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PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF GENETIC

COMPETENCE IN *STREPTOCOCCUS SANGUINIS*

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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List of Abbreviations

α	alpha
β	beta
Δ	delta
%	percentage
°C	degrees Celsius
μ l	microliter
μ g	microgram
ABC	ATP-binding cassette
Arg	arginine
ATP	adenosine triphosphate
BHI	brain heart infusion
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
CFU	colony forming units
CHD	congenital heart disease
Cm	chloramphenicol
CSP	competence stimulating peptide
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid

DNase	deoxyribonuclease
DTT	dithiothreitol
Em	erythromycin
EtBr	ethidium bromide
FDR	false discovery rate
Gly	glycine
h	hour
HK	histidine kinase
HS	horse serum
IE	infective endocarditis
kb	kilobase
kDa	kiloDalton
M	molar
MIC	minimal inhibitory concentration
min	minute
ml	milliliter
mM	millimolar
mm	millimeter
mRNA	messenger RNA
MVP	mitral valve replacement
NaOAc	sodium acetate

ng	nanogram
nm	nanometer
O/N	overnight
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PLG	phase lock gel
qRT-PCR	quantitative real-time PCR
QS	quorum sensing
RAD	ramhorn array database
RHD	rheumatic heart disease
RNA	ribonucleic acid
RR	response regulator
rRNA	ribosomal RNA
SAM	significance analysis of microarrays
TH	Todd Hewitt
U	unit

Abstract

PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF GENETIC COMPETENCE IN *STREPTOCOCCUS SANGUINIS*

By Alejandro Miguel Rodriguez, B.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2008

Major Director: Todd O. Kitten, Ph.D.
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The ability of bacteria to assimilate free DNA from the environment is known as competence. Though many studies have focused on competence regulation in *Streptococcus pneumoniae* and *Streptococcus gordonii*, *Streptococcus sanguinis* has yet to be examined. Physiological characterization of competence in *S. sanguinis* strain SK36 and its *comC* mutant, JFP41, led to the genome-wide transcriptional analysis of cells induced to competence via addition of competence-stimulating peptide (CSP). A total of 128 genes were induced at least 2-fold, 74 of which were classified as either “early” or “late” based on their induction patterns. Expression patterns were verified

using qRT-PCR. This study identified genes not up-regulated in *S. pneumoniae* or *S. gordonii* and lays the foundation for bioinformatic studies to identify conserved binding sites upstream from CSP-regulated genes. These results also shed light on the possible existence and identity of expected CSP exporters in *S. sanguinis*, which have so far eluded discovery.

I. INTRODUCTION

A. Streptococci

Over the past century, the *Streptococcus* genus has undergone vast changes in nomenclature and taxonomy. Much of what is known today has occurred through advances in technology. For example, one of the earliest methods to differentiate between streptococcal species was to observe their phenotypic growth on blood agar plates. The observation, first noted in 1903 by Shotmuller (23), allows for categorization of streptococci into three distinct groups based on their hemolytic properties. Alpha-hemolysis consists of a reduction of iron in hemoglobin resulting in a greenish streak across the plate. The beta-hemolytic species are those that lyse red blood cells and present as clear regions upon streaking. Lastly, the gamma-hemolytic group consists of species that show no hemolysis at all.

A major breakthrough in the categorization of the *Streptococcus* genera was first detailed by Lancefield (55) when she described a method of determining and consequently identifying carbohydrate group antigens that were associated with the beta-hemolytic group. In 1937, Sherman (94) grouped the streptococci into distinct groups based on their hemolytic reactions, carbohydrate antigens, and basic metabolism and tolerance tests. The result was the formation of the four groups which many scientists over the years used to categorize new streptococcal species – the pyogenic, lactic, enterococci, and viridans group. The application of 16S rRNA (2, 33) gene sequencing and ribosomal 16S-23S intergenic spacer region sequencing (106) has

allowed researchers to more accurately revise the classification system and create phylogenetic trees, consequently permitting precise measurements of similarity between streptococcal species.

B. Viridans streptococci

The term “oral streptococci” has been used synonymously in the past with that of the “viridans streptococci”, though great care must be taken when using either as a collective term, seeing as they are not always comparable (111). Most viridans streptococci are in fact oral streptococci residing primarily within the human mouth. The oral and viridans streptococci usually colonize hard and soft tissues, and as such have been isolated not only on tooth surfaces in the oral cavity, but also in the gastrointestinal and genitourinary tracts. Although usually existing as benign commensal agents, some oral streptococcal species have been implicated in dental diseases such as periodontitis (27) and dental caries (26).

The mechanism of adherence to teeth has been extensively investigated. Typically, oral streptococci are the first to compete for colonization sites on tooth surfaces (76). Initial adherence by the early colonizers *S. mitis* and *S. sanguinis* onto tooth surfaces is believed to be the critical factor for the onset of dental plaque. It first requires the presence of salivary glycoproteins (72), such as MG2 (25) and α -amylase (1), among others, which are part of the human salivary pellicle that coats the tooth. Murray *et al.* (72) suggested that bacteria that adhere to these glycoproteins are more likely involved in plaque formation. Once these early colonizers establish a permanent niche in the oral cavity, they behave as receptors and ligands for further attachment by

other bacteria, which then aids in stronger adherence to the tooth surface. For example, Hamada *et al.* (34) observed that *S. sanguinis* (known then as *S. sanguis*), when bound by *S. mutans*, adhered much more strongly to glass surfaces. He also observed that upon this heterospecies interaction, *S. sanguinis* produced large volumes of a gelatinous polysaccharide in the presence of sucrose. This product is the basis of dental plaque, or biofilm, which creates an environment that aids in further adherence by other streptococcal species, as well as protection against the host immune response and antibiotics.

C. Streptococcus sanguinis

In 1997, the name *S. sanguis* drew criticism due to it being grammatically incorrect and was consequently changed to *S. sanguinis* (105). One of the better known members of the viridans group, *S. sanguinis* has been shown to be one of the very first colonizers of the human oral cavity (57). It is benign in its native niche and actually thought to be advantageous due to its antagonistic role against the dental caries inducing bacterium, *S. mutans* (63). Furthermore, it was postulated that a high *S. mutans*/*S. sanguinis* ratio may lead to a predisposition for development of dental caries. However, there is no conclusive data to either confirm or deny this hypothesis at the moment.

Although *S. sanguinis* predominates within the oral cavity, it has also been shown to enter the bloodstream. Streptococci within the oral cavity may enter the bloodstream through invasive dental procedures or through the simple act of practicing proper dental hygiene (61, 62, 93). The problem is exacerbated if an individual is

suffering from periodontal disease where the soft tissue surrounding the tooth easily tears. At this point, a streptococcal invasion is easily cleared by the host immune response in a healthy individual. However, in cases in which a host has suffered heart damage, host platelets aggregate on the damaged endothelium resulting in the inflammation of the heart valve due to the formation of a thrombus called a “vegetation.” As has been previously shown (19), *S. sanguinis* may adhere to the vegetation and enlarge it, resulting in a condition known as infective endocarditis.

D. Infective endocarditis

Though quite rare, infective endocarditis (IE) is a life-threatening condition if left untreated. The incidence of IE in the general population per year is somewhere in the range of 1.7 – 7 per 100,000 (7, 39, 101) and seems to affect men (approximately 1.7:1) more so than woman (73). Approximately 42 – 52% of infections are caused by *Streptococcus spp.* (75, 115). The median age for those suffering from IE has increased to approximately 47 – 69 (68, 109) presumably due to proper dental hygienic practices becoming the norm and average life spans increasing due to advances in the medical field thus leading to longer exposure to bacteremia (73). There has been, however, a recent surge in younger patients who have a history of IV drug abuse.

Development of IE requires a preexisting condition of heart damage causing injury to the endothelial surface of the heart valve. Risk factors include rheumatic heart disease (RHD), congenital heart disease (CHD), mitral valve prolapse (MVP) with regurgitation, and cardiac valve replacement (68). Steckelberg and Wilson (95)

discovered that patients with a prosthetic valve or who have suffered before from endocarditis were 400 times more likely to be afflicted with IE than those who had no such medical history. The likelihood of developing IE for patients suffering from MVP increases approximately 10 fold, which is significant considering the high incidence of the disease. It was also shown that in cases of RHD, the lifetime risk of acquiring IE increases from 5 to 380-440 per 100,000 patient-years (112).

Though the intricate pathogenesis for the development of IE has been extensively investigated, the exact mechanism of complex interactions has yet to be fully elucidated. Generally, platelets aggregate to the site of the damaged endothelium on the heart valve resulting in nonbacterial thrombotic endocarditis (NBTE) (112). Oral streptococci transiently introduced into the bloodstream via trauma to the gingival crevice may adhere to the platelet aggregation through complex interactions between bacterial adhesins and receptors located on platelets. The specific adhesins required for such interactions have also been investigated. Some oral streptococci possess the sialic acid-binding adhesin, Hsa, which has been implicated not only in adherence to tooth surfaces, but also in IE (50, 100). In *S. parasanguinis*, a lipoprotein receptor antigen I (LraI) homolog, FimA, has been suggested to mediate adherence to the salivary pellicle as well as the fibrin platelet matrix (8).

Once adherence to the NBTE has taken place, cells lodge themselves into the complex and stimulate further production of fibrin on their surfaces. Adhered bacteria may also recruit cytokine and tissue factor secreting monocytes to the area which further stimulate the production of procoagulating factor VII (FVII) (5). It was

suggested that streptococcal glucosyltransferases (GTFs) may play a role in the recruitment of monocytes by inducing the assembly of adhesion molecules and stimulating IL-6 production (114). The process continues, resulting in the overall increase of the vegetation allowing bacterial cells to sustain replication, impervious to the host immune response. Adhered cells replicate as rapidly as bacteria in broth cultures (112) resulting in vegetations with a bacterial density of up to 10^8 CFU per gram (74). Just like in any biofilm, cells in the vegetation are mostly metabolically inactive, rendering treatment with antibiotics difficult.

Clinical manifestations of those suffering from IE may be somewhat difficult to interpret. Patients may complain of fever, chills, muscle and joint aches, fatigue, and loss of appetite; all symptoms of infection with the common flu. In cases where the patient suffers from congestive heart or renal failure, or has recently used antimicrobial drugs, fever may be minimal or non-existent (73). However, a more telling sign is the discovery of a heart murmur during physical examination and observation of the peripheral manifestations of IE. Signs include petechiae on the skin or conjunctivae and splenomegaly. Even so, patients with right-sided endocarditis do not exhibit such peripheral symptoms (73).

In patients suspected of suffering from IE, several examinations may be performed for a definitive diagnosis. In 1994, Durack *et al.* (20) at Duke University came up with a method that integrated the clinical symptoms, laboratory work, and echocardiogram examination – consequently known as the “Duke criteria” – to successfully screen patients for IE. The criteria called for the positive identification of

IE via an echocardiogram or the presence of commonly associated bacteria in blood cultures such as *Streptococcus spp.* and *Staphylococcus spp.*, as well as the symptoms detailed above. The criterion has consequently been utilized effectively in numerous studies. However, due to the development of the more sensitive transesophageal echocardiogram (TEE), the increasing number of patients categorized as “possible”, the role of *Staphylococcus aureus* bacteremia in endocarditis, and the improper identification of culture-negative cases, a more recent modification to the Duke criteria was proposed (58).

Treatment of patients who have been diagnosed with IE can be difficult. Blood cultures are taken to identify which species is responsible for IE in the patient, followed up with an antimicrobial susceptibility test to determine the minimal inhibitory concentration (MIC) of the drugs of choice (73). As an example, for patients with native valve endocarditis and an MIC of penicillin $< 0.1 \mu\text{g/ml}$, administering penicillin G and gentamicin for two weeks is usually sufficient (29). For the most part, treatment is usually started in the hospital and continued as an outpatient. More invasive procedures must be followed for patients who suffer from congestive heart failure.

Over the past few decades, the American Heart Association has suggested different prophylaxis treatments in preparation for dental procedures; the penultimate recommendation coming in 1997 (16). However, even with these proposals, the incidence of IE over the years has not been alleviated. Furthermore, very little published data are available suggesting a definitive link between prophylaxis and prevention of IE. Most studies have focused on dental procedures due to the belief that bacteremia

caused by a tooth extraction, for example, was much higher in intensity than that resulting from daily routine cleaning. Studies, have shown a significant increase in bacteremia levels after dental procedures (28, 61, 93). However, one study showed that bacteremia for these procedures was as low as pre-extraction levels (91), suggesting much lower bacteremia than previously thought. Due to the emergence of penicillin resistant strains, there has been a more recent emphasis on good oral hygiene for the prevention of IE, rather than the application of antibiotics before dental treatment (28, 90). Considering the emergence of antibiotic resistant strains, the low incidence of IE in the general population, and the lack of evidence linking antibiotic prophylaxis with prevention of IE, the American Heart Association has made recent alterations to its recommendations, such that only patients with a narrowed set of underlying heart conditions are recommended to be given some form of prophylaxis before dental procedures involving manipulation of gingival tissue or perforation of the oral mucosa (112). These recommendations take into account the conditions for which there exists the highest risk of adverse outcomes from IE.

E. Competence in streptococci

1. Definition and history

The ability of any given bacterium to uptake and incorporate free extracellular DNA from the environment – also known as ‘naked DNA’ – is known as competence and may play a critical role in bacterial adaptation and evolution in its native niche. DNA is usually released upon cell lysis and can be integrated into a host cell genome. If

this results in genotypic changes, the host cell is said to have been transformed. The observation of transformation was first noted by Griffith (32) when he discovered that a pathogenic pneumococcal strain – today referred to as *S. pneumoniae* – could be destroyed and somehow transform nonpathogenic strains into the pathogenic type. In 1944, Avery *et al.* (4) observed that the transforming factor hypothesized by Griffith was actually DNA. This study was one of the earliest examples giving credence to the idea that DNA was in fact responsible for carrying genetic information.

2. Genes associated with competence

Since its first discovery, extensive investigation into the transformation capability of *S. pneumoniae* has been very well documented. It was first noted that transformation appeared to be cell density-dependent, and that it occurred abruptly and decayed over time (104). The idea that a system is governed by cell density fits in line with the quorum sensing (QS) system model whereby bacteria communicate with each other in order to adapt to a given environmental cue. Indeed, many studies have shown a direct link between *S. pneumoniae* competence and the QS system (56, 69, 103). Further observation by Tomasz (103) led to the hypothesis that the system was regulated by some secreted autocatalytic macromolecule. It was also shown that this competence factor could be isolated from filtrates (102). The induction of competence was shown in the presence of this activator and was lacking when the activator was absent (103). Subsequent studies finally isolated a competence stimulating heptadecapeptide (CSP) which was found to be encoded by *comC* (35) and was confirmed to stimulate competence in *S. pneumoniae* (81).

Analysis of the *com* locus, discovered via insertion-duplication mutagenesis (10), revealed two genes, *comA* and *comB*, which encoded proteins of 77 kDa and 49 kDa respectively (11). Deletion of either was shown to abolish natural competence, though function was restored in the presence of exogenous CSP (10). Predictions made via analysis of the *comA* sequence revealed that it belonged to a superfamily of ATP-dependent transport proteins, also termed ATP-binding cassette (ABC-type) transporters (42) suggesting that these genes encode for the transport system responsible for the exportation of CSP into the extracellular space. ComA and ComB, associated with CSP secretion, were shown to resemble transporters that secrete double-Glycylated bacteriocins (43). Initially, CSP in *S. pneumoniae* is produced as a 41 amino acid precursor peptide and is subsequently cleaved yielding a 17 amino acid peptide which is then transported into the external milieu via ComA with the help of the accessory protein ComB (35). Further investigations revealed that the protease component responsible for this cleavage resides within ComA (46).

Sequencing of the *comC* downstream region revealed two putative ORFs designated *comD* and *comE* (81). Homology searches revealed that these two genes belong to a family of previously described two-component regulatory systems (98). ComD shared vast homology with a transmembrane histidine kinase (HK) receptor protein, while *comE* was similar to a response regulator (RR) (81). Briefly, as HK becomes activated, adenosine triphosphate (ATP) phosphorylates the histidine residues on the HK protein which in turn donates a phosphoryl group to an aspartic acid residue in the RR (98). It was postulated that as CSP reaches a critical threshold in the

environment, it binds to the HK receptor, which in turn activates the RR. Therefore, ComE up-regulates transcription of the *com* genes – *comAB* and *comCDE* – behaving in a positive feedback loop (81). The hypothesis was supported in another study by Håvarstein *et al.* (37) which concluded that ComD was in fact the CSP receptor and by Ween *et al.* (110) who demonstrated the DNA binding and autocatalytic function of ComE. Researchers in the latter study also created a model looking at conserved regions based on upstream sequences of *comCDE* in different streptococcal species. It was suggested that the putative ComE binding site, also known as the ComE box, was a 9-bp direct repeat of aCA₁TTcaG, with the two sequences separated by no more than 12 bp.

Genomic sequencing, biochemical characterization, and bioinformatic approaches have allowed investigators to identify many different genes associated with DNA uptake and recombination, including *comEA*, *comEC*, *cilA*, *cilB*, *cilD*, *cilE*, and *coi* (9, 56, 82). Analysis of the upstream section of these genes revealed a highly conserved eight nucleotide long sequence – TACGAATA – known as the *com*-box, at the -10 position, and that it was crucial for the induction of transcription (9). Lee *et al.* (56) noted that this highly conserved sequence preceded only these genes and not in *comAB* or *comCDE* suggesting that transcription was not ComE dependent.

Investigations using purified pneumococcal RNA polymerase holoenzymes revealed a protein associated with *cilA* transcription, named ComX. It was observed that ComX was ComE-dependent, and was not an autoinducer. Furthermore it was suggested that ComX behaved as an alternate sigma factor regulating transcription of downstream

genes responsible for the DNA processing phase of competence (56). The competence genes were distributed into two categories based on their temporal expression. “Early” response genes were shown to be those that were ComE dependent (e.g. *comAB* and *comCDE*), while the majority of “late” *com* genes were those that carry a *com*-box within their promoters and are thus ComX dependent (3, 84, 89, 108).

3. DNA uptake machinery

It has been shown in *S. pneumoniae* that cells induced to competence behave as recipients for foreign DNA, while a sub-fraction of the population lyse and act as donors (96). It was further shown that cells lysing were non-competent and seemed to be more affected as the population of competent cells increased (97). As cells lyse, their DNA becomes subject to uptake by these competent cells. The mechanism for DNA uptake has been extensively studied in both *Bacillus subtilis* and *S. pneumoniae*. Typically, a competence pseudopilus, encoded by the seven-gene *comG* operon (18), allows access of dsDNA to DNA receptor proteins on the surface of these competent cells. ComEA is a transmembrane protein which contains an exposed C-moiety at the outer surface which likely binds with DNA (6, 44), although in *S. pneumoniae*, a transposon insertion in this gene diminished, but did not eliminate, DNA attachment onto cell surfaces (6) suggesting the presence of other DNA receptors. In streptococcal species, the transmembrane complex is encoded by a four-gene operon; *comYA-D* in *S. gordonii* for example (82).

In *B. subtilis*, the attachment of dsDNA to the cell surface is followed immediately by double-stranded cleavages by the nuclease, NucA (86). In *S.*

pneumoniae the endonuclease responsible for the cleavage of dsDNA prior to its transportation across the cell-wall has not been elucidated. However, studies have shown that prior to DNA transport, single-strand nicks are created (53) followed by double-strand breaks (70). It was observed in *S. pneumoniae* that ssDNA was linearly transported with 3' - 5' polarity (67), that it occurred at approximately the same rate as the degradation of the second strand, and that this degradation proceeded with opposite polarity (66). It was discovered that the nuclease responsible for this degradation was encoded by *endA* (54, 87). Linear DNA is then transported into the cytoplasm through ComEC, a polytopic membrane protein postulated to form an aqueous channel for DNA to transverse (17). Once inside the cell, integration into the host genome is thought to be a RecA-dependent process requiring sequence homology between the foreign DNA and host chromosomal DNA. This hypothesis was further supported through studies showing that competence is abolished in a RecA deficient mutant (83). The model of competence regulation used for this study can be seen in Figure 1.

4. Difference in competence regulation in *S. mutans* with respect to *S. pneumoniae*

As stated earlier, much of what is known of natural competence has been learned from studies in both *B. subtilis* and *S. pneumoniae*. Due in part to the emergence of whole genome sequences, there has been an explosion of information regarding competence in different streptococcal species. There is extensive evidence suggesting that the mechanism regulating competence is similar in streptococci belonging to the mitis and anginosus groups (38). Species in these groups contain a similar *comCDE* operon which is typically flanked by the tRNA-Arg or tRNA-Glu genes. Unlike *B.*

Figure 1. Schematic representation of the *S. pneumoniae* competence model adopted for this study.

- 1.) *comC* is transcribed resulting in the translation of the precursor competence stimulating peptide (CSP).
- 2.) CSP binds to its target, the product of *comAB*, where the signal peptide is cleaved. As a result, CSP is transported into the external milieu.
- 3.) As the concentration of CSP builds in the extracellular space, it ultimately binds to the histidine kinase receptor encoded by *comD*. This interaction causes the phosphorylation of the histidine residues and activation of the ComD receptor.
- 4.) ComD then transfers the phosphate group unto an aspartic acid residue in the response regulator encoded by *comE*. ComE then activates transcription of the *comAB* and *comCDE* operons, as well as the alternate sigma factor encoded by *comX*.
- 5.) ComX in turn activates the transcription of genes responsible for the uptake of DNA.
- 6.) Included among these are the *comY* genes encoding a pseudopilus. The pseudopilus is thought to be required for the initial interaction between DNA and DNA receptor proteins on the surface of the cell.

Figure 1

subtilis, competence characteristically occurs at early- to mid- log phase as observed in *S. pneumoniae* (38) and *S. gordonii* (65). There seems to also be a correlation between pH of the media and competence (12, 104) in *S. pneumoniae*.

Extensive study of the competence system in *S. mutans* in recent years has been conducted to observe any differences in the systems between the two groups. For the most part, the mechanism of quorum sensing appears to be similar to that of *S. pneumoniae* (15). A key difference between the two, however, is the product encoded by *comC*. Whereas in *S. pneumoniae* a 17-amino acid competence stimulating peptide is cleaved from a 41-amino acid precursor, in *S. mutans* a 46-amino acid precursor is encoded by *comC* yielding a 21-amino acid mature peptide after cleavage (59). Interestingly, the mature peptide also contains the double Gly-motif exhibited in other streptococcal species.

In general, transformation frequencies in *S. mutans* are much lower than in other streptococci (64, 71). Interestingly, it has been observed that addition of *S. mutans* CSP to *S. mutans* induces coordinated expression of genes involved in competence and mutacin IV (48), a nonantibiotic bacteriocin (88). The findings suggest that during the competent phase, *S. mutans* acquires DNA from its environment by lysing surrounding non competent *S. mutans* cells as well as cells that are phylogenetically similar, such as *S. gordonii* (47).

On the regulatory front, whereas *comABCDE* partake in a positive feedback loop with ComE serving as the auto-catalyst in *S. pneumoniae*, in *S. mutans* non phosphorylated ComE represses *comC* while simultaneously up-regulating production

of the bacteriocin encoding *nlmC* gene (52). In the presence of CSP however, ComE is activated resulting in expression of *comC*. It was later hypothesized that unlike what has been previously described for *S. pneumoniae*, *comC* does not lie in the same operon as *comDE*. Though mutations in *comC* and *comDE* negatively affect competence in *S. mutans* (59) just like in other streptococcal species, the repressive role of ComE suggests a unique mode of competence regulation compared to *S. pneumoniae*.

5. Putative *com* genes in *S. sanguinis*

In 1997, Gaustad and Havardstein (30) located *comC* in *S. sanguinis* and determined the theoretical sequence of the translated protein. Unlike what has been observed in other streptococcal species, the presumed mature 15-amino acid (NH₂-DLRGVPNPWGWIFGR-COOH) competence stimulating peptide lacks the signature double-Gly motif (38). The Gly-Gly motif has been thought to be the site that is cleaved during export by dedicated ABC-type transporters (36). Nevertheless, the presumed mature peptide was synthetically produced in a laboratory, and shown to induce competence specifically in *S. sanguinis* (38). A visual example showing the differences between ComC of *S. sanguinis* and that of other streptococcal species can be seen in Figure 2.

It was previously noted that the supernatant from cultures of group H *S. gordonii* strain Challis would not increase the number of observable transformants in cultures with naturally competent group F strains, indicating a level of pheromone specificity (80). In *S. sanguinis*, CSP synthesized using the predicted mature peptide sequence was shown to specifically induce competence in *S. sanguinis*, but not in *S. gordonii* nor in *S.*

Figure 2. The competence stimulating precursor peptide in seven different *Streptococcus spp.* The leader peptides are underlined signifying the presumed site of cleavage which is represented by a “-“.

Figure 2

<i>S. gordonii</i>	MKKKNKQNLLOKELQQFEILTERKLEQVTGG-DVRSNKIRLWWENIFFNKK
<i>S. mutans</i>	MKKTLSLKNDKFKEIKTDELEIIIGG-SGSLSTFFRLFNRSFTQALGK
<i>S. pneumoniae</i>	MKNTVKLEQFVALKEKDLQIKGG-EMRLSKFFRDFILQRKK
<i>S. mitis</i>	MKNTVKLEQFKEVTEAELQEIRGG-DWRISSETIRNLIFPRRK
<i>S. infantis</i>	MEKTVKLEQFKKLTEKELQEIQGG-DWRFLNSIRD LIFPKR
<i>S. oralis</i>	MKNTVKLEQFVALKEKDLQEIKGG-EMRLPKILRDFIFPRKK
<i>S. sanguinis</i>	MKIYSFQIALNSNSLVQTFW-DLRGVPNPWGWIFGR

milleri (30) giving further support to this observed specificity. In *S. pneumoniae*, two unique variants of CSP and ComD have been identified (CSP1 and CSP2; ComD1 and ComD2) (85). It was observed that addition of synthetic CSP1 to cells carrying ComD2 was 200 times less potent than when it was added to cells carrying ComD1 (49), indicating that not only is CSP species-specific, but that it may also be strain specific. It was later proposed that a hydrophobic region within the peptide which is unique in many different streptococcal species may contribute to the specificity of each CSP for its cognate ComD receptor (49).

The genome of *S. sanguinis* has been recently sequenced (113). It was determined that this streptococcal species had the largest genome of the sequenced streptococci, and that it contained approximately 2,274 putative proteins, 61 tRNAs, and four rRNA operons. It was also shown to share 23 more proteins with *S. mutans* than with *S. pneumoniae*. Though it was discovered to contain orthologs for 20 of the 22 indispensable genes essential for competence in *S. pneumoniae* (83), the most notable absence was the *comAB* operon and *comW*. The ComW protein has been thought to activate and stabilize ComX (99). The absence of *comAB*, at least through initial observation of the genome, is not entirely surprising considering the unique sequence of the precursor peptide. The predicted unique cleavage site shown in Figure 2 for this peptide may indicate a unique transportation system in *S. sanguinis* not seen in any other streptococcal species. However, whether or not this presents the actual site of cleavage or even if *S. sanguinis* CSP is cleaved at all has yet to be determined.

F. Specific Aims

The aim of this project is to characterize genetic competence in *S. sanguinis*. Though extensively studied in *S. pneumoniae*, the regulation of competence at the molecular level has not been fully elucidated in *S. sanguinis*. Considering the implications of a naturally competent organism in the oral environment, and the fact that *S. sanguinis* is one of the first colonizers of the human mouth as well as having been implicated in the cause of infective endocarditis, the necessity to study the adaptable nature of this organism becomes immediately apparent. A transcriptome analysis of competence induction via the addition of exogenous CSP would give great insight into the regulatory mechanism governing competence in *S. sanguinis*. It would also allow the possible identification of the unknown proteins that process and export CSP in *S. sanguinis*.

II. MATERIALS AND METHODS

A. Plasmids and strains

A list of plasmids and strains used in this study is shown in Table 1.

Streptococcus sanguinis wild type strain, SK36, was selected for genome sequencing based on multiple criteria (113), including keeping with common characteristics that define *S. sanguinis* using accepted diagnostic tests, and virulence in the rat model of IE. SK36 was consequently selected for this study based on the availability of the genome sequence. A *comC* knockout strain was created and named JFP41 (see below). Plasmid pJFP16 (78), kindly provided by Dr. Sehmi Paik, contains a chloramphenicol (Cm) acetyltransferase (*cat*) gene within a mini-transposon. The mini-transposon is nestled within approximately 1.9 kb of flanking DNA homologous to *nrdD* (~950 bp on either side). The plasmid also contains a kanamycin resistance gene, *aphA-3*, in the same orientation as *cat*. Plasmid pJFP36 (manuscript in preparation), kindly donated by Lauren Turner, is a 5.5-kb plasmid containing an erythromycin resistance cassette, *pSerm*. *NcoI* restriction sites are integrated at the 3' and 5' end of the *erm* construct which has been situated within SSA_0169. A total of 1.3 kb of flanking DNA (740 bp upstream and 620 bp downstream of the *erm* cassette) are also inserted into the plasmid. Maps of each plasmid are shown in Figure 3.

B. Bacterial cultivation

SK36 and JFP41 were grown at 37°C – either anaerobically for 24 hrs or at 6% O₂ for 48 hrs – on Brain Heart Infusion (BHI) broth (Bacto™, Sparks, MD.)

Table 1. Plasmids and strains used in this study

Table 1

Strains and plasmids	Phenotype	Reference
Strain		
<i>S. sanguinis</i>		
SK36	Human dental plaque isolate, adhesive to saliva-coated HA	(41, 51, 113)
JFP41	$\Delta comC$, derived from SK36	This study
Plasmids		
pJFP16	Cm ^R and Kn ^R encoded by <i>cat</i> and <i>aphA-3</i> genes respectively in the same orientation; containing <i>nrdD1::cat</i>	Dr. Sehmi Paik (78)
pJFP36	Kn ^R and Em ^R ; derivative of pJFP34 with <i>pSerm</i> ligated into SSA_0169	Lauren Turner (manuscript in preparation)

Figure 3. Map of A) pJFP16 and B) pJFP36 reproduced with permission.

Figure 3

supplemented with 1.5% (w/v) agar for colony growth on plates, unless otherwise indicated. For transformation protocols, strains were grown overnight (O/N) at 37°C aerobically in Todd Hewitt (TH) broth (Bacto™, Sparks, MD.) supplemented with 2.5% (v/v) horse serum (HS) (Invitrogen, Grand Island, NY).

C. Transformation assays

Transformation was performed as previously described (79) for mutant creation, or using a slightly modified protocol for competence assessment. For the latter, unless otherwise indicated, O/N cultures of each strain was diluted 1:200 into pre-warmed TH•HS (pH 7.6) and incubated at 37°C until the optical density at 660 nm (OD₆₆₀) reached ~ 0.07. A total of 330µl of culture was then transferred into a pre-warmed 0.7 ml microfuge tube containing 10ng of pJFP16 or pJFP36, and 70ng of *S. sanguinis* CSP (30). In order to eliminate any extracellular DNA, cells were allowed to incubate at 37°C for 20 min before the addition of DNaseI (10.5 ng µl⁻¹). The cells were then incubated an additional 10 min before being serially diluted in PBS and layer plated.

D. Layer plating

For the transformation assays, 12.5 ml aliquots of sterile molten low-melting-point agarose (Promega) dissolved in Tryptic Soy Broth (Bacto™) (1% w/w) were stored overnight at 37°C. After serial dilutions were performed on post-transformed cells, 0.1ml was then transferred to these aliquots, mixed, and dispensed into Petri dishes (100x15mm plates, Fisherbrand®). The agar was allowed to solidify and subsequently incubated for 2 h at 37°C. An additional 12.5ml of TSA ± chloramphenicol (Cm) (10µg ml⁻¹; final concentration 5µg ml⁻¹) or erythromycin (Em)

(20 $\mu\text{g ml}^{-1}$; final concentration 10 $\mu\text{g ml}^{-1}$) was added to each plate. Transformation frequency was determined after 48 h of incubation and expressed as the total number of CFU ml^{-1} from plates with antibiotic divided by the total number of CFU ml^{-1} in plates with no antibiotics.

E. Growth curve in acidic medium

In order to determine whether acidic conditions had adverse effects on SK36, a growth curve assay was performed. SK36 was grown O/N in TH•HS (pH 7.6) at 37°C. The culture was diluted 1:100 in pre-warmed TH•HS at either pH = 7.6 or pH = 6.9 in a 96-well plate and left to incubate at 37°C in a FLUOstar plate reader (BMG LabTechnologies) where the OD₄₅₀ was recorded every 10 minutes immediately following shaking in order to re-suspend the culture. Values were obtained by subtracting the average of three background wells. Blanks were created by adding 2 μl of TH•HS (pH = 7.6) to 198 μl of acidic or neutral TH•HS. Three biological replicates were used and each contained three technical replicates in the 96-well plate.

F. Construction of JFP41

In order to control the competence state in SK36 and study its regulation, *comC* was targeted for deletion. Manipulation of the genome was accomplished via Gene Splicing by Overlap Extension (“gene SOEing”) (40). All primers used for this study are listed in Table 2. In order to generate JFP41, two sets of primers were developed to amplify the upstream and downstream regions of *comC*. Primer ComC-B contained an 18-bp 5’ tail fully complimentary to the first 18-bp of primer ComC-C. Both primers were created with the intention that the final product would ultimately fuse the start

Table 2: Primers used for the production of JFP41 and subsequent screening and sequencing of selected mutants.

Table 2

Primer Name	Sequence (5' – 3')	Primary Use	Used in sequencing
ComC-A	CGAACAAGAATACCTGATT	Deletion of <i>comC</i>	Yes
ComC-B*	<u>TCCAATTATTTCAAGTGACAT</u> <u>TAACTATCTCCTATCTTTT</u> A	Deletion of <i>comC</i>	No
ComC-C	TCACTTGAAATAATTGGATTA	Deletion of <i>comC</i>	No
ComC-D	TGGCACTCTCAATAGCA	Deletion of <i>comC</i>	Yes
FWD-1	TCTAAGCGAACGGTC	PCR screening	Yes
RVS-1	CGGAACTATAATCTCAAGA	PCR screening	Yes
Chkup-1	TCATATAAAAACTCTACTG	Sequencing	Yes
Chkdn-2	ACTAATGTAGTAACATAATTC	Sequencing	Yes
Chkup-3	GATTGCCGTCAAGATTACTT	Sequencing	Yes
Chkdn-4	CTTTTTATGTCTGTCAATTGA	Sequencing	Yes
Chkup-5	AGAGATTTAAAATCAGCC	Sequencing	Yes
Chkup-7	GTGGGTGTTGGTTACCAG	Sequencing	Yes

*Note: underlined region is the 5' tail used as a self-primer in the second round of PCR amplification

codon of the *comC* gene with the second codon of the *comD* gene. In so doing, the *comC* gene would be eliminated. Platinum® PCR Supermix High Fidelity (Invitrogen) was used for the subsequent PCR amplification rounds. A temperature gradient was used for the first PCR round to ensure optimal amplification. PCR profile used for the first amplification process was as follows: one cycle at 95°C for 4 min, followed by 30 cycles of amplification (95°C 45s, 50°C – 60°C 45s, and 68°C 2 min), followed by 68°C for 7 min and 20°C hold. The second round of PCR to piece both products together had a similar PCR profile with the exception of having the annealing temperature set at 55°C and the extension step lasting 3 min.

Before and after the final PCR amplification round, the products were purified using MiniElute® PCR Purification Kit (Qiagen) per manufacturer's protocol. The samples were electrophoresed on a 0.8% Ultrapure™ agarose gel (Invitrogen) and compared to mass standards to determine final concentration. The product was then diluted to working concentrations (5ng μl^{-1}). To incorporate this linear DNA into SK36 genome via homologous recombination, 10ng of product was used. SK36 was transformed using the protocol described above.

G. Screening and identification of mutants

With no marker in place to select for putative mutants via antibiotic selection, PCR screening was required. Whole-cell preps were performed on isolated colonies by inoculating 1.25 ml of BHI per well in a 48-well block and incubating at 37°C O/N in 6% O₂. After vortexing to re-suspend the inoculum, 1ml was then transferred to 1.5 ml microfuge tubes, centrifuged at 18,000 x g for 2 min., and the pellet washed twice in 10

mM Tris-Cl (pH 8.0). After centrifugation, the pellet was once again re-suspended in 10 mM Tris-Cl and stored at -20°C until use.

Two primers, FWD-1 and RVS-1, were utilized that flanked the *comC* gene (Table 2). A total of 1.2µl of the cell-preps were used as template for the PCR amplification step. The PCR profile used was as follows: one cycle at 95°C for 4 min, followed by 30 cycles of amplification (95°C 45s, 51° 45s, and 72°C 30s), followed by 72°C for 7 min and 20°C hold. The samples were then run on a 1.4% agarose gel and stained with ethidium bromide (EtBr).

To ensure that the mutant had no downstream effects on flanking genes, transformation studies were conducted on suspected mutants. Three putative mutants were selected and grown O/N in 2ml BHI at 37°C in 6% O₂. The strains were then diluted 1:200 in TH•HS and grown until an OD₆₆₀ of 0.07 was reached. A total of 330µl of culture was then transferred to 0.7ml microfuge tubes containing ± 10ng pJFP36 and ± 70ng CSP. The cells were allowed to incubate for 1.5 h in a 37°C water bath before being diluted 1:100 into the wells of a 96-well plate containing BHI ± Em (10µg ml⁻¹). The cells were grown O/N at 37°C in a FLUOstar plate reader (BMG LabTechnologies) where the cultures were shaken every 10 minutes and the OD₄₅₀ recorded. Values were obtained by subtracting the average of three background wells. Blanks were created by adding 2µl of TH•HS to 198µl of BHI.

H. RNA isolation

RNA was isolated from *S. sanguinis* using a protocol developed for *S. pneumoniae* (84), with a few modifications. Briefly, JFP41 was grown in TH•HS until

an OD₆₆₀ of 0.068 – 0.074 was obtained. Twenty ml of culture was removed from the inoculated broth and transferred to a tube of equal volume hot acid phenol containing 0.1 M citrate buffer (pH 4.3, Sigma) and 0.1% (v/v) sodium dodecyl sulfate (SDS, Ambion). The rest of the culture was exposed to CSP at a final concentration of 220ng ml⁻¹ before also being transferred to hot acid phenol tubes. These tubes were then incubated in boiling water for 10 min and mixed intermittently. The samples were then cooled in an ice bath and transferred to Phase Lock Gel™ (PLG) tubes (5 Prime). After centrifugation at 1500 x g for 5 min, the supernatant was extracted using an equal volume of acid phenol:chloroform (1:1). The samples were then thoroughly vortexed and transferred to PLG tubes for an additional spin at 1500 x g for 5 min. The supernatant was once again extracted using an equal volume of chloroform, mixed, and centrifuged in PLG tubes. The supernatant was then adjusted to 0.3 M NaOAc using a 3 M stock (Ambion), and an equal volume of isopropanol was added. The sample was chilled for 20 min in an ice bath and centrifuged at 8000 x g for 15 min at 10°C. The resulting RNA pellet was washed with 70% ethanol, dried via tube inversion, and dissolved in 100µl DEPC-treated water.

These samples were then treated with DNase I and purified over RNeasy columns (Qiagen) according to protocols supplied by the manufacturer. Total yield was determined by examining absorbance at 260nm. Any samples with a 260/280 ratio > 2.2 or < 1.8 were discarded. In order to confirm good RNA quality, the remaining samples were run on an Experion™ Chip (BioRad) per manufacturer's protocol. Only RNA that

was shown to maintain a 23S/16S ratio > 1.8 was kept and stored at -80°C until further use.

I. *Streptococcus sanguinis* DNA microarray slides

Microarray slides for *S. sanguinis* were obtained from the J. Craig Venter Institute (JCVI), Pathogen Functional Genomics Resource Center (PFGRC). Unique oligonucleotides 70 bases in length were designed to represent internal regions of all 2274 ORFs predicted from the genome sequence of *S. sanguinis* (113). These probes were printed on 25 x 75 mm aminosilane coated glass slides and then baked for 2 h at 80°C before DNA was cross-linked to the surface via UV exposure. All slides were stored at room temperature in a dessicator until use. For this study, oligonucleotides were printed in 96 grids containing 96 spots each. All genes had four technical replicates on the slide. As a control, each slide contained 500 *Arabidopsis thaliana* probes 70 bases in length.

J. cDNA synthesis and labeling

The protocol was taken from PFGRC and can be seen on their website at <http://pfgrc.jcvi.org/index.php/microarray/protocols.html>. Briefly, 2 μg total RNA was thawed on ice and annealed to 6 μg Random Hexamers (Invitrogen) in a total volume of 18.5 μl and incubated at 70°C for 10 min. The tubes were then snap frozen on ice before the addition of 6 μl of 5x First Strand Buffer (250 mM Tris-Cl, pH 8.3, 375 mM KCl, 15 mM MgCl_2), 3 μl 0.1M DTT (Invitrogen), and 0.6 μl of a dNTP solution (25 mM each dATP, dCTP, and dGTP, 15 mM dTTP (Invitrogen) and 10 mM amino allyl-dUTP

(Sigma)). A total of 400 U of SuperScript III (200 U μl^{-1} , Invitrogen) was added to the mixture and then incubated O/N at 42°C.

The RNA template was then hydrolyzed via the addition of 10 μl 0.5 M EDTA and 10 μl 1 M NaOH and incubated at 65°C for 15 min. The mixture was neutralized with 25 μl 1 M Tris pH 7.0 and 10 μl 3 M NaAc, and purified using MiniElute® PCR Purification Kit (Qiagen) replacing the Tris buffers with phosphate buffers (5 mM KPO_4 , pH 8, 80% EtOH) and (4 mM KPO_4 , pH 8.5) for the wash and elution steps respectively. Samples were analyzed using a NanoDrop® Spectrophotometer where cDNA yield was determined using the formula $(\text{OD}_{260}) \times (33 \mu\text{g/ml})$ and total pmol cDNA yield calculated using the following formula:

$$\frac{(\text{Total ng cDNA}) \times (1000 \text{ pg/ng})}{324.5 \text{ pg/pmol}}$$

The reaction was then dried in a Speed-Vac centrifuge before being resuspended in 4.5 μl 0.1 M Na_2HCO_3 , pH 9.3 and 4.5 μl of the ester dye (Cy3 or Cy5 (Amersham) for the control and experimental samples respectively). The coupling reaction proceeded for 1.5 – 2 h at room temperature in the dark before being stopped via the addition of 35 μl 100 mM NaAc pH 5.2 and then purified over Qiagen MiniElute PCR purification columns per manufacturer's protocol and supplied buffers. The cDNA probes were then analyzed using a NanoDrop® Spectrophotometer and pmol of dye incorporation determined using the general formula of $(A \times e)/b$, where A is the absorbance at 550nm

or 650nm (for Cy3 or Cy5 respectively); e is the extinction coefficient (150,000 liter/mol-cm and 250,000 liter/mol-cm, Cy3 and Cy5 respectively); and b is the path length in cm. The nucleotide to dye incorporation ratio was ascertained via the following formula:

$$\# \text{ nucleotides/dye incorporated} = \frac{\text{pmol cDNA}}{\text{pmol CyDye}}$$

Only samples with > 200 pmol of dye incorporation and a nucleotide to dye incorporation ratio < 40 were kept. The probes were then combined and dried to completion once again.

K. Microarray hybridization

Slides were pretreated for 2 – 3 h at 42°C in 50µl of prehybridization solution (5x SSC, 0.1% SDS, 1% BSA). Slides were maintained in hybridization cassettes (ArrayIt®) throughout the incubation phase. The slides were then washed three times with MilliQ water in a High Throughput Wash Station (ArrayIt®) followed with one wash in isopropanol and dried using a mini-slide centrifuge (Labnet International).

Dried probes were then resuspended in 50µl hybridization buffer (40% formamide, 5x SSC, 0.1% SDS, 0.6µg µl⁻¹ salmon sperm DNA). The solution was heated at 95°C for 10 min while vortexing intermittently and then transferred onto pretreated slides under a LifterSlip™ (Erie Scientific). The probes were allowed to hybridize for 16 – 20 h at 42°C in a hybridization cassette. Humidity was maintained

with the addition of 15µl of unused hybridization buffer in each well. Slides were washed twice in 2x SSC, 0.1% SDS, 0.1 mM DTT set at 55°C for 10 min, twice in 0.1x SSC, 0.1% SDS, 0.1 mM DTT for 5 min, and twice in 0.1x SSC, 0.1 mM DTT for 5 min. The last two washes were performed at room temperature. The slides were then dipped in MilliQ water before being dried in a mini-slide centrifuge. Microarrays were immediately scanned in an Axon GenePix® 4200 scanner (MDS Analytical Technologies).

L. Array analysis

Data was collected and analyzed using GenePix Pro 6.0. In order to identify spots and determine normalization values, annotation information for the microarray slides was obtained from the J. Craig Venter Institute which allows a grid to be drawn around each array element. Each spot on the array was individually checked and flagged if it contained one of the following problems: low level of hybridization where the intensity of the signal was not > two-fold of the local background level; high background streaks due to poor handling of the array that run into the spot; or if > 25% of the element contained poor hybridization where signal was undetectable. Once all values were collected, they were submitted to the Ramhorn Array Database (RAD) at <http://ramhorn.csbc.vcu.edu/ilat/>, created by Dr. Paul Fawcett at the Virginia Commonwealth University, which is essentially similar to the Stanford Array Database <http://genome-www5.stanford.edu/>. RAD stores images as well as the raw and normalized data from microarray experiments and provides a user-friendly method of visualizing and analyzing data.

Once in RAD, the spots were filtered out based on three user-defined parameters. Firstly, the regression correlation for each spot had to be greater than 0.5. Secondly, the mean of the red channel (experimental values) normalized to the green channel (control values) had to be 2-fold greater than the normalized median values of the background for the red channel. Lastly, the mean values of the green channel had to be 2-fold greater than the median values of the background. This allows a more unbiased method of filtering out elements that passed through the initial user-based filtering process as described above. In order to determine the significance of each element on the array, the Significance Analysis of Microarray (SAM) method was applied for each gene passing through the filtering process. SAM, originally developed by Tusher *et al.* (107), is a method by which genes are identified based on statistically significant changes in expression. This is performed by incorporating a set of gene specific *t*-tests and then assigning them a score on the basis of change in gene expression relative to the standard deviation of repeated measurements for that gene. Genes with scores greater than a user-defined threshold are deemed potentially significant. SAM also predicts the false discovery rate (FDR), or false positives, by analyzing permutations for each repeated measurement and estimating the percentages of genes discovered by chance. For this study, a threshold was used so that the FDR was < 0.1 .

Once genes with significant changes in expression were identified for each time point, the data were analyzed using Cluster 3.0 (22). This program performs a variety of clustering methods including hierarchical gene clustering. Once the data set was

analyzed and genes clustered according to similar expression patterns, the data were visualized using TreeView. Both programs were developed and maintained by the Eisen Lab (22) at the University of California at Berkeley and can be downloaded from their website at http://rana.lbl.gov/eisen/?page_id=42.

M. Array validation using real-time PCR

All stored RNA samples were subjected to an additional DNase treatment using TURBO DNA-free (Ambion) and purified per manufacturer's protocol. PCR amplification was performed using primers developed by Primer 3.0 and run on a 1.4% agarose gel to determine the presence of contaminating DNA. Afterwards, cDNA templates were created via Ready-To-Go™ You-Prime First-Strand Beads (Amersham Biosciences) as described in the manufacturer's protocol, using 2µg of total RNA. qRT-PCR reactions were carried out in a 7500 Fast RT-PCR system (Applied Biosystems) using RT² Real-Time™ SYBR Green/Rox PCR master mix (SuperArray) and designed primers. Genes selected for qRT-PCR verification were *comD*, *comE*, *comX*, *comYD*, *comEA*, and *gapA*. For normalization values, *tuf* was used as a control. A list of primers used for this study is shown in Table 3. Data was collected for experimental samples at each time point of the competence induction. The experiment was conducted three times in triplicate. A standard curve was generated using a template of known concentration via serial dilutions. Normalized values were obtained and relative expression levels were determined by comparing mean quantity transcript levels to the control.

Table 3. Primers used for qRT-PCR verification of microarray results

Table 3

Primer Name	Sequence (5' – 3')	Target	Product Size
comD-FWD	CGAGAAAGAAGAGCGTTTGC	<i>comD</i>	206 bp
comD-RVS	AATTTGCATCAGCCAAGACC		
comE-FWD	TCAAGTCAGGATGGAAACGAC	<i>comE</i>	160 bp
comE-RVS	TTCCTCCCCCTTAATGTCAA		
comYD-FWD	TACCAGGAGACGCAGAGGTT	<i>comYD</i>	203 bp
comYD-RVS	ACTGTCCTGTCCTCCGTCTG		
comEA-FWD	CTGAGAGCAGGAGCGAGAGT	<i>comEA</i>	245 bp
comEA-RVS	GCCTCTGTCGCCGTATTTAG		
comX-FWD_3	CGCCAGCCTTATGAAGAAGT	<i>comX</i>	91 bp
comX-RVS_3	TCGCTTCTCTGAAGGCAACT		
gapA-FWD	GCTTTCCGTCGTATCCAAAA	<i>gapA</i>	221 bp
gapA-RVS	ACACCGTCAGTAGCCCAGTC		
tuf-FWD	GCTTCAACTGACGGACCAAT	<i>tuf</i>	181 bp
tuf-RVS	CGTCACCTGGGAAGTCGTAT		

III. RESULTS

A. Density-dependence of natural competence in SK36

The purpose of this study is to characterize competence regulation in *S. sanguinis* strain SK36. Therefore, it was first necessary to determine the cell density at which the optimal level of competence occurs. As first described by Tomasz and Hotchkiss (104), *S. pneumoniae* exhibits a cell-density dependent increase and decay of competence which has not yet been fully elucidated in *S. sanguinis*. In order to determine the effect of cell density on competence in SK36, numerous transformation assays were conducted on cultures. Initially, O/N cultures were diluted 1:200 and then 330 μ l aliquots were withdrawn after varying periods of time. However, varying results were obtained possibly due to inconstant incubation temperatures and repeated exposure to O₂ that resulted from repeated sampling of the same culture. Subsequent experiments were conducted in which serial dilutions were performed on O/N cultures, and the diluted cultures were allowed to incubate for three hours prior to removing only one aliquot from each tube for transformation. All experiments were plated in triplicate.

Figure 4 shows the combined results of multiple experiments. There was a definitive overall rise and fall of competence in SK36 that is related to cell density. Transformation frequencies gradually increased as cell population increased. When the cell density reached approximately a log₍₁₀₎ value of 6 (i.e. 1 x 10⁶ CFU ml⁻¹), competence quickly increased reaching a maximum frequency of 21%. The results show that as cells reached a density of 6.5 – 6.6 logs, competence was at its optimal

Figure 4. Graph depicting the transformation frequency of SK36 relative to cell density expressed as CFU/ml. Each colored line represents an individual experiment.

Figure 4

level. The cell density correlates to an absorbance value at OD₆₆₀ of 0.068 – 0.074. The results also showed that competence was specific at this cell density seeing that once frequency reached a peak, competence levels quickly dropped. As cell density approached 8.4 logs (i.e. $\sim 2.7 \times 10^8$ CFU ml⁻¹), competence decreased to below detection levels.

B. SK36 competence and growth are negatively affected in acidic conditions

In order to control natural induction of competence, investigators working with *S. gordonii* and *S. pneumoniae* have shown that competence may be inhibited by growing these species in acidic conditions with no adverse effects on growth (84, 108). However, no studies have been conducted on *S. sanguinis* to determine the effect of acidic conditions on competence and growth. To determine whether this is also seen in *S. sanguinis*, SK36 was grown O/N in neutral (pH 7.6) and acidic TH•HS (8 mM HCl, pH 6.8). The cultures were diluted 1:200 and a transformation assay was performed. As has been observed in other studies, transformation frequencies dropped to below detection levels suggesting that natural competence was effectively inhibited (Fig. 5A). It was observed, however, that O/N cultures grown in acidic TH•HS had significantly lower OD₆₆₀ readings than those grown in neutral conditions, which suggested a deleterious effect on the growth of SK36.

Acting on this observation, a growth study was performed in which O/N cultures were grown in neutral conditions before being diluted 1:100 in acidic and neutral media. As suspected, growth was significantly affected ($p < 0.001$) in a negative manner by growing in acidic conditions (Fig. 5B). When the experiment was performed again in

Figure 5. Effects of acidic pH conditions on A) competence levels and B) growth rate in SK36. Error bars represent the standard deviation from the mean.

Figure 5

A.

B.

slightly more alkaline conditions, growth was only moderately affected (data not shown), however competence was partially restored (Fig 5A). The results were in stark contrast to what has been observed in other streptococcal species and presents a challenge for experimental design. Rather than repressing competence in SK36 via growth in acidic conditions, an alternate route was needed.

C. Generation of a *comC* mutant

In order to regulate competence in *S. sanguinis* without subjecting cultures to acidic conditions, *comC* was targeted. The *comC* gene encoding competence stimulating peptide lies just downstream of *degP* which is in the opposite orientation, as well as a hypothetical protein, SSA_2380, and a tRNA-Arg² gene in the same orientation. The *comC* gene lies upstream of and is likely in the same operon as *comDE*. In order to ensure that the open reading frames for these downstream genes were maintained, primers were developed that would take the start codon of *comC* and fuse it with the 2nd codon of *comD*. In so doing, *comC* would be deleted as well as the entire 29-bp intergenic region between *comC* and *comD*. Thus, the *comD* gene would presumably be controlled using the transcriptional and translational start signals that normally control *comC* expression. A schematic representation of this work is shown in Figure 6.

After the first round of PCR amplification, two products were recovered. The upstream region was predicted to be approximately 1.9 kb in length while the downstream product was approximately 1.0 kb. Both products were compared to a molecular weight standard and confirmed to be the correct size (Fig 7A). Afterwards, the products were purified and then combined using outer primers in a second round of

Figure 6. Flow chart depicting the construction of JFP41, a mutant deficient in the production of endogenous CSP due to a *comC* knockout.

Figure 6

Figure 7. The images show the results obtained after PCR amplification. A) This gel shows the results after the first round of amplification. The upstream product is shown in lane 4 and 5. The downstream product is shown in lane 6 and 7. B) This image shows the results after a successful gene SOEing experiment. Lanes 3-6 show the final product in varying concentrations.

Figure 7

PCR amplification. The resulting 3 kb product was diluted and run on a gel (Fig. 7B). The image shows four bands at different concentrations which were all compared to mass standards to determine concentrations. They were also compared to a molecular weight standard to confirm the correct size of the final product.

Having attained the correct sized amplicon, the purified product was then introduced into SK36 by transformation, as described in Materials and Methods. Transformed cells were surface-plated on BHI and incubated for 48 hours at 6% O₂. Having no selectable marker for these mutants, PCR-screening was conducted on 80 selected colonies. Primers were developed that flanked *comC* such that the difference between SK36 and mutant products was predicted to be 135 bp. Figure 8A shows the results of the first 20 of 80 colonies examined during the first round of screening. As can be seen, all colonies gave rise to amplicons of the size predicted for the wild-type gene. A more careful inspection revealed that colony number 13 contained what would appear to be a light band of the size expected for the mutant strain (indicated by a white arrow). Since this sample also had a wild-type size band, it was hypothesized that the initial colony selected was a mixture of mutant and wild-type cells.

In order to prove this and subsequently retrieve pure colonies, colony number 13 was re-streaked on BHI plates and grown for 48 hours at 37°C in 6% O₂. A total of 40 colonies were selected and once again screened using the selected primers. This time, single bands were obtained (Fig. 8B) confirming the earlier suspicion that the colony was mixed. Out of these 40 colonies, 8 were positive for the mutant strain and 3 colonies were arbitrarily selected and designated JFP41-1, JFP41-2, and JFP41-3.

Figure 8. Images show the results of the mutant selection based on PCR-screening. In both pictures, the control “WT” and “Mut” lane contain the amplicon recovered after PCR amplification using SK36 gDNA and the purified product attained from gene SOEing respectively. A) The agarose gel showing that colony number 13 contains an amplicon of the size predicted for the mutant. B) After re-streaking colony number 13 and PCR-screening, 8 colonies (four in this image) were shown to yield bands equivalent in size to that expected for the mutant strain.

Figure 8

Since *comC* was deleted in the mutant strain, it was expected that it would lack the ability to become naturally competent, but would regain competence to SK36 levels via the addition of exogenous CSP. In order to confirm this hypothesis, the three selected colonies were once again streaked on BHI plates, and one colony from each plate was taken. A transformation study was conducted on these isolated colonies where they were first grown to an OD₆₆₀ of 0.074 and then transformed using pJFP36 $\pm$ 70ng of exogenous CSP. pJFP36 is a suicide plasmid containing approximately 650-bp of DNA on either side of an erythromycin (Em) resistance cassette that must integrate into the chromosome to confer Em resistance (see Materials and Methods). The cells were incubated for 1.5 h before being diluted into 96-well plates and left O/N to incubate at 37°C.

When grown in normal conditions in the absence of exogenous CSP and antibiotics, all three mutants grew at a slightly faster rate than SK36, although all strains reached stationary phase approximately 7.5 h after dilution (Fig. 9A). When the mutant strains were grown in BHI in the presence of Em and absence of CSP, all three strains lacked any sign of growth in the media (Fig. 9B). Meanwhile, SK36 displayed a small lag before reaching stationary phase approximately 17 h after dilution. This was expected considering that all cells not transformed would have been killed upon contact with the antibiotic. Lastly, when the mutant strains were treated with CSP and then grown in the presence of Em, all strains grew at the same rate as SK36 (Fig. 9C), reaching stationary phase approximately 15 h after initial dilution.

Figure 9. Transformation study analysis of the three putative mutants compared to SK36. A) Growth of the 4 strains in BHI. B) Cells not pretreated with CSP during transformation assays and grown in BHI and Erm (10 μ g ml⁻¹). C) Cells pretreated with CSP during the transformation assay and grown in BHI and Erm (10 μ g ml⁻¹).

Figure 9

A. Growth Curve in BHI **B. Growth Curve in BHI+Abx** **C. Growth Curve in BHI + Abx, CSP**

The results demonstrate two critical factors required for the expected mutant phenotype: first, that natural competence was abolished; and secondly, that competence was restored upon the addition of exogenous CSP. The three selected mutants displayed both characteristics. In order to definitively confirm the phenotype of these mutants, a transformation assay was conducted on JFP41-1 – which will hereinafter be referred to as simply JFP41 – and compared to SK36. Transformation frequency levels obtained from this experiment show that natural competence in JFP41 was below detection levels. However, when the cells were treated with CSP, competence was restored to near SK36 levels (Fig. 10).

Lastly, having confirmed the identity of this mutant, the final analysis was to confirm that the DNA sequence of the mutant had the expected sequence. This was important to determine for future analysis using JFP41. Primers were designed to obtain sequence information that spanned the entire 3-kb region introduced by transformation, paying particular attention to the junction point. A schematic representation of the “strategy view” generated by Seqman™ II is shown in Figure 11. Using these primers, it was confirmed that JFP41 contained no mutations anywhere within the span of DNA introduced into the cell during mutagenesis. Thus, we concluded that a *comC* mutant had been successfully created.

D. Determination of the optimal concentration of CSP for JFP41 competence

Once a *comC* mutant was generated, it was critical to characterize its specific phenotype and behavior in order to further study gene regulation. To do so, it was first necessary to determine the amount of exogenous CSP required in order to induce

Figure 10. Competence levels of WT and JFP41 in standard transformation assays. The graph is the result of one experiment plated in triplicate. The error bars represent the standard deviation from the mean.

Figure 10

Figure 11. A strategy view obtained by Seqman depicting the location of the selected primers as well as the overall length of product obtained via sequencing.

Figure 11

optimal competence in the mutant. For this study, JFP41 was transformed with pJFP16 and treated with varying concentrations of CSP. Plasmid pJFP16 contains approximately 950 bp of DNA homologous to *nrdD* flanking a chloramphenicol (Cm) resistance cassette which must be integrated into the chromosome to confer Cm resistance. Figure 12 shows the results of three separate experiments plated in triplicate. Results showed that there was an initial linear response to CSP concentration and transformation frequency. However, once the cells were treated with CSP at a concentration approximately 200 – 240ng ml⁻¹, competence peaked. Once the concentration of CSP rose to 300ng ml⁻¹ and above, competence began to depreciate, although transformants were still recovered. The results showed that JFP41 can be induced to optimal competence levels via the addition of exogenous CSP at a concentration of approximately 200 - 240ng ml⁻¹. Previous studies have used 70ng of CSP in 334µl of culture. This is equivalent to 210ng ml⁻¹ which falls within the range observed here.

E. Density dependence of competence in JFP41

Natural competence within the cell rose and fell with respect to cell density in the wild-type strain, SK36 (see Fig. 4). This fits the model established for *S. pneumoniae* (104) in which density dependence of competence results from accumulation of CSP. It was therefore of interest to determine whether in strain JFP41, which produces no CSP, competence could be induced at any cell density upon addition of exogenous CSP. To test this hypothesis, a study was performed similarly to the SK36 experiment described previously (See section A) in which O/N cultures were serially

Figure 12. The effect of exogenous CSP concentration on the transformation frequency of the *comC* mutant. The results shown are the average of three individual experiments plated in triplicate. Error bars represent the standard deviation in transformation frequencies between the three experiments.

Figure 12

diluted and allowed to incubate for 3 h before treatment with CSP (210ng ml⁻¹) and transforming DNA. Colonies were counted 48 hours after layer plating.

Interestingly, three individual experiments yielded two unique trends (Fig. 13). The first series (blue line) showed that transformation frequency levels rose and fell with respect to cell density. As cells reached a log₍₁₀₎ value of 7.2 (i.e. a cell density of ~ 1.6 x 10⁷ CFU/ml) an optimal competence level was reached. The other two series showed a different trend. In both cases, transformation frequencies were at their highest when cell density was quite low. As cell density decreased, the number of transformants recovered began to drop. These two seemingly different trends did converge at one point, however. As cell density approached approximately a log₍₁₀₎ value of 8.8 (6.3 x 10⁸ CFU ml⁻¹), competence had dropped yielding a transformation frequency less than 4%.

With such different trends observed in this study, it was difficult to conclude whether or not cell density truly had any effect on the ability of JFP41 to become competent. Whereas in one experiment low cell density produced very low numbers of transformants, another two studies yielded opposite results. In all three cases, competence dropped significantly at > 1 x 10⁸ CFU ml⁻¹; yet even these numbers were significantly higher than what was observed in SK36, where competence had dropped to below detection levels. The results suggest that the density dependence of competence is not due entirely to CSP accumulation. Therefore, when using JFP41 with exogenous CSP to model natural competence in a wild-type strain, it was concluded that

Figure 13. Graph depicting the transformation frequency of JFP41 relative to cell density expressed as CFU/ml. Each colored line represents an individual experiment.

Figure 13

it would be best to use cell densities similar to those at which competence occurs naturally in the wild-type strain. Further investigations characterizing the cell density dependence of competence in the *comC* mutant should be conducted in future studies.

F. Decay of competence after CSP treatment in JFP41

Previous studies have shown that upon the addition of the competence stimulating peptide to cells, *S. gordonii* quickly becomes competent. However, after a short period, competence decays, most likely due to gene repression (108). To determine whether this would also be true for *S. sanguinis*, the duration of competence after exposure to CSP was examined in JFP41. For this experiment, JFP41 was pre-treated with CSP for the length of time indicated and then allowed to incubate with transforming DNA for 10 minutes before being layer plated. As in previous experiments in which layer plating was used, the cells were allowed to incubate for 2 h to allow expression of the antibiotic resistance and then an additional layer $\pm$ Cm was added. The results showed a clear response to CSP (Fig. 14). After 10 minutes of incubation, JFP41 reached maximum competence levels comparable to those that had been observed previously in SK36. After 20 minutes, competence began to drop, and by 30 minutes, transformation frequency had decreased to 5%.

This rise and fall of competence recorded here is similar to that found in *S. gordonii* (108), indicating a strong temporal regulation of competence. Interestingly, while in the former study competence dropped to below detection levels, competence in this study showed observable transformants well above the limit of detection at a transformation frequency $<5.8 \times 10^{-6}\%$. It should be noted that the level of detection in

Figure 14. Activation and repression of competence in JFP41 after induction with CSP. The results are the product of three individual biological replicates plated in triplicate. The error bars shown here represent the standard deviation from the average transformation frequencies between each experiment.

Figure 14

the former study was similar to this study at $<5 \times 10^{-6} \%$. This may indicate that competence in the *S. sanguinis* mutant may not be fully repressed once activated by CSP. However, considering that there is a definitive rise and fall of competence, it may be concluded that temporal expression of competence is indeed occurring in the mutant. Once induced in the cell, the duration of competence is about 30 minutes based on these results. Therefore, any examination of gene expression during induction of competence should be performed within 30 minutes of CSP addition.

G. Microarray analysis of transcriptional regulation in response to CSP.

Based on the results described above, transcription of the regulatory genes activated upon competence induction was analyzed via microarray analysis based on a 30-minute time period. Since genes responsible for the regulation of competence are up-regulated and down-regulated rather quickly, it was concluded that the analysis would be performed at 2.5 minute intervals, and since mRNA levels can vary greatly in such short intervals, it was determined that RNA would be isolated by a hot acid phenol protocol whereby cells are lysed immediately upon exposure to phenol and boiled in a water bath. Cells were grown to an OD_{660} of 0.068 – 0.074 before being induced to competence via the addition of exogenous CSP at 220ng ml^{-1} . Twenty-ml aliquots were removed and used for RNA extractions. The quality of the purified RNA was determined by checking the 23S / 16S rRNA ratio using an Experion™ Chip (see Materials and Methods). Figure 15 demonstrates one such analysis. Degraded or contaminated RNA samples were easily discovered and discarded.

Figure 15. An image created with the Experion™ software looking at the quality of prepared RNA. The ladder is observed in the first lane. The second lane is an example of an acceptable RNA sample, showing very little degradation. 23S rRNA is observed at the top, 16S rRNA is the second band, and 5S rRNA is the bottom band. The third lane shows an example of degraded RNA. RNA degradation was estimated by taking the density of the 23S band and dividing it by the density of the 16S band.

Figure 15**Experion analysis of two RNA samples**

A cDNA library was prepared using isolated RNA samples and then hybridized to microarrays slides constructed by JCVI containing unique oligonucleotides specific for each predicted ORF in SK36. During initial analysis, each spot on the array was individually checked and flagged if it contained one of the following problems: low level of hybridization where the intensity of the signal was $<$ two-fold of the local background level; high background streaks that run into the spot due to poor handling of the array; or if $> 25\%$ of the element contained poor hybridization where signal was undetectable. Examples of each condition are shown in Figure 16. Data was collected from microarrays and submitted and filtered out based on significance using SAM. For this study, a two-class unpaired response format was conducted to calculate values for each gene. SAM was allowed up to 1000 permutations and missing data was imputed based on the K-nearest neighbor method. The delta value of significance was also adjusted so that the False Discovery Rate calculated by SAM was < 0.1 attaining q-values of 0%. Candidate genes were identified if after exposure to CSP there was a greater than twofold increase in expression relative to expression at the 0 min time point (no CSP addition). Table 4 below shows the normalized values obtained by taking the $\log_2$ of the ratio of the value of the samples induced to competence via CSP, to the values of the uninduced samples. Out of 208 genes that made it through the cut-off point of greater than twofold regulation, 128 genes were found to be up-regulated. Out of this pool, only five genes appeared to be regulated greater than 16-fold at 2.5 minutes compared to base levels: *comD*, *comE*, *comX*, SSA_0195, and SSA_1889. For

Figure 16. Examples showing the reasons for flagging and consequently removing microarray spot from being used in the normalization of values. Spots which A) show low levels of hybridization, B) that have high background streaks running through them, or C) do not contain enough data to confidently calculate values.

Figure 16

A.

B.

C.

Table 4. All genes listed were found to be significantly expressed by SAM and were up-regulated a minimum of twofold relative to base levels in at least one time point. The values listed here have been normalized to the control (uninduced) and represent the result of taking the log base two of their initial values. Original values were taken by dividing the median average intensity of the experimental value by the median average intensity of the control. Blank values are due to insufficient data.

Table 4

^a locus	^b Gene Symbol	^c 0 min Average	2.5 min Average	5 min Average	7.5 min Average	10 min Average	12.5 min Average	15 min Average	^d 17.5 min Average	20 min Average	30 min Average	^e Gene Direction
SSA_0016	comX	0.09	4.63	6.36	5.21	5.19	4.40	2.98		2.55	1.84	+
SSA_0023	aspC	-0.05	-0.03	-0.37	-0.20	0.44	0.89	1.07	0.90	0.85	0.25	+
SSA_0025	recO	0.03	-0.12	-0.24	-0.22	0.60	0.80	1.19	1.25	0.76	0.14	+
SSA_0036		0.03	0.41	6.23	5.20	6.16	5.88	5.88	5.20	5.28	4.23	-
SSA_0063	ruvB	0.05	0.04	0.08	1.09	1.39	1.19	1.30	1.22	1.53	0.72	+
SSA_0141		0.41	-0.28	0.48	1.17	1.60	-0.25	-0.14	0.67	-0.11	0.12	+
SSA_0166		0.75	-0.27	0.62	2.67	2.88	1.44	1.09	0.93	1.33	1.64	+
SSA_0184	comYA	-0.10	1.45	5.99	5.31	6.74	6.20	6.46	4.81	5.51	4.91	+
SSA_0185	comYB	-0.57	0.29	5.98	5.24	6.38	6.04	6.07	4.28	5.11	5.08	+
SSA_0186	comYC	0.15	1.02	6.09	4.93	5.60	5.70	6.07	4.72	5.56	4.59	+
SSA_0187	comYD	-0.36	1.13	6.38	5.58	6.34	6.39	6.03	5.01	5.83	5.01	+
SSA_0188			1.23	6.24	5.89	6.69	6.47	6.31	5.16	5.89	5.59	+
SSA_0189		-0.13	0.37	6.44	6.06	6.60	6.21	6.30	5.40	6.02	5.00	+
SSA_0190		-0.14	0.33	5.58	5.40	6.08	5.94	5.94	5.40	5.63	4.76	+
SSA_0191		-0.21	-0.04	4.52	5.25	6.19	5.72	6.18	5.48	5.52	4.54	+
SSA_0192	ackA	0.11	0.21	3.10	3.19	4.50	3.93	4.43	4.16	3.98	3.25	+
SSA_0195			3.98	4.65	3.83	3.44	3.17	2.87	2.60	2.36	2.64	+
SSA_0197	folP	0.36	1.60	2.50	2.17	1.69	1.28	0.79	0.92	0.87	0.74	+
SSA_0198		-0.02	1.19	2.33	1.67	1.47	1.03	0.96	0.89	0.70	0.72	+
SSA_0199	folE	-0.02	1.06	2.24	2.00	1.41	1.00	0.69	0.59	0.50	0.60	+
SSA_0200	folK	0.09	0.77	2.14	1.23	0.86	1.16	0.83	0.56	0.52	0.64	+
SSA_0212		0.23	0.15	0.05	1.01	1.45	0.36	0.19	0.25	0.14	0.49	+
SSA_0214	ssb2		0.94	6.13	6.09	6.74	7.06	6.70	5.47	6.14	4.32	+
SSA_0215	rbsB	-0.19	-0.15	0.85	1.68	2.36	2.33	2.16	1.67	1.42	0.85	+
SSA_0216		-0.10	-0.32	0.72	1.57	2.14	2.18	2.19	1.70	1.14	0.61	+
SSA_0217		-0.26	-0.38	0.25	1.54	2.00	2.06	1.85	1.48	1.17	0.67	+
SSA_0218		0.23	0.17	0.49	1.09	1.70	2.23	2.01	1.64	1.38	1.18	+
SSA_0225	groS	0.24	0.10	-0.10	0.30	0.52	1.04	0.88	0.47	0.32	0.53	+
SSA_0240		0.20	0.40	0.43	1.55	1.07	1.03	0.63	0.37	0.93	0.05	+
SSA_0242		0.04	-0.28	0.58	1.67	1.12	0.70	0.16	0.29	0.14	-0.04	+
SSA_0267		-0.33	-0.29	0.35	1.27	0.15	0.23	-0.11	-0.20	0.17	0.56	-
SSA_0310		1.03	-0.24	2.55	3.97	3.10	3.18	1.86	1.35	2.43	2.20	+
SSA_0316		0.52	-0.23	0.41	1.51	1.37	0.94	0.87	1.25	1.11	0.52	+

^a locus	^b Gene Symbol	^c 0 min Average	2.5 min Average	5 min Average	7.5 min Average	10 min Average	12.5 min Average	15 min Average	^d 17.5 min Average	20 min Average	30 min Average	^e Gene Direction
SSA_0410		0.08	0.07	0.84	1.59	1.46	1.18	1.45	0.44	0.93	0.64	-
SSA_0433		0.15	0.13	0.39	1.55	2.00	0.94	0.85	0.91	1.03	0.73	-
SSA_0504	amiF	-0.12	0.24	0.43	0.90	1.23	0.92	0.27	0.06	0.28	0.42	+
SSA_0563		-0.26	-0.19	0.17	1.31	1.61	0.20	0.09	0.73	0.01	0.44	-
SSA_0589	dapE	-0.38	-0.30	0.13	0.79	1.21	0.67	0.28	0.69	0.40	0.16	+
SSA_0597		0.15	0.23	0.74	0.68	0.52	0.17	0.53	1.03	0.09	0.35	+
SSA_0620		-0.18	-0.19	0.08	1.22	1.88	1.01	0.89	0.82	0.57	0.34	-
SSA_0639		-0.26	0.67	4.74	5.93	5.61	5.22	5.34	4.80	4.77	2.91	-
SSA_0640		-0.24	1.01	5.17	6.04	5.66	5.47	5.34	4.99	4.93	3.20	-
SSA_0642	pilD	0.35	2.22	6.66	6.34	5.94	6.05	6.45	5.55	5.54	3.44	-
SSA_0675	xseB	-0.11	0.02	1.16	1.45	1.85	2.61	2.03	1.40	1.37	0.31	+
SSA_0676	ispA	0.45	0.09	1.69	2.48	3.02	3.25	2.63	2.34	1.55	0.97	+
SSA_0677	hlyA	0.13	-0.04	1.58	2.81	3.13	3.30	3.04	2.22	2.02	0.84	+
SSA_0678	ahrC	-0.14	-0.07	1.38	3.07	3.31	3.15	3.01	2.41	1.91	0.87	+
SSA_0679	recN	0.16	0.31	1.51	2.33	3.17	3.54	3.16	2.54	2.01	1.31	+
SSA_0680	pphA	0.02	0.24	1.28	3.37	4.01	3.85	3.40	3.04	2.36	2.01	+
SSA_0715	comEA	-0.01	1.51	6.01	5.65	6.41	5.98	6.55	5.45	5.57	4.03	+
SSA_0716	comEC	-0.17		5.93	5.69	6.64	5.73	5.95	5.33	6.00	5.19	+
SSA_0718		-0.18	-0.01	0.73	1.21	1.61	0.77	1.08	0.54	0.70	0.76	+
SSA_0720	holA	-0.09	-0.05	0.13	1.00	0.85	1.36	1.07	0.51	0.55	0.27	+
SSA_0743	argR	-0.20	-0.36	1.14	2.77	2.32	1.59	1.17	0.80	1.76	1.25	-
SSA_0749	coiA			2.90		3.35	3.50	2.51	2.31			+
SSA_0750		0.17	0.22	2.23	1.89	2.43	3.47	2.23	2.17	1.37	0.51	+
SSA_0824	dnaG	-0.17	-0.14	0.83	2.06	2.70	2.51	2.15	1.83	1.39	0.67	+
SSA_0825	rpoD	-0.28	-0.25	0.92	2.23	2.99	2.37	2.00	1.71	1.32	0.47	+
SSA_0826		0.29	0.25	1.22	2.36	3.06	3.08	2.64	2.10	1.61	0.94	+
SSA_0981	leuD	-0.13	-0.46	-0.22	0.12	1.41	0.44	0.37	0.81	0.57	0.41	+
SSA_1000	galR	-0.24	-0.47	-0.16	1.00	1.00	0.21	0.08	0.56	0.47	0.55	-
SSA_1009	galT	-0.01	0.59	0.77	0.24	0.62	-0.04	0.39	1.04	-0.10	0.32	+
SSA_1076		0.15	0.07	0.13	0.97	1.04	0.26	0.18	0.41	0.37	0.40	+
SSA_1172			0.24	0.69	1.15	1.15	1.07	0.93	0.75	0.70	1.17	-
SSA_1183		-0.36	-0.12	2.36	3.82	4.10	3.54	3.35	3.04	2.89	1.49	-
SSA_1184	topA	-0.26	-0.12	2.76	4.10	4.39	3.78	3.54	3.11	2.98	1.69	-
SSA_1185	smf	0.23			5.61	5.85	6.80		5.81	4.81	3.42	-
SSA_1207	pta	-0.24	-0.20	0.06	1.75	2.70	2.58	2.47	1.82	1.33	0.56	-
SSA_1208		-0.21	-0.24	0.13	2.21	3.07	3.08	2.68	2.16	1.50	0.60	-

^a locus	^b Gene Symbol	^c 0 min Average	2.5 min Average	5 min Average	7.5 min Average	10 min Average	12.5 min Average	15 min Average	^d 17.5 min Average	20 min Average	30 min Average	^e Gene Direction
SSA_1209	ppnK	-0.17	-0.38	0.12	2.21	3.24	2.74	2.39	2.05	1.55	0.56	-
SSA_1210		0.10	0.31	0.78	2.09	3.04	3.47	3.04	2.40	1.89	1.03	-
SSA_1212	prs	-0.07	0.01	0.28	1.03	1.32	2.20	1.62	1.90	0.98	0.30	+
SSA_1213		-0.09	-0.13	0.32	0.02	0.14	0.29	0.46	1.20	0.61	-0.04	+
SSA_1214		-0.14	-0.19	0.50				0.85	1.15	0.66	-0.03	+
SSA_1216		0.10	0.22	1.41	0.48	0.50	0.70	0.69	0.93	1.06	1.31	+
SSA_1217		-0.03	0.14	0.94	0.27	0.51	0.42	0.04	0.52	0.63	1.20	+
SSA_1218	radC	-0.02	0.87	5.19	5.39	5.74	5.54	5.61	5.29	5.33	2.80	-
SSA_1235	pyrC	0.08	0.37	1.20	2.71	3.63	3.44	2.98	2.78	2.35	0.92	-
SSA_1252		-0.26	-0.35	0.53	1.40	1.27	0.09	-0.67	0.40	0.80	1.31	-
SSA_1314		0.15	0.01	0.08	1.58	0.16	0.46	-0.13	-0.27	0.00	-0.02	-
SSA_1323		0.25	0.25	0.39	1.04	1.32	0.39	0.70	0.75	0.70	1.57	-
SSA_1393			0.35	1.33	2.35	0.69	1.81	0.09	0.02	1.24	0.34	-
SSA_1535			-0.22	0.65	1.08		2.29	1.85	1.83		0.72	-
SSA_1536	bioY	0.21	0.25	0.20	0.26	0.74	1.13	0.83	0.72	0.39	0.25	+
SSA_1537		0.01	0.38	2.68	4.54	4.97	4.81	4.24	3.47	3.40	1.94	-
SSA_1584		-0.06	0.00	0.33	1.27	0.66	0.44	0.25	0.35	0.39	0.07	-
SSA_1681		-0.22	-0.19	0.73	1.15	0.36	0.02	-0.30	0.14	0.62	0.28	-
SSA_1711	nth	-0.28	-0.24	0.23	1.27	0.50	0.27	-0.39	-0.23	0.25	-0.12	-
SSA_1713	serA	0.06	0.12	0.51	1.29	2.07	2.65	2.27	1.71	1.34	1.41	-
SSA_1715	serC	0.08	0.02	0.86	1.45	2.20	2.77	2.58	1.94	1.93	0.94	-
SSA_1716		-0.06	0.44	3.78	4.16	4.79	4.59	4.35	4.38	4.11	2.57	-
SSA_1717	spnIM	0.01	0.58	3.83	4.36	4.86	4.71	4.62	4.36	4.23	2.51	-
SSA_1812		0.03	-0.07	1.31	2.80	3.31	2.90	2.27	1.47	1.13	0.52	-
SSA_1813		-0.05	-0.10	1.13	2.07	2.44	2.13	1.69	0.98	0.57	0.13	-
SSA_1830		0.09	0.13	1.36	1.73	2.55	3.27	2.57	2.00	1.79	0.56	+
SSA_1831	recX		0.07	1.31	2.26	3.04	3.58	2.80	2.01	1.90	0.74	+
SSA_1834	raiA	-0.34	-0.10	2.07	3.35	3.55	3.12	2.71	2.73	2.24	1.14	-
SSA_1835	comFC	0.24	1.27	4.75	5.30	5.00	5.08	4.72		4.77	3.28	-
SSA_1836	comFA	0.03	1.25	5.75	5.48	6.12	5.93	5.75	5.38	5.43	3.47	-
SSA_1879	glyS	-0.01	0.00	0.53	1.64	0.51	-0.11	-0.37	-0.10	0.22	0.60	-
SSA_1889		0.19	4.46	4.97	4.81	4.29	3.49	3.74	2.69	2.66	2.61	-
SSA_1975		0.01	0.14	0.82	0.09	1.04	1.45	1.92	0.89	0.47	0.27	-
SSA_2005	dnaJ	-0.31	-0.15	0.06	0.83	1.37	1.39	1.40	0.90	0.38	-0.10	-
SSA_2006		-0.37	-0.39	-0.12	0.56	1.06	1.00	0.92	0.30	-0.26	-0.57	-

^a locus	^b Gene Symbol	^c 0 min Average	2.5 min Average	5 min Average	7.5 min Average	10 min Average	12.5 min Average	15 min Average	^d 17.5 min Average	20 min Average	30 min Average	^e Gene Direction
SSA_2008	grpE	0.01	-0.04	0.04	0.65	1.18	1.12	0.92	0.09	-0.29	-0.45	-
SSA_2036		-0.14	-0.20	0.43	1.08	0.85	0.68	0.18	0.59	0.38	0.29	-
SSA_2116	cbf	0.06	0.12	1.53	3.04	3.63	3.10	2.65	2.29	1.76	0.99	-
SSA_2117	rmuC	-0.01	0.11	1.65	3.13	3.73	3.42	2.89	2.39	1.97	1.01	-
SSA_2122		0.11	0.81	1.44	3.07	2.30		2.79	2.17	1.63	0.34	-
SSA_2156		-0.14	-0.24	0.81	2.61	3.24	2.60	2.27	2.01	1.48	0.76	-
SSA_2157	radA	0.17	0.15	2.42	3.83	4.04	4.00	3.90	3.04	2.96	1.76	-
SSA_2158		-0.04	0.11	2.61	3.63	4.31	4.31	3.61	2.95	3.00	1.56	-
SSA_2159		-0.11	0.07	2.81	4.37	4.46	4.35	3.79	3.32	2.86	1.37	-
SSA_2160	dut	0.05	-0.02	3.03	4.36	4.96	4.42	4.17	3.68	3.01	1.55	-
SSA_2181		-0.21	-0.16	0.09	1.10	0.58	0.78	0.44	0.14	0.28	-0.11	-
SSA_2189			-0.24	0.48	1.86	1.86	1.43	1.05	0.62	0.83	0.18	-
SSA_2190		0.19	0.41	0.30	0.55	0.92	1.12	1.12	1.06	0.77	0.32	+
SSA_2192		-0.03	1.17	3.40	3.98	4.31	3.93	3.85	3.43	3.53	2.02	-
SSA_2231		-0.32	-0.21	0.95	2.23	2.37	1.90	1.35	0.98	0.58	0.48	-
SSA_2233		0.06	0.34	2.72	2.67	2.90	4.12	3.22	2.64	1.77	1.34	-
SSA_2244		-0.20	0.10	1.64	2.80	3.19	2.92	2.83	2.44	2.11	1.43	-
SSA_2245	recA	-0.14	0.16	2.23	3.07	3.72	3.38	3.34	3.27	2.88	1.99	-
SSA_2246	cinA	-0.04	1.28	4.58	5.36	5.72	5.45	5.59	5.30	5.41	4.67	-
SSA_2253	tag	0.05	0.26	0.06	0.64	1.03	1.14	0.80	0.47	0.35	0.25	-
SSA_2321	czcD	0.04	-0.09	0.22	1.02	0.74	0.33	0.23	0.13	0.08	0.00	-
SSA_2378	comE	-0.12	4.81	5.06	4.67	4.06	3.68	3.50	3.26	3.45	3.40	-
SSA_2379	comD	-0.08	4.29	4.45	4.69	3.72	3.39	3.21	2.82	3.31	3.20	-

- (a) SSA numbers indicate gene loci with SSA_0001 at the chromosomal origin of replication. Each gene was given a UID number and probes representing these regions were on the microarray slides.
- (b) Symbol given to each gene based on the published annotation.
- (c) Values shown are the ratio of the value at each time point (2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, and 30 minutes) after addition of CSP to the value at time 0. Data for 0 minute reported here to show comparison values.
- (d) Data taken for 17.5 minutes post-CSP exposure is the result of only two individual experiments.
- (e) + and - designate the orientation with respect to the origin of replication of the *S. sanguinis* chromosome.

visualization of data, a colorimetric graph was created using numerical values for all up-regulated genes (Figs. 17 and 18).

The values calculated from this study were first clustered based on similar expression patterns using Cluster 3.0. For this study, genes were clustered using the average linkage clustering technique which is a hierarchical agglomerative clustering algorithm. Based on the figures created using the TreeView software, genes were definitively grouped into two categories. Those whose expression was induced at least 4-fold at 2.5 minutes were named “early response genes” due to their immediate expression following CSP exposure. Genes that lagged just behind showing greater than a 3-fold induction no later than 10 minutes post-CSP exposure were termed “late response genes”, due to their relatively delayed response to CSP.

For the early response cluster, nine genes were clustered together including the five listed previously (Fig. 17). The other four genes, SSA_0197, 0198, 0199, and 0200 showed minimal up-regulation throughout the time course. Closer observation revealed that all four of these genes were adjacent to each other and were just downstream of the early response gene SSA_0195. Thus, expression of these genes may in fact be caused via transcriptional read through of the terminator sequence in this gene.

The other five genes up-regulated in the early response cluster show that after initial activation at 2.5 minutes, expression of these genes continued throughout the 30-minute time course. After approximately 7.5 – 10 minutes of relatively constant expression, intensity levels began to decay, although never returning to basal levels. This is an interesting observation since competence genes in other streptococcal

Figure 17. Colorimetric representation of relative expression levels of CSP-induced *S. sanguinis* genes. Expression levels are measured by hybridization to cDNA in four replicate spots on microarray slides. The results are a product of three time courses individually conducted. Shown are the 128 genes up-regulated > 2-times basal levels in at least one time point and have been identified as significantly expressed based on SAM. The first zoomed cluster contains those genes that were classified as early response genes. The second zoomed clusters are genes that were classified as first-class late response genes. Each expression value was designated a color code to indicate levels of expression. The colors varied from black (< 2-fold up-regulation) to bright red (> 32-fold up-regulation).

Figure 17

Figure 18. The same colorimetric graph as Figure 17, only focused on the second-class of late response genes. Genes highlighted in red signify those that showed 3-fold induction 5 minutes post-CSP exposure. Genes in blue showed induction starting at 7.5 minutes after CSP-exposure. Lastly, genes in green were shown to be induced greater than 3-fold after 10 minutes post CSP-exposure.

Figure 18

species have a definitive rise and fall of expression. Expression levels for these genes do fall; however the lag is much longer and expression never completely returns to baseline levels over the 30-minute time course.

The finding that late response genes exhibited two distinct expression patterns led to their classification as either “first-” or “second-class late response genes”. Genes in the first class are those that are highly up-regulated 5 minutes post-CSP exposure, and remain at high levels through the 30-minute time course. Genes that were classified as second-class late response genes are those that were up-regulated between 5 – 10 minutes after CSP exposure. Genes in this class reached 3-fold induction at least once throughout the 30-minutes time course, but their expression levels quickly diminished soon afterwards.

The second cluster in Figure 17 and the expanded clusters in Figure 18 show the first and second classes of “late” response genes respectively based on the criteria described above. Out of the 119 genes that displayed a twofold or greater induction in at least one time point and were not classified as “early” response genes, 69 genes can be characterized as “late” response because up-regulation began no later than 10 minutes post-CSP exposure. Interestingly, out of this pool, seven genes were isolated and not directly adjacent to any other CSP-regulated gene, SSA_0036, 0310, 1537, 2122, *radC*, *pyrC*, and *ispA* (Fig. 19). However, the remaining 62 genes were clustered near or directly adjacent to other genes passing the twofold greater expression filter. Of these 64 late response genes, 9 are putatively situated within the same operon. These include the four *comY* genes, SSA_0188, 0189, 0190, 0191 and *ackA*. The remaining 55 genes

Figure 19. Organization of CSP-induced late response genes. Genes classified as first-class late response genes are represented in red and genes classified as second-class late response genes shown in black. ORF orientation relative to the chromosomal origin of replication is indicated by the direction of the arrow.

Figure 19

are organized in clusters of two or more adjacent genes that lie in similar orientations and exhibit similar temporal expression patterns. Additionally, *ssb2*, *coiA*, *smf*, *spnIM*, *comFA*, and *cinA* are part of unique operons that are clustered together due to their similar expression patterns and categorized as first-class late response genes (Fig. 17). However, these genes lie upstream of other genes that are seemingly co-transcribed and are shown to be up-regulated, yet clustered in the second class of late response genes (Fig. 19), indicating a slightly different temporal regulation.

In the first 26-gene set (Fig. 17), late response genes were activated 5 minutes post-CSP exposure and, just as with the early response genes, remained active throughout the 30-minute time course. Unlike the early genes, these late genes remained up-regulated at high levels with the exception of most notably *comFA* and *comFC* which began to show signs of repression at the 30-minute mark. The second class of late genes activated (Fig. 18) showed induction between 5 and 10 minutes after CSP exposure. Unlike what has been seen in the first class of late genes, expression of these genes rose and fell within the 30-minute time course. This second class contains 11 hypothetical proteins as well as 5 different proteins dealing with DNA recombination and repair. As has been noted before (Fig. 19), many of these genes lie just downstream or directly adjacent to other up-regulated genes observed in the first cluster, yet vary in their expression levels.

Lastly, the remaining 49 genes that showed 2-fold levels of induction in at least one time point were grouped into a final category termed “delayed response genes”, due in part to the lengthy delay between the late genes and these genes, and also because

expression was minimal. Expression of these genes occurred approximately 15 minutes after initial exposure with CSP. Unlike what was observed for the previous two categories, expression barely made the cut-off and quickly dropped to below acceptable levels after a few minutes. Most of these genes are annotated as heat shock proteins, DNA recombination proteins, and co-chaperonins (i.e. SSA_2008, *dnaJ*, and *groS* respectively). The fact that expression of these genes is minimal and their patterns highly different from those characterized previously, suggests that regulation of these genes may not be via a ComE or ComX dependent pathway. Alternatively, their proximity to other highly regulated genes also suggests that regulation may have been incidental.

H. Putative competence genes are shown to be activated in *S. sanguinis* via microarray analysis in response to CSP treatment.

A total of 22 genes that are up-regulated in response to CSP in *S. pneumoniae* have been shown to be indispensable for competence (84). In order to determine if these same genes are present and similarly regulated in *S. sanguinis*, the translated sequences were retrieved, and a BLASTP search was performed with them in an attempt to look for homologs in *S. sanguinis*. In the TIGR4 strain, eight of the genes that were found to be indispensable for competence were classified as early response genes. Out of these eight, only four genes were found to have homologs in *S. sanguinis*: *comCDE*, and *comX* (Table 5). Since a *comC* mutant was used for these arrays, no data were collected for this gene. However, considering that *comDE* were up-regulated and are likely co-transcribed along with *comC*, it is reasonable to assume that *comC* would normally be

Table 5. The results of a BLASTP search in which the protein sequences of genes known to be indispensable for competence in *S. pneumoniae* strain TIGR4 were used as query sequences to search for orthologs in *S. sanguinis*.

Table 5

<i>S. pneumoniae</i> locus	<i>S. sanguinis</i> Ortholog	Upregulated
Early		
SP_0014, <i>comX</i>	SSA_0016, <i>comX</i>	Yes
SP_0018, <i>comW</i>	None	N/A
SP_0042, <i>comA</i>	None	N/A
SP_0043, <i>comB</i>	None	N/A
SP_2006, <i>comX2</i>	None	N/A
SP_2235, <i>comE</i>	SSA_2378, <i>comE</i>	Yes
SP_2236, <i>comD</i>	SSA_2379, <i>comD</i>	Yes
SP_2237, <i>comC</i>	SSA_2394, <i>comC</i>	N/A
Late		
SP_0954, <i>comEA</i>	SSA_0715, <i>comEA</i>	Yes
SP_0955, <i>comEB</i>	SSA_0716, <i>comEC</i>	Yes
SP_0978, <i>coiA</i>	SSA_0749, <i>coiA</i>	Yes
SP_1266, <i>dprA</i>	SSA_1185, <i>smf</i>	Yes
SP_1808, <i>pilD</i>	SSA_0642, <i>pilD</i>	Yes
SP_1908, <i>ssbB</i>	SSA_0214, <i>ssb2</i>	Yes
SP_1940, <i>recA</i>	SSA_2245, <i>recA</i>	Yes
SP_2047, hyp. prot.	SSA_0190, hyp. prot.	Yes
SP_2050, <i>cglD</i>	SSA_0187, <i>comYD</i>	Yes
SP_2051, <i>cglC</i>	SSA_0186, <i>comYC</i>	Yes
SP_2052, <i>cglB</i>	SSA_0185, <i>comYB</i>	Yes
SP_2053, <i>cglA</i>	SSA_0184, <i>comYA</i>	Yes
SP_2207, <i>cflB</i>	SSA_1835, <i>comFC</i>	Yes
SP_2208, <i>cflA</i>	SSA_1836, <i>comFA</i>	Yes

up-regulated in SK36. Orthologs of the remaining four genes have not been identified in *S. sanguinis*. The gene for ComW, a protein believed to be responsible for stabilizing ComX, as well as *comX2* (an identical second *comX* gene) have been shown to be absent in *S. sanguinis* (113); *comAB* has also eluded detection.

The remaining 14 indispensable genes are classified under “late response”. All 14 genes contain homologs in *S. sanguinis*. In all cases, the microarray results showed that these genes were up-regulated in rather strong fashion and were clustered together in the first class of late response genes (Fig.17). Interestingly, the function of SSA_0190 has yet to be determined and is classified as simply a hypothetical protein. Nevertheless, in both the TIGR4 strain and *S. sanguinis*, expression of this gene is high when cells are induced to competence. Although it can be speculated that these genes are essential for competence given their homology to known indispensable genes in *S. pneumoniae*, further investigations specifically targeting these genes must be performed before this is concluded in *S. sanguinis*.

I. Examination of sequences upstream of highly expressed CSP-induced genes reveals a putative “com-box” for late response genes.

In order to look at putative promoter sequences under the control of either ComE or ComX for early and late response genes respectively, the SK36 genome was searched using the fuzznuc program from the EMBOSS suite <http://www.ch.embnet.org/EMBOSS/index.html>. For the late response genes, a model was based on the findings of Campbell *et al.* (9) for *S. pneumoniae*. Based on their findings that a conserved octamer was observed upstream of these late response genes

and that it was likely the region that ComX bound thus promoting transcription, the *S. sanguinis* genome was also searched for a similarly conserved region, also known as the “*com*-box”. Based on a strict interpretation of the model with no tolerance for mismatches, 14 matching sequences were located. Out of these 14, eight were found within genes and were concluded unlikely to be binding sites for ComX. Another two were found upstream of SSA_2192 and *pyrC*, two genes classified as late response genes based on the findings detailed above. The remaining four sites were found to be upstream of *comEA*, *comFA*, *pilD*, and *smf* (Table 6). When the parameters were loosened to allow up to one mismatch, octamers upstream of *comYA*, *coiA*, and *ssb2* was discovered. Thus the only missing gene from the list gathered from before (Table 5) was *recA*. Closer analysis of this gene revealed that it was the second gene in an operon just downstream of *cinA*. Surveillance of the upstream region from *cinA* revealed a putative conserved sequence 177 bp upstream from the start site. However, this putative *com*-box contained two mismatches (Table 6). The *com*-box was not found immediately upstream of SSA_0185, 0186, 0187, or 0190, nor of 0716 or 1835. In summary, all late response genes that share homology to genes in *S. pneumoniae* that have been confirmed as essential for competence, contained conserved putative *com*-boxes upstream of their start sites. The only gene not directly downstream of the putative promoter sequence is *recA*.

Interestingly, when the model created by Ween *et al.* (110) was used to search for possible ComE binding sites, only three regions were located throughout the genome and only *comC* lay just downstream of the putative promoter. *comX*,

Table 6. This table shows the results of a fuzznuc analysis of *S. sanguinis* using the model for the conserved octamer upstream of the late response genes established in *S. pneumoniae* strain, TIGR4. The position of the putative *com*-box relative to the gene start site is listed.

Table 6

TIGR4	TTTT-(0,12)-TACGAATA-(0,212)-ATG
<i>comYA</i>	TTTTT-----0-TgCGAATA-----48-ATG
<i>ssb2</i>	CTTT-----0-TACGAATg-----21-ATG
<i>pilD</i>	TTTTT-----11-TACGAATA-----8-ATG
<i>comEA</i>	TTTTT-----9-TACGAATA-----21-ATG
<i>coiA</i>	TTTT-----0-TgCGAATA-----39-ATG
<i>smf</i>	TTTTT-----0-TACGAATA-----19-ATG
<i>comFA</i>	TTTT-----10-TACGAATA-----8-ATG
<i>cinA</i>	CTTT-----1-TtCaAATA-----177-ATG

SSA_1889, and SSA_0195 lacked this promoter sequence suggesting that regulation of these early genes may not follow the same model that has been established for *S. pneumoniae*. In order to determine if a conserved region was located upstream to the gene start site that could be considered an operator site, the sequences were aligned using the ClustalW program analyzing the first 117-bp upstream from the start site. After alignment, the results showed that there were several regions where all sequences aligned well (Table 7). At the -55 site, the *comC* sequence contains a TATTT which is shared with *comX* and SSA_1889 and only partially with SSA_0195. If the latter two are removed, this conservation is extended further to include CTTTCTATTT. Additionally, 29-bp upstream to this region is another sequence, AAANNAt, which is conserved between all four sequences. Because the microarray result limits us to these four early response genes, it is difficult to create a model based on this small of a pool. Further investigation looking at putative promoter sequences for these early response genes will have to be performed in the future.

J. Examination of temporal regulation of selected competence genes via qRT-PCR.

To confirm the results observed from the microarrays and to obtain more quantitative analysis of transcriptional regulation in *S. sanguinis*, qRT-PCR reactions were performed. RNA samples were taken from each time point, and primers developed to quantitate three early response genes – *comD*, *comE*, and *comX* – and two late response genes – *comYD*, and *comEA*. The data for each gene were normalized to *tuf*. This gene was selected for normalization based on microarray results showing that expression for this gene remained constant and high throughout the 30-min CSP time

Table 7. Sequence alignment of the regions upstream from four early response genes using ClustalW.

Table 7

comC	-----TAATGTCATTT <u>CGGGGA</u> -----T <u>AAAAAAT</u> GACATTT <u>CAGGG</u>
comX	-----ACTTAAAAATAGTTATTCGAGTCAGAAGAGCTCCT <u>AAAAATATG</u> -CTTTT <u>TAGAA</u>
SSA_1889	-----AATGCCTTAATAATTCATTTTTAAAGAG----- <u>AAATAAT</u> CCCCATCCGAAG
SSA_0195	GAAAGTAGAGAATATCTGCTTTTTTTGT <u>CATTTGAGG</u> -CAC <u>AAATTA</u> GGACATTTGAGGG
	* * ** *** * * *
comC	--AAAATTGTCACCTAACT <u>CTTTC</u> <u>TATTTT</u> CTATAATGATAAATGTAAGGTGACTTACAA
comX	--GAAAGAGCCACCTTG <u>CTTTC</u> <u>TATTTA</u> -CAGTAGAATTAA--GTCAAGTAAATTTTAA
SSA_1889	TTAAAGAAATTATCAGGTGGGGAT <u>TATTT</u> TATGTCTTTGATTTTGAAAAGGTGCTATTTCA
SSA_0195	---GATTTCTACCAGCTTGCTCT <u>TACTT</u> -CTTCTATAATGA---TAAGAGAAGAATGGA
	* * * *
comC	GATAAAAAGATAGGAGATAGTTATG
comX	GGAGGAACT-----ATG
SSA_1889	GGGATAAAAA-----ATG
SSA_0195	GAGAAT-----ATG
	*

*Note: underlined region in the *comC* sequence represents the conserved sequences matching the model established by Ween *et al.* (110).

course. As an additional control, the housekeeping gene, *gapA*, was also included in the analysis.

Analysis reveals that, just as in the microarrays, early genes were activated only 2.5 minutes after CSP induction (Fig 20A). Messenger RNA levels reached a maximum approximately 5 minutes post-CSP exposure. Transcript levels then decreased, although never fully returning to baseline levels. Similarly, late response genes exhibited a small 2.5-minute lag before being activated (Fig. 20B). The *comEA* gene showed expression levels that were quite high at 2.5 min, although not as high as *comX*. Both *comEA* and *comYD* showed maximal expression 15 minutes after CSP exposure. When putting the two graphs together, there is a definitive overlap between the two classifications of genes (Fig. 20C). The high transcript levels in both late response genes may also give insight to what was observed in the microarrays. Late response genes in the microarrays were determined to remain at constant high levels. The qRT-PCR results showed transcript levels did in fact drop after reaching maximum levels, but perhaps not enough to have been observed in the microarrays.

In summary, the qRT-PCR analysis reveals two waves of overlapping transcription. The early response genes were turned on starting at 2.5 minutes, but reached maximum levels at 5 minutes. It would appear that the 30-minute time period is not sufficient to reveal the point at which these early response genes return to baseline expression. For the first-class late response genes, it appears that mRNA levels rose dramatically after 2.5 minutes of lag time, reaching a maximum expression level at 15

Figure 20. The results of qRT-PCR analysis on samples prepared at the indicated times. Values were plotted based on relative expression levels compared to the values obtained at time 0. The log base 2 of these results are plotted. A) Results for the early response genes. B) Results for the late response genes. C) The combined results for both waves of transcription including the plotted values for *gapA*. Error bars omitted for simplicity.

Figure 20

A.

B.

C.

minutes. Just as in the early response genes, expression levels began to decay immediately following peak levels. The results here verify the data observed with the microarrays.

IV. DISCUSSION

The evolutionary implication of a competent organism becomes immediately apparent when considering the subsequent adaptability of the cell to environmental cues. Importantly, it has also been shown that competence can benefit bacteria by conferring upon them the ability of picking up antibiotic resistance genes. Besides conferring upon cells new properties, it has also been suggested that the uptake of extracellular DNA can serve as a carbon source for many bacteria (24). As was stated earlier, the presence of platelets aggregated to the damaged endothelium of heart valves predisposes an individual to infective endocarditis. As *S. sanguinis* adheres to these platelets, a vegetation is formed which has some characteristics of biofilms. Biofilms in other streptococcal species have been shown to be affected in competence mutants (60, 77). Therefore, considering that *S. sanguinis* is one of the first colonizers of the oral cavity (57) and that it has been implicated as a causative agent of infective endocarditis (73), the necessity to genetically characterize the regulation of competence becomes evident.

The attainment of competence can be repressed by growing, at least some streptococcal species, in acidic conditions. However, unlike what has been seen before (84, 108) in other streptococcal species, growing SK36 in these conditions not only abolishes competence, but also negatively affects the growth rate of the bacterium. It is not understood why *S. sanguinis* is much more sensitive to pH than other streptococcal species examined, though it is not entirely implausible to believe that these conditions

cause the transcription of stress response genes, allocating cell resources away from growth activators and more towards repairing the cell wall for example. It could very well affect another quorum sensing mechanism entirely devoted to population control such as in *Staphylococcus aureus* which contains a two-component system that regulates the timing of population growth by cross-talking with other cells in the vicinity (reviewed in reference (31)). By sensing these nearby cells, *S. aureus* senses when to start and end exponential growth.

As was originally noted by Tomasz and Hotchkiss (104), competence seems to develop and decay in *S. pneumoniae* based on cell density. The result from Section A of the Results section implicates *S. sanguinis* as an additional streptococcal species with similar characteristics. It should be noted that as cultures reach stationary phase, the media will gradually become acidic. Studies determining if the drop in competence observed in Figure 4 is a result of the acidity of the media are currently ongoing. It has been observed that the density dependence for spontaneous competence in *S. pneumoniae* is eliminated once a large dose of exogenous CSP is applied to cells (35). Thus, competence is altered from that of cell density-dependence to CSP-dependence. It has been hypothesized that the decay of competence may be mediated by genes up-regulated via a ComX-dependent pathway (56). Therefore, as ComX is produced during the competent phase of the streptococcal species, genes required for repression of this stage are also transcribed. Section E of the Results section shows that the mechanism of competence repression may not fit this model entirely. Though no definitive trend was observed at low cell density, when JFP41 was at high cell densities, competence

appeared to be repressed, albeit not as dramatically as it was observed in SK36, even in the presence of saturating concentrations of CSP.

The findings implicate a second regulatory system partially involved in the repression of competence. It has been observed that competence may also be repressed in oxygen limiting conditions via a two-component system (21). However, transformation conditions were essentially performed aerobically in this study, and oxygen limitation thus can not be implicated here. A potential explanation of these results can be made through the observation of the role of LuxS in repression of competence (92). The study showed a hierarchy of regulation whereby LuxS was involved in the repression of competence, but not as highly as CiaRH and StkP in *S. pneumoniae*. Therefore, further investigation into the regulatory mechanism of natural competence in *S. sanguinis* must be performed.

The physiological characterization of competence in the *comC* mutant was critical for the success of the microarray analysis, which was a major component of this project. Unlike what has been seen in *S. gordonii* where varying concentrations of CSP had no effect on competence (108), in JFP41, competence appeared to resemble that of *S. mutans* (47) which showed a more linear dose-like response to CSP (see Results, Section D), plateauing at approximately 200 – 240 ng ml⁻¹. Thus, a CSP concentration within this range was used for the microarray studies. As briefly mentioned above, even though natural competence was abolished in JFP41, transformation frequencies were negatively affected at high cell densities. The microarray studies were therefore performed at a density that was optimal for transformation in SK36, ensuring that

experiments would be performed under relevant conditions (see Results, Section A). Lastly, since the microarrays were examining genes induced by CSP, it was necessary to determine the duration of competence in the mutant strain. It was shown that competence peaked 10 minutes following CSP exposure, and abruptly dropped, reaching low transformation frequencies after 30 minutes. Thus the results suggest that regulation of competence occurs within a 30-minute time period.

Studies have shown that once competence is induced in *S. pneumoniae*, it appears for approximately one hour before transformation frequencies begin to drop below the level of detection (14, 104). Typically, optical density readings will be within 0.1 – 0.2 from the start to end of competence. In both *S. pneumoniae* and *S. sanguinis*, competence occurs at early- to mid-log phase. Comparatively speaking, the results presented here differ somewhat in that competence occurs for a longer period of time in *S. sanguinis* than in *S. pneumoniae*, although still occurring early in the exponential phase of growth. Transformation frequencies of 3% and above can be attained at an OD₆₆₀ of 0.030 – 0.600 (data not shown), indicating cell densities between $6 \times 10^5 - 1 \times 10^8$ CFU ml⁻¹. Assuming a doubling time of 40 minutes, this equates to 4.5 hours of recoverable transformants. For the *comC* mutant with respect to duration of competence, the results in this study are similar to what was observed in *S. gordonii* under acidic conditions in the presence of exogenous CSP (108). That is to say, within the first 30 minutes post-CSP exposure competence abruptly peaked, and similarly dropped, thus suggesting similar regulation in both species.

Two previous studies in *S. pneumoniae* and *S. gordonii* have looked at the genome-wide transcription of genes after induction of competence (83, 108). In both studies, repression of endogenous CSP was obtained by growing the strains in acidic conditions. The microarray studies in this project were performed with a *comC* mutant strain, JFP41. Extensive planning was performed in order to ensure that the reading frames for the flanking genes were maintained and that no polar effects on downstream genes would occur. The use of JFP41 solved a major issue to facilitate the microarray analysis; it allowed for the induction of competence without having to grow cells in any media other than what is normally used for the transformation of SK36, and thus avoided the possible complication of growing the strain in conditions which may alter gene regulation or even up-regulate stress response genes that would otherwise not be affected in normal conditions.

The results of the microarray experiment are similar to what has been observed in *S. pneumoniae* and in *S. gordonii* (83, 84, 108). Transcription of these competence genes follows two waves: genes that are expressed early and are consequently regulated in one manner and late response genes that are regulated via an alternate route. Transcript levels of the early genes were up-regulated within 2.5 minutes of CSP exposure. Within the next 2.5 minutes, ComX had been produced and was presumably actively binding to conserved sequences and increasing late gene mRNA transcript levels seeing as late response genes were being up-regulated. A similar pattern is seen in *S. pneumoniae* where it was observed that transcript levels begin to rise immediately after exposure to CSP, reaching maximum expression 7.5 – 10 minutes after CSP

induction. Late response genes lagged just behind these early responders beginning their up-regulation at 5 minutes, and reaching maximum levels approximately 15 minutes post-CSP exposure. In *S. gordonii*, the early induced genes were similarly expressed. However, late response genes were not up-regulated until 15 minutes after initial induction; a significant lag compared to the previous two.

The results also show that for all genes that are homologs of those essential for competence in *S. pneumoniae* (*comCDEX*, *comEA/C*, *comYA/B/C/D*, *comFA/C*, *coiA*, *recA*, *smf*, *pilD*, *ssb2*, and *SSA_0190*), regulation appears to occur at the transcriptional level. Transcription of all early response genes occur in parallel as well as all late response genes, and mRNA transcript levels rise accordingly, suggesting that each gene classified under one of these headings is regulated by a similar promoter. Searching the *S. sanguinis* genome using Fuzznuc revealed that all late response genes shown to be essential for competence in *S. pneumoniae* contained the same 8 bp region (TACGAATA) preceded by a string of [CT]TTT no more than 12 bp away. The only notable difference was seen in *recA*. For this gene, the putative *com*-box contains two mismatches, does not serve as the immediate upstream promoter sequence, and is greater than 150 bp away from the start site. However, considering that *cinA* is the immediate downstream gene and has been shown to be up-regulated in competence induction in other studies (83, 108), and classified as a late gene, this may in fact be the binding site for activation of transcription for this operon. In *S. gordonii*, a total of 12 CSP-induced genes were found to contain this sequence upstream from their start site;

notably the *comY*, *cinArecA*, *comF* operons, and *pilD* had this sequence upstream from their start sites just as in this study (108).

It should be noted that the model used in *S. pneumoniae* for the *com*-box sequence may not be fully conserved in all competent streptococci and may not represent the actual *com*-box in *S. sanguinis*. As stated in the Results section (see Section I), using a strict interpretation of the model, only 14 sites were discovered throughout the genome; 8 of which lay in the middle of genes. When the model was loosened to allow one mismatch, the number of hits increased dramatically (data not shown). Most of these hits lay in the middle of genes and had no effect on transcription. Interestingly, many genes not found to be up-regulated via microarray analysis contained this putative promoter just upstream of their start sites. The fact that there was no change of expression in these genes indicates that the model being used may not be entirely sound in *S. sanguinis*. Additionally, many genes that were up-regulated also contained an additional string of TTTT. However, when the model was updated to incorporate this new set of T's, it was observed that this sequence was also found in the middle of many genes and also upstream of genes not regulated in competence. These results show that the *com*-box sequence has yet to be fully elucidated in *S. sanguinis* and thus further investigation must be performed to determine all elements of the *com*-box.

Conversely, using the model established by Ween *et al.* (110) for ComE binding sites, only three hits were seen throughout the *S. sanguinis* genome, and only one was upstream of a highly up-regulated operon, *comCDE*. *comX*, SSA_1889, and SSA_0195

did not have this putative promoter sequence. When the upstream sequences of all four early response genes were aligned, multiple regions were found to be similar. Most notable was a 5-bp sequence just upstream of their start sites (TATTT; see Results Section I) and another 7-bp (AAA[AT][AT]A[TG]) region between 28 – 29-bp upstream from that. Including only *comC* and *comX*, the former sequence would expand to a 10-bp CTTTCTATTT. It should be noted that this small 7-bp sequence could in fact represent a putative UP element in this upstream region. It would be quite enticing to suggest that these sequences are in fact the actual ComE binding site for these early response genes, however further investigation must first be performed before this is concluded. In *S. gordonii*, the direct repeats described in the model were missing upstream from their early response genes as well. The fact that in two separate streptococcal species, the expected ComE box sequence was missing indicates the necessity to extensively study the upstream sequences for these early response genes.

Expression of the first class of late genes (see Results, Fig. 17) was different than that of the second class of late genes (see Results, Fig. 18). In many cases, the second class of late genes lay directly adjacent to the first class of late genes suggesting that they may be co-transcribed (see Results, Fig. 19); the fact that expression in both classes begins at the same time supports this hypothesis. However, after 12.5 ± 2.5 minutes of expression, transcript levels drop. This abrupt change in transcript levels, even though they may in fact be transcribed simultaneously, suggests that unique transcriptional regulation for each gene plays a vital role in competence. Indeed, expression of *recA* appeared to fluctuate throughout the 30-min time course which

supports earlier findings detailing the differences in changes of expression in *S. pneumoniae* for *recA* (45). It is important to note, however, that this relatively low expression compared to other genes in the first class may be due to the weak putative promoter sequence discovered using Fuzznuc. It should be noted that the observed drop in transcript levels may also indicate a loss of mRNA stability within the cell. It may also indicate that initial transcription may have occurred due to transcriptional read-through of the terminator sequences just upstream from these first class genes. As transcription of first-class late response genes begins to drop, so does the frequency with which transcriptional read-through occurs and thus transcription levels for these downstream genes is negatively affected.

It is quite interesting to observe the relative expression levels of genes that seem to play no role in the induction of competence, but are in fact up-regulated when the cells become competent. As noted in the Results section (see Section G), a total of 128 genes in *S. sanguinis* were found to be up-regulated in response to exposure with CSP; however only 17 genes (~13.3%) have homologs in *S. pneumoniae* that have been shown to be essential for competence. The vast majority of genes up-regulated by CSP in *S. sanguinis* are probably also not essential for competence. It has been suggested that transcription of genes not essential for competence in *S. pneumoniae* occurs due to their close proximity to other highly expressed genes with a putative *com*-box (83). Many of these genes encode proteins involved in DNA recombination and repair and transcriptional regulators. Though not important for induction of competence, it is apparent how these proteins would be useful when introducing foreign DNA into the

genome. Evolutionarily speaking, the clustering of these genes near CSP-induced genes may confer a Darwinian advantage for the species. Thus, instead of allocating resources such as polymerases and ribosomal proteins to each individual gene with the ultimate goal of acquiring foreign DNA from the environment, only a select few absolutely critical genes are directly regulated via CSP, and the rest are selectively placed in a location to benefit from their transcription. It has also been suggested that many of these clustered genes in *S. pneumoniae* take part in the killing of surrounding cells (13). Whether or not this occurs in *S. sanguinis* has yet to be elucidated and requires further investigation.

Summarizing the microarray results, the number of genes that were up-regulated in response to CSP in this study was shown to be 128. Out of this lot, 69 were classified as late response genes and only 5 classified as early. The remaining 54 genes showed a maximum of 2 to 3-fold expression approximately 15 – 20 minutes after initial exposure with CSP. The number of CSP-induced genes in *S. pneumoniae* and *S. gordonii* was 188 and 177 respectively (~50% more). A total of 19 genes in *S. pneumoniae* were classified as “delayed response”, and nearly half of these were deemed ComX-independent. Interestingly, many of these genes were stress response genes such as *groES*, and *dnaJ*. The acidic conditions SK36 was grown in caused a deleterious effect on growth. This condition could very well account for the number of “delayed” response genes observed in *S. pneumoniae*. However, *S. gordonii* was grown in similar conditions and induced to competence in the same manner; yet very few delayed genes were observed, and expression of these stress response genes was minimal, suggesting

that the observation of delayed response genes may not have to do with growth conditions.

In *S. gordonii*, 127 late response genes were detected. The number of genes in this category compared to *S. pneumoniae* and *S. sanguinis* is quite large. Additionally, the lengthy lag between early and late response genes may also have been caused by the differences in CSP concentration used to induce the cells. Whereas in *S. pneumoniae* and in this study, the concentration of CSP used was 200 and 220ng ml⁻¹ respectively, the concentration used in the former study was only 100ng ml⁻¹; though it should be noted that varying concentrations of CSP showed no increase in number of transformants recovered in *S. gordonii*. Another explanation for the larger pool of categorized “late response” genes may be due to their experimental procedure where only the 5 and 15 minutes after initial exposure with CSP were analyzed. Nevertheless, further investigations dealing with varying growth conditions should be performed to study their specific effects in *S. sanguinis*.

The sequences of the proteins encoded by the 69 late response genes observed in *S. sanguinis* were used to search for similarly up-regulated genes in *S. pneumoniae* and *S. gordonii* (Table 8), which were clustered into 25 unique operons (see Fig. 19). In the former species, homologs of 55 out of the 69 late response genes were identified, 34 of which were shown to be similarly up-regulated. In *S. gordonii*, 61 homologs were identified and 40 of those genes were up-regulated in a similar fashion. Besides the similarities between the three species, the differences are interesting to note. SSA_1713, 1715, and 1716 share no homology to either species, however, SSA_1717 is

Table 8. Orthologs in *S. pneumoniae* and *S. gordonii* of the 69 *S. sanguinis* late response genes initially discussed in Section G of the Results section. “+” denotes genes that were found to be up-regulated in their respective studies. Blank spaces indicate that no orthologs were discovered for this gene.

Table 8

<i>S. sanguinis</i> Ortholog	<i>S. pneumoniae</i> Ortholog	Up- regulated	<i>S. gordonii</i> Ortholog	Up- regulated
SSA_0036	SP_2216	-	SGO_2094	+
SSA_0184, <i>comYA</i>	SP_2053	+	SGO_1924	+
SSA_0185, <i>comYB</i>	SP_2052	+	SGO_1923	+
SSA_0186, <i>comYC</i>	SP_2051	+	SGO_1922	+
SSA_0187, <i>comYD</i>	SP_2050	+	SGO_1921	+
SSA_0188	SP_2049	+	SGO_1920	+
SSA_0189	SP_2048	+	SGO_1919	+
SSA_0190	SP_2047	+	SGO_1918	+
SSA_0191	SP_2045	+	SGO_1917	+
SSA_0192, <i>ackA</i>	SP_2044	-	SGO_1916	+
SSA_0214, <i>ssb2</i>	SP_1908	+	SGO_1902	+
SSA_0215, <i>rrsB</i>	SP_1725	-	SGO_1897	-
SSA_0216	SP_0386	-	SGO_1896	-
SSA_0217	SP_0387	-	SGO_1895	-
SSA_0218	SP_1683	-	SGO_1894	+
SSA_0310			SGO_1332	-
SSA_0639	SP_1808	+	SGO_0664	+
SSA_0640			SGO_0988	-
SSA_0642, <i>pilD</i>	SP_1808	+	SGO_0664	+
SSA_0675, <i>xseB</i>	SP_1206	-	SGO_0694	+
SSA_0676, <i>ispA</i>	SP_1205	-	SGO_0695	+
SSA_0677, <i>hlyA</i>	SP_1204	-	SGO_0696	-
SSA_0678, <i>ahrC</i>	SP_1203	-	SGO_0697	-
SSA_0679, <i>recN</i>	SP_1202	-	SGO_0698	+
SSA_0680, <i>pphA</i>	SP_1201	-	SGO_0699	+
SSA_0715, <i>comEA</i>	SP_0954	+	SGO_1602	+
SSA_0716, <i>comEC</i>	SP_0955	+	SGO_1601	+
SSA_0743, <i>argR</i>	SP_0893	-	SGO_1588	-
SSA_0749, <i>coiA</i>	SP_0978	+	SGO_1575	+
SSA_0750				
SSA_0824, <i>dnaG</i>	SP_1072	+	SGO_1274	-
SSA_0825, <i>rpoD</i>	SP_1073	+	SGO_1273	-
SSA_0826	SP_1074	+	SGO_1272	-
SSA_1183			SGO_1194	+
SSA_1184, <i>topA</i>	SP_1263	-	SGO_1197	+
SSA_1185, <i>smf</i>	SP_1266	+	SGO_1198	+
SSA_1207, <i>pta</i>	SP_1100	+	SGO_1219	-
SSA_1208	SP_1099	+	SGO_1220	-
SSA_1209, <i>ppnK</i>	SP_1098	+	SGO_1221	-
SSA_1210	SP_1097	+	SGO_1222	-
SSA_1218, <i>radC</i>	SP_1088	+	SGO_1229	+
SSA_1235, <i>pyrC</i>	SP_1167	-	SGO_1248	-
SSA_1537	SP_0782	+	SGO_0745	+
SSA_1713, <i>serA</i>				

SSA_1715, <i>serC</i>				
SSA_1716				
SSA_1717, <i>spnIM</i>	SP_1431	-		
SSA_1812	SP_1336	-	SGO_0643	-
SSA_1813				
SSA_1830			SGO_0628	+
SSA_1831, <i>recX</i>	SP_1902	-	SGO_0626	+
SSA_1834, <i>raiA</i>	SP_2206	+	SGO_0610	+
SSA_1835, <i>comFC</i>	SP_2207	+	SGO_0609	+
SSA_1836, <i>comFA</i>	SP_2208	+	SGO_0608	+
SSA_2116, <i>cbf</i>	SP_1980	+	SGO_0201	+
SSA_2117, <i>rmuC</i>	SP_1981	+	SGO_0200	+
SSA_2122			SGO_0194	-
SSA_2156			SGO_0172	+
SSA_2157, <i>radA</i>			SGO_0171	+
SSA_2158				
SSA_2159	SP_0022	+		
SSA_2160, <i>dut</i>	SP_0021	+	SGO_0169	+
SSA_2189				
SSA_2192			SGO_1994	+
SSA_2231	SP_0200	+	SGO_2034	-
SSA_2233				
SSA_2244	SP_0189	-	SGO_2044	+
SSA_2245, <i>recA</i>	SP_1940	+	SGO_2045	+
SSA_2246, <i>cinA</i>	SP_1941	+	SGO_2046	+

homologous to a DNA methyltransferase in *S. gordonii*. This gene was not found to be CSP-induced in their study, however it was found to be strongly up-regulated in this study at 5 minutes post-CSP exposure. SSA_0310 and 0640 are homologous to putative transcriptional regulators not found to be up-regulated in *S. gordonii* but were in fact strongly up-regulated in the microarrays in this study. Lastly, SSA_2122 is homologous to a hypothetical protein in *S. gordonii* which was not found to be up-regulated in their study. Overall, the 69 late response genes encode for 17 hypothetical proteins, 9 DNA modification, recombination, or repair proteins and 3 transcriptional regulators, as well as 14 of the previously discussed genes found to be essential for competence in *S. pneumoniae*.

Observation of relative expression based on abundance of transcript levels using qRT-PCR showed two waves of transcription that increased and dropped at two different stages (see Results, Section J). Whereas transcript levels of the three selected early response genes: *comD*, *comE*, and *comX*, reached peak levels at 5 minutes post CSP-exposure, the selected late response genes – *comYD* and *comEA* – showed a continued increase in expression until expression of both decreased 15 minutes post-CSP exposure. The results may suggest two unique stages of repression, and since transcript levels for all three of the early response genes began to drop well before those of the late response genes, it could be concluded that repression of early genes occurs simultaneously and presumably in identical fashion. Late response genes behave similarly to each other via their continued increase in transcript level until the 15 minute time point. At this point transcript levels begin to drop just as in the early response

genes. It is important to note that although ComX has been implicated in the repression of transcription of late response genes, the decrease in transcript intensity in this study shows only that mRNA levels have fallen, either by transcriptional regulation or by their sudden instability within the cell.

As expected, *comDE* and *comX* were up-regulated in response to CSP. However, what is most interesting to observe are genes up-regulated that were unexpected. The results of this study show two early response genes up-regulated in response to CSP that were clustered together. SSA_0195 is a hypothetical protein whose gene lies just downstream of the *comY* operon and *ackA* gene. The fact that SSA_0195 and the other two genes were categorized under two different responses suggests that SSA_0195 has its own promoter and is transcribed separately. Its regulation could also account for the observation of SSA_0197, 0198, 0199, and 0200 appearing among the early response genes, even though their expression was minimal. Searching upstream from the start site of SSA_0195 reveals sequences similar to those seen upstream of *comC* and *comX* also suggesting that it is regulated by a similar promoter, although further investigation must be performed to confirm this. SSA_0195 shares little homology with other known proteins in closely related species, although it does share partial homology with *S. gordonii* SGO_0474 (E value = 5e-3), an abortive infection protein. The second gene also observed to respond in similar fashion to CSP was SSA_1889. This gene encodes a conserved 279-aa uncharacterized protein. Unlike what was observed in SSA_0195, the upstream sequence of SSA_1889 aligned more neatly with *comC* and *comX*, suggesting a much more strongly conserved promoter sequence. This could account for why

expression levels in this gene appeared higher than in SSA_0195. SSA_1889 is homologous to SMU_1208c (E value = $6e-16$) in *S. mutans*, a hypothetical protein. Just as in *S. gordonii* where it was observed that a unique FeS assembly protein was classified as an early response gene and shared no homology to other known up-regulated genes in *S. pneumoniae* (108), the revelation of two genes in *S. sanguinis* that are strongly up-regulated early underlines the necessity of studying competence regulation in numerous streptococcal species in order to identify genes that are indispensable or nonessential for competence, as well as elucidate the competence mechanism in its entirety.

The purpose of this study was to look at gene regulation in *S. sanguinis* in response to competence induction, and serve as a foundation for future investigations. The amount of data collected in this study should give great insight into this regulation mechanism, and provide information for bioinformatic approaches in the future. It was also hoped that the putative *comAB* operon would be discovered based on studies showing that the ABC-type transporter was also up-regulated early in response to CSP in *S. gordonii* and *S. pneumoniae* (84, 108). However, no obvious transporters were found to be significantly up-regulated in this study. Interestingly, the arrays showed that several ABC-type transporters were being repressed throughout the 30-minute time course. Considering the highly unique sequence of the mature CSP peptide, and the absence of the double-Gly motif, the mechanism for CSP export across the cell wall may also be unique to *S. sanguinis*. Further investigations looking for homologs in other

bacteria based on the findings in this study could prove useful in discovering the CSP transport mechanism.

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