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Exploring the function of the putative fratricins of *Streptococcus mutans* and *Streptococcus vestibularis*

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Henny Lyfjell

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Abstract

Antibiotic resistance is a growing problem in modern society and is largely caused by bacteria being able to exchange genetic material with each other through horizontal gene transfer. Streptococci have been shown to be competent for natural transformation, a process in which bacteria can acquire new genetic material from the environment. The enzyme responsible for making DNA available is called fratricin, and this enzyme has been extensively studied in *Streptococcus pneumoniae*. However, there is limited knowledge about fratricins from other streptococcal species. Therefore, the purpose of this thesis was to investigate the putative fratricins LytF and CbpD from *Streptococcus mutans* and *Streptococcus vestibularis*. The aim was to investigate if the fratricins are competence induced and to identify their function in the transformation process.

Using a luciferase reporter assay it was shown that transcription from P_{lytF} and P_{cbpD} increased upon competence induction. Although the signal strengths were relatively weak, the results indicate that the genes are competence induced. By knocking out *lytF* in *S. mutans*, it was also discovered that LytF has an important role in the DNA-uptake process, as the transformability was severely reduced. CbpD from *S. vestibularis*, on the other hand, had limited impact on this process, as knockout of the fratricin reduced the transformation efficiency minimally. It was therefore speculated that CbpD in *S. vestibularis* only functions as a fratricin, while LytF on the other hand may have an additional role in the DNA-uptake process in competent *S. mutans* cells. Since the transformation pilus is an important part of the uptake mechanism, it has been suggested that the fratricin-like proteins may help the pilus to penetrate the peptidoglycan layer of the cell wall, allowing the pilus to exit the cell. However, immunofluorescence microscopy suggested that the pilus protein ComGC was present on the cell surface of Δ*lytF* cells, although it was present in slightly smaller amounts in the mutant compared to the wildtype. These findings may indicate that certain pores within the cell wall of *S. mutans* are wide enough for unassisted pilus penetration. However, since the activity of LytF increases the transformability of the bacterium, it is possible that the fratricin enlarges the pores to ensure a more efficient pilus penetration through the cell wall. Further experiments are needed to confirm this finding, as there is a possibility that the results are a consequence of another function performed by LytF. Lastly, it was also investigated whether LytF functions as a cell wall hydrolase, but zymography revealed no muralytic activity for this protein.

Sammendrag

Antibiotikaresistens er et økende problem i dagens samfunn og skyldes i stor grad at bakterier kan utveksle genetisk materiale med hverandre gjennom horisontal genoverføring. Streptokokker er blant annet vist å være kompetente for naturlig transformasjon, en prosess der bakterier kan få tilgang til nytt genetisk materiale fra omgivelsene. Enzymet som er ansvarlig for å tilgjengeliggjøre DNA kalles fratricin, og dette enzymet har blitt nøye undersøkt i *Streptococcus pneumoniae*. Kunnskap om fratriciner fra andre streptokokkarter er derimot mangelfull, og denne oppgaven har derfor hatt som formål å undersøke de antatte fratricinene LytF og CbpD fra henholdsvis *Streptococcus mutans* og *Streptococcus vestibularis*. Målet med oppgaven var å undersøke om fratricinene er kompetanse-induserte og å kartlegge deres funksjon i transformasjonsprosessen.

Ved å gjennomføre et luciferase reporter assay ble det vist at P_{lytF} og P_{cbpD} økte ved induksjon av kompetanse. Selv om signalstyrken var relativt svak, indikerer resultatene at genene er kompetanse-induserte. Ved å knocke ut *lytF* i *S. mutans* ble det også oppdaget at LytF har en viktig rolle i DNA-opptaksprosessen ettersom transformasjonsevnen ble kraftig redusert. CbpD fra *S. vestibularis* har derimot liten innvirkning på denne prosessen, da knockout av fratricinet reduserte transformasjonseffektiviteten minimalt. Det ble derfor spekulert i at CbpD i *S. vestibularis* bare fungerer som et fratricin, mens LytF på den andre siden kan ha en tilleggsrolle i DNA-opptaksprosessen hos kompetente *S. mutans* celler. Siden transformasjonspilusen er en viktig del av opptaksmekanismen, har det blitt foreslått at det fratricin-liknende proteinet kan hjelpe pilusen med å penetrere peptidoglykanlaget i celleveggen, slik at pilusen kommer seg ut av cellen. Immunofluorescens mikroskopering antydet derimot at pilusproteinet ComGC i var tilstede på celleoverflaten til Δ*lytF* mutanten, selv om den var til stede i litt mindre grad hos mutanten sammenliknet med villtypen. Resultatene indikerer derfor at visse porer i celleveggen til *S. mutans* er brede nok til at pilusen kan komme seg gjennom uten assistanse. Ettersom aktiviteten til LytF øker bakteriens transformasjonsevne er det imidlertid mulig at det antatte fratricinet forstørrer porene for å sikre en mer effektiv piluspenetrering av celleveggen. Ytterligere eksperimenter trengs likevel for å bekrefte dette, da det er en mulighet for at resultatene er en konsekvens av en annen funksjon som utføres av LytF. Til slutt ble det også undersøkt om LytF fungerer som en cellevegghydrolase, men zymografi avslørte ingen muralytisk aktivitet for dette proteinet.

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1 Introduction

1.1 *Streptococcus*

The genus *Streptococcus* includes over 100 species of bacteria with a broad range of ecological and epidemiological characteristics[1, 2]. This genus consists of Gram-positive bacteria that grow in pairs or chains and are non-spore-forming[3]. Streptococci are part of the normal microflora of the human oral cavity and intestine, as well as being commonly found on the skin, throat and upper respiratory tract[1]. Despite the fact that many streptococci are opportunistic only under the right conditions, streptococcal infections are among the most important problems facing medicine today. Pathogenic streptococcal species were isolated and reported as early as the end of the 19th century, and diseases caused by these bacteria include pneumoniae, meningitis and scarlet fever[1, 3]. Streptococci not only cause disease in humans but are also an important group of pathogens that cause bovine mastitis, one of the most common and costly diseases in the dairy industry[4]. Due to their large medical and economic impact, it is essential to gain a better understanding of how streptococci cause disease and how to combat them.

Streptococci have traditionally been classified according to their hemolytic pattern on blood agar[3]. α-hemolytic colonies perform partial lysis of red blood cells and appear as green or brown colonies on blood agar. β-hemolytic colonies perform complete lysis, resulting in a transparent zone around the colonies, while the colonies that do not lyse blood cells are γ-hemolytic[5]. The Lancefield system can further distinguish some of the β-hemolytic species from each other through carbohydrate antigens found in the bacterial cell wall, but this system cannot be used for accurate identification of specific β-hemolytic species[6]. As more streptococci have been isolated, there has been a need for better approaches to distinguish them both at species- and strain levels. The use of 16S rRNA sequencing has made it possible to identify bacteria at species level in a more accurate way, dividing the streptococci into six phylogenetic groups: pyogenic, anginosus, mitis, salivarius, bovis and mutans[7]. *Streptococcus suis* has also been placed in a separate group, as this species has several phenotypical and genetically diverse strains (**Figure 1.1**)[8].

Streptococcus mutans is a species that was first isolated by Clarke et al in 1924[9]. The bacterium is facultative anaerobic and belongs to a group of mutans streptococci consisting of several closely related species[10]. Within this group, *S. mutans* is one of the species most commonly isolated from humans[11]. The natural habitat of this species is the human oral cavity and infants are colonized soon after the first teeth have emerged[12, 13]. In addition to being a major cause of

dental caries, the bacterium can also cause infective endocarditis if it enters the bloodstream[14]. Another streptococcal species that also has been associated with human infections like bacteremia and endocarditis is *Streptococcus vestibularis*[15, 16]. This species was isolated from the vestibular mucosa of human oral cavities by Whiley and Hardie in 1988[17]. Although both *S. vestibularis* and *S. mutans* inhabits the human oral cavities, *S. vestibularis* has an intermediate cariogenic potential compared to *S. mutans*[18]. *S. vestibularis* belongs to the salivarius group of streptococci and is usually considered as a commensal bacterium, even though it has been found to cause serious human infections[19].

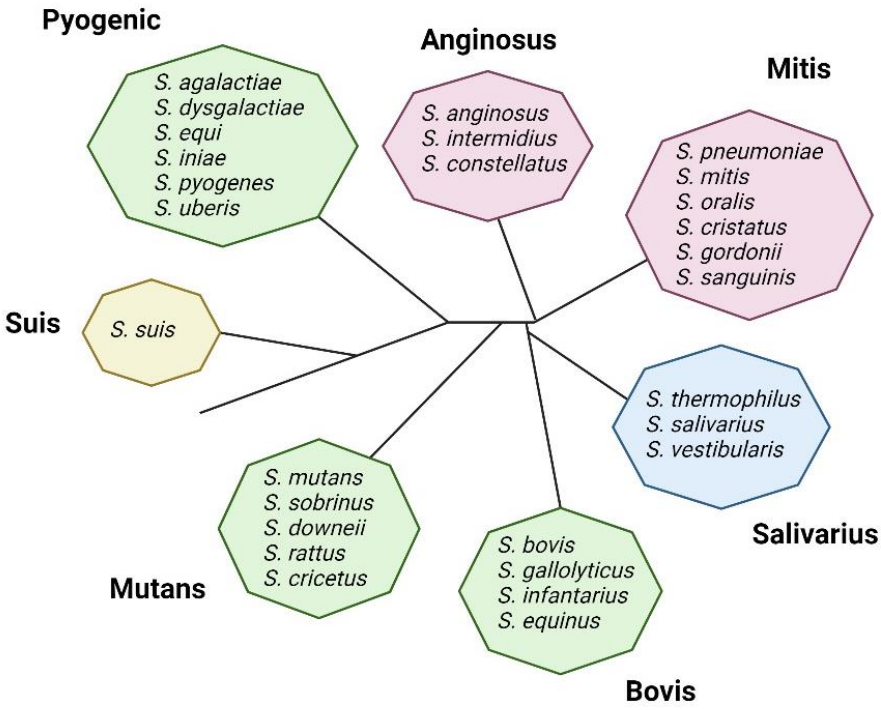

Figure 1.1 Phylogenetic relationship between the seven subgroups of streptococci based on 16S rRNA sequencing. The colors in the figure illustrate what kind of system the different subgroups use for natural transformation: ComCDE (red), ComRS type-I (blue), ComRS type-II (green), and ComRS type-III (yellow). The figure is adapted from Shanker et al and created with BioRender.com[20].

1.1.1 Antibiotic resistance

Antibiotic resistance is a constantly increasing problem and several outbreaks of disease caused by multi-resistant bacteria have occurred in recent years. The increasing level of resistance in bacteria has long been associated with the increasing use of antimicrobials, which has resulted in a selective pressure among the exposed bacteria[21]. The antibiotic resistance crisis has also arisen because the pharmaceutical industry does not consider development of antimicrobials to

be an economically profitable investment, as production of new antibiotics is very time-consuming and development of resistance is almost inevitable[22]. In addition, they still make a huge profit on existing drugs. Since antibiotics are only used for a short period of time, it is also more profitable for the pharmaceutical industry to produce medicines that treat chronic diseases.

In general, antibiotic resistance can be divided into intrinsic and acquired resistance[23]. Intrinsic resistance is a phenomenon where bacterial species are naturally resistant to certain antibiotics, whereas acquired resistance is due to chromosomal point mutations or acquisition of mobile resistance genes[21]. Acquisition of genetic elements occurs through horizontal gene transfer and is a key event in the emergence of multidrug-resistant strains. Various plasmids in streptococci are associated with transfer of antibiotic resistance and large parts of their gene pool also consists of mobile and exogenous DNA[24,21]. Horizontal gene transfer has therefore been important for the genetic diversity in streptococci and is largely responsible for the development and spreading of resistance in these species.

1.1.2 Biofilm

Many streptococcal species are known as biofilm producers. Biofilms are organized communities of microbes embedded in a protective extracellular matrix consisting of polysaccharides, proteins, DNA and lipids[25]. Biofilm formation is often induced by environmental changes that are stressing the cells, such as antimicrobial agents and nutrient limitation[21]. An important property of biofilms is their ability to protect the microorganisms against mechanical forces and the host's immune system, in addition to increase the bacterial tolerance against antimicrobial agents[21]. Biofilm formation can also promote horizontal gene transfer between inhabitants of the biofilm, thereby promoting the uptake of resistance genes. The ability of streptococci to become antibiotic resistant is therefore also related to their ability to produce biofilm.

Dental caries is a condition that affects a large part of the world's population, and its development is associated with the formation of biofilm. *S. mutans* is believed to be the primary agent participating in this serious condition as this bacterium is highly effective at forming biofilm on hard tissues in the oral cavity[26, 27]. One factor that contributes to oral biofilm development in *S. mutans* is its ability to produce glucosyltransferase, an enzyme that synthesizes glucan from sucrose. The synthesized glucan can interact with surface-associated

glucan binding proteins and thereby promote cell-cell aggregation and adhesion to the tooth enamel[28-30]. In the absence of sucrose, however, attachment of *S. mutans* occurs through interactions between salivary agglutinins and adhesins[26]. One of the adhesins produced by *S. mutans* is called streptococcal protein antigen P (also known as antigen I/II) and is responsible for promoting colonization of the human oral cavity[29].

Biofilm formation in the oral cavity is a complex process and involves several salivary components. α-amylase is an abundant protein in the human saliva and binds to α-amylase-binding proteins (ABPs) found on bacterial surfaces[31]. Salivary amylases release glucose and maltose from starch, which are further metabolized by oral bacteria to form the biofilm in dental plaque[31]. α-amylase binding streptococci (ABS) make up a significant proportion of the dental plaque microflora and most of these species express several α-amylase binding proteins[32]. *S. vestibularis* is known to produce only one α-amylase binding protein, which may be one of the reasons why this species is only mildly cariogenic[32, 33]. *S. vestibularis* is also unable to aggregate to sucrose as the bacterium lacks the ability to produce extracellular polysaccharides from this substrate, and this may also affect its cariogenicity[17, 34]. For *S. mutans,* on the other hand, there is some controversy as to whether or not the bacterium can bind to amylase-coated surfaces[31, 32, 35]. Despite this, *S. mutans* can build a potentially more cariogenic biofilm when sucrose is combined with starch, because starch hydrolysates can be incorporated during glucan synthesis and thus increase bacterial attachment[31, 36].

1.1.3 Pathogenesis and virulence factors

In order for a bacterium to cause disease, several basic requirements must be met. Firstly, the bacterium must be able to attach to a tissue surface and compete with the organisms that are present. For *S. mutans*, this involves adherence to the tooth surface and to other bacteria that are present in the biofilm on the tooth[29]. In addition to this, the microbe needs to be capable of harming the host to be considered a pathogen. *S. mutans* is a bacterium that usually affects the tooth surface and thus rarely causes invasive disease[29]. The bacteria produce acids that damage tooth enamel and the metabolism of *S. mutans* is therefore key to the pathogenesis of dental caries. *S. mutans* can metabolize a wider variety of carbohydrates than many other bacteria, and the fermentation of these carbohydrates is the principal source of energy[29]. *S. mutans* has also been shown to metabolize sucrose to lactic acid faster than other oral bacteria, and this property is probably due to the many enzyme systems that can transport and metabolize sucrose[37, 38].

The ability to tolerate acids also serves as a virulence factor in *S. mutans*[28]. The pH value in dental plaque decreases in the presence of fermentable carbon sources, and the bacteria's own production of acids would have resulted in suicide if the bacteria had not shown a remarkable acid tolerance[38, 39]. Inhabitants of dental plaque experience rapid pH fluctuations and must therefore have an acid tolerance mechanism to withstand the acid shock[39]. *S. mutans* is more acid tolerant than many other bacteria and this is largely due to a membrane bound F_1F_0 ATPase proton pump that keeps the intracellular pH at 7.5[14, 28, 29]. The proton pump allows *S. mutans* to cope with the constantly changing pH levels and reduce the denaturing effects of an acidic cytoplasm[39]. It also ensures that enzymes and other cellular processes, which would otherwise be inhibited by an acidic intracellular compartment, function properly[40].

Another virulence factor in *S. mutans* involves the production of bacteriocins[41]. Bacteriocins are antimicrobial peptides and when produced by *S. mutans*, they are more specifically called mutacins. *S. mutans* can produce different types of mutacins belonging to the two major classes of bacteriocins named lantibiotics and non-lantibiotics[42]. The lantibiotic mutacins have a wide spectrum of activity against Gram-positive bacteria, whereas the non-lantibiotic mutacins only are active against closely related species[43]. The mutacins can help the bacteria to compete for limited nutrients in their environment and aid in the establishment by reducing the presence of competitors during tooth colonization. Mutacins are therefore considered an important factor affecting the colonization of *S. mutans* in dental biofilm[44].

1.2 Horizontal gene transfer

Horizontal gene transfer involves the exchange of genetic material between more or less distantly related organisms, and differs from the usual vertical transfer of genes from parent to offspring[45]. This form of genetic exchange has long been seen as an important force in the evolution of bacteria, and has been linked to the development of antibiotic resistance and pathogenicity[46]. Three mechanisms for horizontal gene transfer in bacteria are transduction, transformation and conjugation (**Figure 1.2**). Transduction is the transfer of genetic material from one bacterium to another via bacteriophages. This kind of transfer occurs when a bacteriophage mistakenly encapsulate bacterial DNA , either instead of, or in addition to its own genome[47]. Transformation involves the uptake of free DNA from the environment, while conjugation is the transfer of genetic material between a donor and a recipient through direct contact with a conjugation pilus[46]. Once the DNA has been transported inside the bacterium, it has several possible fates, and can either be degraded by the bacterium's own nucleases or it

can be integrated into the genome. Integration of the incoming DNA depends on the degree of homology to the genomic DNA of the host, or that it is physically associated with other sequences that can be integrated, such as transposable elements and bacteriophage genes[48]. Conjugative plasmids can, however, also exist as separate replicative units without being integrated into the genome. In this way, horizontal gene transfer can contribute to increased genetic diversity in bacteria.

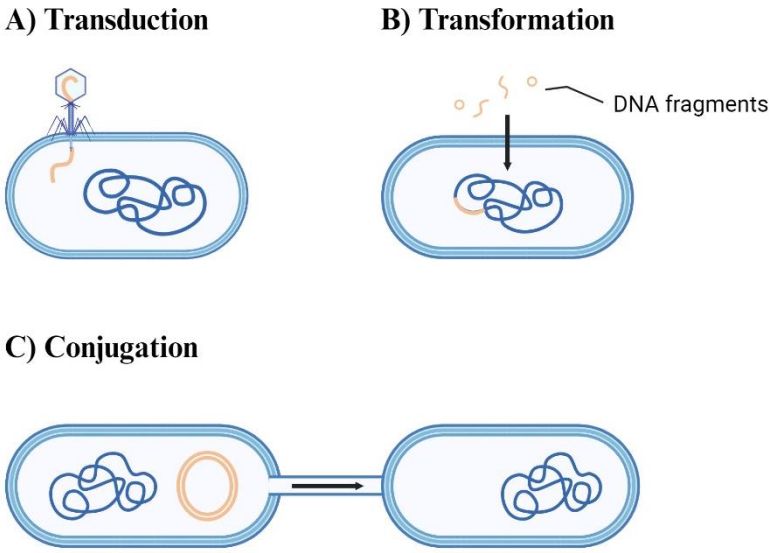

Figure 1.2 Three different mechanisms for horizontal gene transfer. A) Transduction involves the transfer of genetic material from one bacterium to another with the assistance of bacteriophages. B) Transformation refers to the uptake and integration of extracellular DNA from the environment. C) Conjugation involves physical contact between the donor and recipient cell where the genetic material is transferred through a conjugation pilus. The figure is created with BioRender.com.

1.2.1 Natural transformation

Natural transformation is a process where bacteria can take up DNA from the environment and integrate it into their genome[49]. The phenomenon of naturally transforming bacteria was first described for *Streptococcus pneumoniae* in 1928, but it was not known that DNA was the transforming molecule at that time[50]. Since then, more than 80 species have been shown to be naturally transformable[51]. The fact that this trait is conserved across a broad range of bacteria indicates that the ability to perform transformation is functionally important, as it provides access to DNA as a source of genetic information or nutrition[52]. To undergo transformation, bacteria must enter a unique physiological state called competence. This state causes the

bacteria to activate specific genes that encode proteins responsible for transporting and incorporating DNA into the genome[53]. Most naturally transformable bacteria develop a time-limited competence as a response to specific environmental conditions such as changing growth conditions and nutrient availability, cell density and exposure to antibiotics[52, 54].

Two competence inducing signaling systems have been discovered in streptococci[55]. One of the systems is called ComCDE and is found in species in the mitis and anginosus groups and includes the model organism *S. pneumoniae*[56]. The *comDE* genes encode a histidine kinase, ComD, which acts as a receptor for the competence stimulating peptide (CSP), and its corresponding response regulator ComE[49]. CSP is synthesized as a precursor peptide, called ComC, and is further processed into mature form before it is secreted by a transporter[57]. CSP then accumulates extracellularly under appropriate conditions until it reaches the critical amount required to induce competence. At this CSP concentration, sufficient numbers of ComD receptors will bind CSP probably leading to ComD dimerization, which triggers autophosphorylation of the cytoplasmic kinase domain of ComD[58]. The phosphoryl group on ComD is then transferred to ComE proteins, which becomes activated and will bind to the promotor region of the early competence genes and activate transcription[49]. This results in transcriptional activation of the *comX* gene which encodes the alternative sigma factor ComX[49]. This sigma factor is responsible for activating the transcription of the late competence genes involved in DNA uptake and homologous recombination. One of the late competence genes encodes an enzyme called fratricin. Fratricins lyse non-competent cells by breaking down their cell wall and play an important role in the transformation process as they make DNA from viable cells available for uptake[49]. For this mechanism to function optimally, it is important that the competent cells are not damaged by self-produced fratricins. The bacteria therefore express an early competence immunity protein, called ComM, to protect themselves[59]. More details about the fratricide mechanism can be found in **section 1.2.3**.

Species in the pyogenic, salivarius and bovis group have a signaling system called ComRS[56]. Unlike the ComCDE system, competence in the ComRS system is induced by a peptide called XIP (*comX*-inducing peptide)[60]. This peptide is derived from the pre-peptide ComS, which is exported and cleaved by specific proteases to mature form. When the mature peptide is described later in the thesis, it has nevertheless been referred to as ComS. Unlike the ComCDE system, XIP is then transported back into the cytoplasm through an Opp/Ami oligopeptide transporter and binds the transcriptional regulator ComR directly[20, 61]. Binding of XIP activates ComR, which induces transcription of *comX* and *comS*, resulting in a positive feedback

regulatory loop and activation of the late competence genes (**Figure 1.3**)[61]. Due to sequence variations in the XIP sequence and the *comS* and *comX* promoters, three different types of ComRS systems (type I-III) have been identified[20]. ComRS type-I and type-II have different inverted repeats in the *comS* and *comX* promoter sequences, and the XIP peptide in ComRS type-II consists of a C-terminal WW-motif, whereas such a motif is not present in type-I[62]. In ComRS type-III, the WW-motif is also present in the XIP peptide sequence, but the motif is separated by two residues[62]. The ComRS variants are prevalent in different phylogenetic groups of streptococci, as illustrated by the colors in **Figure 1.1**.

The mutans group of streptococci is unique as most strains encode both ComCDE and ComRS[56]. This feature allows the expression of ComX to be activated by both CSP and XIP. Despite this, it has been discovered that CSP and XIP induce competence in *S. mutans* under different environmental conditions and provide different degrees of activation[63]. Exogenous CSP leads to activation of ComX in about one-half of the cells in the population in a chemically defined medium, whereas XIP activates ComX in all the cells. It has also been shown that *S. mutans* becomes non-competent if components in the ComRS system is knocked out[62]. Although the ComRS system can function in the absence of ComCDE, ComCDE does not work in the absence of ComRS[55]. Furthermore, it has been discovered that the ComCDE system in *S. mutans* is orthologous to the bacteriocin producing system BlpABCRH in *S. pneumoniae*[64]. The ComCDE system in *S. mutans* is therefore responsible for regulating the production of several bacteriocins, and it has been speculated that the bacteriocins make the cytoplasmic membrane more permeable to XIP, which leads to ComR activation. Naming the genes *comCDE* is therefore suggested to be a mis-annotation since they are not directly involved in regulating competence in *S. mutans*[51, 63].

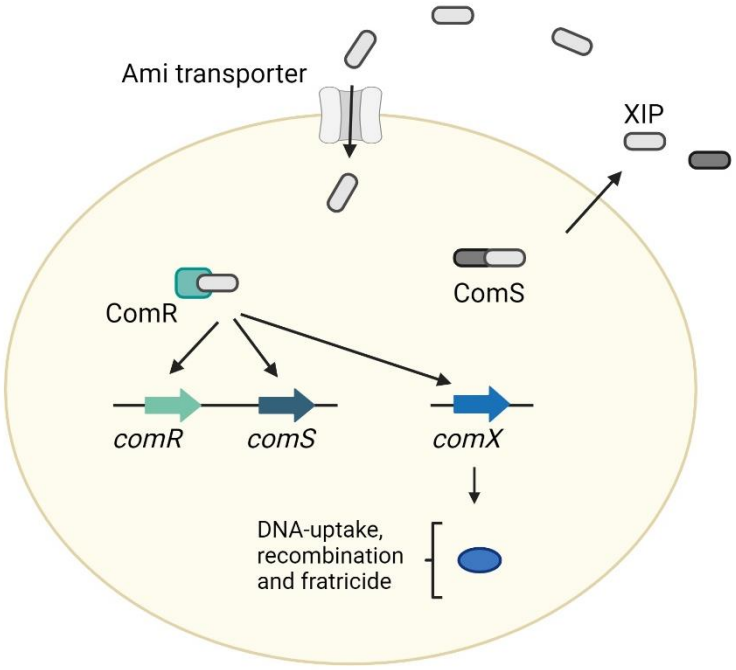

Figure 1.3 Overview of the ComRS system for regulation of natural competence. The precursor peptide ComS is secreted by an unidentified transporter and is processed to the mature form XIP (*comX*-inducing peptide). XIP is then transported into the cell through the Ami transporter and binds to the transcriptional regulator ComR. The activated ComR binds to P_{comR}, P_{comS} and P_{comX}, resulting in a positive feedback loop and activation of the late competence genes. The figure is adapted from Berg et al and created with BioRender.com[65].

1.2.2 DNA-uptake and homologous recombination

After the cells have become competent for natural transformation, the extracellular DNA can be transported through the cell wall and into the cytosolic compartment. This process is believed to be common to all transformable bacteria and is dependent on a set of conserved late competence genes[51]. An important component in this process is the type IV-like competence pilus, which acts as the primary DNA receptor and capture the available double-stranded DNA on the outside of the cell[49, 66]. However, it is still unknown how this pilus penetrates the peptidoglycan layer of the competent bacterium. In *S. pneumoniae*, the pilus is made up of the main pilin-like protein, ComGC, and other smaller pilin-like proteins (ComGD, ComGE and ComGG)[67]. Depolymerization of the pilus allows the DNA to cross the peptidoglycan layer, bringing the double-stranded DNA into contact with the membrane receptor ComEA[67]. The receptor then delivers the DNA to the endonuclease EndA, which further degrades one of the DNA strands, allowing transport of the complementary strand through the transmembrane channel ComEC. Internalization of the single-stranded DNA is facilitated by the ATP-dependent translocase ComFA (**Figure 1.4**)[66].

After the DNA has been transported into the cell, it must undergo homologous recombination to be integrated into the genome of the recipient bacterium. Homologous recombination is a process in which genetic material is exchanged between two DNA-strands containing regions with similar base sequences. Key components in this process include the late competence gene products SsbB, DprA, RecA, CoiA and RadA[49]. The internalized single-stranded DNA is first covered with single-stranded DNA binding protein B (SsbB), which protects the DNA from degradation[68]. A DNA processing protein (DprA) then promotes the binding of the recombinase RecA. RecA performs a homology search of the genome and incorporate the single-stranded DNA into the recipient's genome if a homologous sequence is found[51]. CoiA and RadA are needed for efficient recombination, but their exact function is unknown[69, 70].

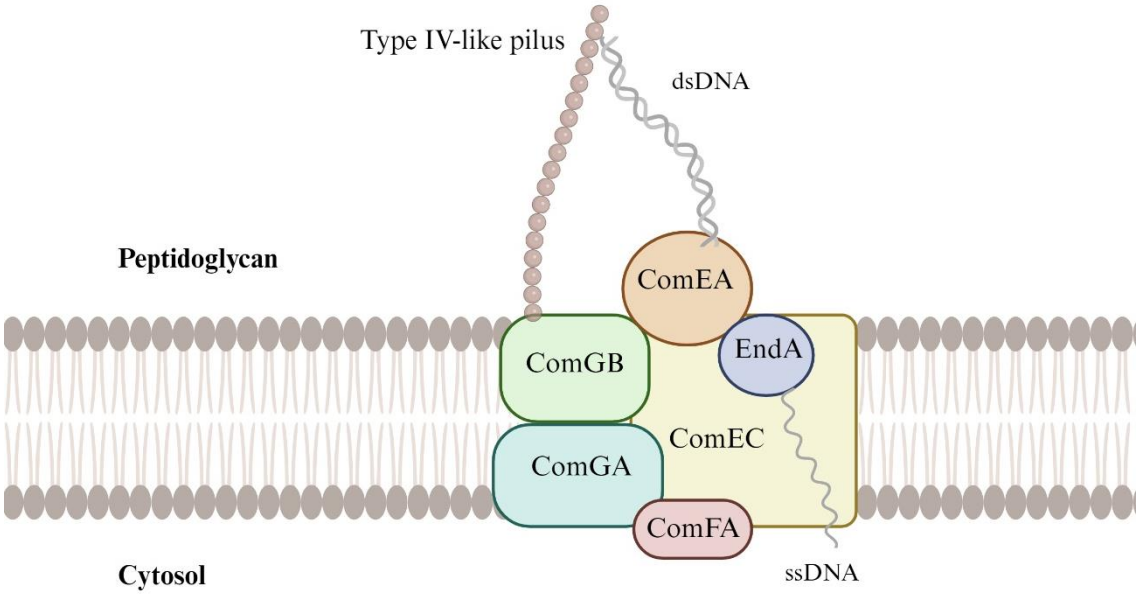

Figure 1.4 Components of the DNA-uptake system in *S. pneumoniae*. A type IV-like transformation pilus binds to double-stranded DNA and depolymerization of the pilus brings the DNA into contact with the membrane receptor ComEA. The endonuclease EndA degrades one of the DNA-strands, while the complementary strand is transported through the transmembrane channel ComEC. ComFA is an ATP-dependent translocase that contributes to the internalization of single-stranded DNA. The figure is adapted from Muschiol et al and created with BioRender.com[71].

1.2.3 The fratricide mechanism

It was thought that natural transformation depended on extracellular DNA being released by dead bacteria in the environment. However, it is now known that several species can trigger the active release of DNA from living bacteria by using a so-called fratricide mechanism[67]. Competent streptococci produce murein hydrolases called fratricins, which are secreted after

the bacterium has entered competent state and serve to lyse nearby non-competent cells[72]. In *S. pneumoniae*, the three choline-binding proteins, CbpD, LytA and LytC, have been found to contribute to the fratricidal mechanism[73]. However, CbpD (choline-binding protein D) has been identified as the key murein hydrolase, since competence induced cell lysis is abolished in the absence of this enzyme[65]. LytA and LytC play a minor role in the fratricidal mechanism as their presence in target cells contribute to the lysis process[74]. Although many streptococcal species have genes encoding CbpD-like proteins, this enzyme is not found in all naturally transformable streptococci[72]. Instead, some streptococci produce a murein hydrolase, called LytF, that is unrelated to CbpD, but presumably has the same function. This fratricin has been detected in most streptococci lacking a *cbpD*-like gene, although a third fratricin variant, called Zoocin A, also has been identified[72]. The fact that all streptococci appear to encode fratricins strongly suggests that these cell wall degrading enzymes have an important function in natural transformation[65].

1.2.3.1 CbpD

CbpD is essential for the fratricide mechanism in *S. pneumoniae* and is secreted after the bacterium has become competent for natural transformation[72]. Pneumococcal CbpD consists of a catalytic domain, in addition to two different cell wall binding domains. The N-terminal region consists of a CHAP domain belonging to a family of cysteine histidine-dependent amidohydrolases/peptidases that hydrolyze peptide bonds in the cell wall. The exact hydrolytic mechanism of CbpD has not been identified, but other muralytic CHAP domains act either as amidases, disrupting the N-acetylmuramyl-L-Alanine bond, or as endopeptidases cleaving within the peptide portion of the peptidoglycan, and it is therefore likely that the CHAP domain in CbpD has a similar function[75, 76]. The C-terminal end of CbpD consists of four choline-binding repeats, that together make up the choline-binding domain, and is responsible for binding CbpD non-covalently to choline-covered teichoic acids in the cell wall[77]. The pneumococcal CbpD also has one or two Src homology 3-binding (SH3b) domains in the center of the fratricin, which binds to the peptidoglycan portion of the cell wall[78]. Since the pneumococcal fratricin only acts against streptococci that have choline in the cell wall, it is only active against closely related species and has a narrow target range[65].

Genes encoding CbpD-like proteins have been identified in the genome of several streptococcal species, such as *S. vestibularis*[79]. These proteins all consist of an N-terminal CHAP-domain, while the C-terminal domain is variable and not choline binding[72]. Naming them CbpD is

therefore misleading and should be changed in the future when the functions of these C-terminal domains hopefully are better described. Nevertheless, the current naming CbpD will be used in the following text. A ComX binding motif has been identified in the promoter region of all the CbpD-like genes and it is therefore reasonable to assume that they are functional analogs of pneumococcal CbpD, and thus can lyse non-competent streptococci[72]. Based on variation in the C-terminal region six different CbpD variants have been identified (**Figure 1.5**)[72]. *S. vestibularis* and other streptococcal species in the salivarius group produce a CbpD-like protein consisting of a conserved but uncharacterized domain, and therefore lack the choline-binding domain and SH3b domain found in *S. pneumoniae*. Consequently, these fratricin variants probably recognize and bind other structures in the cell wall of their target cells than *S. pneumoniae*[65]. Although many streptococcal species have these CbpD-like genes, it has not been demonstrated that all are competent for natural transformation and performs fratricide[72].

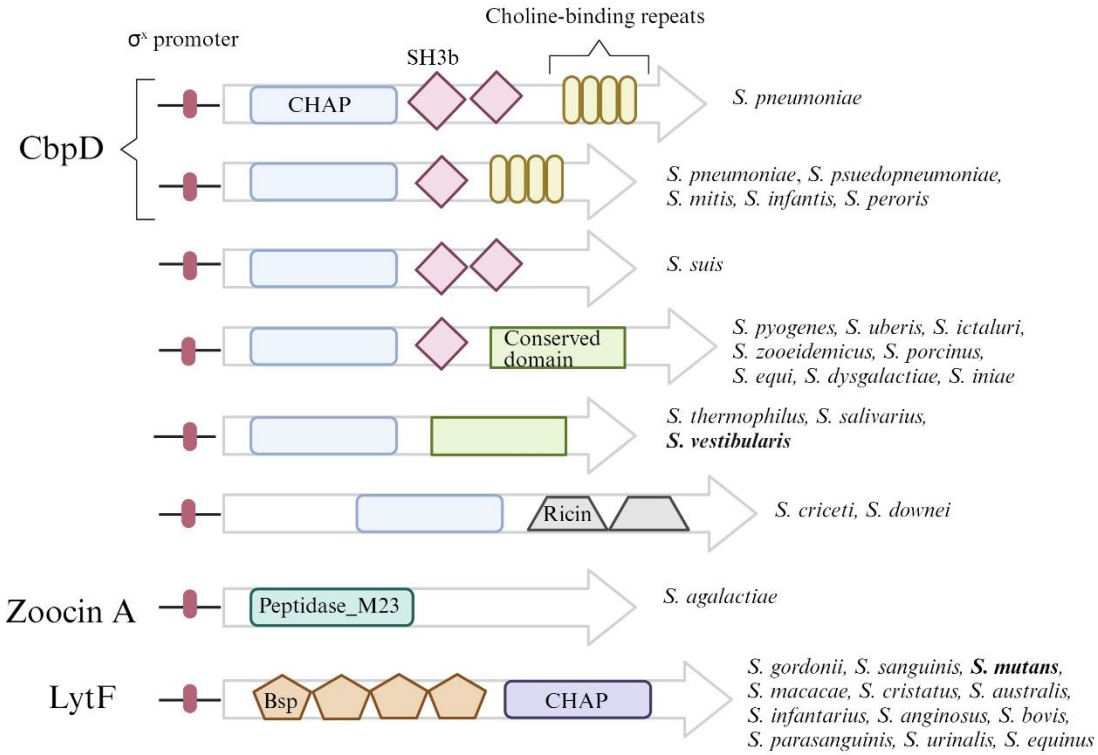

Figure 1.5 Composition of fratricins found in various streptococcal species. CbpD and LytF are the most common fratricins, but a third variant, called Zoocin A, has also been discovered. All the fratricins have a comX promoter region upstream of the gene, in addition to a catalytic cell wall hydrolyzing CHAP domain (except for Zoocin A). The Scr homology 3-binding (SH3b) domain recognizes and binds to peptidoglycan, while the choline-binding domain binds to choline-covered teichoic acids in the cell wall. The figure is adapted from Berg et al and created with BioRender.com[65].

1.2.3.2 LytF

Some streptococcal species lack genes encoding CbpD-like fratricins in their genome. Most of these species produce an alternative competence-regulated murein hydrolase called LytF[72]. This fratricin performs a similar function to the CbpD-like fratricins and is produced by at least 12 streptococcal species, including *S. mutans*[72]. LytF consists of an N-terminal region with two to five consecutive Bsp-like (group B streptococcal secreted protein) domains and a C-terminal CHAP domain. Bsp domains have only been found in streptococcal proteins that also carry a CHAP, amidase or N-acetylmuramidase domain, suggesting that these domains are responsible for attaching the muralytic domain to the substrate[80]. Bsp presumably recognizes structures that are only present in newly synthesized peptidoglycan, and since LytF can consist of a variable number of Bsp domains, it is assumed that these domains are involved in establishing the specificity and target range of the fratricin[72]. Even though the CHAP domain in LytF is highly conserved within the LytF group, this domain shares little sequence similarity with the corresponding domain in CbpD-like fratricins[72].

1.2.3.3 Immunity against fratricide

A critical part of the fratricidal mechanism in competent streptococci is to avoid being lysed by their own fratricins. In *S. pneumoniae*, this is achieved by expressing the immunity protein ComM, which is encoded by an early competence gene[59]. The transcription of *comM* increases immediately after induction of competence and peaks after approximately five minutes, before it is rapidly downregulated[59, 81]. The competent pneumococci therefore develop full immunity before the cells have had time to express CbpD. As ComM is only present in the cells for a limited period of time, and the level of this protein is reduced before the expression of CbpD has reached the maximum rate, it suggests that ComM does not need to be present in the cells at the same time as the fratricin to provide immunity[81]. The mechanism by which ComM protects cells from CbpD is unknown, but since ComM is an integral membrane protein, direct contact with CbpD is unlikely. However, it has been suggested that ComM may confer immunity by altering the cell wall structure of newly synthesized peptidoglycan, or introduces changes that inhibit the attachment of CbpD to the cell wall[81]. Although this immunity mechanism is essential for the competent cells, excessive concentrations of ComM have been shown to inhibit growth[81]. No such immunity protein has been identified against LytF-type fratricins[74].

1.3 Streptococcal cell wall structure

Bacteria often live in unpredictable environments and have developed a complex cell wall structure to survive. The bacterial cell wall is located on the outside of the cytoplasmic membrane and its function is to protect the organism from the environment, as well as stabilizing the cell membrane so it can withstand the internal osmotic pressure[82]. The cell wall not only acts as a first line defense against toxic molecules, but also ensures interaction with the environment by allowing selective transport of nutrients and waste products in and out of the cell[83]. In Gram-positive bacteria, the cell membrane is enclosed by a thick peptidoglycan cell wall, while Gram-negative bacteria have a thinner peptidoglycan layer because the cell wall is enclosed by an outer membrane[82]. However, both Gram-positive and Gram-negative bacteria share the same chemical structure of peptidoglycan. In addition to being the enzymatic substrate for fratricins during transformation, the cell wall also often serves as a target for different types of antibiotics, as it is essential for normal cell division and survival[82]. As antibiotic resistance has become widespread, there is a growing need to understand how the cell wall is synthesized and regulated.

The cell wall, which consists of a peptidoglycan layer, is made up of linear chains of repeating disaccharide units linked by glycosidic bonds. The linear chains consist of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) connected by β-1,4 linkages[84]. These chains are further connected via cross-links consisting of short peptide stems attached to MurNAc (**Figure 1.6**). In the peptidoglycan of Gram-positive bacteria that are not yet cross-linked, the short linear peptide consists of five amino acids[82]. In *S. pneumoniae*, the peptide stem consists of L-alanine, followed by D-isoglutamine and L-lysine[82]. The terminal dipeptide in the peptide stem is made up of D-Ala-D-Ala, but the last residue is not found in the mature peptidoglycan molecule[84]. The cross-linking between the chains occurs between the carboxyl group of D-Ala at position 4 and the amino group of L-Lys at position 3, either directly or indirectly through a short peptide bridge (Ala-Ala or Ser-Ala)[84]. The glycan chains in the cell wall of *S. pneumoniae* also have secondary modifications, including N-deacetylation of GlcNAc residues and O-acetylation of MurNAc residues, which protect the bacterium from antimicrobial peptides and muralytic enzymes[82, 85]. Although the fundamental structure of peptidoglycan is the same in all bacteria, there are variations in glycan strand length and modifications, as well as peptide bridge length and composition[84].

Wall teichoic acids (WTAs) are long polymers that are bound to the peptidoglycan layer of many Gram-positive bacteria, including *S. pneumoniae*. These polymers are important for cell

physiology and cell division, as well as being central to pathogenesis and antibiotic resistance[86]. The structure of WTAs is highly variable, but is often a polymer consisting of glycerol-phosphate or ribitol-phosphate[83]. Several streptococci, including *S. mutans*, however lack the ability to generate WTAs. These species often have rhamnose-glucose polysaccharides (RGPs) covalently bound to MurNAc residues in the cell wall instead[87]. The function of RGPs is poorly defined, but they are thought to have a similar function to WTAs[88, 89]. RGPs in pathogenic streptococcal species have been associated with virulence, immune evasion and resistance to antimicrobial compounds, suggesting that these polysaccharides have an important function for cell survival[88]. RGPs consist of a polyrhamnose chain with variable oligo- or polysaccharide side chains that can consist of glucose, N-acetylglucosamine, galactose, N-acetyl galactosamine or phosphate[88]. The backbone of RGPs is nevertheless relatively conserved, although the number of rhamnose residues can vary both between species and within species[90]. As RGPs are part of the outermost layer of the bacterial cell envelope, these polysaccharides are presumably the cell's initial contact point with the environment[89].

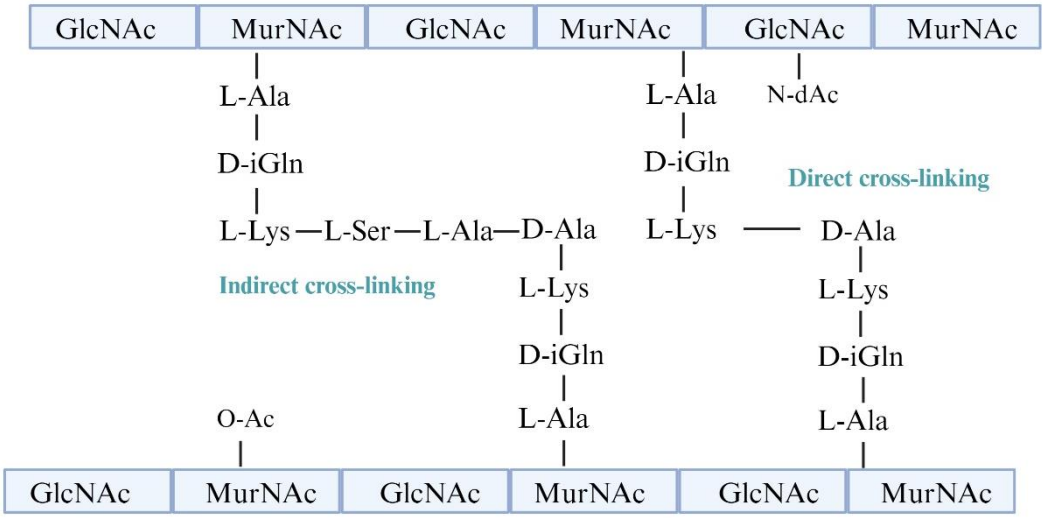

Figure 1.6 Cell wall composition in *S. pneumoniae.* The peptidoglycan layer consists of chains of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) units linked by β-1,4 bonds. The chains are further connected via peptide stems that are attached to MurNAc, through direct or indirect cross-links. The glycan chains also have secondary modifications, with N-deacetylation of GlcNAc residues and O-acetylation of MurNAc residues. The figure is adapted from Vollmer et al and created with BioRender.com[85].

1.4 Aim of the study

Antibiotic resistance in bacteria is a growing problem worldwide, making it more challenging to treat infections. One process that strongly contributes to the spread of antibiotic resistance genes is natural transformation. All streptococci appear to possess the genes needed to undergo transformation, yet this process has been most studied in *S. pneumoniae*. Fratricins are particularly important in this process, as they ensure lysis of closely related species making DNA available for uptake. For streptococcal species outside of the mitis group, there is still a limited understanding of the function of the various fratricins and their contribution to the transformation process. This thesis has therefore been part of a larger project that aims to characterize genes encoding putative fratricins in various streptococcal species. In this work, the putative fratricins of *S. mutans* and *S. vestibularis* have been investigated. It was desirable to investigate if these fratricins are competence induced and whether they have an effect on the transformability of the bacterium. Furthermore, it was also a goal to overexpress and purify the fratricins to investigate their function and to test their lytic activity.

2 Materials

2.1 Bacterial strains and plasmids

Table 2.1 List of bacterial strains used in this work.

Strain	Genotype and relevant characteristics[1]	Source or reference
***S. mutans* UA159**		
HL3	Strep^R	Sara Arbulu Ruiz
HL5	HL3, but Δ*lytF*::Janus	This work
HL20	pRM37	This work
HL21	HL5, but pRM37	This work
RM99	pRM15	Rebekka Moe
RM100	pRM25	Rebekka Moe
***S. vestibularis* ATCC 49124/ NCTC 12166**		
HL6	Strep^R	This work
HL9	HL6, but Δ*cbpD*::Janus	This work
HL11	HL9, but Δ*cbpD*	This work
RM90	pRM30	Rebekka Moe
RM103	pRM20	Rebekka Moe
***S. dysgalactiae* stdys021**		
MM418	pFD116 P_{sigX}-*luc*-GFP	Marita Torrissen Mårli
S. pneumoniae		
RH426	Template for the Janus-cassette	[91]
DS228	Template for the spectinomycin-cassette	Dr. Daniel Straume
E. coli		
Genehogs	pRM37	This work
DH5α	pFD116	Marita Torrissen Mårli
BL21	pRM6	Rebekka Moe

[1] Strep; streptomycin

Table 2.2 List of plasmids used in this work.

Plasmid	Description	Source or reference
pRM6	His-TEV-LytF	Rebekka Moe
pRM15	pFD116 P_{lytF}-*luc*	Rebekka Moe
pRM20	pFD116 P_{cbpD}-*luc*	Rebekka Moe
pRM25	pFD116 P_{comX}-*luc*	Rebekka Moe
pRM30	pFD116 P_{comX}-*luc*	Rebekka Moe
pRM37	pFD116 P_{comG}-*ComGC*	This work

2.2 Primers

Table 2.3 List of primers used in this work.

Name	Sequence (5'-3')	Reference
Janus F	GTTTGATTTTTAATGGATAATGTG	[92]
Janus R	CTTTCCTTATGCTTTTGGAC	[92]
Primers to sequence the *rpsL* gene in *S. vestibularis*		
RM142	ACAATAATTATCAAAAAATGATACTATATTAGACG	Lab collection
RM143	TACCACGTTTACCGTCAAGC	Lab collection
Primers for *lytF* knock-out in *S. mutans*		
RM144	AATGGCAAAAAAACCAATGC	Lab collection
RM145	CACATTATCCATTAAAAATCAAACTCTCAATCGAAATCTCCTTTATTCT	Lab collection
RM146	GTCCAAAAGCATAAGGAAAGGAGAACAAAACTAAGATGCGCG	Lab collection
RM147	ACCTATTGGCCAGCAAGC	Lab collection
RM148	AAGAATAAAGGAGATTTCGATTGAGAGAGAACAAAACTAAGATGCGCG	Lab collection
RM149	CGCGCATCTTAGTTTTGTTCTCTCTCAATCGAAATCTCCTTTATTCTT	Lab collection
Primers for *cbpD* sequencing in *S. vestibularis*		
RM150	GGCCATTATTGGATTAGCTACG	Lab collection
RM151	CGTTTGCCATATAGGCACAGC	Lab collection
Primers for testing transformation efficiency in *S. mutans*		
HL1	CAGATGATGCGATAGTGTCCG	This work
SA1	TGTCACACTTCTGGTCAAACTGTGCC	Lab collection
SA14	AGCTGTCCAGGAACCTTTGTGAAGC	Lab collection
DS717	TTAAATGTGCTATAATACTAGAAAATACTTGTGGAGGTTCCATTGTGAGG AGGATATATTTGAATAC	Lab collection
DS121	TTATAATTTTTTTAATCTGTTATTTAAATAG	Lab collection
HL9	TATTTAAATAACAGATTAAAAAAATTATAAAGGGGCTGATTATTGTAGCT ATCAACGG	This work
Primers for *cbpD* knock-out in *S. vestibularis*		
HL3	CAACATCTGTTGTCGGTCAGATATCC	This work
HL6	CACACTGTCAGACACAGCTGATGC	This work
HL7	GATTTTAATTGTTTTTTTCATCATATTCACCTATAGTTTTGTACTCCATTTA AAATGTTTAAACG	This work
HL8	CGTTTAAACATTTTAAATGGAGTACAAAACTATAGGTGAATATGATGAA AAAAACAATTAAAATCG	This work
HL10	GTATTTAAAGATTATATCACATTATCCATTAAAAATCAAACCGATAGAATA ATTTCTGATTGAGAAACG	This work

HL11	GGTACTAAACGTCCAAAAGCATAAGGAAAGGCGATATACTTTCTCAGGACATGC	This work
Pilus quantification in *S. mutans*		
HL14	ATGAAAAAGATCAAGAGAGTCAGTGTAC	This work
HL15	CCATGGTTACTTGTCGTCATCGTCTTTGTAGTCATTCGCGACATGCCGTGTTT	This work
HL20	ACTAGTGATACCCCTTTTCGTTCCAAATG	This work
HL21	GTACACTGACTCTCTTGATCTTTTTCATAAAACCTCCCATCTTATCTATTCG	This work
MM63	CTCATTAAGCAGCTCTAATGC	Lab collection
RM088	TTTCCTTGGTGTATCCAACG	Lab collection
Primers for testing transformation efficiency in *S. vestibularis*		
HL16	CGGGCTATCAACTCTCATTGC	This work
HL17	CCACAAGTATTTTCTAGTATTATAGCACATTTAAAATTGTTTATGCCATATGATTGTAACACC	This work
HL18	CTATTTAAATAACAGATTAAAAAAATTATAATATATTCAGAAAGCCTAAGTAACTCCCC	This work
HL19	TAGTAGTGAGGAAATCTCCGACG	This work

2.3 Enzymes, chemicals, inducer peptides and pre-made buffers

Table 2.4 List of enzymes, chemicals and pre-made buffers used in this work.

Compounds	**Product number**	**Supplier**
1 kb DNA ladder	N0468	New England BioLabs
5X Phusion High Fidelity Buffer	B0518S	New England BioLabs
Activated charcoal	1.020184.1000	Merck
Agar powder	20767.298	VWR
Agarose	15510-027	Invitrogen
Alexa Fluor™ 488 goat anti-rabbit IgG	A11034	Thermo Fischer Scientific
Ammonium persulfate (APS), $(NH_4)_2S_2O_8$	A3678	Sigma-Aldrich
Ampicillin, $C_{16}H_{18}N_3O_4SNa$	A-9518	Sigma-Aldrich
Anti-rabbit antibodies	NA	NA
Bacto™ Todd Hewitt	249240	BD Diagnostic
Biotin, $C_{10}H_{16}N_2O_3S$	19606	Sigma-Aldrich
Bis-acrylamide	0172	VWR
Bromophenol blue, $C_{19}H_9Br_4NaO_5S$	B-5525	Sigma-Aldrich
Calcium chloride anhydrate, $CaCl_2 \cdot 2H_2O$	1.02381.0500	Merck
Calcium chloride, $CaCl_2$	102378	Merck
Calcium pantothenate, $C_{18}H_{32}CaN_2O_{10}$	P5155	Sigma-Aldrich
Calf Intestinal alkaline Phosphatase (CIP)	M0290	New England BioLabs
Casitone	225930	Gibco
Choline chloride	C1879	Sigma-Aldrich
Copper sulfate pentahydrate, $CuSO_4 \cdot 5H_2O$	61240	Fluka
D-luciferin	NA	NA
DNase	DN25	Sigma-Aldrich
dNTPs	N0447	New England BioLabs
Formaldehyde, CH_2O	NA	NA

Gel Loading Dye Purple (6x)	B7025S	New England BioLabs
Glass wool	1.04086.0250	Merck
Glycerol, $C_3H_8O_3$	1.04094.1000	Merck
Glycine, $C_2H_5NO_2$	A13816	Alfa aesar
Hydrogen chloride, HCl	30721	Riedel-De Haën
Imidazole, $C_3H_4N_2$	I2399	Sigma-Aldrich
IPTG, Isopropyl-β-D-thiogalactisidase	A1008,005	ITW Reagens
Iron (II) sulphate, $FeO_4S \cdot 7H_2O$	44970	Fluka
Kanamycin, $C_{18}H_{36}N_4O_{11} \cdot H_2O_4S$	K4000	Sigma-Aldrich
L-asparagine monohydrate, $C_4H_8N_2O_3 \cdot H_2O$	A4284	Sigma-Aldrich
L-cysteine hydrochloride, $C_3H_7NO_2S \cdot HCl \cdot H_2O$	30130	Fluka
L-tryptophan, $C_{11}H_{12}N_2O_2$	T0254	Sigma-Aldrich
Magnesium chloride hexahydrate, $MgCl_2 \cdot 6H_2O$	63072	Fluka
Manganese (II) chloride tetrahydrate, $MnCl_2 \cdot 4H_2O$	31422	Riedel-De Haën
Methanol, CH_3OH	1.06009.5000	Merck
N,N,N',N'-tetramethylethane-1,2-diamine (TEMED), $C_6H_{16}N_2$	T9281	Sigma-Aldrich
NcoI-HF	R3193L	New England bioLabs
Nicotinic acid, $C_6H_5NO_2$	72309	Fluka
PeqGreen	PEQL37-501	Saveen Werner
Phusion® High-Fidelity DNA Polymerase	M0530	New England BioLabs
Potassium hydrogen phosphate, K_2HPO_4	1.05099.1000	Merck
Pyridoxine hydrochloride, $C_8H_{12}ClNO_3$	95180	Fluka
rCutSmart™ Buffer	B6004S	New England BioLabs
RedTaq® 2X Master Mix	5200300-1250	VWR
Riboflavin, $C_{17}H_{20}N_4O_6$	R7649	Sigma-Aldrich
Skim milk powder	1.15363.0500	Merck
Sodium acetate, $C_2H_3O_2Na$	S3272	Sigma-Aldrich
Sodium Chloride (NaCl)	S9625	Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	05030	Fluka
Spectinomycin, $C_{14}H_{24}N_2O_7 \cdot 2HCl \cdot 5H_2O$	S9007	Sigma-Aldrich
SpeI-HF	R3133S	New England BioLabs
Streptomycin, $C_{21}H_{39}N_7O_{12} \cdot 1.5H_2SO_4$	S6501	Sigma-Aldrich
T4 DNA ligase	M0202L	New England BioLabs
T4 ligase reaction buffer (10x)	B0202S	New England BioLabs
Thiamine hydrochloride, $C_{12}H_{18}Cl_2N_4OS \cdot HCl$	T4625	Sigma-Aldrich
Triton® X-100, $C_{14}H_{22}O(C_2H_4O)_{n\ (n=9-10)}$	X-100	Sigma-Aldrich
Trizma® Base	77-86-1	Sigma-Aldrich
Tryptone	LP0042	Oxoid
Tween® 20	9005-64-5	Sigma-Aldrich
Yeast extract	1.04086.0250	Merck
Zinc sulfate heptahydrate, $ZnSO_4 \cdot 7H_2O$	96500	Fluka

Table 2.5 List of inducer peptides used in this work.

Inducer peptide	Description	Amino acid sequence	Supplier
ComS	Induces competence in *S. mutans*	GLDWWSL	Research genetics, Inc
ComS	Induces competence in *S. vestibularis*	VPFFMIYY	Research genetics, Inc
ComS	Induces competence in *S. dysgalactiae*	QVDWWRL	Research genetics, Inc

2.4 Kits and equipment

Table 2.6 List of kits and laboratory equipment used in this work.

Kit/equipment	Model/Product number	Supplier
96-well polystyrene microtiter plates	82.1581.001	Thermo Fischer Scientific
Amicon® Ultra-15 Centrifugal Filter	UFC9010	Merck
E.Z.N.A.® Plasmid Mini Kit I	D6943-02	Omega Bio-Tek
Gel imager	GelDoc-100	BioRad
Imaging System	Azure c400	Thermo Fischer Scientific
Microplate reader	Synergy H1 Hybrid Reader	BioTek®
NucleoSpin® Gel and PCR Clean-up	740609.250	Macherey-Nagel
PCR Thermocycler	ProFlex PCR systems	Applied Biodynamics
Spectrophotometer	NanoDrop2000	Thermo Fischer Scientific
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	34580	Thermo Fischer Scientific
Trans-Blot® Pure Nitrocellulose Membrane (0.45 µm)	162-0115	BioRad
AnaeroGen™	NA	Oxoid

2.5 Contents of prepared growth media, buffers and solutions

1 kb DNA ladder (50 mg/ml)

50 µl 1 kb DNA ladder, 200 µl 10x loading buffer, 750 µl dH_2O

1x SDS electrophoresis buffer

28.8 g glycine, 6.04 g Tris base, 2 g SDS, dH_2O up to 2 L.

2x SDS sample buffer

0.125 M Tris-HCl (pH 6.8), 4 % (v/v) SDS, 0.30 M (2%) β-2-mercaptoethanol, 20 % (v/v) glycerol, 0.01 % (w/v) bromophenol blue

50 x TAE-buffer

424 g Tris base, 57.1 ml acetic acid, 100 ml 0.5 M EDTA, pH 8.0

Adjusted to 1 L with dH_2O

C-medium

150 ml pre C-medium, 150 µl 0.4 mM $MnCl_2$, 1.5 ml 20 % glucose, 3.75 ml ADAMS III, 110 µl 3 % glutamine, 2.25 ml 2% Na pyruvate, 95 µl 1.5 M sukrose, 1.5 ml 2 mg/ml uridine adenosine, 1.5 ml 8 % albumin/BSA, 3.75 ml 10 % yeast extract. See **Appendix 1** for more details.

Coomassie blue stain

0.5 g Coomassie Brilliant Blue (0.2 % w/v), 200 ml ethanol (40% v/v), 37.5 ml acetic acid (7.5% v/v), 265 ml dH_2O

Coomassie destain

50 ml acetic acid, 500 ml ethanol, 400 ml dH_2O

Lysogeny broth (LB)

10 g/L NaCl, 10 g/L tryptone and 5 g/L yeast extract

Phosphate-buffered saline (PBS)

8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, dH_2O, pH 7.4

Transfer buffer

3.03 g Tris-Base, 4.4 g glycine, 100 ml 100 % methanol, dH_2O up to 1 L

Tris-buffered saline (TBS)

50 mM Tris-HCl (pH 7.5), 150 mM NaCl

TBS with Tween®-20 (TBST)

0.05 % (v/v) Tween®-20 in TBS

3 Methods

3.1 Growth and storage of *S. mutans* and *S. vestibularis*

S. mutans and *S. vestibularis* were grown on Todd-Hewitt (TH) plates and incubated at 37 °C. *S. vestibularis* was grown anaerobically using airtight containers, and the oxygen was removed by using anaerobe atmosphere generating bags (AnaeroGen from Oxoid). TH plates containing 50, 100, 200 and 500 µg/ml streptomycin were used to induce streptomycin resistance. To select for gain and loss of the Janus cassette, TH plates containing 400 µg/ml kanamycin and 200 µg/ml streptomycin were also used. Furthermore, for cultivation in liquid media, TH broth or C-medium was used for growth of *S. vestibularis*, and C-medium for *Streptococcus dysgalactiae* and *S. mutans*. Different sugars were removed from the C-medium of *S. mutans* and is specified when relevant. LB-medium was used for growth of *Escherichia coli* and this bacterium was grown aerobically at 37 °C. Start cultures were made by growing the bacteria at 37 °C to exponential phase (OD_{550} 0.2-0.4). The start cultures were then added sterile glycerol to a final concentration of 15 % and stored at -80 °C. Frozen stocks for long term storage at -80 °C were also made by adding sterile glycerol to a final concentration of 15 %.

3.2 Polymerase chain reaction

Polymerase chain reaction (PCR) is a method that enables amplification of specific DNA fragments[93]. A PCR reaction is divided into three temperature-specific steps that are repeated in 25-35 cycles, and after each cycle, the target sequence doubles in number. The main components of PCR are template DNA, primers, DNA polymerase and the four deoxynucleotide triphosphates (dNTPs) dATP, dCTG, dGTP and dTTP[94]. In the first step of PCR, template DNA duplex is denatured into two single strands by heating up the reaction mixture to 95-98 °C. When the strands are separated, the temperature is lowered to about 45-72 °C (depending on the primer sequence and length), which allows the primers to anneal to complementary regions of the template. The primers are small oligonucleotides that flank the DNA segment to be amplified, and are usually 18 to 30 bases long[94]. In the last step, the DNA polymerase extend the new strands from the annealed primers in 5' to 3' direction[93]. This step is carried out at the temperature optimum for the DNA polymerase being used in the reaction.

In this work, PCRs were performed using two different DNA polymerases, Phusion® High-Fidelity and RedTaq® polymerase. The Phusion® High-Fidelity polymerase has a unique structure where a *Pyrococcus*-like enzyme is fused with a processivity-enhancing domain

(template DNA-binding domain), ensuring increased fidelity and speed[95]. Since the High-Fidelity DNA polymerase provides a low mutation rate, it was used in reactions where the DNA sequence must be correct after amplification. The RedTaq® polymerase, on the other hand, was used in PCR reactions where the accuracy of the final product was not as important. This polymerase has less proofreading capacity and was therefore used for screening of mutants. When Phusion® High-Fidelity polymerase was used, the enzyme was mixed with dNTPs, primers flanking the region of interest, template DNA, dH_2O and 5x Phusion® HF buffer. The concentrations of the components are listed below in **Table 3.1**. The standard thermocycling program used with the Phusion® High-Fidelity polymerase is listed in **Table 3.2**.

Table 3.1 Components in the Phusion® High Fidelity PCR reaction mix.

Component	Final concentration/volume
5x Phusion ® HF buffer	1x
10 mM dNTP	200 µM
10 µM forward primer	0.5 µM
10 µM reverse primer	0.5 µM
DNA template	10-100 ng (depending on type of template)
Phusion® HF DNA polymerase	1 unit
dH_2O	To a final volume of 50 µl

Table 3.2 Standard thermocycling for Phusion® High-Fidelity PCR.

Step	Temperature	Time	Cycles
Initial denaturation	98 °C	30 seconds	1x
Denaturation	98 °C	5-10 seconds	25-35x
Annealing	45-72 °C[a]	10-30 seconds	
Elongation	72 °C	15-30 seconds per kb	
Final extension	72 °C	5-10 minutes	1x
Hold	4-10 °C	∞	

[a] Annealing temperature was adjusted based on the T_m of the primers.

3.2.1 Colony PCR screening

Colony PCR is a rapid method to confirm that transformants have acquired desired DNA after transformation. The screening in this work was performed by using RedTaq® 2x Master Mix, which consists of dNTPs, a buffering solution, the *Taq* polymerase and a loading dye. The RedTaq® 2x Master Mix was mixed with appropriate primers, DNA template and dH_2O as described in **Table 3.3.** Before setting up the PCR reactions, potential transformants were lysed by picking one colony in 5 µl sterile dH_2O which were heated in the microwave on maximum effect (800 watts) for 2-3 minutes. The standard thermocycling program used with RedTaq® polymerase is listed in **Table 3.4**. This program is slightly different from the program used in Phusion® High-Fidelity PCR, as *Taq* polymerase is unstable at temperatures above 95 °C. The extension speed of *Taq* polymerase is also slower, with 1,000 bp per minute.

Table 3.3 Components in the RedTaq® PCR reaction mix.

Component	Final concentration/volume
RedTaq® MasterMix 2x[a]	5 µl
10 µM forward primer	0.1 µM
10 µM reverse primer	0.1 µM
DNA template	1 µl of lysed cells
dH_2O	To a final volume of 10 µl

[a] The RedTaq® 2x Master Mix contains dNTPs, a buffering solution, the *Taq* polymerase and a loading dye.

Table 3.4 Standard thermocycling program for RedTaq® PCR.

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	30 seconds-5 minutes	1x
Denaturation	95 °C	30-60 seconds	25-35x
Annealing	50-65 °C[a]	10-30 seconds	
Elongation	72 °C	1 min per kb	
Final extension	72 °C	5-10 minutes	1x
Hold	4-10 °C	∞	

[a] Annealing temperature was adjusted based on the T_m of the primers.

3.2.2 Overlap Extension PCR

Overlap extension PCR (OE-PCR) is a method where primers in initial PCR reactions create DNA fragments with complementary ends, which can be utilized to fuse the fragments in a

second PCR[96]. The overlapping strands of these products will then hybridize at the 3' end region in a subsequent PCR to generate a preferred full-length product (**Figure 3.1**). In this work, OE-PCR was utilized to assemble gene knockout cassettes. The desired fragments were amplified using Phusion® High-Fidelity polymerase. The primers used in OE-PCR had an additional sequence at the end that was complementary to the fragment to be joined. The PCR products were separated by agarose gel electrophoresis to confirm that the amplification was successful, and that bands of the desired size was obtained (**section 3.3**).

For *lytF* knockout, the flanking regions to *lytF* were amplified from *S. mutans* using RM144 and RM145 as primers for the upstream region, and RM146 and RM147 for the downstream region. The Janus cassette was amplified from a strain of *S. pneumoniae* (RH426) using Janus F and Janus R. Two OE-PCR reactions were then performed to join the three fragments and the samples were placed in the PCR machine when the temperature had reached 98 °C (hot start). OE-PCR was also carried out to create a construct to replace the Janus cassette. First, the up- and downstream regions flanking *lytF* were amplified using RM144, RM149, RM148 and RM147 as primers. The fragments were then fused through OE-PCR to create the desired end product.

The same procedure was done for *cbpD* knockout in *S. vestibularis.* The flanking regions to *cbpD* were amplified from *S. vestibularis* using HL3 and HL4 as primers for the upstream region, and HL5 and HL6 for the downstream region. Two OE-PCR reactions were then performed to join the two fragments with the Janus cassette. For deletion of the Δ*cbpD*::Janus cassette, the upstream and downstream fragments to *cbpD* were amplified using HL3, HL7, HL8 and HL6 as primers. Then, OE-PCR with the fragments from this reaction was used to create an upstream-downstream fragment.

OE-PCR was also used to create a spectinomycin resistance cassette for testing the transformation efficiency in *S. mutans* and *S. vestibularis*. In *S. mutans*, upstream and downstream to an Abi-like protein (SMU_42) were first amplified using SA1, HL1, HL9 and SA14. The spectinomycin resistance cassette was then amplified from DS228 using DS717 and DS121 as primers, and the three fragments were then combined by OE-PCR. The same procedure was carried out for *S. vestibularis*, but in this case the upstream and downstream to a IS110-family transposase (HMPREF9425_1127) was amplified using HL16, HL17, HL18 and HL19.

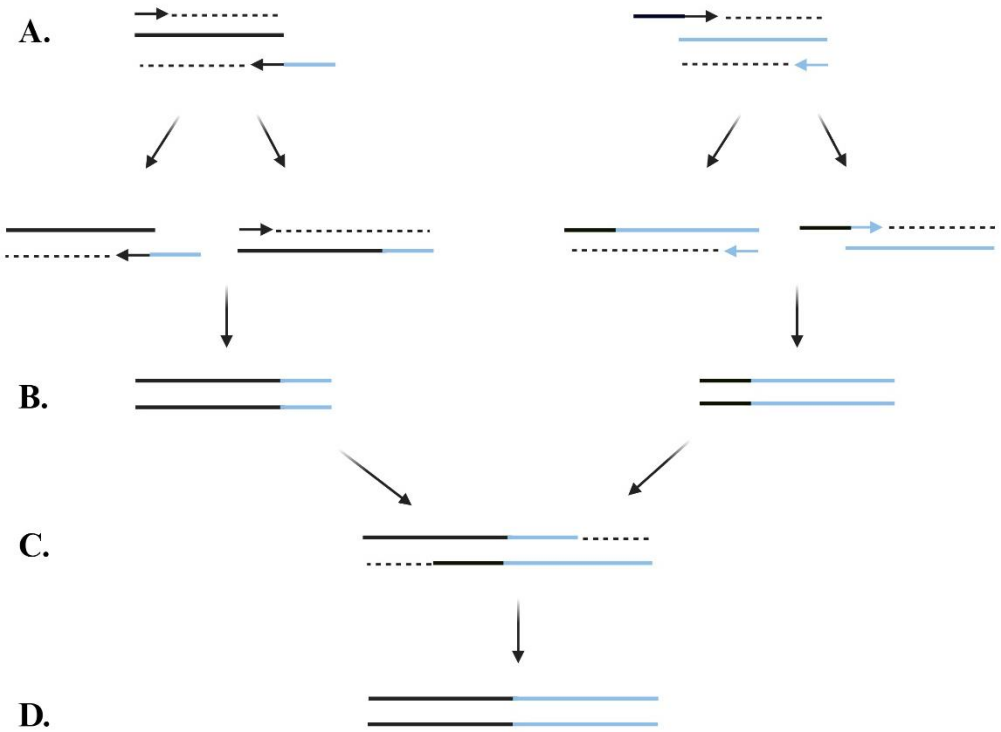

Figure 3.1 Schematic diagram of overlap extension PCR (OE-PCR). **A)** One of the primers (arrows) in each primer pair is designed with overhang that is complementary to the fragment to be joined (black/blue). The dotted lines illustrate DNA polymerase extension. Using the original fragment as template, the DNA polymerase will join the template sequence and the overhanging sequence of the primer. **B)** This results in overhanging sequences being added to both fragments. **C)** The complementary sequences in both fragments can then anneal, allowing the two fragments to be fused together after the sequences are extended (**D**). The figure is created with BioRender.com.

3.3 Agarose gel electrophoresis

Agarose gel electrophoresis is an effective method that can be used to separate DNA fragments based on their sizes[97]. This method uses an electric field to drive charged molecules across the gel, and since the phosphate backbone of the DNA molecule is negatively charged, the DNA fragments will migrate towards the positive pole[97]. The DNA fragments move through pores in the gel, and since the smallest fragments encounter less resistance, they will travel faster than larger DNA molecules. The DNA molecules can be visualized by adding a DNA-binding dye to the gel that fluoresces under UV light when bound to DNA. A ladder, which is a mixture of DNA fragments of known sizes, is also applied to the gel for comparison with the applied samples.

In this work, agarose gel electrophoresis was used to analyze the presence and size of PCR products. For DNA molecules ranging from 500-5000 bp, a 1% (w/v) agarose gel was used, whereas for DNA molecules between 150-500 bp, a 2% (w/v) agarose gel was used. To make a

1% (w/v) agarose gel, 0.5 g agarose was boiled in 50 ml 1x TAE buffer (40 mM Tris-Acetate, 1 mM EDTA), and 1 µl of the fluorescent dye peqGreen was added to the gel solution at 60°C. The solution was poured into a cast, and a comb was placed in the gel to create wells. The gel was left at room temperature for about 20 minutes before it was moved to a gel electrophoresis chamber. Furthermore, the DNA samples were mixed with a 6x gel loading dye to a final concentration of 1x. The loading dye contains glycerol and the dye bromophenol blue, which simplifies loading by making the samples visible in the gel. Loading dye was not added to the PCR samples with RedTaq® polymerase, as there is already a dye present in this PCR mix. Next, 5 µl of a ladder with an appropriate size interval was added to one of the wells. The electrophoresis was performed at 100V for approximately 25-35 minutes. The DNA fragments were visualized by UV light in a GelDoc-1000 (BioRad).

3.3.1 Purification of DNA from agarose gel

DNA fragments were purified from the agarose gel by cutting out the DNA band of interest using a sterile scalpel. The DNA was extracted and purified using the NucleoSpin® Gel and PCR Clean-Up Kit (Macherey-Nagel). The agarose gel piece with the desired DNA fragment was first dissolved in NTI (200µl/100 mg agarose gel) at 55 °C for 5-10 minutes. The solution was then transferred to a NucleoSpin® Gel and PCR- Clean-Up column and centrifuged at 11,000 *g* for 30 seconds. The column consists of a silica membrane that normally will bind to the positively charged hydrogens of water molecules. However, silica can also bind specifically to the negatively charged oxygens of the DNA phosphate backbone if the DNA is dissolved in a solution containing high concentrations of chaotropic salts. The NTI buffer of the purification kit contains a high salt concentration, and the salts will therefore bind to water molecules. The water molecules are thus unavailable to silica, which then will bind the DNA molecules instead. The column was then washed with 700 µl NT3 buffer to remove contaminants from the silica membrane, and the sample was centrifuged again at 11,000 *g* for 30 seconds. After washing, centrifugation was carried out with an empty column to dry the silica membrane. DNA was then eluted in an Eppendorf tube using 15-30 µl of a low-salt NE buffer. The column was incubated at room temperature for 1 minute before a final centrifugation was performed at 11,000 *g* for 1 minute. The purified DNA fragments were stored at -20 °C.

3.4 Plasmid isolation

Plasmids were isolated using the E.Z.N.A.® Plasmid Mini Kit I by following the manufacturer's protocol. A 1-5 ml overnight culture was centrifuged at 13,000 *g* for 1 minute at room temperature, and the supernatant was removed. The pellet was then resuspended in 250 µl solution I which contains RNase A to degrade RNA. Next, 250 µl solution II was added to the tube, and the tube was gently inverted 4-5 times to obtain a clear lysate. Solution II contains Sodium Dodecyl Sulfate (SDS), which solubilize the cell membrane and denatures proteins in the cells. The solution also contains sodium hydroxide (NaOH) which breaks the hydrogen bonds between the DNA bases and converts double-stranded DNA into single-stranded. Furthermore, 350 µl solution III was added and the tube was rotated gently until a flocculent white precipitate was formed. Solution III contains potassium acetate which reduces the alkalinity of the solution, allowing the hydrogen bonds between the bases of the single-stranded DNA to re-establish. Small circular plasmids will easily re-nature, and the double-stranded plasmid can therefore easily dissolve in solution, while single-stranded genomic DNA and other denatured proteins will precipitate. The tube was then centrifuged at maximum speed (≥ 13,000 *g*) for 10 minutes. A HiBind® DNA Mini Column was inserted into a 2 ml collection tube and the cleared supernatant was transferred carefully into the column. The sample was then centrifuged at 13,000 *g* for 1 minute, and the filtrate was discarded. Next, 500 µl HBC Buffer diluted with 100 % isopropanol was added, and the sample was centrifuged at 13,000 *g* for 1 minute. Furthermore, 700 µl DNA Wash Buffer diluted with 100 % ethanol was added, and the sample was centrifuged at maximum speed for 30 seconds. The empty HiBind® DNA Mini Column was then centrifuged at maximum speed for 2 minutes to dry the column and to ensure that all the ethanol was removed. Next, the column was transferred to a nuclease-free Eppendorf tube, and 30-100 µl Elution Buffer was then added. Finally, the sample was incubated at room temperature for 1 minute and centrifuged at 13,000 *g* for 1 minute. The eluted DNA was then stored at -20 °C.

3.5 Restriction digestion and ligation

Restriction enzymes are enzymes that recognize and cleave specific DNA sequences ranging from four to eight base pairs in length. The enzymes make double-stranded cut in the DNA and produce either sticky or blunt ends, depending on whether the cut is staggered or straight in the DNA duplex. In this work, restriction enzymes were used for plasmid construction and all

digestions were carried out with two different enzymes. Restriction digestion reactions were performed by following the manufacturer's protocol (NEB) as described in **Table 3.5**.

Table 3.5 Components in a restriction digestion reaction.

Component	Final concentration/volume
Plasmid DNA	1 µg
10x supplied reaction buffer	5 µl (1x)
Restriction enzyme 1	1 µl
Restriction enzyme 2	1 µl
dH_2O	To a final volume of 50 µl

The restriction digestions were incubated for 1.5-2 hours at optimum temperature for the given restriction enzyme. In this work, all the restriction digestions were incubated at 37 °C. Reactions with digested vectors were added 1 µl CIP (Calf Intestinal alkaline Phosphatase) when 30 minutes of the incubation time remained. CIP catalyze dephosphorylation of 5' ends of DNA and was added to prevent re-ligation of the empty vector. Cleavage products were removed from the vector by agarose gel electrophoresis followed by gel extraction and purification as described in **section 3.3**. For cases where DNA less than 10 bp were cleaved off target DNA fragments, digested DNA was purified directly using the NucleoSpin® and PCR Clean-up kit.

The plasmid pRM37 was constructed by inserting a fragment between SpeI and NcoI restriction sites in plasmid pFD116. Ligation was made possible by cutting both the insert and vector with the same restriction enzymes, resulting in complementary overhangs. SpeI and NcoI are type II restriction enzymes, meaning they cut within their recognition sequence. SpeI recognizes the DNA sequence 5'-ACTAGT-3' and cuts the strand after the 5'-adenine, while NcoI recognizes the DNA sequence 5'-CCATGG-3' and cuts the strand after the first 5'-cytosine. Ligation of the insert and vector were catalyzed by T4 ligase, which forms phosphodiester bonds between the 5' phosphate and 3' hydroxyl termini. The components of the ligation reaction were mixed using a vector to insert molar ratio of 1:5-1:7. This kind of vector to insert ratio seeks to saturate the vector with insert, making it less likely that an empty vector will be ligated. The ligation reaction consisted of 2 µl of the supplied 10x buffer, 1 µl T4 ligase and dH_2O to a final volume of 20 µl and was incubated at 16 °C overnight. The ligation reactions were then stored at -20 °C or directly transformed into the host.

3.5.1 DNA sequencing

DNA sequencing is the process of determining the order of nucleotides in DNA. In Sanger sequencing, the target DNA is copied many times by creating fragments of varying lengths using chain-terminating dideoxynucleotide triphosphates (ddNTPs) in addition to regular dNTPs[98]. As the dNTPs and ddNTPs have an equal chance of attaching to the growing sequence, each sequence will terminate at different lengths[98]. The ddNTPs contain a fluorescent marker, and a laser can therefore be used to detect the nucleotide sequence. In this work, Sanger sequencing was performed to verify correct construction of plasmids. Sanger sequencing was performed by GATC, Eurofins Genomics and the solution sent contained 200-300 ng plasmid DNA, 2 µl of the primer (10µM) and dH_2O to a final volume of 10 µl.

3.6 Genetic modification of *S. mutans* and *S. vestibularis*

3.6.1 Establishment of the Janus cassette system

In this work, gene knockouts were constructed using the Janus cassette system. The Janus cassette system can be used in strains that are resistant to streptomycin due to a recessive mutation in the *rpsL* gene[99]. A spontaneous mutation in *rpsL* usually results in a lysine replacement in protein S12 in the small ribosomal subunit and provides the bacterium with resistance to streptomycin[100]. The lysine replacement usually occurs at position 42 or 86, but amino acids at other positions can also be affected[100, 101]. Streptomycin usually inhibits protein synthesis, but the mutation in *rpsL* prevents binding of streptomycin and thus it's activity. Since the Janus cassette contains a kanamycin resistance marker and an $rpsL^+$ marker, it is possible to select for its acquisition and its loss[99]. The $rpsL^+$ marker expresses a version of RpsL that confers dominant streptomycin sensitivity and strains with the Janus cassette are therefore kanamycin resistant and streptomycin sensitive[99]. Removal of the cassette will restore streptomycin resistance and remove kanamycin resistance. To utilize the Janus cassette system, streptomycin resistance was induced and Sanger sequencing was then performed to verify mutation in the *rpsL* gene.

3.6.2 Transformation of *E. coli*

3.6.2.1 Preparation of chemically competent E. coli

E. coli is not considered a naturally competent bacterium, however, it can be made artificially competent using various methods. One of the methods that can be used to introduce foreign

DNA into *E. coli* is called $CaCl_2$ heat-shock transformation (chemically competent *E. coli*). It is unclear how calcium chloride contributes to the transformation process, but it is believed that it facilitates the binding of DNA on the cell surface by neutralizing the electric repulsion between the negatively charged phospholipids and DNA[102]. Calcium chloride is also believed to increase the permeability of the cell membrane making it easier for DNA to be taken up into the cell[102]. Heat treatment then generates pores in the cytoplasmic membrane, allowing DNA to enter the cell.

To perform transformation in *E. coli*, chemically competent cells first had to be prepared. This was accomplished by incubating a 5 ml overnight culture of *E. coli* in LB medium at 37 °C shaking. After incubation, the culture was diluted 1/100 in a final volume of 100 ml in fresh LB medium in an Erlenmeyer flask. The culture was grown to a OD_{600} of approximately 0.4, and then cooled on ice for approximately 20 minutes. The cells were harvested by centrifugation at 5,000 *g* for 5 minutes at 4 °C, and the supernatant was removed. All the subsequent steps were then carried out at 4 °C. The cells were resuspended in ½ of the culture volume in 0.1 M ice cold $CaCl_2$ and kept on ice for 2 hours. Cells were then harvested by centrifugation at 5,000 *g* for 5 minutes and resuspended in approximately 5 ml 0.1 M $CaCl_2$ with 15 % glycerol. Finally, the competent cells were aliquoted in Eppendorf tubes and stored at -80 °C.

3.6.2.2 Heat-shock transformation of E. coli

Heat shock transformation in *E. coli* was performed by thawing 50 µl chemically competent cells on ice. Plasmids or ligation reaction was added to the cells (usually 2-10 µl) followed by gentle mixing. The mixture was kept on ice for 30 minutes before the cells were heat shocked at 42 °C for 30-40 seconds. Next, 250 µl LB medium was added to the cells and the cells were incubated at 37 °C shaking for 1 hour. After that, 50 and 200 µl was plated out on LB agar plates with antibiotics for selection of transformants. The transformants were verified by colony PCR screening.

3.6.3 Transformation of *S. mutans*

S. mutans was naturally transformed using a protocol adapted from Salvadori et al[103]. An overnight culture of *S. mutans* was diluted to OD_{550} ~ 0.05 in C-medium w/o sucrose. The culture was incubated at 37 °C until the OD_{550} reached 0.08-0.1. Then 1 ml of the culture was transferred to an Eppendorf tube, and ComS was added to a final concentration of 1 µM. The

tube was then incubated for 3 hours at 37 °C before 100 µl of the culture was transferred to two new Eppendorf tubes. One tube was the transformation reaction, while the other was a negative control. Donor DNA (50-200 ng) was added to the transformation reaction, and the tube was mixed gently by tapping the bottom of the tube. The samples were incubated at 37 °C for 20 minutes. Then 200 µl of pre-warmed C-medium w/o sucrose was added to both the transformation reaction and the negative control, and the samples were incubated for another 40 minutes. Finally, 20-200 µl of the transformation mix was spread on selective agar plates and incubated at 37 °C in a CO_2 cabinet (10 % CO_2) over night.

3.6.4 Transformation of *S. vestibularis*

A start culture of *S. vestibularis* was thawed in a 37 °C water bath and centrifuged at 4,000 *g* for 5 minutes at room temperature. The spent growth medium was removed, and the cells were resuspended in fresh C-medium to an OD_{550} of 0.025 and incubated in a 37 °C water bath until OD_{550} was at 0.05-0.1 (preferable closest to 0.05). Then, 1 ml of the cell culture was transferred to two Eppendorf tubes containing 250 ng ComS. One tube was also added 200-250 ng transforming DNA, while the other tube served as a negative control. The tubes were incubated on a 37 °C water bath for 2 hours, before 10-30 µl of the cultures were plated on TH plates with relevant antibiotics. The plates were incubated anaerobically at 37 °C over night.

3.7 Testing transformation efficiency

Transformation efficiency was tested with both recombining (linear DNA) and non-recombining DNA (plasmid). The experiment was carried out using start cultures of *S. mutans* and *S. vestibularis* and the cultures were transformed with the desired fragments as described in **section 3.6.3** and **3.6.4**. A 10-fold dilution series (10^{-1}-10^{-9}) were prepared by filling 9 Eppendorf tubes with 900 µl C-medium. Then 100 µl of the culture was transferred to the 10^{-1} tube, and from the 10^{-1} tube, 100 µl was transferred to the 10^{-2} tube, and so on. Once all the dilutions were made, 50 µl from each dilution was plated onto plates without antibiotics. In addition to this, 50 µl of the original transformed culture and the negative controls were plated out on plates with spectinomycin (100 µg/ml). The plates were incubated at 37 °C for at least 24 hours. After incubation, the number of transformants and the total number of bacteria per ml culture were calculated. The transformation efficiency was also calculated by dividing the number of transformed cells by the total number of cells.

3.8 Luciferase reporter assay

Luciferase reporter assay can be used to investigate the activity of a promoter of interest. In this assay, the promoter of a desired target gene, including any regulatory regions, is placed upstream of the coding sequence of firefly luciferase[104]. If the desired promoter is switched on, the cells will produce luciferase, which acts as a reporter protein and produces light when it oxidizes D-luciferin using ATP[104]. Bioluminescence can then be measured, and the amount of light reflects the degree of transcription from the promoter.

In this work, streptococci examined with the luciferase reporter assay were grown in C-medium or C-medium without sucrose/glucose. ON-cultures were diluted to OD_{550} ~ 0.05 and incubated at 37 °C until they were in exponential phase (OD_{550} ~ 0.2-0.4). The cultures were then diluted to an OD_{550} of 0.025 and transferred to a 96-well Corning NBS clear-bottom plate. D-luciferin was added to the wells to a final concentration of 180 µg/ml. The plate was incubated in a Synergy Hybrid Reader (BioTek) at 37 °C, and OD_{550} and luminescence were measured automatically every 5 minutes. ComS (100 ng/µl) was added to induce expression of the *luc* gene.

3.9 Zymogram

Zymography is a technique that can be used to study hydrolytic enzymes. The method is useful for visualizing the hydrolysis of a substrate, either by detecting the formation of a reaction product, or the disappearance of the substrate[105]. In this way, zymography can be used for any hydrolase acting on a biological substrate. Zymography is based on SDS-PAGE and in this work the substrate used was whole cells of *S. mutans*, which were integrated into the polyacrylamide gel matrix.

The substrate was prepared by growing 300 ml of culture with *S. mutans* to OD_{550} ~ 0.2. The sample was then centrifuged at 7,000 *g* for 10 minutes before the pellet was dissolved in 1.25 ml of 1.5 M Tris-HCl pH 8.8. The cells were then heated at 95 °C for 10 minutes to inactivate the bacteria. The solution was then added to a 10 % SDS-PAG as described in **section 3.10.1**. After electrophoresis, the gel was washed in de-ionized water for 2*30 minutes. Finally, the gel was incubated in refolding buffer (50 mM NaCl, 20 mM $MgCl_2$, 0.5 % Triton X-100 and 20 mM Tris-HCl, pH 7.4) by gentle shaking for 3-12 hours, or until clear lytic zones were observed on the gel.

3.10 Western blotting

Western blotting is a method that can be used to identify and quantify specific proteins in a sample. The technique involves separation of proteins by gel electrophoresis, transfer of proteins onto a membrane and detection of the protein of interest using specific antibodies[106]. A technique that is commonly used to separate proteins is Sodium Dodecyl-Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE). Sodium dodecyl-sulfate is a detergent that binds to and denatures the proteins, giving them a negative charge that is proportional to the molecular weight, and this ensures that the proteins can be separated based on size[107]. The separated molecules are then transferred to a membrane on which a target protein can be detected using a specific primary antibody. A secondary antibody, often conjugated to an enzyme, is used for recognition of the primary antibody and to increase the sensitivity of the assay. By adding a substrate for the antibody conjugated enzyme, the position where the primary antibodies are bound on the membrane can be detected. Depending on the substrate used, it can be converted to a detectable product bound to the membrane, or as used in this work, result in light emission. The chemiluminescence signal can then be detected using a camera.

3.10.1 Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis

For Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE), a 10 or 15 % separation gel and a 4 % stacking gel were casted. These were made as described in **Table 3.6**, **Table 3.7** and **Table 3.8**. To one of the wells in the gel, 5 µl of appropriately molecular weight size marker was added, while in the respective wells 15 µl of each sample was added. The electrophoresis compartments were then filled with 1x SDS electrophoresis buffer. Electrophoresis was performed at 120 V until the samples had reached the separation gel and then at 180 V until the migration front reached the end of the gel.

After electrophoresis, separated proteins were visualized by Coomassie blue staining. The gel was submerged in 0.2 % Coomassie blue and heated to the boiling point of the Coomassie blue staining solution using a microwave. The gel was then incubated with gentle shaking for 10 minutes. Finally, the gel was rinsed in water and destaining solution was added. The gel was again placed in the microwave, as described earlier. The gel was then left to destain overnight, and images of the gel were taken with the Azure Imaging System.

Table 3.6 Components in the 10 % separation gel used in SDS-PAGE.

Solutions in separation gel	Volume used for two gels
dH_2O	4.78 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
10 % SDS	100 µl
40 % acrylamide + 0.8 % bis-acrylamide	2.5 ml
10 % APS	100 µl
TEMED	5-10 µl

Table 3.7 Components in the 15 % separation gel used in SDS-PAGE.

Solutions in separation gel	Volume used for two gels
dH_2O	3.55 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
10 % SDS	100 µl
40 % acrylamide + 0.8 % bis-acrylamide	3.75 ml
10 % APS	100 µl
TEMED	5-10 µl

Table 3.8 Components in the 4 % stacking gel used in SDS-PAGE.

Solutions in stacking gel	Volume used for two gels
dH_2O	3.15 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
10 % SDS	50 µl
40 % acrylamide + 0.8 % bis-acrylamide	500 µl
10 % APS	50 µl
TEMED	5-10 µl

3.10.2 Immunodetection of proteins

Immunodetection is the use of antibodies to detect the presence of specific proteins in a complex sample. In this work, immunoblotting was used to detect specific proteins immobilized onto a PVDF (polyvinylidene fluoride) membrane after being separated by SDS-PAGE. Transfer of proteins from a SDS-PAG to a membrane is called Western blotting. To perform western blotting, the SDS-PAG was transferred to a tray with ice-cold Western blot transfer buffer. The

PVDF membrane was soaked in 100 % methanol for 30 seconds, washed a few times with dH_2O and transferred to a tray with ice-cold transfer buffer. Filter papers (3 mm) were also soaked in ice-cold transfer buffer before blotting. The filter papers, the gel and the membrane were then stacked on top of each other in a specific order in a Trans Blot Turbo machine to transfer the proteins from the gel to the membrane (**Figure 3.2**). The membrane is placed on the anode side of the gel so that the negatively charged SDS-coated proteins will migrate from the gel onto the membrane. Semi-dry electroblotting was performed at 25 V and 1.3 A for 7 minutes. After blotting, the membrane was transferred to a tray with fresh 5 % fat-free skim milk in TBST and the tray was placed at a platform rocker for 1 hour. The tray was then placed at 4 °C overnight.

The following day, the membrane was rinsed with TBST before adding 10 ml of primary anti-Flag antibodies (1:4000 in TBST) from rabbit. The membrane was then incubated at a platform rocker for 1 hour. Next, the membrane was washed for 10 minutes with 20-30 ml TBST. This washing step was repeated three times before 10 ml anti-rabbit antibody (1:4000 in TBST) was added. The membrane was then incubated for another 1 hour at a platform rocker and washing of the membrane was carried out as described previously. The Thermo Scientific SuperSignal™ West Pico PLUS Chemiluminescent substrate kit was used to detect proteins on the membrane. From this kit, 5 ml of Luminol was mixed with 5 ml of stable peroxide. The membrane was incubated in this solution for one minute before images were taken using the Azure Imaging System.

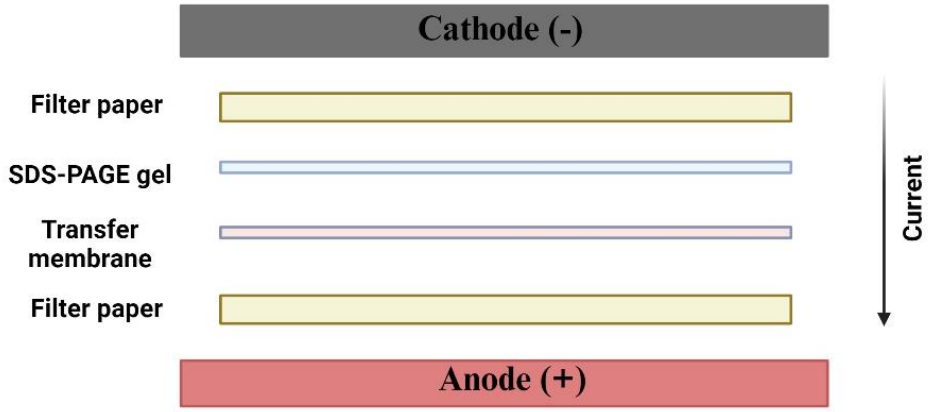

Figure 3.2 Schematic illustration of the stacking order in western blotting. The blot sandwich is composed of a SDS-PAGE gel placed on top of a transfer membrane, which in turn is surrounded by a layer of filter paper. The proteins migrate from the cathode (-) towards the anode (+). The figure is created with BioRender.com.

3.11 Preparation of protein samples for detection of Flag-ComGC

To compare the amount of pilus in the wildtype strain and the fratricin mutant, it was necessary to know at what time point pilus was expressed. For detection of Flag-ComGC in whole cell extracts, ON-cultures were diluted to an OD_{550} ~ 0.05 in a total volume of 30 ml. When OD_{550} was 0.08-0.1, 5 ml was transferred to a new tube on ice. Competence was then induced in the remaining culture by adding ComS to a final concentration of 1 µM, and the culture was incubated at 37 °C. The 5 ml culture that was put on ice was centrifuged at 5,000 *g* for 5 minutes and the supernatant was removed. The pellet was dissolved in 50 µl 1x SDS sample buffer and stored at -20 °C. For the ComS-induced culture, 5 ml was removed after 1, 2, 3 and 4 hours. The cell pellets were dissolved in 50 µl 1x SDS sample buffer and stored at -20 °C for SDS-PAGE and immunoblot analyses.

For detection of Flag-ComGC in culture supernatants, bacteria were grown to OD_{550} 0.08-0.1 in 20 ml cultures. The culture was split in two, where one parallel was added a final concentration of 1 µM ComS, while the other served as uninduced control. After incubation at 37 °C, the cultures were normalized so that OD_{550} was more or less the same in all the samples. The cells were then passed through a 25G needle six times and collected at 5,000 *g* for 5 minutes. The supernatant was concentrated using Amicon® Ultra-15 Centrifugal Filter at 4,000 *g* until the sample volumes were reduced to 500 µl. The samples were then transferred to Eppendorf tubes and stored at -20 °C. Finally, the samples were prepared for SDS-PAGE by mixing 10 µl sample with 10 µl of 2x SDS sample buffer.

3.11.1 Immunoprecipitation of Flag-ComGC

Volumes of 40 µl Anti-FLAG® M2 Affinity Gel beads were equilibrated in 500 µl TBS. Culture supernatants (10 ml) were added to the beads and the samples were incubated on a rotating stand for 1 hour at room temperature. The beads were collected at 2,000 *g* for 2 minutes, the supernatant was removed, and the beads were washed 3 times with 1 ml TBS. Finally, the beads were dissolved in 50 µl 1x SDS sample buffer and thereafter removed by centrifugation at 2,000 *g*. SDS-PAGE and western blotting were then performed.

3.12 Microscopy

Microscopic analyses were performed using a Zeiss AxioObserver and a 100x phase contrast objective. ON-cultures were diluted to OD_{550} ~ 0.05 and were incubated at 37 °C until OD_{550} was 0.1-0.2. The cells were centrifuged for 1 minute at 13,000 *g* and washed with phosphate-buffered saline (PBS). The pellet was then resuspended in 250 µl PBS, and 1 µl of the sample were added to glass plates covered with 1 % agarose (w/v) in PBS.

3.12.1 Fluorescence microscopy

Phase contrast and fluorescence microscopy was performed using an adapted protocol from Laurenceau et al[66]. Bacterial cell pellets were resuspended in PBS to an OD_{550} of approximately 0.1. Then 50 µl of the sample were transferred inside a hydrophobic frame (made using a PAP-pen) on VWT® Polysine® Adhesion slides. The sample was then removed from the frame and the cells that had adhered to the glass were fixed for 30 minutes with 3.7 % formaldehyde. Next, 50 µl of PBS with 1 % BSA (w/v) was added before the samples were incubated for 1 hour with anti-Flag-antibodies (1:300 in PBS) in a humidity chamber. Samples were then incubated for 1 hour in the dark with Alexa Fluor™ 488 Goat anti-rabbit IgG (1:300). The slides were washed with PBS between each of these steps. Pictures were taken by using a Zeiss AxioObserver with ZEN Blue Software, an ORCA-Flash 4.0 V2 Digital Complementary Metal-Oxide-Semiconductor (CMOS) camera (Hamamatsu Photonics), and a 100x phase contrast objective. An HPX 120 Illuminator (Zeiss) served as light source for fluorescence microscopy. The images were then processed in ImageJ.

3.13 Overexpression of recombinant proteins and preparation of cell lysates for protein purification

To purify LytF from *S. mutans*, the recombinant protein first had to be overexpressed in *E. coli*. A 5 ml culture was therefore incubated in LB at 37 °C overnight and was further inoculated in 100 ml LB-medium. When there was sufficient growth, the bacterial culture was placed at 4 °C until the following day. The culture was then reinoculated in 1000 ml LB to an OD_{600} ~ 0.1 and incubated at 37 °C. When the OD_{600} was around 0.4, the bottle was cooled to room temperature and IPTG (0.1 mM) was added to induce protein expression. The bottle was further incubated at 25 °C for 4 hours. The cells were then collected by centrifugation at 12,000 *g* for 5 minutes and the pellet was frozen at -20 °C. Ampicillin 100 µg/ml was used for all the cultivation steps.

For purification of His-tagged proteins expressed in bacteria, the cells were dissolved in 20 mM Tris-HCl pH 7.5, 500 mM NaCl and 20 mM imidazole. In addition to this, DNase was added to make it easier to dissolve the pellet. The solution was then distributed in FastPrep tubes with acid washed glass beads (≤106 µm) from Sigma® Life science. Homogenization was performed using a FastPrep 24® machine at a speed of 6 m/s for 3*20 seconds. The tubes were then centrifuged at maximum speed for 5 minutes before the supernatant was transferred to a Nunc tube. The supernatant was further filtered through a 0.45 µm filter and the sample was then manually applied to a Cytiva HisTrap™ HP His tag column.

3.14 Immobilized metal affinity chromatography

Chromatography is a method that can be used to separate, identify and purify desired components from a sample[108]. Immobilized metal affinity chromatography (IMAC) can be used to purify proteins based on their affinity to specific metal ions, and this technique utilizes the affinity of proteins containing natural surface-exposed histidine, cysteine and tryptophan residues, as well as proteins with engineered affinity tags[109]. In this work, an affinity tag with polyhistidine (His-tag) was used. When the sample material is applied to a column (the stationary phase) charged with metal ions, usually divalent copper ions, the affinity tagged protein will bind to the column. When a solution (the mobile phase) is passed through the column, the proteins that have not bound, or have bound weakly to the column, will be removed. Elution of the desired protein can then be accomplished by adding increasing amounts of imidazole, which will outcompete the His-tag by binding to the metal ions in the column.

IMAC was performed using ÄKTA pure™ chromatography system and 1 ml/min flow. The loaded His-tag column was washed with 10x column volumes of buffer A (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 20 mM imidazole), and elution with buffer B (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 500 mM imidazole) was then performed using a linear gradient from 0-100 % B over 30 ml. The eluate was detected using a wavelength of 280 nm and was further analyzed by SDS-PAGE, see **section 3.10.1**. This was done to investigate if the purification was successful and contained the protein of desired size.

4 Results

In *S. pneumoniae*, the fratricin CbpD has been shown to be important for targeted lysis of close relatives (fratricide mechanism, **section 1.2.3**), making homologous DNA available for uptake by the competent pneumococcus[110]. The fratricide mechanism is therefore important for the spread of antibiotic and virulence genes in this species. For streptococci outside the mitis group, however, the presence of a fratricide mechanism is not well described although most streptococci have a *cbpD*/*lytF*-like gene[72]. Instead, studies have shown that deletion of the fratricin encoding gene has a negative effect on the transformability of the competent cell in *S. suis, Streptococcus thermophilus* and *Streptococcus sanguinis*[111-113]. The present study has therefore been part of a larger project aiming to characterize whether the genes encoding putative fratricins are competence induced, to test their lytic activity, target range and possible effect on transformability. In the work presented here we have chosen to study two different fratricins; the LytF of *S. mutans* (mutans group) and a CbpD-like protein of *S. vestibularis* (salivarius group). First, we wanted to test if expression of *lytF* and *cbpD* is induced during competence in *S. mutans* and *S. vestibularis*.

4.1 Are *lytF* and *cbpD* expression induced during competence?

To test if expression of *lytF* and *cbpD* was induced upon entrance to the competence state, the P_{lytF} and P_{cbpD} promoters were placed in front of the *luc* gene encoding firefly luciferase and cloned into a plasmid. Strains of *S. mutans* and *S. vestibularis* containing the P_{lytF}-*luc* and P_{cbpD}-*luc* plasmid were created by PhD student Rebekka Moe. To ensure that competence was induced when the competence inducing peptides were added, clones containing plasmids with their cognate P_{comX} promoters in front of *luc* was also used (also constructed by Rebekka Moe). Luminescence of the different strains was measured during competence, and if the promoters were switched on, luciferase could act as a reporter protein and produce light by using ATP to oxidize D-luciferin. The assay was performed by growing the cells in different growth media, as it has been shown that the promotor activity of certain competence-associated genes in *S. mutans* are influenced by the type of carbohydrate sources available[114]. For *S. vestibularis* only one growth medium was used.

4.1.1 Addition of ComS did not increase transcription from P_{comX} in *S. mutans*

To control that competence was induced after addition of competence stimulating peptides, a strain with the *luc* gene located behind the P_{comX} promoter was used. However, induction of competence did not result in elevated transcription from the P_{comX} promoter, as no clear increase in RLU (relative luminescence unit) were observed (**Figure 4.1**). The results were highly similar to what was observed in the negative control where a luciferase gene was not inserted (**Figure A.0.1**), indicating that the experiment has not worked optimally. Since there was no successful transcription from the P_{comX} promoter, we are unable to determine if type of carbohydrate source have an effect on transcription. The results may be caused by the slow bacterial growth, as this reduces the number of cells containing the reporter construct. This will in turn lead to a generally lower expression of luciferase, making it challenging to detect and quantify the activity of the promoter.

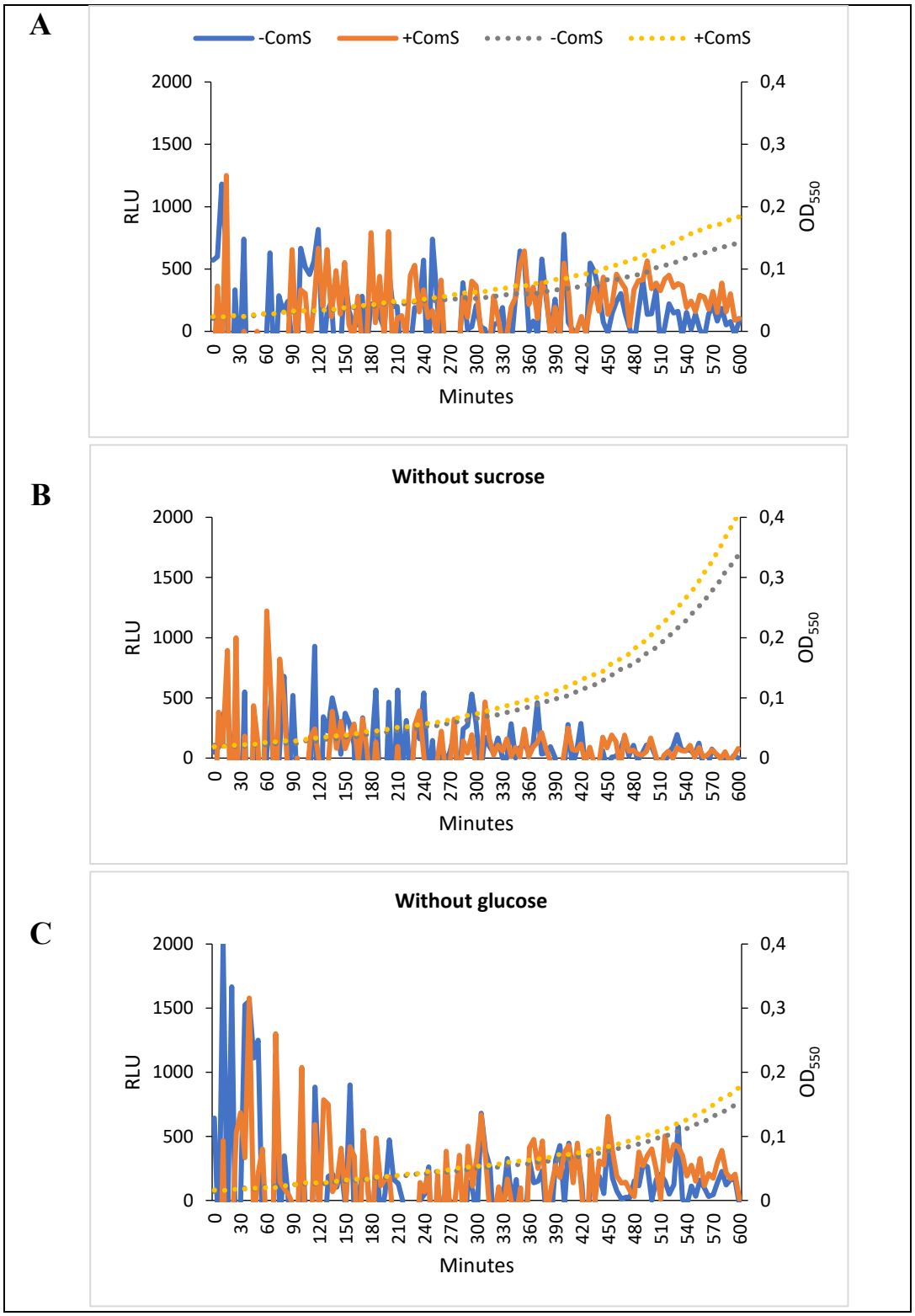

Figure 4.1 Luciferase reporter assay performed with a strain of *S. mutans* in which *luc* expression is driven by the P_{comX} promoter. ComS was added at time 0 to induce cells to competence. Growth curves are represented by dashed lines, while relative luminescence unit (RLU) is represented by solid lines. Different growth media were used: **A)** C-medium **B)** C-medium without sucrose **C)** C-medium without glucose.

4.1.2 Competence induction results in increased transcription from P_{lytF} in *S. mutans*

Although we did not see induction of P_{comX}, (ComX is required for induction of late competence genes such as *lytF*), luciferase reporter assay was also performed to investigate whether *lytF* is competence induced (**Figure 4.2**). Surprisingly, addition of ComS inducer resulted in elevated transcription from the P_{lytF} promoter and highest induction was observed in C-medium without glucose. Although an increase in luminescence was observed after addition of ComS, the signal was low compared to the positive control (induction of P_{comX} in *S. dysgalactiae*, **Figure A.0.2**). Since we have observed large variation in signal strength in different streptococcal species (data not shown), the results still indicate that *lytF* is competence induced, as there was stronger signal in the samples compared to the negative control. The results also indicate that carbohydrate source had an effect on induction of the P_{lytF} promoter, as there was higher transcription from the promotor when the cells were cultured without glucose. Despite the slow bacterial growth in both regular C-medium and C-medium without glucose, clear changes in transcription from P_{lytF} were observed when competence was induced.

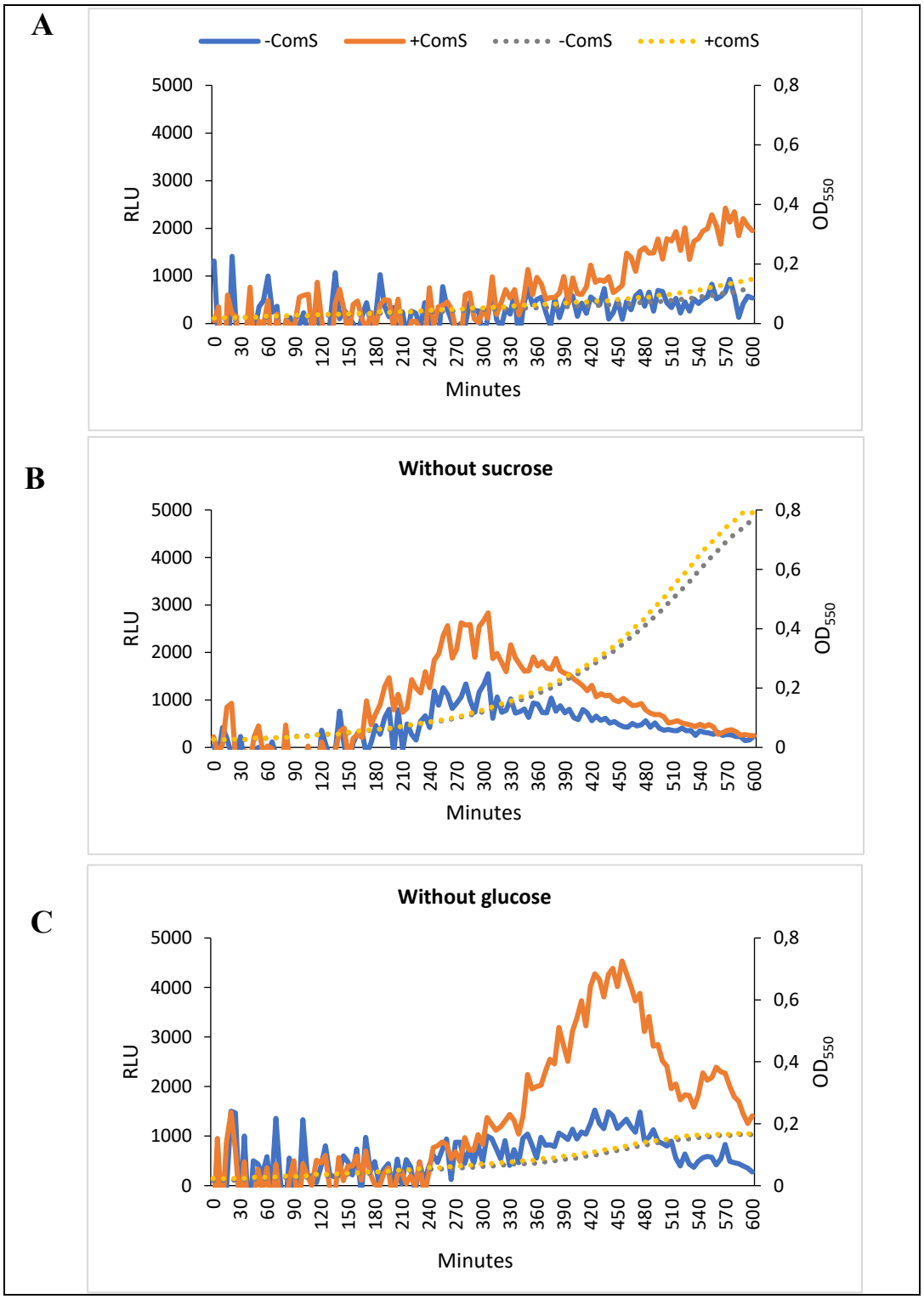

Figure 4.2 Luciferase reporter assay performed with a strain of *S. mutans* in which P_{lytF} is linked to a luciferase encoding gene. ComS was added at timepoint 0 to induce expression of this gene. Growth curves are represented by dashed lines, while relative luminescence unit (RLU) is represented by solid lines. Different growth media were used: **A)** C-medium **B)** C-medium without sucrose **C)** C-medium without glucose.

4.1.3 P_{comX} and P_{cbpD} are induced in *S. vestibularis* after addition of ComS

Similarly as for *S. mutans*, it was investigated whether *cbpD* is competence induced in *S. vestibularis* by measuring the P_{cbpD} activity. For *S. vestibularis*, the P_{comX} promoter was induced after addition of ComS as the RLU increased (**Figure 4.3**), strongly suggesting that the cells entered the competent state. Although RLU increased rapidly after addition of ComS this increase was relatively small. The P_{comX} promoter was also activated at a later stage in the growth phase, and both the induced and non-induced sample had an increase in RLU after 300 minutes, which was also seen in the negative control (**Figure A.0.3**).

Furthermore, the P_{cbpD} promoter was activated in competent *S. vestibularis* cells, showing that expression of *cbpD* indeed is elevated during competence in this species (**Figure 4.4**). The increase in RLU was also elevated after 300 minutes as observed for *S. vestibularis* P_{comX}, suggesting that competence is re-induced during this growth phase. Transcription from the promoter was also increased without addition of synthetic ComS and may be a result of self-induction. Since RLU was measured to be three times larger than the RLU in the negative control, this indicates that transcription from the P_{cbpD} promoter has been achieved.

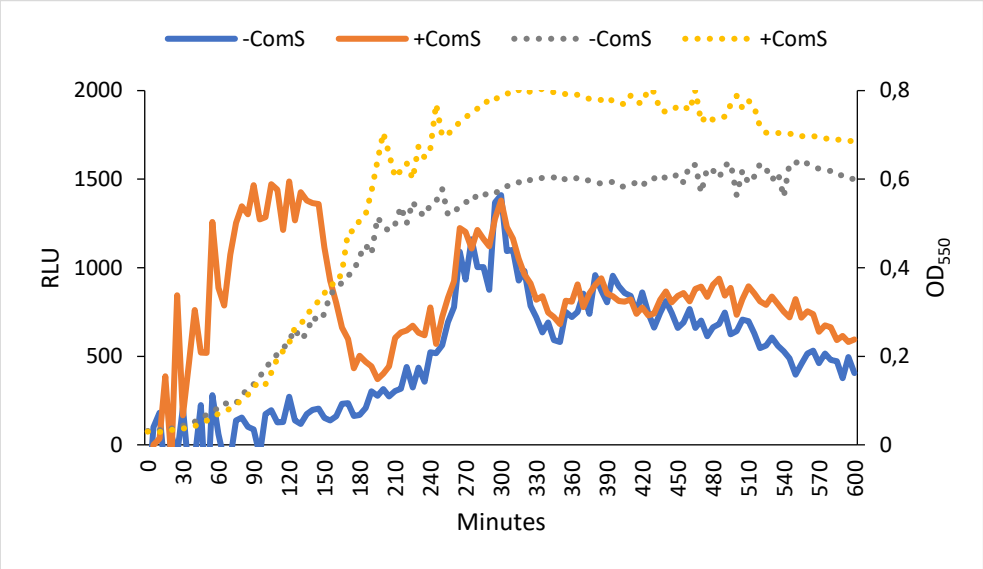

Figure 4.3 Luciferase reporter assay performed with a strain of *S. vestibularis* in which P_{comX} is linked to a luciferase gene. ComS was added at timepoint 0 to induce expression of this gene. Growth curves are represented by dashed lines, while relative luminescence unit (RLU) is represented by solid lines.

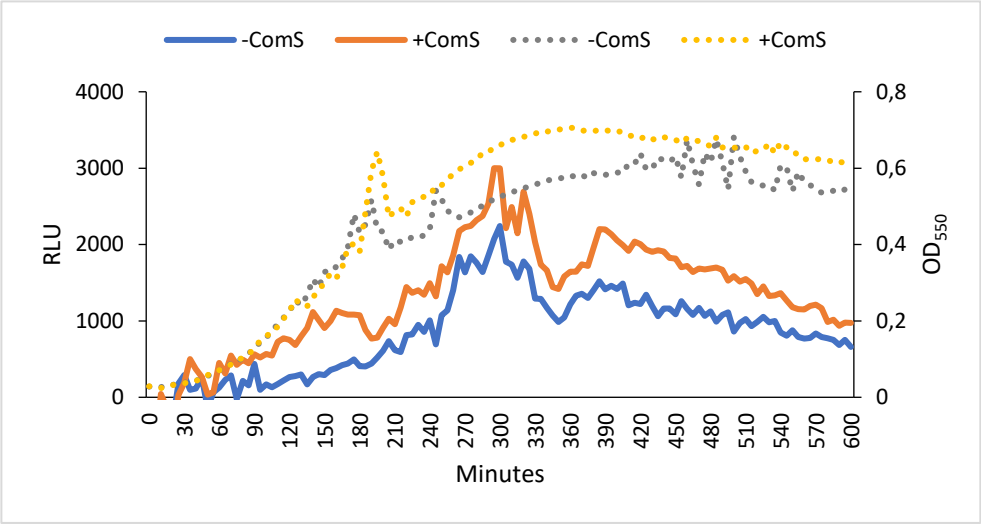

Figure 4.4 Luciferase reporter assay performed with a strain of *S. vestibularis* in which P_{cbpD} is linked to a luciferase gene. Competence was induced at timepoint 0 by the addition of ComS (250 ng/ml). Growth curves are represented by dashed lines, while relative luminescence unit (RLU) is represented by solid lines.

4.2 Transformation efficiency in fratricin deficient *S. mutans* and *S. vestibularis*

Based on the *luc*-reporter assays we concluded that expression of *lytF* and *cbpD* is competence induced in *S. mutans* and *S. vestibularis*, respectively. Since it has been shown previously that deletion of competence-induced *cbpD*/*lytF*-like genes in streptococci reduced their ability to transform, we wanted to test if deletion of *lytF* and *cbpD* had similar effect in *S. mutans* and *S. vestibularis*[112, 113]. To avoid any unwanted polar effects on genes flanking the knock-out cassette, the so-called Janus cassette system was used to knock out *cbpD* and *lytF*. One major advantage of this system is that it can be removed through negative selection in streptomycin resistant strains. For *S. mutans*, a strain that was already resistant to streptomycin was used with the Janus system, whereas for *S. vestibularis*, a mutation conferring streptomycin resistance needed to be introduced in the *rpsL* gene (**section 3.6.1**).

4.2.2 Creating a Janus-compatible strain of *S. vestibularis*

Streptomycin works by inhibiting protein synthesis[115]. However, resistance can be acquired by expressing altered versions of the S12 ribosomal unit, which is accomplished by mutations in the *rpsL* gene[100, 115]. To obtain a streptomycin resistant strain of *S. vestibularis*, the bacterium was grown on TH-agar containing increasing concentrations of streptomycin (50-500 µg/ml). After 5 days, resistant colonies appeared and Sanger sequencing revealed point mutation in position 301 of *rpsL* (**Figure A.0.4**), which resulted in an amino acid replacement from lysine (AAA) to glutamic acid (GAA).

4.2.3 Deletion of *lytF* and *cbpD*

Both *S. mutans* and *S. vestibularis* have been shown to perform natural transformation in laboratory settings[62, 116]. It should therefore be possible to knock out the *lytF* gene in *S. mutans* and the *cbpD* gene in *S. vestibularis* by providing PCR amplified linear gene knockout cassettes to competence induced cells. Indeed, transformants were obtained using Δ*lytF*::Janus and Δ*cbpD*::Janus cassettes, respectively. PCR screening showed that the genes were successfully replaced by Janus. After successful knockout of the putative fratricin encoding genes with the Janus cassette system, the Janus cassette was removed from the genomes to minimize any polar effects on nearby genes. This can be done by transforming with a DNA fragment comprising ~ 1000 bp regions flanking both sides of Janus only. Several attempts were made to remove the Janus cassette in *S. mutans*, but these were not successful as no transformants were obtained. The Janus cassette therefore remained in the genome when transformation efficiency was tested in the Δ*lytF* mutant (see next section). In *S. vestibularis*, however, the Janus cassette was easily removed from the genome.

4.2.4 Knocking out *lytF* in *S. mutans* leads to a dramatic reduction in transformation efficiency

To determine whether LytF and CbpD in *S. mutans* and *S. vestibularis* are important for transformation when the competent cells are given equal amounts of transforming DNA, a transformation efficiency assay was performed. A spectinomycin resistance cassette designed to replace the SMU_42 gene (encoding an Abi-like protein) was constructed and used to transform wildtype *S. mutans* and a Δ*lytF* mutant. The result indicated that the ability of the Δ*lytF* mutant to acquire the spectinomycin resistance gene was strongly reduced compared to that of the wildtype (**Table 4.1**). Few positive clones (0-85 CFU/ml) were present on TH plates with spectinomycin for the Δ*lytF* mutant, which displayed up to 1100-fold decrease in transformation efficiency. Although the results varied, a large reduction in transformation efficiency was generally observed for the Δ*lytF* mutant.

Next, we wondered whether this reduction in transformation was linked to DNA-uptake or to the DNA recombination process. The transformation experiments were therefore repeated using non-recombining DNA, but with the same resistance marker. The plasmid pFD116 was used for this purpose. This plasmid contains a spectinomycin resistance gene and is based on pLZ12, a plasmid shown to replicate in streptococci[117]. No clear impact on the results was observed when non-recombining DNA was used. A T-test was performed to verify that the observed

results were significant, and the analysis yielded p-values of 0.014 and 0.021 for recombinant and non-recombinant DNA, respectively. The results can therefore be considered significant. For further details, see **Table A.0.1** and **Table A.0.2**. The results therefore suggest that LytF is important for the transformation process in *S. mutans*, more specifically for DNA-uptake.

Table 4.1 Transformation efficiency in a *S. mutans* wildtype strain and a Δ*lytF* knockout mutant.

Recombining DNA			**Non-recombining DNA**		
Efficiency WT	**Efficiency ΔLytF**	**Difference in efficiency**	**Efficiency WT**	**Efficiency ΔLytF**	**Difference in efficiency**
$8.80*10^{-5}$	$7.63*10^{-8}$	1153.34	$1.85*10^{-4}$	$1.91*10^{-7}$	968.59
$2.86*10^{-5}$	0	-[a]	$1.39*10^{-4}$	$2.23*10^{-7}$	623.32
$9.10*10^{-5}$	$1.61*10^{-7}$	565.22	$4.35*10^{-5}$	$1.02*10^{-7}$	426.47

[a] It was not possible to calculate the difference in efficiency as there were no transformants for the Δ*lytF* mutant.

4.2.4.1 Microscopy of *S. mutans* revealed normal morphology after *lytF* knockout

Since LytF most probably functions as a cell wall hydrolase that makes cuts in the cell wall, possible changes to the cell morphology of the Δ*lytF* mutant was examined by comparing with wildtype cells. Microscopy was performed to ensure that LytF did not affect chain formation, as this could have led to an over- or underestimation of the transformability of the mutant. As demonstrated in **Figure 4.5**, no abnormalities in morphology or chaining were observed in the Δ*lytF* knockout cells. The estimated transformation efficiency of the Δ*lytF* mutant should therefore be reliable, supporting the assumption that LytF is involved in DNA-uptake during natural transformation.

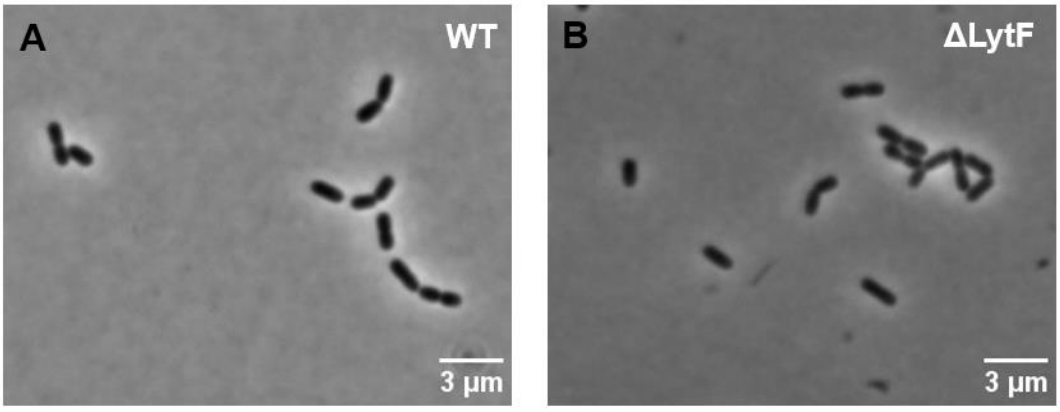

Figure 4.5 Cell morphology in *S. mutans*. The wildtype strain (**A**) and the Δ*lytF* mutant strain (**B**) were grown in C-medium to an OD_{550} of ~ 0.1-0.2. Images were obtained using a Zeiss AxioObserver and a 100x phase contrast objective.

4.2.5 Transformation in *S. vestibularis* is minimally affected when *cbpD* is knocked out

Considering that deletion of the fratricide encoding gene reduced transformation efficiency by up to 1000-fold in *S. mutans* and that the same effect has been observed in other streptococci (*S. suis*, *S. thermophilus* and *S. sanguinis*), it was expected that deletion of *cbpD* in *S. vestibularis* also would significantly reduce the cells' ability to transform. This was, however, not the case. The results from the transformation efficiency assay in *S. vestibularis* using recombining DNA (**Table 4.2**) indicates that the ability to obtain spectinomycin resistance was minimally affected after knocking out *cbpD*, although the same trend of reduced transformation was observed. For one experiment, deletion of the fratricin resulted in approximately 7-fold decrease in transformation efficiency. In fact, it was estimated that the mutant had 3-fold increase in efficiency in one of the experiments, and it was therefore concluded that the differences in transformation efficiency were minimal. Furthermore, no clear impact on the results was observed when non-recombining DNA was used to test the transformation efficiency instead of recombinant DNA. A T-test revealed that the differences were not statistically significant, neither using recombining DNA (p=0.14), nor non-recombining DNA (p=0.13). For more details regarding the statistical analysis, see **Table A.0.3** and **Table A.0.4.**

Table 4.2 Transformation efficiency in a *S. vestibularis* wildtype strain and a Δ*cbpD* knockout mutant.

Recombining DNA			**Non-recombining DNA**		
Efficiency WT	**Efficiency ΔCbpD**	**Difference in efficiency**	**Efficiency WT**	**Efficiency ΔCbpD**	**Difference in efficiency**
$8.96*10^{-4}$	$1.29*10^{-4}$	6.95	$1.32*10^{-5}$	$6.47*10^{-6}$	2.04
$7.64*10^{-4}$	$3.91*10^{-4}$	1.95	$1.13*10^{-5}$	$1.04*10^{-5}$	1.09
$1.79*10^{-5}$	$5.89*10^{-5}$	0.30	$7.34*10^{-6}$	$6.34*10^{-6}$	1.16

4.3 Purification of *S. mutans* LytF

Because of the dramatic reduction in transformability displayed by LytF deficient *S. mutans*, it was decided to focus on investigating the function of this putative fratricin. Like other fratricins studied previously, LytF has a CHAP domain which is known to make cuts in the peptidoglycan layer[76]. To confirm that LytF in *S. mutans* is a cell wall hydrolase, it was attempted to purify large amounts (milligrams) of the protein for subsequent lysis assays. It was also desirable to obtain pure LytF to perform complementation experiments where LytF was added to competent Δ*lytF* cells, to check if addition of extracellular LytF to a Δ*lytF* mutant could restore the

transformation efficiency to wildtype levels. If LytF restored the transformability of a Δ*lytF* mutant to wildtype levels, it would suggest that LytF acts on the outside of the cell surface to promote DNA-uptake. The *E. coli* strain used to overexpress mature LytF contained a plasmid with a His-tagged LytF and a cut site for a TEV protease (performed by Rebekka Moe). The cut site was inserted to allow removal of the His-tag, as the tag could interfere with the function of the fratricin. LytF was purified using IMAC as described in **section 3.14**. The eluted proteins were analyzed using SDS-PAGE to determine which of the eluted fractions that contained His-TEV-LytF.

His-TEV-LytF has a theoretical mass of 59.5 kDa and no distinct band corresponding to this size were detected (**Figure 4.6**). Proteins of approximately the same size were detected, but since the amount of protein was quite low, it was uncertain whether this was LytF. It was also purified too small amounts of these proteins to be able to perform complementation experiments, and there was insufficient time to repeat the overexpression and purification experiment. The fact that many proteins of different sizes were detected may indicate that several of the proteins expressed in *E. coli* have a high content of surface-exposed residues, and thus compete with the His-tagged LytF protein for binding to the column. The results therefore indicate that LytF was not successfully overexpressed and purified.

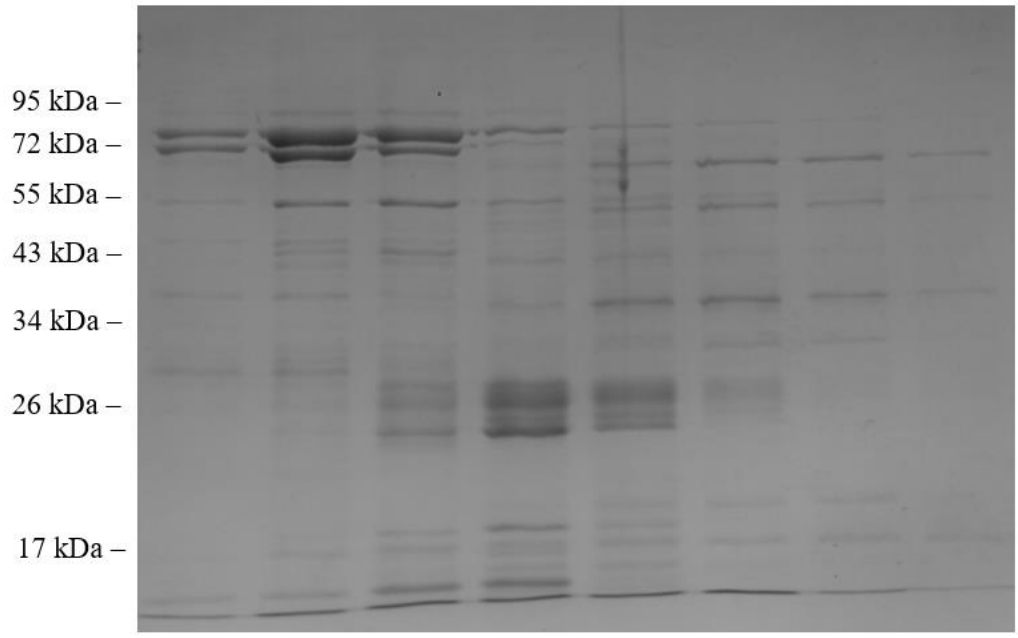

Figure 4.6 Purification of recombinant LytF by using Ni(2+) IMAC. The wells were added eluate from different fractions of the A_{280} chromatogram indicating protein content.

4.4 Zymography revealed no muralytic activity of LytF

Since pure His-tagged LytF was not obtained, zymography was performed to determine if LytF functions as a competence-specific murein hydrolase. Cells from *S. mutans* was incorporated into the SDS-PAG and the analysis were performed with protein extracts from the wildtype and the Δ*lytF* knockout strain. For each of the strains, there was a competence induced and a non-competence induced sample.

The zymogram yielded clearance zones corresponding to a protein with a molecular mass of 95 kDa (**Figure 4.7**). This protein was detected in the wildtype and the fratricin knockout, before and after induction of competence. Wider clearance zones in the induced samples are a result of the samples being incubated for a longer period of time, resulting in more protein in the samples. Since LytF has a theoretical mass of 59.5 kDa, but no hydrolytic protein with this size was detected, the results therefore revealed no lytic activity of LytF.

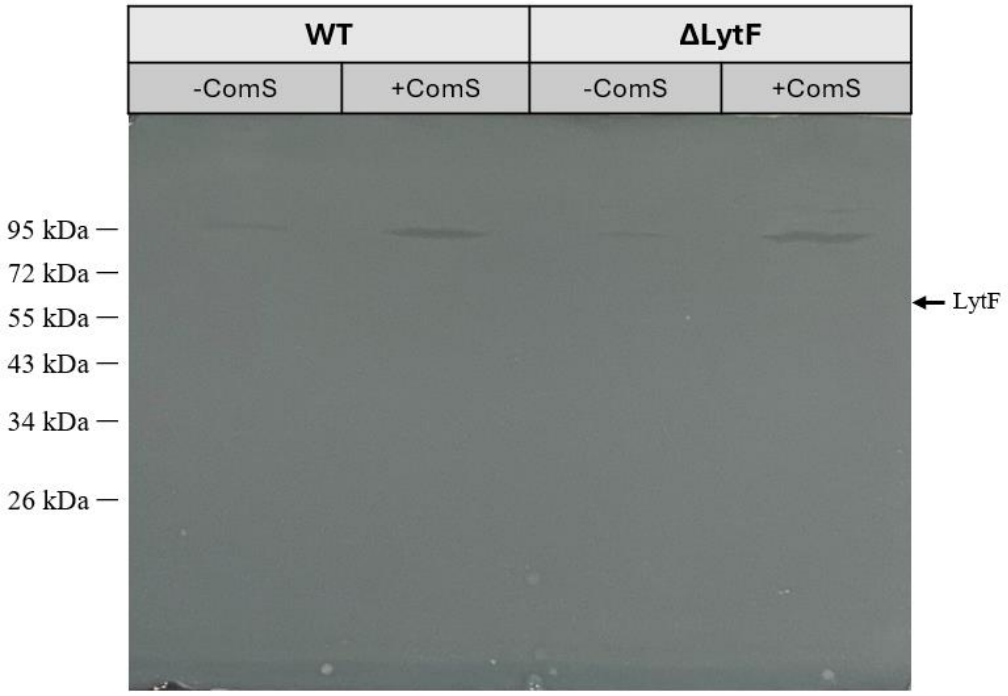

Figure 4.7 Zymogram with incorporation of *S. mutans* cells. The clearance zones are a result of degradation of *S. mutans* cells that have been incorporated into the SDS-PAG. Competence was induced by adding ComS (1µM). Lane 1, non-competence induced *S. mutans* (WT) cells; lane 2, competence induced *S. mutans* (WT) cells; lane 3, non-competence induced *S. mutans* (Δ*lytF*) cells; lane 4, competence induced *S. mutans* (Δ*lytF*) cells. The black arrow indicates the theoretical size of LytF.

4.5 Expression of ComGC in *S. mutans*

The data presented in this thesis so far show that LytF is expressed during competence in *S. mutans* and that it is critical for natural transformation in this bacterium. The use of non-recombining DNA did not improve transformation rates, suggesting that the lack of LytF somehow prevents DNA-uptake. An essential structure for DNA-uptake in streptococci is the type IV-pilus, a transformation pilus that is only expressed during competence[66]. This pilus is extended from the cytoplasmic membrane and needs to cross the cell wall layer in order to bind extracellular DNA. However, the pores in the cell wall may be too narrow (7 nm) for the pilus (diameter of 6-9 nm) to penetrate[118, 119]. In addition, the diameter of the pilus structure increases by 2 nm when bound by DNA. It is therefore believed that a specific cell wall hydrolase creates pores or expands existing pores in the cell wall through which the pilus can cross. This enzyme is however not known. LytF from *Streptococcus gordonii* has been shown to have muralytic activity in zymograms and unpublished zymography data in the MolMik group have shown that LytF from *S. sanguinis* can cleave the cell wall of non-competent *S. sanguinis* cells[72]. However, active lysis of non-competent target cells in liquid cultures (i.e fratricide) has never been observed for this protein. A working hypothesis is therefore that LytF could have a different- or additional function than being part of a fratricide mechanism during competence, i.e. making pores for the transformation pilus. If so, it would be expected that the Δ*lytF* mutant would produce less amounts of pilus. Unpublished data have shown that this is in fact the case for a Δ*lytF* mutant of *S. sanguinis*. Hence, we wanted to determine if the dramatic reduction in transformability of the Δ*lytF* mutant in *S. mutans* also could be linked to reduced amounts of pilus. This would strengthen the hypothesis that LytF somehow contributes to pilus extrusion to the extracellular space.

The pilus protein ComGC constitutes the main part of the competence pilus and can be used as an indicator for the presence and abundance of the entire pilus structure. A Flag-tag encoding sequence was therefore fused at the 5' end of *comGC* to produce a C-terminally tagged ComGC, facilitating visualization of the competence pilus by immunodetection. Since *comGC* is located in the *comG* operon, where several pilus genes overlap, the tagged gene was placed on a plasmid (pRM37) in order to not interfere with the other genes in the operon. pRM37 was then transformed into the wildtype and the Δ*lytF* mutant (strain HL20 and HL21) and the Flag-tagged pilus was then identified using specific antibodies against the Flag-tag sequence.

4.5.1 Flag-ComGC is detected in whole cell extracts 3 hours after competence induction

In order to look at the amount of extracellular Flag-ComGC in *S. mutans*, it was first necessary to identify when ComGC is expressed during competence. *S. mutans* strains HL20 and HL21 were induced to competence at OD_{550} = 0.08-0.1 and samples were collected at 1, 2, 3 and 4 hours post induction. Uninduced cells were used as a negative control. Immunodetection of Flag-ComGC revealed that Flag-ComGC is expressed 3 hours after induction of competence (**Figure 4.8**). The results also indicated that the amount of ComGC from the whole cell extract is greatest 4 hours after competence induction. The proteins detected were about 16 kDa in size, which was slightly larger than expected, as Flag-ComGC is approximately 12.6 kDa. As this protein was not detected in the negative, uninduced control, and since the detected proteins are so close to the theoretical size of Flag-ComGC, it can still be assumed that the detected protein is Flag-ComGC.

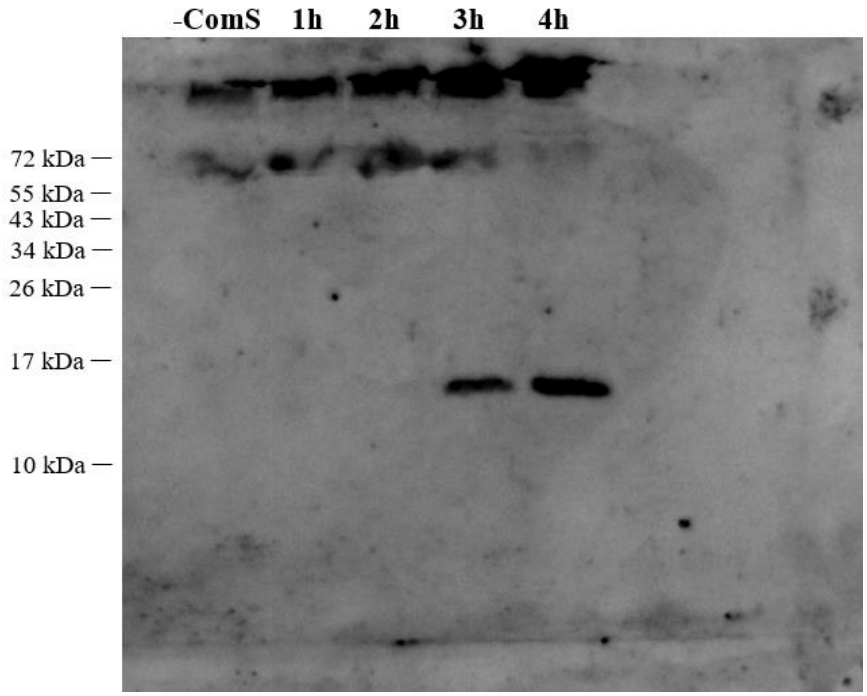

Figure 4.8 Immunodetection of Flag-ComGC in *S. mutans* using whole cell extracts. Competence was induced by adding ComS (1µM) and samples were taken out after 1, 2, 3 and 4 hours. In the negative control (the well on the left), ComS was not added. The image was taken with 40 seconds exposure time.

4.5.2 Flag-ComGC was not detected in culture supernatants

The relative amount of extracellular Flag-ComGC can be used as a measure for the amount of pilus that is able to cross the peptidoglycan layer. Immunodetection of extracellular Flag-ComGC was therefore performed in the wildtype and the Δ*lytF* mutant using culture supernatants. The cells were incubated for 4 hours after induction of competence, as previous

results indicated that Flag-ComGC was expressed in highest quantities at this time point (**section 4.5.1**). Flag-ComGC was then mechanically detached from the cell surface by repetitively passing the cells through a thin needle, causing the protein to end up in the supernatant. The experiment was conducted several times and *S. mutans* was grown in different growth media.

Detection of Flag-ComGC was initially performed by growing *S. mutans* in C-medium without sucrose. Culture supernatants were concentrated 20x using Amicon Ultra centrifugation filters (10 kDa). However, the sample material did not enter the SDS-PAG, but was instead found to concentrate at the very top of the gel, and no proteins were therefore detected (**Figure A.0.5**). Several adjustments were therefore made to optimize immunoblotting of Flag-ComGC. The concentrated supernatants with pilus protein were, among other things, dialyzed to remove excess salt that could interfere with the migration of the samples. Thermo Scientific's Slide-A-Lyzer® Dialysis Cassette was used for this purpose. The sample material was injected into the cassette and the cassette was incubated in TBS for 1 hour in room temperature. The dialyzed samples were then removed from the cassette and the proteins were separated by SDS-PAGE. The results indicated that dialysis had no effect, as the sample material was still unable to enter the SDS-PAG.

Since C-medium contains large amounts of BSA (0.7 mg/ml), it was suspected that the concentration of BSA, which will be even higher after sample preparation, caused the problems during electrophoresis. Immunoblotting was therefore performed removing Bovine Serum Albumin (BSA) from the growth medium. After removal of BSA from the growth medium, the sample material no longer accumulated at the top of the gel. Despite this, no Flag-ComGC were detected, and it was therefore assumed that Flag-ComGC was not expressed in the absent of BSA. An attempt was therefore made to use TH as growth medium instead of C-medium. It was investigated at which timepoint Flag-ComGC is expressed during competence in TH-medium, as competence induction may vary depending on the growth medium. Based on the immunodetection approach used here, Flag-ComGC was not expressed in TH-medium (**Figure A.0.6**), and this growth medium was consequently not used for further investigation of pilus in *S. mutans*.

A final attempt was carried out with C-medium, as previous results confirmed that Flag-ComGC is expressed in this growth medium. Instead of concentrating the culture supernatants using Amicon Ultra centrifugation filters, it was attempted to pull-down (immunoprecipitation) Flag-ComGC from the supernatant using Anti-Flag conjugated agarose beads. It was confirmed that

Flag-ComGC was expressed in these samples using whole cell extracts as described above (**Figure 4.9**). For the immunoprecipitation of Flag-ComGC, no proteins with the same size as the pilus protein were detected, and it was therefore not successful to isolate Flag-ComGC with agarose beads. Proteins with a size of approximately 20 and 26 kDa were, on the other hand, detected in all samples except for the uninduced Δ*lytF* mutant. Flag-ComGC was not detected from the culture supernatant in this study, and relative pilus amount in the wildtype and the Δ*lytF* mutant could therefore not be compared.

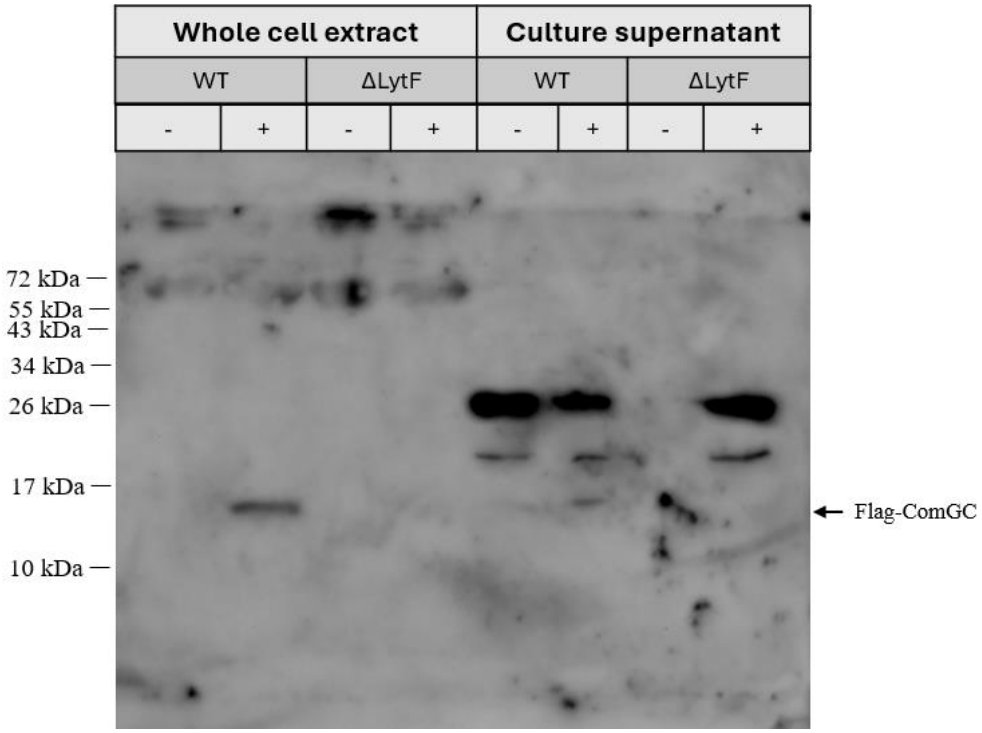

Figure 4.9 Detection of Flag-ComGC from *S. mutans*. For each sample there is an uninduced sample (-) and a sample taken 4 hours after competence induction (+). The four first samples are samples from whole cell extracts, while the last four are from culture supernatants. The image is taken with 5 minutes exposure time. The arrow points at the Flag-tagged ComGC protein detected in the wildtype.

4.6 A ComGC-containing appendage can be observed after knocking out *lytF* in *S. mutans*

The Flag-tagged pilus protein ComGC was also examined by immunofluorescence microscopy to investigate if the relative amount of pilus protein was the same in the wildtype and the Δ*lytF* mutant. The protein was visualized as green light surrounding the cells using primary anti-Flag (rabbit) and secondary Alexa-Fluor488 conjugated anti-rabbit antibodies. A minority of the cells had fluorescent appendages, suggesting the presence of a pilus (**Figure 4.10**). Fluorescent appendages were observed in both the wildtype and the Δ*lytF* knockout mutant, although there

were a few more wildtype cells with such appendages. The results therefore suggest that the competence pilus does not depend on LytF to emerge from the cell, even though it may appear that more pilus is available for DNA-uptake when the putative fratricin is present. For this experiment, an uninduced wildtype was also used as a negative control and fluorescence microscopy image of this is shown in **Figure A.0.7**. Unfortunately, this sample cannot be used to interpret the results, as there is too much fluorescent light and background noise in the image. The experiment should therefore have been repeated to obtain a proper control sample, but there was insufficient time for this. As the experiment was only conducted once, the results are preliminary, and the experiment should therefore be repeated for confirmation.

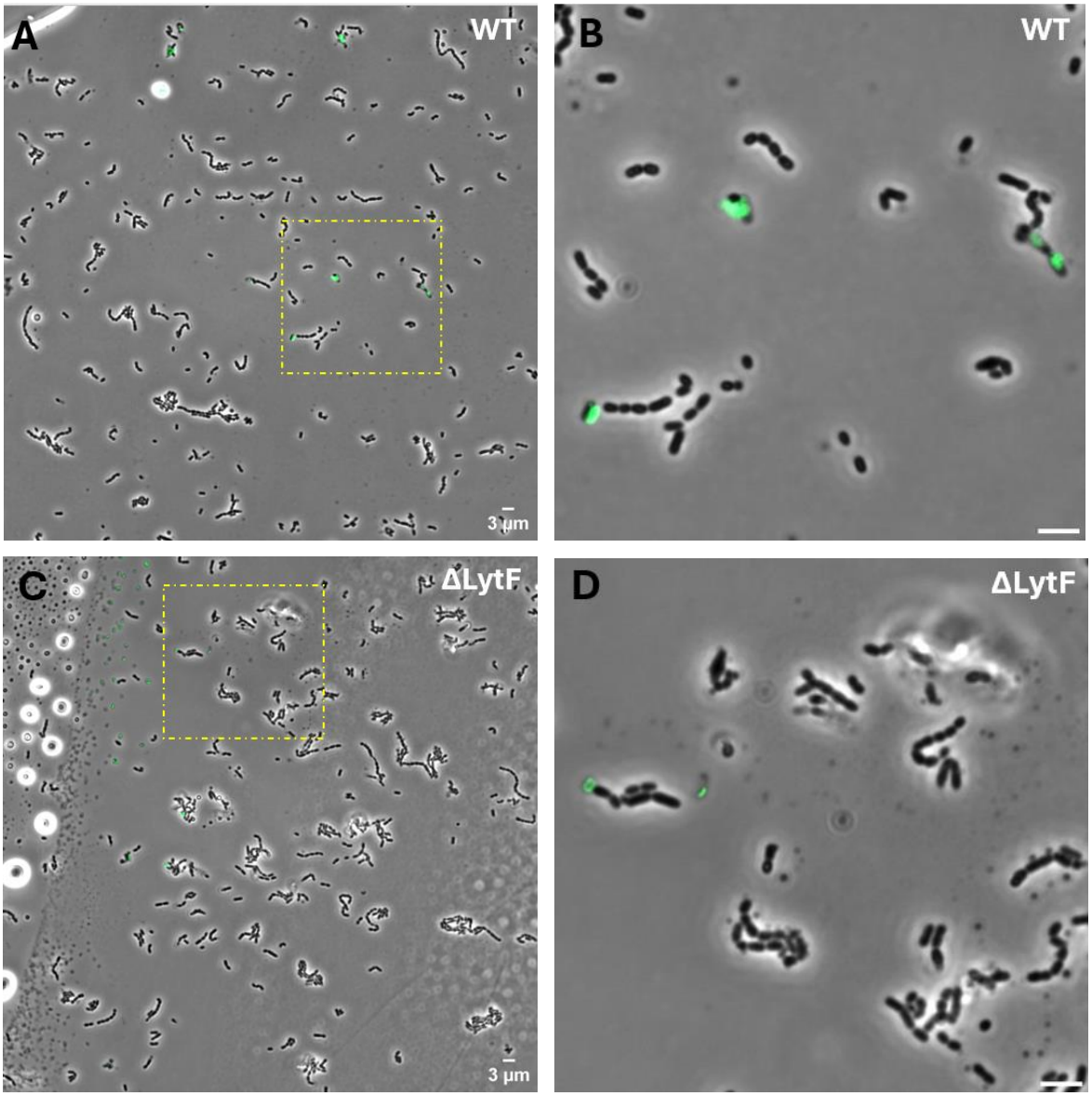

Figure 4.10 Fluorescence microscopy of *S. mutans* expressing Flag-ComGC in the presence (**A/B**) and absence of *lytF* (**C/D**). The microscopy images on the right are enlarged images of the yellow highlighted areas in the images on the left.

5 Discussion

5.1 Is *lytF* expression induced during competence in *S. mutans*?

In competent pneumococci the predatory fratricide mechanism, which depends on expression of the cell wall hydrolase CbpD, has been shown to be important for the cells to acquire genetic material from close relatives. This mechanism is important for the quick spreading of antibiotic resistance genes between pneumococcal strains and other mitis group streptococci[120]. In addition, it is important for capsule switching, resulting in pneumococci that escape vaccine coverage[121, 122]. Interestingly, most streptococci outside the mitis group also have a *cbpD*/*lytF*-like gene encoding putative cell wall hydrolases. However, experimental evidence supporting their involvement in a fratricide mechanism is scarce. In this project, one goal was therefore to investigate whether the gene encoding the putative fratricin, LytF, in *S. mutans* is competence induced and has muralytic activity. The activity of P_{comX} was used as a control that competence was induced when ComS were added, but no transcription was observed from this promoter (**Figure 4.1**). As it has already been established that *comX* is competence induced in this species and is responsible for activating the transcription of the late competence genes, the experiment was not working optimally[62]. The fact that no activity of the promoter was observed after competence induction may be due to the formation of secondary structures on the transcript. P_{comX} is placed in front of a recombinant *luc* gene and a possible hairpin structure of the 5' untranslated region (5' UTR) and *luc* could form, leading to inaccessibility of the shine dalgarno sequence in the ribosomal binding site, thus blocking the translation or terminating the transcription. Another possibility is that a mutation has occurred in the *luc* gene, making the luciferase enzyme non-functional.

Despite no transcription from P_{comX}, it was still discovered that transcription from P_{lytF} increased upon competence induction (**Figure 4.2**). This demonstrates that ComX was probably expressed upon competence induction even though the *luc* reporter assay did not detect P_{comX} induction. The signal strength was nevertheless relatively low, and it may therefore appear that the *lytF* gene is transcribed at low levels during competence in *S. mutans*. However, we are not entirely sure whether the bacteria generally transcribe a small amount of *lytF*, or whether this is due to poor bacterial growth. The transcription of *lytF* could therefore have been influenced by a combination of the type of carbohydrate source available and bacterial growth. However, as *S. mutans* had sufficient growth in C-medium without sucrose, and the signal strength was still low, it suggests that there is generally a low production of LytF. Despite poor growth,

transcription from P_{lytF} increased most in the absence of glucose, suggesting that glucose is not a preferred carbohydrate source for the transformation process. It is possible that the signal strength would have been stronger if the bacterial growth had been better. However, the results still indicate that *lytF* is competence induced, and that the absence of glucose results in highest expression from P_{lytF}. This is consistent with previous findings, where a significant increase in P_{comX} activity was detected after competence induction when sucrose was present, compared to when the cells were grown with glucose[114].

5.2 Is *cbpD* expression induced during competence in *S. vestibularis*?

Similarly to *S. mutans*, it was investigated whether *cbpD* from *S. vestibularis* is competence induced. Elevated transcription of *luc* from P_{comX} was observed after addition of ComS (**Figure 4.3**), indicating that competence was induced. Although the signal strength was weak, the increase in luminescence upon ComS addition was significant and reproducible. Transcription from P_{comX} seemed to be re-activated at the transition from exponential to stationary growth. This was a puzzling observation, but perhaps it is a result of an accumulation of compounds in the medium that can induce the promoter. It is also possible that the promoter is regulated by other intracellular factors produced during the transition to stationary phase, and that *S. vestibularis* therefore has a system that induces *comX* in response to cell density. Entrance to competence in the stationary phase is not an uncommon observation. Both *Haemophilus influenzae* and *Bacillus subtilis* enter the competent state in the stationary phase[123, 124]. In these species, nutritional depletion is an important cue for competence induction[51]. Perhaps a similar mechanism can explain the quick induction of P_{comX} in *S. vestibularis* when entering the stationary phase.

Transcription from P_{cbpD} was also elevated after induction of competence (**Figure 4.4**), although the signal strength was relatively low compared to the relative luminescence observed for other competence promoters studied (approximately 10-100 fold lower than P_{comX} of *S. thermophilus* and P_{ssbB} of *S. pneumoniae*)[125, 126]. However, the negative control, where a luciferase gene was not present, also had an increase in luminescence at the same time point. This could possibly be due to the luminescence signal being transmitted from the adjacent wells, as the signal strength is lower in the control. This may indicate that there is indeed a real induction of the P_{cbpD} promoter upon ComS addition. Self-induction probably occurred in the uninduced sample, inducing transcription from P_{cbpD} upon transition to the stationary phase. This is very likely as the uninduced sample had increased expression from P_{cbpD} at exactly the same time as the

induced sample, suggesting that the bacterium prefers to enter a competent state during this growth phase. This observation supports the statement that the promoter can either be controlled by compounds accumulating in the medium, or that it occurs as a response to cell density or nutrition depletion. To avoid spontaneous competence induction, the experiment should have been repeated with a Δ*comS* mutant.

Based on the *luc* reporter assay, both *cbpD* from *S. vestibularis* and *lytF* from *S. mutans* seems to be transcribed in low quantities during competence. There may be several reasons for this. Since the bacteria do not need to lyse more than one bacterial cell with homologous DNA for the transformation process to be successful, producing a small concentration of fratricin may be an energy-saving strategy. The fact that some fratricin subvariants may cut more efficiently in the cell wall of non-competent cells may also be a possible explanation, since more efficient hydrolysis of peptidoglycan reduces the amount of fratricin required to release DNA. Research has shown that some species produce fratricins with a broader muralytic spectrum, lysing peptidoglycan from more streptococcal species than fratricins with a narrow spectrum[72]. If the fratricin has a broad spectrum of activity, DNA can be made available from potentially more bacterial cells nearby, which might require lower concentrations of the enzyme since it does not necessarily have to diffuse a long distance to reach a target cell. However, the muralytic activity and target range of the putative fratricins of *S. vestibularis* and *S. mutans* remains to be shown. It is also important to keep in mind that the luciferase reporter assay is not a direct measure of how much *cbpD* and *lytF* are transcribed from the native promoter in the genome, but it is a measure of the induction of the promoter ectopically inserted on a plasmid. The fact that the promoter is activated indicates that the promoter is turned on by ComS induction. However, in order to determine the amount of transcript, RNA must be isolated, and qPCR or RNA sequencing must be performed on the native expression of *cbpD* and *lytF*.

5.3 The fratricin variants have varying impact on the transformation process

Fratricins are known to make homologous DNA available for uptake during transformation. However, transformation efficiency testing has shown reduced transformation in fratricin deficient mutants, suggesting that the fratricins in some species are not only involved in the fratricide mechanism, but also have another important function for transformation. In this work, it was shown that the transformation rate of *S. mutans* was significantly reduced (up to 1100-fold) in a Δ*lytF* mutant when the same amount of recombining DNA was available (**Table 4.1**). This may indicate that LytF has a function other than making DNA available for uptake during

the transformation process. The transformation efficiency was tested with both recombinant and non-recombinant DNA to investigate whether it was the homologous recombination or the DNA-uptake that was weakened in the fratricin mutant. As mentioned earlier, there was no apparent difference in efficiency between the group that was given recombinant DNA and the group that was given non-recombinant DNA. This suggests that it is the DNA-uptake mechanism that is affected when *lytF* is knocked out. If LytF had been involved in homologous recombination, the group with non-recombinant DNA would have had a significantly improved transformability, compared to the group with recombinant DNA, which was not the case. As the transformation efficiency of the Δ*lytF* mutant was significantly reduced, it is also not surprising that it was challenging to remove the Janus cassette from the genome of this strain. The results strongly suggests that LytF has an important role in the uptake of DNA during transformation, although it is unknown exactly how the protein contributes to this process.

Although transformation by plasmid is most likely not dependent on the DNA recombination apparatus in the cell, it cannot be ruled out that this process requires some of the DNA recombination proteins. As EndA in *S. pneumoniae* degrades one of the two DNA-strands on the incoming DNA, the cells most probably need to take up the two DNA-strands in the plasmid in two separate transformation events[67]. Inside the cell, the single-stranded forward and the complementary DNA-strand can then form DNA duplexes due to base complementarity, making the plasmid circular again. Exactly which components contribute to this mechanism is uncertain, but a possibility is that RecA, which is responsible for incorporating homologous DNA into the genome of the bacterium, also is involved in the assembly of the plasmid inside the cell. In addition, both DNA-strands in the plasmid have a cut that must be repaired by DNA ligase, an enzyme that is also present during recombination. Therefore, it cannot be completely ruled out that transformation with plasmid requires some of the DNA recombination proteins. Transformation with plasmid should therefore also have been investigated in a Δ*recA* mutant as a control. If deletion of *recA* only reduces transformation efficiency when using linear DNA, but not when using plasmid, it would prove that LytF indeed is important for DNA uptake.

The extreme transformation efficiency reduction in the Δ*lytF* mutant could have been due to changes in the bacterial cell morphology, and not the fact that the fratricin has an additional role in the transformation process. In *Streptococcus sanguinis*, it has been discovered that the fratricin mutant had a slightly higher amount of elongated chains than the wildtype[113]. If the Δ*lytF* mutant formed chains, the calculated transformation efficiency may have been overestimated as a chain may have appeared as a single colony, when the long chain actually

represents several individual bacterial units. However, no differences in morphology between the wildtype and the Δ*lytF* mutant were observed (**Figure 4.5**), strengthening the hypothesis that that LytF has a central function in the transformation process.

For *S. vestibularis*, on the other hand, no reduction in transformation efficiency was observed in the Δ*cbpD* mutant (**Table 4.2**), indicating that CbpD from *S. vestibularis* probably is not important for transformation when the competent cells are provided naked DNA for uptake. Perhaps CbpD in *S. vestibularis* solely functions as a fratricin, similar to its pneumococcal counterpart, while LytF might have a double role in the species expressing this murein hydrolase, functioning as a fratricin and/or somehow facilitating DNA-uptake by the competent cell. Based on these results, it can be concluded that not all fratricin-like proteins function to increase the transformability of the producer cell when naked DNA is available for uptake. This additional function is not specific for LytF either, as it has been shown that deletion of CbpD from *S. thermophilus* and CrfP from *S. suis* also results in reduced transformation efficiency[111, 112]. Since *S. thermophilus* and *S. vestibularis* both belong to the salivarius group of streptococci, but only one of them has reduced transformation efficiency when the fratricin is knocked out, this suggests that streptococcal species with the same origin not necessarily have fratricins with similar functions. Several streptococcal species may have independently developed additional functions for their fratricins through convergent evolution, and this function is therefore likely species-specific.

5.4 It is still unknown if the putative LytF fratricin in *S. mutans* is an autolytic enzyme

Fratricins are important for spreading genetic material between several streptococcal species by breaking down the cell wall of susceptible relatives, leading to lysis and the release of homologous DNA. However, the results in this work did not provide any insight into the cell wall hydrolytic capabilities of LytF in *S. mutans*, as we were unable to overexpress and purify LytF for subsequent lysis assays. Furthermore, the zymogram was also unable to detect muralytic activity of LytF, as no clearance zones with the size of LytF were observed (**Figure 4.7**). The reason why LytF could not be detected in the zymogram may be that the concentration of the putative fratricin is too low, which is consistent with the fact that the *luc* transcription from P_{lytF} was low. Alternatively, SDS may have denatured the secondary and tertiary structures in the protein, destroying active regions or binding sites. If the refolding buffer then was unable to completely refold the protein, it may have caused the enzyme to be non-functional. The activity of LytF may also depend on the presence of specific cofactors or coenzymes. SDS can

potentially interfere with these compounds and disrupt their binding to the enzyme, resulting in a reduction of enzymatic activity. If LytF had been present in high enough concentrations, one would have expected to see a clearance zone only for the competence induced wildtype.

Since it has been established that LytF from streptococcal species such as *S. gordonii* and *S. sanguinis* are murein hydrolases, it is likely that LytF in *S. mutans* has a similar function[72, 113]. The C-terminal CHAP domain is highly conserved, and all LytF-type fratricins have N-terminal regions consisting of Bsp domains. Since the CHAP domain is responsible for hydrolyzing peptide bonds in the cell wall, it is likely that the putative fratricin in *S. mutans* has muralytic properties[65]. However, as the number and structure of the Bsp domains varies, and they presumably determine the binding properties and target range of the fratricin, the properties of LytF in *S. mutans* may nevertheless vary from fratricins in other streptococcal species[72].

Although the hydrolytic activity of LytF was not found in this work, the zymography showed that another hydrolytic enzyme was present. Previous studies have shown that *S. mutans* produces an autolysin called AtlA, which has a molecular mass of approximately 104 kDa[127]. The central part of this autolysin consists of a putative cell wall-binding domain, while the C-terminus has a muraminidase domain. Cleavage of AtlA results in a mature protein with a size of about 90 kDa, having peptidoglycan hydrolysis activity[127]. Since autolysins are peptidoglycan degrading enzymes that contribute to cell growth and division in Gram-positive bacteria, it makes sense that the protein is produced in both induced and non-induced cells of *S. mutans*[128]. Although it may appear that the detected protein is involved in competence, as the clearance zones are larger in the competence induced samples, this is likely due to a longer incubation period resulting in higher cell density and thus protein concentration.

5.5 Expression of the pilus protein ComGC in *S. mutans*

During transformation, competent streptococci produce a competence pilus that transports available DNA into the bacterial cell. However, it is not known how this pilus, which is attached to the cytoplasmic membrane, extends through the cell wall. We speculate whether fratricins can contribute to this process and wanted to investigate if the amount of extracellular pilus protein in *S. mutans* is affected when the fratricin is knocked out. In order to study pilus quantity, it was first necessary to identify when the pilus is expressed during competence. The pilus protein ComGC was used as an indicator for the presence of the entire pilus structure and the protein was found to be expressed 3 hours after competence induction (**Figure 4.8**). Compared

to *S. pneumoniae*, which expresses ComGC already 10 minutes after competence induction, *S. mutans* takes significantly longer to express this protein[129]. This indicates that that the bacterium requires a lot of time to incorporate available DNA into the genome after competence has been induced, or that competence is not induced immediately after ComS is added. If the bacterium uses this mechanism to cope with stress, acquiring genes as soon as possible would be advantageous if this can give the bacterium a greater chance of survival. However, taking time to express the pilus may still be beneficial, as it requires a lot of energy to express all the components involved, and the stress experienced by the bacterium may be temporary.

In an attempt to compare the amount of pilus protein extending beyond the peptidoglycan layer in the wildtype and the Δ*lytF* mutant, it was discovered that ComGC was not expressed in TH-medium, or expressed below the detection limit of our immunoblot assay. As mentioned earlier, it has been discovered that the promoter activity of certain competence-associated genes in *S. mutans* is influenced by the carbohydrate sources available[114]. If the nutritional conditions are not optimal for *S. mutans* in TH-medium, the expression of the pilus protein may be lower than in C-medium. Lower expression of ComGC can make detecting the protein in the sample more challenging, as it is more difficult to distinguish the signal from the background noise. There may also be peptides in the TH-medium that compete with ComS to be transported into the cell via the Ami transporter, resulting in a reduced activation of ComR. If this is the case, ComGC may have been expressed later than 4 hours after competence induction. Consequently, it cannot be completely ruled out that ComGC cannot be expressed in this growth medium.

It was attempted to compare the amount of extracellular ComGC in the wildtype and the Δ*lytF* mutant by using immunoprecipitation to pull down the Flag-ComGC from the growth medium. However, the experiment was unsuccessful as no Flag-ComGC was detected (**Figure 4.9**). For this approach to be successful, the Flag-tag on ComGC needs to be available to the anti-Flag antibodies. A potential explanation for not being able to pull down Flag-ComGC could be that the C-terminal Flag-tag is hidden inside the 3D-structure of the pilus filament, making it inaccessible to the anti-Flag antibody. The lack of ComGC expression in *S. mutans* is an unlikely explanation, as immunoblotting of whole cell extracts from the same samples indicated that Flag-ComGC was present.

Despite that no extracellular ComGC was detected, other components of 26 and 20 kDa were pulled down non-specifically by the anti-Flag antibody. The 26 kDa and 20 kDa proteins were detected in the induced and non-induced wildtype, in addition to the competent induced Δ*lytF* mutant. To exclude that the 26 and 20 kDa components derived from the growth medium, a

pull-down performed on fresh growth medium should have been included. However, the fact that these components were not detected in the uninduced Δ*lytF* culture supernatants, suggests that they are proteins produced by *S. mutans*. It is however, challenging to explain why the 26 and 20 kDa proteins were not detected for the Δ*lytF* mutant, as it is expected that the uninduced wildtype has the same expression pattern as the uninduced mutant. One possible explanation could be that the uninduced wildtype had self-induced and that these proteins represent competence induced proteins. However, this is unlikely as ComGC was not detected when whole cells were used, and the samples were taken from the same sample material. The results may therefore indicate that the detected protein is not competence induced. If the protein is not competence induced, it is expected that this protein is present in an uninduced Δ*lytF* mutant, which it was not. The experiment was not repeated due to time limitations, and it was therefore never investigated whether the results were due to technical issues or whether biological replicates would have given the same outcome.

5.6 LytF may have an additional function in genetic transformation in *S. mutans*

Since LytF has a CHAP domain and is competence induced, the working hypothesis has been that it functions as a fratricin. However, the reduced transformation efficiency of a Δ*lytF* mutant suggests an additional role in natural transformation. We have found that deletion of *lytF* in *S. sanguinis* leads to reduced amounts of transformation pilus (unpublished data) and have therefore hypothesized that LytF cuts the cell wall of competent cells so that the pilus can cross. If true, there should be a reduced amount of pilus protein on the outside of the cell when the fratricin is knocked out. Since we could not detect the pilus by immunoblotting, a more direct approach, i.e. immunofluorescence microscopy, was used to see if the Δ*lytF* mutant had fewer pili. Despite this, the immunofluorescence images showed that ComGC was present in the Δ*lytF* mutant (**Figure 4.10**), indicating that the competence pilus is not completely dependent on the assistance of LytF to escape through the cell wall. However, it is difficult to draw any conclusions as the results could not be compared to an induced wildtype strain. In the absence of such a control, it is challenging to determine whether the observed fluorescent appendages are the pilus protein ComGC or background noise. This discovery should therefore be confirmed with further experiments.

If the observed fluorescent appendages are the pilus protein ComGC, it may indicate that LytF is not necessary for the pilus to penetrate the cell wall in *S. mutans*. However, since the pili was slightly less abundant in the mutant, and unpublished data have shown that this is the case for

a Δ*lytF* mutant of *S. sanguinis*, it may suggest that the fratricin somehow assist the pilus in crossing the cell wall. One speculation is that the fratricin enlarge already existing pores in the cell wall, which may explain why fratricins make DNA uptake more efficient. However, some pores may still be large enough for the pilus to escape unassisted, a statement consistent with a small number of pili still being detectable on the mutant cell surface. The competence pilus in streptococci are morphologically similar to other type IV pili that have a diameter of 6-9 nm[119]. Since the pore size usually is 7 nm, although this is dependent on the degree of cross-linking in the cell wall, this suggests that the pilus, in some cases, will be able to pass through the pores in the cell wall[65]. In *S. pneumoniae*, the competence pilus is 6.4 nm in diameter on average, and since knockout of *cbpD* in this species has not been shown to affect transformability, it is reasonable to think that the pilus manages to get out of the 7 nm pores on its own[92, 130]. However, the pilus may not be able to pass through the pores when it is bound to DNA, as this requires more physical space. It is therefore possible that pneumococci produce alternative hydrolases performing this role. Since the transformability of *S. mutans* is severely impaired when the *lytF* is knocked out, it can be speculated that the cell wall has tighter cross-links, or that the pilus is slightly larger in diameter, and therefore unable to penetrate the cell wall effectively without assistance. It could be interesting to find out where the putative fratricin is found before DNA-uptake, and it can be speculated whether it is located at the tip of the pilus or somewhere else in the periplasm.

The reduced amount of pilus protein observed in the Δ*lytF* mutant could also be a consequence of an alternative function performed by the fratricin. It has been shown that activation of competence during infection is important for cell wall remodeling, resulting in the exposure of specific virulence factors on the surface of competent pneumococci[131]. However, the exposure of these virulence factors is reduced in a *cbpD* mutant, and *cbpD* deficient cells have a weakened ability to adhere to human epithelial cells[131]. This indicates that the fratricin promotes colonization to the target cells by altering the composition of its own cell surface, which indirectly may facilitate the penetration of the transformation pilus out of the peptidoglycan layer. In this way, fratricins can potentially contribute to the virulence mechanism, as well as ensuring that new genomic material can be acquired during natural transformation.

However, it is also possible that LytF in *S. mutans* does not function as a fratricin, as it has not been confirmed that LytF is a part of the fratricidal mechanism. If so, *S. mutans* has the ability to be competent for natural transformation but does not play an active role in the release of DNA for cellular uptake, and therefore must acquire DNA in an alternative way. One possibility

is that *S. mutans* take up DNA that is already available in the environment as a result of various biological processes related to the bacterial life cycle. This approach is probably less advantageous for the bacterium, as it is likely that the available DNA may originate from distantly related species, and therefore cannot be incorporated into the genome due to lack of homology. As a result, gene transfer between LytF-containing species might be slower compared to CbpD-containing species. It could be interesting to explore whether genomic data can yield additional insight into the gene composition of streptococcal species harboring different types of fratricins, as there is a possibility that species with CbpD can access homologous DNA more easily and thus change their gene composition at a faster rate.

6 Concluding remarks and future research

Based on the work done in this thesis, it can be concluded that expression of *lytF* and *cbpD* is induced during competence in *S. mutans* and *S. vestibularis*. Furthermore, by knocking out the putative fratricins in these species, it was also discovered that LytF from *S. mutans* most probably has an important role in the DNA-uptake process. CbpD from *S. vestibularis*, on the other hand, has little impact on this process, as knockout of the putative fratricin reduced the transformation efficiency minimally. The function of LytF was investigated in more detail, however, it was not confirmed that this putative fratricin acts as a cell wall hydrolase. There is a possibility that LytF can assist in getting the competence pilus through the cell wall, allowing it to bind and transport DNA into the cell, but further experiments are needed to confirm this.

In the future, fratricin-like proteins from several streptococcal species should be investigated to get a better understanding of their function. It would be interesting to find out which and how many fratricins have the ability to influence the transformation efficiency, and the reason why this function has been developed in some of the species. It would also be interesting to find out if the fratricin's contribution to the DNA-uptake process is an advantage for the bacteria. These questions may be important to answer in order to obtain a comprehensive understanding of the transformation mechanism. With further knowledge about the mechanisms bacteria use for spreading antibiotic resistance genes, it might be possible to develop strategies that prevent this, which in turn could prolong the clinical relevance of antibiotics.

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Appendix

A1. List of solutions and procedure for making components in C-medium

Pre C-medium	45 mg L-cystein hydrochloride 8 g sodium acetate 20 g casitone 0.024 g L-tryptophan 34 g potassium hydrogen phosphate (K_2HPO_4) The chemicals were dissolved in dH_2O to a total volume of 4 liters. The solution was autoclaved and stored at room temperature.
ADAMS III	128 ml ADAMS I 3.2 ml ADAMS II (10x) 1.6 ml L-asparagine monohydrate 0.16 g choline chloride 0.4 g calcium chloride anhydrate 16 g magnesium chloride hexahydrate The chemicals were dissolved in dH_2O to a total volume of 800 ml and pH-adjusted to pH 7.6. The solution was sterile filtered and stored at 4 °C
ADAMS II (10x)	500 mg iron (II) sulfate heptahydrate 500 mg copper sulfate pentahydrate 300 mg zinc sulfate heptahydrate 200 mg manganese (II) chloride tetrahydrate 10 ml concentrated HCl The chemicals were dissolved in dH_2O to a total volume of 100 ml. The solution was sterile filtered and stored at 4 °C
ADAMS I	0.15 ml 0.5 mg/ml biotin 75 mg nicotinic acid 87.5 mg pyridoxine hydrochloride 300 mg calcium pantothenate 800 mg thiamine hydrochloride 35 mg riboflavin The chemicals were dissolved in dH_2O to a total volume of 0.5 l and pH-adjusted to pH 7.0. The solution was sterile filtered and stored at 4 °C.
10 % yeast extract	40 g yeast extract 360 ml dH_2O 37 % HCl 16 g activated charcoal The chemicals were mixed and incubated for 2 hours at 4 °C. The solution was then filtered over a column packed with glass wool and celite overnight. The solution was then pH-adjusted to pH 7.8 and the final volume was adjusted to 400 ml with dH_2O. The solution was sterile filtered and stored in 4 ml aliquots at -80 °C.

A2. Luciferase reporter assay control samples

Strains without an inserted luciferase gene were used as negative controls for both *S. mutans* and *S. vestibularis*. *S. dysgalactiae* was used as a positive control.

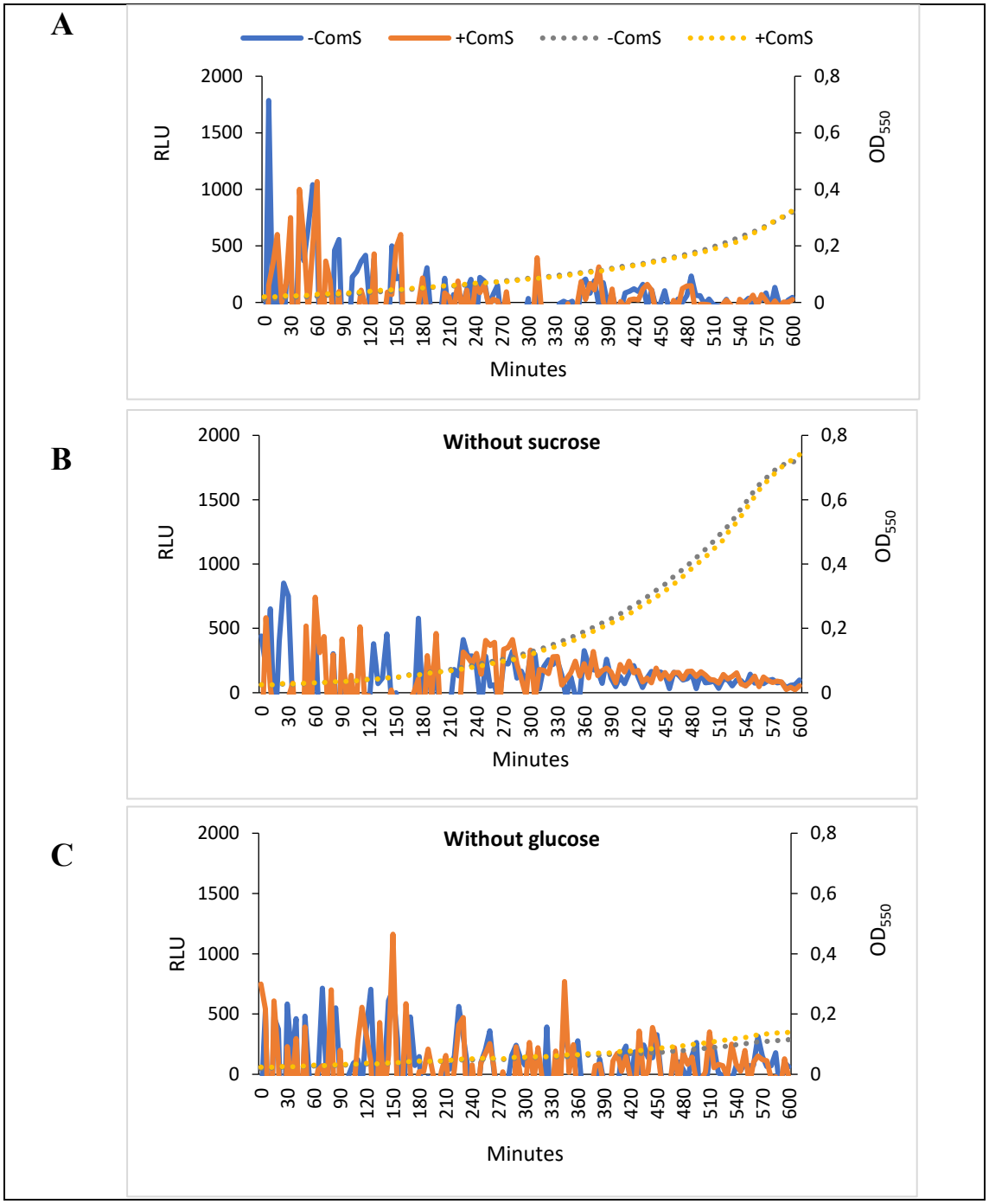

Figure A.0.1 Luciferase reporter assay performed with a strain of *S. mutans* without the luciferase gene (negative control). ComS was added at time 0 to induce competence. Growth curves are represented by dashed lines, while relative luminescence unit (RLU) is represented by solid lines. Different growth media were used: A) C-medium, B) C-medium without sucrose and C) C-medium without glucose.

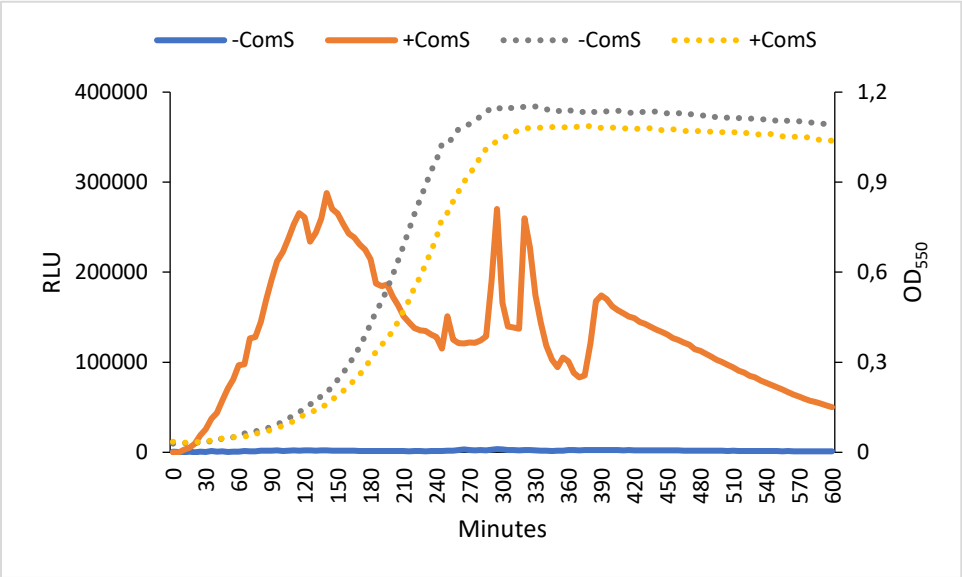

Figure A.0.2 Luciferase reporter assay performed with a strain of *S. dysgalactiae* in which P_{sigX} is linked to a luciferase gene. ComS was added at time 0 to induce expression of this gene. Growth curves are represented by dashed lines, while relative luminescence unit (RLU) is represented by solid lines.

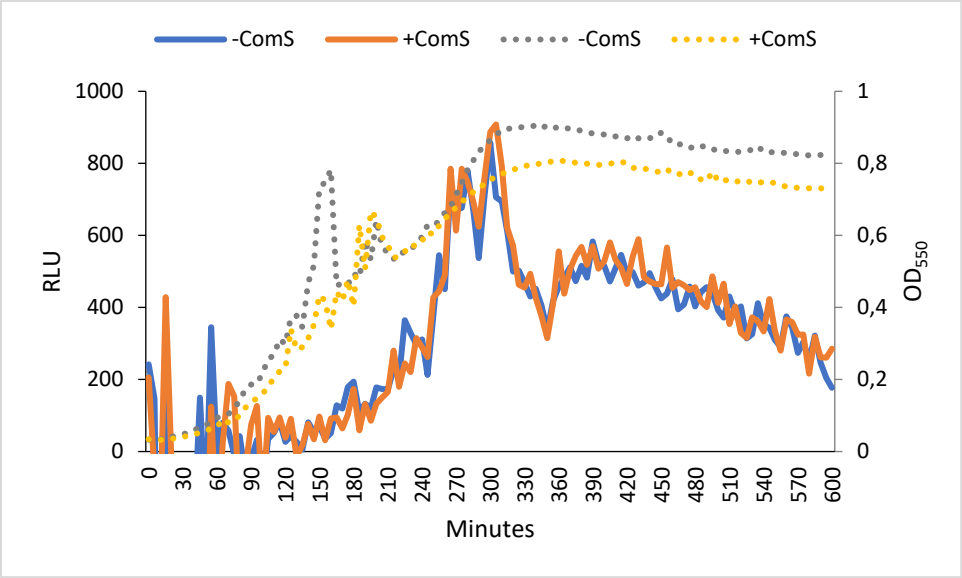

Figure A.0.3 Luciferase reporter assay performed with a strain of *S. vestibularis* without the luciferase gene (negative control). ComS was added at time 0 to induce competence. Growth curves are represented by dashed lines, while relative luminescence unit (RLU) is represented by solid lines.

A3. Mutation in the *rpsL* gene of *S. vestibularis*

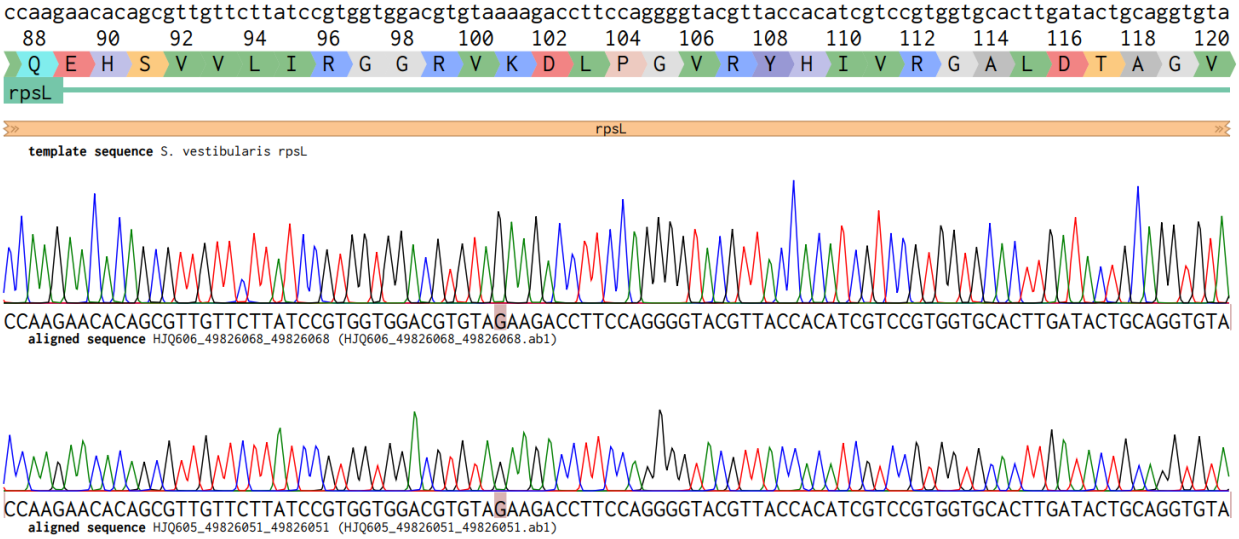

Figure A.0.4 Sanger sequencing of the *rpsl* gene from *S. vestibularis*. Sequencing revealed point mutation in position 301 (highlighted in red), resulting in an amino acid replacement from lysine (AAA) to glutamic acid (GAA).

A4. Two-sample T-test with assumed equal variances

Table A.0.1 Analysis of transformation efficiency with recombinant DNA in *S. mutans*

Recombinant DNA	**Efficiency WT**	**Efficiency ΔLytF**
Mean	6,91943E-05	7,90933E-08
Variance	1,24127E-09	6,48449E-15
Observations	3	3
Pooled Variance	6,20639E-10	
Hypothesized Mean Difference	0	
df	4	
t Stat	3,397816988	
P(T<=t) one-tail	0,013666125	
t Critical one-tail	2,131846786	
P(T<=t) two-tail	0,027332249	
t Critical two-tail	2,776445105	

Table A.0.2 Analysis of transformation efficiency with non-recombinant DNA in *S. mutans*

Non-recombinant DNA	**Efficiency WT**	**Efficiency ΔLytF**
Mean	0,000122614	1,71927E-07
Variance	5,23201E-09	3,91592E-15
Observations	3	3
Pooled Variance	2,61601E-09	
Hypothesized Mean Difference	0	
df	4	
t Stat	2,931944984	
P(T<=t) one-tail	0,021365413	
t Critical one-tail	2,131846786	
P(T<=t) two-tail	0,042730826	
t Critical two-tail	2,776445105	

Table A.0.3 Analysis of transformation efficiency with recombinant DNA in *S. vestibularis*

Recombinant DNA	**Efficiency WT**	**Efficiency ΔLytF**
Mean	0,000559312	0,000193167
Variance	2,24264E-07	3,07261E-08
Observations	3	3
Pooled Variance	1,27495E-07	
Hypothesized Mean Difference	0	
df	4	
t Stat	1,255891223	
P(T<=t) one-tail	0,138754273	
t Critical one-tail	2,131846786	
P(T<=t) two-tail	0,277508546	
t Critical two-tail	2,776445105	

Table A.0.4 Analysis of transformation efficiency with non-recombinant DNA in *S. vestibularis*

Non-recombinant DNA	**Efficiency WT**	**Efficiency ΔLytF**
Mean	1,05898E-05	7,72863E-06
Variance	8,82377E-12	5,26846E-12
Observations	3	3
Pooled Variance	7,04612E-12	
Hypothesized Mean Difference	0	
df	4	
t Stat	1,320136806	
P(T<=t) one-tail	0,128632689	
t Critical one-tail	2,131846786	
P(T<=t) two-tail	0,257265377	
t Critical two-tail	2,776445105	

A5. Detection of Flag-ComGC in *S. mutans*

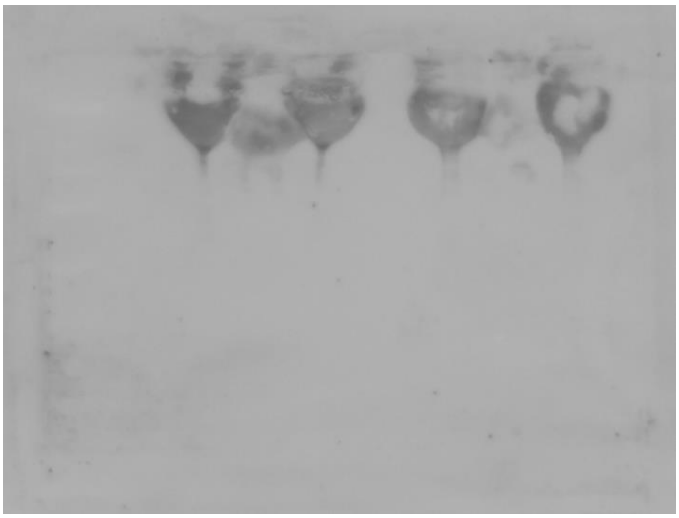

Figure A.0.5 Detection of Flag-ComGC in culture supernatants from *S. mutans*. The cells were grown in C-medium without sucrose and the image was taken with 40 seconds exposure time.

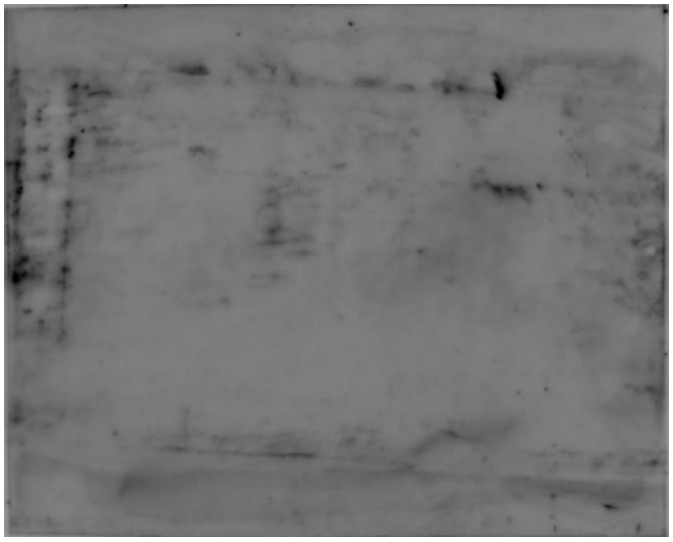

Figure A.0.6 Detection of Flag-ComGC in *S. mutans* using whole cell extracts. The cells were grown in TH-medium and the image was taken with 10 minutes exposure time.

A6. Fluorescence microscopy of non-induced wildtype strain

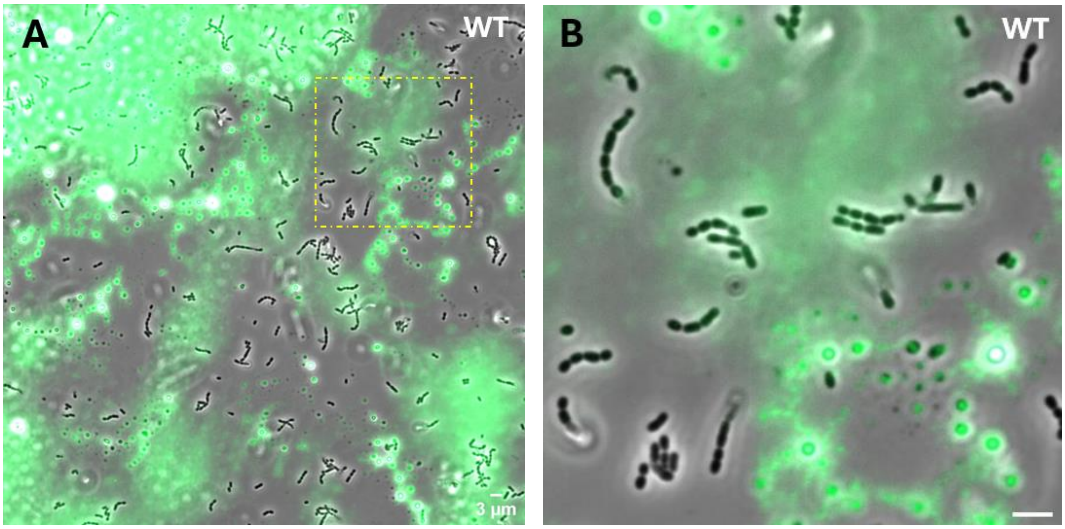

Figure A.0.7 Fluorescence microscopy image of a *S. mutans* wildtype strain which has not been competence induced. The image on the right (B) is an enlarged image of the yellow highlighted area in the microscopy image on the left (A).

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