convey is that the signals observed would fluctuate and were not fixed at one location as, one would expect. It would seem, based on the observation made from this experiment that the signal observed does not represent the true location of ComM.

5 Discussion

ComM has been shown to be a decisive factor in the process of fratricide during competence in *S. pneumoniae.* That is, ComM protects the cell against suicide of their own lytic fratricide enzymes. It is therefore important to fully understand ComM's mode of action and the way competence and the acquisition of exogenous DNA is achieved. In fact, knowledge about the workings of ComM are indeed very limited. ComM allows for the ingenious way by which *S. pneumoniae* acquire homologous DNA that is not too closely similar to its own, as this would be redundant. By using the quorum sensing pheromone CSP, which varies slightly among strains, identical pneumococcal strains are induced to produce the immunity protein ComM while others, not recognizing the secreted CSP, become a target for the bacterial fratricides. It has previously been demonstrated that the immunity granted from ComM is targeted against the key murein hydrolase CbpD. So, if CbpD enables the effects of the autolysins LytA and LytC, this is prevented by initially inhibiting CbpD.

5.1 ComM expression is a stress factor for the cells

By using the ComRS system, the expression of ComM was manipulated without inducing the cells for competence. This experiment demonstrated the extended stationary phase with the LytA mutants one would expect, as a result of no LytA releasing and accumulating in the medium. The experiment was conducted to see if ComM reinforced the cell wall in any way that would halt this sudden decay of cells as can be seen at the end of the stationary phase. While ComM did not extend the stationary phase, it did seem to slow the rate of decay, instead of undergoing lysis after stationary phase, the curves rather flattened out over time. The cells expressing the highest levels of ComM grew somewhat slower than the control cells, and they started to lyse immediately after entering the stationary phase. This would demonstrate that ComM does indeed have an effect on the efficiency of LytA. The reason for this might be that ComM is responsible for some modification of the cell wall, resulting in an increased resilience against the effects of LytA. As the target for LytA is the binding of the first alanine in the stem peptide of the peptidoglycan chain, the modification from ComM might lie in this region. During the production of the peptidoglycan chain, lipid II is transported through the membrane before being modified into peptidoglycan by the PBPs. ComM might be responsible for some

modification to Lipid II or Lipid I during this stage, as this is the only step in the formation of the peptidoglycan layer when ComM has the opportunity to affect the process as it is an integral membrane protein. The experiment also demonstrate that only a small amount of ComM was needed to show a reduced response from the accumulated LytA. At a concentration of 0.015µM ComS* the optical density does not decay as rapidly as the control, slowing down the effects of LytA. Another observation was the decline in the peak optical density for the higher concentrations of ComM, this was seen in both the immune and non-immune cell cultures. The highest levels of ComM seem to trigger the activity of LytA at a much earlier time in the stationary phase. This could be a result of either increased susceptibility towards LytA, or that the ComM expressing cells burst more easily, and thereby release LytA when entering the stationary phase. Either way, this suggests that ComM induce a change in the cell envelope of *S. pneumoniae*. The corresponding LytA negative cells displayed similar reduction in growth when expressing ComM, but no lysis in the stationary phase, demonstrating that the reduced growth rate in ComM expressing cells are independent of LytA, but that the earlier autolysis is. High levels of ComM is therefore toxic to the cells themselves, and this can also be seen in microscope pictures of ComM overexpression strains, where they form long chains and display improper septum formation. This can be seen in Figure 4.3 in the results section, where the cells are overexpressing ComM. This fact supports the hypothesis that ComM modifies the cell wall and is located in the septum, as the synthesis of new pneumococcal cell wall originates from the septum. It has been speculated that ComM might be responsible for a shift from the septal peptidoglycan synthesis to the peripheral. Since ComM overexpression cultures show this elongated cell shape, demonstrating that have difficulties completing septal formation and separating. As CbpD targets the septal region, this shift from septal to peripheral growth might be the cause for immunity, but this has not been tested (Straume; PersComm).

A similar experiment was conducted using the strains RH609-RH612 which are strains that express ComM constitutively at different levels, where RH609 is the overexpression mutant. This experiment showed similar results to the previous experiment, with a gradual drop in optical density over time (data not shown), again demonstrating the stress that overexpression of ComM puts on the cells. If the high and continuous expression of ComM puts stress on the cells, it makes sense that during competence ComM is transiently expressed to avoid such stress. Unpublished data in the laboratory of Prof. Håvarstein have shown that ComM expression peaks 10 minutes after entering the competent state, while after 20 minutes, when expression of CbpD starts, almost no ComM can be detected. Therefore, ComM is present in

the cells at very low levels when CbpD is expressed. The immunity provided by ComM must therefore last longer than the life time of the ComM protein itself for the cells to be immune against CbpD.

5.2 Stem peptide composition in the cell wall of CbpD-immune pneumococci

The current hypothesis about ComM's function is that it performs a modification in the cell wall of competent *S. pneumoniae*, which prevents the lytic activity of CbpD. The nature of this modification is still unknown, however. During the production of peptidoglycan, lipid I and lipid II are constructed inside of the cytoplasm before being transported out of the membrane by a flippase. During this stage it is possible that ComM performs a modification to either lipid I or lipid II while it is anchored to the membrane. Since CbpD most probably cleaves covalent bonds in the stem peptides of sensitive cells, we wanted to see if any modification had been done to the stem peptides in the peptidoglycan of immune cells. First, the cell wall was purified from sensitive and immune cells and then treated with LytA, which cleaves the amide bond between MurNAc and the first L-Ala residue of the stem peptides. The free stem peptides can be analysed by reverse phase HPLC. If any modifications had been made to the stem peptides, such changes should be apparent in the HPLC analysis. Differences in the samples would show up either as new peaks, representing a modified stem peptide, or the same stem peptides would be represented with higher or lower peaks compared to the control, signifying either an increase or a decrease in the amount of that particular stem peptide. The results of these tests are shown in section 4.5.

The HPLC analysis showed no significant differences in the stem peptide composition. This is interpreted as ComM not being responsible for any modifications of the stem peptides nor their composition in the peptidoglycan. If there is no differences in the stem peptides the modifications might lie in the N-acetylglucosamine and N-acetylmuramic acids of the peptidoglycan, e.g. by interfering with the peptidoglycan binding property of the SH3b domains in CbpD. In order to test this hypothesis, mutanolysin was used. Mutanolysin cleaves the 1,4-β links between NAM and NAG resulting in muropeptides consisting of a carbohydrate covalently attached to an amino acid chain. However, the attempt to do an HPLC analysis of cell wall samples proved difficult, and yielded no data.

An alternative experiment was performed to study the results of mutanolysin treatment on the peptidoglycan from sensitive and immune cells. This experiment was a Remazol Brilliant Blue® release assay, with the use of Remazol Brilliant Blue® labelled peptidoglycan. If there was any differences in the amount of muropeptides released from peptidoglycan derived from immune and non-immune cells, it would indicate that ComM has made some unknown modification to the glycan chains, which makes it more difficult for mutanolysin to cleave its target. The results of this experiment is shown in section 4.8. The results did, however, not reveal any significant differences in mutanolysin hydrolytic activity between immune and non-immune cell wall material. Although a slight divergence, if any, was observed after the initial 10 minutes of the mutanolysin treatment. Here, the cell wall from immune cells was slightly more resistant to mutanolysin, however, prolonged incubations with the enzyme produced similar results between the two samples. Since these differences were so insignificant these results cannot be interpreted as any modifications in the muropeptides of the cell wall. It there were to be any modifications done, an HPLC analysis should be able to locate this. As this was not achieved, it is still unclear whether ComM has made any modifications in this area. Unpublished data in the laboratory of Prof. Håvarstein has shown that ComM does not inhibit the binding of CbpD to the cell wall (Straume; PersComm), the inhibition might lie in the murein hydrolase domain of CbpD. Whether this inhibition is located in the binding of the substrate in the active site, or directly inhibiting the activity of the active site by conformational modifications, is still unknown.

5.3 Immunity of cells after extended exposure to ComM

The mureinhydrolase CbpD is responsible for the damage done to the cell wall of non-competent cells during competence, which in turn triggers the activity of LytA and LytC in the target cells. In this work an experiment was conducted with CbpD on *lytA* knock out cells and with *comM* expressed from an early stage. Considering that ComM is such a transient protein, constitutive expression of ComM using the ComRS-system would increase the probability of detecting CbpD resistant cell wall material. Since *lytA* knock out mutants were used, the zymograph experiment would only show the activity of CbpD. The CbpD on the uninduced cell (ComM-) culture show clear lytic zones after only 30 minutes, demonstrating the effectiveness of CbpD alone. The zymograms of cells expressing ComM showed no signs of lytic zones after the same time of incubation in refolding buffer, demonstrating that long exposure to ComM still granted immunity, and that cells expressing ComM outside the competent state become

immune against CbpD. After one hour and 30 minutes, lytic zones appeared for the immune cells, demonstrating that full immunity was not achieved but rather a much delayed reaction from CbpD.

There might be several reasons for this, but two being more probable than other alternatives. As ComM is a most likely an integral membrane protein with no large loops or structures outside of the membrane, the protein probably does not interact with CbpD directly. Rather it is possible that ComM induces changes in the substrate of CbpD. For example, a change in the murein stem peptides of the peptidoglycan layer could cause a decrease in affinity of the catalytic CHAP domain of CbpD to its substrate. This will make it more difficult for CbpD to perform its activity but not impossible, and given enough time or amount of enzyme, its affinity towards its substrate will not matter. An alternative explanation is related to ComM somehow changing the cell surface so that CbpD does not bind properly to the cells for its lytic activity to occur. If ComM could interfere with the level of choline residues on the teichoic acid this might alter the binding specificity of the CBD domain of CbpD and consequently its localization on the peptidoglycan. Similarly, other changes to the peptidoglycan could alter the specificity of the two SH3b domains of CbpD, which are essential for its muralytic activity and are shown to bind peptidoglycan (Eldholm, Johnsborg et al. 2010). This results in the CHAP domain not being able to find its substrate. Either the binding of the CBD and/or the SH3b domains to the peptidoglycan results in a conformational change in CbpD allowing the catalytic CHAP domain to find its substrate, or simply the catalytic domain cannot locate its substrate without the help of CBD and/or the SH3b domains guiding it. If ComM modifies the cell surface, it would be reasonable to assume that this does not necessarily grant full immunity but rather slows the reaction enough during standard competence situations.

If the cell wall binding domains (CBD and SH3b) play a minor role in the immunity function of ComM (first hypothesis, modification of CbpD substrate) then one would assume that the CHAP domain should be catalytically active on sensitive cells even without the CBD and SH3b domains helping it find its substrate. This was tested by purifying the CHAP domain alone, and testing it using zymography on the same two cell cultures as in the previous experiment.

This experiment demonstrated that the CHAP domain was not able to exhibit catalytic activity for either the control or the immune cell culture. This would mean that the CHAP domain by itself is not active without its CBD domain and its SH3b domains. The results of this experiment seem to make the second hypothesis most likely, as CHAP is ineffective alone on both sensitive and immune cells. The CHAP domain never displayed signs of lytic activity even after 24 hour

in refolding buffer. This is in agreement with the notion that the functional role of the CBD domain and the SH3b domains, in addition to facilitating binding to the cell surface, also might help position the CHAP domain close to its substrate. This shows that proper binding of CbpD to the cell wall is crucial for its lytic action. Still, since the CBD domain and the SH3b domains are essential for CbpD activity in general, the observed lack of activity by the CHAP domain alone makes it impossible to rule out the first hypothesis; that the ComM could work by creating changes in the substrate of the CHAP domain.

5.4 Localization of sfGFP-ComM

To identify where in the cell ComM localizes would give important information regarding how it can make *S. pneumoniae* immune to CbpD. Unfortunately, where ComM localizes in *S. pneumoniae* is still unknown. In this thesis a localization study was attempted using the green fluorescent protein and constructing a sfGFP-ComM fusion protein. The current hypothesis is that ComM is located in the septum of the cell, as this is the target of attack for CbpD. Once the fusion protein sfGFP-ComM was constructed and shown to be functional using a microplate reader, the cells were induced for the expression of sfGFP-ComM using the ComRS system and subsequently observed in the microscope. The result of this experiment is shown in section 4.7. The fluorescent signal from the cells displayed only a seemingly random pattern. Several different expression levels of sfGFP-ComM were used (different concentrations of ComS*), as well as varying the time span of expression, but with the same results.

No distinct pattern could be observed, hence the localization of ComM could not be ascertained. There might be several reasons for this. As the sfGFP part of the sfGFP-ComM fusion protein is still active even without the ComM part, what we are observing might be the sfGFP from the fusion protein after ComM has been degraded. The signal from sfGFP would then quickly outnumber the signal from intact sfGFP-ComM. This is consistent with experiments done at Prof. Håvarsteins laboratory that showed that ComM is known to be quickly degraded in the cells (unpublished data from Dr. Daniel Straume). As the sfGFP is located at the N-terminal of ComM, and since the topological prediction of ComM implied that the N-terminal is located in the cytoplasm, any sfGFP freed by ComM degradation could spread inside the cell and form clusters randomly throughout. This implies that ComM is active long enough to grant immunity, but is degraded too rapidly, making the sfGFP-ComM protein difficult to use as a tool to determine its localization. There might still be active sfGFP-ComM, as these cells still show signs of immunity, but the amount of sfGFP-ComM still active might be just high enough to

grant immunity but too low to be observed in the microscope on the background cloud of many more free sfGFP-molecules emitting their fluorescent signals. Another reason for the weak randomly distributed signal could be that the majority of sfGFP-ComM forms misfolded clusters that still emit some fluorescence signal.

Another explanation for the observed spread out signal is that ComM truly is spread throughout the cell membrane, and not specifically located in the septum as indicated by the fact that this is the target location of CbpD. But this is, however, unlikely since it doesn't fit with other observations done by others in the laboratory, such as the chain formation and disrupted septum formation which all point to ComM being responsible for some change in the cell wall in the septum area during cell division (unpublished data). Though the results from this sfGFP-ComM experiment is not decisive enough to discourage that ComM might be located in the septum of the cells, as the two are not mutually exclusive.

The results discussed above was carried out by continuous expression and overexpression of sfGFP-ComM using the ComRS system. This resulted in a random and weak signal pattern, which we wondered could be due to misfolding and mislocalization of sfGFP-ComM due to excess of the protein. Therefore, another experiment was conducted to see if this could be circumvented by replacing the native *comM* gene with the *sfGFP-comM* fusion protein. This would allow expression of sfGFP-ComM to be competence induced. While the immunity test for this mutant proved successful, no fluorescence signal was observed in the microscope (data not shown). The reason for this result could be that the amount of sfGFP-ComM produced during natural competence is too low to be detected.

Another reason for the observed results might be that the signals reflect the true location of ComM in the cell and that they are spread throughout the cell membrane with no specific pattern or affiliation to the septum. If this was the case, one would expect the *sfGFP-comM::nativecomM* to show the same as the others, signal throughout the cell. Instead no signal was observed at all. It is likely that ComM is spread out too thin, and expressed in too small amounts to be measured in this way and that ComM is denatured to rapidly too be observed using this method.

5.5 Pattern of phospho-theronine proteins during competence

We wondered if ComM works as an immunity protein by interfering with phosphorylation of DivIVA by interfering with its kinase StkP (Serine/threonine-protein kinase). StkP plays a

major part in cell division and regulation of cell shape through the control and phosphorylation of DivIVA. DivIVA is found in the septum, where it is involved in the cell division in *S. pneumoniae* and other Gram-positive bacteria. The proteins are most frequently studied in the bacteria *Basillus subtilis*, where it works together with a complex consisting of two proteins, MinC and MinD. This complex inhibits cell division at the poles of the cell. This strongly indicates that DivIVA has a predominant role in the cell morphology and cell division. (Nováková, Bezoušková et al. 2010)

With the use of immunoblotting, proteins having phosphorylated threonines could be detected using an anti-phosphothreonine-antibody. A Horseradish peroxidase-conjugated secondary antibody was used to bind to the primary antibody. The cell cultures were studied at different stages of competence to identify any differences in either the level of phosphorylation or the phosphorylation of proteins during competence. A Δ*comM* mutant (RH420) was used to demonstrate if ComM affects this phosphorylation either indirectly or directly. The results of this experiment is shown in section 4.4. As Figure 4.7 shows, two proteins were discovered to be phosphorylated, DivIVA and an unknown protein.

As both of these proteins are equally phosphorylated both during normal cell growth and competence in both the wild type and in the Δ*comM* mutant, it shows that neither competence nor ComM affects the level of phosphorylation of these proteins.

6 Concluding remarks and further work.

Since *S. pneumoniae* is a major human pathogen, with the capability of accessing a pan-genome through natural transformation endowing it with antibiotic resistance, new capsule genes and virulence factors. The immunity protein ComM is an important factor of the competence state of *S. pneumoniae,* protecting the competent cells from lysis during fratricide. It is therefore a very important feature to study for understanding this important process in the pneumococcal life. Principal objectives should be to figure out its mode of action and its locations in the cell. ComM protects competent cells against the murein hydrolase CbpD, which plays a major role in the acquisition of exogenous DNA from neighbouring cells accessing the pan-genome. As the location and activity is still unknown for ComM, this work has attempted to shed some light on this matter. The attack point for CbpD lies in the septum of the cells, and ComM is therefore hypothesised to be located in the septum. As for the activity of ComM, it is likely that it performs some modification to the cell envelope of the cells, resulting either in CbpDs choline binding domains or SH3b domains losing their affinity to its substrate or in a modification of the murein acids so that the murein hydrolase domain (CHAP) of CbpD is unable to cleave its substrate.

Growth curves and microscope observations of ComM overexpression mutants have demonstrated the stress that is inflicted upon the cells when large amounts of ComM is produced. The microscope pictures especially demonstrated the abnormal morphology and chain formation, Figure 5.1, leading to the hypothesis that ComM interferes with normal cell wall synthesis. To find out if this interference changed the peptidoglycan structure, an HPLC analysis using LytA treated cell wall samples was conducted. This analysis showed little to no change in composition regarding the stem peptides of the peptidoglycan, and it was concluded that ComM is not responsible for any modification of the stem peptides. An analysis using mutanolysin instead of LytA was carried out using HPLC but proved difficult as no results were obtained from this analysis. A different procedure was conducted as a substitute, the Remazol Brilliant Blue® release assay to demonstrate if there was a difference in the efficiency of mutanolysin for the cell wall derived from the ComM expressing cell culture. This experiment did not display any significant difference in mutanolysin efficiency, other than a slight

difference the first 10 minutes, but this does not rule out the possibility of ComM induced mutopeptide modifications. To study what, if any, these modifications might be, a reverse phase HPLC analysis should be performed. This experiment proved difficult to perform in this work. Given more time the analysis should be obtainable and it might be interesting to see if the activity of ComM is located towards this part of the peptidoglycan.

Zymographic tests demonstrated that the CHAP domain of CbpD was unable to cleave peptidoglycan deriving from both immune and non-immune cells, leading to the hypothesis that proper binding of CbpD to the cell wall is crucial for its activity. It is likely that the cell wall binding domains of CbpD guides the catalytic CHAP domain to its substrate. As the CHAP domain alone is inactive, it might be reasonable to speculate that the immunity function of ComM is to alter the binding of CbpD on the cell wall.

The location of ComM was attempted to be determined using the fusion protein sfGFP-ComM. The current hypothesis is that ComM is located in the septum of the cell, as this is where CbpD targets its activity. This hypothesis is supported by the fact that the cells seem to have difficulty in cell division, a problem that might derive from modifications of the cell wall in the septum as this is where the new cell wall is created during cell division. This hypothesis proved difficult to ascertain as there were a lot of conflicting results, which might result from uncoupled sfGFP that had remained active after ComM had degraded. Such uncoupled sfGFPs might be the reason for the random distribution of GFP signal observed throughout the cell. This experiment was also conducted using the native promoter for *comM*. However, no signal was observed in the microscope, even though the cells were immune only expressing sfGFP-ComM from P_{comM}. This experiment could be successful if the false positives could be reduced, there might also be a timing issue for time after induction and the concentration of ComS* used to induce for sfGFP-ComM that was not met during this experiment. If the signal of sfGFP-ComM, in the case of the *sfGFP-comM::nativecomM* strain, is just below detectable range for our microscope, perhaps a microscope with a better camera would be able to detect the signal. Another experiment could be to use single cell microscopy to study one cell in real-time which had been induced with CSP-1. This contention would constitute an interesting follow-up of the current work.

References

Avery, O. T., et al. (1944). "STUDIES ON THE CHEMICAL NATURE OF THE SUBSTANCE INDUCING TRANSFORMATION OF PNEUMOCOCCAL TYPES : INDUCTION OF TRANSFORMATION BY A DESOXYRIBONUCLEIC ACID FRACTION ISOLATED FROM PNEUMOCOCCUS TYPE III." J Exp Med **79**(2): 137-158.

Berg, K. H., et al. (2011). "Peptide-Regulated Gene Depletion System Developed for Use in Streptococcus pneumoniae." Journal of Bacteriology **193**(19): 5207-5215.

Berg, K. H., et al. (2013). "Effects of low PBP2b levels on cell morphology and peptidoglycan composition in Streptococcus pneumoniae R6." J Bacteriol **195**(19): 4342-4354.

Bouhss, A., et al. (2008). "The biosynthesis of peptidoglycan lipid-linked intermediates." FEMS Microbiology Reviews **32**(2): 208-233.

Bui, N. K., et al. (2012). "Isolation and analysis of cell wall components from Streptococcus pneumoniae." Analytical Biochemistry **421**(2): 657-666.

Denapaite, D., et al. (2012). "Biosynthesis of teichoic acids in Streptococcus pneumoniae and closely related species: lessons from genomes." Microb Drug Resist **18**(3): 344-358.

Eldholm, V., et al. (2009). "Fratricide in Streptococcus pneumoniae: contributions and role of the cell wall hydrolases CbpD, LytA and LytC." Microbiology **155**(Pt 7): 2223-2234.

Eldholm, V., et al. (2010). "Pneumococcal CbpD is a murein hydrolase that requires a dual cell envelope binding specificity to kill target cells during fratricide." Mol Microbiol **76**(4): 905-917.

Garrity, G. M., et al. (2004). "Taxonomic outline of the prokaryotes. Bergey's manual of systematic bacteriology." Springer, New York, Berlin, Heidelberg.

Golubchik, T., et al. (2012). "Pneumococcal genome sequencing tracks a vaccine escape variant formed through a multi-fragment recombination event." Nature genetics **44**(3): 352-355.

Griffith, F. (1928). "The Significance of Pneumococcal Types." Epidemiology & Infection **27**(02): 113-159.

Hamilton, H. L. and J. P. Dillard (2006). "Natural transformation of Neisseria gonorrhoeae: from DNA donation to homologous recombination." Mol Microbiol **59**(2): 376-385.

Hardie, J. M. and R. A. Whiley (1997). "Classification and overview of the genera Streptococcus and Enterococcus." Journal of Applied Microbiology **83**(S1): 1S-11S.

Higuchi, R., et al. (1988). "A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions." Nucleic Acids Research **16**(15): 7351-7367.

Holloway, B. W. (1993). "Genetics for all bacteria." Annu Rev Microbiol **47**: 659-684.

Hoskins, J., et al. (2001). "Genome of the Bacterium Streptococcus pneumoniae Strain R6." Journal of Bacteriology **183**(19): 5709-5717.

Håvarstein, L. S., et al. (1995). "An unmodified heptadecapeptide pheromone induces competence for genetic transformation in Streptococcus pneumoniae." Proceedings of the National Academy of Sciences **92**(24): 11140-11144.

Håvarstein, L. S., et al. (1996). "Identification of the streptococcal competence-pheromone receptor." Molecular Microbiology **21**(4): 863-869.

Håvarstein, L. S., et al. (2006). "New insights into the pneumococcal fratricide: relationship to clumping and identification of a novel immunity factor." Molecular Microbiology **59**(4): 1297-1037.

Jedrzejas, M. J. (2001). "Pneumococcal Virulence Factors: Structure and Function." Microbiology and Molecular Biology Reviews **65**(2): 187-207.

Johnsborg, O., et al. (2008). "A predatory mechanism dramatically increases the efficiency of lateral gene transfer in Streptococcus pneumoniae and related commensal species." Molecular Microbiology **69**(1): 245-253.

Johnsborg, O., et al. (2007). "Natural genetic transformation: prevalence, mechanisms and function." Research in Microbiology **158**(10): 767-778.

Johnsborg, O. and L. S. Håvarstein (2009). "Pneumococcal LytR, a Protein from the LytR-CpsA-Psr Family, Is Essential for Normal Septum Formation in Streptococcus pneumoniae." J Bacteriol **191**(18): 5859-5864.

Johnsborg, O. and L. S. Håvarstein (2009). Regulation of natural genetic transformation and acquisition of transforming DNA in Streptococcus pneumoniae.

Kadioglu, A., et al. (2008). "The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease." Nat Rev Micro **6**(4): 288-301.

Kausmally, L., et al. (2005). "Choline-Binding Protein D (CbpD) in Streptococcus pneumoniae Is Essential for Competence-Induced Cell Lysis." Journal of Bacteriology **187**(13): 4338-4345.

Kawamura, Y., et al. (1995). "Determination of 16S rRNA sequences of Streptococcus mitis and Streptococcus gordonii and phylogenetic relationships among members of the genus Streptococcus." Int J Syst Bacteriol **45**(2): 406-408.

Kilian, M., et al. (2008). "Evolution of <italic>Streptococcus pneumoniae</italic> and Its Close Commensal Relatives." PLoS ONE **3**(7): e2683.

Kjos, M., et al. (2010). "The Abi Proteins and Their Involvement in Bacteriocin Self-Immunity." J Bacteriol **192**(8): 2068-2076.

Knutsen, E., et al. (2004). "Two Separate Quorum-Sensing Systems Upregulate Transcription of the Same ABC Transporter in Streptococcus pneumoniae." J Bacteriol **186**(10): 3078-3085.

Krogh, A., et al. (2001). "Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes." J Mol Biol **305**(3): 567-580.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**(5259): 680-685.

Larkin, M. A., et al. (2007). "Clustal W and Clustal X version 2.0." Bioinformatics **23**(21): 2947-2948.

Laurenceau, R., et al. (2013). "A Type IV Pilus Mediates DNA Binding during Natural Transformation in Streptococcus pneumoniae." PLoS Pathogens **9**(6): e1003473.

Leclerc, D. and A. Asselin (1989). "Detection of bacterial cell wall hydrolases after denaturing polyacrylamide gel electrophoresis." Canadian Journal of Microbiology **35**(8): 749-753.

Lee, M. S. and D. A. Morrison (1999). "Identification of a New Regulator in Streptococcus pneumoniae Linking Quorum Sensing to Competence for Genetic Transformation." Journal of Bacteriology **181**(16): 5004-5016.

Marchler-Bauer, A., et al. (2015). "CDD: NCBI's conserved domain database." Nucleic Acids Research **43**(Database issue): D222-D226.

Morlot, C., et al. (2003). "Growth and division of Streptococcus pneumoniae: localization of the high molecular weight penicillin-binding proteins during the cell cycle." Molecular Microbiology **50**(3): 845-855.

Mortier-Barrière, I., et al. (2007). "A Key Presynaptic Role in Transformation for a Widespread Bacterial Protein: DprA Conveys Incoming ssDNA to RecA." Cell **130**(5): 824-836.

Nováková, L., et al. (2010). "Identification of Multiple Substrates of the StkP Ser/Thr Protein Kinase in Streptococcus pneumoniae." J Bacteriol **192**(14): 3629-3638.

Oxoid (2002). "AnaeroGen™, Product Details." Oxoid, Ltd. Basinstoke, Hampshire, England.

Pei, J. and N. V. Grishin (2001). "Type II CAAX prenyl endopeptidases belong to a novel superfamily of putative membrane-bound metalloproteases." Trends in Biochemical Sciences **26**(5): 275-277.

Pinho, M. G., et al. (2013). "How to get (a)round: mechanisms controlling growth and division of coccoid bacteria." Nat Rev Micro **11**(9): 601-614.

Rodriguez, A. M., et al. (2011). "Physiological and molecular characterization of genetic competence in Streptococcus sanguinis." Molecular oral microbiology **26**(2): 99-116.

Scheffers, D.-J. and M. G. Pinho (2005). "Bacterial Cell Wall Synthesis: New Insights from Localization Studies." Microbiology and Molecular Biology Reviews **69**(4): 585-607.

Sham, L.-T., et al. (2012). "Recent advances in pneumococcal peptidoglycan biosynthesis suggest new vaccine and antimicrobial targets." Current Opinion in Microbiology **15**(2): 194-203.

Stamsas, G. A., et al. (2013). "CHiC, a new tandem affinity tag for the protein purification toolbox." J Microbiol Methods **92**(1): 59-63.

Stamsås, G. A., et al. (2013). "CHiC, a new tandem affinity tag for the protein purification toolbox." Journal of Microbiological Methods **92**(1): 59-63.

Straume, D., et al. (2014). "Natural transformation and genome evolution in Streptococcus pneumoniae." Infection, Genetics and Evolution(0).

Sung, C. K., et al. (2001). "An rpsL Cassette, Janus, for Gene Replacement through Negative Selection in Streptococcus pneumoniae." Applied and Environmental Microbiology **67**(11): 5190-5196.

Towbin, H., et al. (1979). "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications." Proceedings of the National Academy of Sciences **76**(9): 4350-4354.

Warrens, A. N., et al. (1997). "Splicing by overlap extension by PCR using asymmetric amplification: an improved technique for the generation of hybrid proteins of immunological interest." Gene **186**(1): 29-35.

Appendix

A. Amino acid- and nucleotide sequence of ComM/comM in *S. pneumoniae* R6

Amino acid sequence – ComM

>gi|15459443|gb|AAL00565.1| Hypothetical protein spr1762 [Streptococcus pneumoniae R6]
MKSMRILFLLALIQISLSSCFLWKECILSFKQSTAFFIGSMVFVSGICAGVNYLYTRKQEVHSVLASKKS
VKLFYSMLLLINLLGAVLVLSDNLFIKNTLQQELVDFLLPSFFFLFGLDLLIFLPLKKYVRDFLAMLDRK
KTVLVTILATLLFLRNPMTIVSLLIYIGLGLFFAAYLVPNSVKKEVSFYGHIFRDLVLVIVTLIFF

Nucleotide sequence - *comM*

>gi|15902044:c1730321-1729701 Streptococcus pneumoniae R6 chromosome, complete genome
ATGAAATCAATGAGAATCTTATTTTTGTTAGCTTTAATTCAAATCAGTTTGAGTAGCTGTTTCCTATGGA
AGGAATGCATCTTGTCCTTTAAACAAAGTACAGCTTTTTTCATCGGAAGCATGGTTTTCGTTTCAGGAAT
CTGTGCTGGAGTAAATTATCTTTATACCCGTAAGCAAGAAGTCCATAGTGTCCTAGCCAGTAAGAAGTCG
GTGAAGCTTTTTTACAGTATGTTACTCTTAATTAATTTGTTAGGAGCTGTTCTTGTTTTGTCAGATAACT
TGTTCATCAAAAATACGCTGCAGCAAGAATTAGTTGACTTTTTATTGCCATCCTTCTTTTTCCTATTTGG
GCTAGATTTGCTGATTTTTTTACCCTTGAAAAAATACGTGCGCGATTTTCTTGCTATGCTGGACAGAAAA
AAGACAGTGTTGGTGACTATTTTAGCAACACTTCTTTTCTTAAGAAATCCAATGACCATTGTCTCACTTC
TGATTTATATTGGACTGGGCTTGTTTTTTGCAGCCTATCTTGTCCCAAATTCGGTTAAGAAGGAAGTTTC
CTTTTATGGTCATATTTTCCGAGATCTTGTATTGGTCATTGTTACGCTCATTTTCTTTTAG

B. Amino acid- and nucleotide sequence of CbpD/cbpD in *S.pneumoniae* R6

Amino acid sequence - CbpD

>gi|9454349|gb|AAF87768.1|AF278686_1 choline binding protein D [Streptococcus pneumoniae]
MKILPFIARGTSYYLKMSVKKLVPFLVVGLMLAAGDSVYAYSRGNGSIARGDDYPAYYKNGSQEIDQWRM
YSRQCTSFVAFRLSNVNGFEIPAAYGNANEWGHRARREGYRVDNTPTIGSITWSTAGTYGHVAWVSNVMG
DQIEIEEYNYGYTESYNKRVIKANTMTGFIHFKDLDGGSVGNSQSSTSTGGTHYFKTKSAIKTEPLASGT
VIDYYYPGEKVHYDQILEKDGYKWLSYTAYNGSYRYVQLEAVNKNPLGNSVLSSTGGTHYFKTKSAIKTE
PLVSATVIDYYYPGEKVHYDQILEKDGYKWLSYTAYNGSRRYIQLEGVTSSQNYQNQSGNISSYGSHSSS
TVGWKKINGSWYHFKSNGSKSTGWLKDGSSWYYLKLSGEMQTGWLKENGLWYYLGSSGAMKTGWYQVSGK
WYYSYSSGALAVNTTVDGYRVNSDGERV

Nucleotide sequence – *cbpD*

>gi|15902044:c2000358-1999012 Streptococcus pneumoniae R6 chromosome, complete genome

ATGAAAATTTTACCGTTTATAGCAAGAGGAACAAGTTATTACTTGAAGATGTCAGTTAAAAAGCTTGTTC
CTTTTTTAGTAGTAGGATTGATGCTAGCAGCTGGTGATAGTGTCTATGCCTATTCCAGAGGAAATGGATC
GATTGCGCGTGGGGATGATTATCCTGCTTATTATAAAAATGGGAGCCAGGAGATTGATCAGTGGCGCATG
TATTCTCGTCAGTGTACTTCTTTTGTAGCCTTTCGTTTGAGTAATGTCAATGGTTTTGAAATTCCGGCAG
CTTATGGAAATGCGAATGAATGGGGACATCGTGCTCGTCGGGAAGGTTATCGTGTAGATAATACACCGAC
GATTGGTTCCATTACTTGGTCTACTGCAGGAACTTATGGTCATGTTGCCTGGGTGTCAAATGTAATGGGA
GATCAGATTGAGATTGAGGAATATAACTATGGTTATACAGAATCCTATAATAAACGAGTTATAAAAGCAA
ACACGATGACAGGATTTATTCATTTTAAAGATTTGGATAGTGGCAGTGTTGGGAATAGTCAATCCTCAGC
TTCAACAGGCGGAACTCATTATTTTAAGACCAAGTCTGCTATTAAAACTGAACCCCTAGTTAGTGCAACT
GTGATTGATTACTATTATCCTGGGGAGAAGGTTCATTATGATCAGATACTTGAAAAAGACGGCTATAAGT
GGTTGAGTTATACTGCCTATAATGGAAGCTATCGTTATGTTCAATTGGAGGCTGTGAATAAAAATCCTCT
AGGTAATTCTGTTCTTTCTTCAACAGGAGGAACTCATTATTTTAAGATCAAGTCTGCTATTAAAACTGAA
CCCCTAGTTAGTGCAACTGTGATTGATTACTATTATCCTGGAGAGAAGGTTCATTATGATCAGATACTTG
AAAAAGACGGCTATAAGTGGTTGAGTTATACGGCTTATAACGGAAGTCGTCGCTATATACAGCTAGAGGG
AGTGACTTCTTCACAAAATTATCAGAATCAATCAGGAAATATCTCTAGCTATGGATCCAATAATAGTTCA
ACTGTCGGTTGGAAGAAAATAAATGGTAGTTGGTATCATTTCAAATCAAATGGTTCTAAATCAACAGGAT
GGCTGAAAGACGGTTCTAGCTGGTATTATTTGAAATTATCTGGTGAAATGCAGACAGGATGGTTAAAGGA
GAATGGCTCGTGGTATTATCTGGGTAGTTCAGGGGCAATGAAAACAGGCTGGTACCAGGTCTCTGGTGAG
TGGTATTATTCTTACTCTTCAGGCGCCTTAGCTATTAATACGACGGTGGATGGCTACAGAGTAAACAGTG
ATGGAGAACGAGTATAG

C. Molecular weight standard

Colour Protein Standard, Broad Range. (NEB #P7712)

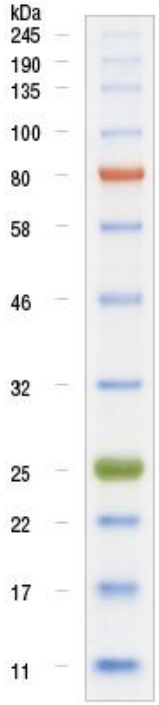


Figure A-1 **Colour protein standard, broad range.** A broad range proteiner marker (NEB) which contains a mixture of different labelled proteins of known sizes used as reference points for protein assays. Figure taken from (https://www.neb.com/products/p7712-color-prestained-protein-standard-broad-range-11-245-kda)

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