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# Characterization of the penicillin-binding proteins in *Streptococcus oralis* Uo5

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Biotechnology

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**Silja Kavlie Fykse**

**Ås, May 2017**

## Abstract

In the search for new antibiotics the mechanism behind cell division has been studied for decades. The extensive use of antibiotics has led to the development of resistant bacteria, a problem that has become a global threat. Obtaining a deeper understanding of cell division is essential in the battle against bacteria. In a Gram positive bacteria the cell wall mainly consists of peptidoglycan, which shapes the cell and withstands turgor pressure. The penicillin-binding proteins (PBP) are responsible of generating and cross-linking the glycan strands. The strain investigated is *Streptococcus oralis* Uo5. The strain has acquired several low-affinity PBPs, which is one of the reasons why Uo5 has gained high resistance levels to penicillin- and cefotaxime. The aim of the study was to characterise the properties of low-affinity PBPs in the high-resistant strain Uo5. The commensal *Streptococcus oralis* is closely related to the opportunistic pathogen *Streptococcus pneumoniae*, and their genes are often referred to as mosaic genes, caused by horizontal gene transfer.

The PBPs were removed using constructed Janus cassettes, through natural transformation. The growth of all constructed mutants was investigated using a microtiter plate, and β-lactam resistance was determined by using E-test for three different β-lactams. The morphology of the cells was investigated using light microscope, and Bocillin FL assay was executed for detecting active PBPs. One of the main findings in this study suggests that it is possible to remove PBP2b in *S. oralis* Uo5, which is an essential protein in pneumococcus. The morphology and growth results have however indicated the importance of the low affinity to PBP2b in *S. oralis* Uo5. Removing *pbp2b* resulted in decreased resistance in all three tested β-lactams, indicating that some of the high resistance in *S. oralis* Uo5 is caused by the altered PBP2b. From the results PBP1b and PBP2a seem to be much more sensitive to β-lactams than PBP1a, PBP2x and PBP2b, and contribute little to the overall resistance of the Uo5 strain against Penicillin G, Amoxicillin and Oxacillin. Other important findings were that the deletion of PBP1a was only possible when PBP1b was removed, and the Bocillin FL assay also showed no interaction with PBP1a when PBP1b was removed. The best way to continue this study would be to subject all the 10 mutants to whole genome sequencing in order to verify that they are correctly made and to identify and characterize possible suppressor mutations.

## Sammendrag

Mekanismene bak celledeling har gjennom flere tiår blitt studert, i håp om å finne nye antibiotika. Overdreven bruk av antibiotika har ført til utvikling av resistente bakterier, dette er et problem som har eskalert til å bli en global trussel. Å oppnå en dypere forståelse av celledeling er essensielt for kampen mot resistente bakterier. I en Gram-positiv bakterie er celleveggen hovedsakelig bygget opp av peptidoglykan, som former cellen og motstår osmotisk trykk. De penicillin-bindende proteinene (PBP) er ansvarlige for å generere og kryssbinde glykantrådene. I dette studiet ble stammen *Streptococcus oralis* Uo5 undersøkt. Stammen har ervervet flere lav affinitets PBPer, og har høy resistens mot penicillin og cefotaxime. Målet med studiet var å karakterisere egenskapene av lav affinitets PBPer i denne resistente *S. oralis* Uo5 stammen. Den kommensale *S. oralis* er nært beslektet med den opportunistiske patogene *Streptococcus pneumoniae*. Genene deres blir ofte kalt for mosaikk gener, som er forårsaket av horisontal genoverføring.

*Pbp* gener ble fjernet ved bruk av konstruerte Janus kassetter, gjennom naturlig transformasjon, og det ble totalt laget . Veksten av alle de konstruerte mutantene ble undersøkt ved bruk av en mikrotiterplate, og β-laktam resistens ble bestemt ved å bruke E-test for tre forskjellige β-laktamer. Morfologien av cellene ble undersøkt ved bruk av lysmikroskop og Bocillin FL assay ble utført for å oppdage aktive PBPer. Et av hovedfunnene i dette studiet antyder at det er mulig å fjerne PBP2b i *S. oralis* Uo5, som er et essensielt protein i pneumokokker. Morfologien og vekst funnene har derimot indikert viktighetsgraden av den lave affiniteten til PBP2B i Uo5. Fjerning av *pbp2b* resulterte i lavere resistens i de tre testede β-laktamene, som indikerer at noe av det høye resistensnivået skyldes den altererte PBP2b. Fra resultatet vises det at PBP1b og PBP2a virker mye mer sensitiv for β-laktamer enn PBP1a, PBP2x og PBP2b, og bidrar derfor lite til den totale resistensen mot Penicillin G, Amoxicillin og Oxacillin. Den beste måten å fortsette dette studiet er ved å genomsekvensere alle de 10 lagde mutantene. Dette vil bekrefte om riktig mutant virkelig er laget, og identifisere og karakterisere mulige suppressormutasjoner.

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# 1. Introduction

## 1.1 *Streptococcus oralis*

*Streptococcus oralis* belongs to the phylum Firmicutes, class *Bacilli*, order *Lactobacillales* and genus *Streptococcus* (Garitty et al. 2004; Salvetti et al. 2013). *S. oralis* is part of the mitis phylogenetic group, which also includes *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus parasanguis*, *Streptococcus pneumoniae* and *Streptococcus sanguis*, displayed in figure 1.1 (Reichmann et al. 2011).

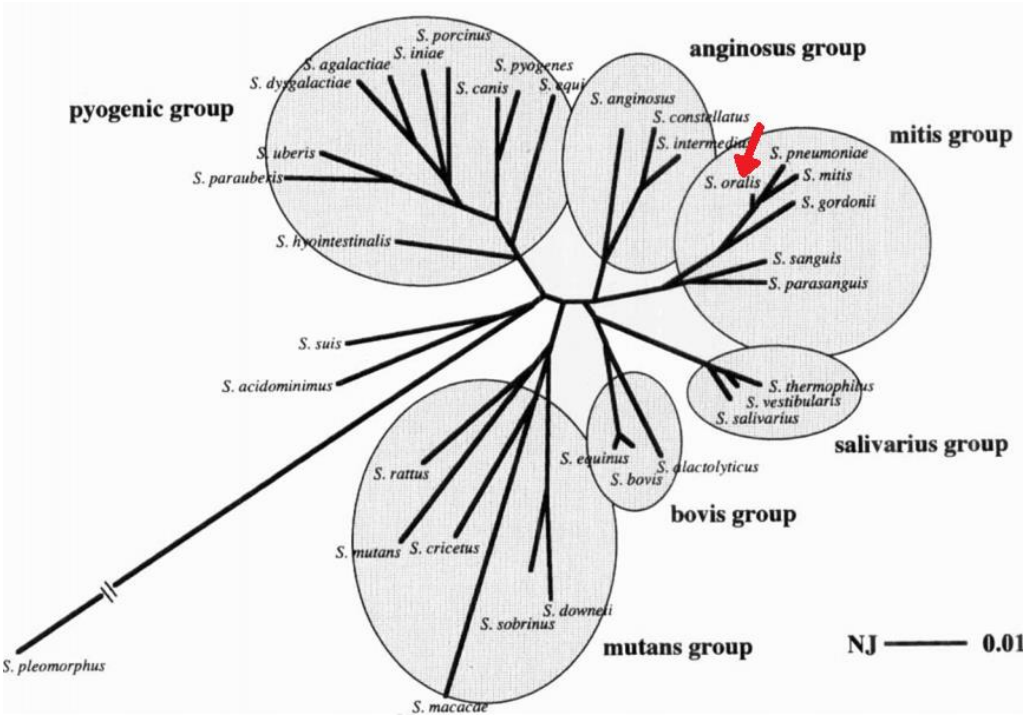


**Figure 1.1 Division of the genus Streptococcus.** The genus is divided into 6 phylogenetic groups, pyogenic, anginosus, mitis, salivarius, bovis and mutans. The distance between species in the neighbour joining tree estimated using 16S rRNA. *S. oralis* is highlighted by the arrow (Kawamura et al. 1995).

The strain investigated in this study is *S. oralis* Uo5. As it has acquired several low-affinity penicillin-binding proteins by horizontal gene transfer, it has become highly resistant to penicillin and cefotaxime. The Uo5 strain was first isolated in the early 1980s in Hungary (Reichmann et al. 1997: Reichman et al 2011). Members of the species *S. oralis* are commensal bacteria that thrive in the oral cavity of humans. They are Gram-positive nonmotile bacteria, facultative anaerobes, and nonsporing. The morphology of *S. oralis* has been described as

ovoid, growing either as singles, pairs or in short chains, resembling *S. pneumoniae*. (Bergey et al. 2009). The morphology of a typical *S. oralis* is viewed in figure 1. 2

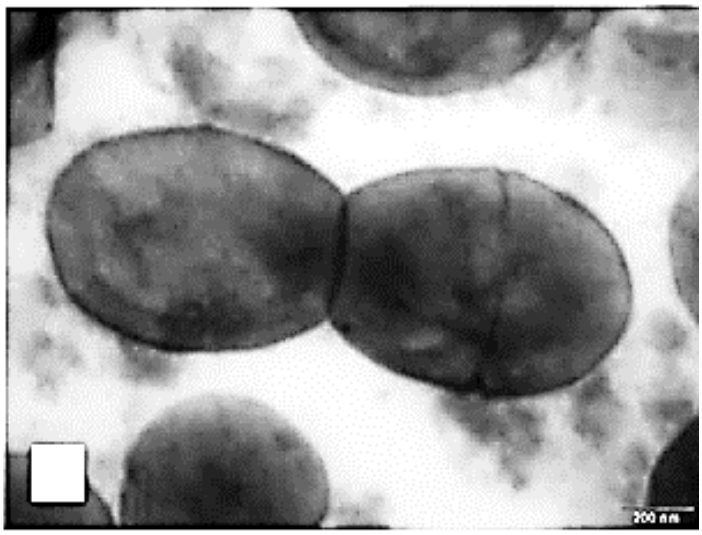


**Figure 1.2 Electron microscope image of *S. oralis,* HCD30**. Scale bar showing 200 nm (Corcuera et al. 2013).

One of *S. oralis'* closest relatives is *S. pneumoniae*, also known as pneumococcus, which has been estimated by the World Health Organization to cause about 1.6 million deaths annually (WHO 2017). Pneumococcus is a major human pathogen that causes diseases such as sinusitis, otitis media, pneumonia, bacteremia and meningitis. It can be transiently carried in the upper airways of healthy hosts (Van der Poll & Opal 2009). *S. mitis* and *S. oralis* are rarely associated with disease, but they are both reported to behave as minor opportunistic pathogens. They may cause inflammation on the inner layer of the heart (endocarditis) and neutropenic bacteremia (Han et al. 2006). Like *S. oralis*, *S. mitis* is also a part of the normal oral human microbiota, and colonizes both the hard surfaces on teeth, and the mucous membranes (Bensing et al. 2001). *S. pneumoniae* colonizes mainly the respiratory tract (Lanie et al. 2007), and is often found in the nasopharynx, the upper region of the throat located behind the nasal airway (Marchisio et al. 2002).

## 1.2 Natural transformation

Natural transformation is a mechanism of horizontal gene transfer that includes uptake, and incorporation of exogenous DNA (Lorenz & Wackernagel 1994). Natural transformation was first discovered in *S. pneumoniae* in 1928 by Frederick Griffith (Griffith 1928), but the regulation and molecular mechanism behind natural transformation remained unknown for many years. In 1995, a 17-residue peptide produced by pneumococcus was discovered, called the competence-stimulating peptide CSP (Håvarstein et al. 1995). For three decades before the discovery of CSP, natural transformation was induced by high cell density, with no knowledge on the underlying mechanism.

Species belonging to the Mitis group are all naturally competent for genetic transformation (Johnsborg et al. 2007). *S. pneumoniae* has been used as a model organism because of this property, its short generation time, and because it is fairly easy to grow. Natural transformation enables the pneumococcus to acquire new traits such as drug resistance and resistance to vaccine-induced immunity. This is done by taking up relevant DNA from the environment, followed by incorporation of this DNA into the genome of the recipient through homologous recombination (Straume et al. 2015; Straume et al. 2016).

CSP induces competence in *S. pneumoniae* by activating a two-component system consisting of the transmembrane histidine kinase ComD and the response regulator ComE. CSP binds to ComD resulting in autophosphorylation of the kinase (Claverys & Håvarstein 2002). The phosphoryl group is transferred to ComE, which in the phosphorylated form activates the transcription of about 20 so called early competence genes. These include comCDE, comM and comX (Ween et al. 1999). The early gene product, ComM, protects the cell against CbpD (Choline-binding protein D), a murein hydrolase expressed during competence. Hence, if competent cells do not express ComM during the competent state, they undergo autolysis. This mechanism is called fratricide (Håvarstein et al. 2005). CbpD is a murein hydrolase, which binds noncovalently to the phosphorylcholine moiety linked to lipoteichoic acids (LTA), and wall teichoic acids (WTA) (Sànchez-Puelles et al. 1990).

The precursor of CSP, ComC, is secreted by the ABC-transporter ComAB. ComC consists of an N-terminal leader peptide, and a C-terminal part corresponding to CSP. The N-terminal leader peptide is cleaved off concomitant with export by a proteolytic domain residing in the N-terminal end of ComA (Biswas et al. 2015; Hui & Morrison 1991). CSP stimulates its own production through an autocatalytic loop consisting of the ComABCDE proteins. Thus,

competence induction leads to secretion of extracellular CSP, which presumably acts as a signal that can be sensed by neighbouring cells. A large diversity of CSPs are produced by different strains and species belonging to the mitis phylogenetic group. Since the ComD receptor is specific for is cognate CSP, streptococci can be divided into different pheromone groups (pherogroups). By definition, members of each pherogroup share and respond to the same CSP, while they are not able to recognize CSPs belonging to other pherogroups (Håvarstein et al. 1997). The protein ComX is an alternative sigma factor that controls the transcription of about 60 late competence genes (Lee & Morrison 1999). The late genes encode the DNA uptake and recombination machinery, plus the fratricin CbpD. CbpD can lyse close related strains, presumably to release homologous DNA that can be taken up by the competent cells (Kausmally et al. 2005). Natural transformation is responsible for the dissemination of virulence and antibiotic resistance genes in streptococci, and gives their genomes a mosaic structure. The mechanism is displayed in figure 1.3 (Johnsborg & Håvarstein 2009).

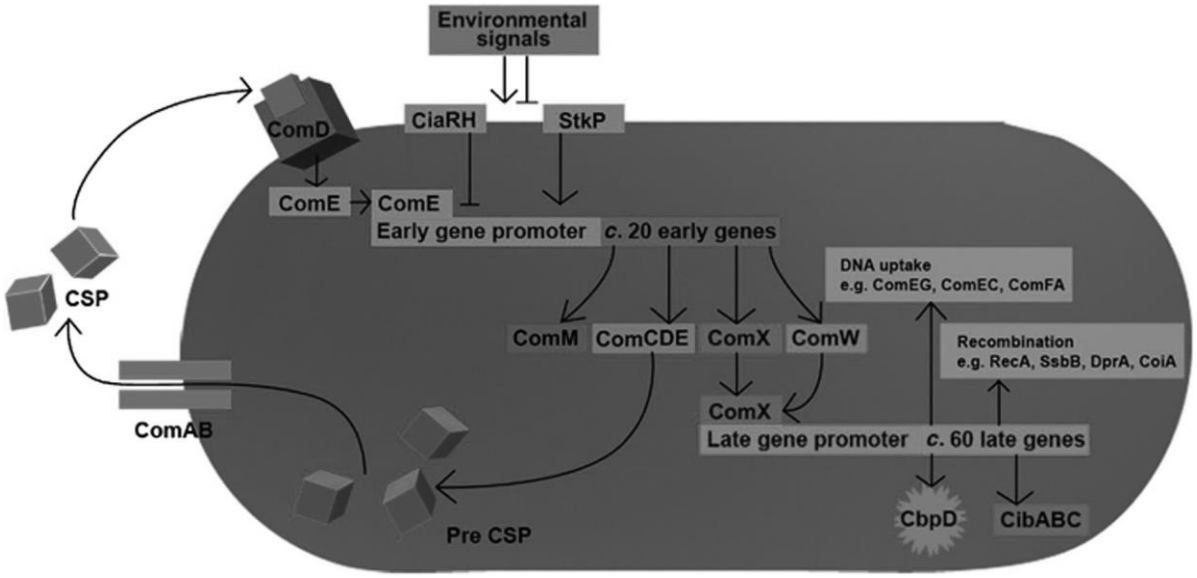


**Figure 1.3 Competence regulation in *S. pneumonia*.** Pre CSP is cleaved and secreted by ComAB. CSP can then bind neighbour cells, or the same cell's ComD, leading to transcription of 20 early genes. Among the early genes are those encoding CSP, fratricide immunity (ComM), and the alternative sigma factor ComX. ComX initiates transcription of about 60 late genes, some of which are responsible for DNA uptake, recombination and (Johnsborg & Håvarstein 2009).

The genome of *S. oralis* Uo5 is sequenced (NC_015291.1), which shows that all the competence genes are present. The synthetized CSP peptide used in this study is based on the *comC* sequence of the Uo5 strain.

## 1.3 Bacterial cell wall

The primary function of the cell wall in bacteria is to protect the cell from its internal turgor pressure and give shape to the cell. The turgor pressure occurs because of a higher protein (and other molecules) concentration inside the cell than in the extracellular environment. Bacteria is characterized as either Gram-positive, or Gram-negative, revealed by a staining method published by Hans Christian Gram in 1884 (Gram 1884). Gram-positive bacteria have a thick peptidoglycan layer outside the cytoplasmic membrane, while Gram-negative only have a thin layer, located between the cytoplasmic and outer membrane (Fig. 1.4)

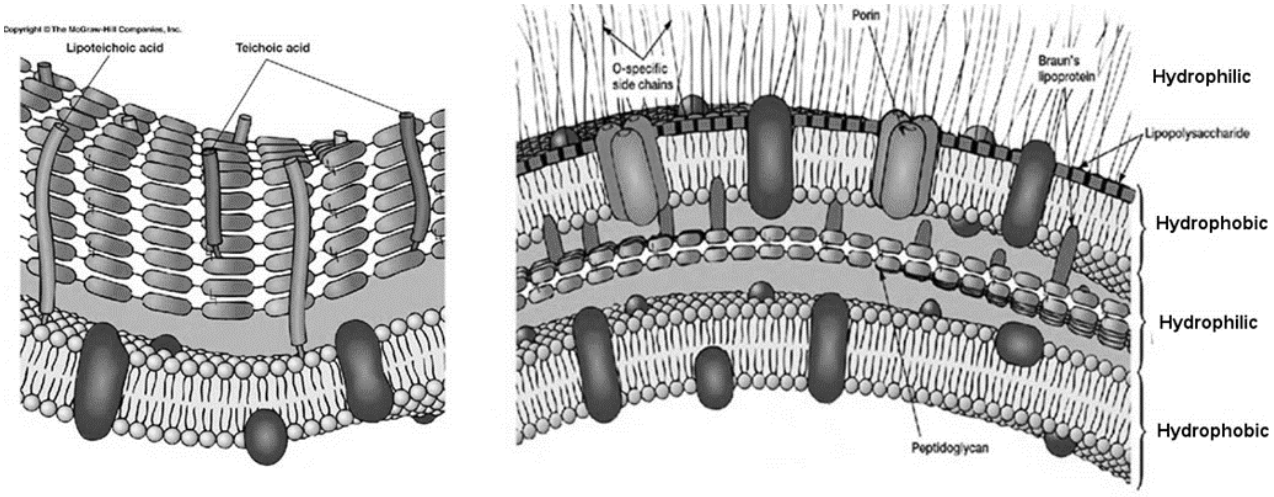


**Figure 1.4 Cell wall composition of Gram-negative and Gram-positive bacteria.** The composition of a Gram-positive cell wall containing a thick peptidoglycan layer, LTA and WTA is shown in the left illustration. The illustration on the right shows a Gram-negative bacterium with a thin peptidoglycan layer located between the cytoplasmic and outer membrane (Prescott et al. 2005).

The peptidoglycan layer (also called murein) is composed of glycan chains cross-linked by short peptides (Fig. 1.5). The glycan chains consists of alternating β-1,4-linked N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG). The muropeptides cross-link the glycan strands as shown in figure 1.6 (5) (Di Guilmi et al. 2003; Turner et al. 2014).

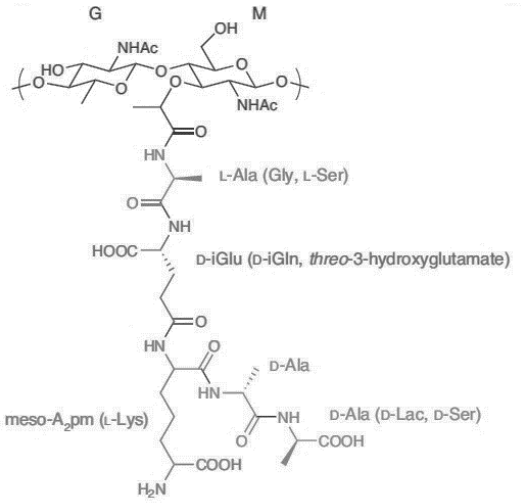


**Figure 1.5 The streptococcal peptidoglycan precursor.** NAG-NAM linked to a pentapeptide (Vollmer 2012).

Analyses of the stem peptide composition show that it varies between streptococcal strains and species. In *S. pneumoniae* linear pentapeptides (L-alanyl-γ-D-glutamyl-L-lysyl-D-alanyl-D-alanine) attached to N-acetylmuramic acid residues on separate glycan strands are cross-linked by formation of a direct bond between L-lysine on one peptide strand and D-alanine on the other. In addition to these directly cross-linked peptide bridges pneumococcal peptidoglycan also contains peptide bridges made from branched muropetides. In these branched muropeptides the ε-amino terminus of L-lysine is substituted by a dipeptide branch consisting of L-alanine or L-serine followed invariably by L-alanine. The sequential addition of L-alanine/L-serine and L-alanine to the ε-amino group of L-lysine takes place at the cytoplasmic side of the membrane and is carried out by MurM and MurN, respectively (Garcia-Bustost et al. 1987; Vollmer et al. 2008) Figure 1.6 shows the five muropeptide types found in the cell wall of the *S. oralis* Uo5 strain (Todorova et al. 2015). For most Gram-positive bacteria like *S. oralis*, lysine localized in position three is linked to alanine in position four. In Gram-negative bacteria lysine is replaced by meso-diaminopimelic acid (Vollmer et al. 2008).

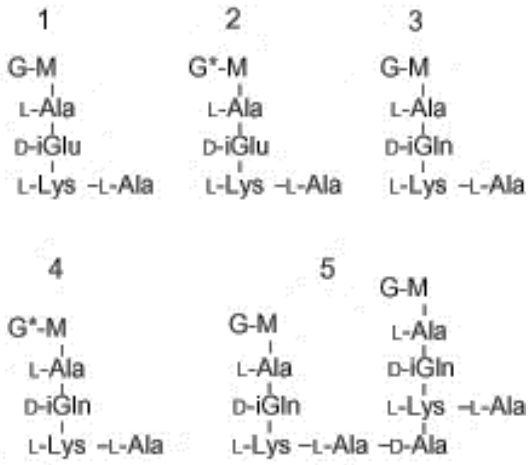


**Figure 1.6 Proposed structures of muropeptide present in the cell wall of *S. oralis* Uo5**. Muropeptide 5 is branched with one alanine attached to lysine instead lysine instead of the serine-alanine or alanine-alanine dipeptide found in *S. pneumoniae*. G stands for NAG, N for NAM, and G* for glucosamine (Todorova et al. 2015).

Other polysaccharides in the Gram-positive cell wall besides peptidoglycan is lipoteichoic acids (LTA) and wall teichoic acids (WTA). WTA is attached to the peptidoglycan, while LTA is anchored to the membrane through a glycolipid (Neuhaus & Baddiley 2003). WTA is hypothesized to recruit cell wall assembly proteins, regulating the peptidoglycan machinery (Brown et al. 2013). The architecture of the peptidoglycan layer surrounding *S. oralis* is similar to that of its close relative *S. pneumoniae*, but analysis of the muropeptide composition of the Uo5 strain revealed a difference which is further described in chapter 1.5. *S. oralis* Uo5 is also reported to have more complex LTAs, but with a high resemblance to pneumococcal LTA (Gisch et al. 2015).

## 1.4 Cell division

For a bacterium to have an ovoid shape like *S. oralis*, it must have both peripheral and septal cell wall synthesis. Synthesis of the cell wall is performed by large multiprotein complexes, which functions as peptidoglycan synthesizing machines (Berg et al. 2013). The multiprotein complex that synthesizes the septal cross-wall, and carries out the cell division is called the divisome (Goehering & Beckwith 2005). The multiprotein complex that synthesizes the lateral cell wall leading to cell elongation is called the elongasome. Although bacterial cell wall synthesis has been studied in various model bacteria for many decades, a lot of uncertainties

and unknowns still exist. It is hoped that a better understanding of the cell division and elongation machineries will lead to the discovery of new targets for antimicrobial drugs.

The cell wall synthesis is described here for *S. pneumoniae*, which is by far the best studied bacterium among the streptococci. It is reasonable to assume that the cell wall synthesis in *S. oralis* is highly similar. At the initiation of the cell division process in *S. pneumoniae*, a tubulin homologue, FtsZ, forms a ring (the Z-ring) at the midcell. FtsZ is the most conserved division protein in bacteria. It polymerizes into a ring structure, which functions as scaffold for the assembly of divisome proteins. The Z-ring is also essential for cell constriction during the division process (Adams & Errington 2009; Harry 2001). A protein termed FtsA tethers FtsZ to the membrane and stabilizes the Z-ring (Egan & Vollmer 2013). Other Z-ring regulators are ZapA, ZapB, EzrA and SepF. Proteins involved in cell division at a later stage includes FtsQ (DivIB), FtsB (DivIC), FtsL, FtsK, FtsW, PBP2x, PBP1a, GpsB, DivIVA, PcsB, LytB. They are mainly responsible for septal peptidoglycan synthesis and cell separation (Massida et al. 2013).

A schematic model is displayed in figure 1.7 with putative localization of the proteins for both the divisome and elongasome. All proteins involved in the septal and peripher cell wall synthesis in an ovoid bacteria like *S. pneumoniae* are located in the septal region of the cell (Scheffers & Pinho 2005) . How the peptidoglycan synthesis is regulated still remains largely unknown, but studies indicate that GpsB is ivolved in coordinating septal and peripheral peptidoglycan synthesis (Lewis 2017). GpsB is shown to form complexes with BPB2b and PBP2a (Rued et al. 2017).

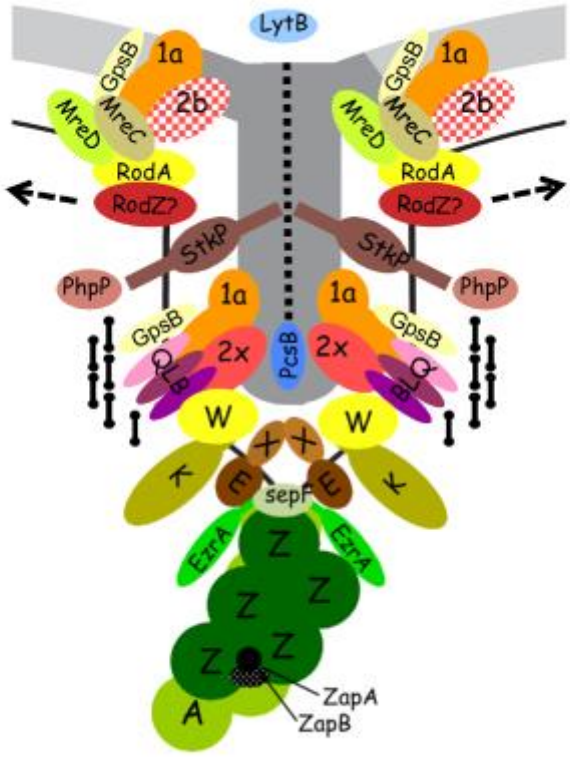


**Figure 1.7 Overview of the peptidoglycan biosynthetic machinery in *S. pneumoniae.*** The dark grey area represents the septal wall which is being synthesized, while the light grey area represents the peripheral wall. The black dashed line represents the splitting of the septal cross-wall by PcsB. The labels 1a, 2b, 2x, Q, L, B, W, X, E, K, Z, A stands for PBP1a, PBP2b, PBP2x, FtsQ, FtsL, FtsB, FtsW, FtsX, FtsE, FtsK, FtsZ, FtsA, respectively. Scale bar shows 0,25 µm (Massidda et al. 2013).

Proteins belonging to the core elongasome are MreC, MreD, RodZ, DivIVA, RodA, PBP2b, PBP1a, and CozE (Spr0777) (Barendt et al. 2011; Fenton et al. 2015; Straume et al. 2017; Tsui et al. 2016). PBP3 (DacA) and DacB are involved in modifying murpeptides by cleaving of the D-alanines at the C-terminal end. Pruning of the last D-Ala residues of the pentapeptides by PBP3 and DacB reduces the availability of donor stem-peptides for the transpeptidase reaction and thereby limits the network formation in the peptidoglycan. The serine/threonine protein kinase StkP and its cognate phosphatase PhpP are key regulators of pneumococcal cell wall synthesis and cell division. Together with GpsB and DivIVA they are believed to orchestrate the switch between septal and lateral peptidoglycan synthesis (Beilharz et al. 2012; Fleurie et al. 2014). MreC, MreD, and RodZ has an unknown role in peripheral peptidoglycan synthesis. RodA, however, was proposed to function as a lipid II flippase (Fadda et al. 2007; Massidda et al. 2013; Mohammadi et al. 2011), but recent research has shown that RodA is an essential peptidoglycan polymerase, playing a key role in bacterial cell wall synthesis, just like the

penicillin-binding proteins (Emami et al. 2017; Meeske et al. 2016). RodA has a vital role in the extension of the lateral walls (Meeske et al. 2016), and is part of the SEDS protein family (shape elongation division and sporulation). RodA is a conserved core part of the elongasome, and is reported to be functional without any other peptidoglycan polymerase in *Bacillus subtilis* (Meeske et al. 2016). Newly PBP2b is shown to interact with RodA (Straume et al. 2017).

### 1.5 Cell wall synthesis

The biosynthesis of the peptidoglycan cell wall is a complex process, which can be divided into three stages. In the first stage, the peptidoglycan precursor, lipid II, is synthesized in the cytoplasm. The second stage occurs in the cytoplasmic membrane, where lipid II is flipped from the cytoplasmic to the periplasmic side. Together with the SEDS proteins RodA and FtsW, the penicillin-binding proteins (PBPs) perform the last stage of peptidoglycan synthesis. Class A PBPs are transglycosylases that polymerize disaccharide units of lipid II into glycan strands. Newly synthesized glycan strands are cross-linked by transpeptidase reactions carried out by class A as well as class B PBPs. The specific pathway depicted in figure 1.8 displays the different steps involved in pneumococcal peptidoglycan synthesis. Starting with the precursor UDP-NAM, five amino acids are sequentially attached in four different steps by MurC, MurD, MurE and MurF, respectively. Then, with the help of MraY, UDP-NAM pentapeptide is anchored to the cell membrane by a bactroprenol hydrocarbon chain, now called lipid I. The conversion to lipid II, happens when MurG attaches NAG to lipid I (Bugg et al. 2011). Next, the bi-enzyme complex MurT/GatD amidates D-Glu to iGln (Münch et al. 2012). MurM and MurN synthesize the pentapeptide branches by adding two amino acids to lysine. MurM adds the first amino acid (L-alanine or L-serine), while MurN adds the second amino acid (L-alanine) of the branch (Lloyd et al. 2008). Of note, *S. oralis* Uo5 does not have *murN* and harbours an unusual *murM* gene, resulting in the formation of a interpeptide bridge consisting of only one L-alanine residue (see Fig. 1.6-5) (Todorova et al. 2015).

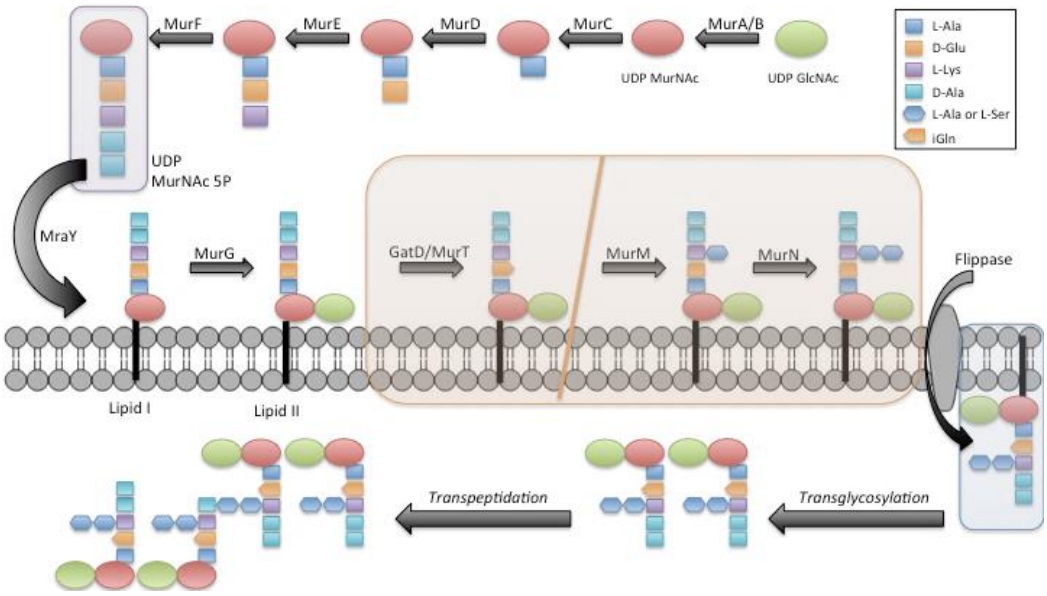


**Figure 1.8 The peptidoglycan synthesis pathway of *S. pneumoniae.*** The cartoon depicts the three different stages of peptidoglycan synthesis, which take place in the cytoplasm, cytoplasmic membrane (lipid-linked steps), and periplasm, respectively. Amino acids are attached to NAM before being anchored to lipid I. After NAG is added, lipid II is flipped to the outside of the cytoplasmic membrane where it is the substrate of PBPs (Rowland 2016).

### 1.5.1 The penicillin-binding proteins

The last extracellular stage of peptidoglycan synthesis, involves the enzymatic activities of the PBPs. The transglycosylase activity of the PBPs polymerizes the NAG-NAM-pentapeptide units into glycan strands where NAM and NAG is linked by a β-1,4-glycosidic bond (Scheffers & Pinho 2005). Glycan strands are cross-linked with other strands through PBP-mediated transpeptidation reactions as illustrated in figure 1.9 a. First the peptide bond between D-alanine four and five on the donor stem peptide is cleaved by a PBP, forming an acyl-enzyme intermediate. Then, the resulting ester bond between enzyme and substrate is attacked by the ε-amino group of L-lysine in the acceptor stem peptide to cross-link neighbouring glycan strands.

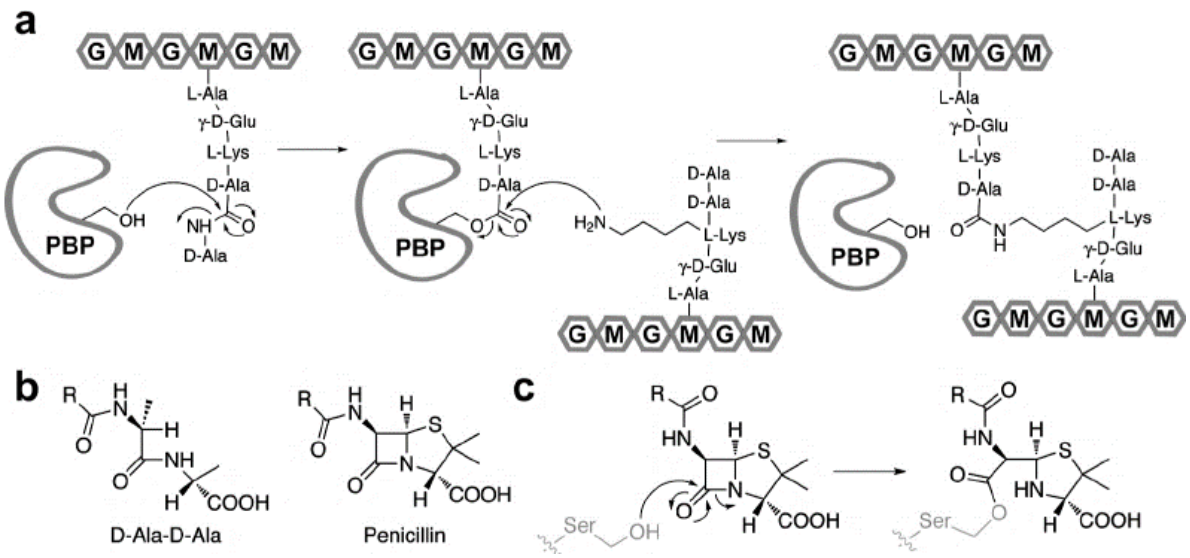


**Figure 1.9 The transpeptidation reaction and its inhibition by β-lactams A)** Illustration of the transpeptidase reaction carried out by penicillin-binding proteins (PBPs). **B)** The structure of the C-terminus of the stem peptide (D-Ala-D-Ala) compared to the structure of a penicillin. **C)** β-lactams are structural mimetics of the natural substrate of PBPs. Hence, the active sites of PBPs attack the beta-lactam ring, leading to an almost irreversible modification that inactivates their catalytic activity (Kocaoglu et al. 2012).

PBPs are divided into three classes called A, B and C. Those belonging to class A and B are high molecular mass (mass > 60 kDa) PBPs, while class C PBPs have lower molecular mass. *S. pneumoniae* produce six different PBPs. Three PBPs belong to class A, namely PBP1a, PBP1b, and PBP2a. These are bifunctional proteins that possess transglycosylase as well as transpeptidase activity. Their transglycosylase domains are located close to the transmembrane segment in the N-terminal part of the proteins, whereas the transpeptidase domain is located at the C-terminal end (Fig. 1.10).

*S. pneumoniae* produce two class B PBPs (PBP2x and PBP2b), which only possess transpeptidase activity. The precise function of the domain termed PBP_dimer (Fig. 1.10) remains uncertain, but it has been suggested that it is required for the formation of PBP2x and PBP2b homodimers. PBP2x and PBP2b are essential for septal and peripheral peptidoglycan synthesis, respectively (Berg et al. 2013; Sham et al. 2012). The precise role(s) of the PASTA domains is not clear, but it has been shown that they are required for correct mid-cell location of PBP2x (Peters et al. 2014)

As shown in figure 1.10, class A and B PBPs are anchored to the cytoplasmic membrane by a single transmembrane segment (M). They have very small N-terminal domains residing in the cytoplasm, while their transglycosylase and transpeptidase domains are located in the

extracellular space (Di Guilmi et al. 2002). The transpeptidase domain has three highly conserved amino acid motifs SXXK, SXN, and KS(T)G (Fig. 1.10), where the SXXK motif contains the active-site serine.

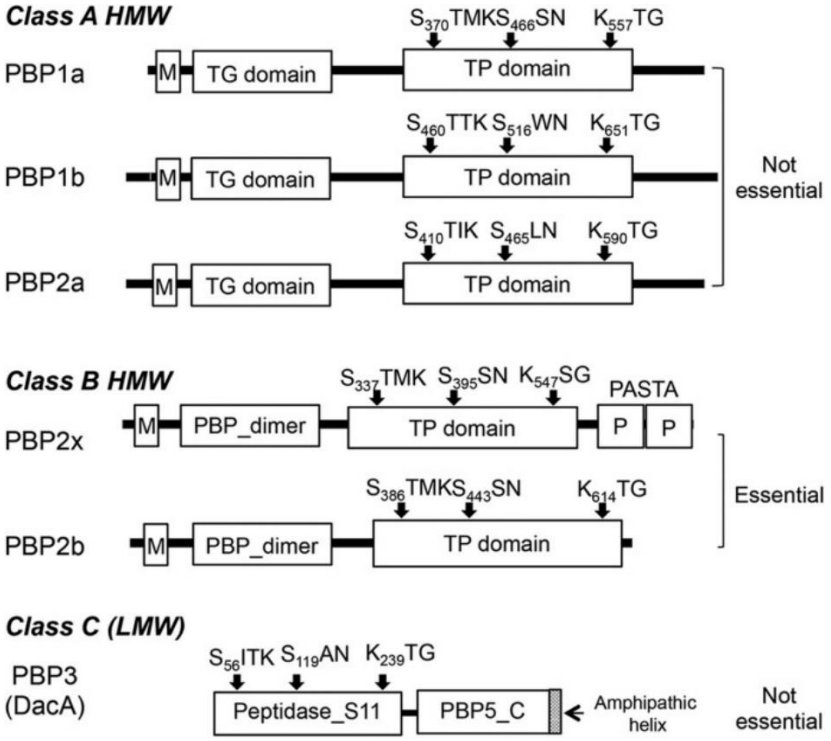


**Figure 1.10 Schematic overview of the domain architecture of the three classes of PBPs in *S. pneumoniae*.** Members of the high-molecular-weight classes all have a transmembrane anchor (M) and a transpepsidase (TP) domain. Class A PBPs are bifunctional and have in addition a transglycosylase (TG) domain. The PBP_dimer domains present in class B PBPs are believed to be involved in dimerization. PBP2x is unique in having two C-terminal PASTA (penicillin-binding protein and serine/threonine kinase associated) domains. PBP3, which belongs to class C PBPs, consists of a N-terminal D, D-carboxypeptidase domain and a C-terminal amphipathic helix which tethers the protein to the outer face of the cytoplasmic membrane (Kocaoglu et al. 2015).

As mentioned above, both class B PBPs are essential in *S. pneumoniae*, while the class A PBPs can be individually deleted. It is also possible two construct double knockouts, with the exception of the PBP1a/ PBP2a combination. PBP1a/PBP2a deletion mutants are not viable (Kjell et al. 1993; Paik et al. 1999). The single class C PBP, PBP3 (DacA), is a D-alanyl-D-alanine carboxypeptidase that is located on the cell surface. PBP3 removes the terminal D-alanine from the pentapeptide side chain, presumably to control the extent of peptidoglycan cross-linking (Massidda et al. 2013; Maurer et al. 2012). As PBP3 degrades the D-alanyl-D-alanine substrate of the high-molecular-weight PBPs, it is believed that most intact pentapeptides are present where there is newly synthesized peptidoglycan, e. g. at the cell

division site (Morlot et al. 2004; Scheffers & Pinho 2005). The *dacA* gene is not described as an essential gene in pneumococcus, but removing the gene causes decreased growth rate, and rounded cell shape with division asymmetry (Barendt et al. 2009; Massidda et al. 2013).

As mentioned above, neither *pbp2b* nor *pbp2x* can be deleted in *S. pneumoniae,* demonstrating that these PBPs are essential for peptidoglycan synthesis. PBP2b is a key component of the elongasome, while PBP2x is a crucial component of the divisome (Berg et al. 2013; Sham et al. 2012). Most of the studies investigating cell wall synthesis and division in ovoid cocci have been performed on *S. pneumoniae*. However, since *S. oralis* is such a close relative, it is reasonable to assume that the mechanisms behind these processes are highly similar in these bacteria.

### 1.6 β-lactam resistance

The first beta-lactam, Penicillin G, was discovered by Alexander Fleming in 1928. The event marks the start of the antibiotic era (Cruickshank 1955). Already in 1945 different versions of penicillin (Penicillin -G, -K, and -F) was mass-produced, and sold on the commercial market (Willcox 1947). Less than a decade later, resistant bacteria was starting to become a significant problem. Resistant strains of *Staphylococcus aureus* were reported, and others were emerging (Chambers & DeLeo 2009). Since then, many different beta-lactams, and other antibiotics have been released for clinical use. The widespread use of antibiotics has led to the development of resistant bacteria, a problem that has become a global threat. Reports show that the resistance level in bacteria is highly correlated with the overuse of antibiotics in medicine and agriculture. Antibiotic has been widely used in the feed of livestock for three main purposes: treating sick animals, preventing disease among animals susceptible to infections, and promoting the growth performance (*Antibiotic Resistance Threats in the United States* 2013).

Resistance against antibiotics can be obtained by different mechanisms. Some bacteria produce enzymes that attack antibiotics by derivatization. Others produce enzymes, as for example β-lactamases, which degrade and inactivate antibiotics by cleaving essential bonds (Bush et al. 1995). Another type of resistance mechanism relies on efflux pumps that transport antibiotics out of the cell (Bambeke et al. 2000). None of these mechanisms give rise to β-lactam resistance in streptococci. Instead, streptococci develops resistance against β-lactams by acquiring PBPs with altered transpeptidase domains that has a much lower affinity for β-lactams than the

corresponding “wild-type” domains. Alterations of the PBPs happens in such a way that the enzymes are still able to bind and process its natural substrate, but it requires larger amounts of beta-lactams to inhibit the catalytic process. The mystery investigated for decades is how low-affinity PBPs can evade the lethal effect of β-lactams while still being active as enzymes?

As previously mentioned, the penicillin-binding proteins (PBPs) are responsible for synthesizing, and cross-linking the peptidoglycan layer. Their name comes from their ability to bind the β-lactam ring of penicillins and other β-lactams. The structure of the β-lactam ring is highly similar to the structure of the D-alanines at the fourth and fifth position of the pentapeptide (Fig. 1.9 b). β-lactams inhibit PBPs by acylation of their active site serine hydroxyl group in a reaction analogous to that with the natural substrate (Fig.1.9 c) (Grebe & Hakenbeck 1996). The formation of a covalent bond between PBPs and β-lactam antibiotics prevents PBPs from binding to their natural substrate. This leads to inhibition and misregulation of cell wall synthesis followed by autolysis (Hakenbeck et al. 1999).

Studies have shown that commensal streptococci inhabiting the oral cavity and nasopharynx constitute a reservoir for genes encoding low-affinity PBPs. These genes can be transferred to *S. pneumoniae* through natural genetic transformation. Genes encoding low-affinity PBPs always have a mosaic structure. This structure results from interspecies gene transfer events followed by homologous recombination, in which sequence blocks from genes encoding β-lactam-sensitive PBPs get replaced by corresponding blocks from genes encoding low-affinity PBPs. In genetically distinct β-lactam-sensitive strains of *S. pneumoniae* the DNA sequence of their *pbp* genes differs by less than 0.1%. In contrast, genes encoding low-affinity PBPs from β-lactam resistant isolates have a mosaic structure containing sequence blocks that are ~ 20% divergent when compared to sensitive strains. Thus, it is likely that the mosaic structure of these genes has evolved through repeated gene transfer and recombination events (Dowson et al. 1993; Martin & Hakenbeck 1992; Sibold et al. 1994) This view is supported by the fact that the same or highly similar mosaic blocks can be found in resistant strains of *Streptococcus mitis* and *Streptococcus oralis* (Reichmann et al. 1997). PBP2b and PBP2x are considered to be the primary resistance determinants in *S. pneumoniae*. Acquisition of low-affinity variants of these PBPs result in low-level resistance. To obtain high-level resistance, acquisition of a low-affinity variant of PBP1a is required as well (Fani et al. 2014; Hackenbeck 2000; Hakenbeck et al. 2012). In highly resistant isolates up to four PBPs are altered (Laible et al. 1991). Only

mutations in the transpeptidase domain has been reported to be important for beta-lactam resistance (Denapaite et al. 2007).

It is likely that β-lactam resistance in streptococci comes at a fitness cost resulting in slower growth and a less functional cell wall. A mutated PBP with low affinity to beta-lactams will not function as efficiently as the wild-type protein. Hence, the cell wall may become structurally weaker, and become more susceptible to peptidoglycan hydrolases.

In addition to low-affinity PBPs, MurM has been shown to play a role in β-lactam resistance in *S. pneumoniae* (Filipe et al. 2000; Todorova et al. 2015). For reasons that are not well understood, deletion of murM in a resistant strain often results in complete breakdown of resistance (Filipe & Tomasz 2000). As mentioned in chapter 1.5, MurM is required for the synthesis of stem peptide branches. *S. oralis* Uo5 lacks the gene encoding MurN, and express an atypical MurM protein. Whether this contributes to the observed β-lactam resistance of *S. oralis* Uo5 is not known.

### 1.7 The aim of the study

β-lactam resistant clinical isolates of *S. pneumoniae* and other mitis group streptococci are often hard, if not impossible, to manipulate genetically. The reason why they do not become competent under laboratory conditions is not known. It is uncommon to have a high-resistance strain like *S. oralis* Uo5 that can be induced to competence for natural transformation. This gave us the opportunity to study the properties of low-affinity PBPs in their natural host. The main goal of this study was therefore to characterize different low-affinity PBPs in *S. oralis* Uo5, with the aim to determine their essentiality, contribution to β-lactam resistance, and effect on growth and morphology.

# 2. Materials

## 2.1 Strains of *S. oralis* Uo5

**Table 2.1 Overview of the strains made and used in this research**

| STRAIN NAME | GENOTYPE/RELEVANT CHARACTERISTICS | CONSTRUCTION/ (REFERENCE) |
|---|---|---|
| ***S. ORALIS*** | | |
| **SPH319** | Wt, *S. oralis* Uo5 | (Gift from Regine Hakenbeck) |
| **GS820** | SPH319, but ΔComA, $Sm^R$ | Unknown, belongs to Gro Anita Stamsås. removed *comA* |
| **GS951** | GS820, but ΔPbp2a::Janus, $Kan^R$, | Constructed by transforming GS820 with a Janus cassette that replaces *pbp2a*. Kanamycin resistance was acquired |
| **GS964** | GS951, but ΔJanus::DEL, $Sm^R$ | Constructed by transforming GS951 with ΔJansus::DEL. $Sm^R$ |
| **GS985** | GS820, but ΔPbp2b::Janus, $Kan^R$ | Constructed by transforming GS9820 with a Janus cassette that replaces *pbp2b*. $Kan^R$ was acquired |
| **GS1012** | GS985, but ΔJanus::DEL, $Sm^R$ | Constructed by transforming GS985 with ΔJansus::DEL. |
| **SF4** | GS820, but ΔPbp1b::Janus, $Kan^R$ | Constructed by transforming GS820 with a Janus cassette that replaces *pbp1b*. $Kan^R$ was acquired |
| **SF5** | SF4, but ΔJanus::DEL, $Sm^R$ | Constructed by transforming SF4 with ΔJansus::DEL. |
| **SF6** | SF5, but ΔPbp1a::Janus, $Kan^R$, | Constructed by transforming SF5 with Janus cassette that replaces *pbp1a* |
| **SF7** | SF5, but ΔPbp2a::Janus, $Kan^R$ | Constructed by transforming SF5 with Janus cassette that replaces *pbp2a* |
| **SF8** | GS1012, but ΔPbp1b::Janus $Kan^R$ | Constructed by transforming GS1012 with Janus cassette that replaces *pbp1b*. $Kan^R$ was acquired |
| **SF9** | GS964, but ΔPbp1b::Janus $Kan^R$ | Constructed by transforming GS964 with Janus cassette that |

| | | |
|---|---|---|
| | | replaces *pbp1b*. Kan$^R$ was acquired |
| **SF10** | GS1012, but ΔPbp2a::Janus Kan$^R$ | Constructed by transforming GS1012 with Janus cassette that replaces *pbp2a*. Kan$^R$ was acquired |
| **SF11** | GS964, but ΔPbp2b::Janus Kan$^R$ | Constructed by transforming GS964 with Janus cassette that replaces *pbp2b*. Kan$^R$ was acquired |
| **SF12** | SF5, but ΔPbp2b::Janus Kan$^R$ | Constructed by transforming SF5 with Janus cassette that replaces *pbp2b*. Kan$^R$ was acquired |
| **SF13** | SF9, but ΔJanus::DEL Sm$^R$ | Constructed by transforming SF9 with ΔJanus::DEL |
| **SF14** | SF7, but ΔJanus::DEL Sm$^R$ | Constructed by transforming SF7 with ΔJanus::DEL |
| **SF15** | SF11, but ΔJanus::DEL Sm$^R$ | Constructed by transforming SF11 with ΔJanus::DEL |
| **S. PNEUMONIAE** | | |
| **OLA235** | RH426, Janus Kan$^R$ | Reference: (Johnsborg & Håvarstein 2009) |

## 2.2 Peptides

**Table 2.2 The amino acid sequence of the competence stimulating peptide CSP for SK304 *S. oralis* used in this work.**

| Peptide | Strain | Amino acid sequence (N→C) | Stock concentration | Manufacturer |
|---|---|---|---|---|
| CSP | *S. oralis* | mkntvkleqfkeveteaelqeirgg**DWRISETIRNL IFPRRK** | 100µg/ml | Research Genetics Inc |

## 2.3 Primers for sequencing

**Table 2.3 Overview of the primers used for sequencing *pbp1a***

| PRIMER NAME | OLIGONUCLEOTIDE SEQUENCE (5'→3') | DESCRIPTION |
|---|---|---|
| 645 | CTACTTATTGCCGGCCGTTG | Fwd sequencing primer, 151bp upstream from pbp1a Uo5, SOR_1641 |
| 646 | CCCAATGAGGTTTATAGTACGC | Rwd 119bp downstream from pbp1a Uo5, SOR_1641 |
| 647 | GACTAAACAAGAGATCTTGACC | Fwd 507 down stream from start codon pbp1a Uo5, SOR_1641 |
| 648 | CGTGACTGGGGTTCTTCTATG | Fwd sequencing primer 119 bp down stream of the start codon in pbp1a Uo5, SOR_1641 |

## 2.4 Primers

**Table 2.4 Overview of all the primers used in this research with its oligonucleotide sequence and a short description**

| PRIMER NAME | OLIGONUCLEOTIDE SEQUENCE (5'→3') | DESCRIPTION | REFERENCE |
|---|---|---|---|
| KAN484F | GTTTGATTTTTAATGGATAATGTG | Fwd Amplify Janus cassette from Ola235 | (Johnsborg et al. 2008) |
| RPSL41R | CTTTCCTTATGCTTTTGGAC | Rwd Janus cassette Ola235 | (Johnsborg et al. 2008) |
| 491 | CGAGAGGGCAGTGAAACTC | Fwd 926 bp upstream *pbp2a* SOR_0190 *S. oralis* Uo5 | This study |

| | | | |
|---|---|---|---|
| 492 | CTCTCTGGGGTTATAATTCTG | Rwd 995 bp downstream *pbp2a* SOR_0190 Uo5 | This study |
| 493 | CACATTATCCATTAAAAATCAAACGCGTTTATTTTATCATCTTCACC | Rwd upstream gene *pbp2a* SOR_0190 Uo5, overhang Janus | This study |
| 494 | CGTCCAAAAGCATAAGGAAAGGAGGCTTGTCAAAGCCTAGG | Fwd downstream gene start *pbp2a* SOR_0190 Uo5, overhang Janus | This study |
| 495 | GCGTTTATTTTATCATCTTCACC | Rwd upstream gene start *pbp2a* SOR_0190 Uo5, used for removing Janus | This study |
| 496 | GGTGAAGATGATAAAATAAACGCGAGGCTTGTCAAAGCCTAGG | Fwd downstream gene *pbp2a* SOR_0190 Uo5, overhang right downstream of gene, used for removing Janus | This study |
| 524 | CAATCTTATGAGCAATTTCTGG | Rwd 1146 bp downstream from *pbp2b* SOR_0561 Uo5 | This study |
| 525 | CACATTATCCATTAAAAATCAAACACAACTAAGACTCTTTTCTAGAA | Rwd right upstream from *pbp2b* SOR_0561, overhang Janus | This study |
| 526 | CGTCCAAAAGCATAAGGAAAGGCAGTTGCAGTAGTCTTTCC | Fwd just upstream the end of gene *pbp2b* SOR_0561 Uo5, the 108 bp in 3' end is not removed due to a gene downstream | This study |
| 527 | ACAACTAAGACTCTTTTCTAGAA | Rwd upstream from *pbp2b* SOR_0561 UO5, used for removing Janus | This study |
| 528 | TTCTAGAAAAGAGTCTTAGTTGTGCAGTTGCAGTAGTCTTTCC | Fwd 108 bp upstream the end of gene *pbp2b* SOR_0561 Uo5, overhang right upstream from the same gene, used for removing Janus | This study |
| 529 | GTGAATGAGTGTCCGGTTTAC | Fwd 508 bp upstream *pbp2b* SOR_0561 Uo5 | This study |

| | | | |
|---|---|---|---|
| 530 | GCGATATGACAAAAGCATTTCC | Fwd 1682 bp upstream from *pbp2b* SOR_0561 Uo5 | This study |
| 603 | TTCCAATTACTGCCATGGGTTTC | Fwd 1021 bp upstream where Janus is inserted over *pbp1a* SOR_1641 Uo5 | This study |
| 604 | AACCATCTTCGTCTTGTTTCCAG | Rwd 982 bp downstream where Janus inserted over *pbp1a* SOR_1641, UO5 oralis | This study |
| 605 | CACATTATCCATTAAAAATCAAACGTAGAGGAAGAGACCTCCACC | Rwd upstream *pbp1a* where Janus is inserted over *pbp1a* SOR_1641 Uo5, overhang Janus | This study |
| 606 | CGTCCAAAAGCATAAGGAAAGCATTTATCACCCAGAATATTCTGG | Fwd downstream pbp1a where Janus is inserted over *pbp1a* SOR_1641 Uo5, overhang Janus | This study |
| 607 | GTAGAGGAAGAGACCTCCACC | Rwd upstream where Janus is inserted over *pbp1a* SOR_1641 Uo5, used to remove Janus. | This study |
| 608 | GGTGGAGGTCTCTTCCTCTACCATTTATCACCCAGAATATTCTGG | Fwd downstream where Janus is inserted over *pbp1a* SOR_1641, overhang upstream, used for removing Janus | This study |
| 609 | CACGGAGTCCTTGTTTGAGTTC | Fwd 1158 bp upstream *pbp1b* SOR_126 Uo5 | This study |
| 610 | TACTGGGCTTGTTTTGCTTCTTC | Rwd 988 bp downstream *pbp1b* SOR_01296 Uo5 | This study |
| 611 | CACATTATCCATTAAAAATCAAACGAATGTCCTCGCTTTCTCTATTAT | Rwd upstream *pbp1b* SOR_0126 Uo5, overhang Janus | This study |
| 612 | CGTCCAAAAGCATAAGGAAAGGAAAGAGTAGTAGCAAGGTTAGTA | Fwd downstream the gene *pbp1b* SOR_0126 Uo5, overhang Janus | This study |
| 613 | GAATGTCCTCGCTTTCTCTATTAT | Rwd upstream gene start *pbp1b* SOR_0126 Uo5, used for removing Janus | This study |

| | | | |
|---|---|---|---|
| 614 | ATAATAGAGAAAGCGAGGACATTCGAAAGAGTAGTAGCAAGGTTAGTA | Fwd downstream *pbp1b* SOR_0126 Uo5, overhang upstream gene start, used for removing Janus | This study |
| 615 | GGAAATTGATGGAATTTGTTATGAC | Fwd 1027 bp upstream *pbp2x* SOR_0341 Uo5 | This study |
| 616 | TCCCCAGAAGAAGAAATCGACC | Rwd 986 bp downstream where Janus is inserted over *pbp2x* SOR_0341 Uo5 | This study |
| 617 | CACATTATCCATTAAAAATCAAACGGCAAAGTGTACTATTTTTTCTTTC | Rwd upstream where Janus is inserted over *pbp2x* SOR_0341 Uo5, overhang Janus | This study |
| 618 | CGTCCAAAAGCATAAGGAAAGGACTAATACAGCTATCAAAAACATTA | Fwd downstream where Janus is inserted over *pbp2x*, SOR_0341 Uo5, overhang Janus | This study |
| 619 | GGCAAAGTGTACTATTTTTTCTTTC | Rwd upstream where Janus is inserted over *pbp2x* SOR_0341 Uo5, used for removing Janus | This study |
| 620 | GAAAGAAAAAATAGTACACTTTGCCGACTAATACAGCTATCAAAAAACATTA | Fwd downstream where Janus is inserted over *pbp2x*, overhang right upstream, used for removing Janus | This study |
| 643 | ATGAACAAACAAACTTTTCTGCG | Fwd start *pbp1a* Uo5, for checking if *pbp1a* is still present | This study |
| 644 | TTATGGTTGTGCTGGTTGAGG | Rwd end *pbp1a* Uo5, for checking if *pbp1a* is still present | This study |

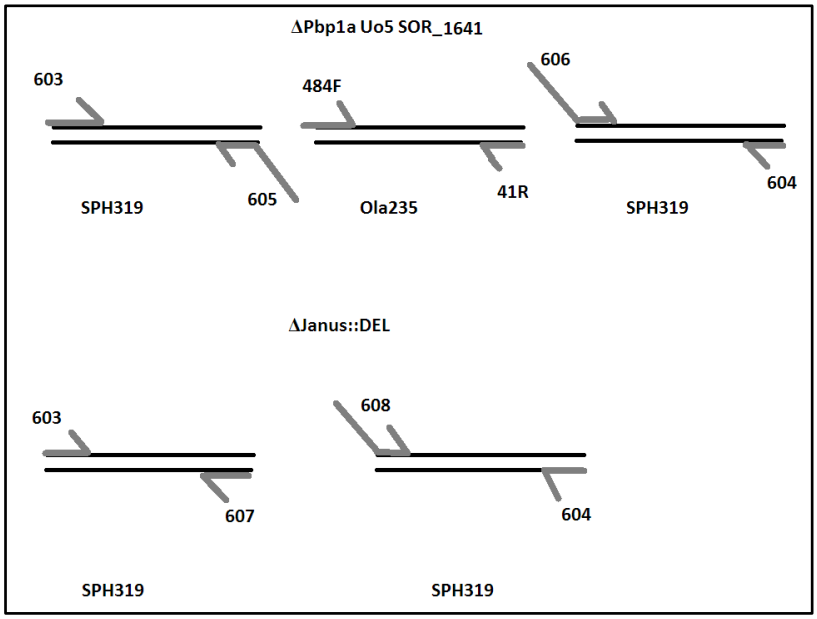


**Figure 2.1 Illustration of primers used to remove *pbp1a***. The figure displaying which primers and strains that were used to make both the construct with Janus, and the construct for removing Janus.

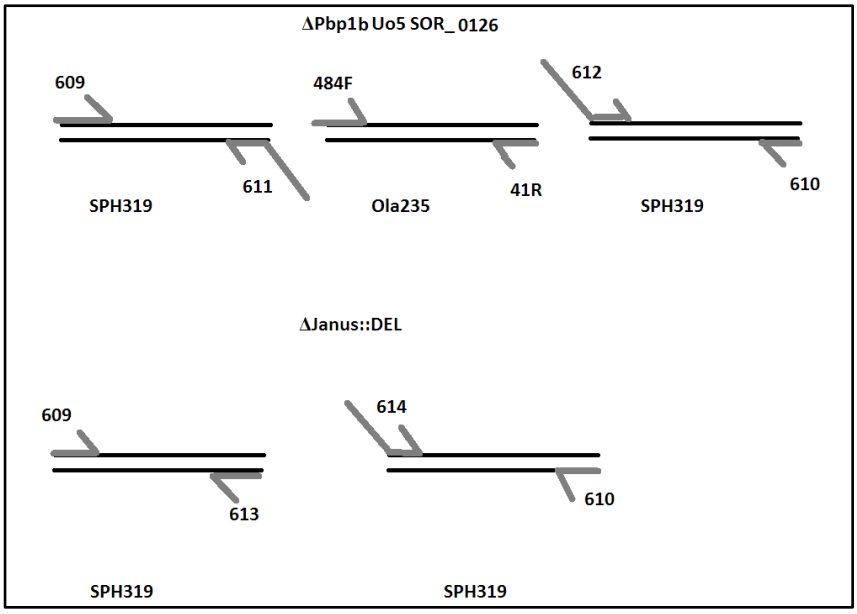


**Figure 2.2 Illustration of the primers used to remove *pbp1b***

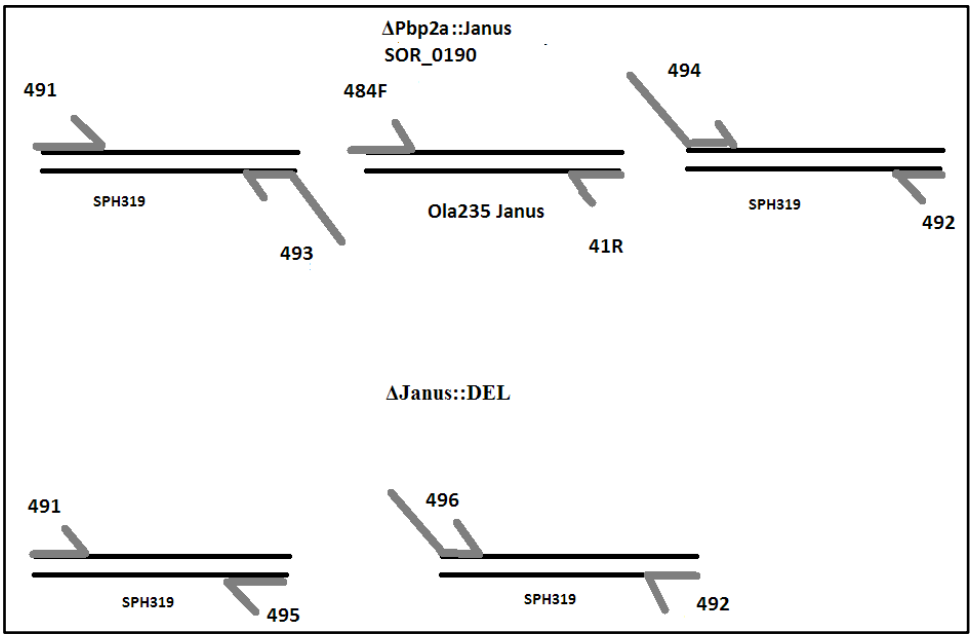


**Figure 2.3 Illustration of the primers used to remove *pbp2a*.**

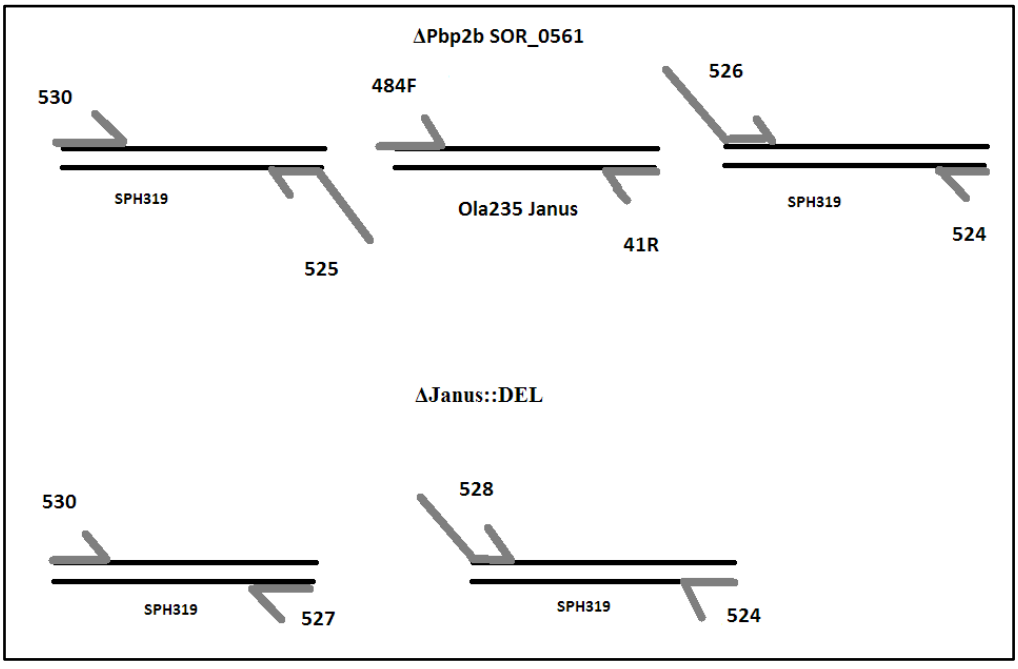


**Figure 2.4 Illustration of the primers used to remove *pbp2b*.**

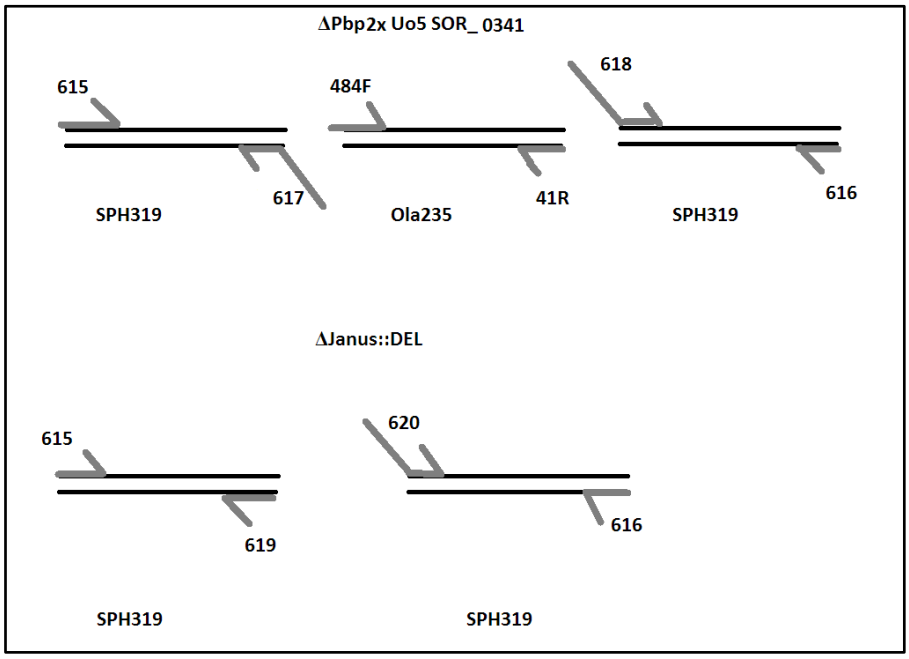


**Figure 2.5 Illustration of the primers used to remove *pbp2x*.**

## 2.5 Standards for molecule weight, enzymes and nucleotides

**Table 2.5 Overview of ladder, enzymes and nucleotides used in this work**

| Name | Stock concentration | Producer | Product number |
|---|---|---|---|
| 1 kb DNA ladder | 50 ng/µl, in MQ-water and loading buffer | Invitrogen | 15615-024 |
| dNTPs (dATP, dCTP, dGTP and dTTP) deoxynucleotide triphosphate | 100 mM | Promega | |
| Phusion® High-Fidelity DNA Polymerase | 2,0 U/ µl | New England BioLabs | M0530L |

| | | | |
|---|---|---|---|
| OneTaq® DNA polymerase | 5,0 U/ml | New England BioLabs | M0267S |

## 2.6 Antibiotics

**Table 2.5 Overview of all antibiotics used in this research**

| Antibiotic | Chemical formula | Producer/supplier | Product number |
|---|---|---|---|
| Etest Amoxicillin (AC) | $C_{16}H_{19}N_3O_5S$ | bioMérieux | 15785 A |
| Etest Oxacillin (OX) | $C_{19}H_{19}N_3O_5S$ | bioMérieux | 15857 A |
| Etest Penicillin G (PG) | $C_{16}H_{17}KN_2O_4S$ | bioMérieux | 15801 A |
| Kanamycin sulfate from Streptomyces kanamycetius (Kan) | $C_{18}H_{36}N_4O_{11} \cdot H_2O_4S$ | Sigma Aldrich | K4000-50G |
| Streptomycin sulfate (Sm) | $C_{42}H_{84}N14O_{36}S_3$ | Sigma Aldrich | S6501-25G |

## 2.7 Kit

**Table 2.6 Kit used in this research**

| Name | Function | Producer | Product number |
|---|---|---|---|
| NucleoSpin® Extract II | DNA extraction | Machinery-Nagel | 740609.250 |

## 2.8 Computer software

**Table 2.7 Overview of the computer software and applications used in this research**

| Program | Application | Available from |
|---|---|---|
| Chromas | Analysing sequencing | http://www.softpedia.com/get/Science-CAD/Chromas-Lite.shtml |
| Clustal Omega | Sequence alignment | http://www.ebi.ac.uk/Tools/msa/clustalo/ |
| Reverse Complement | Finding reverse complements for primer design | reverse-complement.com |

## 2.9 Chemicals

**Table 2.8 Overview of the chemicals used in this research**

| Name | Chemical formula | Producer/supplier | Product number |
|---|---|---|---|
| β-mercaptoetanol | $C_{22}H_6OS$ | Sigma Aldrich | M6250 |
| Agarose | $C_{24}H_{38}O_{19}$ | Invitrogen | |
| Active coal | C | Merck | 1.02182.1000 |
| Acryl/Bis™ (40%) | $C_7H_{10}N_2O_2$ | Amresco | 0311-500ML |
| Agar powder | $C_{14}H_{24}O_9$ | VWR | 20767.298 |
| albumin/BSA 8% | | Thermo scientific | AN0035A |
| Ammonium Persulfate (APS) | $(NH_4)_2S_2O_8$ | SIGMA | A3678-25G |
| Aspargine monohydrate | $NH_2COCH_2CH(NH_2)COOH \cdot H_2O$ | VWR | BDH4508-500GP |
| Bacto™ Casitone | | BD and Company | 211825 |
| Bacto™ Todd Hewitt Broth | | BD and Company | 8X04921 (0903) |
| Biotine | $C_{10}H_{16}N_2O_3S$ | VWR | 89085-280 |
| Bromphenol blue | $C_{19}H_9Br_4O_5SNa$ | Sigma Aldrich | B-5525 |
| $CaCl_2$ dihydrate | $CaCl_2 \cdot 2H_2O$ | Sigma Aldrich | C5080-500G |
| Ca panthothenate | $C_{18}H_{32}CaN_2O_{10}$ | Sigma Aldrich | C8731 |
| Celite | | Acros Organics | 20632500 |
| Choline chloride | $C_5H_{14}CINO$ | Sigma Aldrich | C1879-500G |
| Copper(II) sulphate pentahydrate | $CuSO_4 \cdot 5H_2O$ | Sigma Aldrich | C-2284 |

| | | | |
|---|---|---|---|
| di-potassium hydrogen phosphate | $K_2HPO_4$ | VWR | 1.37010.9100 |
| Ethylenediaminetetraacetic acid (EDTA) | $C_{10}H_{16}N_2Na_2O_8 \cdot 2H_2O$ | VWR | 71003-398 |
| Glass wool | | Supleco | 2-0384 |
| Glycerol solution | $C_3H_8O_3 \cdot H_2O$ | Sigma Aldrich | 49781-5L |
| Hydrochloric acid | HCl | Merck | 1.00317.2500 |
| Iron (II) sulphate heptahydrate | $FeSO_4 \cdot 7H_2O$ | Sigma Aldrich | F8633 |
| L-Cystein hydrogencloride monohydrate | $C_3H_7NO_2S \cdot HCl \cdot H_2O$ | Fluka | 30130 |
| L-Glutamine | $C_5H_{10}N_2O_3$ | Fluka | 49419 |
| L-Tryptophan | $C_{11}H_{12}N_2O_2$ | Fluka | 93660 |
| Magnesium chloride hexahydrate | $MgCl_2 \cdot 6H_2O$ | Sigma Aldrich | M2670-500G |
| Magnesium chloride tetrahydrate | $MnCl_2 \cdot 4H_2O$ | Sigma Aldrich | M8054-100G |
| Monosodium phosphate | $NaH_2PO_4$ | VWR | 0823-1KG |
| Nicotinic acid | $C_6H_5NO_2$ | Sigma Aldrich | N-4126 |
| One Taq® DNA buffer | | New England BioLabs | B9022S |
| PeqGREEN | | VWR | 732-2960 |
| Phusion® HF-Buffer **5x** | | New England BioLabs | B0518S |
| Pyridoxine hydrochloride | $C_8H_{11}NO_3 \cdot HCl$ | Fluka | 95180 |
| Riboflavin | $C_{17}H_{20}N_4O_6$ | Sigma Aldrich | R-7649 |
| SDS ultrapure | $C_{12}H_{25}NaO_4S$ | AppliChem | A1112,1000 (UN1325) |
| Sodium Acetate | $NaC_2H_3O_2$ | Sigma Aldrich | S2889-1KG |
| Sodium Hydroxide | NaOH | VWR | 470302-544 |
| Sodium phosphate dibasic heptahydrate | $HNa_2PO_4 \cdot 7H_2O$ | VWR | 97061-468 |
| Sodium Pyruvate | $C_3H_3NaO_3$ | Thermo Fisher | 11360070 |
| Thiamine hydrochloride | $C_{12}H_{18}Cl_2N_4OS \cdot xH_2O$ | Merck | 3677981 |
| Tris/Tricine/SDS Buffer | | BIORAD | 161-0744 |
| Triton® X-100 | | Sigma Aldrich | X100-100ML |
| Trizma® base | | Sigma Aldrich | T1503-1KG |
| Zinc sulfate heptahydrate | $ZnSO_4 \cdot 7H_2O$ | Sigma Aldrich | Z-0251 |

## 2.10 Equipment

**Table 2.9 Overview of the equipment and machines used in this study**

| Name | Model | Producer |
|---|---|---|
| Anaerobic culturing bag | AnaeroGen™ | Oxoid |
| Autoclave | cv-el 12L/18L | Certoclav |
| Benchtop homogenizer | Fast prep 24 | MP™ |
| Centrifuge 1 | 5430 R | Eppendorf |
| Centrifuge 2 | Multifuge 3 S-R | Heraeus |
| Chamber for anaerobe culturing | | Oxoid |
| Disposable cuvettes 1,5 ml | 759015 | Brand |
| Electrophoresis cell for agarose gel electrophoresis | Mini-Sub®cell GT | BioRad |
| Electrophoresis machine | Power Pal 200 | BioRad |
| Filtropur S 0.2 (0,2µm) | | Sarstedt |
| Gel documentation | GelDoc-1000 | BioRad |
| Glass beads | G4649 | Sigma |
| Incubation cabinet | | Termaks |
| Magnet stirrer | MR 3001K | Heidolph |
| Micro plate reader | Synergy H1 Hybrid Reader | BioTek |
| Microscope | LSM 700 | Zeiss |
| Microtiter plate (96 wells) | 3604 | Corning |
| Microwave | MWO602 | Wirlpool |
| Nanodrop Spectrophotometer | Nanodrop 2000 | Thermo scientific |
| Next generation tip refill | 0.1-10 µl | VWR |
| Next generation tip refill | 1-200 µl | VWR |
| Next generation tip refill | 100-1250 µl | VWR |
| PCR-machine | 2720 Thermal Cycler | Applied Biosystems |
| pH-meter | PHM210 | MeterLab® |
| Pipettes | Div. | Thermo Scientific |
| Scale (0.0001-200g) | CP124S | Sartorious |
| Spectrophotometer | LKB Novaspec II | Pharmacia |
| Sterile bench | AV-100 | Telstar |

| | | |
|---|---|---|
| Syringes (for sterile filtration) | Div. | BD plastipak™ |
| Table centrifuge 1 | 5424 | Eppendorf |
| Table centrifuge 2 | 5415 R | Eppendorf |
| Water bath | 19 | Julabo |
| Water bath | MB | Julabo |
| Water bath | D 3006 | GLF |

## 2.11 Recipes – culture mediums, solutions and buffers

### 2.11.1 TH culture mediums

**Liquid Todd Hewitt solution (TH)**

For 100 ml:

- 3 g Todd Hewitt broth
- 100 ml dH2O

The solution is autoclaved at 121 ºC for 15 minutes.

**Soft agar 100 ml solution**

- 3 g Todd Hewitt broth
- 0,75 g agar
- 100 ml dH2O

Autoclaved at 121 ºC for 15 minutes, stored in 4 ºC.

**Todd Hewitt Agar (THA)**

For 500 ml:

- 15 g Todd Hewitt Broth
- 7,5 g agar

MQ water filled up to an end volume of 500 ml. The solution is autoclaved at 121℃ for 15 minutes. At 60 ºC it's possible to add antibiotics. Plates with Kanamycin 400 µg/ml, and streptomycin 200 µg/ml.

### 2.11.2 Solutions for C-medium

**Pre C-medium:**

For 4 litres:

0,045 g L-cystein HCl

8 g Natrium Acetate

20 g Bacto™ Casitone

0,024 g L-tryptophan

34 g di-kaliumhydrogenphosphate ($K_2HPO_4$)

MQ-water was added to an end volume of 4 l. 150 ml of the solution was distributed in 250 ml pyrex bottles and autoclaved at 121 degrees for 15 minutes. Solution is stored at room temperature.

**ADAMS I:**

0,15 ml Biotine 0,5 mg/ml

75 mg Acide nicotinique

87,5 mg Pyridoxine hydrochloride

300 mg Ca panthothenate

80 mg Thiamine hydrochloride

35 mg riboflavin

500 ml $dH_2O$

pH adjusted to 7,0

**ADAMS II – 10x:**

500 mg $CuSO_4 \cdot 5H_2O$

500 mg $FeSO_4 \cdot 7H_2O$

500 mg $ZnSO_4 \cdot 7H_2O$

500 mg $MnCl_2 \cdot 4H_2O$

10 ml concentrated HCl

Added MQ $H_2O$ to a 100 ml end volume, and sterile filtered with Filtropur S 0.2. Solution was stored in 4 ºC.

**ADAMS III:**

128 ml ADAMS I

3,2 ml ADAMS II – 10x

1,6 mg Aspargin monohydrate

1,6 mg Choline

0,4 g $CaCl_2$ anhydre

16 g $MgCl_2 \cdot 6H_2O$

800 ml $dH_2O$

pH adjusted to 7.6

**Yeast extract:**

40 g yeast extract

360 ml $dH_2O$

6 ml 12N/37% HCl

16 g active coal

- 40 g yeast extract was dissolved in 360ml MQ $H_2O$. pH was adjusted with HCl to 3.0, and 16 g of active coal was added. The solution was stirred for 10 minutes with a magnet stirrer, and stored at 4 ºC for 2 hours before filtrating overnight through a column of glass wool and celite. PH was then adjusted to 7,8 with a high molar NaOH, and adjusted

end volume to 400 ml. Solution was sterile filtrated, and 4 ml transferred to 15 ml falcon tubes, stored at -80 ºC.

### 2.11.3 C-medium

To 150 ml pre- C- medium the following is added

150 µl $MnCl_2$ 0,4 mM

1,5 ml glucose 20%

3,75 ml ADAMS III

110 µl glutamine 3%

2,25 ml Na pyruvate 2%

95 µl Sucrose 1,5 M

1,5 ml uridine adenosine 2 mg/ml

1,5 ml albumin/BSA 8%

3,75 mL yeast extract

Filter the solution with filtropur S 0.2 for elimination of bacteria.

### 2.11.4 Buffer for microscopy

**1 x Phosphate-buffered saline (PBS), pH 7.2**

8,0 g NaCl (137mM)

1,7799 g Na2HPO4.2H2O (10 mM)

0,201 g KCl (2,7 mM)

0,272 g KH2PO4 (2 mM)

pH adjusted to pH 7,2 and end volume adjusted to 1 L. Short time storage at room temperature.

### 2.11.5 Solutions and buffers to agarose gel electrophoresis

**10 x loadingbuffer**

2 ml 1 % bromphenol blue

5 ml 50 % glycerol

8 ml MQ-water

Stored at 4 ºC

**50 x TAE buffer (Tris-Acetate-EDTA)**

242 g Tris base

57,1 ml acetic acid

100 ml 0,5 M EDTA (pH 8,0)

MQ-water added to an end volume of 1 l. Stored at room temperature. 1 x TAE is used for making agarose gels, and as buffer in gel electrophoresis chamber.

### 2.11.6 Buffers for Bocillin FL assay

**0,5 M Tris-HCl pH 6.8 (100 ml)**

Tris base 6.0 g

$dH_2O$ 60 ml

Adjust pH to 6.8 with HCl

$dH_2O$ to 100 ml

**1.5 M Tris-HCl buffer, pH 8.8 (150 ml)**

Tris base 27.23 g

$dH_2O$ 80 ml

Adjust to pH 8.8 with HCl

dH2O to 150 ml

**2x SDS-PAGE**

3.75 ml 0.5 M Tris-HCl, pH 6.8

15 ml 50% Glyserol

0.3 ml 1.0% Bromphenol blue

6 ml 10% SDS

$dH_2O$ to 30 ml

5% β-mercaptoethanol added fresh (50 µl to 950 µl sample buffer)

**Sodium phosphate buffer (20mM, pH 7.2):**

3.27 g $Na_2HPO_4 \cdot 7H_2O$

0.94 g $NaH_2PO_4$

Adjust pH to 7.2 and bring volume to 1 L with $dH_2O$

**2.11.7 Solutions**

**50 % glycerol**

20 g 85% glycerol

100 ml MQ-water

Autoclaved for 15 minutes at 121 ºC

**dNTP-mixture 10 mM**

10 µl 100 mM dATP

10 µl 100 mM dCTP

10 µl 100 mM dGTP

10 µl 100 mM dTTP

60 µl MQ-water

Stored at -20 ºC

# 3. Methods

## 3.1 Cultivation and retention of *S. oralis*

*S. oralis* Uo5 was grown in liquid TH-medium, Todd Hewitt (TH) agar plates, and in fresh C-medium when induced for transformation. Freezing stock was made with TH-medium, grown in 37 ºC water bath to an end $OD_{550}$ of roughly 0,3, then 15% glycerol (80 %) added, stored at -80 ºC. Start cultures are frozen down in nunc tubes, while main freezing stocks are stored in 2 ml cryogenic vials specialized for long term storage. For making an anaerobic environment when growing on TH-agar plates, the plates are inserted in a chamber by oxoid with a AnaeroGen$^{TM}$ bag, which reduces the oxygen level to under 1 %.

## 3.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a method for amplifying many copies of selected fragments of DNA. The principle of PCR is that a polymerase is added for an in vitro amplifying of the template, with designed oligonucleotide primers. By adding a buffer for securing the pH, and deoxynucleotide phosphates (dNTPs) as building blocks, DNA Polymerase and designed primers, the chain reaction can make multiple copies of a wanted sequence. The template can be lysed cells, genomic DNA extracted from cells, previous PCR-product and plasmids, and should contain 100-200 ng DNA per 50 µl reaction. The chain reaction that is repeated contain three different steps:

1. Denaturing. Single stranded DNA gets available by denaturing double stranded DNA by heating samples to 94/98 ºC for five minutes for the initial denaturing step, and 30 seconds when the step is repeated. (If cells are lysed at the initial denaturation step, the initial denaturing durance should be increased to 10 minutes.)
2. Hybridizing primers. The primers are designed to hybridize at 60 ºC, by calculating the melting point at 60 ºC, using the basic rule of adding 4 ºC degrees for every C and G, and 2 ºC A and T.
3. Elongation. By increasing the temperature to 72 ºC DNA polymerase gets activated, and synthesized new DNA. Durance of this step varies by the size of the fragments. Different polymerase have different speeds and proofreading error.

Step 1-3 is repeated 25-30 times.

### 3.2.1 Phusion® High-Fidelity DNA Polymerase

Phusion® DNA Polymerase was used in this research when constructing fragments used for transformation, both for amplifying DNA and overlap PCR. The polymerase is thermostable, and possess polymerase activity at 72 ºC. The DNA polymerase possesses a 5'→3' polymerase activity, and 3'→5'exonuclease activity. The exonuclease activity is what enables us to do overlap PCR, and the error rate in Phusion is more than 50 times lower than for *Taq* DNA Polymerase (*Phusion® High-Fidelity DNA Polymerase* 2017).

#### 3.2.1.1 Protocol for PCR using Phusion ® High-Fidelity DNA Polymerase

1. The following reactants was added in a PCR-tube kept on ice:

| **Reagent** | **End concentration/volume** |
|---|---|
| 10 µl 5x Phusion ® HF-Buffer | 1x |
| 1 µl dNTP (10mM) | 0,2 mM |
| 2,5 µl primer Forward (10 pmol/ µl) | 0,5 µM |
| 2,5 µl primer Reverse (10 pmol/ µl) | 0,5 µM |
| x µl template DNA | 1-100 ng (varying on template used) |
| 0,5 µl Phusion ® DNA polymerase | 0,02 U/ µl |
| X µl $dH_2O$ | To an end volume of 50 µl |

2. The PCR reaction was executed with the thermocycler with suited settings depending on length of the fragment, which type of template used, and primer melting point. Basic procedure:

| **Step** | **Temperature and time** |
|---|---|
| 1. Initial denaturing | 98 ºC, 5 minutes |
| 2. Denaturing | 98 ºC, 30 seconds |
| 3. Primer hybridization | 58 ºC, 30 seconds |

| | |
|---|---|
| 4. Elongation | 72 ºC 1:30 seconds (15 s/kb if DNA is template, and 30 s/kb if cells are used as template) |
| 5. Repeat step 2-4, 25 times | |
| 6. Elongation | 72 ºC 5 min |
| 7. | 4 ºC, ∞ |

When using DNA as template the PCR tubes should not be applied on the thermocycler before sample chamber reaches 80 ºC, in order to avoid unwanted polymerase activity when the temperature is rising. PCR product is kept on ice or at 4 ºC for short time storage, and in freezer for longer storage.

### 3.2.2 One Taq® DNA Polymerase

One *Taq* DNA polymerase is a blend of Deep Vent DNA polymerase which has a low stable 3'→5'exonuclease activity, and *Taq* DNA (exonuclease-free) polymerase for robust amplification (Barnes 1994). One *Taq* Polymerase was used for screening all selected colonies from antibiotic agar plates after transformations.

#### 3.2.2.1 Protocol for PCR using One *Taq* DNA Polymerase

1. The following reactants was added in a PCR-tube kept on ice:

| **Reagent** | **End concentration/volume** |
|---|---|
| 5 µl 5x One *Taq* Standard Reaction Buffer | 1x |
| 0,5 µl dNTP (10mM) | 0,1 mM |
| 0,5 µl primer Forward (10 pmol/ µl) | 0,1 µM |
| 0,5 µl primer Reverse (10 pmol/ µl) | 0,1 µM |
| x µl template DNA (normally colony picked with toothpick and dipped in $dH_2O$) | 1-100 ng (varying on template used) |
| 0,125 µl One *Taq*® DNA polymerase | 5 U/ µl |

X µl $dH_2O$ (17,5µl) To an end volume of 25 µl

2. The screening PCR was executed in a thermocycler, with suited settings for the reaction:

| Step | Temperature and time |
|---|---|
| 1. Initial denaturing | 94 ºC, 10 minutes |
| 2. Denaturing | 94 ºC, 30 seconds |
| 3. Primer hybridization | 58 ºC, 30 seconds |
| 4. Elongation | 68 ºC, X minutes (1 min/ kb) |
| 5. Repeat step 2-4, 25 times | |
| 6. Elongation | 68 ºC, 7 min |
| 7. | 4 ºC, ∞ |

### 3.3 Overlap extension PCR

When making the construct for removing the genes encoding the penicillin-binding proteins, the PCR was done by overlap extension PCR, splicing by overhang extension. By using a high-fidelity DNA polymerase that introduces minimal mutations will allow us to make large constructs over 20 kb (Nelson & Fitch 2011). The primers designed to make fragment A (upstream from gene) and B (downstream from gene) both contain an overlapping region to the Janus cassette, generating a flanking fragment end. The fragments A and B are amplified in the first PCR reaction, and combined with a Janus cassette in a second reaction. Further overlap extension PCR generates the template used for transformation illustrated in figure 3.1.

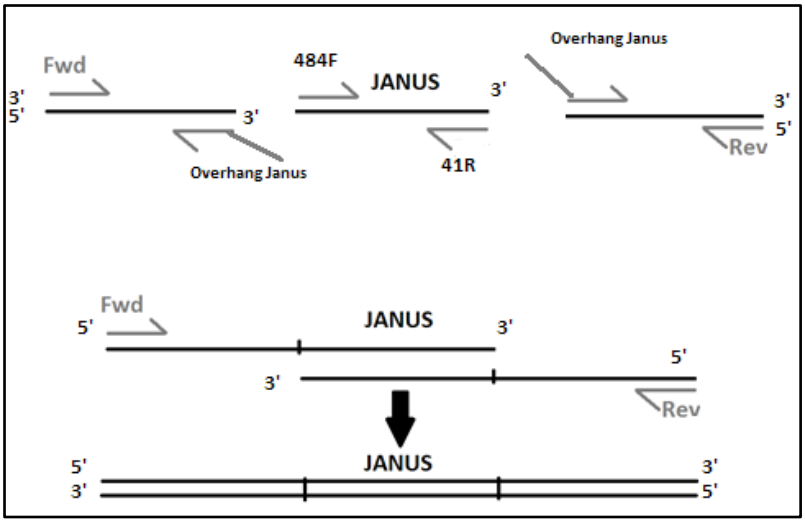


**Figure 3.1 Illustrating of overlap extension PCR,** the last PCR making the construct for removing selected genes.

### 3.4 DNA separation by gel electrophoresis

DNA fragments varying from 0.1- 25 kb in length, can be separated by gel electrophoresis (Sambrook & Russel 2001). The gel consists of a network of agarose polymers, and peqGreen for staining the DNA. The gel is placed in a chamber in an electric field filled with buffer. DNA contains negative charged phosphate groups, permitting the DNA to migrate towards the plus pole. The migration rate is determined by the length of the fragments, concentration of agarose (normally from 0.5-2.0 %), applied voltage and other less significant factors (Lee et al. 2012) The longer the DNA fragments are the slower they move in the gel. A ladder from Invitrogen with known size of fragments are used for determining the size of the separated DNA, visualized under UV-light.

#### 3.4.1 Protocol for agarose gel electrophoresis, 1% gel

For one gel:

1. 0,5 g of agarose was added to 50 ml 1x TAE-buffer.
2. The solution was heated in a microwave at max watt until the agarose was completely dissolved. Solution was cooled down to roughly 60 ºC before adding 2 µl peqGreen.
3. Solution was then transferred to a gel tray, and combs inserted for making wells, set for 30 minutes.
4. Polymerized gel was placed in a gel electrophoresis chamber filled with 1x TAE-buffer.
5. Samples and Invitrogen ladder was then applied, and run 30 minutes on 90 V.
6. The DNA separation was visualized using Gel Doc-1000 (BioRad).

### 3.5 Extraction of DNA

Extraction of DNA was performed using the kit NucleSpin®, by cutting out the desired fragment separated on agarose gel, visualized under UV-light, or from direct PCR product. The gel was dissolved in NTI-binding buffer containing chaotrophe salts which bind the silica membrane in the spin coulomb. Followed by removing all other components, using a washing buffer containing ethanol. DNA was eluted from the membrane using NE (5 mM Tris/HCl, pH 8.5).

### 3.5.1 Extraction of DNA from gel using NucleoSpin®

1. Add 750 µl Binding buffer NTI to eppedorf tube containing DNA in gel

    - 55 ºC water bath shaken until dissolved

    - Transfer 700 µl at the time to the column

    - Centrifuge 1 minute at 11 000 G

        X more times and discard supernatant.

2. Add 700 µl washing buffer NT3 (containing alcohol) to column

    - Centrifuge 1 minute at 11 000 G

    - Discard supernatant

    - Centrifuge 2 minutes at 11 000 G, and insert column into a sterile eppendorf tube.

3. Add 50 µl elution buffer (5 mM Tris/HCl, pH 8.5) to the column

    - wait 1 minute before centrifuging the column at 11 000 G

    - Stored on ice

### 3.5.2 Extraction of DNA from direct PCR product

When sample is being sent for sanger sequencing, the extraction procedure should be extracted from direct PCR product, and not from a gel. The protocol is now the same as for 3.5.1, but with less binding buffer (90 µl for 45 µl PCR product), and reduced amount of elution buffer (about 35 µl depending on the DNA concentration).

### 3.6 Transformation of *S. oralis*

*S. oralis* is competent for natural transformation, and can incorporate extracellular DNA through homologous recombination. We can easily get *S. oralis* competent for natural transformation by adding competence stimulating peptide (CSP) (Håvarstein et al. 1995). The wt strain used in this study has the gene *comA* removed. This gene encodes a transporter allowing CSP to be secreted out of the cell. The removal of *comA* inhibits the bacteria for inducing competence unless synthetic CSP is added.

### 3.6.1 Negative selection by using the Janus cassette

The Janus cassette was constructed by Sung et al. (2001), and is also called *kan-rpsL* cassette. The cassette gives resistance against kanamycin ($Kan^R$), and sensitivity against streptomycin ($Sm^S$). For being able to use the Janus cassette system, your strain must be streptomycin resistant (recessive mutation in *rpsL*), and kanamycin sensitive (Sung et al. 2001). The *rpsL* gene encodes the S12 ribosomal protein, which streptomycin will inhibit. When *rpsL* is expressed both in the introduced cassette and in the wt allele it causes streptomycin sensitivity, making *rpsL* a popular counterstable marker (Dean 1981; Reyrat et al. 1998). The cassette is used in a two-step transformation procedure, making it easy to select transformants.

In the first transformation, the Janus cassette is inserted through homologues recombination, yielding in $Kan^R$ and $Sm^S$. Only transformed bacteria will now be able to survive on petri dishes with TH agar containing kanamycin (400 µg/ml). The second transformation is the deletion of the Janus cassette, by replacing the cassette with constructed template containing only the flanking regions homologue to the target site. By deleting the cassette, the original phenotype is restored ($Sm^R$), and selected transformants can be selected from agar plates containing streptomycin (200 µg/ml). All strains in this research (table 2.1) are constructed by the use of this Janus cassette, and the designed primers for making the different Janus construct are displayed in figure 2.1-5.

### 3.6.2 Protocol for transformation of *S. oralis*

- A start culture of the wildtype GS820 from -80 ºC freezer is left on 37 ºC water bath until defrosted.

- Cells are harvested at 4000 G for 5 min, before supernatant are removed. The pellet is resuspended in 1 ml C-medium. (If start culture contains antibiotics, then dissolve pellet in TH-medium and let it grow for at least an hour in 37 ºC water bath for reducing the antibiotic concentration.)

- Adjust the $OD_{550}$ to 0,06.

- Place the tube containing the cells for 20 minutes on a 37 ºC water bath.

- Two Eppendorf tubes marked plus and minus. In the plus tube 10 µl of template is added and 2,5 µl of CSP. In the minus tube only 2,5 µl of CSP is added, keep on ice.

- 1 ml of the bacteria is then added to each of the eppedorf tubes marked plus and minus.

- Keep the eppendorf tubes on a 37 ºC water bath for about 2,5 hours (3 hours if the bacteria grow slowly).

- Apply 30-40 µl of the cellsuspention on either a Kan 400 µg/ml, or Strep 200 µg/ml agar plate (as described in 3.6.1) and spread it out with a drigalski spatula.

- Let the plates air dry in the sterile bench for about 5 minutes, and then place the plates inside an anaerobic chamber and insert the anaerobic bag

- Place the chamber inside an incubator 37 ºC overnight. Pick and screen colonies the next day. Let the selected colonies grow in the same concentration of either streptomycin or kanamycin in TH-medium. Screening the colonies are done by PCR with One *taq* DNA polymerase.

## 3.7 Microscopy

### 3.7.1 Preparation of samples

- Grow cells until good visible growth

- Add 1 mL of 1% agarose melted in PBS onto a glass slide, and put a second glass slide gently over the agarose, creating a smooth surface. Then remove the slide on top carefully.

- Apply 10 µl of cell suspension on top of the agarose gel, and allow to air dry for 2 minutes.

## 3.8 Testing β-lactam resistance using E-test

β-lactam resistance is often measured by getting the minimal inhibitory concentration (MIC) by adding an antimicrobial agent to a bacterium which determines the lowest concentration that prevents visible growth of a bacterium. This gives a quantitative estimate of the suscebility of the bacterium and it is easy to test (Hendriksen 2003). E-test (epsilometer test) produced by bioMérieux is used to determine MIC values for the different bacteria strains, and comes in strips with readable MIC values.

### 3.8.1 Protocol for testing β-lactam resistance using E-test

- 100 mL soft agar is melted in microwave oven for about 5 minutes.

- While hot, 5 mL is applied in 20 different glass tubes and cooled in a 48 ºC water bath for at least 30 minutes.

- 200 µl of cells in exponential phase grown in TH medium are added to the soft agar, briefly vortexed, and then applied on TH-agar plate, without any antibiotic added.

- Strips containing different type of antibiotics are laid on top of the plates.

- The plates are placed in an airtight chamber with a AnaeroGen$^{TM}$3.5L bag.

- Airtight chamber is incubated in a 37 ºC heating cabinet overnight.

## 3.9 Sanger sequencing

DNA sequencing was first developed in the early 1970s, since then sequencing methods designed to determine the nucleotide order in DNA has progressed a lot over the year. The first methods were based on two-dimensional chromatography, but was fast upgraded to fluorescence based methods. Sequencing since has become much cheaper, more accurate, and with higher throughput. Several different techniques have been developed, called next-generation sequencing.

Sequencing has become a great tool for numerous of applications. Sanger sequencing was discovered in 1975 (Sanger & Coulson 1975), and underwent a breakthrough in 1977, when Sanger sequencing became dideoxy sequencing. Based on the principle of detecting labelled chain-terminating nucleotides, synthesized by DNA polymerase (Sanger & Nicklen 1977). Sanger sequencing requires DNA polymerase, a primer, template, deoxynucleotides (dNTP) and dideoxy nucleotides (ddNTP), and something to separate the fragments, and detect the fluorochromes attached to the ddNTPS. Each ddNTPs are labelled with different fluorochromes, differentiating the four different bases. The property which makes ddNTP special (compared to dNTP), is that it lacks the 3'OH, which inhibits ddNTP of binding the 5'phosphate of the next deoxy nucleotide (or ddNTP), stopping the synthesis (Chidgeavadze & Beabealashivilli 1986). A lower concentration of ddNTPs are added in the sequencing reaction compared to added dNTP, allowing the fragments to grow, and leaving the incorporation of ddNTP at random. This results in the template being synthesized into fragments with different

lengths. These fragments are then subjected to capillary electrophoresis, separated by size in a gel like matrix and fluorescence is detected.

### 3.9.1 Sample preparations

Sequencing *pbp1a* was done for validation of presence of the gene. Samples were sent to a company named GATC. DNA concentration was tested using nanodrop. The amount of PCR product and water was adjusted accordingly. The following protocol was used:

| **Reagent** | **Stock concentration** |
|---|---|
| 3 µl amplified sequence | 100 ng |
| 1 µl Fwd or Rwd primer | 0,2 µM |
| 6 µl $dH_2O$ | |
| Total volume 10 µl | |

### 3.10 Labelling of PBPs with Bocillin FL

Bocillin binds PBPs just like penicillin, but fluoresces. This method allows us to compare affinities of PBP between the different mutants and wildtypes. In a normal pneumococcus, five bands containing the HMW PBP will be separated based on size on the SDS polyacrylamide gel. One band for PBP1a, -1b, -2b and -2x. Bocillin assay is used to check which of the PBS are active.

Protocol:

- Grow cells in a 50 ml TH-medium, adjust OD=0.3

- Resuspend pellet in 100 µl sodium phosphate buffer (20mM, pH 7.2) with 0.2% Triton x-100. Lyse cells by using glass beads and fastprep. Settings on fastprep: 6,5 m/s 0.30 s repeated three times. The lysate can be stored at -80 ºC

- Add 3.3 µM Bocillin to 15µl lysate and incubate at 37 ºC for 30 minutes.

- After the incubation, add 2 x SDS sample buffer and boil for 10 minutes

- Load 10 µl onto a SDS-PAGE with 4 % stacking gel and 10 % separation gel.

- Run gel at 100 V for 15 minutes. Speed up to 200 V and run until the front leaves the gel, then continue running for 90 minutes. Check gel without removing it from the glass plates – if 2b, 2x and 2a have not been separated, run for another 30 minutes.

**10% separation gel:**

6.42 ml 30 % acrylamide

3.5 ml 2 % bisacrylamide

5 ml buffer pH 8.8

4.25 ml $dH_2O$

- Degas for 10 minutes

200 µl 10 % SDS

10 µl TEMED

250 µl 10% APS

- Add 3.2 ml between the plates, level with water

- Set for 15-20 minutes

**4 % stacking gel:**

1 ml 30 % acrylamide

550 µl 2 % bisacrylamide

2.5 ml buffer pH 6.8

5.7 ml $dH_2O$

- Degas for 10 minutes

50 µl bromphenol blue

200 µl 10 % SDS

10 µl TEMED

150 µl APS

- Add combs and set for 20 minutes

# 4. Results

## 4.1 Construction of the GS820 strain from *S. oralis* Uo5 (SPH319)

The GS820 strain was used as a starting point to make all the mutants shown in table 2.1. GS820 is derived from SPH319. It contains no *comA* gene and a mutated *rspL* gene that gives rise to resistance against streptomycin. The parental strain SPH319 was a gift from prof. Regine Hakenbeck. To avoid spontaneous competence induction, the *comA* gene had been deleted by Dr. Gro Stamsås before I started my study. Furthermore, to be able to use the Janus cassette for gene replacement through negative selection (see 3.6.1), the GS820 strain was made resistant to streptomycin. This was done by plating the cells on agar plates containing 1 mg/ml streptomycin and select for spontaneously resistant mutants.

### 4.1.1 Determination of MIC-values for SPH319 and GS820 for three different β-lactams.

Testing of β-lactam resistance was performed by using E-tests from bioMérieux for three different β-lactams; Penicillin G (PG), Amoxicillin (AC) and Oxacillin (OX). The protocol used is described in 3.8.1. The purpose of the experiment was to estimate the level of β-lactam resistance in the GS820 strain compared to the SPH319 parental strain. SPH319 is known to be a highly resistant strain, which expresses three low-affinity PBPs (PBP1a, PBP2x and PBP2b) (Todorova et al. 2015). Although unlikely, there was a chance that the slightly different genetic background of the GS820 strain could influence its MIC-values. The results presented in table 4.1 show a slight decrease in Penicillin G resistance for the GS820 strain compared to the SPH319, while the MICs for the other two β-lactams remain the same.

**Table 4.1 E-test for three different β-lactams, with the observed MIC-values in µg/ml for GS820 and SPH319**

| **Strain** | **Penicillin G 0.02-32 µg/ml** | **Amoxicillin 0.16-256 µg/ml** | **Oxacillin 0.16-256 µg/ml** |
|---|---|---|---|
| GS820 | 6 | 4 | 24 |
| SPH319 | 8 | 4 | 24 |

### 4.1.2 Growth curves for the GS820 and SPH319 strains.

Growth curves for the GS820 and SPH319 are displayed in figure 4.1. Cultures of harvested cells were grown in TH-medium to a $OD_{550}$ = 0.05 before they were transferred to a microtiter plate and incubated in a Synergy Hybrid Reader H1 for 20 hours at 37 ºC. The optical density of the cultures were automatically measured at 492 nm every 10 minutes, and they were shaken every five minutes. The growth curves represent the mean of four parallels for each strain. The experiment was performed twice with highly similar results.

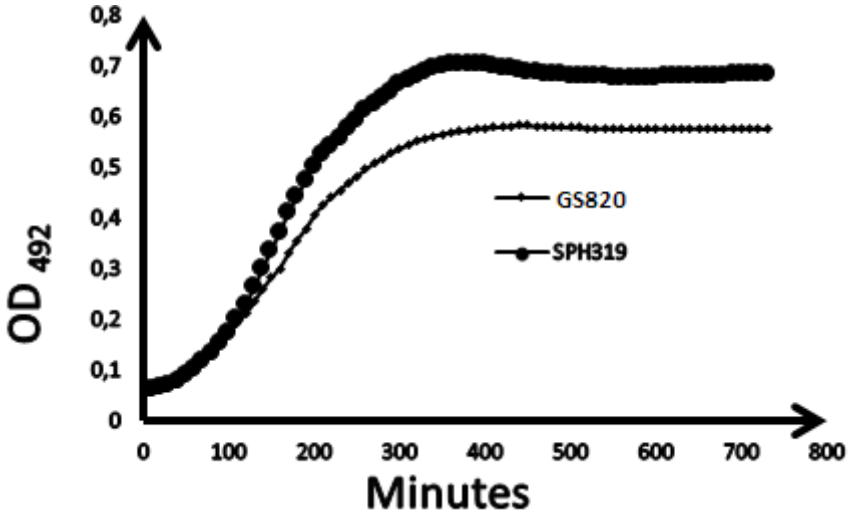


**Figure 4.1 Growth curves for the GS820 and SPH319.** The curves represent the mean of four parallels. The GS820 strain had a doubling time of 64 minutes compared with its SPH319 which had 58 minutes.

The curves depicted in figure 4.1 show a reduced growth rate in the exponential phase for GS820 compared to SPH319.

### 4.1.3 Comparison of the morphologies of GS820 and *S. oralis* Uo5 (SPH319)

To compare the morphologies of the GS820 and SPH318 strains, samples of exponentially growing bacteria were prepared as described in 3.7 and examined with a Zeiss LSM700 microscope**.** Figures 4.2 and 4.3 show cells with an ovoid shape growing in short chains or in pairs. Judging from the light microscope pictures, there is no clear morphological differences between the GS820 and SPH319 strains.

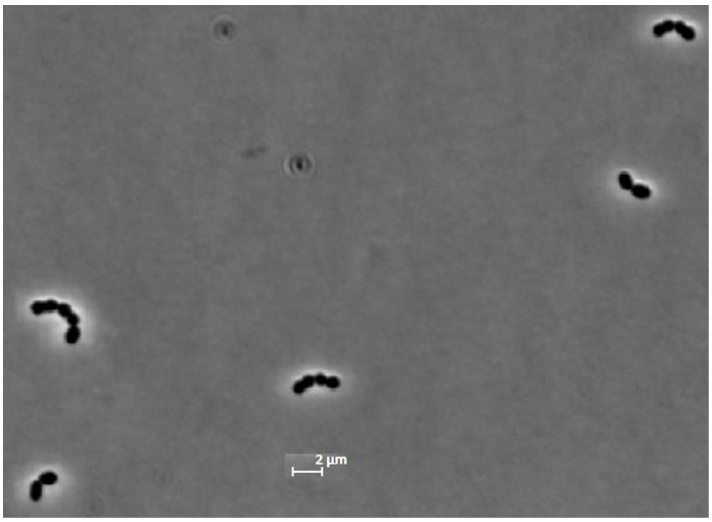


**Figure 4.2 Phase contrast microscopy of the SPH319 strain.**

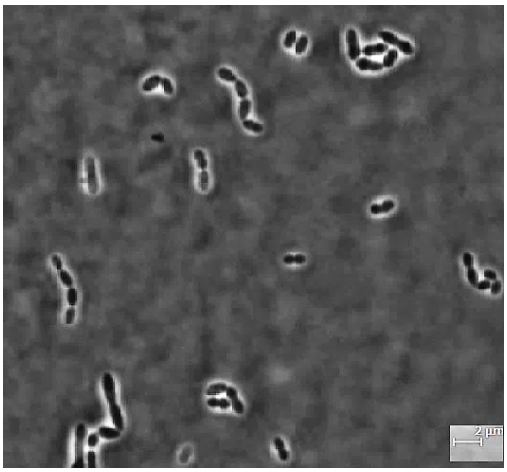

**Figure 4.3 Phase contrast microscopy of the GS820 strain.**

## 4.2 Deletion of penicillin-binding protein 1b

As a first step to identify the PBPs that contribute to the high-resistance profile of *S. oralis* Uo5, PBP1b (SOR_0126) was deleted. The mutant SF4 was constructed by replacing the *pbp1b* gene in GS820 with the Janus cassette through natural transformation induced by synthetic CSP. Next, the Janus cassette was removed by a second transformation with a DNA fragment consisting of only the flanking regions upstream and downstream of *pbp1b*. This gave rise to the strain SF5 (see table 2.1). The protocol is described 3.6.2, and the primers used is given in

figure 2.2. Deletion of PBP1b was done to determine if PBP1b contributes to the high β-lactam resistance level observed for the Uo5 strain. In *S. pneumoniae* the *pbp1b* gene is nonessential. Hence, it was of interest to determine whether *pbp1b* can also be deleted in *S. oralis* Uo5.

### 4.2.1 β-lactam resistance in the Δ*pbp1b* mutant

To find out whether deletion of PBP1b affects the level of β-lactam resistance in the SF5 strain, the MIC-values for Penicillin G, Amoxicillin and Oxacillin were determined. As mentioned above, the goal was to identify the PBPs responsible for the high-level β-lactam resistance in *S. oralis* Uo5. Assessment of the MIC-values was performed with the E-test as described in protocol 3.8.1. The results are given in table 4.2.

**Table 4.2 E-test for three different β-lactams.**

| Strain | Penicillin G (PG) | Amoxicillin (AC) | Oxacillin (OX) |
|---|---|---|---|
| **GS820** | 6* | 4 | 24 |
| **SF5 (Δ*pbp1b*)** | 6 | 8 | 24 |

*MIC-values are given in µg/ml.

The results show that deletion of PBP1b does not affect the level of resistance against Penicillin G and Oxacillin. However, the results suggest that the *Δpbp1b* mutant has become more resistant to Amoxicillin

### 4.2.2 Growth curve for SF5 (Δ*pbp1b*) strain

The SF5 strain was grown in TH medium until the culture reached $OD_{550}$ = 0.05. The culture was then split into four parallels and transferred to a microtiter plate. Growth was measured as described above (4.1.2) for 20 hours in the Synergy Hybrid Reader. The GS820 strain was analysed in parallel for the sake of comparison. The resulting growth curves are presented in figure 4.4.

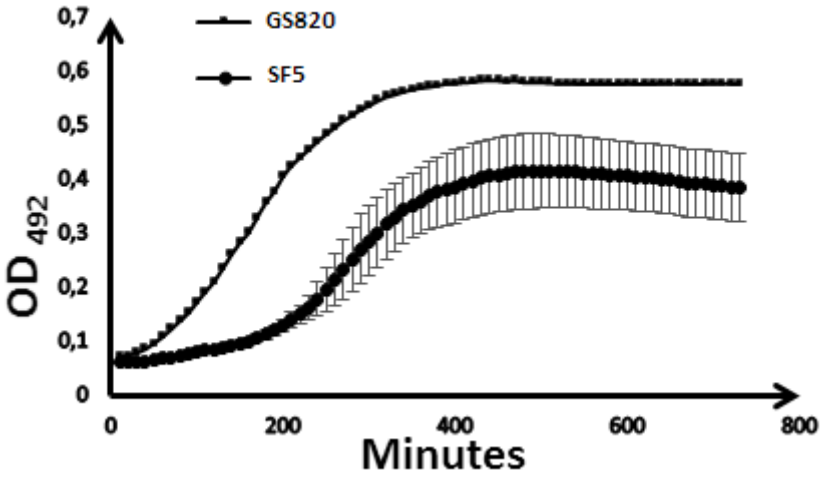


**Figure 4.4 Comparison of growth rates of the GS820 and SF5 (Δ*pbp1b*) strains.** Error **bars** represent standard deviation (n = 4).

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The doubling time during exponential growth was calculated to be ~64 minutes for GS820, while it was ~90 minutes for the SF5 strain.

### 4.2.3 Morphology of SF5 strain

The cells of the SF5 mutant displayed figure 4.5 looks healthy and normal, showing a nice ovoid shape growing as singles, pairs and short chains.

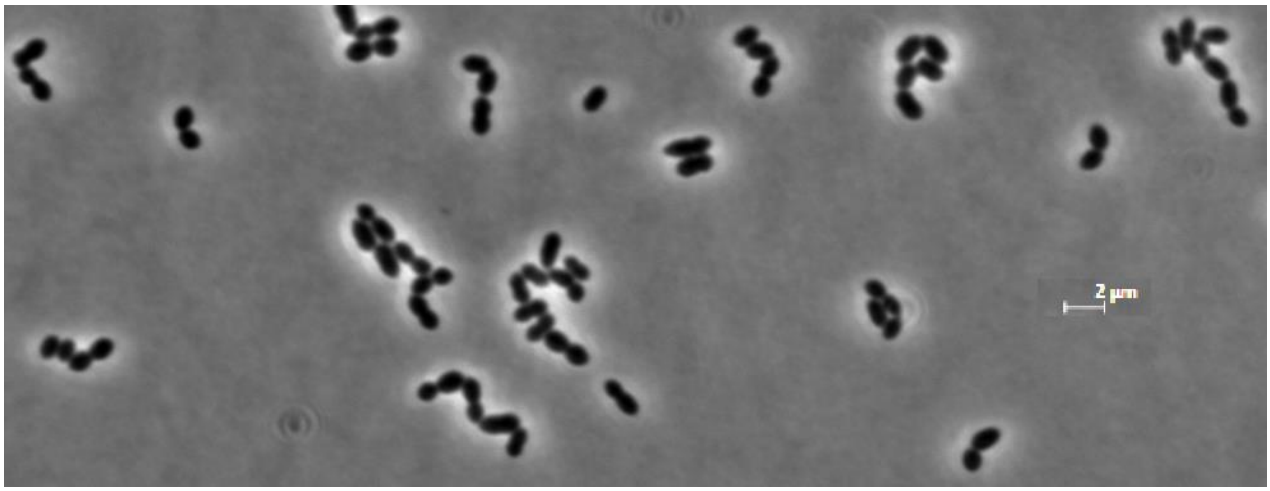


**Figure 4.5 Phase contrast microscopy of SF5 (Δ*pbp1b*).** Scale bar represents 2 µm

## 4.3 Deletion of penicillin-binding protein 2a

The Janus cassette was used to delete the *pbp2a* gene (SOR_0190) of *S. oralis* Uo5 as described in 3.6, giving rise to the GS964 strain. This was done by Dr. Gro Stamsås before I started the present study. The primers and templates employed is illustrated in figure 2.3. PBP2a was deleted for the same reason as PBP1b. It was of interest to determine if PBP2a contributes to β-lactam resistance in the *S. oralis* Uo5 strain, and whether its removal has any impact on growth or morphology of the host. Furthermore, even though PBP2a is not essential in *S. pneumoniae*, we did not know beforehand if it would be possible to delete this PBP in *S. oralis* Uo5.

### 4.3.1 β-lactam resistance in the GS964 (Δ*pbp2a*) mutant

E-test strips were used to determine the MIC-values of Penicillin G, Amoxicillin and Oxacillin for the GS964 strain. The results are displayed in table 4.3. A modest change in resistance is observed for two of the β-lactams tested. The GS964 strain becomes more sensitive to Penicillin G than its parental strain GS820, while it is the other way around for Amoxicillin.

**Table 4.3 MIC-values of three different β-lactams tested on the GS964** *(Δ*pbp2a*)* **strain.**

| Strain | Penicillin G (PG) | Amoxicillin (AC) | Oxacillin (OX) |
|---|---|---|---|
| **GS 820** | 6* | 4 | 24 |
| **GS964 *(Δpbp2a*)** | 4 | 8 | 24 |

*MIC-values are given in µg/ml.

### 4.3.2 Growth curve for GS964 (Δ*pbp2a*) strain

The growth rates of the GS964 strain was determine as described above. The results show that deletion of the *pbp2a* gene has no effect on the growth rate under the growth conditions used (figure 4.6). The doubling time for GS820 was estimated to 64 minutes, while it was estimated to be 65 minutes for the GS964 mutant.

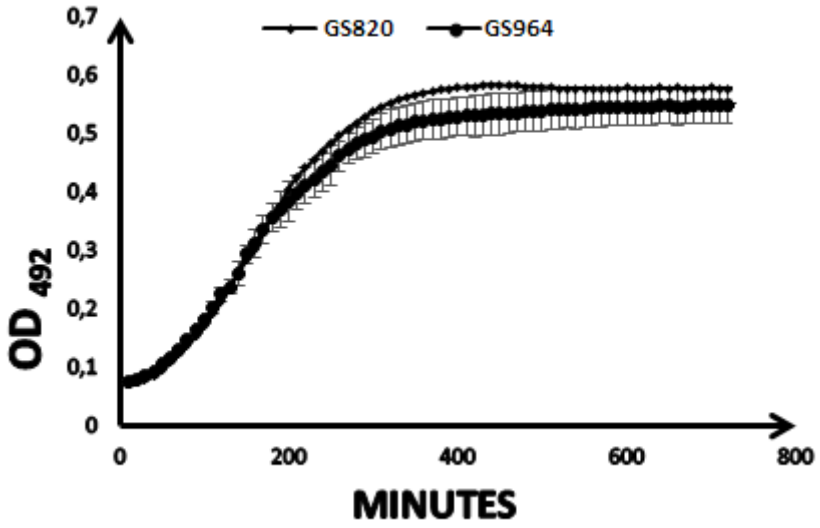


**Figure 4.6 Comparison of growth rates of the GS820 and GS964 (Δ*pbp2a*) strains.** Error bars represent standard deviation (n = 4).

### 4.3.3 Morphology of strain GS964 (Δ*pbp2a*)

Strain GS964 was prepared for phase contrast microscopy as explained above. Most GS964 cells grow in relative short chains and some in pairs. Close examination of the cells revealed that some of them appears swollen. Hence, deletion of PBP2a seems to affect the morphology of *S. oralis* cells.

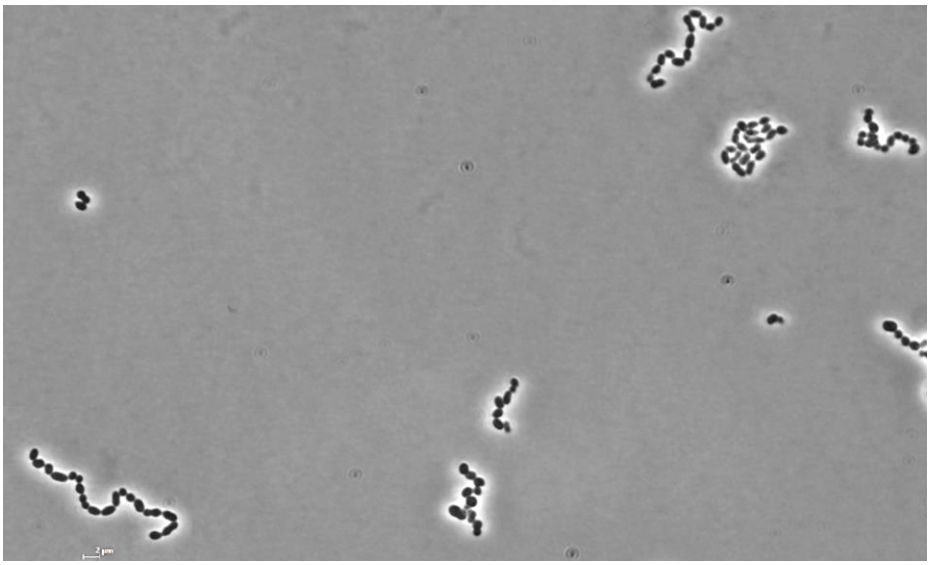

**Figure 4.7 Phase contrast image of strain GS964 (Δ*pbp2a*).** Scale bar represents 2 µm.

### 4.4 Deletion of penicillin-binding protein 2b

Penicillin-binding protein 2b (PBP2b) is an essential transpeptidase in *S. pneumoniae*. It is a key component of the pneumococcal elangosome, and is required for lateral peptidoglycan synthesis. In addition, a low-affinity PBP2b is required for high-level penicillin resistance in *S. pneumoniae*. Surprisingly, when Gro Stamsås used the Janus cassette to delete the *pbp2b* (SOR_0561) gene in *S. oralis* Uo5, a few transformants was obtained. According to Gro Stamsås, fewer transformants were obtained, than in a standard transformation, indicating that the Δ*pbp2b* transformants contain additional mutations that suppress the loss of PBP2b. The primers and templates employed is illustrated in figure 2.3. Sequencing of the regions upstream and downstream of the *pbp2b* gene in one of the Δ*pbp2b* transformants confirmed that the gene had been deleted. This mutant strain was termed GS1012.

#### 4.4.1 Determination β-lactam resistance in the Δ*pbp2b* mutant

The E-tests performed with the GS1012 strain gave the results presented in table 4.4. A huge reduction in resistance against Penicillin G compared to the parental strain GS820 was the most striking result. The MIC-values for Amoxicillin and Oxacillin were also reduced, strongly indicating that deletion of PBP2b causes a general reduction in β-lactam resistance.

**Table 4.4 MIC-values for three different β-lactams tested on the GS1012 (Δ*pbp2b*) strain.**

| Strain | Penicillin G (PG) | Amoxicillin (AC) | Oxacillin (OX) |
|---|---|---|---|
| GS820 | 6* | 4 | 24 |
| GS1012 (*Δpbp2b*) | 1 | 3 | 12 |

*MIC-values are given in µg/ml

#### 4.4.2 Growth curve and estimation of growth rate for the GS1012 (Δ*pbp2b*) mutant

The GS1012 strain grew very slowly after it was transferred to the microtiter plate and incubated in the Synergy Hybrid Reader. After about 300 minutes the growth rate increased dramatically. In addition, the GS1012 strain stopped growing at a lower density that the GS820 reference strain (Fig. 4.8). The doubling time estimated for the GS1012 strain was estimated to

be 85 minutes. The estimate was done in the exponential phase between 0.15-0.3. and 300-385 minutes. Hence, it grows slower than the GS820 strain which has a doubling time of 64 minutes.

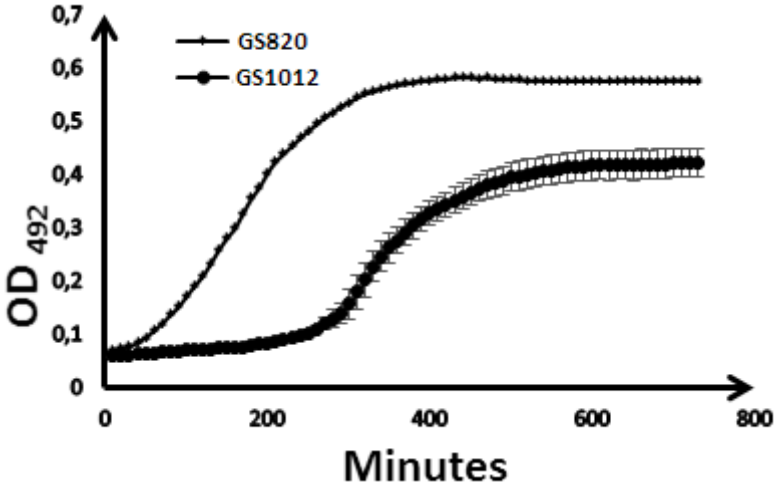


**Figure 4.8 Growth curve of a mutant (GS1012) lacking *pbp2b* compared with the its parental strain (GS820.** Error bars represent standard deviation (n = 4).

### 4.4.3 Morphology of the GS1012 (Δ*pbp2b*) strain

The morphology of the cells lacking *pbp2b* is shown in figure 4.9. Most of the cells have an abnormal morphology. They vary in shape from very small to very large. In addition, both small and large cells have a rounded shape, i.e. they appear less elongated than GS820 cells.

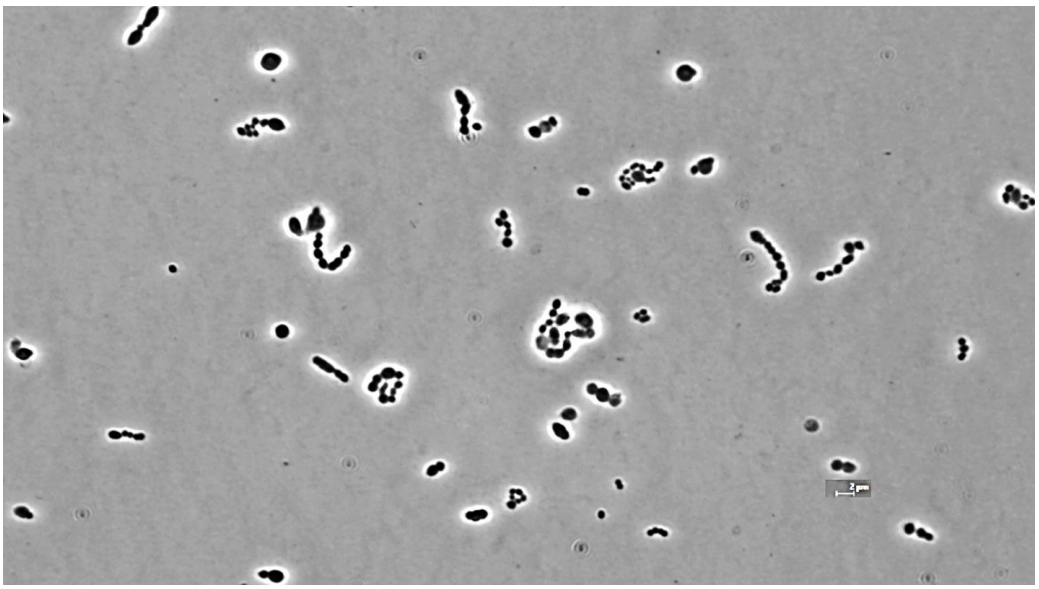

**Figure 4.9 Phase contrast image of the GS1012 (Δ*pbp2b*) mutant.** Scale bare represents 2 µm

In sum, the GS1012 mutant cells look very sick.

## 4.5 Construction of two *pbp1b/pbp2a* double-knockout strains

In *S. pneumoniae* it is possible to construct a *pbp1b/pbp2a* double-knockout strain. Streptococci have three bifunctional class A PBPs, namely PBP1a, PBP1b and PBP2a. *S. pneumoniae* can survive with only PBP1a or PBP2a, but a pbp1a/pbp2a double-knockout strain is not viable. Based on the data published on pneumococcal PBPs, it should be possible to delete the *pbp2a* gene in the SF5 (Δ*pbp1b*) strain (see 4.2). Hence, the SF5 strain was transformed with a DNA fragment consisting of the Janus cassette flanked by ~1000 bp sequences corresponding to the regions immediately upstream and downstream of the *pbp2a* gene. The Janus cassette was subsequently removed through negative selection by transforming with a DNA fragment consisting of the fused flanking regions. The resulting strain was named SF7. A *pbp1b*/*pbp2a* double-knockout strain was also constructed from the GS964 (Δ*pbp2a*) strain by deleting the *pbp1b* gene as outlined above. The resulting strain was called SF9. SF7 and SF9 are both *pbp1b/pbp2a* double-knockout strains, and should be genetically identical.

### 4.5.1 Determination of β-lactam MIC-values for the SF7 (Δ*pbp1b*/Δ*pbp2a*) and SF9 (Δ*pbp2a*/Δ*pbp1b*) strains

The results of the E-tests for the SF7 and SF9 strains are shown in table 4.4. Surprisingly, the MIC-values for these strains are not the same. The SF7 strain is significantly more resistant than the GS820 strain for all β-lactams tested. In contrast, the SF9 strain has become more sensitive against the three antibiotics compared to GS820. This result was unexpected as the same genes were deleted in the two strains. The only difference was the order in which the *pbp2a* and *pbp1b* genes were deleted.

**Table 4.5 MIC-values for three different β-lactams tested on the SF7 (Δ*pbp1b*/Δ*pbp2a*) and SF9 (Δ*pbp2a*/Δ*pbp1b*) strains**

| Strain | Penicillin G (PG) | Amoxicillin (AC) | Oxacillin (OX) |
|---|---|---|---|
| GS820 | 6 | 4 | 24 |
| SF7 | 12 | 12 | 32 |
| SF9 | 6 | 8 | 12 |

*MIC-values are given in µg/ml

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### 4.5.2 Growth curves and estimation of growth rate for the SF7 and SF9 strains

The growth rates of the SF7 and SF9 strains were compared to GS820 (Fig. 4.10). Both strains have a doubling time of about 55 minutes, and consequently grow somewhat faster than the GS820 strain. The latter has a doubling time of about 64 minutes. In all cases the doubling time was estimated from the exponential part of the growth curve. In addition to growing more rapidly than the GS820 strain, the double-mutants reaches a higher OD before they enter stationary phase.

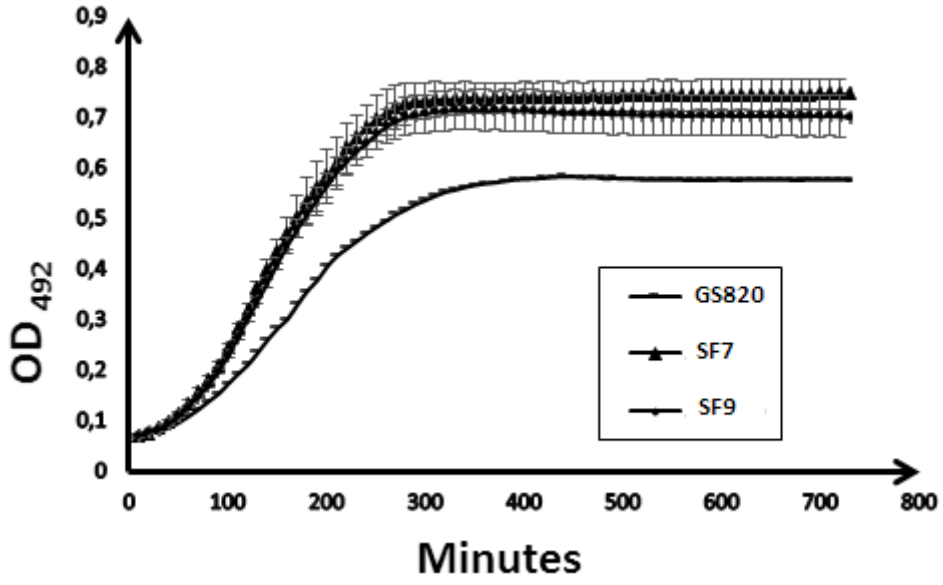


**Figure 4.10 Growth curves for the GS820, SF7 (Δ*pbp1b*/*Δpbp2a*) and SF9 (Δ*pbp2a*/Δ*pbp1b*) strains**. Error bars represent standard deviation (n = 4).

### 4.5.3 Morphology of the SF7 and SF9 strains

The morphologies of the SF7 and SF9 mutants, both of which lack the genes encoding PBP1b and PBP2a, are shown in figures 4.11 and 4.12. Both strains grow in relatively long chains. Up to 50 cells were counted in a single chain. Apart from growing in chains, the morphology of the cells appears normal for the SF7 as well as for the SF9 strain.

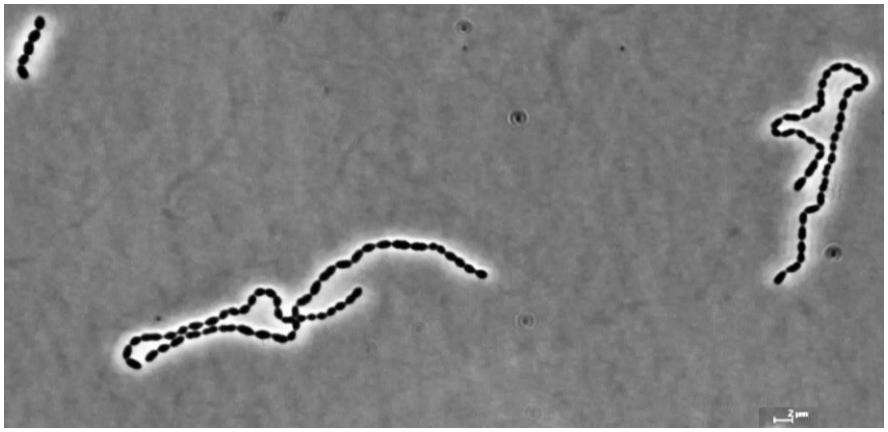

**Figure 4.11 Phase contrast image of the SF7 (Δ*pbp1b*/Δ*pbp2a*) strain. Scale bar represents 2µm.**

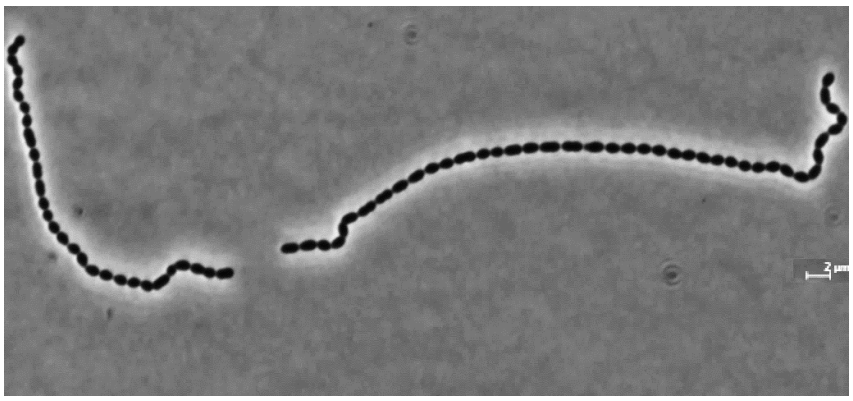

**Figure 4.12 Phase contrast image of the SF9 (Δ*pbp2a*/Δ*pbp1b*) strain. Scale bar represents 2µm.**

### 4.6 Deletion of penicillin-binding protein 1a

Using the same strategy as before, I tried to delete the *pbp1a* (SOR_1641) gene from the GS820 strain. No transformants were obtained, even though the experiment was repeated several times.

### 4.7 Construction of a *pbp1b*/*pbp1a* double-knockout mutant

Although I did not succeed in making a *pbp1a* single-knockout mutant, I tried to delete the *pbp1a* gene in strains where one of the other PBPs had already been deleted. I failed to delete *pbp1a* in strains harbouring a Δ*pbp2a* or Δ*pbp2b* mutation, but surprisingly it was possible to delete the *pbp1a* gene in a Δ*pbp1b* background. The resulting *pbp1b*/*pbp1a* double-knockout mutant was named SF6.

### 4.7.1 MIC-values for three different β-lactams tested on the SF6 (Δ*pbp1b*/Δ*pbp1a*) strain

While removal of the *pbp1b* gene alone did not reduce β-lactam resistance in the SF5 strain, a strong reduction in resistance was observed for the SF6 strain (table 4.6). The reduction was particularly strong for Penicillin G and Amoxicillin. This result clearly shows that the low-affinity PBP1a protein expressed by *S. oralis* Uo5 contributes significantly its ability to grow in the presence of high levels of β-lactams.

**Table 4.6 MIC-values for three different β-lactams tested on the SF6 (Δ*pbp1b*/Δ*pbp1a*) strain.**

| Strain | Penicillin G (PG) | Amoxicillin (AC) | Oxacillin (OX) |
|---|---|---|---|
| GS820 | 6* | 4 | 24 |
| SF6 | 1,5 | 0,75 | 16 |

*MIC-values are given in µg/ml

### 4.7.2 Growth curves and estimation of growth rate for the SF6 strain

The growth curves of SF6 and the GS820 reference strain are similar. However, estimation of their growth rates from the exponential parts of their curves indicates that the SF6 strains grows faster than the GS820 strain. The estimated doubling times for SF6 and GS820 were 51 and 64 minutes, respectively. In contrast to the GS820 strain, the SF6 strain starts to autolyse immediately after reaching stationary phase.

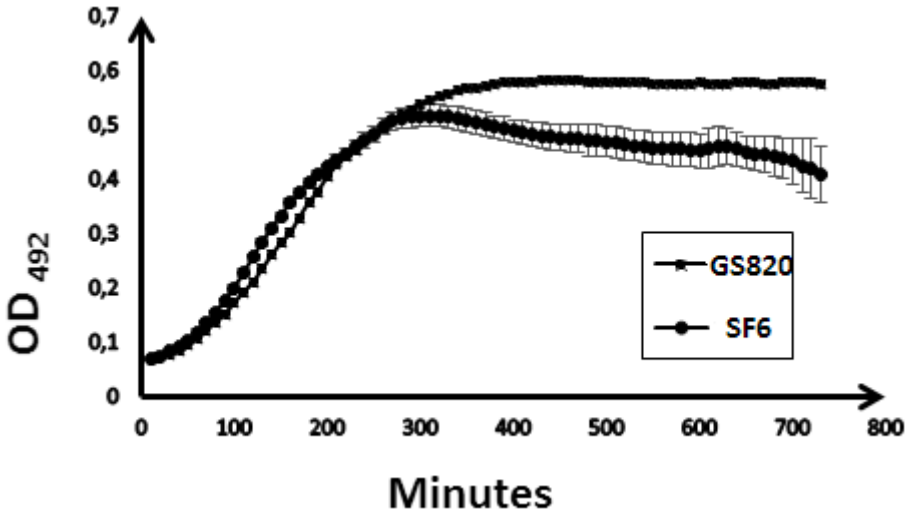


**Figure 4.13 Growth curves for the GS820 and SF6 (Δ*pbp1b*/Δ*pbp1a*) strains**. Error bars represent standard deviation (n = 4).

### 4.7.3 Morphology of the SF6 strain

The phase contrast image of the SF6 strain (Fig. 4.14) shows cells with an odd morphology that form pairs but not chains. Many cells appear elongated and generally enlarged in size, suggesting that they struggle to divide.

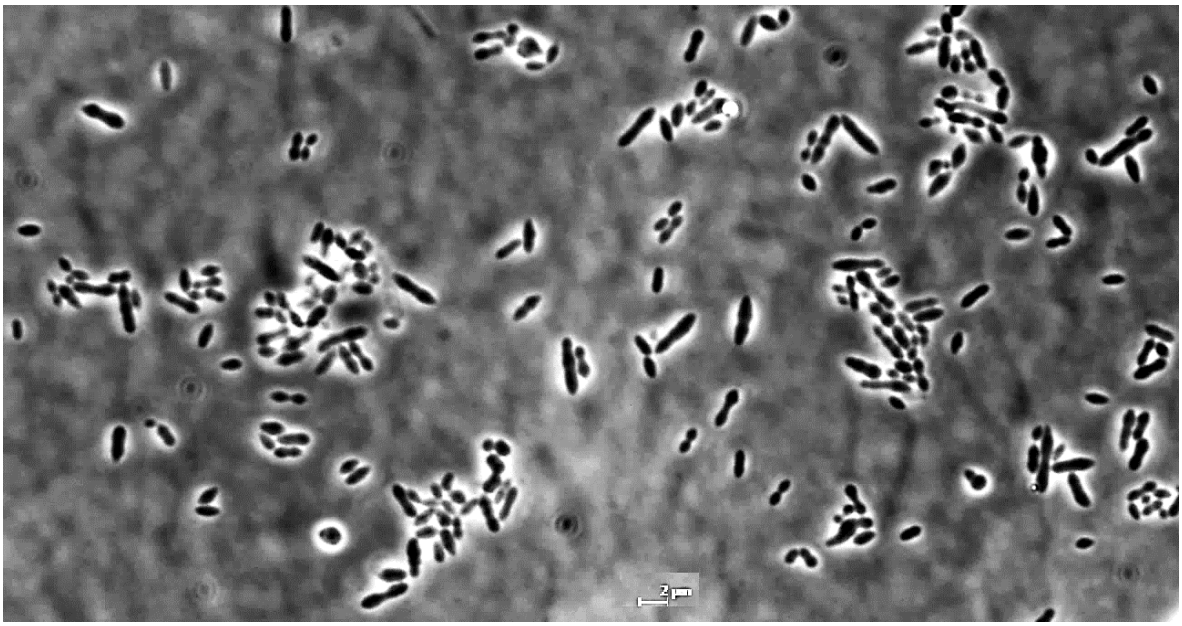

**Figure 4.14 Phase contrast image of the SF6 (Δ*pbp1b*/Δ*pbp1a*) double-knockout strain.** Scale bar represents 2µm.

### 4.8 Construction of a *pbp2a*/*pbp2b* double-knockout mutant

The strain GS964 lacking PBP2a was used as a starting point to make the SF11 (*pbp2a*/*pbp2b*) double-knockout mutant. The 964 strain was transformed with a DNA fragment consisting of the Janus cassette flanked by ~1000 bp sequences corresponding to the regions immediately upstream and downstream of the *pbp2b* gene. Thus, in the resulting SF11 strain the Janus cassette replaced the *pbp2b* through homologous recombination.

#### 4.8.1 Determination of β-lactam MIC-values for the SF11 (Δ*pbp2a*/Δ*pbp2b*) strain

E-test strips containing Penicillin G, Amoxicillin or Oxacillin were used to determine MIC-values for the SF11strain for these antibiotics. As shown in table 4.7, the SF11 strain displayed a strong reduction in resistance against Penicillin G and Oxacillin compared to the GS820 strain, while it had become less sensitive to Amoxicillin.

**Table 4.7 MIC-values for three different β-lactams tested on the SF11 (Δ*pbp2a*/Δ*pbp2b*) strain.**

| Strain | Penicillin G (PG) | Amoxicillin (AC) | Oxacillin (OX) |
|---|---|---|---|
| GS820 | 6* | 4 | 24 |
| SF11 | 1,5 | 8 | 3 |

*MIC-values are given in µg/ml

#### 4.8.2 Growth curves and estimation of growth rate for the SF11 strain

Figure 4.15 show the results of an experiment carried out to compare the growth rates of the SF11 and GS820 strains. Estimation of the growth rates from the exponential part of the curves showed that the doubling time for the GS820 mutant is about 64 minutes while it was calculated to be 66 minutes for the SF11 strain. Thus, it can be concluded that there is no significant difference between the strains with respect to growth rate during the exponential phase. The SF11 strain stopped growing at a somewhat lower density that the reference strain.

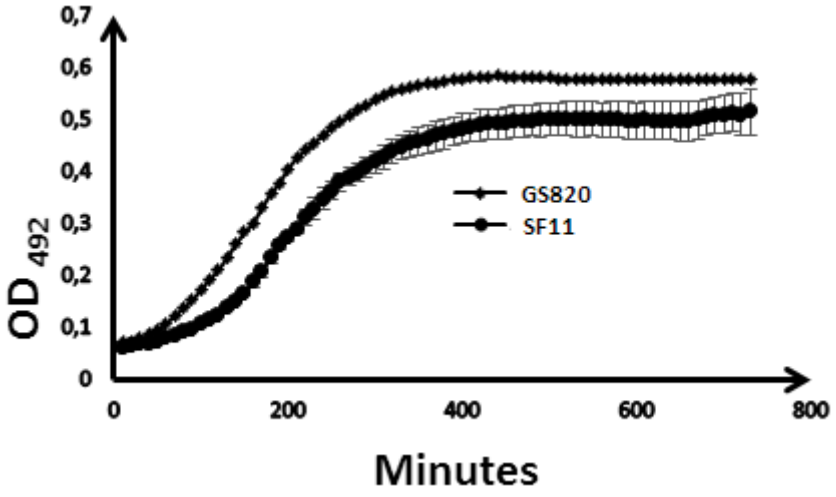


**Figure 4.15 Growth curves for the GS820 and SF11 (Δ*pbp2a*/Δ*pbp2b*) strains**. Error bars represent standard deviation (n = 4).

### 4.8.3 The morphology of the double knock SF11 strain

Phase contrast microscopy of the SF11 strain (Fig. 4.16) revealed small cells that did not look like classic ovoid cocci. Their shape were more spherical than elongated. The cells rarely grew alone, but formed moderately long chains.

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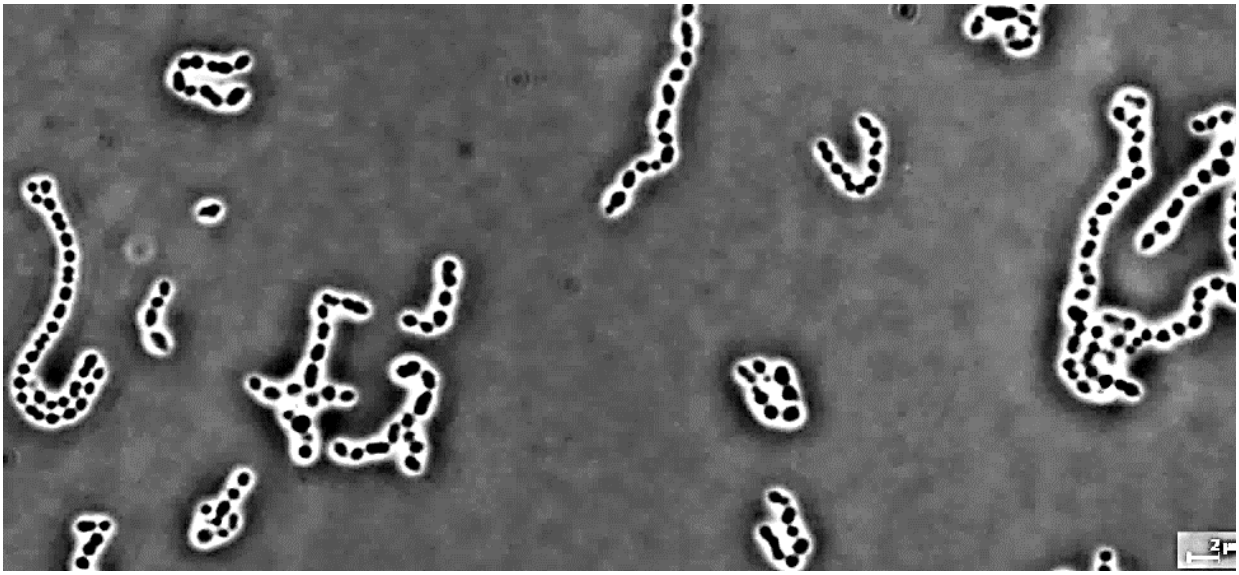

**Figure 4.16 Phase contrast image of the SF11 (Δ*pbp2a*/Δ*pbp2b*) double-knockout strain.** Scale bar represents 2µm

### 4.9 Deletion of penicillin-binding protein 2x

Despite several attempts, I did not succeed in deleting the *pbp2x* (SOR_0341) gene of *S. oralis* Uo5. GS820 cells were transformed with a DNA fragment consisting of the Janus cassette flanked by sequences corresponding to the upstream and downstream regions of the *pbp2x* gene. No transformants were obtained when the transformation mix was plated on agar plates containing the appropriate concentration of kanamycin.

### 4.10 Bocillin FL assay

Bocillin FL is a fluorescent penicillin V derivative that binds tightly to penicillin-binding proteins (PBPs). When added to a bacterial culture the penicillin V part of Bocillin FL will bind covalently to the active site of all PBPs containing a transpeptidase domain, i.e. PBP1a, PBP1b, PBP2a, PBP2x, PBP2b and PBP3. After separation of bacterial proteins on a SDS-PAGE gel the PBPs can be visualized by a FluorImager. To analyze the mutants constructed in the present study I used the protocol described in 3.10. The experiment was done twice, with the same results. An image of the Bocillin FL-stained mutant strains are shown in figure 4.17. It is not possible to separate PBP1a and PBP1b under the conditions used.

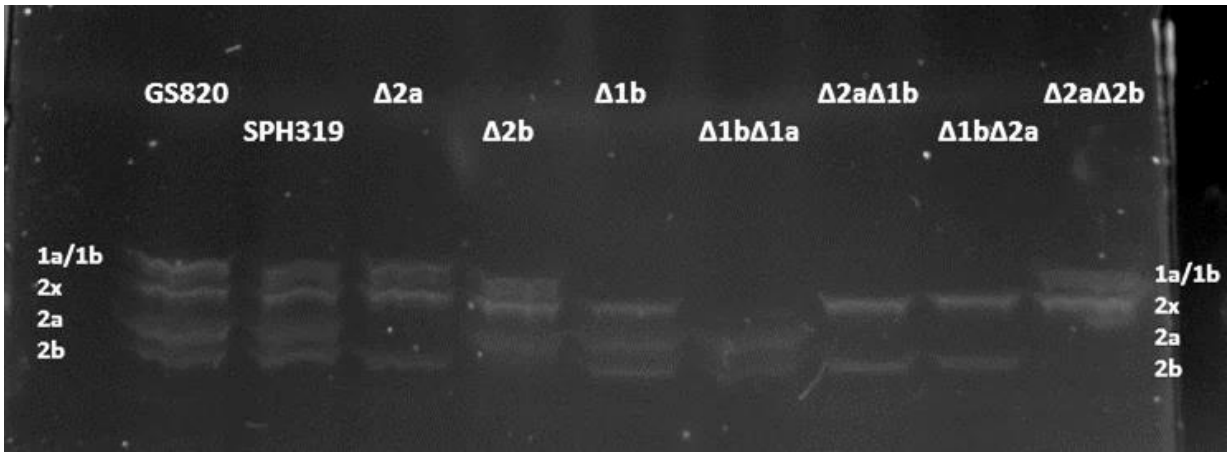


**Figure 4.17** Bocillin FL fluorogram of the various PBP mutant strains constructed in the present study. The picture is taken with an Azure Biosystems C400 FluorImager. The exposure time was 30 seconds. Positions of the bands corresponding to PBP1a (1a), PBP1b (1b), PBP2x (2x), PBP2a (2a) and PBP2b (2b) are indicated.

# 5. Discussion

## 5.1 Comparison of the GS820 strain with its precursor SPH319

Estimation of the doubling times for the GS820 and SPH319 strains revealed that GS820 has a slightly decreased growth rate compared to its precursor SPH319. The GS820 strain mainly grow in pairs and short chains, with a morphology highly similar to SPH319, i. e. *S. oralis* Uo5 (Fig. 1.2). GS820 was derived from the SPH319 strain by deletion of the *comA* gene and selection for resistance towards streptomycin. The latter always involves acquisition of specific amino acid changes in the ribosomal *rpsL* gene. It is conceivable that changes in the *rpsL* gene could have a negative impact on the function of the ribosomes and consequently slightly reduce the growth rate of the GS820 strain. The morphology and β-lactam resistance, on the other hand, was not appreciably effected. The small decrease in Penicillin G resistance for the GS820 strain compared to the SPH319 strain reported in table 4.1, might be an experimental error that occurred when I read the MIC-values. All MIC-values for the three β-lactams are at the high end of the E-test scale, especially for Oxacillin and Penicillin G. Hence, it can be difficult to discern between small differences at the very "top" of the scale.

## 5.2 Deletion of penicillin-binding protein 1b

Deletion of the *pbp1b* gene in *S. oralis* Uo5 did not have a large impact on its β-lactam resistance profile (Table 4.2). A slight increase in Amoxicillin resistance was detected, but again, it could be due to just an error in interpreting the MIC zone on the agar plate. This indicates that PBP1b does not contribute to the high-level β-lactam resistance phenotype of *S. oralis* Uo5. The reason for this could be that PBP1b is a high-affinity PBP (β-lactam sensitive), or that it does not have an important function in the cell wall metabolism of *S. oralis* Uo5. The fact that PBP1b can be deleted shows that it is not essential under laboratory conditions. Another possibility is that the SF5 strain might have acquired suppressor mutations that alleviated the loss of PBP1b. Besides, it is known that class A PBPs in *S. pneumoniae* can have overlapping functions and to a certain degree substitute for each other.

The growth rate of the SF5 strain was significantly reduced compared to GS820. The SF5 strain has a much longer lag phase than GS820, and stops growing at a lower cell density ($OD_{492}$ = 0.4 compared to ~0.6 for GS820). This indicates that deletion PBP1b has an impact on the cell

wall metabolism of *S. oralis* Uo5, suggesting that other PBPs cannot fully compensate for its loss.

The morphology of the SF5 strain is displayed in figure 4.5. It shows ovoid cells growing in pairs and short chains, i. e. with a morphology highly similar to the GS820 strain. To what degree the cells are functioning normally without PBP1b is difficult to evaluate.

### 5.3 Deletion of penicillin-binding protein 2a

Deletion of the *pbp2a* gene led to minimal changes in growth rate and β-lactam resistance. This indicates that removing PBP2a in *S. oralis* Uo5 does not affect resistance, as already described for *S. pneumoniae* (Muños et al. 1992). The cells are growing in chains and as diplococci, and have a somewhat different morphology compared to the GS820 strain (Fig. 4.7). In addition to forming longer chains than the GS820 strain, the cells of the GS964 mutant strain seem to vary in length. Together these results show that removal of the *pbp2a* gene from the Uo5 strain affects its morphology, but does not greatly affect the its ability to grow and proliferate. Similar to PBP1b, PBP2a does not seem to contribute much to the high-level resistance phenotype of *S. oralis* Uo5. This might be because PBP2a is penicillin sensitive, and/or because its function in the synthesis of cell wall peptidoglycan can be easily substituted for by one of the other class A PBPs.

### 5.4 Deletion of penicillin binding protein 2b

Unexpectedly, colonies were obtained on kanamycin agar plates when the GS820 strain was transformed with the Janus cassette containing flaking sequences homologous to the sequences flanking the *pbp2b* gene. This was unexpected because deletion of *pbp2b* is not possible in *S. pneumoniae* (Kjell et al. 1993). The Bocillin FL assay (Fig. 4.17) confirmed that the GS1012 strain lacks PBP2b. Although transformation with the *pbp2b* deletion fragment gave rise to kanamycin resistant transformants, the number of transformants obtained were significantly lower than for a standard transformation. This indicates that deletion of *pbp2b* exerts severe stress on the bacterial host. Presumably, those who survived has acquired suppressor mutations that to a certain degree alleviate the stress imposed by the loss of PBP2b.

Cells in the GS1012 culture show large heterogeneity. Some cells are small with a round shape, while others are large and balloon-shaped. In sum, they look very sick (Fig. 4.9). The growth experiment shown in figure 4.8 confirms that the GS1012 mutant is struggling. The biphasic growth pattern displayed by the GS1012 culture (Fig. 4.8) suggests that a small fraction of the cells in the culture possesses a beneficial mutation that give them an advantage with respect to growth rate. Hence, they grow faster than the rest of the population and eventually dominates the population, giving rise to an overall faster growth rate for the culture. It was recently discovered in *S. pneumoniae* that deletion of MltG, a membrane-bound lytic transglycosylase, also removes the requirements of PBP2b (Tsui et al. 2016). Hence, it is possibility that the GS1012 mutant has acquired a suppressor mutation that inactivates MltG.

Interestingly, the GS1012 mutant has much lower resistance against all three types of β-lactams tested than the GS820 strain (table 4.4). This big reduction in MIC values demonstrates that PBP2b plays a central role in the development of high-level β-lactam resistance in *S. oralis* and presumably other streptococci. This is in accordance with studies in *S. pneumoniae*, which have shown that high-level resistance requires the presence of low-affinity versions of PBP1a, PBP2x and PBP2b (Hakenbeck et al. 2012). In addition, it is reasonable to assume that the cell wall of the GS1012 strain is highly abnormal and probably weakened. This might also contribute to reduced resistance against the β-lactams tested.

### 5.5 Deletion of penicillin binding protein 1b and 2a

When removing the two apparently high-affinity PBPs, PBP1b and PBP2a from Uo5, it is likely that the cell wall biosynthesis machinery does not function normally. Because we feared that construction of a double-knockout mutant would introduce stress that might select for transformants with additional suppressor mutations, removal of *pbp1b* and *pbp2a* was done in two different ways. SF7 was made by first removing *pbp1b* then *pbp2a*, while it was done the other way around for SF9. The morphologies of the SF7 and SF9 strains were identical, displaying long chains of up to 50 cells. The mutants also displayed identical growth curves (Fig. 4.10), but they differed greatly in β-lactam resistance. Because the differences in resistance levels are large, it is unlikely that they are due to experimental errors. Besides, the experiment was conducted twice. Thus, the only explanation for the observed results is that the SF7 and/or SF9 strain have acquired additional mutations that influence their MICs against the β-lactams tested. The SF9 strain is not so different from the GS820 strain, so in this case

difficulties in reading correct MIC-values from the scale on the E-test strip could have influenced the results. In other words, the SF9 strain and the GS820 strain might actually have the same MIC values. If this is correct the SF9 strain does not contain suppressor mutations. In contrast, the SF7 strain almost certainly contains suppressors. Compared to the GS820 strain, it has become more resistant against all three antibiotics, especially Amoxicillin. It would have been very interesting to sequence the whole genome of the SF7 strain to identify the suppressor mutation(s).

The SF7 and SF9 strains have a shorter doubling time than the GS820 strain as well as the SPH319 wild-type strain. This was unexpected, as lacking both PBP1b and PBP1a is not considered to be favourable for growth. Since growth is measured as optical density, it is possible that growing in long chains in contrast to pairs will influence the determination of growth rate. However, it is more likely that the SF7 and SF9 strains have acquired suppressor mutations that somehow compensate for the loss of PBP1b and PBP2a.

### 5.6 Deletion of penicillin-binding protein 1a

PBP1a can be deleted in *S. pneumoniae* (Paik et al. 1999). It was therefore surprising that I did not succeed in deleting PBP1a in S. oralis Uo5. If my result is correct, it indicates that the specific functions of PBP1a and/or the other class A PBPs are not exactly the same in *S. oralis* and *S. pneumoniae*.

### 5.7 Deletion of penicillin-binding protein 1b and 1a

Only when PBP1b was removed it became possible to remove PBP1a, a finding that is difficult to understand. Perhaps PBP1a and PBP1b work together in the wild-type cell in an interdependent manner. PBP1a alone might be tolerated, while PBP1b alone might be harmful.

Deletion of PBP1b gave little or no change in resistance (Table 4.2), while removal of PBP1a in addition to PBP1b in the SF6 strain resulted in a modest decrease in resistance against Oxacillin and large decreases in resistance against Penicillin G and Amoxicillin (Table 4.6). These results are in accordance with data reported for *S. pneumoniae*. As mentioned above, Hakenbeck and co-workers have found that high-level β-lactam resistance in *S. pneumoniae* requires low-affinity versions of PBP1a, PBP2x and PBP2b. Hence, it is not surprising that deletion of PBP1a results in a large drop in MIC-values. When, both PBP1a and PBP1b is

deleted, the only class A PBP left in the cell is PBP2a. PBP2a is most likely a high-affinty PBP. Thus, when PBP1a and PBP1b are absent, the cell is completely dependent on the more β-lactam sensitive PBP2a, and the MIC-values consequently drops.

In the pneumococcus a double-knockout mutant of pbp1b and pbp1a has been reported to be viable, but it exhibit defects in positioning of the septum (Paik et al. 1999). Microscopy images the SF6 strain shows elongated cells, which could indicate that they are struggling with respect to septum formation and cell division (Fig. 4.14). In streptococci that struggles to form septal cross-walls, peripheral peptidoglycan synthesis will dominate and the cells will become more elongated (Morlot et al. 2003).

The initial removal of *pbp1b* did not change the appearance of the cells. In contrast, when *pbp1a* was also deleted, the morphology of the cells changed dramatically. The only remaining PBP with transglycosylase activity present in the SF6 double-knockout mutant is PBP2a. Transglycosylase activity, i.e. polymerization of the NAG-NAM-pentapeptide precursor into glycan strands, is required for cross-wall synthesis as well as peripheral peptidoglycan synthesis (Morlot et al. 2004). Consequently, PBP2a should be involved in both processes in the SF6 strain. However, recently it was discovered that RodA, a member of the SEDS family of proteins, is a peptidoglycan polymerase (Meeske et al. 2016). RodA is part of the elongasome in pneumococci, where it together with the transpeptidase PBP2b probably constitute the core peptidoglycan synthesis machinery (Straume et al., 2017, Molecular Microbiology). Furthermore, RodA has a homologue, FtsW, which together with the transpeptidase PBP2x probably constitute the core peptidoglycan synthesis machinery in the divisome. The function of class A PBPs might therefore be to act as accessory proteins that assist the core machineries in ways that we do not yet understand, or perhaps they are involved in peptidoglycan repair and maintenance. Nevertheless, the finding that the SF6 strain form elongated cells suggest that PBP1b and PBP1a are required for normal cell division, indicating that they work together with FtsW and PBP2x to synthesize the septal cross-wall.

I did not succeed in constructing *pbp1a*/*pbp2b* and *pbp1a*/*pbp2a* double-knockout mutants, strongly indicating that these combinations are lethal in *S. oralis* Uo5. This is in accordance with the results reported by Paik et al. (1999). They found that PBP2b is essential in *S. pneumoniae,* and that a *pbp1a*/*pbp2a* double-knockout mutant is not viable.

### 5.8 Deletion of penicillin-binding protein 2a and 2b

Interestingly, it was possible to construct a *pbp2a*/*pbp2b* double-knockout mutant in *S. oralis* Uo5. The strain was called SF11. The *pbp2a* gene was deleted first due to the very abnormal (sick) cells observed when the GS1012 (Δ*pbp2b*) strain was made. Judging from their morphologies and growth rates the SF11 double-knockout mutants look healthier that the GS1012 single-knockout mutants. This is hard to understand, but it is tempting to speculate that suppressor mutations are involved. Presumably, the GS1012 strain is very sick because the suppressor mutations it has acquired can only partly compensate for the sever stress imposed by the absence of the *pbp2b* gene. The SF11 strain might have been "luckier" with its suppressors resulting in a healthier mutant strain. It is also possible that deletion of the *pbp2a* gene function as a suppressor that alleviates the stress imposed by the loss of the *pbp2b* gene. This question can only be solved by performing whole-genome sequencing of the GS1012 and SF11 strains.

The E-test analysis of the SF11 strain revealed a large decrease in resistance against Penicillin G and Oxacillin. The decrease in MIC against Penicillin G was approximately the same as observed with the Δ*pbp2b* single-knockout mutant GS1012. However, the reduction in Oxacillin resistance is much larger for SF11 than for GS1012. It appears that the combined loss of *pbp2a* and *pbp2b* has made the SF11 mutant much more sensitive to Oxacillin. Again, it is difficult to know whether this phenotype results from the double-knockout mutations alone, or if there is also additional suppressor mutations involved. Another curiosity that is difficult to explain without involving hypothetical suppressor mutations is the observation that resistance to Amoxicillin is significantly reduced in the GS1012 strain while it is unchanged in the SF11 double mutant.

The microscopy images in figure 4.16 show that removal PBP2a and PBP2b results in abnormally small and compressed cells. They resemble the shape of the *pbp2b* single-knockout cells, but grow in longer chains. This is in agreement with the findings of Berg et al. (2013) who recently showed that depletion of PBP2b in *S. pneumoniae* gives rise to long chains of cells that are compressed in the direction of the long axis, resulting in a round or lentil-like appearance. The chains suggest that the cells are not able to separate from each other after cell division. LytB is responsible for the final stage of daughter cell separation in *S. pneumoniae* (De Las Rivas et al. 2002; García et al. 1999). Hence, it is possibility that deletion of *pbp2b* alters the substrate of LytB, resulting in a non-functional or poorly functional enzyme.

### 5.9 Deletion of penicillin-binding protein 2x

In my hands, the *pbp2x* gene was impossible to delete. This indicates that PBP2x is absolutely required for survival. PBP2x is essential in *S. pneumoniae* too (Kjell et al. 1993; Paik et al., 1999). Studies in which the expression of PBP2x has been gradually depleted have shown that pneumococci growing with suboptimal levels of PBP2x become somewhat elongated and lemon shaped, indicating that they struggle to divide (Berg et al. 2013) .

### 5.10 Bocillin FL assay

The Bocillin FL gel depicted in figure 4.17 constitutes strong evidence that the mutants are correct in the sense that the PBPs targeted are actually deleted. This eliminates the possibility that the Janus cassette could have been recombined into a different location in the genome, leaving the targeted *pbp* gene intact. The GS820 and SPH319 strains displays five bands, one for each high-molecular weight PBP. This result differed from Bocillin FL analyses performed on *S. oralis* Uo5 by Todorova et al. (2015). In their analyses, only the bands corresponding to PBP1b and PBP2a were detected. This is a reasonable result considering that *S. oralis* Uo5 is highly resistant to penicillins, and therefore must produce at least some PBPs with low affinity for these antibiotics. They concluded that PBP1a, PBP2x and PBP2b have low affinity for penicillins, while PBP2a and PBP1b bind penicillins with higher affinity and consequently are more sensitive. So why did all PBPs interact with Bocillin FL in our analysis? A possible explanation is the difference in incubation times used. Regine Hakenbeck and co-workers incubated 5 µl cell lysate with Bocillin FL for 20 minutes, while I incubated 15 µl cell lysate with Bocillin FL for 30 minutes. Low-affinity PBPs react more slowly with penicillin. Hence, the longer reaction time would in all likelihood have labelled both low-affinity and high-affinity PBPs.

Judging from the Bocillin FL gel the GS964 (Δ*pbp2a*), GS1012 (Δ*pbp2b*) and SF11 (Δ*pbp2a*/Δ*pbp2b*) mutant strains all seem to be correct with respect to the PBPs deleted.

In case of the SF5 (Δ*pbp1b*) mutant, however, something unexpected has happened. Instead of detecting four high molecular weight PBPs, only three are present. Both PBP1a and PBP1b seem to be lacking. It is very strange that the absence of PBP1a has not affected the level of resistance in the SF5 mutant. This puzzling result could be due to a mix-up of strains, or some other experimental error.

The lane loaded with lysate from the SF6 (Δ*pbp1b*/Δ*pbp1a*) strain lacks bands corresponding PBP1b and PBP1a as expected, but the binding of Bocillin FL to PBP2x is much weaker than in neighbouring lanes. It is unlikely that less SF6 lysate is loaded, as the bands corresponding to PBP2a and PBP2b is clearly visible. A possible explanation could be that the expression of PBP2x is downregulated in the SF6 mutant. This hypothesis is in agreement with the elongated and very abnormal morphology displayed by the SF6 cells. Another imaginable explanation is that PBP2x, which is a low-affinity PBP in *S. oralis* Uo5, has stained poorly in this case. An even longer incubation time might have confirmed or rejected this possibility.

As intended, bands corresponding to PBP2a and PBP1b were missing in the SF7 and SF9 mutants. In addition, the band corresponding to PBP1a appeared to be missing. Loss of all three class A PBPs is highly unlikely, as it would certainly be lethal. Furthermore, it does not fit with the E-test results for the SF7 and SF9 strains. Deletion of PBP1a should give a significant reduction in β-lactam resistance in itself. This is not observed. On the contrary, β-lactam resistance increases in the SF7 strain. Hence, the apparent loss of PBP1a is in all likelihood not real. The most plausible explanation for its disappearance is that its expression level has been reduced, or that it stains poorly because of its low affinity for β-lactams.

# 6. Concluding remarks and future work

The subject of the present work was the highly β-lactam resistant strain *S. oralis* Uo5 and its PBP-proteins. Because some PBPs are essential, while others are more or less functionally redundant, they are notoriously difficult to study. Although, my results were hard to interpret, they are in general agreement with those reported by Todorova et al. (2015). PBP1b and PBP2a seem to be much more sensitive to β-lactams than PBP1a, PBP2x and PBP2b, and apparently contributes little to the overall MIC of the Uo5 strain against Penicillin G, Amoxicillin and Oxacillin. A big problem when studying proteins that exert a lot of stress when they are deleted is the emergence of suppressor mutants that can mask the phenotype of the primary mutations. On the other hand, it is well established that the identification of so-called suppressor mutants and their further phenotypic and molecular analysis represent a very powerful tool for mapping protein pathways/complexes and the interactions between their components. Thus, the best way to continue this study would be to subject all the mutants to whole genome sequencing in order to verify that they are correctly made and to identify and characterize possible suppressor mutations.

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# Appendix A. Bocillin FL assay

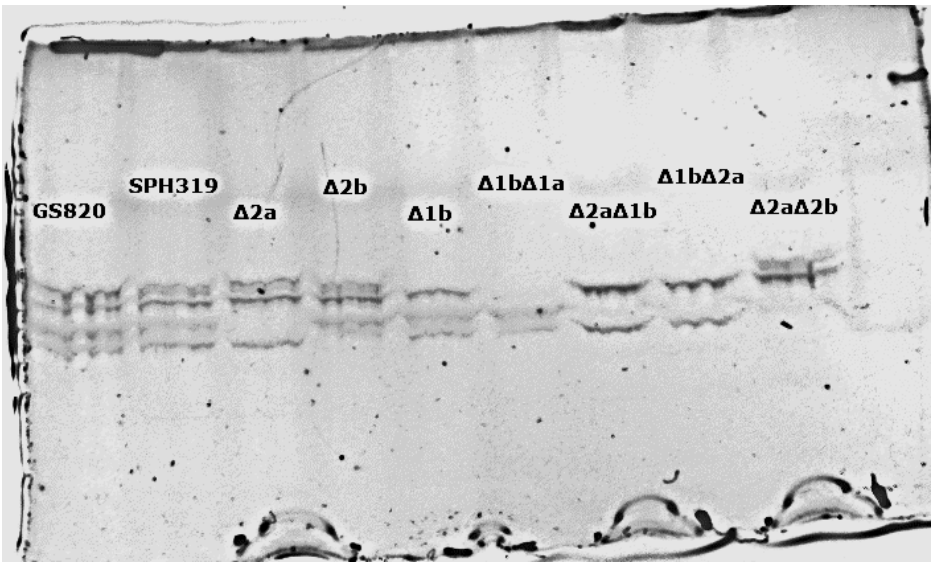


**Figure A.1.** Image of Bocillin FL assay, 10 µl (not 15 µl as the other) cell lysate added.

# Appendix B. E-test β-lactams

## Penicillin G resistance

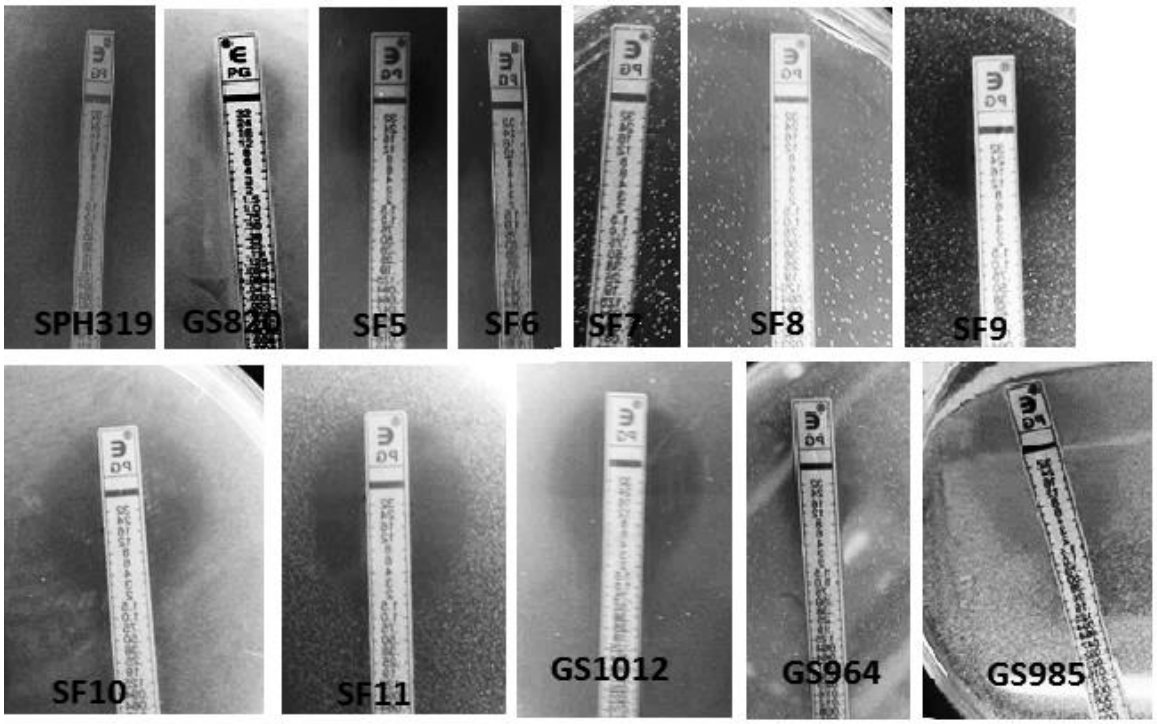


**Figure B.1.** Phase contrast image of the conducted E-test with Penicillin G for the strains described in this study.

## Amoxicillin resistance

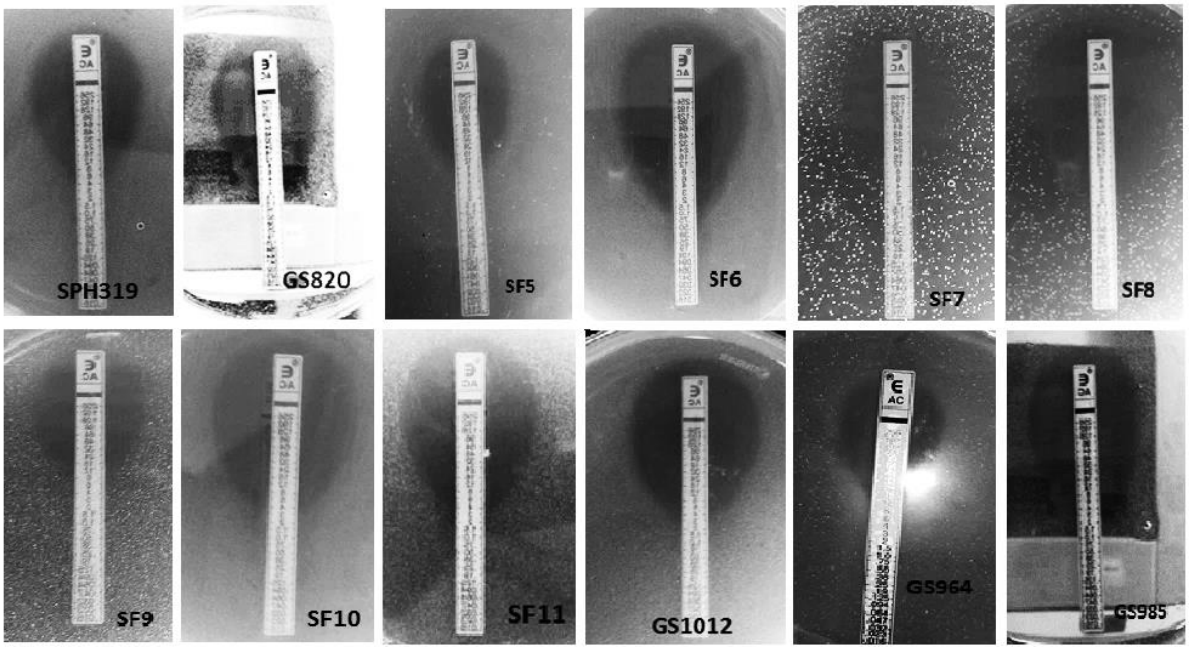


**Figure B.2**. Phase contrast image of the conducted E-test with Amoxicillin for the strains described in this study.

## Oxacillin resistance

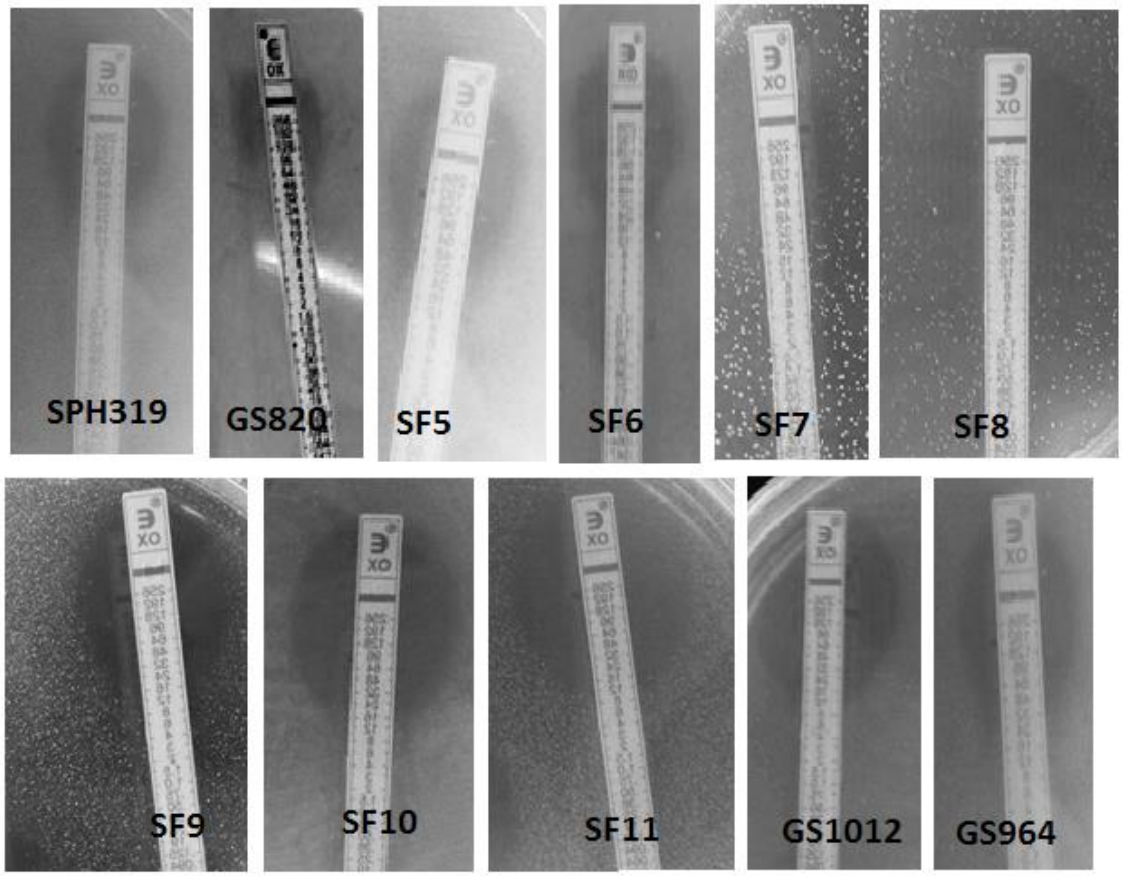


**Figure B.3**. Phase contrast image of the conducted E-test with Oxacillin for the strains described in this study.


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