COMPETENCE HETEROGENEITY IN THE *STREPTOCOCCUS* MILLERI GROUP

INVESTIGATION OF COMPETENCE HETEROGENEITY IN *STREPTOCOCCUS* MILLERI GROUP CLINICAL ISOLATES

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I dedicate this work to my parents who have always taught me the value of hard work, dedication and perseverance

Abstract

The *Streptococcus* Milleri/Anginosus group (SMG) includes *Streptococcus anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius*. The SMG is found in healthy individuals but these bacteria are most known clinically for being associated with invasive disease and more recently, airway infections including cystic fibrosis (CF). The SMG like many other streptococci are naturally competent, being able to actively bind, uptake and integrate extracellular DNA. Competence regulation involves a competence-stimulating peptide (CSP) derived from the ComC precursor and a two-component signaling system (a histidine kinase ComD and its response regulator ComE). In this study, I examined the distribution of CSP/ComD sequences and competence in 170 SMG clinical isolates from CF airways and invasive disease. Five predicted CSP sequences were observed; one represented a newly predicted CSP and two arose from frameshift mutations in *comC* and appeared to be non-functional. The three CSPs fall into two functional groups that do not cross-activate due to receptor specificity. In addition, I observed that the *Streptococcus constellatus* subspecies *pharyngis* strains could not be transformed. However, I demonstrated that the *pharyngis* strains possess a functional ComCDE pathway, suggesting that the CSP regulates genes other than those involved in natural transformation. For many strains, I observed high endogenous competence levels that were only marginally induced by added peptide. These strains appear to be constitutively competent during exponential growth. The high basal level of expression and the heterogeneity in the SMG competence systems could impact how the SMG evolve during colonization and infections and specifically acquire antibiotic resistance

and virulence genes.

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Sean Taylor, a field application specialist at Bio-Rad gave me excellent advice on my real-time PCR experiment and for this I am very grateful to him.

Last but not least, my parents who are my heroes deserve special thanks for their unwavering love, support and understanding. Throughout my degree, I often drew inspiration from their value in hard work and perseverance.

My time in the Surette laboratory has been extremely rewarding both personally and intellectually. I am grateful for all the experiences afforded to me throughout my degree and wish Dr. Surette continued success in his future endeavors.

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List of Abbreviations and Symbols

Ap	ampicillin
ATCC	American Type Culture Collection
BHI	brain heart infusion
CF	cystic fibrosis
CFTR	cystic fibrosis conductance regulator
cfu	colony-forming unit
cfu/ml	colony-forming unit per milliliter
CLSI	Clinical and Laboratory Standards Institute
CO_2	carbon dioxide
comCDE	competence operon
CSP	competence-stimulating peptide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
Ery	erythromycin
g	gram
gDNA	genomic DNA
L	litre
LB	Luria-Bertani
ldh	lactate dehydrogenase
mg	milligram
MHBA	Mueller-Hinton blood agar
ml	millilitre
NaCl	sodium chloride
NCBI	National Center for Biotechnology Information
PCR	polymerase chain reaction
pmol/µl	picomole/microliter
R	resistant
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SMG	*Streptococcus* Milleri Group
ssDNA	single-stranded DNA
SsbB	single-stranded binding protein
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
Spc	spectinomycin
$T_{10}E_{25}$	Tris (hydroxymethyl) aminomethane hydrochloride 10 mM, ethylenediaminetetraacetic acid 25 mM, pH 8.0

Tet	tetracycline
THY	Todd-Hewitt supplemented with 0.5% yeast extract
THY-Ery150	Todd-Hewitt supplemented with 0.5% yeast extract and erythromycin (150 μg/ml)
THY-Spc75	Todd-Hewitt supplemented with 0.5% yeast extract and spectinomycin (75 μg/ml)
THY-Spc75-Ery150	Todd Hewitt supplemented with 0.5% yeast extract, spectinomycin (75 μg/ml) and erythromycin (150 μg/ml)
THY-Spc75-Tet1	Todd Hewitt supplemented with 0.5% yeast extract spectinomycin (75 μg/ml) and tetracycline (1 μg/ml)
THY-Spc75-Tet1-Ery150	Todd Hewitt supplemented with 0.5% yeast extract, spectinomycin (75 μg/ml), erythromycin (150 μg/ml) and tetracycline (1 μg/ml)
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
tRNA	transfer ribonucleic acid
U	units
μg	microgram
μM	micromolar
VGS	Viridans group streptococci

Declaration of Academic Achievement

Anne-Marie Lacroix designed and performed all experiments and analyzed the obtained results. Michelle Pinto designed the primers for real-time PCR as well as the RNA extraction protocol to which a few modifications were made. Dr. Jennifer Stearns helped with the statistical analysis of the real-time PCR data. Sean Taylor, a field application specialist at Bio-Rad, provided advice on real-time PCR. Margot Grinwis carried out the ComD alignments. Dr. Surette provided helpful advice and contributed to the design and conception of all experiments.

CHAPTER ONE: INTRODUCTION

1.1 The *Streptococcus genus*

The streptococci are Gram-positive cocci occurring in pairs or chains and they are non-spore formulating and catalase-negative (Hardie & Whiley, 1997). Their nutritional requirements are varied and as facultative anaerobes, some species require carbon dioxide (typically 5%) for growth (Hardie & Whiley, 1997, 2006). The 80 or so currently described streptococcal species, include many known commensals and pathogens in vertebrates (Hardie & Whiley, 1997). Billroth first coined the term 'streptococcus' to describe the morphologies of chain forming cocci in animal wounds (Billroth, 1874). Since then, several advances have been made to more clearly define and understand the *Streptococcus* genus. Early attempts to differentiate the streptococci were based on phenotypic traits (fermentation and tolerance tests), hemolysis patterns on blood agar (α, β, γ) and the presence of surface antigens (Lancefield groupings, e.g. A, B, C, E, F, and G) (R. Facklam, 2002; Hardie & Whiley, 1997). These classification systems led to exceptions within species definitions and were thus revised with molecular tools including DNA-DNA hybridization and 16S rDNA comparative sequence analysis. Based on these tools, the streptococci have been classified into six phylogenetic groups: pyogenic, mitis, salivarius, bovis, anginosus/milleri and mutans (**Figure 1.1**). The later five groups are collectively referred to as the viridans group streptococci (VGS). The term "viridans" has been problematic as it is based on exclusion criteria. Sherman originally classified the VGS as streptococcal species that are not β-hemolytic, not tolerant to high pH or salt and do not grow at 10^{o}C (Sherman, 1937). Today, viridans continues to

describe a heterogeneous collection of streptococci that can act as both commensals and pathogens in humans.

The streptococci are frequent colonizers of the mouth and upper respiratory tract of healthy individuals and they have also been implicated in disease, often acting as opportunistic pathogens (Hardie & Whiley, 2006). The focus of my research is the *Streptococcus* Milleri group (SMG), also known as the Anginosus group. The SMG are recognized for their association with abscess formation and purulent infections (Hardie & Whiley, 2006).

1.2 The *Streptococcus* Milleri/Anginosus Group

Members of the Milleri/Anginosus group include *Streptococcus anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius*. The SMG are often considered commensals of the oral (Poole & Wilson, 1979; R. A. Whiley, Freemantle, Beighton, Radford, & Hardie, 1993), gastrointestinal (Poole & Wilson, 1979) and female urogenital microbiota (Ahmet, Warren, & Houang., 1995; Poole & Wilson, 1979) and are found in 15-60% of healthy individuals at these sites. However, these bacteria are most known clinically for being associated with brain and liver abscesses (R. A. Whiley, Fraser, Hardie, & Beighton, 1990) and they have been identified in 50% of pleural empyemas (Ahmed, Marrie, & Huang, 2006). A retrospective study carried out in the Calgary Health Region found the SMG to be associated with the highest incidence of pyogenic streptococcal infections, and more than all the other streptococci combined (Laupland, Ross, Church, & Gregson, 2006). The SMG have been implicated in a number of other

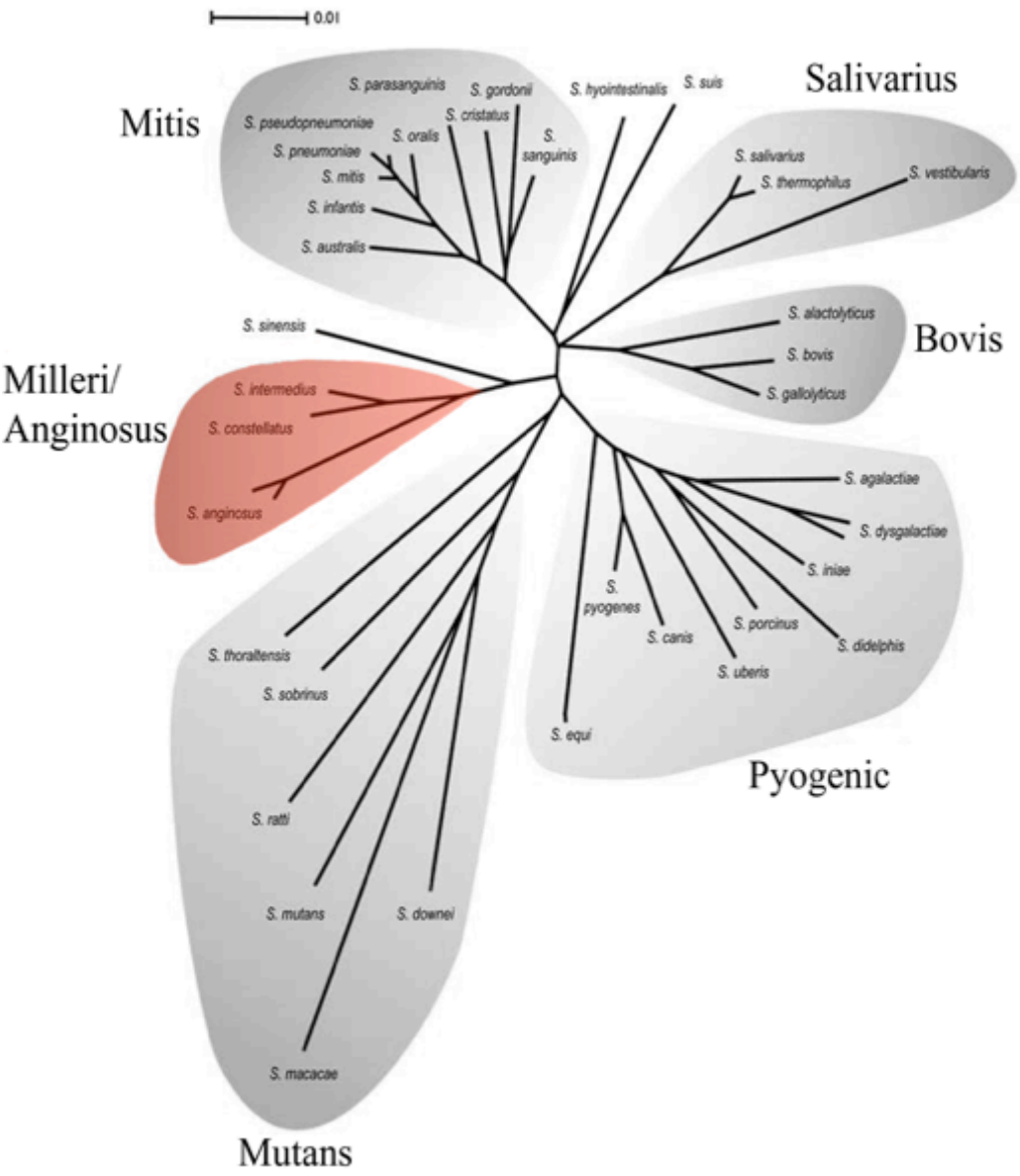

Figure 1.1 Phylogenetic tree representing the six streptococcal groups and representative species determined by 16S rDNA. (Figure modified from Kawamura, Hou, Sultana, H. Miura, & Ezaki, 1995).

infections, some of which include periodontal disease (Haffajee, Socransky, & Ebersole, 1985), endocarditis (Murray, Gross, Masur, & Roberts, 1978; Parker & Ball, 1976), bacteremia (Bert, Bariou-Lancelin, & Lambert-Zechovsky, 1998; Jacobs, Pietersen, Stobberingh, & Soeters, 1994; Salavert et al., 1996), sinusitis (Blayney, Frootko, & Mitchell, 1984), meningitis (Wu & Tsung, 1983), appendicitis (Poole & Wilson, 1977), pharyngitis (R. A. Whiley, Hall, Hardie, & Beighton., 1999), obstetric and neonatal infections (Evaldson, Carlstrom, Lagrelius, Malmborg, & Nord, 1980; Haffar, Fuselier, & Baker, 1983) as well as skin and soft tissue infections (Murray et al., 1978). These infections are often second to abdominal or oral trauma that likely disrupt normal SMG habitats, allowing these microorganisms to travel in the blood to sterile body sites (Gossling, 1988).

Another feature of SMG infections is that they are usually polymicrobial. The SMG are often isolated from mixed culture with other anaerobes and Gram-negative pathogens (Piscitelli, Shwed, Schreckenberger, & Danziger, 1992). Our research has shown that the SMG can be isolated from polymicrobial infections in the airways of adult cystic fibrosis (CF) patients at the onset of pulmonary exacerbations (Parkins, Sibley, Surette, & Rabin, 2008; Sibley et al., 2008; Sibley, Parkins, Rabin, & Surette, 2009). Although the SMG have been associated with pneumonia (Ahmed et al., 2006; Kobashi, Mouri, Yagi, Obase, & Oka, 2008; Shinzato & Saito, 1995; Wong, Donald, & Macfarlane, 1995), they are underappreciated as airway pathogens and have only recently gained recognition in CF.

1.2.1 Characterization of the *Streptococcus* Milleri/Anginosus group

The SMG consists of three closely related species that are phenotypically and serologically diverse (Piscitelli et al., 1992). The SMG vary in Lancefield grouping (A,C, G, F, non-groupable), hemolysis on blood agar (α, β, γ) , sugar fermentation and production of hydrolytic enzymes (chondroitin sulfatase, hyaluronidase, DNAse and proteases) (Grinwis et al., 2010b; Jacobs & Stobberingh, 1995; Piscitelli et al., 1992). This diversity has been a source of misidentification of the SMG in the clinic, with these organisms often being overlooked or underestimated in infections (Piscitelli et al., 1992). Although the SMG are phenotypically diverse, some of their common characteristics include smaller colony sizes (> 0.5 mm in diameter) than other streptococci, a slower growth rate, a characteristic caramel odor due to diacetyl production (Chew & Smith, 1992), the ability to hydrolyze arginine, acetoin production from glucose and an inability to ferment sorbitol (Gossling, 1988).

Approximately half of SMG clinical isolates are non-groupable or Lancefield F (Grinwis et al., 2010b) and only one quarter are β-hemolytic (Gómez-Garcés, Alós, & Cogollos, 1994). Of the three SMG species, *S. anginosus* is the most diverse as isolates are α- or non-hemolytic, are Lancefield types A, C, F, G or non-groupable and possess a wide array of sugar fermentation patterns (Piscitelli et al., 1992; R. A. Whiley et al., 1990). It has been proposed that *S. anginosus* be further divided into subspecies but this has yet to be formally established (Jacobs, Schot, & Schouls, 2000b; R. A. Whiley et al., 1999). *S. intermedius* is more homogeneous as most isolates are non-hemolytic on sheep or horse blood agar, are Lancefield non-groupable, produce β-galactosidase and ferment

trehalose and lactose (R. A. Whiley & Beighton, 1991). *S. constellatus* can be divided into *S. constellatus* subspecies *pharyngis* and *S. constellatus* subspecies *constellatus*. *S. constellatus* subspecies *pharyngis* are β-hemolytic, Lancefield group C, produce β-galactosidase and ferment lactose. *S. constellatus* subspecies *constellatus* are β-hemolytic Lancefield group F or non-hemolytic and non-groupable (R. A. Whiley et al., 1999).

Despite the variable characteristics of the SMG, identification methods have been devised to distinguish these organisms from other streptococci. Currently, the SMG are cultured on Columbia blood agar and identified with a positive result for the Vogues-Proskauer test (arginine hydrolysis, esculin hydrolysis and production of acetoin from glucose), a negative result for sorbitol fermentation, a reaction with the Lancefield group F antigen and glycosidase tests (R. Facklam, 2002; Ruoff & Ferraro, 1986; R. A. Whiley & Beighton, 1991; R. A. Whiley et al., 1990). However, these identification methods have often excluded many SMG isolates and routine culturing on Columbia blood agar does not always support SMG growth (Sibley et al., 2010; Sibley et al., 2008). Rapid commercial kits that contain a variety of biochemical tests such as API 20 Strep and API Rapid ID 32 (bioMérieux, Marcy l'Etoile, France) are useful for group level identification but have been known to overlook the SMG (Belko, Goldmann, Macone, & Zaidi, 2002; Limia, Alarcón, Jiménez, & López-Brea, 2000). Although the SMG have traditionally been characterized with biochemical tests, advances in molecular technology have led to genotyping as the gold standard for microbial identification. Recently, Grinwis et al. demonstrated that the SMG can be classified to the species and sub-species level based on molecular tools such as 16S rDNA sequence comparisons in addition to phenotypic traits

including Lancefield grouping and hydrolytic enzyme production (hyaluronidase and chondroitin sulfatase) (Grinwis et al., 2010b). A multiplex real-time PCR assay was also developed to distinguish SMG species in diagnostic samples using targets for the *cpn60* and *16S* rRNA genes, and this was validated with our CF clinical specimens (Olson et al., 2010).

1.2.2 Confusion surrounding nomenclature

Due to the heterogeneity within the SMG, it should come as no surprise that these organisms have been difficult to characterize with β- , α- and γ- hemolytic strains possessing one of four different Lancefield group antigens or no group antigens. Some of the various terms used for these organisms include the "anaerobic streptococci" (Yoshikawa, Chow, & Guze, 1975), "minute colony streptococci" (Long & Bliss, 1934; Poole & Wilson, 1976), "indifferent and haemolytic streptococci of Lancefield group F" (Ottens & Winkler, 1962) and "the non-hemolytic *Streptococcus* MG" (Mirick, Lewis, Curnen, & Horsfall, 1944). *Streptococcus anginosus* is the oldest name and was used in 1906 to describe microorganisms associated with pharyngitis and the normal alimentary canal (Andrewes & Horder, 1906). The terms "constellatus" and "intermedius" appeared later to describe anaerobic viridans streptococci (Gossling, 1988). It was not until 1956 that Guthof coined the term "*Streptococcus milleri*" to describe oral non-haemolytic streptococci (Guthof, 1956). As this terminology was limiting, Colman and Williams broadened Guthof's *S. milleri* to include minute β-haemolytic streptococci as well as other non-haemolytic streptococci based on phenotypic traits, cell wall composition and

DNA transformation (Colman, 1969; Colman & Williams, 1965; Colman & Williams, 1972). Facklam suggested that the *S. milleri* be further divided into two species based on lactose fermentation, where *Streptococcus* MG-*intermedius* is lactose positive and *Streptococcus anginosus-constellatus* is lactose negative (R. R. Facklam, 1977). Facklam later revised this division to include β-hemolytic strains. Consequently, the Milleri group was divided into the three species currently on the approved list of bacterial names: *Streptococcus constellatus*, *Streptococcus intermedius* and *Streptococcus anginosus* (R. R. Facklam, 1984).

Colman and Williams' nomenclature used mainly by Europeans, characterizes the SMG as one species under the name *S. milleri* whereas Facklam's division used mostly in the United States, divides the SMG into three species under the hierarchical name *Streptococcus* Milleri group (Gossling, 1988). Initially, Facklam's division was met with opposition based on phenotypic heterogeneity and DNA hybridization studies that showed high levels of homology between the three SMG species. Consequently, Coykendall proposed that this group of organisms be unified under the name Anginosus as Milleri was not on the approved list of bacterial names (Coykendall, Wesbecher, & Gustafson, 1987a, 1987b). Eventually, further DNA hybridization studies and phenotypic tests on Anginosus strains proved that there are in fact three distinct species and this in general is not disputed today (R. A. Whiley & Hardie, 1989). Although *S. anginosus* remains the oldest approved name, *Streptococcus milleri* continues to be used in the clinic as it carries significance to clinicians dealing with pyogenic infections (Piscitelli et al., 1992).

1.2.3 Clinical relevance in purulent and respiratory infections

The SMG are associated with a number of infections across the human body with studies commonly reporting their involvement in pyogenic infections (Belko et al., 2002; Siegman-Igra, Azmon, & Schwartz, 2012). In one recent study consisting of 245 patient-unique SMG isolates, the SMG were most often isolated from pus infections representing 35% of cases (Siegman-Igra et al., 2012). The SMG have been isolated from purulent infections ranging from dental to internal organ abscesses, being most known for their association with pus formation in the brain and liver (Mishra & Fournier, 2013; R. A Whiley, Beighton, Winstanley, Fraser, & Hardie, 1992; R. A. Whiley et al., 1990). When isolated from the blood, clinicians are warned of abscess formation as the likely cause of bacteremia (Murray et al., 1978; Ruoff, 1988; Siegman-Igra et al., 2012).

The pathogenic potential of the SMG is underappreciated in airway infections. When respiratory cultures are analyzed for pathogenic streptococci, often only β-hemolytic isolates are identified as relevant (Ruoff, 1988). As the SMG are phenotypically diverse with isolates being both β- and non β-hemolytic, they are often overlooked in the clinic. Furthermore, routine clinical analysis carried out on sheep blood could overlook SMG strains that are β-hemolytic only on human blood (Jacobs, Schot, & Schouls, 2000a; Nagamune et al., 1996). All *S. intermedius* strains tested to date produce intermedilysin, which specifically targets human red blood cells (Grinwis et al., 2010b; Nagamune et al., 2000). Intermedilysin forms pores in human erythrocytes by binding to CD59, a glycosyl-phosphatidylinositol membrane protein involved in inhibiting the formation of the complement membrane attack complex on host cells (Giddings, Zhao, Sims, & Tweten,

2004). Up to now, intermedilysin has been the only virulence factor linked to pathogenesis in human cells (Nagamune et al., 2006; Sukeno et al., 2005). As *S. intermedius* isolates are only β-hemolytic depending on the medium used, their pathogenic potential could be underestimated in infections.

There is now increasing evidence that the SMG are involved in respiratory infections including community acquired pneumonia and pulmonary abscesses (Ahmed et al., 2006; Kobashi et al., 2008; Shinzato & Saito, 1995; Wong et al., 1995). It has been reported that the SMG account for up to one-fifth of thoracic infections with pleural empyemas being the most common (Porta et al., 1998). In developed countries, including Canada, the SMG account for 30-50% of pleural empyema cases (Ahmed et al., 2006; Kobashi et al., 2008; Ripley et al., 2006; Spier, 2008). Some reports suggest that patients with SMG associated empyema are more likely to have comorbidities but this is not a requirement (Brims, Lansley, Waterer, & Lee, 2010; Jerng et al., 1997; Kobashi et al., 2008). In the majority of cases, SMG associated empyemas require surgery (Porta et al., 1998; Ripley et al., 2006). A Colorado study reported that patients that did not undergo surgery had a mortality rate of 31% (Ripley et al., 2006). On average patients with SMG associated empyemas remain in the hospital for 34 days and 24 days when surgery is required (Ripley et al., 2006). Despite the prevalence of the SMG in pleural empyemas, their involvement is often overlooked as they are not usually associated with lower airway infections and are considered commensals of the upper respiratory tract (Brims et al., 2010).

Recently, the SMG have been implicated in cystic fibrosis respiratory infections. Our research has demonstrated that the SMG are a common cause of pulmonary exacerbation in these patients (Parkins et al., 2008; Sibley et al., 2010; Sibley et al., 2008). Only one study previous to this showed the involvement of the SMG in pulmonary exacerbations in a pediatric patient with CF (Cade, Denton, Brownlee, Todd, & Conway, 1999). In CF patients, a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene results in defective epithelial chloride secretion and sodium hyperabsorption across the apical membranes of mammalian cells. Consequently, this reduces the amount of fluid covering the surface of epithelial cells, preventing cilia from clearing microorganisms trapped within mucous layers in the airways (Lyczak, Cannon, & Pier, 2002; Smith, Travis, Greenberg, & Welsh, 1996). Defective mucociliary clearance and an impaired innate immunity lead to constant microbial colonization and ultimately, chronic pulmonary infections. These are characterized by periods of stability (despite high bacterial loads) that are often interrupted by acute exacerbations, which are overt immunological responses resulting in irreversible lung damage accounting for up to 90% of deaths (Lyczak et al., 2002; Sibley, Rabin, & Surette, 2006).

Until recently, only a few bacterial species were believed to be involved in CF airway disease with *Pseudomonas aeruginosa* being the most common, colonizing the airways of 80% of patients by early adulthood (Rajan & Saiman, 2002). Over the past several years, culture dependent and independent methods have shown that airway infections in CF are polymicrobial and that pathogens associated with acute exacerbations are often missed by conventional clinical microbiology methods (Sibley et al., 2008;

Sibley et al., 2009). Traditional techniques have relied upon sputum analysis, biochemical confirmation and antibiotic susceptibility testing (Sibley et al., 2006). These approaches have been problematic because some organisms do not grow on standard media, anaerobic microbes often remain unidentified, and overgrowth by predominate organisms such as *Pseudomonas aeruginosa* limits the ability to detect other bacteria. In addition, many organisms are considered part of the commensal microbiota and if detected, are often dismissed as clinically irrelevant. Due to these limitations, the involvement of SMG group organisms has remained largely unrecognized (Parkins et al., 2008; Sibley et al., 2010).

Research in the Surette laboratory has shown that members of the SMG were linked to approximately 40% of hospital admissions during a six month study (Parkins et al., 2008; Sibley et al., 2010). In SMG infected patients, exacerbations correlate with high levels of the bacterium and resolution with a decrease in the SMG with no observable changes in the levels of the predominant pathogen, *Pseudomonas* (Sibley et al., 2008). Surprisingly Columbia Blood agar, the standard culture medium for CF sputum, was unable to detect the SMG as the numerically dominant pathogen (Sibley et al., 2010). Culture on non-selective media (tryptic soy agar and brain heart infusion agar) was used to detect the SMG (Parkins et al., 2008; Sibley et al., 2008). The development of a semi-selective medium, McKay agar, has allowed for isolation of the SMG from complex clinical samples and has helped in the determination of the course of antibiotic therapy (Sibley et al., 2010). When treated with targeted antibiotics, SMG burden declined, there

was a decrease in the frequency of exacerbations and lung function improved (Sibley et al., 2009).

1.2.4 Resistance to antimicrobial agents

The SMG continues to be an important agent in purulent and airway infections often requiring the prolonged use of antibiotics for treatment. Consequently, the emergence and spread of antibiotic resistant strains could become a concern. The SMG are generally considered susceptible to the majority of antimicrobial agents (Piscitelli et al., 1992; Ruoff, 1988). However, some studies suggest the SMG are intermediately resistant to clindamycin, fluroquinolones and beta-lactams (Asmah, Eberspacher, Regnath, & Arvand, 2009; Tracy et al., 2001; Yamamoto et al., 2002). Antimicrobial resistance is likely underestimated in the SMG as variable methods have been used to determine susceptibility (Gossling, 1988) and Muller-Hinton blood agar (MHBA) does not adequately support SMG growth (Sibley et al., 2010). A study of SMG infections in patients 19 years or younger reported that only 50% of isolates grew on standard MHBA (Belko et al., 2002). In keeping with other studies, the Surette laboratory found that ~60% of tested CF and invasive isolates failed to grow on MHBA (Grinwis et al., 2010a) and this study also demonstrated that Brain Heart infusion (BHI) agar was equivalent to MHBA for strains which grew for the later but had the advantage that all strains grew on BHI.

Antimicrobial susceptibility tests carried out on BHI showed that isolates from CF patients are more resistant to clindamycin and macrolides than invasive isolates from

purulent infections. The higher resistance in CF isolates was associated with the chronic use of antibiotics. The majority of isolates from CF patients on chronic azithromycin therapy were macrolide resistant (84%). In comparison, resistance was reported in only 24% of isolates from CF patients treated for acute infections (Grinwis et al., 2010a). Macrolides such as azithromycin function by inhibiting protein synthesis by interacting with residues in the 23S rRNA of the 50S ribosomal subunit (Gaynor & Mankin, 2003). Mechanisms of macrolide resistance in the streptococci include post-transcriptional methylation of the 23S rRNA target site, active efflux, mutation of the ribosome and macrolide inactivation (Leclercq & Courvalin, 1991; Lynch & Zhanel, 2009). Interestingly, 66% of the Surette laboratory SMG strains that are resistant to macrolides have point mutations in their 23S rRNA gene. This was observed in all three SMG species and this was especially enriched in *S. constellatus* isolates (M. Grinwis & M. G. Surette, unpublished data). This finding was surprising as ribosomal mutations are a rare mechanism of resistance for the streptococci with active efflux and methylation being more common (Lynch & Zhanel, 2009).

The erythromycin ribosome methylation (*ermB* or *ermTR*) and the macrolide efflux pump A or E (*mefA* or *E*) alleles account for > 97% of pneumococcal macrolide resistance worldwide (Lynch & Zhanel, 2009). In the Surette laboratory strain collection, CF and invasive SMG strains that did not possess the 23S rRNA mutation carry the *mefA* or *E* (3%), *ermB* (23%) or *ermTR* (8%) macrolide resistance determinants (M. Grinwis & M. G. Surette, unpublished data). These determinants are likely horizontally transferred between the streptococci as they are carried on conjugative transposons (Zolezzi et al.,

2004). In one of our sequenced SMG (*S. anginosus* strain C238), *ermTR* is located near a putative conjugal transfer protein, which suggests that this determinant could potentially be horizontally transferred on a conjugative transposon. The transfer of resistance genes in pneumococci has been well documented, but less is known in the SMG. The SMG are naturally competent, a state which promotes horizontal gene transfer through DNA uptake from the environment. In addition to mutations, horizontal gene transfer could contribute to the SMG's documented resistance to antibiotics.

1.3 Horizontal gene transfer and DNA uptake

Horizontal gene transfer facilitates bacterial evolution through the acquisition of DNA different from that already present in the recipient's bacterial genome (Dowson et al., 1997). The transfer of genetic material can occur via natural genetic transformation, transduction or conjugation. Natural transformation is less common but as it has been reported in more than 60 bacterial species, this number is likely being underestimated (O. Johnsborg & Håvarstein, 2009). Chromosomal determinants acquired through natural transformation are usually transferred between closely related bacterial species as integration relies on homologous recombination. For recombination to occur, the maximum divergence in the acquired sequence is usually 20-30% (Dowson et al., 1997). Integration results in mosaic genes composed of nucleotides from the donor and recipient. Small DNA fragments may recombine within a gene or the whole gene could be replaced (Dowson et al., 1997). Often these mosaic genes encode a protein with a different

function than the donor, promoting diversity (Dowson et al., 1997; Johnston, Campo, Berge, Polard, & Clarveys, 2014).

The ability to be transformed normally occurs during a short time period termed competence, during which proteins for transformation are produced. In several transformable bacteria, DNA uptake relies on orthologs of the type II secretion and type IV pilus systems (Johnston et al., 2014). In contrast to *Haemophilus influenzae* and *Neisseria* species, DNA uptake in the streptococci is not dependent on a specific uptake sequence (Chen & Dubnau, 2004). In the streptococci, the pilin-like proteins for DNA uptake are encoded by the *comG* operon with ComGC being the major pilin (**Figure 1.2**). The pneumococcal pilus is 2-3 μm in length and extends outside of the cell envelope to provide access for incoming DNA to the membrane bound receptor, ComEA. ComEA binding is followed by cleavage of the double stranded DNA by the EndA nuclease and transportation of the single DNA strand (ssDNA) through the ComEC pore. Once internalized the ssDNA is coated with the single strand binding protein (SsbB) for protection against nuclease degradation. The DprA recombinase loader also binds to the ssDNA, loading RecA for homology searches with the recipient's bacterial genome (Johnston et al., 2014). DNA uptake through competence induction has been an important driving force in genetic diversity within the streptococci, with *Streptococcus pneumoniae* serving as a model organism. Classic examples of inter- and intra-species recombination include the acquisition of penicillin resistance genes and capsular switching (O. Johnsborg & Håvarstein, 2009). Increased resistance to β-lactams has resulted

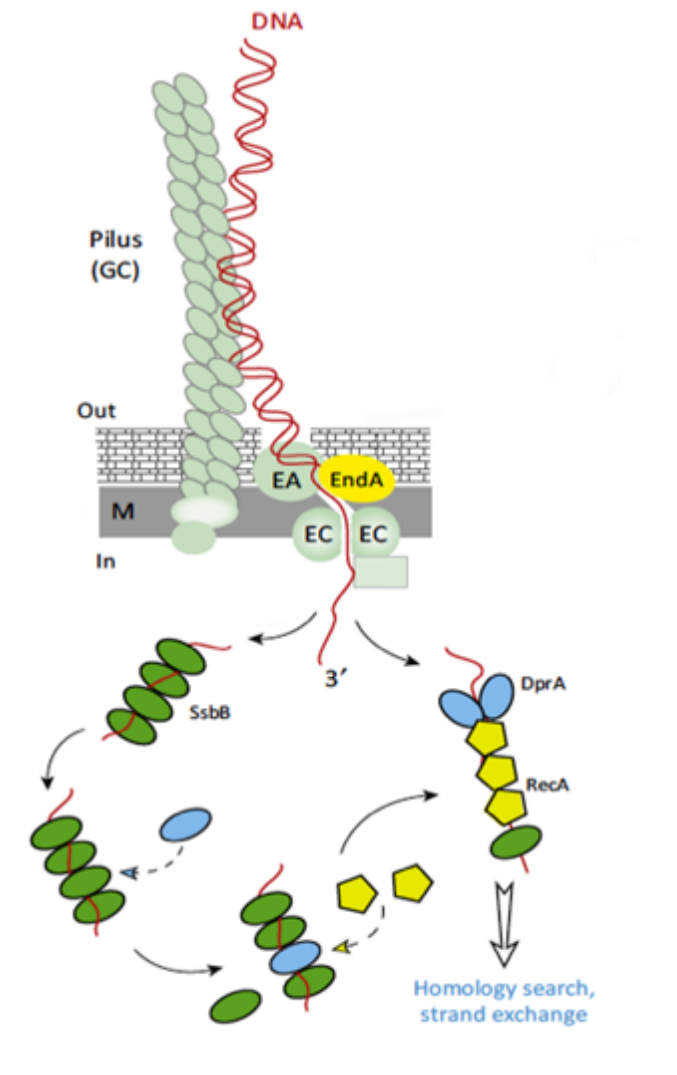

Figure 1.2 Uptake of DNA during pneumococcal transformation. The pneumococcal pilus (ComGC) extends outside of the cell envelope to provide access for incoming DNA to the ComEA receptor. ComEA binding is followed by cleavage of the double stranded DNA by the EndA nuclease and transportation of the single DNA strand (ssDNA) through the ComEC pore. Once internalized the ssDNA is coated with the single strand binding protein (SsbB). The DprA recombinase loader binds to the ssDNA, loading RecA for homology searches with the recipient's bacterial genome. Green lines, host chromosome; red lines, transforming ssDNA; M, membrane; EA, ComEA; EC, ComEC; Green ovals, ssbB; blue ovals, DprA; yellow pentagons, RecA. (Image taken with permission from Johnston et al., 2014)

from their overuse leading to clones with mutated penicillin binding proteins that are transferred between the streptococci (Coffey et al., 1991; Dowson et al., 1989; O. Johnsborg & Håvarstein, 2009). Interestingly, antibiotics themselves have been known to activate competence genes, promoting survival and adaptation to stress (Prudhomme, Attaiech, Sanchez, Martin, & Claverys, 2006). Virulence genes such as those involved in capsule formation are also transferred between the streptococci, making the development of vaccines more challenging (Gherardi et al., 2009; Porat et al., 2004). Horizontal transfer mediated by competence induction has thus been an important mechanism in the evolution of the streptococci.

1.4 Discovery of the competence system

Competence for natural genetic transformation was first discovered in *Streptococcus pneumoniae* (A. Tomasz & Hotchkiss, 1964; A. Tomasz & Mossler, 1966). As early as the 1920s, Griffith observed natural transformation by studying the acquisition of a capsule by un-encapsulated *Streptococcus pneumoniae* strains (Griffith, 1928). Forty years later, vigorously growing streptococci were found to become competent with a proteinaceous compound termed "competence factor" (R. Pakula & Walczak, 1963; A. Tomasz, 1965; A. Tomasz & Hotchkiss, 1964). Purification and sequencing of this signaling molecule revealed it to be a 17 amino acid peptide (L. S. Håvarstein, Coomaraswamy, & Morrison, 1995) . The active naturally cleaved ComC peptide is named competence-stimulating peptide (CSP). The CSP can be made

synthetically and continues to be a staple for the study of pneumococcal competence as it makes transformations easy and reproducible (J.-P. Clarveys & Håvarstein, 2002).

Typically, competence occurs during the early log phase and lasts for a short time period which for *Streptococcus pneumoniae,* is approximately 30 minutes (O. Johnsborg & Håvarstein, 2009). The conditions that determine when a bacterium becomes competent are still poorly understood. In the laboratory, different growth conditions have been shown to affect natural transformation (Desai, Mashburn-Warren, Federle, & Morrison, 2012; Gromkova, Mottalini, & Dove, 1998; Meibom, Blokesch, Dolganov, Wu, & Schoolnik, 2005; Sexton & Vogel, 2004; Spizizen, 1958). Although many streptococci possess the machinery necessary for competence development, the ability to be transformed varies among the six major streptococcal phylogenetic groups. Organisms within all of the streptococcal groups have been shown to be naturally competent except for members of the Pyogenic group (Mashburn-Warren, Morrison, & Federle, 2012) Desai et al., 2012; Fontaine et al., 2010; L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997; Martin et al., 2006; Morrison, Guédon, & Renault, 2013).

1.5 Competence regulation

In many streptococcal species including the SMG, competence is regulated by *comCDE*. The *comCDE* operon encodes the competence-stimulating peptide (CSP) precursor (ComC), a histidine kinase (ComD) and its cognate response regulator (ComE) (J.-P. Clarveys & Håvarstein, 2002; L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997). The ABC transporter, ComAB, cleaves the ComC precursor after a conserved

double glycine motif and secretes the mature peptide (**Figure 1.3**). Once the CSP attains a threshold level in the environment, it binds to ComD, which becomes autophosphorylated. The phosphoryl group is transferred to ComE, which activates the transcription of early competence genes including *comCDE* and *comAB* (Ween, Gaustad, & Håvarstein, 1999). Activation of *comCDE* and *comAB* promotes autoinduction through increased production and secretion of the mature CSP (J.-P. Clarveys & Håvarstein, 2002; Ween et al., 1999). Another early gene activated by phosphorylated ComE includes *comX* which encodes the alternative sigma factor (M. S. Lee & Morrison, 1999). *comX* is involved in the transcription of the late competence genes some of which are involved in natural transformation. Interestingly, of the >100 genes induced by the activation of *comCDE*, only approximately 22 are required for natural transformation and a few others for competence mediated cell lysis termed fratricide (O. Johnsborg & Håvarstein, 2009; S. N. Peterson et al., 2004). Fratricide is a process whereby a competent cell will release muralytic enzymes to lyse related non-competent cells to promote DNA release and gene exchange (Berg, Biornstad, Johnsborg, & Håvarstein, 2012). Other than fratricide and natural transformation, the role of several other CSP-regulated genes remains largely unknown (O. Johnsborg & Håvarstein, 2009).

1.6 Competence in the *Streptococcus* Milleri/Anginosus group

Håvarstein et al. reported that competence is as ubiquitous in the SMG as it is in the Mitis group which includes *S. pneumoniae* (L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997). A genomics study of seven SMG genomes from the Surette laboratory

identified orthologs for 21 out of the 22 genes involved in natural transformation in *S. pneumoniae* (Olson et al., 2013). Thus far, one study has characterized two CSPs in the SMG and these were not found to cross-react (L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997). As peptides are specific for their receptors (ComD), a CSP from one group may or may not induce competence in a strain from a different peptide group (L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997). Variability in the CSPs and their receptors could facilitate or be a barrier to intra and inter-species genetic exchanges, which could directly impact how the SMG evolve during colonization and infections.

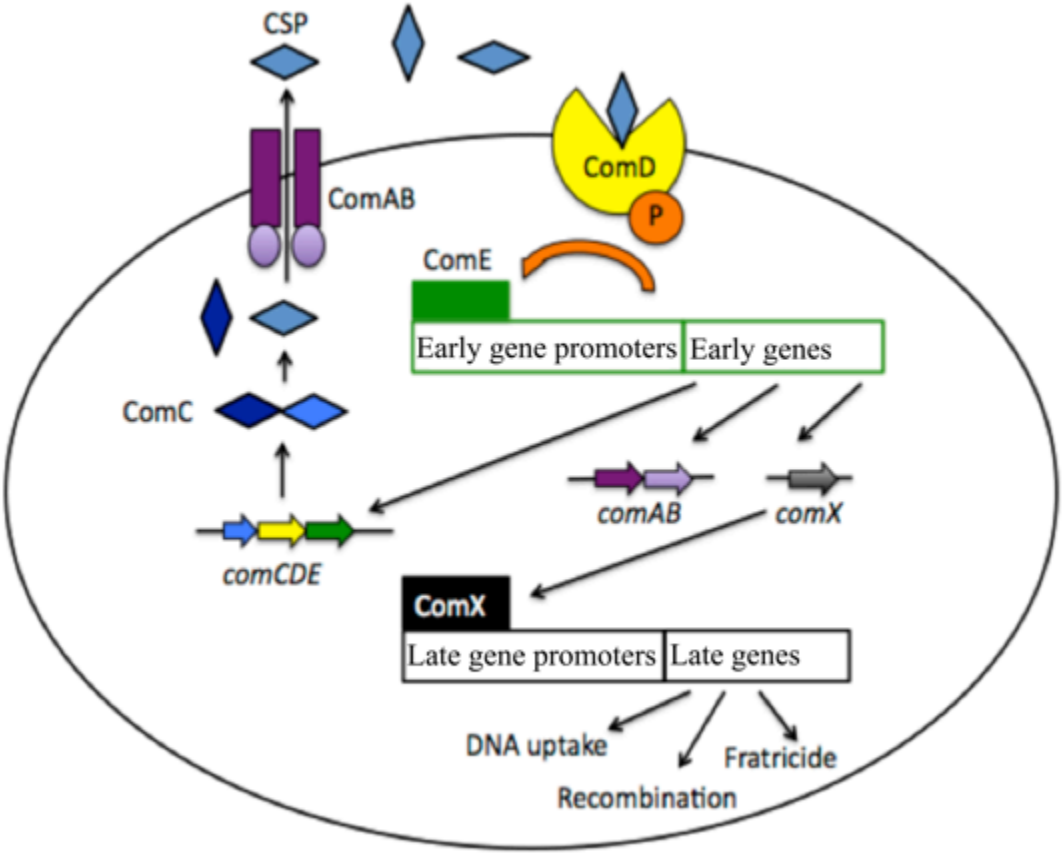

Figure 1.3 Schematic representing competence regulation by the *comCDE* system. The competence stimulating peptide (CSP) precursor is encoded by *comC*, which is cleaved after a double glycine by the ComAB transporter, which exports the CSP to the extracellular medium. Extracellular accumulation of the mature CSP causes it to bind to the histidine kinase ComD, which becomes autophosphorylated. A phosphoryl group is transferred to the ComE response regulator. ComE binds to the early gene promoters, upregulating the transcription of early competence genes one of which includes *comX*. In turn, *comX* upregulates the transcription of the late competence genes involved in DNA uptake, recombination and fratricide. (Image adapted from Johnsborg & Håvarstein, 2009, and Suntharalingam & Cvitkovitch, 2005).

1.7 Objectives and hypothesis

Members of the *Streptococcus* genus are among some of the most commonly found bacteria across the human body (The Human Microbiome Project Consortium, 2012). Several streptococcal species are part of the normal microbiota, while others are known pathogens often colonizing hosts asymptomatically (Beck, V.B. Young VB, & Huffnagle, 2012; Cho & Blaser, 2012; Dewhirst et al., 2010). There is substantial evidence that the streptococci exchange genetic information as many species are naturally competent. This exchange has been linked to the transfer of antibiotic resistance determinants and virulence factors (O. Johnsborg & Håvarstein, 2009). Research thus far has focused on competence in the Mitis and Mutans groups, with only one study to date published on the SMG (L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997).

Given the importance of competence in other streptococci and the lack of knowledge on its role in SMG associated infections, we sought to study the *comCDE* system in our cystic fibrosis and invasive SMG clinical isolates. This collection includes 125 pulmonary SMG strains from the airways of adult cystic fibrosis patients and 45 invasive strains isolated from liver, hip, brain and lung abscesses, blood, empyema and unknown sites (Grinwis et al., 2010b). With this strain collection, we were interested in studying variability in the ComD histidine kinase and C-terminus of ComC, which encodes the CSP. The N-terminus of ComD and C-terminus of ComC are hypervariable and these regions are important for determining receptor-ligand interactions (L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997). It is hypothesized that strain-to-strain

variability in the SMG CSP alleles and ComD will facilitate or interfere with horizontal gene transfer. In order to evaluate this hypothesis, three objectives were undertaken:

1. Evaluate strain-to-strain variability in the CSPs of the SMG and other streptococci through sequence analysis. Sequencing of *comC* has revealed a large diversity of CSPs among naturally competent streptococci, with peptides differing even within species (O. Johnsborg & Håvarstein, 2009). Previously two CSP sequences had been described for the SMG (L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997) but only a small number of strains were examined. Variability was investigated in CSPs from 170 clinical SMG isolates and sequences were compared to CSPs from other streptococci.

2. Investigate CSP specificity. Competence peptides are often specific to the strain producing them and can induce competence in only strains that respond to and produce this same CSP (L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997). This specificity is due to the complementarity between the CSP and its ComD receptor, which are thought to co-evolve (O. Johnsborg & Håvarstein, 2009). Despite this specificity, there are examples of streptococcal strains responding to non-cognate CSPs (O. Johnsborg, Eldholm, Bjørnstad, & Håvarstein, 2008). The two peptides characterized in the SMG were not found to cross-react but only four strains were tested in this study (L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997). We therefore sought to investigate how specific the CSP peptides were for their ComD receptors in our collection of clinical isolates since this could impact intra- and inter-species genetic exchanges.

3. Determine whether *S. constellatus* subspecies *pharyngis* isolates have a functional *comCDE* pathway despite being non-transformable. In all of our *Streptococcus constellatus* subspecies *pharyngis* strains, a three amino acid deletion was identified in the N-terminus of ComD, the region involved in binding to the CSP. Despite having an intact *comCDE*, the *pharyngis* strains were non-transformable with their cognate CSP. The identified deletion was further explored to determine its impact on competence and the acquisition of antibiotic resistance determinants.

Variability in the competence pathway of the SMG could facilitate the horizontal gene transfer of antibiotic resistance genes through intra- and inter-species genetic exchanges. This has important implications in the treatment of SMG infections, particularly in cystic fibrosis where management of chronic infections requires long-term antibiotic use.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Bacterial strains, growth conditions and plasmids

A collection of 173 *Streptococcus* Milleri group strains described previously (Grinwis et al., 2010b) was used in this study. This includes 125 respiratory isolates, 45 isolates from patients with invasive infections (blood, empyema, unknown sources, brain abscess, liver abscess and hip abscess) (**Table 2.1**) and reference strains *S. intermedius* ATCC 27335, *S. constellatus* ATCC 27823, and *S. anginosus* ATCC 33397. All SMG bacterial strains were grown in Brain Heart Infusion medium (BHI, Bacto) or Todd-Hewitt broth (Bacto) supplemented with 0.5% yeast extract (THY, Bacto) and incubated at 37^{o}C in 5% CO_2. Bacterial stocks were maintained at -80^{o}C in 10% skim milk. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37^{o}C and stocks were maintained in 20% glycerol at -80^{o}C. The plasmids, primers and some of the strains used in this study are outlined in **Tables 2.2** and **2.3**. Antibiotic concentrations for selection were as follows: spectinomycin (Spc), 75 μg/ml; tetracycline (Tet), 1-10 μg/ml and erythromycin, 150 μg/ml for the SMG; ampicillin (Ap), 100 μg/ml; erythromycin (Ery), 150 μg/ml and spectinomycin (Spc), 100 μg/ml for *E. coli*.

2.2 The *comCDE* operon

2.2.1 Isolation of genomic DNA

An enhanced enzymatic lysis protocol was carried out to obtain higher quality genomic DNA for amplification of *comCDE*. The SMG were recovered from frozen

stocks on BHI containing 0.01 mg/ml colistin sulfate salt and 0.005 mg/ml oxolinic acid (Sigma-Aldrich; St. Louis, MO). Overnight THY cultures (5 ml) were grown at 37°C with 5% CO_2 and harvested by centrifugation at 2000 rpm for 20 minutes at 4°C (Sorvall® Intruments). The pellets were re-suspended in 500 µl of $T_{10}E_{25}$ (Tris-HCl 10 mM pH 8.0, EDTA 25 mM pH 8.0) and lysis was carried out with 50 µl lysozyme (Sigma-Aldrich, 100 mg/ml), 10 µl mutanolysin (Sigma-Aldrich, 10 U/ µl) and 10 µl RNase A (Qiagen, 10 mg/ml) for 1 hour at 37°C. Once lysis was complete, 25 µl of 25% SDS, 25 µl of Proteinase K (Invitrogen, 20 mg/ml) and 62.5 µl of 5M NaCl were added and microcentrifuge tubes were incubated at 65°C for 30 minutes. For the organic extraction, an equal volume of 25:24:1 (phenol:chloroform:iso-amyl alcohol) was added to each microcentrifuge tube, which were spun for 10 minutes at maximum speed (Eppendorf). The aqueous phase was transferred to 25 µg capped Zymo columns and DNA purification was performed according to the manufacturer's instructions (Zymo Research Corporation, Irvine, CA). DNA concentrations were measured with a Nanodrop Spectrophotometer ND-1000 and standardized to 25 ng/µl with ultra-pure distilled water (Life Technologies).

2.2.2 PCR amplification of the *comCDE* operon

The *comCDE* operon is located upstream of an Arg-tRNA and downstream of a Glu-tRNA gene. Primers based on these conserved tRNAs, were used to amplify *comCDE* in the SMG. Recombinant Taq (Invitrogen) and primers at a final concentration of 10 pmol/µl were used in a standard PCR reaction. PCR fragments of the expected size (2.6 kb) were obtained after 30 cycles at an annealing temperature of 67°C. PCR products

were sequenced with tArg2 in the forward direction by Macrogen (Korea). Nucleotide sequences for *comCD* were examined with the Scanner v1.0 program (Applied Biosystems). Sequences were translated to protein using ExPASY translate (http://web.expasy.org/translate/) to identify the predicted CSP and N-terminal regulon of ComD.

2.3 Phylogenetic analysis of competence peptides

An NCBI search (http://www.ncbi.nlm.nih.gov/) for ComC in "All Databases" was carried out. Protein hits for ComC were limited to ≤ 60 amino acids and *Bacillus* peptides were filtered out. Unpublished ComC sequences were also collected from (Kilian et al., 2008) (**Table 2.4**). A multiple sequence alignment of ComC was undertaken with Muscle version 3.8 (http://www.drive5.com/muscle/). The alignment was viewed with Jalview 2.8 (http://www.jalview.org/) and a neighbor joining tree was constructed with SplitsTree4 (Huson & Bryant, 2006). Bootstrap support of 1000 repetitions was performed.

2.4 Transformation assays

2.4.1 Preparation of synthetic competence peptides

Peptides were purchased from RS Synthesis (Louisville, KY) at a >80% purity grade. Stock solutions were diluted in ultra-pure distilled water (Life Technologies) at a concentration of 10 mg/ml. Peptide sequences were as follows:

CSPA: DSRIRMGFDFSKLFGK; CSPB: DRRDPRGMIGIGKKLFG, CSPC; DRRDPRGIIGIGKKLFG, CSPE: DRRDPRGMIGIGKNYLVRRFYHYESG.

2.4.2 Saturation curves for CSP and pDL278 plasmid

To determine the optimum amount of plasmid and CSP to use for the transformation assays, saturation curves were completed. Concentrations of CSP ranged from 0.5 ng/ml to 512 ng/ml. Concentrations of plasmid ranged from 0.001 μg/ml to 10 μg/ml. After incubating the plates at 37^{o}C in 5% CO_2 for 48 hours, transformation frequency was calculated as follows: Frequency = transformants (transformants/ml)/total bacteria (cfu/ml).

2.4.3 Transformations with synthetic CSP

Competence was investigated by determining the number of Spc resistant transformants after exposure to pDL278 (LeBlanc, Lee, & Abu-Al-Jaibat, 1992) plasmid DNA in the presence and absence of added exogenous competence-stimulating peptide (CSP). Unless otherwise stated, bacterial colonies were harvested from THY agar and grown in THY broth for 24 hours at 37^{o}C with 5% CO_2. Cultures were diluted 1,000-fold and incubated until they reached early logarithmic phase (approximately 2 hours) to avoid the induction of endogenous competence. In all cases, pDL278 (1 μg/ml) and synthetic CSP (20 ng/ml or 200 ng/ml) were added to cultures, which were incubated for an additional hour. Cultures were plated on THY agar with and without 75 μg/ml

spectinomycin (Sigma-Aldrich). Transformants and the total number of bacteria were counted after 48 hours.

2.5 Endogenous competence

2.5.1 Investigating dilutions and growth phase for strains with high endogenous competence levels

For the endogenous competence experiment, cells were either: 1) spun down at 24 hours and washed with fresh THY, 2) CSP and vector were added directly to cells in stationary phase without washing or 3) cells were diluted 1,000 to 10,000 fold and grown for 2 hours in a 5% CO_2 incubator at 37^{o}C before the addition of peptide and/or pDL278. (**Figure 2.1**). Transformants and the total number of bacteria were counted after 48 hours of incubation in 5% CO_2 at 37^{o}C.

2.5.2 Co-culture assay

B196, a strain that does not become competent under these standard growth conditions without added CSP, was incubated with strains that exhibit high endogenous competence levels (M431, C1392 and C1391). The standard transformation protocol was carried out with a 1,000-fold dilution, followed by a 2-hour incubation and growth for 1 hour with CSP and/or pDL278. Strains were grown separately for 2 hours and added together before the addition of CSP and/or vector or cultures were incubated together after the 1,000-fold dilution (**Figure 2.2**). Cultures were plated on THY agar and THY agar with selective antibiotics including 75 μg/ml spectinomycin (Sigma-Aldrich), 150

ug/ml erythromycin (Sigma-Aldrich) or 10 μg/ml tetracycline (Sigma-Aldrich). The antibiotic concentrations chosen were based on antimicrobial susceptibility tests carried out according to the Clinical and Laboratory Standard Institute (CLSI) guidelines (CLSI, 2007) or as described by Grinwis et al. (Grinwis et al., 2010a). After incubating the THY plates at 37^{o}C in 5% CO_2 for 48 hours, transformation frequencies were calculated.

2.5.3 Construction of Δ*comC* and Δ*comCDE* SMG strains

To delete *comCDE*, the flanking regions (± 1 kb) of the operon were amplified from *S. constellatus* strain C1392 with primer pairs C1392comCfw2Xba/ C1392protrv2Hind and HPrv1Eco/ C1392comEfw1Bam (**Table 2.3**). For *S. intermedius* strain B196, flanking regions of *comCDE* (± 1 kb) were amplified with B196comCfw1Xba/ B196 protrv1Hind and B196ComEfw1Bam/ HPrv1Eco. The spectinomycin resistance (Spc^{R}) gene was amplified from pDL278 with pDL278fwBam and pDL278rvXba. The three resulting PCR fragments for B196 and C1392, were digested with the appropriate restriction enzymes (*Xba*I, *Hind*III, *Eco*RI and *Bam*HI), purified with the PureLink Gel Extraction kit (Life Technologies) and ligated one at a time into the multiple cloning site of pUC19 (Life Techologies) using the Roche Rapid DNA ligation kit. The ligation product was transformed into Top10 *Escherichia coli* cells (Life technologies) and transformants were selected on LB agar plates containing 100 μg/ml of ampicillin and spectinomycin. The resulting pComCDE1392 and pComCDE196 vectors were used as templates for the amplification of the knockout constructs with comCDEdw/B196comCDEup and comCDEdw/C1392comCDEup. The PCR products

were purified with 25 μg capped Zymo columns according to the manufacturer's instructions (Zymo Research Corporation, Irvine, CA) and 1 μg/ml was transformed into C1392 and B196 cells treated with CSP (20 ng/ml). The *comCDE* knockout strain was obtained via a double crossover event resulting in the replacement of *comCDE* with spectinomycin. The insertions were verified by PCR and sequenced with C1392ComErvSeq and B196ComErvSeq.

To delete *comC*, a similar protocol to that used for the *comCDE* knockout was carried out. Flanking regions of *comC* were amplified from C1392 and B196 genomic DNA with PCR primer pairs listed in **Table 2.3**. Upstream *comC* pieces were the same as those for the *comCDE* knockout. Downstream flanking *comC* pieces were amplified with C1392comCrv2Bam/C1392comDfw4Eco and B196comDfw1Eco/B196comCrv1Bam. The 2 fragments and spectinomycin resistance gene from pDL278 were digested with the appropriate enzymes (*Xba*I, *Hind*III, *Eco*RI and *Bam*HI), purified with the PureLink Gel Extraction kit (Life Technologies) and ligated into the multiple cloning site of pUC19 (Life Technologies) with the Roche Rapid DNA ligation kit. The ligation product was transformed into Top10 *E. coli* cells (Life Technologies) and transformants were selected on LB agar plates containing 100 μg/ml of ampicillin and spectinomycin. The resulting pComC1392 and pComC196 vectors were used as templates for the amplification of the knockout construct with primers B196comCdw/B196comCDEup and C1392comCdw/C1392comCDEup. The PCR product was purified with 25 μg capped Zymo columns (Zymo Research Corporation, Irvine, CA) and 1 μg/ml was transformed into C1392 and B196 cells treated with CSP (20 ng/ml). The *comC* knockout strain was

obtained via a double crossover event resulting in the replacement of *comC* with spectinomycin. The insertion was verified by PCR and sequenced with tArg2.

2.5.4 Construction of complementation vectors

Plasmid PAL2 (Beard, Salisbury, Lewis, Sharpe, & MacGowan, 2002), which contains *luxABCDE* was digested with *Eco*RI to remove the luciferase genes. The larger fragment containing the erythromycin resistance gene was re-ligated to form pAM2. The *Bam*HI and *Eco*RI sites in pAM2 were used to ligate in the *comDE* complementation piece. Digested products were purified with the PureLink Gel Extraction kit (Life Technologies), the Roche Rapid ligation kit was used for all ligations and transformations were carried out in Top10 or DH5alpha *E. coli* cells (Life Technologies).

To make the *comDE* complementation vector, the *comCDE* promoter of C1392 was amplified with C1392comCfw1Bam/B196tArgrv1Eco. The PCR fragment was purified, digested with *Bam*HI and *Eco*RI and ligated to pDL278 digested with the same restriction enzymes. The *comDE* genes were amplified from C1392 with B196ComDfw1Bam/ C1392ComErv1Hind and ligated to pDL278 to form pDL*comDE.* The promoter and *comDE* genes were amplified from pDL*comDE* using primer pairs B196tArgrv1Bam/C1392comErv1Eco. The resulting PCR fragment was digested with *Bam*HI and *Eco*RI and ligated to pAM2 to form pAM2DE. The insertions were verified by restriction analysis and sequencing.

For the *comCDE* complementation vector, *comCDE* and its promoter were successfully amplified from C1392 with primers rlmhBam/HP1Eco. The PCR product remains to be ligated to pAM2 to form the final pAM2CDE product.

2.5.5 Electroporation with the pAM2DE complementation vector

As C1392 Δ*comCDE* was no longer competent, pAM2DE was electroporated into the knockout strain. The electroporation protocol was modified from (Buckley, Vadeboncoeur, LeBlanc, Lee, & Frenette, 1999). From a frozen skim milk stock, the C1392 Δ*comCDE* strain was streaked and grown on THY agar with spectinomycin (75 μg/ml) and incubated for 48 hours in a 5% CO_2 incubator at 37°C. Colonies were inoculated in 5 ml of BHI and 0.5 M sorbitol with spectinomycin (75 μg/ml). After 48 hours of growth at 37°C in a 5% CO_2 incubator, the culture was diluted 100 fold and incubated for an additional 12-15 hours. A second 100 fold dilution was carried out in 18 ml of BHI with 0.5 M sorbitol and incubated until reaching an $OD_{600} \approx 0.8$. Once the desired OD_{600} was reached, 2 ml of 2 M glycine was added to the culture which was incubated for an additional 1.5 hours. The culture was then spun down at 4°C for 10 minutes at 4000 rpm. The supernatant was discarded and the pellet was re-suspended three times with 1 ml of cold electroporation solution (10% glycerol and 0.5 M Sorbitol). After the final wash step, cells were re-suspended in 200 μl of electroporation solution. Cells (50 μl) were then combined with 1 μg of DNA in a chilled BioRad 0.2-cm electroporation cuvette and electroporated as follows: 25 μF, 200 Ω, 2.00 kV. Immediately after electroporation, 950 μl of BHI with 0.5 M sorbitol was added to the

cuvette and transferred to a 1.5 ml microcentrifuge tube which was incubated for 2-2.5 hours in a 5% CO_2 incubator at 37°C. The culture was plated on BHI with erythromycin (150 µg/ml) and spectinomycin (75 µg/ml) and incubated for 3 days in a 5% CO_2 incubator at 37°C.

2.5.6 Transformations with Δ*comC* and Δ*comCDE* SMG strains

For transformations into the C1392 Δ*comCDE* and Δ*comC* strains, pAM2 or genomic DNA from B196 (Ery^R) and M431 (Tet^R) were used. All transformations were undertaken with 1 µg/ml of pAM2 or 1 µg/ml of gDNA in the presence or absence of added CSP (200 ng/ml). As a control, transformations into the C1392 wild type strain were undertaken. Transformations were plated on THY, THY-Ery150, THY-Spc75-Ery150, THY-Tet1, THY-Spc75-Tet1, THY-Spc75-Erm150-Tet1. Transformation frequencies and cfu/ml were calculated after 48 hours of incubation at 37°C in 5% CO_2.

2.6 Real-time PCR

2.6.1 CSP treatment and RNA extraction

Overnight bacterial cultures in THY, were diluted to an OD_{600} ~ 0.05 then incubated for 1 hour at 37°C with 5% CO_2. Samples of 1.5 ml were collected for RNA purification at 0, 5, 10 and 20 minutes after CSP addition (200 ng/ml). The bacterial pellets were treated with RNAprotect Bacteria Reagent (Qiagen) and stored at -80°C until used. Bacterial pellets were re-suspended in 700 µl RNAse free water, 10 µl of 100 mg/ml lysozyme (Sigma-Aldrich) and 5 µl of 10 U/µl mutanolysin (Sigma-Aldrich). RNA

extraction was carried out with TRIzol reagent (Invitrogen) in combination with column purification using the Qiagen RNeasy Kit. An on-column DNAse digestion step was also carried out according to the Qiagen manufacturer's instructions. RNA extraction was completed in triplicate for all four bacterial strains at each time point and quality was assessed spectrophotometrically (at 260 and 280 nm) and with the Agilent Bioanalyzer.

2.6.2 Reverse transcriptase and real-time PCR

cDNA was generated from 50 ng of RNA at each time point with the iScript Select cDNA synthesis kit (BioRad) according to the manufacturer's instructions. Random primers were used for conversion of RNA to cDNA and samples were stored at -20^{o}C. The genes selected for this study included the *comX* target and *16S* rRNA and *ldh* reference genes. Primers for these three genes can be found in **Table 2.3**. Primers were validated first with genomic DNA to confirm the specificity and amplicon size before being used in quantitative real-time PCR.

Real-time PCR reactions were carried out in a CFX96 Real-time PCR machine (BioRad). Primers were validated with a thermal gradient PCR and 55^{o}C was determined to be optimal. Standard curves were carried out to investigate the efficiency of amplification of each primer set. Efficiencies ranged from 90%-110% and R^2 values were at least 98%. For the samples tested, 10 µl reactions were performed in triplicate using Sso Fast Evagreen supermix (BioRad), 4 µl cDNA and 500 nM of primer. The specificity of the product was determined by melt curve analysis. Appropriate controls, including a no-template control and an RT-free RNA control, showed either no amplification or were

>4 Ct above that of target sequences. The stability of the *16S* rRNA and *ldh* reference genes was also verified with the CFX manager software. Gene expression was analyzed with the comparative CT method ($2^{-\Delta\Delta CT}$) using the following calculation: Ratio = $(E_{target})^{\Delta Ct\ target\ (control - treated)}/(E_{ref})^{\Delta Ct\ ref\ (control - target)}$. *comX* expression was calculated relative to time point 0 min (before CSP addition) and normalized to *ldh* and *16S* reference genes.

2.6.3 Statistical analysis

A One-Way ANOVA was carried out to compare *comX* expression across the bacterial strains at each time point in order to obtain a p value.

Table 2.1. Sources of SMG isolates

Site	***S.anginosus***	***S.constellatus***	***S.intermedius***	**All Strains**
CF Sputum	48	43	37	128
Hip Abscess			1[a]	1
Brain Abscess		1	4	5
Liver Abscess		1	3	4
Blood	2	5	8[a]	15
Empyema	3	3	5	11
Unknown[b]	3	4	2	9

[a] An isolate was obtained from a CF patient with invasive *S. intermedius* infection coincident with pulmonary exacerbation

[b] Invasive infections where details are unavailable

Table 2.2 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
SMG		
C1392	Wild type, Em^S, Tet^S, Spc^S	This study
C1391	Wild type, Em^S, Tet^S, Spc^S	This study
M431	Wild type, Em^S, Tet^R, Spc^S	This study
B196	Wild type, Em^R, Tet^S, Spc^S	This study
B196 Δ*comCDE*	*comCDE* knockout, Em^R, Tet^S, Spc^R	This study
B196 Δ*comC*	*comC* knockout, Em^R, Tet^S, Spc^R	This study
C1392 Δ*comCDE*	*comCDE* knockout, Em^S, Tet^S, Spc^R	This study
C1392 Δ*comC*	*comC* knockout, Em^S, Tet^S, Spc^R	This study
C1392CDE - pAM2DE	C1392 Δ*comCDE*:: pAM2DE Em^R, Tet^S, Spc^R	This study
Plasmids		
pDL278	*E. coli-Streptococcus* shuttle vector, Spc^R	LeBlanc et al., 1992
pAL2	pVA838 derivative with *luxABCDE*, Erm^R	Beard et al., 2002
pAM2	Cloning vector derived from pAL2 with *luxABCDE* removed, Em^R	This study
pUC19	*E. coli* cloning vector, Ap^R	
pComCDE1392	pUC19 harboring the C1392 *comCDE* knockout construct	This study
pComCDE196	pUC19 harboring the B196 *comC* knockout construct	This study
pComC1392	pUC19 harboring the C1392 *comC* knockout construct	This study
pComC196	pUC19 harboring the B196 *comC* knockout construct	This study
pAM2DE	pAM2 harboring C1392 *comDE*	This study
pDL*comDE*	pDL278 harboring C1392 *comDE*	This study

Table 2.3 Primers used in this study with restriction sites in bold

Primers	Oligonucleotide Sequence (5'→3')	Source
tArg2	CATAGCTCAGCTGGATAGAGCATTCGCCTTC	Håvarstein et al., 1996
tGlu	GGCGGTGTCTTAACCCCTTGACCAACG	Håvarstein et al., 1996
comEF1	GGTGGAGAAGTTAGAATG	This study
comER1	AGATTTATGAGGTGAAAAG	This study
comCF1	AGAGGTAGTAAAAAAGGAAG	This study
comCR1	AGAAAAATCAAATCCCATTC	This study
Real-time PCR		
ldhF1	GGTTCTTCTTACGCATTTG	This study
ldhR1	TCTTCAGCATCCCCTTC	This study
16SF1	AAGTAGAACGCACAGGATG	This study
16SR1	CAGTAAATGTTCTTATGCGGTATTAG	This study
comXF2	GAAGAGGTAAGTGAGATTG	This study
comXR4	TGGTCTTATAGTAAACATAGAG	This study
ΔcomCDE		
C1392comCfw2Xba	GCG**TCTAGA**CACTTTAGTTATTTCCTTTTTGC	This study
C1392protrv2Hind	GCG**AAGCTT**AATAAACCACCAATAAAACCTATAGC	This study
HPrv1Eco	GCG**GAATTC**TAGTTCTATTCTTGATAAACTCGG	This study
C1392comEfw1Bam	GCG**GGATCC**ACTCGAATGAAAATTGGAAAGTTG	This study
B196comCfw1Xba	GCG**TCTAGA**TAGCAAACAACTTTTTCATAATTTCTC	This study
B196 protrv1Hind	GCG**AAGCTT**TCCTCCTAGTTATTTCAATTACTG	This study
B196ComEfw1Bam	GCG**GGATCC**TAGAGGAGTCAAAAGGATG	This study
comCDEdw	TAGTTCTATTCTTGATAAACTCGG	This study
B196ComCDEup	TCCTCCTAGTTATTTCAATTACTG	This study
C1392comCDEup	AATAAACCACCAATAAAACC	This study
ΔcomC		
C1392comCrv2Bam	GCG**GGATCC**TGG GAT TTG ATT TTT CTA AAC	This study
C1392comDfw4Eco	GCG**GAATTC**CAAAATCTTTTACTTCAAATG	This study
B196comDfw1Eco	GCG**GAATTC**AATCTTTTACTTCAAATGTTAATTC	This study
B196comCrv1Bam	GCG**GGATCC**TGGTAAATAAATGTTCAGGAGTAAG	This study
C1392comCdw	CAAAATCTTTTACTTCAAATG	This study
B196comCdw	AATCTTTTACTTCAAATGTTAATTC	This study
Complementation		
comDE		
Promoter		
C1392comCfw1Bam	GCG**GGATCC**ATAAATTCTCCTTAATGTTTGTTTTTTG	This study
tArgrv1Eco	GCG**GAATTC**AATCCTGCTGGGATCATATAAG	This study
tArgrv1Bam	GCG**GGATCC**AATCCTGCTGGGATCATATAAG	This study
comDE		
ComDfw1Bam	GCG**GGATCC**TGGGATTTGATTTTTCTAAAC	This study
C1392ComErv1Hind	GCG**AAGCTT**CTTAACCCCTTGACCAAC	This study
C1392comErv1Eco	GCG**GAATTC**CTTAACCCCTTGACCAAC	This study

comCDE		This study
rlmhBam	GCG**GGATCC**ATGATTCAACAAGGAAGTCC	This study
HP1Eco	GCG**GAATTC**ATTGACATTTATCCCGAATGGTC	
Spectinomycin		This study
pDL278 fw1Bam	GCG**GGATCC**GATTTTCGTTCGTGAATACATG	This study
pDL278rv1Xba	GCG**TCTAGA**ACTGGTCGTGAAATTGTTGC	
Sequencing		
pDL278Seq	GAT TAA GTT GGG TAA CGC CAG G	This study
C1392ComErvSeq	CAACTTTCCAATTTTCATTCGAGT	This study
B196ComErvSeq	CATCCTTTTGACTCCTCTA	This study

Table 2.4 Amino acid sequences of streptococcal competence stimulating peptides

Phylogenetic group Species Strain	References/Accession numbers	Amino acid sequence of CSP
Milleri/Anginosus		
CSP A	This study	DSRIRMGFDFSKLFGK
NCTC 10713	CAA04341.1	
NCDO 2227	CAA04354.1	
NCTC 11325	CAA04350.1	
ATCC 27823	This study	
ATCC 33397	This study	
ATCC 27335	This study	
CSP B	This study	DRRDPRGMIGIGKKLFG
CSP C	This study	DRRDPRGIIGIGKKLFG
NCTC 10708	CAA04352.1	
CSP D	This study	DRRDPRGIIGIGKNYLVRRFYCYESG
CSP E	This study	DRRDPRGMIGIGKNYLVRRFYHYESG
Mitis *S. mitis*		
B6	CAA04344.1	EMRKPDGALFNLFRRR
SK575	EID28488.1	EMRKMNEKSFNFFNIFRRR
SK601	Kilian et al., 2008	EMRKMNEKSFNIFNIFSIFRRR
SK597[a]	ZP_07642740.1	EMRRIDEKLVGIFNFFRRR
SK615	Killian et al., 2008	EMRKSNNTFFNFLRRI
Hu8	CAA04347.1	EMRKSNNNFFHFLRRI
SK262	Kilian et al., 2008	EMRRSNNNFFNFLRRI
SK579	ZP_14293572.1	EIRKSNSALVNFFKRR
NCTC12261	ZP_07644369.1	EIRQTHNIFFNFFKRR
SK609	Killian et al., 2008	EIRKTSNSLLNFFKRR
SK599	Killian et al., 2008	EMRRIGSVLLNFFKRR
SK1080	ZP_12496250.1	EMRRIDKIFNFLKRR
SK616	ZP_13519962.1	EMRRIDKIFINFLKRR
SK1073	ZP_12493733.1	ESRLSRLLRDFIFQIKQ
Col18	CAB39414.1	ESRVSRIILDFLFQRKK
SK564	ZP_07646796.1	ESRVSDILLDFLFRRKK
Col16	CAB39412.1	ESRISDILLDFLFQRKK
SK611	Kilian et al., 2008	EMRLPKILRDFIFPRKK
B5	CAA04359.1	ESRLPKIRFDFIFPRKK
F0392	ZP_12577722.1	EWRIPELIRNLIFPKRK
ATCC6249	ZP_07461794.1	DLRISETIRNLIFPRKKK
SK145	Kilian et al., 2008	DMRISESIRNLIFPRKKK

S. pneumoniae		
2082170	ZP_15702938.1	IFFSYEKSNEIRRKSNGFTWIWDSYCSFFNY
SP19-BS75	ZP_01834096.1	EMRKMNEKSFNFFNFFNFFRRR
GA47373	ZP_12824775.1	EMRKMNEKSFNIFNFFNFFRRR
GA13224	ZP_13983665.1	EMRKMNEKSFNIFNFFNFFNFFNFFRRR
SK305	CAB39529.1	EMRKMNEKSFNIFNFFNFFNFFRRR
A66	AAF70541.1	EMRISRIILDFLFLRKK
101/87	CAB39523.1	ESRLPKILLDFLFLRKK
AP200	YP_003877815.1	EMRLSKFFRDFIFESSIMKLATLP
GA13723	ZP_14064082.1	EMRLSKFFRDFILQRKSNEIRGKSNGFIWI
SP11-BS70	EDK63014.1	EMRLSKFFRDFILQRKK
Rx	AAC44440.1	EMRLSKFFRDFILQRKK
SK676	Kilian et al., 2008	ERRIPDVIRSLLFQKRK
S. oralis		
SK79	Kilian et al., 2008	DKRGLMDLFKQIPIFRRK
SK1074	ZP_13522198.1	EIRKENNFLFYFFKRK
SK597[a]	Kilian et al., 2008	EMRRIDEKLVGIFNFFRRR
Col19	CAB39419.1	EMRLPKILRDFIFPRKK
SK96	Kiliab et al., 2008	EWRIPELIRNLIFPKRK
DSM 20066	O33690.1	DWRISETIRNLIFPRRK
ATCC35037	ZP_06611237.1	DKRLPYFFKHLFSNRTK
S. infantis		
SK283	Kilian et al., 2008	DIRSFKFLNKIFPKK
SK140	EIF38756.1	DIRLPHIIKKLFSK
SK350	Kilian et al., 2008	EWRPMYTINNFLFSKSK
SK970	ZP_12582150.1	DWRFLGSIRELIFPKKNK
SK348	AAM18092.1	DWRFLNSIRDLIFPKRK
X	ZP_12580090.1	DKRLTYFITNLFPKRKNRKHYRET
ATCC700779	EIG39655.1	DKRLTYFITNLFPKRKK
S. sanguinis		
133-79	ADF56053.1	DIRGIPNPWDWIFGR
SK36	ABN45737.1	DLRGVPNPWGWIFGR
ATCC49296	ZP_07887078.1	DWRISETIRNLIFPRRK
S. gordonii		
NCTC3165	CAA04356.1	SQKGVYASQRSFVPSWFRKIFRN
Challis	YP_001451370.1	DVRSNKIRLWWENIFFNKK
NCTC7865	CAA66791.1	DIRHRINNSIWRDIFLKRK
S. cristatus		
ATCC51100	CAA04366.1	DLRNIFLKIKFKKK
S. oligofermentans		
AS 1.3089	ABF72115.1	DSRNIFLKIKFKKK

S. pseudopneumoniae		
SK674	Kilian et al., 2008	EWRPPYTINNFLFPKRK
IS7493	YP_004769537.1	EMRLPKILRDFIFPRKK
S. peroris		
ATCC700780	ZP_08065889.1	DRRITSFFANLFQKRKK
Mutans		
S. mutans		
N1b	CAL29394.1	SGTLSTFFRLFNRSFTQA
NN2025	BAH87262.1	SGSLSTFFRLFNRSFTQALGK
FP051	ABE02379.1	SGSLSTFFRLFNRSFTQALGKIR
S. species		
2_1_36FAA[b]	EEY80904.1	DLGVLVGIFAAAYVIGSDLARRK
2_1_36FAA	EEY80792.1	DIRHRINNSIWRDIFLKRK
F0407	EHI76098.1	EVRKENNFLFYFFKRK
SK140	EIF38756.1	DIRLPHIIKKLFSK

[a]SK597 appears for *S. mitis* and *S. oralis* as this CSP has been reported to be a hybrid of these two species (Kilian et al., 2008)

[b]Two different sequences were recovered from PubMed for the non-speciated *Streptococcus* strain 2_1_36FAA

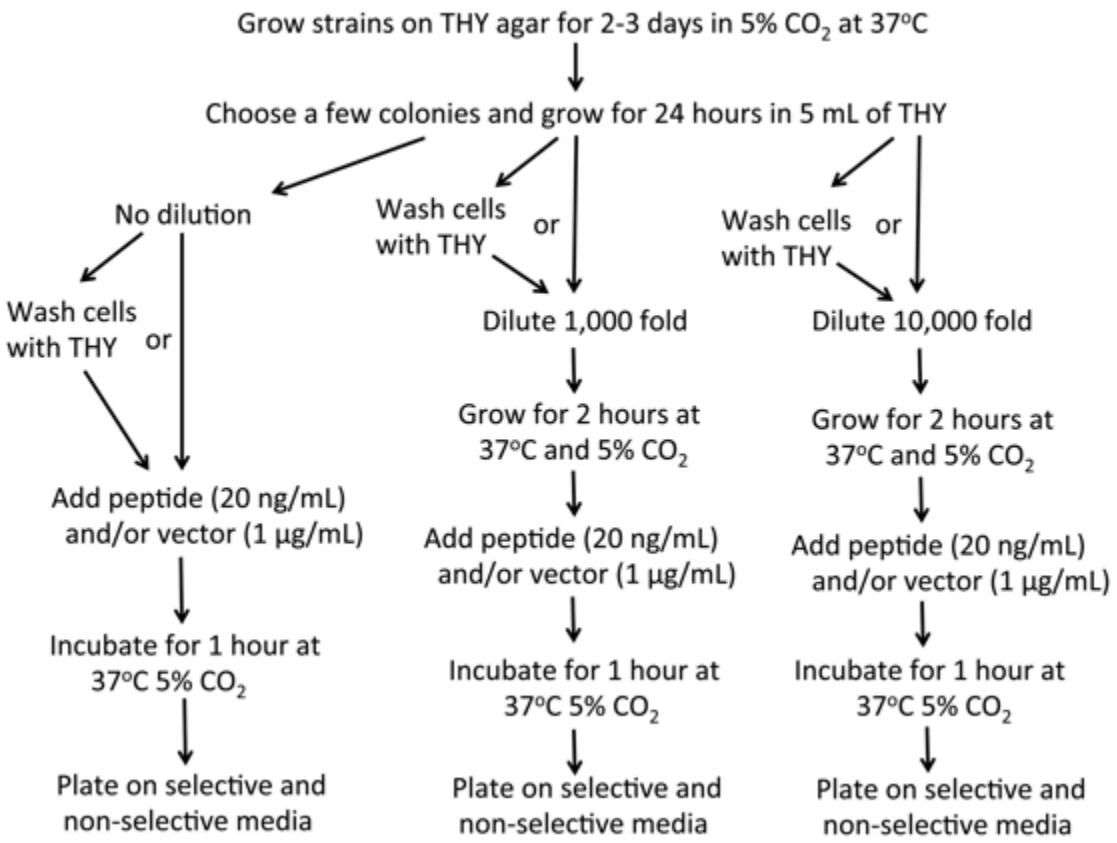

Figure 2.1 Dilution and growth phase experimental workflow.

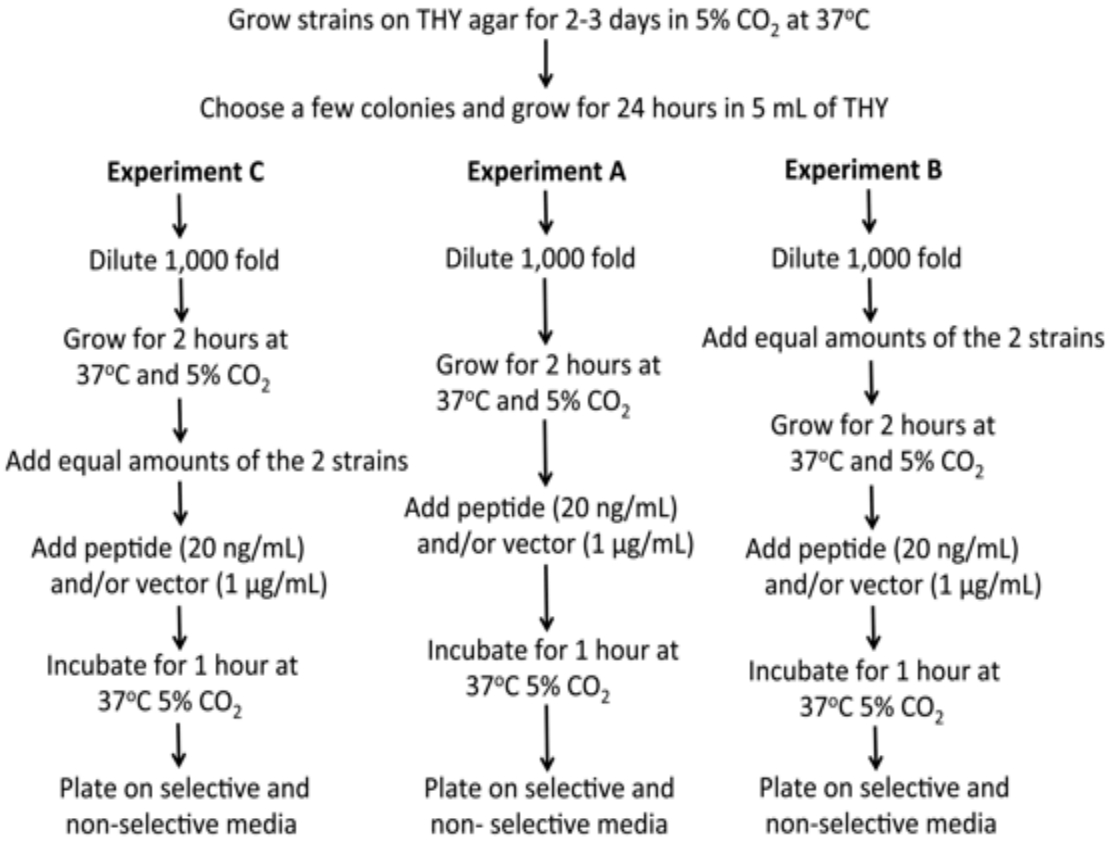

Figure 2. 2 Co-culture assay workflow.

CHAPTER THREE: RESULTS

3.1 Heterogeneity in SMG Competence Peptides

Bacterial CSP is synthesized as a precursor peptide. Upon processing by the ABC transporter ComAB, the ComC leader is cleaved after a conserved double glycine and exported as the mature CSP (J.-P. Clarveys & Håvarstein, 2002). Two CSP sequences have been described for the SMG (L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997) but, until now, only a small number of strains had been examined. Here, variability was investigated in CSPs from a collection of 170 clinical SMG isolates, derived from adult cystic fibrosis patients and invasive disease. Identified SMG CSPs were aligned and compared to other streptococcal CSPs from NCBI and the literature.

3.1.1 Identifying CSPs in our SMG strain collection

PCR amplification of *comCDE* using primers based on the conserved Arg-tRNA and Glu-tRNA which flank the operon allowed for the successful recovery and sequencing of *comC* genes from 166 SMG strains. This includes CSP genes from reference strains ATCC27335 (*S. intermedius*), ATCC27823 (*S. constellatus*) and ATCC33397 (*S. anginosus*). In four SMG strains no PCR product for *comCDE* (approximately 2.6 kb) could be recovered with the tRNA primers (**Figure 3.1**). In order to examine whether *comCDE* is organized differently in these strains, internal primers for the operon were designed (**Table 2.3**). Only portions of the operons could be amplified, including one *comC* sequence with primers for the Arg-tRNA and *comE*, suggesting that

comCDE is interrupted in different locations by insertions.

The predicted CSPs for strains whose *comC* could be sequenced fell into five groups (Groups A, B, C, D and E) that did not correlate with the clinical source of the bacterial isolate (**Table 3.1**). In other words, each CSP group was not specific to only CF or invasive isolates. Interestingly, different CSP types were found to co-colonize some of our CF patients, specifically CSPs A and B, or A and C (**Figure 3.2**). Invasive SMG strains are represented by a single isolate per patient and it was therefore unknown whether mixed CSP types colonized these individuals. CSP sequences from Groups A and C were originally identified by Håvarstein et al. (L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997). Most of the CSP sequences from SMG belong in Group A (87%) with all three species represented. Amino acid sequences for Groups B, C, D and E CSPs are similar to each other and very different from those in Group A. Interestingly within Group A, species specific differences in the nucleotide sequences can be observed. This is likely due to genetic drift (**Table 3.2**). Group B and C sequences appear to be specific to *S. anginosus* and *S. constellatus* respectively. Examination of the nucleotide sequences for Group B and C peptides indicate that they differ by a methionine to isoleucine substitution. Group D and E peptides are frameshift variants of C and B, respectively, both with an adenine deletion (**Table 3.2**), and our results suggests that these CSPs are not functional.

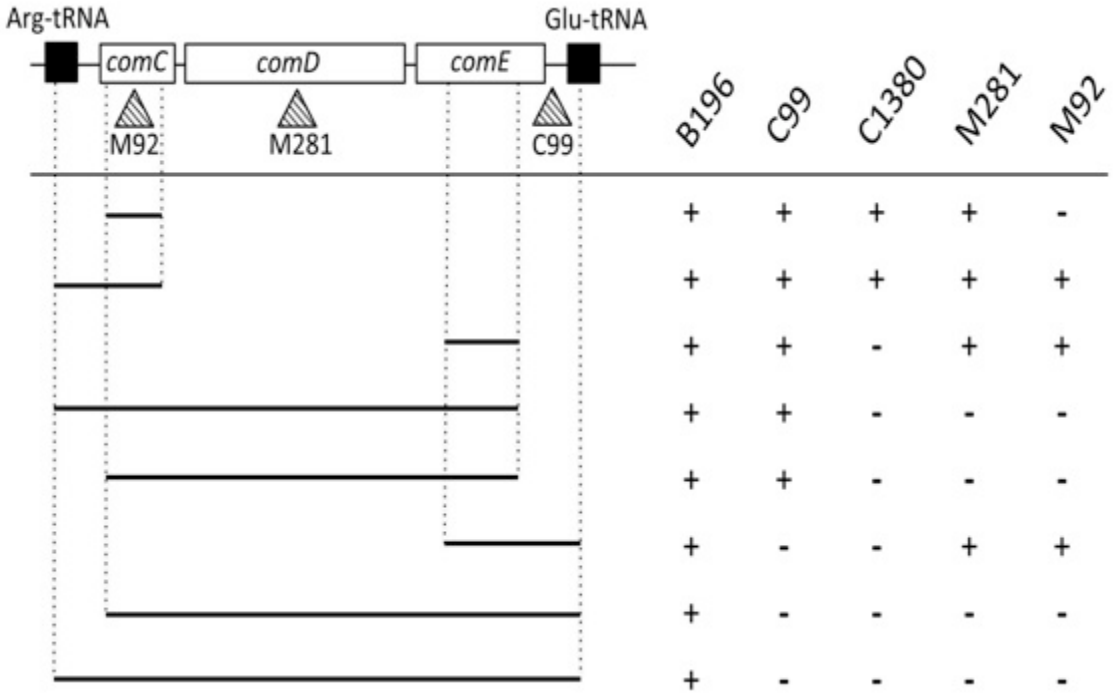

Figure 3.1 Polymerase chain reactions carried out on strains whose *comCDE* could not be amplified with primers based on the conserved tRNAs. Solid black lines represent PCR products amplified with primers in Table 2.3. Triangles represent areas where the insertions are thought to be present in C99, M281 and M92. C1380 has an insertion somewhere between *comC* and the Glu-tRNA. B196 was used as a positive control.

TABLE 3.1 Distribution of CSPs from 167 SMG isolates and three ATCC typed strains. Amino acid substitutions are in bold.

CSP	Amino acid sequence	*S. anginosus*		*S. constellatus*		*S. intermedius*		Total
		Invasive[c]	Airway	Invasive	Airway	Invasive	Airway	
A[a,b]	DSRIRMG-FDFSKLFGK	4	35	11	35	23	37	148 (87.1%)
B	DRRDPRG**M**IGIGKKLFG	4	6	-	-	-	-	10 (5.9%)
C	DRRDPRG**I**IGIGKKLFG	-	-	4	5	-	-	9 (5.3%)
D	DRRDPRG**I**IGIGKNYLVRRFYCYESG	-	-	-	1	-	-	1 (0.6%)
E	DRRDPRG**M**IGIGKNYLVRRFYHYESG	-	2	-	-	-	-	2 (1.2%)

[a]No PCR product of *comC* could be recovered for three strains from CSP Group A using the standard primers based on the conserved tRNAs.
[b]CSP group A also includes 3 ATCC typed strains: *S. anginosus* ATCC33397, *S. constellatus* ATCC27823 and *S. intermedius* ATCC27335.
[c]Invasive strains were isolated from liver, hip, brain and lung abscesses, empyema and unknown sources

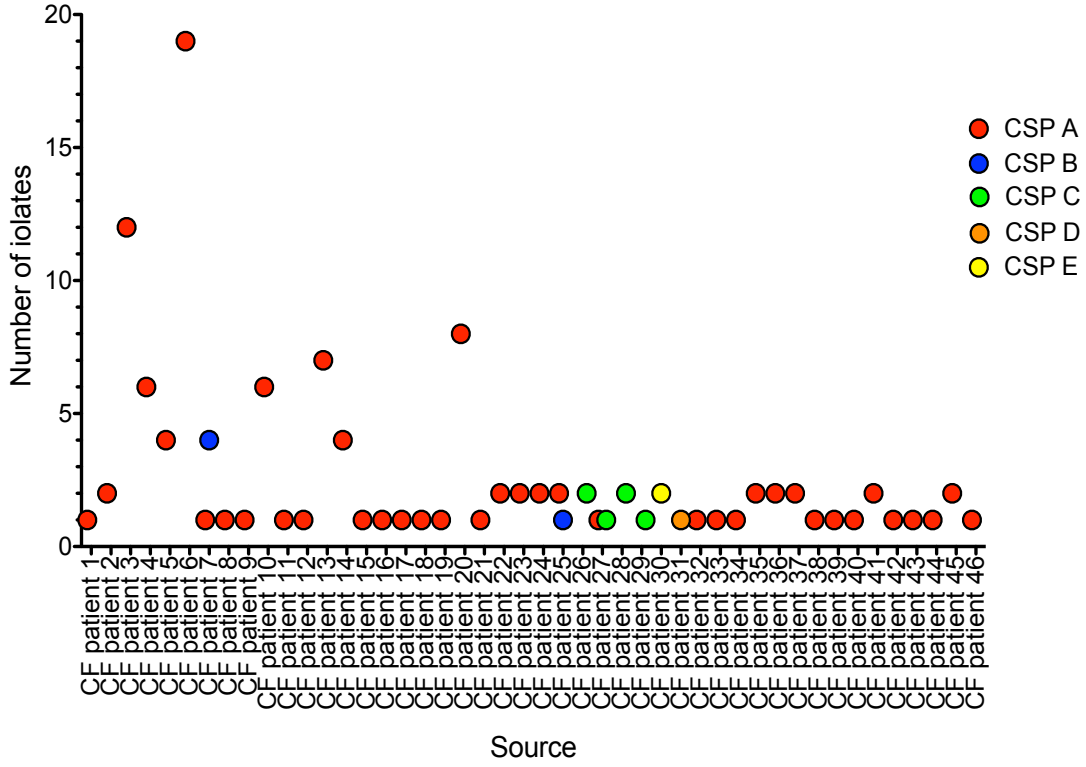

Figure 3.2 SMG CSP types per patient. Two CSPs could not be included since their sequences were not recovered with the standard primers used to amplify *comCDE*.

Table 3.2 Distribution of CSP nucleotide sequences from 167 SMG isolates and three ATCC typed strains. Nucleotide substitutions are bolded.

CSP	Nucleotide sequences	*S. anginosus*	*S. constellatus*	*S. intermedius*	Total
A.1	GATAGTCGAAT**A**AGAATGGGATTTGATTTTTC**T** AAACTTTTTGGTAAA	1	47	-	48 (28.2%)
A.2	GATAGTCGAAT**C**AGAATGGGATTTGATTTTTC**T** AAACTTTTTGGTAAA	1	-	61	62 (36.5%)
A.3	GATAGTCGAAT**C**AGAATGGGATTTGATTTTTC**A** AAACTTTTTGGTAAA	38	-	-	38 (22.4%)
B[a]	GATAGGAGGGATCCTAGAGGA**ATG**ATTGGAATT GGGAAAAAATTATTTGGT	10	-	-	10 (5.9%)
C[a]	GATAGAAGAGATCCTAGAGGA**ATA**ATTGGAATT GGGAAAAAATTATTTGGT	-	9	-	9 (5.3%)
D[b]	GATAGAAGAGATCCTAGAGGA**ATA**ATTGGAATT GGAAAAAA<u>T</u>TATTTGGTTAGGAGATTTTATTGT TATGAATCTGGA	-	1	-	1 (0.6%)
E[b]	GATAGGAGGGATCCTAGAGGA**ATG**ATTGGAATT GGGAAAAA<u>T</u>TATTTGGTTAGGAGATTTTATCAT TATGAATCTGGA	2	-	-	2 (1.2%)

[a] The arginine substitution for guanine ('ATG' to 'ATA') results in a methionine to isoleucine amino acid substitution.
[b] Nucleotide differences that result in a frameshift mutation are underlined. Deletion of an adenine that is present in B and C CSPs, results in a frameshift mutation in D and E peptides.

3.1.2 Neighbour joining tree of streptococcal CSPs

In this study, three novel SMG CSPs were identified. A neighbor joining tree was constructed to gain a better understanding of how closely these peptides resemble other streptococcal CSPs, collected from NCBI and the literature (**Figure 3.3**). CSP sequences included those from the Mitis group, the Milleri group and *Streptococcus mutans*. Most of the sequences are from the Mitis streptococci since the *comCDE* system has been well characterized in this group. For the SMG, the three novel CSPs as well as those characterized by Håvarstein et al. (1997) were included in the CSP alignments. Interestingly, *S. mutans* possesses two competence systems, the ComCDE and closely related ComRS systems. The ComRS system is also found within the Salivarius, Bovis and Pyogenic streptococci (L. S. Håvarstein, 2010). For this analysis, only ComC peptides related to the ComCDE system were included. In total 1357 sequences were collected and from this, 63 unique CSP sequences were aligned and compared (**Table 2.4**). The neighbor joining tree shows that Groups B, C, D and E cluster on their own branch. CSP A shares a common branch with *S. oralis* and a non-speciated *Streptococcus*.

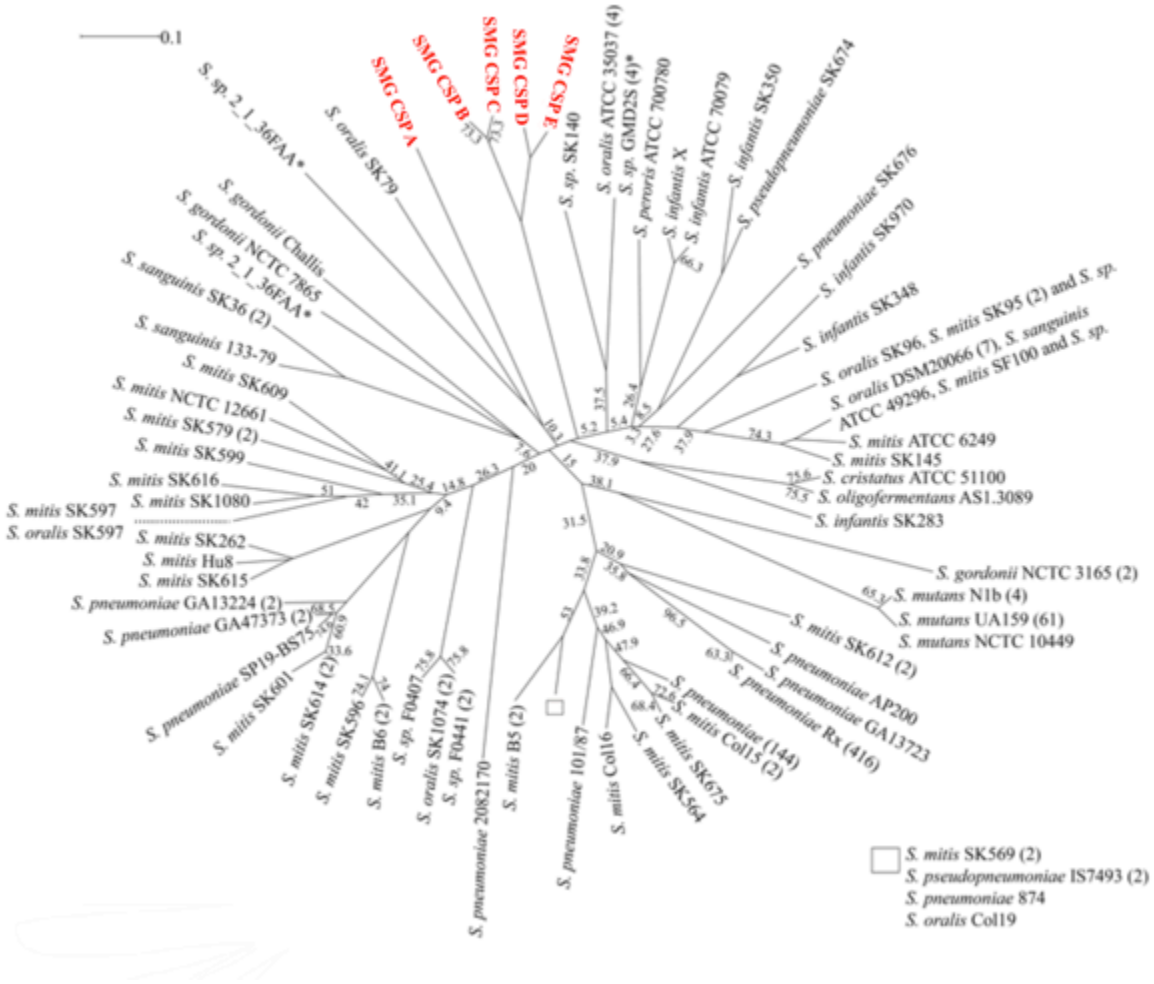

Figure 3.3. Neighbour-joining tree of CSP amino acid sequences from the Milleri group (in bold), Mitis group and *Streptococcus mutans* collected from NCBI and the literature. For strains with an asterisk (*), more than one competence peptide for the same strain was collected from NCBI. Where multiple strains for the same CSP was found, the number is indicated in brackets. The phylogenetic tree was built with 1,000 bootstrap repetitions in SplitsTree4 with bootstrap values <80 indicated. The ruler indicates amino acid substitutions per site.

3.2 Competence Stimulating Peptide Cross-Activation

Competence peptides are specific for their receptors (ComDs) as a CSP from one group may or may not induce competence in a strain from a different peptide group. The two previously characterized SMG CSPs (designated CSPs A and C in this thesis) were not found to cross-react. However, only four SMG strains were tested (L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997). We sought out to test cross-activation with strains from our five SMG CSP groups (A, B, C, D and E). Results from these experiments suggested that there are two functional peptide groups, CSPs A and B/C, and that the longer frameshift peptides D and E are non-functional. To test the two functional groups hypothesis, cross-activation was further investigated with CSPs A and B with 41 SMG isolates.

3.2.1 CSP and vector saturating levels.

Experimental parameters for competence induction were explored by determining peptide and vector saturation levels for a strain from Group A (*S. intermedius* strain B196) and one from Group B (*S. anginosus* strain M423). Concentrations of CSP varied from 0.5 ng/ml to 512 ng/ml. Strain M423 had higher endogenous competence levels than B196 and reached saturation at 16 ng/ml, whereas B196 reached saturation of CSP at 64 ng/ml (**Figure 3.4 A**). A concentration of 20 ng/ml was chosen for further experimentation since higher concentrations of peptide could mask CSP specificity. Plasmid saturation was also tested with concentrations of pDL278 varying from 0.001 μg/ml to 10 μg/ml (**Figure 3.4 B**). Although plasmid saturation occurred at 0.1 μg/ml for

both B196 and M423, experiments for CSP specificity were carried out with 1 μg/ml of pDL278.

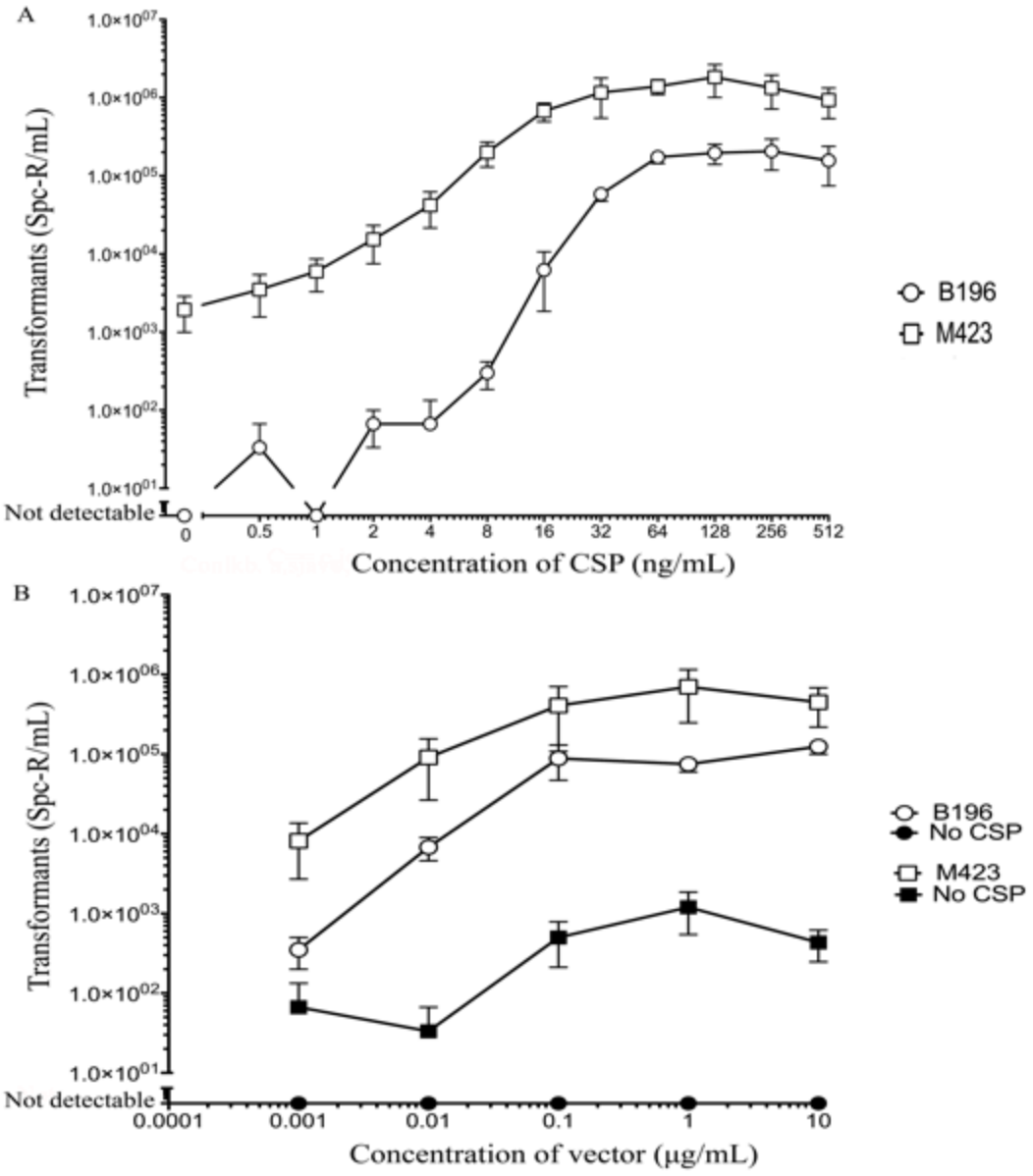

Figure 3.4 Investigating parameters for competence induction. A) Effect of different concentrations of CSP A and B on competence using a constant concentration of pDL278 plasmid (1 µg/ml). B) Effect of different concentrations of pDL278 vector on competence with a constant concentration of peptide (200 ng/ml). Total spectinomycin resistant transformants were determined after a one hour incubation with CSP and/or pDL278. M423 (Group B CSP) and B196 (Group A CSP) SMG strains were used. This experiment was done in triplicate and plated in duplicate. Standard error of the mean values are plotted with corresponding error bars.

3.2.2 Synthetic CSP induces competence in the SMG

A variety of CSPs have been identified in naturally competent streptococci (L. S. Håvarstein, 2010). These peptides are specific to the strain producing them and can induce competence in other strains that respond to and produce this same CSP (L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997). As peptides are specific for their receptors (ComDs), a CSP from one group may or may not induce competence in a strain from a different peptide group. In order to analyze the specificity of the five peptides identified in our strain collection, 20 ng/ml of CSP from Groups A, B, C and E were used to induce competence in SMG strains from these same groups (Groups A to E), allowing them to take up pDL278 plasmid DNA. A Group A CSP was found to induce competence in strains within its own group, whereas Group B and C peptides could activate competence in strains from groups B, C as well as the only strain in group D (**Table 3.3**). A CSP D was not tested since it differs from CSP E by one amino acid substitution and both are frameshift peptides. It was therefore hypothesized that cross-activation results with CSP E would likely be applicable to CSP D. Both strains in Group E were not competent and CSP E could not induce competence in any strains from Groups A-D above endogenous competence levels. Based on transformations carried out with CSPs A, B, C and E, two functional groups were identified: Group A and Group B/C.

TABLE 3.3 Cross-activation across the five SMG CSP groups with synthetic peptide

Strain	Species	CSP Group	Fold change			
			CSP A	CSP B	CSP C	CSP E[a]
B196	*S. intermedius*	A	875	1	3	2
M423	*S. anginosus*	B	2	838	1110	2
M459	*S. constellatus*	C	1	5187	5484	2
M414	*S. constellatus*	D	<1	3923	4283	<1

[a]No transformants were obtained for the two strains in CSP group E.

3.2.3 Transformations with CSP A and B to investigate specificity

To further investigate competence and CSP specificity, transformations were carried out on a subset of the strain collection with CSPs A and B. Transformations were carried out with 117 SMG strains in our collection but only 41 had comparable growth rates and were transformable. Of the 41 SMG strains tested, 27 had noticeable levels of competence induction with CSPA or B (**Figure 3.5**). High levels of induction were seen for M410 with a 3000-fold increase with CSP A versus without. For CSP B, the highest levels of competence induction were achieved in strain M423 with a 218-fold increase with peptide B. As expected, cross-activation was not observed with CSP A and B. CSP B differs from CSP C by one amino acid substitution at their C-terminus. Due to this sequence similarity, CSP B could activate competence in its own strains (C261 and M423) as well as those in group C (M459 and M510). These results suggest that synthetic CSP A and B were able to induce competence in their respective strains and that these peptides cannot cross-activate.

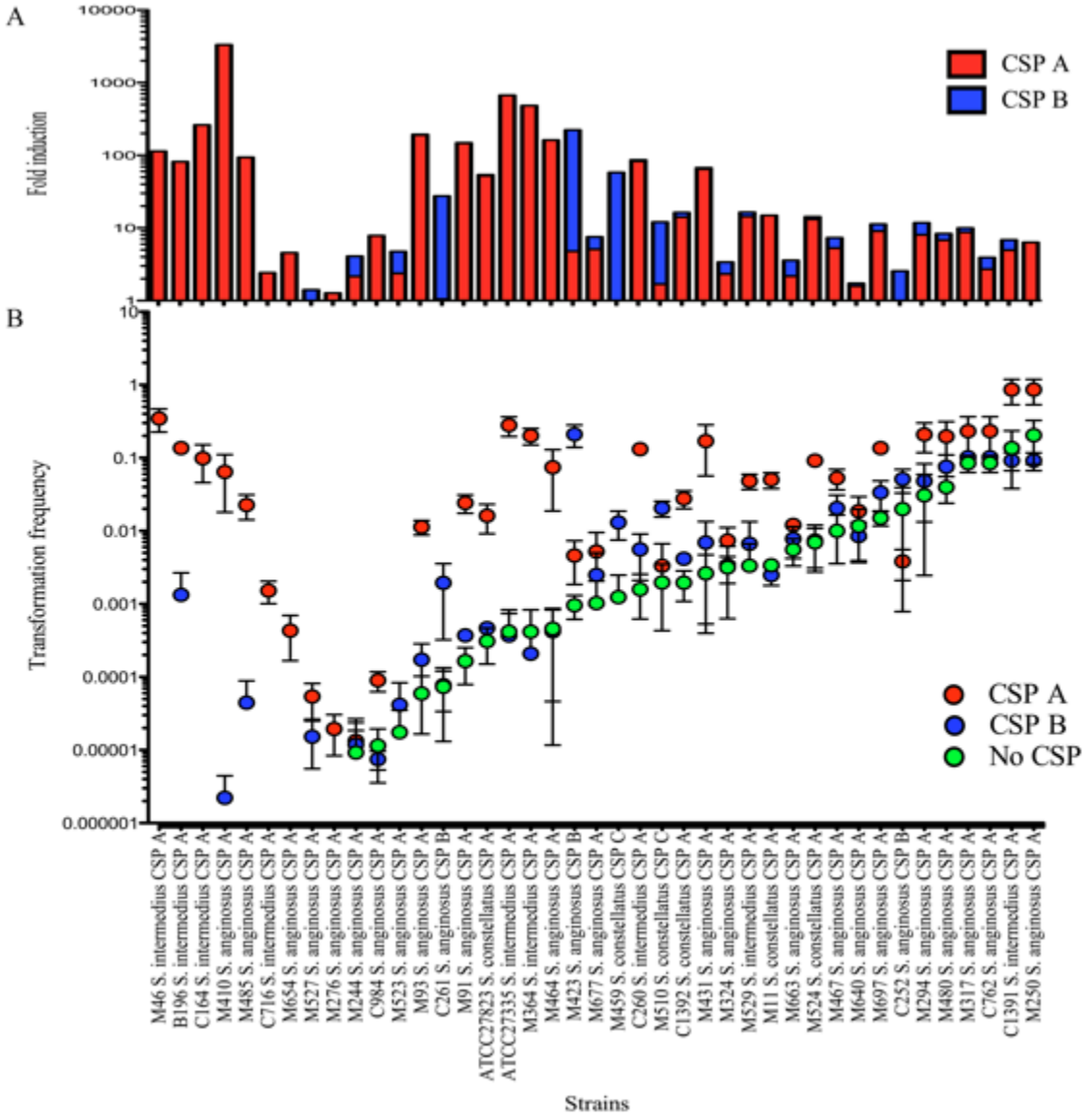

Figure 3.5 Competence induction with CSPs A and B. A) Fold induction calculated as the number of transformants with peptide/transformants without peptide. B) Transformation frequencies with CSP A (red circles), CSP B (blue circles) and just vector (green circles). Transformations were carried out in triplicate with 20 ng/ml of CSP and/or 1 µg/ml pDL278 vector and standard error of the mean was calculated.

3.3 Endogenous competence

The ability to acquire DNA from the extracellular environment varies across bacteria with more than 70 species reported to be competent (O. Johnsborg, Eldholm, V. and Håvarstein, L. S., 2007; Mashburn-Warren et al., 2012). There is currently research on the distribution of natural competence across the six streptococcal phylogenetic groups, but less is known about how transformation frequencies vary within species (Evans & Rozen, 2013). We observed high transformation frequencies in many of our SMG strains without added CSP. Under the conditions tested, some of the SMG could be constitutively competent or saturating levels of CSP could already be present. To test these hypotheses, preliminary experiments involved further diluting the SMG to reduce endogenous competence levels and carrying out competence assays during stationary phase to investigate if *com* genes are constitutively expressed. In addition, strains were co-cultured to investigate whether highly endogenously competent strains were producing saturating levels of CSP that could activate DNA uptake in an SMG strain with no observable competence induction in the absence of added CSP. And finally, knockouts of *comC* and *comCDE* were undertaken to examine if high endogenous competence levels were CSP-dependent.

3.3.1 Dilutions and growth phase to investigate endogenous competence

In an effort to reduce endogenous competence levels, bacterial strains with high (M250 and C1392) and no (B196) detectable endogenous competence levels were spun down after 24 hours to remove any peptide present prior to dilution and before the

addition of CSP and vector (**Figure 3.6**). SMG strains were also further diluted 10 fold on top of the original 1,000 fold dilution. Neither method reduced endogenous competence levels suggesting that under the conditions tested, the competence pathway was turned on independent of cell density.

Competence varies over the growth of a culture and has been shown to reach a maximum during early log phase in *S. pneumoniae* (L. S. Håvarstein, Coomaraswamy, et al., 1995; A. Tomasz & Hotchkiss, 1964). To test whether some of the SMG strains were constitutively competent, CSP and pDL278 were added to cultures during stationary phase. If some of the SMG were constitutively competent, the transformation frequencies at 24 hours should be the same as those calculated for the cultures grown to early log phase. No transformants were obtained at 24 hours above detectable limits of 10^{-6} to 10^{-7}. Interestingly, when cells were washed at 24 hours and re-suspended in fresh THY media, transformants were obtained after addition of peptide and vector for 1 hour. This was apparent especially for B196 and M250 with transformation frequencies of 10^{-3} and 10^{-2} respectively. Upon washing the cells with fresh THY media, the activation of competence could have been caused by a change in conditions (such as pH) or growth as the cells are no longer in stationary phase.

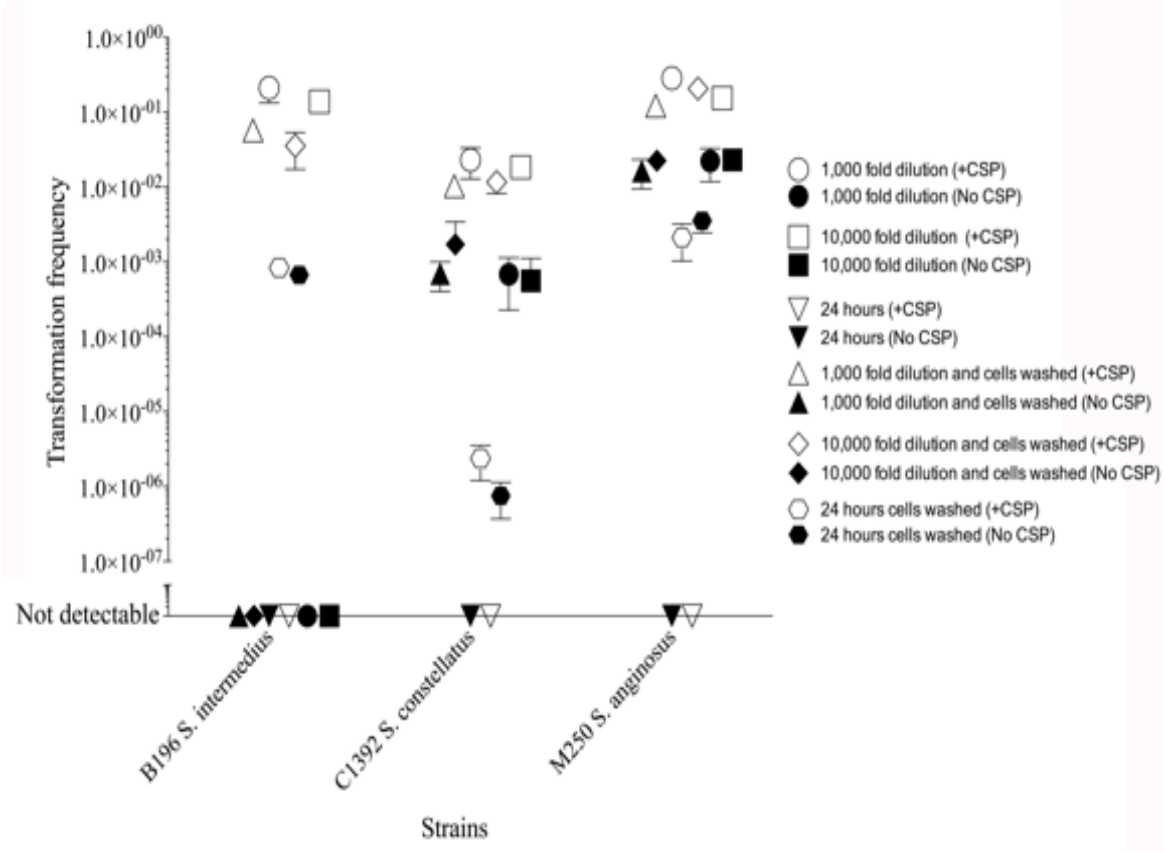

Figure 3.6 Investigation of high endogenous competence levels in the SMG. Transformations were carried out with 20 ng/ml of CSP and/or 1 µg/ml of pDL278 vector at 24 hours and during early log phase. At 24 hours, cells were washed with fresh THY medium and diluted 1,000 or 10,000 fold or directly diluted without washing and grown for 2 hours. CSP and vector were also added to cells at 24 hours directly or after washing with fresh THY medium. Transformations were carried out for 1 hour in a 5% CO_2 incubator at 37^{o}C and frequencies were calculated as follows: (transformants/ml)/total number of bacteria (cfu/ml). Figure shows standard error of the mean from three independent experiments.

3.3.2 Co-culture assays

To investigate if the high level of competence in some of the SMG strains was due to constitutive CSP production, we looked for cross-activation between strains with high endogenous competence levels (C1392, C1391 and M431) and B196, a strain with no observable competence induction in the absence of CSP A. For the co-culture assays, selection was carried out with the differing antibiotic resistances of the chosen clinical strains. In experiment A, strains were transformed individually for comparison of growth and transformation frequencies to values obtained in co-culture. In experiment B, equal amounts of the two strains were grown together after the 1,000 fold dilution before the 1 hour incubation with CSP and/or vector. In experiment C, the two strains were grown separately for 2 hours after the 1,000 fold dilution and added together for the 1 hour incubation with CSP and/or vector (**Figure 2.2**).

No transformants of B196 were recovered in co-culture unless exogenous CSP A was added (**Table 3.4**). These findings were the same irrespective of whether strains were grown together after the 1,000 fold dilution (Experiment B) or grown separately and then added together for the 1 hour incubation with CSP and vector (Experiment C). Transformation frequencies for B196 decreased in co-culture in the presence of added CSP and this was not found to be a result of one strain inhibiting the growth of the other. B196 (erythromycin resistant) and M431 (tetracycline resistant) could be recovered on selective media even in co-culture. Similar colony forming units per milliliter on THY agar with erythromycin were also obtained for B196 grown individually as in co-culture with C1392 or C1391 (**Table 3.5**). These results suggest that the high levels of

endogenous competence observed in C1392, C1391 and M431 is due to high levels of expression of competence genes. If C1392, C1391 and M431 were producing saturating amounts of CSP we reasoned that B196 would become competent in the presence of these strains. Since B196 did not become competent in co-culture, these results suggest that the high baseline levels observed are independent of CSP.

Table 3.4 Transformation frequencies for the co-culture and monoculture assays

		Transformation frequencies					
		Ery^R and Spc^R		Tet^R and Spc^R		Spc^R	
Experiment	Strains	**+CSP**	**No CSP**	**+CSP**	**No CSP**	**+ CSP**	**No CSP**
A	B196 (Ery^R)	0.2 ± 0.06	None	-	-	0.2 ± 8 x 10^{-2}	None
	C1392	-	-	-	-	4 (± 2) x 10^{-2}	3 (± 2) x 10^{-3}
	C1391	-	-	-	-	5 (± 3) x 10^{-1}	2 (± 1) x 10^{-3}
	M431 (Tet^R)	-	-	6 (± 3) x 10^{-2}	0.001 ± 5 x 10^{-4}	8 (± 2) x 10^{-2}	3 (± 1) x 10^{-3}
B[c]	B196 + C1392	5 (± 1) x 10^{-2}	None	-	-	4 (± 2) x 10^{-2}	0.002 ± 4 x 10^{-4}
	B196 + C1391	5 (± 4) x 10^{-2}	None	-	-	2 (± 2) x 10^{-1}	0.002 ± 6 x 10^{-4}
	B196 + M431	3 (± 1) x 10^{-4}	None	3 (± 4) x 10^{-2}	2 (± 1) x 10^{-3}	8 (± 3) x 10^{-2}	0.001 ± 2 x 10^{-4}
C[d]	B196 + C1392	6 (± 1) x 10^{-2}	None	-	-	0.05 ± 3 x 10^{-3}	0.004 ± 3 x 10^{-4}
	B196 + C1391	7 (± 6) x 10^{-2}	None	-	-	3 (± 3) x 10^{-1}	0.002 ± 7 x 10^{-5}
	B196 + M431	7 (± 5) x 10^{-4}	None	3 (± 1) x 10^{-2}	0.0008 ± 6 x 10^{-5}	4 (± 2) x 10^{-2}	0.002 ± 5 x 10^{-4}

[c]Strains were grown together after the 1,000-fold dilution for 2 hours.
[d]Strains were grown separately for 2 hours after dilution then combined before CSP and vector addition.
This experiment was completed in triplicate and transformation frequencies were calculated as follows: transformants/total number of bacteria ± SEM. For strains where no transformants were obtained ("None"), the minimum detection level is between 10^{-2} and 10^{-3}. The '-' indicates that the cultures were not plated on the indicated selective medium as no transformants were expected.

Table 3.5 Cfu/ml for the co-culture and monoculture assays

		cfu/ml					
		Erythromycin		Tetracycline		No selection (THY agar)	
Experiment	Strains	**+ CSP**	**No CSP**	**+ CSP**	**No CSP**	**+ CSP**	**No CSP**
A	B196 (Ery^R)	7 (± 2) x 10^4	5 (± 1) x 10^4	No growth	No growth	$1x10^5 \pm 6x10^4$	6 (± 3) x 10^4
	C1392	No growth	No growth	-	-	3 (± 1) x 10^5	$2x10^5 \pm 8x10^4$
	C1391	No growth	No growth	-	-	9 (± 3) x 10^4	$1x10^5 \pm 3x10^4$
	M431 (Tet^R)	No growth	No growth	5 (± 2) x 10^6	4 (± 1) x 10^6	4 (± 1) x 10^6	6 (± 2) x 10^6
B[c]	B196 + C1392	4 (± 1) x 10^4	$2x10^4 \pm 8x10^3$	-	-	$1x10^5 \pm 5x10^4$	$2x10^5 \pm 8x10^4$
	B196 + C1391	$2x10^4 \pm 3x10^3$	$2x10^4 \pm 5x10^3$	-	-	7 (± 1) x 10^4	9 (± 2) x 10^4
	B196 + M431	$2x10^4 \pm 4x10^3$	$2x10^4 \pm 7x10^3$	$3x10^6 \pm 5x10^5$	$3x10^6 \pm 9x10^5$	$3x10^6 \pm 6x10^5$	$3x10^6 \pm 7x10^5$
C[d]	B196 + C1392	$3x10^4 \pm 6x10^3$	$3x10^4 \pm 9x10^3$	-	-	$1x10^5 \pm 5x10^4$	$2x10^5 \pm 6x10^4$
	B196 + C1391	$2x10^4 \pm 1x10^3$	$1x10^4 \pm 2x10^3$	-	-	$5x10^4 \pm 5x\ 10^3$	$1x10^5 \pm 3x10^4$
	B196 + M431	$3x10^4 \pm 3x10^3$	$3x10^4 \pm 6x10^3$	4 (± 2) x 10^6	$3x10^6 \pm 5x10^5$	5 (± 2) x 10^6	$3x10^6 \pm 7x10^5$

[c]Strains were grown together after the 1,000-fold dilution for 2 hours.
[d]Strains were grown separately for 2 hours after dilution then combined before CSP and vector addition.
This experiment was completed in triplicate and the cfu/ml ± SEM values were calculated.
The '-' indicates that no growth was expected as the strains tested are not resistant to the indicated antibiotics.

3.2.3 Preliminary experiments with *comC* and *comCDE* knockout mutants

Results from the co-culture assay suggested that competence could be independent of CSP in the strains with high transformation frequencies in the absence of added peptide. To further investigate, we created *comC* and *comCDE* knockouts in an *S. constellatus* strain (C1392) with high endogenous competence levels. We reasoned that if the high endogenous competence levels observed in C1392 are CSP independent then a *comC* knockout and a *comCDE* knockout in the presence of a *comDE* complementation vector, will be transformable with efficiencies similar to those of the wild type strain. For comparison, *comC* and *comCDE* knockouts were also created in an *S. intermedius* strain (B196), which does not become competent without added CSP. However, B196's resistance to a number of antibiotics prevented further experimentation with the knockout strains. Transformations were therefore only undertaken with the C1392 wild type strain and knockout strains in the presence of the pAM2 vector or genomic DNA from B196 (Ery^R) and M431 (Tet^R).

For the C1392 wild type strain, no transformants were obtained with genomic DNA and thus conditions will need to be optimized for this assay. Only results for transformations with M431 genomic DNA are shown (**Table 3.6**). On the contrary, C1392 was successfully transformed with pAM2. However, transformation frequencies with pAM2 were lower in comparison to pDL278, the vector used for all the other DNA uptake assays. As the knockout strains were created with the spectinomycin marker from pDL278, transformations could no longer be carried out with this vector. In the C1392 wild type strain, transformation frequencies were only 10 fold lower with pAM2 than

pDL278. Although endogenous competence levels were also lower with pAM2, these could still be measured. We observed a 100 fold difference between transformation frequencies with and without CSP in the presence of pAM2.

One apparent difference between the wild type strain and knockouts is that the latter had 10 fold lower cfu/ml values. Furthermore, no transformants were obtained for the *comC* and *comCDE* knockout strains in the presence of pAM2 or genomic DNA. This was to be expected for the entire operon knockout. On the other hand, transformants for the *comC* knockout were expected in the presence of added exogenous CSP. As *comC* is part of an operon, it is likely that polar effects on *comDE* could have resulted in the lack of transformability in this strain. To account for this likely effect, the pAM2DE complementation vector was electroporated into the *comCDE* knockout strain. The *comCDE* knockout strain was chosen instead of *comC* to carry out experiments in the same genetic background. Furthermore it was thought that complementation in the *comCDE* knockout would be easier due to likely polar effects in the *comC* knockout. As the complementation strain is spectinomycin and erythromycin resistant, transformations were carried out with genomic DNA from a tetracycline resistant SMG strain (M431). No transformants were obtained for both C1392 and the complemented strain.

These preliminary experiments were an important starting point for investigating if competence is CSP independent in C1392. Further experimentation will be needed to optimize transformations with the mutants and a *comCDE* complementation strain will also need to be created.

Table 3.6 Transformations with C1392 wild type and knockout strains

	CSPA + pDL278		pDL278 (No CSP)		CSPA + pAM2		pAM2 (No CSP)		CSPA + M431 gDNA		M431 gDNA (No CSP)	
Strain	cfu/ml	Spc^R	cfu/ml	Spc^R	cfu/ml	Ery^R	cfu/ml	Ery^R	cfu/ml	Tet^R	cfu/ml	Tet^R
C1392	$3x10^5 \pm 6x10^4$	$6 (\pm 2) \times 10^{-2}$	$4x10^5 \pm 6x10^4$	$1x10^{-3} \pm 8x10^{-4}$	$3x10^5 \pm 2x10^4$	$1x10^{-3} \pm 1x10^{-4}$	$4x10^5 \pm 6x10^4$	$5 (\pm 2) \times 10^{-5}$	$6x10^5 \pm 2x10^4$	None	$2x10^6 \pm 4x10^5$	None
C1392 Δ*comC*	-	-	-	-	$2x10^4 \pm 4x10^3$	None	$1x10^4 \pm 4x10^3$	None	-	-	-	-
C1392 Δ*comCDE*	-	-	-	-	$2x10^4 \pm 3x10^3$	None	$4x10^4 \pm 6x10^3$	None	-	-	-	-
C1392 Δ*comCDE* + *comDE* complement	-	-	-	-	-	-	-	-	$1x10^5 \pm 6x10^3$	None	$6x10^4 \pm 3x10^3$	None

Transformation frequencies and cfu/ml were calculated from three independent experiments and ± SEM values are shown. For strains where no transformants were obtained ("None"), the minimum detection level is between 10^{-4} and 10^{-6}.
The '-' indicates that the cultures were not plated on the indicated medium.

3.4 Heterogeneity in the N-terminus of ComD

A comparison of ComD receptors from closely related streptococci revealed that the first 60-100 amino acids are heterogeneous and that this region is likely involved in determining CSP specificity (J.-P. Clarveys & Håvarstein, 2002; L. S. Håvarstein, Gaustad, P., Nes, I. F. and Morrison, D. A., 1996). As receptor and ligand are likely to co-evolve (O. Johnsborg & Håvarstein, 2009), we were interested in investigating CSP group and ComD complementarity. We were able to investigate sequence differences in the N-terminus of ComD since sequencing of *comC* extended into the start of *comD*. Through sequence alignments, ComDs were found to be complementary to their CSPs. Of interest within one ComD sequence, was a three amino acid deletion that was identified in all *S. constellatus* subspecies *pharyngis* strains (n=33). Of the *pharyngis* strains tested, none could be transformed with their cognate CSP. It was therefore hypothesized that a mutation in ComD could have affected their transformability. We further explored whether the competence pathway was still functional in these strains by measuring *comX* expression in two *pharyngis* and two non-*pharyngis* strains.

3.4.1 Variability in the N-terminus of ComD within each CSP group

Quality sequence for 5' end of *comD* was obtained for 157 SMG with primers specific for the conserved transfer RNAs flanking the *comCDE* operon. For sequence comparisons, ComDs were trimmed to 97 amino acids. Sequence alignments revealed that the N-terminal regions of ComD are mostly conserved within each CSP group with some species-specific amino acid substitutions and deletions (**Figure 3.7**). Only unique

ComDs from each CSP group were used for comparison. The frameshift peptides were therefore excluded since CSP D and E have the same ComD sequences as *S. constellatus* strain C1366 (CSP group B) and *S. anginosus* strain C238 (CSP Group C), respectively. This was the opposite of what would be expected since CSP D is a frameshift variant of CSP C and CSP E is a frameshift variant of CSP B. Nevertheless, CSPs B and C could still equally induce competence in the only strain from Group D (**Table 3.3**).

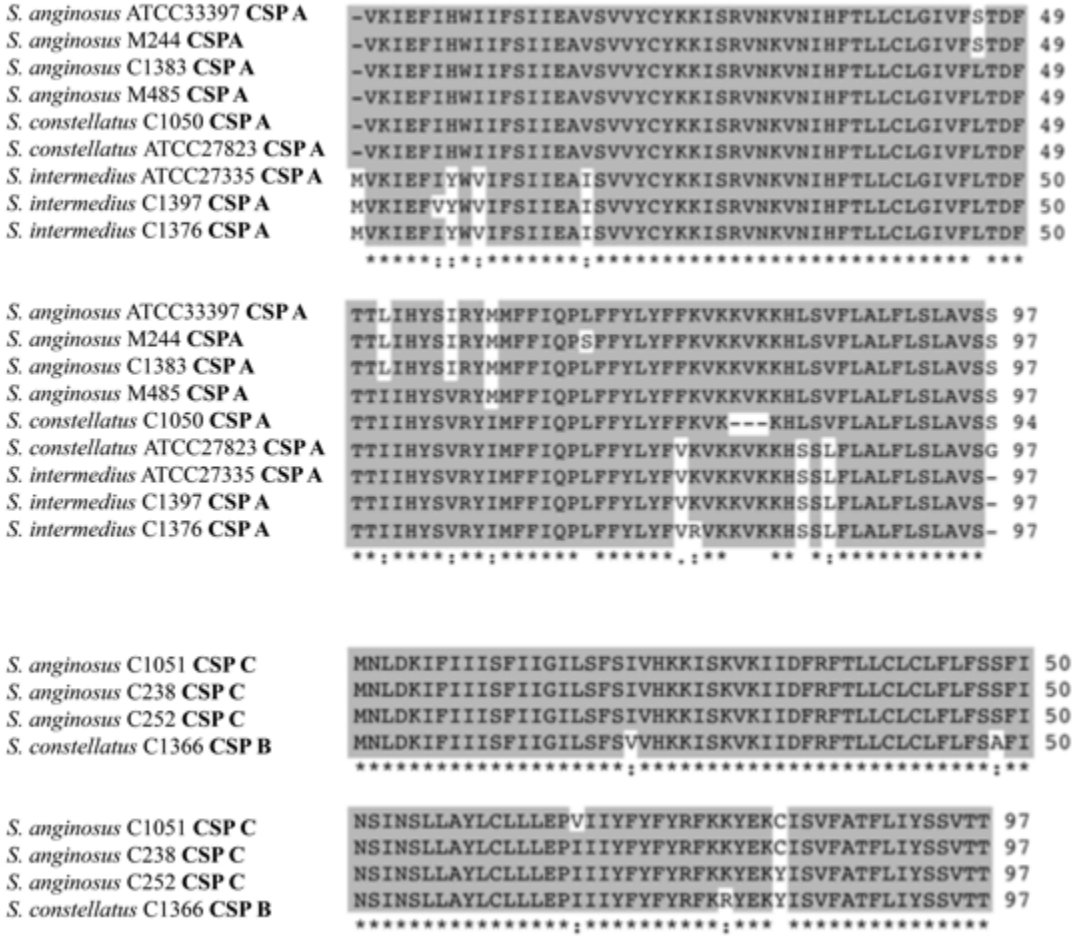

Figure 3.7 ClustalW alignments of unique ComDs from CSP groups A, B and C (in bold). CSP D and E have the same ComD sequences as *S. constellatus* strain C1366 (CSP Group B) and *S. anginosus* strain C238 (CSP Group C) respectively.

3.4.2 The N-terminus of ComD and *comCDE* pathway in the *pahryngis* strains

Another interesting observation from the ComD alignments was a Lysine-Valine-Lysine deletion after amino acid 77 of C1050's histidine kinase (**Figure 3.7**). We observed that all *S. constellatus* subspecies *pharyngis* isolates (n=33) have this same deletion that is not present in the *S. constellatus* subspecies c*onstellatus*, *S. intermedius* or *S. anginosus* strains (**Figure 3.8**). The observed deletion warranted further analysis due to the importance of the N-terminus of ComD for receptor-ligand interactions. We had previously noted that one of these strains (C1050) could not be transformed with exogenous peptide (Grinwis and Surette, unpublished data). To explore this further, we added synthetic CSP A (200 ng/ml) and 1 μg/ml of pDL278 plasmid to six representative *pharyngis* isolates (C1050, C232, C256, C266, M21 and M290) and six non-*pharyngis* isolates (M11, M245, M54R, C1371, C1393, C1387 and B196) grown in rich medium (THY). The six *pharyngis* strains tested could not be transformed with their cognate CSP, whereas most of the *Streptococcus constellatus* subspecies *constellatus* strains became competent with the exception of M245 (**Table 3.5**).

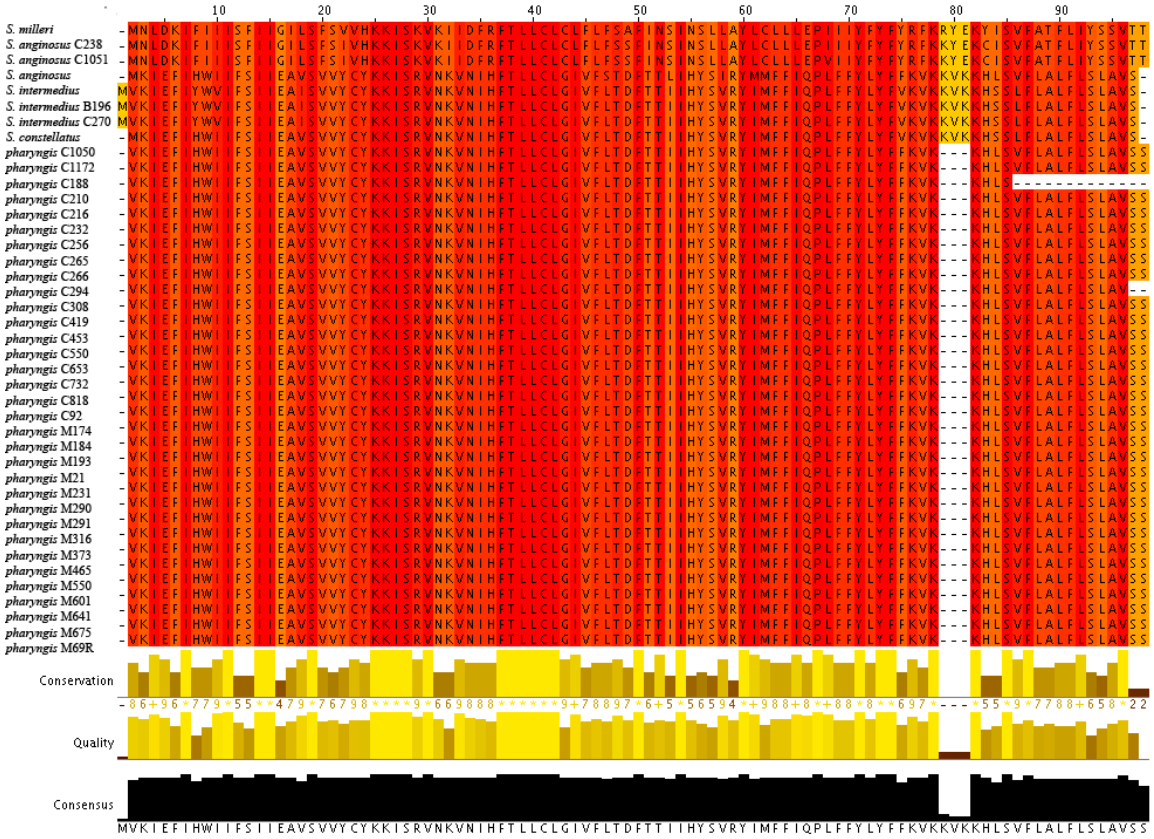

Figure 3.8 Alignments with the first 97 amino acids of ComD viewed with Jalview 2.8. SMG include the *pharyngis* strains (n=33), strains from Håvarstein, et al. (1997) (*S. anginosus* NCTC 10713, *S. constellatus* NCTC 11325, *S. milleri* NCTC 10708, *S. intermedius* NCDO 2227) and B196 (*S. intermedius*), C270 (*S. intermedius*), C1051 (*S. anginosus*) and C238 (*S. anginosus*). Nucleotides are colored by conservation.

TABLE 3.7 Transformation frequencies of *pharyngis* versus non-*pharyngis* SMG

	Transformation frequencies	
Strain	CSP A	No peptide
S. constellatus subspecies *pharyngis*		
C1050	No transformants	No transformants
C232	No transformants	No transformants
C256	No transformants	No transformants
C266	No transformants	No transformants
M21	No transformants	No transformants
M290	No transformants	No transformants
S. constellatus subspecies *constellatus*		
M11	0.01 ± 0.002	3 (±1) x 10^{-5}
M245	No transformants	No transformants
M54R	1 (± 1) x 10^{-1}	2 (± 2) x 10^{-4}
C1371	7 (± 1) x 10^{-3}	7 (± 7) x 10^{-6}
C1387	3 (± 3) x 10^{-1}	1 (± 1) x 10^{-2}
C1393	5 (±3) x 10^{-6}	No transformants
S. intermedius		
B196	0.1 ± 0.03	6 (±3) x 10^{-5}

Transformation frequencies were calculated as follows: transformants (transformants/ml)/total number of bacteria (cfu/ml) ± SEM. This experiment was repeated three times. For strains that could not be transformed, the minimum detection level is between 10^{-2} and 10^{-5}.

3.4.3 Real-time PCR to measure *comX* induction

To further investigate whether ComD was causing the lack of transformability in the *pharyngis* strains, real-time PCR was carried out to measure *comX* expression. ComX is a sigma factor which is involved in regulating the late *com* genes, some of which are important for natural transformation (J.-P. Clarveys & Håvarstein, 2002). We reasoned that if the deletion in ComD reduces CSP binding, there would be lower expression of *comX* in the *pharyngis* strains and this would not be induced by addition of exogenous peptide. In order to test this, cell cultures were grown to early-logarithmic phase in THY and RNA was collected at 0, 5, 10 and 20 minutes after addition of 200 ng/ml of CSP A. Uninduced cells were used as a reference and were assigned a value of 1. Interestingly, the *pharyngis* strains had higher CSP dependent *comX* expression than the non-*pharyngis* strains (**Figure 3.8**). This was noticeable in C232 with a 95-fold increase in gene expression at 10 minutes after CSP addition. This increase in expression was higher than that seen in the other three SMG strains tested as well as what has been reported for *S. pneumoniae* (S. Peterson, Cline, Tettelin, Sharov, & Morrison, 2000). In the non-*pharyngis* strains, B196 and C1392, *comX* expression was maximal at 5 minutes both reaching 5-fold increases. On the other hand, maximal *comX* expression was variable in the *pharyngis* strains reaching a 95-fold increase at 10 minutes for C232 and 16-fold increase at 20 minutes for C1050. This data suggests that the deletion in ComD in the *pharyngis* strains does not affect the response to the CSP and activation of the response regulator ComE.

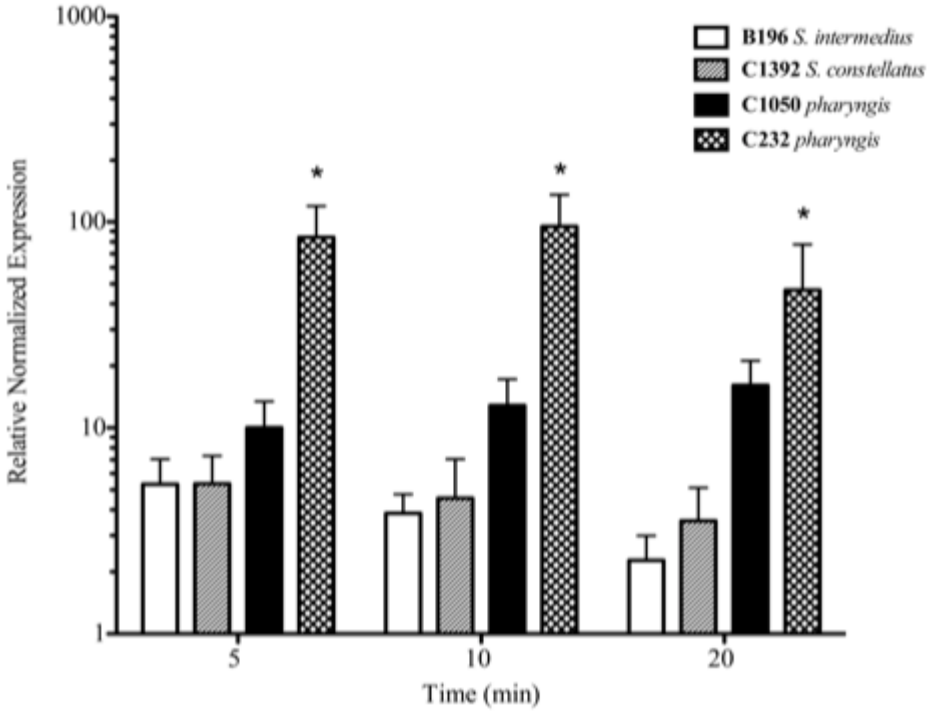

Figure 3.8 *comX* expression in the *pharyngis* and non-*pharyngis* SMG strains. Expression is relative to 0 minutes and normalized to the *16S* rRNA and *ldh* reference genes. The means ± SEM of three experiments are shown. A One-Way ANOVA was carried out to compare *comX* expression across the bacterial strains at each time point, *$p<0.05$.

CHAPTER 4: DISCUSSION AND CONCLUSION

The present study aimed to give new insights on competence in the *Streptococcus* Milleri/Anginosus group (SMG), members of which are considered commensal of the upper respiratory tract, gastrointestinal and urogenital tracts but have also been associated with disease. It is not clear what causes the shift from a commensal to a pathogenic lifestyle. Natural competence for genetic transformation could contribute to the dissemination of virulence and antibiotic resistance genes as this has been shown to occur in other streptococci (Dowson et al., 1989; Eran et al., 2007; Sibold et al., 1994). In many streptococci including the SMG, competence is regulated by a secreted CSP encoded by *comC* and a two-component system (ComDE). Natural transformation has been extensively described in the Mitis group, with *S. pneumoniae* being the first species studied (Griffith, 1928). However, competence has been less well characterized in the SMG.

To study competence in the SMG, I investigated variability in *comCD* since this region is important for receptor-ligand interactions. ComD receptors have been known to discriminate between CSPs (L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997) so I was interested in the specificity of the SMG peptides for their receptors. Through CSP specificity studies, I found that several strains in our collection had high levels of competence in the absence of added peptide. Preliminary experiments were undertaken to investigate if the high endogenous competence levels were CSP dependent. Although a number of strains in our collection were highly competent, others remained non-transformable. In particular, the *S. constellatus* subspecies *pharyngis* strains were non-

transformable despite having an intact *comCDE*. A mutation in the *comEA* DNA uptake gene in 2 of our sequenced SMG genomes could be affecting transformability in the *pharyngis* strains (Olson et al., 2013).

This is the first study to look at variation in *comCD* and transformation rates across a large collection of clinical SMG isolates. Heterogeneity in the CSPs, variability in transformation rates and future directions are discussed.

4.1 Heterogeneity in the *comCD* of the SMG

Sequencing of a large number of *comC* alleles from different streptococcal strains and species has revealed that there is a lot of CSP diversity, especially within the Mitis group (O. Johnsborg, Blomqvist, Kilian, & Håvarstein, 2007; Pozzi et al., 1996; Whatmore, Barcus, & Dowson, 1999). CSP diversity has not been extensively investigated in the SMG with only four strains studied thus far (L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997). I therefore amplified and sequenced CSPs associated with *Streptococcus anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius* from our collection of invasive and CF patient isolates. In particular, I was interested in the central regions of the CSPs as these are variable and likely determine specificity (Whatmore et al., 1999). Most of the SMG clinical isolates tested possessed *comC*, which encodes the mature CSP. A PCR product for *comC* was obtained for 167 of the 170 strains. Three *comC* sequences could not be obtained due to potential insertions in different locations across *comCDE*. Of the recovered CSP sequences, five SMG peptides were observed, two of which were previously characterized by Håvarstein et al. (1997)

(CSP's A and C) and 3 novel CSP sequences (CSP's B, D and E).

In keeping with previous observations, the SMG CSPs are cationic with a negatively charged N-terminus, a positively charged C-terminus and a double glycine leader (L. S. Håvarstein, Diep, & Nes, 1995; L. S. Håvarstein, Holo, & Nes, 1994). Due to frameshift mutations caused by a deletion of adenine at position 42 in the nucleotide sequences of CSPs D and E, these novel peptides are longer than expected. However, they were only identified in three strains with one strain possessing CSP D and two strains with CSP E. The majority of SMG possess CSP A (87%) and approximately 10% possess CSPs B and C, the latter two peptides differing by only a single amino acid substitution (methionine to isoleucine at position 8). These findings conform with studies in *S. pneumoniae* and *S. mutans*, which found one CSP sequence to be more common than other variants (Allan et al., 2007; Whatmore et al., 1999).

In comparison to the Mitis streptococci, diversity in the SMG CSPs was low. However, more diversity was identified than previously reported (L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997). This diversity could be a result of genetic exchanges within the SMG and with other streptococci or an accumulation of point mutations (L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997; O. Johnsborg & Håvarstein, 2009). Random mutations are more likely to be selected against due to the complementarity between the CSP and ComD with changes in one resulting in changes in the other. Furthermore, mutations causing amino acid changes can affect CSP functionality (O. Johnsborg & Håvarstein, 2009). This seems to have been the case for D

and E CSPs. No changes in the N-terminus of ComD were observed when compared to those of strains in groups B and C. However, a single nucleotide deletion in D and E CSPs caused them to be non-functional frameshift variants of C and B peptides, respectively.

The single amino acid substitution difference between CSPs B and C could have been acquired through horizontal gene transfer or mutation. Through horizontal gene transfer, it is possible that *comC* and *comD* could have been acquired with single nucleotide polymorphisms. On the other hand, it is equally likely that the nucleotide difference between B and C peptides could be the result of a mutation. As the substitution does not affect functionality, it can be considered a neutral mutation that may have occurred through drift in the nucleotide sequence. Interspecies recombinational exchanges through natural transformation are likely to have led to the differences between CSPs A and B/C. The neighbor-joining tree revealed that CSP A is more similar to CSPs from a Mitis group strain (*S. oralis*) and to a non-speciated *Streptococcus* strain than to the other four SMG peptides. This suggests that Mitis group strains could be a possible source of DNA for recombination in the SMG. Given some sequence similarity, it would be interesting to investigate if CSPA could cross-activate competence in certain Mitis group strains.

4.2 Cross-activation with the 5 SMG CSPs

Different streptococcal strains and species produce CSPs with varying sequences. This restricts communication to streptococci that can recognize and respond to a certain

CSP (O. Johnsborg et al., 2007). Although CSPs are specific to their ComD receptors, there are cases of non-cognate CSPs being sensed by a non-producer strain (O. Johnsborg & Håvarstein, 2009). In one reported case, a pneumococcal R6 strain was able to sense and respond to two *Streptococcus mitis* CSPs (O. Johnsborg et al., 2008). Although cross-activation studies with CSPs from different streptococcal groups is intriguing, our focus was on investigating CSP specificity with strains from the five identified SMG groups.

To gain insight into the specificity of the five SMG CSPs, synthetic peptides A, B, C and E were used to induce competence and allow for transformation in the presence of a plasmid. Consistent with previous findings by Håvarstein et al. (1997), CSPs from Groups A and C did not cross-activate. Two functional CSP groups were identified in this study: 1) CSP A and 2) CSPs B/C. CSPs B and C could cross-react, most likely due to a single amino acid difference between the two peptides. Due to a lack of sequence similarity, CSP A could not cross-activate strains in CSP groups B and C. A CSP D was not tested but B and C peptides could activate competence in the only strain in this group. Cross-activation was not undertaken with CSP D since it only differs from CSP E by one amino acid substitution and both are longer frameshift peptides. It is likely that CSP D would also be non-functional as observed for CSP E, which was unable to induce competence within its own group or in any of the SMG strains tested.

The N-terminus of the CSP is known to be important for determining its activity and specificity (O. Johnsborg, Kristiansen, Blomqvist, & Håvarstein, 2006). Although the N-terminus of CSP E and D are the same as active CSP B and C respectively, the

frameshift mutations cause CSP E and D to be 26 amino acids in length instead of 17. This could hinder protein folding and ultimately binding to the ComD receptor. In *S. pneumoniae*, the CSP adopts an amphiphilic α-helical conformation in the presence of membrane mimicking environments (O. Johnsborg et al., 2006). This conformation could potentially be disrupted in the frameshift peptides, preventing binding to ComD. Alternatively, the longer frameshift peptides could be binding to ComD and not activate the competence pathway.

4.3 High endogenous competence levels in several SMG strains

Interestingly, cross-activation studies revealed that several strains in our collection are transformable in the absence of added CSP. This high level of endogenous competence was not reduced by dilution or washing of cells, making us question whether competence is always active in these strains. Competence for transformation in the streptococci is not usually constitutive as observed for *Neisseria* species (Solomon & Grossman, 1996). In one reported case of constitutive competence, a mutation was identified in the carboxyl terminal of ComD in *S. pneumoniae*. This resulted in competence occurring at low cell densities even in the presence of proteases that attack the CSP and at low pH, a growth condition that is non-conducive for DNA uptake (Lacks & Greenberg, 2001). This study seems to have been the exception as most investigations on DNA uptake in the streptococci have reported competence as a transient property (O. Johnsborg & Håvarstein, 2009; Morrison, Guédon, & Renault, 2013; Piotrowski, Luo, & Morrison, 2009). Nevertheless, we were interested in determining whether competence

could be constitutive in SMG strains with high endogenous competence levels.

Competence during stationary phase was investigated in two SMG strains with high endogenous competence levels and one strain with no detectable competence induction without added CSP. Bacterial cells in stationary phase were not competent unless they were re-suspended in fresh culture medium. A change in growth condition such as pH or washing the cells with new media, could be necessary to activate the competence pathway in the absence of CSP or during stationary phase when the conditions for competence induction are unfavorable. A change in pH is known to affect competence in *S. pneumoniae* independent of cell density (J.-P. Clarveys, Prudhomme M. and Martin B, 2006). Under the conditions tested with added CSP, the results obtained suggest that competence in the SMG occurs throughout active growth.

As competence was determined not to be a constitutive state in the strains with high endogenous competence levels, we wanted to know whether these strains were producing saturating amounts of CSP during early to mid log phase. To investigate this, a strain that is non-transformable without added CSP (B196 under the growth conditions of our experiments) was grown with strains that exhibited high endogenous competence levels (M431, C1392 and C1391). In theory, if saturating levels of CSP were being produced by M431, C1392 and C1391, then B196 should become competent in co-culture and take up added plasmid. This was not found to be the case and the results obtained suggest that M431, C1392 and C1391 already have a high basal activation of *comCDE* during exponential growth that is independent of CSP production. One interesting finding

was that transformation frequencies decreased in co-culture especially for B196 in the presence of M431. This decrease in transformation frequency could not be explained by a change in growth since similar cfu/ml were obtained in co-culture and in monoculture. As the strains tested all respond to CSP A, this reduced transformation frequency could be caused by proteases degrading CSP A in co-culture. If competence is CSP-independent in M431, the presence of proteases would only affect competence in B196.

Thus, one theory on the high endogenous competence levels is that competence could be independent of CSP in these strains. Another theory is that the strains with high endogenous competence levels could be producing local concentrations of CSP that do not diffuse into the medium to activate competence in B196. It is often assumed that cells in a particular population, will always sense nearby signals. Youk and Lim (2014) demonstrate that in yeast cells, self-communication occurs in the presence of signals from other cells. The authors attribute this phenomenon to the incomplete mixing of the culture medium resulting in a local environment surrounding each cell. At low cell densities in the absence of mixing, the local concentration of a signal is high so that each cell senses its own signal and not that of neighboring cells (A. J. Lee & You, 2014). In the SMG which are known to form aggregates, it is possible that during early log phase at a relatively low cell density, a higher local concentration of CSP could surround the endogenously competent strains. Under these circumstances, communication with B196 would be less likely to occur.

In order to test these interesting theories, *comC* and *comCDE* knockouts were

created for a strain with high endogenous competence levels (C1392) and a strain that is non-transformable in the absence of CSP under the growth conditions used (B196). We reasoned that if competence in C1392 were CSP-independent, a *comC* knockout as well as a *comCDE* knockout complemented with *comDE* would have similar transformation frequencies with and without added CSP. In the knockout strains, competence induction was not detected as plasmid DNA was not taken up. For the *comC* mutant this could be due to polar effects to *comDE*. As the SMG are resistant to a number of different antibiotics, we were limited in what could be chosen for the transformations. Genomic DNA would be an easy solution and has been used in the original transformation studies with the SMG (L. S. Håvarstein, Hakenbeck, R. and Gaustad, P., 1997). As no transformants were obtained with genomic DNA from the SMG, conditions will need to be optimized. Further experimentation will therefore be required to ascertain which theory is more accurate in explaining the high levels of endogenous competence.

4.4 The *pharyngis* strains have an intact comCDE pathway

Many SMG strains in our collection could be transformed in the absence of added CSP, whereas other strains remained non-transformable. Although competence genes are thought to be widespread amongst the streptococci, many strains remain non-transformable in the laboratory. This lack of transformability could be a result of mutations or non-ideal growth conditions (Mashburn-Warren et al., 2012). Of note, the *Streptococcus constellatus* subspecies *pharyngis* strains could not be transformed with their cognate CSP. For all the *pharyngis* strains in which quality *comD* sequence could be

obtained, sequence alignments revealed a 3 amino acid deletion in the N-terminus of the histidine kinase. As the N-terminus of ComD is involved in binding to the CSP, we hypothesized that the deletion could be affecting the transformability of the *pharyngis* strains. To test this hypothesis, we investigated whether the ComDE signaling system was still intact in the *paryngis* strains by measuring *comX* expression. The *comX* sigma factor is downstream to ComDE but upstream of DNA uptake genes. To our surprise, *comX* expression was higher in the two *pharyngis* strains tested than in non-*pharyngis* strains. These results suggested that the *comCDE* pathway is intact in the *pharyngis* strains and that it is likely downstream genes to *comX* that are affected. In two of our sequenced *pharyngis* genomes, a frameshift mutation was identified in *comEA* (Olson et al., 2013), which is a membrane receptor involved in binding incoming DNA (Johnston et al., 2014). Mutations in DNA uptake genes have also been reported to occur in *S. pneumoniae*. In one study, transformability was affected by a mutation in the competence pilus structural gene *comYC* (Croucher et al., 2011). Although mutations in DNA uptake genes are the most probable cause for the lack of transformability I observed, with *comEA* as the most likely candidate, this will need to be further explored with all the *pharyngis* strains.

One interesting question that arose from studies in the *pharyngis* strains is that if *comCDE* is not involved in regulating DNA uptake, what other phenotypes could this pathway be regulating in these strains? Of the >100 genes regulated by *comCDE* in *S. pneumoniae* only approximately 22 are involved in natural transformation and the roles of several other genes remain unknown (S. N. Peterson et al., 2004). The possibility that *comCDE* could be regulating other phenotypes in the *pharyngis* strains is an intriguing

future direction that could be explored.

4.5 Future Directions

Based on the current studies, many future directions could be undertaken. One immediate direction will include completing the experiments with the *comC* and *comCDE* knockout strains to investigate the variability in transformation rates across the SMG. Another future direction could involve investigating whether the CSP modulon regulates phenotypes other than natural transformation in the *pharyngis* strains. Lastly, fratricide is a process that could be explored in the SMG. Competence pathways are co-regulated with proteins that mediate cell lysis through a process termed fratricide. It is thought that this may promote horizontal gene transfer by providing DNA for uptake (O. Johnsborg & Håvarstein, 2009).

4.5.1 Completion of the mutant experiment to investigate endogenous competence

For the mutant experiment, a few additional steps will need to be carried out to determine if the high endogenous competence levels observed are CSP-independent. The *comCDE* operon and its promoter will need to be complemented in the C1392 *comCDE* knockout background. The success of *comCDE* and *comDE* complementation could be investigated in C1392 with a plasmid or genomic DNA from SMG strains carrying a tetracycline resistance marker. If genomic DNA is pursued, different amounts of transforming DNA and growth conditions will be explored. Further experimentation with B196 knockout strains will require the use of ampicillin, penicillin G or tetracycline

resistance markers for complementation and downstream transformations as B196 is resistant to erythromycin, which was used as a selection marker for complementation in C1392.

4.5.2 Does the CSP modulon regulate other phenotypes in the *pharyngis* strains?

Despite having an intact *comCDE*, DNA uptake was not observed for the *S. constellatus* subspecies *pharyngis* isolates with their cognate CSP. We observed significant induction of *comX* in the presence of CSP, suggesting that the *comCDE* pathway could be important in the *pharyngis* strains for regulating phenotypes other than those involved in natural transformation. A comparison could be made between gene expression in a *pharyngis* versus a non-*pharyngis* strain in the presence and absence of CSP to predict and later test potential phenotypes. In a microarray study carried out with *S. pneumoniae* to investigate CSP responsive genes, 188 genes were expressed and only 22 are required for natural transformation. Some of the other genes encode hypothetical proteins, DNA repair proteins, autolysins, heat shock proteins and putative bacteriocins (S. N. Peterson et al., 2004). These genes could be activated as a consequence of competence and may not have direct roles in natural transformation or they may contribute to other phenotypes that rely on the same pathway. If similar genes are present in the *pharyngis* strains, they may not be expressed as these strains are unable to take up DNA.

One method to test this hypothesis would be to carry out RNA-sequencing to investigate gene expression in response to CSP in *pharyngis* and non-*pharyngis* strains

from our collection. As some of our clinical SMG isolate genomes have been sequenced and annotated, RNA-sequencing reads can be mapped back to these genomes. Preparation of RNA has been optimized and RNA-sequencing was carried out on one SMG strain (Michelle Pinto, unpublished data). These established methods would provide a basis for the proposed experiment. Any results obtained will be of interest since global analysis of gene expression in response to CSP has yet to be undertaken in the SMG.

4.5.3 Fratricide in the SMG

In addition to regulating natural genetic transformation, the competent state in the streptococci controls the secretion of muralytic enzymes termed fratricins (Berg, Biornstad, et al., 2012). Studies have shown that the pneumococci compete in co-culture through a process termed fratricide, whereby competent cells attack non-competent sibling cells with muralytic enzymes meanwhile protecting themselves with immunity proteins (Guiral, Mitchell, Martin, & Claverys, 2005). As natural transformation and fratricide are co-regulated, competence mediated cell lysis could promote the acquisition of DNA from other related bacterial species (Berg, Biornstad, et al., 2012). Fratricide involves two processes that include heterolysis and allolysis.(O. Johnsborg & Håvarstein, 2009) (**Figure 4.1**). Of interest is whether inter-CSP group lysis (heterolysis) is more likely to occur than self-lysis (autolysis) or intra-CSP group lysis (allolysis). In other words, will strains/species from two different peptide groups be more likely to lyse themselves than those from the same CSP group.

Due to the large number of existing CSPs in nature, strains/species with differing CSP types are likely to co-exist resulting in mixed populations of competent and non-competent cells for fratricide. In our study, SMG strains with more than one CSP type were found in cystic fibrosis patients. Other studies have identified strains with differing CSP types co-habiting the nasopharynx and middle ear (Brugger, Frey, Aebi, Hinds, & Muhlemann, 2010; Valente, Lencastre, &´-Leao, 2012; Vestrheim, Gaustad, Aaberge, & Caugant, 2011). Although strains/species with different CSPs types can co-exist, there is still debate over whether these restrict recombination (Valente et al., 2012). Some authors suggest that heterolysis would encourage more genetic diversity through inter-CSP group genetic exchanges (Cornejo, McGee, & Rozen, 2010). Others argue that inter-CSP group genetic exchanges are limited so that differentiation of CSP populations occurs preferentially over genetic exchanges (Carrolo, Pinto, Melo-Cristino, & Ramirez, 2009, 2014; J.-P. Clarveys & Håvarstein, 2007).

To uncover whether heterolysis is more or less likely to occur in the SMG than allolylis and/or autolysis, co-culture assays could be undertaken. The LytA, LytC and LytF muralytic enzymes (Berg, Solheim, & Håvarstein, 2012; Eldholm, Johnsborg, Haugen, Ohnstad, & L. S. Håvarstein, 2009) are present in our sequenced SMG genomes, suggesting a potential role for fratricide. Investigating cell lysis could involve propidium iodide assays to measure nucleic acid release in co-culture versus monoculture. Real-time PCR could allow for the measurement of the strain specificity of the DNA release. Results from these experiments would provide novel insights on how the SMG lyse and acquire DNA from similar or related bacteria during co-colonization.

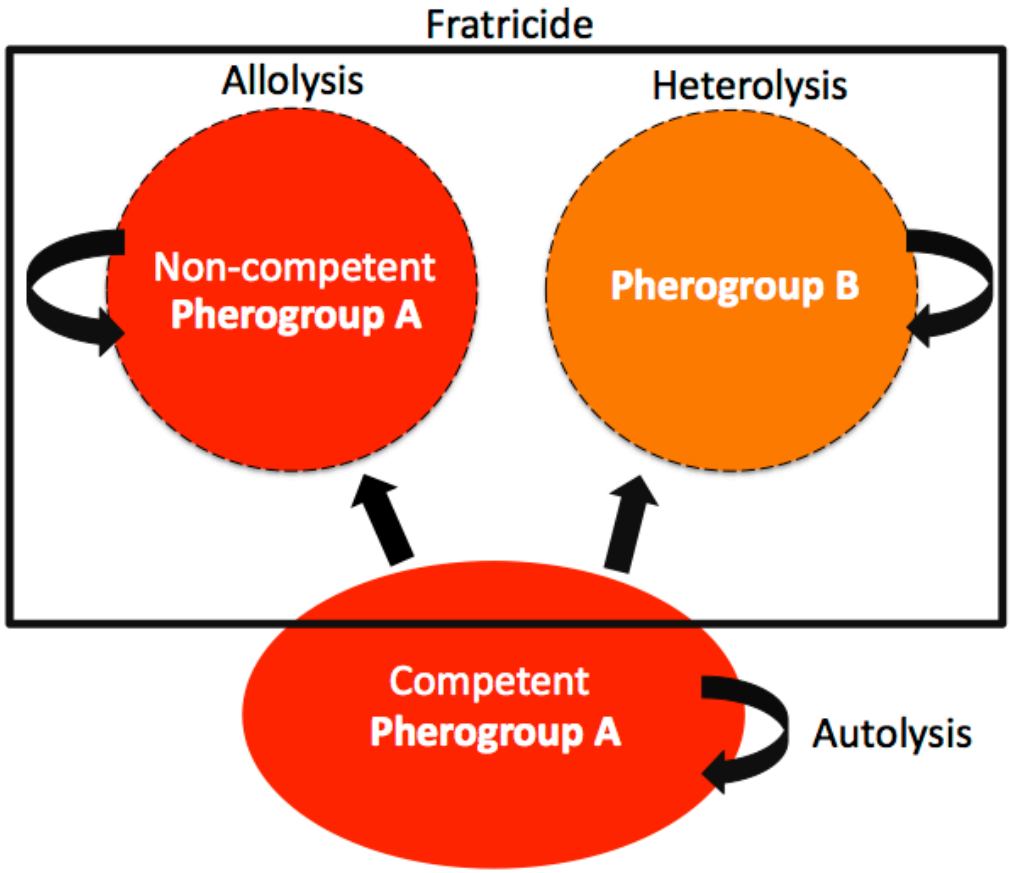

Figure 4.1 Diagram explaining the different mechanisms involved in cell lysis. Allolysis is the lysis of a cell of the same CSP group as the competent attacker cell. Heterolysis is the lysis of a cell of a different CSP group as the competent attacker cell. The red colour represents a bacterial cell of the same species/strain or closely related species of the same CSP group as the competent attacker cell. Orange represents species or strains that respond to a different peptide than the competent attacker cell. Autolysis is the lysis of the competent cell itself.

4.6 Conclusion

Naturally transformable bacteria become competent to take up DNA, which recombines into the bacterial chromosome or becomes digested on entry (Chen & Dubnau, 2004). Transformation can be beneficial by providing nutrients in the form of nucleotides (Redfield, 1993), the DNA taken up could be involved in genome repair (Bernstein, Byers, & Michod, 1981) and/or recombination could be used to adapt to different or changing environments (Didelot & Maiden, 2010; Vos, 2009). In many streptococci, transformation relies on a bacterial communication mechanism consisting of a competence stimulating peptide (CSP) encoded by *comC* and two-component system (ComDE) (O. Johnsborg & Håvarstein, 2009). In this study I sought out to better understand the *comCDE* system in the Milleri/Anginosus group with our collection of cystic fibrosis and invasive SMG clinical isolates. I identified more variability in CSP/ComD than previously reported for the SMG, with three novel peptides characterized (CSPs B, D and E). This diversity could be a result of genetic exchanges within the SMG and with other streptococci or mutation. Through transformation assays in the presence of vector DNA, two distinct CSP populations were uncovered, CSPA and CSP B/C which do not cross-react. CSPs D and E were identified as non-functional variants of C and B respectively.

I found transformation frequencies to be variable across the SMG with some strains having high endogenous competence levels, while others remained non-transformable. Despite having an intact *comCDE* pathway, the *S. constellatus* subspecies

pharyngis strains were non-transformable. A mutation in the DNA uptake gene *comEA* was found in two sequenced *pharyngis* strains (Olson et al., 2013) and this could be affecting competence in these strains. Other strains in our collection had high basal levels of activation of *comCDE* during exponential growth. Diluting the cells did not reduce these high levels of competence. Furthermore in co-culture assays, CSP naturally produced by a highly endogenously competent strain could not activate competence in a strain with no observable transformants without added CSP. These results suggested that the high endogenous competence levels could be CSP-independent but this will need to be further explored.

To summarize, more variability in the CSPs was identified in the SMG with two functional peptide groups characterized. This is the first study to investigate variability in transformation rates across a collection of SMG clinical isolates. Many strains had high levels of competence induction in the absence of added CSP. These high endogenous competence levels could contribute to the SMG's documented resistance to antibiotics including macrolides and clindamycin (Grinwis et al., 2010a), which could render treatment of chronic infections such as CF, more challenging.

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