Master thesis odontology

Mechanism of DNA release during competence in *Streptococcus mitis*

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PREFACE

In the autumn of 2012, with the wish to learn more about science, I contacted Håkon Valen Rukke, who at that current time was a PhD candidate, at the Department of Oral biology, Faculty of Dentistry, University of Oslo. After assisting me in arranging a meeting with his supervisor and the leader of his group, Professor Fernanda Cristina Petersen, I was fortunate to be accepted into Professor Petersen's group. During the summer of 2013 I worked in the lab and performed five different experiments. The following year I organized and analyzed the results and also got to meet PhD candidate Roger Junges. After finishing the results we decided that additional experiments were needed and during the winter, spring and summer of 2015 three more experiments were done. In winter 2015 I was fortunate to meet the PhD Candidate Gabriela Salvadori da Silva who assisted me with the last three experiments. Looking back this has been a challenging, exciting and extremely informative process which would have been impossible without the guidance from my supervisors and the other people that I have worked with. Together they have given me insight into the world of science and guided me through an unfamiliar field, always with the best intentions to assist me whenever needed.

Therefore I would like to express my appreciation and special thanks to Professor Fernanda Cristina Petersen and PhD Candidates Roger Junges and Gabriela Salvadori da Silva for all their time and work and for supporting me throughout the whole process. I also want to thank former supervisor Håkon Valen Rukke for his expertise and for believing in me from the very beginning, senior engineer Heidi Aarø Åmdal for her assistance in the lab, and Postdoc Stian Andre Engen for providing me with epithelial cells and for being there along the way. At last I want to say sincere thanks to the University of Oslo, Faculty of Dentistry for granting me with the summer scholarships.

ABSTRACT

Streptococcus mitis is a Gram positive facultative anaerobic bacterium commonly found in the human oral cavity where it colonizes all surfaces and forms the basis of biofilms. *S. pneumoniae* is a close relative of *S. mitis* and despite sharing about 83% of their genomes, *S. pneumoniae* has a much higher pathogenic potential. Both species are competent for natural genetic transformation, which is induced by pheromones known as competence stimulating peptides (CSPs). The recognition and binding of CSP causes the expression of numerous genes, including the expression of killing factors targeting non-competent closely related streptococci living in the same environment. In *S. pneumoniae*, the killing mechanism termed fratricide is accomplished by a murein hydrolase; Choline-binding protein (CbpD). A CbpD homologue is also found in S. mitis, but it is function in competence is not completely understood. In the present study we investigated the effect of CSP and cbpD deletion on *S. mitis* growth, signaling and release of DNA. To be able to explore these effects, we have used optical density assays, luciferase reporter assays, and real-time PCR, respectively. Our results demonstrated that CbpD plays a key role in the lysis of competent *S. mitis.* However, lysis was not abolished in the absence of cbpD, indicating that other factors are involved in growth inhibition, lysis and subsequent DNA release in competent *S. mitis*. Furthermore, we showed that a *cbpD* deletion mutant presented a stronger response to the *S. mitis* competence pheromone. The ability to lyse other bacteria and take up their genetic information might be crucial for *S. mitis* survival and tolerance to different stressors *in vivo.* Understanding *S. mitis* pheromone signaling might reveal reasons why this bacterium is such a successful colonizer and may shed light into how the exchange of important bacterial genetic information, *e.g.* antibiotic resistance genes, takes place in the oral cavity.

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INTRODUCTION

Pneumonia is responsible for the death of more children than any other disease in the world. Every year, more than 2 million children die from pneumonia. Approximately one-fifth of the under five years-old deaths worldwide (1). *Streptococcus pneumoniae*, often referred to as pneumococcus, is the leading cause of this disease. This gram-positive bacterium is alpha-hemolytic and a facultative anaerobe. Most of the time, it resides asymptomatically in the nasopharynx of healthy carriers (2). In immunocompromised individuals, elderly and children, *S. pneumoniae* can become pathogenic and spread to other locations, causing pneumonia, middle ear infection, meningitis, and septicemia (3). Antibiotics are used for the treatment of pneumonia but sadly a great portion of children suffering from this illness do not get the medication. Furthermore, certain areas struggle with high levels of resistance to some antibiotics, leading to disturbances in the treatment strategy (1). Recently, awareness on antimicrobial resistance has been raised as a response to the increase in the number of cases each year in several countries (4). Understanding the disease process the how antibiotic resistance is developed is a major concern in medicine and dentistry.

Streptococcus mitis, which is a member of the Mitis group of streptococci, colonizes all surfaces of the oral cavity including teeth, tongue and mucosal surfaces, in addition to the tonsils and nasopharynx (5). It is referred to as an “early colonizer” in which it forms the basis of biofilm during its formation; it is one of the few bacteria that can colonize the teeth at early stages of dental plaque formation in adults and it is one of the initial microbes that colonize the oral cavity of newborns (5, 6). Consequently, bacteria that adhere to the early colonizers, called “secondary colonizers”, will continue the development of the biofilm (7). *S. mitis* is most frequently found as a commensal in the human host, but rare events of septicemia in neutropenic cancer patients following chemotherapy have been reported (3, 8, 9). Moreover, it is one of the oral streptococci that are associated with endocarditis (10, 11). *S. pneumoniae* and *S. mitis* are both member of the Mitis group of streptococci. They share about 83% of the same genome, being genetically very similar, therefore it is striking that

they behave so differentially (12). From current evidence, these two streptococci have evolved from a common ancestor that has a great similarity to the present *S. pneumoniae*. In comparison to *S. pneumoniae* and their common ancestor, it is suggested that in *S. mitis* loss of virulence determinants may be the reason for the decrease in its pathogenic potential (13).

Bacterial communities consist of different populations that are able to communicate with each other. This way of sharing information within- and across populations is based on intercellular chemical signaling termed quorum sensing (QS). QS depends on pheromones; small substances secreted by bacteria, operating as signals that can be detected by other bacterial cells. In Gram positive bacteria the secreted pheromones are small peptides. The importance of quorum sensing relies in its ability to control numerous fundamental systems including regulation of virulence factors, conjugation, biofilm formation and natural competence for transformation. The pheromones interact with processes between bacteria and host and these signaling pathways can possibly be interfered. Since this will target virulence/pathogenicity in a different way than antibiotics, QS and its signaling pathways may be targets for novel therapeutic strategies to fight antibiotic-resistant bacteria (14).

Horizontal gene transfer (HGT) is a process in which an organism transfers genetic material to another cell that is not its descendant, and has played a major role in the evolution of bacteria. HGT is not a passive process such as natural selection, since it is due to processes that depend only on the bacterial cells, such as natural genetic transformation, conjugation and transduction. Natural genetic transformation occurs in some types of bacteria that can take up DNA fragments dispersed in the environment and incorporate them into their DNA. This fragment will be incorporated into the DNA of the bacteria through the exchange of bases between the original and the absorbed DNA fragment, also known as homologous recombination. If there is compatibility in this exchange, the fragment becomes part of the genetic material of the bacteria, being duplicated and passed during the binary fission (15). As stated previously, this phenomenon enables bacteria to select and incorporate different external genetic material, thus, creating a big pool of genetic information

in different environments. This common gene pool that, for instance *S. pneumoniae*, shares with other members of its own species and closely related commensal streptococci, can give them advantages when under certain types of external stress, leading to “favorable” genes spreading in high speed (16). Originally, natural genetic transformation was discovered in *S. pneumoniae* (17) in 1928, when Frederick Griffith observed that a non-virulent strain was able to obtain the virulence from another one; he called it the “transforming principle”. Later, in 1944, Oswald Avery and coworkers identified the “transforming principle” as being DNA (18). From then on, the competence system in the pneumococcus has been studied extensively (19-22). There is little knowledge, however, regarding transformation and the competence system in *S. mitis*.

In *S. pneumoniae,* competence for natural genetic transformation is a transient state: it spontaneously arises during the exponential growth phase (Figure 1) at a certain cell density and reaches its top peak after approximately 20 minutes, before it quickly shuts down.

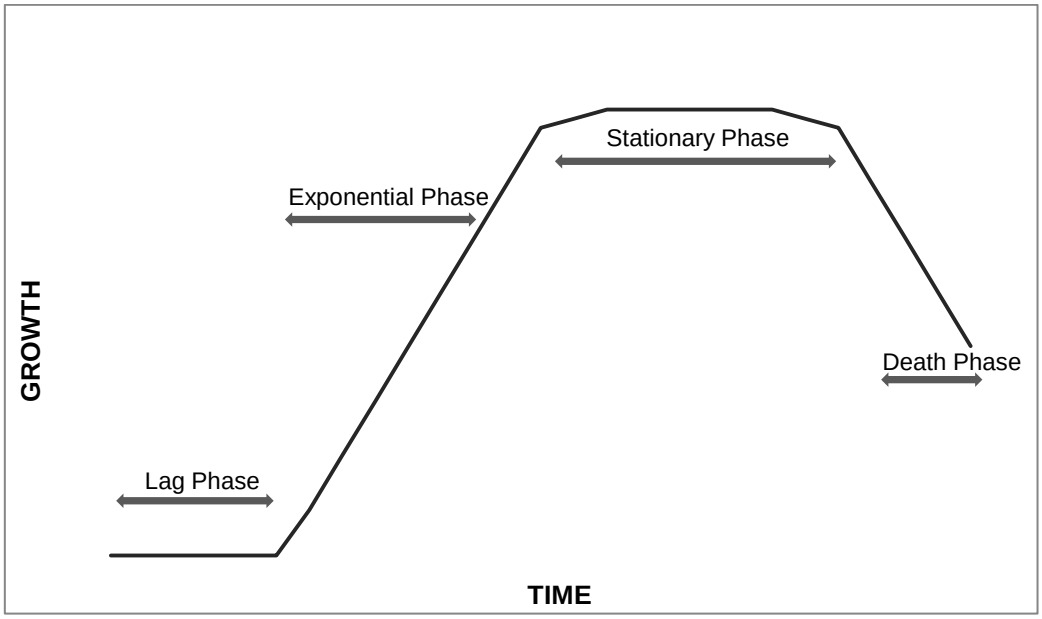

Figure 1: The figure demonstrates the different bacterial growth phases. Lag phase; bacteria are preparing for the cells to divide, RNA, proteins and other factors are synthesized. The exponential growth phase; bacteria now are dividing by binary fission, when reaching the stationary phase, the dividing of bacteria ends. In the death phase, accumulation of toxic products in addition to lack of nutrients is leading to bacteria- death with subsequent lysis (23).

Competence development can be divided into two main parts consisting of the QS pathway and the competence-promoting machinery (19). The QS pathway initiates the competence development cascade: *comC* is activated, and its activation will lead to the initiation of transcription and translation of the gene into a pro-peptide before it is cleaved and exported out of the cell as the competence-stimulating peptide (CSP) by an ABC transporter, ComAB (20). When a threshold concentration of CSP in the external environment is reached, CSP binds to its membrane receptor histidine kinase ComD leading to phosphorylation of the response regulator ComE. After phosphorylation of ComE, the alternative sigma factor SigX is activated, (21, 22) in addition to more 20 early genes. Together, ComD and ComE make up a two-component signal transduction system that senses and responds to the extracellular CSP concentration. This system activates expression of both *comAB* and *comCDE* operons, forming a positive feedback loop. The alternative sigma factor*,* SigX*,* is a response regulator that controls transcription of the competence genes involved in DNA uptake and recombination and also genes related to fratricide and immunity to fratricide. These genes are also known as the “late competence genes”. For most of the species in the mitis and anginosus phylogenetic groups, the regulation of the competence system is described to be strikingly similar (Figure 2) (15).

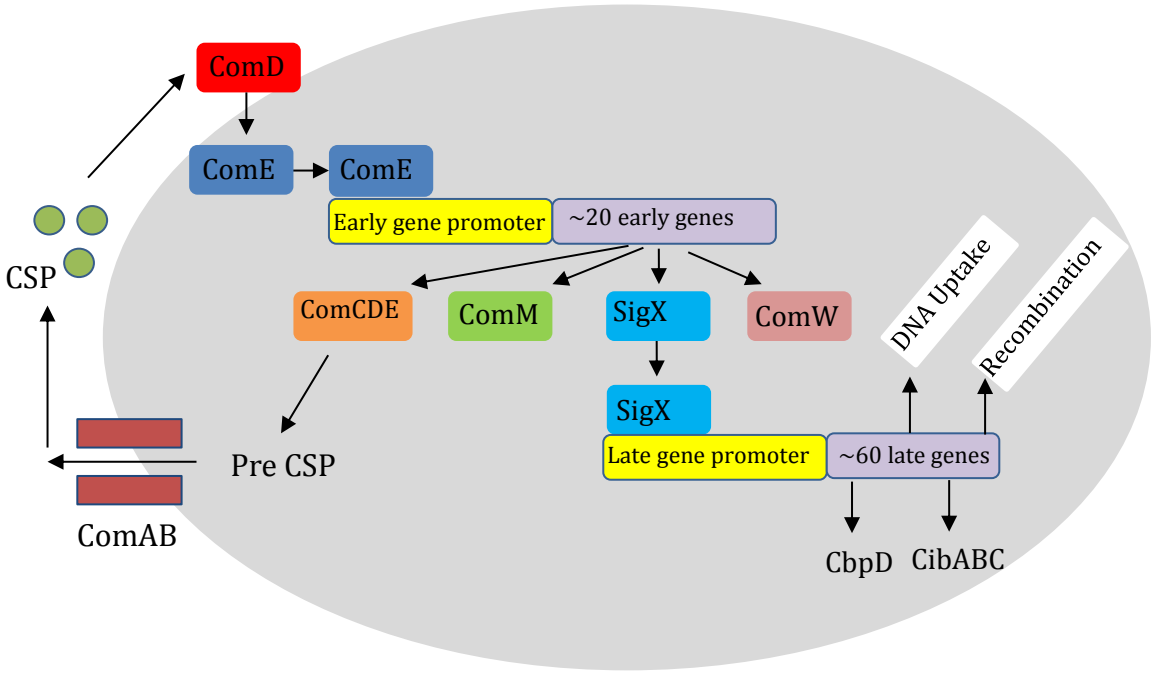

Figure 2: Figure illustrating competence regulation in *S.pneumoniae*. Pre CSP is a result of *comC* gene activation. The ComAB transporter processes and excretes pre CSP, leading to CSP accumulation extracellularly. Upon a certain concentration, CSP is recognized by its receptor ComD, which results in phosphorylation of the response regulator, ComE. Phosphorylated ComE binds and then activates the expression of various early genes; including transcription of the *comCDE* operon. This event leads to an accumulation of CSP, ComD and ComE, resulting in a positive feedback loop that gives rise to a boost in the production of the alternative sigma factor SigX, as well as ComM and ComW. Sig X activates the late genes, ensuring the production of a murein hydrolase CbpD; the main enzyme responsible for the fratricide mechanism. The presence of ComM ensures that the competent cells are protected against fratricide. Also, the proteins responsible for uptake of foreign DNA and recombination are a product of activation of the late genes. Environmental signals act as activators or repressors of the *comCDE* operon (15).

In *S. pneumoniae*, only 23 of more than 100 CSP-inducible genes are necessary for transformation. Two dispensable genes are *cbpD* and *comM* which are key players in a competence-associated mechanism called fratricide. When pneumococci become competent, they synthesize and secrete the late gene product, CbpD. This murein hydrolase acts as a weapon, which lyses and kills not competent cells. Hence, the competent pneumococci behave as predators that attack and lyse non-competent cells in the same culture. The competent cells that produce CbpD protect themselves by secreting an immunity protein, ComM, which is embedded in the membrane and neutralizes the muralytic activity of CbpD via an unknown mechanism (Figure 3). Since ComM is a product of an early competence gene, the non-competent pneumococci lacking this immunity protein are thus susceptible to CbpD (24, 25). Fratricide triggers lysis of non-competent cells resulting in the

release of intracellular material, and this has been shown to result in the release of transforming DNA that can be taken up by competent cells. Unlike other substances that contribute to the lysis of noncompetent cells (*e.g.* LytA and LytC) CbpD is expressed from a late gene promoter, hence CbpD is synthesized only when the cells are competent, demonstrating that CbpD is a key player in fratricide mechanism. Fratricide is not unique to pneumococci, given this mechanism has also been demonstrated in competent *S. mitis* and *S. oralis* (24). However, little is known about the role of CbpD and cell lysis in *S. mitis*.

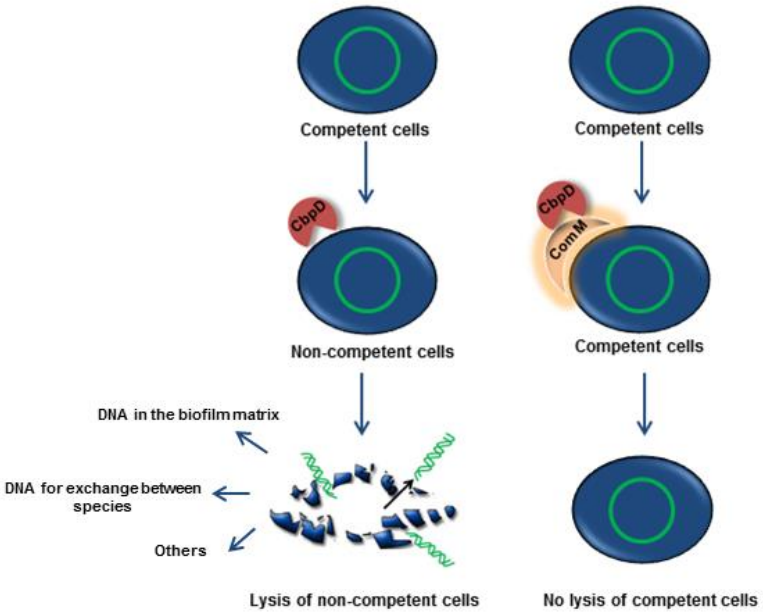

Figure 3. Figure demonstrating the fratricide mechanism. Competent cells produce and secrete CbpD which leads to lysis of the non-competent target cells in the same environment and subsequent DNA release to the extracellular environment. On the other hand, competent cells exposed to this murein hydrolase will protect themselves from suicide by its lytic activity by production of the membrane embedded immunity protein ComM.

As previously mentioned, CbpD of *S. pneumoniae* is a murein hydrolase and a product of the SigX-controlled late gene promoter. Different streptococci produce distinct types of SigX-controlled murein hydrolases, CbpD produced by *S. pneumoniae* is classified as a cell-wall-degrading toxin (class III bacteriocin), also called a bacteriolysin (26). It is further recognized as a 449 residue hydrolase consisting of three different basic components: the CHAP domain, the SH3b domains and a choline-binding domain

(compromising four choline-binding repeats) (Figure 4). The CHAP domain (cysteine, histidine,-dependent aminohydrolases/peptidases) with its active site cysteine, has been demonstrated to be required for fratricide. It acts as peptidases or as amidases degrading the cell-wall cross links. CbpD is therefore believed to cleave essential peptides of the cell wall peptidoglycan (27). The function of the SH3b domain is dual; it first guides and then binds the catalytic CHAP domain to the cell wall peptidoglycan (28). Finally, the choline binding domain ensures binding to teichoic-acid present in the cell envelope of targeted cells (24). CbpD of *S. mitis* only differs from the pneumococcal in the number of SH3b domains, *S. mitis* has one, while *S. pneumoniae* has one or two (24, 27). Nevertheless, CbpD produced by *S. mitis* is identical to several other streptococci, including the CbpD of *S. oralis* (24) (Figure 4).

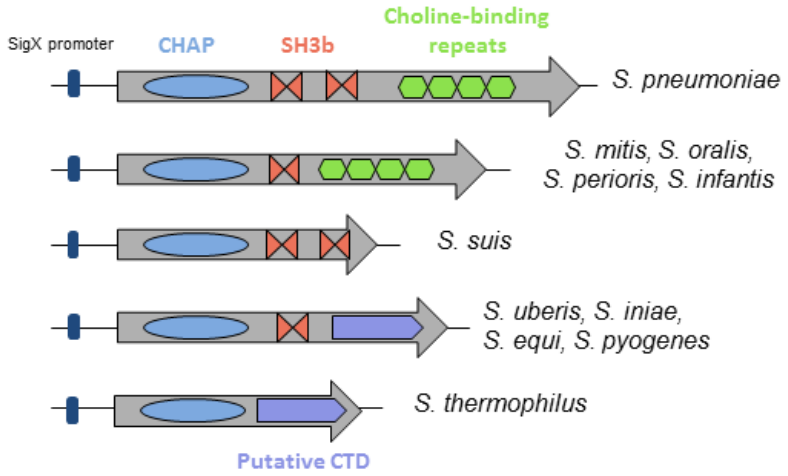

Figure 4. Streptococcal CbpD-like proteins encoded by the early gene SigX. Light blue: CHAP, cysteine, histidine-dependent amidohydrolases/peptidases; Orange: SH3, binds peptidoglycan; Green: choline-binding repeats, binds choline residues linked to teichoic acid; Lilac: Putative C-terminal domain, uncharacterized domain that probably mediates binding to the cell wall of target cells.

It is well established that CbpD is involved in the killing of sister cells, cells that are genetically identical, or nearly identical to the producer cells. The question is: how related do the streptococcal species need to be for CbpD to kill? To get access to DNA from other species cross-species lysis is acquired, since the composition of CbpD differs between the various streptococcal species (Figure 4). Cross-species lysis can only occur if different

species are able to respond to each other's CbpD. Johnsborg and co-workers demonstrated that both competent *S. pneumoniae* and *S. mitis* were able to cause CbpD-dependent lysis of the non-competent version of each other in addition to non-competent *S. oralis*. Also, they showed that competent *S. pneumoniae* was unable to attack and kill a more distant related species, as non-competent *S. gordonii* did not respond to its CbpD. Therefore, CbpD of *S. mitis* and *S .pneumoniae* is able to act towards closely related streptococcal species (29). Another crucial factor for fratricide to occur is the coexisting of competent and non-competent cells in the same culture. This is possible because different CSPs are produced by different cells both intra- and interspecies. Cells with the same type of CSP are known to be part of the same pherogroup, making them sensitive to the same type of CSP. Thus, not all the cells will respond to the CSP secreted, rendering a fraction of the culture non-competent and further sensitive to CbpD and subsequent lysis (29).

Aim

The aims of this study were to investigate whether *Streptococcus mitis* communication via competence pheromone affects growth, the pheromone signaling and cell lysis and whether such an effect depends on the choline-binding protein D (CbpD). Ultimately, it is important to understand how the interplay between *S. pneumoniae*, a major human pathogen, and *S. mitis*, an oral commensal, occurs during competence.

Material and Methods

Bacterial strains and media. All used strains of *S. mitis* and *S. pneumoniae* are listed in Table 1. Bacteria were stored at −80°C in Todd Hewitt Broth (THB, Becton Dickinson and Company, Le Pont de Claix, France) or Tryptic Soy Broth (TSB, Soybean-Casein Digest medium , BactoTM) supplemented with 15% glycerol. Pre-cultures used in the experiments described below were made from fresh liquid cultures incubated at 37°C in a 5% CO_2-supplemented atmosphere and grown until the cultures reached an absorbance at 600 nm (optical density at 600 nm [OD_{600}]; Biophotometer; Eppendorf) of 0.5 or 0.3, before storage at −80°C in 15% glycerol. For plating, blood agar base No. 2 (Oxoid, Hampshire, England) supplemented with 5% defibrinated sheep blood (TCS Biosciences Ltd., Buckingham, United Kingdom) was used. Selective levels of antibiotics were 500 µg ml^{-1} kanamycin, 20 µg ml^{-1} erythromycin and 500 µg m^{-1} spectinomycin.

Transformation. Transformation of *S. mitis* was carried out as previously described (30). Briefly, pre-cultures of *S. mitis* MI017 and MI039 were diluted 1:10 in TSB. Cultures were grown to an OD_{600} of 0.03 before addition of chromosomal DNA from MI055 at a concentration of 10 µg per ml culture and CSP for *S. mitis* CCUG31611^T (EIRQTHNIFFNFFKRR; GenScript) at a final concentration of 250 nM. Cultures were incubated under aerobic conditions at 37°C for 4 h. Mutants were selected on blood agar

plates supplemented with the appropriate antibiotics after 24 hours of incubation at 37°C, 5% CO_2.

Growth Curve and Luciferase Reporter. Pre-cultures of *S. mitis* wild type, MI017, MI039, MI055, MI062 and MI063 were diluted 1:50 in TSB. Cultures were split in two and induced with 100ng ml^{-1} CSP or kept untreated. 200µL were transferred to microtiter wells of a 96-well plate suitable for optical density (OD_{600}) and luminescence measurements. 100µM of luciferin was added to each well. Readings were made using a plate reader (BIO-TEK, SynergyttT). Results were analyzed using the software Gen 5 1.10 (BIO-TEK).

Real-time PCR (RT-PCR). Pre-cultures of MI081, MI082 were diluted 1:10 in TSB grown until an OD_{600} of 0.03. At this point, 100ng ml^{-1} CSP was added. Sample '0' was taken immediately after the addition of CSP, whereas remaining samples were taken at the following time points: 15, 30, 60, 120, 240, 300, 360, 420 and 480 min after CSP induction. In each time point, 1mL of culture was centrifuged at 10,000g for 4 minutes in a temperature of 4°C, following the protocol for DNA release described by Itzek, Zheng (31). Supernatants were collected, frozen immediately in liquid nitrogen and stored at -80°C. For the use in real time PCR (Thermo Scientific (Fermentas), MaximaSYBR Green/ROX qPCR, K0222), 1µL of supernatant of each mutant in each time point was added to 9,9µL of RNase free water, 12,5µL of SYBR Green/ROX and 0,1µL for each primer forward and reverse for *gyrA* gene (FP369 -FP370) (Table 1). Samples were always used in duplicates. Standard DNA concentrations of 1ng, 10ng and 100ng were also used as a control. PCR conditions included an initial denaturation at 95°C for 10 min, followed by a 40-cycle amplification consisting of denaturation at 95°C for 30 s and annealing and extension at 55°C for 1 min.

Statistical Analysis. Repeated measures analysis of variance (rANOVA) was used as statistical approach to repeated measure experiments like growth assays and luciferase reporters. All statistical calculations were performed using the GraphPad Prism 6 program. A *p* value <0.05 was considered to be statistically significant.

Table 1. Mutants, peptides and primers used in this study.

Mutant	**Description**	**Source**
S.mitis NCTC12261	Wild-type *S. mitis* biovar 1 type strain, which corresponds to NCTC 12261; encapsulated, transformable strain; sequenced genome; Km^S Erm^S	CCUG® 31611
MI017	CCUG31611, but Δ*cbpD*::Kan; Kan^R	Not published
MI039	CCUG31611, but Δ*cps*::Kan, Δ*cbpD*::Erm; Kan^R Erm^R	Not published
MI055	CCUG31611, but p_{comX}-luc::Spec: $Spec^R$	Not published
MI062	CCUG31611, but Δ*cbpD*::kan. p_{comX}-luc::Spec; Kan^R $Spec^R$	Not published
MI063	CCUG31611, but Δ*cps*::Kan, Δ*cbpD*::Erm, p_{comX}-luc::Spec; Kan^R Erm^R $Spec^R$	Not published
MI081	CCUG31611, but Δ*comC*:Kan, p_{ldh}-*luc*:Spec; Kan^R $Spec^R$	Not published
MI082	CCUG31611, but but Δ*comC*:Kan, Δ*cbpD*::Erm, p_{ldh}-*luc*::Spec; Kan^R Erm^R $Spec^R$	Not published
SP001	BAA334, but Δ*comA*::Kan; Kan^R	Not published
SP037	BAA334, but Δ*cbpD*::Erm; Erm^R	Norwegian Institute of Public Health
Competence Stimulating Peptide	Sequence	Source
S. mitis 12261	EIRQTHNIFFNFFKRR	GenScript
S. pneumoniae BAA334	EMRISRIILDFLFLRKK	GenScript
Primers	Sequence (5' to 3')	Source
FP017	TTTTGTTCATGTAATCACTCCTTC	Sigma-Aldrich
FP018	CACGCCAAAGTAAACAATTTAAG	Sigma-Aldrich
FP037	TCATTTTCTCCCACCAGCTT	Sigma-Aldrich
FP109	CAAGCCTGATTGGGAGAAAA	Sigma-Aldrich
FP369	GCCGTTCGTGGTATGAGTCG	Sigma-Aldrich
FP370	GGTCGCAACTGTGCGCTTAC	Sigma-Aldrich

Results

S. mitis and *S. pneumoniae* are both known to be competent for natural genetic transformation (24, 32). Natural genetic transformation is regulated by a QS-system that uses the competence-stimulating peptide (CSP) as its pheromone, when CSP has reached a threshold concentration it will bind to its receptor which leads to activation of the competence specific genes, and competence is induced (15). Fratricide is a competence associated mechanism where competent cells lyse non-competent sibling cells in the same culture. The three different choline-binding murein hydrolases, CbpD, LytA and LytC, are responsible for fratricide in *S. pneumoniae*. While CbpD is recognized as the principal component for this process, LytA and LytC are accessory lytic proteins that contribute to the muralytic function of CbpD (24). Six years ago, Johnsborg and co-workers (29) detected that the deletion of *cbpD* abolished *S. pneumoniae* and *S. mitis* ability to lyse target cells, and by that they showed that CbpD is employed as a killing mechanism in *S. mitis* also.

The study mentioned previously is the only one that has explored the mechanism of lysis during competence in *S. mitis,* thus more investigation is required in order to understand how this system is regulated during competence in the referred bacteria.

Growth inhibition during competence in *S. mitis* is present even in the absence of CbpD. Three independent experiments were conducted to assess the effect of the competence pheromone on the *cbpD* deletion mutant (Figure 5). Growth rate at the exponential growth phase was inhibited by 42.2 % in Δ*cbpD* mutant in the presence of the competence pheromone (CSP). Likewise, for the wild-type (WT), the growth rate was also inhibited in the presence of the competence pheromone (Figure 5). Growth inhibition even in absence of CbpD indicates that others factors are involved in cell lysis during competence in *S. mitis*. This finding provided us the evidence that something else is happening and motivated a deeper investigation in the subject.

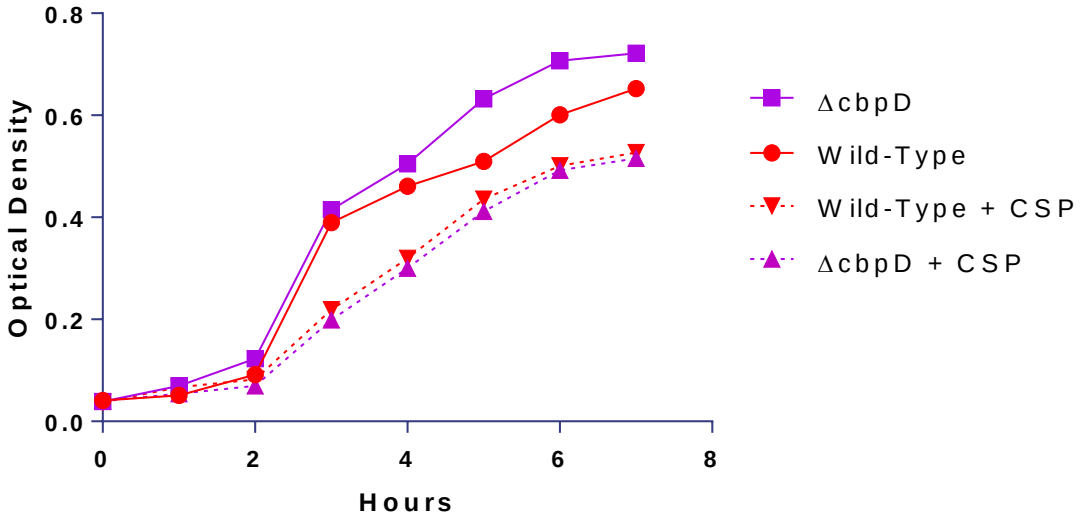

Figure 5. Growth curves of *S. mitis* Δ*cbpD*, and WT in TSB. Cultures were induced with 100ng ml^{-1} CSP12261 or kept untreated. Incubation time of 8 hours at 37°C, 5% CO_2. Representative of three independent experiments.

Construction of reporter and deletion strains for the study of the interplay between natural competence and *cbpD* expression in *S. mitis.*

As mentioned in the introduction, during competence, the alternative sigma factor SigX that plays a key role in competence development is up-regulated. Thus, we constructed *sigX* promoter reporters to monitor the response to the competence pheromone of a mutant without CbpD (Δ*cbpD*). The DNA donor used in the transformation assays was from a previously constructed p_{comX}-luc-reporter in a wild-type background. The recipient strain was a *cbpD* deletion mutant (Δ*cbpD)*. After selection in plates with the antibiotic marker, colonies were screened two times more for the detection of the desired phenotype. Confirmation of *cbpD* deletion and insertion of the marker was also conducted by regular PCR followed by placing primers inside the new insert.

***cbpD* deletion resulted in a stronger response to the competence pheromone.** While CbpD in *S. pneumoniae* mediates cell lysis during the competent state, in *Streptococcus thermophilus* its role is in CSP signaling. To investigate whether CbpD in *S. mitis* may have a role in signaling, we performed a *sigX* reporter assay using both a wild-type and a *cbpD* deletion strain. Figure 6 shows that Δ*cbpD* mutant presented a significantly higher expression of *sigX* when the synthetic CSP was added in comparison to the wild-type strain from 2 to 8 hours ($p<0.0001$). The peak of competence in Δ*cbpD* mutants lasted for almost 3 hours after addition of synthetic CSP, while it lasted for 2 hours in the wild-type. These results show that the deletion of the murein hydrolase CbpD increases *sigX* expression in S. mitis by an unknown mechanism.

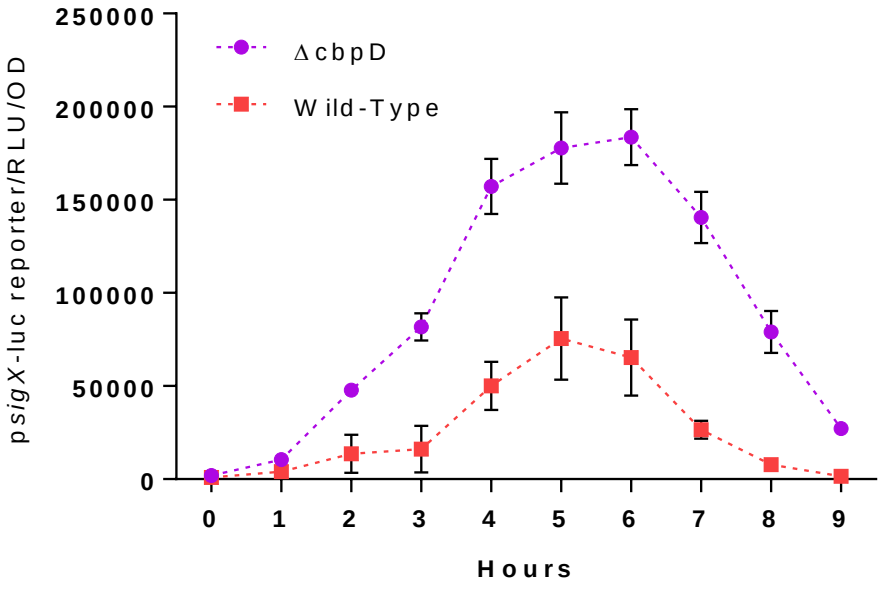

Figure 6. Repeated measures of *sigX* expression in the mutant Δ*cbpD* p_{comX}-luc and in the wild-type (p_{comX}-luc) in TSB normalized by the optical density of the cultures (mean and standard error). Cultures were induced with 100ng ml^{-1} CSP12261. 100μM luciferin was added all cultures. After this, cultures were incubated at 37°C for 9 hours. Representative results from three independent experiments are shown. The dots represent the mean and error bars represent the standard error of the mean.

CSP induces DNA release in Δ*comA S. pneumoniae* mutant. Competence-induced cell lysis increases the efficiency of horizontal gene transfer by providing extracellular DNA that can be taken up by competent recipient cells. We considered important to develop a quantitative assay that allows us to measure DNA release from lysing cells in competent cultures of *S. mitis* and *S. pneumoniae*. Real time PCR is a technique that allows the accurate measurement of extracellular DNA in competent and non-competent cultures. We used a *S. pneumoniae* strain with a disrupted *comA* gene as a control, because this strain is not able to export endogenous CSP, therefore it is possible to control when competence is induced by adding the synthetic peptide. Furthermore, we have strong evidence of how the lysis system works in that species (25, 33) and thus, we were able to draw comparisons regarding *S. mitis*. Likewise, we used a *S. mitis* mutant strain lacking *comC* gene, rendering them unable to develop spontaneous competence.

At all times after 30 minutes following CSP addition, the concentration of DNA in culture supernatants was higher in the CSP treated culture than in the control (Figure 7A). Our results show that the content of extracellular DNA in the Δ*comA* competent culture released 75% more DNA than the non-competent culture of the same strain after 30 minutes after CSP addition ($p=0.02$) (Figure 7B). Likewise, during the exponential growth phase, the competent culture released 47,5% more DNA when compared to the untreated culture. Nevertheless, this pattern remained in the stationary phase, when the CSP induced culture release 48,2% more DNA than the non-induced culture (Figure 7A). These findings are aligned with what have been reported by Kausmally et al. (25) and Moscoso & Claverys (33), which confirms reliability in the method used for DNA release detection in this study.

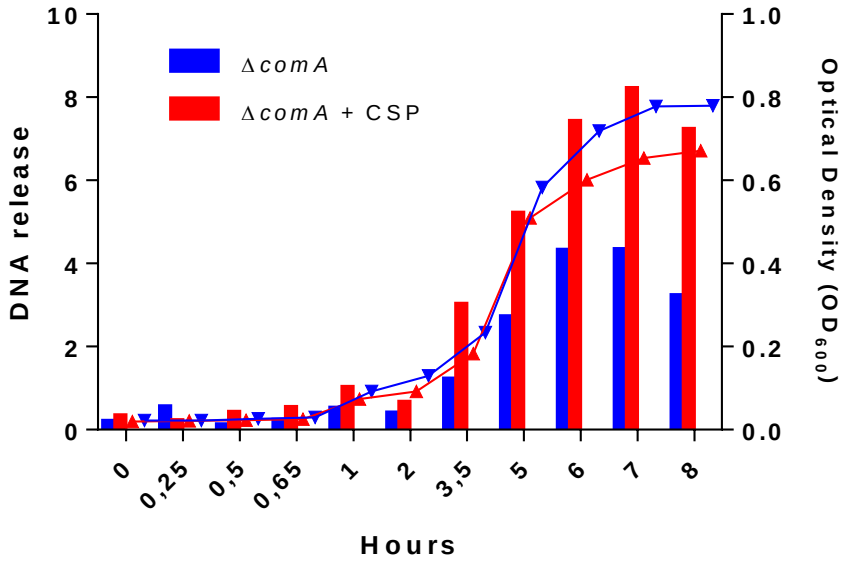

Figure 7A. DNA release (ng/µL) in S. *pneumoniae* Δ*comA* mutant is represented by blue and red bars and growth by blue and red lines. When the bacteria culture reached an OD_{600} 0,035 it was split in two, and half of the culture was induced to competence by addition of 250 ng ml^{-1} of synthetic CSP, while the other was kept untreated. After 0-, 15-, 30-, 45-, 60-, 120-, 240-, 300-, 360-, 420- and 480 minutes of incubation at 37°C, OD_{600} was measured, supernatants were collected by centrifugation, frozen immediately in liquid nitrogen and stored at -80°C. The collected supernatants were used in a real-time PCR assay with primers amplifying *gyrA*, a constitutive gene.

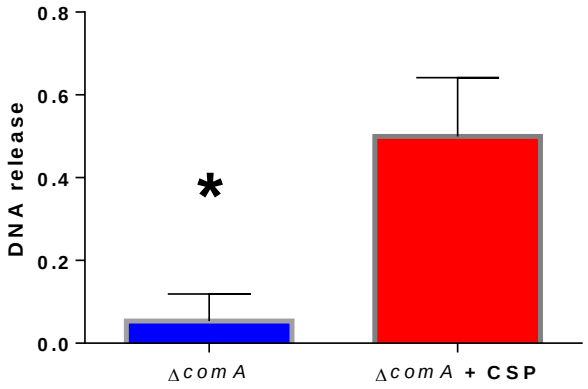

Figure 7B. DNA release (ng/µL) in *S. pneumoniae* Δ*comA* mutant after 30 minutes of incubation is represented by blue and red bars. When the bacteria culture reached an OD_{600} 0,035 it was split in two, and half of the culture was induced to competence by addition of 250 ng ml^{-1} of synthetic CSP, while the other was kept untreated. After 30 minutes of incubation at 37°C, supernatants were collected by centrifugation, frozen immediately in liquid nitrogen and stored at -80°C. The collected supernatants were used in a real-time PCR assay with primers amplifying *gyrA*, a constitutive gene.

Competence-induced cell lysis in *S. mitis* is similar to *S. pneumoniae.*

To elucidate how competence induced cell-lysis and subsequent DNA release is led in *S. mitis,* we constructed a mutant lacking both *comC* and *cbpD genes*. The DNA donor used in the transformation assay was from a previously constructed Δ*cbpD* strain in a wild-type background. The recipient strain was a *comC* deletion mutant (Δ*comC),* which is not able to produce endogenous CSP therefore it is possible to control when competence is induced by adding the synthetic peptide. After selection in plates with the antibiotic markers, colonies were screened two times more for the detection of the desired phenotype. Confirmation of both *comC* and *cbpD* deletion, as well as the insertion of the markers were also conducted by regular PCR followed by placing primers inside the new insert. Then, we set up an identical experiment as performed to investigate lysis in *S. pneumoniae.*

Figure 7C shows that, differently from *S. pneumoniae*, *S. mitis* did not present any difference in the amount of released DNA between induced and non-induced samples after 30 minutes of incubation. It was possible to see, however, a consistent pattern of increased extracellular DNA concentration in CSP-induced samples after 120 minutes of incubation. In the exponential growth phase, released DNA present in the competent culture followed a progressive pattern, reaching a 64% difference between competent and non-competent cultures. (Figure 7D).

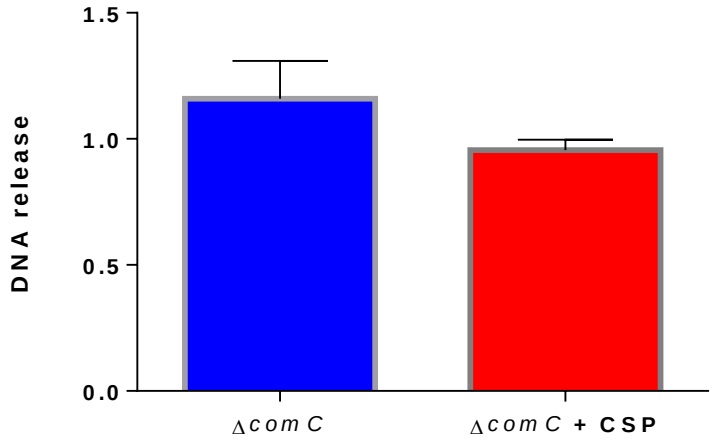

Figure 7C. DNA release (ng/µL) in *S. mitis* Δ*comC* mutant is represented by blue and red bars. When the bacteria culture reached an OD_{600} 0,035 it was split in two, and half of the culture was induced to competence by addition of 100 ng ml^{-1} of synthetic CSP, while the other was kept untreated. After 30 minutes of incubation at 37°C, supernatants were collected by centrifugation, frozen immediately in liquid nitrogen and stored at -80°C. The collected supernatants were used in a real-time PCR assay with primers amplifying *gyrA*, a constitutive gene.

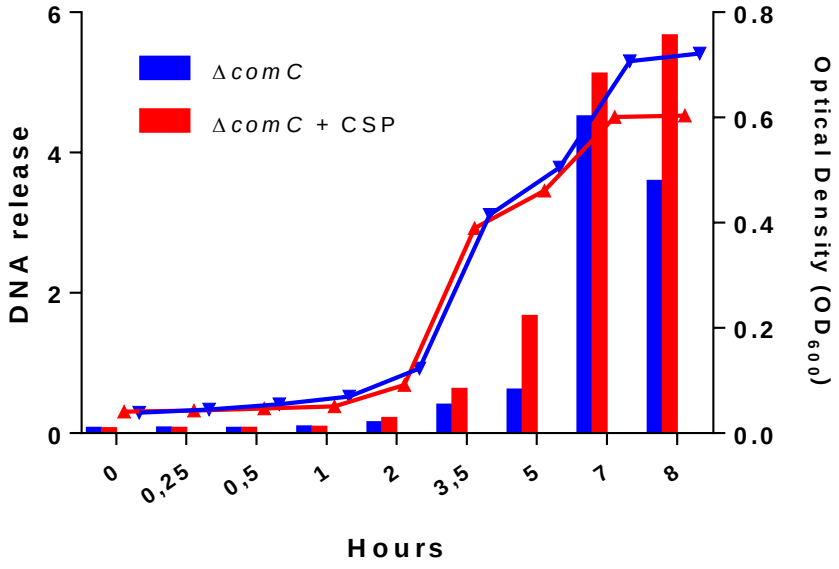

Figure 7D. DNA release (ng/µL) in *S. mitis* Δ*comC* mutant is represented by blue and red bars and growth by blue and red lines. When the bacteria culture reached an OD_{600} 0,035 it was split in two, and half of the culture was induced to competence by addition of 100 ng ml^{-1} of synthetic CSP, while the other was kept untreated. After 0-, 15-, 30-, 60-, 120-, 240-, 300-, 420- and 480 minutes of incubation at 37°C, OD_{600} was measured, supernatants were collected by centrifugation, frozen immediately in liquid nitrogen and stored at -80°C. The collected supernatants were used in a real-time PCR assay with primers amplifying *gyrA*, a constitutive gene.

Presence of CbpD is related to higher DNA release. After observing that cell lysis is similar in *S. mitis* and in *S. pneumoniae*, we proceeded to investigate the role of CbpD in this unique phenomenon. Figure 8 shows DNA release in two different *S. mitis* mutants (Δ*comC* and double mutant Δ*comC*Δ*cbpD*). During the first hour of incubation, it was not possible to see any difference regarding competence-induced DNA release between the two mutants. From 120 minutes and further, *S. mitis* mutant lacking *cbpD* presented a pattern that consisted in less lysis compared to the strain with an intact *cbpD*, and for this deletion mutant lysis was not depended on the presence of CSP, since at 5 hours of incubation the untreated culture presented higher DNA concentration. Furthermore, we could infer that competence induced cell lysis in *S. mitis* starts occurring when sigX expression increases. (red and purple lines). Even though lysis decreased when CbpD was not present, it was still not abolished, indicating that other factors are involved in the regulation of cell lysis and transformation of this bacterium.

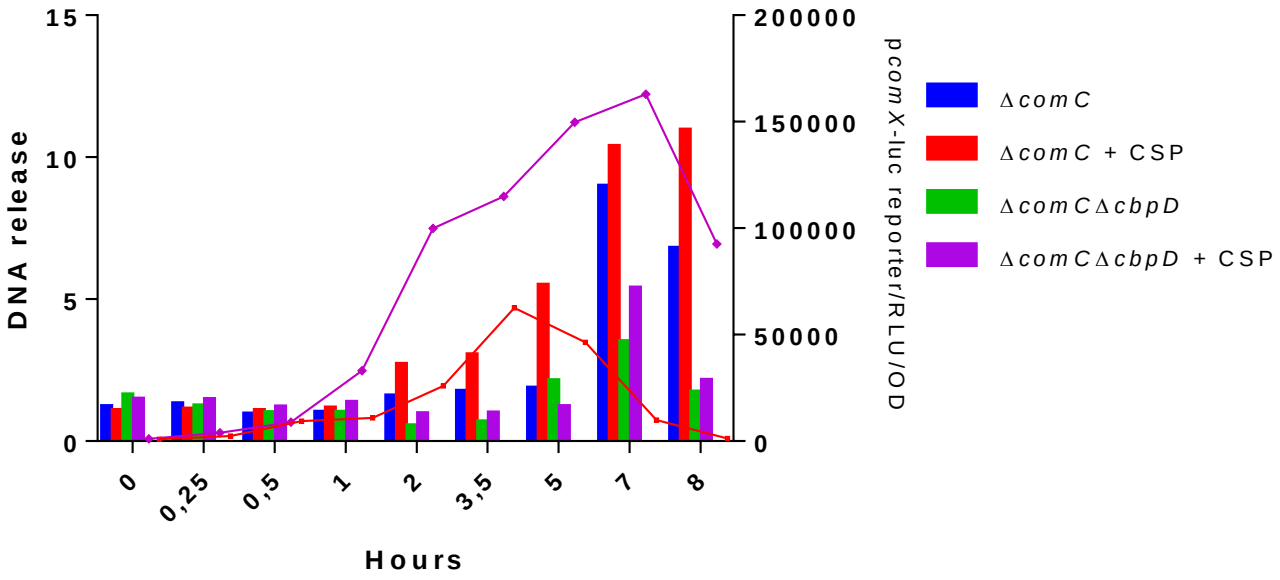

Figure 8: DNA release (ng/µL) normalized by optical density and competence (RLU/OD) in S. *mitis* Δ*comC* mutant (blue and red bars) and Δ*comC* Δ*cbpD* mutant (green and purple bars). When the bacteria cultures reached an OD_{600} 0,035 they were split in two, and half of the cultures was induced to competence by addition of 100 ng ml^{-1} of synthetic CSP, while the others were kept untreated. After 0-, 15-, 30-, 60-, 120-, 240-, 300-, 360-, 420- and 480 minutes of incubation at 37°C, supernatants were

collected by centrifugation, freeze immediately in liquid nitrogen and stored at -80°C. The collected supernatants were used in a real-time PCR assay with primers amplifying *gyrA*, a constitutive gene.

Discussion

In the present study, we have shown that the mechanism of competence-induced cell lysis in *S. mitis* is depended on CbpD, but that it also involves other factors than the action of this murein hydrolase. We observed that deletion of CbpD reduces lysis in *S. mitis*, however, in contrast with previous findings (25), it does not abolish this process. In addition, our results showed that CSP inhibits growth even when CbpD is not present. From this, it is possible to conclude that CbpD is present in the process of lysis; though, we can infer that it is not the only player in this competence-associated killing mechanism in *S. mitis*.

In *S. pneumoniae*, the state of natural competence inhibits growth. This cell lysis effect has been attributed to CbpD (34, 35). As mentioned earlier, pneumococci possess three different muralytic proteins, CbpD, LytA and LytC, responsible for the fratricide mechanism. Thus, while LytA and LytC have auxiliary functions, CbpD is essential for competence-induced cell lysis in this species. In the present work, when we deleted *cbpD* in *S. mitis*, lysis still existed but not mediated by CSP. We know that *S. mitis* does not produce LytA and LytC (29) and that CbpD possibly leads to competence-induced lysis in *S. mitis*. Furthermore, CbpD of *S. pneumoniae* and *S. mitis* have a great homology of 73%; only differing in the number of the peptidoglycan binding SH3 domains (24).

The relationship between competence development and the release of chromosomal DNA into culture media in *S. mitis* is unclear. Given that fratricide is a known competence related mechanism, we also looked at the peak of competence (by using a *sigX* reporter), and we were able to follow DNA release in relation to the pattern of *sigX* expression. A group that has investigated the correlation between competence-induced DNA release and the peak of competence is Moscoso and collaborators, they did so by looking at a number of time points from 0 to 240 minutes in *S. pneumoniae* Δ*comC* strain. Their results showed that the release of DNA in competent culture started after the peak of *comX* expression, achieving its highest point when pneumococci no longer were expressing *sigX* (33). This result is also aligned with what Dagkessamanskaia and co-workers reported (36). According

to the present study, the link between the pattern of competence and DNA release follows the same design in *S. mitis*. Thus, we observed that the Δ*comC* strain treated with CSP differentiated from the untreated cells first when the peak of competence had been reached (at 120 minutes), and still increasing even though *comX* expression decreased, thereby lasting for a longer period after the peak of competence was reached. Kausmally et al. have also looked at DNA release during competence in *S. pneumoniae* and in distinction to what was done in the present study; they used only one time point, measuring the amount of released DNA after 30 minutes of CSP induction. Their results showed that for the wild-type strain the extracellular DNA content when the cells were competent was a 100-fold greater that an identical non-competent strain. In a mutant lacking CbpD*,* only a fivefold increase of DNA release was seen in the CSP-induced culture. Nevertheless, when comparing with the cbpD deleted strain to the wild-type, when both were in the competent state, the results showed a tremendous increase in DNA release for the strain with a functional *cbpD* gene. In conclusion, they showed that removal of the *cbpD* gene nearly abolished cell lysis in competent pneumococci.

As previously mentioned, the features related to the competence system in *S. mitis* have not been explored yet, which led us to further explore the kinetics of this process. The great distinction from what Kausmally and co-workers did, compared to our study was that we looked at growth and DNA release during 8 hours, which gave us an overview of what was happening during all growth phases in competent and non-competent *S. mitis*. We could also observe a difference between the treated wild-type and *cbpD* mutant strains when it came to reduction of lysis in the *cbpD* mutant culture, but not before 120 minutes. Even though *cbpD* was absent, the cultures still presented some considerable degree of lyses both with and without CSP. Hence, this leads us to believe that another mechanism of lysis is present, and it can or cannot be related to competence.

Regarding the window of competence in *S. pneumoniae*, we know from the literature that competence development (relative *sigX* expression) starts increasing immediately after the addition of CSP to the respective medium, the peak is reached 30 minutes after before it

decreases (33). Since this has not been explored in *S. mitis*, this was of interest to be investigated this further. By using *sigX* promoter reporters we were able explore competence development in the wild-type and Δ*cbpD* mutant strains. Our results demonstrated that in the *cbpD* deletion mutant, *sigX* expression was much higher than in the wild-type, showing that the presence of this membrane murein hydrolase may influence how *S. mitis* competent cells are responding to the CSP signal. Furthermore, recent results from our laboratory also revealed a relationship between the deletion of *cbpD* and higher transformation efficiency of *S. mitis.* However, the mechanisms by which CbpD may affect CSP signaling on competent cells still needs to be clarified.

As described previously, competence induced cell lysis in *S. mitis* occurred even though the *cbpD* gene was absent, a rather unexpected result compared to how it is organized in its close relative *S. pneumoniae.* This indicates that there is a possible additional factor contributing to competence related cell lysis in *S. mitis*, and if so, what could that be? Recently, our group has performed a transcriptome analysis of *S. mitis* in response to CSP (Salvadori *et al.* unpublished results), and we identified ~100 genes up-regulated by the pheromone. All core competence genes (*comAB, comCDE, comX, comW*) were found to be up-regulated, as well as the key players of fratricide, *cbpD* (116.7-fold) and *comM* (23.5-fold). These results ensure that the competence system is present and active in *S. mitis* in addition to the classical mechanism of fratricide. Interestingly, we identified an operon (SM12261_0044-0047) that presented all its genes in at least 195.19-fold induction, and two of these genes seem to be involved in bacteriocin activities while the others are annotated as hypothetical proteins. Furthermore, for two of the four genes that compose this operon (SM0044 and SM0047), it was not possible to find orthologues in any *S. pneumoniae* strain, which means they are genes unique of *S. mitis* and may possibly be involved in the process of competence-induced cell lysis. Nevertheless, the fact that CSP did not have the expected influence on cell lysis of a *S. mitis* mutant strain with a disrupted *cbpD* gene in the present study, leads to the hypothesis that there could be an additional process or other murein

hydrolase not associated to competence that is also triggering cell lysis. This highlights an interesting area for future research.

Fratricide is co-regulated with natural genetic transformation. It ensures that competent streptococci are given access to a common gene pool that is very much larger than their own genomes, and it has been demonstrated among different streptococcal species like *S. mitis*, *S. pneumoniae* and *S. oralis* (29). Since *S. pneumoniae* is a frequent cause of illness and lethality in human, the use of antibiotics remains high, resulting in growing prevalence of penicillin resistance. Mutations in the penicillin-binding proteins (PBPs) and further recombination events mediated by natural transformation intra- and inter-species, are most likely to be the responsible these important outcomes (15). It is well demonstrated that commensal bacteria like *S. mitis* contains a bulk of penicillin- resistant elements that can easily be transported to *S. pneumoniae* by natural genetic transformation (29). Furthermore, in clinical dentistry, the use of antibiotics is at times needed, *e.g.* prophylactic use before surgery to prevent endocarditis, and *S. mitis* is known as one of the oral bacteria most commonly associated with this disease (10, 11)

Natural genetic transformation is a major mechanism of horizontal gene transfer in bacteria, most often triggered by signals known as pheromones. This highlights the relevance of exploring the role of such signaling systems. The ability to lyse other bacteria and take up their genetic information might be crucial for *S. mitis* survival and tolerance to different stressors *in vivo*. Of particular relevance is the fact that *S. mitis* can also harbor important genes, *e.g.* antibiotic resistance, which can eventually be incorporated by other species in the oral cavity. Understanding *S. mitis* pheromone signaling might reveal reasons why this bacterium is such a successful colonizer, being a major component of the biofilm formed in all surfaces of the oral cavity, and may shed light into how the exchange of bacterial genetic information takes place in the human mouth.

Conclusion

In the present work, we showed that the murein hydrolase; Choline-binding protein (CbpD) plays a crucial role in cell lysis during competence in *S. mitis*. Furthermore, we show that this process also requires the activity of other factors than CbpD, which are up to this moment, unknown. In addition, we provided evidence of how the kinetics of DNA release is developed in *S. mitis*. These findings shed light on the horizontal gene transfer process that takes place with an important oral commensal and a close relative to a major human pathogen. Future research should focus on how gene transfer occurs *in vivo*.

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