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**ROLE OF TWO-COMPONENT SYSTEM RESPONSE REGULATORS IN  
VIRULENCE OF STREPTOCOCCUS PNEUMONIAE TIGR4 IN INFECTIVE  
ENDOCARDITIS**

A thesis submitted in fulfillment of the requirements for the  
degree of Master of Science at Virginia Commonwealth University

By

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**LIST OF ABBREVIATIONS**

BHI	brain heart infusion
CFU	colony forming units
CSP	competence stimulating peptide
hrs	hours
HBSS	Hank's buffered salt solution
HK	histidine kinase
HS	horse serum
kb	kilobase
M	molar
min	minute
ml	milliliter
ng	nanogram
nm	nanometer
O/D	optical density
PCR	polymerase chain reaction
qPCR	quantitative real-time polymerase chain reaction
rpm	rotation per minute
RR	response regulator
sec	second
TCS	two component system
TH	Todd-Hewitt
THY	Todd-Hewitt + yeast

TSA tryptic soy agar

$\mu\text{g}$  microgram

$\mu\text{l}$  microliter

## ABSTRACT

### ROLE OF TWO-COMPONENT SYSTEM RESPONSE REGULATORS IN VIRULENCE OF STREPTOCOCCUS PNEUMONIAE TIGR4 IN INFECTIVE ENDOCARDITIS

By My N. Trinh, B.A.

A thesis submitted in partial fulfillment of the requirements for the Degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, 2011

Director: Ping Xu, Ph.D.  
Associate Professor of Oral & Craniofacial Molecular Biology and Microbiology & Immunology

Streptococci resident in the oral cavity have been linked to infective endocarditis (IE). While viridans streptococci are commonly studied and associated with IE, less research has been focused on *Streptococcus pneumoniae*. Two-component systems (TCSs), consisting of a histidine kinase (HK) protein and response regulator (RR) protein, are bacterial signaling systems that may mediate *S. pneumoniae* TIGR4 strain virulence in IE. To test this hypothesis, TCS RR mutants of TIGR4 were examined in vivo through use of rabbit models. There were 14 RR proteins identified and 13 RR mutants synthesized because SP\_1227 was found to be essential. The requirement of the 13 RRs for *S. pneumoniae* growth in IE models was assessed by quantifying mutants after overnight inoculation in IE infected rabbits through use of real time PCR (qPCR), colony enumeration on antibiotic selection plates, and competitive index assays. Real time PCR pinpointed several candidate virulence factors. Candidate RR SP\_0798 was selected to be further examined. In the in vivo model, mutant SP\_0798 grew significantly less than our control mutant SP\_1678, which encodes a hypothetical

protein and grew at a comparable rate to wild-type TIGR4 strains. Literature and databases identified SP\_0798 as the *ciaR* gene, which has roles in regulating many diverse cellular functions. Our data suggests that RR SP\_0798 is a virulence factor of *S. pneumoniae* TIGR4 strain in IE. This research may place more emphasis on virulence factors and lead to novel methods to combat pneumococcal endocarditis.

## INTRODUCTION

### Infective Endocarditis

Infective Endocarditis (IE) is an infection of the heart valves or endocardium that affects 10,000-20,000 people every year. Lesions of the heart cause a mass of cells to form vegetation, thereby allowing bacteria in circulation to colonize to further block blood flow and immune responses. Complications from IE can lead to congestive heart failure, aneurysm, or stroke [1]. Various streptococci commonly found in the oral cavity have been linked to IE, especially in patients with pre-existing endocardial lesions. Treatment is difficult, often involving hospitalization and surgery, which still has a high rate of failure with a mortality rate of approximately 25% and can result in future morbidity [2]. If left untreated, IE will progress and is uniformly fatal. Antibiotic prophylaxis is a common preventative method because it was first believed that invasive dental procedures disrupting mucosal surfaces facilitated streptococci access into the bloodstream for infection [3]. However, more recently, most cases of IE are shown to occur in the absence dental manipulation so antibiotic prophylaxis is ineffective [4]. This has led to speculation on links between natural human oral flora and their role in disease. More specifically, studies are performed to identify virulence factors of streptococci that allow their success in infection of the blood and heart. Of these studies, many have been performed on the IE models concerning the virulence of viridans streptococci such as *Streptococcus sanguinis* and *Streptococcus mutans*, which are responsible for 30-40% of cases, while little has been studied about the virulence of *Streptococcus pneumoniae*, which is more rarely the cause of IE. However,

there are unique characteristics to pneumococcal endocarditis. For example, Austrian Syndrome is a condition consisting of meningitis, pneumonia, and endocarditis and most often observed in middle-aged men with pre-existing conditions such as alcoholism. This syndrome is caused *S. pneumoniae* infection with meningitis symptoms first seen and endocarditis later diagnosed. Although Austrian Syndrome is clinically rare, it is highly aggressive and often fatal because endocarditis is recognized too late [5].

### **Streptococcus pneumoniae**

*S. pneumoniae* is a gram-positive, alpha-hemolytic strain of oral streptococci. It is natively present in the upper respiratory tract of humans, but may be pathogenic under certain conditions. Upon infection, *S. pneumoniae* causes many problems in human health such as pneumonia, bacteremia, otitis media, meningitis, sinusitis, peritonitis and arthritis. Several strains of *S. pneumoniae* have been identified based on the chemical composition of its polysaccharide capsule, virulence factors, and antigens produced. The particular TIGR4 strain has a polysaccharide capsule, which prevents phagocytosis by the host immune system, and can be up to  $10^5$  times more virulent compared to unencapsulated strains such as R6. Virulence may also be attributed to the ability of the bacteria to sense conditions differing from the normal and inducing cellular changes that allow it to thrive and cause disease.

## Two-component systems

Two-component systems (TCSs) are signaling pathways commonly found in bacteria. Studies have shown that TCSs in streptococci play an important role in virulence [6, 7, 8]. They are composed of a membrane bound protein called a histidine kinase (HK) that senses environmental changes and autophosphorylates its own histidine residue. The HK signals to its corresponding cytosolic response regulator (RR) protein by transferring the phosphate to an aspartate residue. The RR undergoes a conformational change and induces a response by regulating gene transcription. TCSs have been shown to modulate responses such as osmoregulation, chemotaxis, sporulation, photosynthesis, and pathogenicity [9]. In disease, TCSs may regulate expression of virulence factor genes in response to specific environmental situations.

## Specific Aims

We are interested in identifying TCS RR genes of *S. pneumoniae* TIGR4 involved in infective endocarditis (IE) virulence and investigating their gene regulons. To do so, we constructed mutant DNA of RRs of TCSs with sequence tags by replacing the target gene with antibiotic resistance cassettes. We wanted to generate mutant cells by transforming TIGR4 cells with mutant DNA of the RR genes by homologous recombination, however, due to its capsule, the TIGR4 strain is difficult to transform at a reported efficiency of ~0.02% compared to highly transformable strains such as R6 at ~1%. In an effort to optimize the transformation efficiency of TIGR4, we tested different conditions outlined in the basic protocol by Bricker et al. [10]. After experimentation on source of cells, type of media, incubation time, oxygen environment, pH, and donor

DNA concentration, we modified the protocol slightly to result in the same reported transformation efficiency, but was more time efficient. We created 13 RR mutants and found that the RR Spn\_1227 was an essential gene because mutant colonies were not able to form. We used these mutants for *in vitro* studies, as well as *in vivo* studies in rabbit endocarditis models. We examined growth patterns of mutants independently by optical density measurements and mutants when co-inoculated by real-time PCR. Competitive Index calculations showed statistically significant decreases in quantity of a particular mutant *in vivo* endocarditic models identified candidate virulence factors. We confirmed candidates in additional animal experiments, as well as analysis in blood killing assays.

### **Significance**

We hope to identify specific TCS response regulator mutants that affect growth of *S. pneumoniae* in the rabbit endocarditis models and that this recognition of regulons and pathways involved in virulence will put further emphasis on TIGR4 research in IE and establish new strategies to combat pneumococcal bacteria.



## MATERIALS & METHODS

### Bacterial strains

Bacterial strains used in the following experiment are listed in Table 1. *Streptococcus pneumoniae* strain TIGR4 is an original isolate from human blood and was obtained from the American Type Culture Collection (ATCC). All other *S. pneumoniae* mutant strains used were derived from TIGR4.

Table 1. Bacterial strains for study

Table 1

Strain	Phenotype	Source
<i>S. pneumoniae</i> TIGR4	Human blood isolate	American Type Culture Collection
ΔSP_0083	Erm <sup>r</sup>	this laboratory study
ΔSP_0156	Erm <sup>r</sup>	this laboratory study
ΔSP_0376	Erm <sup>r</sup>	this laboratory study
ΔSP_0387	Erm <sup>r</sup>	this laboratory study
ΔSP_0526	Erm <sup>r</sup>	this laboratory study
ΔSP_0603	Erm <sup>r</sup>	this laboratory study
ΔSP_0661	Erm <sup>r</sup>	this laboratory study
ΔSP_0798	Erm <sup>r</sup>	this laboratory study
ΔSP_1633	Erm <sup>r</sup>	this laboratory study
ΔSP_2000	Erm <sup>r</sup>	this laboratory study
ΔSP_2082	Erm <sup>r</sup>	this laboratory study
ΔSP_2193	Erm <sup>r</sup>	this laboratory study
ΔSP_2235	Erm <sup>r</sup>	this laboratory study
ΔSP_1678_Erm	Erm <sup>r</sup>	this laboratory study
Δ SP_1678_Km	Km <sup>r</sup>	this laboratory study

## **Bacterial cultivation and cell preparation**

Frozen cells of *S. pneumoniae* strains were grown in 5ml of liquid medium of THY+HCl (Todd-Hewitt broth (Difco) infused with 0.5% yeast extract, 0.5% glycine, and 11mM hydrochloric acid) at 37°C under microaerophilic conditions (6% O<sub>2</sub>, 80% N<sub>2</sub>, 7% CO<sub>2</sub>, 7% H<sub>2</sub>) for 18 hrs. These cells were then diluted 100x's (56µl culture + 5.54ml THY+HCl) in the same medium and incubated at 37°C under microaerophilic conditions for 6 hrs to reach mid-log phase or O/D<sub>600nm</sub> = 0.3, as read by a spectrophotometer. Mid-log phase cultures were stored as stock with sterile glycerol (10% glycerol + 90% culture) at -80°C. Mid-log phase stock cultures were used in all subsequent experiments.

## **Mutant construction**

To study the virulence of TCSs in IE, mutants were made by deleting putative RR genes of TIGR4. Through the use of bioinformatics and homology comparisons among databases of streptococci, putative RR genes were found as well as their predicted functions. To construct mutants, three sets of primers (F1/R1, F2/R2, and F3/R3), shown in Table 2, were designed for use in polymerase chain reaction (PCR) amplification of TIGR4 genomic DNA. Primers F1/R1 amplify the fragment upstream of the target gene, primers F2/R2 amplify the antibiotic (erythromycin or kanamycin) resistance gene to replace the target gene, and primers F3/R3 amplify the fragment downstream of the target gene. We used Invitrogen high fidelity Taq DNA polymerase in our PCR reaction mixture. The PCR conditions to amplify each fragment were 94°C for 1 min., followed by 30 cycles at 94°C for 30 s, 54 °C for 30 s, 68 °C for 1 min. 30 s, and

finally 68 ° C for 4 min. The 5' ends of the F2/R2 primers were complimentary to the upstream and downstream sequences so that the final PCR product produced was linear recombinant DNA containing all three fragments. The final linear DNA construct was used for homologous recombination into the TIGR4 genome as shown in Figure 1. The upstream and downstream had long flanking sequences that were approximately 1 kb in length so as to ensure accurate homologous recombination for transformation in to the TIGR4 genome. Individual fragments were visualized as bands on 1% gel electrophoresis and purified as described below. The PCR conditions for ligation were 94°C for 2 min., followed by 30 cycles at 94°C for 30 s, 54 ° C for 30 s, 68 ° C for 3 min. 30 s, and finally 68 ° C for 10 min. This approximately 3 kb linear DNA product was again visualized by 1% gel electrophoresis, purified, and further PCR amplified using the F1/R3 primers. The concentration of the mutant donor DNA product was measured by NanoDrop technology.

Table 2. Primers used for bacterial strains

Table 2

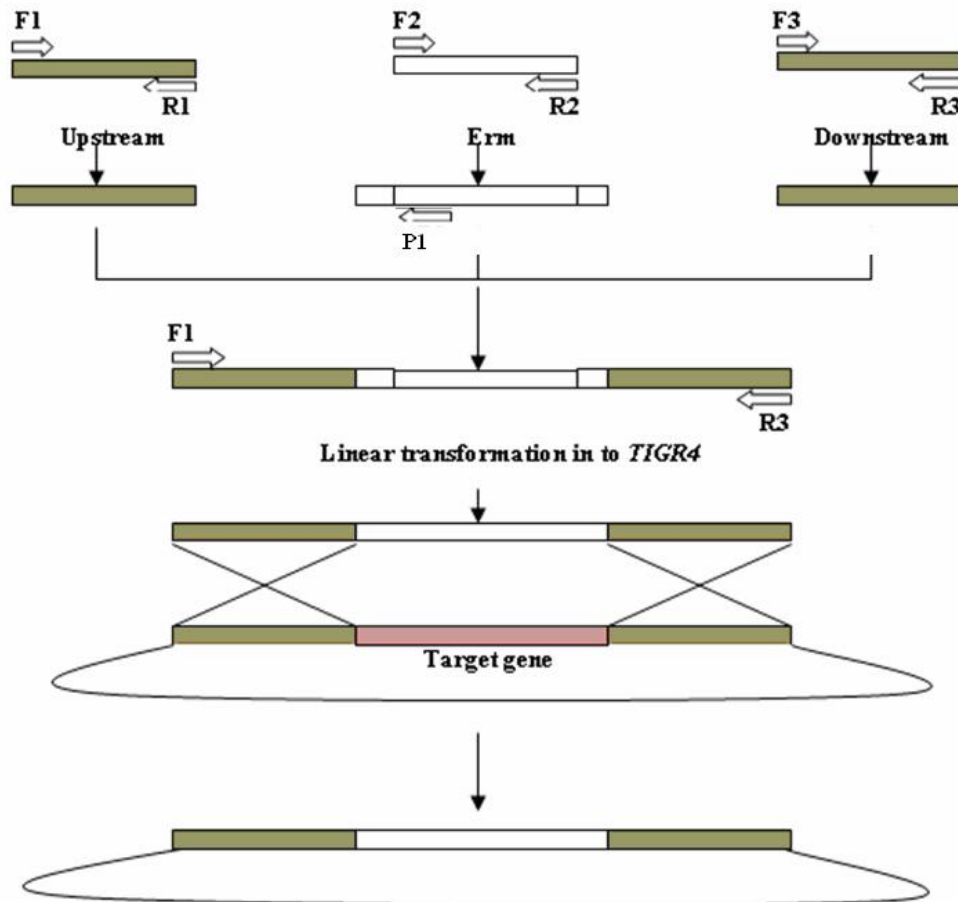
Primers	Nucleotides sequences (5'to 3')
---------	---------------------------------

SP_0083 F1	CAT GTG ATT CCA TAC GAA CTC TTC
SP_0083 R1	CCT TCT CAC TAT TTA GTC ATC CAA CTC CCA TCT GTC TCT CCT TTG AT
SP_0083 F3	AAA GGA GGA AAA TCA CAT GTC CAA CTA TAA GAT AGA GAA ACC GAG AGG
SP_0083 R3	CAT CGG TCA CAC TGA TTG AAA G
SP_0156 F1	CCA AAA GCA GGT AGT GGA TTT AGT A
SP_0156 R1	CCT TCT CAC TAT TTA GTC ATC CAA CAT ACA TTT TCT CCC TTT CTA CTC A
SP_0156 F3	AAA GGA GGA AAA TCA CAT GTC CAA CTA CCG AAA ACA GGT AGA AAC TAT A
SP_0156 R3	GGT CCA TTT CAT AGA AAT TTT TGC
SP_0376 F1	GAA AGC TAT GAC TTG ATG CAA CAC T
SP_0376 R1	CCT TCT CAC TAT TTA GTC ATC CAA CCC CCA TGG CTG ACC TAC TTA TT
SP_0376 F3	AAA GGA GGA AAA TCA CAT GTC CAA CCG TGG TGT TGG ATA TAC CAT GC
SP_0376 R3	ATA CCA TTT GCC TCG TACT ATA TTT C
SP_0387 F1	TCT TTT TAT TGT TGG TTT TCA GCA T
SP_0387 R1	CCT TCT CAC TAT TTA GTC ATC CAA CTT TCA TCT TTA CTC CTT TAT CAT TCC
SP_0387 F3	AAA GGA GGA AAA TCA CAT GTC CAA CCG CCA CCA TTT GGT GGG GCA AGA GG
SP_0387 R3	CCC TCC ATT ATC ACA TAA ACA GGT A
SP_0526 F1	ATG AGA AGA CTG GAA GTC TGG TAA A
SP_0526 R1	CCT TCT CAC TAT TTA GTC ATC CAA CTC TCA TCT TCT TAC TCT CCC TC
SP_0526 F3	AAA GGA GGA AAA TCA CAT GTC CAA CGT GTC TGA GGC CAT CAA TAA AT
SP_0526 R3	TAT CTG CTC CAT ATC CTC CTC TTC
SP_0603 F1	GCT CTT GAG CAA TCT ATC TCT GGT A
SP_0603 R1	CCT TCT CAC TAT TTA GTC ATC CAA CTT TCA TAC TTT AAC TGC TCT CTA TTT
SP_0603 F3	AAA GGA GGA AAA TCA CAT GTC CAA CCG CAA TGT TGG TTA TAA ATT GGA G
SP_0603 R3	AAA GTA TCT GCA GAA CAT GGT GAT T
SP_0661 F1	AGG AAA ACT TGA AGA ATT TTG TGG T
SP_0661 R1	CCT TCT CAC TAT TTA GTC ATC CAA CGG TCA TGC TCT GCT CCT TTA CC
SP_0661 F3	AAA GGA GGA AAA TCA CAT GTC CAA CCC TCG TCA GTT TAA GAA GGG AG
SP_0661 R3	ACA GAC AAG AAG AGA TGT GAC ACT G
SP_0798 F1	GTT GCT AAA TTG GCT CGT CAT AAC T
SP_0798 R1	CCT TCT CAC TAT TTA GTC ATC CAA CTA TCA TGA GAA ACT CCT CCT TAT T
SP_0798 F3	AAA GGA GGA AAA TCA CAT GTC CAA CAC TTT GCG TAG TGT TGG GTA TC
SP_0798 R3	ATC ATC TCT CCG AGC TAA GTT CA
SP_1227 F1	AGT TCC ACA TCT AGG TGA CTG GTA G
SP_1227 R1	AAA GGA GGA AAA TCA CAT GTC CAA CCG CCG TGG TGT AGG GTA TTA CA
SP_1227 F3	CCT TCT CAC TAT TTA GTC ATC CAA CTT TCA TAT GTT CAC CTT TTT CTC TAC
SP_1227 R3	AGC CAT TTC CAG TAT TGC TTT TAA C
SP_1633 F1	TAG ATA GAT AAA GGC CAA GTC CAG A
SP_1633 R1	AAA GGA GGA AAA TCA CAT GTC CAA CAC CAA GAA AGG AAT AGG GTA CG
SP_1633 F3	CCT TCT CAC TAT TTA GTC ATC CAA CGT GCA TGC GCT TCT CCT TTT CC
SP_1633 R3	ATA ATG GAA CCT TGT GGA ATG AAT A
SP_2000 F1	GAG CGA ATT TTA TCT GTC AAG TGA T
SP_2000 R1	AAA GGA GGA AAA TCA CAT GTC CAA CAA TAT CGC GAA AGA ATC TGG TTG
SP_2000 F3	CCT TCT CAC TAT TTA GTC ATC CAA CTT TCA TCT ACT TTC TCT CT TAT AAA
SP_2000 R3	CTG GTT TTT CTT TTT CCT ATC CAA T
SP_2082 F1	GAT ATT TCT GCG ACT CAT TTT GAA C
SP_2082 R1	CCT TCTCAC TAT TTA GTC ATC CAA CTG TCA TCT ATT ATC TCC TAT TGG T
SP_2082 F3	AAA GGA GGA AAA TCA CAT GTC CAA CCG TTA TGG TTA TAA CTT CAA GGA G
SP_2082 R3	AGA TGC TCA ACA ATA TGC TCA AGA C
SP_2193 F1	CTA ATA AGT GGC TCA TCT GGT CAA T
SP_2193 R1	AAA GGA GGA AAA TCA CAT GTC CAA CGT GAA AAA TGT TGG GTA TAA GAT TAG
SP_2193 F3	CCT TCT CAC TAT TTA GTC ATC CAA CGT TCA TCT CTC TCC CTT TCT AC
SP_2193 R3	CAA GCT TCA GCT CTG AAA TTG TTA
SP_2235 F1	AAA TTG CTT TCC ATT CTT TAA ACT T
SP_2235 R1	AAA GGA GGA AAA TCA CAT GTC CAA CGA TAT TTT AGA GAA AAA ATC TCA AAA GT
SP_2235 F3	CCT TCT CAC TAT TTA GTC ATC CAA CAA CTT TCA TTC AAA TTC CCT CTT AAA
SP_2235 R3	GCC TAT TTT GAC AAG GAC TAC CTT T
SP_1678 F1	CTG GAA TGA CAC CTT CGT ATT TTT
SP_1678 R1_erm	AA GGA GGA AAA TCA CAT GTC CAA CTT ACA AAG AAA AAT GAT GGA GGA G
SP_1678 F3_erm	CCT TCT CAC TAT TTA GTC ATC CAA CGA ACA AAT CTA TTT TTT CTT TTG GAC
SP_1678 R3	GTA TCA AAG ACG AGG CAG TAG CAT
SP_1678 R1_km	GTT TTA GTA CCT GGA GGG AAT AAT GTT ACA AAG AAA AAT GAT GGA GGA G
SP_1678 F3_km	GCC ATT TAT TCC TCC TAG TTA GTC AGA ACA AAT CTA TTT TTT CTT TTG GAC
Erm_F	GTT GGA TGA CTA AAT AGT GAG AAG GAG TGA TTA CAT GAA CAA
Erm_R	GTT GGA CAT GTG ATT TTC CTC CTT TTT ATT TCC TCC CGT TAA ATA ATA G

Figure 1. Schematic of synthesis of DNA fragments for mutant construction by PCR amplification and homologous recombination for integration of antibiotic resistance cassette into chromosome of *S. pneumoniae* TIGR4.\*



Figure 1\*



\*Patel, Jenishkumar. Environmental responses of two-component systems in *Streptococcus sanguinis*. 2010 Aug.

### **Purification of PCR amplified fragments**

PCR amplified DNA was purified using QIAGEN Sample and Assay Technologies. Essentially, 5 volumes Buffer PB (105µl Buffer PB) was added to 21µl PCR sample and placed in a QIAquick spin column. The column was placed in a 2ml collection tube and centrifuged for 1 min. The flow through was discarded. To wash away unbound DNA, 750µl of buffer PE with ethanol was added to the column and again centrifuged for 1 min. The flow through was discarded and the column was placed in a clean 2ml Eppendorf tube. DNA was eluted with 20µl of distilled water, which sat at room temperature for 1 min, and then centrifuged for 1 min. The concentration of DNA fragments was measured using NanoDrop technology. Fragments were combined in equivalent concentrations for ligation.

### **THY+HCl preparation**

To prepare the THY+HCl media, 14.8g brain heart infusion (37g/1L BHI), 2g yeast extract (0.5%), and 400µl HCl (11M) was added to 400ml of distilled H<sub>2</sub>O and mixed on a stir plate. This solution was then autoclaved for 20 min, allowed to cool, and kept for working stocks at 4°C for weeks.

### **Agar plate preparation**

To prepare agar plates, 14.8g brain heart infusion (37g/1L BHI) and 6g agar was added to 400ml of distilled H<sub>2</sub>O and mixed on a stir plate. This solution was autoclaved for 20 min and cooled to 55°C. To make plates without antibiotic, we poured approximately 10ml of this solution onto new Petri dishes, while working in the hood,

and allowed plates to dry. For antibiotic selection plates, we added and mixed erythromycin to a final concentration of 10µg/ml or kanamycin to a final concentration of 500µg/ml into the autoclaved solution. Approximately 10ml of this solution was poured onto new Petri dishes and allowed to dry. Plates were stored in -20°C.

## Transformation

Once all mutant DNA was synthesized, *S. pneumoniae* TIGR4 parent strain had to be transformed into mutant strains for study. The transformation protocol followed for the TIGR4 strain was optimized from that outlined by A.L. Bricker et al. [10]. Mid-log phase stock cultures were thawed and diluted 50-fold with media (20µl culture + 980µl THY+HCl) in 14 separate conical tubes (one for each putative response regulator gene). Tubes were incubated at 37°C under microaerophilic conditions for 3 hrs to reach  $O/D_{600nm} = 0.03$ , as read by a spectrophotometer. To induce competency of cells, 10N (1µl) NaOH, 10% (10µl) BSA, 1M (1µl) CaCl<sub>2</sub>, and 2.8µl CSP-2 (competence stimulating peptide type 2) was added to each tube and incubated at a 37°C under microaerophilic conditions for 14 min. 50ng of mutant donor DNA was then added to tubes and incubated at 37°C under microaerophilic conditions for 90 min. After 90 min, cells were diluted 200-fold in media (5µl cells + 995µl THY+HCl). Transformed mutant bacteria were selected by spreading 100µl of diluted cultures onto antibiotic (10mg/ml erythromycin or 500mg/ml kanamycin) selection agar plates. Plates were incubated at 37°C under microaerophilic conditions for 48 hrs. Colonies were counted and transformation efficiency was calculated using the formula: (CFU on plates with antibiotic / CFU on plates with no antibiotic).

## **Mutant confirmation and sequencing**

To confirm mutant bacteria, a single colony from the antibiotic selection plate was picked up using a Pipette tip and inoculated in 4ml of THY medium (without HCl) with erythromycin (10 $\mu$ g/ml) or kanamycin (500 $\mu$ g/ml), depending on the antibiotic cassette used to replace the target gene, and incubated at 37°C under microaerophilic conditions, overnight until turbulence was observed. Colony PCR was run using the overnight culture and primers F1/R3 and reaction conditions of 94°C for 2 min., followed by 30 cycles at 94°C for 30 s, 54 ° C for 30 s, 68 ° C for 3 min. 30 s, and finally 68 ° C for 10 min. Mutant bacterial cells were confirmed when bands were observed on 1% gel electrophoresis. Also, to further confirm correct mutants, DNA upstream of the antibiotic cassette that is specific to all strains was sequenced by Virginia Commonwealth University's Nucleic Acids Research Facilities. Sequence data was cross-referenced to the TIGR4 genome database to confirm that the sequences matched for the correct location of the target gene. Mutant strains were stored in 30% glycerol in -80°C.

## ***In vitro* studies**

To elucidate growth rates of mutants, growth curves of each mutant, individually, were constructed. Each mutant strain from frozen stocks was inoculated in 5ml THY+HCl and incubated at 37°C under microaerophilic conditions overnight. Overnight cultures were diluted 100-fold in media (56 $\mu$ l culture + 5.54ml THY+HCl). 200 $\mu$ l of each mutant diluted culture was pipetted in triplicate in wells of 96-well PCR plates so that each plate had repeats of every mutant. One plate was made for every time point to be

measured for the growth curve, therefore, eight plates were made for hourly time points of 0, 4, 5, 6, 7, 8, 9, and 10. Each plate was incubated in separate Anoxomat (technology used to culture anaerobes and microaerobes) jars at 37°C under microaerophilic conditions. At each time point, a corresponding plate was removed from incubation and the cell growth was measured using the FLUOstar plate reader (BMG Technologies) at  $O/D_{600nm}$ . After 10 hrs of measurements, a growth curve using the average of the hourly repeats for each mutant was constructed from the data collected.

To examine how RR mutants grew when inoculated together, in vitro co-inoculation was performed. Frozen mid-log phase stocks of each mutant were thawed and 100 $\mu$ l was streaked out onto antibiotic selection plates. Plates were incubated at 37°C under microaerophilic conditions for 48 hrs. 1-2 colonies from plates were inoculated into tubes with 5ml BHI (brain-heart infusion). These tubes containing mutant cells as well as one corresponding tube for each mutant of 12.6ml of BHI were incubated at 37°C under microaerophilic conditions overnight. Overnight mutant cell cultures were confirmed to have an  $O/D_{660nm} \sim 0.9$ . 1.4ml of each mutant culture was transferred into the tube of 12.6ml pre-warmed BHI and quickly returned to incubate at 37°C under microaerophilic conditions for 3 hrs. After 3 hrs, tubes were centrifuged at 4000 rpm for 10 min at 4°C and supernatant discarded, twice. Pellets were resuspended and vortexed in 7ml cold sterile PBS. The cell mixture was serially diluted in PBS (400 $\mu$ l cells:400 $\mu$ l PBS; 300 $\mu$ l cells:500 $\mu$ l PBS; 100 $\mu$ l cells: 700 $\mu$ l PBS) and cell growth was measured. From serial dilution data, all mutant cell cultures were adjusted to an  $O/D_{660nm} = 0.8$  ( $1 \times 10^8$  CFU) by adding PBS. Finally, to combine all mutants in

culture to make what is referred to as the inoculum, 1ml of each mutant culture was pulled together into one conical tube and vortexed. 100µl of inoculum was transferred into 10ml of BHI and incubated at 37°C under microaerophilic conditions overnight. The in vitro inoculum was six times serially diluted by a 10-fold factor each time in PBS. Also, 100µl of each dilution was streaked onto antibiotic selection plates and incubated at 37°C under microaerophilic conditions for 48 hrs, at which point a colony count was made. The remaining inoculum was centrifuged with the supernatant being discarded and pellet stored in -80°C. Once the inoculum has been incubated overnight, it is now the output. The in vitro output was diluted and plated, just as the inoculum, to make a colony count. The bacterial colonies from inoculum and output plates of approximately 5,000 CFU (colony forming units) were isolated by adding 10ml of PBS and gently scraping the surface of plates to detach colonies. The liquid suspension was collected in a conical tube and centrifuged to store the pellet and discard supernatant. To isolate DNA from bacterial pellets, the QIAamp DNA Mini and Blood Mini Kit (QIAGEN) for purification of genomic DNA from gram-positive bacteria was followed. Pellets were resuspended in 180µl of an enzyme solution of 20mg/ml lysozyme, 1x Tris/EDTA (pH 8.0), and 1.2% Triton, incubated at 37°C for 30 min, and transferred to Eppendorf tubes. 20µl Proteinase K and 200µl Buffer AL was added to samples, mixed, and allowed to incubate at 56°C for 1 hr with intervals of vortexing until the samples appeared clear in color. Tubes were briefly centrifuged before adding 200µl Buffer AL, then mixed, and incubated at 70°C for 10 min. Samples were briefly centrifuged before adding 200µl of 100% ethanol and mixed. This mixture was applied to the QIAamp mini spin column and centrifuged at 8000 rpm for 1 min. The filtrate was discarded while the spin column

was placed in a new collection tube. 500µl Buffer AW1 was added and centrifuged. The filtrate was discarded while the spin column was placed in a new collection tube. To the spin column, 500µl Buffer AW2 was added, centrifuged at 14,000 rpm for 3 min, filtrate was discarded, and centrifuged again at 14,000 rpm for 1 min. 50µl of distilled water was added to the column, which was placed in a new collection tube, and allowed to sit at room temperature for 5 min. Finally, the tube was centrifuged at 8,000 rpm for 1 min and the flow through was kept as the mutant DNA samples.

These samples were diluted 10x to be used for real-time PCR (qPCR) as a way of quantifying DNA of interest. Primers of approximately 25 bases for qPCR were designed using Primer Express 3.0 software to amplify the specific sequence ligating the erythromycin cassette and downstream fragment. The forward primer was complimentary to the erythromycin sequence, while the reverse primer was complimentary to the specific sequence of the downstream fragment of each mutant. The amount of target DNA was quantified based on a standard curve created by serially diluting, by a 10-fold factor, a PCR amplified product of the gene of interest. The PCR product, which the standard curve was based on, was of known DNA concentration as determined by NanoDrop and adjusted to 0.5026ng/µl, which corresponded to  $2 \times 10^5$  copies/µl. We used 2µl of DNA as template, 1µl dH<sub>2</sub>O, 1µl of each primer, and 5µl 2x PCR Master Mix (Applied Biosystems) in our qPCR reaction mixture. Quantified DNA was applied in the competitive index formula: (mutant output/control output) / (mutant input/control input), to analyze growth of each mutant when co-inoculated.

## **In vivo endocarditis model**

To simultaneously screen all mutants for virulence effects in IE, rabbit models as described in previous studies [11, 12] were used for in vivo infection and study. To induce endocarditis and valve damage, catheters were inserted into the internal carotid artery, past the aortic valve of six anaesthetized rabbits. Two days after catheterization, the same inoculum prepared for in vitro studies ( $O/D_{660nm} = 0.8$  or  $1 \times 10^8$  CFU, each mutant) was loaded at 0.5ml volume into syringes for injection into the ear vein of rabbits. Twenty hours after injection, necropsy was performed. Rabbits were sacrificed by injection and their hearts were removed. Tissue and vegetation were recovered, grinded up, and homogenized. In vivo output is referred to as the homogenate, which after harvesting was vortexed, diluted and streaked onto selection plates in the same manner as in vitro output. We performed colony counts from the in vivo homogenates and inoculum and harvested the cells for qPCR. A competitive index assay was done just as for our in vitro samples. Standard error and significance were calculated to analyze growth of mutants in endocarditis models.

Once we had identified candidate virulent RRs, we confirmed our results by performing an additional experiment in our animal IE model. A second antibiotic selection marker (Km) was used to replace the SP\_1678 gene to form a control strain [13]. We chose one candidate RR mutant, which resulted in a CI significantly less than 1, and mixed it with the control mutant in equal amounts ( $1 \times 10^7$  CFU). This inoculum was injected into the ear vein of three catheterized rabbit to evaluate the relative fitness in IE. Again, the bacteria was recovered from infected heart valves 20 hrs later, diluted, and enumerated on selection plates with either Km or Erm [11]. Colonies that grew on



Km plates were the control strain and colonies that grew on Erm plates were the candidate RR mutant. The colony counts were applied to the CI formula and significance was calculated to confirm virulence of our candidates.

### **Statistical analysis**

To calculate statistical significance of CI assays, we performed paired t-tests comparing the CI results of each strain to 1, which is the value that would suggest the response regulator has no effect in endocarditis virulence. We used a cutoff p-value of 0.05 so that a p-value < 0.05 suggested that CI results were significant.

### **Blood killing assay**

To examine possible virulence mechanisms of candidate RRs, a CI assay was performed using mutants in human blood. All 13 RR mutants with Erm resistance, as well as the control mutant SP\_1678 with Km resistance were inoculated in 200 $\mu$ l THY+HCl media at 37°C in microaerophilic conditions, overnight. 50 $\mu$ l of overnight cultures were transferred into 450 $\mu$ l THY+HCl media in an autoclaved deep 96-well plate and incubated at 37°C in microaerophilic conditions for 4 hrs. After incubation, cultures were at  $O/D_{660nm} = 0.8$  or  $1 \times 10^8$  CFU, so that 0.5ml of control mutant could be transferred into the wells containing RR mutant cells and mixed well by pipetting up and down. Cultures were then harvested by centrifuging at 4000 rpm for 10 min at 4°C, discarding supernatant, resuspending pellets in 1ml HBSS (Hank's Buffered Salt Solution), and repeating these steps once. The bacterial mixture was serially diluted (once by 100-fold and subsequently four times by 10-fold) in autoclaved deep 96-well

plates and plated onto Erm and Km selection plates. In addition, 10 $\mu$ l of the remaining bacterial mixture was added to 90 $\mu$ l of human blood, which was collected no longer than 4 hrs prior to experimentation, in an autoclaved deep 96-well plate. This plate was incubated at 37°C for 1 hr, while gently mixed. Following incubation, the blood and bacterial mixture was serially diluted (once by 100-fold and subsequently four times by 10-fold) and plated onto Erm and Km plates. All plates were allowed to dry and incubated at 37°C in microaerophilic conditions for 2 days. The colonies were enumerated for CI calculations.

#### **Literature and database search**

We searched NCBI databases for papers and their GEO databases for microarray data on gene expression, regulons, and disease models relating to candidate virulence factors that we have identified in our experiments.

## RESULTS

### Identification of TCS genes in *S. pneumoniae* TIGR4 strain

There were 46 streptococcal genomes completed as of April 2010, of which 11 were of *S. pneumoniae* strains. From the databases of these genomes and use of comparative genomics [14], we found all pneumococcal strains had either 13 or 14 TCSs. In the TIGR4 strain, we identified 13 putative HKs and 14 putative RRs listed in Table 3, including 1 orphan RR (SP\_0376), which is in agreement with previous studies and its homologous genes. Also from our preliminary bioinformatic analysis, we identified a gene, SP\_1678, encoding a hypothetical protein that we used to synthesize a mutant strain to be used at a control in subsequent experimentation.

Table 3: A List of mutated genes used for study and their putative function

Table 3

Gene	Putative Function	TCS	Possible Regulatory Function <sup>15</sup>
SP_0083	DNA-binding response regulator	08	
SP_0156	DNA-binding response regulator	07	
SP_0376	DNA-binding response regulator RitR	orphan	
SP_0387	DNA-binding response regulator	03	
SP_0526	Response regulator BlpR	13	pheromone/peptide sensing
SP_0603	DNA-binding response regulator VncR	10	
SP_0661	DNA-binding response regulator ZmpR	09	nutrient perception
SP_0798	DNA-binding response regulator CiaR	05	competence/penicillin susceptibility
SP_1227	DNA-binding response regulator	02	redox/energy sensing
SP_1633	DNA-binding response regulator	01	
SP_2000	DNA-binding response regulator	11	
SP_2082	Response regulator	04	phosphate sensing
SP_2193	DNA-binding response regulator	06	
SP_2235	Response regulator ComE	12	quorum sensing/competence
SP_1678	Hypothetical protein		

<sup>15</sup> Lange R., Wagner C., Saizieu A., Flint N., Molnos J., Steiger M., Caspers P., Kamber M., Keck W., Amrein K.E. Domain organization and molecular characterization of 13 two-component systems identified by genome sequencing of *Streptococcus pneumoniae*. *Gene*. 1999 Sep; 237 (1): 223-234.

## **Construction of mutants**

To create mutants for genes of interest, we routinely use PCR associated site-directed mutagenesis in our lab and we found this method to be useful in producing consistent results for our *S. pneumoniae* experiments. We used an antibiotic resistance gene flanked by regions homologous to the chromosomal target gene as the cassette for mutants. An erythromycin-resistance cassette was used to synthesize all 14 RR mutants, as well as SP\_1678, which was our mutant control strain. A kanamycin-resistance cassette was also used to make a Km resistant SP\_1678 mutant control strain, which was used in later experiments so that RR mutants and control mutants could be co-inoculated in vivo and selectively enumerated on different antibiotic plates for competitive index assays. [13]. 1% gel electrophoresis confirmed that PCR combined and amplified the three fragments (upstream, antibiotic-resistance cassette, and downstream) for our final mutant DNA product to be used as donor DNA in transformation. Trials of PCR were repeated because not all mutants were successfully amplified with the first attempt. Also, oftentimes gels showed that the DNA synthesized was impure because several other bands were observed in addition to the correct sized band, so we repeated the PCR to get the cleanest results we could achieve. Eventually, all RR mutant DNA of approximately ~3 kb were successfully made, as displayed in Figure 2.

## **Confirmation of mutants by colony PCR and sequencing**

After TIGR4 cells were transformed using our RR mutant DNA and cultured for 48 hrs on antibiotic selection agar plates, we found colonies grew for all of our mutants

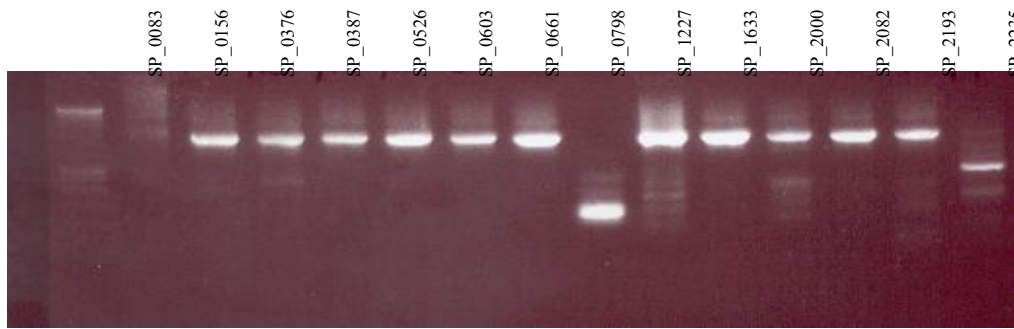
except SP\_1227. We wanted to confirm that these bacterial colonies were indeed the correct mutant cells with the antibiotic-resistance cassette replacing the gene of interest in the genome. To do so, we performed colony PCR on cells from the isolated colonies using F1 and R3 primers for each mutant, respectively, as described previously. The correct sized band of approximately ~3 kb can be visualized on the 1% gel in Figure 3. Another method we employed to confirm our mutants were correct was sequencing DNA from mutant colonies by use of internal primers P1 (Figure 1). The sequences of 60 bp obtained from our mutants are upstream of the target gene and can be seen in Table 4 and matched the genomic database for TIGR4 RR genes. Since all sequences precisely matched, we were confident our mutants were correct and did not need to further sequence downstream of the target gene.

Figure 2. (A) 1% agarose gel electrophoresis of PCR amplified products from the ligated upstream, antibiotic resistance cassette, and downstream fragments. SP\_0083 (lane 2), SP\_0156 (lane 3), SP\_0376 (lane 4), SP\_0387 (lane 5), SP\_0526 (lane 6), SP\_0603 (lane 7), SP\_0661 (lane 8), SP\_1227 (lane 9), SP\_1633 (lane 10), SP\_2000 (lane 11), SP\_2082 (lane 12), SP\_2193 (lane 13), SP\_2235 (lane 14), and 1kb DNA marker (lane 1). Bands are shown in each lane at ~3 kb, which confirms that all cassettes were successfully amplified except SP\_0083, SP\_0798 and SP\_2235, which are later amplified as shown in the second gel (B). SP\_0083 was synthesized in additional PCR reactions (data not shown).



Figure 2

(A)



(B)

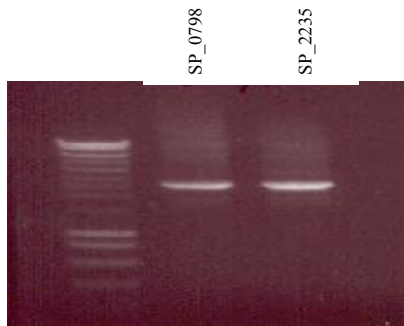


Figure 3. (A) 1% agarose gel electrophoresis of DNA fragments from colony PCR.  $\Delta$ SP\_0083 (lane 2),  $\Delta$ SP\_0156 (lane 3),  $\Delta$ SP\_0376 (lane 4),  $\Delta$ SP\_0387 (lane 5),  $\Delta$ SP\_0526 (lane 6),  $\Delta$ SP\_0603 (lane 7),  $\Delta$ SP\_0661 (lane 8),  $\Delta$ SP\_1227 (lane 9),  $\Delta$ SP\_1633 (lane 10),  $\Delta$ SP\_2000 (lane 11),  $\Delta$ SP\_2082 (lane 12),  $\Delta$ SP\_2193 (lane 13),  $\Delta$ SP\_2235 (lane 14), and 1kb DNA marker (lane 1). Bands are shown in each lane at ~3 kb, which confirms that all TCS RRs replaced by antibiotic-resistance cassettes into *S. pneumoniae* TIGR4 chromosome except  $\Delta$ SP\_0526,  $\Delta$ SP\_2082 and  $\Delta$ SP\_2193, which are later replaced as shown in the second gel (B)

Figure 3

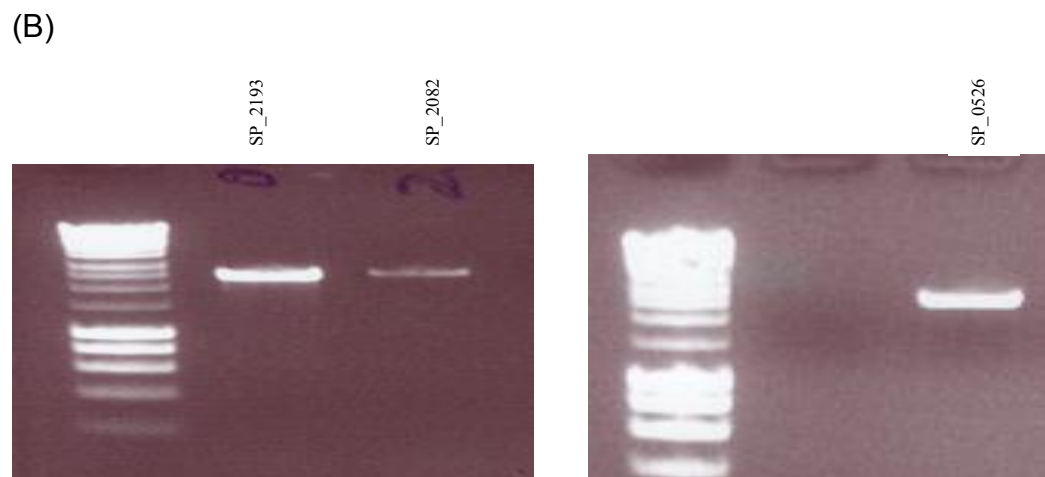
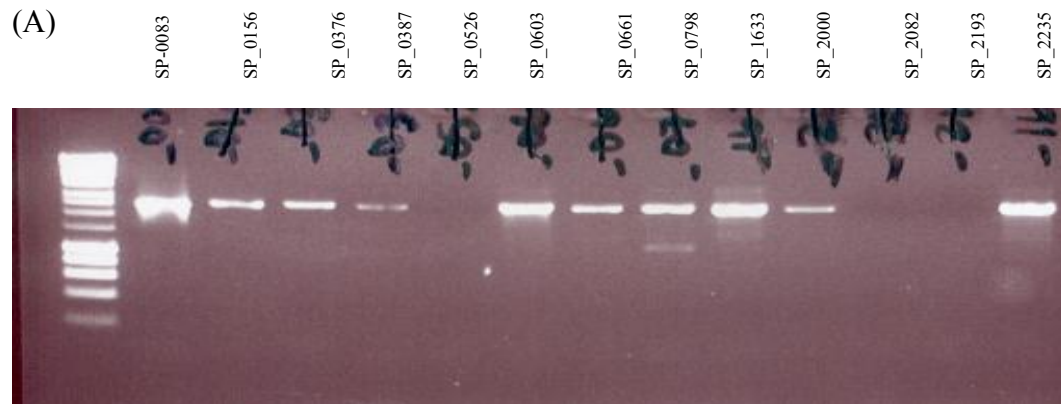


Table 4. Sequence data upstream of target RR gene. Sequencing performed by Virginia Commonwealth University Nucleic Acids Research Facilities using internal erythromycin P1 primer designed by our lab.

Table 4

Strain	Upstream sequence and genome region
ΔSP_0083	89041 CCATCTGTCTCTCCTTTGATAAAAACAGTGCTATACTGCTTTAAGTATAACACTATTTTT 88982
ΔSP_0156	153585 ATACATTTTCTCCCTTTCTACTCATCTTGAATTGTAATACGATACTGAACACCGGCTTGC 153526
ΔSP_0376	355164 CCCCATGGCTGACCTACTTATTTTTCGTCATACCAAGAGTAGTGAAGGTTCTTCTTTG 355105
ΔSP_0387	366905 TTTTCATCTTACTCCTTTATCATTCTTATCTAACAGGGGAATACGGATATCAACCGCCA 366846
ΔSP_0526	505566 TCTCATCTTCTACTCTCCCTTTCAACCATTTTTGACGAATTTCTCTATAGCGACGTC 505507
ΔSP_0603	569487 TTTCACTTTAACTGCTCTCTATTTTTATTTTTCTTAGAATAAATACCTACCCTATTT 569428
ΔSP_0661	631660 GGTTCATGCTCTGCTCCTTTACCACTACTAGTATCAGTATAGCAAAATTTCTCCTCTAACT 631601
ΔSP_0798	751967 TATCATGAGAACTCCTCCTTATTAATAACTATTATACCAAATTTGCCTTAAAAAAACTC 751908
ΔSP_1633	1534188 TGCATGCGCTTCTCCTTTCCATTATTATAACAGATTTTTCCATGCTAGATGGTCTGAAA 1534247
ΔSP_1678	1578831 AAATCTATTTTTCTTTGGACTTTTTCTATTTTTATCTATGGGCTTATAATCATATAC 1578890
ΔSP_2000	1904343 TTTTCATCTACTTTCTCTCCTTATAAAGTAGTCGAACCTGCACTTCAGTTGGATGTTTCTG 1904402
ΔSP_2082	1991246 TGTCATCTATTATCTCCTATTGGTAACATTATAACACAATTATCAGAAATCCTAACATTG 1991187
ΔSP_2193	2114758 AATGTTTCATCTCTCTCCCTTTCTACTACCAGAACTCCATACATCTATTTCTGTATGCTA 2114817
ΔSP_2235	2156409 AACTTTCATTCAAATTCCTCTTAAATCTAATGATTTGTCTAAATGTACTGCCTTCCATC 2156468

## **Establishment of *S. pneumoniae* TIGR4 cell cultures**

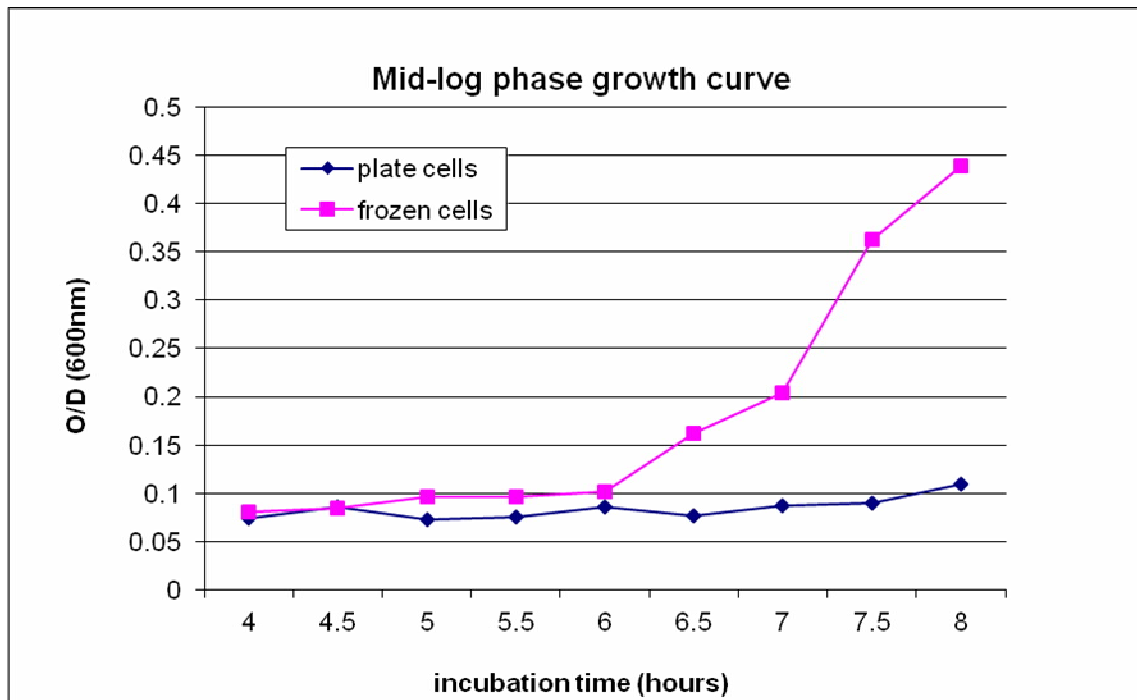
Once mutant DNA was synthesized, we tested various conditions to prepare wild-type TIGR4 cells and to induce competence. Competence is the ability of cells to take up foreign DNA from the surrounding environment into their own genome. Previous studies have reported low transformation efficiencies for the TIGR4 strain, therefore, we optimized a protocol originally published by A.L. Bricker et al. We first needed to culture our stock cells to mid-log growth phase cells because it was reported that cells in this phase are more competent. We tested the incubation time required to reach mid-log phase and whether the time was dependent upon source of stock cells. Two sources of stock cells were tested, frozen cells taken directly from the  $-80^{\circ}\text{C}$  and cells from colonies that were streaked on TSA plates and incubated at  $37^{\circ}\text{C}$  for 48 hrs. These two samples of TIGR4 cells were separately incubated in THY+HCl media at  $37^{\circ}\text{C}$  in microaerophilic conditions. The cell growth was measured by a spectrophotometer at 600nm at every 30 min for 8 hrs, at which point the stationary phase of growth was reached. The growth curve is shown in Figure 4 and we found that frozen cells incubated for 7-7.5 hrs grew best. Growth media was another variable tested to optimize efficiency. The original protocol reported using THY+HCl media to culture cells, so we tested this media against TH+HS (Todd-Hewitt + horse serum) media, which we regularly use in our lab to culture other streptococci. Our results confirmed THY+HCl is better for TIGR4 transformation because it yielded an efficiency of 0.03%, while TH+HS media yielded 0.00% efficiency. Lastly, we tested the oxygen condition that was best for cell growth. After 7 hrs of incubation, the optical density at 600nm of cultures were as follows aerobic = 0.22, microaerophilic = 0.55, and anaerobic

= 0.61. Cells did not grow sufficiently in aerobic conditions, while there was not a significant difference between anaerobic and microaerophilic conditions. We ultimately grew cultures in microaerophilic conditions similar to the candle jars employed by Bricker et al. and because it was more feasible, technically. Finally, we settled on a protocol that was most efficient and reproducible in our lab and resulted in an average transformation efficiency of 0.02%, which was equivalent to that reported by Bricker et al [10]. From colony PCR and sequencing data, all of our mutants were confirmed except for RR SP\_1227. No colonies grew for the SP\_1227 mutant and database research showed that this gene is homologous to an essential gene, SSA\_1565, of *Streptococcus sanguinis*, therefore, we concluded that SP\_1227 is essential in TIGR4.

Figure 4: Growth curve of stock TIGR4 cells grown to mid-log phase. Two sources of cells, frozen stocks vs. colonies on TSA plates, were inoculated at in THY+HCl media at 37°C in microaerophilic conditions for 8 hrs. Culture growth was measured using a photospectrometer at 600nm at 30 min intervals.



Figure 4



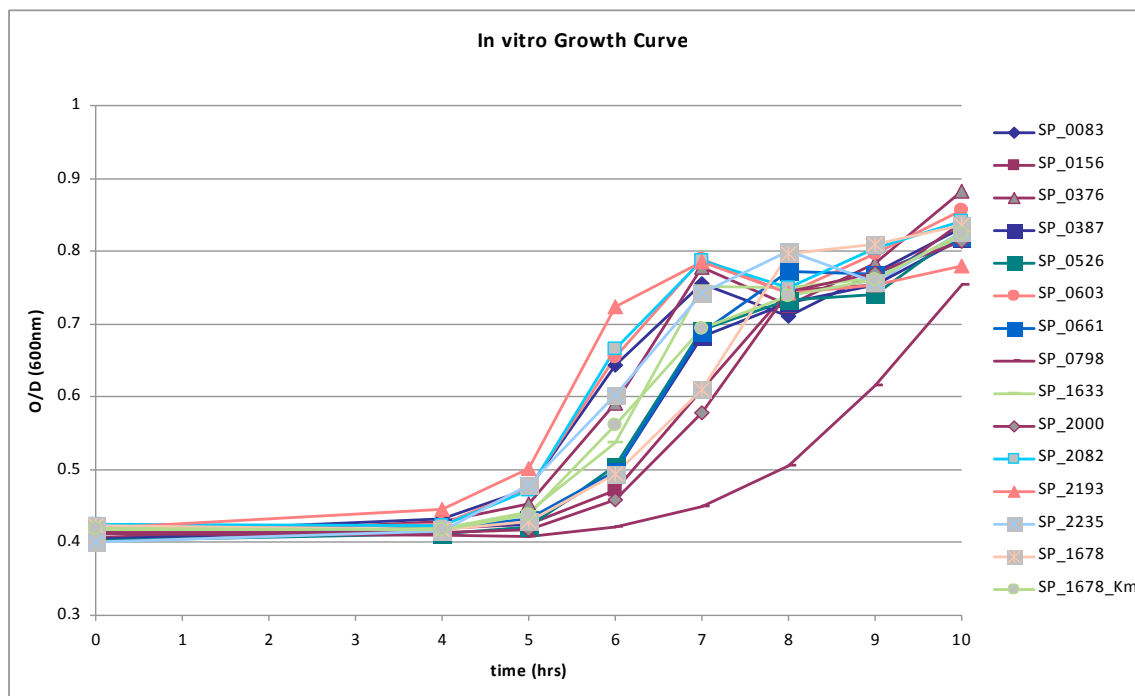
## Examination of in vitro studies

As a preliminary step and reference for further study in our in vivo IE model, we conducted in vitro experiments to elucidate growth rates of our mutants. After measuring growth of mutants inoculated individually, we found from the growth curve shown in Figure 5 that all the RR mutants grew at similar rates to the control mutant, SP\_1678, except for RR SP\_0798. Data shows that SP\_0798 grew slower than the other 12 RR mutants.

We also wanted to examine growth rates of RR mutants when inoculated together, as would be done when studied in our in vivo models. To do so, we co-inoculated mutant and quantified growth using specific primers and qPCR. We analyzed the data gathered from qPCR by calculating the competitive index for each mutant. A  $CI > 1$  suggests that the RR mutant grew faster than the control and a  $CI < 1$  suggests that the RR mutant grew slower than the control. The CI values are shown in Table 5 (B). The majority of response regulator mutants had a  $CI > 1$ , with the exception of mutant strains SP\_0376 and SP\_2000, which had CI values less than 1.

Figure 5: Growth curve of all 13 RR mutants with Erm-resistance cassettes, control mutant  $\Delta$ SP\_1678 with Erm- and Km-resistance cassette. Bacterial cultures were individually grown at 37°C in microaerophilic conditions over the course of 10 hrs. Growth was measured by O/D readings at 4 hrs and every hr thereafter on a FLUOstar plate reader at 600nm. Mutant  $\Delta$ SP\_0798 showed significantly slower growth than all other mutants.

Figure 5



## Examination of in vivo studies

After our in vitro examination, we were prepared to screen mutants to observe their role in virulence in an in vivo IE model. To do so, we first co-inoculated all of our RR mutants, including our control mutant into six rabbit IE models. qPCR was performed to quantify the behavior of mutants. The data from qPCR, which are the average of three repeats, along with the calculated CI and p-values can be seen in Table 5. From the CI information and statistics (cutoff p-value of 0.05), we saw that SP\_0156, SP\_0376, SP\_0603, SP\_0661, SP\_0798, SP\_2082, and SP\_2235 appeared to have some role in virulence in endocarditis. However, due to limitations on the number of rabbit models we had available we could only choose one candidate RR to further examine.

Table 5. Real-time PCR and competitive index results

Table 5

## A. Quantitative real-time PCR results

strain	inoculum	in vitro output	in vivo homogenate
ΔSP_0083	3.14E+06	4.18E+07	1.36E+05
ΔSP_0156	2.35E+06	1.20E+07	2.93E+05
ΔSP_0376	5.91E+05	2.82E+03	undetected
ΔSP_0387	6.94E+06	1.68E+08	1.27E+05
ΔSP_0526	2.06E+07	5.09E+07	3.98E+05
ΔSP_0603	1.58E+05	2.25E+06	1.83E+03
ΔSP_0661	7.19E+05	6.97E+06	1.14E+04
ΔSP_0798	9.73E+05	5.37E+06	undetected
ΔSP_1633	1.09E+06	8.73E+06	1.83E+04
ΔSP_2000	1.75E+05	1.82E+06	1.01E+04
ΔSP_2082	2.56E+05	1.16E+06	1.46E+03
ΔSP_2193	9.17E+05	4.55E+06	6.18E+04
ΔSP_2235	1.60E+05	5.58E+05	1.84E+03
ΔSP_1678	1.81E+06	2.57E+06	2.83E+04

## B. Competitive Index results and p-values

strain	in vitro CI	in vitro p-value	in vivo CI	in vivo p-value
ΔSP_0083	9.4	0.0012	2.78	0.0122
ΔSP_0156	3.59	0.0014	0.663	0.0129
ΔSP_0376	0.003	0.0089	n/a	n/a
ΔSP_0387	17.07	0.0001	1.17	0.0866
ΔSP_0526	1.74	0.0007	1.24	0.0006
ΔSP_0603	10.87	0.0014	0.743	0.0513
ΔSP_0661	6.78	0.0067	0.4	0.0132
ΔSP_0798	3.88	0.0011	n/a	n/a
ΔSP_1633	5.67	0.0035	1.07	0.3388
ΔSP_2000	0.732	0.0036	0.366	0.1712
ΔSP_2082	3.21	0.0016	0.367	0.0289
ΔSP_2193	3.47	0.0029	4.28	0.0084
ΔSP_2235	2.41	0.024	0.723	0.013

## **Examination of CiaR mutant in endocarditis virulence**

Based on previous literature and putative functions as well as our in vivo CI results from co-inoculation, we chose to test candidate RR SP\_0798, also known as gene *ciaR*. This mutant strain showed significant decreased growth in rabbit endocarditis models, which suggests that its response regulator gene played an important role in virulence, so we wanted to further examine it. The results of our candidate RR mutant and the control mutant inoculation in rabbit IE models, as well as the CI numbers and P-values are shown in Figure 6.

## **Blood killing assay**

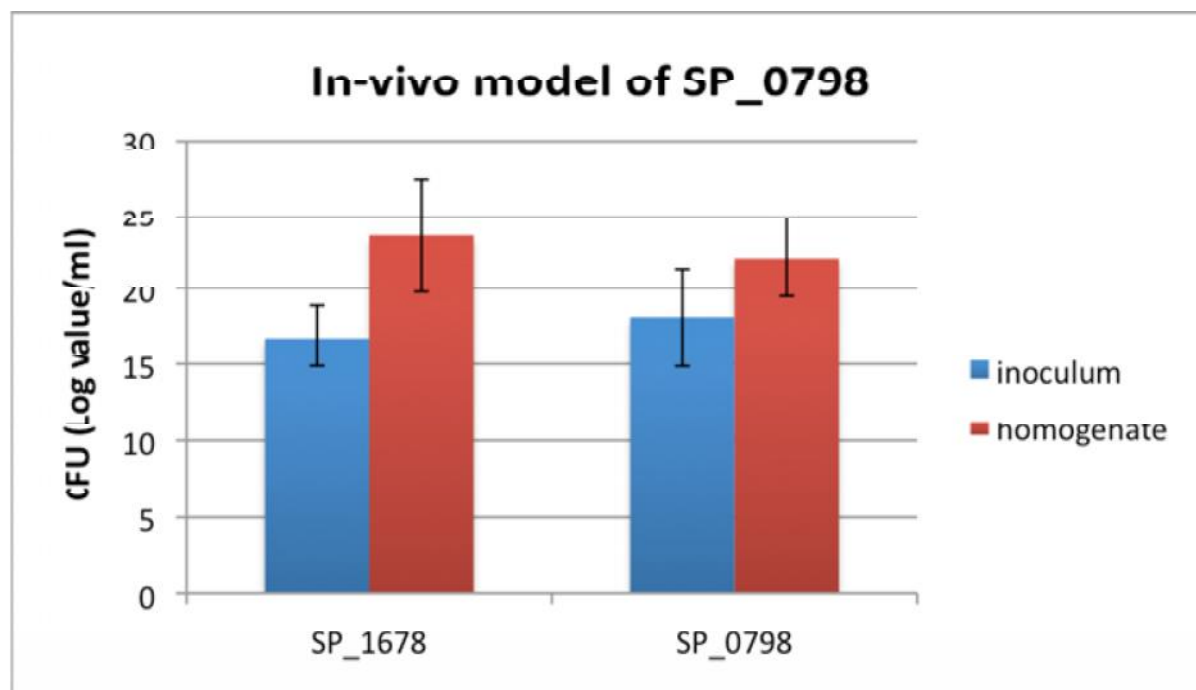
The results of our blood killing assay were inconclusive. Although our control mutant grew successfully on kanamycin selection plates before and after inoculation in human blood, our RR mutants did not have consistent growth on erythromycin selection plates.  $\Delta$ SP\_0083 and  $\Delta$ SP\_0661 displayed consistent growth on input and output selection plates, but the remaining 11 RR mutants did not grow on plates at all (data not shown).



Figure 6. In-vivo competitive index experiment with candidate virulence factor RR  $\Delta$ SP\_0798 and control  $\Delta$ SP\_1678. (A) Graph displays log value CFU per 100ul spread onto selection plates. Output CFU shown is average of three repeats. Error bars are the distribution of the repeats from the average. (B) CI was calculated for the three repeats of *in vivo* homogenate. The average CI as well as the P-value is also shown.

Figure 6

(A)



(B)

Sample	$\Delta$ SP_0798 CI	P-value
in vitro output	0.18	
in vivo homogenate-568	0.07	
in vivo homogenate-569	0.08	
in vivo homogenate-570	0.12	
avg in vivo homogenate	0.09	0.000282

## DISCUSSION

*Streptococcus pneumoniae* is a gram-positive, alpha-hemolytic bacterium that is native in the upper respiratory tract of humans. It has been subject to much scrutiny due its prevalent role in many diseases such as pneumonia, bacteremia, otitis media, meningitis, sinusitis, peritonitis and arthritis. However, its role in infective endocarditis is less studied because it is not a common cause of the disease. Although the cause of IE is mostly attributed to viridans streptococci, it has been reported that *S. pneumoniae* is the cause of 1-3% of adult cases of IE [16] and 3-5% in children [17]. It is notable that pneumococcal endocarditis in association with diseases such as meningitis and pneumonia is unique to Austrian Syndrome, which is a highly aggressive and often fatal condition [5]. When the pneumococcal pathogen accesses the bloodstream, it can infect the heart valves and complicate IE.

Although researchers have found that the encapsulated isolates are up to  $10^5$  more virulent in invasive diseases, than those that are nonencapsulated [18], it is unclear what specific genes of *S. pneumoniae* cause it to facilitate IE, so we set out to study these virulence factors. The chemical structure and thickness of the capsule is related to the ability of the bacteria to survive in the blood of hosts. There have been 90 different serotypes [18] of *S. pneumoniae* identified based on the presence and composition of the polysaccharide capsule. Our study focuses on the highly virulent encapsulated TIGR4 strain known as *S. pneumoniae* serotype 4. Research on TIGR4 is more tedious in comparison to the avirulent, nonencapsulated R6 strain because a correlating characteristic of a polysaccharide capsule is that the bacteria are much more difficult to transform into mutants for study. Specifically, the R6 strain has a

transformation efficiency of ~1%, while the TIGR4 strain has an efficiency of only 0.02% [10]. Other notable differences between the strains that may contribute to virulence relate to amino acid biosynthesis, cellular processes and envelope, central metabolism, and signal transduction [18]. Genes involved in signal transduction through two-component systems (TCSs) have been characterized as virulence factors in disease models such as pneumonia and meningitis in various studies [9], but no studies have included IE. TCSs are composed of a histidine kinase (HK) protein that senses the environment and a corresponding response regulator (RR) that regulates gene transcription for the bacterium to appropriately adapt, thrive, and in the case of disease, escape host defenses. The whole genome of TIGR4 has been sequenced and has 2,160,837 base pairs with 2236 coding regions, of which 1440 have putative biological functions [19]. There are 14 RRs and 13 HKs, including an orphan regulator, identified in *S. pneumoniae* TIGR4.

The primary goal of this study was to place more research emphasis on strategies to combat pneumococcal bacteria in IE by analyzing the role of RRs and their regulons in virulence of *S. pneumoniae* TIGR4 strain. Although there are presently two commonly administered vaccines against *S. pneumoniae*, they are aimed towards disease models such as pneumonia and meningitis, not endocarditis. It would be better if we could identify virulence factors in IE for vaccine design against this disease model. Also, the current vaccines target surface proteins that are specific to a limited number of serotypes. Since we are examining virulence roles intracellular signaling transduction proteins and target genes that are common in *S. pneumoniae*, development of a vaccine aimed at these proteins may provide pleiotropic protection.

We established a system of synthesizing 14 RR mutant strains by replacing target genes with an antibiotic-resistance cassette using PCR amplification and integrating this DNA into the genome by homologous recombination. We found one RR gene SP\_1227, which is part of TCS02, to be essential in that mutants were not viable. Behavior of all other mutants was tested by mixing and inoculating them in both in vitro and in vivo rabbit endocarditis models. Virulence was assessed by the growth rates of mutants as measured by quantitative real-time PCR and competitive index assays. We found several mutants with decreased growth in the rabbits, but picked one candidate virulence factor in SP\_0798 and further tested it, individually, in rabbit endocarditis models. Mutant SP\_0798 was confirmed to have significantly reduced growth based on its CI. We searched literature and databases to elucidate the regulon of this regulator and found that it had many functions.

Of the 11 *S. pneumoniae* completed genomes, all contained either 13 or 14 TCSs. To synthesize our 14 TIGR4 RR mutants, we optimized the protocol outlined by Bricker et al. TIGR4 cells undergo cycles of competency and refractory, therefore, they must be cultured for 7-7.5 hrs to mid-log phase when they are most competent. The THY+HCl media used to culture bacteria was ideal because the acid slows entry into competency so that natural competency and addition of endogenous CSP do not overcompensate each other and reduce efficiency. The glycine supplemented in media partially disrupts the cells wall to facilitate CSP and exogenous DNA entry for transformation. After transformation, we confirmed what was reported in previous papers [9], by background database searches, and experiments performed in our lab by Jenishkumar Patel that SP\_1227 is homologous to SSA\_1565 in *S. sanguinis* and *ycyG*

in *B. subtilis*. These genes are essential in their respective organisms. SP\_1227 mutants formed no viable colonies and led us to believe that this TCS regulates genes that are required for *S. pneumoniae* viability. RR SP\_0526 was once thought to be essential because no colonies appeared to grow in mutants, however, it was later shown that this mutant has a very low transformation efficiency [15]. Using our optimized transformation protocol, it was still difficult to synthesize mutant SP\_0526. Our colony PCR results, as seen in Figure 3, show that it took repeated trials for mutant colonies to finally grow and collected. All other TCS RR formed several apparent colonies and were successfully transformed.

In previous studies with regards to diseases such as pneumonia and bacteraemia, TCS01, TCS02, TCS04, TCS05, TCS06, TCS07, TCS08, TCS09, TCS12, TCS13, and TCS14 were found to have some role in virulence [9]. TCS14 is composed of an orphan RR without a correlating HK and is identified as SP\_0376 in TIGR4. The virulence of TCS14 appears to be tissue specific and due to the fact that it regulates iron transport [20]. Iron is essential for bacterial growth, however, it can be detrimental at high levels in certain tissue when it is converted to reactive oxygen species from hydrogen peroxide. TCS09 consisting of RR SP\_0661 in TIGR4 has been demonstrated to have a major role in virulence in pneumonia and bacteraemia, however, the extent of its virulence is dependent on strain and site of infection [9]. Similarly, TCS04 consisting of RR SP\_2082 in TIGR4 is a virulence factor in pneumonia that is strain specific and reported to show the most virulence in the TIGR4 strain. As a virulence factor, TCS04 regulates the *psa* operon, which contributes to resistance to oxidative stress [21]. Resistance to oxidative stress can be beneficial to bacterial

survival against host immune defenses that employ reactive oxygen species (ROS) to kill pathogens. TCS12 encodes HK *comD* and RR *comE*, which are responsible for the natural competency of *S. pneumoniae*, however, this TCS has also been linked to virulence in pneumonia and bacteraemia [22]. In addition to regulating competency genes, TCS12 also regulates virulence factors autolysin LytA, stress response protein HtrA, and choline binding protein CbpD. TCS13 consisting of RR SP\_0526 in TIGR4 is a virulence factor that regulates synthesis and export of bacteriocin-like peptides, which are predicted to kill competing microbes and/or have a cytotoxic effect on hosts, allowing a growth advantage in its environment [23].

From our in vitro growth curve we found that most of our response regulator mutants grew at comparable rates to our control strain, with the exception of the SP\_0798 mutants, which grew slower. However, for our in vitro competitive index assay, we observed that most mutants, including the SP\_0798 strain, had a CI significantly greater than 1, which indicated that the mutants grew more than the control strain. In the case of SP\_0798, we hypothesize that the other mutants were compensating for the missing function of  $\Delta$ SP\_0798, and therefore, allowed it to grow at faster rates than what was observed when we performed the individual growth curve. Also, it is possible that the increased growth of the other response regulator mutants when compared to the control is because the response regulator mutants were slower to reach their stationary growth phase than the control, and that this was just not observed in the span of the growth curve experiment. In the cases where mutants had a  $CI < 1$  as seen with strains SP\_0376 and SP\_2000, which indicated they grew slower than the control strain. This result contrasts the growth patterns observed in the growth

curve of individual strains, and we suppose that may be because when strains were co-inoculated they were competing and some factors inhibited the growth of strains SP\_0376 and SP\_2000.

For study on endocarditis virulence, we co-inoculated all 13 TIGR4 RR mutants along with 1 control mutant in rabbit models. DNA was isolated from vegetation formed on heart valves of rabbits. Growth of RR mutants was compared to growth of the control mutant, which behaved analogously to wild type, by real-time PCR (qPCR) with specific primers and competitive index assays.  $CI < 1$  suggested that a mutant had reduced virulence in IE, therefore, the RR was a possible virulence factor. Based on our model, we found SP\_0156/TCS07, SP\_0376/TCS14, SP\_0603/TCS10, SP\_0661/TCS09, SP\_0798/TCS05, SP\_2082/TCS04, and SP\_2235/12 were possible virulence factors. Of these, SP\_0603/TCS10 was of particular surprise because it had not been reported as having a role in virulence in previous studies of other diseases. It was reported that TCS10, also annotated as *vncRS*, had some role on vancomycin tolerance [24] but its biological functions are still to be determined. The study did perform microarray analysis of SP\_0603 (data not shown), which revealed that mutants had more than two-fold upregulation of a ribosomal protein L31 and three cell wall anchor proteins (SP\_0462-SP\_0464) as well as down-regulation of purine metabolism genes, *pbuX* and *purK*. The significance of these gene functions in virulence has yet to be determined.

Due to time limitations and availability of rabbit models, we were only able to select one candidate virulence factor for further examination. Based on the CI assay, we chose SP\_0798 because the mutant showed significantly decreased growth in IE



models and previous literature on its functions led us to believe it may be a virulence factor. We individually inoculated  $\Delta$ SP\_0798 with our control mutant and confirmed that it did indeed have a role in virulence. From these results we searched microarray databases and literature relating to the regulon and function of SP\_0798, which is also known as the *ciaR* gene. It appears the *ciaR* regulates many target genes with findings that its gene product binds various promoter sites. Microarray data also identified several genes that were significantly upregulated or downregulated by the mutant in comparison to the wild type. General features of target genes include sugar metabolism, competence, stress response, autolysis, and virulence. Table 6 was put together by Mascher et al. [26] and summarizes the genes they identified as part of the *ciaR* regulon. We observed that SP\_0798 had diverse functions including those in competence, antibiotic (beta lactam) resistance, stress responses, autolysis, polysaccharide metabolism and transport, and virulence. Several genes were transcribed from promoters regulated by *ciaR* including those for modification of teichoic acids (*lic*), sugar metabolism and transport (*mal*, *man*), temperature stress response (*htrA*), chromosome segregation (*parB*), and protease maturation (*ppmA*). Regulation of sugar metabolism and transport is likely to have a role in virulence as it would contribute to cell wall and capsule synthesis and composition. The cell wall and capsule protects the entire cell, as discussed earlier, and therefore is important for its ability to survive any environment. Also, it is reported that in *S. pneumoniae* the *htrA* or high-temperature requirement A gene, also known as DegP/DO protease, confers tolerance to temperature shifts that pathogens often encounter. HtrA is a serine protease that can act as a molecular chaperone and have proteolytic activity, which usually serve for

quality control of other proteins in situations of high temperatures. In addition to heat resistance, HtrA can prevent protein degradation and assist in protein folding in situations of oxidative and osmotic stress [25]. The ability of HtrA to aid the cell in response to the environment through the TCS RR SP\_0798 signaling may explain its role in virulence. Interestingly, five strongly regulated promoters by *ciaR* were for the expression of small non-coding RNAs, otherwise known as *cia*-dependent small RNAs or *csRNAs*. It was reported that *csRNA4* and *csRNA5* affected stationary phase autolysis [26]. Another study reported that the *ciaR* target site affected biological

Table 6. Putative target genes of *ciaR* from Mascher et al [26]

Table 6

Region	Gene(s) <sup>a</sup>	TIGR4 annotation
1	Hypothetical; <i>ugd</i> *	SP0133-SP0137
2	<i>gpmB</i> *, <i>pflE</i> *, <i>deoR</i> *	SP0239-SP0247
3	<i>ruvB</i> *, <i>uppS</i>	SP0256-SP0262
4	<i>manLMN</i> *	SP0281-SP0285
5	<i>spiAB</i> , <i>pnc</i> ( <i>blp</i> ) cluster	SP0524-SP0533
6	<i>fibB</i> ( <i>murN</i> )	SP0611-SP0617
7	<i>deoAC</i> *	SP0839-SP0845
8	<i>pit2ABCD</i>	SP1030-SP1037
9	Hypothetical	SP1057-SP1067
10	<i>orf2</i> and -3, <i>licABC</i>	SP1266-SP1274
11	<i>pgm</i> *, <i>bta</i> *	SP1694-SP1499
12	<i>axe</i> *	SP1694-SP1697
13	<i>sacA</i>	Downstream of SP1795
14	<i>cyl</i>	SP1945-SP1954
15	Hypothetical; 23S RNA	SP2001-SP2005
16	<i>malPM</i>	SP2104-SP2108
17	<i>dltABCD</i> *	SP2173-SP2178
18	<i>orfL</i> , <i>htrA</i> *, <i>spo0J</i> *	SP2234-SP2240

\*homologs

peptide production including bacteriocin and cytolysin [27]. Bacteriocins are protein toxins and their secretion from one strain of bacteria can kill competing microbes or damage host defenses so it may be advantageous for *S. pneumoniae* in hosts and a way to complicate IE. Cytolysins are proteins secreted by bacteria that cause the death of other cells such as red blood cells by lysis, and therefore, their role in virulence may be similar to bacteriocins, as mentioned previously, in that they provide bacteria a growth advantage by inhibiting host defenses.

It is of interest to uncover the pathways and their correlated phenotypes regulated by the *ciaR* gene and other virulence factors against hosts to determine better methods to combat bacteria. A study focused on Group B Streptococci (GBS) reported that *ciaR* mutants had significant decreases in intracellular survival in neutrophils, macrophages, and human brain microvascular endothelial cells. These mutants showed less viability because they were more susceptible to killing by antimicrobial peptide, lysozymes, and reactive oxygen species of the immune system [28]. Although this study is on a different organism than our study, we can turn our attention to the possibility that *S. pneumoniae* may use similar mechanisms in hosts. We can study these mechanisms by performing blood killing assay and phagocytosis assays. Our blood killing assay produced inconclusive results and needs to be repeated. We hypothesize that the erythromycin concentrations used for selection plates were too high and killed most bacterial strains that were sensitive and that  $\Delta$ SP\_0083 and  $\Delta$ SP\_0661 strains were able to grow because they had higher expression of the erythromycin cassette and tolerated the antibiotic. A phagocytosis assay was

performed by our lab member Jenishkumar Patel with the protocol routinely used for *Streptococcus sanguinis*, which is commonly studied in our lab. This assay is an in vitro experiment to test how RR mutants behaved in the presence of macrophage cells, which are cells part of the immune system that kill pathogens. We hypothesized that mutant strains that had a role in virulence reduced colonies numbers in comparison to the control strain because deletion mutants were susceptible to macrophage killing. However, we were not able to confirm or deny our hypothesis because mutants did not grow consistently and results were not reliable. We believe this we because the growth rate of *S. pneumoniae* is much slower than that of *S. sanguinis*, therefore, the protocol must be adjusted to account for this difference for better results.

Much of our results are preliminary and further experimentation needs to be performed for more definite conclusions. Our inoculation of RR mutant strains in endocarditis rabbit models for in vivo study needs to be repeated so that results can be proven to be reproducible. We also need more rabbit endocarditis models to individually test the other TCS RRs (SP\_0156/TCS07, SP\_0376, SP\_0603/TCS10, SP\_0661/TCS09, SP\_0798/TCS05, SP\_2082/TCS04, and SP\_2235/12) that we identified as candidate virulence factors. In addition to repeating our experiments, future experiments for analysis on the regulons of TCS RRs that are virulence factors may be performed. Chromatin immunoprecipitation (ChIP) and sequencing can identify DNA-binding sites of candidate TCS RRs and reveal what genes are being regulated by the protein. Once putative binding sites have been identified, a method to examine protein-DNA binding affinity would be by way of an Electrophoretic Mobility Shift Assay (EMSA). In the EMSA, competing probes with known sequences along with a

radioactive labeled probe of interest would be incubated with the candidate TCS RR protein. These experiments may elucidate the regulons of RRs, and therefore, by identifying the gene functions and the pathways they are involved in we can understand their contribution to virulence.

In conclusion, this study is a preliminary step in demonstrating that a number of TCS RRs in *S. pneumoniae* TIGR4 strain have some role in virulence in infective endocarditis. Further experiments may confirm our candidate response regulator SP\_0798, as well as other candidates, as virulence factors so that they themselves or genes within their regulon could potentially be vaccine candidates for IE. Therefore, identification of regulatory networks of these TCSs may reveal novel signaling pathways related to IE and lead to better preventative methods against the disease. Altogether, results will hopefully bring more attention to *S. pneumoniae* as a causative agent for IE and lead to more research on the subject.

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