Norwegian University of Life Sciences

Department of Chemistry, Biotechnology and Food Science

Master's Thesis 2015

60 credits

A functional study of the essential amidotransferase complex MurT/CobQ in *Streptococcus pneumoniae*

En funksjonell studie av det essensielle amidotransferasekomplekset MurT/CobQ i *Streptococcus pneumoniae*

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Acknowledgements

This work has been carried out working with the Molecular Microbiology group at The Department of Chemistry, Biotechnology and Food Sciences (IKBM), Norwegian University of Life Sciences from August 2014 to May 2015.

I would like to thank professor Leiv Sigve Håvarstein for allowing me to write my thesis in collaboration with the Molecular Microbiology group. It has been an incredibly educational and humbling experience.

Big thanks to my supervisor Dr. Daniel Straume for always giving excellent advice and allowing me to work independently with such an exiting topic. With your door always open and always taking your time to listen to my inquiries, you have been a true inspiration and working with you has been a true pleasure.

To Dr. Kari Helene Berg, thank you for showing me the ropes, giving good advice and making me feel included in the group. Thank you to Dr. Gry Anita Stamsås for your infectiously good mood, and for helping me with loads of technical problems even though you didn´t have to. Thanks you to Zhian Salehian for always being happy to help me. Your energetic, non-stop working attitude is amazing.

Also thank you to fellow students Karoline Wahlstrøm and Vegard Osa Lie for loads of fun and interesting conversations, both on and off topic.

And last but not least, big thanks to my friends and family for being supportive, for showing an interest in my work and for being a source of relief in challenging times.

Olav Aaseth Hegnar
Ås, May 2015

Abstract

The bacterial cell wall is responsible for maintaining cell shape and gives protection from osmotic lysis caused by turgor pressure. The major component of the cell wall in Gram-positive bacteria is the structurally complex biopolymer peptidoglycan. *Streptococcus pneumoniae* (Pneumococcus) is a Gram-positive human pathogen responsible for an estimated 1-2 million deaths annually worldwide. Studies of its cell wall synthesis machinery are of high academic interest and it can contribute to drug target discoveries, which have the potential to improve treatments in the future. The recently discovered essential amidotransferase complex MurT/CobQ, encoded by the operon *murTcobQ*, is in *S. pneumoniae* responsible for the amidation the peptidoglycan precursor lipid II. The amidation of the second residue γ-glutamate to isoglutamine in lipid II has previously been shown to be necessary for the transpeptidase activity of the peptidoglycan synthesising proteins, known as penicillin binding proteins (PBPs). What biological role this amidation plays is currently not known. In the present work depletion of MurT/CobQ expression has been used extensively to study how low levels of amidated lipid II affects the phenotype of *S. pneumoniae*.

The sensitivity against the β–lactam antibiotics cefotaxime and ampicillin did not appear to be significantly affected by MurT/CobQ depletion, and neither did lysozyme resistance. The non-essential PBP1a is the only PBP to have reported residual transpeptidase activity with non-amidated lipid II *in vitro*. This proved difficult to demonstrate *in vivo*, and as such the results of these experiments were inconclusive. It was shown that depletion of MurT/CobQ severely affected the pneumococcal cells ability to properly divide, with septal cell wall synthesis being inhibited. The cells were still able to synthesize cell wall peripherally, strongly indicating that there is a difference between the septal and peripheral cell wall synthesising machineries in their ability to utilize non-amidated lipid II as substrate. The depletion of MurT/CobQ also affected the ability of the muralytic fratricide protein CbpD to successfully lyse cells, further strengthening the existing theory that this enzyme attacks the septal region of dividing cells. Furthermore this work demonstrated that *in vivo,* the PBPs are able to cross-link the stem peptides of the cell wall using non-amidated lipid II as substrate. Here it was shown that while the cell walls of normal pneumococcal cells contained a small amount of non-amidated stem-peptide dimers, cells depleted of MurT/CobQ contained significantly higher amounts of non-amidated stem-peptide dimers.

Sammendrag

Den bakterielle cellveggen gir bakteriecellene sin form og beskytter dem fra osmotisk lysis. Hovedkomponenten i celleveggen hos grampositive bakterier den komplekse biopolymeren peptidoglykan. *Streptococcus pneumoniae* er en grampositiv, humanpatogen bakterie som er ansvarlig for mellom 1-2 millioner dødsfall årlig på verdensbasis, og studier av celleveggssyntesen kan potensielt lede til forbedrede behandlingsmetoder i fremtiden. Det nylig oppdagede essensielle amidotransferasekomplekset MurT/CobQ, kodet av operonet *murTcobQ*, er ansvarlig for amideringen av peptidoglykanforløperen lipid II i *S. pneumoniae*. Amideringen av aminosyren γ–glutamat til isoglutamin i lipid II er tidligere vist å være nødvendig for transpeptidaseaktiviteten til de peptidoglykansyntetiserende enzymene (PBPer). Hvilken biologisk rolle denne amideringen spiller er for øyeblikket ukjent. I dette arbeidet har depletion (underuttrykk) av MurT/CobQ uttrykk blitt brukt for å studere hvordan lave konsentrasjoner av amidert lipid II påvirker fenotype hos *S. pneumoniae*.

Sensitiviteten mot β–laktam antibiotikaene cefotaxim og ampicillin, samt lysozym ble ikke signifikant påvirket av MurT/CobQ depletion. Det ikke-essensielle enzymet PBP1a er det eneste som tidligere har vist en viss aktivitet med uamidert lipid II *in vitro*. Dette viste seg å være vanskelig å demonstrere *in vivo*, og resultatene fra disse forsøkene var mangelfulle. Arbeidet har vist at depletion av MurT/CobQ påvirker streptokokk-cellenes evne til å dele seg ved at den septale celleveggssyntesen blir inhibert. Cellene evnet fremdeles å syntetisere ny cellevegg i lengderetningen, noe som indikerer at der er en forskjell mellom de septale og perifere celleveggssyntesemaskinerienes evne til å bruke uamidert lipid II. Depletion av MurT/CobQ førte også til at det muralytiske fratricidproteinet CbpD ikke klarer å lysere celler, noe som bidrar til å styrke den rådende teorien om at dette proteinet angriper septum hos pneumokokker i delingsfasen. Videre viser denne studien at *in vivo* så evner PBPene å inkorporere og kryssbinde uamidert lipid II til en viss grad i celleveggen. Det ble vist at mens celleveggen til normale celler inneholdt en liten mengde uamiderte peptid-dimerer, så inneholdt MurTCobQ-depleted celler et signifikant høyere nivå av uamiderte peptid-dimerer.

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1 Introduction

1.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae (Pneumococcus) is a major human pathogen and a scientifically and historically important microorganism. It is a natural commensal of the upper respiratory tract in humans, in particular the nasopharynx. The species has been a model organism in biology for well over a century, played a crucial role in the discovery of DNA as the carrier of genetic information and has greatly contributed to our understanding of molecular biology (Avery et al. 1944, Lederberg 1994). *S. pneumoniae* was independently discovered in the late 19th century by American physician George Sternberg and French chemist Louis Pasteur and was the first organism in which horizontal gene transfer was described, in the famous experiments of Frederick Griffith in 1928 (Griffith 1928). Griffith demonstrated that by co-inoculating heat killed virulent pneumococci with a living avirulent strain in mice, the avirulent strain became virulent, killing the mice. Mice injected with only heat killed bacteria or with the avirulent strain did not cause pneumoniae and death. This transfer of phenotypic traits was later revealed to involve DNA uptake and homologous recombination (Avery et al. 1944). *S. pneumoniae* is the leading cause of community-acquired pneumonia, with an estimated 1-2 million deaths annually worldwide (Hoskins et al. 2001, Croucher et al. 2011). The species is the subject of intensive research and further insight into its biology could lead to improved treatments for infections as well as provide fundamental information about bacterial biology in general.

1.1.1 Phylogeny, morphology and metabolism

S. pneumoniae is a low GC (39.7%), Gram-positive bacterium within the phylum Firmicutes (Paul De Vos et al. 2009). The currently accepted taxonomy is presented in table 1.1

Table 1.1: The taxonomic classification of *S. pneumoniae*

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

The bacterium is one of more than 50 recognized species within the genus *Streptococcus* (Kilian et al. 2008). These 50+ species are placed within 6 major groups: mitis, salivarius, mutans, anginosus, bovis and the pyogenic group, based on 16S rDNA analysis (Hardie and Whiley 1997). Figure 1.1.1, taken from work done by Kawamura et al. (1995), shows the phylogenetic relationship between 34 of the most studies *Streptococcus* species, with *S. pneumoniae* being placed within the mitis group.

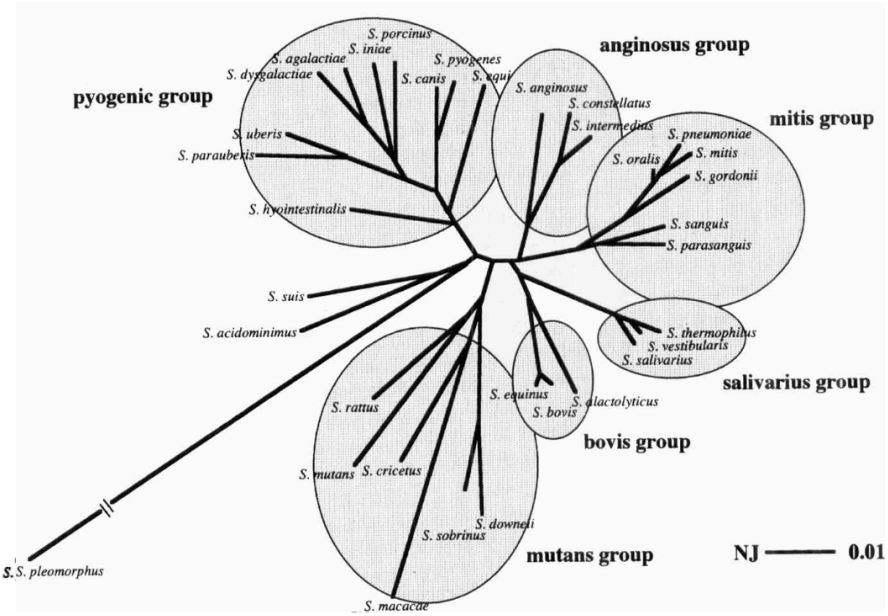


Figure 1.1.1: Phylogenetic relationships between 34 *Streptococcus* species. The tree is based on 16S rDNA data, with *S. pneumoniae* placed within the mitis group. Figure from Kawamura et al. (1995)

Most members within the mitis group, including *S. pneumoniae*, exhibit a natural ability to take up extracellular DNA and incorporate it into their genome through homologous recombination (Kilian et al. 2008). This process was coined natural transformation by Avery, MacLeod and McCarty in 1944, and species possessing it are said to be naturally competent. This ability has most likely contributed greatly to the evolution of the species within the mitis group (Kilian et al. 2008, Croucher et al. 2011).

S. pneumoniae cells are oval, spherical or cocci like in shape, usually 0.25-1.25 μm in diameter and are found in pairs or as short chains (figure 1.1.2.). The distal ends tend to be slightly pointed or lance shaped, and the cells usually appear encapsulated with a polysaccharide (Paul De Vos et al. 2009). This capsule is generally associated with virulence and the lab strains used in this work lack the capsule genes, rendering them avirulent

(Hoskins et al. 2001). Pneumococci are strongly α-haemolytic on blood agar when incubated aerobically and β-haemolytic when incubated anaerobically due to the production of pneumolysin O (Paul De Vos et al. 2009). They are catalase negative and oxidase positive, and the white colonies appear smooth and dome shaped.

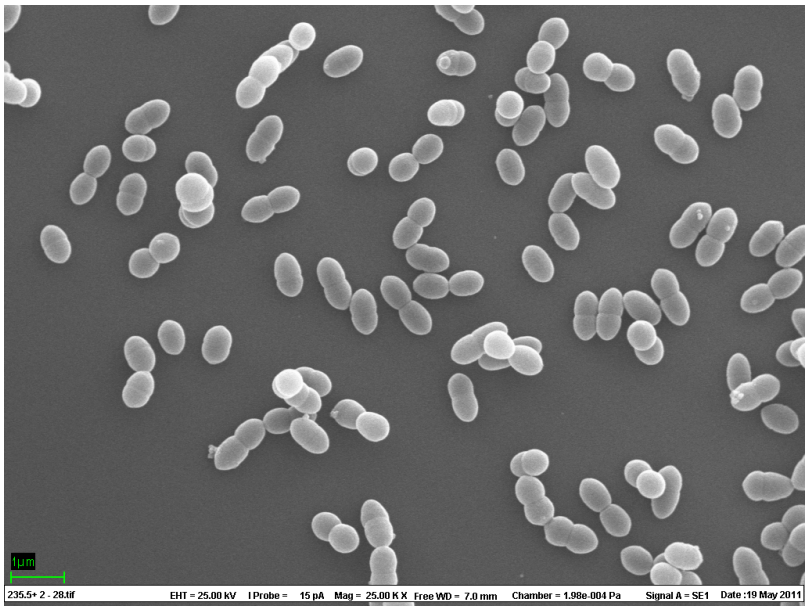


Figure 1.1.2 ***Streptococcus pneumoniae.*** Scanning electron micrograph taken at 25,000x magnification, showing typical diplococci shape. Image courtesy of Dr. Daniel Straume of the Molecular Microbiology group, NMBU.

S. pneumoniae is a chemoheterotroph and lacks the genes of the electron transport chain and the TCA cycle. It gets its energy through the fermentation of carbohydrates, producing lactic acid as a byproduct (Hoskins et al. 2001). Pneumococci grow best under anaerobic, acidic conditions (pH 5.0), and require a rich growth medium supplemented with peptides, purines, pyrimidines, salts, and vitamins for optimal growth (Lacks and Hotchkiss 1960).

1.1.2 Epidemiology

As previously mentioned, *S. pneumoniae* can be found as a commensal in upper respiratory tract of healthy individuals, usually without any adverse effects. It is carried by approximately 10% of healthy adults, 20-40% of healthy children, while more than 60% of infants can be carriers (van der Poll and Opal 2009). The bacterium is an opportunistic pathogen estimated to cause between 1 and 2 million deaths annually, with the highest mortality being in developing countries in Asia and Africa (Croucher et al. 2011, Kim et al. 2012). In addition to being the leading cause of community-acquired pneumoniae, it is also

known to cause a wealth of other serious diseases including sepsis, meningitis, arthritis and endocarditis, as well as less serious infections like sinusitis and otitis media (Hoskins et al. 2001, Hiller et al. 2007, Sham et al. 2012). Pneumococcal diseases are particularly common among immune compromised patients such as infants, the elderly and people with HIV, as well as in otherwise healthy individuals following viral infections like influenza. The major virulence factors of *S. pneumoniae* are the polysaccharide capsules preventing phagocytosis by the immune system (Sham et al. 2012). There are currently more than 90 different recognized capsule serotypes, making the construction of a single effective vaccine difficult. Yet 23-valent (covering 23 serotypes) and 7-valent vaccines have proven successful (Sham et al. 2012). The species is also notorious for being 100% resistant to human lysozyme, a bacteriolytic enzyme of the mucosa and saliva responsible for the hydrolysis of peptidoglycan in the bacterial cell wall (Yoshimura et al. 1988, Bera et al. 2005). Increasing antibiotic resistance in pneumococci is of growing concern to the medical community, with a reported 31% of all isolated strains in the US being resistant to one or several antibiotics (CDC 2013). This poses a major threat to human health and is made more complicated by the ease of which resistance genes are spread through horizontal gene transfer (Croucher et al. 2011).

1.2 Natural transformation in *S. pneumoniae*

The acquisition of genetic materials through lateral gene transfer has been a major driving force in the evolution of prokaryotic genomes (Johnsborg et al. 2007). There are three known mechanisms of horizontal gene transfer in bacteria: conjugation, transduction and natural transformation. Natural transformation differs from conjugation and transduction, as it is initiated by the recipient cell and not reliant on extrachromosomal elements promoting their own maintenance and distribution (Johnsborg et al. 2007). Through natural transformation cells acquire exogenous DNA and incorporate it into their genome by homologous recombination. More than 60 bacterial species, including *S. pneumoniae*, are known to be naturally transformable (Johnsborg et al. 2007). Because *S. pneumoniae* takes up DNA regardless of source, it does not discriminate between homologous and foreign DNA.

In the pneumococci competence for natural genetic transformation is a tightly regulated, transient process that lasts for approximately 30 minutes. *S. pneumoniae* becomes

spontaneously competent during the exponential growth phase (OD_{550} 0.15-0.2) when grown in a competence-promoting medium (Tomasz 1966). Competence is initiated by the production the alternative σ–factor ComX, which induces a transcriptional reprogramming of the cells (Lee and Morrison 1999, Johnsborg and Håvarstein 2009). A schematic overview of the regulation of competence in *S. pneumoniae* is shown in figure 1.2.1.

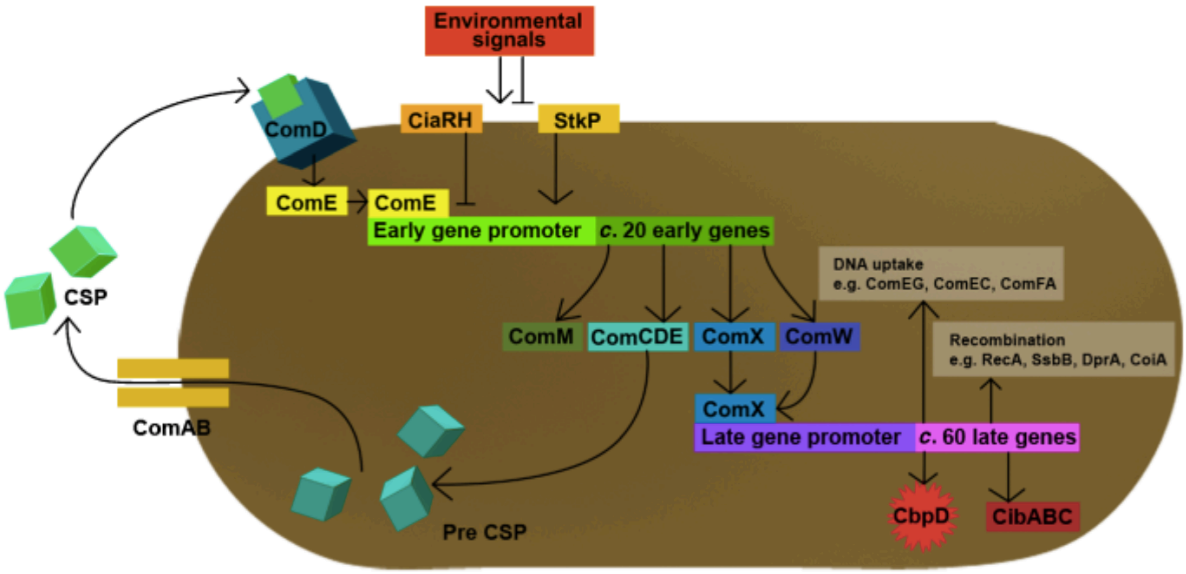


Figure 1.2.1: Schematic presentation of competence regulation in *S. pneumoniae*. The CSP precursor is processed and secreted out of the cell by the ComAB transporter. Mature CSP accumulates outside of the cell where it is sensed by ComD, resulting in autophosphorylation and the subsequent transfer of the phosphoryl group to the cytoplasmic response regulator ComE. ComE then binds to and activates several early competence gene promoters, among them increasing transcription of *comCDE* generating an auto-induced loop as the cell produces more Pre-CSP, ComD and ComE. Also activated is the transcription of the fratricide immunity protein ComM and of the alternative σ–factor ComX. The latter activates the transcription of the late competence genes. Many of these genes are responsible for the uptake and recombination of extracellular DNA as well as the muralytic fratricide protein CbpD. Figure from Johnsborg & Håvarstein (2009).

Induction of competence is regulated by an auto-inducing regulatory system encoded by the *comCDE* operon. The *comC* gene encodes a competence-stimulating peptide (CSP), while *comD* and *comE* encodes a two-component system comprising a membrane embedded histidine kinase receptor (ComD) and its cognate response regulator (ComE) (Håvarstein et al. 1995, Johnsborg and Håvarstein 2009). Pre-CSP is processed to mature CSP when it is transported out of the cell via the ATP-binding ComAB transporter. Here CSP can bind to ComD of the CSP-producing cell or nearby cells. The interaction with CSP leads to the

autophosphorylation of ComD, which transfers its phosphoryl group to the response regulator ComE (Håvarstein et al. 1996). ComE binds to a tandem repeat motif present in all promoters of the early competence genes (Ween et al. 1999). Phosphorylated ComE is found to form oligomers, which are required for efficient expression of early competence genes. These genes include the *comCDE* operon, resulting in an autoregulatory circuit, in addition to the fratricide immunity gene *comM*, and the alternative σ–factor ComX (Johnsborg and Håvarstein 2009). Competence is initiated by ComX which induces a transcriptional reprogramming of the cells (Johnsborg and Håvarstein 2009). ComX activates approximately 60 late competence genes, including those required for DNA uptake and homologous recombination. Also expressed is the peptidoglycan hydrolase CbpD (Choline binding protein D), a protein involved in the lysis of closely related non-competent neighbouring cells in a process called fratricide. The late competence gene product called DprA, which binds and prevents the function of phosphorylated ComE, turns off competence (Mirouze et al. 2013).

1.2.1 Fratricide in *S. pneumoniae* and the importance of CbpD

Fratricide, meaning "the killing of ones brother", is a term used for describing the competence-induced lysis of non-competent sister cells or closely related species by competent pneumococcal cells (Johnsborg and Håvarstein 2009, Eldholm et al. 2010). This mechanism plays a significant role in the horizontal transfer of genes between pneumococci in laboratory cultures, and it has been proposed that the purpose of this phenomenon is to capture genetic material from other cells in order to facilitate repair of damaged genes and acquire novel features (Johnsborg et al. 2008, Berg et al. 2012). The competence-induced peptidoglycan hydrolase CbpD is a key component during fratricide as it binds to and attacks the cell wall of non-competent cells, causing a release of nutrients and DNA (Figure 1.2.2) (Kausmally et al. 2005, Eldholm et al. 2010). Two other cell wall hydrolases called LytA and LytC also contribute to cell lysis during fratricide. LytA and LytC are produced in both competent and non-competent cells. The contribution to fratricide by LytA and LytC from competent cells is not clear, but they are believed to participate in target cell lysis (Eldholm et al. 2010). LytA and LytC in non-competent cells on the other hand have been shown to accelerate the lysis reaction once CbpD has made damage to the cell wall (Eldholm et al. 2009). Furthermore, on solid medium, the two-peptide bacteriocin CibAB has also been shown to contribute to target cell lysis during fratricide (Guiral et al. 2005). Deletion of *cbpD*

in competence-induced cells abolishes lysis of non-competent cells, demonstrating that CbpD is the key player in competence-induced fratricide (Johnsborg et al. 2008).

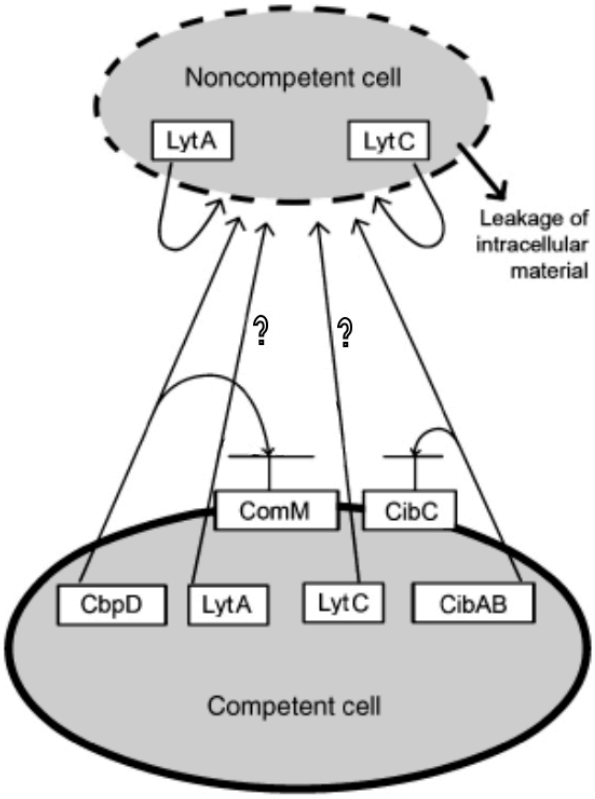


Figure 1.2.2 Factors influencing cell lysis during fratricide in pneumococcus. CbpD and CibAB lyse non-competent cells, while the competent cells remain immune due to the production of ComM and CibC, respectively. The role of LytA and LytC in this process is unclear. While CbpD is the main contributing factor in liquid cultures, the two-peptide bacteriocin CibAB appears to be the main trigger factor on solid media. Modified from Johnsborg & Håvarstein (2009).

The transmembrane early-gene proteins ComM and CibC are needed for immunity against CbpD and CibAB respectively. A Δ*comM* strain commits suicide when induced to competence with CSP because it is unprotected from its own CbpD (Håvarstein et al. 2006). The mechanism of ComM has yet to be established.

CbpD most probably catalyses the hydrolysis of peptide bonds in the stem peptides of the pneumococcal cell wall and is thus classified as an amidase/endopeptidase. The modular CbpD enzyme consists of an N-terminal hydrolytic CHAP domain, two SH3b domains and a C-terminal choline-binding domain (CBD) (Eldholm et al. 2010). The choline-binding domain recognizes the phosphorylcholine decorating the teichoic acids in the bacterial cell wall (see 1.4.1), and localizes the enzyme to the septal region of the pneumococcal cells. The

two SH3b domains have been shown to be essential for proper CbpD function, and evidence indicates that they direct the CHAP domain to its peptidoglycan substrate by binding to an unknown part of the peptidoglycan complex (Eldholm et al. 2010, Berg et al. 2012).

1.3 The bacterial cell wall

Most bacteria have a cell wall surrounding their cytoplasmic membrane that gives the bacteria their shape and protects them against osmotic lysis from turgor pressure (Cabeen and Jacobs-Wagner 2005). There are two general classes of cell walls in bacteria, the Gram-positive cell wall and the Gram-negative cell wall (figure 1.3.1). This classification dates back to 1884 when Hans Christian Gram developed his technique (Gram-staining) for distinguishing between the two major classes, and was for a long time an important taxonomic tool before the advent of genetic analysis (Woese 1987).

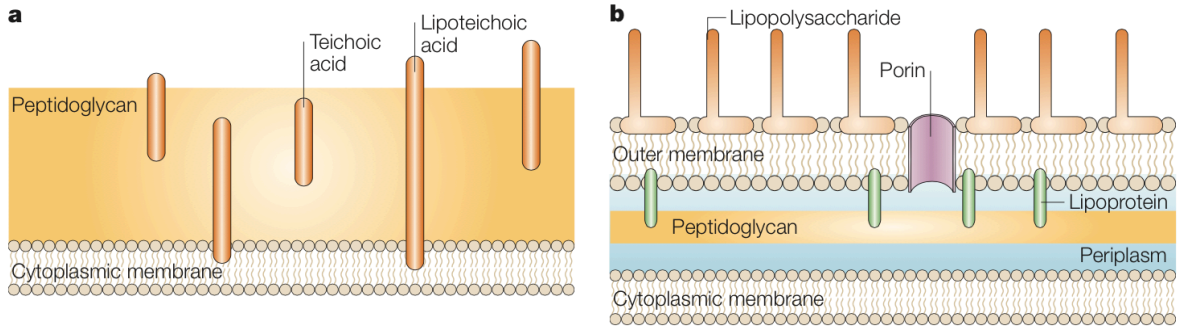


Figure 1.3.1: The bacterial cell wall. a: The gram-positive cell wall is composed of a thick layer of peptidoglycan with embedded teichoic and lipoteichoic acids. Proteins are also embedded in and attached to the cell wall (not shown in illustration). **b:** The gram-negative cell wall is composed of a thin layer of peptidoglycan attached to the outer membrane via lipoproteins. The outer membrane consists of a lipid bilayer with lipopolysaccharides and proteins. Figure from Cabeen and Jacobs-Wagner (2005).

The main constituent of the bacterial cell wall is called peptidoglycan. This is a giant macromolecule composed of glycan chains that are cross-linked by stem peptides, forming a mesh-like structure that completely envelops the cell. The Gram-negative bacteria have a cell wall composed of a thin layer of peptidoglycan located between the cytoplasmic membrane and an outer membrane, in an area called the periplasm. The cell wall in Gram-negative bacteria serves as structural support and is linked to the outer membrane by lipoproteins. The outer membrane anchors several proteins and lipopolysaccharides and serves an important role in adherence, transport of substances in and out of the cell and as virulence factors. In Gram-positive bacteria, the cell wall is composed of a thick peptidoglycan sheath with

anchored proteins, teichoic acids and lipoteichoic acids, located outside of the cell membrane (Cabeen and Jacobs-Wagner 2005).

1.3.1 Peptidoglycan

The main constituent of the bacterial cell wall is the structurally complex peptidoglycan, also known as murein. It is composed of linear glycan chains of alternating *N*-acetylglucoseamine (Glc*N*ac) and *N*-acetylmuramic acid (Mur*N*ac) linked by β-1,4 glycosidic bonds, which in turn are cross-linked by short peptides attached to the Mur*N*ac residues (Figure 1.3.2) (Vollmer et al. 2008). The cross-linking peptides usually consist of the amino acid sequence L-Ala, D-Glu, meso-A2pm (diaminopimelic acid) and D-Ala. The cross-linking generally occurs between the carboxyl group of D-Ala at position 4 and the amino group of the diamino acid at position 3. There are several know alternate variants of the tetrapeptide in different species, including an amidation of D-Glu to D-iGln in *Streptococcus*, but the basic architecture of peptidoglycan is shared among all eubacteria (Scheffers and Pinho 2005, Vollmer et al. 2008, Zapun et al. 2013). The cross-linked peptides can make further cross-links with neighbouring peptides by DD-transpeptidation, forming dimeric, trimeric or tetrameric structures (Bui et al. 2012). All of this, along with covalently bound teichoic and lipoteichoic acids, contributes to give peptidoglycan a highly complex 3D structure in *S. pneumoniae*. The length of the glycan chains varies greatly among different bacterial species, from 6 disaccharide units in *S. aureus* to up to 500 in *B. subtilis* (Ward 1973, Hughes et al. 1975, Harz et al. 1990, Hayhurst et al. 2008, Vollmer et al. 2008). In *S. pneumoniae* the glycan chains are usually approximately 25 disaccharide units in length, with shorter glycan chains being almost entirely absent (Bui et al. 2012). The gram-positive peptidoglycan layer can be 20-80 nm thick, while it is considerably thinner in gram-negative bacteria at approximately 7-8 nm (Vollmer et al. 2008).

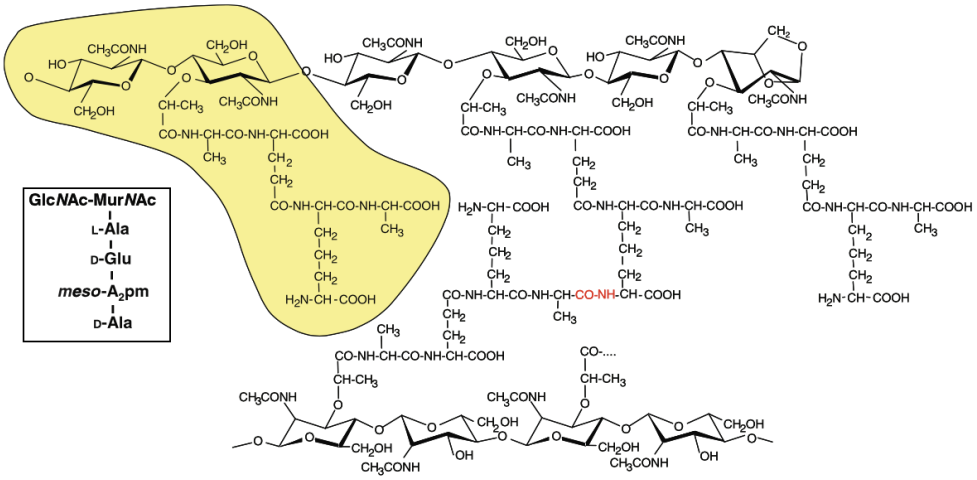


Figure 1.3.2: General peptidoglycan structure. The major component of the gram-positive cell wall is the polymer peptidoglycan. It is composed of alternating β–1,4 linked *N*-Acetylglucoseamine and *N*-Acetylmuramic acid cross-linked by short peptide chains attached to the *N*-Acetylmuramic acid residues. The composition of the peptide side chains shows considerable variation between species and may also appear non-cross-linked. Figure from Vollmer et.al (2008).

1.4 The pneumococcal cell wall

The cell wall of *S. pneumoniae* is a multi-layered peptidoglycan complex, with covalently attached teichoic acids (WTA) and membrane bound lipoteichoic acids (LTA). In addition to determining cell shape and giving mechanical strength, the cell wall components are required for a multitude of biological processes including uptake of nutrients, binding to external macromolecules, adhesion to surfaces, interaction with the human host organism and uptake of DNA during transformation (Vollmer 2007). Some of the capsular polysaccharides mentioned in section 1.1.2 are covalently attached to the cell wall, while others are not. Through genome analysis done with the laboratory strain R6, more than 470 proteins have been predicted to be secreted to the medium or attached to the cell wall (Hoskins et al. 2001). Of these proteins, 69 are likely exposed to the cell surface according to analysis done on the TIGR4 strain (Tettelin et al. 2001).

1.4.1 Teichoic and lipoteichoic acids of the pneumococcal cell wall

Teichoic acids are polysaccharides that are rich in phosphodiester bonds and are only found in gram-positive bacteria (Neuhaus and Baddiley 2003). They play an integral part in cell function as they bind cell surface proteins that are involved in regulation of cell wall elongation and division, transport processes and the regulation of cell wall hydrolases (Ton-

That et al. 2004, Hakenbeck et al. 2009). They are also important for resistance to antimicrobial peptides, lysozymes and interaction with the host (Neuhaus and Baddiley 2003, Weidenmaier and Peschel 2008). The pneumococcal cell wall contains an unusually complex peptidoglycan-bound teichoic acid (WTA) consisting of multiple repeating units. The membrane bound lipoteichoic acid (LTA) is made up of the same repeating unit (Denapaite et al. 2012). This contrasts to most other Gram-positive bacteria, where the WTA and LTA have different primary structures. Pneumococcal LTAs are attached to the cytoplasmic membrane through a glycolipid anchor. The repeating unit of WTAs and LTAs in pneumococci comprises the rare amino sugar 2-acetamido-4-amino-2,4,6-trideoxygalactoce, glucose, Rib-P and two Gal*N*Ac residues each carrying a phosphorylcholine moiety (Figure 1.4.1) (Denapaite et al. 2012). In pneumococci the LTAs and WTAs can contribute to up to 50% of cell wall dry weight (Neuhaus and Baddiley 2003).

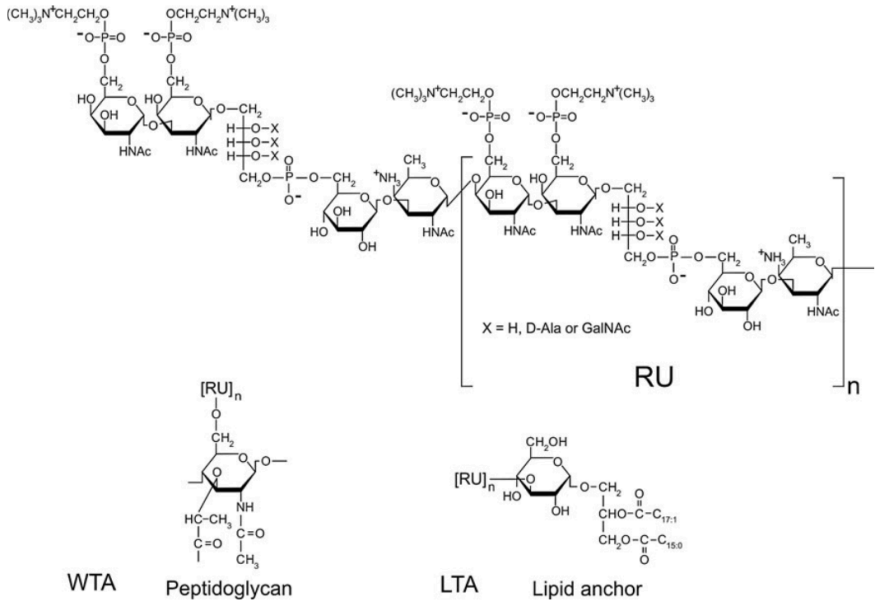


Figure 1.4.1: The structure of *S. pneumoniae* teichoic and lipoteichoic acid repeating unit. The WTA and the LTA consists of the same repeating unit containing the rare amino sugar 2-acetamido-4-amino-2,4,6-trideoxygalactoce, glucose, Rib-P and two Gal*N*Ac residues, each carrying a phosphorylcholine moiety. Binding of the repeating unit to Mur*N*ac yields the WTA, while binding to the glycolipid glucose-diacetylglycerol yields the LTA. Figure from Denapaite et.al. (2012).

The pneumococci require choline in the growth medium, as it is metabolized to decorate the teichoic acids as phosphorylcholine (Tomasz 1967, Denapaite et al. 2012). Phosphorylcholine serves as anchoring point for several proteins (choline binding proteins), including the previously described CbpD (see 1.2.1).

1.4.2 Peptidoglycan synthesis in *Streptococcus pneumoniae*

The machinery involved in the synthesis of the pneumococcal cell wall is complex, and unravelling its components and their functions is an area of intensive research. The proteins involved in synthesizing and modifying peptidoglycan are called Penicillin Binding Proteins (PBPs), as they also are the targets of β–lactam antibiotics (Zighelboim and Tomasz 1980, Zighelboim and Tomasz 1981). All bacterial species that have a peptidoglycan cell wall have one or several PBPs, and they are found either in the cytoplasm or as membrane bound proteins. *S. pneumoniae* has 6 PBPs that can be divided into three sub-classes according to their activity (Sauvage et al. 2008, Zapun et al. 2008a). The class A PBPs; PBP1a, PBP1b and PBP2a are bifunctional enzymes having both a transpeptidase domain (TP) and a glycosyl transferase domain (GT). PBP2b and PBP2x are class B PBPs, which only have a TP domain, while the class C PBP DacA (PBP3) has an identifiable TP domain sequence-wise, but is in fact a carboxypeptidase that removes the fifth D-Ala residue from the stem peptide by hydrolysis (Zapun et al. 2013). The recently discovered carboxypeptidase DacB is structurally unrelated to DacA, and is responsible for the removal of the fourth residue D-Ala from the stem peptides (Abdullah et al. 2014). PBP2b and 2x are essential enzymes (Kell et al. 1993), while all class A PBPs can be deleted individually, indicating some overlap in function (Hoskins et al. 1999, Zapun et al. 2013). The reason for the apparent surplus of PBPs in *S. pneumoniae* and other ovococci (i.e. *Lactococcus* and *Enterococcus*) is likely due to a need for different machineries or activity in the septal and peripheral cell wall synthesis (Sham et al. 2012, Philippe et al. 2014).

The GT domain polymerizes the glycan chains (Glc*N*Ac-Mur*N*Ac), while the TP domain is involved in the cross-linking of the stem peptides. In the transglycosylation reaction the C-4 carbon of Glc*N*Ac of the peptidoglycan precursor Lipid II is added to the reducing end of the Mur*N*Ac residue of the nacent lipid-linked peptidoglycan strand, followed by the release of undecaprenyl pyrophosphate (Scheffers and Pinho 2005). Prior to transpeptidation, the D-Ala-D-Ala bond of one stem peptide is cleaved, providing the necessary energy for the transpeptidation reaction which takes place on the outside of the cytoplasmic membrane, where no energy donors such as ATP is present (Scheffers and Pinho 2005). β–lactam antibiotics are structurally analogous to the D-Ala-D-Ala motif of the stem peptide, which is the natural substrate for the TP domain of the PBPs. β–lactams therefore bind the active site of the TP domain irreversibly to form a stable adduct (Tipper and Strominger 1965). This

results in inactivation of the cell wall synthesis and inhibition of cell growth. β–lactam resistance in *S. pneumoniae* is a result of mutations in the *pbp* genes causing conformational changes in tertiary PBP structure, yielding functional PBPs with low β–lactam affinity (Zighelboim and Tomasz 1981, Hakenbeck et al. 1999, Sauvage et al. 2008). β–lactam resistance poses a serious threat to public health, as resistance is on the rise worldwide (Moreno et al. 1995, Bedos et al. 1996, Musher 2000, Croucher et al. 2011, CDC 2013).

Two models for cell wall synthesis have been proposed for ovococci bacteria (Berg et al. 2013). One model suggests that there are two independent machineries, where one is responsible for peripheral cell wall synthesis, while the other makes the septal cell wall. The other model is based on single machinery that can switch between peripheral and septal synthesis by activating different PBPs throughout the cell cycle. Although different, it is clear for both models that PBP2b is involved in peripheral cell wall synthesis, while the role of PBP2x is dedicated to septal cell wall synthesis (Deghorain et al. 2010, Perez-Nunez et al. 2011, Berg et al. 2013). Figure 1.4.2 presents a scheme of the septal and peripheral peptidoglycan synthesis machinery as a two complex model.

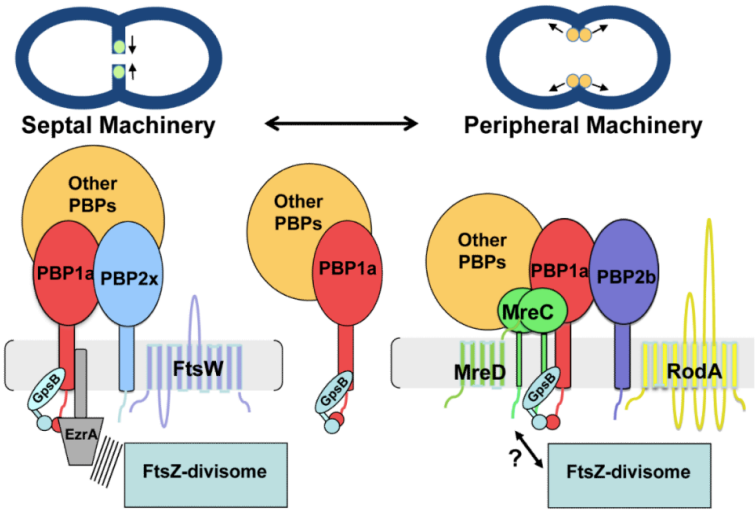


Figure 1.4.2: The septal and peripheral peptidoglycan synthesis machinery in *S. pneumoniae*. It is postulated that the septal machinery consists of EzrA, FtsW and PBP2x, and the peripheral machinery of MreC, MreD, RodA and PBP2b (Claessen et al. 2008, Zapun et al. 2008b, Sham et al. 2012). GpsB and PBP1 are present in both machineries. The septal machinery is in contact with the FtsZ-divisome, and it is possible the peripheral machinery also is initially. Figure from Sham et al. (2012).

In *S. pneumoniae* current evidence shows that PBP2b mediates the peripheral cell wall synthesis along with the proteins MreC, MreD, RodA and GpsB, while PBP2x along with FtsZ, EzrA, GpsB, FtsW and DivIB/FtsL/DivIC are involved in the septal synthesis (Sham et al. 2012), and that these two processes can occur independently of each other (Berg et al. 2013). The roles of the class A PBPs 1a, 1b and 2a remain unclear, but the fact that *S. pneumoniae* only needs 1a or 2a for viability points to some overlap in function. An involved discussion of the other proteins involved in cell wall synthesis and cell division is beyond the scope of this thesis. See Claessen et al. (2008), Zapun et al. (2008b) and Sham et al. (2012) for detailed descriptions. The differences between PBP2b and PBP2x give rise to the characteristic morphology of ovococci (Zapun et al. 2008b). It has been shown that specific shapes confer advantages for environmental challenges like nutrient access, predation avoidance, diffusion and defence against stress, and the morphology of pneumococci is probably an adaptation to a multitude of environmental factors (Philippe et al. 2014). The substrate for the PBPs is the amphipatic macromolecule lipid II, whose synthesis and biological role is discussed in the next section.

1.4.3 Lipid II metabolism in *S. pneumoniae*

The membrane anchored peptidoglycan precursor Lipid II (figure 1.4.3) is a minor component of the cytoplasmic membrane of bacteria, and constitutes less than 1mol% of the membrane phospholipids in gram-positive bacteria (de Kruijff et al. 2008). The synthesis of Lipid II starts in the cytoplasm where UDP-Mur*N*Ac (uridine diphosphate Mur*N*Ac) is formed through the condensation of phosphoenolpyruvate with UDP-Glc*N*Ac, followed by a reduction. The amino acids are added in sequence, resulting in the formation of UDP-Mur*N*Ac-Mur*N*Ac-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala. The addition of the amino acids require the ATP-dependent amino acid ligases MurC, MurD and MurE, with the final two D-Ala-D-Ala units added as a dipeptide by MurE (Blewett et al. 2004). All enzymes involved in these reactions are cytoplasmic. A membrane bound translocase then transfers the Mur*N*Ac-pentapeptide to the isoprene undecaprenyl phosphate (also known as bactoprenol) on the cytoplasmic side the membrane, yielding lipid I. Finally a transferase adds the Glc*N*Ac residue to lipid I, using UDP-Glc*N*Ac-pentapetide as substrate, yielding lipid II (Scheffers and Pinho 2005, Jeffrey D Esko 2009). In *Streptococcus* lipid II is subjected to an amidation of the second residue D-Glu to D-iGln by the enzyme complex MurT/CobQ before it is used in peptidoglycan synthesis (Zapun et al. 2013). The importance of this amidation and

MurT/CobQ is the main focus of the present work and is described in detail in the next section. FtsW is a flippase that is involved in the translocation of lipid II across the cell membrane where it is added to the growing peptidoglycan chain via transglycosylation and transpeptidation as previously described in 1.4.2 (Mohammadi et al. 2011, Typas et al. 2012). Lipid II is the target of several antimicrobial compounds like vancomycin, ramoplanin and the recently discovered teixobactin (Breukink and de Kruijff 2006, Ling et al. 2015).

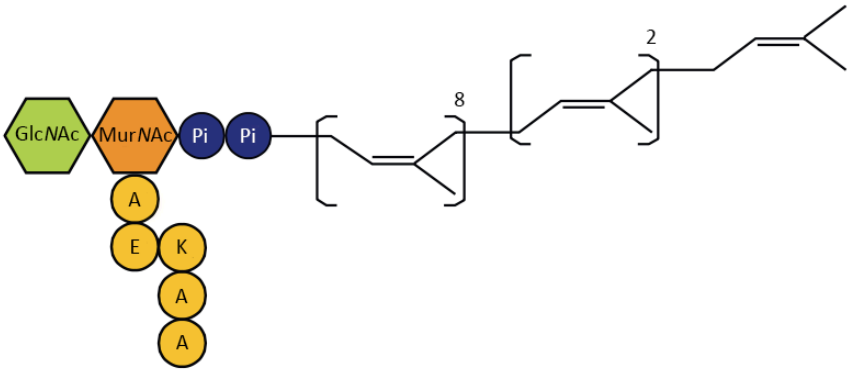


Figure 1.4.3: Lipid II structure. The membrane-anchored peptidoglycan precursor lipid II is composed of a Glc*N*Ac-Mur*N*Ac disaccharide with a pentapeptide attached to the Mur*N*Ac-residue. A pyrophosphate-attached polyisoprenoid composed of eight isoprene units in the *cis*-conformation, followed by two isoprene units in the *trans*-configuration and a final isoprene unit is attached to the Mur*N*Ac-residue (Breukink and de Kruijff 2006). The pentapeptide sequence tends to vary between species, and in *S. pneumoniae* it is L-Ala-D-Glu-L-Lys-D-Ala-D-Ala. The second residue D-Glu is modified to D-iGln before peptidoglycan synthesis. Figure modified from de Kruijff et al. (2008).

1.4.4 MurT/CobQ: Amidation of Lipid II in *S. pneumoniae*

The fact that the second residue γ–glutamate in the peptidoglycan stem-peptide in *S. pneumoniae* is amidated to form isoglutamine has been known for some time, but it was only recently that the enzyme complex catalysing this reaction was discovered (Zapun et al. 2013). The same amidation is also seen in other gram-positive pathogens like *Clostridium perferingens*, *Mycobacterium tuberculosis* and *Stapylococcus aureus*, and the enzymes responsible were first described in the latter in two studies published in 2012 (Figueiredo et al. 2012, Munch et al. 2012). The enzyme complex is composed of two proteins: a glutamine amidotransferase-like protein (GatD) and a Mur ligase homologue (MurT). The complex was given the name MurT/GatD (Munch et al. 2012). The role of the amidation of lipid II has been proposed to play a role in the polymerization of peptidoglycan, being correlated with the cross-linking of neighbouring stem-peptides (Munch et al. 2012). In 2013 the *murT/gatD* orthologous genes *spr1443* and *spr1444* were identified in the *S. pneumoniae* R6 strain, in a

study by André Zapun and colleagues aiming to do the complete *in vitro* reconstruction of peptidoglycan synthesis (Zapun et al. 2013). The Zapun et al. paper uses the same nomenclature for the genes and proteins as the ones found in *S. aureus*, but the gene *spr1444* is already annotated as *cobQ* in GenBank and as such is the name that will be used in this thesis. The *spr1443* gene has no existing GenBank annotated name and will still be referred to as *murT*. Figure 1.4.1 shows the pneumococcal lipid II structure and the site of amidation by MurT/CobQ. The two genes form an operon with one overlapping base pair at the stop codon of *murT* and the start codon of *cobQ*. Both genes are essential for cell viability in pneumococci (Zapun et al. 2013). This makes MurT/CobQ very interesting, not only academically, but also as a potential antibiotic target. Considering the increase in antibiotic resistant isolates, new ways of fighting pathogenic bacteria will be needed.

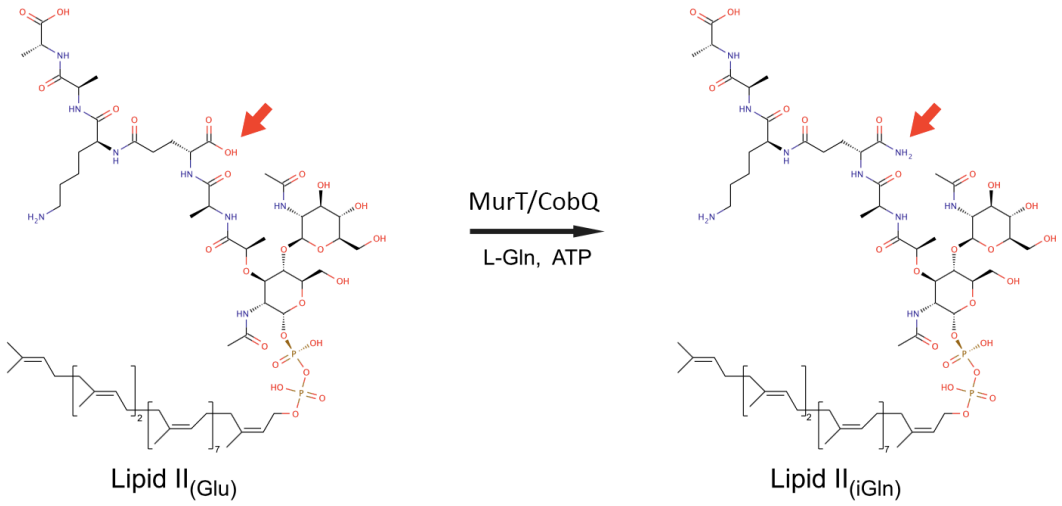


Figure 1.4.4: Structure of pneumococcal lipid II and the activity of MurT/CobQ. The MurT/CobQ enzyme complex catalyses the amidation of the second residue glutamate to isoglutamine on lipid II. The reaction is ATP dependent and uses L-glutamine as the amino-group donor. Figure modified from Zapun et al. (2013).

Previous studies have shown that only a small portion (~2%) of the peptides in the peptidoglycan of *S. pneumoniae* contains glutamate, most of it being in non-cross-linked monomers (12.6% of monomers), while being nearly absent in cross-linked dimers (1.8% of dimers) and is not detectable in trimers (Bui et al. 2012). The research group of André Zapun studied the substrate specificity of PBP1a, 2a, 2b and 2x *in vitro* and found that the enzymes are dependent on the isoglutamine-containing lipid II to successfully carry out transpeptidation. The only PBP that showed some residual TP activity with non-amidated lipid II was the non-essential PBP1a, although this activity was much lower than with amidated lipid II. The GT activity of PBP1a and 2a, on the other hand did, not appear to be

affected by the type of lipid II being used as substrate (Lipid $II_{(Glu)}$ or lipid $II_{(iGln)}$), indicating that the main reason for the amidation is related to the binding specificity or activity of the TP domain (Zapun et al. 2013). Why the cells are able to incorporate a small amount of cross-linked non-amidated peptides into the cell wall, knowing that the PBPs require the amidated form of lipid II, is not known, but it is possible that it is due to the residual PBP1a activity.

1.4.5 Stem peptide composition of the pneumococcal cell wall

The network of stem peptides in the pneumococcal peptidoglycan is structurally complex, as it is composed of a variety of different cross-linked peptides and monomers (Garcia-Bustos et al. 1987). The pentapeptide (see 1.4.3) usually becomes branched at the lipid II level by attaching an L-Ala-L-Ala or L-Ser-L-Ala dipeptide to the ε–amino side group of the L-Lys residue at position 3 prior to peptidoglycan synthesis (Vollmer 2007). The enzyme MurM attaches either an L-Ser of L-Ala before MurN attaches the second L-Ala (Filipe and Tomasz 2000, Smith and Klugman 2001). Garcia-Bustos et al. (1987) found that the main constituents of the pneumococcal cell wall peptides (accounting for 77%) were the 10 mono-, di- and trimers shown in figure 1.4.5. The monomers are not parts of cross-bridges like the dimers and trimers are. Two main types of cross-links exist, with a bond either between the D-Ala in position 4 of the donor peptide and the L-Ala in position 3 of the acceptor peptide, or between the D-Ala in position 4 in the donor peptide and the second branched L-Ala in the acceptor peptide. Further cross branching can occur, yielding trimers, tetramers and pentamers (Garcia-Bustos et al. 1987, Vollmer 2007). As mentioned in the previous section, minor fractions of the monomer and dimer stem peptides have detectable D-Glu instead of D-iGln at position 2 (Bui et al. 2012). The stem peptide compositions can vary dramatically between different pneumococcal strains, with the main difference being in the extent of branching (Vollmer 2007). Around 36% of the total peptide composition is estimated to be monomers, 26% dimers and ~10% trimers in *S. pneumoniae* strain R6. The remaining fractions are thought to be tetramers and a small amount of pentamers (Bui et al. 2012). In some penicillin resistant strains the percentage of highly branched peptides is abnormally high, indicating that some mutations in PBPs garnering resistance has lead to an altered activity and specificity in regards to transpeptidation (Vollmer 2007).

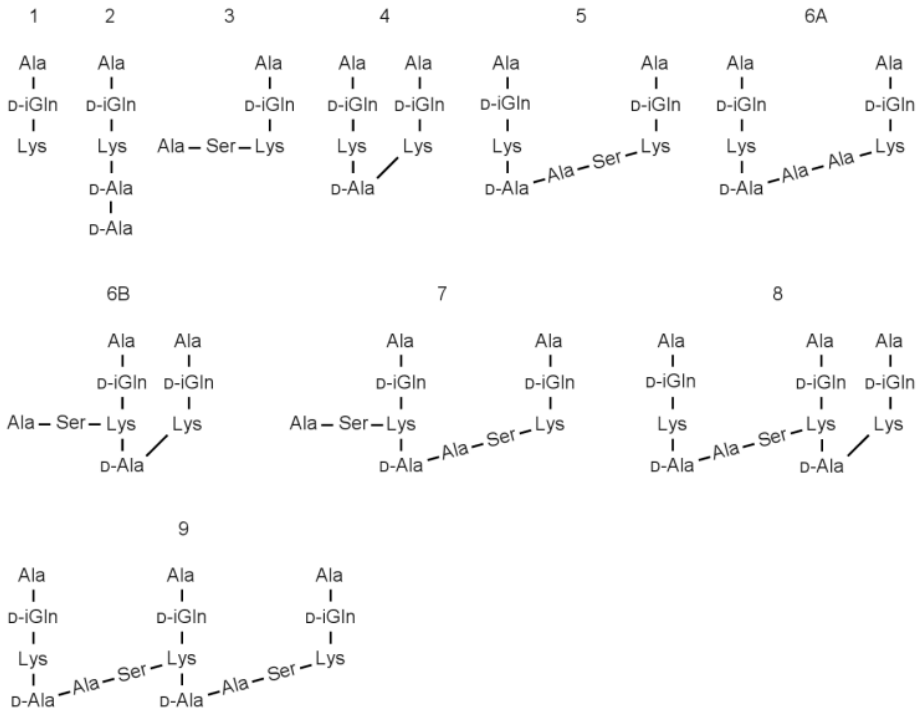


Figure 1.4.5: Major pneumococcal peptidoglycan peptides. The structures of the ten most common monomeric, dimeric and trimeric stem peptides in the peptidoglycan of *S. pneumoniae*. 1-3 are monomers, 4-7 are dimers and 8-9 are trimers. Figure from Vollmer (2007).

1.5 Studies of essential genes

An essential gene is a gene which function is entirely necessary for survival of the cell. Studying essential genes in bacteria present a challenge, as potential phenotypic changes cannot be examined by conventional knockout studies. One solution is to overexpress or underexpress the genes instead. This can be done by ectopically expressing the gene using a tightly titratable promoter, allowing for the deletion of the native gene (Berg et al. 2012). *S. pneumoniae* is estimated to have >132 essential genes, including the previously described *murTcobQ* operon (1.4.4) (Song et al. 2005). Several systems have been developed for ectopic over/underexpression of genes in *S. pneumoniae*. Amongst the most extensively used systems is one based on a Zn^{2+} regulated promoter (P_{czcD}), one based on a fucose regulated promoter and the so-called ComRS system, the latter of which utilizes the signal transduction pathway regulating transformation in *Streptococcus thermophilus* (Berg et al. 2011). Gene expression from these promoters can be controlled by varying the amount of inducer (Zn^{2+}, fucose or ComS*) in the growth medium. There also exist other systems for ectopic expression in *S. pneumoniae.* They include nisin and tetracycline induced promoter systems,

none of which are discussed here as they are beyond the scope of this thesis (Eichenbaum et al. 1998, Apfel et al. 1999, Chan et al. 2003).

1.5.1 The ComRS system: A heterologous peptide-regulated gene depletion system for use in *S. pneumoniae*

In *S. thermophilus* the ComRS system is the signal transduction pathway for genetic transformation and includes the small signal peptide ComS*, the transcriptional activator ComR and the inducible promoter P_{comX} (Fontaine et al. 2010). In species belonging to the Mitis group (i.e. *S. pneumoniae*) regulation of the P_{comX} promoter depends on a two-component system, as previously described in 1.2, where CSP is sensed by the membrane bound histidine kinase ComD. In *S. thermophilus* on the other hand (a species belonging to the salivarius group), the competence inducing peptide (ComS*) is imported into of the cell by the oligopeptide transporter Ami. Inside the cell ComS* will bind to the transcriptional activator ComR, causing a conformational change. ComR recognizes two inverted repeats in the P_{comX} promoter called the ECom box, initiating transcription of the genes necessary for transformation (Fontaine et al. 2010). None of the components in the ComRS system have any homologs in *S. pneumoniae*, and so introduction of this system into pneumococcal lab strains probably does not interfere with normal cell function (Berg et al. 2011). The use of Zn^{2+} and fucose in the growth medium on the other hand might cause unwanted effects on the expression of native genes in *S. pneumoniae.* This makes the ComRS system a preferred choice for over/underexpression studies.

Berg et al. (2011) created a pneumococcal strain having both the *comR* gene and the P_{comX} promoter from *S. thermophilus* in its genome. When studying essential genes in *S. pneumoniae* using the ComRS system, this strain is used. A recombinant version of the gene is inserted behind P_{comX}. Following this, the native gene is removed using an antibiotic selection marker, while the ectopic version of the gene is expressed in the presence of synthetic ComS*. ComS* is imported by the endogenous Ami transporter. The level of ectopic gene expression can be fine tuned depending on the amount of ComS* added. Considering this advantage for the ComRS system and the long experience with this system in the laboratory in which the present work was carried out, the ComRS system was chosen to study the effect of *murTcobQ* underexpression/depletion in *S. pneumoniae*. The ComRS system in *S. pneumoniae* has proven useful in a handful of studies since its conception, most

notably with genes involved in cell wall synthesis and cell division (Berg et al. 2013, Bartual et al. 2014, Berg et al. 2014).

1.6 Thesis objectives

The aim of this work has been the study of the amidotransferase complex MurT/CobQ in *S. pneumoniae*, which is responsible for the amidation of the second residue D-Glu to D-iGln in the peptidoglycan precursor lipid II. The role of this amidation and why it is needed is unclear, but it has been shown to be essential for cell viability. By doing gene depletion studies, this work has focused of how the lack of amidated lipid II affects:

- Antibiotic sensitivity
- Lysozyme sensitivity
- Sensitivity in a Δ*pbp1a* or Δ*pbp2a* background
- CbpD sensitivity in a Δ*comM* background
- Morphology
- Peptidoglycan stem peptide composition

2 Materials

2.1 Bacterial strains

Table 2.1.1: *S. pneumoniae* strains. The table shows the strains used in this work and a description of their genotype.

Strain	Genotype/relevant characteristic	Reference
RH1	Δ*egb::spc*, Ery^R, Spc^R	Johnsborg et al. (2008)
RH420	Δ*comM*::Janus, Ery^R, Kan^R, Cm^R, Spc^R	Eldholm et al. (2009)
SPH131	P_{comR} inserted between *amiR* and *treF*. P_{comX} inserted downstream of *cpsO*. Janus inserted between *cpsO* and *cpsN*, Ery^R, Kan^R	Berg et al. (2011)
KHB104	Δ*pbp1a*::Janus	Berg, unpublished
KHB105	Δ*pbp2a*::Janus	Berg, unpublished

Table 2.1.2: *S. pneumoniae* mutants constructed in this work. The relevant genotype/characteristic are shown.

Strain	Genotype/relevant characteristic
OH6	SPH131 but ΔJanus::$murTcobQ_{ectopic}$, Sm^R
OH7	OH6 but Δ$murTcobQ_{native}$::Janus, Kan^R
OH8	OH7 but ΔJanus, Sm^R
OH9	OH8 but Δ*comM*::Janus, Kan^R
OH10	OH8 but Δ*pbp1a*::Janus, Kan^R
OH11	OH8 but Δ*pbp2a*::Janus, Kan^R

Table 2.1.3: Other bacterial strains. A short description of genotype/characteristic is shown.

Strain	Genotype/characteristic
Lactococcus lactis NZ9000	pNZ8037-CbpD, expresses CbpD when induced with nisin

2.2 Peptide pheromones

Table 2.2.1: Peptide pheromones. The table shows the amino acid sequence of the peptide pheromones used in this work.

Pheromone name	AA sequence (N→C)	Stock concentration
CSP	EMRLSKFFRDFILQRKK	100 μg/ml
ComS*	LPYFAGCL	500 μM

2.3 Primers

Table 2.3.1: Primers. The table shows the nucleotide primers used in this work and their sequence. Stock solutions of all primers were 10 pmol/μl. Underlined sequences indicate overlapping regions.

Primer name	Sequence (5´→3´)	Description	Reference
Kan484F	GTTTGATTTTTAATGGATA ATGTG	Forward primer for Janus Cassette	Johnsborg et al. 2008
RpsL41R	CTTTCCTTATGCTTTTGGAC	Reverse primer for Janus Cassette	Johnsborg et al. 2008
Janus P1 R	AAGTATTTTCTAGTATTATA GCACATTTAACTTTCCTTAT GCTTTTGGAC		
ds188	ATTTATATTTATTATTGGAG GTTCAATGAACTTAAAAAC TACTTTGGG	Binds at start codon of *murT*, 5´-end complementary to khb36	This study

ds189	ATTGGGAAGAGTTACATATTAGAAATTAAGAAAAGTCAGCCTTGCTT	Binds at stop codon of *cobQ*, 5'-end complementary to khb33	This study
ds190	GTCTTTGACTCAACAGGTATC	Binds 1 kb upstream of *murT*	This study
ds191	CACATTATCCATTAAAAATCAAACGTTTCTATTATATCACAAAAGAG	Binds first base upstream of *murT* start codon, 5´-end complementary to Janus	This study
ds192	TTAAATGTGCTATAATACTAGAAAATACTTACAAAGGAAAATGATATCAAAGAAC	Binds the first base downstream of *cobQ* stop codon, 5´-end complementary to janus. Forward	This study
ds193	GTCCACTTCATGAGCAGTCAC	Binds 1 kb downstream of *cobQ*. Reverse	This study
ds194	CCTCTTTTGTGATATAATAGAAACACAAAGGAAAATGATATCAAAGAAC	Binds first base downstream of *cobQ* stop codon, 5´-end complementary to ds195. Forward.	This study
ds195	GTTTCTATTATATCACAAAAGAGG	Binds first base upstream of *murT* start codon. Reverse	This study
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

kbh34	CATCGGAACCTATACTCTTTTAG	Reverse primer for *cpsN*	Berg et al. 2011
109_comMF	GACCGAACTTACCTTGAATGGA	Binds 1 kb upstream of comM. Forward.	
112_comMR	TGCCCCACGCTCTTGG	Binds 1 kb downstream of comM. Reverse.	
khb43	GCATTGCGCTTGATAAGTTTGAGGATAATCAAGATTTATCTTATAAA	*comR* forward primer with 5´ *amiR* overhang	Berg et al. 2011
khb29	GGGTAAATCCCTTATAGATATTATGGAGTTTCTATAAACCATCTGCCAATT	*comR* reverse primer with *treF* overhang	Berg et al. 2011

2.4 Enzymes, nucleotides and molecular weight size markers

Table 2.4.1: Enzymes, nucleotides and molecular weight size markers.

Name	Stock concentration	Manufacturer	Product number
1 kb DNA ladder		New England BioLabs® Inc.	N3232
CbpD	-	-	-
ColorPlus Prestained Protein Ladder	-	New England BioLabs® inc.	P7711S
DNase	10 μg/ml	Sigma	R-6513
dNTPs	10 μM		
Lysozyme, chicken (egg)		Sigma-Aldrich	L6976
LytA	3.7 μg/ml	-	-
Phusion™ High-Fidelity DNA Polymerase	2U/μl	New England BioLabs® Inc.	M0530

RNase	50 μg/ml	Sigma	DN25
Taq DNA polymerase	5U/μl	New England BioLabs® Inc.	M0273
Trypsin, pig	1 mg/ml	Fluka	93615

2.5 Antibiotics

Table 2.5.1: Antibiotics. The table shows the antibiotics used in this work and the concentration of stock solutions.

Antibiotic	Stock solution	Manufacturer	Product number
Ampicillin	100 mg/ml	Sigma-Aldrich	A-9518
Cefotaxime	100 mg/ml	Sigma-Aldrich	C7039
Kanamycin	100 mg/ml	Sigma-Aldrich	K4000
Streptomycin	100 mg/ml	Sigma-Aldrich	S6501

2.6 Kits

Table 2.6.1: Kits. The table shows the kits used in this work.

Name	Application	Manufacturer	Product number
NucleoSpin® Gel and PCR Clean Up	Purification of DNA from agarose gel	Macherey-Nagel	740609

2.7 Software

Table 2.7.1: Software. The table shows the software used in this work and their applications.

Software	Application	Available from
BLAST	Sequence alignments	http://blast.ncbi.nlm.nih.gov/Blast.cgi
Clustal W	Sequence alignments	http://www.ebi.ac.uk/Tools/msa/clustalw2/
Readseq	Reads and converts biosequences	http://www.ebi.ac.uk/Tools/sfc/readseq/
Reverse Complement	Reverse complements DNA sequences	http://www.bioinformatics.org/sms/rev_comp.html

2.8 Chemicals

Table 2.8.1: Chemicals. Alphabetical overview of the chemicals used in this work, chemical formula, manufacturer and product number.

Name	Chemical formula	Manufacturer	Product number
2-mercaptoethanol	$C_{22}H_6OS$	Sigma-Aldrich	M6250
Acetic acid	CH_3COOH	Merck	1.00063.2500
Acetone	$(CH_3)_2CO$	Merck	1.00014.2500
Acetonitrile	CH_3CN	Sigma	34998
Acrylamide	C_3H_5NO	Saveen Werner	B1AC41
Active coal	C	Merck	1.02182.1000
Adenosine, 99%	$C_{10}H_{13}N_5O_4$	Sigma	A9251
Agar		Merck	1.01614.1000
Agarose		Merck	1.01614.1000
Ammonium persulphate (APS)	$(NH_4)_2S_2O_8$	Sigma	A3678
Ammonium sulphate	$(NH_4)_2SO_4$	Merck	1.01217.1000
BactoTM Casitone		BD Diagnostic Systems	225930
BactoTM Todd Hewitt Broth		BD Diagnostic Systems	249240
BactoTM Yeast Extract		BD Diagnostic Systems	212750
Biotin	$C_{10}H_{16}N_2O_3S$	Sigma-Aldrich	19606
Bovine serum albumin		Sigma	A7906
Brilliant Blue	$C_{37}H_{34}N_2Na_2O_9S_3$	Sigma	B-7920
Bromophenol blue	$C_{19}H_9Br_4O_5SNa$	Sigma	B-5525
Calcium Chloride anhydrous	$CaCl_2$	Fluka	21075
Calcium pantothenate	$C_{18}H_{32}CaN_2O_{10}$	Sigma	C8731
Celite		Acros Organics	206352500
Choline chloride	$C_5H_{14}ClNO$	Sigma	C1879
D(+) Glucose anhydrous	$C_6H_{12}O_6$	VWR	101176K

DEAE-cellulose		Sigma	D3764
di-Potassium phosphate	K_2HPO_4	Merck	1.05104.1000
di-Sodium phosphate	Na_2HPO_4	Merck	1.06580.1000
DTT			
Ethylenediaminetetraacetic acid (EDTA)	$C_{10}H_{16}N_2Na_2O_8.2H_2O$	VWR	20 296.360
Formaldehyde	CH_2O	Sigma	F-8775
Glutardialdehyde	$C_5H_8O_2$	Merck	1.06009.2511
Glycerol solution 86-89%	$C_3H_5(OH)_3$	Sigma	49781
Glycine	$C_2H_5NO_2$	Merck	1.04201.1000
Hydrochloric acid, 37%	HCl	Merck	1.00317.2500
Iron(II) sulphate heptahydrate	$FeO_4S.7H_2O$	Fluka	44970
L-Asparagine monohydrate	$C_4H_8N_2O_3.H_2O$	Sigma-Aldrich	A8381
L-Cysteine hydrogenchloride monohydrate	$C_3H_7NO_2S.HCl.H_2O$	Fluka	30130
L-Glutamine	$C_5H_{10}N_2O_3$	Fluka	49419
L-Tryptophan	$C_{11}H_{12}N_2O_2$	Fluka	93660
Lithium chloride	LiCl	Sigma	62476
M17 broth		OXOID	CM0817
Magnesium Chloride, hexahydrate	$MgCl_2(H_2O)_6$	Fluka	63072
Magnesium sulphate	$MgSO_4$	Sigma	M2643
Manganese(II) chloride tetrahydrate	$MnCl_2.4H_2O$	Riedel-de Haën	31422
Methanol	CH_3OH	Merck	1.06009.2500
Methanol for HPLC	CH_3OH	Sigma	34885
Methylene blue	$C_{16}H_{18}N_3SCl$	Sigma	M9140
Nicotinic acid	$C_6H_5NO_2$	Fluka	72309
Paraformaldehyde (PFA)	$OH(CH_2O)_nH$	Sigma	15.812-7
PeqGREEN		peqlab (VWR)	37-5000
Phosphoric acid	H_3O_4P		

Potassium di-hydrogen phosphate	KH_2PO_4	Merck	1.04873.1000
Pyridoxine hydrochloride	$C_8H_{11}NO_3.HCl$	Fluka	95180
Riboflavine	$C_{17}H_{20}N_4O_6$	Sigma-Aldrich	R-7649
SDS, Sodium dodecyl sulphate	$NaC_{12}H_{25}SO_4$	AppliChem	UN1325
Sodium acetate	$C_2H_3NaO_2$	VWR	27650.292
Sytox	-		
Sodium chloride	NaCl	Merck	1.06404.1000
Sodium hydroxide	NaOH	Merck	1.06469.1000
Sodium pyruvate	$C_3H_3NaO_3$	Sigma	P8574
Sucrose	$C_{12}H_{24}O_{12}$	BHD	102754C
TEMED	$C_6H_{16}N_2$	Sigma	T9281
Thiamine hydrochloride	$C_{12}H_{17}N_4OS.HCl$	Sigma	67038
Trifluoracetic acid (TFA)	$C_2HF_3O_2$	Sigma	302031
Trizma® base, min. 99%	$NH_2C(CH_2OH)_3$	Sigma	T1503
Urdine, 99%	$C_9H_{12}N_2O_6$	Sigma	U6381
Zink sulphate heptahydrate	$ZnSO_4.7H_2O$	Fluka	96500
Nisin			

2.9 Equipment and instruments

Table 2.9: Equipment and instruments. The table shows the equipment and instruments used in work. Common laboratory equipment may also have been used but not listed.

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 homogenizer	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 μm)		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
Micro plate reader	FLUOstar OPTIMA	BMG LABTECH
Microtiter plates, 96 wells	3604	Corning
Multi channel pipette	Finnpipette, 30-300 μl	Thermo Scientific
pH-meter	PHM210	MeterLab®
Pipettes	Finnpipettes (0.5-10μl, 10-100μl, 50-200μl, 100-μl, 1-5 ml)	Thermo Scientific
Power supply for electrophoresis	Power pac 1000	Bio-rad
Power supply for electrophoresis	Power pac 300	Bio-rad
Refrigerated 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

2.10 Growth media

2.10.1 Pre C-medium

Recipe for 4 litres:

45 mg L-cysteine HCL

8 g Sodium Acetate

20 g Bacto™ Casitone

24 mg L-tryptophan

32 g di-potassium hydrogen phosphate, K_2HPO_4

MQ water was added to a final volume of 4 litres.

The solution was autoclaved at 121°C for 15 minutes and stored at room temperature.

2.10.2 C-medium

To 150 ml pre C-medium the following was added:

0.4 mM $MnCl_2$	150 µl
20% Glucose (w/v)	1.5 ml
ADAMS III	3.75 ml
3% Glutamine (w/v)	110 µl
2% Sodium pyruvate (w/v)	2.25 ml
1.5 M Sucrose	95 µl
2 mg/ml Uridine/Adenosine	1.5 ml
8% BSA (w/v)	1.5 ml
Yeast extract	3.75 ml

The solution was kept at 4C and needed to be made fresh the same day as it was used.

2.10.3 Todd Hewitt Agar (THA)

15 g Todd Hewitt broth

7.5 g agar

MQ water was added to a final volume of 500 ml.

The solution was autoclaved at 121°C for 15 minutes, cooled to approx. 60°C, antibiotic added and then transferred to sterile plates. The plates were stored in sealed plastic bags at 4°C.

2.10.4 GM17 medium

37.25 g M17 broth

950 ml MQ water

50 ml 10% Glucose (w/v) solution

The solution was autoclaved at 121°C for 15 minutes and stored at 4°C.

2.11 Solutions and buffers

2.11.1 Solutions for C-medium

Yeast extract

40 g yeast extract

360 ml MQ water

6 ml 12 N/37% HCl

16 g active coal

40 g yeast extract was solubilized in 360 mL MQ water and the pH adjusted to 3.0 with HCl. 16 g active coal was added, the solution stirred for 10 minutes on a magnetic stirrer and then kept at 4°C for 2 hours. The solution was filtered trough a column packed with glass wool and celite. pH was adjusted to 7.8 with NaOH and the final volume adjusted to 400 ml. The solution was sterilized by microfiltration, allocated to sterile 15 ml falcon tubes and kept at -80°C.

ADAMS I

0.15 ml 0.5 mg/mg Biotin

75 mg Nicotinic acid

87.5 mg Pyridoxine hydrochloride

300 mg Calcium pantothenate

80 mg Thiamine hydrochloride

35 mg Riboflavin

MQ water was added to a final volume of 500 ml and the pH adjusted to 7.0.

The solution was sterilized by microfiltration and kept at 4°C.

ADAMS II – 10x

500 mg Iron sulfate heptahydrate

500 mg Copper sulphate pentahydrate

500 mg Zinc sulphate heptahydrate

200 mg Manganese(II) chloride tetrahydrate

10 ml concentrated HCl

MG water was added to a final volume of 100 ml. The solution was sterilized by microfiltration and kept at 4°C.

ADAMS III

128 ml ADAMS I

3.2 ml ADAMS II – 10x

1.6 g Asparagine monohydrate

160 mg Choline chloride

400 mg Calcium chloride (dehydrated)

16 g Magnesium chloride hexahydrate

MG water was added to a final volume of 800 ml and the pH adjusted to 7.6. The solution was sterilized by microfiltration and kept at 4°C.

2.11.2 Solutions and buffers for agarose gel electrophoresis

50x TAE (Tris-Acetate-EDTA)

242 g Tris base

57.1 ml acetic acid

100 ml 0.5 M EDTA, pH 8.0

MQ water was added to a final volume of 1litre. The solution was kept at room temperature. 1x TAE buffer was used for agarose gel electrophoresis.

6x DNA loading buffer

10 mM Tris-HCl, pH 8.0

1 mM EDTA

40% Sucrose (w/v)

0.01% Bromophenol blue (w/v)

MQ water

2.11.3 Solutions and buffers used for SDS-PAGE

10x Tris-Glycin running buffer

30 g Tris Base (0.25 M)

144 g glycin (1.92 M)

50 ml 20% SDS (w/v), final concentration 1 (v/v)

Final volume was adjusted to1 litre and the solution was kept at room temperature. 1x running buffer was used when doing SDS-PAGE

2x SDS sample buffer

0.125 M Tris-HCl, pH 6.8

4% SDS (v/v)

0.30 M BETA-mercaptoethanol from a 14 M stock

20% Glycerol (v/v)

0.01% Bromophenol blue (w/v)

MQ water

Solution was kept at 4C

Separation gel (for 2 gels)

Reagent	8%	10%	12%	15%	18%
dH_2O	5.3 ml	4.78 ml	4.3 ml	3.55 ml	2.8 ml
1.5M Tris-HCl, pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml
10% SDS (w/v)	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
40% Acrylamide+0.8% bisacrylamide	2 ml	2.5 ml	3 ml	3.75 ml	4.5 ml
10% APS (w/v)	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
TEMED	5ml	5ml	5ml	5ml	5ml
Total volume	**10 ml**	**10 ml**	**10 ml**	**10 ml**	**10 ml**

Stacking gel (for 2 gels)

Reagent	4%
dH_2O	3.15 ml
0.5M Tris-HCl, pH 6.8	1.25
10% SDS (w/v)	50 ml
40% acrylamide+0.8%bisacrylamide	0.5 ml
10% APS (w/v)	50 ml
TEMED	5 ml
Total volume	**5 ml**

2.11.4 Solutions used for Coomassie staining of gel

Staining solution

0.5 g Coomassie Brilliant Blue (0.1% w/v)
200 ml methanol (40% v/v)
50 ml acetic acid (10% v/v)
250 ml MQ water

Destaining solution

50 ml acetic acid
200 ml methanol
xx ml MQ water up too 500 ml

2.11.5 Refolding buffer for zymography

20 mM Tris-HCl pH 7.6
50 mM NaCl
20 mM $MgCl_2$
0.5% Triton X-100

2.11.6 Fixing solution for TEM and SEM

2% paraformaldehyde (v/v)
2.5% gluatrdialdehyde (v/v)
0.1 M PBS, pH 7.4

3 Methods

3.1 Storage and cultivation of *S. pneumoniae*

3.1.1 Storage of *S. pneumoniae*

The stocks and start cultures used in this work were added 50% glycerol (v/v), to a final concentration of 15%, and kept at -80°C. They were frozen during the exponential phase (OD_{550} 0.3-0.4).

3.1.2 Anaerobic cultivation of *S. pneumoniae*

S. pneumoniae has been grown at 37°C on TH agar plates or in fresh C-medium. The bacterium grows best under anaerobic conditions, and a sterile/autoclaved container with a tightly fastened cap has been used when growing in C-medium. TH agar plates have been incubated in airtight containers with AnaeroGen™ bags that reduce the oxygen level in the container to <1% within 30 minutes by absorbing the oxygen and releasing CO_2 in an exothermic reaction (Oxoid 2002).

3.2 Storage and cultivation of *Lactococcus lactis*

3.2.1 Storage of *L. lactis*

The *L. lactis* expression strain used in this work was added 50% glycerol (v/v), to a final concentration of 15%, and kept at -80°C. It was frozen during exponential phase (OD_{550} 0.3-0.4).

3.2.2 Anaerobic cultivation of *L. lactis*

L. lactis has been grown and incubated at 30°C in GM17 medium. The bacterium grows best under anaerobic conditions and a sterile/autoclaved container with a tightly fastened cap has been used.

3.3 PCR: The Polymerase Chain Reaction

PCR has been used for amplification of DNA fragments for use in transformation, and for the construction of new DNA fragments. The principles of the method were outlined in the 1960s by Prof. Kjell Kleppe, however Kary Mullis and colleagues simplified the method by the use of a thermostable polymerase in the 1980s. It is a technique used for the exponential amplification of DNA molecules (Mullis et al. 1986). The reaction depends on the following reagents:

- A thermostable DNA polymerase for DNA synthesis.
- Deoxynucleotide triphosphates (dNTPs).
- A buffer solution to maintain pH, creating a suitable environment for the polymerase.
- Divalent cations required by the polymerase for activity.
- Monovalent cations.
- A pair of synthetic oligonucleotides with a free 3´ end, to prime DNA synthesis.

The method is composed of three main steps: denaturation, annealing and extension. During the denaturing step the sample is heated to 94-96°C, usually for 30 seconds. The heating of the sample breaks the hydrogen bond base paring in the dsDNA, which enables the binding of primers. Next, the temperature is lowered to between 50-60°C. This facilitates annealing of the primers to the ssDNA target sequence. For the extension step the temperature is raised to 68-72°C, the temperature being set for the optimal temperature of the polymerase being used. This allows the polymerase to synthesize double stranded DNA starting at the free 3´end of the primer. The length of the third step depends on the efficiency of the polymerase and on the length of the target sequence. These three steps are then repeated, usually 25-30 times, resulting in an exponential amplification of the target sequence. In theory one molecule of the target sequence can result in several million copies in 25 cycles ($1x2^{25}$). Usually the setup also includes a preheating step at 94-96°C for 5 minutes, and a final extension step at 68-72°C for 7 minutes (Sambrook and Russell 2001).

For this work, Phusion® High-fidelity DNA polymerase (New England Biolabs® inc.) and Taq DNA polymerase (New England Biolabs® inc.) have been used.

3.3.1 Taq DNA polymerase

Used for screening of colonies after transformation.

Protocol for 20 µl PCR reactions

1. Mix the following reagents in a PCR-tube in order:

Reagents	Final concentration/volume
x µl MQ water	Final volume 20 µl
2 µl Thermo-pol reaction buffer (10x)	1x
0.5 µl dTNP (10 mM)	0.2 µM
0.5 µl primer A (10 pmol/ml stock)	0.5 µM
0.5 µl primer B (10 pmol/ml stock)	0.5 µM
x µl DNA template	50-100 ng pr. 20 ml reaction
0.5 µl Taq DNA polymerase (2 U/ml)	0.02 U/ml

2. PCR reaction is performed in a thermo cycler on a program adapted to
   - The template in question, the melting temperature of the primers (Tm)
   - The length of the fragment being amplified (1 min per 1kb using whole cells/gDNA as template, 30 seconds per 1kb using PCR-fragments/plasmids as template)

Step	Temperature, time
1. Initial denaturation	94°C, 5 min
2. Denaturation	94°C, 30 sec
3. Annealing	x°C, 30 sec (in this work 58°C)
4. Extension	68°C, x sec
5. Go to step 2. x times	25-35 cycles
6. Final extension	68°C, 5 min
7. Final hold	4°C, ∞

3.3.2 Phusion DNA polymerase

Used for amplification of DNA fragments used in transformation, and for the construction of recombinant DNA sequences (overlap extension PCR, see 3.3.4).

Protocol for 50 µl reactions

1. Mix the following reagents in a PCR-tube in order:

Reagents	Final concentration/volume
x ml MQ water	Final volume 50 ml
10 µl 5x Phusion® HF-buffer	1x
1 µl dTNP (10 mM)	0.2 µM
2.5 µl primer A (10 pmol/ml stock)	0.5 µM
2.5 µl primer B (10 pmol/ml stock)	0.5 µM
x µl template DNA	100-200 ng pr. 50 ml reaction
0.5 ml Phusion® DNA polymerase (2 U/ml)	0.02 U/ml

2. PCR reaction is performed in a thermo cycler on a program adapted to
   - The template in question, the melting temperature of the primers (Tm)
   - The length of the fragment being amplified (30 seconds per 1kb using whole cells/gDNA as template, 15 seconds per 1kb using PCR-fragments/plasmids as template)

Step	Temperature, time
1. Initial denaturation	94°C, 5 min
2. Denaturation	94°C, 30 sec
3. Annealing	x°C, 30 sec (in this work 58°C)
4. Extension	72°C, x sec
5. Go to step 2. x times	25-35 cycles
6. Final extension	72°C, 5 min
7. Final hold	4°C, ∞

3.3.3 Primer design

For successful PCRs it is essential to have designed primers that bind specific to their target sequences. The primers used in this work have been designed using an approximated approach were each A-T base pair adds 2°C to the melting temperature (Tm) and each G-C base pair adds 4°C to the Tm, with a target Tm of 60°C. Ideal primers should be between 18-30 nucleotides in length, and have melting temperatures in the range of 52-60°C. It is also desirable to avoid palindrome sequences to avoid the formation of hairpin structures (Innis and Gelfand 1990). For primers used to amplify functional genes, the 5´ends of the forward primers were been designed to start at first codon of the gene (usually ATG), whereas the 5' ends of the revers primers were designed to be complementary to the stop codons TAG, TAA or TGA.

3.3.4 Overlap extension PCR

In the present work overlap extension PCR has been used to construct recombinant DNA molecules. The method, first described by Higuchi and colleagues in 1988, is based on the principles of PCR and can be used as a method of site directed mutations as well as combining DNA of different origins (Higuchi et al. 1988, Ho et al. 1989). In this work the method has been used to 1) create a Janus cassette (se 3.6.1) for deletion of the native *murTcobQ* locus, 2) a fragment for the insertion of *murTcobQ* between *cpsO* and *cpsN,* and 3) a fragment for the removal of the Janus cassette from the native *murTcobQ* locus.

To splice two DNA sequences one needs to synthesize DNA fragments whose ends are complementary to each other. This is achieved by using special primers that have 5´ ends that are complementary to the end of the to-be-joined DNA molecule (figure 3.3.1). It is enough for one of the two to-be-joined DNA sequences to be amplified with primers containing the 5´overlap (Sambrook and Russell 2001). The next step is to join the two fragments, which is done by using PCR products with overlap as templates and only using flanking primers at the non-joined ends. This causes the complementary regions of the two molecules to anneal during the annealing step of the PCR, where the complementary regions of each molecule function as primers for the DNA polymerase, and the two sequences will be fused following the principles of the reaction (Ho et al. 1989).

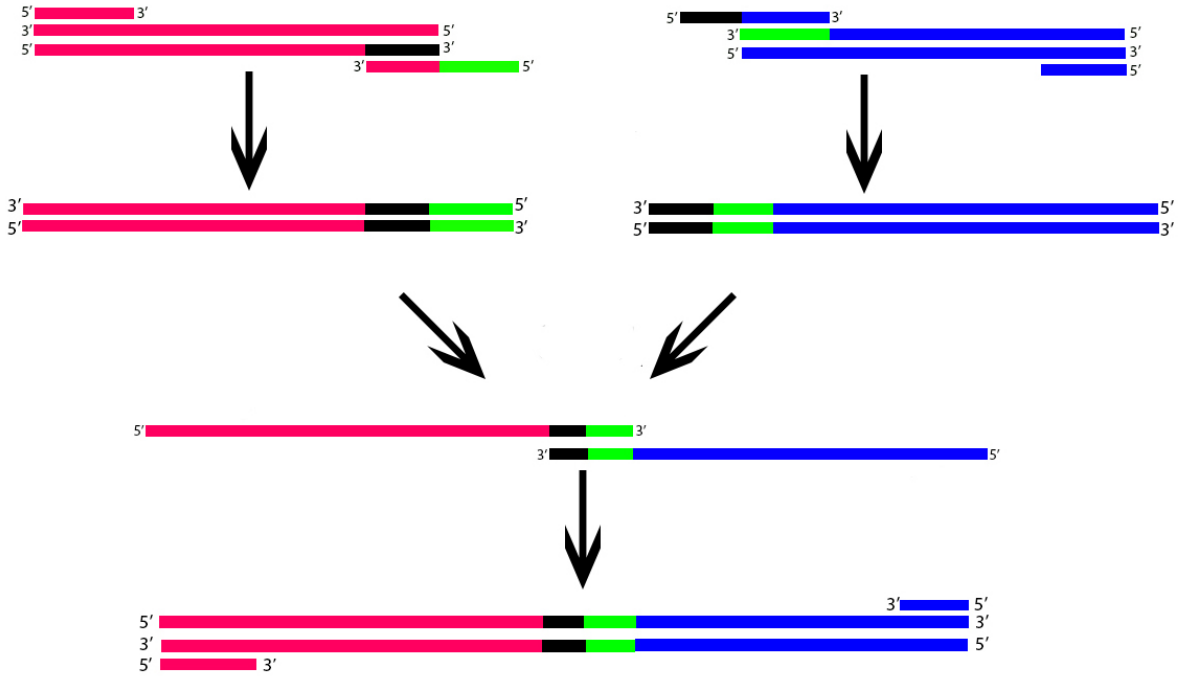


Figure 3.3.1: Overlap extension PCR. The first part of the technique involves generating PCR products that have complementary 3´ regions. This is achieved by using special primers with 5´ overhangs that are complementary to the to-be-joined molecule. In a second PCR the DNA fragments are joined as the overlapping 3´regions acts as primers for extension, and only primers for the distal ends are needed.

3.4 Agarose gel electrophoresis

Agarose gel electrophoresis is a method for the separation and analysis of charged molecules based on size by having them migrate through an agarose gel that is in contact with a cathode in one end and an anode in the other. The method is primarily used to separate DNA and RNA fragments (Sambrook and Russell 2001). In this work agarose gel electrophoresis has been used to analyse and purify PCR products. The PCR product is mixed with a loading buffer that contains a dye that will migrate with the sample in the gel (bromphenol blue), and glycerol, which makes the sample more viscous and easier to apply to the gel. For the recipe of the 6x DNA sample loading buffer see 2.11.2. The pore size of the gel is dependent on the concentration of agarose used (usually 0.7%-2%), where increasing the amount of agarose will decrease the pore size. The gel is submerged TAE-buffer, which conducts electricity. Because of the negatively charged phosphate groups of the DNA (and RNA) molecules they will migrate towards the positively charged anode when an electric current is applied. The smaller DNA molecules migrate faster than the larger ones, and the result is a separation based on size. Other factors deciding migration speed are voltage and the conformation of DNA molecules (Sambrook and Russell 2001). To be able to detect the DNA molecules, an

intercalating DNA binding agent is added when casting the gel. In this work peqGreen from PEQLAB has been used, which will give of a fluorescent signal only when bound to DNA and exposed to UV light (302-366 nm). To be able to qualify the size of the fragments a molecular weigh size marker with DNA fragments of known size is also applied (DNA-ladder for short). Here, the Invitrogen 1kb ladder has been used, shown in the appendix.

3.4.1 Protocol for agarose gel electrophoresis

For 1% agarose gel

1. Agarose (0.5 g) was added to 50 ml TAE-buffer and heated in a microwave oven until all agarose was dissolved.
2. The solution was cooled to approximately 60°C and added pegGreen to a final concentration of 0.5 μg/ml.
3. The solution was transferred to the cast and a comb with appropriate well size added to the still liquid solution, which was then left to solidify.
4. The solid gel was transferred to an electrophoresis tank, the combs removed and TAE-buffer added. It is important that the buffer covers the gel entirely, filling up all the wells.
5. A volume of 10 μl 1kb ladder (Invitrogen) was used as standard and appropriate amounts of the samples were mixed with 6x DNA loading buffer and added to the appropriate wells.
6. Electrophoresis was done at 90 V until sufficient migration could be observed, and the fragments where then visualized under UV light in a Gel Doc-1000 (BioRad)
7. Fragment size was determined using the ladder as reference and extracted from the gel using a sterile scalpel.

3.5 Purification of DNA fragments from agarose gel

PCR-products have been extracted from agarose gel using the NucleoSpin® Gel and PCR Clean Up kit from Macherey-Nagel. The gel fragments were solubilized in a binding buffer (NT) and added to a silica column that will bind the DNA molecules. The chaotrophic salt guanidine thiocyanat in the binding buffer solubilizes the agarose and the DNA, and contributes to the binding of DNA molecules to the positively charged silica material on the column by removing water and making available the negative charges on DNA. The column

was washed with a wash buffer (NT3) containing ethanol, which removes salts, primers and other contaminants. The PCR-product was eluted with an elution buffer, NE (5 mM Tris-HCl pH 8.5), free from chaotrophic salts where the alkaline pH, combined with the absence of the salts, elutes the DNA from the column. The elution buffer volume used was 15-50 µl, depending on the amount of DNA in the sample. The amount of DNA was roughly estimated by visual inspection according to the intensity of the fragment under UV light exposure compared to the Invitrogen 1-kb ladder with known DNA amounts. The protocol from the manufacturer was followed without modification (Macherey-Nagel 2014). The eluate was stored at -20°C.

3.6 Transformation of *S. pneumoniae*

Genetic transformation is the uptake of extracellular DNA and the subsequent incorporation of DNA through homologous recombination. *S. pneumoniae* is a naturally transformable bacterium, a trait that has been exploited in this work to introduce different mutations into the genomes of laboratory strains. Competence is induced in *S. pneumoniae* by the competence stimulating pheromone CSP. The strains used in this study do not express the exporter gene *comA* and is not able to secrete CSP, thus synthetic CSP is needed in the growth medium for the cells to become competent (Håvarstein et al. 1995). When competence is induced the cells remain competent for approximately 20 minutes, with most cells returning to the non-competent state after about 40 minutes (Tomasz 1966, Håvarstein et al. 1995). The culture was induced with synthetic CSP at the beginning of the exponential growth phase (OD_{550} 0.05-0.1) to a final concentration of 250 ng/ml.

3.6.1 Negative selection using the Janus cassette

When doing knockouts or replacements of genes in *S. pneumoniae* strains, the Janus cassette developed by Sung et al. has been used. The bi-functional 1.3-kb cassette is composed of a kanamycin resistance marker (Kan^r), and a counterselectable $rpsL^+$ marker conferring dominant streptomycin (Sm) sensitivity (Sung et al. 2001). By constructing Janus cassettes using, overlap extension PCR, that have flanks that are homologous to the upstream and downstream regions of the target gene, this allows for two-step transformation procedures where target genes can be knocked out using the cassette, and transformants selected for on kanamycin. Subsequently the cassette can then be removed, either by replacement or

deletion, reintroducing streptomycin resistance and consequently allowing for selection of transformants on streptomycin (figure 3.6.1). *S. pneumoniae* strains used in this work are naturally resistant to streptomycin due to a mutation in the gene *rpsL* that causes a lysine replacement in protein S12 of the small ribosomal subunit (Sung et al. 2001). This mutation is recessive, so introducing a streptomycin sensitive $rpsL^+$ allele provides the drug-sensitive phenotype.

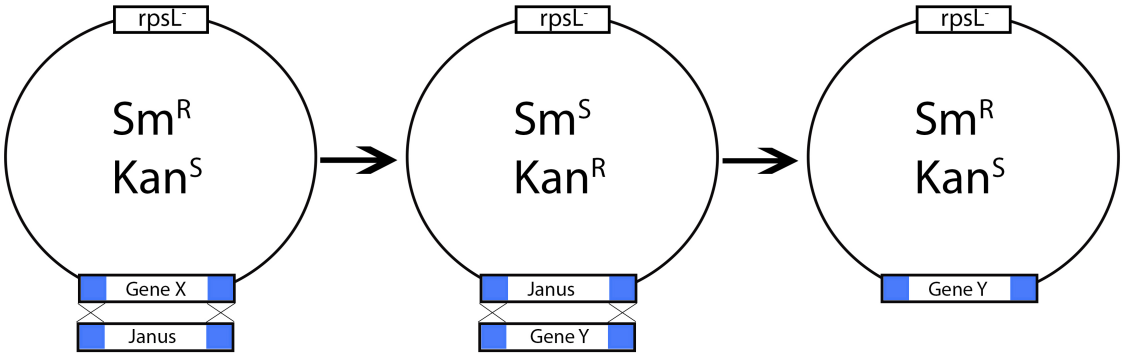


Figure 3.6.1: Simplified illustration of the introduction of the Janus cassette into the circular genome of *S. pneumoniae*. The cassette is incorporated into the genome by homologous recombination, which results in the introduction of kanamycin resistance (Kan) and the loss of streptomycin resistance (Sm). The Janus cassette replaces the native target gene X. Gene Y can then be introduced by replacing Janus, with the cell regaining Sm^R and losing Kan^R. The introduction and removal of the Janus cassette can be selected for using the appropriate antibiotic.

3.6.2 Protocol for transformation of *S. pneumoniae*

1. Bacterial cultures were inoculated in fresh C-medium to OD_{550} 0.05 and incubated in a water bath at 37°C for 15 minutes.
2. 1 ml of cell culture was induced to competence with CSP to a final concentration of 250 ng/ml, and added 0.5-1 µg/ml DNA for transformation. Also, a negative control induced with 250 ng/ml CSP without added DNA was made.
3. The cultures were then incubated in a water bath at 37°C for 2 hours.
4. Transformants were selected for by plating 20 µl culture on TH-agar plates with the appropriate antibiotic, and incubated anaerobically in air tight containers over night at 37°C.
    - Streptomycin: (Sm): 200 µg/ml
    - Kanamycin (Kan): 400 µg/ml

5. The following day, transformants were picked using sterile toothpicks and grown in fresh C-medium with the appropriate antibiotic. At OD_{550} ~ 0.3-0.5 stocks were made as described in 3.1.1. The number of colonies picked depended on the amount of background on the negative plates.
6. Control of transformants was carried out using PCR.

3.6.3 PCR-screening of transformants

After transformation it was necessary to examine whether the acquired antibiotic resistance (Kan or Sm) was due to the successful incorporation of the DNA fragment, and not because of a spontaneous mutation. Spontaneous mutations in the *rpsL*$^+$ gene of the Janus cassette, resulting in a reacquisition of streptomycin resistance, are the most common. The control plates had on average 1/3 of number of colonies relative to the transformant plates. Spontaneous kanamycin resistance is much more rare, but can occur.

In short, primers for screening of transformants where chosen as to only give a PCR-product if the desired fragment had been incorporated into the genome. Usually a forward primer that would bind 1kb upstream of the target, as well as a reverse primer that would bind the end of the inserted gene, was chosen.

3.7 Ectopic gene expression in *S. pneumoniae* using the ComRS system

When studying essential genes it is not possible to do simple knockout mutations, as these knockouts will kill the bacteria. By expressing an essential gene of interest ectopically behind a titratable promoter, one can delete the corresponding native gene. The ComRS system described in section 1.5 is designed for this purpose. It can be used for the overexpression or for the gradual depletion or suboptimal expression of desired genes. The system originates from *Streptococcus thermophilus* and does not have any homologues in *S. pneumoniae* (Berg et al. 2011).

Berg et al. (2011) has cloned the ComRS genes of *S. thermophilus* into neutral areas in the genome of *S. pneumoniae* laboratory strain R6 for use in essential gene studies (figure 3.7.1). The R6 strain has a non-functional capsule locus (*cps* locus), and so insertion of a foreign genetic element between the capsular genes *cpsO* and *cpsN* does not interfere with normal

cell function (Berg et al. 2011). In the strain used in this work (sph131) the P_{comX} promoter from *S. thermophilus* has been inserted between *cpsO* and *cpsN,* together with the Kan^r-$rpsL^+$ cassette (Janus). This cassette confers kanamycin resistance whilst simultaneously making the cells streptomycin sensitive (Sung et al. 2001). The *comR* gene, together with its own promoter (P_{comR}) as well as the constitutive promoter P_1 (Johnsborg et al. 2008) has been inserted between *amiF* and *treR*. The P_1 promoter is used to ensure sufficient *comR* expression. The gene existing between *amiR* and *treF* is a degenerated transposase, and so the insertion is not harmful to the cell in any way. Because it was considered likely that a native oligopeptide permease in *S. pneumoniae* would be able to transport ComS* into the cell, the Ami translocase from *S. thermophilus* was not inserted into the R6 genome (Berg et al. 2011).

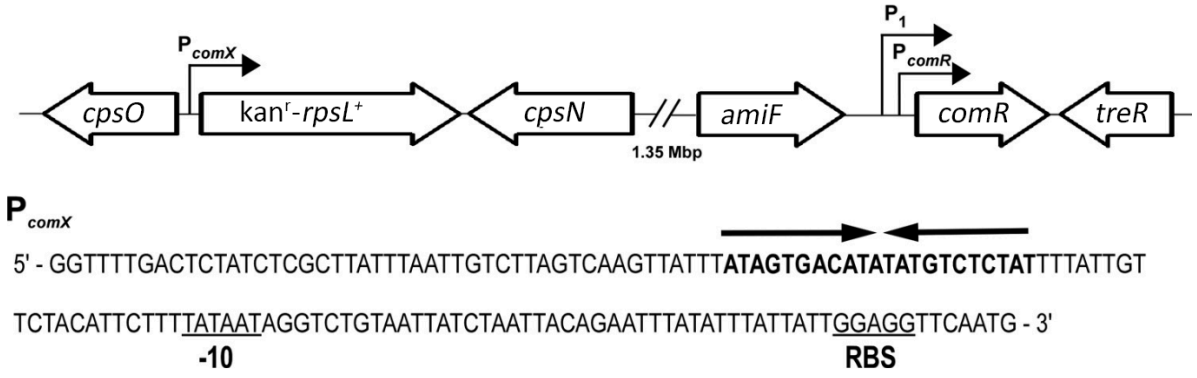


Figure 3.7.1: The ComRS system cloned into the genome of *S. pneumoniae* strain sph131. The Janus cassette (kan^r-$rpsL^+$), together with the promoter P_{comX} has been inserted between the two non-functional capsular genes *cpsN* and *cpsO*. The ComR protein is expressed from the *comR* gene inserted between the genes *amiF* and *treR* together with the constitutive promoter P_1 and its own promoter P_{comR}. Figure modified from Berg et al. (2011).

The expression of the genes downstream of P_{comX} is regulated by adding the synthetic peptide ComS* to the growth medium. One of the two main techniques for underexpression used in this work has been to gradually deplete the expression of the target gene (the operon *murTcobQ*), by doing 2-fold dilutions of cell culture (figure 3.7.2) after washing the cells free from ComS*. By washing and diluting the cells in ComS*-free C-medium, the only ComS* available is what is left intracellularly. In theory, each subsequent dilution will have half the available ComS* compared to the previous dilution in the series. As the cells grow and divide, they will gradually use up the intracellular ComS* and the expression of the genes behind P_{comX} will decrease with time. The term depletion will be used in the rest of this text when describing this technique.

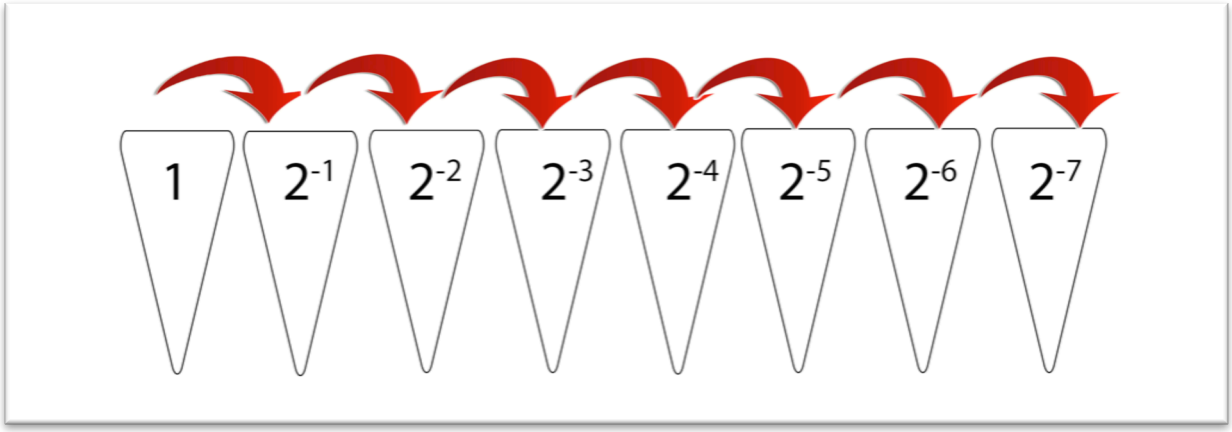


Figure 3.7.2: 2-fold dilution of cell culture. Each tube (2^{-1}-2^{-X}) contains 750 µl fresh C-medium. 750 µl bacterial culture is removed from the previous tube in the series, and added to the next and mixed by pipetting, making the total volume 1.5 ml. This is repeated for each tube in the dilution series.

Another technique used, instead gradual depletion, has been to grow the cells in suboptimal concentrations of ComS*, and in that way have suboptimal expression of *murTcobQ*. In this work the ComRS system has been used extensively to study what effects decreased expression of *murTcobQ* has on morphology, CbpD sensitivity, antibiotic sensitivity, lysozyme sensitivity, changes in cell wall composition and growth in Δ*pbp1a* and Δ*pbp2a* mutants.

3.7.1 Protocol for depletion of gene expression in *S. pneumoniae* using the ComRS system

1. The bacterial cultures were inoculated to OD_{550} ~ 0.05 in fresh, sterile C-medium containing 0.2 μM ComS*.
2. The cell cultures were incubated in a water bath at 37°C to OD_{550} ~ 0.3.
3. The cells were harvested by centrifugation (5000 g) for 5 minutes and all supernatant was removed with a pipette.
4. The cell pellet was resuspended in 1 ml of fresh C-medium, reallocated to an Eppendorf tube and centrifuged (5000 g) for 5 minutes, and all supernatant removed.
5. The washed cell pellet was reinoculated in 1.5 ml fresh C-medium to OD_{550} ~ 0.03-0.05 and used as dilution #1 in a 2-fold dilution series.
6. From each dilution, 300 μl (280 μl when using SYTOX®) was added to two rows of wells in a Corning® 96 Well Microplate, one row containing 0.2 μM ComS* (control) and the other without ComS*.
7. The microtiter plate was incubated at 37°C in a Syngergy H1 microplate reader for 20 hours, measuring OD (and florescence when necessary) every 5 minutes. Absorbance was measured at 492 nm and fluorescence at excitation/emission 504/523 nm.

3.7.2 Ectopic gene expression with the ComRS system using suboptimal ComS* concentrations

For several experiments in this work, having a lower (compared to the wild type gene) but constant expression of the ectopic gene was more preferable than the depletion described in 3.7.1. This was achieved by adding suboptimal concentrations of ComS* to the growth medium. To find suitable concentrations for this purpose, cells were grown in a 2-fold dilution series of ComS*. Then, to further narrow down to appropriate ComS* concentrations, a window of the most promising concentrations were tested. The ComS* concentrations that gave a slower, but steady growth rate when, compared to the control strains grown with 0.2 μM ComS*, were used for further experiments. Suboptimal expression was chosen because it gave more predictable control over growth compared to depletion, and because it was desirable to have the cells growing at a steady rate. This is a fairly crude method, and the ComS* concentrations needed vary depending on what gene is

being studied, as different gene products will have different half-lives and are expressed/required at different amounts in the cell.

3.7.3 Protocol for suboptimal gene expression in *S. pneumoniae* using the ComRS system

1. The bacterial cultures were diluted to OD_{550} ~ 0.05 in fresh, sterile C-medium with 0.2 μM ComS.
2. The cell culture was incubated in a water bath at 37°C to an OD_{550} ~ 0.3
3. The cells were harvested by centrifugation (5000 g) for 5 minutes and all supernatant removed by pipette.
4. The cell pellet was resuspended in 1 ml fresh C-medium, reallocated to an Eppendorf tube and centrifuged (5000 g) for 5 minutes, and all supernatant removed by pipette.
5. The washed cell pellet was reinoculated in fresh C-medium to an OD_{550} ~ 0.01 and added the desirable ComS* concentrations.
6. The cell cultures were incubated in a water bath at 37°C to OD_{550} ~ 0.3, and then centrifuged (5000 g) for 5 minutes and resuspended to OD_{550} ~ 0.01.
7. The cell cultures were added the same ComS* concentrations as in step 5 and incubated in a water bath at 37°C to OD_{550} ~ 0.3
8. The pneumococcal cell cultures were then centrifuged (5000 g) for 5 minutes, resuspended to OD_{550} ~ 0.03-0.05 and then 300 μl from each culture was added to a Corning® 96 Well Microplate. The same ComS* concentration as in step 5 was added to the appropriate wells.
9. The plate was incubated at 37°C in a Syngergy H1 microplate reader for 20 hours measuring OD (and fluorescence when necessary) every 5 minutes. Absorbance was measured at 492 nm and fluorescence at excitation/emission 504/523 nm.

3.7.4 Synergy H1 microplate reader: monitoring growth and lysis of depletion mutants

Experiments were done using the Synergy H1 Hybrid Multi-Mode Microplate Reader from BioTek. The instrument was used to monitor growth (OD_{492}) and cell lysis (fluorescence). Cell lysis was monitored using the SYTOX® Green Nucleic Acid Stain. The SYTOX® stain will give a fluorescent signal when bound to DNA and is not able to permeate the cell membrane, thus it will only give a signal when the cells lyse (Invitrogen 2006).

3.8 Construction of the *murTcobQ* depletion strain OH8

The *murTcobQ* depletion strains used in this work were derived from the strain sph131 described in 3.7. This strain has the ComRS-system inserted into non-functional regions of the genome (Berg et al. 2011). All primers used are presented in table 2.3.1. The strains constructed in this work are shown in table 2.1.2, with a description of the relevant genotype. To do functional studies of *murTcobQ* it was necessary to do ectopic expression in a strain where the wild type gene had been deleted. The following section is a description of the construction of this strain, named **OH8**.

First *murTcobQ* was placed behind the ComS* inducible P_{comX} promoter. The *murTcobQ* operon was amplified from the genome of strain RH1 using the primers ds188 and ds189. The forward primer ds188 contains a sequence at its 5´ that is complementary to the end of the P_{comX} promoter sequence. The reverse primer ds189 has a 5´ sequence that is complementary to the end of the *cpsN* gene. *cpsO* (including P_{comX}) and *cpsN* was amplified from genomic DNA derived from strain sph131 using primer pairs khb31/khb36 and khb33/khb34 respectively. These fragments were then joined, using overlap extension PCR, resulting in a fragment comprising the *cpsO* gene followed by P_{comX}, *murTcobQ* and the *cpsN* gene. The Janus cassette in strain sph131 was replaced with this fragment by natural transformation following the protocol described in section 3.6.2. Transformants were selected for on TH-agar plates containing streptomycin (200 μg/ml). This strain was named **OH6**.

Next, the native *murTcobQ* operon was deleted by replacement with a Janus cassette. Fragments corresponding to the ~ 1kb regions upstream and downstream of the native *murTcobQ* was amplified from genomic sph131 DNA, using primer pairs ds190/ds191 and ds192/ds193 respectively. Janus (Kan^r-$rpsL^+$) was amplified from the genome of strain sph131 with the primer pair Kan484F/RpsL41R, and fused with the upstream and downstream *murTcobQ* fragments using overlap extension PCR. Strain **OH7** was constructed by introducing this Janus cassette in strain OH6, replacing $murTcobQ_{wt}$ by homologous recombination. For ectopic expression of *murTcobQ,* 0.2 μM ComS* was added to the growth medium during transformation, and when selecting for transformants. Transformants were selected for on TH-agar plates containing kanamycin (400 μg/ml).

To avoid a polar effect of downstream genes, the Janus placed in the native *murTcobQ* locus in OH7 was removed. In addition, removal of The Janus cassette enabled further knockouts using the same technique. To do this, the fragments corresponding to the ~ 1kb regions upstream and downstream of *murTcobQ* was amplified from the genome of strain sph131 using primer pairs ds190/ds195 and ds193/ds194 respectively, where ds194 has a 5´ overlap complementary to ds195. These two fragments were joined using overlap extension PCR and introduced into strain OH7 via transformation, resulting in replacement of Janus by homologous recombination. Transformants were selected for on TH-agar plates containing streptomycin (200 μg/ml). This strain was given the name **OH8**. Just like OH7 this strain needed 0.2 μM ComS* in the growth medium.

Further mutants have been constructed in this study derived from OH8, namely a Δ*comM* mutant (**OH9**), Δ*pbp1a* mutant (**OH10**) and a Δ*pbp2a* mutant (**OH11**). The construction of these strains is handled in the results chapter.

3.9 Purification of CbpD using DEAE-cellulose affinity chromatography

Column chromatography is a method used for separating the different components of a chemical mixture by allowing is to pass through a solid phase that has specific affinity for one or several components in the mixture. In this work, diethylaminoethyl cellulose (DEAE-cellulose) affinity chromatography has been used to purify the pneumococcal muralytic fratricide protein CbpD (Choline binding protein D), following the protocol of Sanchez-Puelles et al. (1991). Some modifications to the original protocol have been made. The CbpD enzyme has been used to study what effects underexpression of the *murTcobQ* operon has on this protein's ability to lyse the cells. CbpD is a modular protein and a key enzyme of the late competence process called fratricide described in the introduction (see 1.2.1). It consists of an N-terminal enzymatic CHAP domain, two SH3 domains and a C-terminal choline-binding domain (CBD). DEAE-cellulose (figure 3.9.1) is structurally similar to the choline moieties of the teichoic acids of the streptococcal cell wall (see 1.4.1), which causes the choline-binding domain of CbpD to bind reversibly to DEAE-cellulose under appropriate conditions.

The protein was expressed in the lactococcal expression strain *Lactococcus lactis* NZ9000, which had been transformed with the plasmid pNZ8037-CbpD in a previous study at the

Molecular Microbiology lab (not published). The pNZ8073 plasmid is a nisin regulated expression system (*nisRK*) containing a nisin responsive promoter (De Ruyter et al. 1996). In addition the plasmid contains a selection marker for chloramphenicol resistance, Cam_r.

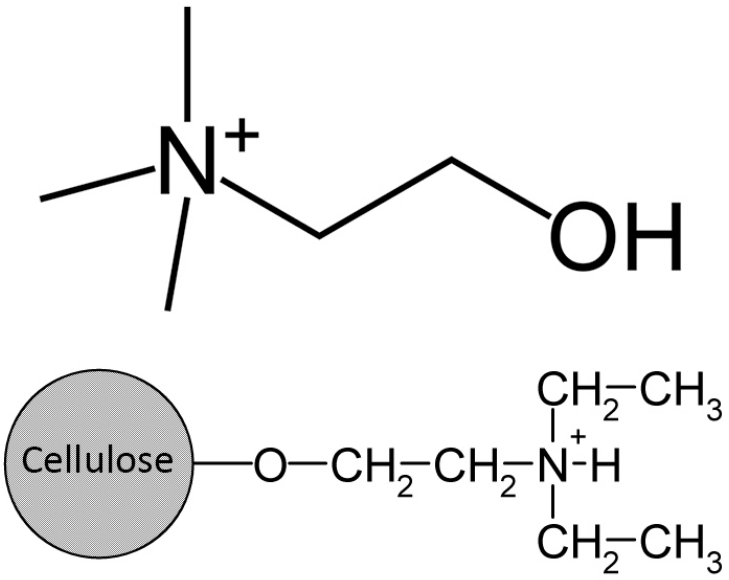


Figure 3.9.1: Structures of choline and DEAE-cellulose. The choline-binding domain of CbpD binds reversibly to the quarternary ammonium cation in choline (top) and the tertiary ammonium cation in DEAE-cellulose (bottom).

3.9.1 Protocol for DEAE-cellulose purification of CbpD

1. Lactococcal cells were inoculated in 5 ml of GM17 medium containing 10 μg/ml chloramphenicol (Cam) and incubated over night at 30°C.
2. The cells were reinoculated (OD_{550} ~ 0.1) in 500 ml fresh GM17 medium with 10 μg/ml Cam, and incubated at 30°C.
3. When the OD_{550} of the cell culture reached 0.3, expression of CbpD was induced by adding 20 ng/ml nisin to the growth medium, and the culture was incubated for 4 hours at 30°C.
4. The cells were harvested by centrifugation (5000 g) for 5 minutes and resuspended in 10 ml 10 mM Tris-HCl pH 7.4 containing 100 mM NaCl.
5. The cells were lysed with acid washed glass beads (0.5g per ml cells) in a Fast Prep 24® benchtop homogenizer at 6.5 m/s for 3 x 20 seconds. The lysate was kept on ice for 30 seconds between each pulse. The glass beads were left to sediment and the

supernatant was collected. Cell debris was then removed by centrifugation (20,000 g), and the supernatant collected.

6. The DEAE-cellulose column was prepared by suspending 3 ml dry DEAE-cellulose in 10 ml 10 mM Tris-HCl pH 7.4 and then left to sediment. Excess Tris-HCl buffer was then removed after sedimentation had taken place.
7. The cell lysate was added to the DEAE-cellulose column, resulting in the reversible binding of the CbpD protein to the DEAE-cellulose.
8. 4 x 10 ml of a high NaCl wash buffer (10 mM Tris-HCl pH 7.4, 1.5 M NaCl) was used to remove other proteins and cellular components bound by anionic interactions to the column.
9. The CbpD protein was eluted using a 10 mM Tris-HCl pH 7.4 buffer with 0.14 M choline, in 1.5 ml fractions. The presence of CbpD in the eluates was controlled by examining the protein content in each fraction using sodium dodecyl sulphate polyacrylamide gel electrophoresis. The purified CbpD was kept at -80°C.

3.10 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a common biochemical technique used for separating proteins according to their size. The gel is composed of acrylamide, bisacrylamide, a denaturing agent such as SDS and a suitable buffer. The acrylamide monomers are cross-linked by bisacrylamide, and the polymerization is initiated by adding tetramethylenediamine (TEMED) and the free radical donor ammonium persulfate (APS) (Sambrook and Russell 2001). The pore size of the gel is inversely proportional to the acrylamide concentration, and the concentration should be chosen according to the size (kDa) of the protein(s) one is studying.

Before the protein sample is applied to the gel, it is denatured in a sample buffer containing the strongly ionic detergent SDS in combination with a reducing agent and heat (Sambrook & Russel). SDS denatures the proteins by disrupting nearly all non-covalent bonds in tertiary structure, and the reducing agent (β-mercaptoethanol) reduces disulfide bonds between cysteine residues. The sample buffer also contains a dye to track the migration of the sample through the gel, and glycerol to make the sample easier to apply to the gel. The denatured polypeptides bind SDS and become negatively charged, where the amount SDS bound is proportional to the molecular weight of the peptide and independent of sequence. The gel

also contains SDS to maintain the denatured state of the proteins. Much like the principles of agarose gelelectrophoresis (3.4), the sample will migrate towards the positively charged anode when an electric current is applied to the gel. Because the denatured proteins have a charge proportional to size, and independent of native charge, the proteins in the sample will migrate according to size (Sambrook and Russell 2001). A molecular weight standard with proteins of known size is used to identify the size of the sample proteins.

Electrophoresis in this work has been done using a discontinuous buffer system where the gel is segmented into a stacking gel (pH6.8) and a separation gel (pH8.8) (Davis 1964, Ornstein 1964). The large pores of the stacking gel, combined with the pH of 6.8, ensure that the protein sample reaches the separating gel in a thin, sharp band. This happens because the SDS-bound proteins become trapped between the leading chloride ions and the trailing glycine ions of the buffer (Laemmli 1970). The proteins are then separated according to size in the separation gel, as the smaller pores makes the sieving effect of the gel become the determining factor for migration. Also, the higher pH of the separation gel makes the ions in the buffer outrun the SDS-bound proteins, eliminating the stacking effect seen in the stacking gel (Sambrook and Russell 2001).

3.10.1 Protocol for SDS-PAGE

For the recipes of buffers, stacking gel and separation gel see 2.11.3

1. Glass plates for 10 x 7.5 cm gels were assembled in the casting frame according to manufacturers instructions (BioRad).
2. The separation and stacking gel solutions were prepared, with APS and TEMED being added last to avoid premature polymerization, and mixed well
3. A volume of 3.2 ml of the separation gel was added between the glass plates immediately following the addition of APS and TEMED using a 5 ml automatic pipette.
4. The stacking gel was added on top of the separation gel, filling the space between the glass plates, followed by the insertion of a comb suitable for the samples being added.
5. The gel was left to polymerize at room temperature for approx. 20 minutes until solid.
6. The protein sample was mixed with a 2X protein sample buffer and incubated at 100°C for 15 minutes.

7. The solid gel was transferred to an electrophoresis tank with 1X running buffer, making sure the running buffer covered all wells in the gel, and protein samples were added in a desirable volume (5-15 ml)
8. Finally, the proteins were separated using electrophoresis at 1 V/m^2 for 10 minutes, followed by 2 V/m^2 for 45 minutes.

3.10.2 Coomassie Brilliant Blue staining of SDS-Polyacrylamide gels

Proteins that were separated by SDS-PAGE were stained using Coomassie Brilliant Blue. The dye stains proteins in the gel by binding to the proteins through electrostatic and hydrophobic interactions, and is one of the most commonly used dyes for detection of proteins separated by SDS-PAGE (Sambrook and Russell 2001, Westermeier and Marouga 2005). For the recipe of the staining and destaining solutions see 2.11.4.

3.10.3 Protocol for Coomassie Brilliant Blue staining:

1. The SDS-Polyacrylamide gel was carefully removed from the glass plates and submerged in the Coomassie staining solution.
2. The gel and staining solution was heated to boiling point using a microwave oven to speed up the binding of the Coomassie molecules to the proteins in the gel.
3. The gel was incubated at in the staining solution at room temperature for 15 minutes or until bands were clearly visible in the gel.
4. The staining solution was then removed, and the gel washed with dH_2O and subsequently incubated in a destaining solution at room temperature until sufficient destaining was achieved (usually 1-2 hours). Images of the stained gel were obtained by using the Azure c400 Gel Documentation System.

3.10.4 Zymography

Zymography is a technique for detecting the activity of hydrolytic enzymes in a sample, after they are separated by SDS-PAGE. An enzyme substrate is suspended in the separation buffer during preparation of the gel and is cast into the separation gel (Vandooren et al. 2013). In this work, zymography has been used to detect the activity of the muralytic fratricide enzyme CbpD, with pneumococcal cells as the substrate. The enzyme sample was loaded onto the gel and the enzyme separated as described in 3.10.1. Following electrophoresis the gel was

incubated 2 x 30 minutes in water and then in a refolding buffer (20 mM Tris-HCl pH 7.6, 50 mM NaCl, 20 mM MgCl and 0.5% Trition X-100). Triton X-100 is a non-ionic surfactant that removes SDS from the gel, restoring protein tertiary structure. In that way the proteins in the sample are re-activated and will start to hydrolyse the substrate in the gel, where areas of digestion will show up as clear bands (Leclerc and Asselin 1989).

3.10.5 Preparation of pneumococcal cells for zymography

Cells were grown in 300 ml fresh C-medium and harvested by centrifugation at (10,000 g) for 10 minutes. The cell pellet was resuspended in 1.25 ml separation gel buffer and the cells were heat killed by boiling for 10 minutes.

3.11 Pneumococcal peptidoglycan isolation

In this work, potential changes in peptidoglycan stem peptide composition of *S. pneumoniae* when depleting the expression of the *murTcobQ* operon has been examined. For the isolation of the pneumococcal cell wall, a protocol adapted from "The Molecular Biology of Streptococci" has been used (Vollmer 2007). The protocol is a multi step process involving the stepwise removal of other cellular components. The bacterial cells were boiled in an SDS solution to solubilize the cell membrane and a high proportion of the proteins. The insoluble cell wall was recovered by centrifugation and washed free of SDS before being mechanically broken using glass beads. It is vital that centrifugation is done at room temperature to avoid precipitation of SDS. DNA, RNA and proteins were removed enzymatically by incubating the sample with DNase, RNase and protease (trypsin). Further purification steps included treatment with lithium chloride, EDTA, and acetone to yield pure peptidoglycan (murein). The peptidoglycan was then enzymatically broken down and analysed by high-performance liquid chromatography (3.12) and Orbitrap mass spectroscopy (3.13).

3.11.1 Protocol for isolation of pneumococcal cell wall

1. *S. pneumoniae* cells were grown in 1 litre of sterile C-medium to an OD_{550} of 0.3-0.5 and harvested by centrifugation (10,000 g) for 10 minutes, at room temperature.
2. To solubilize the cell membrane, the acquired pellet was resuspended in 40 ml ice cold 50 mM Tris-HCl pH 7.4, and added drop wise to 120 ml of boiling 5% SDS

(w/v) while stirring with a magnetic stirrer. The solution was boiled for 15 minutes and then left to cool to room temperature.

3. The crude cell wall material was collected by centrifugation (12,000 g) for 10 minutes at room temperature and washed free of SDS with repeated centrifugations, first 2 x 20 ml 1 M NaCl, followed by 4 x 20 ml dH_2O.
4. After washing, the cell wall material was resuspended in 2 ml dH_2O and mechanically broken in a FastPrep® 24 benchtop homogenizer using acid washed glass beads. 8 x 20 second pulses at 6.5 m/s were used, with 30 seconds rest on ice between every other pulse.
5. The glass beads were left to sediment and the supernatant was reallocated to a new centrifuge tube. The glass beads were washed 2-3 times with 2 ml of dH_2O and left to sediment and the supernatant transferred to the same centrifuge tube to collect as much of the cell wall material as possible. The volume was adjusted with dH_2O to 25 ml and unbroken the cell walls sedimented by centrifugation (12,000 g) at room temperature for 15 minutes.
6. The cell wall pellet was resuspended in 2 ml 100 mM Tris-HCl pH 7.4, and added 20 mM $MgSO_4$, 10 μg/ml DNase and 50 μg/ml RNase and incubated at 37°C for 2 hours while stirring. After 2 hours, 10 mM $CaCl_2$ and 100 μg/ml trypsin was added and the sample was further incubated at 37°C over night.
7. SDS was added to the sample from an 8% stock (w/v) to a final concentration of 1% and incubated at 80°C for 15 minutes. The volume was adjusted with dH_2O to 20 ml and the cell walls were then sedimented by centrifugation (25,000 g) for 30 minutes at room temperature.
8. The pellet was resuspended in 10 ml 8 M LiCl and incubated at 37°C for 15 minutes and then sedimented as before.
9. The pellet was resuspended in 10 ml 100 mM EDTA, pH 7.0 and incubated at 37°C for 15 minutes and then sedimented as before.
10. The pellet was washed successively with 20 ml of dH_2O, 20 ml of acetone and 20 ml dH_2O in glass tubes, centrifuged at 13,000 g for 30 minutes at room temperature. Finally, the pellet was resuspended in approximately 1 ml dH_2O, transferred to an Eppendorf tube and dried in a vacuum centrifuged. The dried pellet was then resuspended in water to a concentration of 50 mg/ml.

3.11.2 Preparation of the stem peptides from pneumococcal cell wall: Digestion of peptidoglycan with the hydrolytic autolysin LytA

The pneumococcal cell wall was hydrolysed using the pneumococcal autolysin LytA. This enzyme had been purified in the lab in a previous work. The enzyme hydrolyses the link between Mur*N*ac residues and the first L-Ala residue of the stem peptides in peptidoglycan.

1. Two mg of purified cell wall was mixed with 10 µl of 100 mM phosphate buffer pH 7.15, 1 µg/ml LytA and dH_2O to a total volume of 100 µl, and incubated overnight at 37°C.
2. The sample was then incubated at 95°C for 20 minutes in a water bath to precipitate LytA, and then centrifuged (20,000 g) for 10 minutes at room temperature to remove undigested peptidoglycan and LytA precipitates.
3. The supernatant was transferred to a new Eppendorf tube and added 100 µl of 100 mM phosphate buffer pH 7.15.
4. The sample was centrifuged (20,000 g) for 5 minutes at room temperature and the supernatant then reallocated to a new Eppendorf tube.
5. The pH of the sample was adjusted to 3.0 using 20% ortho phosphoric acid (approximately 2 µl). The pH was evaluated by applying 1 µl of the sample to pH paper strips.

3.12 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) has been used to analyse the stem peptide composition of the hydrolysed peptidoglycan of pneumococcal cells (see previous section). The technique, which is based on the principles of chromatography where one can separate molecules according to chemical properties, utilizes high pressure to pass a solvent containing the sample through a column with a solid absorbent material (Vollmer 2007). As the different components of the sample will have varying properties (i.e. hydrophobicity, size, etc) they will interact differently with the absorbent material, and as such will lead to a separation as they flow through the column. In this study, reverse phase chromatography has been used to separate the components of the sample (stem peptides from peptidoglycan) according to hydrophobicity. The hydrophobic material of the column results in the binding of the molecules in the sample according to column. By adding an organic solvent as an eluent in linearly increasing concentrations, the peptides in the sample will gradually release

from the column according to hydrophobicity. The eluted peptides are then passed through a detector measuring absorbance at 206 nm.

3.12.1 Reverse phase HPLC protocol

1. The mobile phase (0.05% trifluoracetic acid (TFA) (v/v)) was degassed the day it was used to avoid formation of gas bubbles in the buffers when mixed with the organic solvent used for elution, as gas bubbles would interfere with the A206 measurements.
2. The column was equilibrated with 0.05% TFA for 60 minutes, with a flow rate of 0.5 ml/min.
3. The sample was injected (30 μl) on the column and eluted with a linear gradient of the elution buffer (15% acetonitrile (v/v), 0.05% TFA (v/v)) according to the following program:
    a. Flow 0.5 ml/min
    b. 5 min wash
    c. 100 min elution from 0-15% acetonitrile
    d. 15 minutes regeneration with the mobile phase.

When not using the column it was stored with 50% methanol (v/v).

3.13 Orbitrap

In this study the peptidoglycan stem peptide composition of *S. pneumoniae* has been analysed using an Orbitrap mass analyser. The work has been done externally and so only a brief description of the technique will be given here. For an in depth description see Hu et al. (2005). The instrument consists of an outer barrel-like electrode and an inner spindle electrode that traps ions in an orbit around the inner spindle. By trapping the ions in elliptical orbits around the central electrode they move back and forth along its axis. The axial motion of the ions is harmonic and depends only on the mass-to-charge ratio (m/z). By detecting the axial oscillation of the ion rings mass can be determined by using the Fourier transform of the frequency signal (Hu et al. 2005). The peptidoglycan of the laboratory strain RH1 and the *murTcobQ* depletion strain OH8 has been digested using LytA following protocol 3.11.2, and the freed stem peptides then analysed by Orbitrap mass spectroscopy. The samples were loaded with 0.1% TFA, while the analysis was done with 0.1% formic acid.

3.14 Microscopy

Microscopy of pneumococcal cells has been done in this study to ascertain potential morphological abnormalities when underexpressing the *murTcobQ* operon. For quick analysis, light microscopy has been used (not described). For more thorough examinations, transmission electron microscopy and scanning electron microscopy have been used.

3.14.1 Transmission electron microscopy and scanning electron microscopy

One of the major limitations of traditional light microscopy is the resolution achievable when using the wavelengths of visible light. Electron microscopy solves this issue by using electrons, which can have wavelengths 100,000 times shorter than visible light. Whereas traditional light microscopy gives a resolution >0.2 μm, an electron microscope has the ability to give resolutions as low as 50 pm (Erni et al. 2009). A scanning electron microscope (SEM) produces an image by scanning a sample with a focused beam of electrons and then detects the spread of the electrons, giving an image of the surface of the sample. A transmission electron microscope on the other hand beams the electrons *through* the sample, detecting the spread and gives information about the internal structures of the sample.

The pneumococcal cells needed to be prepared before SEM and TEM, first by fixing the cells using a solution consisting of paraformaldehyde and glutardialdehyde in a PBS buffer (for recipe see 2.11.6). The fixation solution kills the cells by cross-linking proteins and preserves the cells with undisturbed morphology (Morris 1965). For TEM the fixed cells were then dehydrated using ethanol, molded into a resin and cut into 50-70 nm thin sections using a diamond bladed microtome. The sections cannot be more than 100 nm thick or else the electrons will not pass through. For SEM, the samples were dried and coated with gold to make them electrically conductive. This prohibits the build up of heat and charge in the sample.

3.14.2 Protocol for *S. pneumonia* sample preparation for TEM and SEM

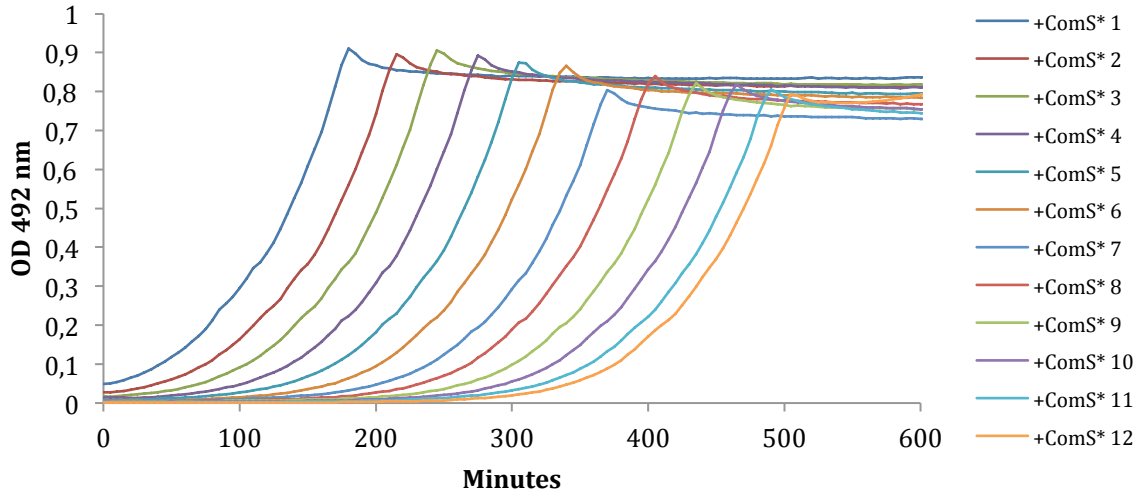
1. The pneumococcal cell cultures were harvested by centrifugation and then fixed in a fixing solution (2% paraformaldehyde (v/v), 2.5% glutardialdehyde (v/v) and 0.1 M PBS, pH 7.4) in a 1:1 ratio.
2. The solution was incubated at room temperature for 1 hour before incubation at 4°C overnight.
3. The solution was centrifuged (10,000 g) for 2 minutes washed in PBS buffer (0.1 M, pH 7.4) to remove the paraformaldehyde and glutardialdehyde. The wash was repeated 3 times.
4. Further preparation was performed by personnel at the Imaging Center (Campus Ås) and involved:
    a. Preparation for TEM:
        i. The samples where molded into 0.5% agarose and fixed over night in a fixing solution.
        ii. The samples were washed in a cacodylate buffer (0.1 M, pH 6.8) and fixed in 1% osmium in cacodylate buffer for 1 hour at 4°C, followed by a second wash.
        iii. The samples were dehydrated using increasing concentrations of ethanol and molded into LRWhite resin.
        iv. Finally the samples were segmented using an ultramicrotome, mounted on a grid and stained with 4% uranyl acetate and 1% potassium permanganate.
    b. Preparation for SEM:
        i. The samples were dehydrated with increasing ethanol concentrations (50%, 70%, 90%, 100%) and then dried with critical point drying.
        ii. The samples were mounted and coated with gold-palladium (Au/Pd).

4 Results

4.1 Depletion of MurT/CobQ in *S. pneumoniae* using the ComRS system

In *S. pneumoniae* the MurT/CobQ enzyme complex is responsible for the amidation of L-Glu to L-iGln of the peptidoglycan precursor lipid II. *In vitro* studies have shown that this modification of lipid II is essential for it to function as a substrate for the PBPs in peptidoglycan synthesis. Consequently, depletion of *murTcobQ* expression using the Zn^{2+} system has previously been demonstrated by to inhibit pneumococcal cell growth (Zapun et al. 2013). To verify these data, and to have a *murTcobQ* depletion strain in our laboratory for further studies of this operon, the strain OH8 was created using the ComRS system (for construction of this strain see 3.8). Depletion of MurT/CobQ was done as described in 3.7.1, and growth was monitored in a 96-well Corning® microtiter plate using a Synergy H1 microplate reader. In brief, the cell cultures were diluted 2-fold in fresh C-medium with (0.2 μM) and without ComS* present. Cells cultures diluted in ComS*-free growth medium will gradually reduce the expression of *murTcobQ* as the level of ComS* decreases throughout the dilution series. The results showed that *murTcobQ* depletion mutant grew well when expression of the operon was ectopically driven with 0.2 μM ComS* (Figure 4.1.1 A). In contrast, when ComS* was removed from the growth medium, *murTcobQ* expression reached a critically low level which severely reduced the growth rate (Figure 4.1.1 B). The most MurT/CobQ-depleted cells displayed more or less no growth at all.

A



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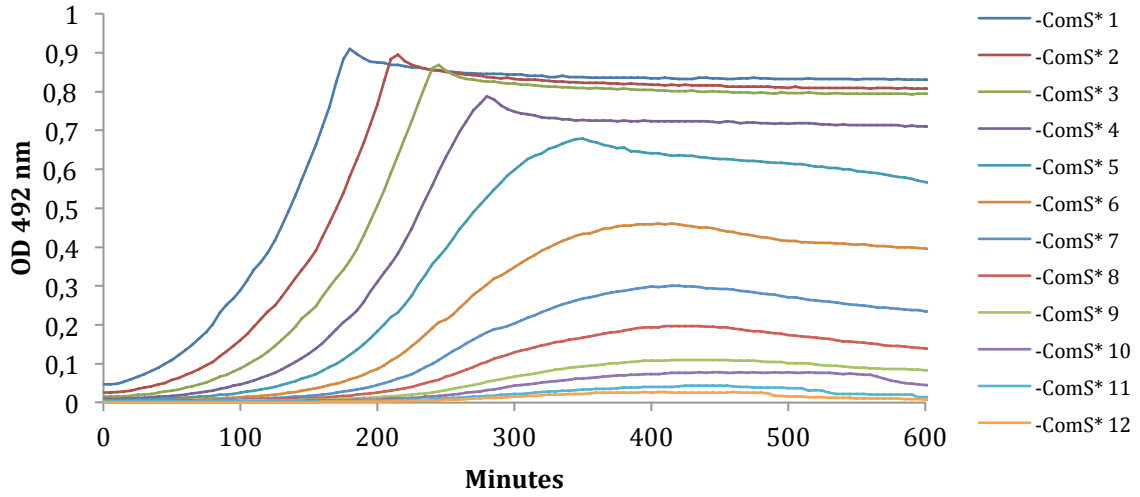


Figure 4.1.1: Figure 4.1.1: Growth of depletion strain OH8 with and without added ComS*. A: 2-fold dilution series (+ComS*1–+ComS*12) of washed OH8 cell culture was grown for 20 hours in a 96 well Corning® microtiter plate. Each well contained 0.2 µM ComS*. OD was measured every 5 minutes at 492 nm. **B:** 2-fold dilution series (-ComS*–-ComS*) of washed, ComS*-free OH8 cell culture was grown for 20 hours in a 96 well Corning® microtiter plate. OD was measured every 5 minutes at 492 nm.

4.2 Suboptimal ComS* concentrations for expression of *murTcobQ*

For certain experiments it was more desirable to have a steady, but impaired growth rate of the depletion strains instead of the gradual depletion described in the previous section. The reasoning for this was that a constant, but suboptimal expression of the *murTcobQ* operon would give more predictable experimental results. When doing gene depletion studies, gene

expression gradually decreases over time. In contrast growing cells in suboptimal ComS* concentrations could in theory give a constant, non-lethal suboptimal gene expression.

To find ideal suboptimal ComS* concentrations, OH8 cells washed free from ComS* was diluted in a 2-fold series of 12, and cells from the 12th dilution was used in a microtiter plate assay. A 2-fold dilution series of ComS* was added to these cells in a 96-well Corning® microtiter plate, with 2.0 μM ComS* in the first well, with the final well containing 0 μM ComS*. Cells that reached an OD_{492} of at least 0.5, with a significantly decreased growth rate was the target. A selection of the most appropriate candidates is presented in figure 4.2.1. The window between growth and no growth was narrowed down to be between 7.8 and 1.95 nM ComS* in the growth medium (dilutions 9, 10 and 11 contains approx. 7.8, 3.9 and 1.95 nM ComS*). As can be seen in the figure, the three parallels grew slightly different, which can most likely be attributed slight variations when making the 2-fold ComS* dilutions.

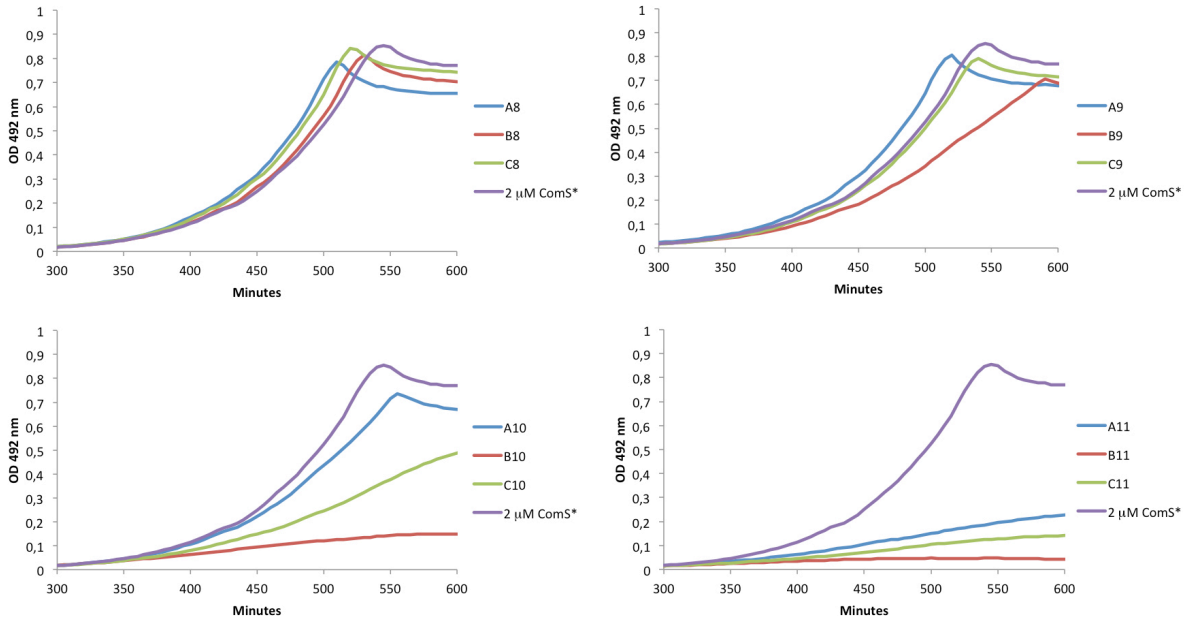


Figure 4.2.1: Growth of *S. pneumoniae* expressing suboptimal levels of MurT/CobQ. The figure shows the growth of the *murTcobQ* depletion strain OH8 in ComS* dilutions 8-11 in a 2-fold dilution series of 12. Dilutions 8, 9, 10 and 11 contain approx. 15.6, 7.8, 3.9 and 1.95 nM ComS*. The experiment was done with three parallels (A-C), and the figure shows slight variations between each parallel. The growth curve of OH8 grown with 2.0 μM ComS* is show for comparison.

Based on the findings presented in figure 4.2.1 it was decided to try to grow the *murTcobQ* depletion strain OH8 in the presence of specific levels of ComS*. By having cells expressing suboptimal levels of MurT/CobQ during growth, it would give us the opportunity to test how

reduced amidation of the L-Glu in the stem peptide affects the cells morphology and physiology. The OH8 strain was washed free from ComS* as described in 3.7.3 and grown from OD_{550} =0.01 to 0.3 in ComS* concentrations ranging from 20 nM to 0.156 nM, resuspended to OD_{550} 0.01 and grown to 0.3 once more in the same ComS* concentrations. The bacterial cultures were then transferred to a 96-well Corning® microtiter plate, where each well contained the appropriate ComS* concentrations, and growth was monitored by measuring OD_{492} every 5 minutes for 20 hours in a Synergy H1 microplate reader (Figure 4.2.2). Using this approach for suboptimal growth, ComS* concentrations between 3.5 and 1 nM was chosen as ideal for further experiments, with a sharp cut off with no growth below 1.25 nM.

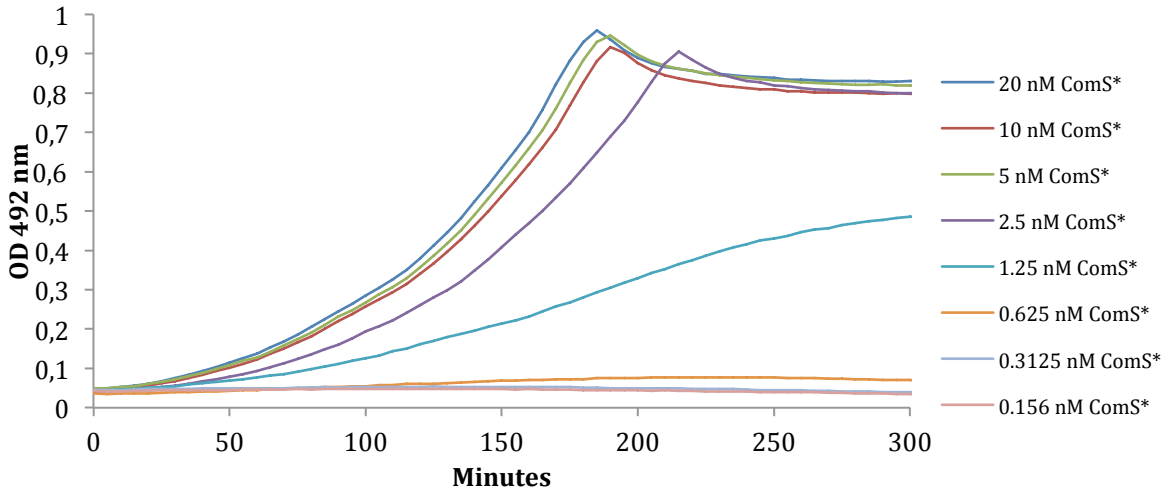


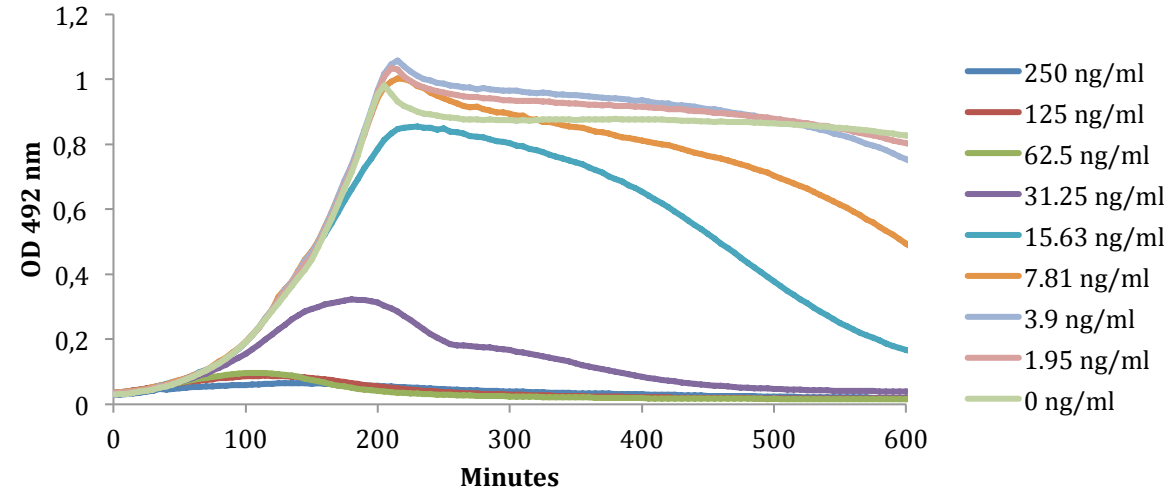
Figure 4.2.2: OH8 growth curves for cultures grown in specific ComS* concentrations. The *murTcobQ* depletion strain OH8 was grown in ComS* concentrations ranging from 20 to 0.156 nM to further narrow down a suitable window for suboptimal growth. The figure shows a narrow window between 2.5 and 0.625 nM for growth vs. no growth.

4.3 β-lactam antibiotic sensitivity in MurT/CobQ-depleted *S. pneumoniae*

Because of *S. pneumoniae*´s role as a human pathogen, it was desirable to see whether pneumococcal cells having reduced levels of amidated lipid II would display changes in sensitivity to two of the most commonly used β-lactam antibiotics, cefotaxime and ampicillin. It was reasonable to hypothesize that a reduced level of the natural substrate of the PBPs would affect the sensitivity to β–lactams. This was done by growing the strain OH8 with ComS* concentrations that provided suboptimal expression levels of *murTcobQ*, as described in as described in 3.7.3 and 4.2. A 2-fold dilution series of the antibiotic being tested was added to the cells. The strain sph131 was chosen as a reference for wild type

antibiotic sensitivity with both cefotaxime and ampicillin experiments. The results showed no significant differences in sensitivity to either antibiotic when compared to sph131 and to OH8 grown in the presence 0.2 µM ComS* (data for OH8 in 0.2 µM ComS* not shown) (Figures 4.3.1 and 4.3.2). Note the difference in maximum optical density between sph131 and OH8. The minimum inhibitory concentration (MIC) for both the control strain and the depletion strain OH8 was the similar with both cefotaxime and ampicillin. MIC with cefotaxime was 62.5 ng/ml for both strains and 156.25 ng/ml with ampicillin for both strains.

A



B

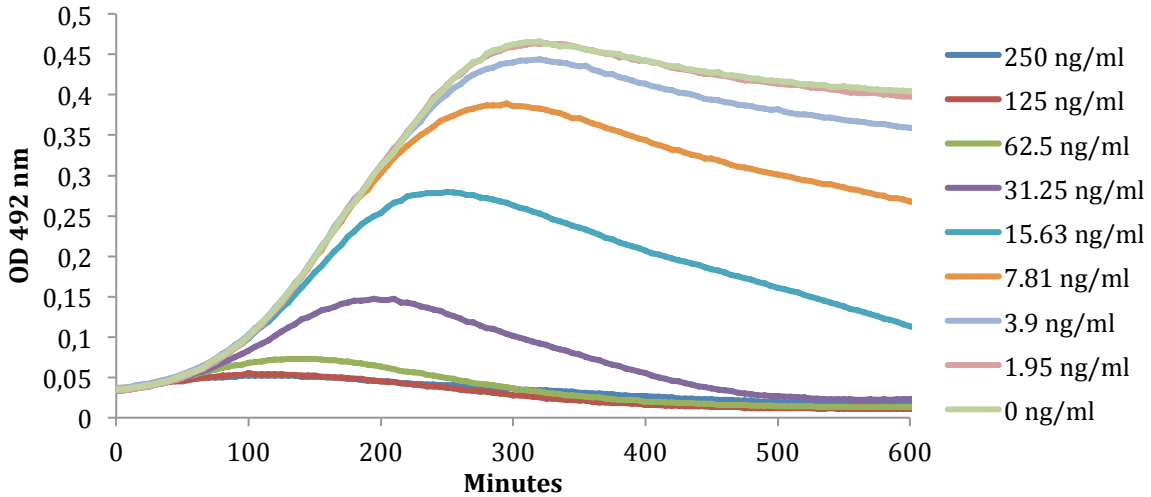
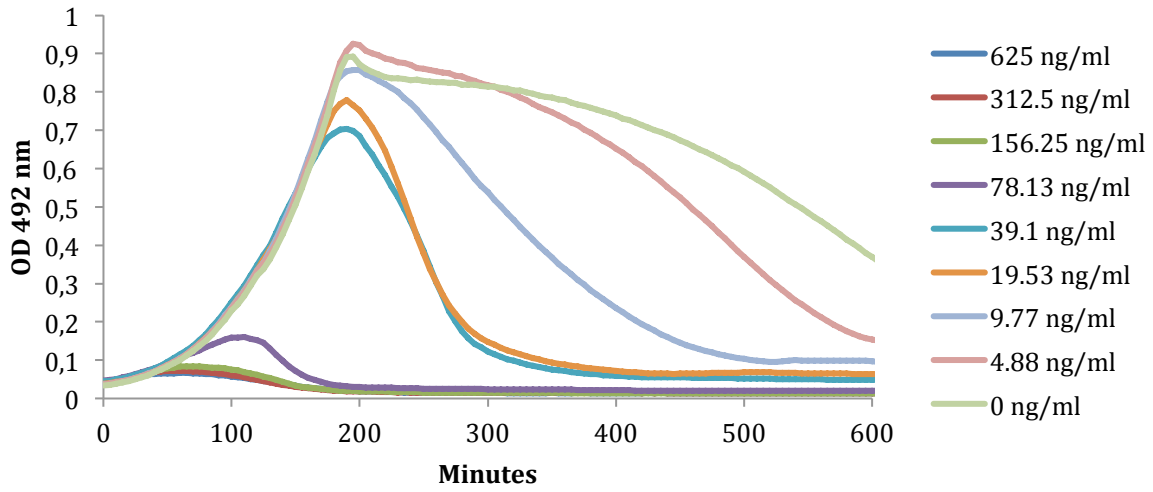


Figure 4.3.1: Cefotaxime sensitivity in strains sph131 (A) and OH8 (B). OD_{492} was measured at 5 minute intervals for 20 hours. The legend shows the different cefotaxime concentrations in each well, and is an approximation based on a 2-fold dilution series where the first well contained 2 µg/ml. The three highest cefotaxime concentrations have been left out of the figure. The experiment was repeated three times.

A



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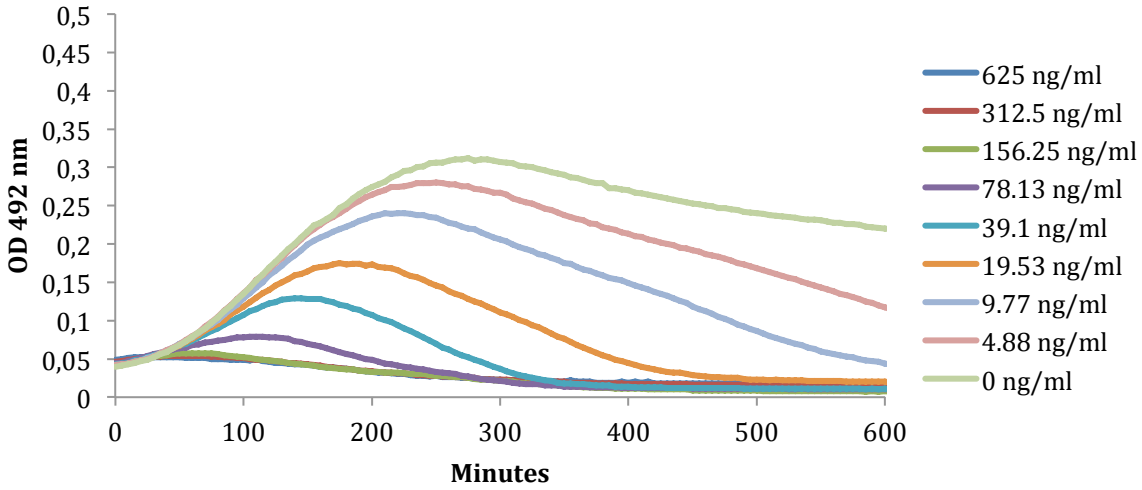

Figure 4.3.2: Ampicillin sensitivity in strains sph131 (A) and OH8 (B). OD_{492} was measured at 5 minute intervals for 20 hours. The legend shows the different ampicillin concentrations in each well, and is an approximation based on a 2-fold dilution series where the first well contained 5 µg/ml. The three highest cefotaxime concentrations have been left out of the figure. The experiment was repeated three times.

4.4 Depletion of MurT/CobQ in a Δ*pbp1a* or Δ*pbp2a* background

When André Zapun and colleagues in 2013 did the complete *in vitro* synthesis of peptidoglycan using recombinant PBPs, they showed that PBP1a had some residual transpeptidase activity with glutamate containing lipid II, whereas PBP2a did not (Zapun et al. 2013). Based on these data it was of interest to examine whether this could be detected using a simple microtiter growth assay by depleting MurT/CobQ in PBP1a and PBP2a knockout mutants. The strains OH10 (OH8, but Δ*pbp1a*::Janus) and OH11 (OH8, but

Δ*pbp2a*::Janus) were made for this purpose, and a depletion of MurT/CobQ was done as described in 3.7.1. The strains KHB104 (Δ*pbp1a*::Janus), KBH105 (Δ*pbp2a*::Janus) and sph131 were used as control strains. The strains OH10 and OH11 were constructed by amplifying the knockout fragments (Janus) from the genomes of KHB104 and KHB105 respectively, transforming them into OH8 following protocol (3.6.2). As can be seen in figure 4.4.1, deletion of *pbp1a* seems to be more harmful than deletion of *pbp2a*, with the *pbp2a* deletion mutant KHB105 having a very similar growth curve to the control strain sph131.

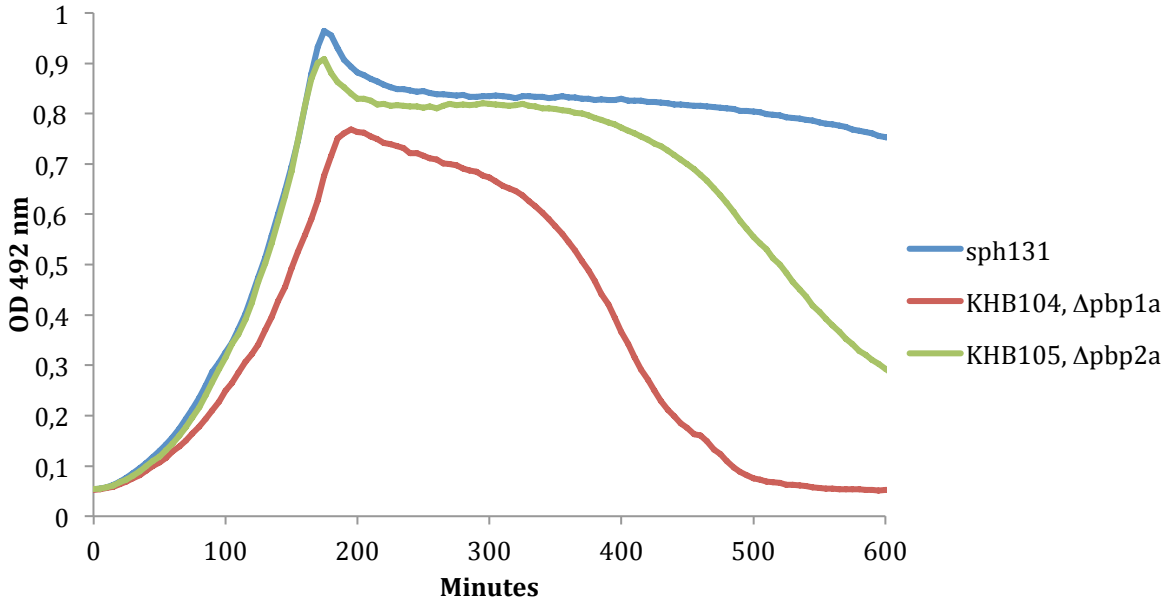

Figure 4.4.1: Growth of sph131, KHB104 and KHB105. A comparison of the growth curves of the three control strains: sph131, KHB104 (Δ*pbp1a*) and KHB 105 (Δ*pbp2a*).

If the mentioned residual transpeptidase activity that PBP1a has with glutamate containing lipid II was to be detected, an increased sensitivity to MurT/CobQ depletion would be expected in the *pbp1a* knockout strain OH10, when compared with the *pbp2a* knockout strain OH11. No significant changes in growth were observed in this experiment, and the relative difference in growth rate between the two strains remained more or less the equal in all dilutions. Figure 4.4.2 shows the growth curves of dilutions 2-7 in 2-fold dilution series of strains OH10 and OH11.

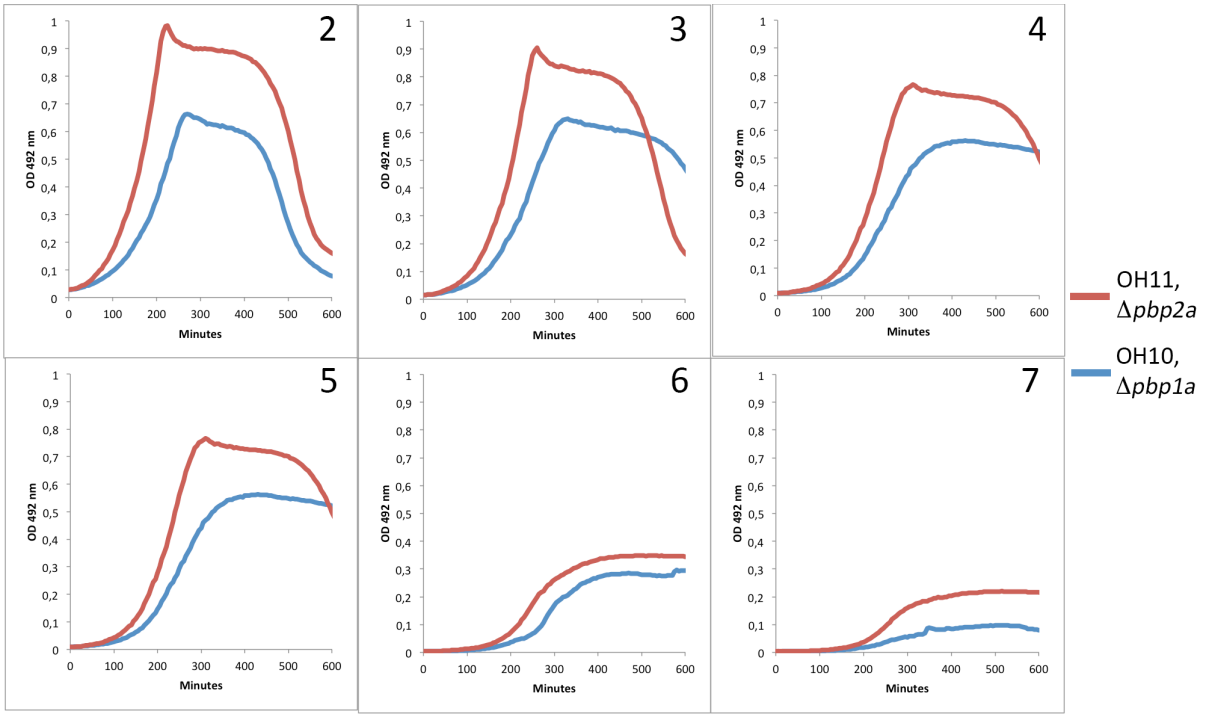

Figure 4.4.2: Depletion of MurT/CobQ in PBP1a and 2a knock out mutants OH10 and OH11. Dilutions 2-7 in a 2-fold dilution series of OH10 and OH11 cells washed to remove all residual ComS*. The differences in growth curves between the two strains stayed approximately the same in all dilutions. The experiment was carried out twice with similar results.

4.5 Lysozyme resistance in MurT/CobQ-depleted *S. pneumoniae*

S. pneumoniae is notoriously resistant to lysozyme, an enzyme present in saliva and mucus which catalyse the hydrolysis of the β-1,4 glycosidic bond between N-acetylmuramic acid and N-acetylglucoseamine in peptidoglycan (Bera et al. 2005). Resistance is obtained through modifications of peptidoglycan. The two enzymes PgdA (*N*-acetylglucoseamine deacetylase) and Adr (O-acetyl transferase) perform *N*-deacetylation of Glc*N*Ac and O-acetylation of Mur*N*Ac respectively, which gives resistance to lysozyme (Vollmer and Tomasz 2000, Crisostomo et al. 2006). To test whether reduced levels of amidated lipid II could interfere with these processes and induce sensitivity to lysozyme, MurT/CobQ-depleted cells were treated with the enzyme. A 2-fold dilution series of OH8 cells washed free from ComS* was added to a 96-well Corning® microtiter plate, and lysozyme from egg white was added to each well to a final concentration of 100 µg/ml. A parallel dilution series of OH8 was also grown without lysozyme as control. OH8 with 0.2 µM ComS* and sph131 were grown with and without lysozyme in the growth medium as lysozyme resistance controls. Figure 4.5.1 shows the growth curves of control strain sph131 grown with and without lysozyme. Strain OH8 displayed the same pattern as sph131 when grown with 0.2 µM ComS* and treated with

lysozyme (data not shown). The cells displayed full resistance against lysozyme during the exponential phase, but started to autolyse earlier in the stationary phase when lysozyme was present.

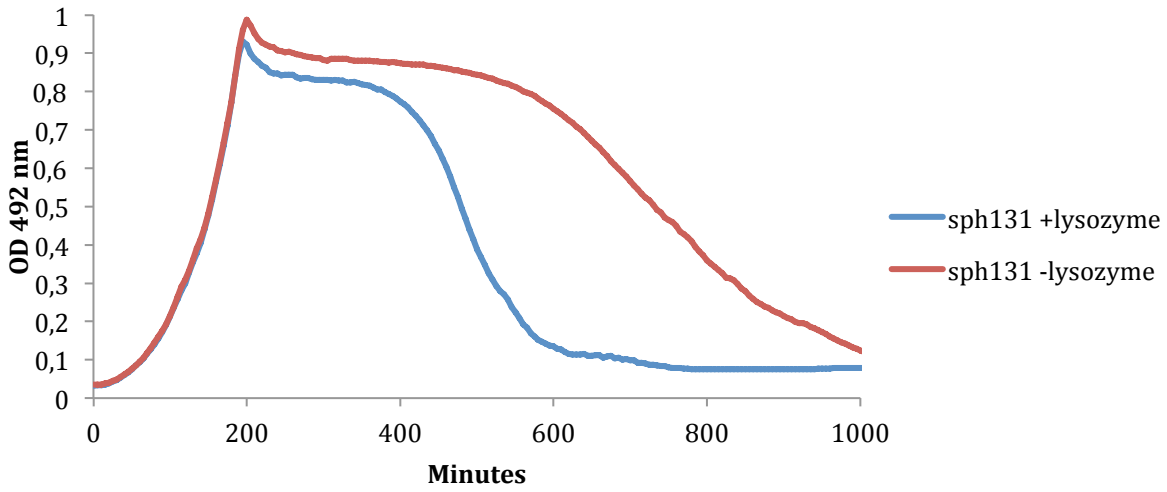

Figure 4.5.1: Lysozyme resistance in sph131. *S. pneumoniae* strain sph131 grown with 100 µg/ml lysozyme, and without lysozyme. The bacteria remained resistant to lysozyme throughout the entire exponential growth phase.

Similarly for the MurT/CobQ-depleted cells, resistance to lysozyme was seen for all dilutions in the series during the entirety of the exponential growth phase, but also here the cells started to autolyse at an earlier time point in the stationary phase when lysozyme was added. Figure 4.5.2 shows dilutions 5-8 in the 2-fold dilution series of MurT/CobQ-depleted OH8 cells.

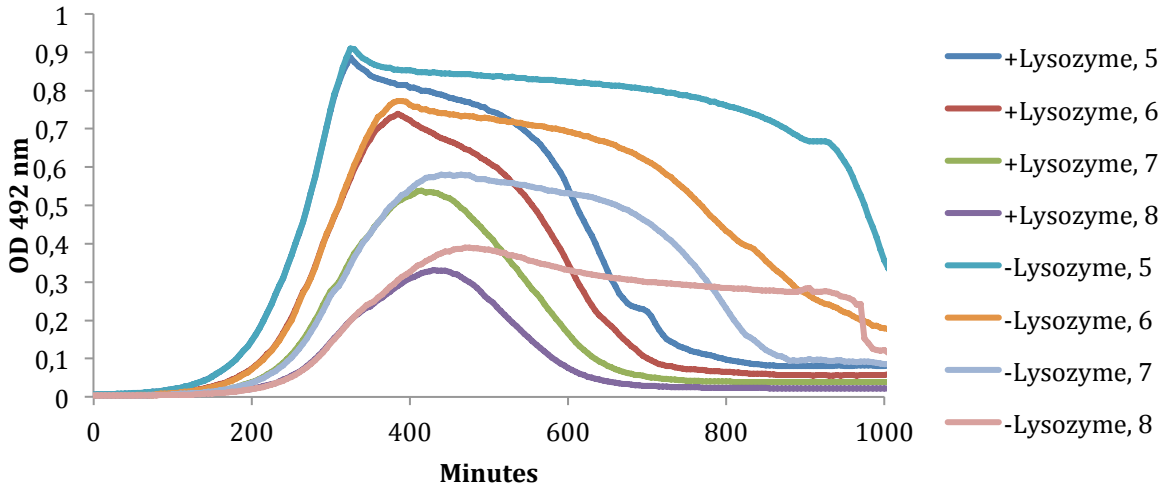

Figure 4.5.2: Lysozyme resistance in OH8. A 2-fold dilution series of OH8 cells washed free from ComS* grown in the presence of 100 µg/ml lysozyme. The same cell dilution series was also grown without lysozyme. Shown are dilutions 5-8, where the depletion of MurT/CobQ caused inhibited growth. Despite depletion of MurT/CobQ, the cells remained resistant to lysozyme during the entire exponential growth phase. The experiment was carried out twice with similar results.

4.6 MurT/CobQ depletion and resistance to the murein hydrolase CbpD

To study whether the depletion of MurT/CobQ could lead to changes in the stem peptide composition of the pneumococcal cell wall, sensitivity against the muralytic fratricide enzyme CbpD was tested, as this enzyme cleaves the stem peptides of the cell wall. Hydrolytic activity of CbpD can be detected by zymography, as previously described by Eldholm et al. (2010). Degradation of cell wall material is then seen as clearing zones in the zymogram. A zymogram containing MurT/CobQ-depleted OH8 cells and a zymogram containing control OH8 cells grown with 0.2 μM ComS* was therefore made (described in 3.10.2). *S. pneumoniae* is only immune against CbpD during competence, when the immunity protein ComM is expressed (Håvarstein et al. 2006). To deplete OH8 cells of MurT/CobQ, the strain was washed free from ComS* as previously described and inoculated in 300 ml fresh C-medium to a final OD_{550} ~ 0.001. An initial OD_{550} between 0.0005 and 0.001, when working with depletion of *murTcobQ* expression in OH8, had previously been established to stall growth at OD_{550} 0.3-0.5 when working with large volumes (data not shown). The MurT/CobQ-depleted cells were harvested at the tail end of the exponential phase (OD_{550} ~ 0.5) and the control at the same OD_{550}. These cells were used as substrate for CbpD in the zymography assay. Recombinant CbpD isolated from *L. lactis* (see 3.9) was added to the gels in 15, 7.5, 3.75, 1.9, 0.9 and 0.5 μl volumes. The concentration of CbpD had not been established. The results showed, rather surprisingly, that the depleted cells became immune to the activity of CbpD while the control remained sensitive (Figure 4.6.1).

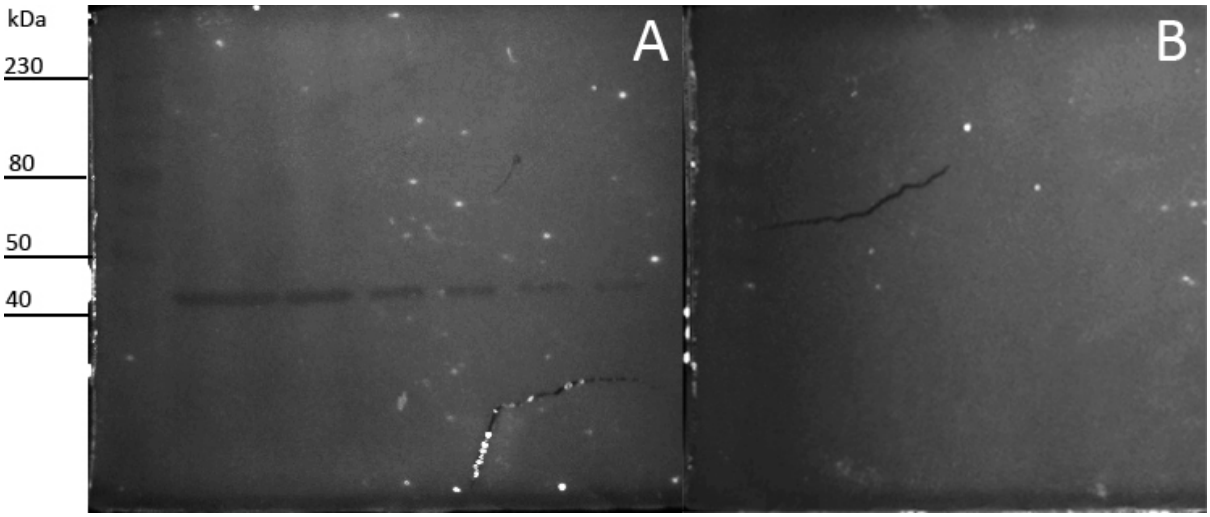

Figure 4.6.1: Zymography of MurT/CobQ-depleted and non-depleted OH8 cells with CbpD. A: Zymogram with OH8 cells induced with ComS*. **B**: Zymogram of the MurT/CobQ-depleted OH8 cells. CbpD is approximately 41 kDa, fitting with the digestion zones visible in zymogram A. The same bands are not visible on the depleted cells, indicating that CbpD cannot hydrolyse the cell walls of these cells.

4.7 Sensitivity to CbpD during competence in a Δ*comM* mutant underexpressing *murTcobQ*

Based on the observation that CbpD was unable to lyse the MurT/CobQ-depleted OH8 cells, it was of interest to see whether this could be observed *in vivo* during competence. To achieve this, it was necessary remove the native *comM* gene from the strain OH8 (with the *comM* gene, *S. pneumoniae* is immune against CbpD during competence). The Δ*comM*::Janus cassette was amplified from the genome of the Δ*comM* knockout strain RH420 (Eldholm et al. 2009), and the gene was then removed from OH8 using this cassette following protocol. The Δ*comM* mutant was named OH9. OH9 cell cultures used in this experiment were grown with suboptimal ComS* concentrations (as described in 3.7.3), ranging from 2.5 nM to 1.0 nM. The strain RH1 was used as control for full immunity, while the Δ*comM* mutant RH420 was used as control for CbpD-sensitive cells. Growth was monitored by measuring OD_{492}, and cell lysis was detected using the fluorescent DNA binding Sytox® Green Nucleic Acid Stain (excitation/emission = 504/523 nm). Sytox® can not enter living pneumococcal cells and will only give a fluorescent signal when bound to extracellular DNA that has been freed as a result of cell lysis (Invitrogen 2006). The experiment was carried out using a 96-well Corning® microtiter in a Synergy H1 microplate reader, measuring OD_{492} and fluorescence every 5 minutes for 20 hours.

Competence was induced after 125 minutes in all strains, when RH1 OD_{492} reached 0.3 (figure 4.7.1), by adding CSP to a final concentration of 250 ng/ml. As expected, RH1 became immune, while the Δ*comM* mutant RH420 lysed. OH9 expressing suboptimal amounts of MurT/CobQ all lysed as well, but gave off a weaker fluorescent signal than the RH420 counterpart. Adjusting for relative fluorescence (figure 4.7.2), the picture remained the same: OH9 lysed to a lesser extent, with the culture expressing lowest levels of MurT/CobQ giving of the weakest fluorescent signal. Although not 100% immune, this indicates that a change in cell wall structure decreases the efficiency of CbpD.

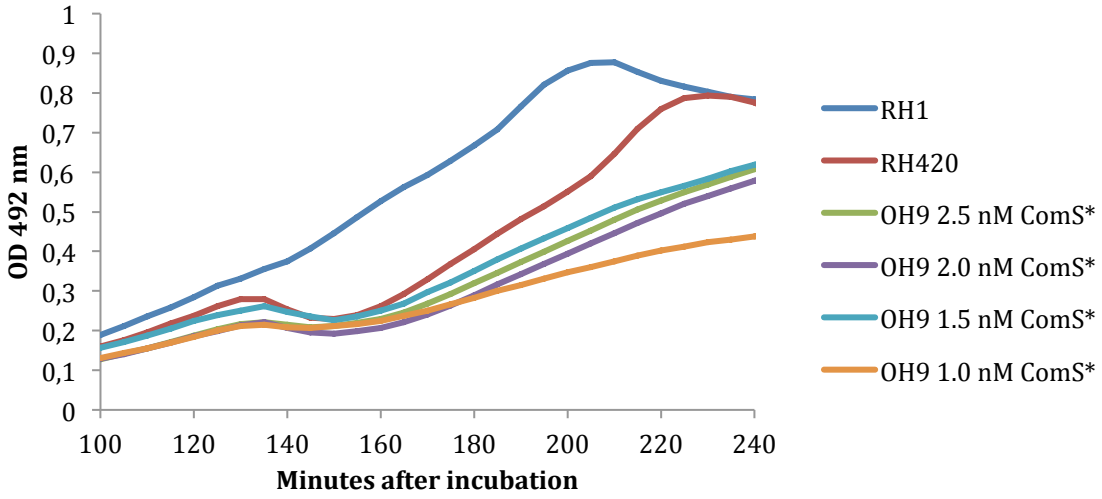

Figure 4.7.1: Growth curves of RH1, RH420 and OH9 induced to competence after 125 minutes. The dip in optical density at 135 minutes is due to the lysis of cells lacking ComM. Note that the dip in the growth curve of "OH9 1.0 nM ComS*" is not as severe as that of RH420, indicating partial immunity. The experiment was repeated three times.

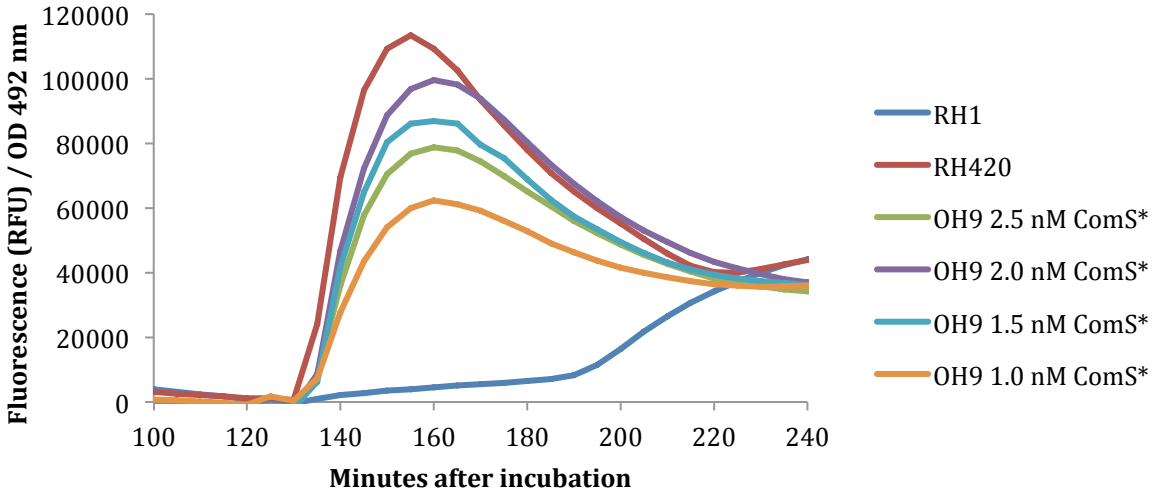

Figure 4.7.2: Relative fluorescence after induction of competence in RH1, RH420 and OH9. Fluorescent signal caused by Sytox® binding to DNA during cell lysis relative to the optical density at 492 nm. The relative fluorescence of the slowest growing OH9 culture (1.0 nM ComS*) is almost half that of RH420. This is most likely due to CbpD not being able to lyse the cells as effectively because of the underexpression of *murTcobQ*. The experiment was repeated three times.

4.8 Depletion of MurT/CobQ in *S. pneumoniae* causes gross morphological abnormalities

Clearly, inhibiting amidation of the secondary residue glutamate in lipid II by depleting MurT/CobQ results in severe growth defects (see sections 4.1 and 4.2). To see whether the inhibition of growth also caused any morphological changes in *S. pneumoniae*, OH8 cells were studied using scanning electron microscopy (SEM) and transmission electron

microscopy (TEM). OH8 cell culture grown in 1.5 nM ComS* according to 3.7.3 was inoculated to OD_{550} ~ 0.003 in 50 ml of C-medium containing 1.5 nM ComS*. The culture was harvested by centrifugation at OD_{550} ~ 0.1, when the culture had shown very little growth for approximately 40 minutes. Control cells grown with 0.2 µM ComS* were harvested at OD_{550} ~ 0.3. The cells were prepared for microscopy as described in section 3.14.2. SEM of the MurT/CobQ-depleted OH8 cells revealed dramatic morphological abnormalities (figure 4.8.1), with the most abundant morphology being the severely elongated cells seen in Figure 4.8.1C. Other morphological abnormalities included bloated, irregular cells as well as "pear" shaped cells. Also worth noticing is that not all cells in the MurT/CobQ-depleted culture are equally stressed (i.e. there are normal-looking cells in the culture as well) indicating that the MurT/CobQ depletion affects the individual cells at different rates.

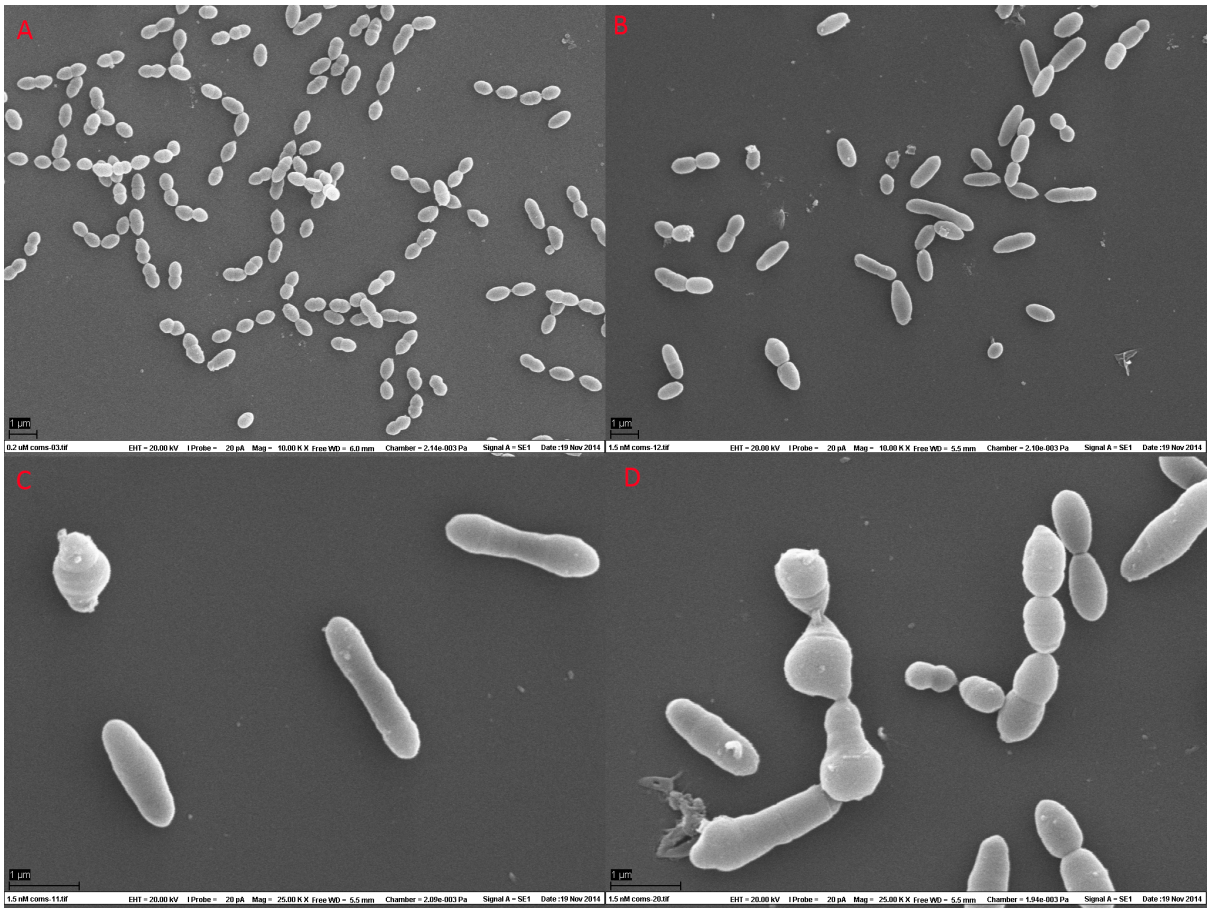

Figure 4.8.1: Scanning electron microscopy of OH8. A: 10,000x magnification of OH8 grown with 0.2 µM ComS* showing normal cell morphology. **B**: 10,000x magnification of MurT/CobQ-depleted OH8 cells, showing abnormal cell morphology, the most abundant being the slightly bloated and elongated cells. **C, D**: 25,000x magnification of MurT/CobQ-depleted OH8 cells.

Further examination by TEM revealed that elongation of the MurT/CobQ-depleted cells was due to the lack of proper septal formation (Figure 4.8.2). This strongly suggests that the divisome is hit hardest by the lack of amidated lipid II as a result of MurT/CobQ-depletion. MurT/CobQ-depleted OH8 cells were seen with up to four incomplete septa, and the cells appeared to be able to grow peripherally but not divide properly.

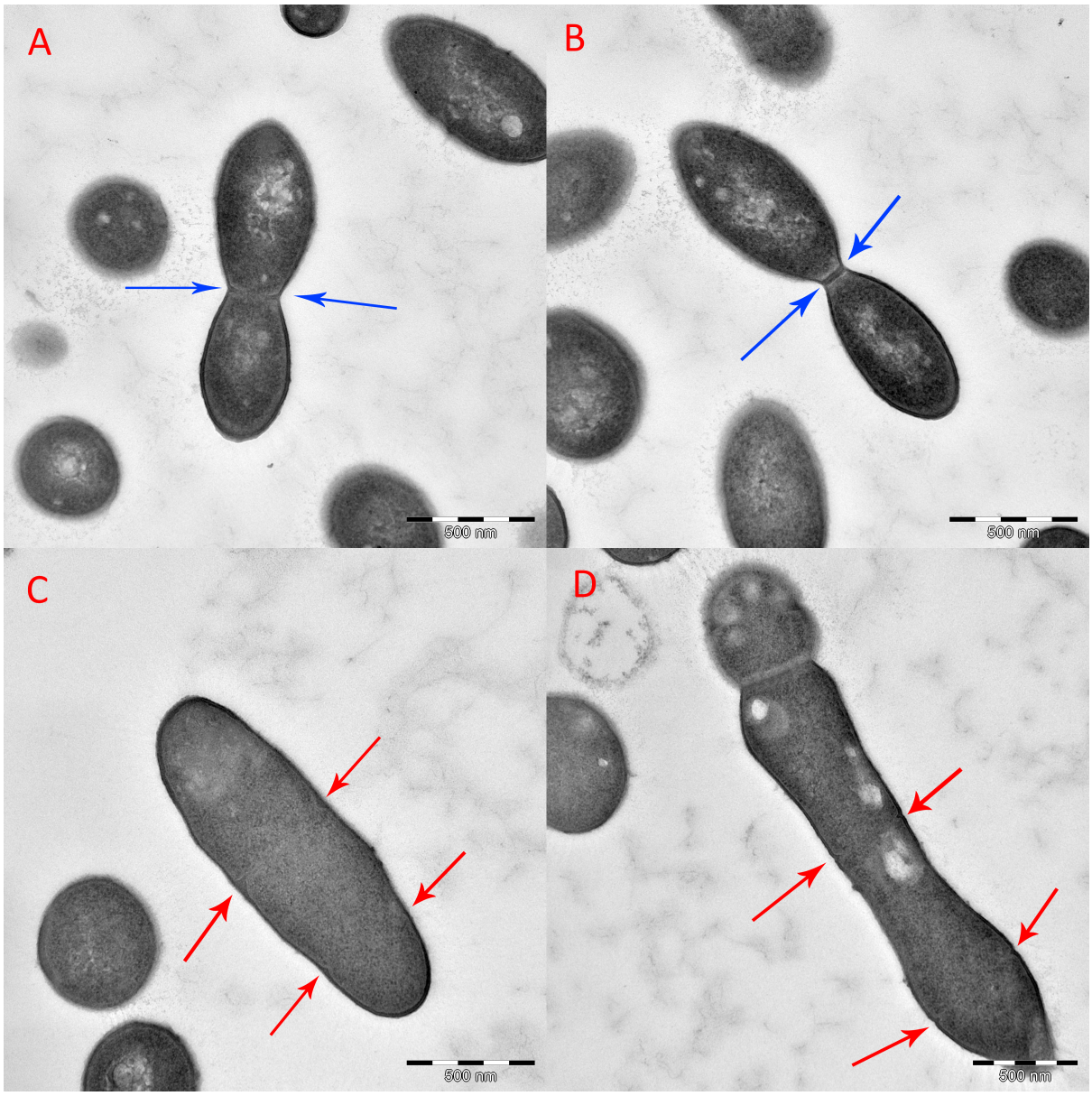

Figure 4.8.2: Transmission electron microscopy of OH8. A+B: OH8 cells grown with 0.2 µM ComS*. The blue arrows indicate normal septal formation. **C+D**: MurT/CobQ-depleted OH8 cells, showcasing abnormal growth. The red arrows indicate incomplete septal formation. The cells appear to be unable to divide properly, but are still able to grow in the longitudinal direction.

4.9 Depletion of MurT/CobQ changes the stem peptide composition of the pneumococcal cell wall

It was of interest to study whether *S. pneumoniae* is able to incorporate glutamate-containing lipid II into the cell wall, and in particular if the depletion of *murTcobQ* expression would lead to any detectable changes in stem peptide composition. Previous studies have revealed that a certain amount of non-amidated lipid II is incorporated into the cell wall, but mostly as non-cross-linked monomers (12.6% of monomer). Non-amidated cross-linked peptides were only detected in dimers (1.8%) and not in trimers (Bui et al. 2012).

Peptidoglycan from MurT/CobQ-depleted OH8 cells was isolated as described in 3.11, as well as peptidoglycan from OH8 cells grown in the presence of 0.2 μM ComS*. RH1 peptidoglycan previously isolated in the laboratory was also used as wild type control. To deplete OH8 of MurT/CobQ, cells washed free from ComS* was inoculated in 2 litres of C-medium to a final OD_{550} of 0.001 (similar to the method for growth used in 4.6). The cells were harvested at the point where growth was observed to level off (OD_{550} ~ 0.5).

4.9.1 HPLC analysis of the stem peptide composition in MurT/CobQ-depleted *S. pneumoniae*

The peptidoglycan derived from MurT/CobQ-depleted and control cells were digested with LytA as described in 3.11.2. The LytA enzyme hydrolyses the link between N-acetylmuramoyl residues and the stem peptides in peptidoglycan, freeing the stem peptides. The peptides were analysed using reverse phase HPLC, revealing a significant change in the stem peptide composition when comparing the stem peptides from MurT/CobQ-depleted cells with that of the control cells (figures 4.9.1). The most striking changes found for MurT/CobQ-depleted OH8 cells, when compared to RH1 and ComS* induced OH8, are two large peaks eluted after 21 and 24 minutes. These are present in RH1 cell wall as well, but are much smaller relative to the peak at 27 minutes. Also, the large peak at 48 minutes in the MurT/CobQ-depleted OH8 chromatogram is not present in RH1 chromatogram. The chromatogram of the peptide composition of OH8 cells induced with 0.2 μM ComS* was similar to that of RH1 (not shown here, see appendix). This indicates that dramatic changes occur in the pneumococcal cell when depleting the expression of *murTcobQ*. Cell wall from

MurT/CobQ-depleted cells was isolated two separate times, and HPLC analysis done three times on each isolate produced similar chromatograms.

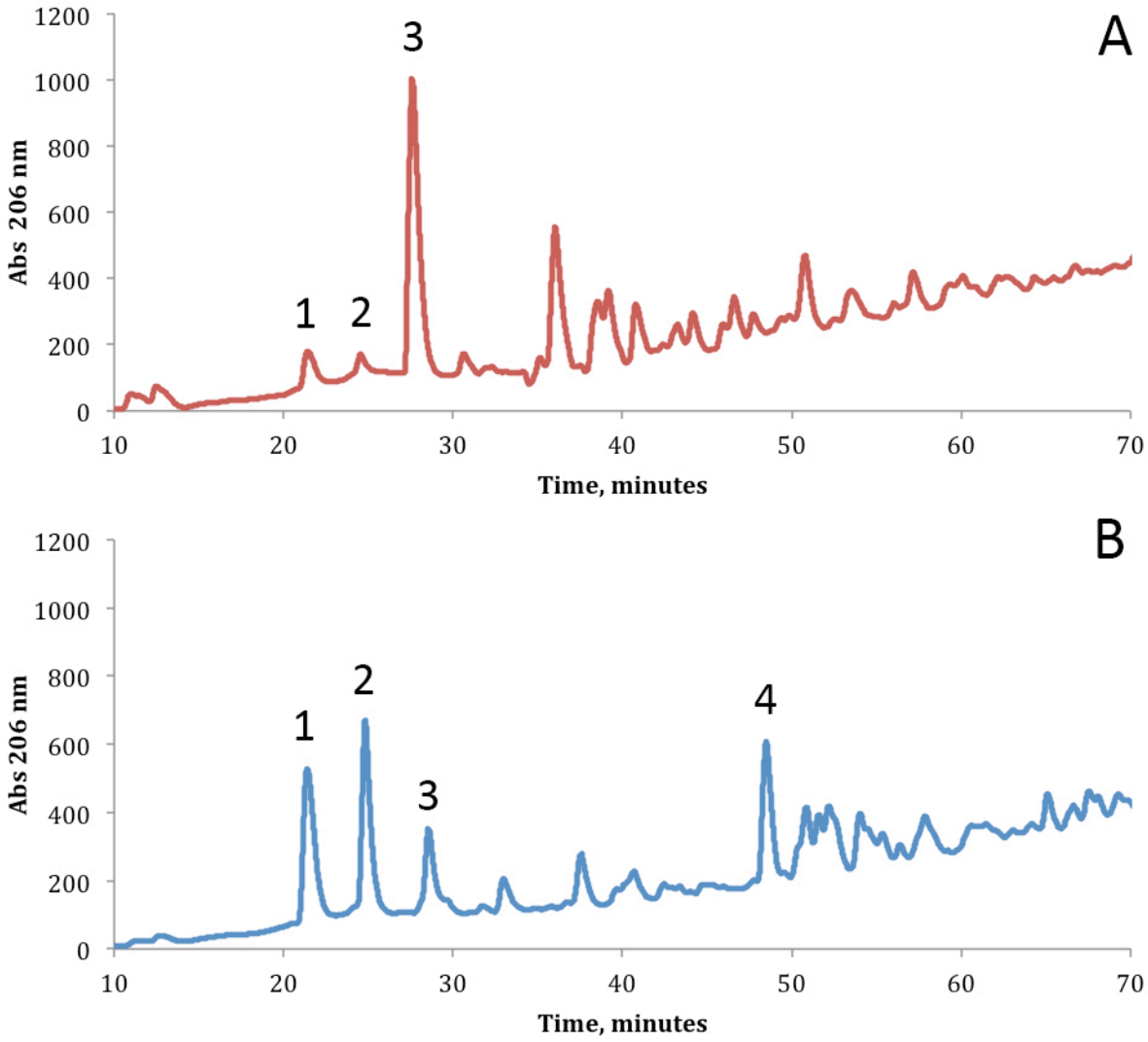

Figure 4.9.1: HPLC of RH1 and MurT/CobQ-depleted OH8. A: Stem peptides of RH1 cell wall analysed by reverse phase HPLC. The chromatogram of OH8 grown with 0.2 μM ComS* was similar (see appendix) **B:** Stem peptides of OH8 cell wall analysed with reverse phase HPLC. The chromatogram deviates from that of RH1 and non-depleted OH8, in particular the two large peaks eluted at 21 (**1**) and 24 (**2**) minutes, as well as the large peak at 48 minutes (**4**).

4.9.2 Analysis of stem peptides in MurT/CobQ-depleted cells

The peptide composition of the isolated peptidoglycan was also analysed by mass spectroscopy using orbitrap. A set of mono-, di-, and trimer peptides, containing glutamate, isoglutamine or both, were the targets of the analysis. The analysis was done externally at the core facility for MS/Proteomics at Campus Ås, IKBM, NMBU. No trimers were detected, which could be attributed to limitations of the method. The mono- and dimer composition of MurT/CobQ-depleted OH8 and RH1 is presented in figure 4.9.3. The analysis showed a small amount of glutamate-containing peptides in the cell wall of RH1, as had been previously demonstrated (Bui et al. 2012). Interestingly, the amount of glutamate containing stem peptides in the MurT/CobQ-depleted OH8 peptidoglycan was drastically increased in comparison. Of particular interest is the fact that it appears that the bacterium is able to utilize non-amidated lipid II as substrate and successfully cross-link the stem peptides. The largest fraction of cross-linked stem peptides containing glutamate was in the form of one amidated and one non-amidated peptide chain joined together. A small portion (3.5-13.2%) of the cross-linked stem peptides was made up of two glutamate containing peptide chains.

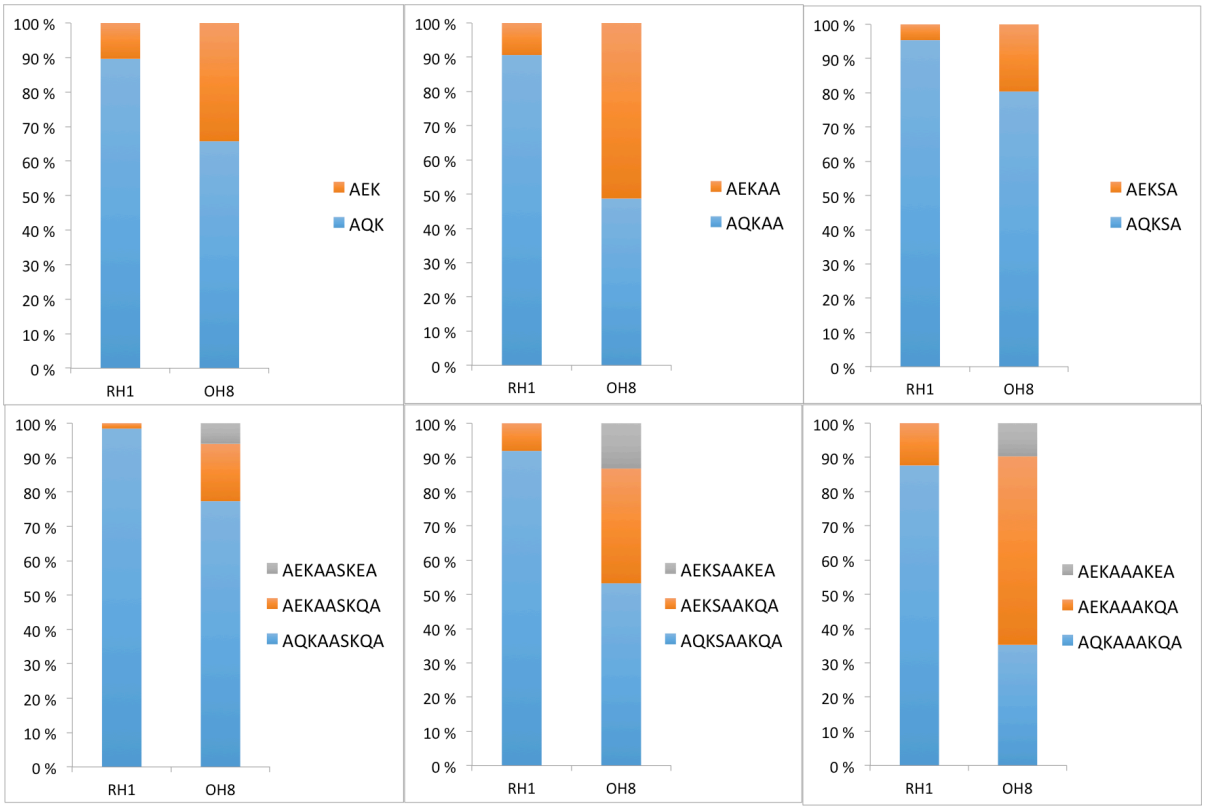

Figure 4.9.3: Stem peptide composition of the pneumococcal cell wall. Relative stem peptide composition of RH1 and MurT/CobQ-depleted OH8 derived from orbitrap data. The top three diagrams are the monomers detected, while the bottom three diagrams show the dimers. No trimers were detected.

5 Discussion

5.1 The effects of MurT/CobQ depletion on general growth in pneumococci

As expected, depletion of *murTcobQ* proved lethal in *S. pneumoniae* when the expression level reached a critical low point, as had been shown previously (Zapun et al. 2013). While Zapun and colleagues used the Zn^{2+} system to do preliminary research on the *murTcobQ* operon, the ComRS system has been used in this work. The main drawback of using the Zn^{+2} system is that zinc plays an important role in pneumococcal physiology, and high levels can potentially affect a number of gene products (Dintilhac et al. 1997, Kloosterman et al. 2008, Eberhardt et al. 2009). Utilizing the ComRS system is likely to interfere less with regular cell function.

The depletion mutants all grew normally in the presence of ComS*, but growth was inhibited in the absence of ComS*. When depleting gene expression of *murTcobQ* using 2-fold dilution series of cell culture, the 4^{th} dilution and onward showed clear growth inhibition as a consequence of decreased MurT/CobQ. Previous studies have shown that the rate of depletion dependents on the genes being studied. This is because different transcripts and proteins will have different half-lives in the cell, and because some proteins are required at very small amounts, while others must be vastly expressed for the cells to survive (Berg et al. 2011, Berg et al. 2013). In certain cases in this work it was more preferable to have the cells expressing a suboptimal, but constant level of MurT/CobQ to have better control over growth rate. This proved more challenging then first anticipated, as the window between reduced growth and no growth was quite narrow (within a few nM of ComS* inducer). The original target was cells that grew to OD_{550} ~ 0.8-1.0, but at slower growth rate. This turned out not to be possible, as the cell cultures all grew at a normal rates to reach this OD when exposed to suboptimal ComS* concentrations. Because of this, a new target OD_{550} of ~ 0.3-0.5 was chosen, since OH8 cultures underexpressing *murTcobQ* reaching these densities showed an obvious decrease in growth rate when compared to OH8 induced with ComS*.

The method for growing the cells in suboptimal ComS* concentrations described in 3.7.2 and 3.7.3 is crude, and it is possible that slightly altering the amount of inoculum would lead to different suitable suboptimal ComS* concentrations. Moreover it was observed that if the

depletion strains were diluted and grown in suboptimal ComS* concentrations three times, as opposed to the two times used, the bacteria were not able to survive a 4th dilution. This indicates that the suboptimal ComS* concentrations are in fact just prolonging the depletion of *murTcobQ* expression, and do not reflect stable suboptimal concentrations. Nonetheless, strictly following the protocol described in section 3.7.3 was used as it represented the best alternative.

5.2 The effects of MurT/CobQ depletion on antibiotic sensitivity

The MurT/CobQ complex was first identified and described in the human commensal and opportunistic pathogen *Stapylococcus aureus* (Figueiredo et al. 2012). *S. aureus* is generally not considered to be a naturally transformable bacterium, although recent evidence has come to challenge this view (Fagerlund et al. 2014). The bacterium has shown a remarkable ability to gain antibiotic resistance trough mutations in target proteins, and multi-drug resistant *S. aureus* (MRSA) has become a major health concern in hospitals world wide, with reports of some strains being resistant to most β-lactam antibiotics in use today. In a study published by Münch et al. in 2012 where the MurT/CobQ enzyme complex was studied in detail, it was reported that gene depletion leads to a regained sensitivity against β-lactam antibiotics in resistant *S. aureus* strains (Munch et al. 2012). It was therefore of interest to examine whether depletion of MurT/CobQ in *S. pneumoniae* would lead to an increase in sensitivity.

Ampicillin and Cefotaxime were chosen because they are among the first choices in the treatment of pneumococcal infections (FDA 2009, WHO 2013). Both are β-lactams, meaning that they bind irreversibly to PBPs, inhibiting peptidoglycan synthesis. Even when fairly stressed by MurT/CobQ depletion, i.e. not being able to reach an OD_{492} >0.5, there was no observable increase or decrease in MIC β–lactam sensitivity based on the growth curves. MIC strip tests were also carried out, but finding a suitable ComS* concentration when doing the assay on a solid medium proved difficult within the limited time frame of this work, and were thus abandoned in favour of microtiter assays. The MIC did not differ between the control strain, non-depleted OH8 cells and MurT/CobQ-depleted OH8 cells in the assays done in this work. Given the fact that the depletion of MurT/CobQ is detrimental to cell growth, one could expect an additive effect when treating the cells with these antibiotics at the same time. Why this is not the case is unclear, although it appears that sub-lethal

concentrations of both ampicillin and cefotaxime is more effective with MurT/CobQ-depleted cells in reducing the maximum optical density of the culture.

5.3 Lysozyme resistance in MurT/CobQ-depleted pneumococci

S. pneumoniae is one of a few known bacterial species to be completely resistant to lysozyme, and being able to avoid lysis in its host is an important contributing factor to its success in colonizing human hosts (Bera et al. 2005, Davis et al. 2008). Lysozyme is a major component of the mucous and saliva of humans and other mammals, and plays a key role in the innate immune system as it hydrolyses glycosidic bonds in the peptidoglycan of bacteria (Davis et al. 2008). The major contributing factor of lysozyme resistance in *S. pneumoniae* has been attributed to modifications of the glycan backbone of peptidoglycan. In particular, two proteins have been identified in contributing lysozyme resistance: PgdA and Adr. PgdA is an N-acetylglucoseamine deacetylase and Adr is an O-acetyl transferase. The former is responsible for the deacetylation of 40-80% of Glc*N*Ac and 10% of Mur*N*Ac residues, while the latter is responsible for the acetylation of Mur*N*Ac residues (Vollmer and Tomasz 2000, Crisostomo et al. 2006).

Even though these are modifications of the glycan chains of the peptidoglycan, it was of interest to test whether the lack of amidation of lipid II could affect the level of peptidoglycan acetylation in a way that would trigger sensitivity to lysozyme. If inhibition of stem peptide amidation could have such a side effect, it would certainly be an interesting observation when considering MurT/CobQ as a possible antibiotic target. However, as there was no observable difference during the exponential growth phase with the MurT/CobQ-depleted cells when grown with lysozyme, it can with a high degree of certainty be concluded the amidation does not influence lysozyme resistance. In this work lysozyme from chicken egg was used, which shows a very high degree sequence similarity with human lysozyme. Nevertheless it cannot be excluded that there might be a difference between these two enzymes in their ability to lyse MurT/CobQ-depleted cells, and so this should be tested in the future.

The cells grown with lysozyme (both the control-strain and the depletion-strain) did however show a sharper decline in optical density when they reached stationary phase compared with the cells grown without lysozyme. This is most likely caused by release of the major

autolysin LytA as the cells autolyse, resulting in a chain reaction. It is quite possible the cell wall of stationary phase cells is less resistant to lysozyme than exponentially growing cells. If so, it is reasonable to think that lysozyme does damage to the cell wall of stationary phase cells, which results in an earlier leakage of LytA. LytA is a highly potent cell wall hydrolase that rapidly lyse stationary phase cells (Tomasz et al. 1988). Perhaps lysozyme then is able to contribute to the lysis of already dying cells in concert with LytA, gaining access to previously inaccessible parts of the peptidoglycan.

5.4 MurT/CobQ depletion in a Δ*pbp1a* or Δ*pbp2a* background

Previous research has shown that *in vitro* PBP1a has some residual transpeptidase activity when using glutamate-containing lipid II, while PBP2a does not (Zapun et al. 2013). The previously mentioned observation (1.4.4) that a small amount of the stem peptides in the pneumococcal cell wall contains glutamate instead of glutamine might be due to this residual activity. If this residual activity of PBP1a is of any biological significance, and what role it may play is not currently known. The study by Zapun et al. was done purely with recombinant PBPs *in vitro*, and so it was of interest to see whether this activity could be distinguished *in vivo* by depletion of *murTcobQ* expression. PBP1a and PBP2a are both non-essential individually, but the deletion of both is lethal to *S. pneumoniae*, indicating some overlap in activity (Zapun et al. 2013). As can be seen in figure 4.1.1 deletion of *pbp1a* is more detrimental to growth than the deletion of *pbp*2a. Thus if the residual PBP1a activity with glutamate-containing lipid II was to be assessed *in vivo* one would expect to see an increase in sensitivity to depletion of MurT/CobQ in the Δ*pbp1a* mutant compared to the Δ*pbp2a* mutant. That is, the Δ*pbp1a* mutant would be more severely affected, as it is not able to use the non-amidated lipid II.

However, this was not observed in the microtiter assays carried out in this work. There might be several reasons for this: a) the conditions *in vivo* and *in vitro* are not the same, and thus the previously reported residual activity might not be valid in a living pneumococcal cell, b) the residual activity might be too low to be detectable or c) the method chosen is not applicable for this purpose. The results in this work proved inconclusive and further work should be done in order to draw any conclusions. One method that might provide better answers is to

analyse the stem peptide composition in cells depleted of MurT/CobQ in a Δ*pbp1a* or Δ*pbp2a* background. Because of time constraints, this was not made a priority in this work.

5.5 CbpD and MurT/CobQ depletion in *S. pneumoniae*

The muralytic hydrolase CbpD is one of the key components of competence-induced fratricide in pneumococcus. CbpD lyse neighbouring non-competent cells while the competent cells are immune against CbpD due to production of the fratricide immunity protein ComM (Eldholm et al. 2009). Experimental data has shown that CbpD needs a dual cell envelope to efficiently kill target cells, and that the choline-binding domain (CBD) acts as a localization target directing the enzyme to the septal region of the pneumococcal cell wall (Eldholm et al. 2010). The two SH3b domains have been established to specifically recognize and bind peptidoglycan. By binding to the septal region during daughter cell separation, the protein lyses cells by hydrolysis of the peptidoglycan cell wall stem peptides. The mechanism with which ComM protects the cells against CbpD is not known, but it is predicted to be a polytopic integral membrane protein (Håvarstein et al. 2006). The only homologs of ComM (based on peptide sequence) are in other closely related *Streptococcus* species in the mitis group, where it serves the same function. Without any similar proteins in the online databases, bioinformatics prediction of mechanism is difficult. As CbpD is an amidase, it has been suggested that ComM might be responsible for a change in the composition or a modification of the cell wall stem peptides, either directly or by activating other proteins (D. Straume, pers. comm.). There is currently work being done in our lab on the localization of ComM, but so far the results have been inconclusive (not published).

MurT/CobQ was one of the more interesting candidates as a potential target of ComM, as local inhibition of this MurT/CobQ in the septal region could lead to a change in peptide composition resulting in the CbpD immunity observed in competent cells. Zymography of MurT/CobQ-depleted cells showed no lysis by CbpD, indicating a possible immunity mechanism (see 4.6). Further more, the cells became less sensitive to CbpD when depleting MurT/CobQ in a Δ*comM*-background (see 4.7). The relative fluorescence was almost half that of the control in the most heavily depleted cells in the microtiter assay. Although this at first might seem like indications of a link between ComM and MurT/CobQ, the explanation probably lies elsewhere: in the inability of the cells to successfully form the septal cross wall. As was observed using TEM and SEM, the MurT/CobQ-depleted cells showed several

invaginations present in abnormally elongated cells that did not lead to complete septal formations. This means that CbpD is unable to bind properly to its target via its choline binding domain and two SH3b domains, and the apparent immunity is likely a result of this and not because of an inability of the catalytic domain to lyse the because of a change in the substrate. When doing microscopy, both apparently healthy and obviously sick cells were observed in the MurT/CobQ-depleted cell cultures. This is to be expected as depletion by removal of ComS* is not an all or nothing approach, but gradual and thus some will deplete faster than others. Because of this we still see lysis in the microtiter assays (see figure 4.7.2) as not all cells are in the same state of MurT/CobQ-depletion.

5.6 The effects of MurT/CobQ depletion on cell morphology and stem peptide composition in *S. pneumoniae*

What parts of the pneumococcal cell wall would be hit hardest by depletion of MurT/CobQ was unknown prior to this work. The fact that the septal and peripheral cell wall synthesising machineries are composed of different sets of proteins, made it possible that a lack of amidated lipid II could affect them in different ways. As shown in 4.8, microscopy revealed that daughter cell separation was severally affected by MurT/CobQ depletion. It appears that a lack of amidated lipid II more or less completely inhibits the septal cell wall synthesis in pneumococcus, with several immature/aborted septa existing in severely elongated cells. It has been shown that PBP2x and PBP2b localize differently during the mid to later stages of cell division, with PBP2x being part of the septal machinery and PBP2b in the peripheral machinery (Berg et al. 2013, Tsui et al. 2014). Inhibition of PBP2x with antibiotics has shown to give elongated cells, which is consistent with inhibition of the septal peptidoglycan synthesis (Perez-Nunez et al. 2011). Depletion of PBP2b gives rounded cells growing in chains, supporting the current model that it is part of the peripheral peptidoglycan synthesis machinery (Berg et al. 2013). Based on this it is likely that septal peptidoglycan synthesis is inhibited by the inability of PBP2x to utilize non-amidated lipid II. Whether PBP2b is able to use non-amidated lipid II *in vivo* remains unclear, but *in vitro* studies have shown that it is not able to do so (Zapun et al. 2013).

As previously mentioned, PBP1a has residual transpeptidase activity with non-amidated lipid II *in vitro*, something which proved difficult do demonstrate *in vivo* (4.2). PBP1a has been

shown to be part of both the peripheral and septal machineries in *Bacillus subtilis* (Claessen et al. 2008), and is postulated to be part of both complexes in *S. pneumoniae* as well (Sham et al. 2012). As shown in 4.9.2 the cells are able to use non-amidated lipid II as substrate, and incorporates it into the cell wall to a greater extent when MurT/CobQ is depleted. Whether this is mainly due to the activity of PBP1a or if other PBPs also are responsible can't be determined by the data collected in this work. One possible way to study this would be to analyse the stem peptide composition of pneumococcal cells depleted of MurT/CobQ in a Δ*pbp1a* background, and then examine to what extent the amount of glutamate-containing stem peptides decrease.

When analysing the stem peptide composition of the control strain and the MurT/CobQ-depleted strain using orbitrap no internal standard was used. This means that the data derived only shows the relative amount of amidated and non-amidated stem peptides within the samples, and no quantitative comparison between the two strains could be made. It is likely that the peptide composition changes in other ways than just the amidation pattern, as for example in the relative amount of the different peptides shown in figure 1.4.5, and is something that should be further explored. This might be one of the explanations for the vastly different HPLC chromatograms from these two strains (figures 4.9.1 and 4.9.2). There is also a possibility that the specificity of LytA might change when the amidation pattern changes, and this can't be excluded as an explanation as to why the HPLC chromatograms differ so.

No trimers were detected in either the control or the *murTcobQ* depletion mutant, most likely because of limitations of the method chosen. And so no conclusions can be drawn as to whether there also are glutamate-containing trimers when depleting MurT/CobQ, and to what extent depletion affects the cells ability to form trimers. Refining the analytical method could potentially give an answer in the future. The most interesting peaks from the chromatograms were collected, but for some as of yet unknown reason did not yield any usable data when analysed by Orbitrap. This might be because the peptides were too small to bind properly to the column prior to mass spectrometry. Nonetheless, this is something that should be further explored in the future, as this will show what the major changes in peptide composition are when depleting MurT/CobQ. Ideally, all well separated peaks from the HPLC chromatograms should be analysed, but this was unfortunately beyond the scope of this work.

6 Concluding remarks

In this work, the essential operon *murTcobQ* has been studied extensively to gain further insight into an important part of pneumococcal biology, namely cell wall synthesis. *S. pneumoniae* is a major human pathogen responsible for over 1 million annual deaths worldwide, and with antibiotic resistance on the rise, the need for new ways to treat these infections is of utmost importance (van der Poll and Opal 2009, Croucher et al. 2011, Hackel et al. 2013). Both basic and applied research can contribute to the development of new treatments, as well as give us important insight into bacterial biology.

The main findings presented in this thesis are the ways in which critically low levels of MurT/CobQ affects growth, morphology and composition of stem peptides in the peptidoglycan in the pneumococcus. The proteins MurT and CobQ are only found in closely related Gram-positive genera like *Staphylococcus, Enterococcus, and Lactococcus*, and why the PBPs in these bacteria are dependent on the amidated form of lipid II to be able to use it as a substrate for peptidoglycan synthesis remains unclear. What is shown in this work is that pneumococci are to some extent able to utilize the non-amidated form of lipid II as a substrate, and successfully cross-link the stem peptides during peptidoglycan synthesis. What PBPs are responsible for this activity is unknown, but *in vitro* synthesis has shown that only PBP1a showed residual transpeptidase activity with non-amidated lipid II (Zapun et al. 2013). The septal cell wall synthesis appears to be hit hardest by the lack of amidated lipid II, indicating that there is some difference in the PBPs of the septal and peripheral cell wall synthesising machineries in their ability to utilize the non-amidated lipid II.

Further work on the MurT/CobQ enzyme complex will be carried out at the Molecular Microbiology group at NMBU, in particular more detailed analysis of the peptide composition when underexpressing the enzyme complex. Also, separate analysis of the stem peptide composition of Δ*pbp1a* and Δ*pbp2a* mutants underexpressing MurT/CobQ is necessary to study whether the observed transpeptidase activity is mostly due to PBP1a.

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Appendix A

Standards

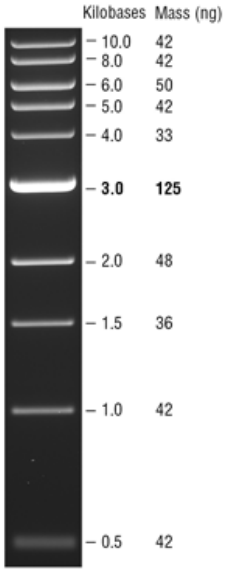

Figure A.1: 1 kb DNA ladder. Useful for estimating size of DNA fragments between 0.5 kbp and 10 kbp. Ladder visualized by ethidium bromide staining on a 0.8% TAE agarose gel. Mass values: 0.5 µg/lane. (Figure from New England Bio Labs, https://www.neb.com/products/n3232-1-kb-dna-ladder).

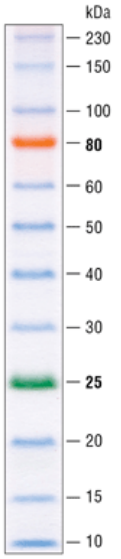

Figure A.2: ColorPlus™ PrestainedProtein Ladder, Broad Range. Used for estimating protein size when doing SDS-PAGE and zymography. Useful for proteins 10-230 kDa in size. Figure from New England Bio Labs (https://www.neb.com/products/p7711-colorplus-prestained-protein-ladder-broad-range-10-230-kda)

Appendix B

murTcobQ sequence:

CTACAAGCAAAAACGTCTTTGACTCAACAGGTATCCAAAGCTTGGATAAATTGCCTGAAAAACTTGGAGTCCTTGGTGGCGG
AAATATCGGTCTTGAATTTGCTGGCCTTTACAATAAACTAGGAAGCAAGGTTACAGTCCTAGATACCTTGGATACATTCCTAC
CTCGTGCAGAACCTTCCATCGCAGCTCTTGCTAAACAATACCTGGAAGAAGACGGTATTGAATTGCTTCAAAATATCCATACT
ACTGAAATCAAAAACGATGGTGACCAAGTGCTTGTCGTAACTGAAGACGAAACTTACCGTTTCGACGCCCTTCTCTACGCAA
CTGGACGCAAACCAAATGTAGAACCACTTCAACTTGAAAATACAGATATTGAACTAACTGAACGTGGTGCTATTAAAGTAGA
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TGACTGCTCATGAAGTGGACCGCGCTGCCCGTGAGGTCATCGAAAAAGCTGGTTATGGTGAGTACTTCAACCACCGTCTCGG

The DNA sequence of the *murTcobQ* operon, including 1kb of the upstream and downstream regions. The *murT* coding sequence is indicated in yellow, while *cobQ* coding sequence is indicated in green. The Stop codon of *murT* (TAA) shares one overlapping base with the start codon of *cobQ* (ATG). The start codons are indicated in blue, while the stop codons are indicated in red.

Appendix C

Additional TEM and SEM micrographs

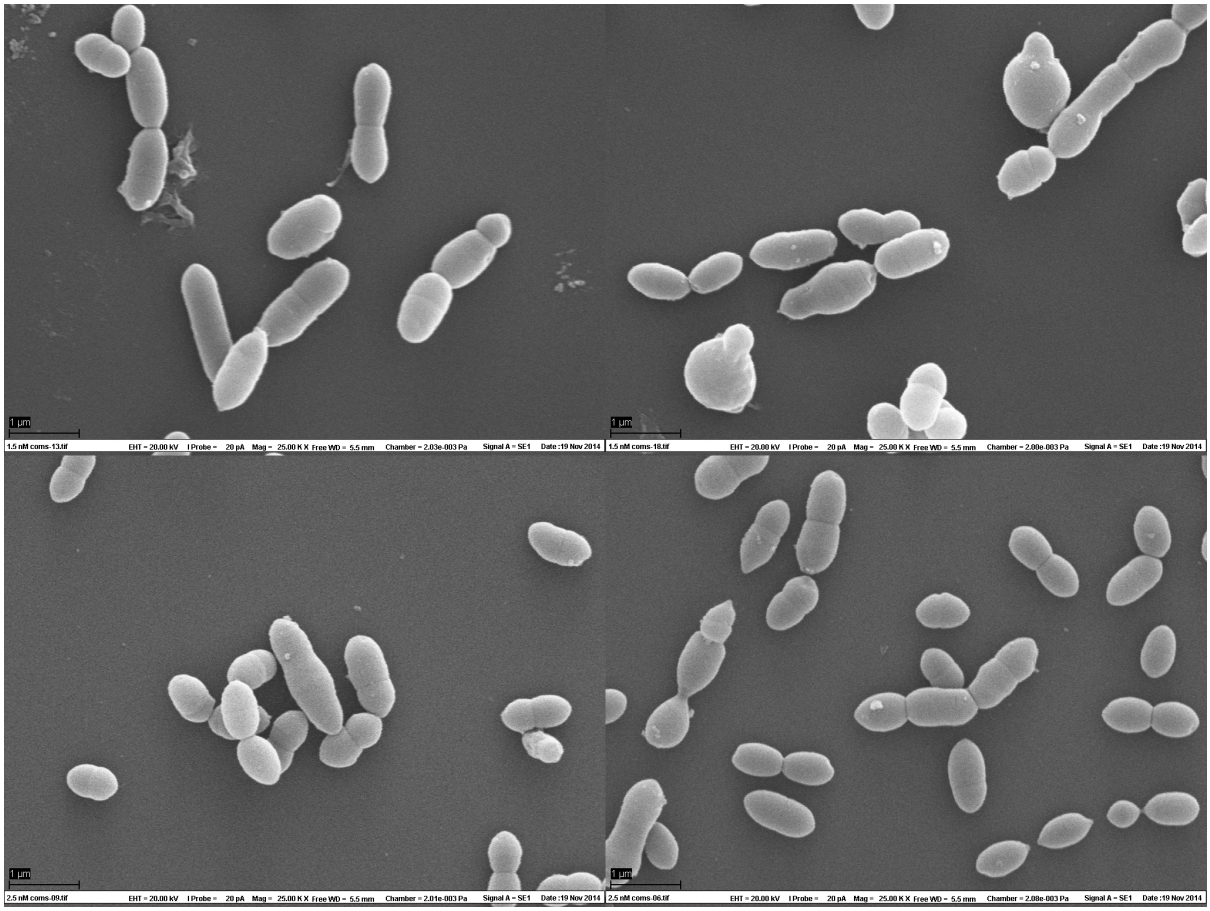


Figure C.1: Additional SEM micrographs of *murTcobQ*-depleted OH8 cells. In addition to grossly elongated cells, pear-shaped and other malformed cells were observable. The depleted cell cultures consisted of a mixture of normal looking cells and sick cells.

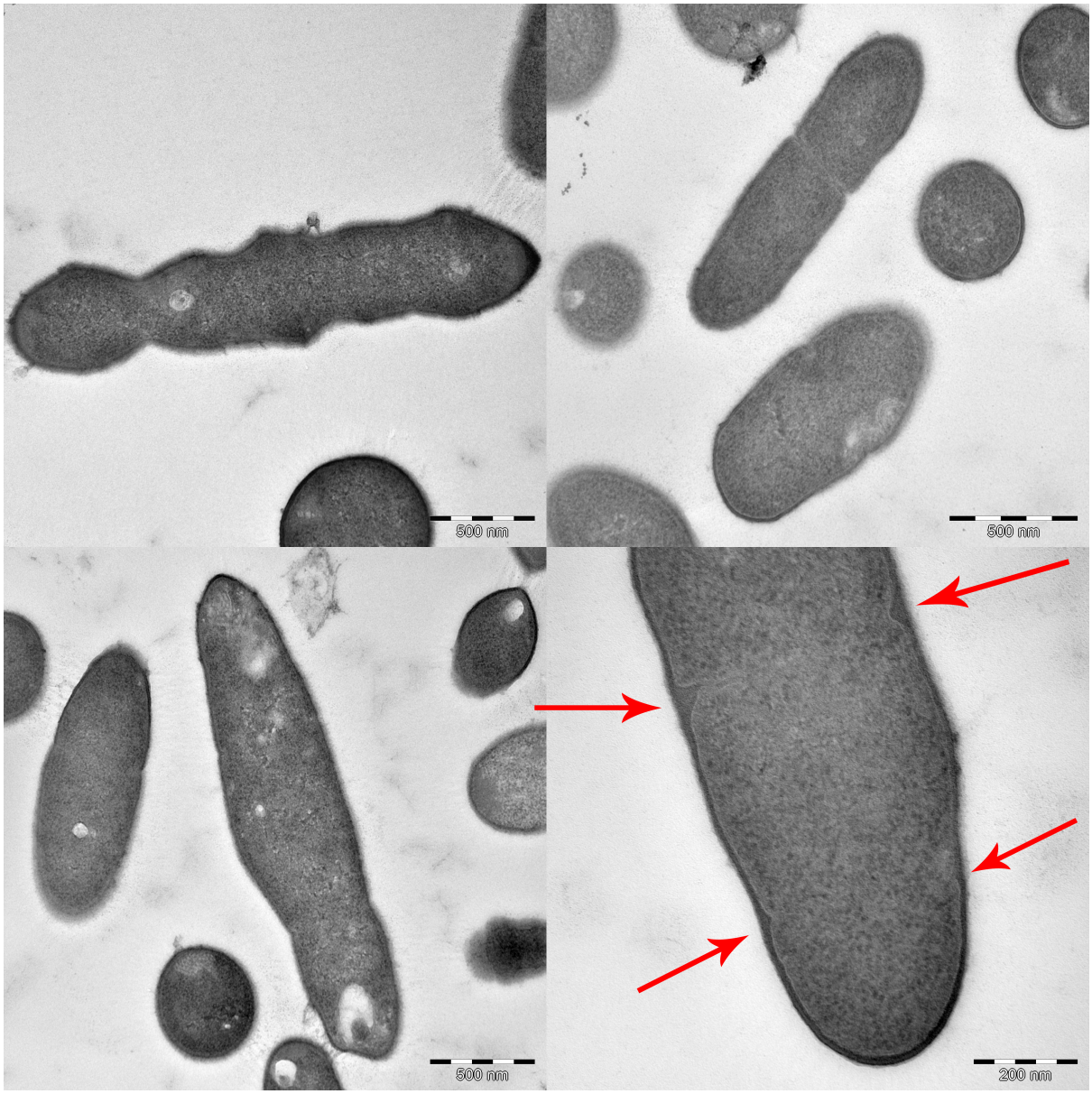


Figure C.2: Additional SEM micrographs of *murTcobQ*-depleted OH8 cells. The red arrows indicate incomplete septal formations. Note the difference in scale on the bottom right picture.

Appendix D

Additional figures and tables

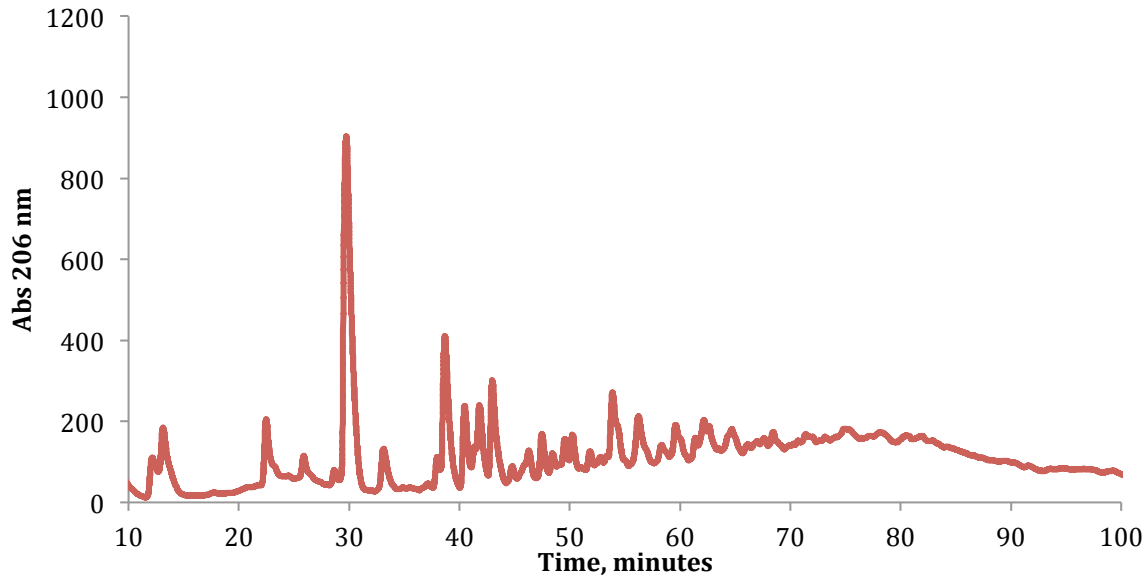
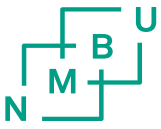


Figure D.1: Stem peptide composition in OH8. HPLC chromatogram of the stem peptide composition in the peptidoglycan of strain OH8, grown in the presence of 0.2 µM ComS*. The isolated peptidoglycan was digested using LytA.

Table D.1: Raw data from orbitrap analysis of pneumococcal stem peptide composition. Isolated peptidoglycan from *murTcobQ*-depleted OH8 cells and RH1 was digested with LytA, and analysed by orbitrap.

	sequence	m/z			max intensity, RH1	max intensity, OH8	rt, RH1/OH8	MS/MS	comment
		z=1	z=2	z=3					
1	AQK	346,2085	173,6079	116,0743	4570000	2820000	12.07/12.09		
	A**E**K	347,1925	174,0999	116,4023	529000	1470000	12.08/12.09		
2	AQKAA	488,2827	244,645	163,4324	4160000	17100000	13.83/14.03	RH1&OH8	
	A**E**KAA	489,2667	245,137	163,7604	429000	18000000	15.07/15.31	OH8	
3	AQKSA	504,2776	252,6424	168,7641	19900000	82400000	13.72/13.74	OH8	
	A**E**KSA	505,2616	253,1344	169,0921	960000	20100000	13.72/13.85		
4	AQKAKQA	744,4362	372,7218	248,8169	800000000	130000000	13.72/13.74	RH1&OH8	base peak

									(100-1000)
	A**E**KAKQA	745,4202	373,2138	249,1449	152000000	?	14.01/?	RH1	
	A**E**KAK**E**A	746,4043	373,7058	249,4729	?	434000	?/15.31		
5	AQKAASKQA	902,5054	451,7563	301,5066	617000000	213000000	14.83/15.08	RH1&OH8	base peak (100-1000)
	A**E**KAASKQA	903,4894	452,2483	301,8346	9830000	46000000	15.71/15.95	OH8	
	A**E**KAASK**E**A	904,4734	452,7403	302,1626	?	16500000	?/18.44	OH8	coeluting with isobar (6a)
6a	AQKSAAKQA	902,5054	451,7563	301,5066	430000000	66400000	15.24/15.63	RH1&OH8	
	A**E**KSAAKQA	903,4894	452,2483	301,8346	37800000	41800000	16.54/16.55	RH1&OH8	
	A**E**KSAAK**E**A	904,4734	452,7403	302,1626	?	16500000	?/18.44	OH8	coeluting with isobar (5)
6b	AQKAAAKQA	886,5105	443,7589	296,175	105000000	304000000	16.34/16.40	RH1&OH8	base peak (100-1000)
	A**E**KAAAKQA	887,4945	444,2509	296,503	148000000	475000000	18.52/18.08	RH1&OH8	
	A**E**KAAAK**E**A	888,4785	444,7429	296,831	?	83700000	?(20.30	OH8	
7	AQKSAAASKQA	1060,5745	530,7909	354,1964					
	A**E**KSAAASKQA	1061,5585	531,2829	354,5244					
	A**E**KSAAASK**E**A	1062,5425	531,7749	354,8524					
8	AQKAASAQKAKQA	1300,7331	650,8702	434,2492					
	A**E**KAASAQKAKQA	1301,7171	651,3622	434,5772					
	A**E**KAASA**E**KAKQA	1302,7012	651,8542	434,9052					
	A**E**KAASA**E**KAK**E**A	1303,6852	652,3462	435,2332					
9	AQKAASAQKAASKQA	1458,8023	729,9048	486,9389					
	A**E**KAASAQKAASKQA	1459,7863	730,3968	487,2669					
	A**E**KAASA**E**KAASKQA	1460,7703	730,8888	487,5949					
	A**E**KAASA**E**KAASK**E**A	1461,7543	731,3808	487,9229					



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