A GENE CLUSTER INVOLVED IN STRESS TOLERANCE, (P)PPGPP METABOLISM AND GENETIC COMPETENCE IN *Streptococcus mutans*

By

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To my baby Kymani, you changed my world, Miguel, mom, dad, sister and family.

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LIST OF ABBREVIATIONS

ΩKm	Polar kanamycin resistance gene cassette
ABC	ATP-binding cassette
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCAA	Branch chained amino acids
BHI	Brain heart infusion
BIP	Bacteriocin immunity protein
BM	Base media
BP	Base pairs
CAT	Chloramphenicol acetyl transferase
CSP	Competence stimulating peptide
DTNB	Dithionitrobenzoic acid
DTT	Dithiothreitol
dsDNA	double stranded DNA
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
GTP	Guanosine triphosphate
HK	Histidine Sensor Kinase
IDV	Integrated density values
kDA	kiloDalton
MAMA	Mismatch amplification mutation assay
MarR	Multiple antibiotic resistance regulator
NPKm	Non-polar kanamycin resistance cassette
O/N	Overnight

OD	Optical density
ONPG	ortho-Nitrophenyl-β-galactosidase
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEP-PTS	Phosphoenolpyruvate:sugar phosphotransferase system
ppGpp/GP4	Guanosine 3', 5'-bispyrophosphate / tetraphosphate
pppGpp/GP5	Guanosine 3'-diphosphate, 5-'triphosphate / pentaphosphate
qRT-PCR	Quantitative real-time polymerase chain reaction
QS	Quorum sensing
RBS	Ribosome binding site
RNAP	RNA polymerase
ROS	Reactive oxygen species
RR	Response regulator
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
ssDNA	Single stranded DNA
SOE	Splice extension overlap
TCA	Tricarboxylic acid
TCS	Two-component system
TLC	Thin layer chromatography
XIP	ComX inducing peptide

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Dental caries is one of the most common infectious diseases, and costs associated with oral care can exceed $70 billion annually. *Streptococcus mutans*, the primary etiological agent of dental caries, has evolved multiple strategies to become established as a constituent of pathogenic biofilms and to cause caries. One of the key factors contributing to the virulence of *S. mutans* is its ability to tolerate environmental stresses and to thrive at low pH, when the growth of many other plaque bacteria is inhibited. The studies provided here identified a previously uncharacterized gene locus, *SMu0835-7*, that was renamed *rcrRPQ* for *r*el-*c*ompetence-*r*elated that is involved in stress tolerance, (p)ppGpp metabolism and genetic competence. *RcrR (SMu0835)* encodes a *m*ultiple *a*ntibiotic *r*esistance family transcriptional regulator (MarR), an autogenous dominant regulator of the operon, and *rcrPQ* (*SMu0836-7*) encode ATP-dependent efflux pumps. Mutations in *rcrPQ* affected the ability of the organism to grow, especially at low pH and in the presence of oxidative stresses. Optimal expression of *relP,* which encodes the synthetase that is the primary source of (p)ppGpp during exponential growth, required *rcrRPQ* and the levels of (p)ppGpp accumulated in exponentially

growing cells were also affected in *rcrRPQ* mutants. It was also found that oxidative stressors caused an increase in (p)ppGpp pools in a RelPRS-dependent manner.

Various mutations made in the *rcrRPQ* operon led to changes in the ability of the cells to be transformed with exogenous DNA. Replacement of *rcrR* with a non-polar antibiotic resistance cassette resulted in over expression of *rcrPQ* s and rendered the strain non-transformable with exogenous DNA. Transcriptional analysis revealed that the expression of *comYA, comX* and *comS,* which are critical for competence and DNA uptake, were dramatically altered in these mutants. Global transcriptional profiling of the various *rcrR* mutants also revealed that the genes in the competence pathway were those that were most differentially regulated. Collectively, the data support that the *rcrRPQ* gene products play a critical role in physiologic homeostasis and stress tolerance by linking (p)ppGpp metabolism, acid and oxidative stress tolerance, and genetic competence of *S. mutans*.

CHAPTER 1
INTRODUCTION

Streptococcus mutans and Dental Caries

Streptococcus mutans is a Gram-positive facultative anaerobe belonging to the viridans streptococci group. The name “viridans” streptococci is derived from the green halo formed around colonies on blood agar plates, indicative of their α-hemolytic activity (49). The viridans streptococci are catalase-negative cocci that form chains and can be further divided into the mutans, mitis, salivarius, anginosus and sanguinus groups. The mutans group consists of *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus cricetus*, *Streptococcus rattus*, *Streptococcus downeii*, *Streptococcus ferus*, *Streptococcus macacae,* and can be differentiated from the other groups by their ability to ferment mannitol and sorbitol. S. *mutans* can be further sub- divided into 4 serotypes *c, e, f* and *k* based on the cell wall rhamnose–glucose polysaccharides (134, 204).

The oral microbiome is diverse with over 600 taxa present (56). Oral streptococci constitute approximately 23% of cultivable bacteria, and the mutans streptococci comprise 2-5% of the population isolated from healthy individuals (29). Dental caries is considered one of the most common bacterial infections in humans, and studies show that 90% of Americans over the age of 20 have dental caries in their permanent teeth (25). In addition to the pain and discomfort associated with dental caries, the disease poses an economic burden for treatment in the United States, with costs for oral health care treatment exceeding $70 billion annually (134). *S. mutans* is the one of the etiological agents of dental caries and is implicated in infective endocarditis (80, 92, 104). It was originally isolated from carious lesions by Clarke in 1924 (43). In Western

populations and developed countries, the most common serotype isolated from the oral cavity is serotype *c* (70-80%), followed by serotype *e* (20%), while *f* and *k* account for less than 5% (162). However, serotype *f* and *k* are the most common serotype isolated from endocarditis plaques (161). Abranches *et al.* showed that strains that were serotype *e* and *f* were more invasive of coronary endothelial cells than strains that were serotype *c* (2). *S. mutans* is able to cause disease primarily based on its metabolism, whereas some other bacteria have typical virulence factors such as toxins or effectors that cause disease and damage to the host.

S. mutans has mechanisms to adhere to the tooth surface, accumulate in biofilms, produce acid and tolerate the acid it generates (20). *S. mutans* is highly effective at producing acids from the fermentation of a wide range of dietary carbohydrates, causing a decrease in pH of the oral biofilms and demineralization of the tooth. It is the accumulation of acids that causes demineralization of the tooth enamel that leads to dental caries and tooth decay (20, 134). In addition, *S. mutans* is particularly acid tolerant (aciduric), and able to grow and to carry out glycolysis at pH values that are well below that needed to damage the tooth mineral. In fact, the property of aciduricity is considered a major contributor to the role of *S. mutans* in the initiation and progression of carious lesions, and aciduricity is a general property of caries-associated bacteria.

S. mutans utilizes many systems to adapt to the oral environment and outcompete other species in a biofilm to cause disease when the conditions are favorable. The oral cavity is continuously changing and can become a hostile environment for bacteria to survive, with rapid variations in pH, oxygen tension and nutrient availability. Bacteria must be able to respond to these fluctuations in order to survive. Carlsson first

described the “feast and famine” lifestyle of oral bacteria to illustrate that these organisms are confronted with extended periods during which saliva is the primary nutrient source, interspersed with comparatively short intervals where nutrients from dietary sources are abundant (38). *S. mutans* lives on the tooth surface at high cell density in dental plaque, and the structure and composition of the plaque are strongly influenced by factors such as pH and nutrient availability. *S. mutans* aggregates to form a protective biofilm and hence contributes to the pathogenicity of *S. mutans* to establish itself as one of the dominant bacteria in cariogenic dental plaque (33, 217). There are four main requirements for *S. mutans* to become cariogenic. The bacteria have to be able to adhere to the tooth surface and form biofilms, accumulate in sufficient numbers to produce damage to the host, generate acid by fermentation and tolerate the acidic environment it generates.

Virulence Factors of *S. mutans*

Adhesion and Biofilm Formation

Bacteria are able to adhere to the tooth pellicle, which is primarily formed from salivary glycoproteins that adhere to the tooth surface and form a thin film. *S. mutans* adheres to the tooth surface through sucrose-dependent and sucrose-independent interactions. Sucrose-independent adhesion is mediated through a surface adhesin P1(SpaP) also known as antigen I/II, which facilitates binding of *S. mutans* to the salivary pellicle (51, 115, 139), and mutants deficient in SpaP were not able to adhere to saliva-coated surfaces (30). The antigen I/II family of proteins share 7 domains, and Bleiweis *et al.* demonstrated that the alanine-rich region or A-region was able to bind salivary agglutinin (50). The proline-rich domain was also thought to be responsible for the interaction of P1(SpaP) with salivary components (20). Other surface-associated

proteins such as WapA (Antigen III) and BrpA have been implicated in the formation of biofilm independently of sucrose. A mutant that lacks the gene encoding the WapA protein had perturbed biofilm architecture (244), and Wen *et al.* showed that mutants deficient in *brpA* had significantly lower biofilm formation when cells were grown in glucose (230).

In the presence of sucrose, dental plaque biofilm becomes irreversibly bound by the formation of glucans and fructans (59). Sucrose is the primary substrate for the glucosyltransferases (GTFs) GtfB, GtfC and GtfD. The GTF enzymes consist of a glucan binding domain (20) and possess sucrase activity that split sucrose into glucose and fructose (159, 240). The glucose moiety is added to a growing polymer of glucan to synthesize water-soluble and water-insoluble glucans (160). The water-insoluble glucans have a higher degree of branching and are predominantly α-1,3-linked whereas the water-soluble glucans are linked primarily by α-1,6-glycosidic linkages. GtfB and GtfC synthesize the water insoluble glucans, while GtfD primarily synthesizes water soluble glucans. Mutants lacking GTF enzymes had diminished cariogenicity in rodents as well (111). Glucans help to facilitate adhesion to the tooth through hydrogen bonding of the glucan polymers to the salivary pellicle (20). *S. mutans* can become coated with glucans in the presence of sucrose, and it is hypothesized that *S. mutans* can attach to glucans within the dental plaque (20). Other glucan-binding proteins (GBPs) found on the cell surface are thought to assist in the adherence and biofilm formation process by binding glucans or mediating dextran-dependent aggregation (20, 21). The levels of GbpB correlate with biofilm formation (151), and a mutant that had the *gbpA* gene inactivated had changes to the architecture of sucrose-dependent biofilm (21).

Carbohydrate Metabolism

There is a correlation of dental caries development and dietary sugar intake (36). Evidence accumulated in the mid 1800s showed a link between bacterial sugar metabolism to acid production and tooth decay (140). Therefore, proteins that are involved in sugar metabolism are considered potential virulence factors of *S. mutans*. These include fructosyltransferase (Ftf), which catalyzes the synthesis of fructans that function as a nutrient reserve (234, 235), a fructanase (FruA), which breaks down fructans for energy use (35) and an extracellular dextranase (DexA), which may help contribute to the synthesis and breakdown of glucans (210). During periods of excess sugar intake, *S. mutans* accumulates intracellular and extracellular polysaccharides (IPS and EPS). IPS are glycogen-like storage polymers and contribute to caries formation and survival during nutrient starvation (34, 208), and EPS are rapidly synthesized when sucrose is present in the diet. The DltA-D enzymes, which are orthologous to the D-alanine-activating enzymes in *Bacillus subtilis*, are involved in the accumulation of intracellular polysaccharides that can be used as an energy reserve (82).

At low sugar concentrations, the phosphoenolpyruvate (PEP) sugar: phosphotransferase system (PTS) is the major system for the uptake of sugars in the cell (97, 118, 224). An incoming sugar must be phosphorylated by the PTS. A phosphate group is transferred to the sugar-specific enzyme and finally to the incoming sugar. The PEP-PTS internalizes a wide variety of sugars including glucose, fructose, mannose, sucrose and lactose. The PEP-PTS consists of two proteins, Enzyme I (E1) and HPr, which is a heat stable phosphocarrier protein. There are also sugar-specific permeases known as Enzyme II (EII) complex, which consist of the EIIA, EIIB and EIIC

domains. A phosphate group from a PEP molecule is transferred to EI, which phosphorylates HPr at His-15. The phosphate group is then transferred to the sugar-specific EIIA and B domains, and then transferred to the incoming sugar for transport by EIIC membrane domain (118, 182). The internalized sugar can be metabolized by various enzymes to end-products which include glucose, and can enter the glycolytic pathway (242).

Carbon catabolite repression (CCR) involves networks that activate or silence genes in response to carbohydrate source and availability. CCR is controlled by HPr and CcpA, which is a transcriptional regulator, in low-G+C content Gram-positive bacteria. During conditions that can induce CCR such as excess glucose, an HPr kinase that is activated by specific glycolytic intermediates, such as fructose-1,6-bisphosphate (F-1,6-bP) or glucose-6-phosphate, can phosphorylate HPr at serine-46 at the expense of ATP. HPr-(Ser-46-P) forms a complex with CcpA that stimulates CcpA binding to conserved catabolite responsive elements (CRE) in the promoters of a variety of genes to control their activity (3, 120). CcpA can regulate sporulation, antibiotic resistance and expression of virulence attributes.

Acidogenicity

In 1940 Stephan showed a rapid decline in plaque pH after a sugar rinse that was linked to the production of lactic acid by bacteria (212). *S. mutans* can produce lactate, formate, acetate and ethanol as fermentation by-products from glycolysis. The acidogenicity of *S. mutans* causes a reduction of plaque pH, and sustained plaque pH values below 5.5 favor the demineralization of enamel and dental caries (120). Lactic acid has a low pKa and is able to demineralize the tooth surface more effectively than other end products such as formate or acetate. The distribution of the fermentation

products changes as growth conditions change. When there is an excess of carbohydrate source, lactic acid is the dominant glycolytic end-product whereas formate, acetate and ethanol are predominant in glucose limiting conditions (238). *S. mutans* has the ability to open a lactate gate which protects the bacteria from sugar killing. The opening of the lactate gate enables the rapid movement of carbohydrates through glycolysis and more efficient movement of lactic acid out of the cells. The production of lactate is controlled by the NAD-dependent lactate dehydrogenase (LDH) (38). When there is an excess of carbohydrate source, NADH levels can build-up as a result of glycolysis. LDH is activated by fructose 1,6-bisphosphate, a glycolytic intermediate, to catalyze the conversion of pyruvate to lactate, generating NAD^+ from NADH. Mutations in *ldh* appear to be lethal in *S. mutans*, probably because of the accumulation of glycolytic intermediates and an imbalance in NADH/NAD^+ (42, 88). Strains that have reduced LDH activity have reduced cariogenicity (42, 65, 102).

In carbohydrate-limiting conditions, *S. mutans* is able to survive and the major end products shift from lactate to formate, acetate and ethanol and *ldh* is not induced. The shift in glycolytic end-products is controlled by the pyruvate formate lyase enzyme (PFL), which can convert pyruvate and coenzyme A (CoA) into formate and acetyl-CoA. However, PFL is not active when oxygen is present, and the pyruvate dehydrogenase complex (PDH) is active (39). Pyruvate can then be converted to acetyl-CoA and CO_2 and NADH is generated (18). Acetyl-CoA can be converted to acetyl-phosphate (acetyl-P). Acetate can then be produced from acetyl-P yielding one molecule of ATP by the enzyme acetate kinase (39). The inactivation of *pdh* impairs the survival of the bacteria in limiting sugar (37).

Aciduricity

In addition to the generation of acids, *S. mutans* is able to tolerate low pH, which distinguishes it from many other oral bacteria and gives it a selective advantage to become a dominant colonizer over other oral bacteria that cannot endure acid as well (118). The bacteria are able to retain glycolytic capabilities at pH values as low as 4.4 (27). The pH of saliva is around neutral, which is optimal for the growth of most oral bacteria (143). However, after an intake of dietary sugars, the pH rapidly decreases as a result of the metabolism of the dietary sugars. *S. mutans* has many strategies to cope and function at this low pH.

F-ATPase. The major mechanism for coping with the low pH is through the extrusion of protons by the F-ATPase (H^+-translocating ATPase), which maintains the internal pH more alkaline compared to the environment (26). Acid-sensitive glycolytic enzymes are protected through the acid tolerance response, and a ΔpH for bioenergetic processes is maintained. Studies have shown that as the pH falls, the activity of the membrane bound F_1F_0-ATPase proton pump increases, helping to maintain the pH at Δ0.5-1 relative to the external environment (81, 215). The optimal pH for the F-ATPase enzyme in *S. mutans* is 6.0, compared to less acid-tolerant bacteria where the optimal pH for the ATPase enzymes is more neutral (184). Also, it has been shown that the F-ATPase can function as an ATP synthase in starved cells at low pH. A sudden drop in pH causes a rapid increase in ATP levels, which was demonstrated to be the result of the F-ATPase acting as an ATP synthase (203). Therefore, the F-ATPase may not only be playing role in proton extrusion, but may play a role in generating ATP for growth (203).

Shift in membrane profile. The fatty acid profile of the membrane shifts from short-

chained saturated fatty acids to mono-unsaturated fatty acids and longer chains as the pH falls (69). The change in the fatty acid membrane profile leads to decreased permeability to protons, which may influence the activity of the F-ATPase (188). The *fabM* gene product is responsible for the generation of mono-unsaturated fatty acids (68), and mutants lacking this gene, were more sensitive to low pH and unable to maintain ∆pH. The *fabM* mutants also exhibited reduced virulence in a rat caries model compared to the parental strain (68).

Membrane biogenesis is critical to stress tolerance as well. Strains that had mutations to genes encoding proteins involved in D-alanyl-lipoteichoic acid synthesis and phospholipid metabolism were incapable of surviving at low pH (31, 241). A strain with a mutation in the *ffh* gene, which encodes a homologue of the eukaryotic signal recognition particle (SRP), was incapable of growing at pH 5 (109). In addition, the membrane-localized chaperone YidC, which is involved in the assembly of membrane proteins, had impaired growth in a variety of stress conditions, including low pH (84, 120).

Alkaline generation. Some oral bacteria are able to produce ammonia by urease enzymes or the arginine deiminase system (ADS) in response to a drop in pH (120). These organisms are able to convert urea or arginine to produce CO_2 and ammonia. *S. mutans* lacks urease and the ADS pathway, so it is not able to generate alkali as efficiently, but it does possess an analogous system. The agmatine deiminase system (AgDS), which is able to convert agmatine, a derivative of arginine, to produce putrescine, ammonia and CO_2. The AgDS is expressed at relatively low levels and does not appear to cause a significant rise in the pH of the environment, as is seen with

the ADS system (72, 73). The ammonia produced from the AgDS in *S. mutans* may be important in increasing the cytoplasmic pH and generating ATP to extrude protons, especially when the bacteria are faced with an acid challenge (73, 74).

Induction of genes for DNA repair. The glycosidic bonds of deoxyribonucleotides are unstable at low pH, so a buildup in acid can cause loss of purines and pyrimidines from DNA due to protonation of the base followed by the cleavage of the glycosyl bond that leave abasic sites (AP) (132). There is an induction of the DNA repair AP endonucleases, which recognize these AP sites in response to low pH (77). Molecular chaperones which are induced for bacteria to cope with different stresses, prevent aggregation and accumulation of improperly folded proteins that may be toxic for the bacteria. In *S. mutans,* the molecular chaperones GroEL and DnaK are rapidly induced by acid shock (99), and mutants that had low levels of the genes encoding these chaperones had impaired capacity to grow at low pH, and even hydrogen peroxide. The induction of DnaK is maintained throughout acidic conditions, and it is proposed that DnaK has a role in the biogenesis of F-ATPase (100, 121). There is also the induction of another stress protein, ClpP peptidase, which may be involved in acid tolerance by preventing the accumulation of denatured proteins and modulating the stability of transcriptional regulators (121). Trigger factor RopA, is a ribosome-associated peptidyl-proly *cis-trans* isomerase molecular chaperone that is conserved in most bacteria. Trigger factor in *S. mutans* is upregulated in response to a deficiency of *luxS*, which affects acid and oxidative stress tolerance and biofilm formation. The expression of *ropA* was also increased in cells stimulated by the competence stimulating peptide and in populations grown in biofilms which suggest that it may have

a role in stress tolerance, competence development, and biofilm formation. Mutants deficient in *ropA* also had diminished tolerance to low pH and oxidative stress (232).

Environmental Stresses in the Oral Cavity and Strategies of *S. mutans* to Overcome These Stresses

Oxidative Stress Tolerance

Oxygen is a critical environmental factor that affects the composition of oral biofilms. Exposure to oxygen severely impaired biofilm formation in *S. mutans* and altered cell surface biogenesis, which may be due to changes in the exopolysaccharide metabolism or in cell-to-cell adherence (5). *S. mutans* is a facultative anaerobe and can metabolize oxygen, but it is catalase-negative, lacks a complete TCA cycle and respiratory chain, and has a limited capacity to metabolize reactive oxygen species (ROS). Streptococci do not possess cytochromes and do not carry out oxidative phosphorylation. Most of the respiration is carried out by NADH oxidases, which reduce molecular oxygen to oxidize NADH to NAD^+ and H_2O_2 (142). *S. mutans* is constantly exposed to oxidative stress agents. These stresses include ROS from host defenses, peroxide-containing oral hygiene products, Fenton chemistry [(1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$; (2) $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH^{\bullet} + H^+$] and the production of hydrogen peroxide by other oral bacteria (118). ROS are generated inside the cell during respiration from single cell electron reductions of oxygen from the host and other oral bacteria. Even though *S. mutans* lacks a catalase enzyme, it does have a superoxide dismutase, NADH peroxidase, glutathione reductase and alkyl hydroperoxide reductase to help cope with oxygen stress (142). NADH peroxidase can convert NADH +H+ H_2O_2 to NAD^++ $2H_2O$ (142). Iron ions stimulate the generation of toxic ROS such as hydroxyl radicals and hydrogen peroxide from the Fenton reaction. Dpr is a member of the iron-

binding protein family, which allows the concentration of free iron ions to be kept low and hence plays an important role for oxygen tolerance by *S. mutans* (239). There are other genes found in the *S. mutans* genome that encode proteins belong to the OxyR, PerR and OhrR families that have been implicated in responding to oxidative stress (118).

Two Component Systems

Two-component systems (TCS) are important for *S. mutans* to monitor and adapt to changing environmental conditions. TCS allow bacteria to modulate gene expression based on a wide variety of environmental signals, such as osmotic shock, pH variations, host-pathogen interaction and other stresses (24). TCS consist of a membrane-bound histidine sensor kinase (HK) that detects environmental signals and undergoes autophosphorylation. The other component in TCS is usually a cytosolic DNA-binding response regulator (RR) that binds to the promoter regions of genes to alter their expression. The HK domain detects environmental signals, which results in autophosphorylation at a specific histidine residue, creating a high-energy phosphoryl group that is transferred to a specific aspartate residue within the N-terminal half of the cognate RR. Phosphorylation induces a conformational change in the regulatory domain resulting in activation of the RR. The activated RR then regulates gene expression by acting as a DNA-binding transcriptional regulator to activate or repress genes (214). There are fourteen putative TCS found in the *S. mutans* UA159 genome. *ComCDE* encodes one of the TCS, which are involved in the development of competence, biofilm formation, bacteriocin production and (p)ppGpp metabolism (9, 205).

Quorum Sensing

Bacteria can communicate in a cell-cell dependent manner through quorum sensing (QS). Quorum sensing bacteria interact with each other by releasing and responding to the accumulation of chemical signal molecules called autoinducers (228). Bacteria utilize these signals to coordinate their behavior on a population-wide basis. The first quorum-sensing system was described in the bioluminescent organism, *Vibrio fischeri* (85). In this bacterium, quorum sensing is controlled by LuxR and LuxI, where LuxI is the autoinducer synthase that produces an acyl-homoserine lactone (AHL). When the signal reaches a critical threshold extracellularly, it is internalized and bound by LuxR, and the complex activates the transcription of the operon encoding luciferase (228). In Gram-negative bacteria, QS is controlled by small molecules called autoinducers (AI), which can be AHLs or other molecules whose production is dependent on *S*-adenosylmethionine (SAM) as a substrate (228). AIs are produced intracelluarly and are able to diffuse across the inner and outer membranes freely. When the concentrations of AI reach a critical level, they can bind cytoplasmic receptors. The receptors that have AI bound can then regulate genes in the QS regulon. Some Gram-negative bacteria are also able to detect AIs by TCS.

In Gram-positive bacteria, QS is controlled by TCSs. There are no known regulatory processes that involve the *N*-acyl homoserine lactone-like signal molecule (AHL) or the LuxI-LuxR system found in Gram-negative bacteria (228). The signaling peptides are referred to as autoinducing peptides (AIP). AIPs are produced intracellularly, processed and secreted, unlike Gram-negative bacteria where the AIs are diffusible across the membrane. When the extracellular concentrations reach a critical level they are detected by the HK domain of TCSs to activate the RR and genes

in the QS regulon.

The LuxS system, which can mediate communication within and between species, is controlled by the *luxS* gene encoding the autoinducer AI-2. The *luxS* gene encodes the AI-2 synthetase and is highly conserved across Gram-negative and Gram-positive bacteria and present in about half of the sequenced bacterial genomes (228). LuxS is involved in the catabolism of *S*-adenosylmethionine and converts ribose homocysteine into 4,5-dihydroxy-2,3-pentanedione, which is the precursor for AI-2. The *luxS* gene in *S. mutans* appears to have an important regulatory role and impacts virulence. Strains that were defective in *luxS* had impaired biofilm formation compared to the wild-type strain (231) and the production of bacteriocins was affected (154).

Some Gram-positive organisms such as *Bacillus subtilis*, have two autoinducing peptides that function in a network that allow the organism to commit to either competence or sporulation, which are two mutually exclusive lifestyles (228). The competence pathway, which will be described in more detail below, is activated by a secreted factor ComX, that is detected by a histidine sensor kinase ComP, which autophosphorylates the response regulator ComA (141). Phosphorylated ComA regulates the transcription of genes necessary for the development of competence. ComA has an antagonist protein, RapC, which when bound to ComA inhibits the development of competence (206). Another autoinducer, competence and sporulation factor (CSF) encoded by *phrC* is secreted and at low internal concentrations CSF binds to the RapC promoter and disrupts RapC binding to ComA, thus promoting the development of competence (206). At high levels, CSF inhibits the ComP-ComA signaling cascade through an unknown mechanism, decreasing the development of

competence and promoting sporulation. So the same CSF signal can cause the bacteria to commit to either competence or sporulation depending on its internal concentration.

In *S. mutans* the induction of competence is one of the main QS systems and is controlled by the accumulation and sensing of peptides by the ComCDE TCS system described below. Biofilm formation, the acid tolerance response and bacteriocin production are controlled by quorum sensing as well.

Genetic Competence

Some bacteria encode genetically programmed machinery to take up DNA from their environment, which is known as genetic competence. The phenomenon was first described by Griffith in 1928 where he observed that he could transfer a virulence factor from a virulent strain to an avirulent strain of *Streptococcus pneumoniae*. The substance extracted from *S. pneumoniae* isolated from diseased mice was able to change the morphology of an avirulent strain (71). Avery *et al.* noted that the substance was DNA (17). Genetic competence and DNA uptake have been linked to adaptation and survival by providing resources and increasing genetic diversity in bacterial species. Competence has been shown to increase the survival of *S. pneumoniae* when challenged with an antibiotic stress, and the induction of competence is also regulated by increased frequency of translations errors (61, 213).

In *S. pneumonia*e, one of the better studied model organisms for the regulation of competence, the competence regulon is controlled in a quorum sensing-like manner, mediated by a 17-aa peptide signaling molecule called competence stimulating peptide (CSP) (87, 144). CSP is derived from ComC, which is a 45-aa protein that is processed post-translationally. ComC is exported by an ATP-binding cassette transporter ComAB,

which cleaves the leader peptide after the Gly-Gly motif to the processed 17-aa peptide CSP (94). CSP is sensed by the TCS system consisting of *comD,* which encodes the histidine kinase sensor, and *come,* which encodes the response regulator (176). ComE controls the expression of *comX,* which encodes an alternative sigma factor (114), potentially through a direct interaction at the ComE-binding site present within the *comX* promoter. The ComX alternative sigma factor is able to activate genes that have the *comX*- box consensus sequence in their promoter region. These genes include the late competence genes encoding proteins involved in DNA uptake and internalization such as *comYA* (136, 138). ComW was identified as another factor that plays a role in regulating competence by controlling the stabilization and activation of ComX. ComW prevents ClpP-dependent degradation of ComX, thereby activating competence (144). ComW is also believed to activate the competence pathway through unknown mechanisms.

The genes encoding the proteins involved in DNA uptake are considered late competence genes and include *comEA*, *comFA*, the *comYA* operon and the genes encoding the proteins involved in the processing of internalized single-stranded DNA such as *coiA*, *recA* and *ssbB* (53, 112). In Gram-positive bacteria, the ComG proteins span the cell wall and form the assembly of a pseudopilus and ATPase. In *S. mutans*, *comG* is part of a nine-gene operon with *comYA* that make up the genes encoding the proteins for forming the pseudopilus (52). Disassembly of the pseudopilus opens a cell wall channel and enables dsDNA to diffuse from the surface to the DNA binding protein, ComEA, which is found in the cytoplasmic membrane (112). In *S. pneumoniae* and *B. subtilis*, dsDNA is bound preferentially over ssDNA and has about 200–fold higher

transformation activity in *S. pneumoniae* for reasons that are not completely understood (45). Once the dsDNA is bound, fragmentation of the dsDNA to ssDNA by EndA occurs (52) and ssDNA fragments are transported across the membrane and the non-transported strand is degraded (45). A highly conserved cytoplasmic membrane channel ComEC, which is present in all known competent bacteria, enables the transport of ssDNA across the cytoplasmic membrane. The ComFA protein is a DEAD-box helicase that is involved in DNA translocation into the cytoplasm in *B. subtilis* (112). Other proteins such as DprA are involved in protecting the ssDNA from endonucleases, and one of the putative functions of SsbB is to prevent back diffusion by binding the ssDNA (15). The RecA protein is required for recombination of the ssDNA. The CoiA protein is also a late competence protein that is implicated in DNA uptake in *S. pneumoniae (54).* Plasmid DNA that has no homologous regions to the transforming bacteria is still transported and processed the same as chromosomal DNA in streptococci. However, it requires recirculization, which makes it less efficient to transform compared to chromosomal DNA (52).

In *S. mutans,* competence, biofilm formation and stress tolerance have been intimately linked. Mutations in the genes of the competence pathway caused attenuated virulence and reduced cariogenicity in rodents (130), and similar mutations in the competence pathway caused reduced biofilm formation (129, 178). The competence pathway in *S. mutans* has some similarity to the competence pathway in *S. pneumonia,* but the *comCDE* genes in *S. mutans* appear to have evolved from a different ancestral gene and appear to be more closely related to genes in *S. pneumonia*e and related organisms that control the production of bacteriocins (144). In

fact, the ComCDE system has been shown to regulate the bacteriocins (which are described in more detail below) (110). Additionally, there are no ComE binding sites in the promoter region of *comX,* and studies thus far indicate there is no direct binding of ComE to the *comX* promoter (95), so the precise mechanism of the ComDE-dependent regulation of *comX* and competence is enigmatic. The active form of CSP is considered a 21-mer (128, 130). However, a processed 18-aa peptide form that has 3 amino acids cleaved is more active in inducing competence (177). Recently, it has been shown that a membrane-associated protease SepM is responsible for the final processing of the 21-mer to the 18-mer form (93).

There are also other signals and factors that influence competence development and DNA uptake in *S. mutans*, many of which are unknown (Figure 1-1). In *Streptococcus thermophilus,* a ComR/S system, which consists of an Rgg-type regulator and autoregulatory peptide ComS, was shown to be the main regulator of *comX (66)*. Morrison *et al.* identified a similar ComR/S system in *S. mutans* and *Streptococcus pyogenes* (149), and it has been shown that ComRS acts as the proximal regulator of *comX* in these streptococci. The ComR regulator is required to activate *comX* and induce competence through an autoregulatory signal, com*X* *i*nducing *p*eptide (XIP), which is processed from the ComS protein. The *comS* gene is located immediately downstream of *comR.* ComS is a 17-aa precursor that is exported through unknown exporters, but is processed extracellularly to the heptamer XIP. XIP is imported through the OPP permease and complexes with ComR to regulate *comX* and activate competence. ComR activated by XIP can regulate *comS*, to induce an autoregulatory feedback loop. The Morrison group showed that the promoter activity of

comX required both XIP and *comR* (149). They could not detect *comX* promoter activity in strains that had *comS* or *comR* mutated. Khan *et al.* were able to identify the heptamer XIP in late exponential phase culture supernates of *S. mutans* that were grown in defined media further demonstrating that the processed heptamer is generated (105). There is a *comX* consensus sequence present in the promoter regions of both *comR* and *comX*, suggesting that there is direct interaction of ComR with the promoter region of *comX.* A recent publication shows that ComR is able to bind to the promoter of *comS* and *comX* in the presence of XIP and other peptides in *S. thermophilus* (67). A potential ComR binding site was also identified in the promoter region of *comX* and *comS* in *S. mutans*. Of note, the mutans group is the only *Streptococcus* group to have both the ComCDE and ComRS system. The other competent streptococci either have the ComCDE system or the ComRS system exclusively (86). In fact, the two systems are activated in different conditions in *S. mutans*. The ComCDE and CSP pathway is able to activate *comX* and competence in rich medium, whereas the ComRS pathway and XIP are active in chemically defined media (55, 207). This further demonstrates that the competence pathway in *S. mutans* is regulated by diverse signals and environmental conditions.

Other pathways that control competence in *S. mutans* include the serine protease HtrA, which has a negative effect on competence, but these effects can be suppressed by the addition of CSP to cultures (6). In *S. pneumonia*e the negative effects of HtrA on competence could be attenuated when there is an increased frequency of translational errors and misfolded proteins (213). It was hypothesized that the structure of CSP resembled misfolded proteins and thus becomes a target for HtrA (63), causing a

negative effect on competence. The addition of excess CSP was able to overcome the negative effects of HtrA degradation. Ahn *et al.* also showed that the development of competence and the genes in the competence pathway are influenced by the CiaRH two component system and may be regulating competence through unknown mechanisms (8). Deletions in *ciaRH* caused loss of transformability and affected the expression of some of the genes in the competence pathway. The late competence genes in *S. mutans,* such as *comYA* are also under the influence of the IrvR and IrvA transcriptional regulators, which act independently of *comX* and are dependent on the cell density of the population. IrvR is a transcriptional regulator that represses the expression of *irvA,* which encodes another transcriptional regulator that directly represses the late competence genes (167). So the IrvA and IrvR regulators serve as another mechanism of control of competence in *S. mutans*. Other factors, including the VicRK TCS, HdrMR, also influence transformation efficiency and *com* gene expression in ways that have not been completely elucidated (200).

Bacteriocins and Immunity Proteins

Bacteriocins are ribosomally-produced antimicrobial peptides produced by some bacteria, to compete against bacteria of similar species for common resources (48). Unlike other antibiotics, such as penicillin and tetracycline, bacteriocins have a relatively narrow spectrum. Most bacteriocins are very potent and can exhibit antibacterial activity at nM concentrations. In Gram-positive bacteria, bacteriocins are pore-forming and are generally classified into two classes, lantibiotics (class I) and non-lantibiotics (class II) (49). The lantibiotic group usually interacts with lipid II and inhibits cell wall synthesis, whereas the non-lantibiotic group disrupts membrane potential and causes essential molecules to escape (166). Lantibiotics contain post-translationally modified

residues and are characterized by dehydrated amino acids and intramolecular thioether bonds that create lanthionine and methyllanthionine residues within the bacteriocin molecule. Lantibiotics are very stable and highly resistant to inactivation under a wide range of environmental stresses (166). The lantibiotics may be further classified based on their tertiary structures such as Type A, which are linear or Type B which are globular. The type A lantibiotics kill susceptible cells primarily through membrane pore formation. Non-lantibiotics consist of non-modified residues except the presence of disulfide bridges. The non-lantibiotics group is further divided; the Class IIa, IIb, IIc and IId bacteriocins. The Class IIa bacteriocins contain an N-terminus consensus sequence (YGNGVxCxxxxCxVxWxxA). Class IIb consists of bacteriocins whose activity is dependent on the action of two different peptides. Class IIc bacteriocins are cyclic bacteriocins with a ring structure formed in a head-to-tail manner. The Class IId bacteriocins consist of linear non-pediocin-like one-peptide bacteriocins (49).

Bacteriocins produced by *S. mutans* are usually referred to as mutacins (79) and may help it compete against other oral streptococci in early dental plaque and maintain colonization of the tooth surface (155). The mutacins are from both the lantibiotic and non-lantibiotic group, but are primarily from the non-lantibiotic group (11, 103). The lantibiotic mutacins have a wide spectrum against Gram-positive bacteria, and the non-lantibiotic group is primarily active against closely related species. The lantibiotic group of mutacins can be divided into mutacin I, II and II (89, 186), whereas the non-lantibiotic group are mutacins IV, V, VI and N (19, 78, 185, 237) and are present in every *S. mutans* strain analyzed thus far (155). One of the main identifying features is the presence of a conserved peptide leader region with a glycine-glycine motif. The non-

lantibiotic group is primarily controlled in a quorum sensing like manner and is regulated by the ComCDE pathway, whereas the lantibiotic mutacins are probably regulated by the ScnRK-like sensory system. The non-lantibiotic bacteriocins and the regulation of competence have considerable overlap. Dufour *et al.* hypothesized that the non-lantibiotic bacteriocin CipB functions as a regulator for the transcription of genes in the competence pathway, and a *cipB* mutant was poorly transformable (57). In addition, some of the mutacins were shown to be activated by CSP, which included the gene encoding CipB (110, 175, 225). Cvitkovitch *et al.* show that purified ComE protein is able to interact with the promoter of a number of bacteriocins which include *nlmAB* and *nlmD* (95).

There are other proteins, such as HdRM and BrsRM, which are referred to as LytTR regulatory systems that regulate mutacin production independently of ComCDE *(168)*. The LytTR systems consist of a membrane-bound protein inhibitor that antagonizes the activity of an associated LytTR family transcriptional regulator (R). The mutacins are influenced by environmental cues such as nutrient availability and oxygen. Transcriptional analysis shows that the bacteriocins are the most upregulated genes in response to aeration (7).

To prevent the bacteria from killing themselves with their own bacteriocins, the organism has genes encoding bacteriocin immunity proteins (BIPS), often found in the same operon or downstream of the bacteriocin-encoding gene. In general, BIPS act specifically towards their cognate bacteriocin (166). The mechanisms for conferring immunity are not clear, and there appears to be a variety of ways in which immunity proteins can act. For class II bacteriocins, transporter proteins that can extrude the

bacteriocins seem to also be involved in conferring immunity. For example immunity to nisin is conferred through the sequestering of the bacteriocins and expulsion through ABC exporters (189, 211). Some BIPS bind to the bacteriocin-receptor complex to block pore formation by the bacteriocins (169). In *S. mutans,* the BIPS affect antimicrobial sensitivity (150). There is also the induction of the gene encoding an immunity protein, CipI, in response to high levels of CSP, to protect the cells against CipB, which is also induced in response to high levels of CSP (175).

Nutrient Limitation and (p)ppGpp Production

During periods of amino acid starvation, there is an induction of the stringent response, which involves the accumulation of the nutritional alarmones, guanosine 3'-diphosphate, 5'-triphosphate (pppGpp or GP5), and guanosine 3',5'-bispyrophosphate (ppGpp or GP4) collectively known as (p)ppGpp (40). (P)ppGpp is synthesized by enzymatic phosphorylation of GDP or GTP from ATP [(197) (Figure1-2]. The induction of (p)ppGpp synthesis is important in regulating the physiology and metabolism of bacteria that are sensitive to constantly changing environments (183). In the absence of this functional stringent response there is an increase in translational errors and the limited energy resources are rapidly depleted. The accumulation of (p)ppGpp alters the expression of a large number of genes in bacteria by downregulating genes for macromolecular biosynthesis while upregulating genes for protein degradation, amino acid biosynthesis and stress tolerance (22, 23, 190, 218, 223). Therefore, the accumulation of (p)ppGpp is important in maintaining genomic stability by inhibiting DNA replication and serves as a mechanism to limit excessive protein synthesis during nutrient limitations until favorable conditions are restored (218). In *Escherichia coli*, (p)ppGpp helps maintain genomic integrity by resolving conflicts between replication

and transcription, by stalling RNAP in concert with DksA (108) and by inhibiting primase, which is essential for the DNA replication machinery (226). The induction of (p)ppGpp also inhibits translation by repressing transcription of tRNA, rRNA and ribosomal proteins.

The accumulation of (p)ppGpp causes a reduction in GTP levels and an increase in ATP levels. The reduction in GTP levels occurs through the inhibition of IMP dehydrogenase by (p)ppGpp, which is the first enzyme in GTP biosynthesis. This leads to an increase in IMP, a precursor for ATP synthesis, resulting in elevated levels of ATP. GTP and ATP are well known gauges for the energy capacity of the cell, and one group demonstrated in *B. subtilis* that the position of either an adenine (A) or guanine (G) at the transcriptional start site determined the regulation of a gene by (p)ppGpp accumulation (222). Since the levels of ATP are increased during a (p)ppGpp-induced stringent response, the genes where transcription started with an A were positively regulated by (p)ppGpp levels, whereas those that started with G were negatively regulated by (p)ppGpp pools. Interestingly, some of these genes involved in positive regulation by (p)ppGpp accumulation and started with A were those involved in branched-chain amino acid synthesis, whereas genes involved in energy metabolism were under negative regulation by (p)ppGpp levels (222).

CodY is a GTP-sensing global nutritional repressor that is highly conserved in Firmicutes. In *B. subtilis*, CodY regulates branched- chain amino acid operon expression, and there have been reports that show a connection between CodY and (p)ppGpp levels (96). The reduction of GTP levels during a stringent response is sensed by CodY, and causes its deactivation leading to derepression of genes such as

the BCAA operon (96). However, *in S. mutans* inactivation of the (p)ppGpp synthetase enzymes did not alleviate CodY repression (124).

Enzymatic Control of (p)ppGpp Metabolism

In Gram-negative bacteria, there are two enzymes responsible for controlling (p)ppGpp production: a synthetase enzyme called RelA and a hydrolase enzyme called SpoT, with weak synthetase activity (70, 183) (Figure 1-2). RelA synthetase enzyme activity is induced by the binding of uncharged tRNAs to the ribosomal A-site during amino acid starvation (83, 147). The cell is able to sense the inability of tRNA aminoacylation to keep up with protein synthesis as is seen in amino acid starvation or inhibitors of aminoacyl tRNA synthases. SpoT appears to be responsible for basal (p)ppGpp production and (p)ppGpp degradation, but is responsible for (p)ppGpp synthesis in response to other stresses such as fatty acid starvation or carbon source starvation(201). Bacteria need to balance the levels of (p)ppGpp to regulate growth; an overproduction of (p)ppGpp inhibits growth by inducing a stringent response while too little production (p)ppGpp impairs the ability of the cells to respond effectively to nutritional stresses. Both the N-terminal and C-terminal domains of Rel/SpoT enzymes (RSH) enzymes are responsible for regulation of enzyme activity, particularly, the C-terminal domain, which is involved in oligomerization and regulation of the RelA protein (75). The catalytic sites of monofunctional RelA enzymes have a conserved acidic triad of residues ExDD (183, 194).

In most Gram-positive bacteria there is a single bi-functional RelA enzyme which has both (p)ppGpp synthetase and (p)ppGpp hydrolase activity (158, 195). The RelA enzyme is regulated by conformational switching between synthetase on/hydrolase off and synthetase off/hydrolase on states (91). Unlike the monofunctional RelA enzyme

found in Gram-negative bacteria, the bifunctional RelA enzyme found in Gram-positive bacteria has a conserved basic RxKD triad in its synthase catalytic site. The N-terminal domain of the bifunctional RelA protein has both synthase and hydrolase activity (98). The C-terminal domain plays a role in regulation through interaction with the N-terminal domain so that only one activity remains on while the other is switched off, and deletions in the C-terminus resulted in higher synthase activity (153). It appears that the function of RelA is not dependent on ribosomes and uncharged tRNAs like the Gram-negative RelA enzyme, but the presence of uncharged tRNAs upregulates the activity of the enzyme (16, 152). Recently it has been shown that *S. mutans* has two additional (p)ppGpp enzymes, RelP and RelQ, which have synthetase domains but no hydrolase domains (122) (Figure 1-3). Subsequently homologues of RelQ and RelP were found in *B. subtilis (163)* and identified in other streptococci and other Gram-positive bacteria (122). In *Enterococcus faecalis*, the RelQ homologue appears to be linked to vancoymycin resistance, where the *relQ* mutant was more sensitive to vancomycin than the parental strain(1, 106). In *S. mutans*, the *relQ* operon is cotranscribed with other genes, *ppnK*, *pta* and *rluE,* whose products are involved in acetate metabolism in the TCA cycle. The *ppnK* gene encodes an NAD kinase, *rluE* encodes a psuedouridine synthetase and *pta* encodes a phosphotransacetylase. Deletions of the various genes in the *relQ* operon caused the cells to grow slower than the parental strain in oxidative and acid stresses. The accumulation of (p)ppGpp was also decreased in the *pta* mutant, whereas higher levels of (p)ppGpp accumulation were seen in the *ΔrelQ* mutant (106).

RelP appears to be responsible for the bulk of the (p)ppGpp production under non-stringent conditions, whereas RelA is required for a mupirocin-induced stringent

response (123). Of note, *relP* is part of an operon encoding a TCS, called *relRS*, and a mutant lacking the *relRS* genes grows faster than the wild-type strain (122). The accumulation of (p)ppGpp by RelPRS plays a significant role in growth control of *S. mutans* and is regulated by different environmental signals than RelA. It appears that *S. mutans* has a different mechanism of regulating (p)ppGpp production from other bacteria by combining intracellular and extracellular signals through a TCS and multiple synthetases. In order for *S. mutans* to survive and thrive in the hostile and variable conditions of the oral cavity, one hypothesis suggests that (p)ppGpp modulation through RelPRS is a mechanism for *S. mutans* to sense environmental signals to limit growth and balance the production of storage compounds. However, much is still unknown about how these two novel synthetases are regulating (p)ppGpp production in *S. mutans*.

Summary

Comparisons of the transcriptional profiles of *S. mutans* growing with aeration versus anaerobically revealed that many genes are regulated by oxygen or redox potential, including bacteriocin-encoding genes, *comX* and *relP* (7) and the *SMu0835-SMu0839* gene cluster (Oralgen database http://www.oralgen.lanl.gov) (SMU.921-SMU.925 GeneBank Locus tag) located immediately upstream of *relP*. In addition, our lab reported that the uncharacterized *SMu0835-837* genes were up-regulated in response to mupirocin treatment in a (p)ppGpp-dependent manner (164), identifying a possible connection of this gene cluster to (p)ppGpp and stress tolerance.

Given the importance of (p)ppGpp metabolism, competence and stress pathways in the regulation of critical homeostatic and virulence pathways in *S. mutans* and the potential regulatory overlap of *relP* and *SMu0835-0839*, we investigated the role of the

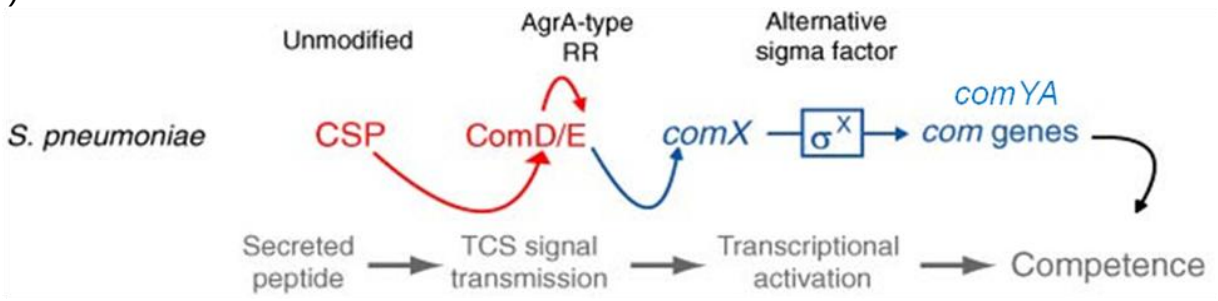
SMu0835-0837 gene cluster in growth, (p)ppGpp metabolism, stress tolerance and genetic competence (199).

Specific Aims

- Assess the role of the *SMu0835-0839* gene cluster in growth, biofilm formation and stress tolerance.
- Identify the basis for regulation of *relPRS* and the effects of *SMu0835-0837* on *relPRS* and (p)ppGpp levels.
- Determine the role of the *SMu0835-0837* on the competence regulon

Some of the work presented here has already been published by the author of the dissertation, Kinda Seaton, in the Journal of Bacteriology.

A)



Adapted from Claverys et al, Microbiol. 2006

B)

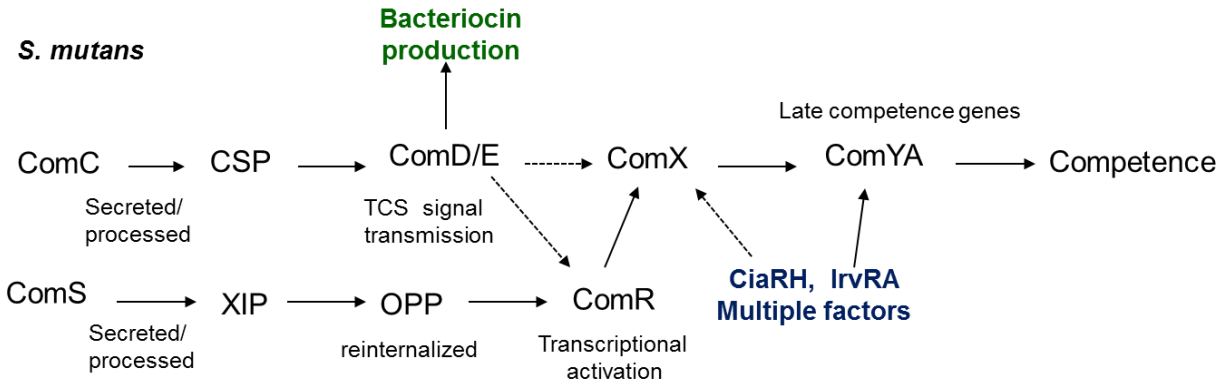
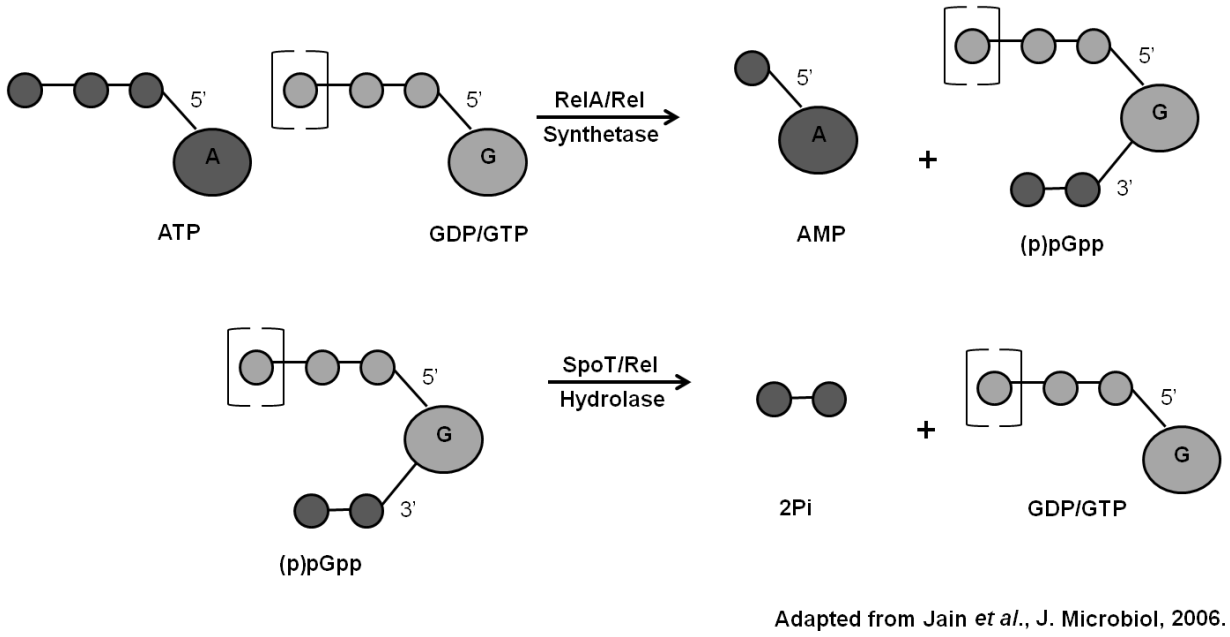


Figure 1-1. Comparison of the competence pathways between *Streptococcus pneumoniae* and *Streptococcus mutans.* A) Competence pathway in *Streptococcus pyogenes*. B) Competence pathway in *Streptococccus mutans.*



Adapted from Jain *et al.*, J. Microbiol, 2006.

Figure 1-2. Schematic showing the regulation of (p)ppGpp by RelA and SpoT in Gram-negative bacteria. RelA can synthesize the production of (p)ppGpp from GDP or GDP and ATP. SpoT can hydrolyze (p)ppGpp to GDP or GTP.

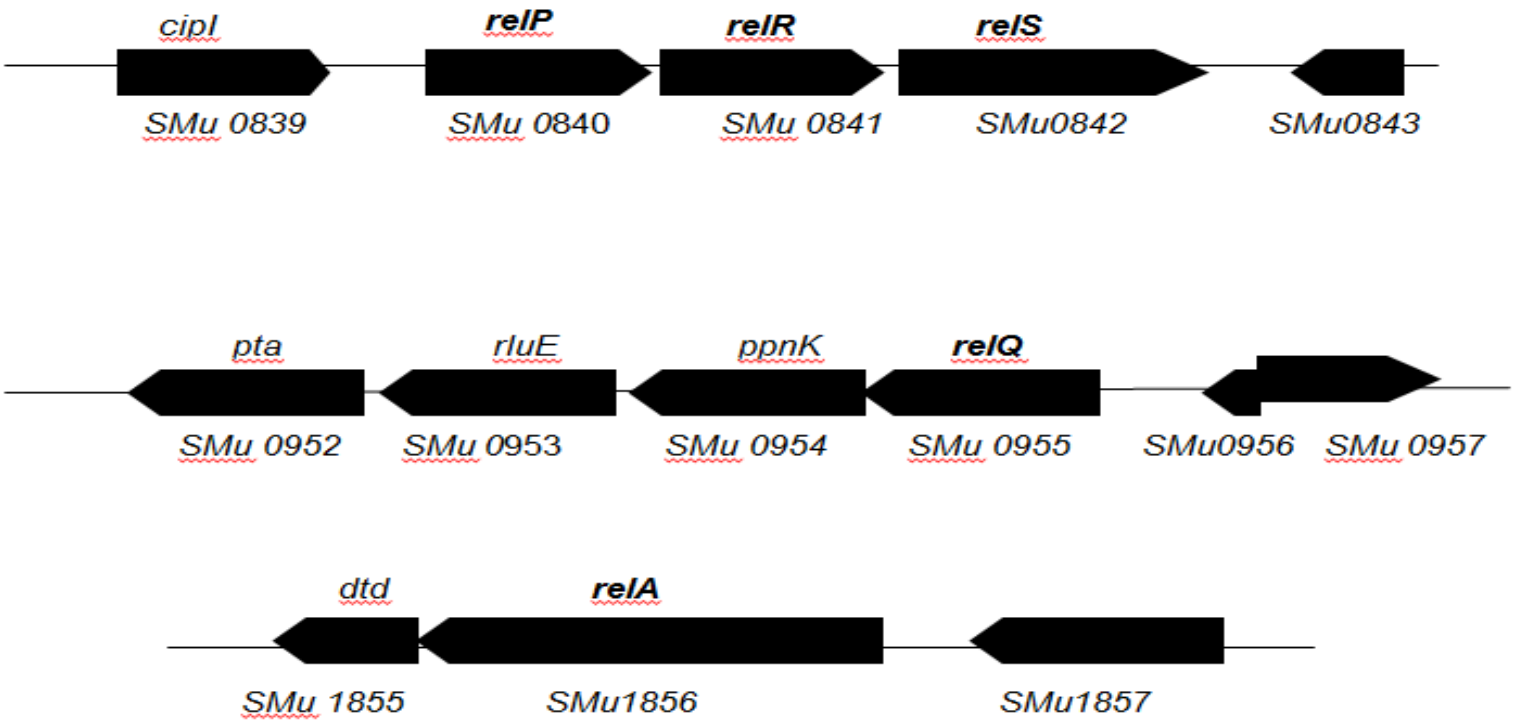


Figure 1-3. Schematic of the genes encoding the three (p)ppGpp enzymes in *S. mutans*

CHAPTER 2
MATERIALS AND METHODS

Growth Conditions

Escherichia coli strains were grown in Luria broth supplemented with chloramphenicol (20 μg ml^{-1}) or ampicillin (100 μg ml^{-1}), when necessary. *E. coli* strains were grown at 37°C with continuous shaking at 250 rpm. *S. mutans* UA159 and its derivatives were maintained in brain heart infusion (BHI) medium (Difco Laboratories, Detroit, MI) supplemented with kanamycin (1 mg ml^{-1}), spectinomycin (1 mg ml^{-1}) or erythromycin (2 μg ml^{-1}) when necessary at 37°C in a 5% CO_2 atmosphere.

For biofilm assays, a semi-defined biofilm medium (BM) (135) supplemented with 10 mM sucrose or 20 mM glucose was used.

For (p)ppGpp accumulation assays, a chemically defined medium, FMC (221), supplemented with 0.3% glucose was used.

Growth Assays

For growth studies, cultures were grown overnight in BHI broth at 37ºC in a 5% CO_2 atmosphere with antibiotics when appropriate, then diluted 1:50 into fresh BHI broth and grown to mid-exponential phase (OD_{600} = 0.5). The cultures were then diluted 1:100 in 350 μl of BHI broth in multi-well plates, overlaid with sterile mineral oil and placed in a Bioscreen C growth monitor (OY Growth Curves AB Ltd., Helsinki, Finland) at 37ºC. The optical density (OD_{600}) was measured every 30 min for 24 hours with shaking for 10 s before each reading.

Low pH. To assess the ability of the mutants to grow at low pH, cells were grown overnight in BHI broth with appropriate antibiotics when necessary for mutants, diluted 1:50 into fresh BHI and grown to mid-exponential phase at 37ºC in a 5% CO_2

atmosphere. The cells were then diluted 1:100 in BHI broth that was acidified to pH 5.5 with HCl, 350 µl of each sample was added to multi-well plates, with a sterile mineral oil overlay and growth was monitored in a Bioscreen C.

Oxidative stress. When effects of air on growth were assessed, no mineral oil overlay was utilized. For analysis of oxidative stress tolerance, the cells were grown to OD_{600} = 0.5 in BHI broth at 37ºC in a 5% CO_2 atmosphere. The cultures were then diluted 1:100 in BHI broth containing 25 mM paraquat (methyl viologen; Sigma-Aldrich, St. Louis, MO) or 0.003% hydrogen peroxide, each well was overlaid with sterile mineral oil and growth was monitored in a Bioscreen C.

Growth in CSP. To analyze growth in CSP, the procedure described above was used. When the cells reached OD_{600} = 0.5 in BHI broth, the cultures were diluted 1:100 in BHI broth containing 2 µM synthetic CSP (174) (97% purity), overlaid with sterile mineral oil and growth monitored in the Bioscreen C.

Biofilm Assays

S. mutans strains were grown to mid-exponential phase in BHI broth at 37ºC in a 5% CO_2 atmosphere. The cells were diluted 1:100 in BM supplemented with either 20 mM glucose or 10 mM sucrose and 200 µl transferred to polystyrene microtiter plates. The cells were incubated in a 5% CO_2 atmosphere at 37ºC for 24 or 48 h. The medium was decanted, and the plates were washed twice with 200 µl of sterile water to remove planktonic and loosely-bound cells. The adherent bacteria were stained with 60 µl of 0.1% crystal violet for 15 min. After rinsing twice with 200 µl of water, the bound dye was extracted from the stained biofilm using 200 µl of ethanol:acetone (8:2) solution, twice. The extracted dye was diluted into 1.6 ml of ethanol:acetone solution. Biofilm formation was quantified by measuring the absorbance of the solution at OD_{575} nm.

DNA Manipulation and Construction of Mutants.

Plasmid DNA was isolated from *E. coli* using the QIAprep Spin Plasmid Kit (Qiagen Inc., Valencia, CA). Cloning was carried out using established protocols (196) . Restriction and DNA modifying enzymes were purchased from Life Technologies Inc. (Rockville, MD) or New England Biolab (Beverley, MA).

PCR Ligation: Insertion Mutagenesis

A series of mutant strains (Table 2-1) was derived from *S. mutans* UA159 using PCR ligation mutagenesis procedures with the insertion of either a polar (ΩKm) or non-polar (NPKm) kanamycin resistance cassette to replace the genes (8). Primers were used to amplify the regions flanking the genes of interest (Table 2-3). The PCR products and pALH123 or omega Km (Table 2-2) when desired, were digested with *Bam*HI and ligated. The ligated mixture was transformed into competent *S. mutans* (Figure 2-1). Transformants were selected on BHI agar with 1 mg ml^{-1} kanamycin. Screening for mutants was done via PCR using A and D primers (Table 2-3) to observe for the correct size on 0.8% agarose gels. Colonies that had the correct size were restreaked on fresh BHI agar plates with 1 mg ml^{-1} kanamycin. DNA sequencing using primers outside the region of cloning (Table 2-3) was done to ensure that the correct mutation had been introduced and that no secondary mutations were created in the genes immediately upstream or downstream of the insertion site during recombination.

Splice Overlap Extension Mutagenesis

A series of mutant strains (Table 2-1) was derived from *S. mutans* UA159 using splice overlap extension (SOE) to make point mutations in different regions (90). Briefly, primers with the desired base changes (Table 2-3) and primers that matched a sequence located 0.5 kbp upstream and downstream of the sequence of interest were

used to amplify DNA and generate two PCR products (Figure 2-2). Selected pairs of PCR products with 20-bp identity that included the desired mutations were subjected to PCR for 5 cycles in the absence of added primers. A second PCR of 30 cycles using the A and D outer primers (Table 2-3, Figure 2-2) was performed to generate a 1-kbp product that had the desired mutations. The final PCR product was run on an agarose gel to ensure correct size then the fragment was excised, gel purified (Qiagen) and transformed into competent *S. mutans* with a suicide plasmid harboring an internal fragment of the *lacG* gene and an erythromycin (Em) resistance (Em^r) determinant (242). Transformants were selected on BHI agar with erythromycin. Mismatch Amplification Mutation Assay (MAMA) PCR was done to screen for isolates that have the desired mutations (41). The MAMA PCR reaction included 20 nM of a primer that has a mismatch in the 3' end that can only amplify wild-type DNA, along with10 nM of the A primer and 30 nM D primer (Table 2-2). If the desired mutation is present, then a longer product (~ 1kb) will be made, but if the wild-type is present, a shorter PCR product (~0.5kb) is made (Figure 2-3). PCR products were run on an agarose gel, and isolates that had the correct size were restreaked. DNA sequencing was used to ensure that the correct mutation had been introduced and that no mutations were created in the genes immediately upstream or downstream of the insertion site during recombination.

Complementation

To complement some of the mutations made in the *rcrRPQ* operon, the genes that were mutated were amplified from a wild-type strain. The amplified DNA was cloned into the pDL278 vector and introduced into the mutant strains. A PCR amplification which included approximately 200 bp 5' of the ATG start codon and 50-bp 3' of the stop

codon of the gene was generated from the wild-type strain (Table 2-3). The regions 5' of the ATG start codon were included to ensure all the regulatory elements including the promoter and transcription initiation site were present. The PCR product and the pDL278 plasmid were digested with *Bam*HI and *Sac*I. The digested PCR product and plasmid were gel purified with a Qiagen DNA purification kit, ligated and transformed into appropriate *S. mutans* strains. Transformants were selected on BHI plates containing 1 mg ml^{-1} of spectinomycin. The isolates were screened using primers specific to pDL278 and run on an agarose gel to check for the correct size and presence of the insert. Colonies with the correct size were sequenced to ensure that complementation of the gene(s) was correct. For strains that had severely impaired transformation efficiency, the complementation and mutagenesis of the genes were done concurrently and plated with 1mg ml^{-1} kanamycin to select for the presence of the kanamycin resistance cassette and 1mg ml^{-1} spectinomycin to select for the complementation on the plasmid. DNA sequencing was done to verify the integrity of the strains.

Construction of Promoter Fusions

For construction of the CAT strains, various DNA regions containing 350 bp and 150 bp upstream of the ATG start site, which included the promoter regions of the *SMu0835* and *relP* genes, were amplified via PCR. The PCR products along with the pJL105 plasmid were digested with *Sac*I and *Bam*HI and purified using a Qiagen Gel purification kit. The digested PCR products were ligated with the digested pJL105 integration vector, which has a staphylococcal chloramphenicol acetyltransferase gene (*cat*) gene that lacks a promoter and ribosome binding site (RBS) (243). The pJL105 vector has sequence homology to the *mtlA* and *phnA* genes, which facilitates double

cross-over recombination and integration of the inserted DNA in single copy in the *S. mutans* chromosome. The *cat*-promoter fusions were transformed into wild-type and mutant strains of *S. mutans* and plated on BHI agar plates containing 1 mg ml^{-1} spectinomycin. The integrity of the strains was verified by PCR and DNA sequencing.

For construction of the LacZ strains, a similar technique was used as described above for constructing the CAT strains. However, the promoter regions were cloned into the pMZ integration vector which carries a staphylococcal beta-galactosidase gene (*lacZ*) gene that lacks a promoter and ribosome binding site (RBS) (243). The vector also has homology to the *mtlA* and *phnA* genes which also facilitates double cross-over recombination and integration of inserted DNA (133). The *lacZ* promoter fusions were transformed into *S. mutans* and plated on BHI plates containing 1 mg ml^{-1} kanamycin. The integrity of the strains was verified using PCR and DNA sequencing.

Transformation Assays.

Overnight cultures were diluted 1:20 in 200 µl of BHI broth in polystyrene microtiter plates. The cells were grown to OD_{600} = 0.15 in a 5% CO_2 atmosphere. When desired, 100 nM of synthetic competence stimulating peptide (CSP) (8), was added, cells were incubated for 10 min and 0.5 µg of purified plasmid pDL278, which harbors a spectinomycin resistance (Sp^r) gene, was added to the culture. After 2.5 h incubation at 37°C, transformants and total CFU were enumerated by plating appropriate dilutions on BHI agar plates with or without the addition of 1 mg ml^{-1} spectinomycin, respectively. CFU were counted after 48 h of incubation, and transformation efficiency was expressed as the percentage of transformants among the total viable cells. In some cases, these experiments were repeated with pMSP3535 (Em^r) and JL105 (Sp^r) to

assess the ability to uptake various types of plasmid DNA and to analyze homologous versus non-homologous recombination.

Measurement of (p)ppGpp Accumulation

The measurement of accumulation of (p)ppGpp in *S. mutans* was done as described elsewhere (122). Briefly, cells from an overnight culture were diluted 1:25 into the chemically-defined medium FMC to OD_{600}= 0.2. Cells were labeled with ^{32}P-orthophosphate for 1 hour at 37ºC, harvested, and (p)ppGpp was extracted with formic acid (122). If desired, 0.003% hydrogen peroxide was added to the samples at the same time as the label. To harvest the labeled cells, the samples were centrifuged at 1400 x*g* for 2 min and the supernates decanted. The cell pellets were then washed with 500 µl of FMC to remove any excess ^{32}P-orthophosphate. To extract the (p)ppGpp, the cells pellets were resuspended in equal volumes of 13 M formic acid and FMC (~20 µl each), followed by a series of 3 free thaw cycles on a dry ice/ethanol and thaw 42°C bath, and the cells centrifuged at 1000 x*g*. Following the extraction, the CPM/µl of the supernatant fraction was measured in a scintillation counter, and 2.0 x 10^5 CPM of each sample was spotted onto polyethyleneimine (PEI) cellulose plate (Selecto Scientific, Suwanee, GA) for thin-layer chromatography (TLC). Solutes were resolved using 1.5 M KH_2PO_4 buffer that had been acidified to pH 3.4 with phosphoric acid. Labeled compounds were detected by exposing the PEI plate with a maximum sensitivity film (Kodak, Rochester, NY) overnight at -80°C (122). The accumulation of GP4 and GP5 was quantified using the AlphaEase FC Fluorochem 8900 (Alpha Innotech, USA) Imaging System Spot Denso Analysis tool. The density of the spots was quantified as integrated density value per area (IDV).

RNA Manipulation

RNA Extraction.

Three colonies from each strain were grown overnight in BHI broth, diluted 1:50 in fresh BHI, grown to OD_{600} = 0.5, harvested and immediately treated with RNAprotect ® reagent from Qiagen (Qiagen Inc., Valencia, CA). Total RNA was extracted using phenol (pH 4.3) (1), DNaseI treated and further purified with the RNeasy mini kit (Qiagen Inc., Valencia, CA). RNA concentration was measured in triplicate using a spectrophotometer and run on a gel to assess integrity.

qRT-PCR

Real time PCR was done as detailed by Ahn *et al*. (6). Purified RNA (1 µg) was used to generate cDNA from gene specific primers according to the Superscript III first-strand synthesis (Invitrogen, Gaithersburg, MD) reverse transcription protocol. The gene specific primers were designed with Beacon Designer 4.0 software and standard curves for each gene were prepared (Table 2-4). The standard curve was generated using eight 10-fold serial dilutions of the PCR products to determine the starting amount for each cDNA template, based on its threshold cycle. The concentrations of purified PCR products were measured at OD_{260}, and the copies/ml for standard curves were calculated according to the formula: copies/ml = $(6.023 \times 10^{23} \times C \times OD_{260}) / MWt$, where $C = 5 \times 10^{-5}$ g/ml for DNA and MWt = the molecular weight of the PCR product (base pairs x 6.56×10^{2} g). The standards were prepared in the concentrations of 10^{8} copies/µl. Triplicates of each cDNA sample along with cDNA controls for each of the triplicate isolates analyzed were subjected to Real-time PCR. Real-Time PCR reactions were carried out using an iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA) and iQSYBR green supermix (Bio-Rad) according to the protocol

provided by the supplier. The thermocycling program was set for 40 cycles of 95°C for 10 s and 60°C for 45 s, with an initial cycle at 95°c for 30 s. The accumulation of PCR products was detected after each cycle by monitoring the increase in fluorescence of the reporter dye from dsDNA binding SYBR green. Data were collected and analyzed using the software and graphics provided by iCycler iQ.

Semi-quantitative-Reverse Transcriptase PCR

Reverse Transcriptase PCR (RT-PCR) was done using gene specific real-time primers (Table 2-4) on 1 µg of RNA that was purified as described above. A PCR reaction of 25 cycles was performed on the cDNAs generated using various combinations of primers (Table 2-4) to analyze the different transcripts made in the *SMu0835-0839* gene cluster. A positive control using wild-type chromosomal DNA and the same primer sets was also used. The PCR reactions were run on a 0.8% agarose gel to analyze size and quantity.

RNA Sequencing

The RNA-sequencing protocol and analysis described below was published by Lin Zeng *et al.* ("Gene regulation by CcpA and Catabolite Repression Explored by RNA-seq in *Streptococcus mutans*", *In Press*)

Total RNA was isolated and purified from the various strains as described above (Qiagen). To remove 16S and 23S rRNAs, 10 µg of high-quality total RNA was processed using the MICROB*Express*™ Bacterial mRNA Enrichment Kit (Ambion of Life Technologies, Grand Island, NY), twice, before precipitating with ethanol and resuspending in 25 µl of nuclease-free water. The final quality of enriched mRNA samples was analyzed using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). cDNA libraries were generated from the enriched mRNA samples using the

TruSeq Illumina kit (Illumina, San Diego, CA), following instructions from the supplier. Deep sequencing was performed at the Cornell University Life Sciences Core Laboratories Center (Ithaca, NY).

Short-Read Alignments

Approximately 20 million short-reads were obtained for each sample. Because the aligner BWA (125) allowed a few gaps for efficient alignment of millions of reads of approximately 100 bp, shorter reads consisting mostly of sequencing adapters would not be mapped. After removing adapter sequences from each short-read (146) and trimming of the 3'-ends by quality scores (198), the resulting sequences were mapped onto the reference genome of strain UA159 (GenBank accession no. AE014133) using the short-read aligner. Mapped short-read alignments were then converted into readable formats using SAMTOOLS (126).

Transcript Predictions

RNA transcripts were inferred by applying a hidden Markov model to site-wise expression levels (145). A pileup command of BWA was used to convert the short-read alignments into pileup values, which were taken as the site-wise expression levels along the genome. Site-wise expression levels are a list of non-negative integers that represent numbers of short-reads mapped to a particular genomic position. For the transcript inference program, ParseRNAseq, the following options were used: "-c 10 -b 25 -force gp", which binned expression levels into 25 parts and allowed 10 emission states for relative expression intensity. Genes that were annotated in close proximity were used as the predicted parts of a transcript, while no information regarding the precise transcription initiation- or stop-sites were pursued in this study. For similar reasons, the orientation of each transcript could not be verified solely based on RNA-

seq data; instead we used the information of annotated genes to determine the strandedness of predicted transcripts.

Prediction of Small RNAs and Targets

Although our RNA-seq protocol did not specifically enrich non-coding small RNAs in the cDNA preparation, small RNAs were retained in the RNA samples as only ribosomal RNAs were depleted using specific oligonucleotides. Consequently, it was difficult to discriminate cDNA originated from small non-coding RNAs from that of mRNAs, as expression of non-coding RNA is often masked by the expression of neighboring background mRNAs. Therefore, we utilized RNAz (76), a program that uses homologous sequences and RNA secondary structures to predict putative non-coding small RNAs. Because sequence alignment was a critical step for finding small RNAs via this approach, intergenic regions that also included up- and down-stream sequences were extracted, BLAST-searched against a database of bacterial genomes (12), and the resultant sequence alignments were further refined using a program named MUSCLE (58). Subsequently, RNAz was applied to the alignments for scoring intergenic regions for putative small RNAs (76, 227). Target genes for each candidate small RNA were predicted using RNAplex (219) and RNAplfold (28), and the resultant genes were then used to perform functional category enrichment tests based on their scores by these two programs. In addition, we employed a method of Rho-independent terminator (RIT) identification to help identify candidate small RNAs (107), which were subsequently scored using RNAz, and a transcriptional signal-based method to identify intergenic sRNA transcription units (TUs) (209).

Statistical Analysis for Differential Expression

The R package DESeq (13) was used to determine differential gene expression on the basis of the negative binomial model (193). Detailed steps for analyzing RNA-seq data for differentially expressed genes were utilized as described elsewhere (170). Briefly, short-reads aligned to a particular annotated gene in the reference genome were counted, generating a table of read counts of all the open-reading frames. Statistical software R of the R package DEseq (220) was then employed to infer differentially expressed genes in various biological conditions. To normalize expression levels among different samples, total sequencing depths for each sample were estimated as the median of the ratios of the sample's counts to geometric mean across all samples, as detailed elsewhere (13, 192).

Microarray Experiments.

S. mutans UA159 microarrays were provided by The Institute for Genomic Research (TIGR). The microarrays consisted of 1,948 70-mer oligonucleotides representing 1,960 open reading frames printed four times on the surface of each microarray slip. A reference RNA that had been isolated from 100 ml of UA159 cells grown in BHI broth to an OD_{600} of 0.5 was used in every experiment. The experimental strains consisted *of S. mutans* UA159, and mutant derivatives *Δ835np*, *Δ836p*, *Δ835-837np* (Table 2-1) grown in BHI to OD_{600}=0.5 in quadruplicate for each strain. All RNA was purified as described above and used to generate cDNA by the protocol provided by TIGR (http://pfgrc.tigr.org/protocols.shtml) with minor modifications. The 2:1 dTTP to aminoacyl-dUTP was used. Superscript III reverse transcriptase (Invitrogen) was used to increase cDNA yields. Purified cDNAs from experimental groups were coupled with indocarbocyanine (Cy3)-dUTP, while reference cDNA was coupled with

indodicarbocyanine (Cy5)-dUTP (Amersham Biosciences, Piscataway, NJ). The amount of in co-operated dye was measured for each sample using the spectrophotometer. The Cy3-labeled experimental cDNA samples were mixed in equal quantity to the Cy5-labeled reference cDNA samples and the subsequent mixture hybridized to the microarray slides. Hybridization was carried out with a Maui four-chamber hybridization system (BioMicro Systems, Salt Lake City, UT) for 16 h at 42°C. The slides were then washed by using TIGR protocols and scanned using a GenePix scanner (Axon Instruments Inc, Union City, CA).

***S. mutans* Microarray Data Analysis.**

After the slides were scanned, single-channel images were loaded into TIGR Spotfinder software (http://www.tigr.org/software/) and overlaid. A spot grid was created according to TIGR specifications and manually adjusted to fit all spots within the grid, and then the intensity values of each spot were determined. Data were normalized using Microarray Data Analysis Software (MIDAS) (http://www.tigr.org/software/) by used LOWESS and iterative log mean centering with default settings, followed by in-slide replicate analysis. Statistical analysis was carried out using BRB Array Tools (http://linus.nci.nih.go/BRB-ArrayTools.html) with a cutoff *P* value of 0.001 for class prediction and class comparison.

Biochemical Assays

Chloramphenicol Acetyltransferase Assay.

Strains carrying promoter-*cat* gene fusions were grown from overnight cultures that were inoculated with appropriate antibiotics and diluted 1:30 in 50 ml of fresh BHI broth in a 5% CO_2 atmosphere at 37ºC to OD_{600} = 0.5. Cells were centrifuged, and the pellets were resuspended in 750 µl of 10 mM Tris pH 7.8. Protein was extracted by

bead-beating in the presence of 500 µl glass beads (0.1mm diameter) for 20 s twice, with a 2 min interval on ice. The cell lysates were centrifuged at 4000 x *g* for 10 min, and the supernates were used for measuring CAT activity by the method of Shaw (202). Briefly, 10-50 µl of the cell lysates were added to 950-990 µl of a prewarmed solution containing 0.4 mg ml^{-1} of DTNB and 200 µl Acetyl-coA (4 mg ml^{-1}). Chloramphenicol (5 µl from a 5 mM stock) was added to the samples immediately before reading, and the rates were tabulated via the spectrophotometer which measures the rate of change in OD of the samples per second over a 2 min interval. The concentration of protein was measured using the bicinchoninic acid assay (Thermo Scientific). CAT activity was expressed as nmoles of chloramphenicol acetylated min^{-1} (mg protein)$^{-1}$. CAT specific activity = (Average Rate/13.6) x (1/Sample volume) x (1/protein concentration) x 10^6

β-Galactosidase Assay

β-galactosidase activity was measured according to the protocol of Miller (245). Strains carrying promoter-*lacZ* gene fusions were grown in 5 ml of BHI broth in a 5% CO_2 atmosphere at 37ºC to OD_{600} = 0.5 in triplicates. Cells (1.5 ml) were centrifuged, and the pellets were resuspended in 1.3 ml of Buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, with freshly added 50 mM β-mercaptoethanol). Cells (500 µl) were permeabilized by high speed vortexing for 1 min with 25 µl of toluene:acetone solution (1:9 v/v), and the 800 µl left over was used to measure OD_{600}. The permeabilized cells were transferred to a 37ºC water bath, and 100 µl of prewarmed ONPG (4 mg ml^{-1} in 0.1M Na-Phosphate buffer pH 7.5) was added to the cells and the time recorded. After a yellow color change was observed, the reaction was stopped with 1 M Na_2CO_3 solution and the time for the color change to occur

recorded. The cells were briefly centrifuged, and the OD_{550} and OD_{420} measured. The specific activity was calculated as Miller units. Miller units = 2000 x (OD_{420} – 1.75 x OD_{550}) /Time (min) / OD_{600}

To assess the promoter activity of *relP* in hydrogen peroxide, the wild-type strain carrying the *relP-lacZ* fusion was grown in triplicates in 5 ml of BHI broth in a 5% CO_2 atmosphere at 37ºC to OD_{600} = 0.2. Hydrogen peroxide (0.003%) was added to 2.5 ml of each sample and the other 2.5 ml was left untreated. The cells were incubated for 1 h in a 5% CO_2 atmosphere at 37ºC before harvesting. The measurement of β-galactosidase activity was performed as described above.

Protein Manipulation

Protein Purification

An N-terminally His_6-tagged RcrR protein was obtained by amplifying the entire *rcrR* structural gene from *S. mutans* UA159 and cloning it in-frame in pQE30 (Qiagen) using DH10B *E. coli* cells. Clones were sequenced to ensure no errors had been introduced. The cells were grown from an overnight culture to an OD_{600} of 0.5 with ampicillin to ensure no loss of the plasmid. The protein was overproduced by induction with 100 mM sterile isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hours and purified as a soluble protein in a Ni^{2+} affinity column using the protocol recommended by the supplier (Qiagen). Briefly, to obtain the purified protein, the induced *E. coli* cells were harvested and resuspended in a lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole adjusted to pH 8 with NaOH. The cells were lysed using mechanical force with 500 µl of glass beads for 30 s twice. The cells were centrifuged at 14, 000 x *g* for 10 min at 4°C. The cell lysates were incubated with 1 ml of Ni^{2+} -NTA resin (Qiagen) for ~1 h at 4°C, then the slurry was loaded on to a column. The column was

then washed 2 times with 4X the volume of the slurry with a wash solution containing 50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole, followed by 2 washes 4X the volume of the slurry with a solution containing 40 mM imidazole to remove nonspecific binding. An elution buffer (450 µl) containing 500 mM imidazole was added to the column 6 times, for a total of 6 eluate fractions. The protein concentration of the different eluate fractions were then assessed via Bradford assay, and the fractions were run on a 12% SDS denaturing polyacrylamide gel to check for purity and size of the protein. The proteins were then dialyzed using a Slide-A-Lyzer 10 kDA Dialysis Cassette (Thermo Scientific, Waltham, MA) in 2.1 L of Binding Buffer to exclude fragments smaller than 10 kDA. The dialyzed protein was quantified via Bradford assay and 1 µg of protein was run on a 12% denaturing polyacrylamide gel to check for integrity of the protein.

Electrophoretic Mobility Shift Assay (EMSA)

An electrophoretic mobility shift assay (EMSA) was carried out by using a previously published protocol (3). DNA fragments containing the promoter region of *rcrR* and other genes were amplified via PCR using biotinylated primers (Table 2-5). The PCR product was gel purified (Qiagen) and quantified using a spectrophotometer. Five fmol of biotinylated DNA probe was used with different concentrations of purified recombinant His_6-tagged *S. mutans* RcrR protein in a 10 µl reaction mixture containing 10 mM HEPES (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, 2 µg poly (dI-dC) and 10% glycerol. After incubation at room temperature for 40 min, the DNA-protein samples were resolved in a 4% [(30:1) acrylamide:bis-acrylamide] non-denaturing, low–ionic strength polyacrylamide gel at 65 V for about 1 hour. The samples were transferred to Genescreen plus hybridization transfer membrane

(Perkinelmer Life Sciences, Boston, MA) that was preincubated for 10 min in TBS buffer using a semi-dry transfer apparatus. The signals were detected using a chemiluminescent nucleic acid detection kit (Thermo Scientific) and an AlphaEase FC (Fluorochem 8900) imaging system as recommended by the supplier.

Fluorescent Polarization Assay

Forward and reverse complementary oligos that were 38 bp and 55 bp, which included the RcrR binding site were synthesized and labeled with 6-FAM (IDTDNA) at the 5' end (Table 2-5). The forward and reverse oligos were annealed using equal amounts (12.5 pmoles) of oligos. The annealing was done in an annealing buffer (10 mM Tris pH 7.5, 1 mM EDTA, 50 mM NaCl) @ 95ºC for 2 min, then gradual cooling to room temperature over a 45 min period. Annealed fluorescently labeled probe (10 fmoles) was used with different concentrations of purified recombinant His_6-tagged *S. mutans* RcrR protein in a 200 µl reaction mixture containing 10 mM HEPES (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, 2 µg poly (dI-dC) and 10% glycerol in a microtiter plate. After incubation at room temperature for 10 min, the samples in the microtiter plate were analyzed in a Synergy 2 plate reader (Biotek, Winooski,VT). The change in fluorescence polarization of the DNA with the addition of increasing concentrations of RcrR protein was measured. Binding constants were then calculated based on non linear regression analysis tools using the Gene5 program (Biotek) and the Prism statistical software.

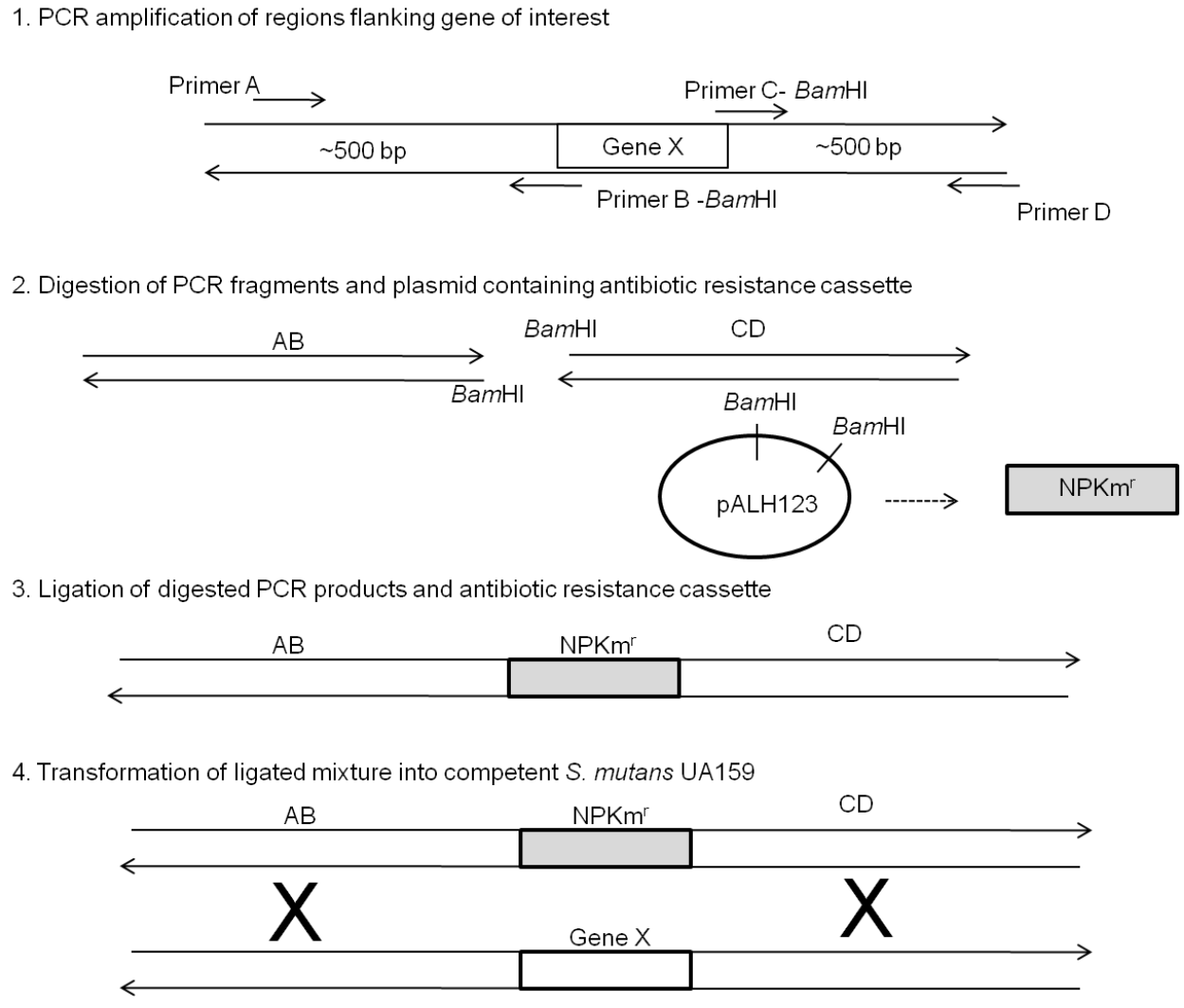


Figure 2-1. Schematic showing the process for making insertion:deletion mutants via PCR ligation mutagenesis

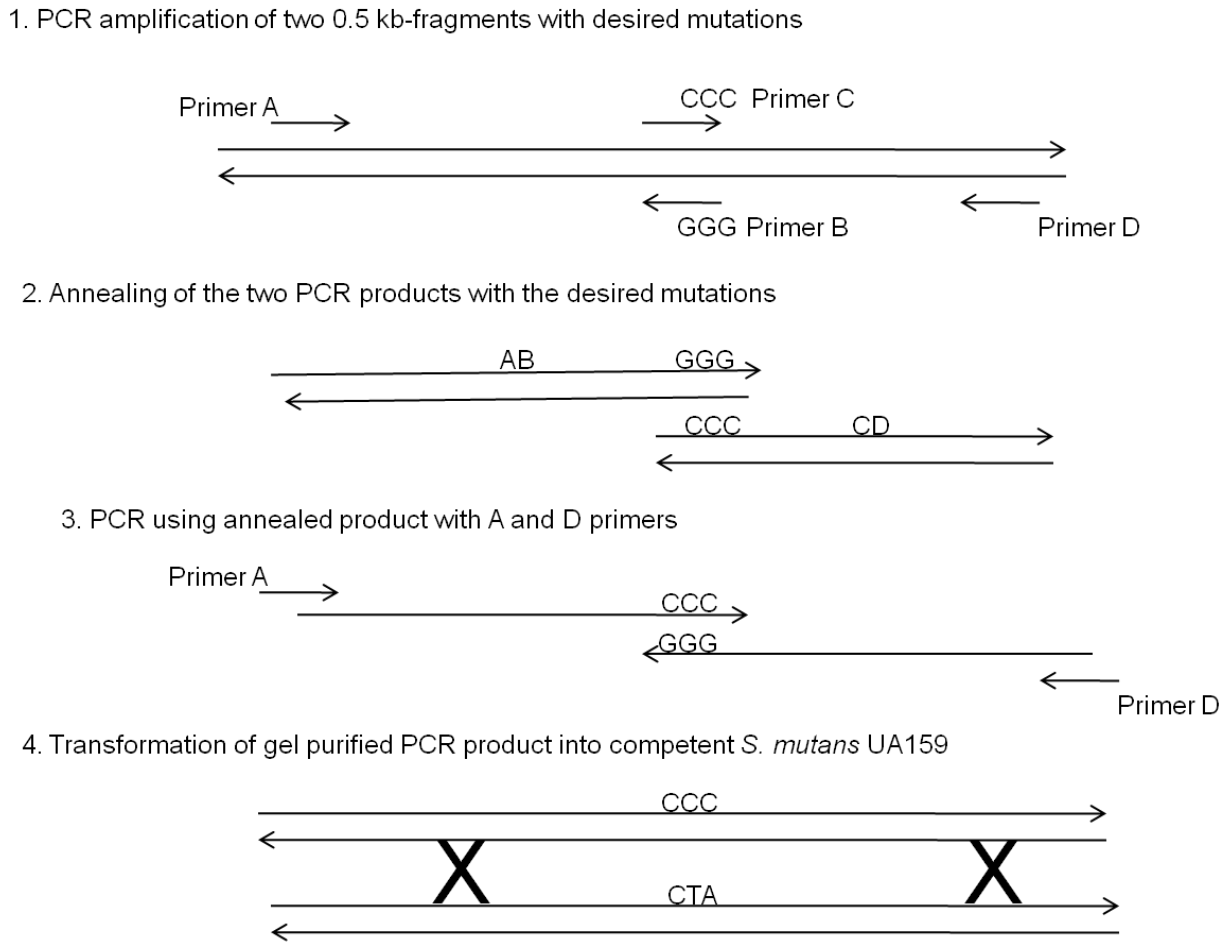


Figure 2-2. Schematic showing the process for making point mutations via splice overlap extension PCR

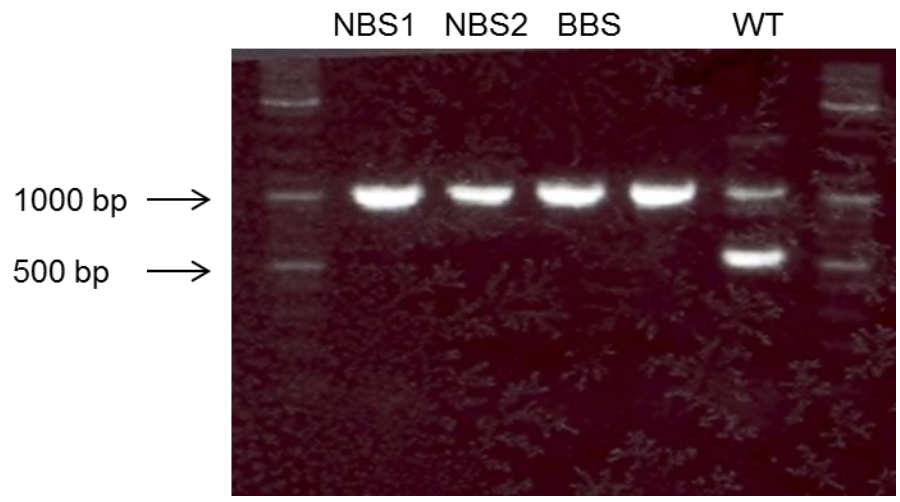


Figure 2-3. Gel image showing size-difference in PCR products generated from the SOE mutants vs. WT using MAMA primers.

Table 2-1. List of strains

Strain	Relevant Characteristics	Source or reference
E. coli strains:		
DH10B	General cloning strain	Invitrogen
S. mutans strains:		
UA159	Wild-type	ATCC 700610
Δ835np	Δ*SMu0835(rcrR)*::NPKm^r	This study
Δ835p	Δ*SMu0835 (rcrR)*::ΩKm^r	This study
Δ836np	Δ*SMu0836(rcrP)*:: NPKm^r	This study
Δ836p	Δ*SMu0836 (rcrP)*:: ΩKm^r	This study
Δ837np	Δ*SMu0837 (rcrQ)*::NP Km^r	This study
Δ837p	Δ*SMu0837 (rcrQ)*:: ΩKm^r	This study
Δ835-837np	Δ*SMu0835,* Δ*SMu0836*, Δ*SMu0837*:: NPKm^r,	This study
Δ836-837np	Δ*SMu0836,* Δ*SMu0837*:: NPKm^r	This study
835$^+$/Δ835np (SJ361)	Δ*SMu0835::*NPKm^r harboring pDL278-*SMu0835*	This study
835$^+$/Δ835p (SJ362)	Δ*SMu0835::*ΩKm^r harboring pDL278-*SMu0835*	This study
835$^+$/Δ835-7np (SJ360)	Δ*SMu0835-7::*NPKm^r harboring pDL278-*SMu0835*	This study
835$^+$/wt (*SJ354)*	UA159 harboring pDL278-*SMu0835*	This study
Δtpx	Δ *tpx*:: NPKm^r	This study
ΔrelA	Δ*relA*:: Em^r	(122)
ΔrelP	Δ*relP*:: NPKm^r	(122)
ΔrelRS	Δ*relRS*:: NPKm^r	(122)
ΔrelAPQ	Δ*relA,* Δ*relP,* Δ*relQ* ::Em^r NPKm^r Tet^r	(122)
NBS1	GAGAACTA-TTTTCCCC	This study
NBS2	AGGAATCA-TTTTCCCC	This study
BBS	TCA-CCC; CTA-CCC	This study
UA159::P$_{835}$-*cat*	Sp^r, Km^r;	This study
ΔrelP ::P$_{835}$-*cat*	Sp^r, Km^r	This study
UA159::P$_{relP}$-*cat*	Sp^r, Km^r	This study
Δ836p ::P$_{relP}$-*cat*	Sp^r, Km^r	This study
UA159 :: P$_{835}$-*lacZ*	Km^r	This study
UA159 ::P$_{NBS1}$-*lacZ*	Km^r	This study
UA159 ::P$_{NBS2}$-*lacZ*	Km^r	This study
Δ835p:: P$_{835}$-*cat*	Km^r	This study
Δ835p:: P$_{NBS1}$-*cat*	Km^r	This study
Δ835p:: P$_{NBS2}$-*cat*	Km^r	This study

Table 2-2. List of plasmids used

Plasmid	Phenotype or description	Reference or source
pJL105	Sp^r, Km^r; Em^r, CAT fusion integration vector based on pJL84 (243)	This study
pDL278	Sp^r *; E.coli-Streptococcus* shuttle vector	(8)
pMSP3535	Em^r; *E.coli-Streptococcus* shuttle vector	(32)
pvT924	Km^r, plasmid encoding the polar Ω kanamycin gene	(173)
pALH123	Km^r; plasmid encoding the non-polar kanamycin gene from Tn1545	(173)
pMZ	Km^r, Em^r, LacZ fusion integration vector based on pMC_{195} and $pMC_{340}B$	(133)
pQE30	Amp^r, *E. coli* expression vector for 6xHis-tagged protein	Qiagen

Table 2-3. List of primers

Primer	Sequence	Application
SMu0835- A	5'-GATGGCTTCTCCCAAACATCC-3'	Inactivation of *SMu0835 (rcrR)*
*SMu0835-B- Bam*HI	5'-TTAAATTCA**GGATCC**GGCTCCTTCA-3'	Inactivation of *SMu0835 (rcrR)*
*SMu0835-C- Bam*HI	5'-GGAAGGTTT**GGATCC**AAATCAAACA-3'	Inactivation of *SMu0835 (rcrR)*
SMu0835-D	5'-CACCTGACGCTGTCTTGGA-3'	Inactivation of *SMu0835 (rcrR)*
SMu0836- A	5'-GCGCCCCTAGGTCTTACCAA-3'	Inactivation of *SMu0836 (rcrP)*
*SMu0836-B- Bam*HI	5'-CCCATTCTT**GGATCC**TTAGACGTT-3'	Inactivation of *SMu0836 (rcrP)*
*SMu0836-C- Bam*HI	5'-ATGACTAAG**GGATCC**GTAGCCCAAC-3'	Inactivation of *SMu08356 (rcrP)*
SMu0836-D	5'-GTCGCTGCAAGAGCTCCATT-3'	Inactivation of *SMu0836 (rcrP)*
SMu0837- A	5'-GTTCCACTGGCTCAGGAAAA-3'	Inactivation of *SMu0837 (rcrQ)*
*SMu0837-B- Bam*HI	5'-CATCTCT**GGATCC**CGGTCAG-3'	Inactivation of *SMu0837 (rcrQ)*
*SMu0837-C- Bam*HI	5'-TGGCTTC**GGATCC**GATCTCT-3'	Inactivation of *SMu0837 (rcrQ)*
SMu0837-D	5'-ACGGAGTCAAAAATCCCAAT-3'	Inactivation of *SMu0837 (rcrQ)*
SMu0839- A	5'-TTGTTATTTTTGGCTGCTTTTT-3'	Inactivation of *SMu0839* (*cipI*)
*SMu0839-B- Bam*HI	5'-CATATCTTT**GGATCC**ACCTAAAATA-3'	Inactivation of *SMu0839* (*cipI*)
*SMu0839-C- Bam*HI	5'-TTATTGTCT**GGATCC**TTTTATTATGG-3'	Inactivation of *SMu0839* (*cipI*)
SMu0839-D	5'CGCACAGCGCTTTAATTCTT-3'	Inactivation of *SMu0839* (*cipI*)
SMu0835-seq FW	5'-CCGCTAAAGACGACAAGAGC-3'	Sequencing of ∆*SMu0835* strains
SMu0835-seq- RV	5'-AGCCCGAACAACACGAACAC-3'	Sequencing of ∆*SMu0835* strains
SMu0836-seq FW	5'-CCGCTAAAGACGACAAGAGC-3'	Sequencing of ∆*SMu0836* strains
SMu0836-seq RV	5'-AACCAAAGCACCAGCAAAGT-3'	Sequencing of ∆*SMu0836* strains
SMu0837-seq FW	5'-TTTACCGCGTGCTTTAGTTTCT-3'	Sequencing of ∆*SMu0837* strains
SMu0837-seq RV	5'-ACTTTCTTGTGCATTTGCCTCT-3'	Sequencing of ∆*SMu0837* strains
P*835* FW- *Sac*I	5'-GCCAGTCTTA**GAGCTC**AGTCAGAAG-3'	*SMU0835* promoter amplification
P*835* RV- *Bam*HI	5'-CTCCTTCAT**GGATCC**ACCTCACTTT-3'	*SMU0835* promoter amplification
P*RelP* FW- *Sac*I	5'AGATAATGCT**GAGCTC**TTTGTGGTT-3'	*relP* promoter amplification
P*RelP* RV- *Bam*HI	5'-CTTGTGACAT**GGATCC**TCCTTCTTA-3'	*relP* promoter amplification
phnA-AS	5'-GATTCCATTCATAAGCACAT-3'	Sequencing of promoter fusion strains
lacZ RV	5'- TCAGAAAATTCTGCAAGAGATTCA-3'	Sequencing of *lacZ* promoter fusion strains
Cat-5'LT RV	5'-TCGTTTGTTGGTTCAAATAA-3'	Sequencing of *cat* promoter fusion strains

Table 2-3. Continued

Primer	Sequence	Application
SMu0835 FW-*Bam*HI	5'-AAGTGAGG**GGATCC**TATGAAGGAG-3'	Amplification of *SMu0835* gene to make recombinant protein
SMu0835 RV-*Hind*III	5'-ATTTTAAAC**AAGCTT**TACTCCTTCTT-3'	Amplification of *SMu0835* gene to make recombinant protein
BS A	5'-ACAAGAGCTGATTGACGTTTCATA-3'	Point mutations in $P_{SMu0835\ (rcrR)}$
BS D	5'-ATGCCCCATTCTTTAGCATTTAGA-3'	Point mutations in $P_{SMu0835\ (rcrR)}$
NBS1 B	5'-TGAGAATATTATAA**GGGGAAAAA**TGAAAACTATTA-3'	Point mutations in $P_{SMu0835\ (rcrR)}$
NBS1 C	5'-TAATAGTTTTCA**TTTTTCCCC**TTATAATATTCTCA-3'	Point mutations in $P_{SMu0835\ (rcrR)}$
BBS B- RV	5'-AGAATATTATAA**GGG**TTCTCATGAAAACTATTATAATGA**GGC**CTTAAACTATTG-3'	Point mutations in $P_{SMu0835\ (rcrR)}$
BBS C- FW	5'-CAATAGTTTAAG**GCC**TCATTATAATAGTTTTCATGAGAA**CCC**TTATAATATTCT-3'	Point mutations in $P_{SMu0835\ (rcrR)}$
BS1 MAMA	5'-ATCATTATAATAGTTTTCATGAGAACT**G**-3'	Screen for point mutations in $P_{SMu0835\ (rcrR)}$
BS2 MAMA	5'-TTCTCCTTGACAATAGTTTAAGGAATC**G**-3'	Screen for point mutations in $P_{SMu0835\ (rcrR)}$
BS seq FW	5'-ATCTCTTATCTTGCGCTGTTTG-3'	Sequencing of strains
BS seq RV	5'-CGACTGCTCCTGTCCATTCAT-3'	Sequencing of strains

Table 2-4. List of real-time primers

Primer	Sequence
RT *835* Sense	5’-TGTTTTAACGCCATTAGGTCAGG-3’
RT *835* Anti Sense	5’-TCCGAGCAACTGATAAGTCTTCC-3’
RT *836* Sense	5’-ATCTGTTTGGCTGTCTGGATGG-3’
RT *836* Anti Sense	5’-ATAATATCTGAGGCGGTCGTTCC-3’
RT *837* Sense	5’-GACAGATACCATGACCAAAGGG-3’
RT *837* Anti Sense	5’-AGAAACCAAAGCACCAGCAAAG-3’
RT *838 (tpx)* Sense	5’-GACACTTGCTGGTAAGAAATTGC-3’
RT *838 (tpx)* Anti Sense	5’-ATAGATGGCACAACGCTAATCAC-3’
RT *839* Sense	5’-TCCTCTGCTTGTTCAGGTTTGC-3’
RT *839* Anti Sense	5’-TCTTGTGCATTTGCCTCTCTAGC-3’
RT-NP Kanamycin Sense	5’-TGACGGACAGCCGGTATAAAGG-3’
RT-NP Kanamycin Anti Sense	5’-CAGATTGCTCCAGCCATCATGC-3’
RT-*relP* Sense	5’-AGACACGCCATTTGAGGATTGC-3’
RT-*relP* Anti Sense	5’-GGTGCTCCAAACTAGCCCAAG-3’
RT-*relA* Sense	5’-’CGCTGAGGCATTTACGCAAGG-3’
RT-*relA* Anti Sense	5’-GCGACTAATCCCCAGCCGATG-3’
RT *comYA* Sense	5’-ATTATCTCTGAGGCATCGTCCG-3’
RT *comYA* Anti Sense	5’-ACCATTGCCCCTGTAAGACTTG-3’
RT *comX* Sense	5’-CGTCAGCAAGAAAGTCAGAAAC-3’
RT *comX* Anti Sense	5’-ATACCGCCACTTGACAAACAG-3’
RT-*comS* Sense	5’-TCAAAAAGAAAGGAGAATAACA-3’
RT-*comS* Anti Sense	5’-TCATCTGACATAAGGGCTGT-3’

Table 2-5. List of primers and oligos for EMSA and FP analysis

Primer	Sequence
P*835*-FW_biotin	5'-/5Biosg/GCCAGTCTTAGAAAGTAGTCAGAAG-3'
P*835*-RV	5'-CTCCTTCATAAAAACACCTCACTTTT-3'
P*relP*-FW_biotin	5'-/5Biosg/AGATAATGCTAGGCTTTTTGTGGTT-3'
P*relP*-RV	5'-CTTGTGACATAATTCATCCTTCTTA-3'
P*comX*-FW_biotin	5'-/5Biosig/CATACCCTGCTTTATCTTGAAT-3'
P*comX*-RV	5'-CTATTACGATGACCTCCTTTTATAAT-3'
RS1+13 FW	5' –AGCCGTTACGTAGTTTTCATGAGAACTAA- 3'
RS1 plus RV	5'- AATATTATAATAGTTCTCATGAAAACTATTATAATGAT- 3'
RS 1 plus FW	5'-ATCATTATAATAGTTTTCATGAGAACTATTATAATATT-3'
RS 1 plus RV	5'-AATATTATAATAGTTCTCATGAAAACTATTATAATGAT-3'
RS 1 plus RV-biotin	5'-/5Biosg/AATATTATAATAGTTCTCATGAAAACTATTATAATGAT-3'
RS 1 plus RV-6FAM	5'-/56FAM/AATATTATAATAGTTCTCATGAAAACTATTATAATGAT-3'
RS 2 plus FW	5'-TGACAATAGTTTAAGGAATCATTATAATAGTTTTCATGAGAACTATTATAATATT-3'
RS 2 plus RV	5'-AATATTATAATAGTTCTCATGAAAACTATTATAATGATTCCTTAAACTATTGTCA-3'
RS 2 plus RV-biotin	5'-/5Biosg/AATATTATAATAGTTCTCATGAAAACTATTATAATGATTCCTTAAACTATTGTCA-3'

CHAPTER 3
CHARACTERIZATION OF THE *SMU0835-837* OPERON AND ITS ROLE ON GROWTH, BIOFILM FORMATION AND STRESS TOLERANCE IN *Streptococcus mutans*

Introduction

S. mutans is able to survive, persist and compete with other bacteria, and eventually cause disease when conditions are favorable. Therefore, it is critical to understand the mechanisms that *S. mutans* utilizes to adapt to stress. The metabolism of (p)ppGpp is an important stress adaptation pathway. The amount of (p)ppGpp accumulated plays a significant role in controlling the physiology of the cell and affects certain virulence attributes in *S. mutans* (98, 122, 123). The discovery of the additional synthetase enzymes, particularly RelP, the dominant producer of (p)ppGpp under non-stringent conditions, shows that there are other signals and factors regulating (p)ppGpp metabolism than was previously known. The signals regulating RelA-dependent (p)ppGpp production are pretty well known, but the signals regulating RelP-dependent (p)ppGpp production have not been revealed. However, it was found that the expression of the *relPRS* operon was upregulated in cells grown with aeration (7). The *SMu0835-0839* gene cluster was upregulated in aeration as well and in a mupirocin-induced stringent response (4, 123). The genes in the *SMu0835-0839* cluster encode products that are typically involved in stress tolerance as well. The linkage to stress tolerance and response to similar environmental signals provided the basis for potential regulatory overlap of *relP* and *SMu0835-0837*. In order to elucidate pathways that are involved in stress responses and factors influence RelP-dependent (p)ppGpp production in *S. mutans*, the *SMu0835-0839* gene cluster was closely examined. We characterized a variety of mutants lacking some or all of these genes. Figure 3-1 shows

a schematic of the operon. *SMu0835,* which we designated *rcrR* for *r*el *c*ompetence *r*elated *(rcrR*) gene is predicted to encode a cytoplasmic transcriptional regulator with a winged helix-turn-helix DNA binding motif and is identified (Oralgen, Los Alamos) as a member of the *m*ultiple *a*ntibiotic *r*esistance (MarR) family of transcriptional regulators (10). MarR-type regulators are widely distributed in bacteria and regulate many functions, including resistance to xenobiotics and oxidative stressors, and expression of virulence genes (64). For example, the *E. coli* MarR protein modulates the expression of multiple genes that impact resistance to a variety of antibiotics and to oxidative stress (14, 181). *SMu0836 (rcrP) and SMu0837(rcrQ)* are apparently co-transcribed with *SMu0835* and are annotated (Oralgen, Los Alamos) as transmembrane ATP-Binding Cassette (ABC) transporters that function as multidrug/protein/lipid transport systems. The ATP-binding cassette and transmembrane domains of both proteins are located on a single polypeptide and are classified as Type 6 transporters (62), which include homo- and hetero-meric ABC transporters that typically function as exporters involved in the externalization of toxic compounds or peptides. The highly conserved ATP-binding Walker A sequence and Walker B magnesium-binding sequences, as well as the signature LSGGQ sequence, found in ABC transporters are conserved in both ORFs. Notably, we could identify MarR-like regulators that are linked to two similar ABC transporters in multiple other streptococcal species and in other Firmicutes (Table 7-1).

SMu0838 (tpx) encodes a thiol peroxidase that was shown by microarray analysis to be up-regulated in response to growth with aeration, compared to anaerobically-growing cells (7). Thiolperoxidases can protect bacteria against oxidative stress by breaking down hydrogen peroxide and organic hydroperoxides. The Tpx protein

contains the 2 cysteine residues that form the conserved CXXC motif and that are oxidized by hydroperoxides to form a sulfenic acid. Notably, there are other proteins in *S. mutans* that contain the FX4 CXXC motif that are involved in responses to aeration, including SMu0629, which modulates the activity of the AtlA autolysin (7). *SMu0839* (*cipI*) was shown by Levesque and co-workers (175) to encode a bacteriocin immunity protein. Bacteriocin immunity proteins are generally integral membrane proteins that confer protection against certain classes of antimicrobial agents and often enhance stress tolerance (150). In *S. mutans*, CipI plays a protective role against the CipB bacteriocin-like protein, which appears to function intracellularly and contributes to cell death following exposure to high levels of competence stimulating peptide (CSP) (175).

Results

Transcriptional Organization of the *SMu0835-837* Operon.

A collection of mutants was made by allelic exchange using either polar or non-polar antibiotic resistance cassettes (Table 2-1). Reverse transcriptase PCR (RT-PCR) and quantitative RealTime® RT-PCR were used to determine if genes could be co-transcribed and to measure stable transcript levels of the genes in the *SMu0835-0839* operon in the *Δ835np*, *Δ836np, Δ836p* and *Δ837np* mutants, respectively. RT-PCR revealed that *SMu0835* can be co-transcribed with *SMu0836* and *SMu0837* as a polycistronic operon (Figure 3-2, Figure 3-3).

Consistent with the RT-PCR results, expression of *SMu0837(rcrQ)* was down-regulated nearly 1000-fold in the polar *Δ836p* mutant compared to the parental strain (Figure 3-4). In contrast, expression of *SMu0838* (*tpx)* or *SMu0839* (*cipI*) was not significantly altered in the *Δ836p* mutant compared to the wild-type strain (Figure 3-5). The lack of influence of polar insertions in *SMu0836-7* on *tpx* mRNA levels was

consistent with our inability to detect a product in RT-PCR reactions using primers that would amplify across the *SMu0837-tpx* intergenic region (data not shown), indicating that these genes are not part of the *SMu0835-7* operon.

Interestingly, the expression of *SMu0836(rcrP)* and *SMu0837(rcrQ)* was significantly up-regulated (~100-fold) in the mutant carrying a non-polar insertion in the *SMu0835* gene (*Δ835np*) compared to the wild-type strain (Figure 3-4, Figure 3-6), adding further support that these three genes constitute an operon and revealing that the MarR-like protein may repress expression of this operon. To further demonstrate that *SMu0835* was autogenously regulated, we measured mRNA levels of the non-polar kanamycin resistance gene that was used to replace *SMu0835* in the Δ*835np* strain, lacking the MarR-like regulator, and compared it to expression levels in the *Δ836np* mutant, which has an intact MarR-like regulator. This non-polar kanamycin resistance cassette lacks a promoter (8), so its expression was driven by the *SMu083*5 promoter in both the ∆*835np* and ∆*836np* strains. Consistent with the RealTime-PCR results, the expression of the non-polar kanamycin marker was about 100-fold higher in the *Δ835np* mutant than in the *Δ836np* mutant (Figure 3-7), adding additional support for the hypothesis that the SMu0835 protein is a negative regulator of the *SMu0835-0837* operon. Also of interest is that the magnitude of induction of the operon associated with loss of the SMu0835 protein indicates that the genes are significantly repressed during exponential growth in rich medium. Notably, though, the expression of this operon was not differentially expressed as a function of growth phase (data not shown).

SMu0835 (RcrR) Is the Dominant Regulator of the Operon .

Based on the qRT-PCR analysis and sRT-PCR experiments it appears that RcrR is the dominant regulator of the operon and therefore EMSAs were done to analyze

whether purified RcrR protein (rRcrR) was able to bind to DNA amplified from the region 5' of the ATG start site of *rcrR*. Purified RcrR protein was able to impede the migration of a DNA fragment that included 140-bp in front of the ATG start site of *rcrR* at low quantities (2 pmoles) (Figure 3-8). The shift of the 140-bp DNA fragment was observed as little as 0.65 pmoles of RcrR protein. Other DNA fragments that included regions containing 250 and 200–bp upstream of the *rcrR* ATG start site were all were impeded by 5 pmoles of purified RcrR protein (data not shown). Cold competition assays were done using biotinylated DNA and unlabeled DNA of the same region in different ratios with 1.25 pmoles of rRcR protein. The amount of biotinylated DNA that was shifted decreased with increasing concentrations of unlabeled DNA, indicative of competitive binding to the purified protein (Figure 3-9). When 50 times more unlabeled DNA was added there was only an approximate 30% shift in biotinylated DNA compared to an approximate 88% shift with no competition (Table 3-1).

Growth Characteristics of the Mutants.

The growth of the various mutants (Table 2-1) was monitored in BHI broth. All of the mutants constructed using the non-polar cassette displayed a modestly extended lag phase and slightly slower growth rate than the wild-type strain. The wild-type strain had a doubling time of about 46 min ± 1.5 min, whereas the non-polar mutants had doubling times of about 51 min ± 3.5 min (Figure 3-10). However, the final optical density attained by the non-polar mutants was consistently lower than the parental strain. Notably, the *Δ836p* polar mutant, which would not express either ABC transporter, exhibited much slower growth (67 min ± 7min) and lower final yields than the non-polar mutants or parental strain (Figure 3-11). Both the polar and non-polar *tpx*

mutants had growth rates similar to the non-polar mutants, so the effects of the loss of the ABC transporters were independent of the level of expression of *tpx* or *cipI*.

To test if the mutants were sensitive to low pH, the strains were grown in BHI broth that was acidified to pH 5.5 with HCl (Figure 3-12). All of the mutants had a slower growth phenotype than the wild-type strain. Importantly, the Δ*836p* mutant grew very poorly at pH 5.5 and showed very little cell accumulation even after 26 hours (Figure 3-13). Exposure to oxygen has been shown to alter growth and biofilm formation by *S. mutans* (4) and some of the genes in the *SMu0835-839* gene cluster were up-regulated in response to oxygen (7). The growth phenotype of the mutants was assessed in BHI without an oil overlay, thus exposing the cells to air during growth, or in medium containing 25 mM paraquat, a superoxide generating agent, with an oil overlay. Because cells could not grow in the presence of paraquat without oil overlay was added. The *Δ836p* polar mutant again had a slower growth rate than the other strains. In addition, the *Δ836np* and *Δ837np* mutants displayed a slow growth phenotype when the cells were exposed to air (Figure 3-14). Interestingly, the *ΔrelP* mutant exhibited significantly faster growth and a shorter lag phase than the wild-type strain in medium containing paraquat or in cultures that were grown with exposure to air (Figure 3-15).

S. mutans has the ability to form biofilms, an essential process in establishment, persistence and pathogenesis. Efflux pumps have been shown to be required for biofilm formation in certain organisms and compounds that can inhibit efflux pumps can affect the ability of bacteria to form biofilms (113). We evaluated if the various mutants had impaired biofilm formation in microtiter plates in BM broth containing sucrose or glucose. Biofilm formation was quantified at 24 and 48 h to take into account the effects

of the slower growth phenotypes of some of the mutants. There were no significant differences seen in biofilm formation after 24 or 48 h when the strains were grown in sucrose (data not shown). However, when the strains were grown in glucose, the Δ*835np* mutant, which overexpresses the ABC exporters and the Δ*836p* mutant, lacking both ABC transporters, formed less biofilm than the other strains at 48 h (Figure 3-16). Collectively, these data highlight the requirement of the ABC transporters for stress tolerance and biofilm formation, but also show that loss of both transporters is required to observe changes in the phenotypes of interest.

Discussion

The ability of *S. mutans* to survive and persist in the continually-varying conditions in the oral cavity is intimately associated with its pathogenicity (33), so understanding how the organism adapts to these often challenging conditions can facilitate the development of novel strategies to compromise the persistence or virulence of this important human pathogen. The results presented here show that the products of the previously uncharacterized *SMu0835-0837* operon, which we now designate as the *rcrRPQ* operon, for *r*el-*c*ompetence-*r*elated genes, play a major role in the regulation of growth and stress tolerance. Although *tpx* and *cipI* participate in particular aspects of stress tolerance and the response to CSP, respectively (175), we focused on the *rcrRPQ* operon because deletion of *tpx* or *cipI* had no significant effects on growth, stress tolerance or gene expression under the conditions we tested. We also found no evidence to support that *tpx* or *cipI* were part of the *rcrRPQ* operon or had an influence on *relP* expression.

The data presented here demonstrate that the RcrP (SMu0836) and RcrQ (SMu0837) exporters are critical for tolerance of the two environmental stresses that

have the greatest influence on the composition (120), biochemical activities and pathogenic potential of oral biofilms, oxidative and acid stress. Strains lacking the RcrPQ transporters grew substantially slower than the parental strain in air or in the presence of the superoxide generator paraquat. Likewise, mutants lacking these transporters were more acid-sensitive than the parental strain, as evidenced by slower growth at pH 5.5. The RcrPQ transporters were also necessary for proper biofilm formation, another attribute associated with the pathogenicity of *S. mutans*. While it is not yet established if the RcrP and RcrQ pumps are able to function as a heterodimeric complex, as many ABC porters do (62), RcrP and RcrQ clearly have redundancy in function, since deletion of only one of the exporters caused modest phenotypic changes compared to those seen in strains carrying mutations that affected the expression of both gene products.

ABC efflux transporters play many roles in bacteria, including extrusion of antibiotics and export in a wide range of compounds, including, metals, peptides and lipids (148). As noted earlier, RcrPQ have a structure typical of ABC export proteins and are annotated as multi-drug/protein/lipid transport systems. The simplest explanation for the growth defects of strains lacking both exporters would be that RcrP and RcrQ are each capable of externalizing a substance or class of compounds that accumulates in growing cells, particularly in aerobic conditions or at low pH. Interestingly, the RcrQ protein is predicted to have a fumarate lyase regulatory domain (Oralgen). Growth of *S. mutans* in air alters metabolism toward heterofermentative growth and impacts the transcriptional profiles of the cells, including enhancing the expression of the genes for pyruvate dehydrogenase and the partial TCA cycle, which in

the case of *S. mutans* could lead to enhanced fumarate production (7). We tested if mutants lacking the ABC transporters were more sensitive to the presence of fumarate, but this was not the case (data now shown). Notably, aeration also increases the production of peptide antibiotics (mutacins) by *S. mutans*, so the transporters could play a role in externalization of peptides with antimicrobial activity, which may account for the smaller zone of inhibition with the strain lacking the RcrPQ porters (Appendix A). We are currently testing the hypothesis that RcrQP participate in the transport of selected peptide-based moieties.

Notwithstanding, multiple findings presented in this chapter reveal that the RcrRPQ system in *S. mutans* has key functions in cellular homeostasis, gene regulation and quorum sensing that extend well beyond simply pumping a deleterious substance from inside the cell. RcrR (SMu0835) is the dominant regulator of the *rcrRPQ* operon, repressing the production of the genes for the RcrP and RcrQ transporters under all conditions tested here. Overexpression or uncontrolled expression of efflux pumps can affect bacterial homeostasis and physiology by imposing a metabolic burden on the bacteria or by hyper-secretion of signaling molecules (131), so it is critical that genes encoding efflux pumps are properly regulated. Of note, the Δ*835np* (*rcrR*$^{-}$) strain had poor biofilm formation in glucose after 48 hours, which may be attributable to the overexpression of the ABC pumps, although we cannot exclude that RcrR is influencing the expression of other genes, such as the *com* genes, to affect biofilm formation. We did not find evidence to support that derepression of the *rcrR* operon occurs as a function of growth phase (data not shown), so we propose that accumulation of a

specific compound or class of compounds, perhaps those exported by the ABC pumps, could serve as an allosteric regulator of the RcrR transcriptional repressor (Figure 3-17).

Figure 3-1. Schematic diagram of the *SMu0835-0839 (rcrRPQ)* gene cluster and the *relPRS* operon in *S. mutans* UA159. *SMu0835 (rcrR)* encodes for a predicted transcriptional regulator of the MarR family; *SMu0836 (rcrP)* and *SMu0837* (*rcrQ)* are annotated as ABC-type multidrug/protein/lipid transport; *SMu0838* encodes a thiol peroxidase; *SMu0839* encodes a predicted bacteriocin immunity protein; *SMu0840 (relP)* encodes a GTP pyrophosphokinase; *SMu0841 (relR)* encodes a response regulator and *Smu0842 (relS)* a sensor histidine kinase of a classic two-component system.

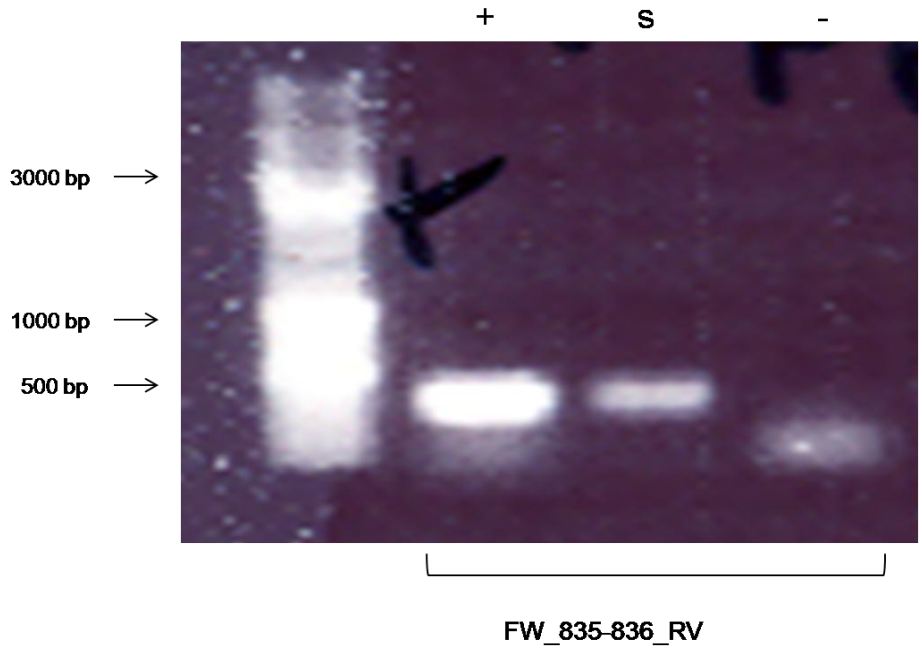


Figure 3-2. RT-PCR using cDNA generated from the *SMu0836* reverse primer. Reverse transcription was performed on 1 µg of purified RNA using the *SMU0836* real-time reverse primer. PCR amplification was performed on the cDNA generated using the SMu0835-FW and the SMu0836-RV primers. Chromosomal DNA from the wild-type strain was also amplified as a positive control. + is the positive control; S is the PCR product from the cDNA generated; - is a negative control from a reaction with no RT enzyme.

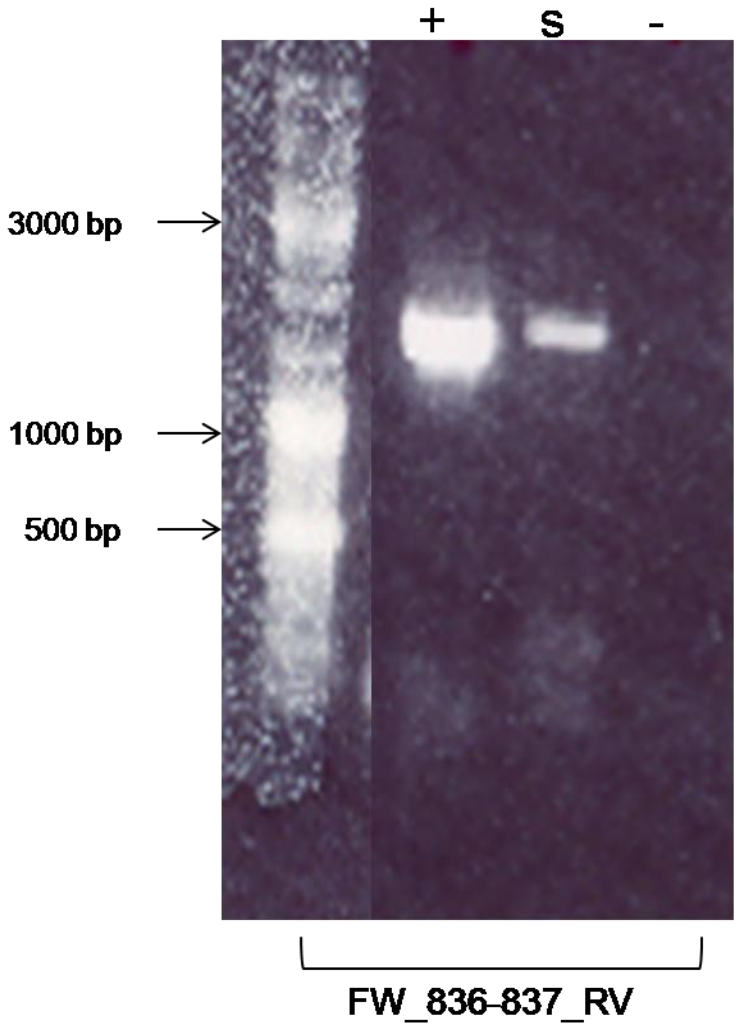


Figure 3-3. RT-PCR using cDNA generated from the *SMu0837* reverse primer. Reverse transcription was performed on 1 µg of purified RNA using the *SMU0837* real-time reverse primer. PCR amplification was performed on the cDNA generated using the SMu0836-FW and the SMu0837-RV primers. Chromosomal DNA from the wild-type strain was also amplified as a positive control. +, is the positive control; S is the PCR product from the cDNA generated, - is a negative control from a reaction with no RT enzyme.

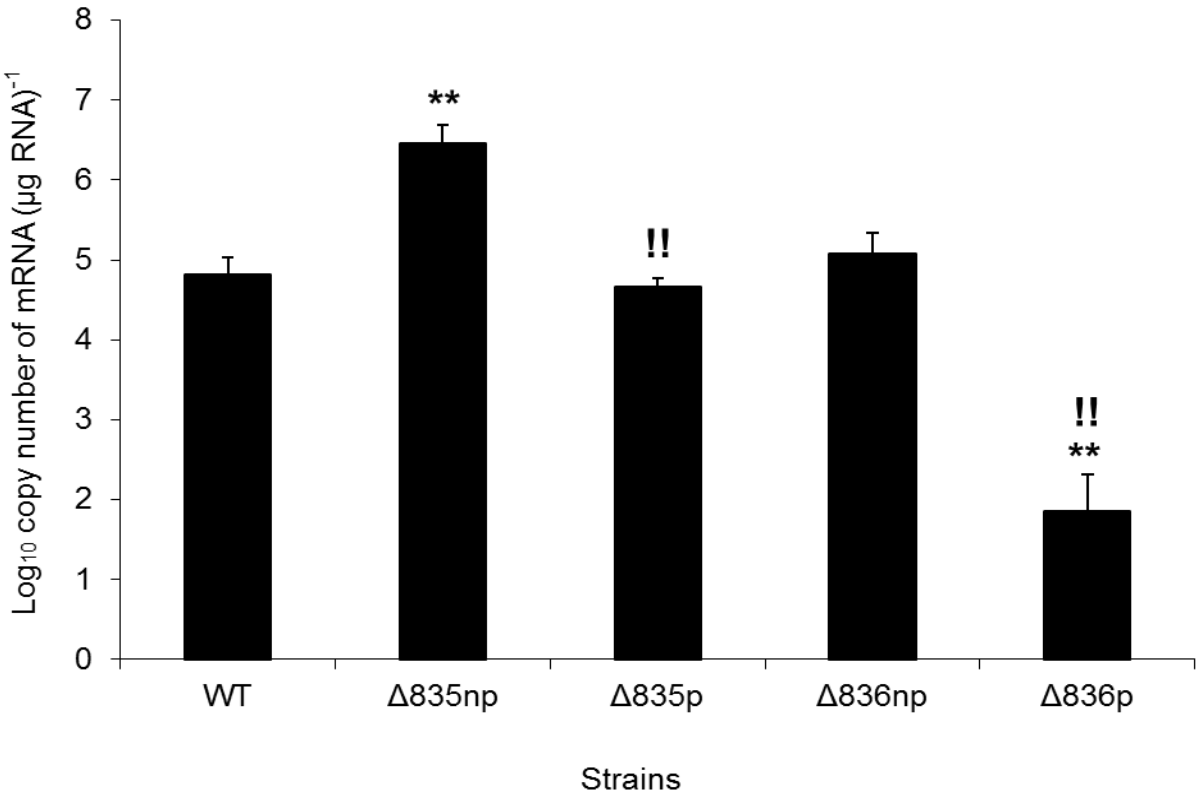


Figure 3-4. RealTime-RT-PCR showing *rcrQ* mRNA levels. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data are presented as the log of the copy number of each gene per µg of input RNA. **, Differs from the wild-type strain at $p<0.005$ (Student's *t*-test). !!, Differs from the *Δ835np* strain at $p<0.005$.

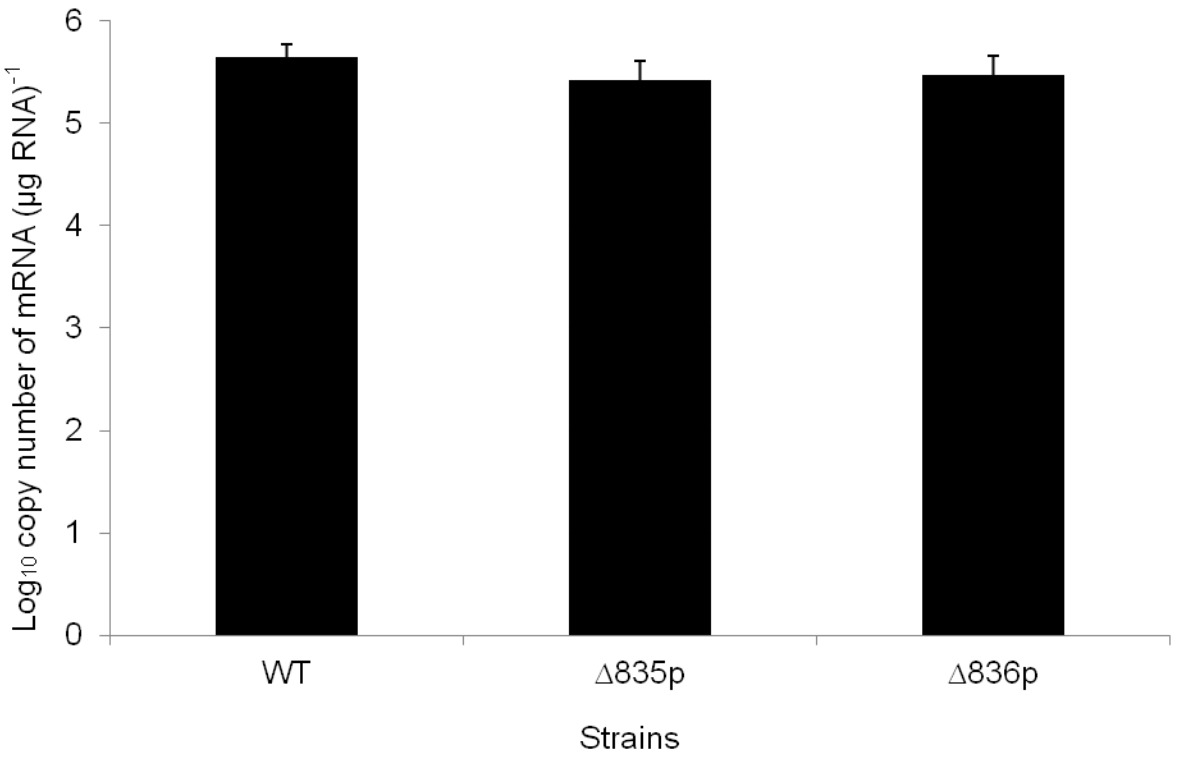


Figure 3-5. RealTime-RT-PCR showing *tpx* mRNA levels. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data are presented as the log of the copy number of each gene per µg of input RNA.

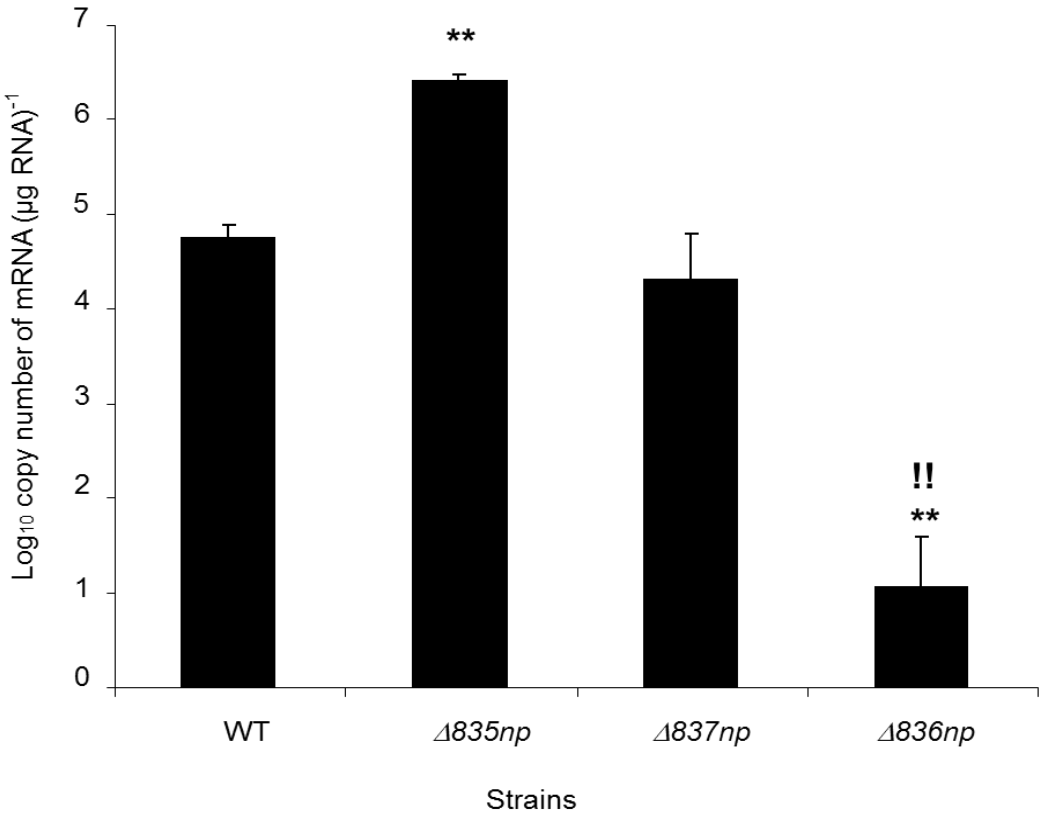


Figure 3-6. RealTime-RT-PCR showing *SMu0836(rcrP)* mRNA levels. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data are presented as the log of the copy number of each gene per µg of input RNA. **, Differs from the wild-type strain at $p< 0.005$ (Student's *t*-test). !!, Differs from the *Δ835np* strain at $p<0.005$.

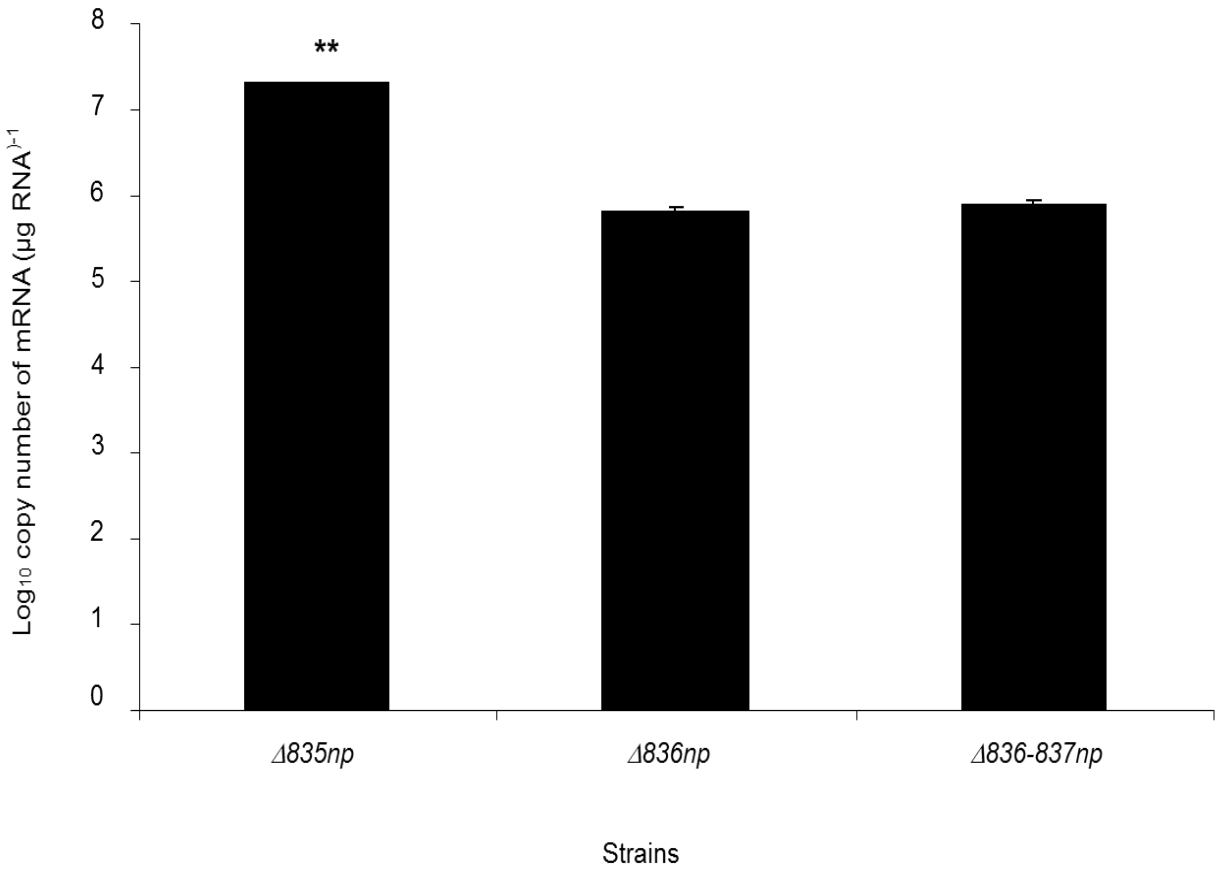


Figure 3-7. RealTime-RT-PCR showing NP Kanamycin mRNA levels. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data are presented as the log of the copy number of each gene per µg of input RNA. **, Differs from the *Δ836np* and Δ*836-7np* strains at $p < 0.005$ (Student's *t*-test).

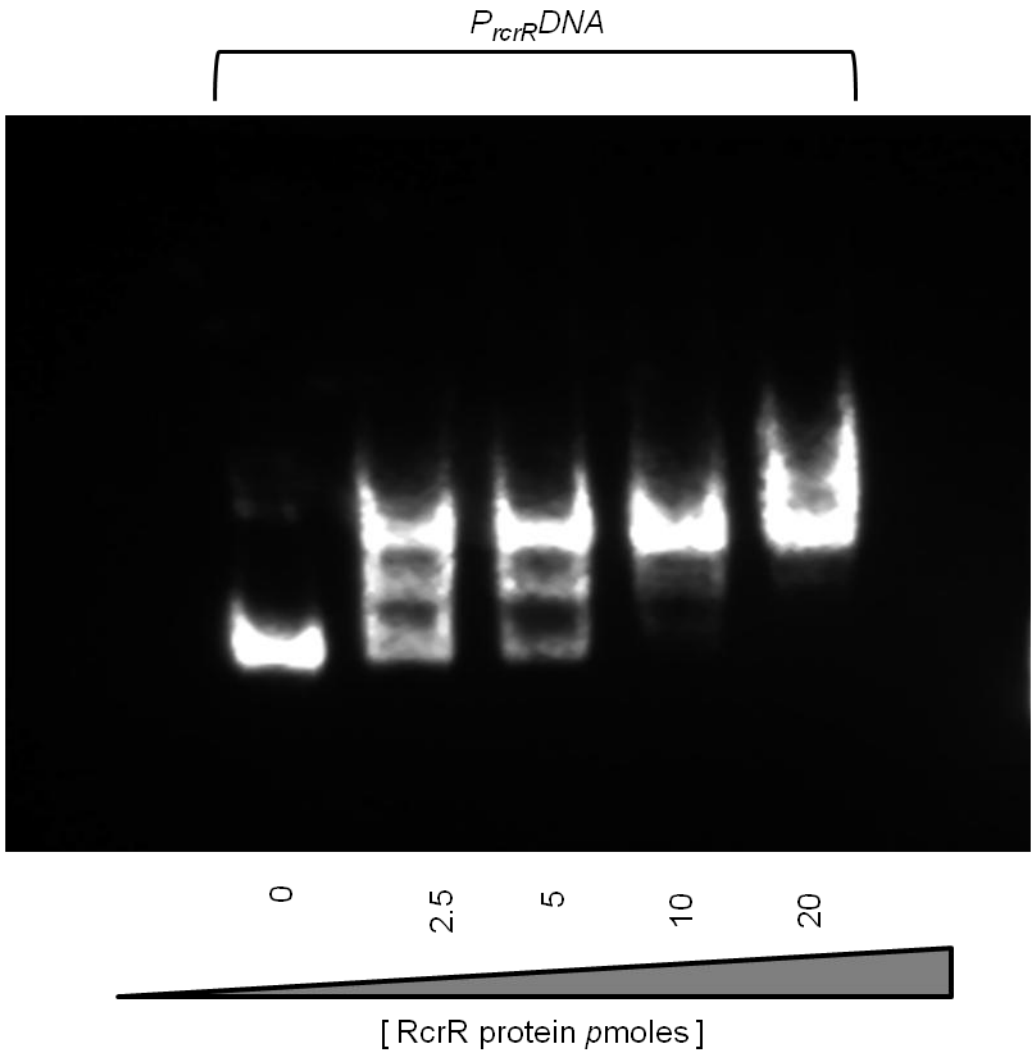


Figure 3-8. EMSA showing binding of biotinylated P_{rcrR} DNA with purified RcrR protein. Purified RcrR protein of various concentrations (0, 2.5, 5, 10, 20 pmoles) was added to 5 fmoles of biotinylated P_{rcrR} DNA in a binding reaction for 40 min. The reactions were run on a non-denaturing polyacrylamide gel and the signal observed via chemiluminescence. The data are a representative of n= 7 EMSAs. There was a similar trend observed in all cases.

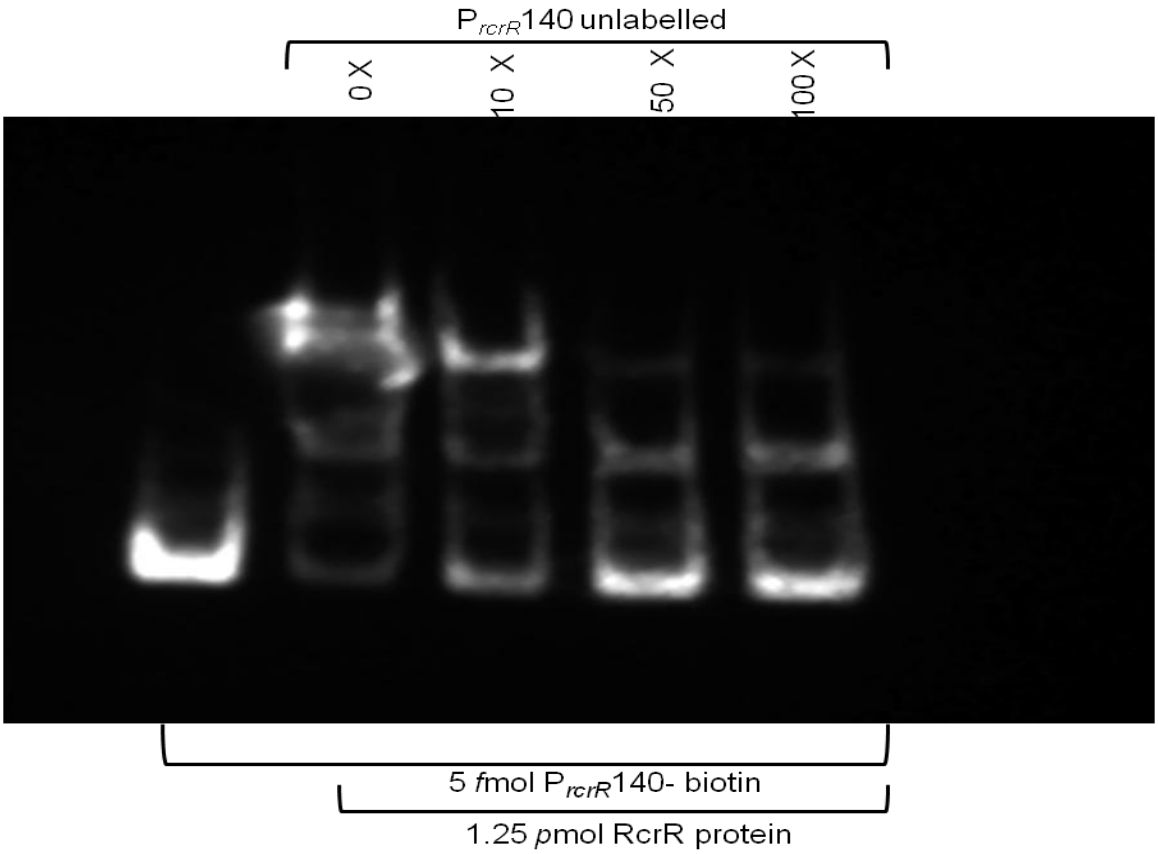


Figure 3-9. EMSA showing competitive binding of 5 fmol biotinylated P_{rcrR} DNA and unlabeled P_{rcrR} DNA with 1.25 pmoles of purified RcrR protein. Various concentrations of unlabeled DNA containing the same region as the biotinylated region were used. The first lane is the no protein control. The reactions were done for 40 min at room temperature. The reactions were run on a non-denaturing polyacrylamide gel and the signal observed via chemiluminescence. The data are a representative of n=3 EMSAs. There was a similar trend observed in all cases.

Table 3-1. Percentage of labeled PrcrR DNA shifted with unlabeled competitor P_{rcrR} DNA

0 x unlabeled DNA	10 x unlabeled DNA	50 x unlabeled DNA	100 x unlabeled DNA
88% shift	71% shift	30% shift	30% shift

% shift calculated as % reduction in the integrated density value (IDV) of the DNA bands compared to the IDV of unshifted DNA band with no protein.

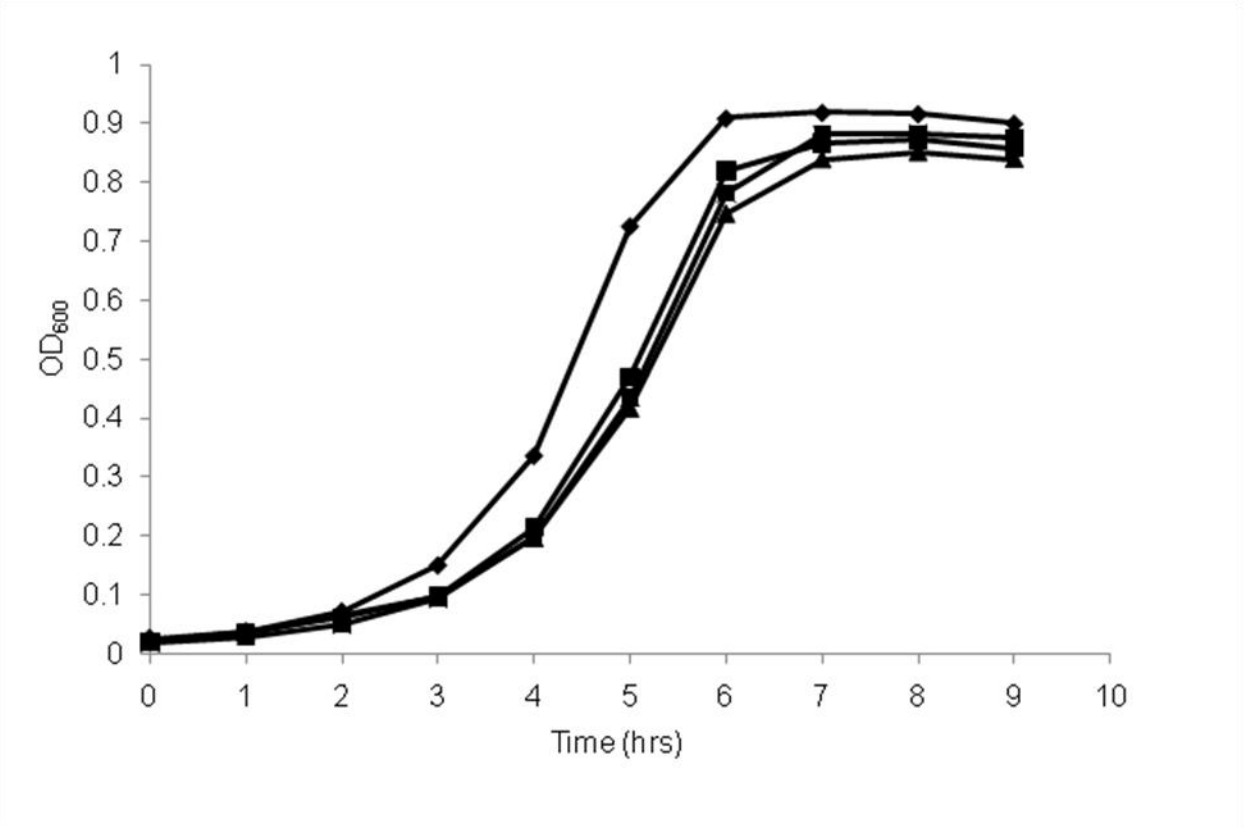


Figure 3-10. Growth comparison of wild-type and non-polar mutant strains in BHI. The strains were grown in triplicate to mid-exponential phase in BHI broth, diluted 1:100 and transferred to fresh BHI broth, overlaid with sterile mineral oil and placed in a Bioscreen C at 37°C to monitor growth. WT, diamonds; *Δ835np*, squares; *Δ837np* X's; *Δ836np*, triangles. The results are representative of three independent experiments performed triplicate.

Table 3-2. Table showing growth characteristics of the non-polar mutants versus the wild-type strain in BHI

Strain	WT	*Δ835np*	*Δ836np*	*Δ837np*
Final OD	0.9 ± 0.017	0.84 ± 0.05	0.80 ± 0.08	0.82 ± 0.03
Doubling Time (min)	46 ± 1.5	47 ± 1	50 ± 0.7	48 ± 3
Lag time (h)	2	3	3	3

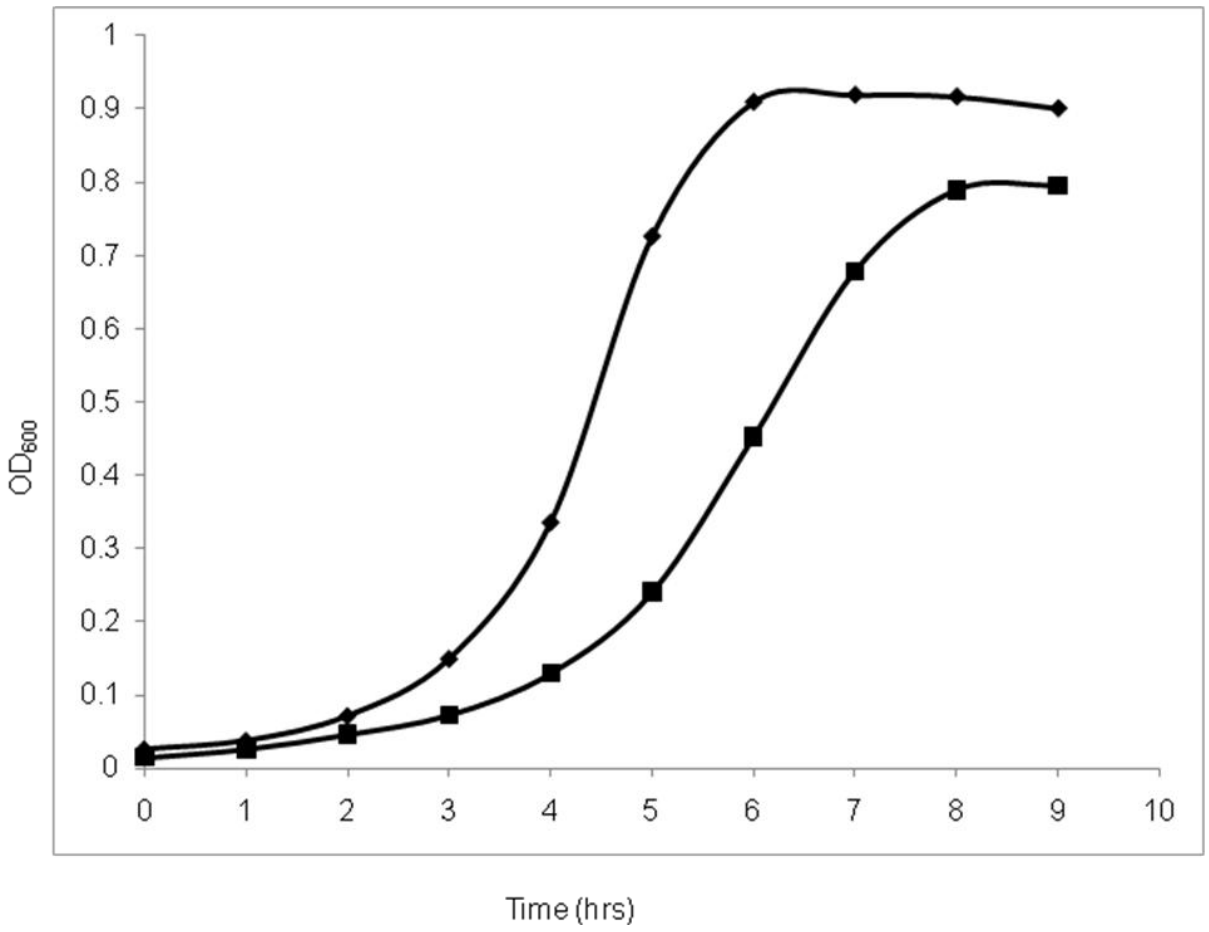


Figure 3-11. Growth comparison of wild-type and polar mutant strains in BHI. Strains were grown as described in Figure 3-10. WT, diamonds; *Δ836p*, squares. The results are representative of three independent experiments performed triplicate.

Table 3-3. Table showing growth characteristics of the polar mutant versus the wild-type strain in BHI.

Strain	WT	*Δ836p*
Final OD	0.9 ± 0.02	0. 78 ± 0.03
Doubling Time (min)	46 ± 1.5	57 ± 7 *
Lag time (h)	2	3

*, Differs from the wild-type strain at $p < 0.05$ (Student's *t*-test)

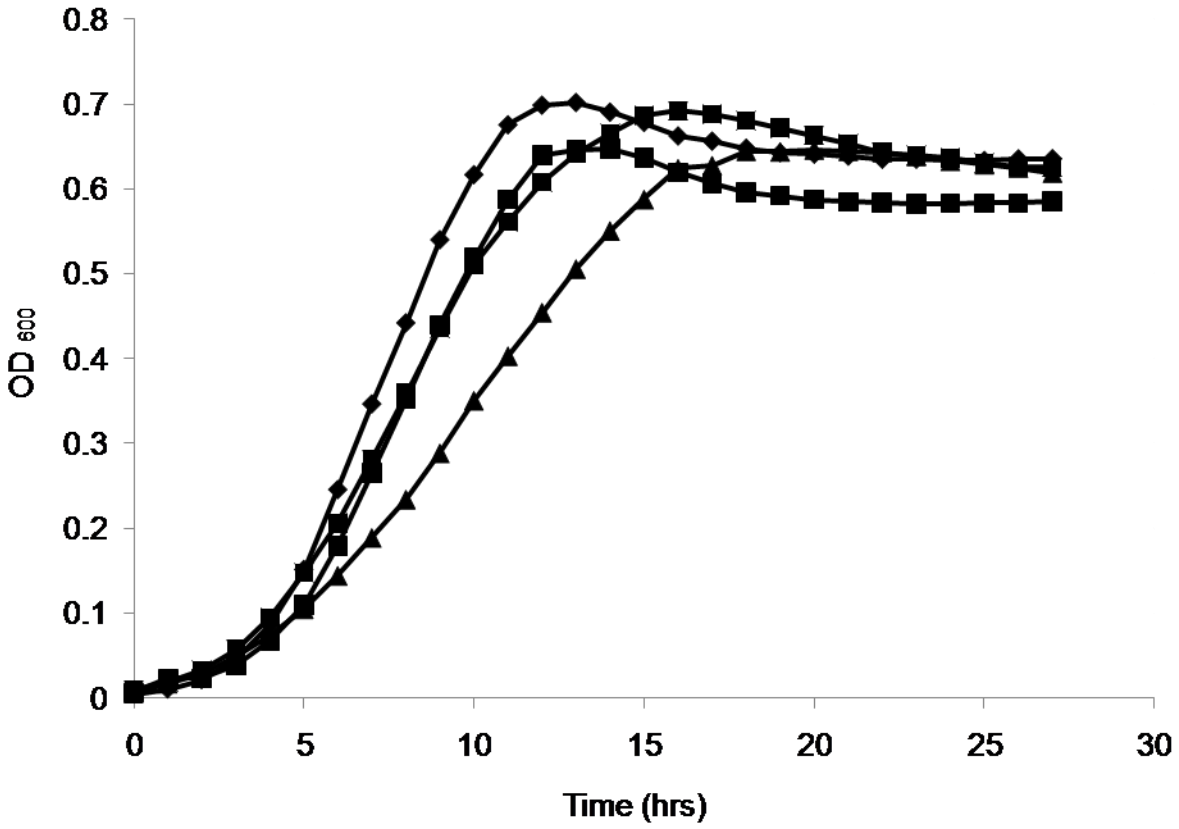


Figure 3-12. Growth of the non-polar mutants versus the wild-type strain at pH 5.5. Cells were grown in triplicate to mid- exponential phase in BHI broth, diluted 1:100 in BHI broth acidified to pH 5.5, covered with sterile mineral oil and placed in a Bioscreen C at 37°C to monitor growth. WT, diamonds; *Δ835np* , squares; *Δ837np* X's; *Δ836np*, triangles. The results are representative of three independent experiments performed triplicate

Table 3-4. Table showing growth characteristics of the wild-type versus the non-polar strains at pH 5.5

Strain	WT	*Δ835np*	*Δ836np*	*Δ837np*
Final OD	0.64 ± 0.03	0.65 ± 0.04	0.62 ± 0.19	0.65 ± 0.05
Doubling Time (min)	98 ± 7	109 ± 29	135 ± 13, *****	103± 24

*, Differs from the wild-type strain at $p < 0.05$ (Student's *t*-test)

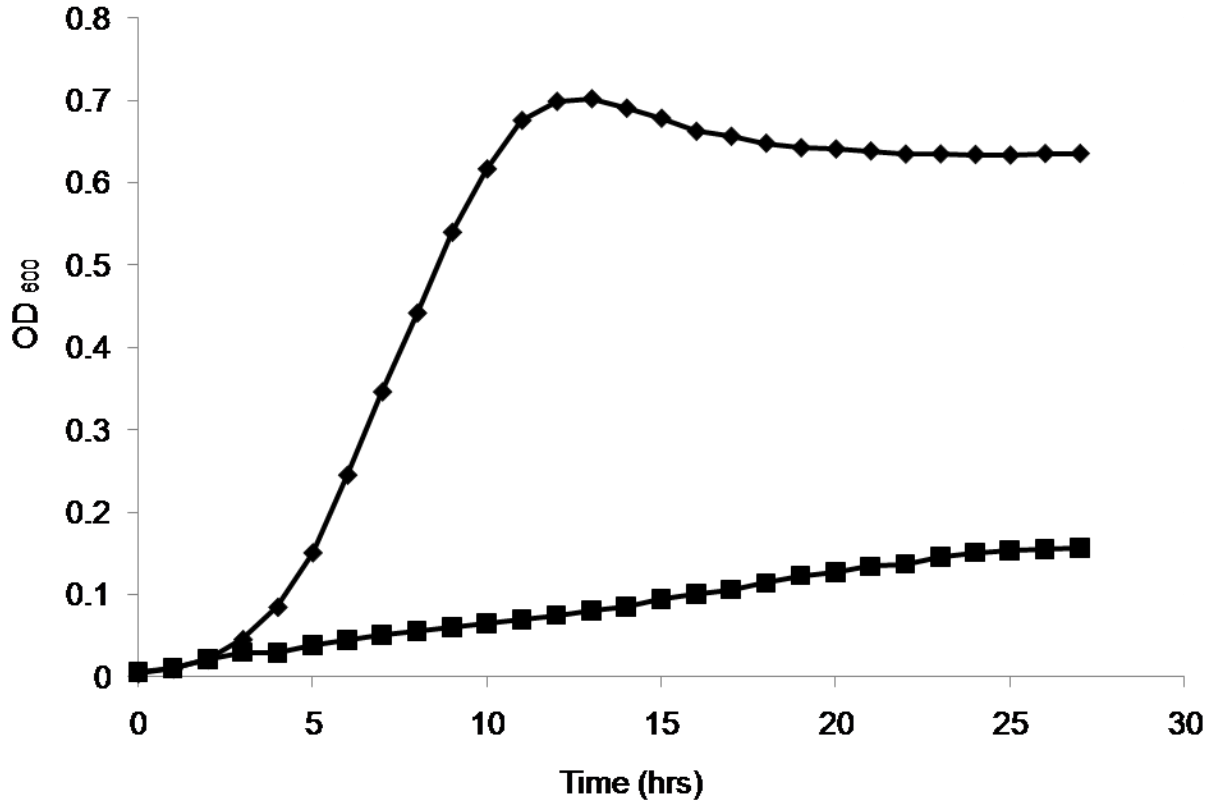


Figure 3-13. Growth of the polar mutant strain versus the wild-type strain at pH 5.5. Strains were grown as in Figure 3-10. WT, diamonds; *Δ836p*, squares. The results are representative of three independent experiments performed triplicate.

Table 3-5. Table showing growth characteristics of the polar mutant versus the wild-type strain at pH 5.5

Strain	WT	*Δ836p*
Final OD	0.64 ± 0.03	0.09 ± 0.01
Doubling Time (min)	98 ± 7	412 ± 117**

**, Differs from the wild-type strain at $p < 0.005$ (Student's *t*-test).

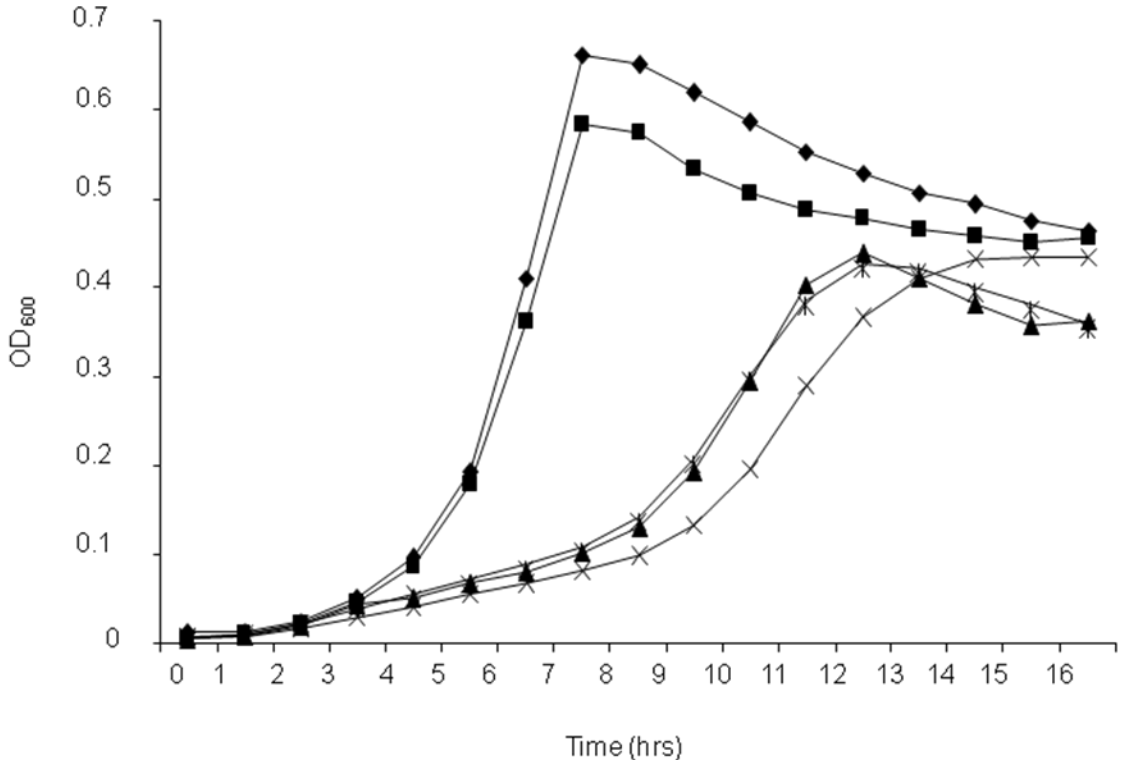


Figure 3-14. Growth of the mutants versus the wild-type strain with aeration. Cells were grown in triplicate to mid-exponential phase in BHI broth then diluted 1:100 in fresh BHI broth and placed in a Bioscreen C at 37ºC to monitor growth without the use of mineral oil. WT, diamonds; Δ*835np*, squares; *Δ836np*, triangles; *Δ837np*, *; *Δ836p,* Xs. The results are representative of three independent experiments performed in at least triplicate.

Table 3-6. Table showing growth characteristics of the mutants versus the wild-type strain with aeration

Strain	WT	*Δ835np*	*Δ836np*	*Δ836p*	*Δ837np*
Final OD	0.63 ± 0.03	0.56 ±0.04	0.39 ± 0.06	0.36 ± 0.07	0.40 ± 0.07
Doubling Time (min)	38 ± 2	42 ± 2	83 ± 3 **	88 ± 13 **	95 ± 15 **

**, Differs from the wild-type strain at $p < 0.005$ (Student's *t*-test)

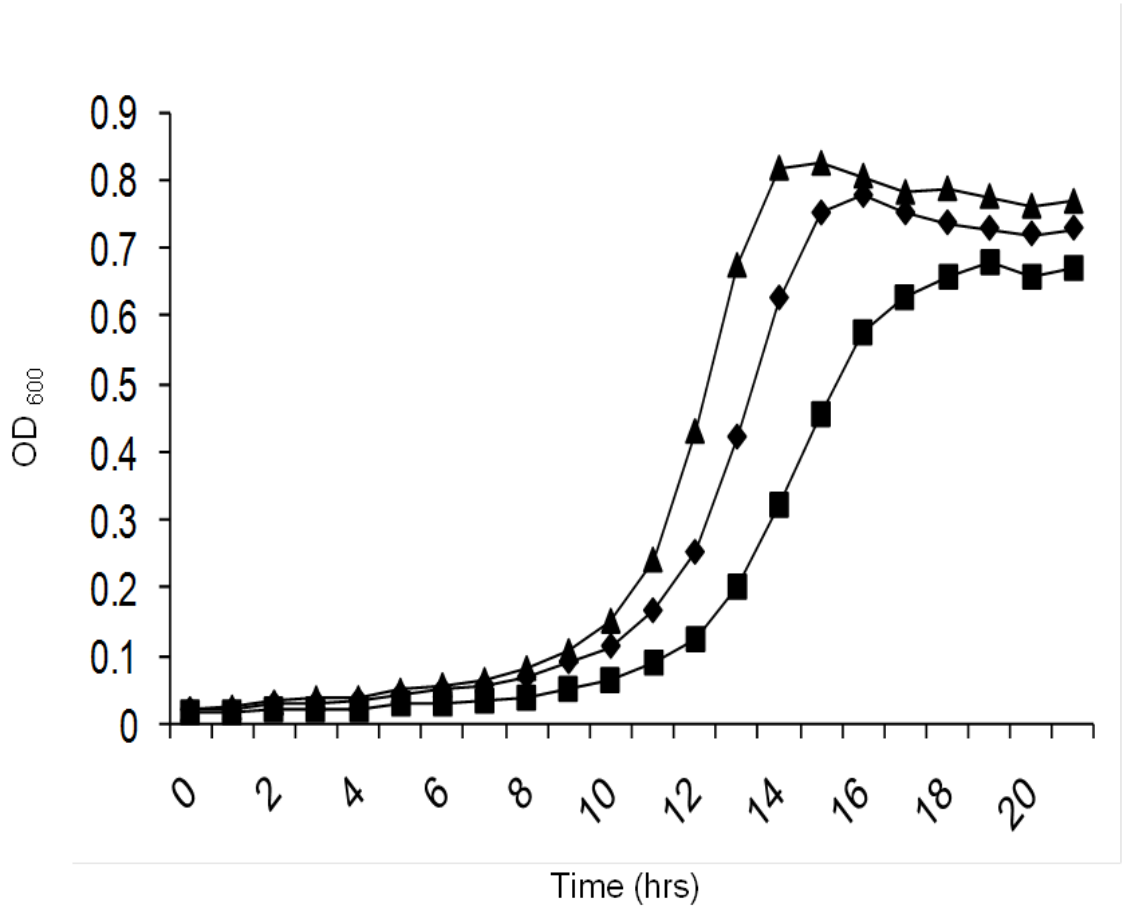


Figure 3-15. Growth of the mutants versus the wild-type strain in 25 mM paraquat. Cells were grown to mid-exponential phase in BHI broth then diluted 1:100 in BHI that contained 25 mM paraquat. WT, diamonds; Δ*836p*, squares; *ΔrelP*, triangles. The results are representative of three independent experiments performed in at least triplicate.

Table 3-7. Table showing growth characteristics of the mutants versus the wild-type strain with 25 mM paraquat

Strain	WT	*ΔrelP*	*Δ836p*
Final OD	0.7 ± 0.04	0.82 ± 0.017	0.7 ± 0.18
Doubling Time (min)	100 ± 21	76 ± 1.8	125 ± 40
Lag time (h)	9	8	10

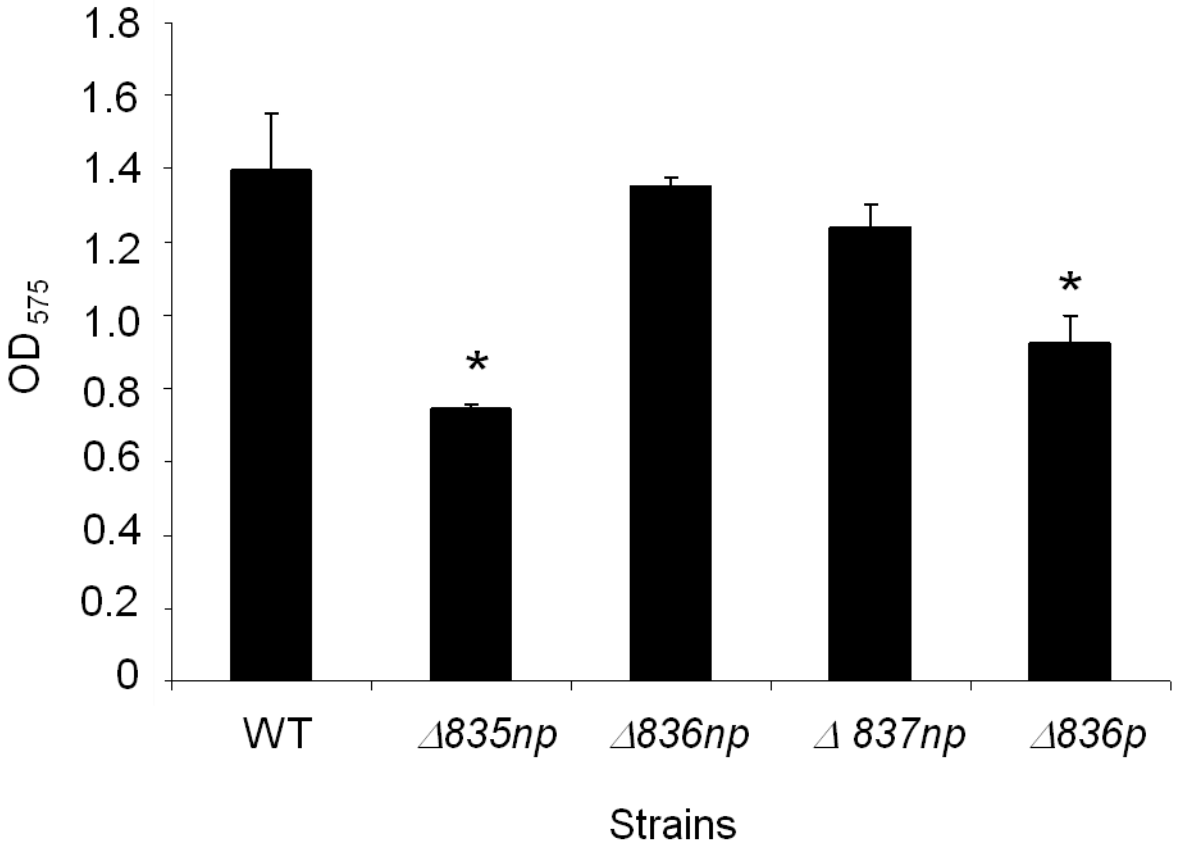


Figure 3-16. The differences in biofilm formation of the mutants compared with the wild-type strain in glucose. Cells were grown to OD_{600} =0.5 in BHI broth then diluted 1:100 into BM supplemented with 20 mM glucose in microtiter plates. Biofilm formation was quantified after 48 h as described in the methods section. The results are representative of three independent experiments performed in at least triplicate. *, Differs from the wild-type at $p<0.05$.

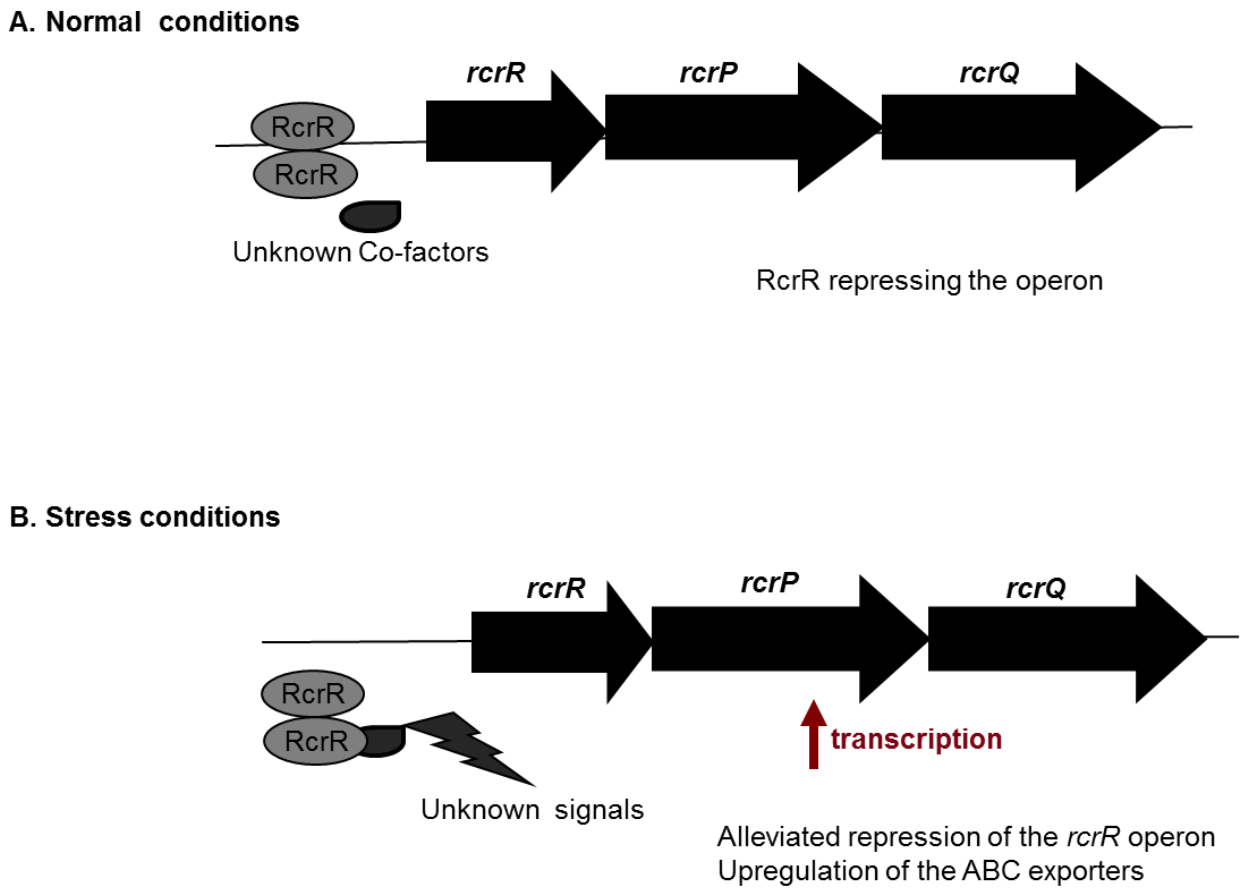


Figure 3-17. Schematic of the proposed regulation of the *rcrPQ* operon by the RcrR protein. A. The RcrR protein represses the operon under normal conditions. B. Under stress conditions, the accumulation of signals causes the RcrR protein to lose its repressing capabilities leading to derepression of the operon.

CHAPTER 4
REGULATION OF *relPRS* AND THE EFFECTS OF *SMU0835-0837* ON *relPRS* AND (p)ppGpp LEVELS

Introduction

The regulation of (p)ppGpp metabolism is critical for maintaining homeostasis in the cell and adapting to changes in nutrient availability. Virulence and other stress pathways have been linked to (p)ppGpp production in a variety of bacterial species (98). In addition, deletions of *relA* affected biofilm formation, a virulence property of *S. mutans* (119). The fact that there are three enzymes governing (p)ppGpp production in *S. mutans* suggests that (p)ppGpp metabolism is based on diverse signals. It was previously thought that nutrient availability was the main signal regulating (p)ppGpp metabolism, but the identification of the additional synthases, RelP and RelQ, suggests that other environmental signals regulate (p)ppGpp metabolism (122). The accumulation of (p)ppGpp in the absence of *relA* under non-stringent conditions also indicates that there are other factors influencing (p)ppGpp production in a dominant way (122). RelP was the major producer of (p)ppGpp under non-stringent conditions, and Lemos *et al.* also showed that overexpressing *relP* in a strain that lacks the (p)ppGpp hydrolase, caused growth arrest of the cells (122). These data demonstrate that production of (p)ppGpp by RelP is of significant importance in regulating the physiology of the cell. *relP* is co-transcribed with *relRS,* which encodes a TCS, and (p)ppGpp accumulation was also affected in a *relRS* mutant under non-stringent conditions (122) . The *relRS* mutant grew faster than the wild-type strain, indicating that (p)ppGpp levels were lower in this strain (122). The linkage of the RelRS TCS to RelP and (p)ppGpp production demonstrates that RelP-dependent production of (p)ppGpp is probably controlled by *relP* transcript levels unlike RelA which is regulated allosterically at the

enzymatic level. Since RelP-dependent production of (p)ppGpp is linked to the RelRS TCS, it also suggests that extracellular signals control RelPRS- dependent (p)ppGpp metabolism. However, these signals have not yet been identified. The upregulation of *relPRS* in aeration detected via microarray analysis (4) indicates that *relPRS* is regulated at the transcriptional level, and oxidative stress may be one of the signals to regulate (p)ppGpp metabolism.

Interestingly, members of our group showed that *SMu0838 (tpx)* and *SMu0839 (cipI)* were upregulated in aeration as well (4). The *SMu0835-837* operon was up-regulated during a mupirocin-induced stringent response in a (p)ppGpp-dependent manner (164), i.e., with no induction of these genes in a *relA* mutant. Therefore, the expression of the *relP* and *SMu0835-7* operons may be coordinated with one another in response to particular stresses or signals.

Results

SMu0835-837 Influence *relP* Expression and Vice Versa.

In order to test if the *SMu0835-0837* operon exerted an effect on the expression of *relPRS*, qRT-PCR was performed to compare the amount of mRNA expressed from the *relPRS* operon in the wild-type strain with that in various mutant strains. The expression level of the *relP* operon in the *Δ835np* and *Δ836p* mutants, which either overexpress or do not express the exporters, respectively, was approximately half that measured in the wild-type strain, whereas the expression of *relP* and *relRS* in the *Δ837np* and *Δ839* mutant was similar to that in wild-type strain (Figure 4-1, Figure 4-2). Similarly, qRT-PCR analysis was done to measure the expression of *SMu0835* and *SMu0836* in a *relP* mutant strain and it was noted that the expression these genes was approximately three-fold lower in the strains lacking *relP* or *relRS,* compared to the wild-

type strain (Figure 4-3, Figure 4-4). In contrast, the expression of *SMu0835* and *SMu0836* was similar in a *ΔrelA* mutant and the wild-type strain (data not shown), an expected finding given the knowledge that RelA does appear to contribute significantly to (p)ppGpp pools under non-stringent conditions in exponentially growing cells (122).

To verify the qRT-PCR results, the promoter for *relP* was fused to a promoterless chloramphenicol acetyltransferase (*cat*) gene and CAT activity was measured in exponentially-growing cells. Consistent with the qRT-PCR data, *relP* promoter activity was lower in the *Δ836p* mutant than in the wild-type background (Figure 4-5). Using a *cat*-gene fusion as described above, *SMu0835* promoter activity was analyzed in the *ΔrelP* genetic background and found to be 1.5-fold lower than in the wild-type genetic background (Figure 4-6). Thus, the *cat* fusion data were consistent with the qRT-PCR data showing that expression of the *SMu0835-7* operon was decreased significantly in the *ΔrelP* and *ΔrelRS* mutants, and vice versa.

Effect of SMu0835-0837 on (p)ppGpp Levels

To verify that the changes in *relP* expression noted in the *SMu0835-7* operon mutants were associated with alterations in the levels of (p)ppGpp in cells, alarmone production was monitored in exponentially-growing cells in the wild-type strain and selected mutants. The results demonstrated that (p)ppGpp production was adversely affected by loss of constituents encoded by the *SMu0835-7* operon, consistent with the changes in *relP* mRNA levels and *relP* promoter activity in these same mutants (Figure 4-7, Table 4-1).

Effect of Oxidative Stressors on (p)ppGpp Pools

RelP is the principal source of (p)ppGpp in exponentially-growing cells and it appears that the RelPRS system functions to slow the growth of the bacteria in

response to signals associated with stress, especially oxidative stress (Figure 3-15). The strain lacking *relP* and *relRS*, grew considerably faster than the wild-type strain when exposed to paraquat and aeration as noted in Figure 3-15. The accumulation of (p)ppGpp in the presence of hydrogen peroxide was tested. There was a significant increase in both ppGpp (GP4) and pppGpp (GP5) levels when 0.003% hydrogen peroxide was present during the labeling of the cells (Figure 4-8). The accumulation of GP4 and GP5, appeared to be dependent on RelPRS. There was no accumulation in GP4 or GP5 in mutants lacking *relP* or *relRS* in response to hydrogen peroxide (Figure 4-8). However, there were no significant changes in the Δ*relA* and Δ*relQ* mutant, suggesting that this accumulation of (p)ppGpp in response to aeration is predominantly *relPRS* dependent.

Effect of Oxidative Stressors on *relP* Promoter Activity

Since there was an increase in the amount of (p)ppGpp accumulated in response to hydrogen peroxide and the transcription of *relP* was up in aeration, β-galactosidase experiments examining the effects of hydrogen peroxide on the *relP* promoter were done. Consistent with the (p)ppGpp accumulation data, there was a significant increase in *relP* promoter activity when the cells were exposed to hydrogen peroxide compared to the cells that were not (Figure 4-9). The activity of the *relP* promoter was 164 ± 9 Miller units when exposed to hydrogen peroxide compared to an activity of 112 ± 5 Miller units when not exposed to stress.

Discussion

The overlap between control of expression of the *rcr* operon and the *relP* operon is of considerable interest. The underlying basis for the retention of multiple (p)ppGpp synthases in *S. mutans* and in certain other prokaryotes remains largely enigmatic, but

a reasonable explanation for the retention or acquisition of RelP and the RelRS system during evolution has been disclosed in this study. In particular, the evidence supports that the RelRS two-component system may sense oxidative stressors or by-products of oxidative metabolism as a signal to stimulate (p)ppGpp production by activating production of RelP. In previous studies done in our lab, it was shown that the production of (p)ppGpp by RelP plays a significant role in regulating the growth of *S. mutans* in exponentially growing cells (122). It was also shown that overexpression of *relP* in a strain lacking (p)ppGpp hydrolase activity could induce growth arrest (122). Of particular interest here was the substantially faster growth rate of the *ΔrelP* mutant strain in the presence of paraquat (Figure 3-15), likely arising from a failure of the mutant to accumulate (p)ppGpp through RelP activity. There is also a clear induction of (p)ppGpp in response to hydrogen peroxide stress, and the data show that RelPRS is the major producer of (p)ppGpp under these conditions. We propose that RelP-dependent production of (p)ppGpp in cells exposed to oxidative stress functions to slow the growth of the cells. Since *S. mutans* does not have a complete respiratory chain, has a limited repertoire of enzymes to detoxify reactive oxygen species and displays much better growth in conditions of lower oxygen tension, RelPRS may help to protect the cells from exhausting limited resources, from overproducing metabolites that are toxic, and from acquiring deleterious mutations.

In *E. coli*, it has been shown that (p)ppGpp plays a role in resistance to an intrinsic peptide by inducing efflux pumps to extrude the peptide (180). Interestingly, the RcrPQ pumps in *S .mutans* are also induced in response to an increase in (p)ppGpp pools during a mupirocin-induced stringent response (164). Both the *rcrRPQ* and *relPRS*

gene products appear to be modulating growth in response to aeration and, based on our CAT assays and real-time PCR analysis, the products of these two operons can exert an effect on the expression of each other. Thus, as part of our working model, we hypothesize that the RcrPQ export apparatus could play a role in externalizing the compound(s) that is detected by RelRS in quorum-sensing fashion to control the growth of *S. mutans* populations in response to elevated redox or reactive oxygen species (Figure 4-10). Such a feedback loop could at least partially explain the interdependence of *relP* and *rcr* operon expression and the decrease in (p)ppGpp levels in the mutant lacking the RcrPQ porters. This and other potential mechanisms controlling *rcr* and *relP* cross-regulation are presently under investigation. We propose, as part of our working model that the regulation of *relP* by *rcrRPQ* may be through the RcrR regulator binding to the *relP* promoter as well. Since *rcrRPQ* is responsive to high levels of (p)ppGpp, the regulation of the operon may be tied to RelP dependent (p)ppGpp levels.

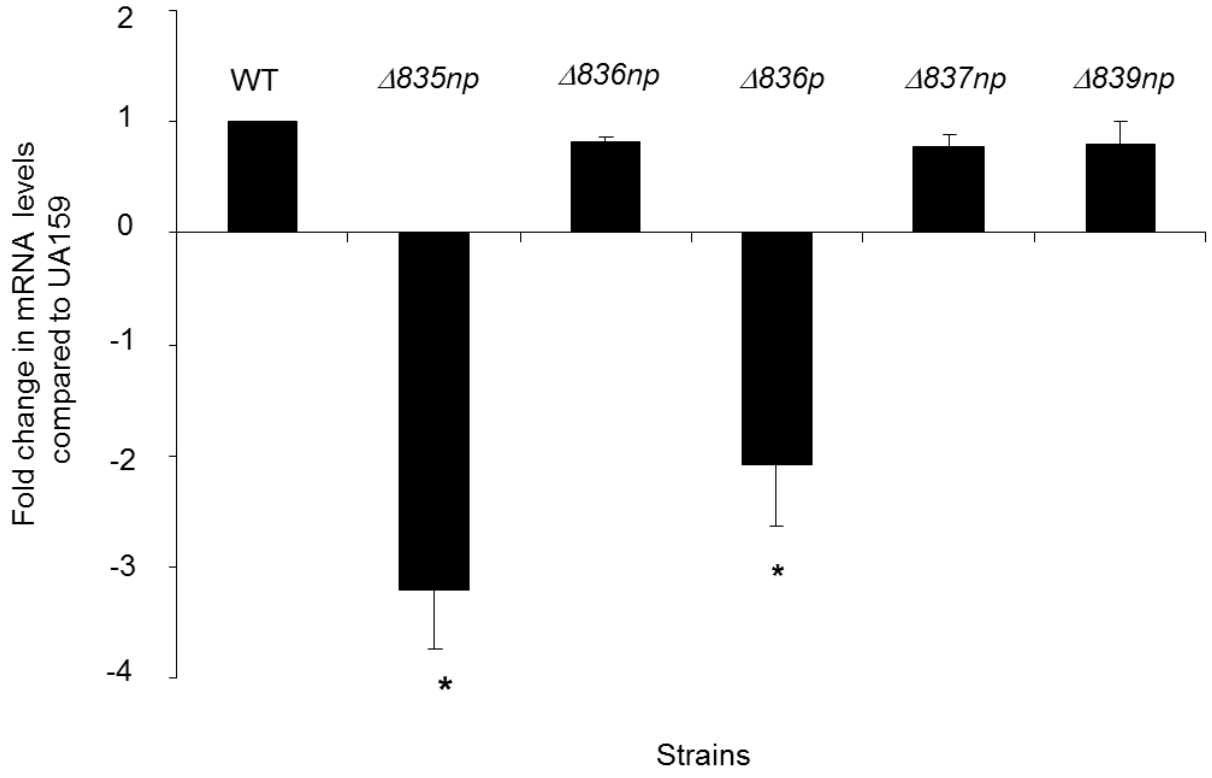


Figure 4-1. RealTime-RT-PCR showing RelP mRNA levels. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data represent the fold-change in copy numbers of the mutant strains compared to the wild-type strain where the wild-type value was set to 1.0 *, Differs from the wild-type at $p< 0.05$ (Student's *t*-test).

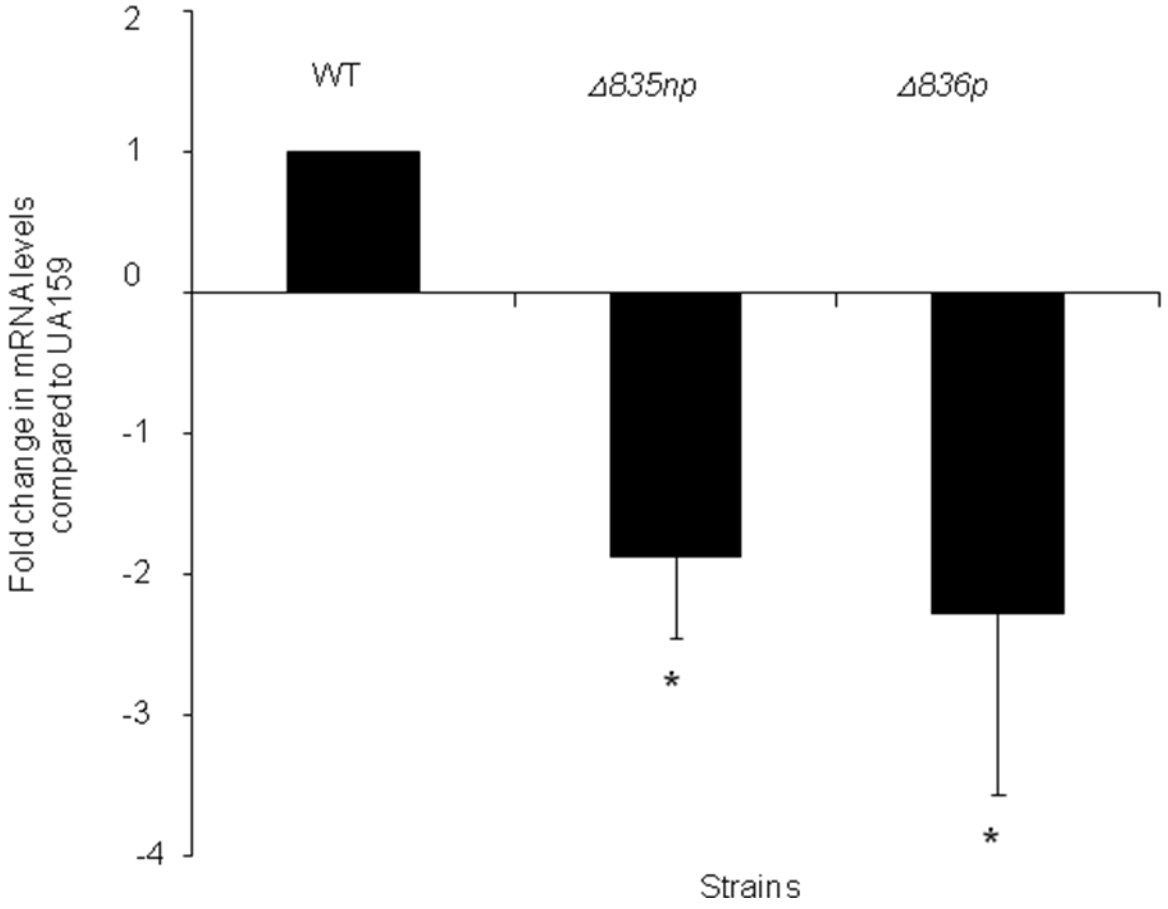


Figure 4-2. RealTime-RT-PCR showing RelRS mRNA levels. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data represent the fold-change in copy numbers of the mutant strains compared to the wild-type strain where the wild-type value was set to 1.0 *, Differs from the wild-type at $p< 0.05$ (Student's *t*-test).

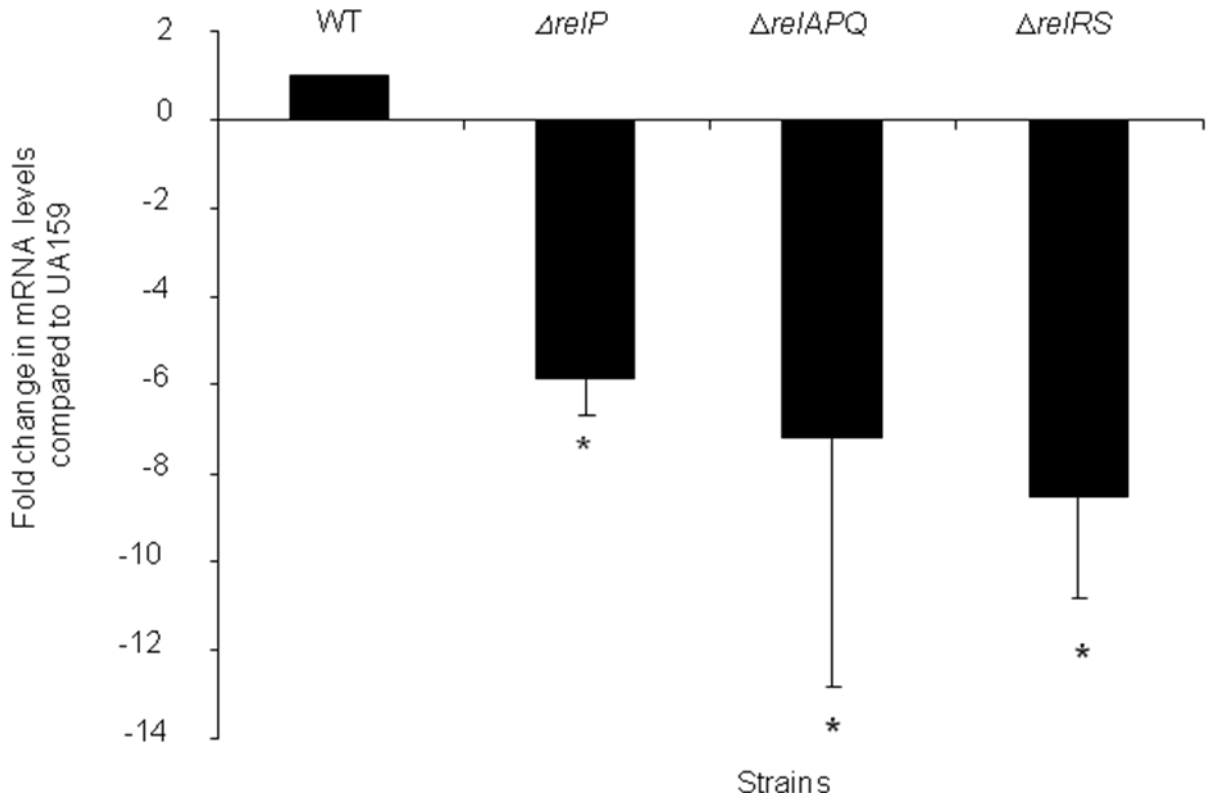


Figure 4-3. RealTime-RT-PCR showing RcrR mRNA levels. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data represent the fold-change in copy numbers of the mutant strains compared to the wild-type strain where the wild-type value was set to 1.0 *, Differs from the wild-type at $p < 0.05$ (Student's *t*-test).

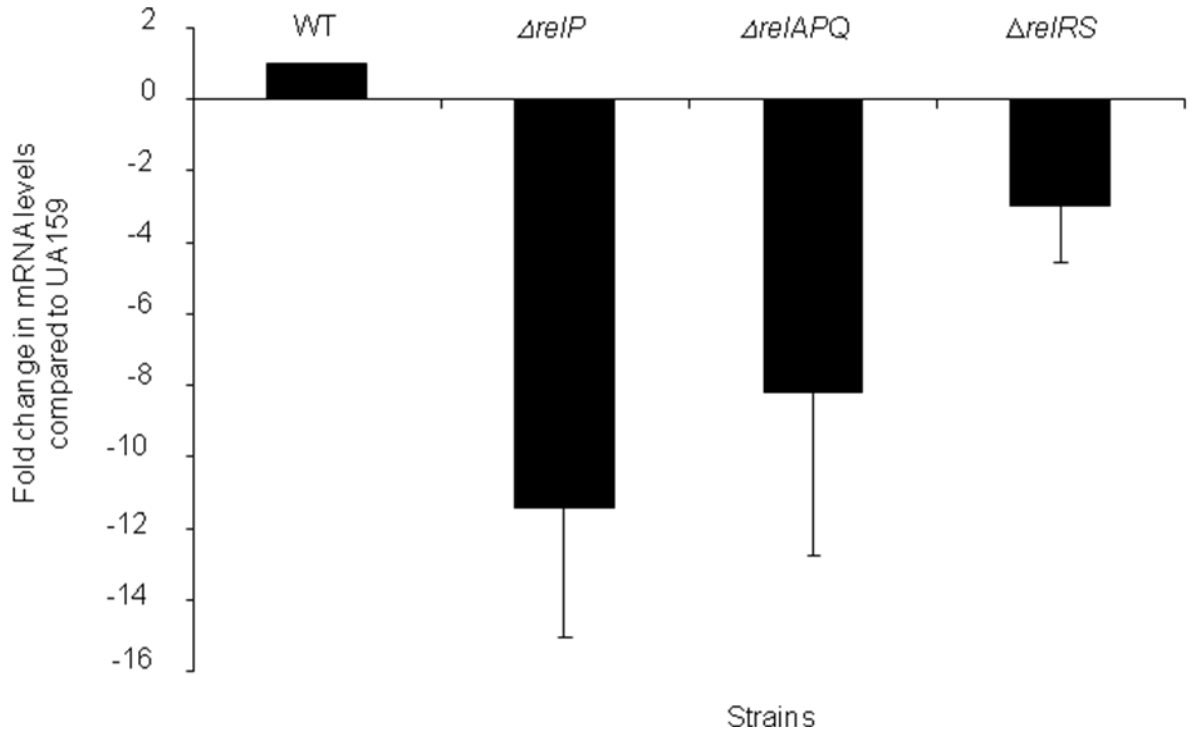


Figure 4-4. RealTime-RT-PCR showing *SMu0836* mRNA levels. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data represent the fold-change in copy numbers of the mutant strains compared to the wild-type strain where the wild-type value was set to 1.0.

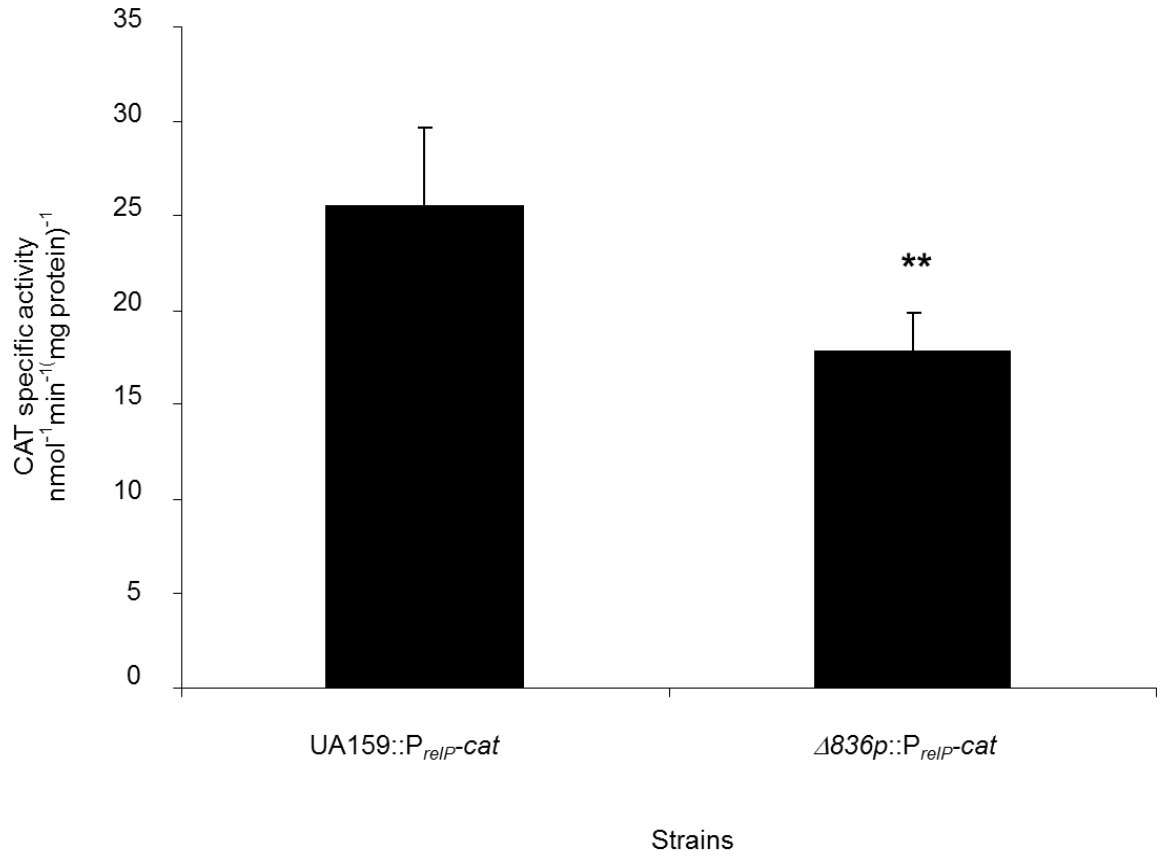


Figure 4-5 CAT activity from the *relP* promoter. The *relP* promoter was fused to a promoterless *cat* gene in the pJL105 integration vector. The *cat*-promoter fusion was transformed into the wild-type and *Δ836p* strains. In all cases cells were grown to mid -exponential phase (OD_{600} = 0.5) and CAT specific activity was measured as described in the methods section. The results are from three independent experiments performed in at least triplicate. *, Differs from the wild-type, $p < 0.05$ (Student's *t*-Test)

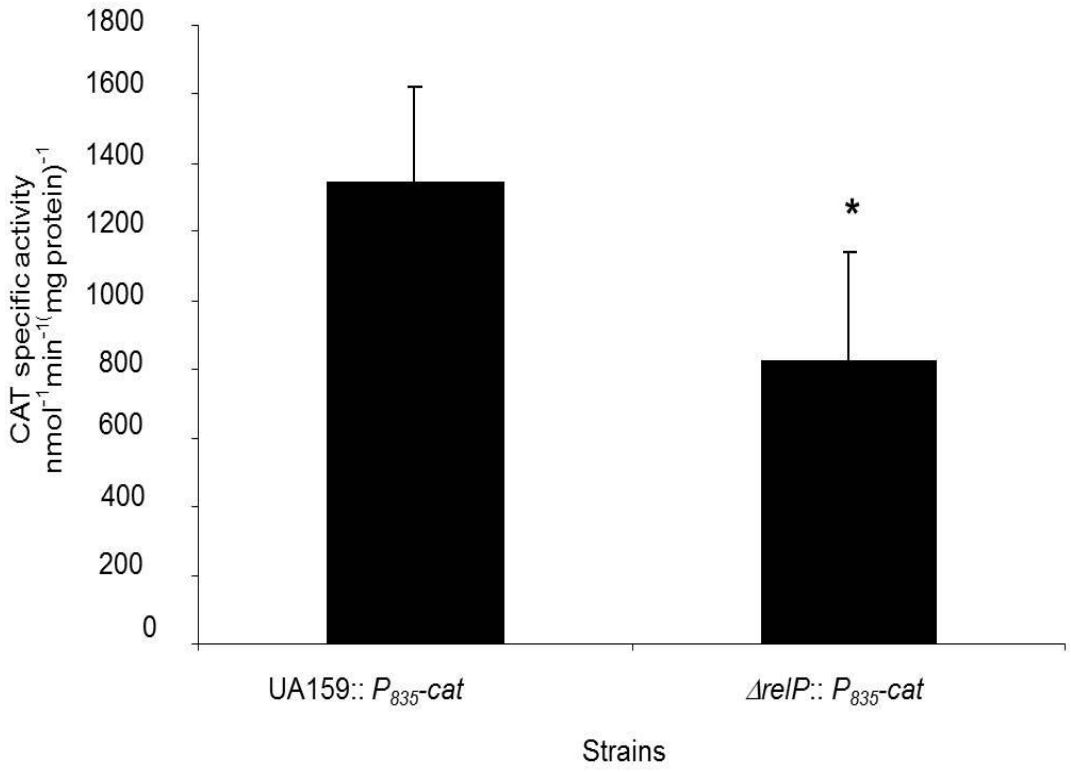


Figure 4-6 CAT activity from the *SMu0835* promoter. The *SMu0835* promoter was fused to a promoterless *cat* gene in the pJL105 integration vector. The *cat*-promoter fusion was transformed into the wild-type and *Δ836p* strains. In all cases cells were grown to mid -exponential phase (OD_{600} = 0.5) and CAT specific activity was measured as described in the methods section. The results are from three independent experiments performed in at least triplicate. *, Differs from the wild-type, $p < 0.05$ (Student's *t*-Test).

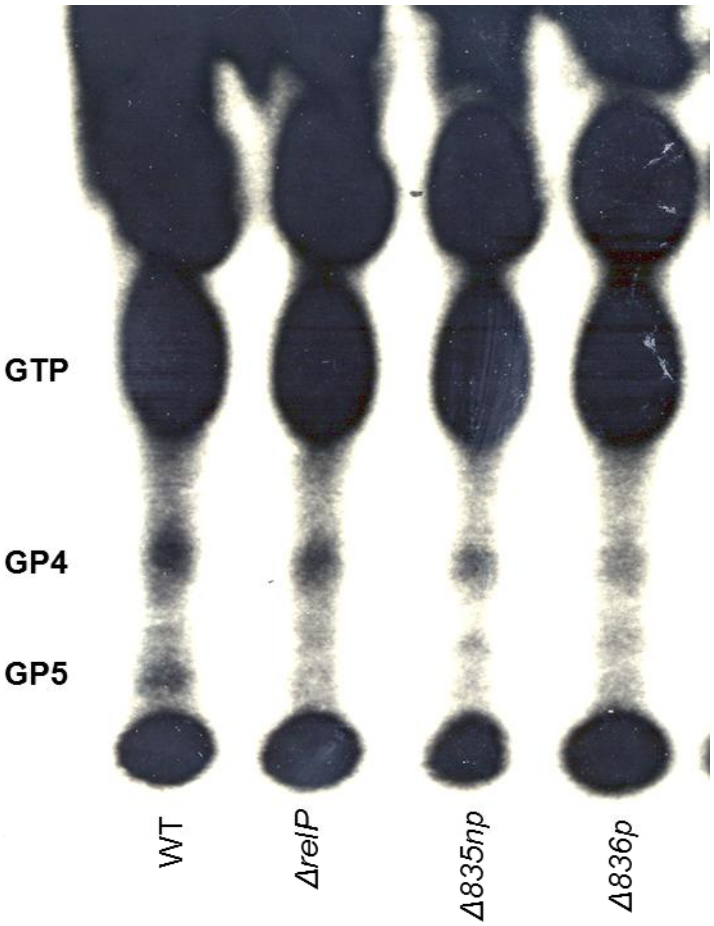


Figure 4-7. (p)ppGpp accumulation in mutant versus wild-type strains. Cells were grown to OD_{600} = 0.2 in FMC and labeled with ^{32}P-orthophosphate. The cells were incubated for 1 h and (p)ppGpp was extracted using 13M formic acid. 2 x 10^5 CPM of each sample was spotted to PEI-cellulose plates for TLC in 1.5 M KH_2PO_4 buffer. The data are a representative of n= 4 experiments. There was a similar trend observed in all cases.

Table 4-1. Percent change in GP4 (ppGpp) and GP5 (pppGpp) accumulation of mutant strains compared to the wild-type strain.

Strain	% Change in IDV* of GP4 compared to the wild-type strain	% Change in IDV* of GP5 compared to the wild-type strain
Δ*relP*	10% Reduction	43% Reduction
Δ*835np*	26% Reduction	62% Reduction
Δ*836p*	30% Reduction	45% Reduction

*****IDV= Integrated Density Value of the Spots

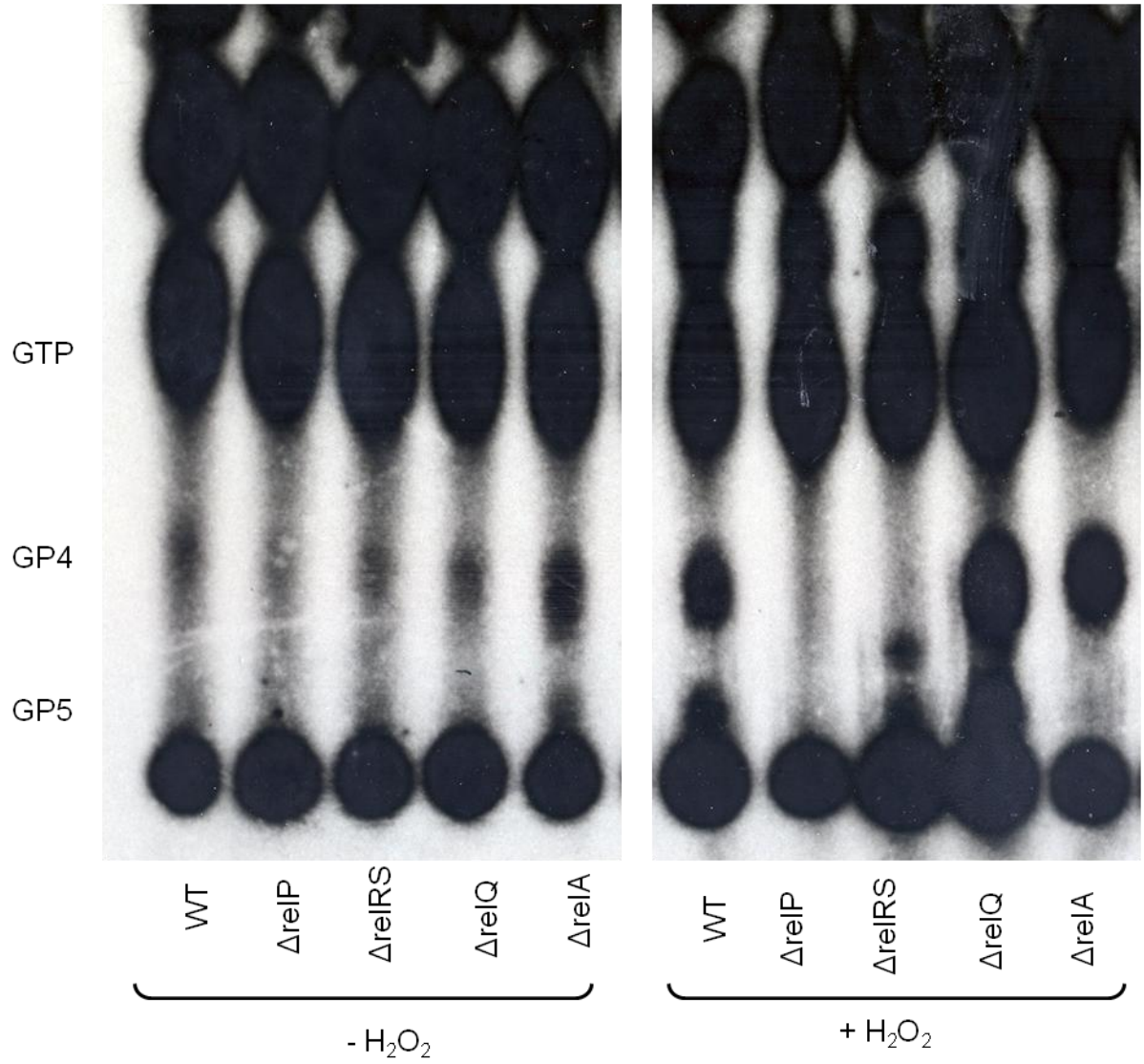


Figure 4-8. Accumulation of (p)ppGpp in hydrogen peroxide. Cells were grown to OD_{600} = 0.2 in FMC and labeled with ^{32}P-orthophosphate. H_2O_2 (0.003%) was added where noted during the labeling. The cells were incubated for 1 h and (p)ppGpp was extracted using 13M formic acid. 2 x 10^5 CPM of each sample was spotted to PEI-cellulose plates for TLC in 1.5 M KH_2PO_4 buffer. The data are a representative of n= 7 experiments. There was a similar trend observed in all cases. The spots observed in the Δ*relRS* deletion strain are an anomaly from the TLC.

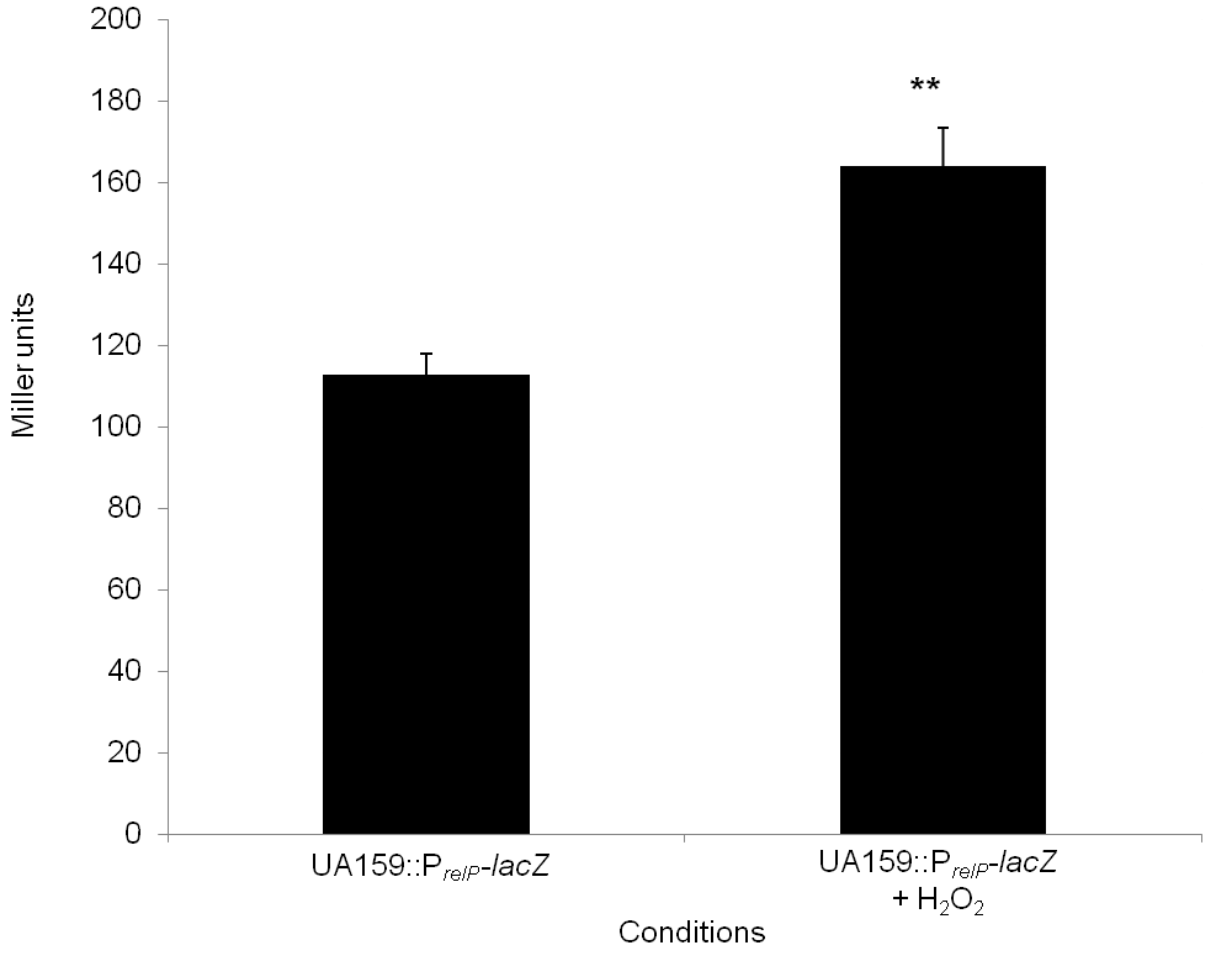


Figure 4-9. The effect of hydrogen peroxide on LacZ activity from the *relP* promoter. The *relP* promoter was fused to a promoterless *lacZ* gene in the pMZ integration vector. The *lacZ*-promoter fusion was transformed into the wild-type strain. In all cases cells were grown to OD_{600} = 0.2. H_2O_2 (0.003%) was added to some of the samples and incubated for 1 hr. LacZ activity was measured as described in the methods section. The results are from three independent experiments performed in at least triplicate. **, Differs from the wild-type, $p < 0.005$ (Student's *t*-Test)

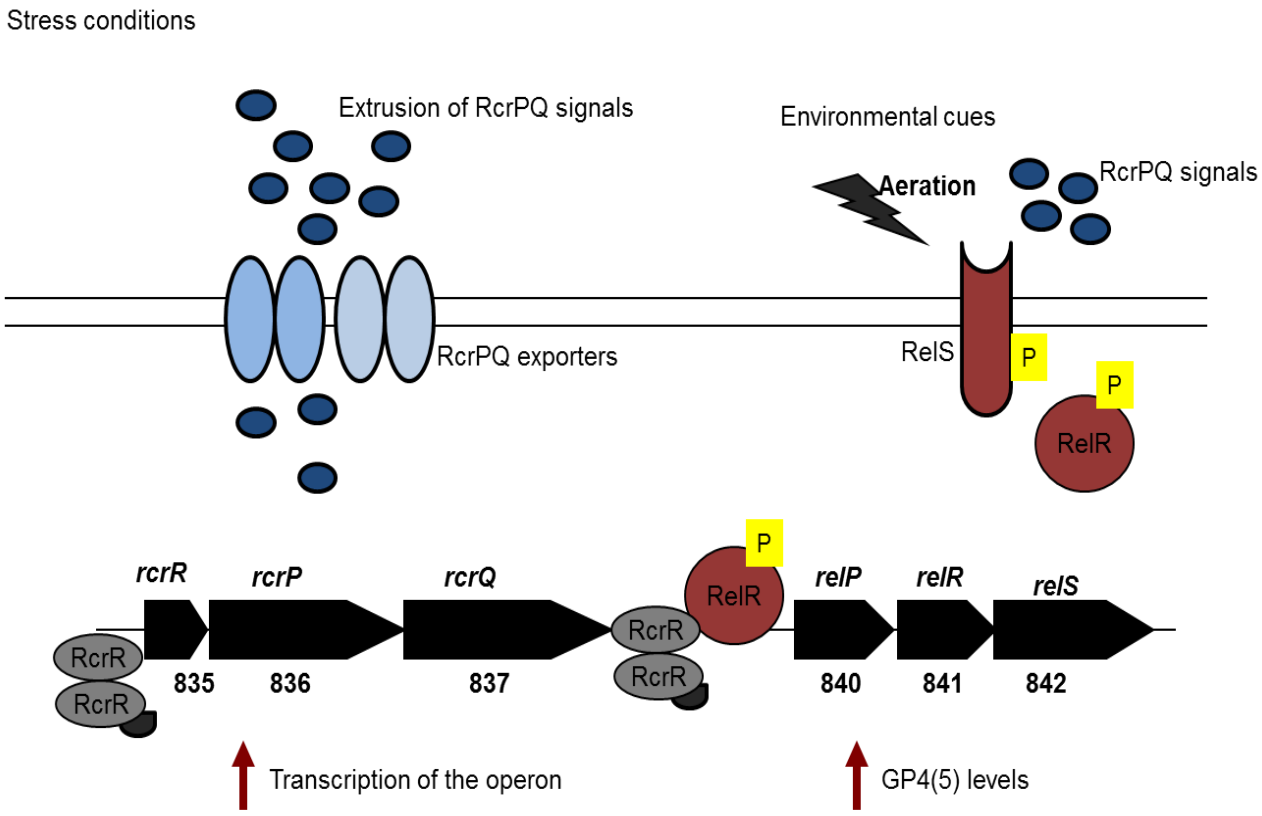


Figure 4-10. Schematic of the potential regulation of the *relPRS* operon by different environmental cues and potential cross regulation with the *rcrRPQ* operon. Under stress conditions, the *rcr* operon is derepressed to upregulate *rcrPQ* encoding the exporters causing the extrusion of substances. The RelRS TCS may be sensing these substances in addition to oxidative stress to regulate (p)ppGpp production by RelP.

CHAPTER 5
THE EFFECT OF *SMU0835-0837* ON THE COMPETENCE REGULON

Introduction

S. mutans is naturally competent, and elements of the competence regulon have been linked to stress tolerance and biofilm formation (127, 129). The induction of competence is a specifically timed event linked to the accumulation of peptide signals and environmental stresses. Studies done with *S. mutans* have shown that competence and acid tolerance, one of the key virulence factors of *S. mutans*, are intimately linked. In particular, mutants lacking components of the competence signaling pathway had a diminished ability to grow at low pH (127). The simplest description of the model for development of competence in some streptococci involves quorum sensing of a secreted peptide by a two-component system, which triggers a cascade of expression of early and late competence genes involved in DNA uptake and recombination. However, recent studies reveal that the mechanisms underlying the regulation of the competence pathway in *S. mutans* are more complex, and that much remains to be understood about the signals, signal transduction systems and additional regulatory pathways affecting competence (8). It is now known that another peptide signaling pathway, ComRS, is the proximal regulator of *comX* in *S. mutans* (149). Previous work done in our lab revealed that multiple signals are required for efficient activation of competence genes through the CiaRH and ComDE two-component systems. ComDE regulates competence by sensing CSP levels, whereas CiaRH appears to be involved in sensing as-yet-undefined signals in a CSP-independent manner. The expression of *comYA* and *comX* are only highly induced when both CSP and horse serum (HS) are present, compared to the addition of CSP or HS alone,

indicating that signals present in HS, possibly additional peptides, regulate competence genes in conjunction with CSP. (8). Therefore, there are multiple parallel systems regulate the competence network in *S. mutans*.

In addition to the slow growth phenotype observed in the various strains with *rcrRPQ* mutations, a loss in the ability to take up DNA was seen in the ∆*835np* strain, and a hyper transformable phenotype was observed in the ∆*835p* strain. Therefore, the mutations in the *rcrRPQ* operon appeared to affect the ability of the cells to uptake DNA. In addition, the CipI immunity protein, which is encoded downstream of the *rcrRPQ* operon, is upregulated in high levels of CSP. The *relPRS* operon, which based on our data is linked to *rcrRPQ* opero, is also affected by high levels of CSP. Based on these data, the linkage of the *rcrRPQ* operon to the competence regulon and the ability of the *rcrRPQ* mutants to take up DNA were carefully assessed.

Results

Competence Defect in *rcrRPQ* Mutants.

The transformation efficiency of the mutants was strongly dependent on the expression of *rcrR* and on the expression levels of the *rcrPQ,* which encode the exporters (Table 5-1). Specifically, when compared with the wild-type strain, the *Δ836p* mutant, lacking both ABC exporters, had lower transformation efficiency when CSP was provided (~10 fold decrease), but higher transformation efficiency than the parental strain in the absence of CSP (~100 fold increase) (Table 5-1, 5-2). Of particular interest, we were unable to obtain even a single transformant of the *Δ835np* strain, which lacks the MarR-like regulator and overexpresses the ABC porter genes (Figure 5-1, Figure 5-2), as well as the strain lacking all three genes (*Δ835-7np*), regardless of whether exogenous CSP was added or not (Table 5-1, 5-2). We also attempted to

transform the *Δ835np* and *Δ835-7np* mutants with plasmid pMSP3535 (32), which like pDL278 is a shuttle vector, but it carries an erythromycin marker instead of spectinomycin resistance, as well as with the pJL105 integration vector (Table 2-1). In no case were transformants isolated (data not shown).

Interestingly, we also analyzed a mutant in which the ΩKm marker (8) was used to replace the *SMu0835* gene (*Δ835polar*). qRT-PCR analysis of the *Δ835p* polar mutant revealed that *rcrP* and *rcrQ* were expressed at levels similar to the wild-type strain (Table 5-1, Figure 3-2, Figure 3-3, Figure 5-1). We determined that the expression of the genes downstream of the polar marker did not arise from an internal promoter in *rcrR*, since cloning of the 3' portion of the *rcrP* gene behind a reporter gene gave no detectable expression in *S. mutans* (data not shown). Instead, since loss of *RcrR* caused roughly a 100-fold increase in the transcription of *SMu0836-7* (Table 5-1, Figure 3-2, Figure 5-1), we believe that high level activation of the *SMu0835* promoter in the strain lacking the MarR-like regulator leads to more read-through of the terminator in the ΩKm element. Thus, if the ΩKm terminator was roughly 99% efficient, then the observed results would be expected. Importantly, the *Δ835polar* strain, lacking the MarR regulator but expressing the exporters at levels that were essentially the same as the wild-type strain, was hyper-transformable (Table 5-1, Table 5-2). In particular, even in the absence of exogenously added sCSP nearly 10^4-fold more transformants were obtained than with the wild-type strain (Table 5-1, Table 5-2).

To determine if the results were due to loss of the MarR-like transcriptional repressor, as well as to changes in the transcription of the ABC porter genes, we introduced a wild-type copy of the *SMu0835* gene expressed from its own promoter on

the shuttle plasmid pDL278 into the Δ*835p*, Δ*835np* and Δ*835-837np* mutant strains (Table 1, Figure 5-2). Complementation of the Δ*835np* and Δ*835p* mutants, strains *835*$^{+}$*/*Δ*835np (SJ361)* and *835*$^{+}$*/*Δ*835p (SJ362),* restored wild-type transformation efficiency and wild-type levels of *SMu0836* gene expression (Figure 5-1, Figure 5-2, Table 5-2). However, introduction of the MarR-like regulator into the strain lacking all three genes in strain *835*$^{+}$*/*Δ*835-7np (SJ360)*, which still lacked the SMu0836/7 transporters, did not restore transformation (Figure 5-3, Table 5-1). Clearly, then, the absolute expression levels of the ABC transporter genes, as well as the presence of the MarR-like regulator, are critical for competence development. Since it is not possible to control the level of expression of complementing genes in *S. mutans* with any precision, we did not complement the mutant strains with the ABC transport genes in this study. Thus, a more reliable picture of the contribution of these gene products to stress tolerance and their impact on competence was garnered by contrasting the behaviors of the various polar and non-polar mutants, and complemented strains. Once the substrate(s) for the porters is identified, efficiency of translocation of the substrate(s) by particular mutants can be correlated with phenotype.

***SMu0835-7(rcrRPQ)* Affect *comX* and *comY* Expression.**

To begin to understand the basis for the changes in the transformation efficiency in strains with aberrant expression of the genes for the MarR-like regulator and ABC transporters, we measured the expression of *comD, comX* and *comYA* in the different mutant strains by qRT-PCR (8, 156). ComD is the response regulator in the two-component system involved in sensing of CSP, *comX* encodes an alternative sigma factor that is under the control of ComD, and *comYA* is required for competence development and is transcriptionally activated by ComX. The qRT-PCR analysis

showed that the expression of *comD* was not affected in the mutant strains compared to the wild-type strain (data not shown). In contrast, the expression of c*omYA* was down-regulated nearly 15-fold in the non-transformable *Δ835np* and *⊿835-837np* mutants, and up-regulated by more than 100-fold in the hyper-transformable *Δ835p* polar strain (Figure 5-4, Figure 5-5). Interestingly, the expression of *comX* was up-regulated nearly 100-fold in both the *⊿835np* and *Δ835p* mutant strains (Figure 5-6, Figure 5-7), but the effect on *comY* expression in these strains was markedly different. Therefore, the effects of mutations in, or changes in the expression levels of, *Smu-0835-7* may affect competence by interfering with ComX activation of *comY*. Also relevant is that complementation analysis revealed that the *835*$^{+}$*/Δ835np* and *835*$^{+}$*/Δ835p* strains expressed wild-type levels of *comX* and *comYA* (Figure 5-5, Figure 5-7).

Differences in Growth of Mutant Strains in the Presence of CSP

High levels of CSP (2 μM) have been shown to cause growth inhibition of the wild-type strain of *S. mutans*. The CSP-induced growth inhibition has been shown to be linked to *comX* levels, the induction of CipB bacteriocin and its immunity protein CipI, which is encoded downstream of the *rcrRPQ* operon (175). In *S. pneumoniae* competent cells produce lytic factors, such as bacteriocins which are targeted against non-competent cells, as a phenomenon known as “pneumococcal fratricide”(44). However, some of the mechanisms for growth inhibition are not known. The growth of the mutants lacking *rcrRPQ* was tested with and without the addition of 2 μM CSP. Interestingly, the *Δ835p* strain, which is hypertransformable, was more sensitive to CSP-induced growth inhibition than the wild-type strain (Figure 5-8, Figure 5-9). The wild-type strain had a lag time of 5 hours whereas the *Δ835p* strain had a lag time of 15

hours. However, the Δ*835np* strain, which is non- transformable, and the Δ*836p* strains were more resistant to growth inhibition by CSP than the wild-type strain (Figure 5-8, Figure 5-9). In fact, high levels of CSP impacted the growth of these strains very little. The wild-type strain had a final OD of 0.5 whereas the Δ*835np* strain had a final OD of 0.7.

Discussion

Work presented herein adds a novel dimension to the control of genetic competence in *S. mutans*, and likely in other naturally-competent streptococci, by demonstrating that the RcrRPQ system plays a dominant role in modulating *com* gene expression and transformation in *S. mutans.* Notably, even after the addition of HS and CSP, no transformants could be obtained in the *Δ835np* mutant strain. The transformation deficiency of the Δ*835np* strain was associated with decreased expression of *comYA* and aberrant regulation of *comX* (Figure 5-4, Figure 5-6). The addition of *SMu0835 (rcrR)* back into the Δ*835np* and Δ*835p* strains restored wild-type levels of *comX* and *comYA* expression and wild-type transformation efficiency, whereas complementation of the Δ*835-837np* strain did not restore transformability. One explanation for the lack of transformation in the *835*$^{+}$*/*Δ*835-837np* strain could be due to the expression levels of *rcrR*. In the *835*$^{+}$*/*Δ*835-837np* stain, *rcrR* was not expressed at wild-type levels, but was actually overexpressed when present on plasmid pDL278 (Figure 5-1). Interestingly, we also constructed a strain in which we overexpressed *rcrR* in a wild-type genetic background (Table 5-2, Figure 5-2). In this case, a reduction in transformation efficiency (data not shown) and *comYA* expression (Figure 5-5) were noted. Therefore, the complete lack of transformability in the *835*$^{+}$*/*Δ*835-837np*

complemented strain, versus the behavior of the strain lacking only the ABC porter genes (Δ*835-837np*) may be related to overexpression of *rcrR*. Collectively, then, the data support the idea that RcrR has the ability to influence the expression of the *com* genes and transformation independently of the ABC transporters.

We propose that the way in which RcrR impacts competence is by negatively regulating the expression of a factor that interferes with ComX activation of *comY*. Specifically, it is established that ComX is required for activation of *comYA* expression in response to CSP. However, particular mutants lacking RcrR (Figures 5-4 to 5-7) display up-regulation of *comX* with concurrent down-regulation of *comY*. The simplest explanation for these findings is that RcrR represses the expression of an anti-sigma factor, similar to the model in *Streptococcus pneumoniae* in which ComX activity is negatively controlled by the ComW anti-sigma factor, blocking activation of late *com* gene expression (137, 216). Also, the lytic behavior of the cells in the presence of high concentrations of CSP was previously shown to be dependent on ComX (175). However, in both the Δ*835np* and Δ*835p* strains, *comX* levels were high but had opposite growth phenotypes in the presence of high CSP (Figure 5-8, Figure 5-9). Of note, the levels of *comYA* and transformation efficiency were more indicative of the lytic behavior. High levels of *comYA* corresponded with higher transformation efficiency and more susceptibility to growth inhibition by CSP.

The effect of the *rcr* system on *comY* and competence appears not only to be affected by RcrR, but also to depend strongly on the level of expression of the *rcrPQ* genes. In fact, one of the more intriguing aspects of this study is that defects in transformation are evident both with the loss of, and the overproduction of, the ABC

porters. In particular, the Δ*835polar* mutant, which has no RcrR but expresses levels of *rcrPQ* that are similar to the wild-type strain, for reasons explained in the results section, has an opposite phenotype to that of the Δ*835np* strain, which overexpresses the *rcrPQ* genes by >100-fold. In this case, the Δ*835p* polar mutant was hypertransformable and displayed up-regulation of *comY*, whereas the Δ*835np* strain could not be transformed, even when exogenous CSP was provided, and had low *comY* expression. Furthermore, the Δ*836p* polar mutant, which had lost both porters, had reduced transformation efficiency compared to the wild-type strain, reinforcing that the appropriate expression of the exporters is necessary for efficient transformation.

The complementation data with the *rcrR* gene provide further evidence that appropriate levels of the RcrPQ porters and proper regulation of the *rcrRPQ* operon are necessary for efficient transformation. In addition, overexpression of the genes encoding the porters, displayed a complete loss of transformation, as was seen in the Δ*835np* strain where the ABC porters are also overexpressed (199). Consequently, whatever the RcrPQ exporters are externalizing is necessary for proper regulation and development of competence. Of note, we tested the transformation efficiency of a *ΔcomC/Δ835p* double knockout strain and observed a hypertransformable phenotype, as was seen in the strain carrying only the *Δ835p* mutation, so the RcrPQ porters are probably not affecting the production or secretion of ComC. Also, the signal(s) being exported by these transporters have the potential to act from inside and/or outside the cell, since *rcrPQ* expression can neither be too high or too low if efficient transformation of cells is to occur.

Table 5-1. Summary showing mutant strains, *SMu0835-837* mRNA expression levels and transformation efficiency compared to the wild-type strain.

Strain	*SMu0835* Expression	*SMu0836* Expression	*SMu0837* Expression	Transformation Efficiency +CSP	Transformation Efficiency –CSP
Δ835np	Deleted	100-fold Increase	100-fold Increase	None*	None
Δ835p	Deleted	Wild-type levels	Wild-type levels	10-fold Increase	10^4-fold Increase
Δ835-837np	Deleted	Deleted	Deleted	None	None
Δ836p	Wild-type levels	Deleted	1000-fold Decrease	10-fold Decrease	100-fold Increase
Δ836-837np	Wild-type levels	Deleted	Deleted	10-fold Decrease	100-fold Increase
835$^+$/Δ*835np*	Wild-type levels	Wild-type levels	Wild-type levels	Wild-type levels	Wild-type levels
835$^+$/Δ*835p*	Wild-type levels	Wild-type levels	Wild-type levels	Wild-type levels	Wild-type levels
835$^+$/Δ*835-837np*	10-fold Increase	Deleted	Deleted	None	None

***** None **=** Zero transformants were detected when 0.04 ml of the transformation mixture was plated directly onto selective medium

Table 5-2. Transformation efficiency of the wild-type and various mutants strains in the presence or absence of added CSP

Strain	% Transformants* + CSP	% Transformants – CSP
UA159	1.7×10^{-3}	1.44×10^{-6}
Δ835np	0	0
Δ835p	4.0×10^{-2}	1.0×10^{-2}
Δ835-837np	0	0
Δ836p	1.87×10^{-4}	2.39×10^{-4}
Δ836-837np	1.61×10^{-4}	1.24×10^{-4}
Δtpx np	3.25×10^{-3}	2.26×10^{-6}

% transformants = (number of transformants/total viable bacteria) x 100. +CSP, exogenous CSP added; -CSP, no CSP added.

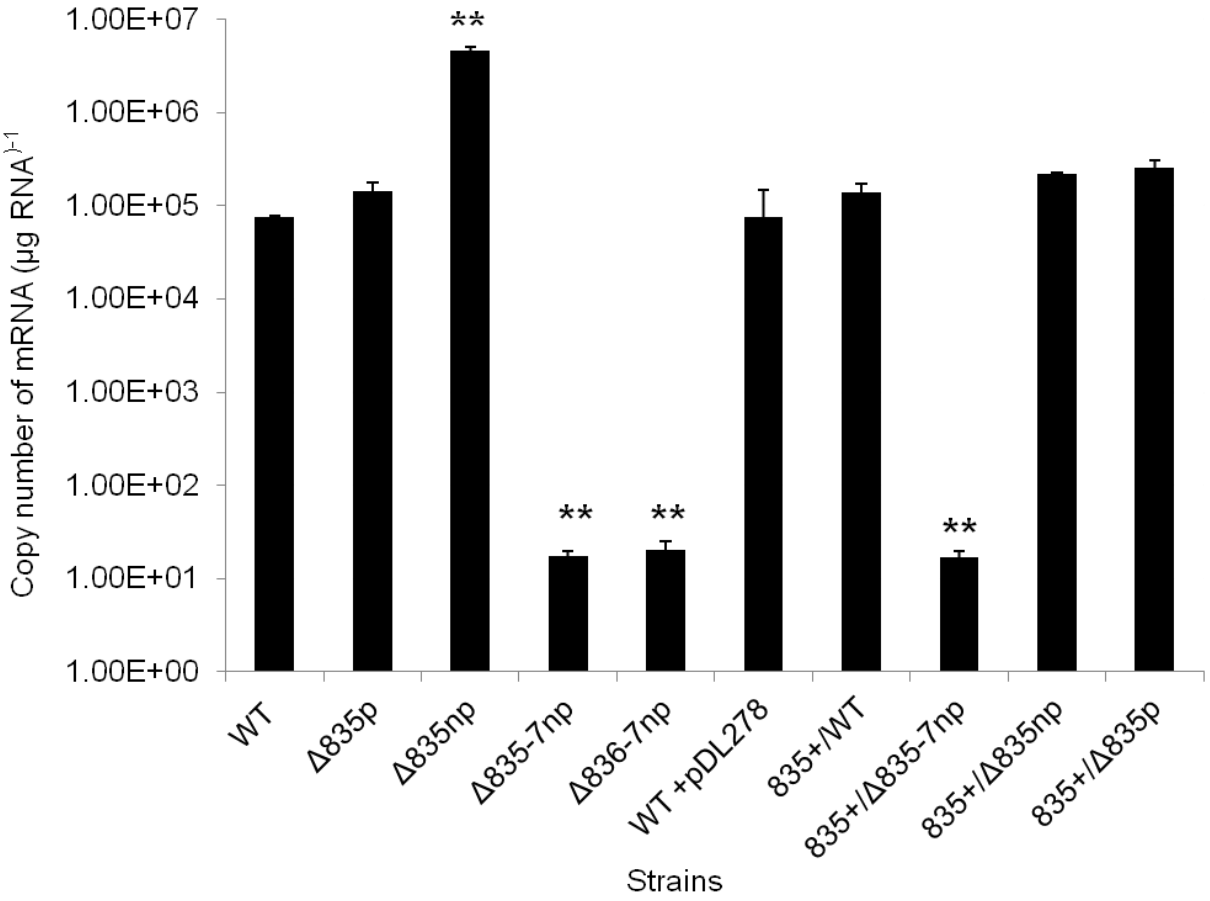


Figure 5-1. RealTime-RT-PCR showing *SMu0836 (rcrP)* mRNA levels. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data presented as the copy number of each gene per µg of input RNA. *, Differs from the wild-type at $P < 0.05$ (Student's t-test). These data were generated by Ann Sagstetter Decker in the summer of 2010, who was a dental student doing research on the *rcrRPQ* operon and competence

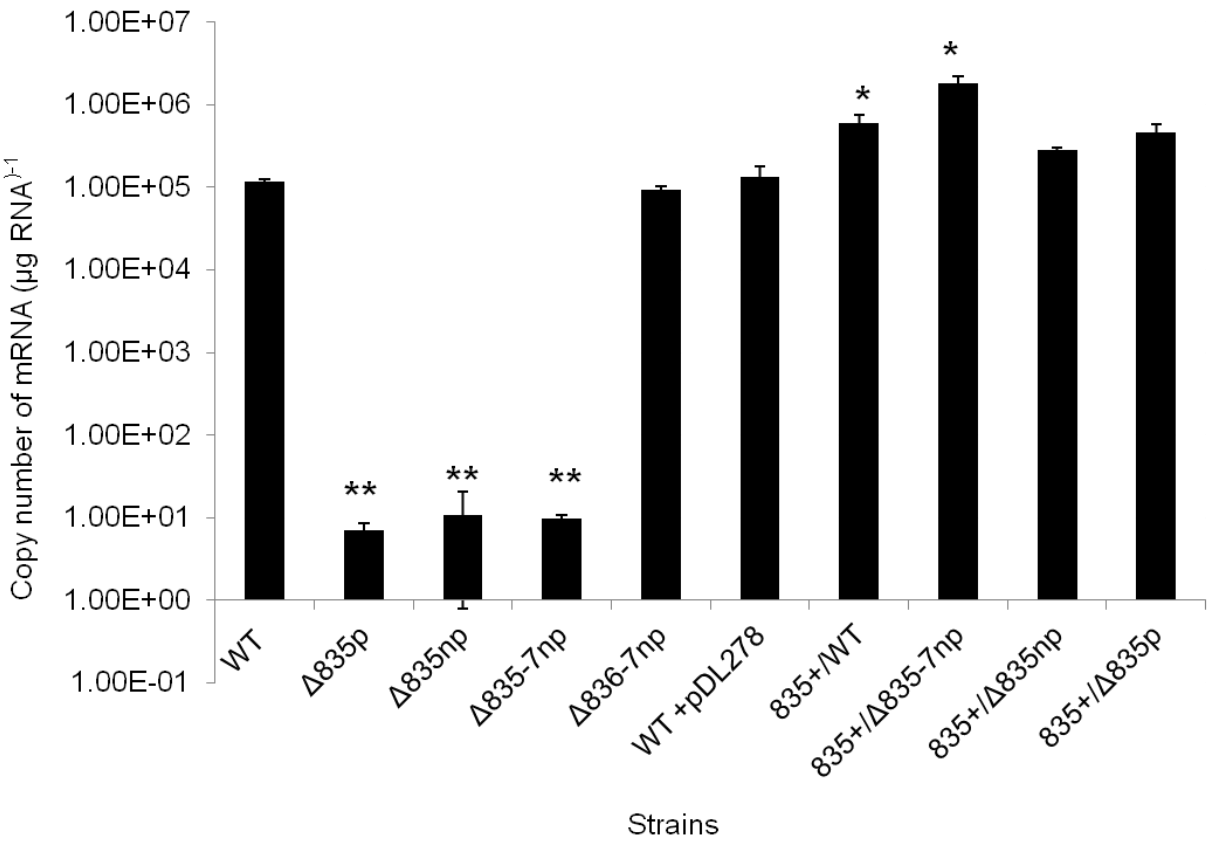


Figure 5-2. RealTime-RT-PCR showing *SMu0835 (rcrR)* mRNA levels. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data presented as the copy number of each gene per µg of input RNA.*, Differs from the wild-type *at p*< 0.05 (Student's *t*-test). **, Differs from the wild-type at *p*<0.005. These data were generated by Ann Sagstetter Decker in the summer of 2010, who was a dental student doing research on the *rcrRPQ* operon and competence.

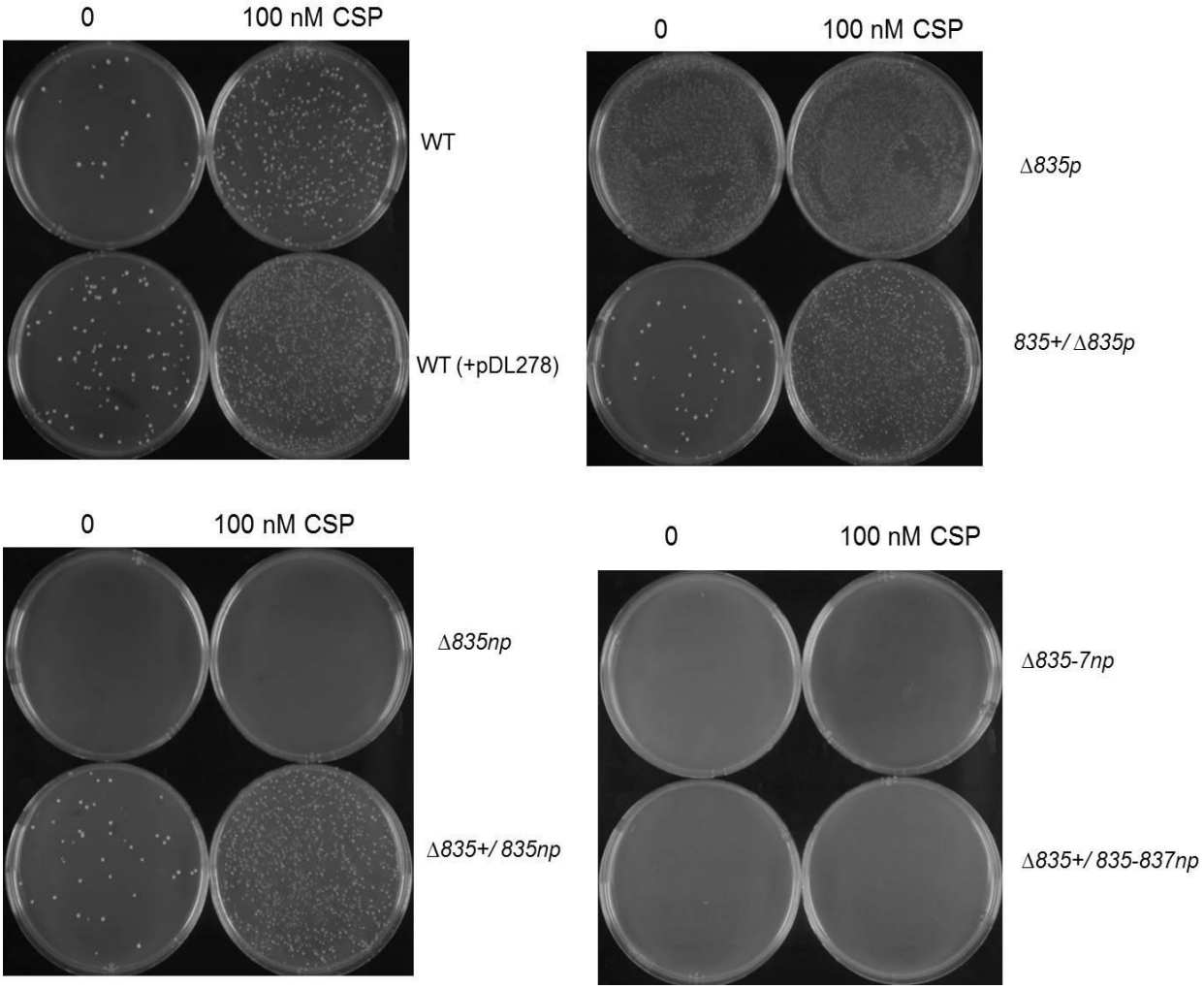


Figure 5-3 Transformability of the complemented strains compared to the mutant and wild-type strains. Cultures were grown to OD_{600} =0.15 and either 0 or 100 nM CSP was added to the cells for 15 mins. Plasmid pPMSP3535 (500 ng) was added. Cells were incubated for 2.5 h and 50 µl of the undiluted reaction was plated on BHI agar with erythromycin. This data were generated by Sang-Joon Ahn, Ph.D.

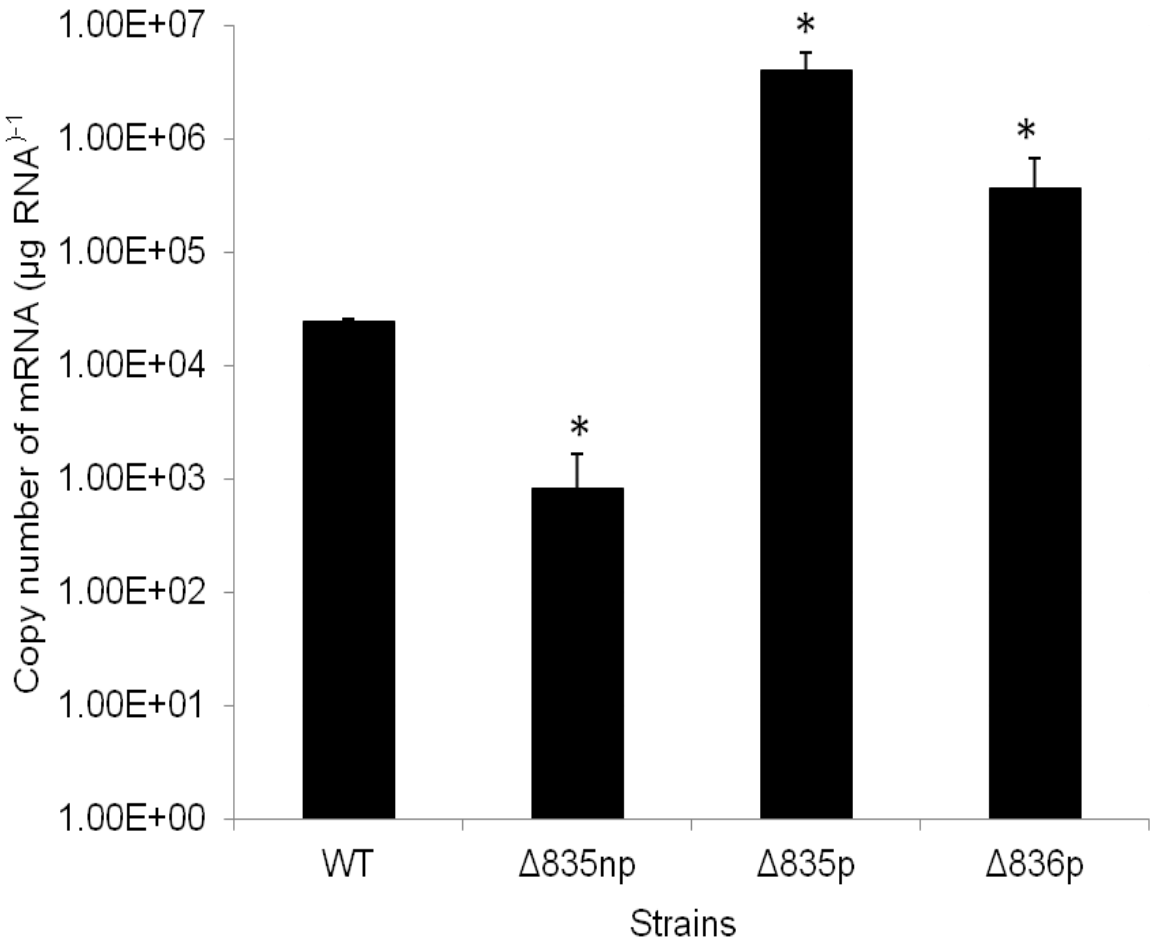


Figure 5-4. RealTime-RT-PCR showing *comYA* mRNA levels. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data presented as the copy number of each gene per µg of input RNA. *, Differs from the wild-type at $p < 0.05$ (Student's *t*-test).

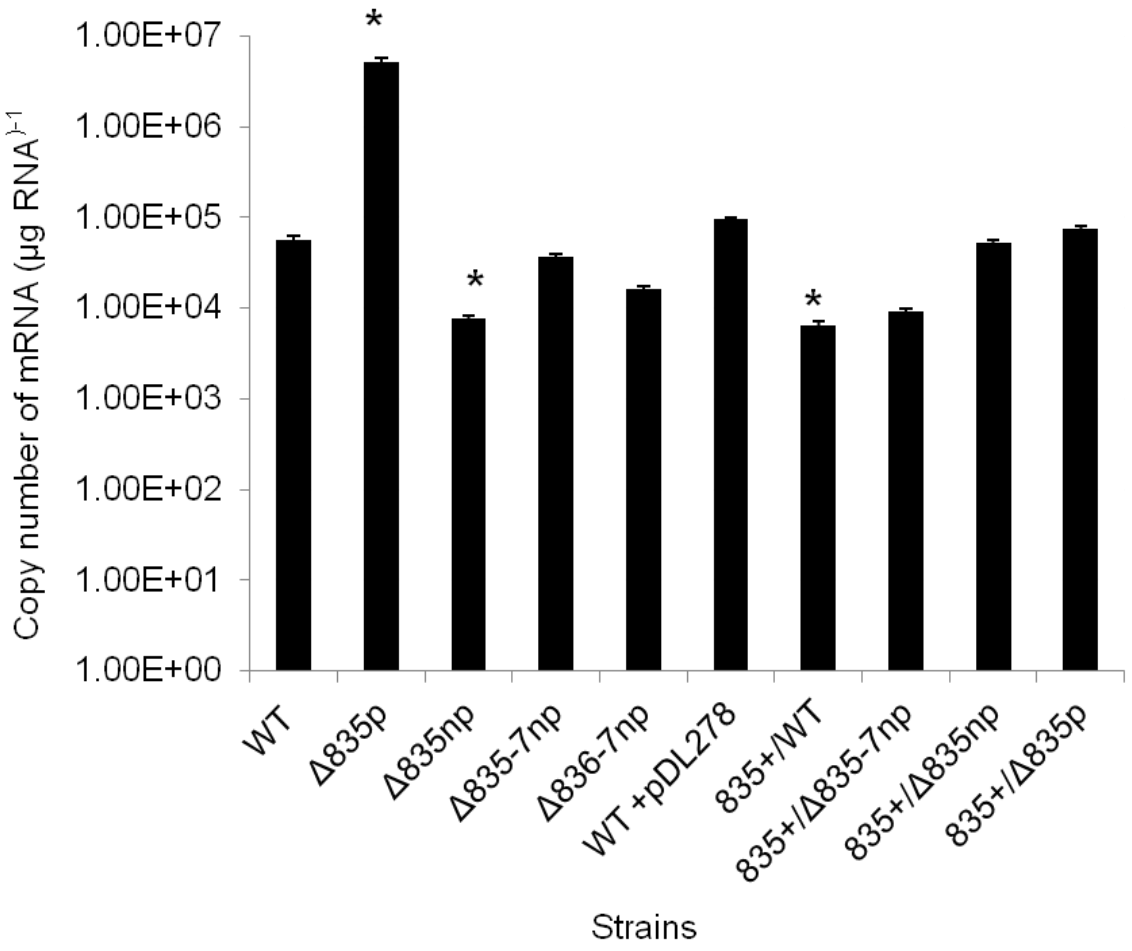


Figure 5-5. RealTime-RT-PCR showing *comYA* mRNA levels in complemented strains. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data presented as the copy number of each gene per µg of input RNA. *, Differs from the wild-type at $p<0.05$. These data were generated by Ann Sagstetter Decker in the summer of 2010, who was a dental student doing research on the *rcrRPQ* operon and competence

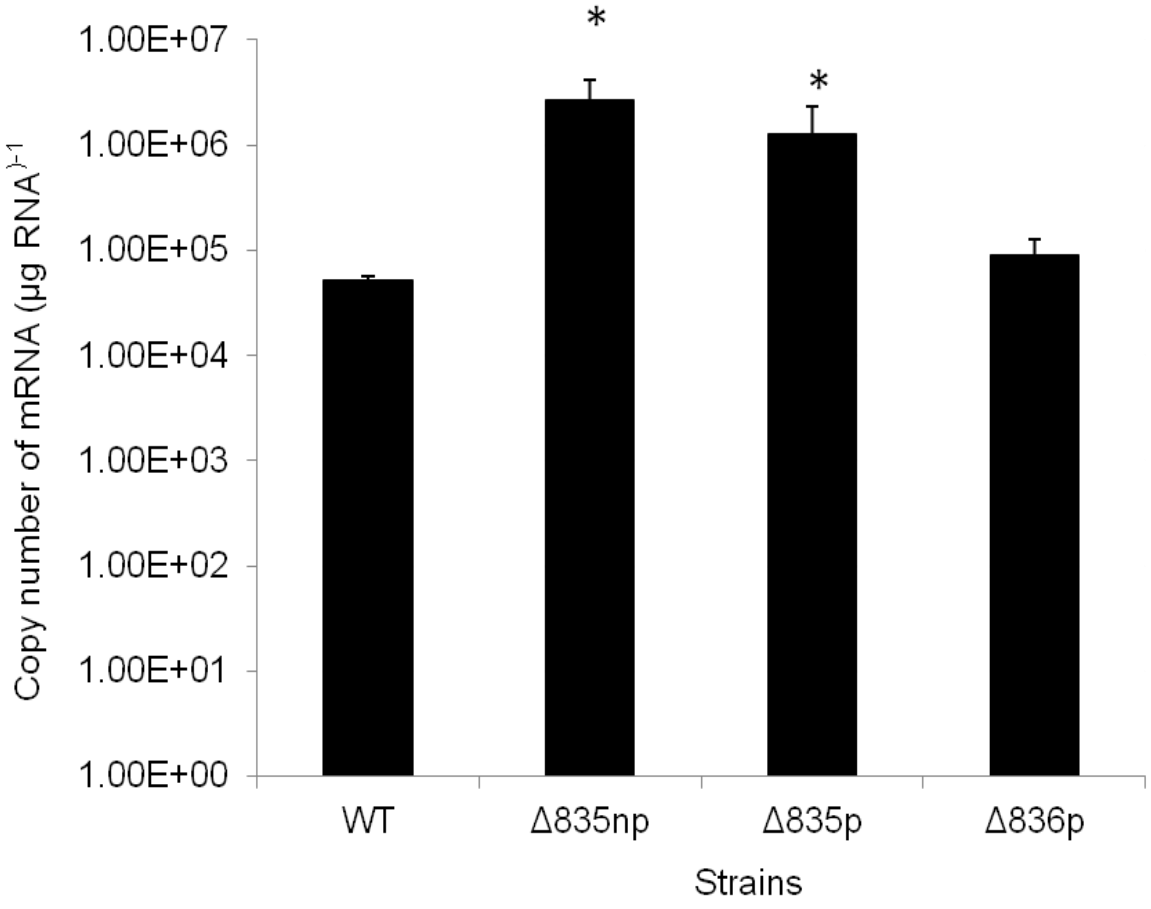


Figure 5-6. RealTime-RT-PCR showing *comX* mRNA levels. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data presented as the copy number of each gene per µg of input RNA. *, Differs from the wild-type at $p < 0.05$ (Student's *t*-test).

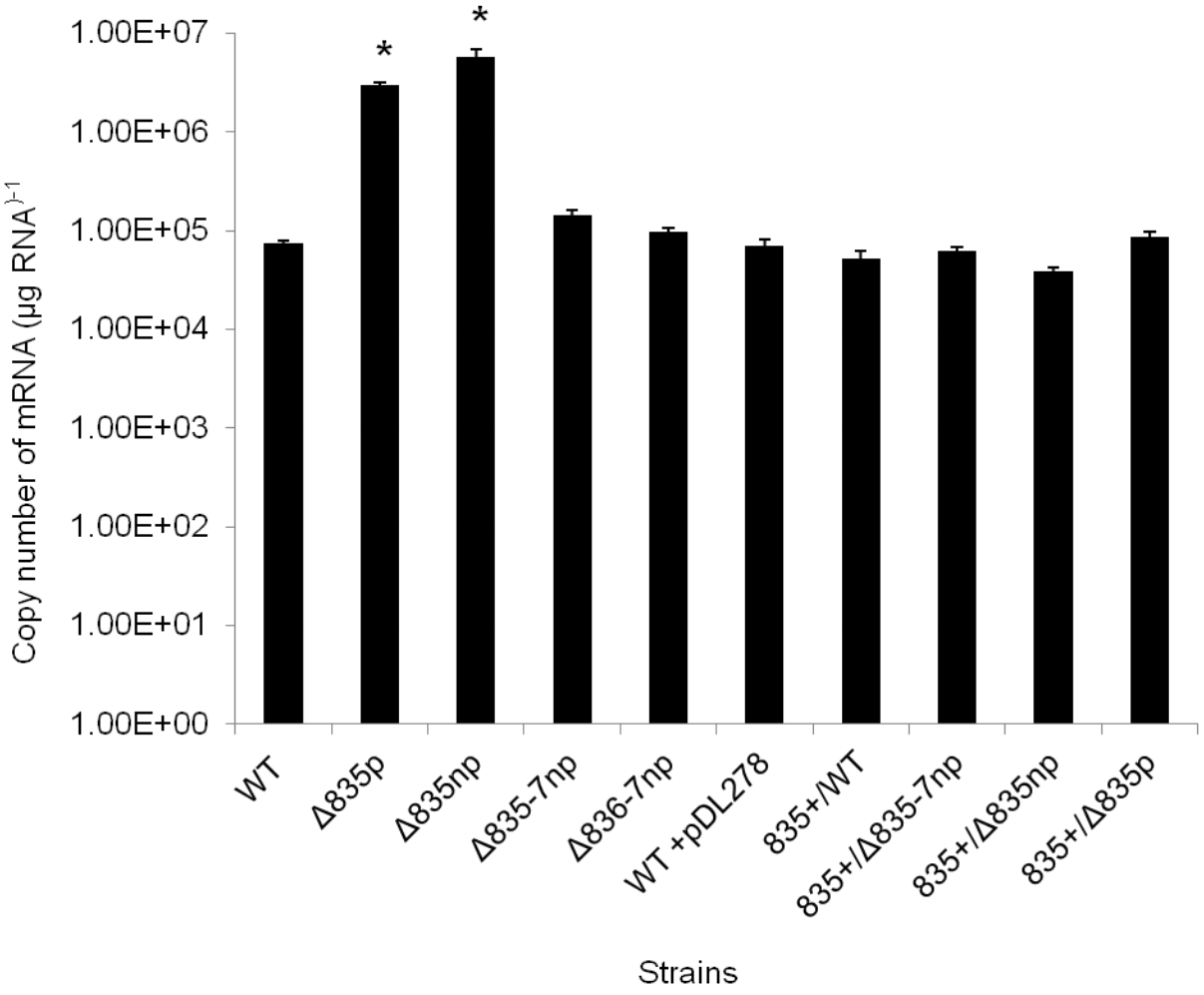


Figure 5-7. RealTime-RT-PCR showing *comX* mRNA levels in complemented strains. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data presented as the copy number of each gene per µg of input RNA. *, Differs from the wild-type at $p < 0.05$ (Student's *t*-test). This data were generated by Ann Sagstetter Decker in the Summer of 2010, who was a dental student doing research on the *rcrRPQ* operon and competence.

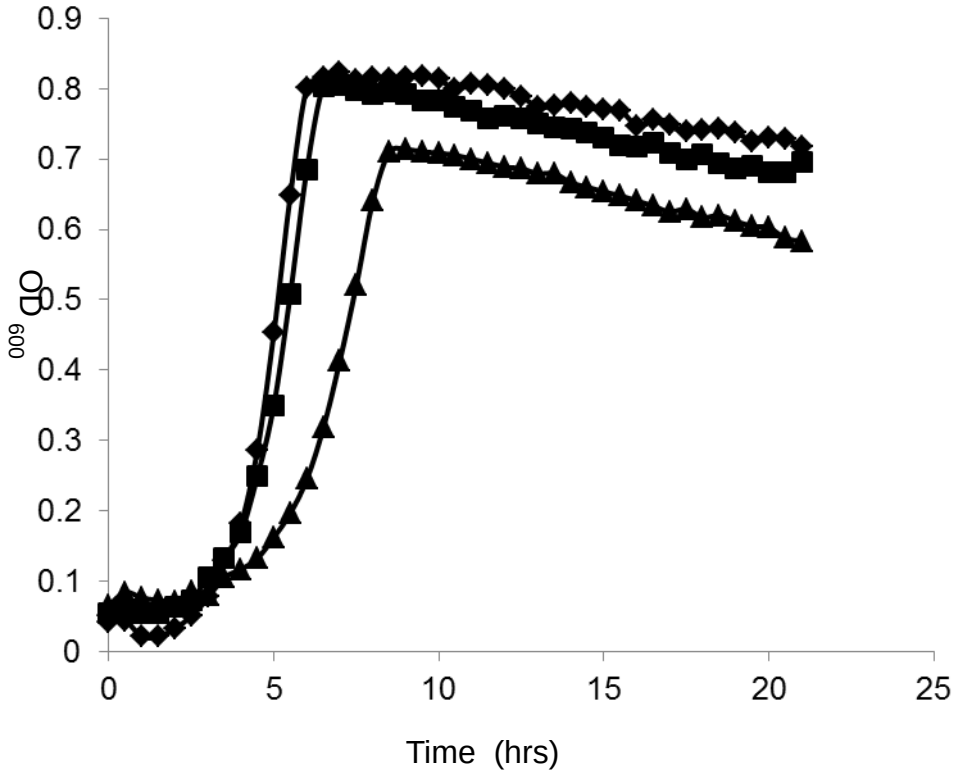


Figure 5-8. Growth comparison of wild-type versus mutant strains in BHI. The strains were grown in triplicate to mid-exponential phase in BHI broth, diluted 1:100 and transferred to fresh BHI broth, overlaid with sterile mineral oil and placed in a Bioscreen C at 37°C to monitor growth. WT, diamonds; *Δ835np* , squares; *Δ835 p* triangles. The results are representative of three independent experiments performed triplicate.

Table 5-3. Table showing growth characteristics of the mutants versus the wild-type strain in BHI.

Strain	WT	*Δ835np*	*Δ835p*
Final OD	0.8 ± 0.02	0.78 ± 0.02	0.68 ± 0.027
Doubling Time (min)	41 ± 1.7	61± 8.5	101 ± 19.4
Lag time (h)	3	3	5

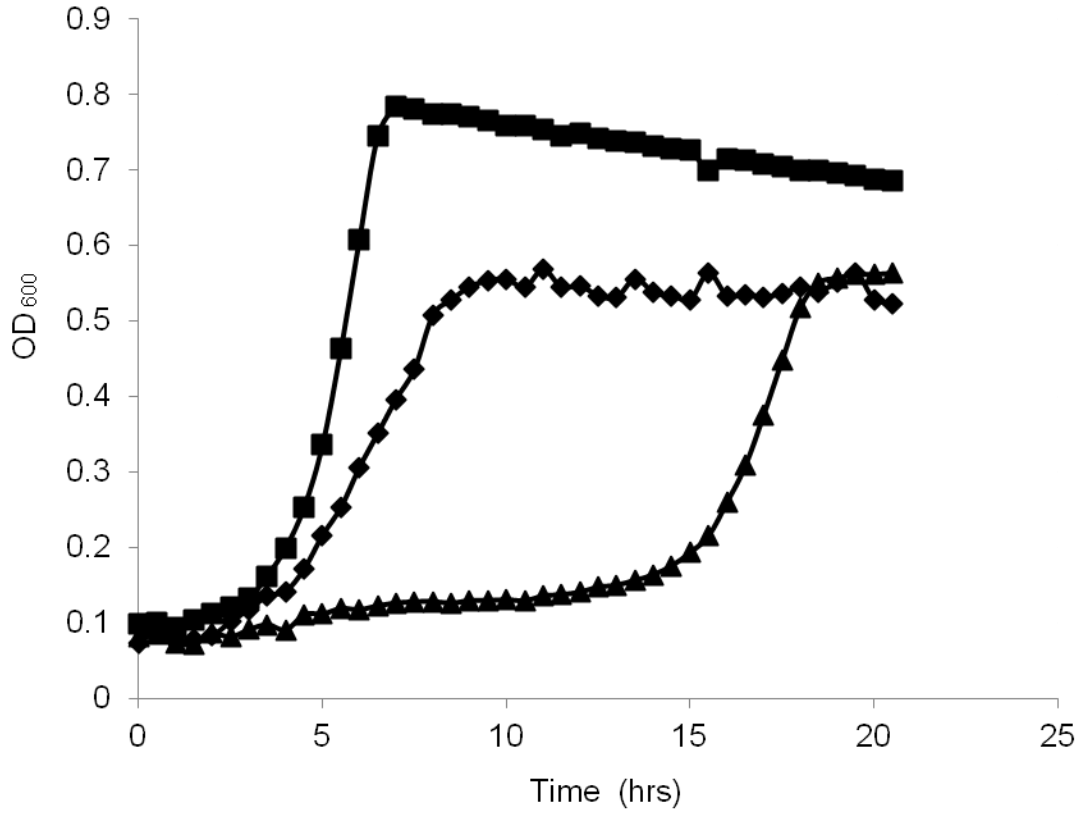


Figure 5-9. Growth comparison of wild-type versus mutant strains in 2 µM CSP. The strains were grown in triplicate to mid-exponential phase in BHI broth, diluted 1:100 and transferred to fresh BHI broth containing 2 µM CSP, overlaid with sterile mineral oil and placed in a Bioscreen C at 37°C to monitor growth. WT, diamonds; *Δ835np* , squares; *Δ835p* triangles. The results are representative of three independent experiments performed triplicate.

Table 5-4. Table showing growth characteristics of the mutants versus the wild-type strain in BHI + 2µM CSP.

Strain	WT	*Δ835np*	*Δ835p*
Final OD	0.51 ± 0.06	0.73 ± 0.02	0.54 ± 0.07
Doubling Time (min)	136 ± 9	69 ± 3, **	143 ± 34
Lag time (h)	3	3.5	15

**, Differs from the wild-type strain, at P<0.005.

CHAPTER 6
REGULATION OF GENE EXPRESSION AND COMPETENCE BY THE SMU0835 (RcrR) PROTEIN

Introduction

Our studies thus far have identified an uncharacterized operon which we named *rcrRPQ* for *r*el *c*ompetence *r*elated, which is involved in stress tolerance, (p)ppGpp metabolism and competence. The *rcrRPQ* operon encodes a predicted DNA binding protein from the MarR family of transcriptional regulators (RcrR) and two predicted ABC efflux pumps are encoded by *rcrP* and *rcrQ*. It was found that loss of or changes in the expression levels of the *rcrRPQ* operon elicited profound effects on the ability of the bacteria to be transformed with chromosomal or plasmid DNA and on the expression of selected early and late competence genes. RcrR was shown to be the dominant regulator of the operon, and a deletion of the *rcrR* gene with a non-polar kanamycin resistance marker (Δ*835np*) caused the upregulation of the genes encoding the *rcrPQ* efflux pumps by ~100-fold. Of note, the Δ*835np* mutant strain was no longer able to take up DNA and the expression of *comYA*, which is critical for the development of competence, was greatly reduced compared to the wild-type strain; even though *comX* mRNA levels were very high. In contrast, a strain carrying a replacement of *rcrR* with a polar kanamycin resistance cassette (Δ*835p*) was constitutively hypertransformable, and the *comX* and *comYA* genes were both significantly upregulated. Interestingly, expression of the *rcrPQ* genes encoding the exporters in the Δ*835p* strain was similar to wild-type levels for reasons explained in Chapter 5 (199). Therefore, based on the phenotype displayed by the Δ*835p* and Δ*835np* strains, the tight regulation of the production of the RcrPQ efflux pumps by RcrR and also RcrR itself appear to play critical roles in the regulation of competence development. Additionally, in a strain

where extra copies of the gene encoding the *rcrR* regulator were expressed on the pDL278 plasmid in a wild-type background (SJ354, Table 2-1), no transformants were obtained in the absence of exogenous CSP (Figure 5-3), suggesting that this regulator plays a key role in regulating the *rcrRPQ* operon and potentially other genes. However, the mechanisms by which the *rcrRPQ* operon exerts multiple effects on competence gene expression and how these gene products integrate competence with stress tolerance and (p)ppGpp metabolism have not yet been identified.

MarR-type regulators have been shown to control the expression of many genes, often those encoding efflux pumps, and have been shown to be important in stress tolerance and adaptation to environmental stresses (60). Most MarR regulators prevent gene expression by sterically interfering with RNA polymerase binding to the promoter to block transcription (172). Since the previous findings show that RcrR has such a profound effect on the *rcrRPQ* operon, DNA uptake and expression of competence genes, it is essential to understand the mechanism by which RcrR regulates gene expression and the physiology of the organism. Here, we examine the interactions of the RcrR protein with potential target sequences in *S. mutans* and show that RcrR has both direct and indirect roles in controlling competence, growth and stress tolerance.

Results

Identification of RcrR Binding Sites

Given the critical impact of the *rcrRPQ* genes on key virulence-related phenotypes, dissecting the basis for regulation of the *rcr* operon and competence genes by the RcrR protein is an essential step toward understanding alternative mechanisms controlling the development of competence and regulating key aspects of cellular physiology. Using EMSAs with purified recombinant RcrR (rRcrR) protein, it was shown

that a relatively small amount (2 pmoles) of rRcrR was able to impede the migration of a 140-bp PCR product containing the promoter region of *rcrRPQ* (Figure 6-1). To examine more directly the interaction of RcrR with the *rcr* operon promoter region, we demonstrated that DNA fragments that included as little as 87-bp immediately upstream of the *rcrR* ATG start codon (data not shown) could be shifted with apparent high affinity by purified rRcrR in EMSAs.

To further understand the interaction of RcrR with the promoter region of *rcrRPQ*, we utilized RegPrecise (http://regprecise.lbl.gov/RegPrecise/browse_regulogs.jsp) to identify a potential psuedopalindromic RcrR target ,TAGTTTTCATGAGAACTA (Figure 6-2) 51-bp upstream of the start codon of *rcrR,* designated here as RS1. Another 15-bp region (TAGTTTAAGGAATCA), designated RS2, was identified directly upstream of RS1 (Figure 6-2). A biotinylated DNA fragment that included only RS1 was not shifted by rRcrR (Figure 6-3), but rRcrR was able to alter the electrophoretic mobility of a PCR product that included RS1 plus 13 random base pairs added to the 5' end of the probe (Table 2-5, Figure 6-3). When the RegPrecise predicted binding site RS1 and the potential weaker binding site RS2 were both included in the target DNA, >80% of the DNA shifted when10 pmoles of rRcrR protein were present (Figure 6-3, Table 6-1). There was also a shift in DNA when an annealed 38-bp biotinylated oligonucleotide that only included the RegPrecise predicted site RS1, or a 55-bp oligonucleotide that included both predicted sites (Table 2-5), was combined with purified rRcrR protein (Figure 6-4, Table 6-2). The 55-bp fragment appeared to be bound slightly more efficiently than the 38-bp fragment as >80% of the former shifted with 5 pmoles of RcrR protein compared with approximately 46% of the latter. Unlabeled, annealed 38-bp and

55-bp probes were added in increasing proportions to 5 fmol of biotinylated 55-bp probe and 1 pmol of purified protein. Both unlabeled probes were able to decrease the amount of biotinylated DNA that was shifted (Figure 6-5, Table 6-3). In addition, preliminary results assessing binding via fluorescence polarization using the same 38- and 55-bp annealed probes that had been tagged with 6-FAM at the 5' end of the reverse primer indicated that the calculated binding constants for the two fragments exposed to identical concentrations of rRcrR did not differ substantially; the estimated K_d for the 55-bp fragment was 0.1 μM and the K_d for the 38-bp fragment was 0.06 μM.

Mutations in the Predicted Binding Sites Affect Binding of the RcrR Protein

Based on the results of the EMSAs detailed above, various mutations were introduced into the RS1 and RS2 binding sites in the 5' region of the *rcrRPQ* operon in the *S. mutans* chromosome via splice overlap extension PCR and transformation (Table 2-1), and all mutations were confirmed by PCR and sequencing. Subsequently, DNA from the promoter regions of the various mutant strains was obtained by PCR to investigate the effect of the mutations on the ability of rRcrR protein to bind. When the PCR product derived from the *NBS1* strain, which had mutations in RS1, was used, a decrease in the amount of shifting was observed (Figure 6-6, Table 6-4). When DNA from the promoter region of the *BBS* strain, containing mutations in both binding sites was used, there was about a 50% shift with 2.5 pmoles of protein and less than 50% shift with 1.5 pmoles of protein, compared to 90% of the product derived from the wild-type organism (Figure 6-7, Table 6-5). However, when the probe was derived from the *NBS2* strain, which had mutations only in the secondary predicted binding site (RS2), there was no difference in the proportion of DNA shifted compared to the wild-type DNA (Figure 6-8).

Mutations in the Binding Site of the *rcrR* Promoter Affect Competence.

Given the impact that perturbations in expression of any of the *rcrRPQ* genes has on competence and the relatively inefficient binding observed for rRcrR to the mutated binding sites *in vitro*, the transformation efficiency of the strains harboring the mutated binding sites was assessed with and without addition of exogenous synthetic CSP. The *NBS1* strain, which has mutations in RS1 only (Table 2-1), displayed a 23-fold increase in transformation efficiency in the absence of added CSP compared to the wild-type strain (Table 6-6). This hypertransformable phenotype was similar to that observed for the Δ*835p* strain (199). However, there was no difference between the *NBS1* mutant and the parental strain in transformation efficiency when 100 nM CSP was added to the cultures (Table 6-6). Strain *BBS*, which had mutations in both RS1 and RS2, yielded no transformants in the absence of exogenous CSP (Table 6-6), but a similar number of transformants as the wild-type strain when CSP was included. Thus, mutation of the predicted binding sites for RcrR influenced the transformation phenotypes. We hypothesized that the different effects of the binding site mutations were associated with different expression levels of the operon, because some mutations affect only RcrR binding while others affect RcrR binding and promoter activity.

Effect of the Mutations on Promoter Activity.

To test the hypothesis that the mutations might impact both RcrR binding and promoter activity, *lacZ*-promoter fusions were made with DNA from the promoter regions of strains WT, *NBS1* and *BBS*. β-galactosidase assays showed that there was increased activity with the P_{BBS} promoter from the strain with mutations in both RS1 and RS2 (159 ±15 Miller units) compared to the P_{WT} promoter (69 ±7.3 Miller units) (Figure 6-9), but only a slightly elevated level of expression from the P_{NBS1} promoter (88 ± 7.4

Miller units). Therefore, the activity of the *rcrRPQ* promoter is affected differently in the binding site mutants, and this in turn influences the competence phenotype. We also concluded that the way that RNA polymerase interacts with the promoters in the BBS and NBS1 strains is different resulting in variations in the promoter activity.

Mutations in the *rcrR* Binding Site Affect *comX*, *comS* and *comYA* Expression

To determine if the phenotypes of the binding site mutants could be associated with *com* gene expression levels, we used qRT-PCR to measure the mRNAs for *comX, comS* and *comYA*, which are critical genes in the development of competence. The *NBS1* strain, which was hyper-transformable, had an approximate 30-fold increase in the expression of *comYA* and about a 20-fold increase in expression of *comS*, compared to the wild-type strain (Figure 6-10, Figure 6-11). The magnitude of the change in expression of *comYA* and *comS* was similar to that observed in the Δ*835p* strain [(199), Figure 6-10]. There was also about a 4-fold increase in *comX* expression in the *NBS1* strain compared to the wild-type strain (Figure 6-12). The levels of *comYA* were slightly lower in the *BBS* strain, albeit not statistically different from the wild-type strain (Figure 6-10), following a trend similar to that observed for the Δ*835np* strain (199).

Effect of the Mutations in the *rcrR* Binding Site on *rcrR* and *rcrP* Expression

Previously, we showed that the expression of the *com* genes was dependent on the expression of the *rcrRPQ* operon (199). Mutations in the RcrR binding site affected the ability of rRcrR to bind efficiently, and β-galactosidase activity from promoters with mutations in the RcrR binding site was significantly higher than activity from the wild-type promoter. Therefore we used qRT-PCR to measure the mRNAs for *rcrR* and *rcrP* in the *NBS1* strains and the *BBS* strains. There was no significant difference in the

expression of *rcrR* or *rcrP* in the *NBS1* strain or the *BBS* strain compared to the wild-type expression levels (Figure 6-13, Figure 6-14).

Mutations in the Predicted RcrR Binding Site Affects Growth in CSP

Physiologically high concentrations of CSP (2 μM) have been shown to cause apparent inhibition of growth of *S. mutans* (57, 187, 233), in part due to cell lysis and the induction of the CipB bacteriocin. Of note, the CipI protein confers resistance to CipB and is encoded about 1 kbp downstream of *rcrRPQ.* Our previous findings show that strains with mutations in the *rcrRPQ* operon grew differently than the wild-type strain in the presence of 2 μM CSP. In particular, and consistent with the transformation phenotypes, the Δ*835np* strain was resistant to CSP-induced growth inhibition, whereas the Δ*835p* strain was hypersensitive to CSP. Interestingly, the *NBS1* strain was more sensitive to CSP, as noted by a slight decrease in growth rate with a minimum doubling time of 87 ± 6 min, compared to the wild-type strain with a minimum doubling time of 82 ± 10min (Figure 6-15, Figure 6-16). However, the wild-type strain attained a final OD_{600} = 0.55 ± 0.012, whereas the *NBS1* strain reached a final OD_{600} of only 0.45 ± 0.07. The *BBS* strain was more resistant to CSP induced growth inhibition (Figure 6-16) when compared to the parental strain, with a final yield of OD_{600} = 0.7 ± 0.011 in CSP and minimum doubling time of 56 ± 3 min (p-value <0.005 compared to the wild-type doubling time). Of note, expression of *rcrR* from its cognate promoter on a multi-copy plasmid in a wild-type genetic background also conferred resistant to CSP-induced growth inhibition (Figure 6-17), with the overexpressing strain achieving a final yield of OD_{600} = 0.85, compared to the wild-type strain with a final yield of OD_{600} = 0.65**.**

Global Regulation by the RcrR Regulator

Mutations of *rcrR* and aberrant expression the RcrPQ exporters affect genes in the competence pathway and impacts (p)ppGpp metabolism (199). Here, we utilized RNA -seq and microarray analysis to show that numerous genes that are critical for the development of competence are regulated by the *rcrRPQ* genes (Tables 6-7,6-8,6-9). The most upregulated regulated genes in the hypertransformable Δ*835p* strain were c*omX, comY, comF,* and *comEA,* but in the non-transformable Δ*835np* strain (Table 6-9) *comYA* was among the most downregulated genes in a microarray study (Table 6-7). Our previous qRT-PCR studies also showed that *comYA*, *comX* and *comS* are affected by mutations in the *rcrRPQ* operon (199) and that a strain that overexpresses *rcrR* also causes a decrease in transformation and diminished sensitivity to growth inhibition by CSP (Figure 6-16, Figure 6-17).

RcrR Has A Weak Interaction With the *comX* and *relP* Promoter Region

Since the deletion of *rcrR* had dramatic effects on the expression of many of the genes in the competence pathway and other genes, we scanned for genes with RS1/2 sites and conducted EMSAs to determine if the effects on the expression of particular genes could be exerted directly by the binding of RcrR to the promoter regions of these genes. The promoter regions of *relP*, *comX* and *comY* had sequences with weak similarity to the RS1 binding site based on the wMATCHER software analysis to find target sequences. Purified rRcrR protein was able to bind to the promoters of *comX,* and *relP*, albeit with apparently lower affinity than to the promoter of the *rcrRPQ* operon (Figure 6-18, Figure 6-19) as well as *comYA* (data not shown). Specifically, there was approximately a 10% shift of the *comX* and *relP* promoter regions when 20 pmoles of rRcrR was utilized, compared to 100% shift with 10 pmol of rRcrR when the *rcrRPQ*

promoter was the target. As a negative control, we utilized the promoter of *fruA*, encoding a polysaccharide hydrolase that is not affected by mutations in the *rcr* genes, and observed no shift in mobility even with as much as 100 pmol of purified protein.

Discussion

The development of competence by *S. mutans* is a tightly regulated process that is influenced by multiple regulatory factors and inputs, many of which have yet to be characterized. Competence is considered to be a stress response and its induction in bacteria has been linked to translational errors, (p)ppGpp production, antibiotic stress, bacteriocin production and oxidative stress (61, 175, 199). The *rcrRPQ* operon is also involved in stress tolerance, particularly oxidative stress, and has a profound effect on competence; constituting a regulatory circuit for competence that was previously undiscovered (199). The studies described in this chapter provide novel insights into the mechanisms by which the products of the *rcrRPQ* operon integrate competence stress tolerance and growth regulation with genetic competence.

The simplest model for regulation of the *rcrRPQ* operon is that the RcrR regulator binds the promoter region of *rcr* to repress the operon. Based on the EMSA data, the Regprecise predicted binding site (RS1) TAGTTTTCATGAGAACTA is the major binding site, since oligonucleotides containing that region were sufficient to support rRcrR binding. The sequence of RS1 is similar to typical MarR binding sites, which usually consist of palindromic or psuedopalindromic sequences (236). Further support for the model was gleaned by demonstrating that mutations in RS1 caused attenuated binding of the rRcrR protein *in vitro* and had effects *in vivo* on transformation efficiency, growth and gene expression that were similar to what would be predicted for loss of RcrR. The weaker predicted binding site (RS2), TAGTTTAAGGAATCA does not appear to be

necessary for binding, at least *in vitro*, since mutations made only in that site did not affect rRcrR binding. Mutations in RS2 also did not affect the organism in any discernable way. However, when both predicted binding sites are present, a greater percentage of DNA is shifted, perhaps reflecting that binding can be enhanced by the second binding site through cooperative interactions. We also noted that rRcrR binding, like that of many regulators of the MarR family, bound more efficiently when additional sequences of 5 to 30-bp were provided adjacent to the binding site (236).

Mutations to the binding site in the promoter region of *rcrRPQ* operon caused the strains to have differences in transformability and aberrant expression of the *com* genes compared to the wild-type strain. Based on our previous findings that revealed a dominant role for the RcrR protein on transformation and expression of the *rcr* operon and *com* genes (199), we conclude that the inability of RcrR to bind effectively to its target sequences to control *rcrRPQ* operon expression levels resulted in the changes in transformability, *com* gene expression and growth. However, we did not detect any significant changes in the expression of the *rcrRPQ* operon in the *NBS1* and *BBS* strains via qRT-PCR (Figure 6-13, Figure 6-14) even though we detected higher promoter activity in these strains compared to the wild-type strain. We posit using gene fusion that the qRT-PCR was not sufficiently sensitive to measure biologically significant changes in the expression of the operon, and that even small fluctuations in the expression levels of *rcrRPQ* may account for observable differences in transformation efficiency and *com* gene expression. A large increase in the expression of *rcrPQ* was seen in the Δ*835np* strain because the gene encoding RcrR was disrupted which resulted in complete derepression of the operon (199). Notably, the *BBS* strain which

had the highest promoter activity as a result of derepression, had a similar phenotype to the Δ*835np* strain. In addition, in the SJ354 strain, where there were extra copies of *rcrR* expressed in a wild-type genetic background, there was not a huge impact on the expression of *rcrPQ,* but there was a significant effect on growth in CSP, transformation efficiency and *comYA* levels (199). Our previous findings also indicate that any changes to the levels of the gene encoding RcrR affected transformability and the expression of some of the *com* genes (199). Collectively, these data show the important role that RcrR and efficient binding of RcrR to its cognate DNA sequences have on transformation and expression of the competence genes.

Consistent with the phenotype displayed with strains harboring various mutations in *rcrR*, microarray and RNA-seq revealed that genes in the competence pathway are the most differentially regulated when there are mutations in *rcrR*. The basis for this change in expression could be due to a weak interaction of RcrR with the promoters of these genes, since weak binding of rRcrR protein *in vitro* to the *comX* and *comYA* promoters was observed. Many MarR type regulators have co-factors, such as biochemical intermediates or other small molecules that may be present *in vivo* that affect binding to DNA to control gene expression (172). Therefore, there may be other factors *in vivo* that affect binding of RcrR to the *com* genes that were absent in the EMSA analysis so RcrR could probably play a significant role in regulating *com* genes. Importantly, the *rcrRPQ* operon was shown to be involved in stress tolerance and its expression was enhanced in response to environmental insult (123, 199). Consequently, as part of our working model (Figure 7-1), the impact of RcrR on *com* gene expression may be enhanced when *rcrRPQ* become derepressed.

The difference in growth phenotype of the mutant strains in CSP is more than likely linked to the changes in expression of the competence genes in these strains. It has been previously shown that inactivation of certain *com* genes analyzed in this study cause the cells to become insensitive to CSP induced growth inhibition (175). Also, we have observed that expression of the *com* genes becomes insensitive to added CSP in the *rcrRPQ* mutants, unlike in the wild-type strain where *com* genes are induced by CSP (data not shown). Of note, *comX* mRNA levels were high in strains that were both resistant and sensitive to CSP-induced growth inhibition, which is a surprising finding since it is believed that the induction of *comX* correlates with lysis and growth inhibition by CSP (117). However, the levels of *comY* correlate well with the sensitivity to CSP in the strains tested here, as does transformation. The sensitive strains such as Δ*835p* and *NBS1* were hypertransformable and had high levels of *comYA* compared with the resistant strains, such as *BBS* and Δ*835np,* which had lower levels of *comYA* and were poorly- or non-transformable. Our previous findings (199) and the transcriptional profiling with *rcrR* mutants presented in this study clearly show that the activity of ComX is affected in the CSP-resistant Δ*835np* strain, even though *comX* expression is high *comY* levels are low (199). Therefore, it appears that ComX-dependent activation of *comY* and other downstream genes is critical for growth sensitivity to CSP. It is known that fratricide or cell death is necessary for the induction of competence in *S. pneumoniae* biofilms (229) and we also see a correlation with transformability and sensitivity to CSP. The induction of bacteriocins was also linked to cell lysis and growth inhibition with high levels of CSP (175, 233). The *rcrRPQ* operon also appears to be impacting the expression of the bacteriocin genes and bacteriocin production (Ahn et

al.. manuscript in preparation) and the RNA-seq data show that at least two bacteriocin-related genes were upregulated in *the* Δ*835p* strain (Table 6-9). Therefore, the effect that the *rcrRPQ* operon exerts on the bacteriocins and their immunity proteins may be part of the explanation for the differences in growth in CSP.

While the data clearly provide evidence of a direct role for RcrR in competence regulation, the expression levels of the genes encoding the *rcrPQ* pumps, which are under the dominant control of RcrR, must be acknowledged as a major factor controlling late *com* gene expression and transformation efficiency (199). An Δ*836p* strain, which lacks *rcrP* and expresses 1000-fold lower levels of *rcrQ* ABC pumps than the Δ*836np* strain, had higher levels of *comYA* and higher transformation efficiency than the wild-type strain without additional CSP (199). In addition, we have data showing that overexpression of the ABC exporters causes a dramatic reduction in transformation efficiency and affects the expression of the competence genes (Ahn *et al.*, manuscript in preparation). These data further support our hypothesis that the efflux pumps may be extruding a compound necessary to activate competence. This signal could function in a similar manner to the competence sporulation factor (CSF) in *B. subtilis* (141, 206) or act as an anti-anti-sigma factor that interferes with a factor, such as ComW, that impairs ComX stability and activation of *comYA,* as seen in *S. pneumoniae* (137, 216). The inability of RcrR to bind efficiently to the promoter of the *rcr* operon and differences in promoter activity could result in slight alterations in the amount of localization of the substrate(s) in the *NBS1* and *BBS* strains. The signal(s) probably need to be balanced carefully where slight fluctuations in their localization can result in differences in the expression of the competence genes and transformation efficiency. Current efforts are

focused on identifying small molecules that RcrPQ are extruding and other factors that interact with RcrR.

In conclusion, the current findings in this chapter in addition to our previous findings show that tight control of the *rcrRPQ* operon by RcrR interacting with its binding site in the promoter region strongly influences the expression of the competence genes and transformation. We also show RcrR is a master regulator and may directly influence *com* gene expression, as well as *relP,* by interacting with their promoters. The potential for RcrR to interact with the *relP* promoter may be one of the explanations for the linkage of the *rcrRPQ* operon to (p)ppGpp metabolism, as previously reported (199). As part of our working model, we propose that competence and RelP-dependent (p)ppGpp metabolism are both controlled by RcrR and the *rcrRPQ* operon to fine tune how the organism responds to different environmental signals (Figure 7-1,7-2). It is also reasonable to hypothesize that RcrRPQ and the RcrPQ substrate(s) could play an important role in bi-stable behavior associated with CSP and competence, as well as influencing decisions by the cell to become competent or lyses. Thus, RcrRPQ may provide the potential missing link between the ComRS and CSP signaling pathways, as well as between bacteriocin production and competence development, as proposed by Lemme *et al.,* Levesque and co-workers, and the Morrison group (57, 63, 116).

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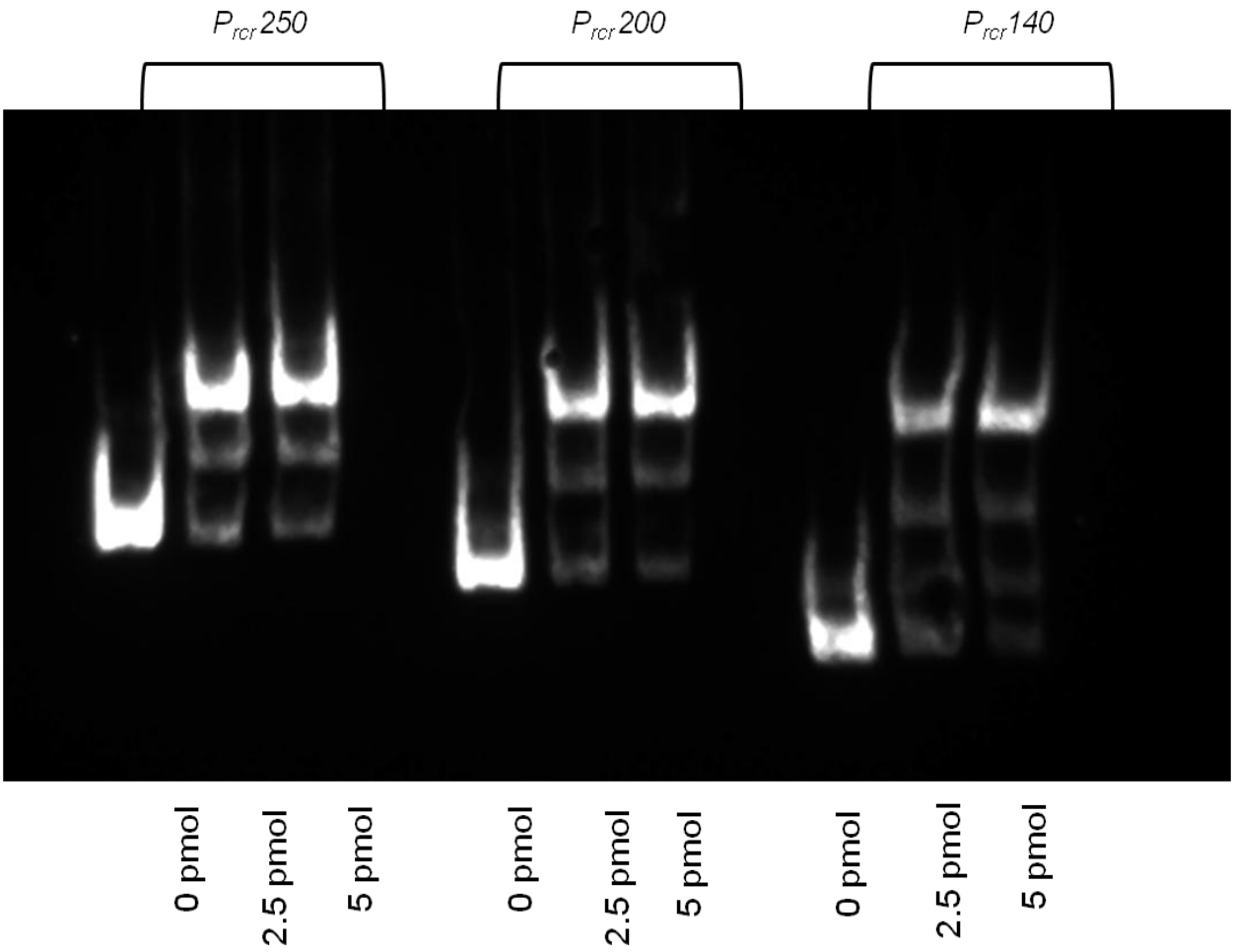

Figure 6-1. EMSA showing binding of biotinylated regions of P_{rcrR} DNA with purified RcrR protein. Purified RcrR protein of various concentrations (0, 2.5, 5, 10, 20 pmoles, Lanes 1-5) was added to 5 fmoles of biotinylated PrcrR DNA in a binding reaction for 40 min. The reactions were run on a non-denaturing polyacrylamide gel and the signal observed via chemiluminescence.

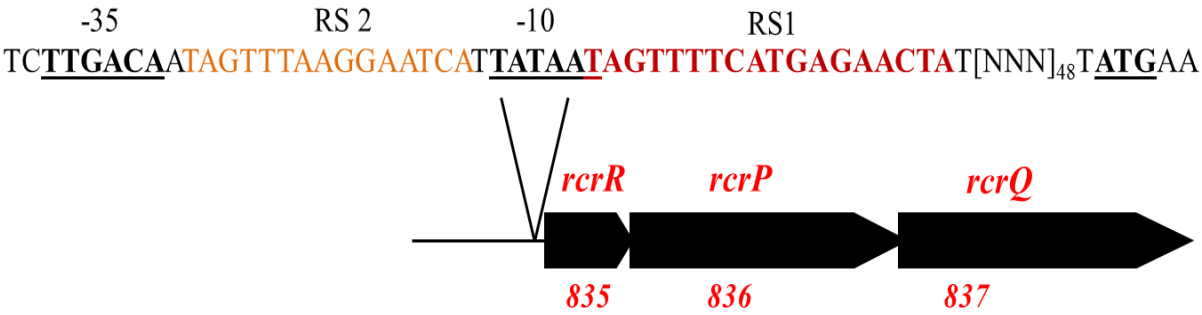

Figure 6-2. Schematic of the promoter region of the *rcrRPQ* operon in *S. mutans* UA159. The ATG start site is highlighted and underlined. The -10 region (TATAAT) is located 56-bp upstream of the start site and the -35 region (TTGACA) is located 79-bp upstream. An 18-bp binding site located 51-bp upstream of the start site was identified via RegPrecise (http://regprecise.lbl.gov/RegPrecise/browse_regulogs.jsp) software prediction tool. The predicted binding site sequence RS1 is TAGTTTTCATGAGAACTA. The secondary predicted site RS2 is TAGTTTAAGGAATCA.

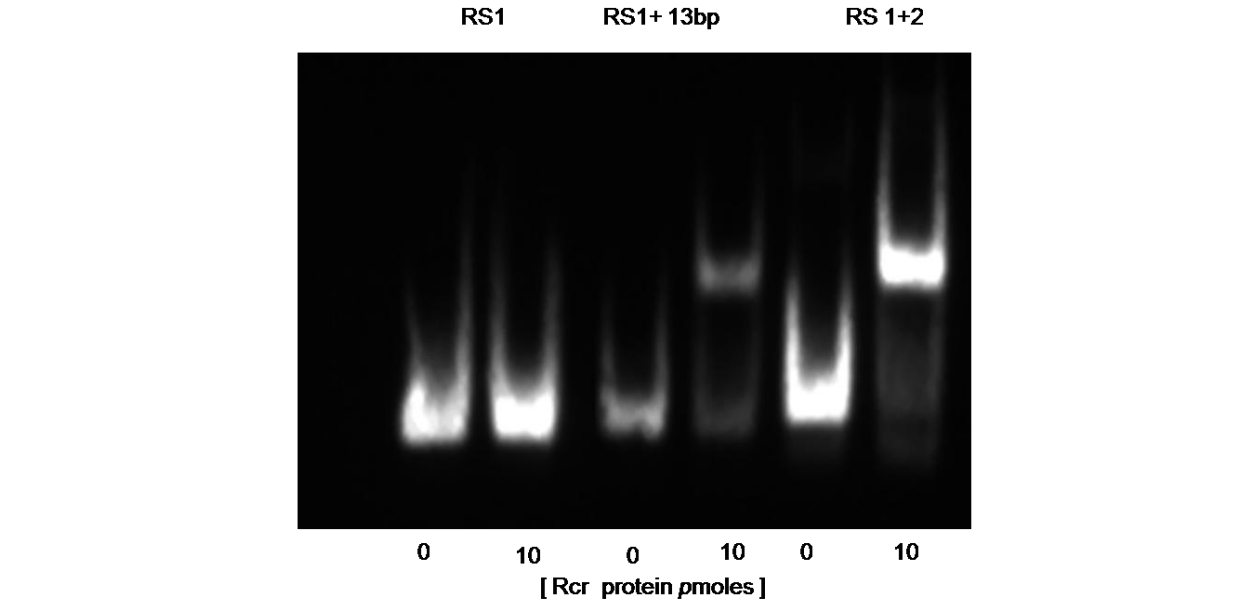

Figure 6-3. EMSA showing binding of biotinylated PCR products of P_{rcrR} with purified RcrR protein. Purified RcrR protein(10 pmol) was added to 5 fmoles of biotinylated P_{rcrR} PCR products RS, RS1+ random and RS1+2 in a binding reaction for 40 min. The reactions were run on a non-denaturing polyacrylamide gel and the signal observed via chemiluminescence. The data are a representative of n=3 EMSAs. There was a similar trend observed in all cases.

Table 6-1. Percentage of DNA shifted with different PCR products of the promoter of *rcrR*

RS1	RS1 + random	RS1 + 2
0% shift	61% shift	83% shift

% shift calculated as % reduction in the integrated density value (IDV) of the DNA bands compared to the IDV of unshifted DNA band with no protein.

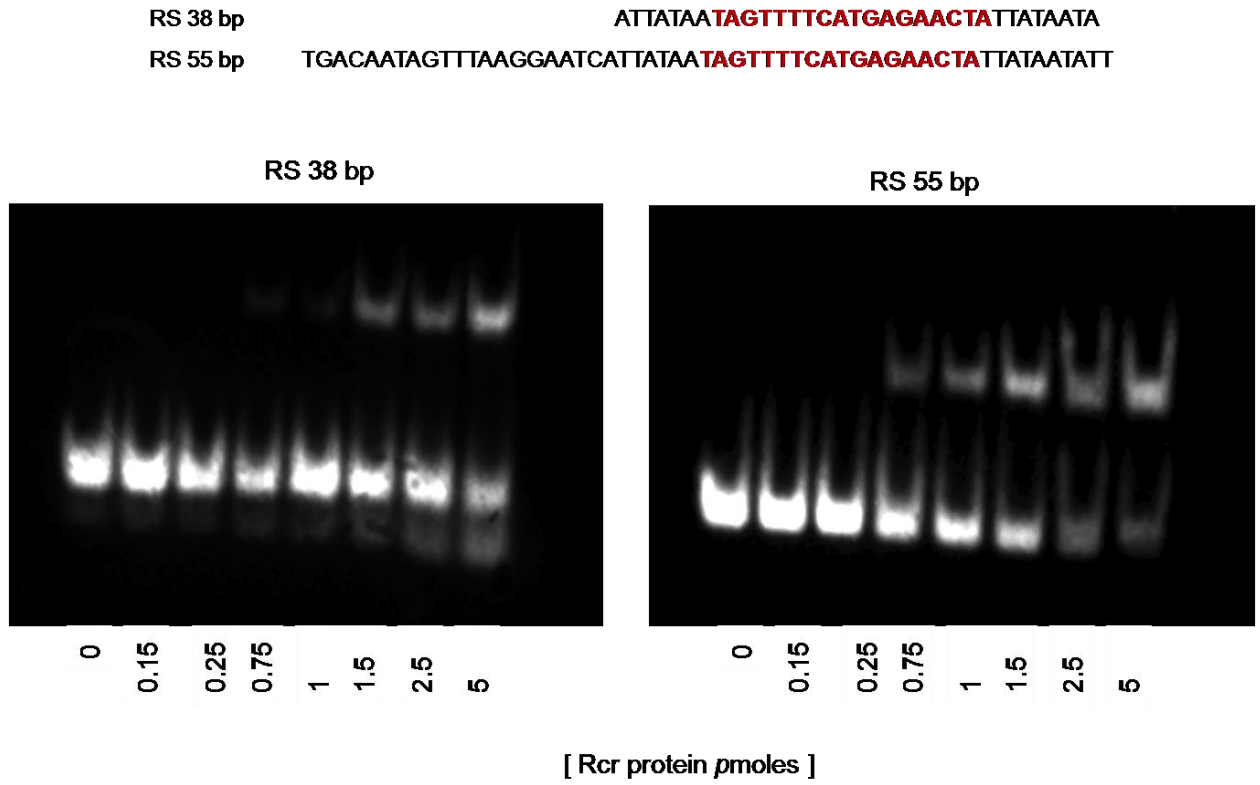

Figure 6-4. EMSA showing binding of biotinylated oligos of P_{rcrR} DNA with purified RcrR protein. Purified RcrR protein of various concentrations was added to 5 fmoles of biotinylated P_{rcrR} DNA fragments 38 bp and 55 bp in a binding reaction for 40 min. The reactions were run on a non-denaturing polyacrylamide gel and the signal observed via chemiluminescence. The data are a representative of n=2 EMSAs. There was a similar trend observed in both cases.

TABLE 6-2. Percentage of DNA shifted with different annealed oligos in the promoter of *rcrR*

Region	1.5 pmol RcrR	2.5 pmol RcrR	5 pmol RcrR
38- bp	20% shift	20% shift	46% shift
55- bp	43% shift	74% shift	83% shift

% shift calculated as % reduction in the integrated density value (IDV) of the DNA bands compared to the IDV of unshifted DNA band with no protein.

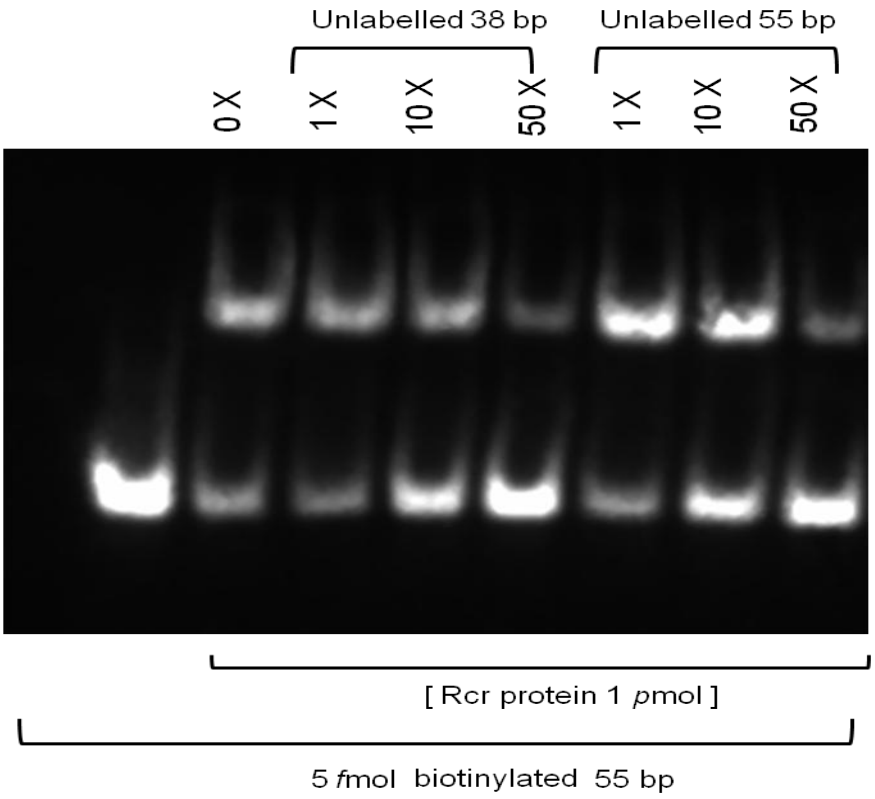

Figure 6-5. EMSA showing competitive binding of biotinylated regions of P_{rcrR} DNA with unlabeled regions of P_{rcr} DNA and purified RcrR protein. Purified RcrR protein (1 pmol) was added to 5 fmoles of biotinylated P_{rcrR} DNA fragment 55 bp with increasing concentration of unlabeled P_{rcr} DNA fragments in a binding reaction for 40 min. The reactions were run on a non-denaturing polyacrylamide gel and the signal observed via chemiluminescence. The data are a representative of n=2 EMSAs. There was a similar trend observed in both cases.

Table 6-3. Percentage of labeled P_{rcrR} DNA shifted with unlabeled oligo competitors of P_{rcrR} DNA

Unlabeled region	0 x unlabeled DNA	10 x unlabeled DNA	50 x unlabeled DNA
38-bp	59% shift	31% shift	10% shift
55-bp	59% shift	27% shift	14% shift

% shift calculated as % reduction in the integrated density value (IDV) of the DNA bands compared to the IDV of unshifted DNA band with no protein.

TTTTCCCC

TTGACAATAGTTTAAGGAATCAT**TATAA**TAGTTTTCATGAGAACTA [NNN]$_{50}$ **ATG**AAGGA

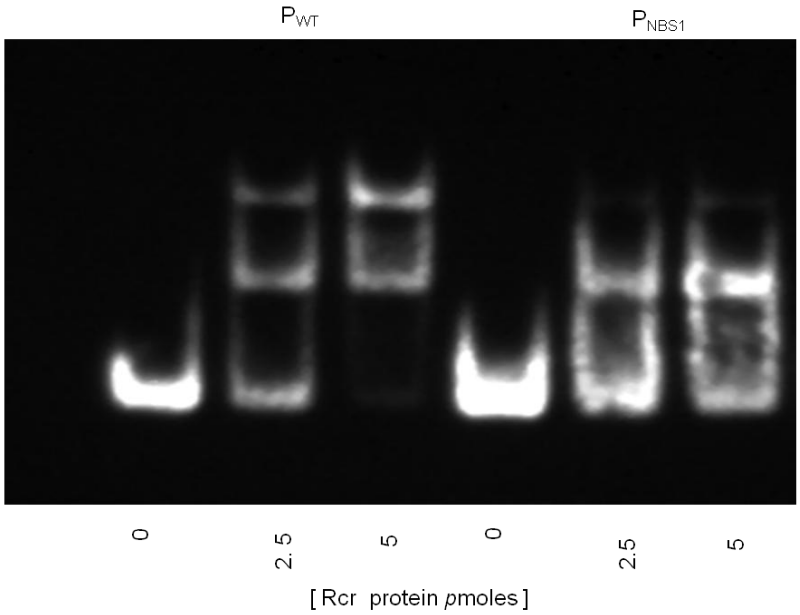

Figure 6-6. EMSA showing binding of biotinylated P_{NBS1} mutated regions of P_{rcrR} DNA with purified RcrR protein. Various concentrations of RcrR protein was added to 5 fmoles of biotinylated wild-type P_{rcrR} DNA fragment (P_{WT}) and mutated P_{rcr} DNA fragment (P_{NBS1}) in a binding reaction for 40 min. The reactions were run on a non-denaturing polyacrylamide gel and the signal observed via chemiluminescence. The data are a representative of n=3 EMSAs. There was a similar trend observed in all cases.

Table 6-4. Percentage of P_{WT} DNA vs. P_{NBS1} DNA shifted at different protein concentrations

Protein concentration	% shift of PWT DNA	% shift of PNBS DNA
2.5 pmol	54% shift	27% shift
5 pmol	90% shift	46% shift

% shift calculated as % reduction in the integrated density value (IDV) of the DNA bands compared to the IDV of unshifted DNA band with no protein.

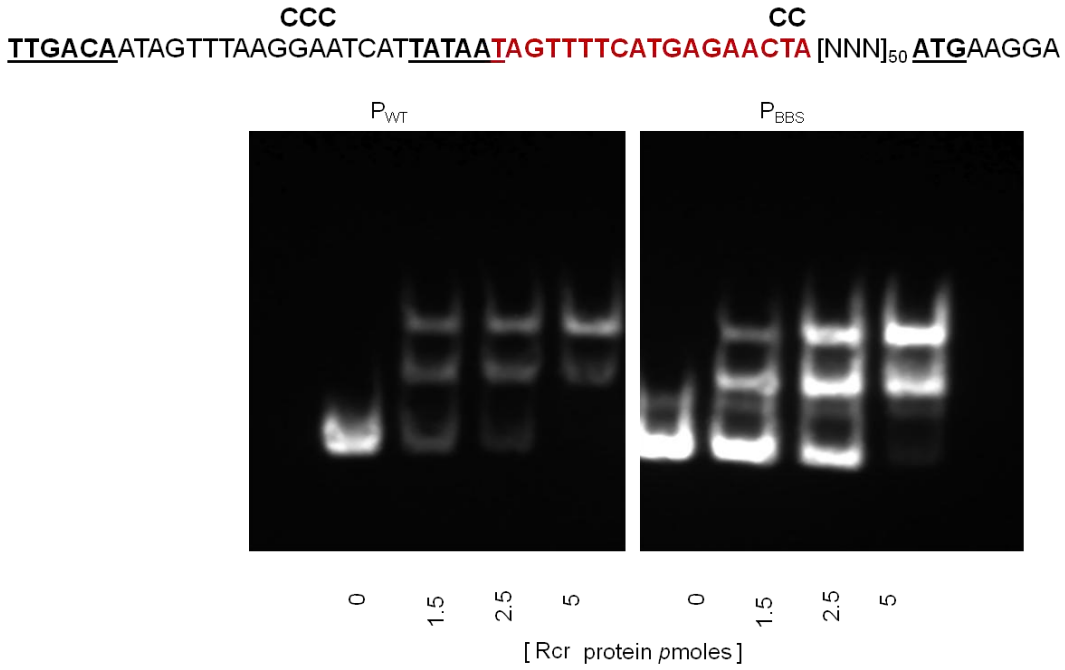

Figure 6-7. EMSA showing binding of biotinylated P_{BBS} mutated regions of P_{rcrR} DNA with purified RcrR protein. Various concentrations of RcrR protein was added to 5 fmoles of biotinylated wild-type P_{rcrR} DNA fragment (P_{WT}) and mutated P_{rcr} DNA fragment (P_{BBS}) in a binding reaction for 40 min. The reactions were run on a non-denaturing polyacrylamide gel and the signal observed via chemiluminescence. The data are a representative of n=3 EMSAs. There was a similar trend observed in all cases.

Table 6-5. Percentage of P_{WT} DNA vs. P_{BBS} DNA shifted at different protein concentrations

Protein concentration	% shift of PWT DNA	% shift of PBBS DNA
1.5 pmol	76% shift	5% shift
2.5 pmol	87% shift	29% shift
5 pmol	95% shift	92% shift

% shift calculated as % reduction in the integrated density value (IDV) of the DNA bands compared to the IDV of unshifted DNA band with no protein.

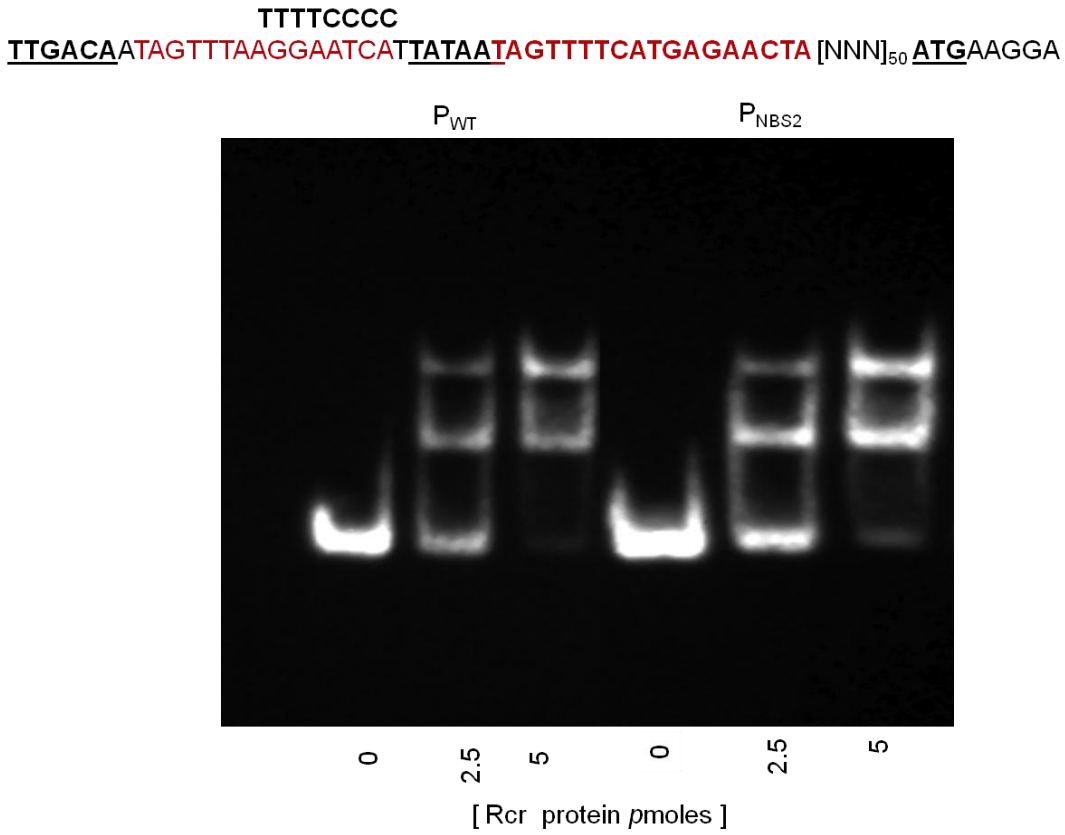

Figure 6-8. EMSA showing binding of biotinylated P_{NBS2} mutated regions of P_{rcrR} DNA with purified RcrR protein. Various concentrations of RcrR protein was added to 5 fmoles of biotinylated wild-type P_{rcrR} DNA fragment (P_{WT}) and mutated P_{rcr} DNA fragment (P_{NBS2}) in a binding reaction for 40 min. The reactions were run on a non-denaturing polyacrylamide gel and the signal observed via chemiluminescence. The data are a representative of n=2 EMSAs. There was a similar trend observed in both cases.

Table 6-6. Transformation efficiency of the wild-type and SOE mutants strains in the presence or absence of added CSP

Strain	% Transformants + CSP	% Transformants - CSP
UA159	5.29×10^{-3}	9.33×10^{-6}
NBS1	3.03×10^{-3}	2.18×10^{-4}
NBS2	2.19×10^{-3}	4.76×10^{-6}
BBS	1.1×10^{-3}	0

% transformants = (number of transformants/total viable bacteria) x 100. +CSP, exogenous CSP.

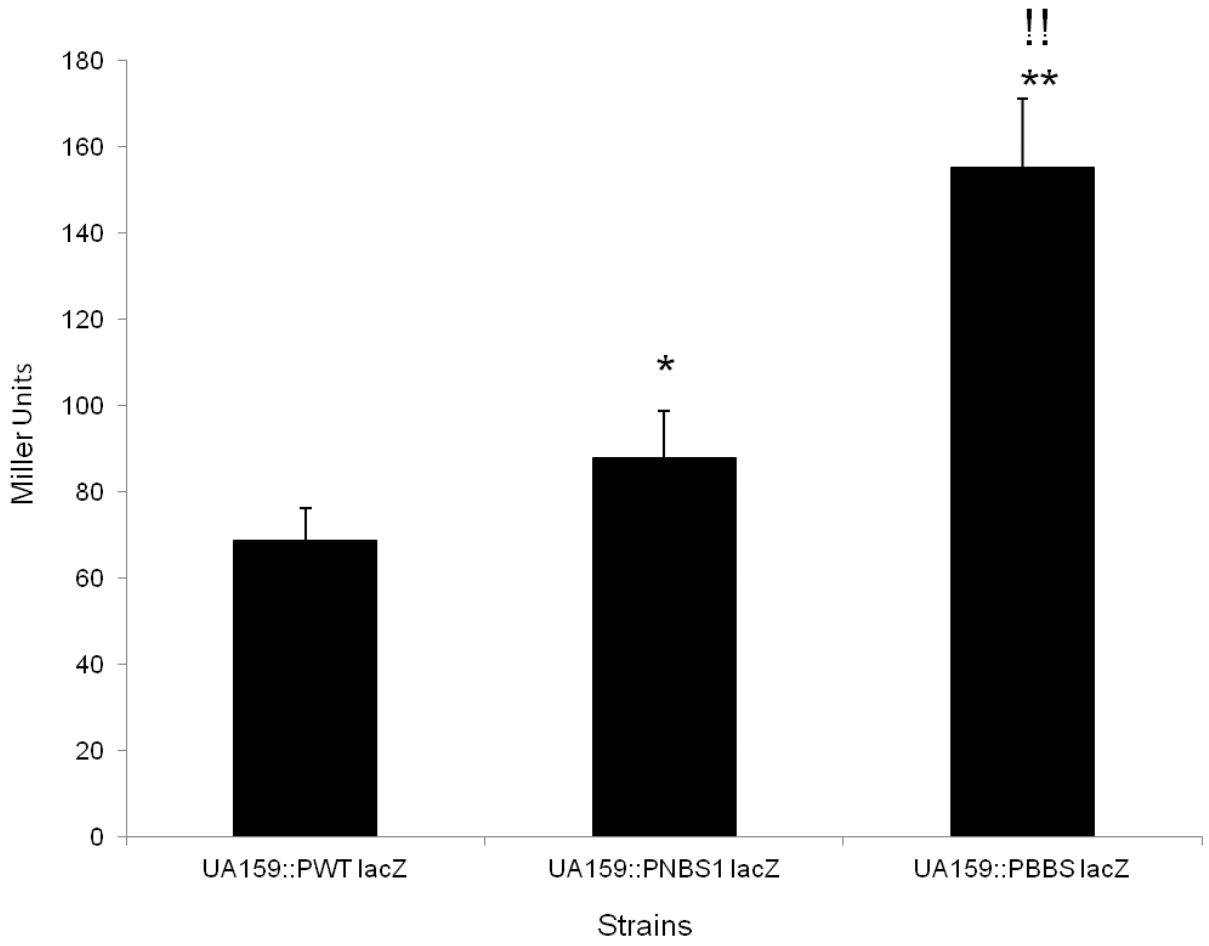

Figure 6-9. β-galactosidase activity from *rcrR* promoters that contained various mutations. The *rcrR* promoters with the different mutations were fused to a promoterless *lacZ* gene in the pMZ integration vector. The *lacZ*-promoter fusion was transformed into the wild-type strain. In all cases cells were grown to mid -exponential phase (OD_{600} = 0.5) and β-galactosidase activity measured as reported in the methods section. The results are from three independent experiments performed in at least triplicate. *, Differs from the wild-type at $p< 0.05$ (Student's *t*-Test). **, Differs from the PWT at $p<0.005$. !!, Differs from PNBS1 at $p<0.005$.

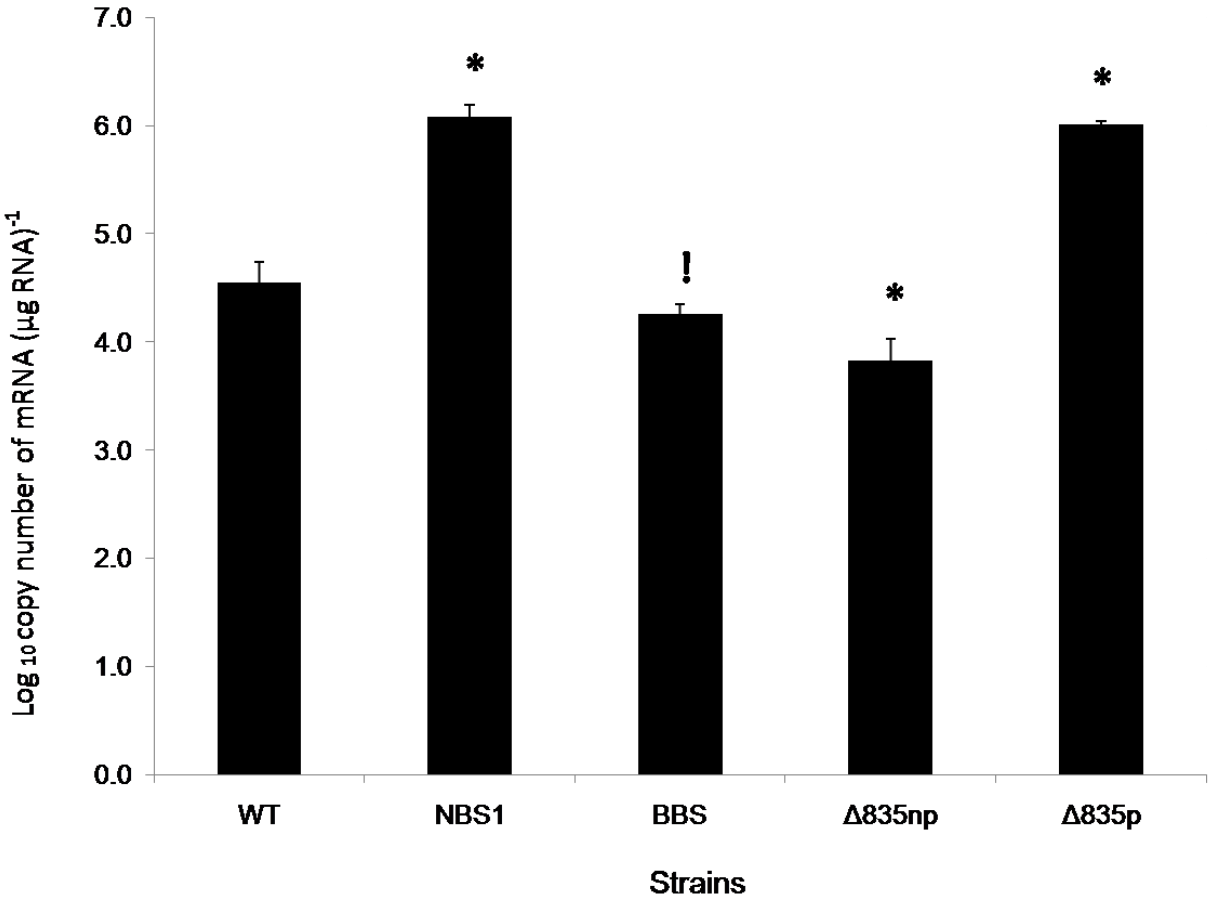

Figure 6-10. RealTime-RT-PCR showing *comYA* mRNA levels in strains with mutations in the RcrR binding site. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data are presented as log of the copy number of each gene per µg of input RNA. *, Differs from the wild-type at $p< 0.05$ (Student's *t*-test). !, Differs from the NBS1 strain at $p<0.05$ (Student's *t*-test).

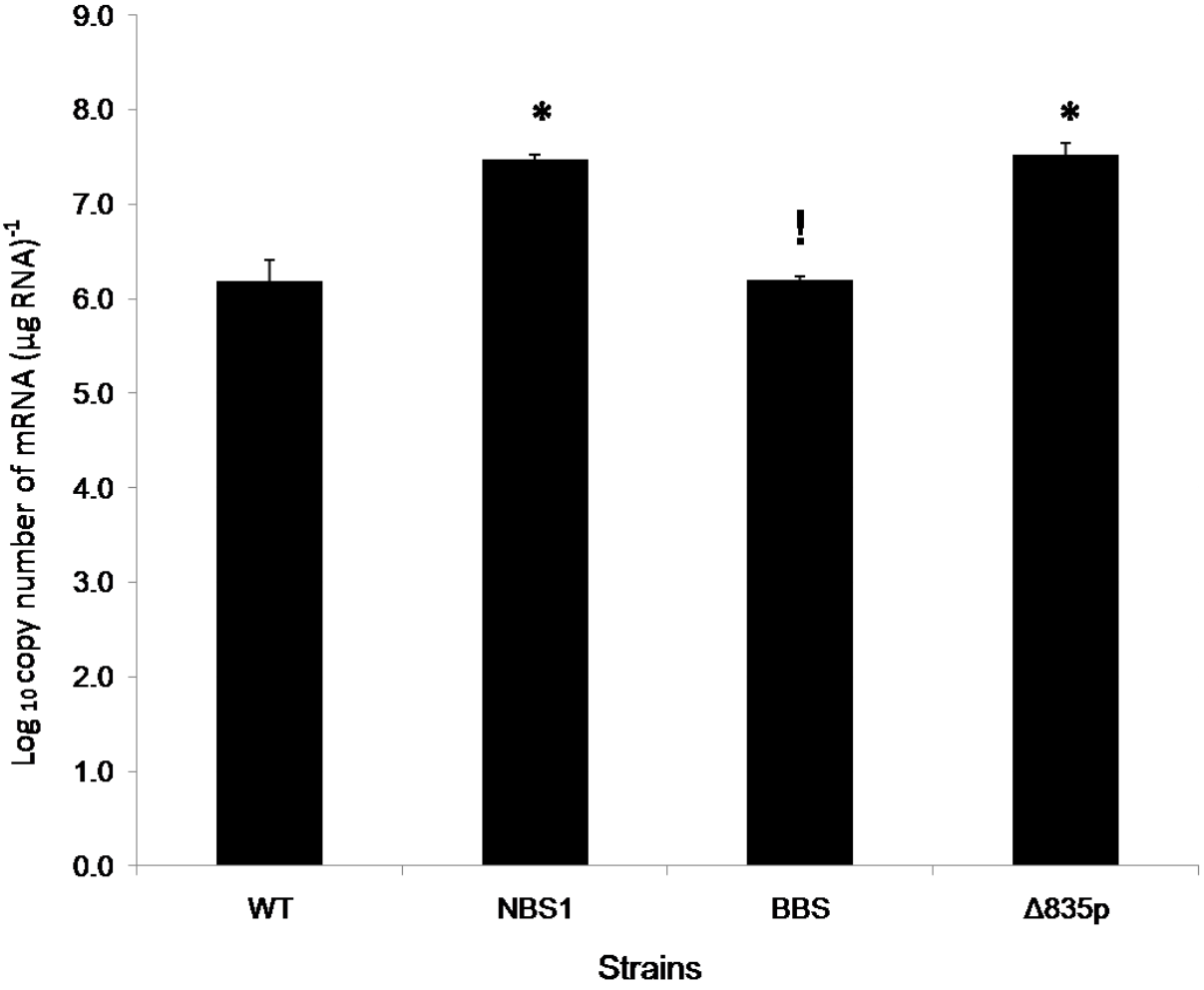

Figure 6-11. RealTime-RT-PCR showing *comS* mRNA levels in strains with mutations in the RcrR binding site. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data are presented as log of the copy number of each gene per µg of input RNA. *, Differs from the wild-type at $p<0.05$ (Student's *t*-test). !, Differs from the NBS1 strain at $p<0.05$.

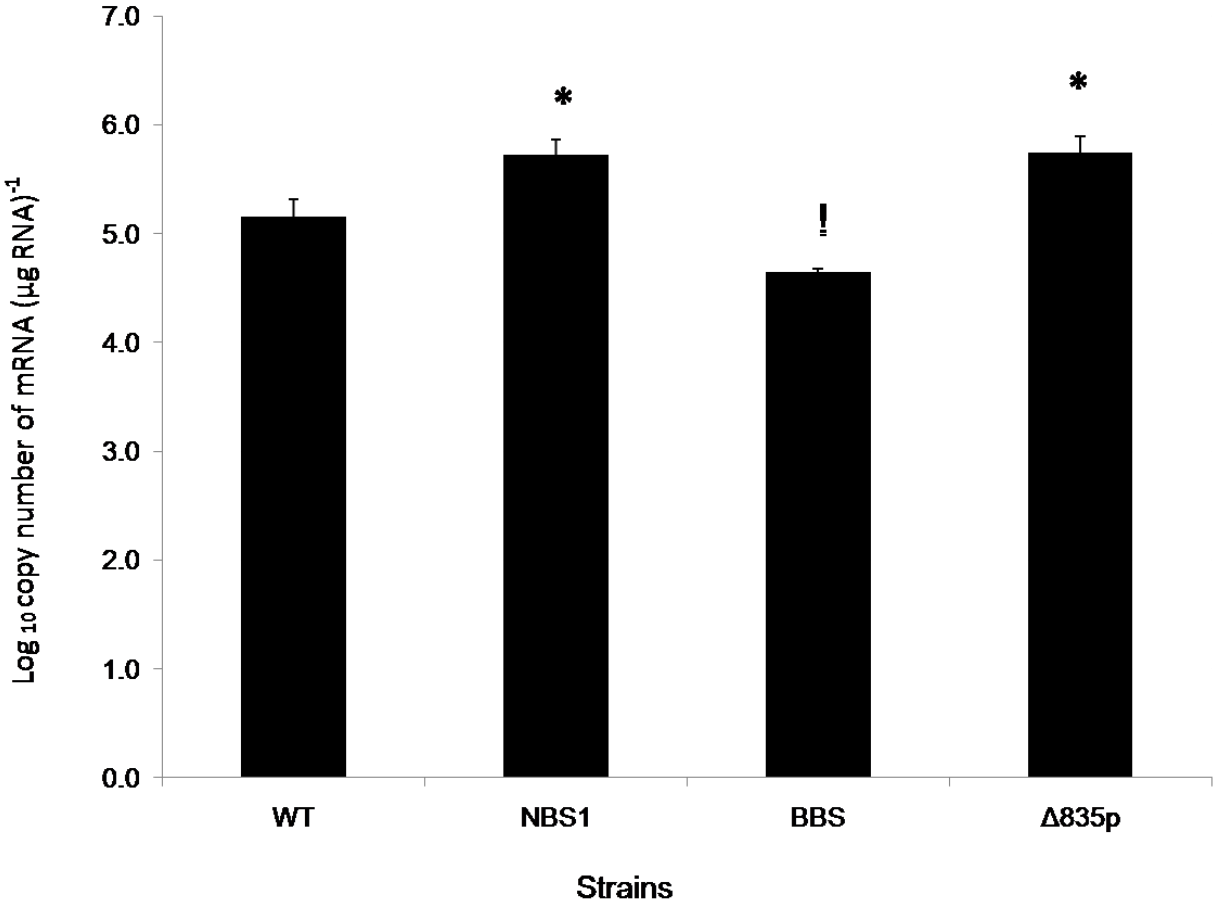

Figure 6-12. RealTime-RT-PCR showing *comX* mRNA levels in strains with mutations in the RcrR binding site. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data are presented as the copy number of each gene per µg of input RNA. *, Differs from the wild-type at $p< 0.05$ (Student's *t*-test). !, Differs from the NBS1 strain at $p<0.05$ (Student's *t*-test).

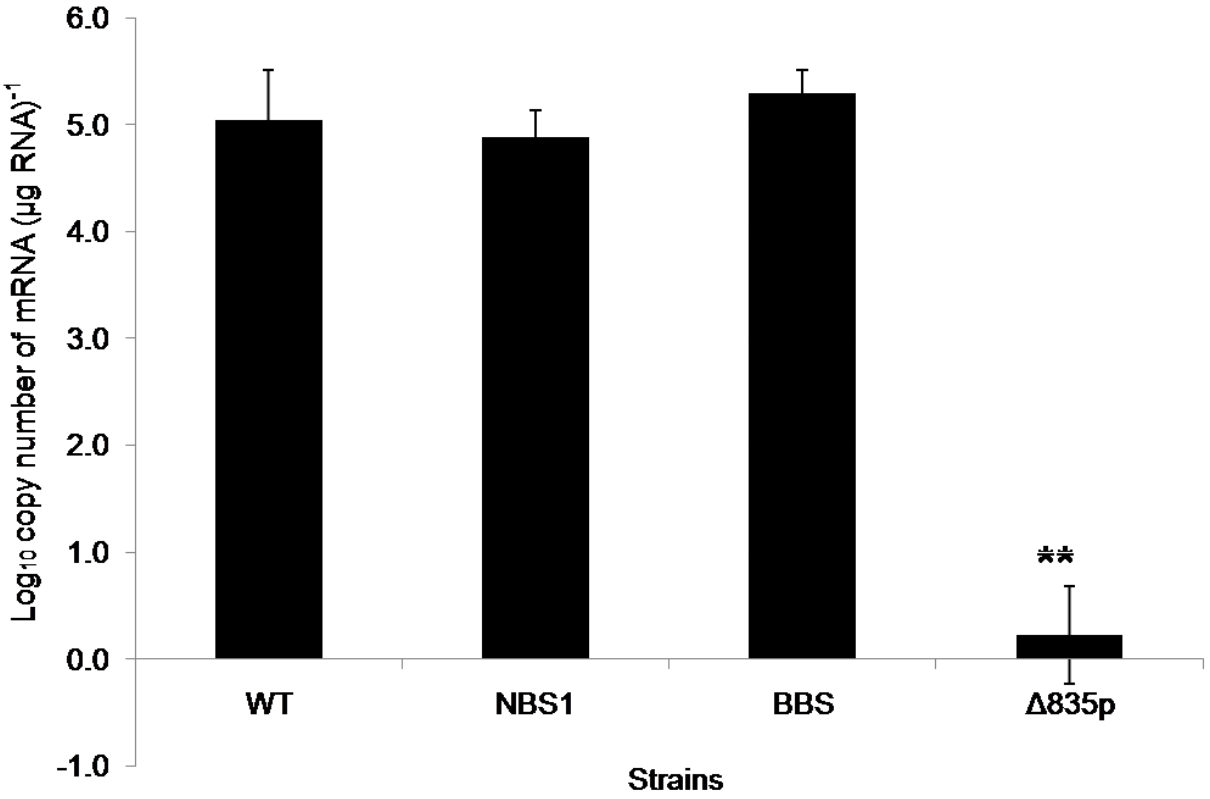

Figure 6-13. RealTime-RT-PCR showing *rcrR* mRNA levels in strains with mutations in the RcrR binding site. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data are presented as the log of the copy number of each gene per µg of input RNA. **, Differs from the wild-type at $p<$ 0.005 (Student's *t*-test).

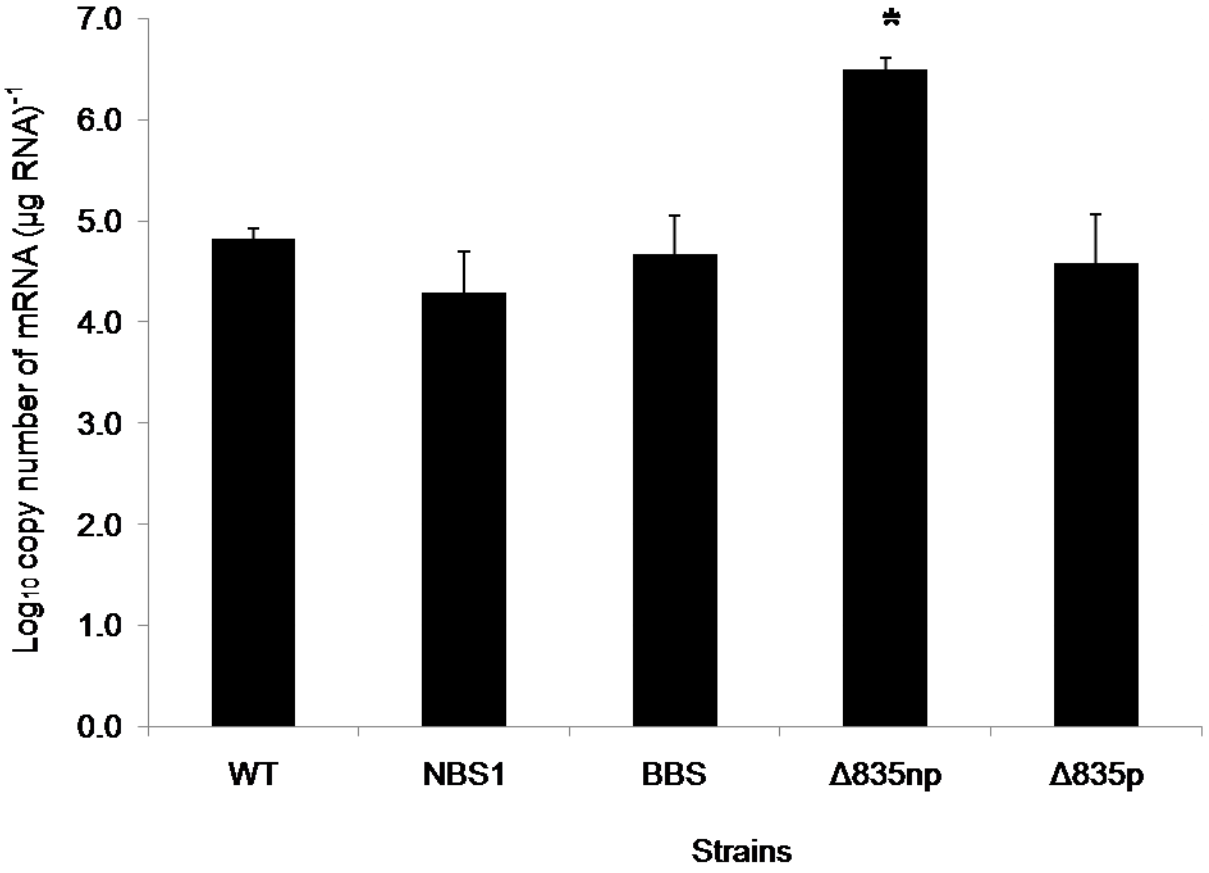

Figure 6-14. RealTime-RT-PCR showing *rcrP* mRNA levels in the strains with mutations in the RcrR binding site. Cells were grown to mid-exponential phase (OD_{600} =0.5), total RNA was extracted and RT was done using gene specific primers followed by qReal-Time PCR. The data are presented as the log of the copy number of each gene per µg of input RNA. *, Differs from the wild-type at $p<$ 0.05 (Student's *t*-test).

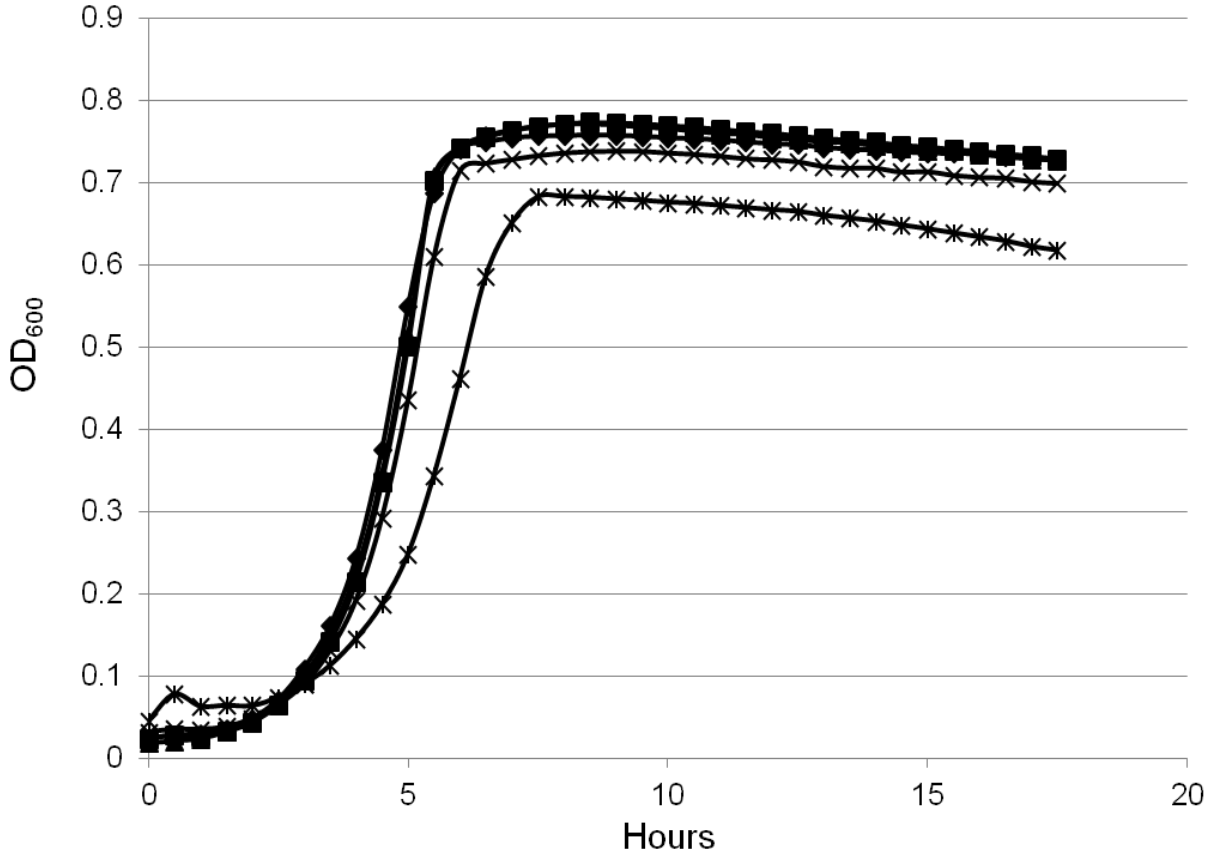

Figure 6-15. Growth comparison of wild-type and mutant strains in BHI. The strains were grown in triplicate to mid-exponential phase in BHI broth, diluted 1:100 and transferred to fresh BHI broth, overlaid with sterile mineral oil and placed in a Bioscreen C at 37 °C to monitor growth. WT, diamonds; *NBS1* , squares; *BBS* triangles, *Δ835np* x's and *Δ835p* *'s. The results are representative of three independent experiments performed triplicate.

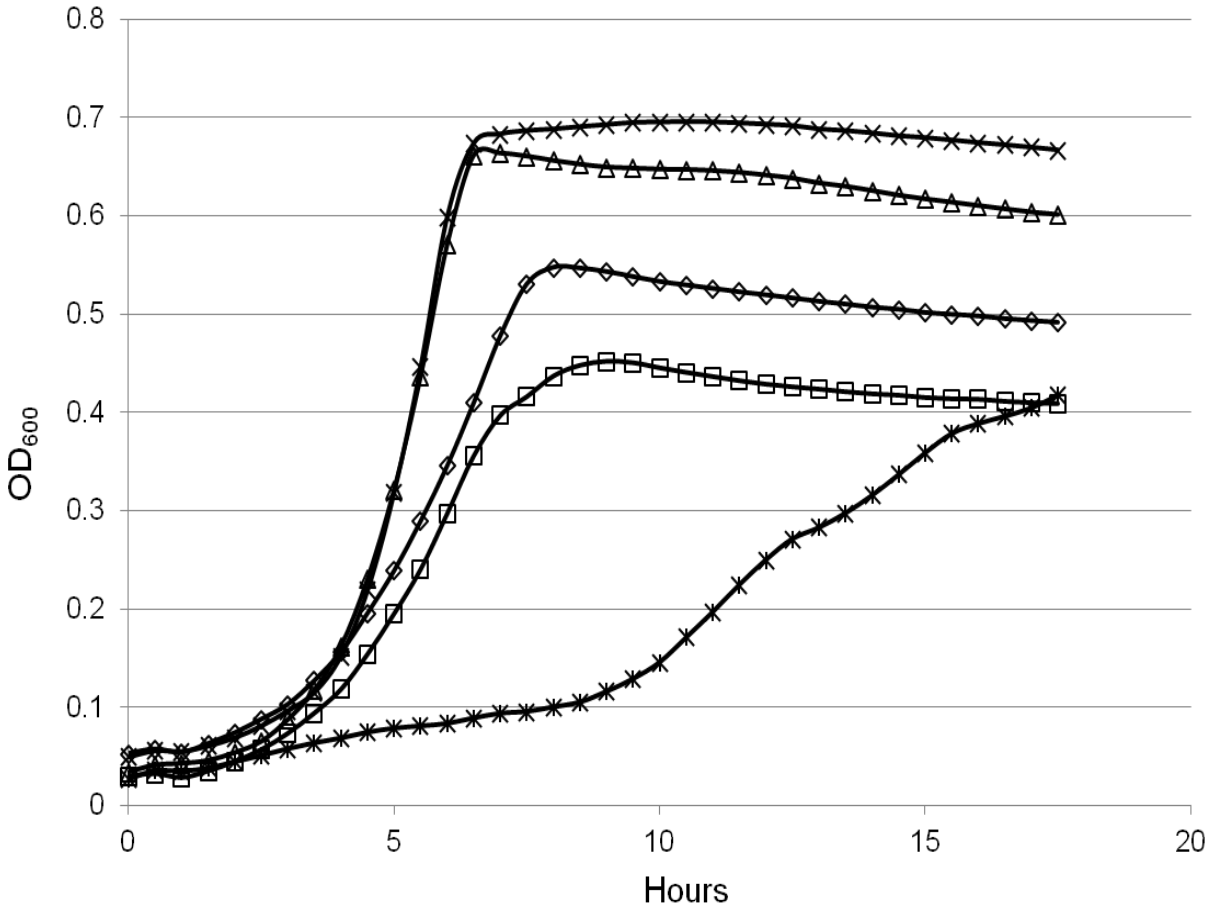

Figure 6-16. Growth comparison of wild-type vs. mutant strains in 2 µM CSP. The strains were grown in triplicate to mid-exponential phase in BHI broth, diluted 1:100 and transferred to fresh BHI broth containing 2 µM CSP, overlaid with sterile mineral oil and placed in a Bioscreen C at 37 °C to monitor growth. WT, diamonds; *NBS1* , squares; *BBS* triangles, *Δ835np* x's and *Δ835p* *'s. The results are representative of three independent experiments performed triplicate. The statistical analysis on the doubling times and final ODs are detailed in the results section describing the growth phenotype.

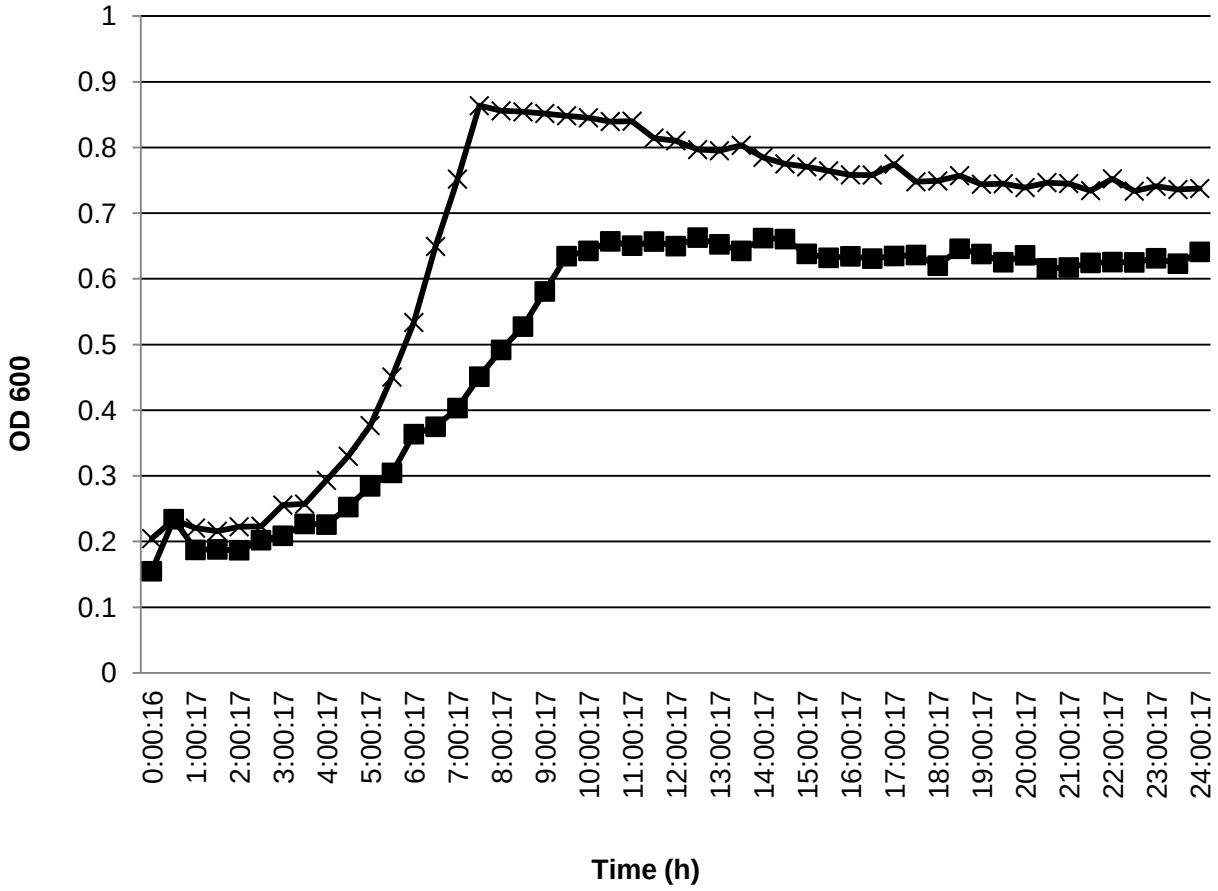

Figure 6-17. Growth comparison of wild-type vs. SJ354 in 2 µM CSP. The strains were grown in triplicate to mid-exponential phase in BHI broth, diluted 1:100 and transferred to fresh BHI broth containing 2 µM CSP, overlaid with sterile mineral oil and placed in a Bioscreen C at 37 °C to monitor growth. WT, squares; *SJ354* . The results are representative of three independent experiments performed triplicate. These data were generated by Ann Sagstetter Decker in the summer of 2010, who was a dental student doing research on the *rcrRPQ* operon and competence.

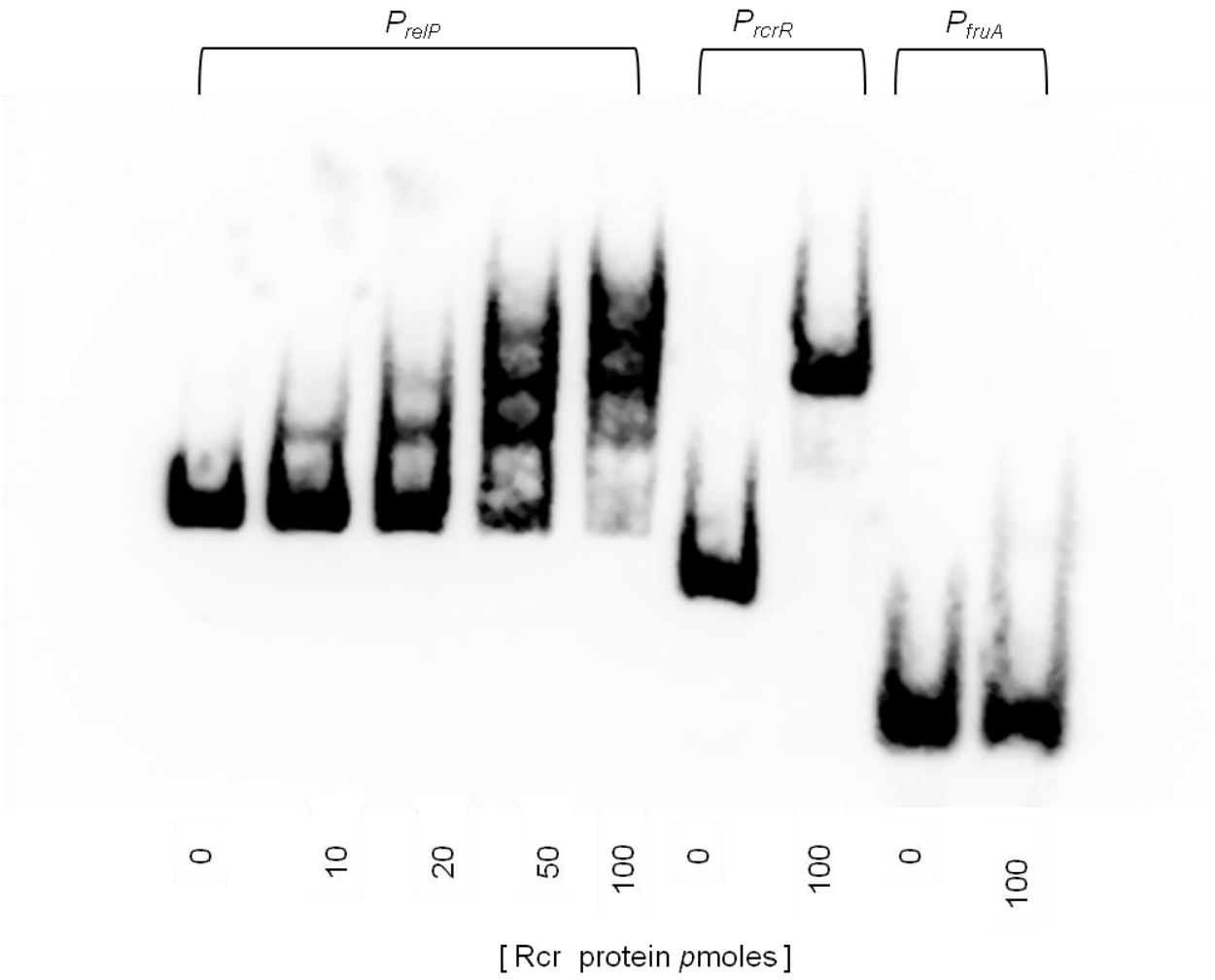

Figure 6-18. EMSA showing binding of the *relP* promoter with purified RcrR protein. Various concentrations of RcrR protein was added to 5 fmoles of biotinylated P_{relP} DNA fragment in a binding reaction, P_{rcrR} and P_{fruA} were added as positive and negative controls for RcrR protein. The reactions were run on a non-denaturing polyacrylamide gel and the signal observed via chemiluminescence. The data are a representative of n=2 EMSAs. There was a similar trend observed in both cases.

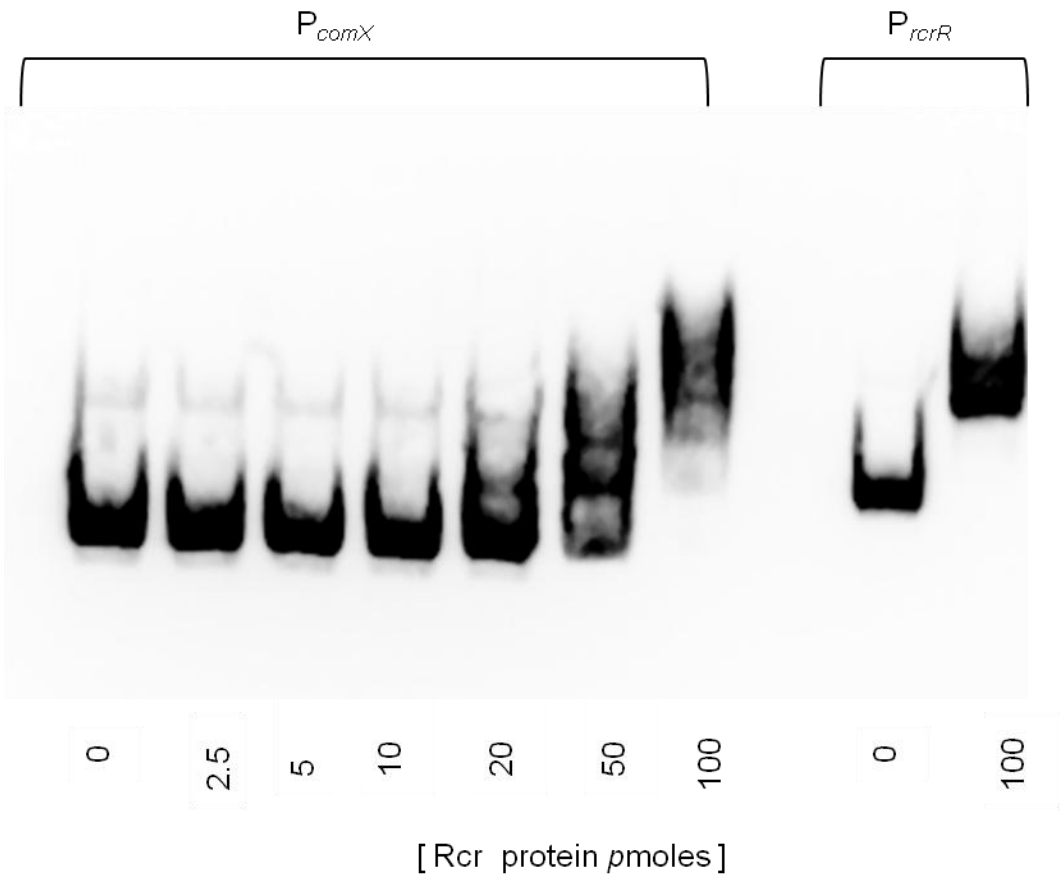

Figure 6-19. EMSA showing binding of the *comX* promoter with purified RcrR protein. Various concentrations of RcrR protein was added to 5 fmoles of biotinylated P_{relP} DNA fragment in a binding reaction, P_{rcrR} and P_{fruA} were added as positive and negative controls for RcrR protein. The reactions were run on a non-denaturing polyacrylamide gel and the signal observed via chemiluminescence. The data are a representative of n=4 EMSAs. There was a similar trend observed in all cases.

Table 6-7. Genes differentially regulated in the Δ*835np* strain vs. wild-type strain via microarray analysis

Parametric p-value	Fold-change	Unique id	Description	Gene symbol
1.00E-07	24.8	SMU.1997	putative ComX1, transcriptional regulator of competence-specific genes	comX1
< 1e-07	21.5	SMU.922	putative ABC transporter, ATP-binding protein	
1.47E-05	11.0	SMU.923	putative ABC transporter, ATP-binding protein	
1.15E-05	6.21	SMU.64	Holliday junction DNA helicase RuvB	ruvB
1.60E-06	5.31	SMU.65	putative protein tyrosine-phosphatase	
0.0014	3.14	SMU.1568	putative maltose/maltodextrin ABC transporter, sugar-binding protein MalX	malX
0.0048	3.11	SMU.574c	hypothetical protein	
0.0006	3.00	SMU.67	putative acyltransferase	
0.0004	2.91	SMU.537	tryptophan synthase subunit beta	trpB
0.0004	2.90	SMU.66	hypothetical protein	
0.0007	2.61	SMU.1056	hypothetical protein	
0.003	2.59	SMU.924	thiol peroxidase	tpx
0.002	2.54	SMU.534	anthranilate phosphoribosyltransferase	trpD
0.0015	2.52	SMU.1079c	putative ABC transporter, ATP-binding protein	
0.001	2.41	SMU.535	indole-3-glycerol-phosphate synthase	trpC
0.0039	0.344	SMU.1968c	hypothetical protein	
0.002	0.336	SMU.493	formate acetyltransferase (pyruvate formate-lyase 2)	pfl2
0.0028	0.041	SMU.1982c	hypothetical protein	
7.16E-05	0.013	SMU.1985	ABC transporter ComYB	comYB

Table 6-8. Genes differentially regulated in the Δ*835np* strain vs wild-type strain via RNA-seq analysis.

Gene ID	Gene Description	Fold Change (Δ835np/WT)	P value
SMU.1997c	competence-specific sigma factor	37.1	1.80e-273
SMU.922	ABC-type multidrug / protein/ lipid transport system	16.6	9.31e-280
SMU.923	ABC-type multidrug/protein/lipid transport system	13.6	5.04e-246
SMU.09	small RNA binding protein	7.91	9.83e-56
SMU.43	conserved hypothetical protein (possible site-specific DNA-methyltransferase/restriction modification enzyme)	7.76	3.71e-31
SMU.1576c	hypothetical protein	7.62	3.26e-21
SMU.44	conserved hypothetical protein	7.39	1.07e-25
SMU.64	Holliday junction DNA helicase	7.30	2.58e-139
SMU.1644c	conserved hypothetical protein	7.11	1.20e-96
SMU.46	hypothetical protein	5.36	3.01e-07
SMU.65	protein tyrosine-phosphatase	4.98	1.51e-77
SMU.10	cell-division protein DivIC	4.55	0.0001
SMU.1343c	polyketide synthase	4.02	9.74e-07
SMU.1250c	hypothetical protein	3.90	6.81-22
SMU.66	conserved hypothetical protein; possible phosphatidylinositol-4-phosphate 5-kinase	3.71	2.65e-36
SMU.533	anthranilate synthase	3.62	3.81e-33
SMU.1161c	conserved hypothetical protein	3.49	2.27e-11
SMU.438c	(R)-2-hydroxyglutaryl-CoA dehydratase activator-related protein	3.46	6.53e-27
SMU.68	hypothetical protein	3.43	6.04e-12
SMU.534	phosphoribosyl anthranilate transferase	3.25	2.74e-39
SMU.1643c	conserved hypothetical protein	3.24	1.06e-08
SMU.1340c	bacitracin synthetase 1/ tyrocidin synthetase III	3.22	2.71e-07
SMU.537	tryptophan synthase	3.22	2.24e-43
SMU.532	anthranilate synthase	3.09	3.93e-39
SMU.928	sensor histidine kinase	0.476	1.09e-06
SMU.463	thioredoxin reductase (NADPH)	0.438	6.04e-26
SMU.1692c	pyruvate-formate lyase activating enzyme	0.426	4.65e-25
SMU.540	peroxide resistance protein / iron binding protein	0.422	1.16e-36
SMU.924	thiol peroxidase	0.414	2.42e-24
SMU.179	conserved hypothetical protein (possible oxidoreductase)	0.365	4.21e-10
SMU.494	transaldolase family protein	0.361	5.46e-16

Table 6-8. continued

Gene ID	Gene Description	Fold Change (Δ835np/WT)	P value
SMU.491	transcriptional regulator	0.356	2.23e-11
SMU.838	glutathione reductase	0.348	1.10e-41
SMU.148	alcohol-acetaldehyde dehydrogenase	0.329	3.05e-10
SMU.1955c	co-chaperonin 10kDa	0.322	1.03-27
SMU.495	glycerol dehydrogenase	0.319	2.13e-27
SMU.460	amino acid ABC transporter	0.319	5.13e-22
SMU.629	superoxide dismutase	0.316	7.12e-64
SMU.996	ABC transporter	0.304	7.23e-06
SMU.120	50S ribosomal protein L28	0.302	4.94e-05
SMU.1703c	conserved hypothetical protein	0.299	3.45e-07
SMU.961	macrophage infectivity potentiator-related protein	0.297	2.30e-19
SMU.1425c	ATP-dependent Clp protease	0.295	4.72e-12
SMU.929c	conserved hypothetical protein	0.292	3.35e-40
SMU.82	chaperone protein	0.285	4.56e-14
SMU.493	formate acetyltransferase (pyruvate-formate lyase)	0.282	7.74e-43
SMU.962	acyl-CoA dehydrogenase	0.239	6.88e-45
SMU.496c	cysteine synthetase A	0.237	7.36e-17
SMU.1701c	conserved hypothetical protein	0.236	0.0001
SMU.80	heat-inducible transcription repressor	0.229	1.43e-18
SMU.594	hypothetical protein	0.210	3.26e-05
SMU.81	co-chaperone protein GrpE	0.209	7.74e-21
SMU.930c	transcriptional regulator	0.203	2.52e-09
SMU.457	hypothetical protein	0.146	7.25e-05
SMU.1861c	hypothetical protein	0.140	6.12e-05
SMU.934	amino acid ABC transporter	0.112	4.65e-31
SMU.1185c	mannitol PTS EII	0.110	5.79e-66
SMU.935	amino acid ABC transporter	0.095	4.32e-30
SMU.933	amino acid ABC transporter	0.094	4.63e-39
SMU.936	amino acid ABC transporter	0.094	2.31e-27
SMU.921	transcriptional regulator	0.080	3.40e-39
SMU.932	conserved hypothetical protein	0.080	1.40e-44
SMU.1395c	hypothetical protein	0.038	1.52e-10

The data were generated by Sang-Joon Ahn and collaborator Sang-Chul Choi in the laboratory of Michael Stanhope at the University of Cornell (Ithaca, NY).

Table 6-9. Genes differentially regulated in the Δ*835p* strain vs wild-type strain via RNA-seq analysis.

Gene ID	Gene Description	Fold change Δ835p/WT	P value
SMU.1001	DNA processing protein	279	0
SMU.1987c	late competence protein; type II secretion system protein E	247	1.58e-32
SMU.1980c	conserved hypothetical protein	227	5.36e-86
SMU.498	late competence protein F	210	0
SMU.1983c	competence protein ComYD	209	1.99e-145
SMU.1984c	competence protein ComYC	203	4.35e-165
SMU.1981c	competence protein G	203	1.62e-34
SMU.1982c	conserved hypothetical protein	200	2.26e-134
SMU.1985c	competence protein; general (type II) secretory pathway protein	191	3.47e-26
SMU.499	late competence protein required for DNA uptake	170	2.81e-164
SMU.626	competence protein; possible integral membrane protein	143	8.63e-299
SMU.625	competence protein	112	2.48e-172
SMU.1967c	single-stranded DNA-binding protein	84.5	7.18e-50
SMU.644	competence protein CoiA	53.0	3.21e-220
SMU.836	hypothetical protein	41.5	5.78e-174
SMU.1979c	conserved hypothetical protein	36.1	4.57e-93
SMU.1912c	hypothetical protein	25.7	7.58e-16
SMU.1910c	hypothetical protein	24.7	3.37e-17
SMU.1997c	competence-specific sigma factor	22.8	2.18e-219
SMU.539c	prepilin peptidase type IV	21.0	4.04e-07
SMU.1909c	hypothetical protein	20.1	1.51e-13
SMU.837	Oxidoreductase	19.1	1.68e-97
SMU.1907	hypothetical protein	18.0	6.48e-13
SMU.1906c	bacteriocin-related protein	16.0	4.95e-06
SMU.151	non-lantibiotic mutacin IV B	10.0	3.89e-05
SMU.1576c	hypothetical protein	8.66	1.46e-23
SMU.1055c	DNA repair protein RadC	8.65	3.37e-101
SMU.506	type II restriction endonuclease	8.51	2.58e-18

Table 6-9. continued

Gene ID	Gene Description	Fold change Δ835p/WT	P value
SMU.769	conserved hypothetical protein	7.70	1.65e-32
SMU.10	cell-division protein DivIC	7.30	8.20e-07
SMU.46	hypothetical protein	7.27	2.39e-09
SMU.2086c	competence damage-inducible protein A	6.59	2.66e-58
SMU.196c	immunogenic secreted protein (transfer protein)	4.10	1.99e-07
SMU.2085c	recombinase A	3.23	7.55e-62
SMU.1915	S. mutans specific competence stimulating peptide	3.23	5.48e-05
SMU.1345c	peptide synthetase similar to mycA	3.25	7.34e-07
SMU.1828	universal stress protein family	0.652	1.46e-08
SMU.132	amino acid amidohydrolase (hippurate amidohydrolase)	0.390	1.46e-20
SMU.1692c	pyruvate-formate lyase activating enzyme	0.385	8.13e-32
SMU.540	peroxide resistance protein / iron binding protein	0.385	2.55e-45
SMU.923	ABC-type multidrug/protein/lipid transport system	0.368	1.11e-51
SMU.932	conserved hypothetical protein	0.335	6.55e-11
SMU.629	superoxide dismutase	0.301	3.04e-71
SMU.924	thiol peroxidase	0.218	1.35e-68
SMU.1185c	mannitol PTS EII	0.176	2.69e-43
SMU.921	transcriptional regulator	0.086	4.55e-38

The data were generated by Sang-Joon Ahn and collaborator Sang-Chul Choi in the laboratory of Michael Stanhope at the University of Cornell (Ithaca, NY).

CHAPTER 7
SUMMARY AND FUTURE DIRECTIONS

The Role of RcrRPQ in Stress Tolerance

S. mutans is able to gain a competitive advantage over many commensal organisms because of its ability to thrive under conditions that are favorable for caries development such as low pH (80, 118). However, *S. mutans* is still able to overcome many environmental conditions that are less than optimal for its growth such as aeration and limited nutrient availability. Therefore, it is critical to understand the mechanisms that *S. mutans* utilizes to respond to stress and eventually cause disease. Transcriptional regulators of the MarR family linked to the expression of genes encoding efflux pumps are one mechanism bacteria use to overcome antibiotic stress and environmental stresses in general (157). MarR proteins linked to efflux have been characterized in other *Streptococcus* species (191). However, there are limited published studies which characterize MarR regulators linked to efflux pumps in *S. mutans* and their role in stress tolerance.

The previously uncharacterized *rcrRPQ* operon, which encodes a MarR regulator linked to two efflux pumps, plays a dominant role in stress tolerance and two other stress adaptation pathways: the metabolism of (p)ppGpp and genetic competence. Specifically, the RcrPQ efflux pumps play a significant role in the ability of the cells to grow in a variety of stresses. The *Δ836p* strain, which has the gene encoding the RcrP (SMu0836) efflux pump deleted and the gene encoding the RcrQ (SMu0837) efflux pump expressed at 1000-fold lower than wild-type levels, exhibited a poor growth phenotype. The ability for *S. mutans* to grow at low pH is a key virulence property (20) and the *Δ836*p mutant had very little cell accumulation even after 24 h at pH 5.5. Efflux

pumps are not only associated with the extrusion of antibiotics but are seen as playing an important role in controlling the physiology of the cell and externalizing a wide range of compounds including, metals, peptides and lipids (148, 179). Therefore, we concluded that the RcrPQ efflux pumps are capable of extruding growth inhibitory compounds that accumulate in growing cells that may be increased in low pH or aerobic conditions. The expression of genes encoding efflux pumps is usually carefully regulated by MarR regulators and uncontrolled expression of genes encoding efflux pumps can eventually pose a metabolic burden on the cell (148). The data accumulated show that the expression of *rcrPQ* is under dominant control by the RcrR (SMu0835) regulator which belongs to the MarR family, and the expression of the genes encoding the pumps had to be carefully regulated. Various strains where *rcrPQ* were aberrantly expressed had an altered physiology compared to the wild-type strain. Strains where *rcrPQ* were overexpressed were not transformable or had defects in transformation efficiency, and many genes in the competence pathway were affected. Therefore it is critical for RcrR to bind to the promoter of *rcrRPQ* to control the expression of the operon. The data detailed in Chapter 1 and 6 show that RcrR controls the expression of *rcrRPQ* by binding to a specific pseudopalindromic sequence, TAGTTTTCATGAGAACTA, in the promoter region of the *rcr* operon, which is consistent with the mechanisms of how MarR-type regulators bind DNA (236). Mutations in the binding site affected the ability of RcrR to bind efficiently, and transformation efficiency and the expression of genes in the competence pathway were perturbed. MarR-type regulators are well known gauges for bacteria to respond to environmental cues. The binding affinity of MarR type regulators to bind promoter-DNA can be altered in

response to different signals to control gene expression and cause a physiological response in the cells(172). It is part of our working model that under non-stress conditions RcrR is able to bind to the promoter of the *rcr* operon to repress the expression of *rcrPQ* (Figure 7-1, 7-2, 7-3). However, the *rcrRPQ* operon is derepressed in response to a stress signal(s) that accumulates inside the cell so the RcrPQ pumps can extrude potentially harmful or growth inhibitory substances.

An interesting finding emerged from these studies when we made various deletions in *rcrQ (SMu0837).* Competence and growth in CSP in these mutants were highly dependent on sequences in the 3' region of *rcrQ* (Table 7-2). We hypothesized that the 3' region of *rcrQ* may have some regulatory elements or encode a putative peptide(s) (Figure 7-4, Figure 7-5) (Ahn *et al.*, manuscript in preparation). The data thus far indicate that the genes encoding the putative peptides are under the control of RcrR as well, since no promoter activity could be detected in the region 5' to the ATG start codon of the genes encoding the peptides via *lacZ* or *cat* fusions. In addition, a mutant was made where the DNA encoding the putative peptides was deleted in the background of the *Δ835n*p strain (Ahn *et al.*, manuscript in preparation). The *Δ835n*p strain, was not transformable and insensitive to growth inhibition by CSP. However, when the DNA encoding the peptides was deleted in the *Δ835n*p strain, the strain exhibited a phenotype more similar to the *Δ835p* strain and was hypertransformable (Ahn *et al.*, manuscript in preparation). Our hypothesis is that the putative peptide(s) in the region 3' of *rcrQ* interferes with either RcrR or may be the signal being exported by the RcrPQ exporters. We have also been able to FLAG-tag these peptides and detect them in the cell wall and cytoplasmic fractions using Western blot analysis. However,

their functions are still unknown. Studies are currently being done investigating the role of the 3' region and the putative peptides on competence development and other pathways such as (p)ppGpp metabolism . These data raise the possibility of other peptide signals involved in the regulation of competence.

Novel Factors Affecting (p)ppGpp Metabolism

The production of (p)ppGpp is critical for bacteria to overcome environmental stresses, particularly nutrient limitation, and bacteria that cannot mount an efficient stringent response acquire deleterious mutations (183). The levels of (p)ppGpp accumulated need to be carefully regulated since overproduction of (p)ppGpp can also shift the physiology of the cell to grow slower, which would negatively affect competitive fitness. Previously, it was thought that RelA was the sole regulator of (p)ppGpp metabolism in Gram-positive bacteria. It is now known, however, that there are other synthetases, RelP and RelQ, that produce (p)ppGpp in *S. mutans,* and their orthologues are fairly widely distributed in other Gram-positive bacteria (122). Based on the studies done by Lemos *et al.*, RelP produces the bulk of (p)ppGpp under non-stringent conditions and RelP-dependent (p)ppGpp accumulation can influence the growth of *S. mutans* (122). Notably, the regulation of RelP-dependent (p)ppGpp production was linked to the RelRS TCS, probably by sensing external signals. However, these external signals were not identified. The studies presented here revealed at least one of the signals that influence RelP-dependent (p)ppGpp production in a dominant way. The RelRS TCS detects oxidative stress to regulate (p)ppGpp production and control the metabolism of the cell. There was a significant increase in the amount of (p)ppGpp accumulated when the cells were exposed to hydrogen peroxide. Importantly, the accumulation of (p)ppGpp in hydrogen peroxide was dependent on both RelP and

RelRS and the levels of (p)ppGpp accumulated were minimally affected by RelA or RelQ. Furthermore, the promoter activity of *relP* was increased when cells were exposed to hydrogen peroxide. *S. mutans* encounters oxidative stress, especially in early biofilm formation in the oral cavity from host defense mechanisms and other oral bacteria, and it has somewhat limited capacity to deal with oxidative stress compared to other oral streptococci such as *S. sanguinus* (142, 165). We posit that the accumulation of (p)ppGpp under oxidative stress can serve as mechanism to slow the metabolism of the cells, so they do not use up resources and acquire deleterious mutations. Therefore, *S. mutans* can persist until conditions become more favorable such as in a mature biofilm. Notably, preliminary data show that (p)ppGpp accumulation was not affected by low pH, and furthermore the expression of *relPRS* was not changed in response to low pH (data not shown). Therefore, we conclude that aeration and oxidative stress are major environmental signals to regulate RelPRS-dependent production of (p)ppGpp.

An interesting finding was the effect of the *rcrRPQ* operon on the expression of *relP* and (p)ppGpp production. The data detailed in Chapter 4 show that the expression of the *rcrRPQ* operon affects the amount of (p)ppGpp accumulated, where the *Δ835np* and *Δ836p* mutants had reductions in levels of (p)ppGpp accumulated in exponentially growing cells. Notably, the expression of *relP* was significantly downregulated in the *Δ835np*, *Δ835p* and *Δ836p* strains as well. The recombinant RcrR protein was also shown to have weak interaction with the promoter of *relP.* Therefore we propose that RcrR may bind weakly to the *relP* promoter under specific conditions such as oxidative stress or when the *rcr* operon is derepressed to positively regulate the expression of

relP and increase (p)ppGpp production to control population growth. Based on the reduced levels of (p)ppGpp accumulated in the *Δ836p* strain, which has *rcrP* deleted and *rcrQ* expressed 1000-fold lower than wild-type levels, we also propose that the RelRS TCS can sense the signals externalized by RcrPQ. We posit that the Rel RS TCS can sense population density via the substance(s) being exported by the RcrPQ exporters when it reaches a critical level to activate RelP-dependent (p)ppGpp production (Figure 4-9) in a quorum sensing-like manner. We have not investigated the role of the putative peptide(s) encoded in the *rcrQ* 3' region, but it is reasonable to hypothesize that if the peptides are being exported by RcrPQ, they may also play a role in the expression of *relPRS* and (p)ppGpp production.

The Role of RcrRPQ on Genetic Competence and DNA Uptake

Genetic competence has nutritional advantages (46) and has been linked to adaptation and survival by increasing genetic diversity in bacterial species. Mutations to genes in the competence pathway attenuated virulence and biofilm formation in *S. mutans* (129, 130). The regulation of competence in *S. mutans* differs from other naturally competent bacteria and *S. mutans* is the only naturally competent bacterium studied thus far that utilizes both of the main peptide signaling systems, ComRS and ComCDE, to regulate competence (86). It appears that there is cross-regulation of the ComCDE and ComRS systems by as-yet-undefined mechanisms, although they both converge at ComX (63). Despite the fact that the alternative sigma factor ComX is required for activation of late competence genes, other factors influence the development of genetic competence, often in a dominant way (8, 63, 167). However, in

most cases the mechanisms by which these other factors affect signaling of competence have not been characterized.

These studies reveal components of the competence pathway that were not previously identified. The *rcrRPQ* operon influences the expression of *comX* and the late competence genes and DNA uptake in a dominant way. Based on the qRT- PCR analysis and transcriptional profiling of various mutants of *rcrRPQ,* RcrR negatively regulates the expression of genes in the competence pathway and may interact with the promoters of genes in the competence pathway directly. The expression of the genes encoding the RcrPQ exporters was also critical in controlling the expression of genes in the competence pathway and DNA uptake. Strains in which the genes encoding the RcrPQ exporters were overexpressed were either non-transformable or had reduced transformation capabilities and had lower *comYA* mRNA levels than in the wild-type cell. The *Δ835p* strain, which had wild-type levels of the genes encoding the RcrPQ exporters, was hypertransformable, whereas the *Δ835np* strain overexpressed *rcrPQ* and was not transformable under any of the conditions tested. The *Δ835np* strain displayed an interesting gene expression profile, where the expression of *comX* was about 100-fold higher than wild-type levels, but the expression of *comYA* was significantly lower. This was a novel finding since ComX is required to activate *comY* and other late competence genes. It is therefore our hypothesis that RcrPQ extrude a signal(s) that is necessary for the activation of ComX (Figure 7-1, Figure 7-2, Figure 7-3). We predict that the signal(s) acts as an anti-anti-sigma factor that impacts ComX protein stability and/or activity. Analogous components that impact ComX stability have been described in other naturally competent Gram-positive bacteria such as *S.*

pneumoniae and *B. subtilis* (228). We hypothesize that the signals being externalized by RcrPQ that affect ComX stability, might be the peptides encoded in the 3' region of *rcrQ*. Current efforts in the lab are focused on generating antibodies to ComX to examine ComX stability in the various *rcrRPQ* mutants generated and use the His_6-tagged purified ComX to perform pull down assays to identify proteins and components such as the peptides that may interact with it.

Linkage of RcrRPQ to Stress Tolerance, (p)ppGpp and Competence

S. mutans has evolved and fine tuned multiple systems to integrate and regulate its gene expression to respond to different environmental challenges (121). Stress tolerance, (p)ppGpp metabolism and competence are all important pathways for *S. mutans* to survive and establish itself and cause caries when the conditions are favorable. The competence pathway and (p)ppGpp metabolism have been described more extensively in other bacteria, but the regulation of both of these systems differs significantly in *S*. *mutans* from other Gram-positive bacteria. An inappropriate induction of these stress response systems can cause the cells to grow inefficiently and cause an unnecessary burden on the cell (101, 183). Consistent with this idea we have some preliminary evidence where a strain that overexpresses *comX* grows poorly (Sang-Joon Ahn, personal communication). Therefore, it is necessary to tightly regulate the expression of *comX*. The findings presented show the important role that the previously uncharacterized *rcrRPQ* operon plays in stress adaptation pathways and links (p)ppGpp metabolism and competence.

We propose that (p)ppGpp metabolism and the development of competence are linked through central control by the RcrR regulator and the substances being localized by the RcrPQ exporters (Figure 7-1, Figure 7-2, Figure 7-3). *S. mutans* has master

regulators such as CcpA (3) which can up- or down-regulate the expression of many genes enabling the bacteria to adapt efficiently to rapid changes in the environment such as those encountered in the oral cavity (120, 205). RcrR appears to serve as another important regulator by influencing both (p)ppGpp metabolism and competence. The upregulation of the *rcrRPQ* operon in the presence of mupirocin (122) indicated that the expression of the operon can be altered in response to stress. An interesting finding from the microarray analysis done by Ahn *et al.*, revealed that the expression of *comX* was downregulated in aeration, whereas the expression of *relPRS* was upregulated in response to aeration (7). Therefore oxidative stress, nutrient limitation or other unidentified stress may be some of the signals to integrate RcrR-dependent control of (p)ppGpp production and the induction of competence. As part of our working model, we propose that in conditions which cause derepression of the *rcr* operon, RcrR can bind more efficiently to the promoters of both *relP* and *comX* to positively regulate the expression of *relP* and (p)ppGpp production, and negatively impact the expression of *comX* and the uptake of DNA (Figure 7-1, 7-2). It is known that the ComCDE system can activate *comX* even though the mechanisms are not clear. However, the expression of *comX* is lower in aeration(7) even though the expression of genes encoding the bacteriocins and ComD were highly upregulated in cells that were aerated (7). Therefore it is reasonable to hypothesize that RcrR can serve as a mechanism to control the expression of *comX* and DNA uptake even when the expression of the genes encoding the bacteriocins and ComDE are upregulated and may serve as the decision whether cells lyse or become competent.

The substances that RcrPQ extrude, which are also under the dominant control of RcrR, play a significant role in the regulation of both (p)ppGpp metabolism and the development of competence. We propose that the substances RcrPQ export can act in a quorum sensing-like manner to regulate RelPRS-dependent production of (p)ppGpp to control the growth of the population while internal concentrations impact ComX stability and activity (Figure 7-1, 7-2). Therefore, localization of the substances being exported by RcrPQ need to be carefully balanced, and slight differences in the localization of the substances can alter the physiology of the cells. The induction of (p)ppGpp production causes the cells to shift their physiology to conserve energy; we posit that the RcrRPQ system serves to delay the induction of competence so as not to utilize unnecessary resources. However, under other conditions where the *rcr* operon is repressed, RcrR may not bind as effectively to the *relP* or *comX* promoters. The amount of the substance externalized by RcrPQ will also be reduced thereby causing lower amounts of (p)ppGpp production by RelP and increasing ComX stability and/or activity (Figure 7-1).

Preliminary studies also show a linkage of (p)ppGpp metabolism to ComS/XIP. A *ΔrelA* mutant is less sensitive to XIP-induced growth inhibition compared to the wild-type strain and had defects in transformation (Justin Kaspar, personal communication). However, the explanation for these observations is not known and further experiments linking the two pathways are in progress. Data also show that the *rcrRPQ* operon exerts an effect on the ComRS/ XIP system as well, where *comS* levels are high in some of the Δ*rcrRPQ* mutant strains. However, the mechanisms underlying the linkage of *rcrRPQ* to ComRS are currently being investigated.

Future Studies

One of the interesting findings in these studies is the significance of the RcrPQ exporters and the signals being exported to regulate the physiology of the cell. Current efforts are focused on identifying the substrates being extruded by the RcrPQ porters through mass spectrometry on concentrated supernates from the various mutants. We are also interested in the putative peptides encoded in the 3' region of *rcrQ*. Studies investigating the localization of these putative peptides through Western blot analysis using antibodies specific to the peptides are currently in progress. The RcrR regulator has a dominant role in regulating the *rcr* operon and other genes through RcrR binding to the promoters of various genes. It is known that MarR regulators can change their binding affinity by the presence of substances such phenols or antibiotics (236). Therefore the identification of substances that can impact the expression of the *rcrRPQ* operon and influence the binding activity of RcrR to various promoters would provide much needed insight into the mechanism of control by the RcrR regulator.

Summary

The RcrRPQ system has not been identified or characterized in other bacterial species. However, other *Streptococcus* species have genes that encode orthologues of RcrRPQ linked to RelPRS (Table 7-1). Interestingly, the species that have orthologues of RcrRPQ linked to RelPRS do not have the ComCDE system, only the ComRS system [Table 7-1, (86)]. Notably, an orthologue of RcrRPQ was identified in *S. pyogenes* (Table 7-1), an organism that has not been transformable in laboratory conditions even though it has all the components of the competence machinery, such as the genes encoding ComG (ComYA). The orthologues of RcrRPQ may have a similar function to control competence in other bacterial species such as *S. pyogenes*.

Current projects in the lab are focused on characterizing other clinical isolates of *S. mutans* and investigating genes that play an important role in stress tolerance. Interestingly, a clinical isolate that does not have the gene encoding the RcrR regulator (47) is not transformable under the conditions tested thus far (Lin Zeng, personal communication), suggesting that this operon is not only important in the strain UA159, but may be a critical regulator in other clinical isolates and contribute to fitness of other strains of *S. mutans.* The transformation capabilities of other strains of *S. mutans* differ from the strain UA159 and some of the clinical isolates are not readily transformable under the conditions tested thus far (171). Therefore, studies characterizing the *rcrRPQ* operon in other strains of *S. mutans* would be interesting to see if they impact the physiology of the organisms and if they function in a similar manner as in the strain UA159.

The findings reveal novel factors that influence the development of competence and (p)ppGpp metabolism, which are two important pathways *S. mutans* utilizes to adapt to various environmental stresses to cause disease. The production of (p)ppGpp and the development of competence are regulated by a MarR-type regulator and the accumulation of other peptides and signals that were not previously identified. The discovery of the peptides encoded in the *rcrQ* 3' region that influence the physiology of the cell may have therapeutic value by impacting the growth of the population or perturbing other physiological pathways. Since the expression of *rcrRPQ* operon affects the physiology of the cells, especially growth at low pH, a key virulence attribute of *S. mutans*, RcrRPQ may also be a good target for therapies to limit the growth of *S. mutans* and the development of caries.

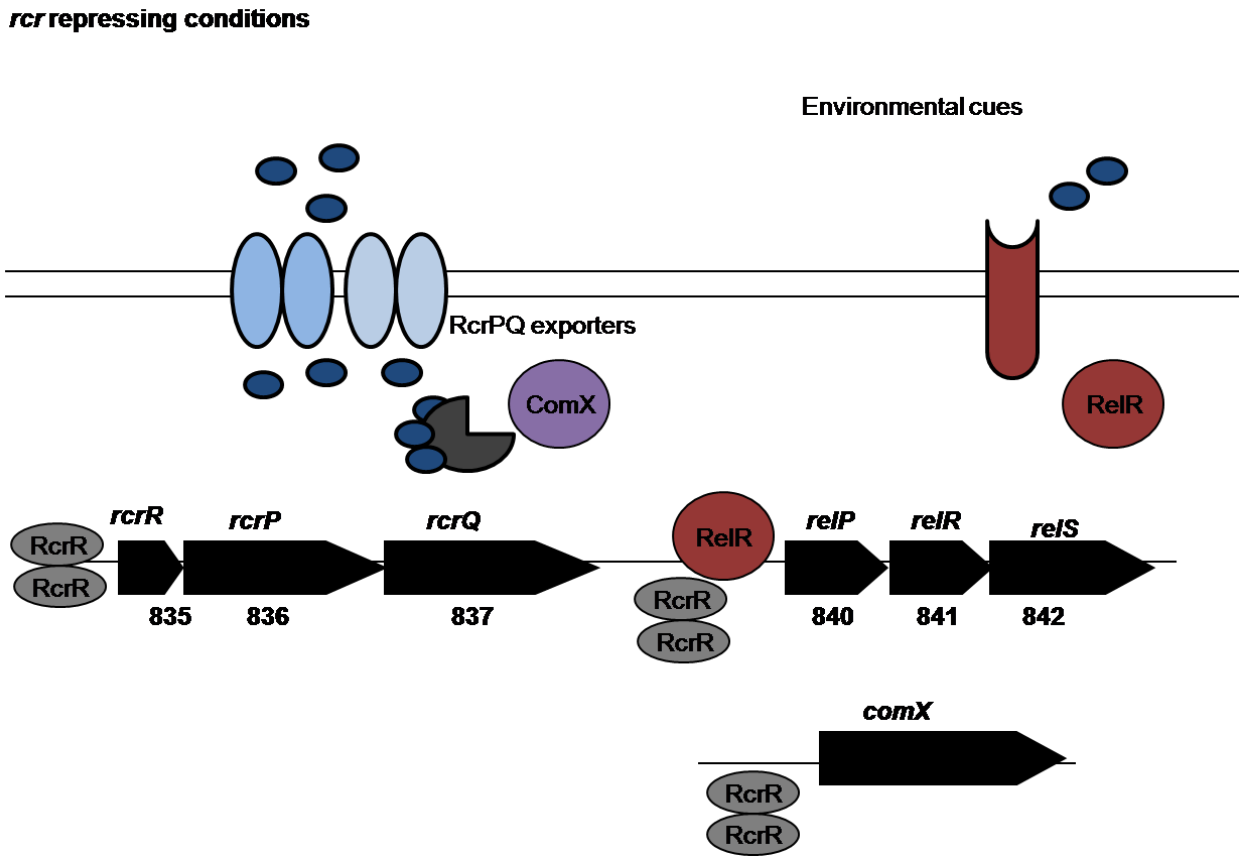

Figure 7-1. Schematic of the working model showing the regulation of *relP* and *comX* by RcrRPQ under *rcr* repressing conditions. RcrR can bind to the promoter of the *rcr* operon and repress expression of the operon. RcrR also has weak interaction with the *relP* and *comX* promoters. The RcrPQ signals can interact with a factor that interferes with ComX stability.

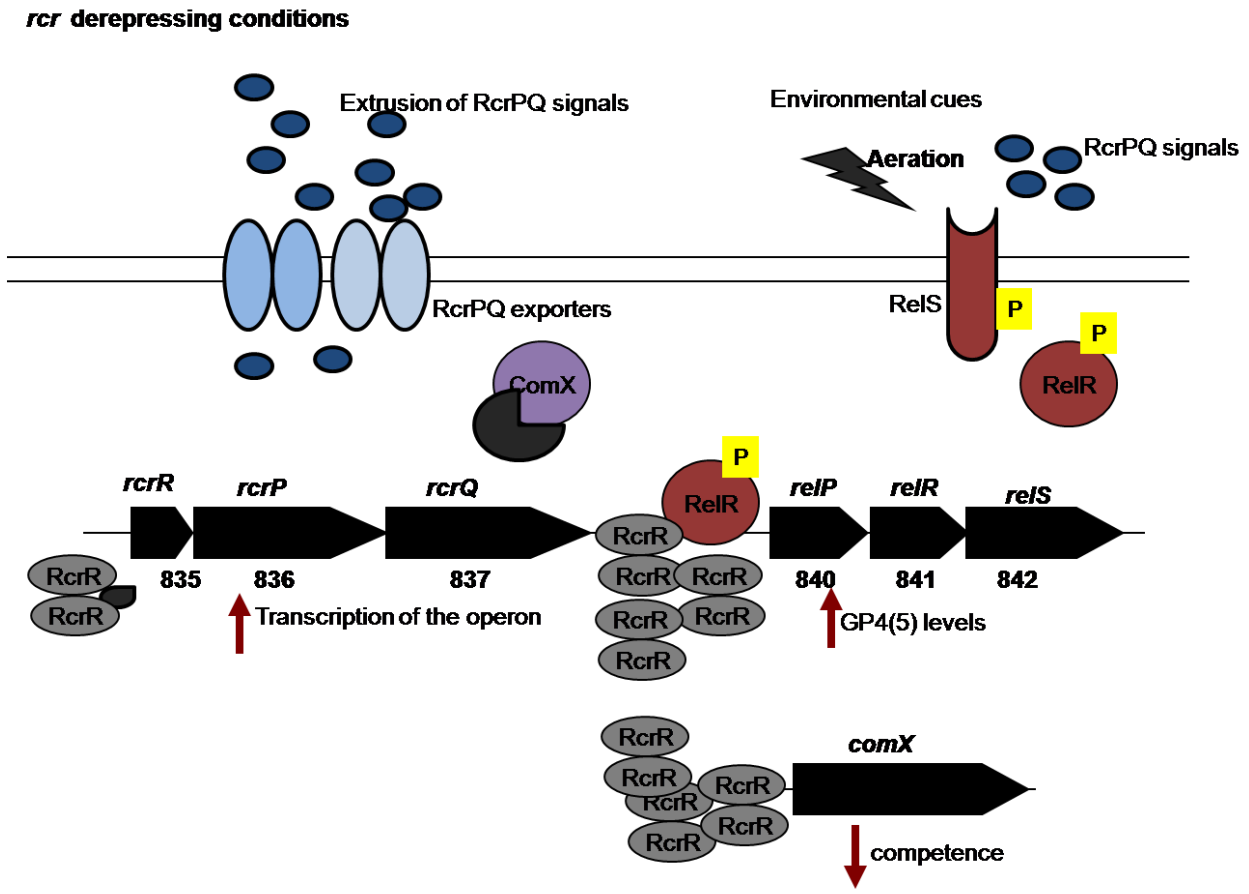

Figure 7-2. Schematic of the working model showing the regulation of *relP* and *comX* by RcrRPQ under *rcr* derepressing conditions. Derepression of the operon can lead to higher levels of RcrR. High concentrations of RcrR can bind to the *relP* promoter to activate (p)ppGpp production as well as bind to the *comX* promoter to impair competence. Upregulation of the genes encoding the RcrPQ efflux pumps also results in externalization of the signal/s interacting with the factor that impairs ComX stability causing its activity to be impaired and impacting competence.

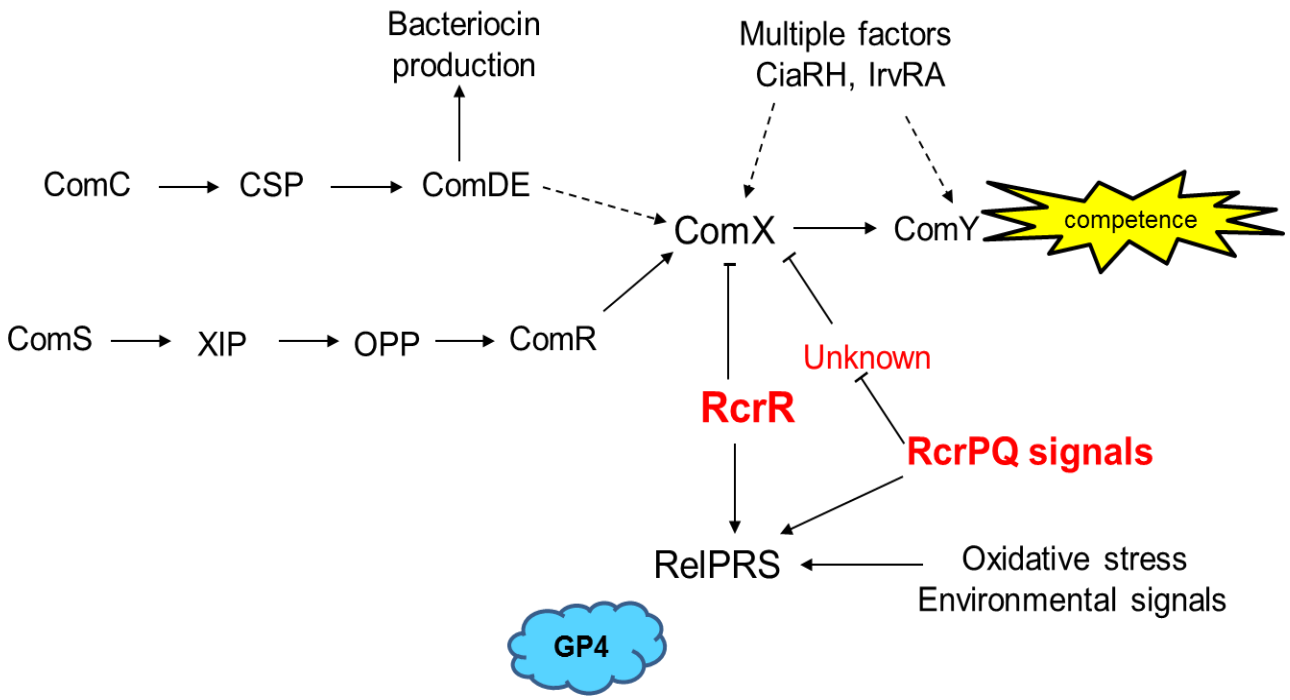

Figure 7-3. Schematic of the working model showing the regulation of the competence pathway and the RelPRS –dependent production of (p)ppGpp by *rcrRPQ*.

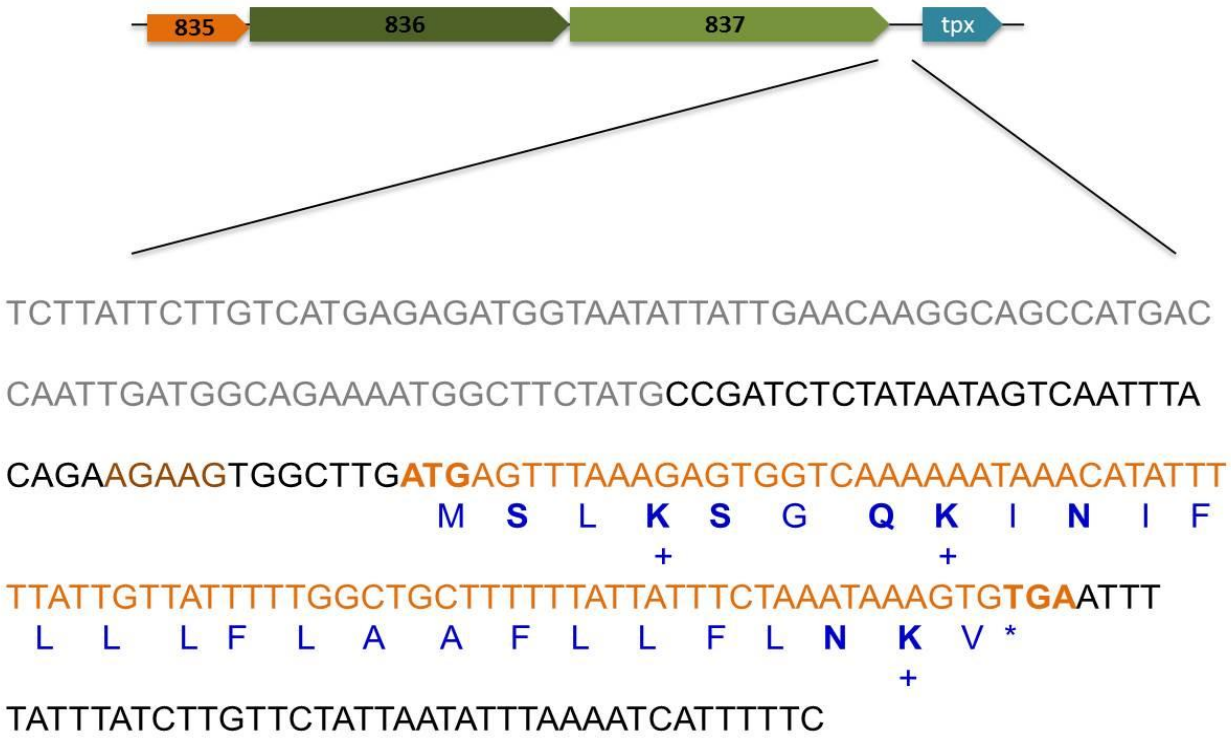

Figure 7-4. Schematic showing the presence of a putative peptide. Peptide 1 in the 3' region of *SMu0837 (rcrQ).*

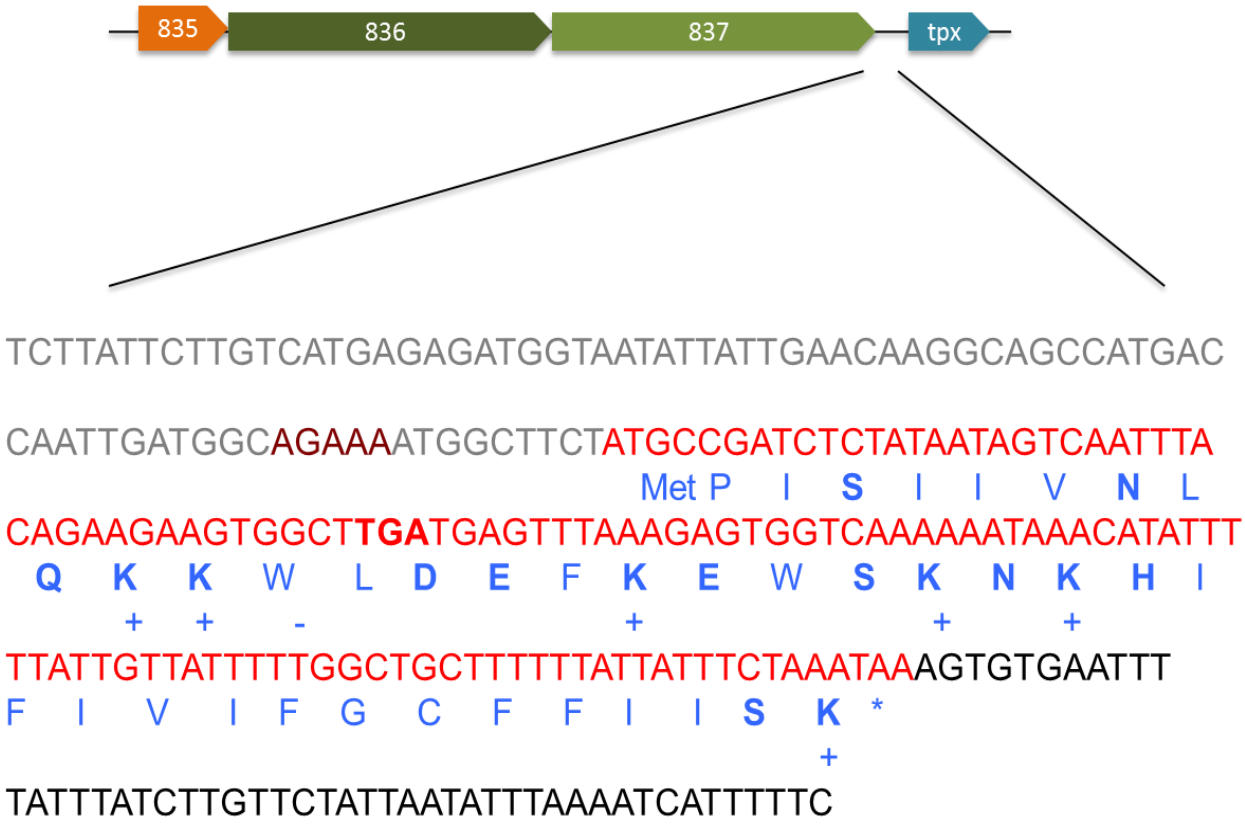

Figure 7-5. Schematic showing the presence of a putative peptide. Peptide 2 in the 3' region of *SMu0837 (rcrQ).*

Table 7-1 List of organisms with genes encoding RcrRPQ homologues linked to RelPRS homologues and their GeneBank Locus ID

Organism	MarR linked to ABC transporters	RelPRS homologues present	RelPRS linked to MarR
Streptococcus gallolyticus	YES GALLO_1282-1286	YES GALLO_1279-1281	YES
Streptococcus salivarius	YES STRASA001_623-625	YES STRASA001_619-621	YES
Streptococcus infantarius	YES STRINF_00326-00328	YES STRINF_00329-00332	YES
Streptococcus thermophilus	YES STU0432-434	NO	NO
Streptococcus pyogenes	YES spyM18_0213-0215	YES spyM18_0934-0936	NO
Streptococcus agalactiae	YES Gbs1400-1402	YES Gbs1397-1399	YES
Streptococcus equisimilis	YES Sez_0219-221	NO	NO
Streptococcus suis	YES SsuiDRAFT_2132-2134	NO	NO
Streptococcus uberis	YES SUB1688-1690	NO	NO
Streptococcus sanguinis	YES SSA_0460-0462	YES SSA_1793-1795	NO
Streptococcus gordonii	YES SGO_1750-1752	YES SGO_0484-0486	NO
Streptococcus pneumoniae	YES CGSSp11BS70_11141-1146	NO	NO
Streptococcus mitis	YES smi_1834-1836	NO	NO

Table 7-2. Summary of mutants with deletions in the *rcrQ* 3' region showing transformation efficiency compared to the wild-type strain

Strain	RcrR levels	RcrP levels	RcrQ levels	No. of peptides present in the *rcrQ* 3' region	Growth with CSP	Transformation efficiency compared to WT
Δ836-7 np V3	Not tested	Deleted	Deleted	0	Resistant	0
Δ837np V2	Not tested	Not tested	Deleted	2	Resistant	0
Δ837 p V2	Not tested	Not tested	Deleted	2	Resistant	0
Δ836-7np V2	Not tested	Deleted	Deleted	2	Resistant	10 -fold increase
Δ836-7p V2	Not tested	Deleted	Deleted	2	Hypersensitive	1000 - fold increase
Δ837np V3	Not tested	Not tested	Deleted	0	Resistant	100- fold increase
Δ835-7p npV2	Deleted	Deleted	Deleted	2	Resistant	1000 -fold increase
Δ835-7 npV3	Deleted	Deleted	Deleted	0	Resistant	1000- fold increase

APPENDIX
ZONE OF INHIBITION

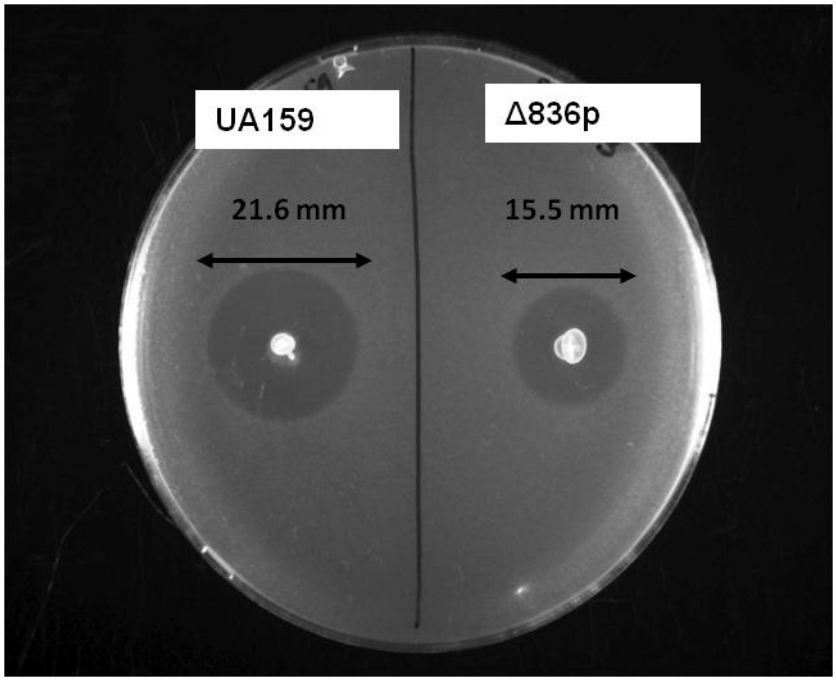

Figure showing the difference in size for the zone of inhibition for the wild-type and *Δ836p* mutant strains of *S. mutans* overlaid with *Streptococcus sanuginus* strain SK150. The diameter for the zone of inhibition of the wild-type strain was 21 mm ± 0.34 mm. The diameter for the zone of inhibition of the *Δ836p* strain was 15.5 mm ± 1.3 mm.

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BIOGRAPHICAL SKETCH

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