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ENVIRONMENTAL RESPONSES OF TWO-COMPONENT SYSTEMS IN STREPTOCOCCUS SANGUINIS

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ENVIRONMENTAL RESPONSES OF TWO-COMPONENT SYSTEMS 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 ABBREVIATION

%	Percentage
ABC	ATP-binding cassette
BHI	Brain heart infusion
BLAST	Basic local alignment search tool
CFU	colony forming unit
CSP	competence stimulating peptide
DNA	deoxyribonucleic acid
Hrs	hours
HK	histidine kinase
HS	horse serum
Kb	kilo base
M	molar
min	minute
ml	milliliter
mm	millimeter
ng	nanogram
nm	nanometer
O.D.	Optical density
O/N	overnight
^{o}C	degrees Celsius
PBS	phosphate buffered saline

PCR	polymerase chain reaction
RNA	ribonucleic acid
RR	response regulator
Sec.	Second
TCS	Two Component System
TH	Todd Hewitt
U	Unit
Wt/Vol	Weight/ volume
µg	microgram
µl	micro liter

Abstract

ENVIRONMENTAL RESPONSEOF TWO-COMPONENT SYSTEMS IN STREPTOCOCCUS SANGUINIS

By Jenishkumar R. Patel, M.S.

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

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The gram-positive bacterium *Streptococcus sanguinis* is a member of human indigenous oral microbialflora and has long been recognized as a key player in the bacterial colonization of the mouth. *S. sanguinis* is also the most common viridians streptococcal species implicated in infective endocarditis. Although many studies have focused on two-component systems in closely related *Streptococcus* species such as *S. mutans, S. pneumoniae* and *S. gordonii;* the mechanism of the response regulator in *S. sanguinis* is still unknown. The ability of *S. sanguinis* to adapt and thrive in hostile environments suggests this bacterium is capable of sensing and responding to various environmental stimuli.

The present study clearly demonstrates that a number of RR genes, SSA_0204, SSA_0217, SSA_1810, SSA_1794, and SSA_1842, in *S. sanguinis* are essential to the

recognition and response to various environmental stresses. Results from this study also identified genes SSA_0260, SSA_0261, and SSA-0262, involved in acidic tolerance and suppressed by SSA_0204 response regulator.

1. INTRODUCTION

S. sanguinis are facultative anaerobic bacteria that show a coccal chain shape in morphology. It is a normal human indigenous oral inhabitant. *S. sanguinis* binds to saliva and coat teeth by a variety of mechanisms and serve as a binder for other micro flora to attach to the tooth surface, forming a plaque [1]. *S. sanguinis* was formally known as *Streptococcus sanguis* until 1997. The name was grammatically corrected [2]. As a member of the viridans group of streptococci, *S. sanguinis* has been found to be one of the pioneer colonizers of the human oral cavity [3]. Viridans, named for the Latin 'viridis,' meaning green, are a group of α-hemolytic streptococci producing a green color in blood agar due to the hemolysis of erythrocytes by oxidizing hemoglobin and secreting H_2O_2 [4].

S. sanguinis is predominantly found in the oral cavity and may enter the bloodstream by various ways such as invasive dental procedures as well as instrumentation on the mucosal surface [5]. *S sanguinis* is an important cause of endocarditis. Endocarditis is a term that describes an inflammation and/or infection in to the inner layer of the heart. It commonly affects the heart valves, but it may also involve non-valvular areas. Each year 10,000 to 20,000 people are affected by infective endocarditis. It is an important disease because endocarditis may result in serious complications such stroke, heart valve damage require surgery, or death [6].

S. mutans is major causative microbial agent of dental caries. Its ability to tolerate acidic stress and the production of acid from carbohydrates is an important mechanism for its carciogenicity [7].Continuous changes in pH inside the dental plaque require rapid

adaptation mechanisms for any microbial inhabitants in this environment. *S. mutans* is considered to be among the most carciogenic pathogens because it has developed various mechanisms to survive against acidic stress. *S. mutans* is capable of performing glycolysis at extracellular pH values as low as 4.0 [8]. In these organisms the F-ATPase functions to transport protons out of the cell, thereby maintaining a pH gradient across the cell membrane and contributing to the acid tolerance. It has also been recorded that *S. mutans* grown at a low pH has increased ATPase activity [7] so that it can maintain the pH difference across the cell membrane. *S. mutans* is more acid resistant than *S. sanguinis*. It has been proven that *S. sanguinis* competes with *S. mutans* for survival. Therefore we are interested in finding more acid tolerant *S. sanguinis* mutants than the wild type strain in order to antagonize *S. mutans* and reduce caries.

The ability of *S. sanguinis* to adapt and thrive in the hostile environments of the oral cavity and of the heart valves suggests that this microorganism is capable of sensing and responding to different environmental stimuli. To understand the survival of *S. sanguinis* in different environments, we investigated the roles of two-component systems (TCS) because they are reported to respond to environmental changes in many prokaryotic bacteria [9]. TCS are highly sophisticated systems involving various signaling molecules inside the cell. Bacterial adaptation to changing environmental conditions is often accomplished by TCS, which regulate gene expression in response to various stimuli [10]. TCS serve as a response coupling mechanism, allowing an organism to sense a wide variety of signals and respond against stress conditions. Researchers have found hundreds of such systems in various organisms, including Eubacteria, Archaea, and a few Eukaryotic

organisms. A typical TCS consists of a histidine kinase (HK) with a conserved kinase core and a response regulator (RR) with a conserved regulatory domain. After stimulating its sensing domain from outside of the cytoplasm, HK gets phosphorylated on its His residue on the conserved kinase core. HK then transfers a phosphate to an Asp residue on the conserved regulatory domain on the RR. In addition, many HKs also possess phosphatase activity, enabling them to dephosphorylate their cognate RRs. Phosphotransfer to the RR results in activation of a downstream effector domain that elicits the specific response. RR then returns to its inactivated state by releasing phosphate, so it does not remain active throughout its lifetime [9]**.**

The sequence analysis of the complete *S. sanguinis* genome shows that it has 2,388,435 bp consisting of 43.4% G + C content, which is relatively higher than the other streptococci. The genome encodes 2,274 predicted proteins, sixty-one tRNAs, and four rRNA operons, from which we have identified twenty-nine genes that putatively encode TCS. These included fifteen putative response regulators and fourteen putative kinase sensors[11]. We are interested in the role of regulators in responding to environmental stimuli. Even today, there haven't been any systematic studies that characterize the two-component signal transduction systems responding to stress tolerance in *S. sanguinis.* In an effort to gain a more comprehensive view of the roles of these response regulators, we systematically inactivated each of the genes coding for the fourteen putative response regulators. We have replaced the genes by an antibiotic resistance gene cassette, except the SSA_1565 encoding gene, which was later found to be an essential gene. Fourteen mutants of TCS response regulators were created and confirmed by sequencing. The TCS mutants

were assessed for their ability to resist various environmental stresses including acidic, alkaline, and osmotic conditions. Microarray analysis was performed to examine the gene regulation in one acidic tolerance TCS mutant. The corresponding regulated gene mutants were examined for their acidic tolerance.

2. MATERIALS AND METHODS

Bacterial strain and growth conditions

All strains used in this study are described in Table 1. *S. sanguinis* SK36, obtained from Kilian & Holmgren, was isolated from human dental plaque. The strains are maintained and routinely grown microaerobic (7.2% H_2, 7.2% CO_2, 79.6% N_2 , and 6% O_2) condition at 37°C in brain heart infusion broth (BHI; Difco Inc., Detroit, MI) supplemented with 1.5% (wt/vol) agar and long term storage at -80°C with 30% glycerol solution. Bacterial growth was determined using a Fluostar plate reader (BMG Labtechnologies, Offenburg, Germany) with the OD 450 nm recorded every 10 minutes immediately following shaking in order to resuspend the cultures. BHI was used as a blank.

Table 1 Bacterial strains used for study

Strain	Phenotype	Source
S. sanguinis		
SK 36	Human Plaque Isolate	Kiliam &Holmgren(1981)
ΔSSA_0169	Km^r ; Δ*0169*::*aphA-3*	from this laboratory
ΔSSA_0204	Km^r ; Δ*0204*::*aphA-3*	this study
ΔSSA_0217	Km^r ; Δ*0217*::*aphA-3*	this study
ΔSSA_0401	Km^r ; Δ*0401*::*aphA-3*	this study
ΔSSA_0516	Km^r ; Δ*0516*::*aphA-3*	this study
ΔSSA_0896	Km^r ; Δ*0896*::*aphA-3*	this study
ΔSSA_0959	Km^r ; Δ*0959*::*aphA-3*	this study
ΔSSA_1113	Km^r ; Δ*1113*::*aphA-3*	this study
ΔSSA_1119	Km^r ; Δ*1119*::*aphA-3*	this study
ΔSSA_1685	Km^r ; Δ*1685*::*aphA-3*	this study
ΔSSA_1794	Km^r ; Δ*1794*::*aphA-3*	this study
ΔSSA_1810	Km^r ; Δ*1810*::*aphA-3*	this study
ΔSSA_1842	Km^r ; Δ*1842*::*aphA-3*	this study
ΔSSA_1972	Km^r ; Δ*1792*::*aphA-3*	this study
ΔSSA_2378	Km^r ; Δ*2378*::*aphA-3*	this study

Identification of the candidate genes for the TCS regulators

To understand the effect of TCS regulators, we deleted the putative regulator genes and studied the mutant tolerance under different stress conditions and compared the tolerance to wild type *S. sanguinis* SK36. Candidate genes were analyzed with BLAST search from gene data base for TCS genes from different closely related Streptococcal spp. We selected E- value cut off 10-5, and than we did reciprocal search with *S. sanguinis* genome for TCS regulators. The homology search confirmed the TCS regulators and provided information on their potential responses to environmental conditions. A list of RR candidates genes and their functions are described in Table 2.

Construction of the mutants

Three sets of primers (F1/R1, F2/R2 and F3/R3) listed in Table 3 were designed to amplify the upstream sequence, the drug resistance gene and the downstream sequence respectively. Both 5' ends of F2 and R2 primers contain sequences complementary with *S. sanguinis* flanking sequences of the target gene. A final PCR recombinant DNA product containing the antibiotic selection marker flanked with *S. sanguinis* genomic DNA is produced using F1/R3. The recombinant DNA was integrated into *S. sanguinis* genome via double crossover recombination. Three different fragments were amplified with appropriate primers by using high fidelity Taq polymerase. The cycling condition for each amplification were 94 ^{o}C for 2 min., followed by the 30 cycle at 94 ^{o}C for 30 sec., 55 ^{o}C for 30 sec., 68 ^{o}C for 4 min., and finally 68 ^{o}C for 4 min. Purity was checked by 1 % gel electrophoresis.

Table 2: A List of RR Genes Used for the Study and Their Putative Functions

Gene `	Putative function
SSA_0204	Nisin biosynthesis two-component response transcriptional regulator nisR
SSA_0217	Two-component system transcriptional regulator (CheY domain and HTH-like DNA-binding domain)
SSA_0401	Two-component response transcriptional regulator (CheY-like receiver and winged-helix DNA-binding domains)
SSA_0516	AmiR two-component response regulator with transcriptional antiterminator output domain
SSA_0896	Two-component system transcriptional regulator (CheY domain and HTH-like DNA-binding domain)
SSA_0959	Two-component response transcriptional regulator (CheY-like receiver domain and a winged-helix DNA- binding domains)
SSA_1113	Two-component response transcriptional regulator (CheY-like receiver domain and a winged-helix DNA- binding domains)
SSA_1119	Two-component response transcriptional regulator (CheY-like receiver and AraC-type DNA-binding domains)
SSA_1565	Two-component response transcriptional regulator (CheY-like receiver and winged-helix DNA-binding Domains)
SSA_1685	Two-component response transcriptional regulator (CheY-like receiver and winged-helix DNA-binding Domains)
SSA_1794	Two-component response transcriptional regulator (CheY-like receiver and winged-helix DNA-binding domains)
SSA_1810	Two-component response transcriptional regulator (CheY-like receiver and winged-helix DNA-binding domains)
SSA_1842	Two-component response transcriptional regulator (CheY-like receiver and HTH DNA-binding domains)
SSA_1972	Two-component system transcriptional regulator (CheY domain and HTH-like DNA-binding domain)
SSA_2378	Two-component system transcriptional regulator, LytR/AlgR family

Table 3: A list of Primers used for study

Primers	Nucleotides sequences (5'to 3')
SsX_0204 F1	TATCTCTAACCCTATTGGGAATCCT
SsX_0204 R1	**GCCATTTATTCCTCCTAGTTAGTCA**TTCCATTATAGCATACCTCTAGC
SsX_0204 F3	**GTTTTAGTACCTGGAGGGAATAATG**ATCGCAACAGTGAGAGGAGTAG
SsX_0204 R3	TTCTGTCCCACTCTCTTTTATCATC
SsX_0217 F1	GACCATTATTGAAATCCTGCTCAT
SsX_0217 R1	**GCCATTTATTCCTCCTAGTTAGTCA**TAAAACCTTAATCATGTCTTCCTC
SsX_0217 F3	**GTTTTAGTACCTGGAGGGAATAATG**CAGAGAGATTTTAGTAAGGAAAGC
SsX_0217 R3	GACTTTTCACCACAGATGGTAAGAC
SsX_0401 F1	AGGATAGATGCTAGTGATGAAATGG
SsX_0401 R1	**GCCATTTATTCCTCCTAGTTAGTCA**AGCCATAAACACTCGCCTCCAG
SsX_0401 F3	**GTTTTAGTACCTGGAGGGAATAATG**GGACTGGGTGTCCGGCTCAAGG
SsX_0401 R3	CTTTTCAAATCACAAAACAAAAAGG
SsX_0516 F1	AGCTTATGTATCGACCAAGGCTAC
SsX_0516 R1	**GCCATTTATTCCTCCTAGTTAGTCA**CTTCATACTTCCCCTCGACAGT
SsX_0516 F3	**GTTTTAGTACCTGGAGGGAATAATG**AAATTGCTGGTGCTCCAAGATG
SsX_0516 R3	TTTTCGATAACTCTCTTCAGCAGAT
SsX_0896 F1	ATTCCAGAGAAATCTCAAGCTCAG
SsX_0896 R1	**GCCATTTATTCCTCCTAGTTAGTCA**GTGCATACTTAGCTTTTTCTCC
SsX_0896 F3	**GTTTTAGTACCTGGAGGGAATAATG**ATGGGCTACCAGCTTGGAGGAG
SsX_0896 R3	GATTCTTGATAGCCTTGAGCAGATA
SsX_0959 F1	AGGAGCAGGAAAAGGTGTATTTAAC
SsX_0959 R1	**GCCATTTATTCCTCCTAGTTAGTCA**AATCATTTTTCTTTCCTCCAATAAAAC
SsX_0959 F3	**GTTTTAGTACCTGGAGGGAATAATG**ACTCTTCGTAGTGTCGGCTACA
SsX_0959 R3	TAGAATGGTCATCAGTTGTTTGAGA
SsX_1113.F1	CTTGGCAAAAGGTCAATACTTCTAC
SsX_1113.R1	**GCCATTTATTCCTCCTAGTTAGTCA**TCCCATTGCCAATCTCCTCATA
SsX_1113.F3	**GTTTTAGTACCTGGAGGGAATAATG**TATAAAATGGAAAGATCACGAGGT
SsX_1113.R3	TTGTGGATTTATGGTAAGTTGAGAG
SsX_1119.F1	TCTCAACAAAAACAAGAAGAAGTCA
SsX_1119.R1	**GCCATTTATTCCTCCTAGTTAGTCA**ATACACAAGATTCAACTCCTTATTTA
SsX_1119.F3	**GTTTTAGTACCTGGAGGGAATAATG**CCTCGACAGTTTAAAAAAGGAG
SsX_1119.R3	ATTTTATTGAACTGCTGAGCCAAGT
SsX_1565.F1	TCTGTTGTCTTGATGGAAACAGTAA
SsX_1565.R1	**GTTTTAGTACCTGGAGGGAATAATG**CGCCGCGGAGTTGGCTACTATA
SsX_1565.F3	**GCCATTTATTCCTCCTAGTTAGTCA**CTTCATATGTTCACCTTAACATAAG
SsX_1565.R3	CGAGGTTCAGTAGGATTATCAAAGA
SsX_1685.F1	TCCCAGACTATTAGCAAACTTCTTG
SsX_1685.R1	**GTTTTAGTACCTGGAGGGAATAATG**ACCAAAAAAGGAATAGGATACGG
SsX_1685.F3	**GCCATTTATTCCTCCTAGTTAGTCA**ATGCATCTTTCCCTCCTAATTTC
SsX_1685.R3	GTCTTTAAGTTCTTCATCTGAAACG
SsX_1794.F1	ACGGCTATAATCAACATTCTTACCA
SsX_1794.R1	**GTTTTAGTACCTGGAGGGAATAATG**TTCACTCTGGTGCCGCTGGAAG
SsX_1794.F3	**GCCATTTATTCCTCCTAGTTAGTCA**CATCATCTTACTCACTTTCTCG
SsX_1794.R3	GAGATTGTAAACAGTGACGCTGTAG
SsX_1810.F1	ATTCTGTGTAGTTCATTGGAATGGT
SsX_1810.R1	**GTTTTAGTACCTGGAGGGAATAATG**CGCGGTGTTGGTTATGCCATGC
SsX_1810.F3	**GCCATTTATTCCTCCTAGTTAGTCA**TGCCATAGGCCTATCCTATTTT

SsX_1810.R3	AACTCTGGTATCAATTTCATCGGTA
SsX_1842.F1	CCCTGAATCATAAAGTCCTGAATAA
SsX_1842.R1	**GTTTTAGTACCTGGAGGGAATAATG**CACCATCTTGTCCCACAAGAAG
SsX_1842.F3	**GCCATTTATTCCTCCTAGTTAGTCA**CTGATGGTCATCCACTAACAAT
SsX_1842.R3	CTTCTTAGGAAATGTGGAGGTTGT
SsX_1972.F1	TAAATAGCATCATAAAGCGGCAGTA
SsX_1972.R1	**GTTTTAGTACCTGGAGGGAATAATG**CGAATCGGCCAAGAGAAAGGCT
SsX_1972.F3	**GCCATTTATTCCTCCTAGTTAGTCA**AAGTTTCATGCTTCCTTCCCTC
SsX_1972.R3	GCTGGTCTTTATCATCTTTCCAGT
SsX_2378.F1	TGAATCAATTAACTGGCTTTGATTT
SsX_2378.R1	**GTTTTAGTACCTGGAGGGAATAATG**AATAGAGGGAAAAGAGTTGACAAA
SsX_2378.F3	**GCCATTTATTCCTCCTAGTTAGTCA**TTTCATAAATTCTATCTCCTAATTGTTA
SsX_2378.R3	CTTTCCTTCTTTTTATGTCTGTCAA
Kan_F:	TGACTAACTAGGAGGAATAA
Kan_R:	TACCTGGAGGGAATAATG
Kan_P_R(5')	GCT TATATACCT TAGCAGGAGACA
Kan_P_F(3')	GTATGACATTGCCTTCTGCGTCC

Purification of PCR amplified fragments

After each PCR cycle the products were purified to get pure amplified fragments without any unwanted DNA. 100 µl of Buffer PB was added to 25 µl of PCR product and mixed. A QIAquick spin column was placed into a 2 ml collection tube and applied the sample to the column and centrifuged for 60 sec. flow-through discarded and placed the QIAquick column back into the same tube. 750 µl of PE (alcohol added) added to wash unbound DNA fragments from the column and centrifuged for 30-60 sec. to remove the residual ethanol centrifuged the column for an additional minute. It was then placed into a QIAquick spin column in a clean 2 ml eppendorf tube. Next, the DNA was eluted with 30 µl of water and centrifuged for 1 minute, then stored at -20^{o}C (QIAGEN Sample and Assay technologies).

TH+HS preparation

To prepare the TH +HS, 250 ml of fresh 1x Todd Hewitt broth was adjusted to a pH of 7.6. The broth was then heated on a stir plate until it came to a rolling boil and allowed to cool to near room temperature. The medium was filter sterilized and transferred to sterile 50 ml conical tubes. Working stocks solution was kept at 4°C for weeks.

***S. sanguinis* SK36 transformation**

The Streptococcus sanguinis SK36 cells were cultured in 2 ml of TH-HS broth and incubated overnight at 37°C, either in an anaerobe jar or with the tube's cap tightly closed. The tube containing 10 ml TH+HS was also placed in the incubator. In the morning 50 µl

of the overnight culture were transferred to 10 ml TH+HS. The O.D. 660 nm of the overnight culture vs. TH was also measured, normally it is ≤ 0.9. The diluted culture was incubated at 37^{o}C for ~ three hours or until OD660 reached 0.07-0.08. The transformed DNA and 2 µl (70 ng) of CSP were added to sterile Eppendorf tubes, which were then placed in a 37°C water bath to pre-heat at which point, 330 µl of cell culture was transferred to each tube. The tubes containing the cells and the DNA were incubated for one hour at 37°C in a water bath. The cells were then plated immediately on BHI agar containing kanamycin and the plates were incubated under microaerobic conditions at 37°C for 48 hours. After two days, the single colony from each plate was collected and the colony PCR was performed under the following conditions: 94^{o}C for five minutes, followed by the 35 cycle at 94^{o}C for 30 sec., 55^{o}C for 30 sec., 68^{o}C for four minutes, and finally 68oC for four minutes. PCR amplification has been performed with primer F1 and R3 shown in table 2. for each respective mutants.

Mutant Confirmation and Sequencing

To confirm the correct fragments were transformed, we verified sequences using internal Km_P_R(5') and Km_P_F(3') primers. The sequencing was done at the DNA core facility in VCU**.**

Growth rate determination

The wild type *S. sanguinis,* SK36 and each of the response regulator mutant strains were grown in a BHI medium. The TCS response regulator mutants were checked for growth rate and compared with the wild type strain SK36. Mutant strains from -80^{o}C were inoculated into 2 ml of BHI medium supplemented with 500 µg/ml kanamycin and incubated overnight at 37^{o}C under microaerophilic conditions in an Anoxomat jar. The next day, each 140 µl sample from the incubated culture is transferred into 15 ml plastic narrow bottomed screw cap tubes containing 14 ml of BHI medium and incubated at 37 C. Every hour 1 ml liquid from each tube was transferred to a cuvette and its O.D. determined at 660 nm (Thermo electron corporation, Biodmate 5)

Acid stress treatment

Routinely, the *S. sanguinis* were grown in microaerobic condition at 37°C in BHI broth overnight. The next day, fresh BHI medium was prepared at pH 6.3 (adjusted with filter sterilized HCl). 2 µl of O/N grown culture of all 14 RR mutants and wild type *S. sanguinis* SK36 were inoculated in to a 96 well plate of 198 µl of BHI medium at pH 7.4 or pH 6.3. Bacterial growth was determined using a FLUOstar plate reader (BMG Labtech, Offenburg, Germany) where the OD 450 nm was recorded every 10 minutes immediately following shaking in order to re-suspend the culture. BHI was used as a blank.

Alkaline stress treatment

To analyze the growth of the *S. sanguinis* cultures at high pH, the initial pH of sterile BHI medium was adjusted to 9.1 with sterile NaOH. Cultures of all mutants that had been grown overnight as well as SK36 strains were incubated at pH 7.4 (standard) and pH 9.1 overnight in 96-well plates at 37°C. Bacterial growth was determined as described above in the acidic stress treatment.

Osmotic stress treatment

Overnight growth cultures of all TCS regulator mutants and wild type strain Sk36 were grown under the standard condition with NaCl 0.5%. The next morning cultures were transferred into fresh BHI medium containing 3.8% NaCl concentration. Growth characteristics were monitored as mentioned above.

Acidic, Alkaline and Osmotic shock treatments

Bacterial strains of the *S. sanguinis* SK36 and the RR mutants were inoculated into BHI medium containing 500 μg/ml of Kanamycin in microaerophilic conditions at 37 ^{o}C. The next morning, we transferred 100 μl of the culture, grown overnight, into 1.9 ml fresh BHI broth at pH 3.6 for acid shock treatment, at pH 10.8 for alkaline shock treatment, and in 10% NaCl for osmotic shock. We took 100 μl samples from each shock treatment before it was incubated and diluted 10-10^4 in cold PBS buffer. We immediately transferred 10 μl from each dilution and put it onto the BHI agar plate containing kanamycin. Each plate was allowed to dry for 15 minutes in a laminar air flow chamber and then incubate for 48

hours in a microaerobic condition at 37 ^{o}C. Each plate was marked for zero minute shock treatments. After 60 minutes, a 100 µl sample was taken and we followed the same procedure as described for the zero minute incubation and 48 hours in a microaerobic condition at 37 ^{o}C. After two days, we observed the plates and counted the colonies before and after shock treatment**.**

Microarray Analysis

DNA microarray analysis was used to determine any changes in the expression levels of different genes. We selected ΔSSA_0204 to check the regulatory network. In brief, both the SK36 and SSA_0204 were cultured overnight. They were then inoculated into a fresh BHI medium with 1% inoculation under microaerobic conditions (7.2% H2, 7.2% CO2, 79.6% N2, and 6% O2) in an Anoxomat jar (Spiral Biotech) at 37°C. After five hours, the cells were collected and lysed by lysozyme treatment and mechanical disruption. RNA was isolated using FastPrep lysing matrix B (Qbiogene) and RNeasy mini kit (Qiagen). DNA was removed from the RNAeasy minikit column by DNase I treatment. Total RNA was quantified using a NanoDrop ® ND 1000 Spectrophotometer. The reverse-transcription, labeling and hybridization were performed according to the protocols provided by http://pfgrc.jcvi.org/index.php/microarray/protocols.html. The significance analysis was performed employing MeV MultiExperiment Viewer

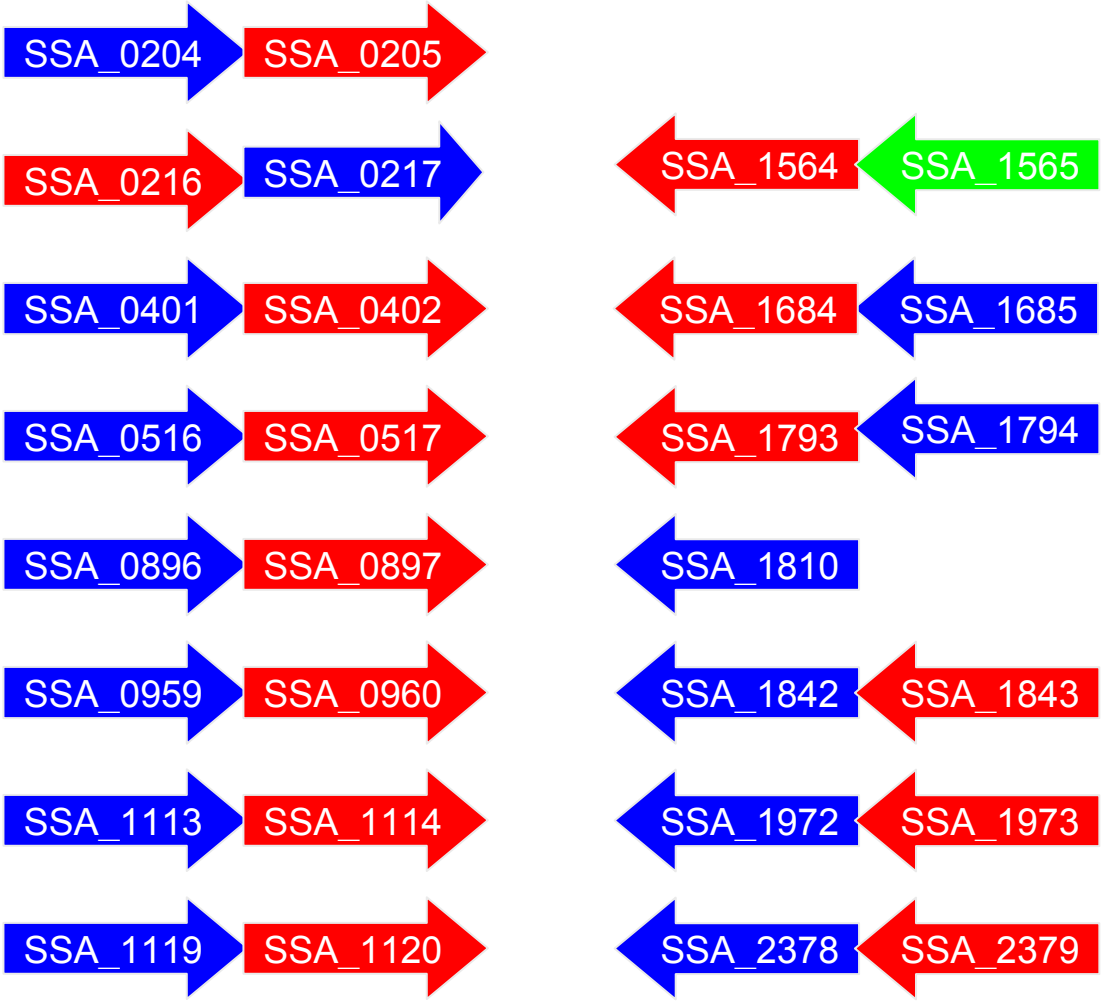
3. RESULTS

Identification of TCS candidates in the genome

Candidate genes were analyzed with BLAST search from gene database for TCS genes from different closely related Streptococcal spp. We selected E- value cut off 10-5, and than we did reciprocal search with *S. sanguinis* genome for TCS regulators. The homology search confirmed the TCS regulators and provided information on their potential responses to environmental conditions. Based on our sequence analysis, we identified 29 genes involved in TCS (Figure 1); they include 15 putative response regulators and 14 putative kinase sensors [11]. For one of the RR genes, SSA_1810, we did not find any adjacent sensor kinase genes. This may be because SSA_1810 gets a signal from other HK, but it is still unknown. Another interesting finding is that SSA_1565 was later found to be an essential gene. We tried to make mutations of this gene several times, but were not successful.

Figure 1 This figure shows TCS response regulators (blue) and sensor kinases (red). The arrow indicates the direction of the gene from 5' to 3'. The green arrow represents the candidate gene SSA_1565, which was found to be an essential gene.

Figure 1


SSA_0204
SSA_0205
SSA_0216
SSA_0217
SSA_1564
SSA_1565
SSA_0401
SSA_0402
SSA_1684
SSA_1685
SSA_0516
SSA_0517
SSA_1793
SSA_1794
SSA_0896
SSA_0897
SSA_1810
SSA_0959
SSA_0960
SSA_1842
SSA_1843
SSA_1113
SSA_1114
SSA_1972
SSA_1973
SSA_1119
SSA_1120
SSA_2378
SSA_2379

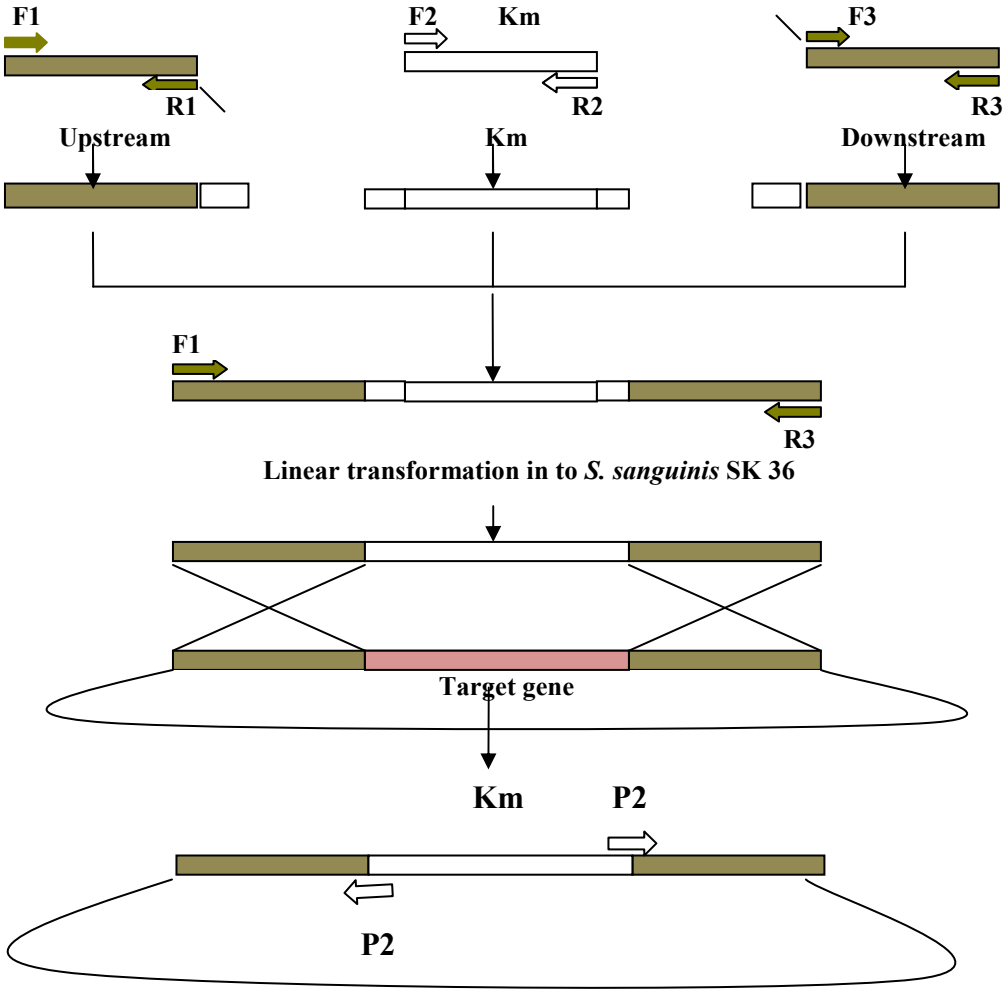
Construction of TCS mutants

There are various ways to make mutations. We have found that using PCR-associated, site-directed mutagenesis works well for deletion of the complete ORF of gene of interest, and it produces a clean genetic background. We used this method to construct the mutations of genes that encode the various putative response regulators of *S. sanguinis* strain SK36. We utilized a cassette, which consists of an antibiotic-resistance gene flanked by regions homologous to the chromosomal target locus. Three sets of primers (F1/R1, F2/R2 and F3/R3) were designed to amplify the upstream sequence, a drug resistance gene and the downstream sequence, respectively. All of the primers used in this study are listed in Table 2. Three different sets of PCR have been performed by using appropriate primers and high fidelity thermostable Taq polymerase to amplify each of these fragments. Both 5' ends of F2 and R2 primers contain sequences that are complementary to *S. sanguinis* flanking sequences of the target gene. A final PCR recombinant DNA product that contains the antibiotic selection marker flanked with *S. sanguinis* genomic DNA (upstream and downstream fragments) was produced using F1/R3 primers. The linear DNA was transferred into *S. sanguinis* SK36 wild type cells. In order to uptake DNA, *S. sanguinis* cells must become competent. The competence is the ability of the cells to take up foreign DNA from the surrounding environment. To increase this ability, we treated cells with a synthetic competence stimulating peptide (CSP). This peptide is a small, stable molecule that induces cell competence under a wide variety of conditions. The use of CSP circumvents many of the limitations to the expression of transformability in oral streptococci. It also expands the opportunities to apply the tools of molecular genetics to

many strains of the species without prior genetic manipulation. The antibiotic resistance marker gene was inserted into the chromosome to replace the target genes by homologous recombination, and a new deletion strain of bacteria was created.

Figure 2: Schematic representation of integration of kanamycin resistance gene cassette into the chromosome of *S. sanguinis* SK36.

Figure 2:


F1
F2
Km
F3
R1
R2
R3
Upstream
Km
Downstream
F1
R3
Linear transformation in to S. sanguinis SK 36
Target gene
Km
P2
P2

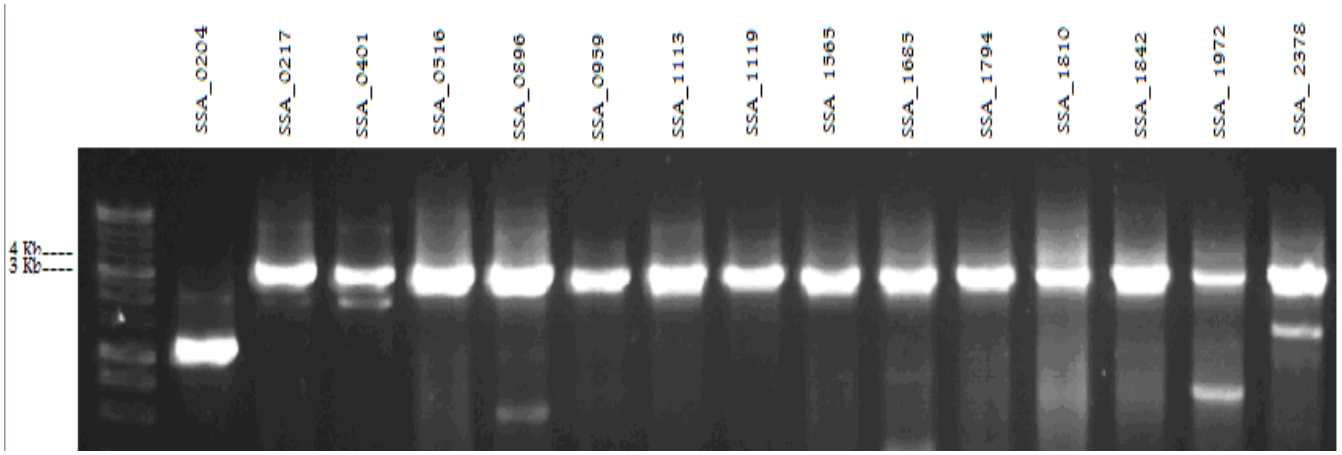
Amplification of cassettes and mutant creation

1% gel electrophoresis confirmed that the three fragments containing the antibiotic resistance gene were successfully fused and amplified by PCR. Here we used previously amplified upstream and downstream regions with their respective primers as shown in Table 2. All of the response regulator gene cassettes of ~3.5 kb were successfully amplified and are shown in Figure 3(A) and 3(B). In the first trial not all of the cassettes were successfully amplified. ΔSSA_0204, ΔSSA_0896, ΔSSA_1685, ΔSSA_1972, and ΔSSA_2378 had impure or small bands in the first trial of amplification as seen in Figure 3(A). We attempted to eliminate these bands by repeating PCR several times, decreasing the amplification time and primer annealing temperature. Finally we successfully amplified all remaining cassettes except ΔSSA_2378. After several processes of amplification we could eliminate this band in case of SSA_2378, so we went ahead and purified PCR products by a PCR product purification protocol as mentioned in the Materials and Methods. Finally, 14 cassettes were successfully transformed, all except ΔSSA_1565, into wild type SK36 cells.

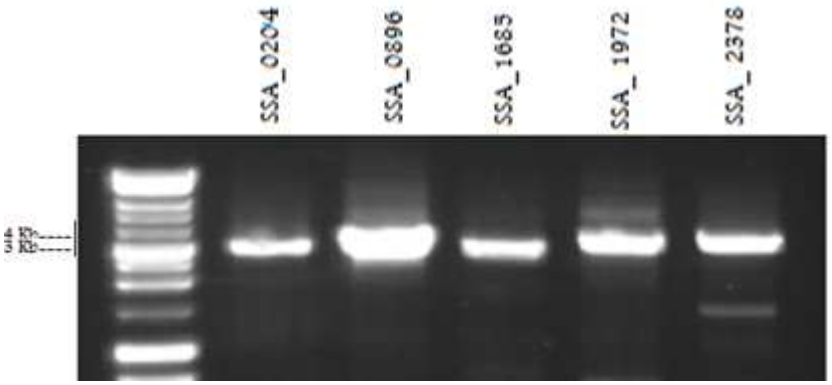
Figure 3: PCR products from the three combined upstream-antibiotic resistance-downstream genes were separated on 1% agarose gel by electrophoresis. ΔSSA_0204 (lane 2), ΔSSA_0217 (lane 3), ΔSSA_0401 (lane 4), ΔSSA_0516 (lane 5), ΔSSA_0696 (lane 6), ΔSSA_0959 (lane 7), ΔSSA_1113 (lane 8), ΔSSA_1119 (lane 9), ΔSSA_1565 (lane 10), ΔSSA_1685 (lane 11), ΔSSA_1794 (lane 12), ΔSSA_1810 (lane 13), ΔSSA_1842 (lane 14), ΔSSA_1972 (lane 15), ΔSSA_2378 (lane 16) and 1kb DNA marker (lane 1). Single bands are shown in each lane at ~3.5 kb, which confirms that all cassettes were successfully amplified except ΔSSA_0204, ΔSSA_0896, ΔSSA_1685, ΔSSA_1972, and ΔSSA_2378, which are later amplified as shown in the second gel (B).

Figure 3:

A.


SSA_0204
SSA_0217
SSA_0401
SSA_0516
SSA_0896
SSA_0959
SSA_1113
SSA_1119
SSA_1565
SSA_1685
SSA_1794
SSA_1810
SSA_1842
SSA_1972
SSA_2378

B.

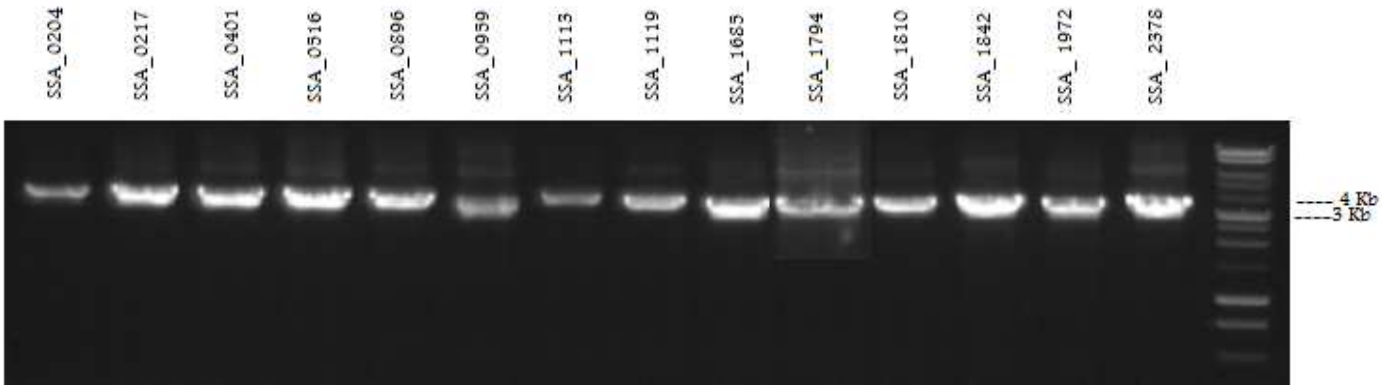

SSA_0204
SSA_0896
SSA_1685
SSA_1972
SSA_2378

Confirmation of mutants by colony PCR and sequencing

After 48 hours of incubation, many colonies appeared on kanamycin containing BHI agar plates for all fourteen response regulator mutants. To further check the correct deletion, we confirmed it by performing colony PCR using the same primers, F1 and R3, for each respective regulator mutant. Results from the colony PCR are shown in Figure 4. It appeared that every band is correct in size and matched with previously amplified PCR fragments. We reconfirmed inserts by sequencing using internal primers P1 and P2 (Figure 2.), at the Nucleic Acids Research Facilities of Virginia Commonwealth University. DNA sequences were analyzed using SeqMan II software (DNASTAR Inc, Madison, WI). Finally, the same colony was used to inoculate BHI medium at 37^{o}C. The next morning, the cultures of mutants that had grown overnight were stored in 30% glycerol at -80^{o}C for additional study.

Figure 4: DNA fragments from the colony PCR were separated on 1% agarose gel by electrophoresis. ΔSSA_0204 (lane 1), ΔSSA_0217 (lane 2), ΔSSA_0401 (lane 3), ΔSSA_0516 (lane 4), ΔSSA_0696 (lane 5), ΔSSA_0959 (lane 6), ΔSSA_1113 (lane 7), ΔSSA_1119 (lane 8), ΔSSA_1685 (lane 9), ΔSSA_1794 (lane 10), ΔSSA_1810 (lane 11), ΔSSA _1842 (lane 12), ΔSSA_1972 (lane 13), ΔSSA_2378 (lane 14) and 1kb DNA marker (lane 15). Bands are shown here at ~3.5 kb, which confirms that all of the TCS response regulators were successfully replaced with an antibiotic resistance genes cassette

Figure 4:

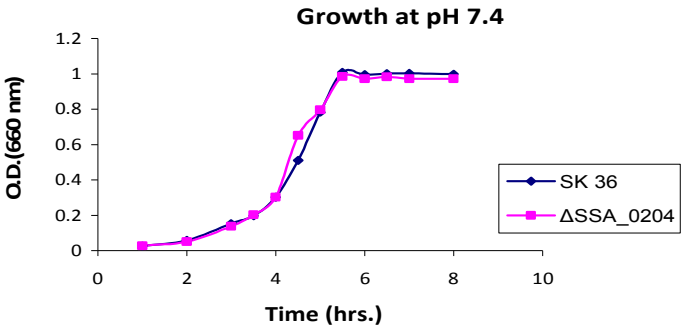

SSA_0204
SSA_0217
SSA_0401
SSA_0516
SSA_0896
SSA_0959
SSA_1113
SSA_1119
SSA_1685
SSA_1794
SSA_1810
SSA_1842
SSA_1972
SSA_2378
4 Kb
3 Kb

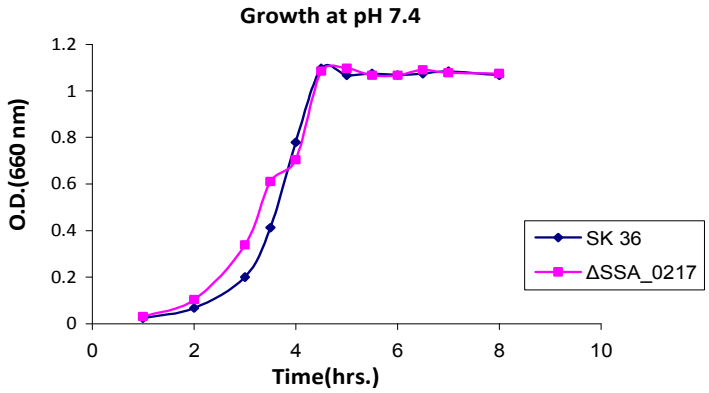
Growth study of response regulator mutants at normal pH 7.4

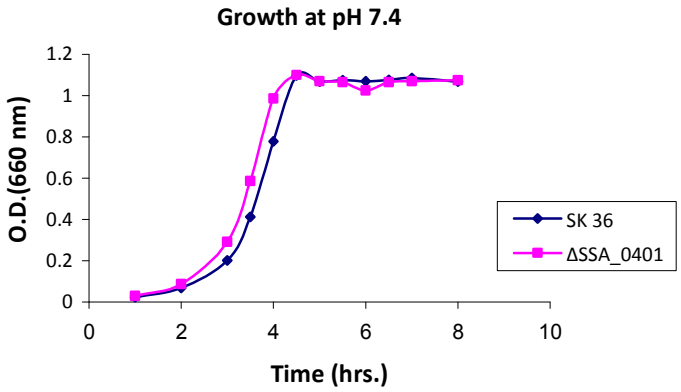
The growth rates of SK36 and each of the response regulator mutants were examined during incubation in 15 ml BHI broth (pH 7.4) at 37^{o}C. The cell density was monitored every half an hour by using spectrophotometer at 660nm wavelengths. The results in Figure 5 show that all of the response regulator mutants except ΔSSA_0959 grew with similar generation time and also had similar cell densities at a stationary phase. Different growth characteristics of SSA_0959 strongly suggest that this response regulator mutant indeed plays a vital role in cell growth and development.

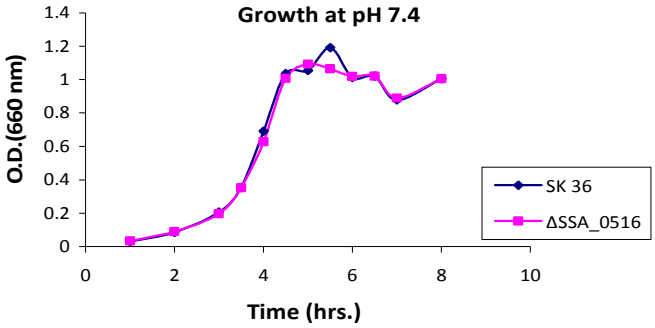
Figure 5: The growth characteristics are shown of all response regulator mutant strains and their comparison with wild type strain *S. sanguinis* SK36 at pH 7.4 in BHI medium. Cells were incubated at 37^{o}C and cell density was monitored every half hour using spectrophotometer at 660 nm wavelengths. Results were obtained from averages of three individual repeats for each mutant strain.

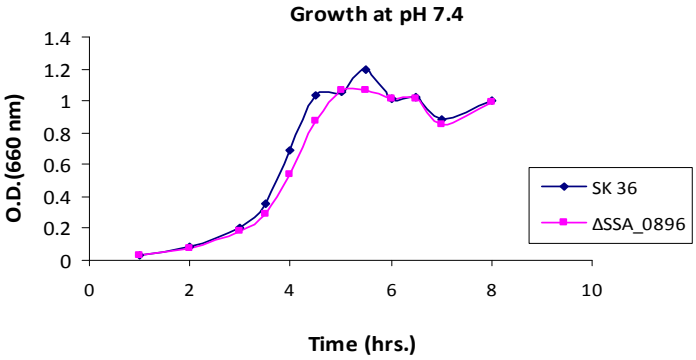
Figure 5

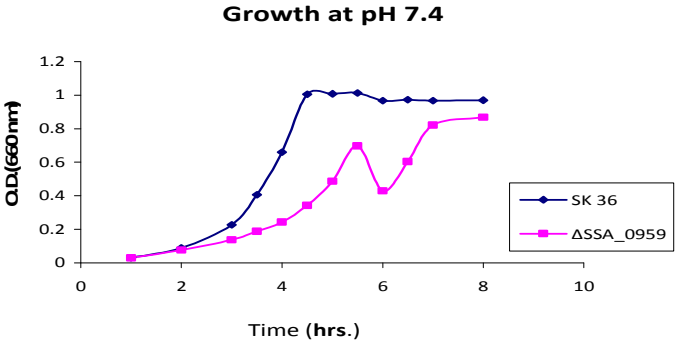

Growth at pH 7.4
O.D.(660 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Time (hrs.)
SK 36
ΔSSA_0204

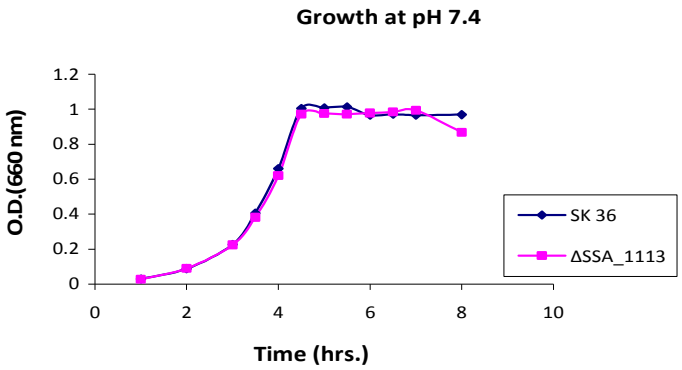

Growth at pH 7.4
O.D.(660 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Time(hrs.)
SK 36
ΔSSA_0217

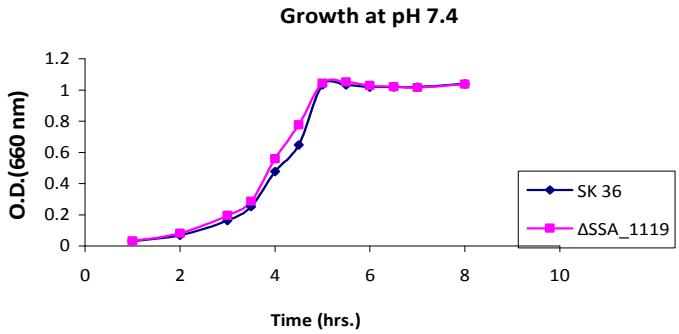

Growth at pH 7.4
O.D.(660 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Time (hrs.)
SK 36
ΔSSA_0401

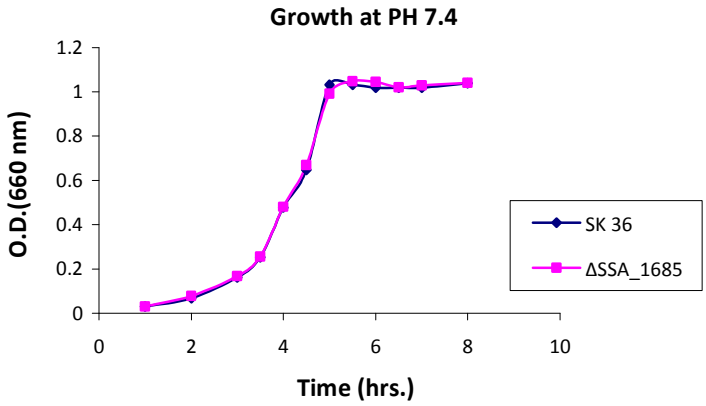

Growth at pH 7.4
O.D.(660 nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Time (hrs.)
SK 36
ΔSSA_0516

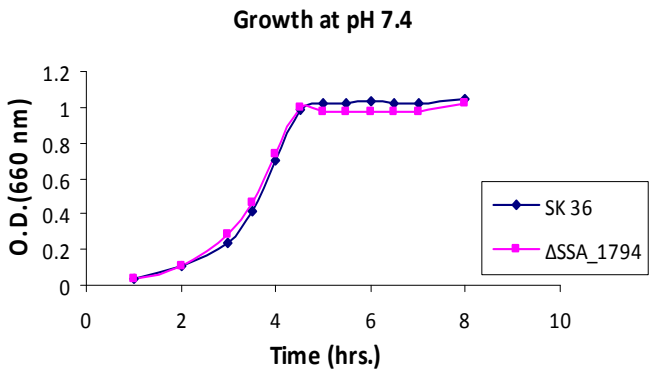

Growth at pH 7.4
O.D.(660 nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Time (hrs.)
SK 36
ΔSSA_0896

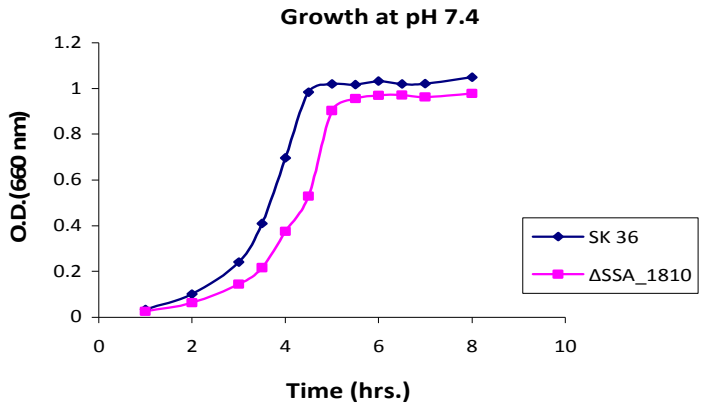

Growth at pH 7.4
OD(660nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Time (hrs.)
SK 36
ΔSSA_0959

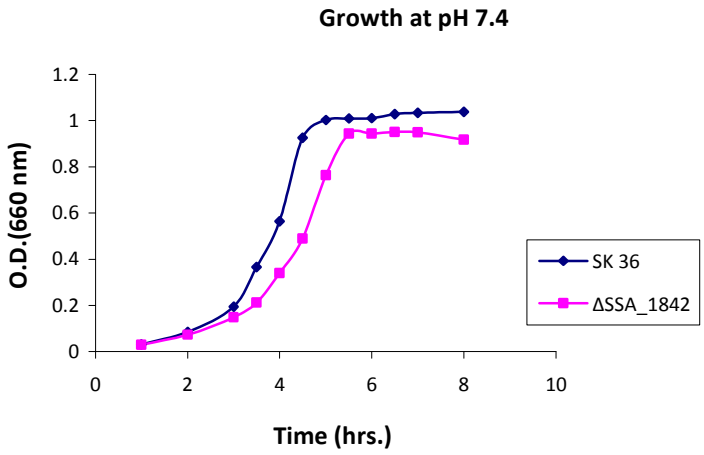

Growth at pH 7.4
O.D.(660 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Time (hrs.)
SK 36
ΔSSA_1113

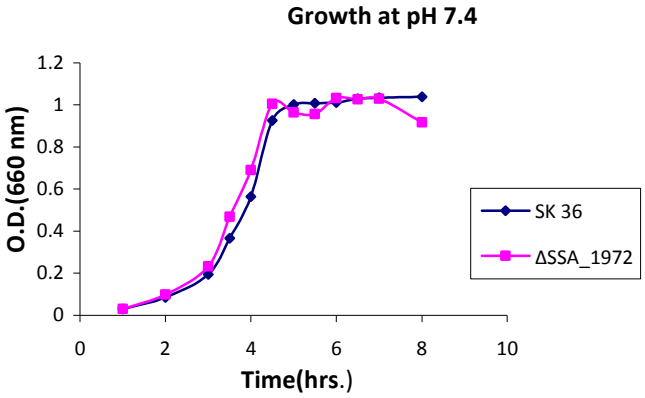

Growth at pH 7.4
O.D.(660 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Time (hrs.)
SK 36
ΔSSA_1119

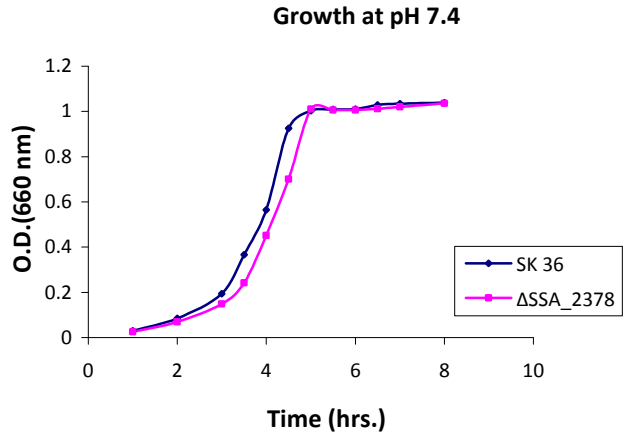

Growth at PH 7.4
O.D.(660 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Time (hrs.)
SK 36
ΔSSA_1685


Growth at pH 7.4
O.D.(660 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Time (hrs.)
SK 36
ΔSSA_1794


Growth at pH 7.4
O.D.(660 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Time (hrs.)
SK 36
ΔSSA_1810


Growth at pH 7.4
O.D.(660 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Time (hrs.)
SK 36
ΔSSA_1842


Growth at pH 7.4
O.D.(660 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Time(hrs.)
SK 36
ΔSSA_1972


Growth at pH 7.4
O.D.(660 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Time (hrs.)
SK 36
ΔSSA_2378

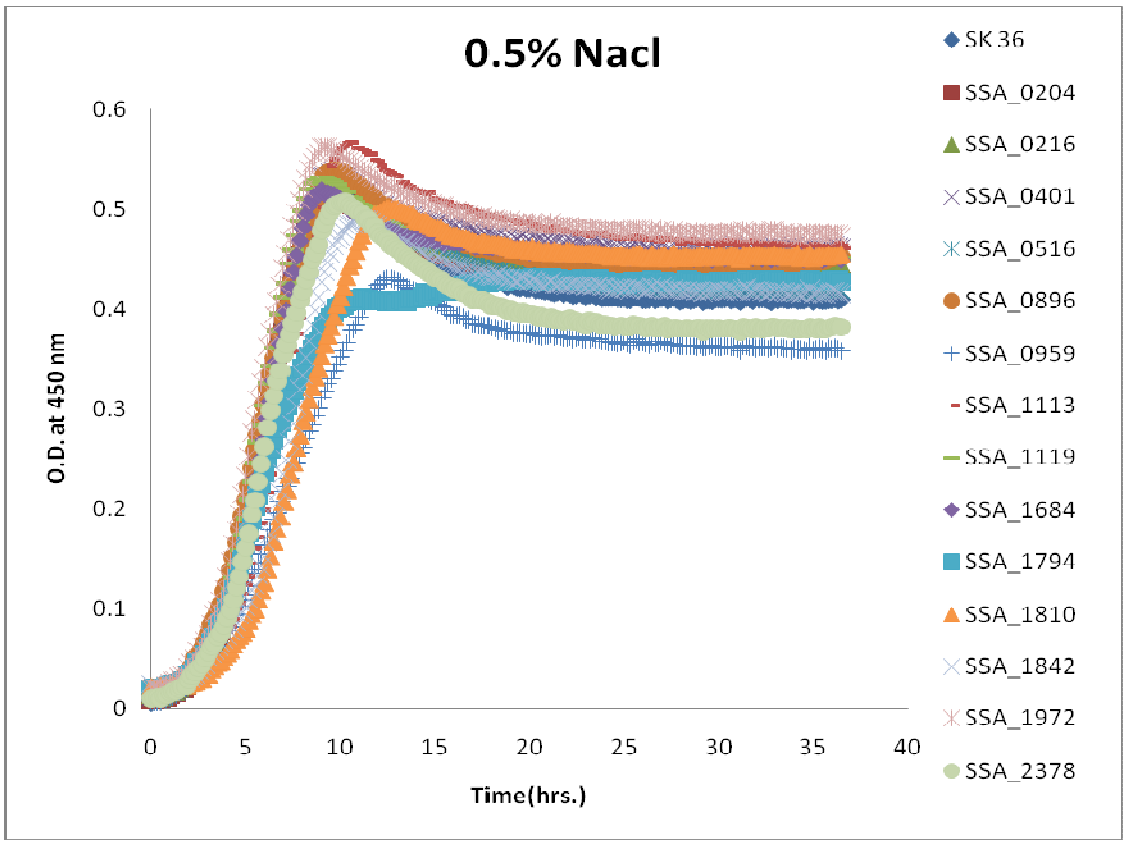
Osmotic stress treatment

Sensor kinases are important for eliciting osmotic stress tolerance in many bacteria, [12], but until now there has not been any systemic study performed to check the osmotic stress tolerance by response regulators in *S. sanguinis*. We began by treating the cells with various concentrations of NaCl starting with 5% NaCl and then we gradually decreased the concentration of NaCl. We found that the maximum NaCl concentration that still allowed SK36 growth was 3.8%. In this study, response regulator mutants were tested for their ability to grow in BHI broth medium containing 3.8% NaCl at 37 ^{o}C. The response of 14 RR mutants against 3.8% NaCl concentration is shown in figure 6. As described in the Materials and Methods, wild type SK36 and all 14 response regulators mutants were inoculated in a microaerobic condition and in a fresh BHI medium at normal conditions overnight at 37^{0}C. The next morning, each of the strains were inoculated in fresh BHI medium containing 3.8% NaCl and tested for stress tolerance. For the next 36 to 40 hrs of incubation, the OD was measured at 450 nm and plotted on a graph of OD 450 nm vs time. The results are shown in Figures 6 and 7, which show that only the ΔSSA_1810 response regulator has a lower growth rate than the wild type SK36 strain in 3.8% NaCl.

Figure 6: Response of 14 RR mutants at 0.5% NaCl concentration (A) and 3.8% NaCl Concentration (B)

Figure 6:

A.


0.5% Nacl
O.D. at 450 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
Time(hrs.)
SK 36
SSA_0204
SSA_0216
SSA_0401
SSA_0516
SSA_0896
SSA_0959
SSA_1113
SSA_1119
SSA_1684
SSA_1794
SSA_1810
SSA_1842
SSA_1972
SSA_2378

B.

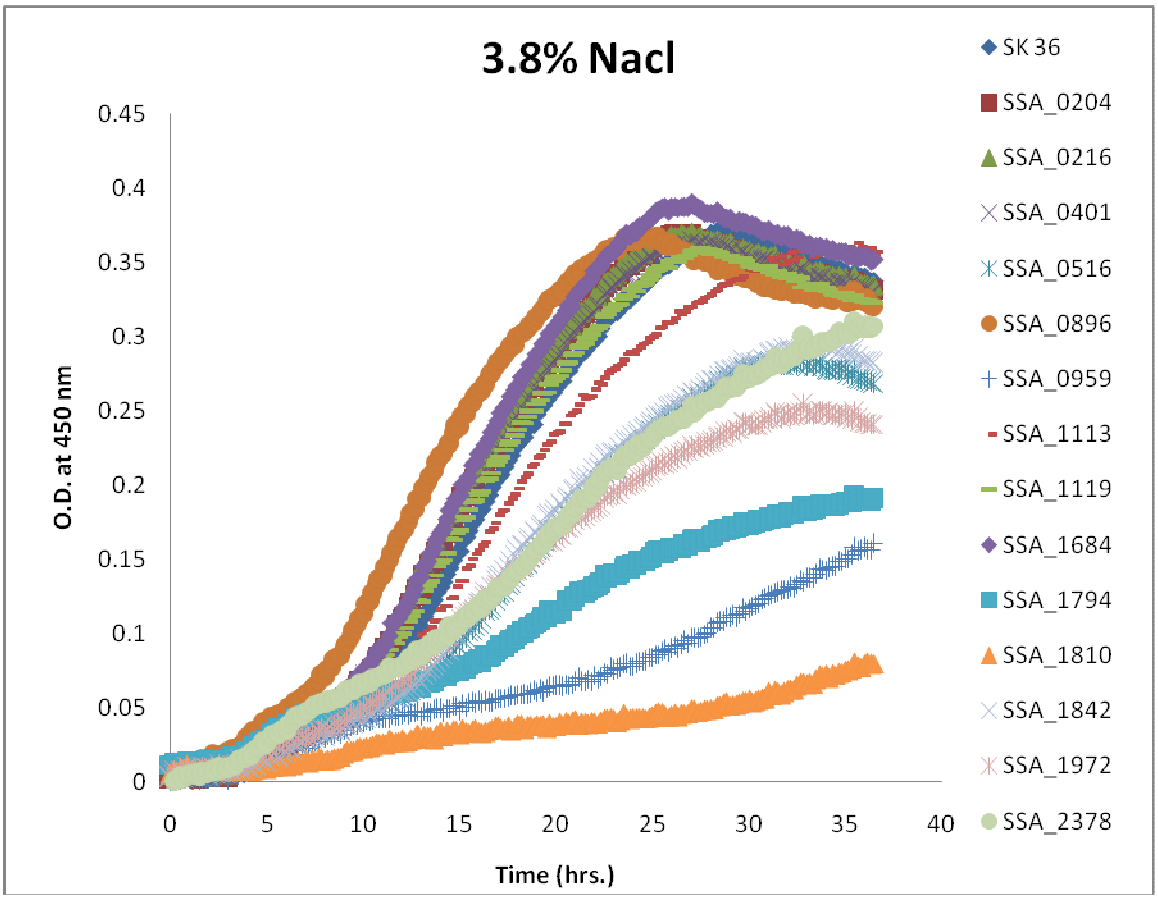
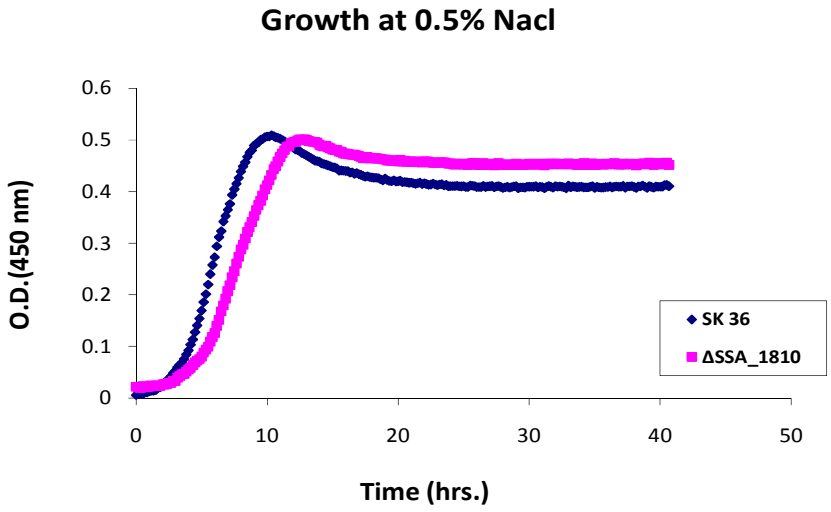

3.8% Nacl
O.D. at 450 nm
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
30
35
40
Time (hrs.)
SK 36
SSA_0204
SSA_0216
SSA_0401
SSA_0516
SSA_0896
SSA_0959
SSA_1113
SSA_1119
SSA_1684
SSA_1794
SSA_1810
SSA_1842
SSA_1972
SSA_2378

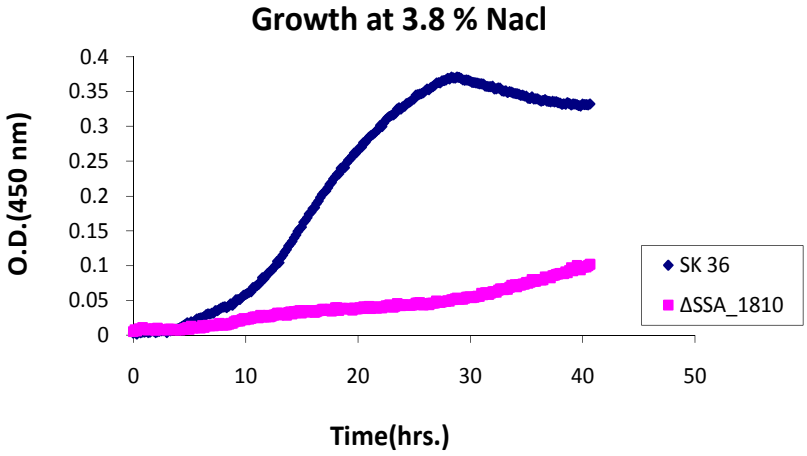
Figure 7: Osmotic stress assay. *S. sanguinis* SK36 (blue) and ΔSSA_1810 (pink) were grown in BHI medium under standard conditions of (A) 0.5% NaCl and (B) 3.8% NaCl. The experiments were repeated three times, and the results obtained were consistent

Figure 7:

A


Growth at 0.5% Nacl
O.D.(450 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
Time (hrs.)
SK 36
ΔSSA_1810

B


Growth at 3.8 % Nacl
O.D.(450 nm)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
Time(hrs.)
SK 36
ΔSSA_1810

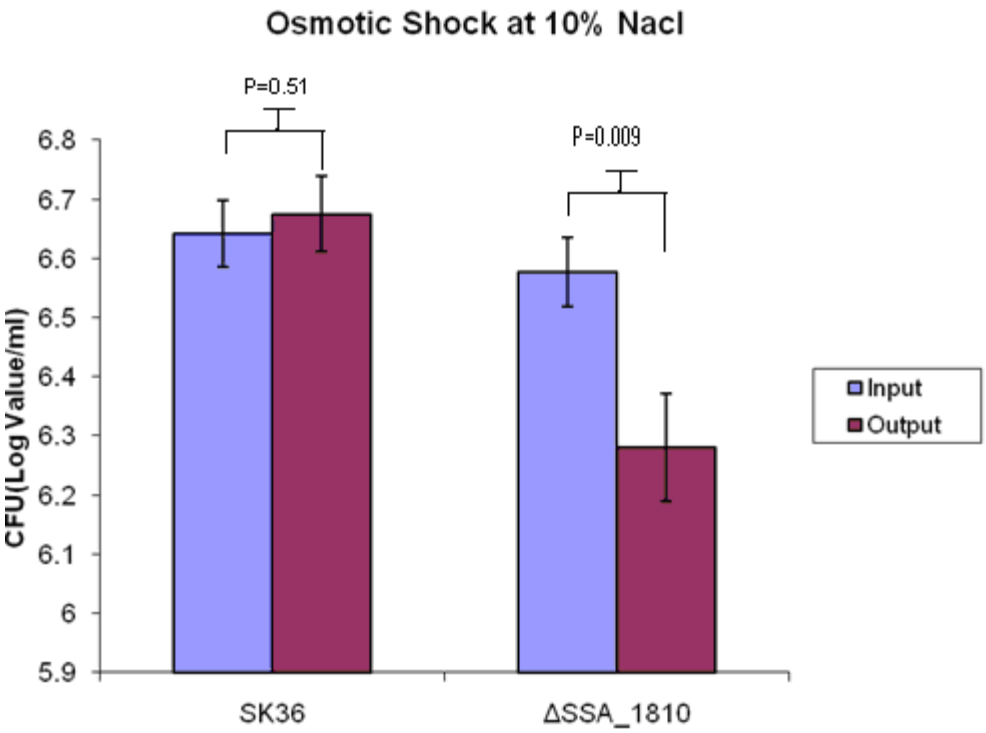
Osmotic shock treatment

After checking that the ΔSSA_1810 has a low growth rate in osmotic stress condition compare to wild *S. sanguinis* SK36, we explored whether this mutation contributes sensitivity to high osmotic shock in BHI medium at 10% NaCl concentration. ΔSSA_1810 and SK36 strains were incubated in BHI medium containing 10% NaCl for 1 hour. Results are shown in Figure 8, which describes the output and input of CFU before and after 1.00 hour of inoculation in BHI medium plus 10% NaCl. The output CFU of ΔSSA_1810 is less than the input CFU (P=0.009), but in case of SK36 there is not significant different in input and output CFU. This indicates that the CFU we obtained after treating the cells in high osmotic shock treatment is less for ΔSSA_1810 than it is for wild type *S. sanguinis* SK36.

The CI was calculated as the mutant: control output ratio (CFU after the shock treatment) divided by the input ratio (CFU before shock treatment). A value less than 1 would indicate reduced mutant cells compared to wild type cells, whereas values greater than 1 would indicate an increased competitiveness, meaning more mutant cells count than the wild type cells. CI was also found to be 0.46, which means that the number of cells surviving osmotic shock was lower than with the wild type SK36. As mentioned earlier in Figure 1, our data suggests that this SSA_1810 is a response regulator that does not have a sensor kinase.

Figure 8: Osmotic shock treatments with 10% NaCl. Input and Output CFU in the above graph show standard deviation based on an average of three repeats. Experiments were conducted twice with consistent results. We have also counted the Competitive Index (CI) of CFU from before and after the shock treatment.

Figure 8:



$$CI = \frac{\text{Output } (CFU_M/CFU_{SK36})}{\text{Input } (CFU_M/CFU_{SK36})}$$

Whereas,

CFU_M, Colony forming unit of mutant's cells

CFU_{SK36}, Colony forming unit of *S. sanguinis* SK36

	ΔSSA_1810
CI	**0.46**

Alkaline stress treatment

TCS have been found to regulate diverse metabolic processes elsewhere, including the bacterial cell cycle, virulence factors and cell-cell communication in a wide range of bacterial species [13]. However, we treated cells with various alkaline conditions starting from 10 pH. We gradually decreased pH until we came to pH 9.1, which is the most alkaline condition SK36 can tolerate. We treated all response regulator mutants under the pH 9.1 compared to the wild type *S. sanguinis* SK36 strain (figure 9). All RR mutants grew at a similar rate compared to the wild type strain SK36 except the three mutants shown in Figure 10 and Figure 11. ΔSSA_1794, ΔSSA_1842, and ΔSSA_1810 were found to be more sensitive to high alkaline environments than the wild type strain SK36. SSA_1810 is less sensitive than the other two RR mutants are, it grows slower in comparison to SK36, but it showed consistent results in every treatment. Our next step was to see if these mutants can tolerate more alkaline stress conditions. To check more alkaline stress conditions, we performed alkaline shock treatments.

Figure 9: Response of 14 RR mutants at pH 7.4. (A) and pH 9.1 (B)

Figure 9:

A.

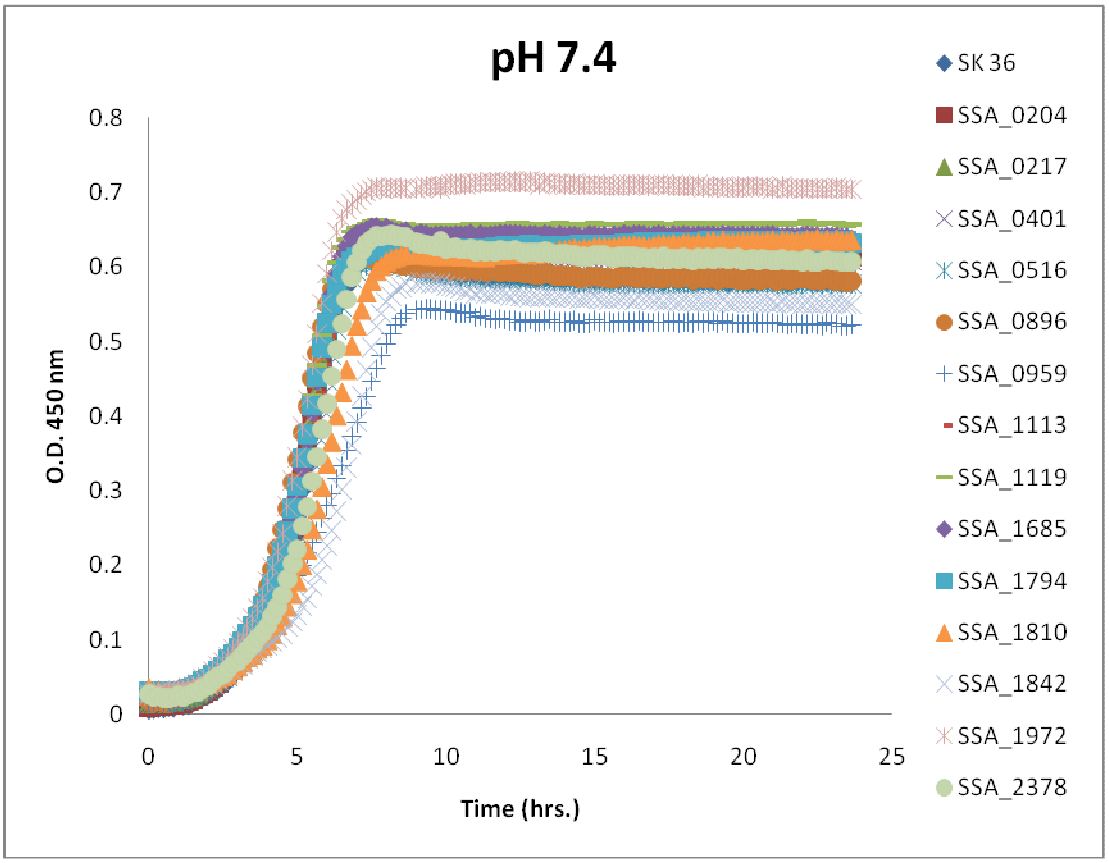

pH 7.4
O.D. 450 nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
Time (hrs.)
SK 36
SSA_0204
SSA_0217
SSA_0401
SSA_0516
SSA_0896
SSA_0959
SSA_1113
SSA_1119
SSA_1685
SSA_1794
SSA_1810
SSA_1842
SSA_1972
SSA_2378

Figure 9:

B.

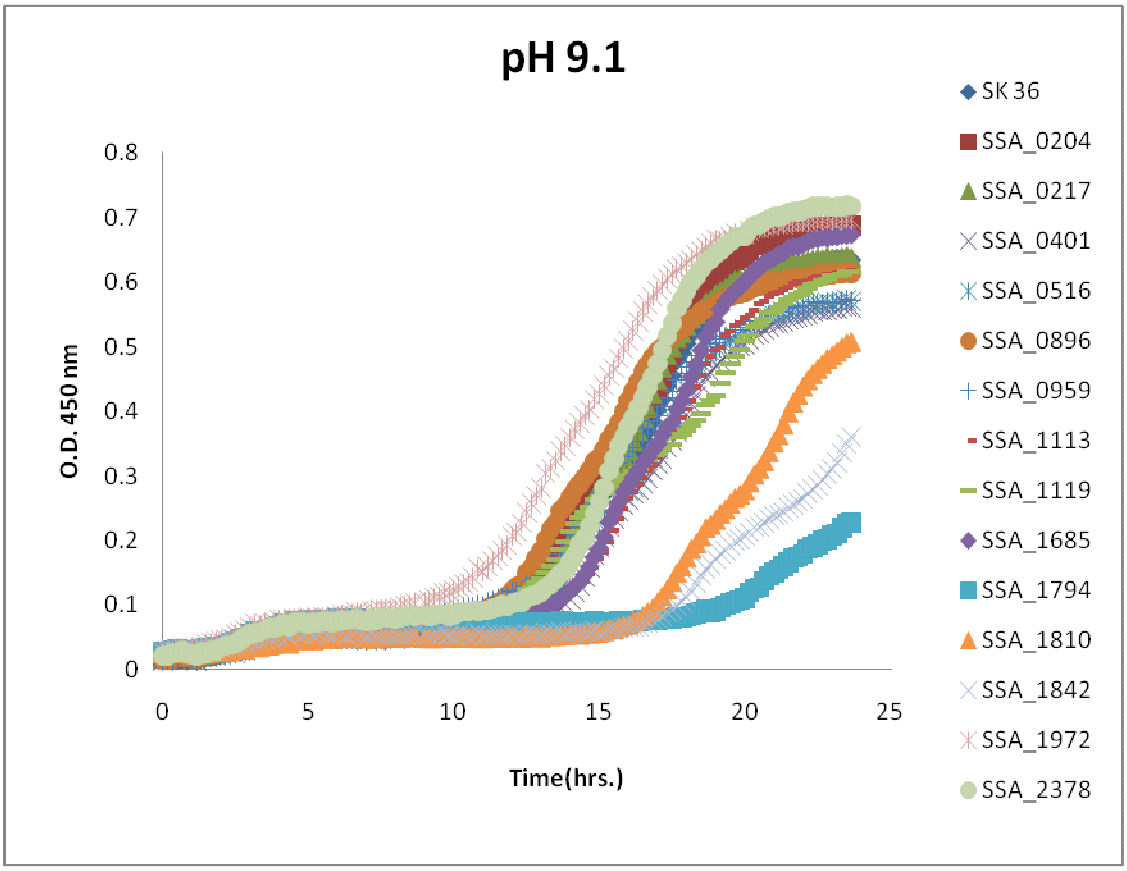
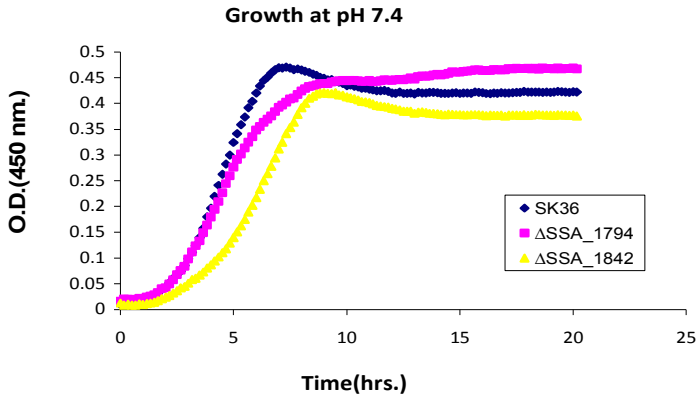

pH 9.1
O.D. 450 nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
Time(hrs.)
SK 36
SSA_0204
SSA_0217
SSA_0401
SSA_0516
SSA_0896
SSA_0959
SSA_1113
SSA_1119
SSA_1685
SSA_1794
SSA_1810
SSA_1842
SSA_1972
SSA_2378

Figure 10: *S. sanguinis* SK36 (blue), ΔSSA_1794 (pink), and ΔSSA_1842 (yellow) were grown in BHI medium at standard condition with (A) 7.4 pH and (B) pH 9.1. Results are shown here. The experiments were repeated twice with three repetitions and consistent results were obtained.

Figure 10:

A


Growth at pH 7.4
O.D.(450 nm)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
Time(hrs.)
SK36
ΔSSA_1794
ΔSSA_1842

B

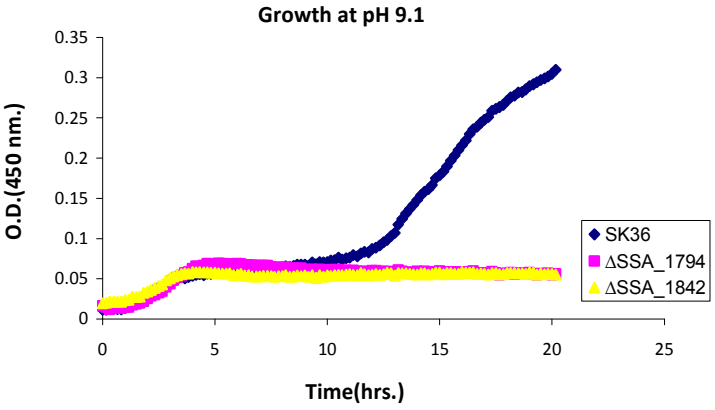
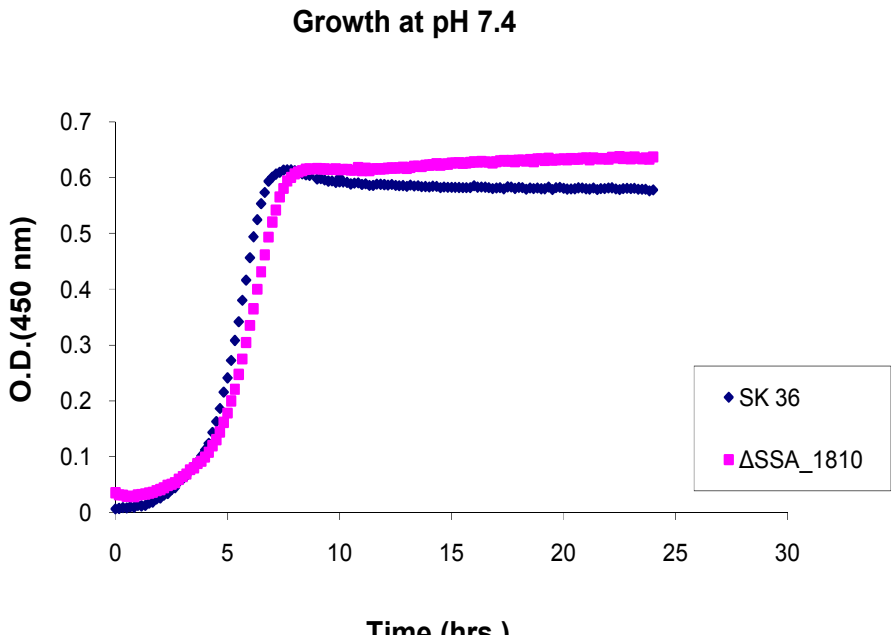

Growth at pH 9.1
O.D.(450 nm.)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
Time(hrs.)
SK36
ΔSSA_1794
ΔSSA_1842

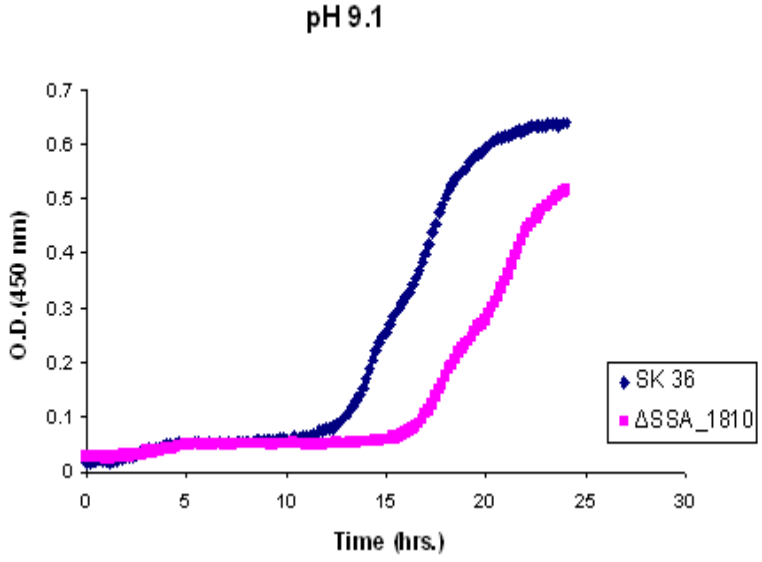
Figure 11: *S. sanguinis* SK36 (blue) and ΔSSA_1810 (pink) grown in BHI medium at standard condition with (A) 7.4 pH and (B) pH 9.1. Experiments were repeated twice with three repeats and consistent results were obtained

Figure 11:

A


Growth at pH 7.4
O.D.(450 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
SK 36
ΔSSA_1810

B.


pH 9.1
O.D.(450 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
Time (hrs.)
SK 36
ΔSSA_1810

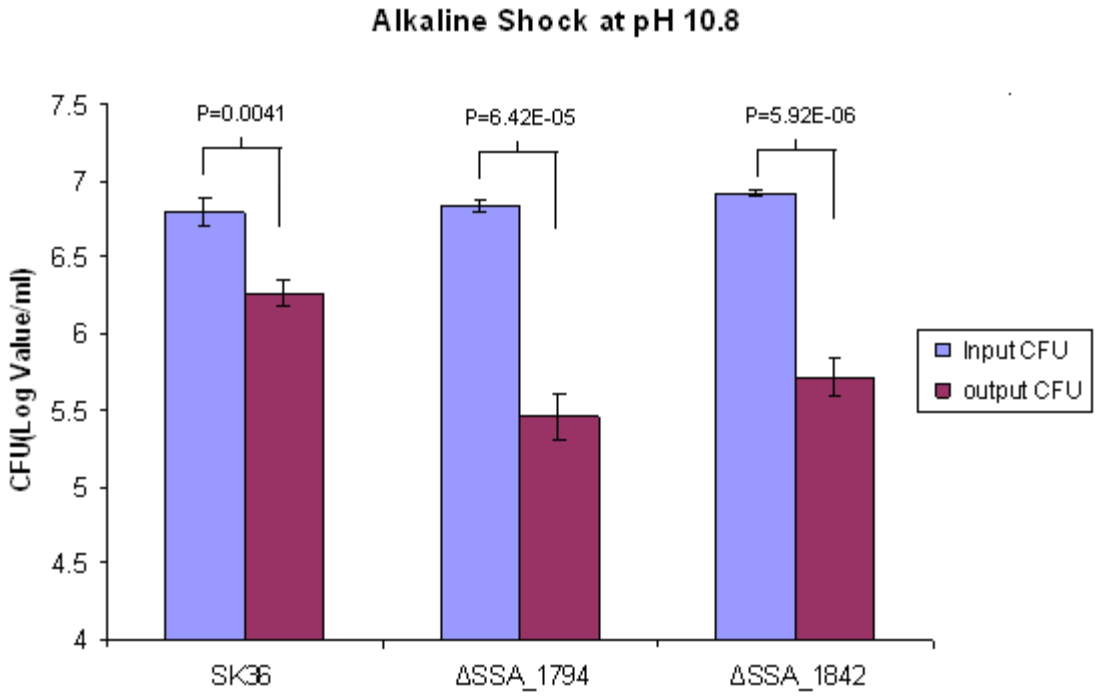
Alkaline shock treatment for response regulator mutants

After checking that the ΔSSA_1794 and SSA_1842 have low growth rate in alkaline stress condition compared to wild *S. sanguinis* SK36 (pH 9.1), we further explored these mutants in high alkaline shock in BHI medium at pH 10.8. Similar to previous tests to check the resistance to osmotic shock, experiments were performed for ΔSSA_1794, ΔSSA_1842 and SK36 strains. They were incubated in BHI medium containing a pH of 10.8 for 1.30 hours. Results are shown here in Figure 12, which describes the output and input CFU before and after osmotic shock in BHI medium which contains NaOH (sodium hydroxide). The output CFU of ΔSSA_1794 and ΔSSA_1842 is less than the input CFU (P=6.42E-05, P=5.92E-06 respectively), the ratio of output CFU to input CFU in both mutant strains is lower than the SK36 ratio. This indicates that the CFU we obtained after treating the cells in high alkaline shock treatment is less for ΔSSA_1794 and ΔSSA_1842 than it is for wild type *S. sanguinis* SK36.

In addition, CI was also found to be 0.14 for SSA_1794 and 0.21 for SSA_1842, which means that the number of cells surviving after the alkaline shock was lower than wild type SK36.

Figure 12: Alkaline shock treatment at pH 10.8. Input and Output CFU is shown in the graph with standard deviation from the average of three repeats. Experiments have been done twice with consistent results. We have also counted the Competitive Index (CI) of CFU from before and after shock treatment.

Figure 12:



	ΔSSA_1794	ΔSSA_1842
CI	0.14	0.21

Alkaline shock treatments of RR-HK double mutants

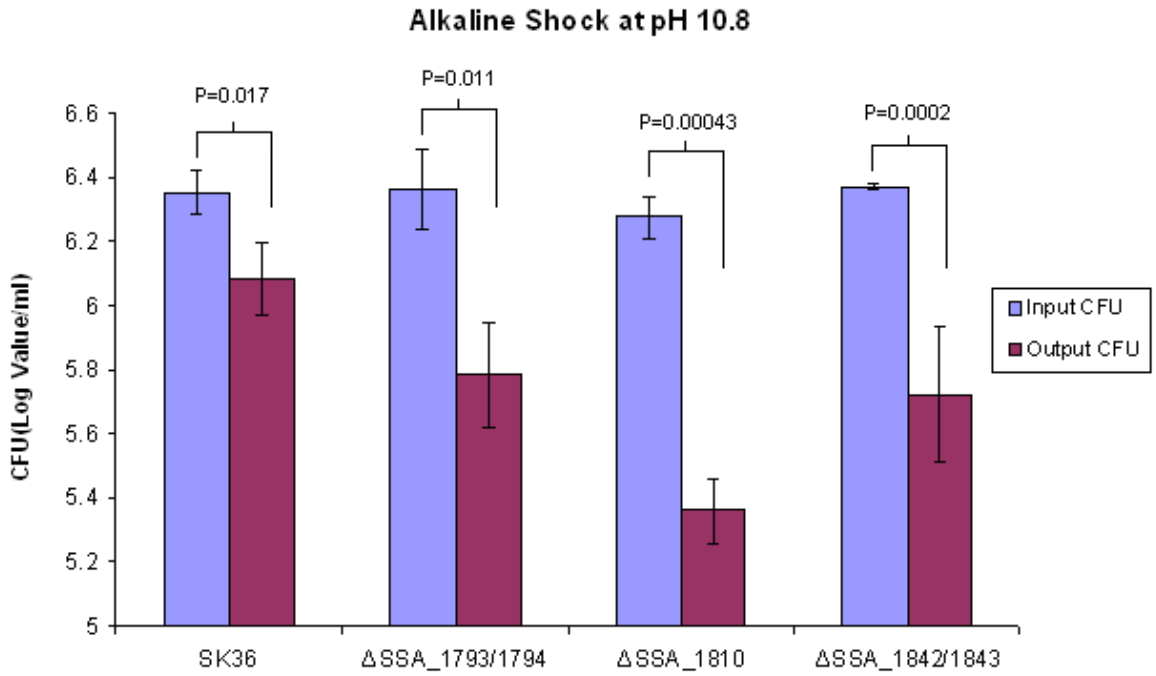
Most TCS are composed of both response regulators and sensor kinases. The question arises as to whether the whole TCS is showing the same response or not. In order to answer this question, we first needed to inactivate the whole TCS system including both response regulator and sensor kinase, at the same time. Secondly, we needed to treat the TCS within the same environmental stress conditions for all cases. We used double TCS mutants, which were already created by Dr. Lei Chen, one of the post doctorates in our lab. He has created all TCS double mutants by replacing a sensor kinase and a response regulator gene with a kanamycin resistance gene. We treated for consequences when both genes (RR-HK) were mutated for each TCS. In terms of acidic and alkali resistance in endodontic pathogens, researchers have found that *S. sanguinis, S. mutans* and *E. faecalis* are more resistant in alkaline than in acidic conditions [14].

TCS double mutants ΔSSA_1793/1794, ΔSSA_1842/1843 and single response regulator mutant ΔSSA_1810 were treated under the same alkaline shock treatment at pH 10.8. The results are shown in Figure 13. Again, the output CFU for both of the TCS mutants and SSA_1810 are less than the input CFU (SSA_1793/1794 P=0.011, SSA_1842/1843 P=0.0002 and SSA_1810 P=0.00043). CI was also found to be 0.49 for ΔSSA_1793/1794, 0.21 for ΔSSA_1810, and 0.43 for ΔSSA_1842/1843 which means that the number of cells surviving after the alkaline shock was lower than wild type SK36.

After observing the response RR and double mutants RR-HK with alkaline conditions, we can speculate that TCS shows the same response either with RR mutant or HK-RR double mutants.

Figure 13: Alkaline shock treatment of double mutants at pH 10.8. The Input and Output CFU in the above graphs shows a standard deviation based on an average of three repeats. The experiments were conducted with three individual repeats for each strain. We have also counted the Competitive Index (CI) of CFU from before and after the shock treatment for each of the mutants.

Figure 13:



	SSA_1793/1794	SSA_1810	SSA_1842/1843
CI	0.49	0.21	0.43

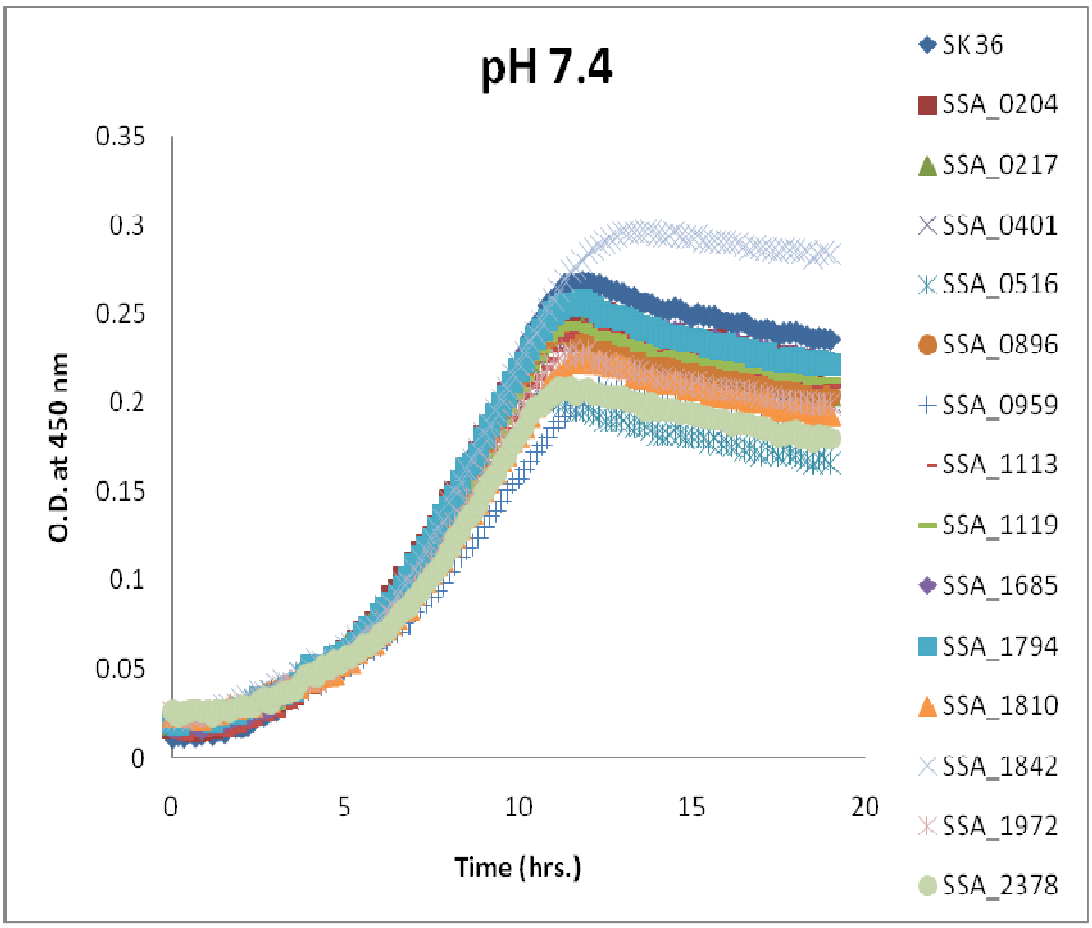
Acidic stress treatments

Researchers have found that many TCS are involved in various forms of stress responses in different bacteria. Three sensors, kinases Smu486, Smu1128, and Smu1516, play a significant role in various stress responses in the *S. mutans* strain UA159 [12]. We have checked different acidic pH's, starting at pH 5.0 and gradually increasing the value. Surprisingly, we found ΔSSA_0204 and ΔSSA_0217 at pH 6.3 grew at a higher rate compared to wild type strain SK36 (Figure 15). All response regulator mutans were treated with acidic stress treatment as describe in materials-methods. Acidic stress treatment of the 14 RR mutants at pH 6.3 is illustrated in Figure 14. The two RR mutants ΔSSA_0204 and ΔSSA_0217 reached higher OD than other TCS mutants or SK36.

To confirm our results, we checked the resistance against more acidic shock treatment.

Figure 14: Responses of 14 RR TCS mutants to Acidic stress at pH 7.4 (A) and at pH 6.3 (B).

Figure 14:
A.


pH 7.4
O.D. at 450 nm
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
Time (hrs.)
SK36
SSA_0204
SSA_0217
SSA_0401
SSA_0516
SSA_0896
SSA_0959
SSA_1113
SSA_1119
SSA_1685
SSA_1794
SSA_1810
SSA_1842
SSA_1972
SSA_2378

B.

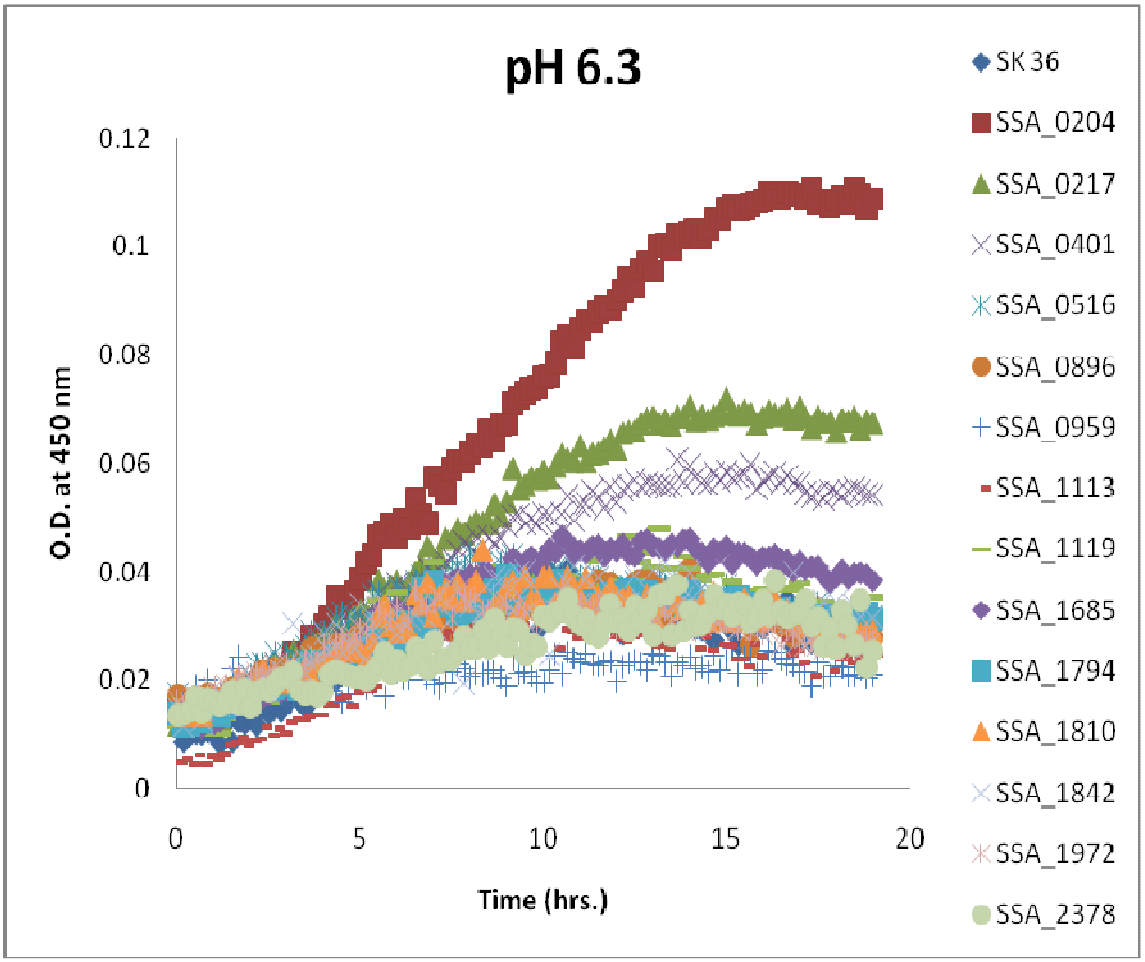
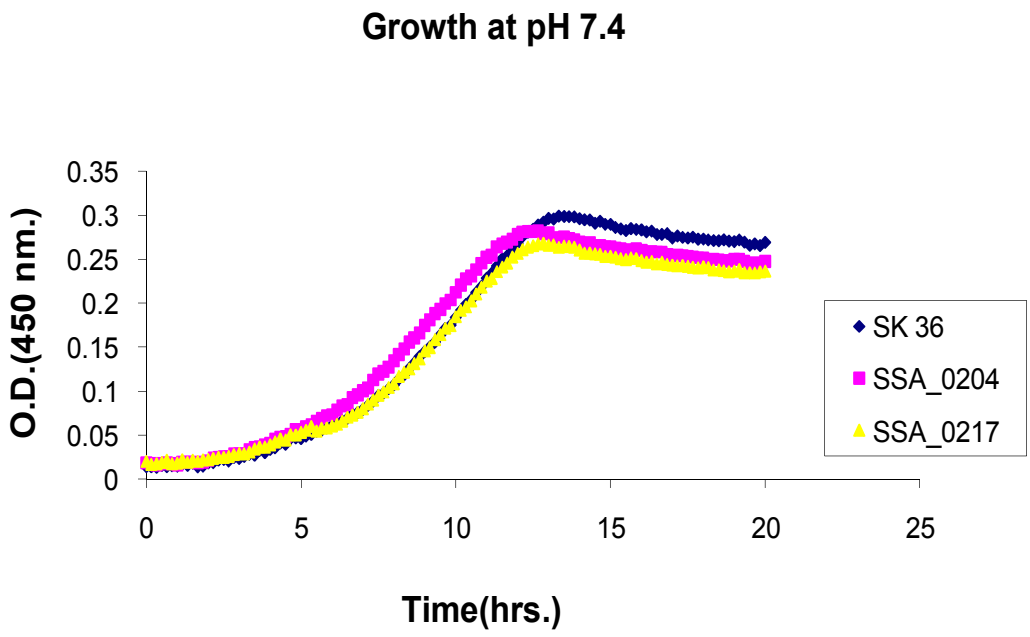
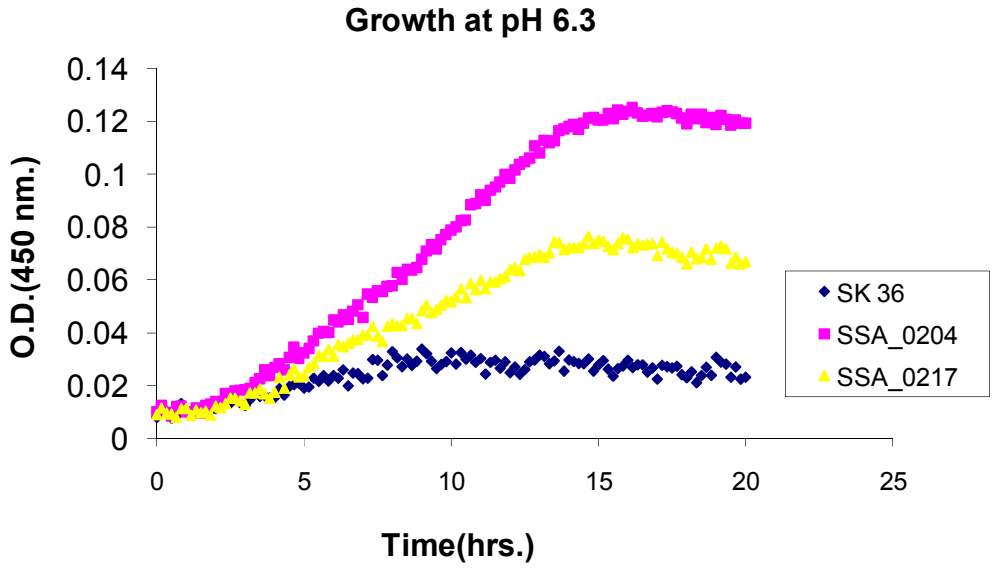

pH 6.3
O.D. at 450 nm
0.12
0.1
0.08
0.06
0.04
0.02
0
0
5
10
15
20
Time (hrs.)
SK 36
SSA_0204
SSA_0217
SSA_0401
SSA_0516
SSA_0896
SSA_0959
SSA_1113
SSA_1119
SSA_1685
SSA_1794
SSA_1810
SSA_1842
SSA_1972
SSA_2378

Figure 15: Acid tolerance assay. *S. sanguinis* SK36 (blue), ΔSSA_0204 (pink), and ΔSSA_0217 (yellow) were grown in BHI medium at pH 7.4 (A) and pH 6.3 (B). The results were obtained from conducting the test at three separate intervals.

Figure 15:
A.


Growth at pH 7.4
O.D.(450 nm.)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
Time(hrs.)
SK 36
SSA_0204
SSA_0217

B.


Growth at pH 6.3
O.D.(450 nm.)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
5
10
15
20
25
Time(hrs.)
SK 36
SSA_0204
SSA_0217

Acidic shock treatment of RR mutants

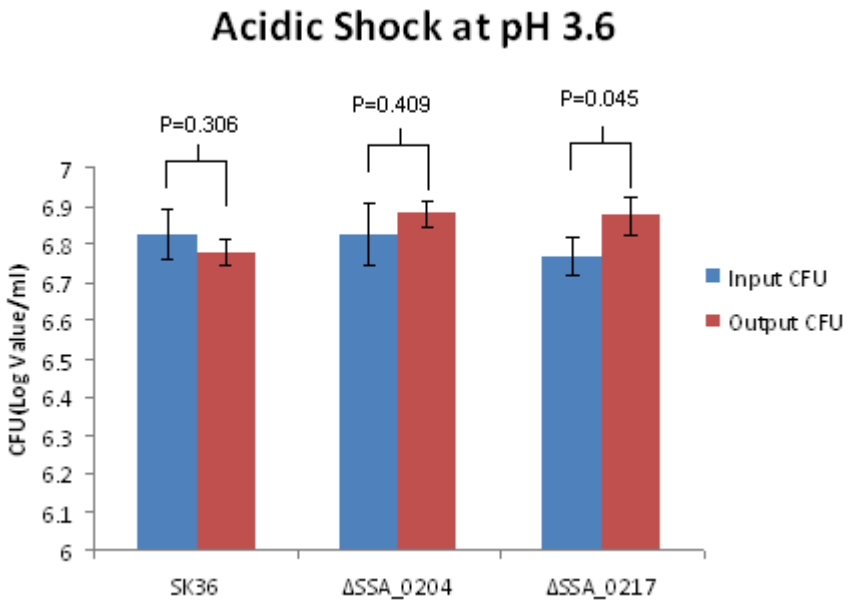
Results shown in Figure 16 describe the output and input CFU, In the case of SSA_0204, there was not a significant difference in input CFU and output CFU. This may be due to not enough exposure to acidic shock treatment at pH3.6, but the overnight growth exposure to pH 3.6 clearly indicates that this mutant shows resistance compared to wild type SK36. In the case of SSA_0217 (P=0.045), there was a clear difference in input and output CFU.

From our experiments, we have calculated that the CI in both response regulator mutants SSA_0204 and SSA_0217 was 1.24 and 1.26 respectively (Figure 16).

After confirming the acidic shock treatments, we next wanted to test the acidic resistance for the above positive mutants ΔSSA_0204 and ΔSSA_0217, since the whole TCS system is mutated.

Figure 16: The acidic shock treatment at pH 3.6. Input and Output CFU are shown in the above graph with standard deviation. We have also counted the Competitive Index (CI) of CFU from the before and after shock treatment.

Figure 16 :



	ΔSSA_0204	ΔSSA_0217
CI	1.24	1.26

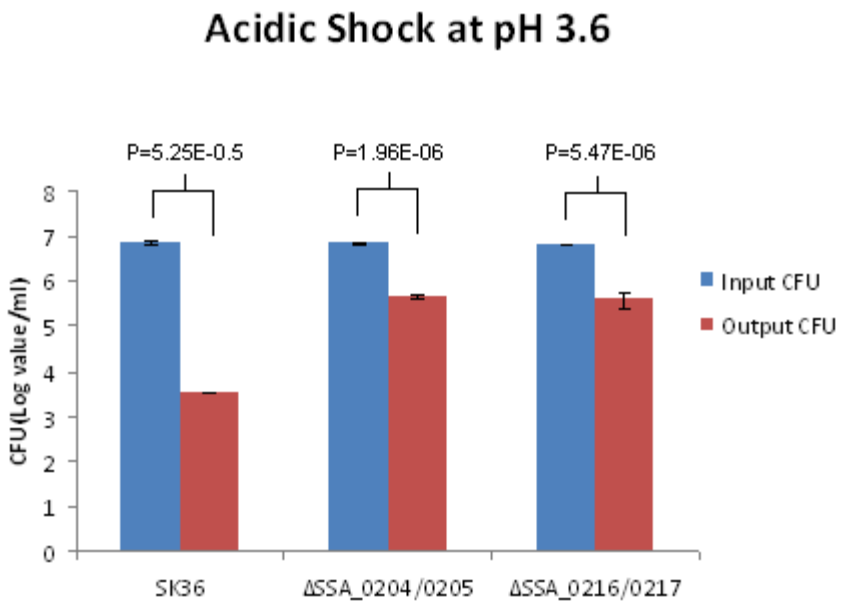
Acidic shock treatments of RR-HK double mutants

A number of studies have connected acid tolerance in *S. mutans* with expression of various TCS including CovR, CiaRH, VicRK, and ComDE [10]. Until now, there has not been a systemic study concerning the relation of TCS in acidic resistance in *S. sanguinis*. To explore this further, all the TCS double mutants were checked for acid resistance. The TCS double mutants ΔSSA_0204/0205 and ΔSSA_0216/0217 were treated under the same acidic shock treatment at pH 3.6. The results are shown here in Figure 17. The input CFU in both TCS mutants were same as SK36, but output CFU in SSA_0204/0205 and SSA_0216/0217 is greater in compare to SK36. From our experiments, we have calculated that the CI in both TCS mutants SSA_0204/0205 and SSA_0216/0217 was 1.96 and 2.22 respectively (Figure 16).

This suggests that TCS ΔSSA_0204/0205 and ΔSSA_0216/0217 were involved in acidic stress resistance.

Figure 17: Acidic shock treatment of double mutants at pH 3.6. CFU shown in above graphs with standard deviation before and after shock treatment. Experiments have been done with three individual repeats for each strain. We have also counted the Competitive Index (CI) of CFU before and after shock treatment.

Figure 17:



	ΔSSA_0204/0205	ΔSSA_0216/0217
CI	1.96	2.22

Microarray analysis of ΔSSA_0204

In order to look at the regulatory network of these TCS, we needed to find out how many genes are regulated by TCS. After treating the response regulator mutants with different environmental stress conditions, our next step was to perform a microarray for each of these respond regulator mutants and check the involvement of clusters of genes. We started with ΔSSA_0204, which was involved in acidic resistance. Dr. Lei Chen performed the microarray analysis. In brief, both the SK36 and ΔSSA_0204 were cultured overnight at pH 7.4. They were then inoculated into a fresh BHI medium with 1% inoculation under microaerobic conditions at 37°C. After five hours, the cells were collected and lysed after lysozyme treatment and mechanical disruption. RNA was isolated and total RNA was quantified using a Nano Drop ® ND 1000 Spectrophotometer. The reverse-transcription, labeling and hybridization were performed according to the protocols provided in material and method. The results shown in Table 4 were taken from his analysis, and reflect that by selecting two-fold cutoffs for the expression of gene in ΔSSA_0204 relative to SK36, he found 55 genes were down-regulated and 88 genes were up-regulated in the SSA_0204 mutant strain related to wild type SK36. For further study and to understand the functional role and the regulatory function of SSA_0204, we used all these mutants and checked their resistance in an acidic environment at pH 6.3, as discussed below.

Table 4: Microarray Analysis of ΔSSA_0204

Genes	Putative Function	*Fold value
SSA_2342	SPX domain-containing protein	0.048349
SSA_0425	Glycosyltransferase	0.082681
SSA_0818	SPX domain-containing protein	0.095732
SSA_1982	LytR/AlgR family transcriptional regulator putative	0.119995
SSA_2382	chromosome partitioning protein ParB or transcriptional regulator Spo0J, putative	0.144664
SSA_1893	N-acetylglucosamine-6-phosphate deacetylase, putative	0.183532
SSA_1924	TetR/AcrR family transcriptional regulator	0.227624
SSA_1165	GntR family transcriptional regulator	0.2314
SSA_2161	NTP pyrophosphohydrolases including oxidative damage repair enzymes, putative	0.236112
SSA_0047	NAD(P)H dehydrogenase (quinone), putative	0.237065
SSA_1481	FmtA-like protein, putative	0.254789
SSA_1205	Bta, putative	0.257776
SSA_2381	DegP protein, putative	0.260039
SSA_1583	6-O-methylguanine-DNA methyltransferase, putative	0.280318
SSA_0909	AbrB family transcriptional regulator	0.289829
SSA_2295	phage integrase family integrase/recombinase	0.290767
SSA_0959	two-component response transcriptional regulator	0.301769
SSA_0004	putative lipoprotein	0.302285
SSA_0614	transporter, putative	0.302473
SSA_0794	Zn-dependent protease, putative	0.319495
SSA_2244	transcriptional regulator Spx	0.321634
SSA_2052	thioredoxin, putative	0.329021
SSA_0910	ABC-type multidrug transporter, ATPase component, putative	0.33856
SSA_2380	SPOUT methyltransferase superfamily protein	0.341999
SSA_2208	preprotein translocase subunit SecE	0.360116
SSA_2240	Holliday junction resolvase-like protein	0.368247
SSA_0610	LemA-like protein, putative	0.38063
SSA_0728	protease, putative	0.385071
SSA_2009	heat-inducible transcription repressor	0.393005
SSA_2005	chaperone protein dnaJ, putative	0.402697
SSA_0822	large conductance mechano-sensitive ion channel, putative	0.403702
SSA_2265	maltodextrin phosphorylase, putative	0.404567
SSA_0858	dTDP-L-rhamnose synthase, putative	0.40891
SSA_0638	tryptophan synthase subunit alpha	0.416365
SSA_0065	low molecular weight phosphotyrosine protein phosphatase, putative	0.420176
SSA_0460	multiple antibiotic resistance operon transcription repressor (MarR), putative	0.423104
SSA_0503	peptide ABC transporter, ATP-binding protein, putative	0.42833
SSA_0746	glucosamine-6-phosphate deaminase, putative	0.430145
SSA_2378	two-component system LytR/AlgR family transcriptional regulator putative	0.430443
SSA_1956	ABC-type Fe-S cluster assembly transporter, ATPase component, putative	0.437012
SSA_2353	ABC-type nitrate/sulfonate/bicarbonate transport system, permease component, putative	0.43783
SSA_0989	transcriptional regulator	0.447011
SSA_1150	4-oxalocrotonate tautomerase	0.450139

SSA_1978	dihydroxyacetone kinase family protein	0.450962
SSA_2371	Zn-dependent peptidase, putative	0.455784
SSA_1790	SpoU rRNA methylase family protein, putative	0.459451
SSA_1385	multiple antibiotic resistance operon transcription repressor (MarR), putative	0.472629
SSA_0259	Thiol peroxidase	0.483158
SSA_0771	Glutaredoxin-like protein, putative	0.483939
SSA_0226	Chaperonin GroEL	0.488688
SSA_0014	Hypoxanthine guanine phosphoribosyltransferase, putative	0.488969
SSA_0849	Signal peptidase I, putative	0.496749
SSA_1731	ATP-dependent Clp protease proteolytic subunit	0.496859
SSA_0816	Copper transport operon or penicillinase transcription repressor, putative	0.498119
SSA_2148	Alkaline shock stress response protein, putative	0.499026
SSA_1050	ABC transporter membrane-spanning permease-spermidine/putrescine transport, putative	2.006008
SSA_0655	Cell division protein FtsA, putative	2.009463
SSA_0491	Alpha-ribazole-5'-phosphate phosphatase, putative	2.011371
SSA_1835	Late competence protein, putative	2.023132
SSA_1699	Galactose-6-phosphate isomerase subunit LacA	2.024175
SSA_2146	Metallo-beta-lactamase, putative	2.037434
SSA_1934	Acetyl-CoA carboxylase biotin carboxyl carrier protein subunit	2.049706
SSA_1961	Amino acid ABC transporter permease/amino acid-binding protein	2.052704
SSA_0836	Preprotein translocase subunit SecA	2.055027
SSA_0784	F0F1 ATP synthase subunit B	2.060158
SSA_1948	Oligopeptide-binding lipoprotein precursor, putative	2.074008
SSA_2139	Membrane protein (preprotein translocase) oxaA 1 precursor, putative	2.076045
SSA_1508	ABC-type lipopolysaccharide transport system, permease component, putative	2.076732
SSA_2217	Cps9H, putative	2.084622
SSA_0837	Glucosyltransferase, putative	2.089073
SSA_0796	ABC transporter ATPase	2.09125
SSA_1641	MutT/nudix family protein, putative	2.095708
SSA_0352	Ribonuclease HIII	2.097718
SSA_2246	Competence damage-inducible protein A	2.101033
SSA_1125	NADPH-dependent FMN reductase, putative	2.118546
SSA_0621	SOS responce UmuC protein, putative	2.135947
SSA_1927	Ex-foliative toxin, putative	2.143348
SSA_0631	Tryptophan synthase subunit beta	2.163569
SSA_1204	Phosphoglucomutase	2.168684
SSA_1035	Pyrimidine-nucleoside phosphorylase	2.182002
SSA_1698	Galactose-6-phosphate isomerase subunit LacB	2.189228
SSA_0214	Single-strand DNA-binding protein	2.215056
SSA_1946	Oligopeptide transport system permease protein, putative	2.215096
SSA_1567	Polar amino acid ABC transporter amino acid-binding protein	2.228212
SSA_2109	Elongation factor G	2.232582
SSA_0712	P-type ATPase-metal cation transport (calcium efflux), putative	2.236459
SSA_2230	Anaerobic ribonucleoside triphosphate reductase	2.236574

SSA_0148	Sugar ABC transporter, ATP-binding protein, putative	2.242236
SSA_0832	Preprotein translocase subunit SecY	2.248351
SSA_0068	Bifunctional acetaldehyde-CoA/alcohol dehydrogenase	2.251359
SSA_1947	Oligopeptide transport system permease protein, putative	2.253426
SSA_1345	Pyrimidine regulatory protein PyrR	2.253605
SSA_0500	Peptide ABC transporter, permease protein, putative	2.254636
SSA_1839	Cysteine synthase, putative	2.266391
SSA_2302	Type IV fimbrial biogenesis protein, prepilin cysteine protease (C20) PilD, putative	2.285512
SSA_2117	DNA recombination protein RmuC, putative	2.30806
SSA_0342	Pyruvate formate-lyase, putative	2.317836
SSA_0862	MurM, putative	2.318405
SSA_2245	RecA protein, putative	2.32939
SSA_1507	ABC-type lipopolysaccharide transport system, ATPase component, putative	2.332089
SSA_1259	Purine nucleoside phosphorylase	2.342853
SSA_0132	DNA-directed RNA polymerase subunit alpha	2.343085
SSA_0715	DNA uptake protein, putative	2.379688
SSA_1928	Acyl-CoA dehydrogenase, putative	2.401293
SSA_0198	Dihydrofolate synthetase, putative	2.40716
SSA_0127	Preprotein translocase subunit SecY	2.413088
SSA_1036	Deoxyribose-phosphate aldolase, putative	2.462744
SSA_2219	UDP-glucose 4-epimerase, putative	2.46963
SSA_1100	Hemolysin exporter, ATPase component, putative	2.502726
SSA_2121	Cell wall surface anchor family protein, putative	2.565713
SSA_1174	Dihydrolipoamide dehydrogenase, putative	2.609676
SSA_2218	Glycosyltransferase (cell wall biogenesis) Cps9G, putative	2.611283
SSA_0186	Competence protein ComYC, putative	2.619938
SSA_1929	Macrophage infectivity potentiator protein, putative	2.632466
SSA_0185	Competence protein ComYB, putative	2.672073
SSA_1226	DNA topoisomerase IV subunit A	2.675734
SSA_1038	Putative lipoprotein	2.72715
SSA_1039	Sugar ABC transporter, ATP-binding protein, putative	2.747168
SSA_1797	Aminodeoxychorismate lyase, putative	2.762536
SSA_1261	Ribose-5-phosphate isomerase A	2.781345
SSA_0192	Acetate kinase, putative	2.785867
SSA_0738	Ornithine carbamoyltransferase	2.823248
SSA_0177	DNA-directed RNA polymerase subunit beta'	2.836511
SSA_1040	Sugar ABC transporter, permease protein, putative	2.862722
SSA_1900	Transcription elongation factor NusA	2.921388
SSA_0770	Ribonucleotide-diphosphate reductase subunit alpha	3.08075
SSA_0514	PduQ protein, putative	3.103629
SSA_0184	Competence protein ComYA, putative	3.382321
SSA_1258	Purine nucleoside phosphorylase	3.38717
SSA_1037	Cytidine deaminase	3.620354
SSA_0260	Manganese/Zinc ABC transporter substrate-binding protein	3.63577
SSA_1260	Phosphopentomutase	3.788152
SSA_0829	Platelet-binding glycoprotein	3.798399

SSA_0191	Adenine-specific DNA methylase	3.816708
SSA_0716	Competence protein, putative	3.846027
SSA_1920	Phosphotransferase system, mannose-specific EIID, putative	3.928878
SSA_0886	Phosphopyruvate hydratase	3.984793
SSA_0189	Competence protein ComGF, putative	4.082164
SSA_1918	Phosphotransferase system, mannose-specific EIIAB, putative	4.154799
SSA_0523	Aldehyde dehydrogenase	4.174382
SSA_1919	Phosphotransferase system, mannose-specific EIIC, putative	4.704057
SSA_0261	ABC-type Mn2+/Zn2+ transport systems, permease component, putative	4.72323
SSA_0187	Competence protein ComYD, putative	5.812876

***Fold Value:** ratio of the expression of genes in ΔSSA_0204 to SK36

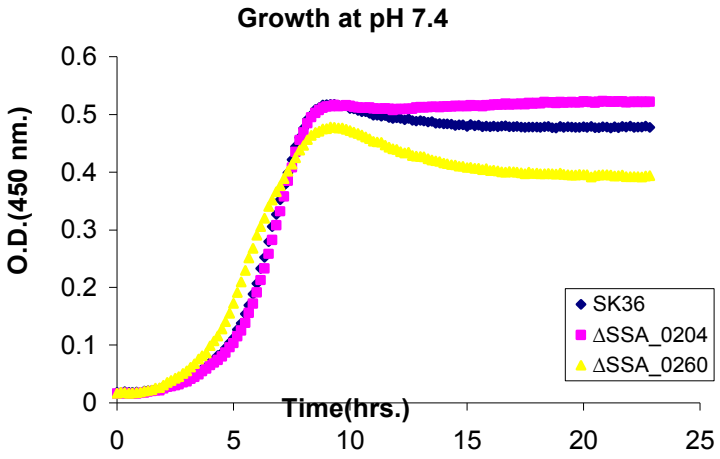
Mutant growth curves of the ΔSSA_0260, ΔSSA_0261, and ΔSSA_0262 mutants at pH 7.4 & pH 6.3.

After microarray analysis of 143 genes from the mutants previously checked for their resistance in acidic pH 6.3, figure 19 compares the growth of 143 mutants at pH 7.4 and pH 6.3. We have found only three strains with mutation in genes related to ABC transport system showing differential growth compared to SK36 or ΔSSA_0204 strains (Figures 18 A and B). Results show that the mutant strains SSA_0260 (ssaB), and SSA_0261 (ssaC) were sensitive to acidic pH6.3. All of the genes were found to be up-regulated when SSA_0204 was dysfunctional. SSA_0260 was 3.6 fold up-regulated and SSA_0261 was 4.7 fold up-regulated and they were found to be involved in an ABC type metal transport mechanism as shown in Table 4. After observing these two genes were involved in acidic resistance, we also treated a mutant strain of the closest gene SSA_0262 (ssaA) with acid stress; fortunately it was also sensitive to acidic stress (Figure 18 C). It has also been proven by the microarray analysis shown in Table 4 that all of these genes are up-regulated when SSA_0204 is mutated. This describes the function of SSA_0204 in a wild type cell. It may be possible that SSA_0204 in a wild type strain suppresses the expression of SSA_0260, SSA_0261, and SSA_0262.

Figure 18: Growth of *S. sanguinis* mutant strains was monitored in microplate reader at pH 7.4 and pH 6.3. OD at 450 nm was measured every 10 min. Values of three replicates are shown (A) ΔSSA_0260, (B) ΔSSA_0261, (C) ΔSSA_0262, compared with *S. sanguinis* SK36 and ΔSSA_0204.

Figure 18:

A


Growth at pH 7.4
O.D.(450 nm.)
0.6
0.5
0.4
0.3
0.2
0.1
0
Time(hrs.)
0
5
10
15
20
25
SK36
ΔSSA_0204
ΔSSA_0260

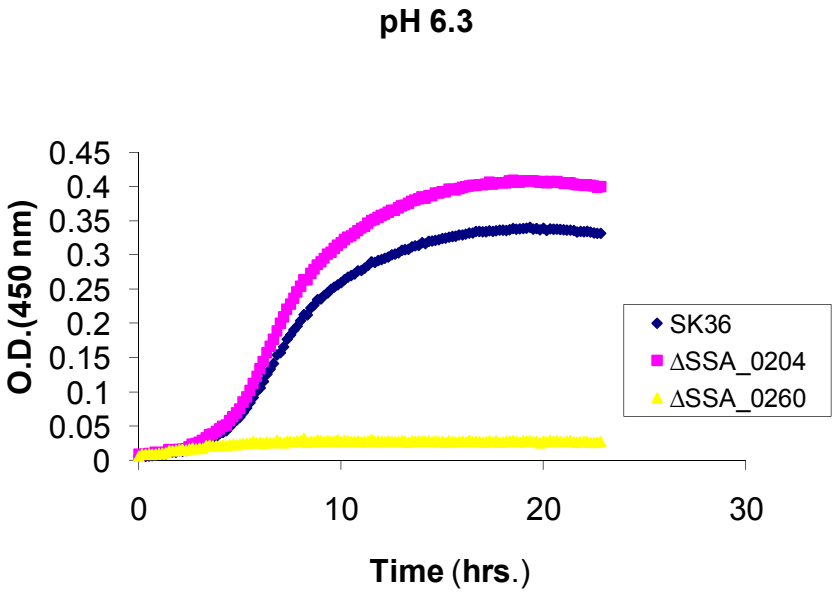

pH 6.3
O.D.(450 nm)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
Time (hrs.)
SK36
ΔSSA_0204
ΔSSA_0260

Figure 18:

B

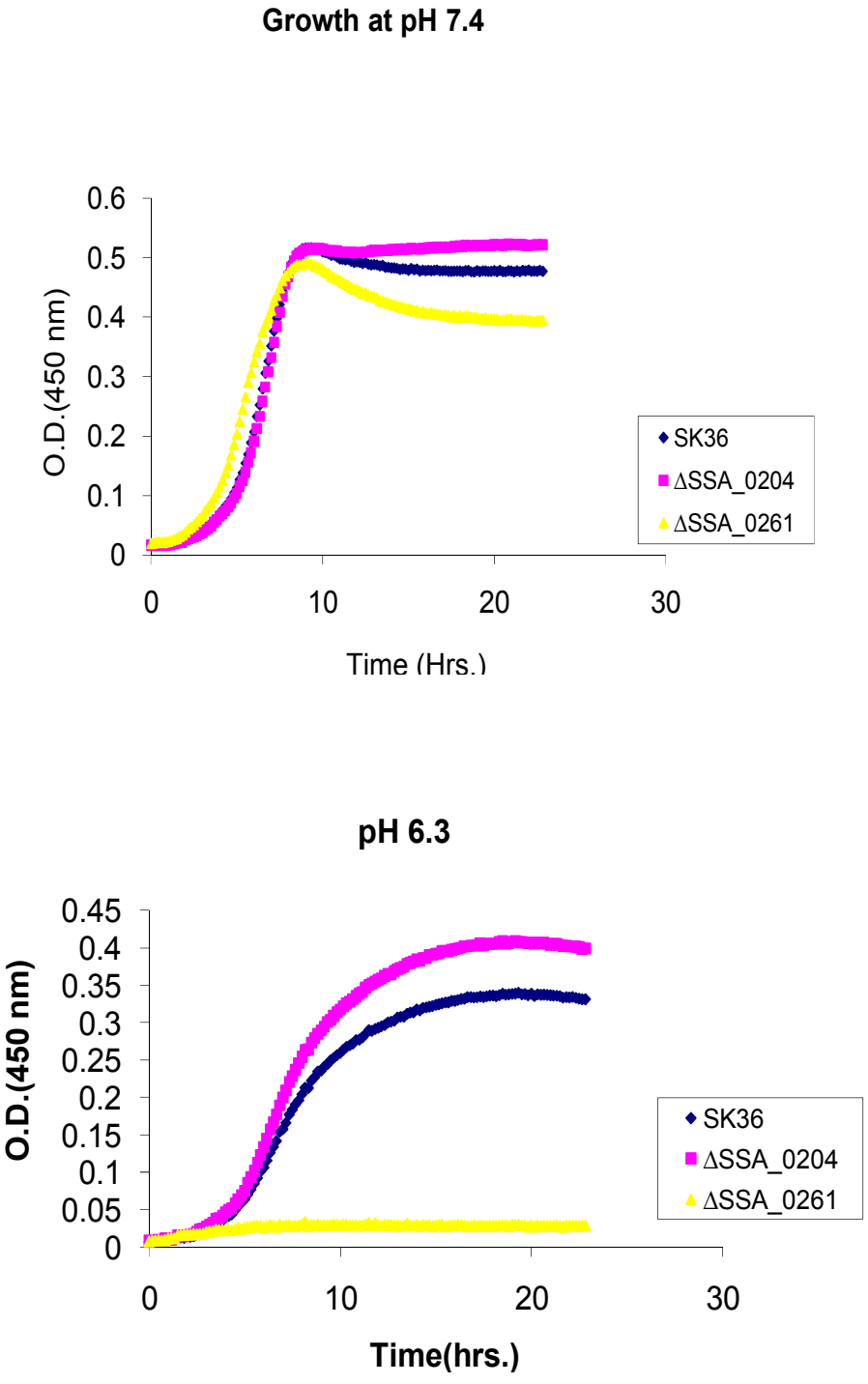
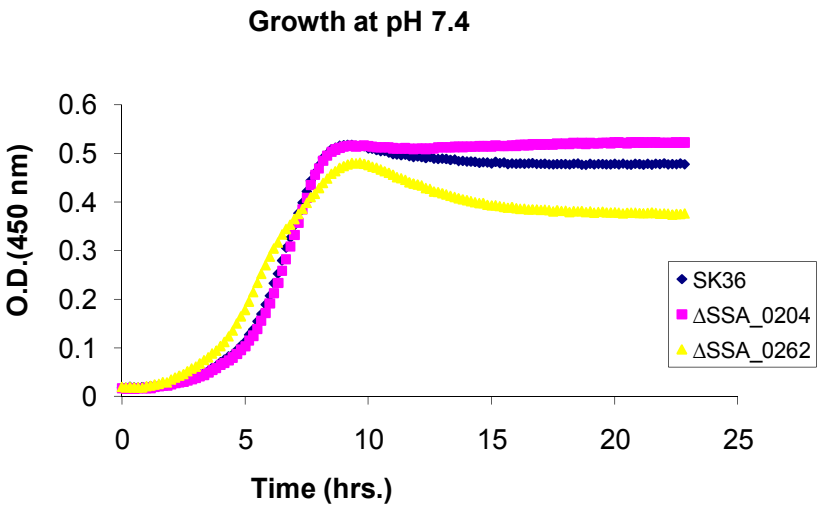

Growth at pH 7.4
O.D.(450 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
Time (Hrs.)
SK36
ΔSSA_0204
ΔSSA_0261
pH 6.3
O.D.(450 nm)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
Time(hrs.)
SK36
ΔSSA_0204
ΔSSA_0261

Figure 18:

C.


Growth at pH 7.4
O.D.(450 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
Time (hrs.)
SK36
ΔSSA_0204
ΔSSA_0262

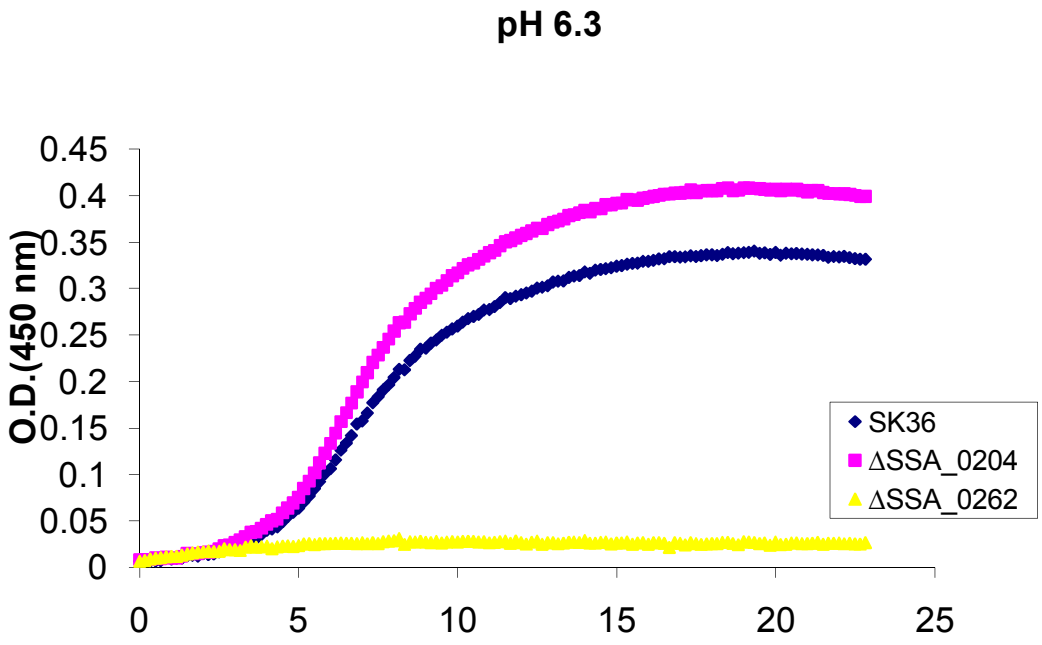
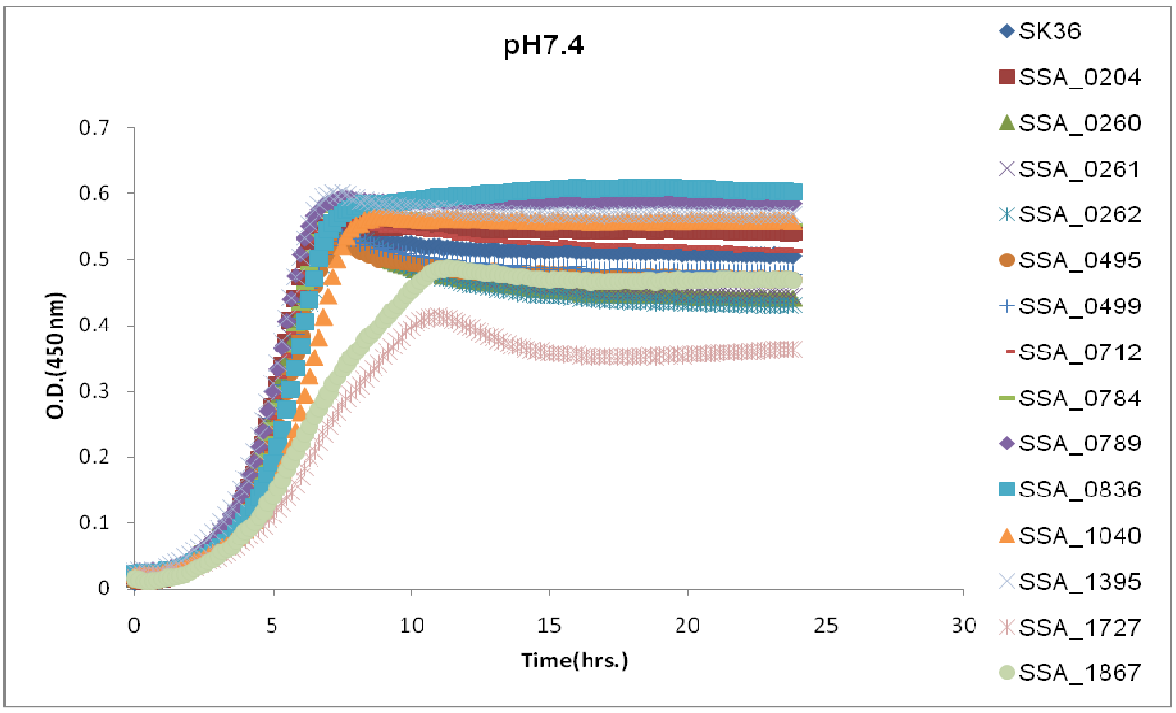

pH 6.3
O.D.(450 nm)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
SK36
ΔSSA_0204
ΔSSA_0262

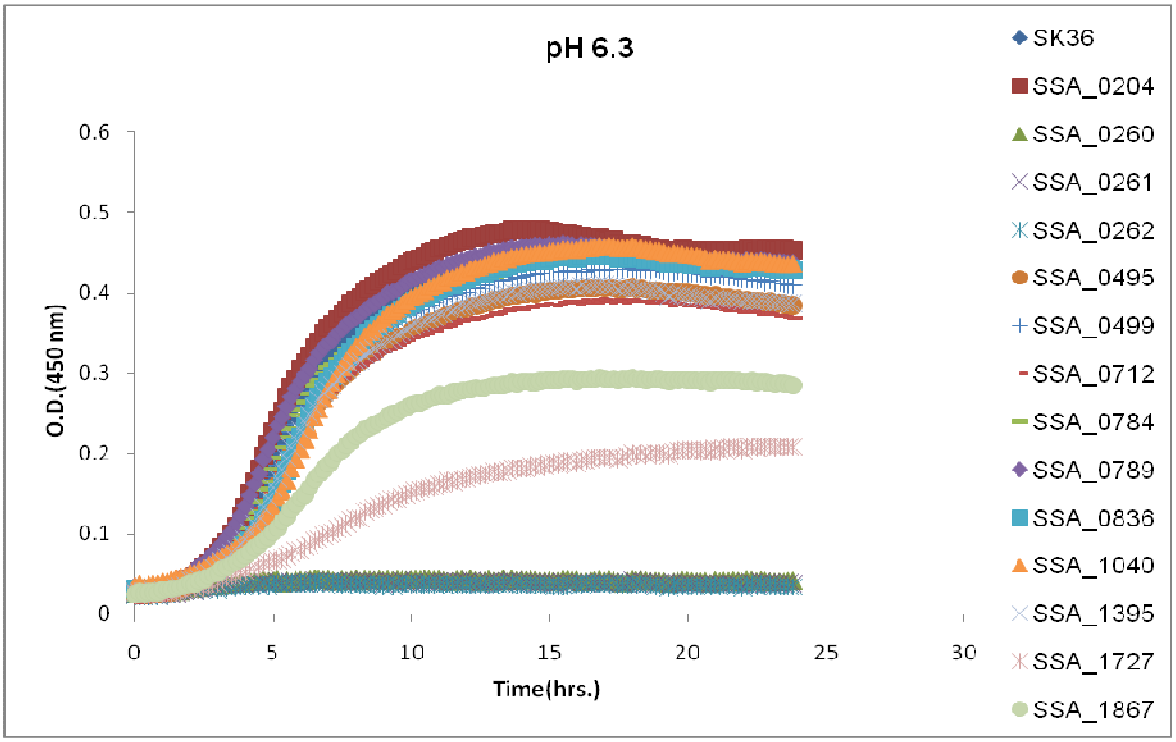
Figure 19: Growth of *S. sanguinis* mutant strains was monitored in microplate reader at pH 7.4 and pH 6.3. OD at 450 nm was measured every 10 min. Mean values from three replicate wells are shown. A1 to A10 at pH 7.4 and B1 to B10 at pH 6.3.

Figure 19:

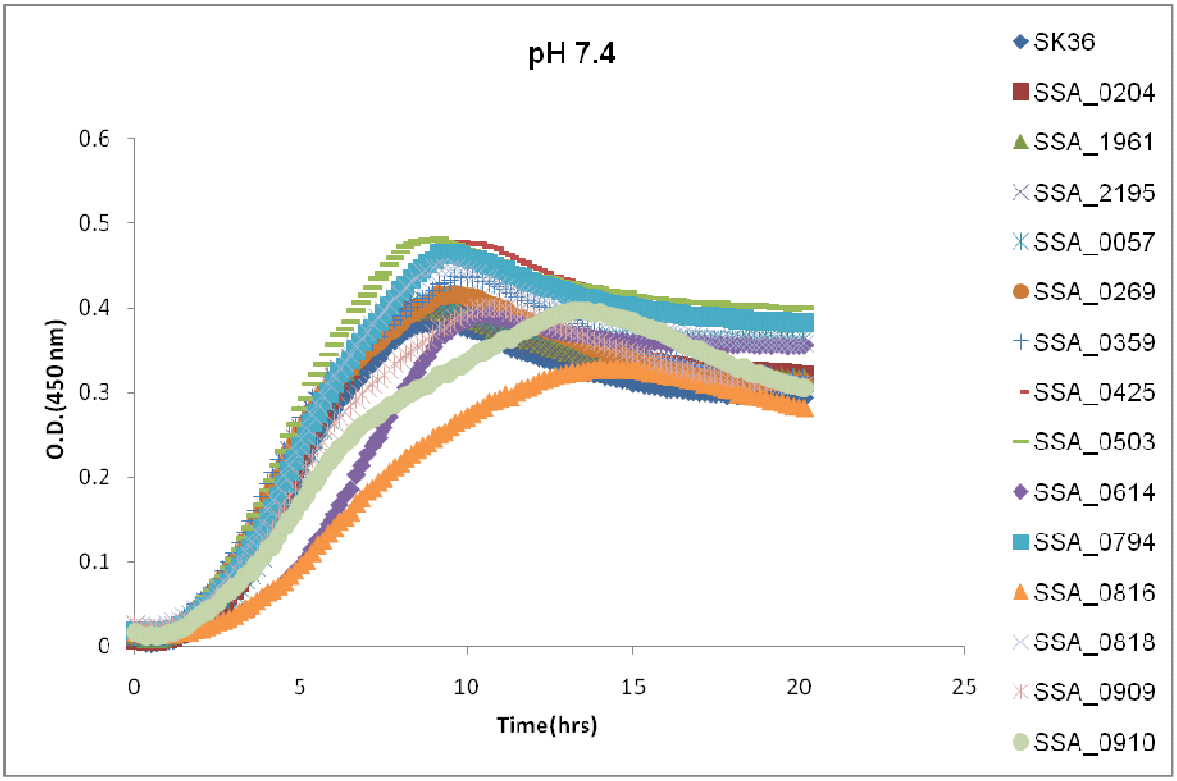
A1



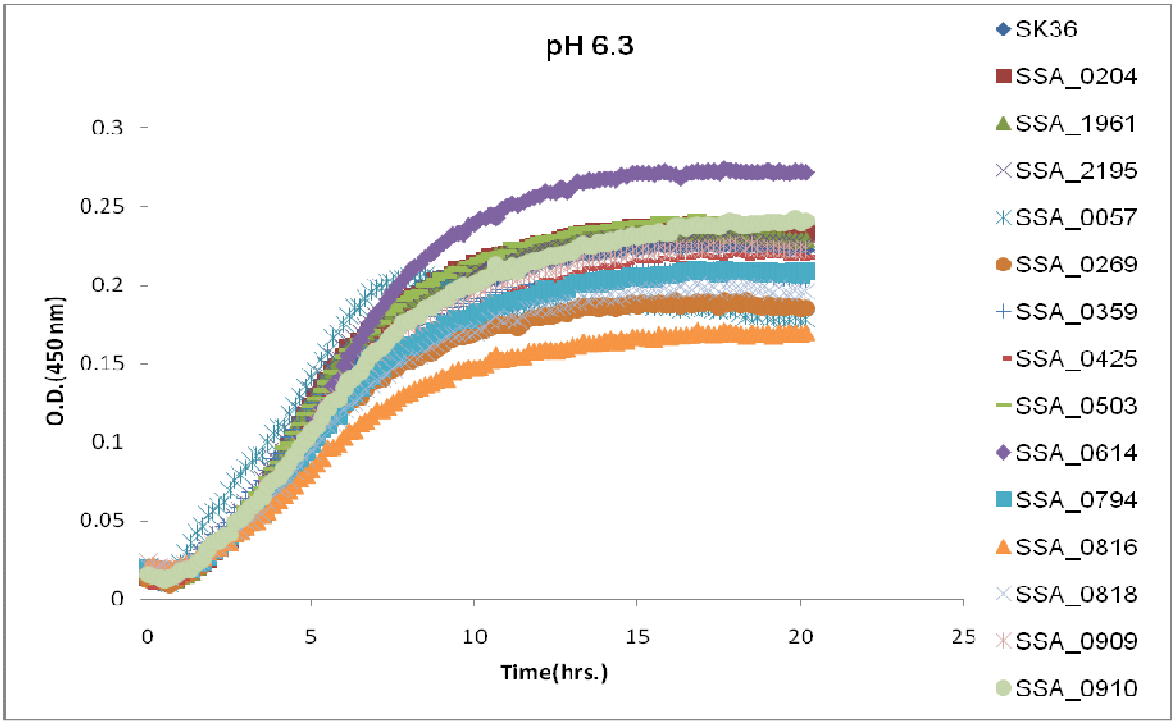
B1



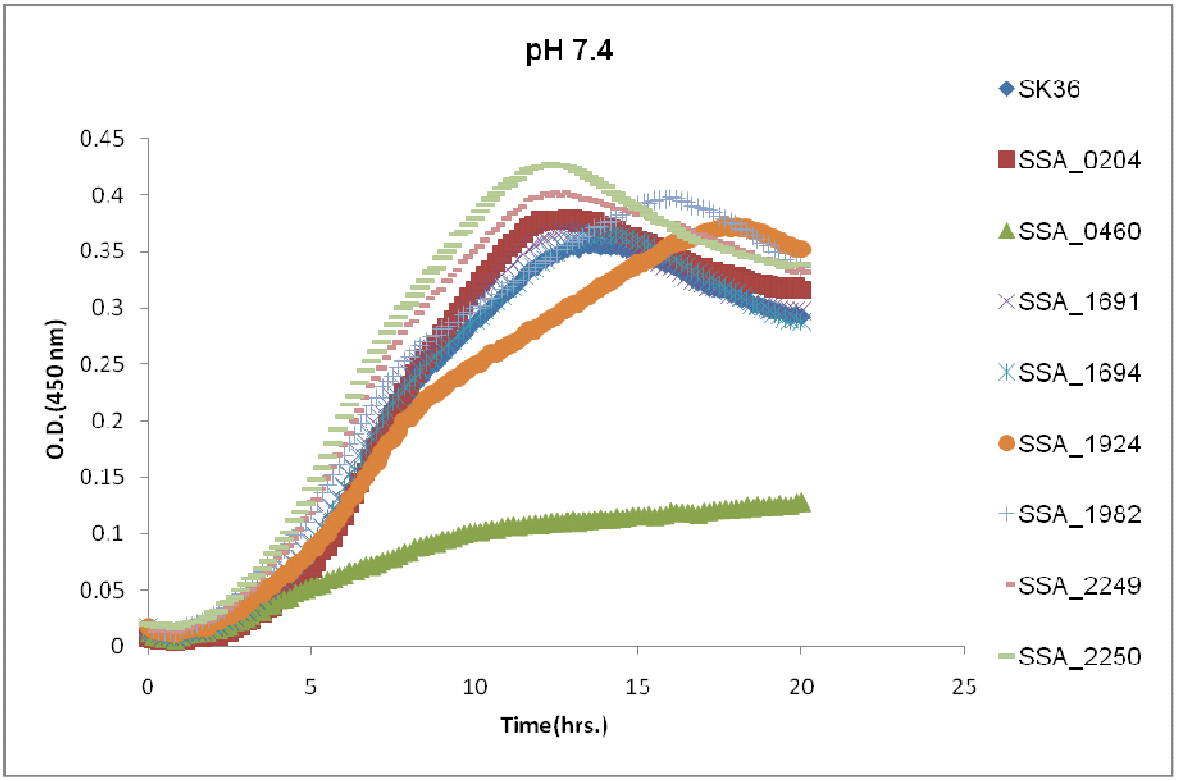
A2


pH 7.4
O.D.(450 nm)
Time(hrs)
SK36
SSA_0204
SSA_1961
SSA_2195
SSA_0057
SSA_0269
SSA_0359
SSA_0425
SSA_0503
SSA_0614
SSA_0794
SSA_0816
SSA_0818
SSA_0909
SSA_0910

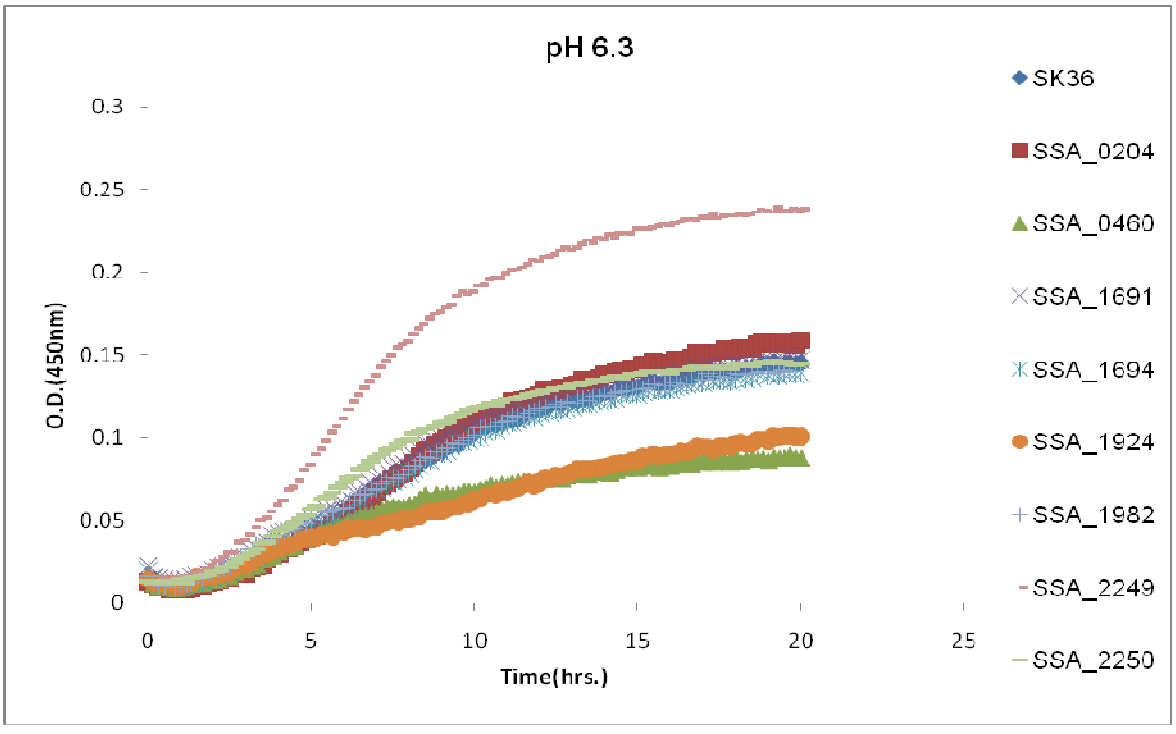
B2


pH 6.3
O.D.(450 nm)
Time(hrs.)
SK36
SSA_0204
SSA_1961
SSA_2195
SSA_0057
SSA_0269
SSA_0359
SSA_0425
SSA_0503
SSA_0614
SSA_0794
SSA_0816
SSA_0818
SSA_0909
SSA_0910

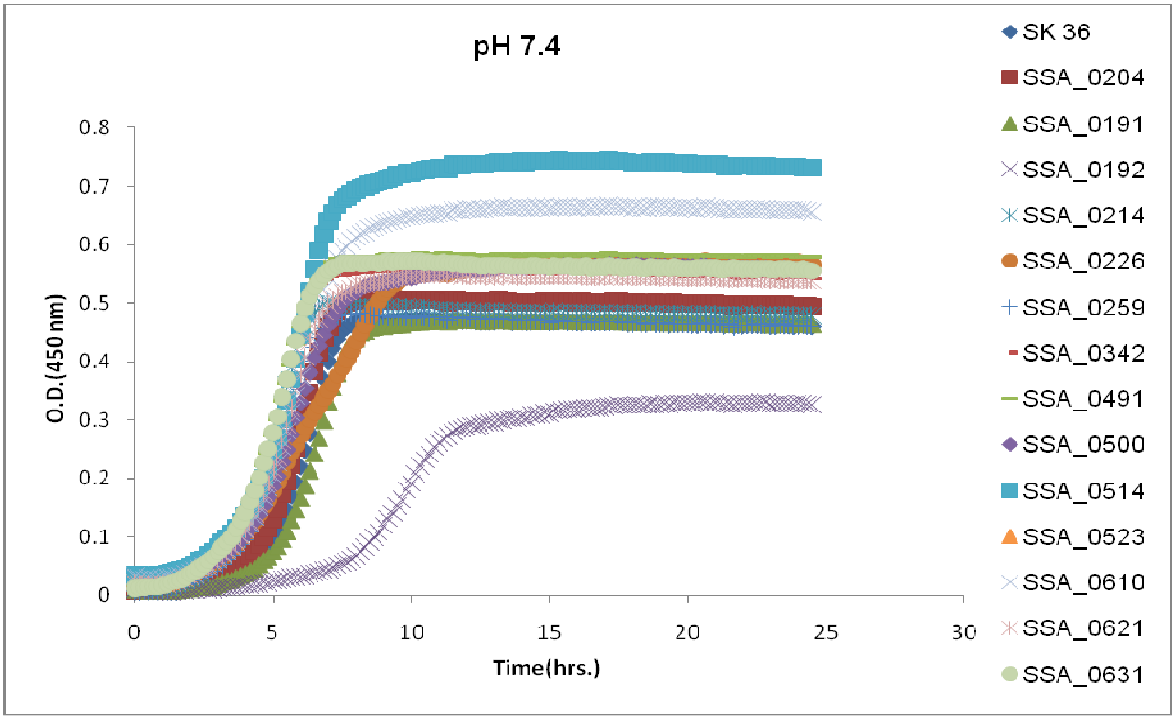
A3


pH 7.4
O.D.(450nm)
Time(hrs.)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
SK36
SSA_0204
SSA_0460
SSA_1691
SSA_1694
SSA_1924
SSA_1982
SSA_2249
SSA_2250

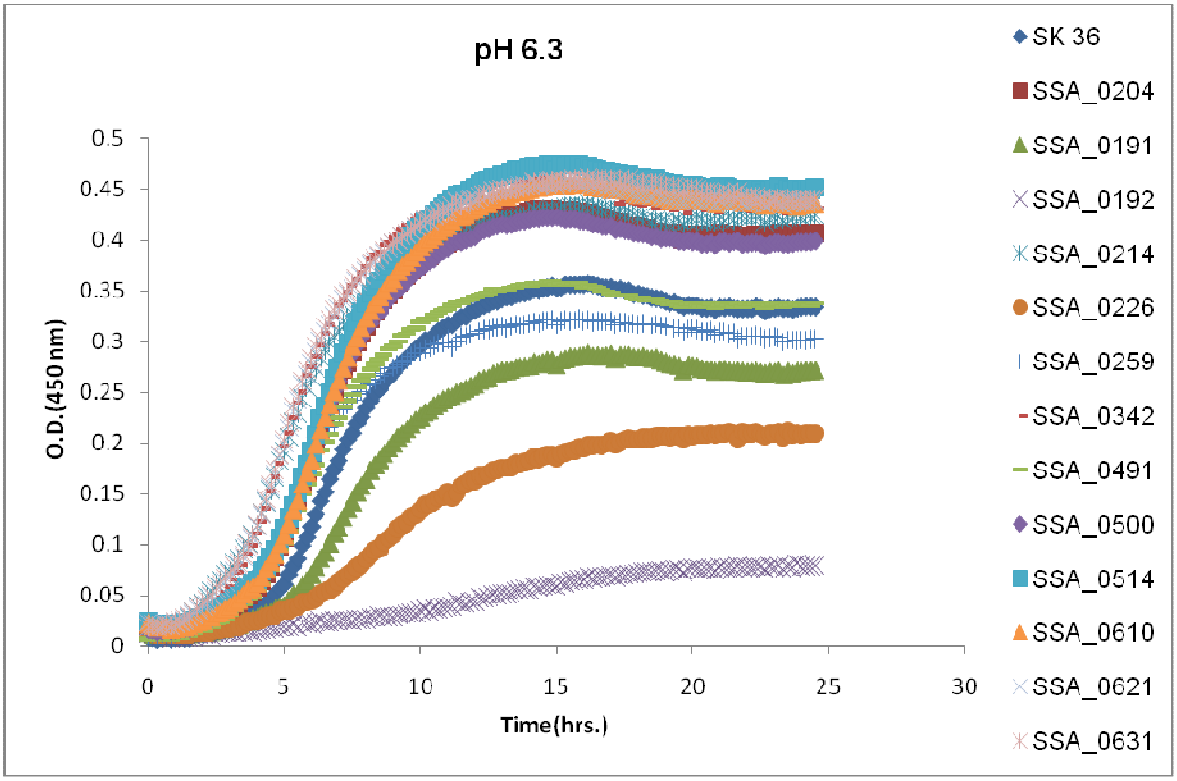
B3


pH 6.3
O.D.(450nm)
Time(hrs.)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
SK36
SSA_0204
SSA_0460
SSA_1691
SSA_1694
SSA_1924
SSA_1982
SSA_2249
SSA_2250

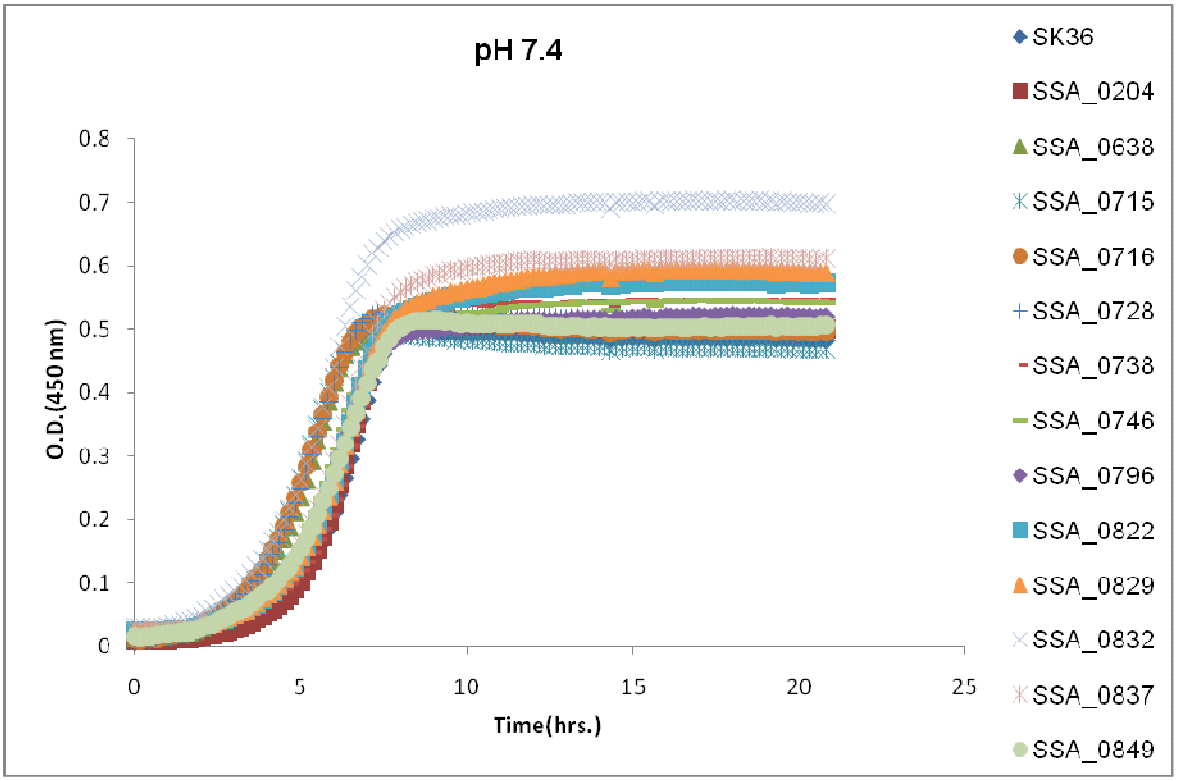
A4


pH 7.4
O.D.(450 nm)
Time(hrs.)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
5
10
15
20
25
30
SK 36
SSA_0204
SSA_0191
SSA_0192
SSA_0214
SSA_0226
SSA_0259
SSA_0342
SSA_0491
SSA_0500
SSA_0514
SSA_0523
SSA_0610
SSA_0621
SSA_0631

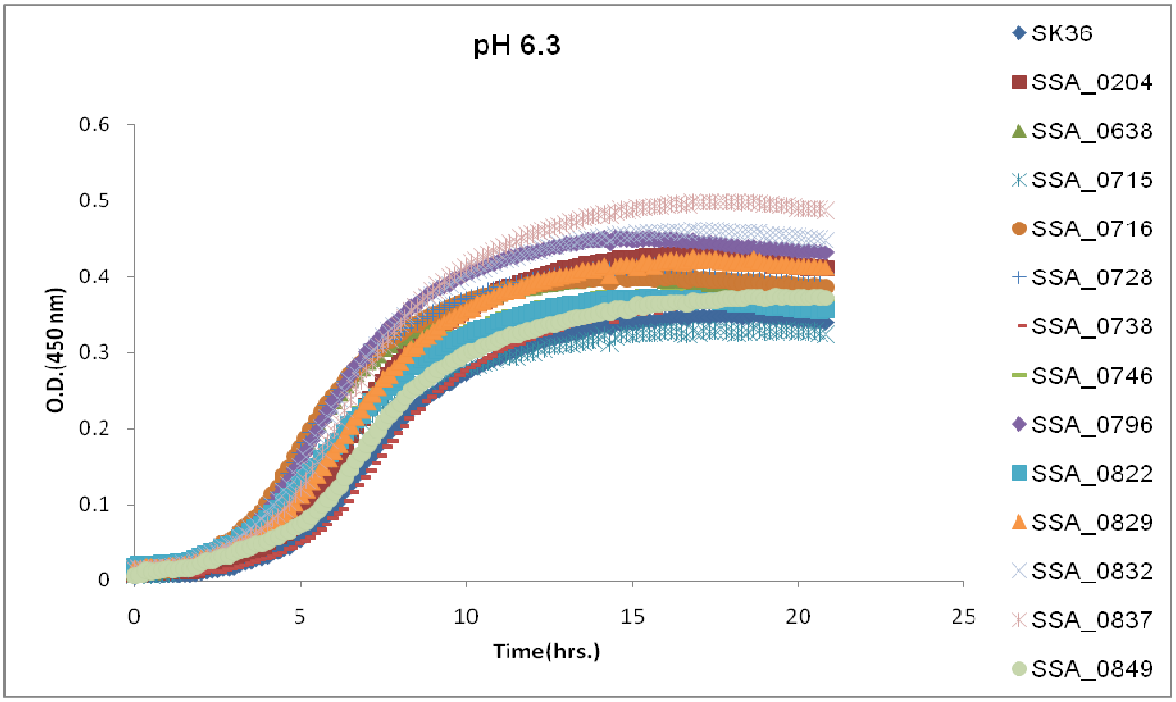
B4


pH 6.3
O.D.(450 nm)
Time(hrs.)
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0.5
0
5
10
15
20
25
30
SK 36
SSA_0204
SSA_0191
SSA_0192
SSA_0214
SSA_0226
SSA_0259
SSA_0342
SSA_0491
SSA_0500
SSA_0514
SSA_0610
SSA_0621
SSA_0631

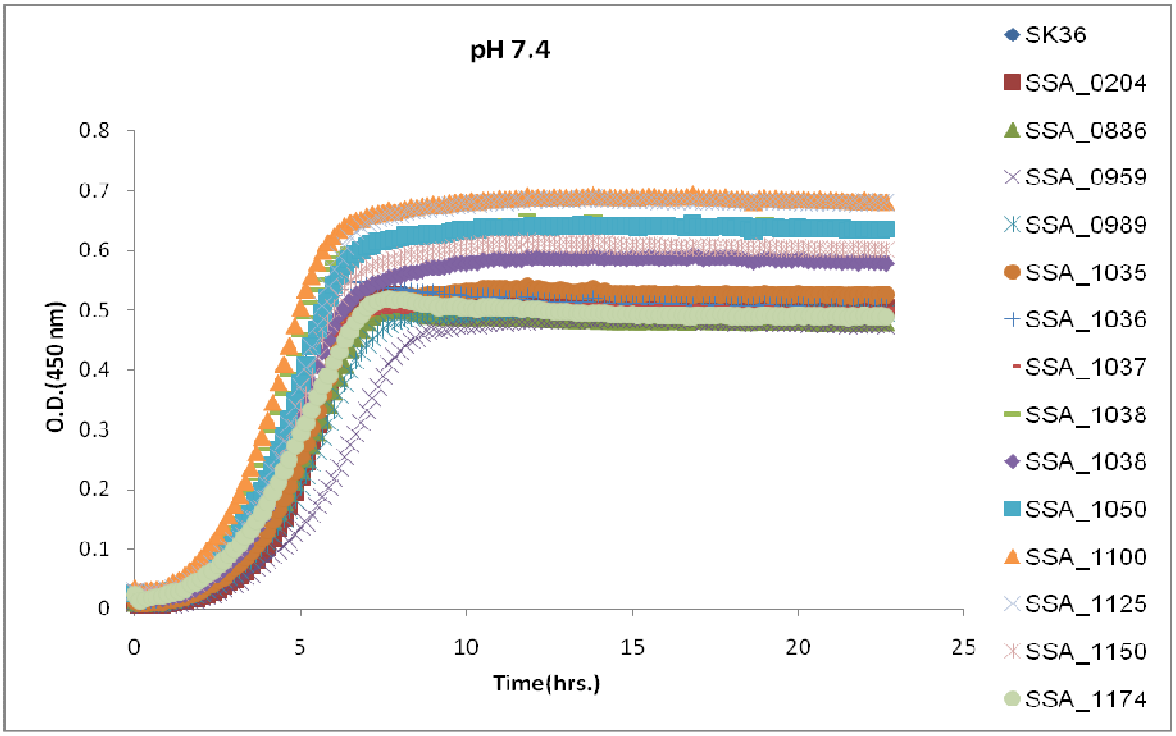
A5


pH 7.4
O.D.(450 nm)
Time(hrs.)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
5
10
15
20
25
SK36
SSA_0204
SSA_0638
SSA_0715
SSA_0716
SSA_0728
SSA_0738
SSA_0746
SSA_0796
SSA_0822
SSA_0829
SSA_0832
SSA_0837
SSA_0849

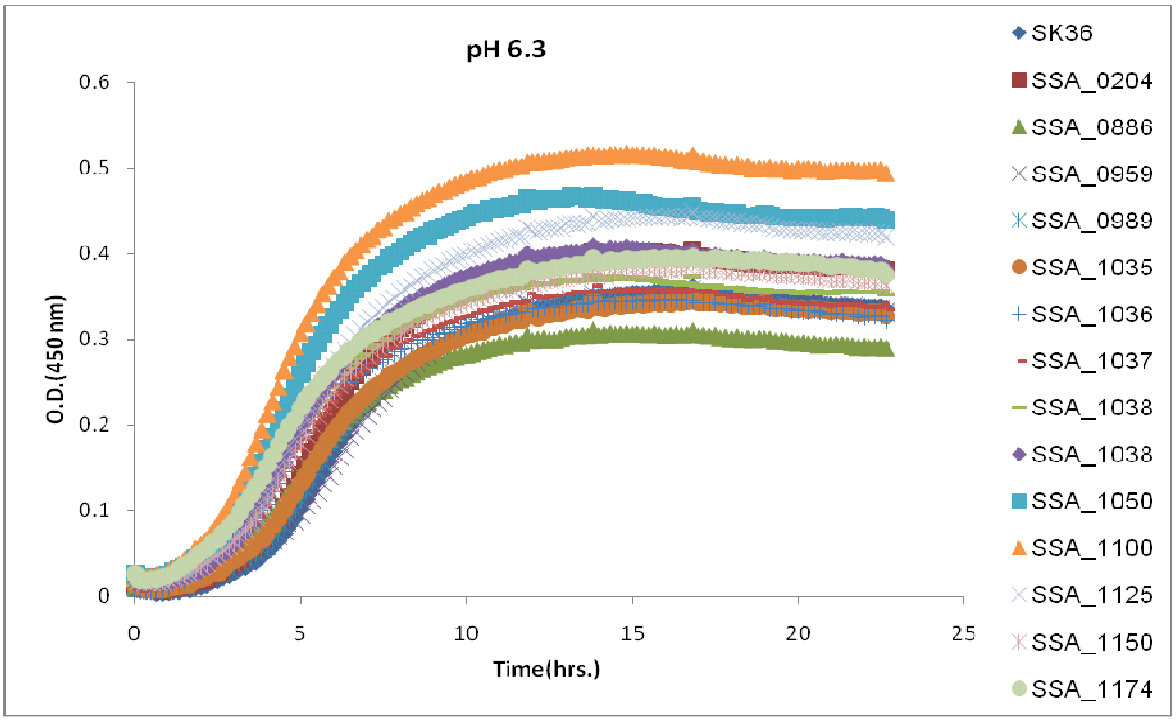
B5


pH 6.3
O.D.(450 nm)
Time(hrs.)
0
0.1
0.2
0.3
0.4
0.5
0.6
0
5
10
15
20
25
SK36
SSA_0204
SSA_0638
SSA_0715
SSA_0716
SSA_0728
SSA_0738
SSA_0746
SSA_0796
SSA_0822
SSA_0829
SSA_0832
SSA_0837
SSA_0849

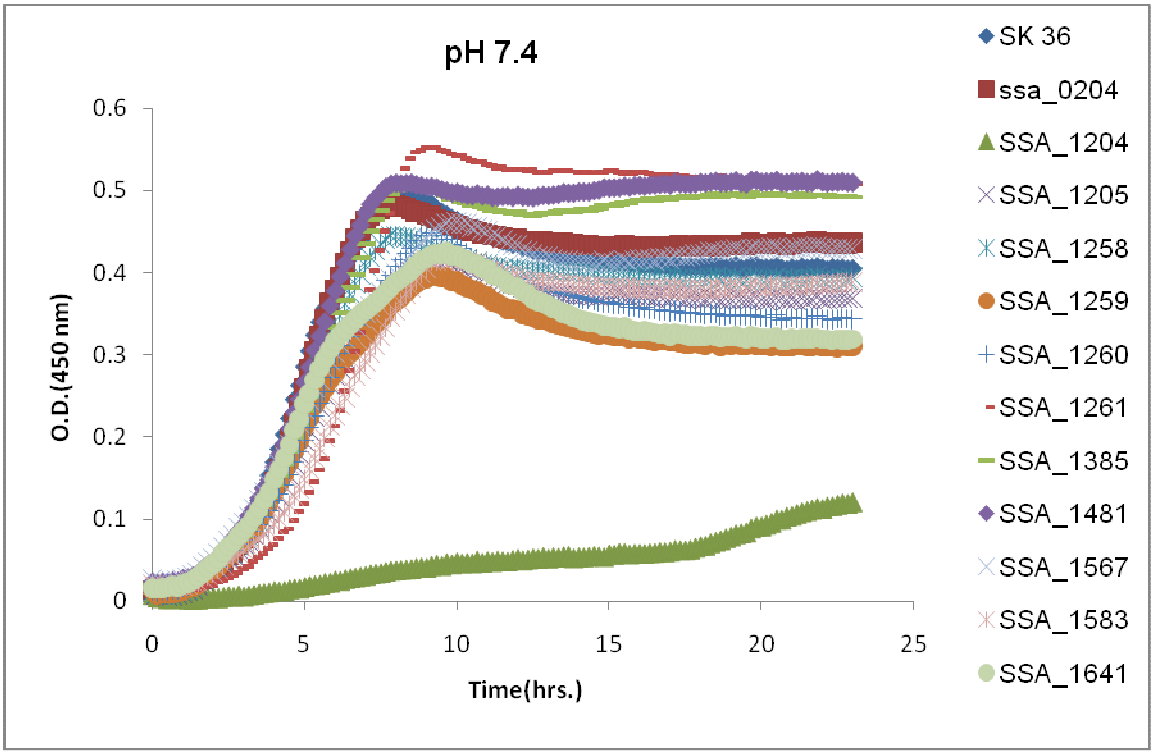
A6


pH 7.4
O.D.(450 nm)
Time(hrs.)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
5
10
15
20
25
SK36
SSA_0204
SSA_0886
SSA_0959
SSA_0989
SSA_1035
SSA_1036
SSA_1037
SSA_1038
SSA_1038
SSA_1050
SSA_1100
SSA_1125
SSA_1150
SSA_1174

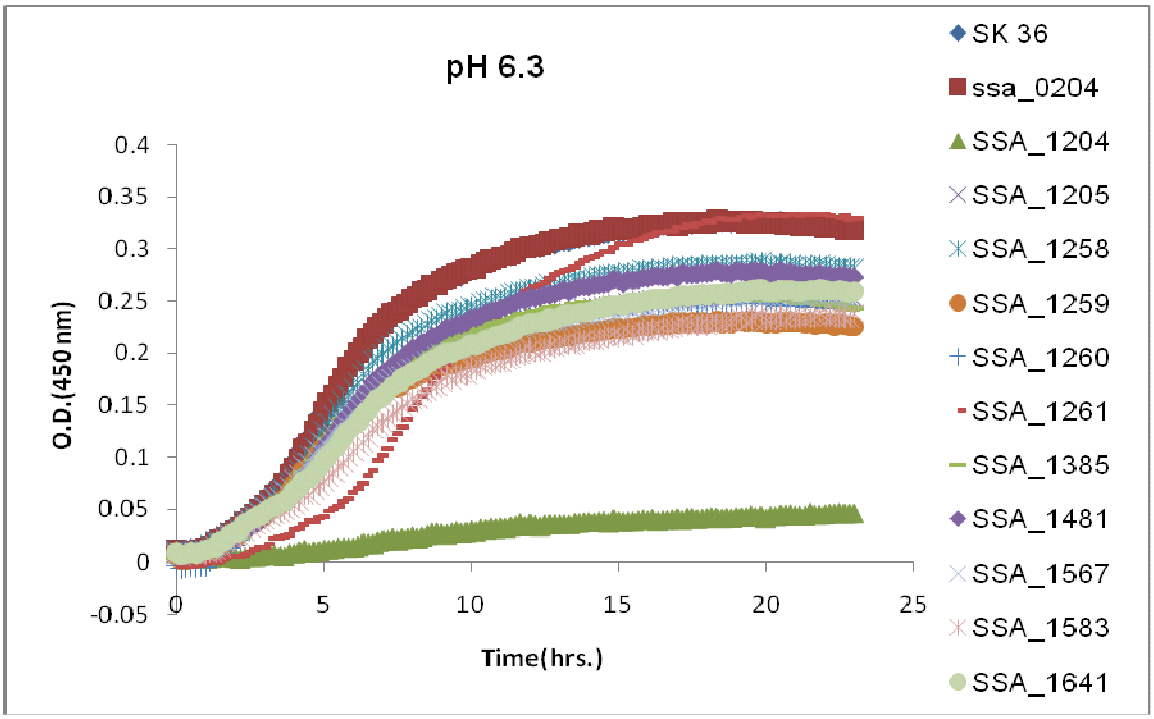
B6


pH 6.3
O.D.(450 nm)
Time(hrs.)
0
0.1
0.2
0.3
0.4
0.5
0.6
0
5
10
15
20
25
SK36
SSA_0204
SSA_0886
SSA_0959
SSA_0989
SSA_1035
SSA_1036
SSA_1037
SSA_1038
SSA_1038
SSA_1050
SSA_1100
SSA_1125
SSA_1150
SSA_1174

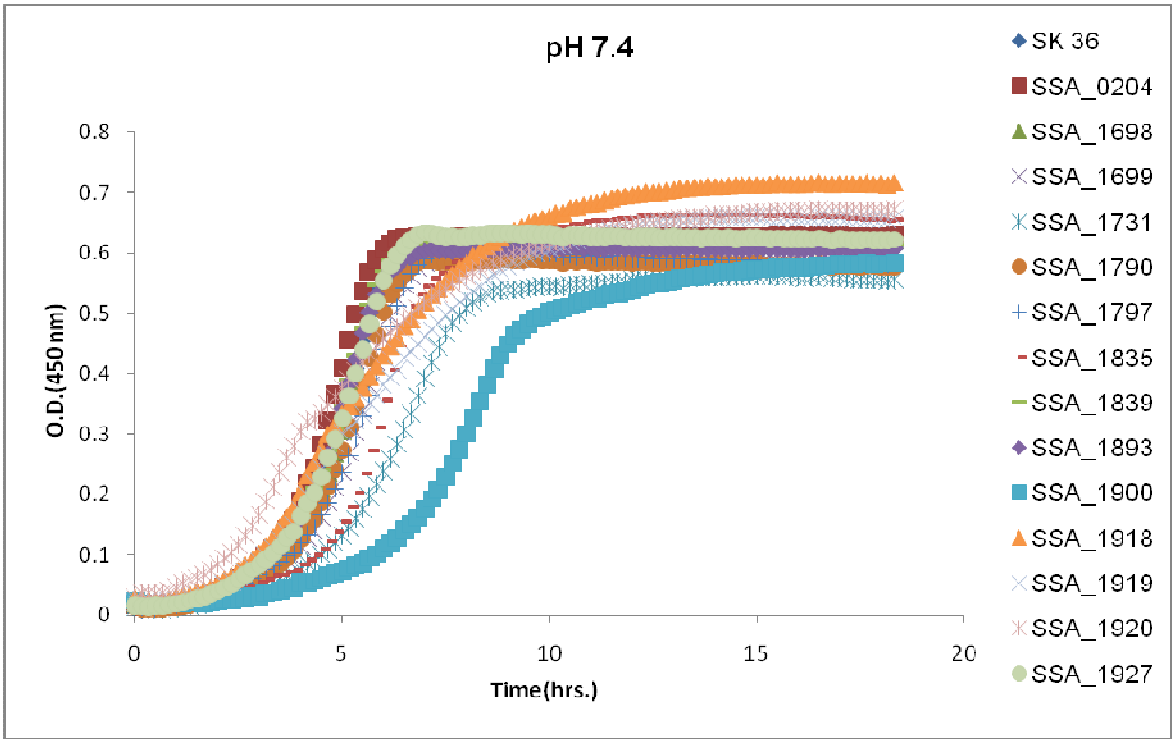
A7


pH 7.4
O.D.(450 nm)
Time(hrs.)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
SK 36
ssa_0204
SSA_1204
SSA_1205
SSA_1258
SSA_1259
SSA_1260
SSA_1261
SSA_1385
SSA_1481
SSA_1567
SSA_1583
SSA_1641

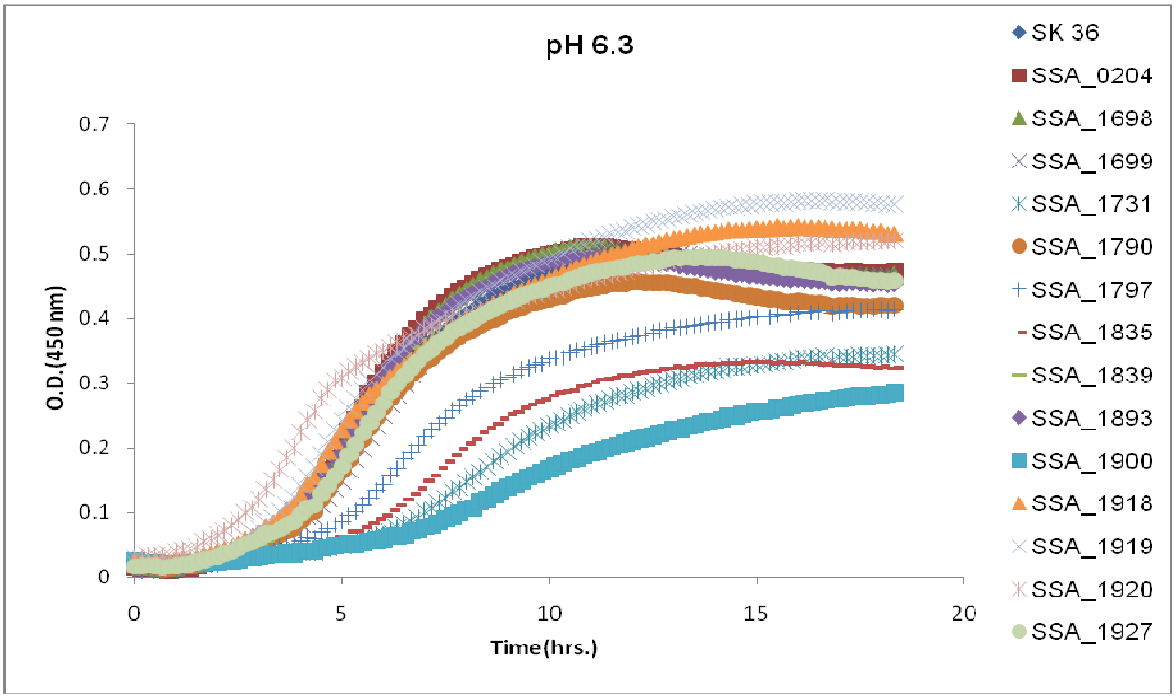
B7


pH 6.3
O.D.(450 nm)
Time(hrs.)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
0
5
10
15
20
25
SK 36
ssa_0204
SSA_1204
SSA_1205
SSA_1258
SSA_1259
SSA_1260
SSA_1261
SSA_1385
SSA_1481
SSA_1567
SSA_1583
SSA_1641

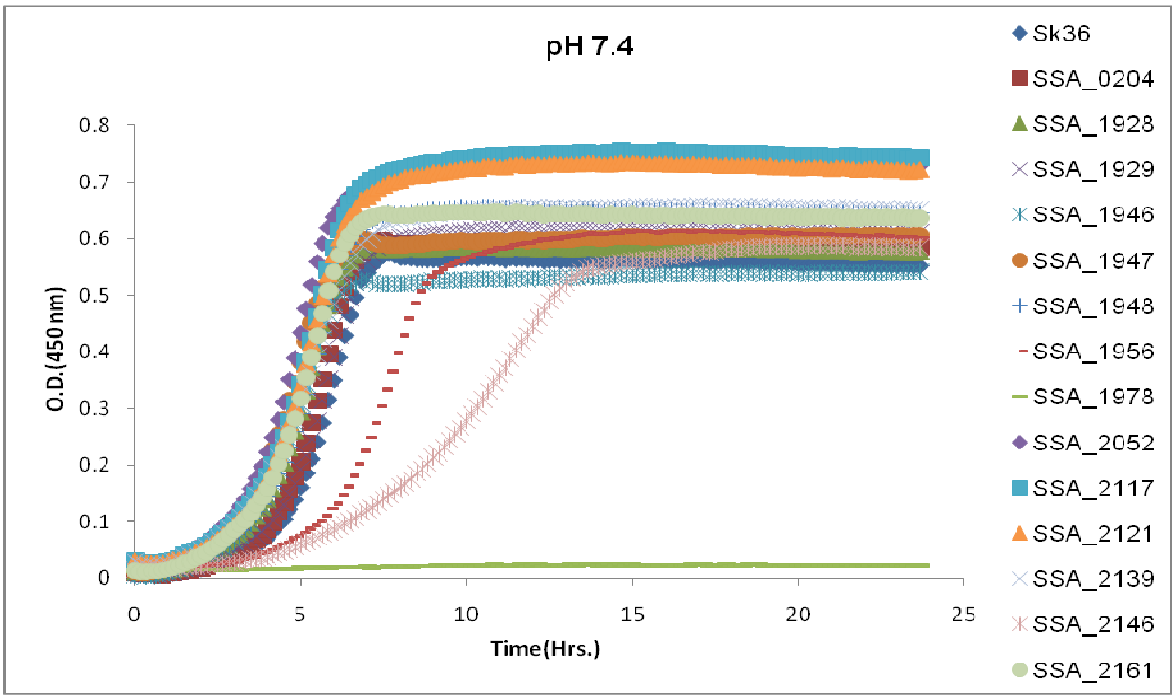
A8


pH 7.4
O.D.(450 nm)
Time(hrs.)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
5
10
15
20
SK 36
SSA_0204
SSA_1698
SSA_1699
SSA_1731
SSA_1790
SSA_1797
SSA_1835
SSA_1839
SSA_1893
SSA_1900
SSA_1918
SSA_1919
SSA_1920
SSA_1927

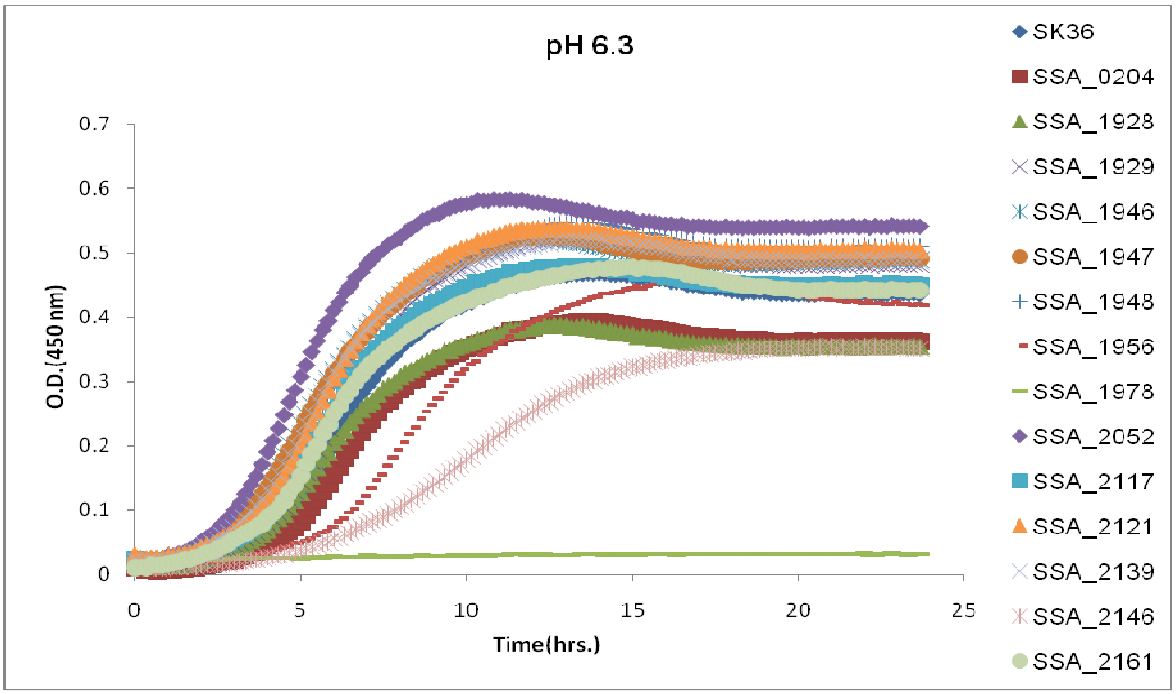
B8


pH 6.3
O.D.(450 nm)
Time(hrs.)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
5
10
15
20
SK 36
SSA_0204
SSA_1698
SSA_1699
SSA_1731
SSA_1790
SSA_1797
SSA_1835
SSA_1839
SSA_1893
SSA_1900
SSA_1918
SSA_1919
SSA_1920
SSA_1927

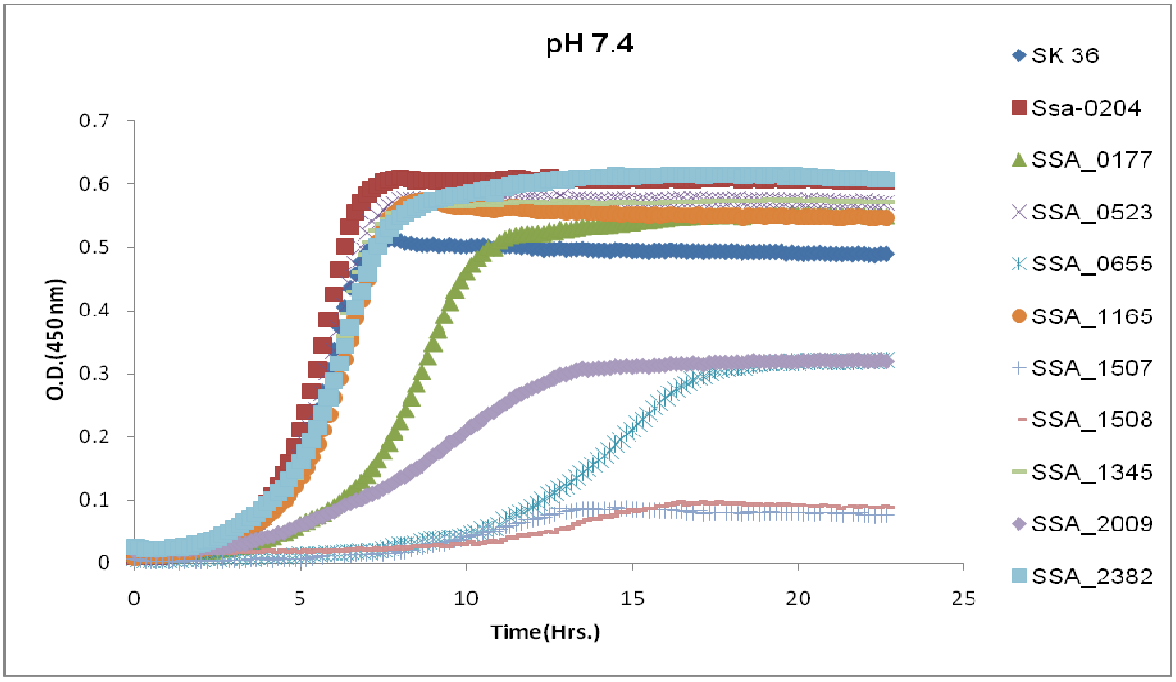
A9


pH 7.4
O.D.(450 nm)
Time(Hrs.)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
5
10
15
20
25
Sk36
SSA_0204
SSA_1928
SSA_1929
SSA_1946
SSA_1947
SSA_1948
SSA_1956
SSA_1978
SSA_2052
SSA_2117
SSA_2121
SSA_2139
SSA_2146
SSA_2161

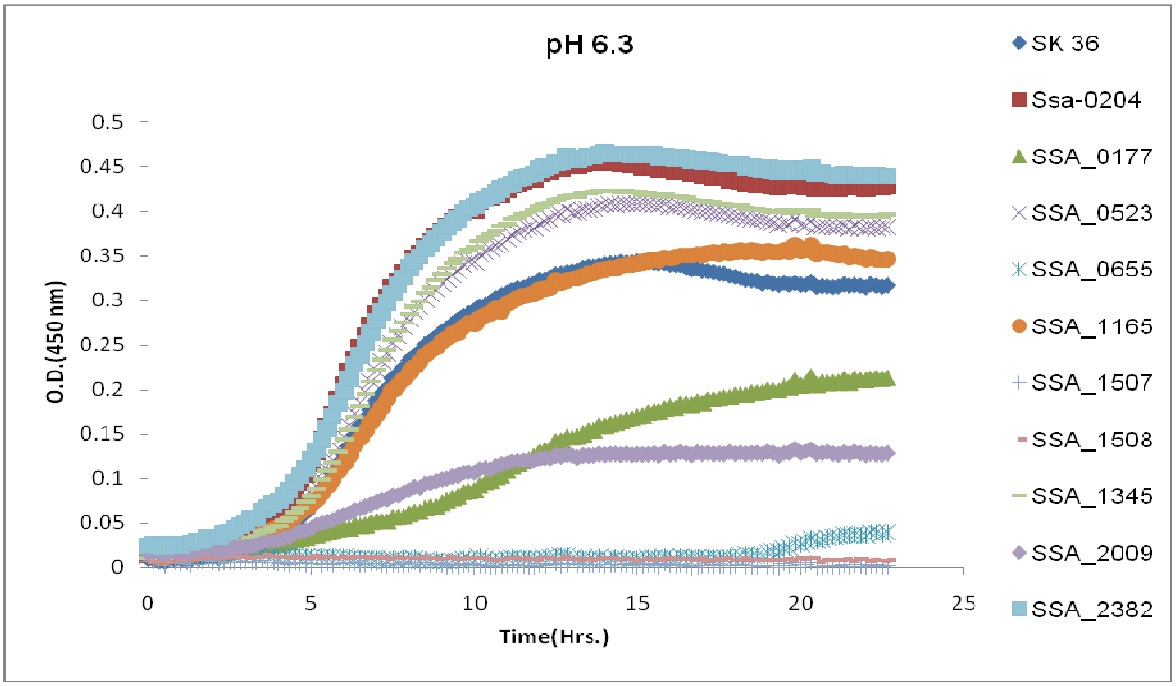
B9


pH 6.3
O.D.(450 nm)
Time(hrs.)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
5
10
15
20
25
SK36
SSA_0204
SSA_1928
SSA_1929
SSA_1946
SSA_1947
SSA_1948
SSA_1956
SSA_1978
SSA_2052
SSA_2117
SSA_2121
SSA_2139
SSA_2146
SSA_2161

A10


pH 7.4
O.D.(450 nm)
Time(Hrs.)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
5
10
15
20
25
SK 36
Ssa-0204
SSA_0177
SSA_0523
SSA_0655
SSA_1165
SSA_1507
SSA_1508
SSA_1345
SSA_2009
SSA_2382

B10


pH 6.3
O.D.(450 nm)
Time(Hrs.)
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0.5
0
5
10
15
20
25
SK 36
Ssa-0204
SSA_0177
SSA_0523
SSA_0655
SSA_1165
SSA_1507
SSA_1508
SSA_1345
SSA_2009
SSA_2382

4. DISCUSSION

The gram-positive bacterium *Streptococcus sanguinis*, a member of human indigenous oral microflora, has long been recognized as a key player in the bacterial colonization of the mouth. *S. sanguinis* is also the most common viridans streptococcal species implicated in infective endocarditis. A number of studies have shown that *S. mutans* is extremely well-adapted to the various adverse environmental conditions of the mouth such as temperature, high acidic condition, high oxygen stress, and nutrient-limited condition. The molecular mechanism that allows bacteria to resist these conditions is an important virulence trait [15], but is very poorly understood. Various mechanisms are involved in bacterial resistance against the stress environments. Proton translocation by F_1F_0 ATPase is one of the key enzymes for regulating intracellular pH or synthesizing ATP in many enteric bacteria [22]. Researchers have found that F_1F_0 ATPase plays a major role in *H. pylori* survival in a wide pH gradient from approximately two to seven [22]. Virulence factors in the *S. sanguinis* help protect the bacterium against possible host defenses and maintain its ecological niche in the oral cavity while at the same time contributing to its ability to cause host damage. Probable virulence factors include adhesins, glucan-producing and binding exoenzymes and proteases and cytokine stimulating molecules. In addition to these factors, several other gene products were found in the SK36, which may also contribute to the virulence of the *S. sanguinis.* Acid tolerance in plaque bacteria is necessary for the formation of caries. *Streptococcal species* such as *S. sanguinis, S. gordonii* and *S. oralis* in a plaque are capable of fermenting carbohydrates into metabolic acid, consequently lowering the plaque pH [21]. Constant changes in the

environment inside the mouth require the *S. sanguinis* to adapt and thrive in the hostile environment of the oral cavity, which suggests that this microorganism is capable of sensing and responding to different environmental stimuli through specific mechanism.

One such mechanism is the TCS, primarily a signal transduction system, which allows a sensor kinase to sense environmental signals and pass the signal onto the response regulators, which then regulate the gene expression that allows the adaptation for changing environments [9]. The number of TCS that have been found in bacteria varies depending upon the genome size and the complexity of the particular organism. Based on sequence analysis of the complete *S. sanguinis* genome, we identified 29 genes involved in TCS including 15 putative response regulators and 14 putative kinase sensors.

The primary goal of present study was to analyze the contribution of each response regulator to the stress tolerance response of the *S. sanguinis,* but we also intended to check the effect of the entire system by excluding both genes, response regulators (RR) and the Histidine kinase (HK) sensor. We treated all RR mutants with different environmental stress. To do so, mutants of *S. sanguinis* SK36 were generated by systematically knocking out the genes that encode for each response regulator. We inactivated each of them by inserting an antibiotic resistance gene cassette. One of the response regulators, SSA_1565, which was later found to be an essential gene, could not be mutated. Fourteen mutants of TCS response regulators were created and confirmed by sequencing. The TCS mutants were assessed for their ability to withstand various environmental stresses like high osmotic shock, alkaline stress, and acidic stress. The properties of RR mutants we

examined in this study are summarized in Table 5. We found out mutating the only RR or RR-HK showing the same response to different stress conditions.

Table: 5 Growth of RR mutants under various stress condition

	Growth under various stress condition		
Strains	Osmotic Stress	Alkaline Stress	Acidic Stress
SK36 (wild type)	+	+	+
ΔSSA_0204	+	+	++
ΔSSA_0217	+	+	++
ΔSSA_0401	+	+	+
ΔSSA_0516	+	+	+
ΔSSA_0896	+	+	+
ΔSSA_0959	+	+	+
ΔSSA_1113	+	+	+
ΔSSA_1119	+	+	+
ΔSSA_1565		Essential gene	
ΔSSA_1685	+	+	+
ΔSSA_1794	+	-	+
ΔSSA_1810	-	-	+
ΔSSA_1842	+	-	+
ΔSSA_1974	+	+	+
ΔSSA_2378	+	+	+

A number of studies have been performed to check the response against environmental stress in different bacteria. There are various data showing the involvement of TCS in the bacterial resistance against different stress conditions, but in the case of *S. sanguinis,* many of the TCS functions are still unknown. Until now, there was no systematic study to characterize the involvement of TCS in these different stress conditions.

In *S. mutans,* it has been reported that there are 13 TCS. It has been discovered that three sensor kinase genes, Smu486, Smu1128, and Smu1516, are involved in various stress responses [12]. Many of these TCS are homologous to closely related bacteria including *S. sanguinis* SK36. We found orthologs of TCS genes of *S. sanguinis* in other bacteria, as described in Table 6. There are a number of studies that have identified the involvement of TCS in various stress responses in *S. mutans*. One important study of *S. mutans* has shown that TCS vicRK is important in counteracting Antibiotic stress [12], oxidative stress [17], competence development, biofilm formation, regulation of virulence gene expression [18] and it was found to be an ortholog of TCS SSA_1564/1565 in *S. sanguinis* (Table 6). In addition to that, the lack of the Vic gene system in *S. mutans* resulted in a modified genetic competence development, lack of adherence, and biofilm formation. Moreover the system was also found to be involved in the regulation of virulence gene expression such as *gtfBCD, ftf,* and *gbpB*. [18]. Another interesting study in *S. mutans* shows that the mutation in the TCS RR gene *tcbR* led to a tenfold reduction in biofilm formation and it was found to be an ortholog of SSA_2378 (comD)[19].

Based on othologs search we found two more essential genes in different species, rr02 in *S. pneumoniae* and yycG in *B. subtilis* are orthologs of SSA_1565 (Table 6).

Table 6. Orthologs of TCS *S. sanguinis* in other bacteria and their functions.

S. sanguinis TCS genes	Bacterium	Ortholog	Description of orthologous protein
SSA_0204(*nisR*) SSA_0205(*nisK*)			
SSA_0216 SSA_1217			
SSA_0401 SSA_0402			
SSA_0516 SSA_0517			
SSA_0896 SSA_0897			
SSA_0959(*ciaR*) SSA_0960(*ciaH*)	*S. gordonii* *S. mutans*	*ciaR* *ciaH(Smu.1128)*	Arginine deiminase system expression in acidic pH[20] Oxidative stress, superoxide stress, acidic stress, thermal stress, mutacin production[12]
SSA_1113 SSA_1114			
SSA_1119 SSA_1120			
SSA_1564 SSA_1565	*S. mutans* *S. pneumoniae* *B. subtilis* *S. mutans*	*vicK(Smu1516)* *rr02* *yycG* *vicR*	Antibiotic stress[12] Essential gene[30] Essential gene[30] Oxidative stress[17], Competence development, biofilm formation, and regulation of virulence genes expression [18]
SSA_1684 SSA_1685			
SSA_1793 SSA_1794			
SSA_1810(*csrR*)	*S. pyogenes*	*covR*	Mutant of covR increase virulence in mice and resistance to phagocytosis[29]
SSA_1842 SSA_1843	*S. mutans*	*Smu486*	Oxidative stress and thermal stress resistance[12]
SSA_1972 SSA_1973			
SSA_2378(*comE*) SSA_2379(*comD*)	*S. mutans*	*tcbR(smu1917)*	Biofilm formation [19]

However, in the case of *S. gordonii,* it has been determined that TCS modulate alkali generation in response to environmental stress. In this study, it was demonstrated that the CiaRH (CiaR is an ortholog of SSA_0959 in SK36) and ComDE component systems are required for the Arginine Deiminase System (ADS) expression in acidic pH. [20]. In our observation based on growth study of SSA_0959 strongly suggest that this response regulator indeed plays a vital role in cell growth and development.

Work has been done to identify that the sensor kinases are important to the response of the high osmotic shock in *Synechocystis* sp. [23]. Based on our analysis we have found one RR mutants, ΔSSA_1810, to be sensitive to osmotic stress, but the ortholog of this gene covR in *S. pyogenes* found that mutation in covR increase the virulence in mice and resistance to phagocytic killing [29].

We treated all of the regulator mutants in high alkaline conditions and compared them to the wild type SK36 strain. We identified that TCS response regulators ΔSSA_1810, ΔSSA_1794, and ΔSSA_1842 are more sensitive to alkaline stress than wild type *S. sanguinis* SK36 cells. ΔSSA_1810 was also identified as alkaline stress sensitive. It surprised us because earlier analysis shown in figure 1 suggests that SSA_1810 does not have any sensor kinases nearby. The question still arises is how does this RR get its signal? One of the possibilities for this is that it may get a response from another HK gene with different TCS, but it is still unknown. We can speculate that it may involve in osmotic stress tolerance.

Their sensitivity was also confirmed with alkaline shock treatment at a pH of 10.8. After confirming the RR mutation's response to alkaline stress conditions, we wanted to check how the entire TCS would respond to the same condition. So, we tried the double mutations (RR-HK) with the same shock treatment. This suggests that the response regulators SSA_1794, SSA_1810 and SSA_1842 are involved in the alkaline stress resistance. The exact mechanism by which this system is regulated is not yet known. Additional studies are warranted not only to understand the strain-dependent phenotypic changes, but also to explore the network of genes that are regulated by TCS in *S. sanguinis*.

So far, research studies have shown that *S. mutans* is able to rapidly adopt an acidic tolerance response [24]. Previous studies on *S. mutans* have shown that sensor kinase ΔSMU_1128 shows significant reductions of growth rate in acidic pH compared to wild type *S. mutans* UA159 [12] and it is an ortholog of SSA_0960(Table 6). The role of TCS does not always have to be to induce expression in order to protect the bacterium. In the case of *S. pyogenes*, a regulatory system with two components, CsrR and CsrS, was found to repress virulence, gene activity, hyaluronic acid capsules, streptolysin S, and pyrogenic exotoxin B [25]. This correlates with our findings that SSA_0204 and SSA_0217 are capable of repressing the acidic resistance of *S. sanguinis* SK36 strain. The mutant strains of SSA_0204 and SSA_0217 show better resistance to acidic stress than does the wild type *S. sanguinis* SK36.

This surprised us, and it motivated us to focus more study on these two RR mutants. In order to find the acid resistance gene regulated by SSA_0204, we have done a

microarray for ΔSSA_0204 (Dr. Lei Chen). The results from the microarray analysis showed that 55 genes were down-regulated, and 88 were up-regulated.

We used mutants for all 143 genes that were previously made in our lab, and we treated them with the same acidic pH of 6.3. We observed that genes related to the ABC type metal transport mechanism, including ΔSSA_0260 (SsaB), ΔSSA_0261, and ΔSSA_0262, were found to be sensitive to acidic stress. Another interesting observation in regards to SsaB was that the loss of SSA_0260 dramatically reduced endocarditic virulence [26]. To correlate with this study, our observations say that SsaB is involved in acidic resistance. In relation to this family of proteins this group also examined the *sloABCR* operon *of S. mutans* required for endocarditic virulence [27]. In addition to this, ABC type iron transporters are also involved in the oxidative stress resistance of *S. pyogenes* [28]. Based on our observations, SSA_0204 may regulate the activity of SSA_0260, SSA_0261, and SSA_0262 allow *S. sanguinis* to resist the acidic stress. Unfortunately we could not found any orthologs for SSA_0204 and SSA_0217.

In conclusion, the present study clearly demonstrates that a number of TCS in *S. sanguinis* are essential for recognition of and response to various environmental stresses. Since TCS are involved in the regulation of virulence genes and biofilm formation, a complete picture of the regulatory network of TCS-regulated virulence genes may reveal novel signaling pathways. *S. sanguinis* has the ability to bind to saliva coated teeth during the progression of dental caries and create a less hospitable environment for other species that may cause dental caries. Therefore, we can potentially use ΔSSA_0204 or

ΔSSA_0217 mutant strains to allow bacterial growth in stress condition and prevent dental caries caused by other bacteria.

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