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Regulation of competence and gene expression in *Streptococcus mutans* by the RcrR transcriptional regulator

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SUMMARY

An intimate linkage between the regulation of biofilm formation, stress tolerance and genetic competence exists in the dental caries pathogen *Streptococcus mutans*. The *rcrRPQ* genes encode ABC exporters (RcrPQ) and a MarR-family transcriptional repressor of the *rcr* operon (RcrR) play a dominant role in regulation of the development of genetic competence and connect competence with stress tolerance and (p)ppGpp production in *S. mutans*. Here we identify the target for efficient RcrR binding in the *rcr* promoter region using purified recombinant RcrR (rRcrR) protein in electrophoretic mobility shift assays and show that DNA fragments carrying mutations in the binding region were not bound as efficiently by rRcrR *in vitro*. Mutations in the RcrR binding site impacted expression from the *rcr* promoter *in vivo* and elicited changes in transformation efficiency, competence gene expression, and growth inhibition by competence stimulating peptide; even when the changes in *rcrRPQ* transcription were minor. An additional mechanistic linkage of RcrR with competence and (p)ppGpp metabolism was identified by showing that the rRcrR protein could bind to the promoter regions of *comX*, *comYA* and *relP*, although the binding was not as efficient as to the *rcrRPQ* promoter under the conditions tested. Thus, tightly controlled autogenous regulation of the *rcrRPQ* operon by RcrR binding to specific target sites is essential for cellular homeostasis, and RcrR contributes to the integration of genetic competence, (p)ppGpp metabolism, and acid and oxidative stress tolerance in *S. mutans* through both direct and indirect mechanisms.

Keywords

dental caries; genetic competence; autolysis; peptide signaling; transcriptional regulator

INTRODUCTION

Streptococcus mutans, a primary etiological agent of dental caries (Loesche 1986), adapts efficiently to the frequent and substantial fluctuations in environmental conditions in the oral cavity; a trait that is considered critical to persistence and pathogenesis (Burne 1998). Key virulence attributes of *S. mutans* include its ability to form biofilms and to grow in environmental conditions that can inhibit the growth of competing organisms (Hamada and

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Slade 1980; Lemos and others 2005). *S. mutans* is particularly effective at producing acids from dietary carbohydrates, which directly causes caries by lowering the pH of oral biofilms and demineralizing the tooth (Banas 2004). *S. mutans* is also especially acid tolerant (aciduric), and can grow and continue to engage in glycolysis at pH values that are well below the point at which significant damage to tooth mineral occurs (Jensen and others 1982; Loesche 1986). Not surprisingly, acid tolerance is considered to be an essential virulence attribute of cariogenic organisms.

A variety of physiologically and genetically diverse bacteria, including *Streptococcus mutans*, encode the machinery to internalize DNA from the environment, a trait known as genetic competence. Genetic competence can enhance persistence and pathogenesis by virtue of its ability to augment adaptation and survival by increasing genetic diversity via lateral gene transfer, as well as because DNA can be utilized as a nutrient (Claverys and others 2006; Cvitkovitch 2001; Johnston and others 2014). In *S. mutans*, the pathways regulating genetic competence have a strong influence on biofilm formation and stress tolerance (Li and others 2002; Li and others 2008; Petersen and others 2005). Further, mutations in the genes of the competence signaling cascade resulted in attenuated virulence in a rodent model (Li and others 2002; Petersen and others 2005).

The development of competence in bacteria is controlled at many levels, but is initiated by small molecule signals and signal transduction pathways. One critical control point for commitment to competence is activation of the expression of the gene for the alternative sigma factor ComX (sometimes called SigX), which guides RNA polymerase to a group of late competence genes that encode the proteins for DNA uptake and recombination. In many streptococci, including *S. mutans*, the proximal regulator of *comX* expression is the Rgg-family transcriptional activator ComR. The *comS* gene encodes a 17-aa peptide that is processed and secreted as the 7-aa SigX-inducing peptide (XIP), a small hydrophobic peptide that is reinternalized by the Opp oligopeptide permease transporter and complexes with ComR to activate *comX* transcription (Mashburn-Warren and others 2010). XIP is a particularly effective activator of genetic competence in chemically defined media. Competence stimulating peptide (CSP) is the product of the *comC* gene and is also a potent activator of competence in complex media. CSP is secreted by a dedicated ABC transporter and processed to 21- and 18-aa derivatives that stimulate the activity of the ComDE two-component system. In *Streptococcus pneumoniae* and some related streptococci, ComE binds directly to the *comX* promoter to activate transcription (Johnston and others 2014; Lee and Morrison 1999; Luo and Morrison 2003; Martin and others 2013). The ComCDE system of *S. mutans* is primarily responsible for direct activation of bacteriocin gene expression and although CSP can activate *comX* transcription, this occurs indirectly through various proposed pathways (Hung and others 2011; Kreth and others 2007).

We recently characterized the *rcrRPQ* operon (rel competence related; Oralgen Gene ID SMu0835-0837, <http://www.oralgen.lanl.gov>; GeneBank Locus tag SMU.921-SMU.923), demonstrating that these gene products profoundly influence acid and oxidative stress tolerance, (p)ppGpp metabolism and competence (Seaton and others 2011). The *rcrRPQ* operon encodes a predicted DNA binding protein of 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, particular *rcrRPQ* genes elicited dramatic effects on the ability of the bacteria to be transformed with chromosomal or plasmid DNA, as well as 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 (*rcrR-NP*, formerly *835-np*) caused upregulation of the genes encoding the *rcrPQ* efflux pumps by over 100 fold. Of note, the *rcrR-NP* mutant strain was no longer able to take up DNA and the expression of *comYA*, a late competence gene that encodes part of the machinery for uptake of DNA, was greatly reduced compared to the wild-type strain. In contrast, a strain carrying a replacement of *rcrR* with a polar kanamycin resistance cassette (*rcrR-P*, formerly *835-p*) was constitutively hyper-transformable and the *comYA* genes were significantly upregulated. Importantly, expression of the *rcrPQ* porters in the *rcrR-P* strain was similar to wild-type levels, and therefore 100-fold lower than in *rcrR-NP* mutant, for reasons explained elsewhere (Seaton and others 2011). Based on the phenotypes displayed by the polar and non-polar *rcrR* strains, we concluded that the tight regulation of the production of the RcrPQ efflux pumps by RcrR, and probably RcrR itself, play critical roles in the regulation of competence development. However, the mechanisms by which the *rcrRPQ* gene products exert multiple effects on competence gene expression and how these gene products integrate competence with stress tolerance and (p)ppGpp metabolism have not yet been disclosed.

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 adaptation to environmental stresses (Ellison and Miller 2006). Most MarR regulators prevent gene expression by sterically interfering with RNA polymerase binding to the promoter to block transcription (Perera and others 2009). Collectively, the data support that RcrR has a profound effect on *rcrRPQ* operon expression, stress tolerance, DNA uptake and expression of competence genes. All of these RcrR-influenced phenotypes can have a direct impact on establishment, persistence and virulence, so 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.

METHODS

Bacterial strains and growth conditions

Escherichia coli strains were grown in Luria broth supplemented with chloramphenicol (20 $\mu\text{g ml}^{-1}$) or ampicillin (100 $\mu\text{g ml}^{-1}$), when necessary. *S. mutans* UA159 and its derivatives were maintained in brain heart infusion (BHI) medium (Difco) supplemented with kanamycin (1 mg ml^{-1}), spectinomycin (1 mg ml^{-1}) or erythromycin (2 $\mu\text{g ml}^{-1}$), when necessary. For growth studies, cultures were grown overnight in BHI at 37°C in a 5% CO_2 aerobic atmosphere, then diluted 1:50 into fresh BHI broth and grown to mid-exponential phase ($\text{OD}_{600} = 0.5$). The cultures were then diluted 1:100 into 350 μl of BHI broth with 2 μM synthetic CSP (Perry and others 2009a) in multi-well plates, overlaid with sterile mineral oil to reduce exposure to air, and placed in a Bioscreen C growth monitor at 37°C.

The optical density (OD₆₀₀) was measured every 30 min for 24 hours with shaking for 15 s before each reading.

DNA manipulation and construction of mutants and reporter gene fusions

A series of mutant strains (Table 1) were derived from *S. mutans* UA159 using splice overlap extension (SOE) to make point mutations in different regions (Ho and others 1989). Briefly, primers (Table S1) with the desired base changes and primers that matched sequences located 0.5 kbp upstream and downstream of the sequence of interest were used to amplify DNA and generate two PCR products. Selected pairs of PCR products with 20 bp of homology that included the desired mutations were subjected to PCR for 5 cycles in the absence of added primers. Then, a second PCR of 30 cycles using the outer primers (Table S1) was performed to generate a 1-kbp fragment that had the desired mutations. The final PCR products were run on agarose gels to confirm the correct size, then the fragment was excised, purified and transformed into competent *S. mutans* along with a suicide plasmid harboring an internal fragment of the *lacG* gene and an erythromycin (Em) resistance (Em^r) determinant (Zeng and others 2010). Transformants were selected on BHI agar with Em. Mismatch Amplification Mutation Assay (MAMA) PCR was used to screen for isolates that carried the desired mutations (Cha and others 1992). Isolates that yielded a PCR product of the correct size were restreaked and cured of the suicide plasmid. DNA sequencing was used to insure that the correct mutation(s) had been introduced and that no mutations were created in the genes immediately upstream or downstream of the insertion site during the transformation.

The promoter regions of the *rcrR* operon that contained the various mutations were amplified and cloned 5' behind a promoterless *lacZ* gene derived from *Streptococcus salivarius* on plasmid pMZ, which is designed to allow for double cross-over recombination and integration of the gene fusion in *S. mutans* as a single copy in the *mtlA-phnA* locus (Chen and others 2002; Liu and others 2009; Zeng and others 2006). The *lacZ*-promoter fusions were transformed into wild-type and mutant strains of *S. mutans* and the integrity of the gene fusions was verified by 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 OD₆₀₀ = 0.15 in a 5% CO₂ aerobic atmosphere. When desired, synthetic CSP (Ahn and others 2006) was added to a final concentration of 100 nM, cells were incubated for 10 min and 0.5 µg of purified plasmid pDL278, which harbors a spectinomycin (Sp) resistance 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 Sp. CFU were counted after 48 h of incubation and transformation efficiency was expressed as the percentage of transformants among the total viable cells.

RNA extraction and qRT-PCR

Three colonies from each strain were grown overnight in BHI broth, cultures were diluted 1:50 in fresh BHI and incubated until the OD₆₀₀ reached 0.5. Cells were harvested, then

total RNA was extracted, DNaseI treated and further purified with the RNeasy mini kit (QIAGEN) (1). RNA concentration was measured in triplicate using a spectrophotometer. The purified RNA (1 µg) was used to generate cDNA from gene specific primers according to the Superscript[®] III first-strand synthesis reverse transcription protocol (Invitrogen). The gene specific primers were designed with Beacon Designer 4.0 software and standard curves for each gene were prepared. Real-Time PCR reactions were carried out using an iCyclerQ Real-Time PCR detection system (Bio-Rad) and iQSYBR green supermix (Bio-Rad) according to the protocol provided by the supplier. Triplicates of each cDNA sample along with cDNA controls for each of the triplicate isolates analyzed were subjected to Real-time PCR. Controls for contaminating DNA (no reverse transcriptase), normalization (µg input RNA and 16S rRNA) and statistical analysis of the data were performed as detailed elsewhere (Ahn and others 2005).

EMSA and protein purification

Electrophoretic mobility shift assays (EMSA) were carried out as detailed elsewhere (Abranches and others 2008). An N-terminally tagged RcrR protein was obtained by amplifying the entire *rcrR* structural gene from *S. mutans* UA159 and cloning it in-frame in pQE30 (Qiagen). The protein was overproduced in *Escherichia coli* by induction with isopropyl-β-D-thiogalactopyranoside (IPTG) and purified as a soluble protein using a Ni²⁺ affinity column as recommended by the supplier (Qiagen). DNA fragments of the 5' region of *rcrR* were amplified via PCR using biotinylated primers (Table S1). The PCR product was gel purified (Qiagen) and quantified using a spectrophotometer. Five femtomoles (fmol) of biotinylated probe was used with different concentrations of purified recombinant 6His-tagged *S. mutans* RcrR protein (rRcrR) in a 10 µl reaction mixture containing 10 mM HEPES (pH 7.9), 50 mM KCl, 5 mM MgCl₂, 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% non-denaturing, low ionic strength, polyacrylamide gel [(30:1) acrylamide:bis-acrylamide]. The samples were transferred to Genescreen plus hybridization membranes (PerkinElmer, Waltham, MA) using a semi-dry transfer apparatus. Signals were detected and quantified using a chemiluminescent nucleic acid detection module (Thermo Scientific) and an AlphaEase FC (Fluorochem 8900) imaging system.

β-galactosidase assays

β-galactosidase activity was measured according to the protocol of Miller (Zubay and others 1972). Strains carrying promoter-*lacZ* gene fusions were grown in 5 ml of BHI broth in a 5% CO₂ aerobic atmosphere at 37°C to OD₆₀₀ = 0.5. Cells from 1.5 ml of culture were collected by centrifugation and the pellets were resuspended in 1.5 ml of Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, freshly-added 50 mM β-mercaptoethanol). Cells from 500 µl of the culture were permeabilized by high speed vortexing for 1 min with 25 µl of a toluene:acetone solution (1:9 v/v) and the OD₆₀₀ of the cell suspension was measured. The permeabilized cells were transferred to a 37°C water bath, 100 µl of prewarmed ONPG (4 mg ml⁻¹ in 0.1 M Na-phosphate buffer, pH 7.5) was added, and the time was recorded. After the color began to change to yellow, the reaction was halted by the addition of 500 µl of a 1 M Na₂CO₃ solution and the time was again

recorded. The cells were briefly centrifuged and OD₅₅₀ and OD₄₂₀ were measured. LacZ specific activity was calculated in Miller units (Zubay and others 1972).

RESULTS

Identification and characterization of RcrR binding sites

We previously demonstrated that inactivation of *rcrR* with a non-polar marker lead to a greater than 100-fold increase in *rcrPQ* mRNA levels, providing evidence that RcrR is an autogenous repressor of *rcrPQ* operon expression. Using electrophoretic mobility shift assays (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 *rcrPQ* (Fig. 1A). Thus, RcrR can influence expression of the *rcrPQ* operon by direct interaction with the promoter region. RegPrecise (http://regprecise.lbl.gov/RegPrecise/browse_regulogs.jsp) was used to identify a potential psuedopalindromic target for RcrR (TAGTTTTTCATGAGAACTA, Fig. 1B), designated here as RS1, located 34 bp upstream of the ATG start codon of *rcrR*. Notably, MarR binding sites usually consist of palindromic or inverted repeat (IR) motifs (Wilkinson and Grove 2006). The sequences immediately upstream of RS1 contain a -10 element (TATAAT) and -35 element (TTGACA) that are appropriately spaced and exactly match the consensus sequence for σ^{70} -type promoters. The most 3' T of the -10 sequence is the same as the 5' T of RS1. In addition, a second predicted binding site (TAGTTTAAGGAATCA), designated RS2, was identified in the spacer region between the -35 and -10 elements, with no overlaps with the -35 or -10 sequences (Fig. 1B).

Using a 101-bp biotinylated DNA fragment that contained only RS1, but no upstream sequences, no shift was observed in EMSAs in the presence of rRcrR (Fig. 2A). When RS1 and the potentially weaker binding site RS2 were both included in the target DNA, >80% of the DNA shifted in the presence of 10 pmoles of rRcrR protein (Fig. 2A). It was previously reported that a MarR protein bound more efficiently to its target when additional sequences were provided adjacent to the binding site (Wilkinson and Grove 2006). Consistent with this report, when a 13-bp random sequence of DNA was added to the 5' end of the RS1 probe, rRcrR was able to bind efficiently to the RS1-containing fragment (data not shown). Likewise, there was a shift in DNA migration when an annealed 38-bp biotinylated oligonucleotide that only included RS1 flanked by extra bases was combined with purified rRcrR protein (Fig. 2B). A 55-bp sythetic biotinylated double-stranded oligonucleotide containing both predicted sites was also shifted very efficiently by rRcrR (Fig. 2B). Morespecifically, rRcrR caused a mobility shift of both the 38- and 55-bp fragments in a protein concentration-dependent manner, and the 55-bp fragment appeared to be bound more efficiently than the 38-bp fragment; >80% of the former shifted with 5 pmoles of RcrR protein compared to about 45% of the latter (Fig. 2B, Table S2). Unlabeled 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 (Fig. S1, Table S3). Of note, we have utilized fluorescence polarization to estimate the binding affinity of the RcrR protein for its target(s). The calculated K_d for the 55-bp fragment was estimated to be 0.1 μ M and the K_d for the 38-

bp fragment was 0.06 μ M (K. Seaton, PhD Dissertation, University of Florida). While the differences in dissociation constants were not statistically significant, the affinity of rRcrR for its target appears high. Collectively, these results indicate that rRcrR may repress *rcrRPQ* operon expression by interacting with two binding sites; one that overlaps by a single nt with the -10 element and one located between the -10 and -35 elements of the promoter.

Mutations in the predicted binding sites affect binding of the RcrR protein

To explore whether the predicted binding sites were targets for RcrR *in vitro* and *in vivo*, various mutations were introduced into RS1 and RS2 by substituting three or eight nucleotides in the 3' end of each binding sequence using splice overlap extension PCR and establishing the markerless mutations in the chromosome of *S. mutans*, as detailed in the methods section. A 0.35-kbp amplicon was used for the mutagenesis and was generated using primers described in Table S1. 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 the mutated sequence *in vitro*. When the PCR product derived from the NBS1 strain, which had mutations in RS1 (thus NBS1), was used in mobility shift assays, a decrease in the amount of shifting was observed (Fig. 3A, Table S4). When DNA from the promoter region of the BBS (both binding sites) 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 87% shift of the product derived from the wild-type organism (Fig. 3B). However, when the probe was derived from the NBS2 strain, which had mutations only in the predicted secondary binding site (RS2, binding site 2 – NBS2), there was no difference in the proportion of DNA shifted compared to the wild-type DNA (Fig. S2). These results provide support for the idea that RS1 functions as a primary RcrR binding site and show that the weaker predicted binding site RS2 is not necessary for RcrR binding to the promoter region *in vitro*. However, it cannot be excluded that RS2 may enhance RcrR-dependent repression, perhaps by fostering cooperative binding or enhancing oligomerization of the repressor.

To determine whether the inefficient binding of rRcrR to the NBS1 and BBS strains impacted promoter activity of the *rcrRPQ* operon *in vivo*, *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 region derived from the strain with mutations in both RS1 and RS2 (159 ± 15 Miller units), compared to the wild-type (P_{WT}) promoter (69 ± 7.3 Miller units) (Fig. 4). In contrast, only a slightly elevated level of expression from the P_{NBS1} promoter (88 ± 7.4 Miller units) was noted. Therefore the activity of the *rcrRPQ* promoter is differentially impacted by the various binding site mutants and RS2 may be sufficient *in vivo* to allow for RcrR to interfere with efficient RNA polymerase binding. It must also be considered that the proximity of promoter elements to certain mutations introduced into the binding sites could affect promoter activity measured from the gene fusions.

Mutations in the RcrR binding sites affect transformation and growth in the presence of synthetic competence stimulating peptide (sCSP)

To evaluate whether less efficient binding of rRcrR influenced competence of *S. mutans*, 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, displayed a 23-fold increase in transformation efficiency in the absence of added sCSP, compared to the wild-type strain (Table 1). However, there was no difference between the *NBS1* mutant and the parental strain in transformation efficiency when 100 nM sCSP was added to the cultures (Table 1). Importantly, strain *BBS*, which had mutations in both RS1 and RS2 and showed a substantial increase in promoter activity and yielded no transformants in the absence of exogenous sCSP (Table 1), but did produce a similar number of transformants as the wild-type strain when sCSP was included.

A recent finding showed that the hyper-transformable *rcrR-P* strain exhibited more severe growth inhibition in the presence of sCSP than did the wild-type strain, whereas the *rcrR-NP* mutant was almost completely resistant to growth inhibition by sCSP (Ahn and others, *In press*). Interestingly, the *NBS1* strain was more sensitive to CSP, with a minimum doubling time of 60 ± 6 min, compared to the wild-type strain with a minimum doubling time of 54 ± 6 min (Fig. 5). However, the wild-type strain attained a final OD₆₀₀ of 0.55 ± 0.012 , whereas the *NBS1* strain reached a final OD₆₀₀ of only 0.45 ± 0.07 . In contrast, the *BBS* strain was more resistant to CSP-induced growth inhibition (Fig. 5) when compared to the parental strain, consistent with the decrease in transformation efficiency, reaching a final OD₆₀₀ = 0.7 ± 0.01 in CSP with a minimum doubling time of 36 ± 1 min. Thus, the inability of RcrR to bind efficiently to its target sequences to control *rcrRPQ* operon expression levels can alter CSP-induced growth inhibition and transformability.

Mutations in the RcrR binding sites can affect *comY* expression

To determine if the phenotypic consequences of binding site mutations could be correlated with *rcr* operon expression, we measured the expression of the *rcrRPQ* operon in the *NBS1* and *BBS* strains via qRT-PCR, but detected no statistically significant changes (Fig. S3), even though we could consistently measure higher promoter activity in these strains than in the wild-type strain (Fig. 4). A possible explanation for this could be that qRT-PCR was not sufficiently sensitive to measure biologically significant changes in the expression of the operon, so that even small fluctuations in the expression levels of *rcrRPQ* may account for observable differences in transformation efficiency. Therefore, we measured expression levels of *comYA*, which are strongly correlated with the development of competence and with the transformation phenotypes of the *rcrR-P* and *rcrR-NP* mutant strains. As expected, the *NBS1* strain, which was hyper-transformable, had about a 30-fold increase in the expression of *comYA*, compared to the wild-type strain (Fig. 6). The magnitude of the change in expression of *comYA* was similar to that observed in the *rcrR-P* strain (Seaton and others 2011). The levels of *comYA* were slightly lower in the *BBS* strain, albeit not statistically different from the wild-type strain (Fig. 6); following a trend similar to that observed for the *rcrR-NP* strain (Seaton and others 2011). Therefore, these results show that the levels of *comYA* correlate well with the transformation phenotypes in these strains, and that the binding site mutations and changes in *rcrRPQ* promoter activity result in

aberrant expression of competence genes, even with relatively small changes in *rcrRPQ* expression.

rRcrR can interact with the promoter regions of *comX*, *comY*, and *relP*

To identify other potential targets for RcrR binding, we scanned the *S. mutans* UA159 genome sequence for RS1- and RS2-like sites and found that the promoter regions of *comX*, *comY*, and *relP* had sequences with weak similarity to the RS1 binding site, based on wMATCHER software analysis. We conducted mobility shift assays to determine if the effects on the expression of the *comX*, *comY*, and *relP* genes could be exerted by the binding of RcrR to the promoter regions of these genes. As shown in Figures 7A and 7B, purified rRcrR protein was able to bind to the promoters of *comX* and *relP*, albeit with apparently lower affinity than for the promoter region of the *rcrRPQ* operon. We also were able to demonstrate binding to the *comYA* promoter region at similar concentrations of rRcrR and probe as for *comX* and *relP* (data not shown). Specifically, there was approximately a 10% shift of the *comX* and *relP* promoter region when 20 pmoles of rRcrR was utilized, compared to a 100% shift with 10 pmol of rRcrR when the *rcrRPQ* promoter was the target. As a negative control, we utilized the promoter region of the *fruA* gene, 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 rRcrR protein. Collectively, these results reveal a potential direct regulatory function for RcrR on competence gene expression, as well as linking RcrR to the control of RelP-dependent (p)ppGpp production. Importantly, the observations are consistent with the competence phenotypes and with the finding that *rcrR* mutations affected *relP* gene expression and promoter activity (Seaton and others 2011).

DISCUSSION

The development of competence by *S. mutans* is a tightly regulated process that is influenced by multiple regulatory elements and inputs, many of which have yet to be characterized. Competence is often considered a stress response and its induction in bacteria has been linked to translational errors, (p)ppGpp production, antibiotic stress, bacteriocin production and oxidative stress (Engelmoer and Rozen 2011; Perry and others 2009b; Seaton and others 2011). MarR-like regulators, such as RcrR, and ABC exporters, like RcrPQ, are involved in regulating many essential functions, including resistance to xenobiotics and oxidative stressors, and influencing the expression of virulence genes in many bacteria. In *S. mutans*, a primary function for RcrRPQ appears to be to monitor environmental inputs and/or the physiologic state of the cell and to integrate this information into the decision to respond to competence signaling or to activate late competence genes. In fact, mutations in *rcrRPQ* compromise acid and oxidative stress resistance and impact RelP-dependent (p)ppGpp production (Seaton and others, 2011). Similarly, the *rcrRPQ* operon is induced in *S. mutans* when the stringent response is triggered by the isoleucyl-tRNA synthetase inhibitor mupirocin (Lemos and others 2007). Given the dominant role of RcrRPQ in competence regulation and its connection to key stress response pathways (Seaton and others 2011), characterization of the regulation and function of these gene

products should provide new insights into the mechanisms connecting competence with stress tolerance and growth regulation.

The foundation for this study was established by the observation that polar (*835p* or *rcrR*-P) and non-polar (*835np* or *rcrR*-NP) mutations in the *rcrR* gene have profound, yet opposite, effects on transformation efficiency; the polar mutant being hyper-transformable and the non-polar mutant being non-transformable. While these phenotypes are strongly associated with *rcrPQ* expression levels, a direct role for RcrR has not been explored. By utilizing *in vitro* and *in vivo* methodologies to identify RcrR binding sites and potential targets of RcrR within the competence regulon, this study begins to shed light on additional mechanisms by which *rcr* mutants may influence transformation efficiency. The simplest model for regulation of the *rcrRPQ* operon by RcrR is that this MarR-like regulatory protein binds with apparent high affinity to sites in the *rcrR* promoter region to repress the operon, probably by preventing RNA polymerase from engaging the promoter given the close proximity of the binding sites to the -35 and -10 elements. The RegPrecise-predicted binding site (TAGTTTTTCATGAGAACTA, RS1) is the preferred binding site, since oligonucleotides containing that region were sufficient to support rRcrR binding. Also, the sequence of RS1 is a nearly-perfect 18-nt inverted repeat, typical of MarR binding sites, whereas the apparently weaker binding site RS2 is not predicted to form a stable stem or stem:loop structure. Thus, from the results presented here and information about other MarR-like regulators, one would predict that *rcrRPQ* expression is repressed by RcrR binding strongly to RS1 and with lower affinity to RS2. Assuming that the binding activity of RcrR is allosterically regulated like other MarR-family repressors, one would also predict that binding of RcrR to a small molecule or class of small molecules, perhaps peptides, would elicit derepression of the operon. As is the case for many autogenously regulated circuits, derepression of the operon would lead to increased RcrR production and a negative feedback loop. We are currently investigating potential signals that may modulate RcrR DNA binding activity. However, data presented here supports that transient induction of the *rcrRPQ* operon in response to certain environmental inputs or conditions could serve as a potent signal to turn off competence or to block the development of competence in *S. mutans*.

While the pathway for autogenous regulation of *rcrRPQ* by RcrR seems straightforward, the way in which *rcrR* polar and non-polar mutations affect competence is far more complex. One interesting finding revealed here was that the expression of *rcrRPQ* must be very tightly controlled, since relatively small changes in *rcrRPQ* promoter activity, as seen with the binding site mutants (*NBS1* vs. *BBS*), can lead to dramatic changes in transformation efficiency and sensitivity to CSP. Based on the behavior and gene expression profiles of *rcrPQ* genes in the polar and non-polar *rcrR* mutants (Seaton and others, 2011), this influence on competence is likely to be correlated with changes in the expression levels of the *rcrPQ* genes encoding ABC exporters. In fact, the strain (*BBS*), which showed high levels of *rcrRPQ* promoter activity, displayed dramatically decreased competence and sensitivity to CSP. As would be expected, the sensitivity to CSP and competence in these strains correlated well with the levels of *comYA*. In particular, the CSP-sensitive *NBS1* strain was hyper-transformable and had high levels of *comYA* expression. In contrast, the CSP-

resistant *BBS* strain had lower levels of *comYA* and was poorly transformable. We cannot readily explain why the mutation of the RcrR binding sites did not lead uniformly to a decrease in transformation efficiency, as one might predict based on the behavior of the non-transformable, highly CSP-resistant phenotypes of the non-polar *rcrR* mutant. Further studies are underway to determine whether other factors influence *rcrRPQ* expression, and in turn competence and CSP sensitivity.

It may seem surprising that *comX* levels were not altered in the *BBS* strain, since *comX* expression is strongly associated with sensitivity to CSP (Fig. S4). However, it is also apparent from the analysis of the polar and non-polar *rcrR* mutants that a dominant control point for *rcrRPQ* mutations on competence occurs at the level of ComX production (Ahn and others, *In press*). Considering that the data presented here demonstrates that RcrR has the potential to interact with the promoter regions for *comX* and *comYA*, it must therefore be considered that the behaviors of the strains and the gene expression patterns could be influenced by overproduction of RcrR in the various binding site mutants. Elevated levels of RcrR could lead to changes in expression in *comX* or *comYA* that influence competence and CSP sensitivity. It should also be reinforced that the changes in *rcrPQ* levels, which were likely induced as a result of binding site mutations, were not nearly as dramatic as those seen in the polar and non-polar *rcrR* mutants, which completely lack a functional RcrR protein and, in the case of the non-polar mutant, produce >100-fold more *rcrPQ* mRNA than the strains analyzed in this study. Therefore, while RcrR likely exerts its primary influence on competence through alterations in *rcrPQ* transcript levels, it must be considered that some of the behaviors observed here are associated with direct interactions of RcrR with genes in the *com* regulon. While the *in vitro* interactions of RcrR with *comX* and *comYA* promoter regions appear far less efficient than with the *rcrRPQ* binding sites, overproduction of RcrR or changes in the binding affinities of RcrR as a result of its interaction with certain allosteric effectors could occur *in vivo* in a way that significantly impacts *com* gene expression.

It is also of interest that RcrR was able to interact with the *relP* promoter *in vitro*, which may in part explain how certain *rcr* mutants influence *relP* expression and modulate RelP-dependent (p)ppGpp accumulation (Seaton and others 2011). The *relP* gene is part of an operon encoding the RelRS two-component signal transduction system (TCS) and this operon resides about 1.5 kbp downstream of *rcrRPQ*. The RelP enzyme produces the bulk of (p)ppGpp that is present during exponential growth (Lemos and others 2007). As previously reported, the *rcrRPQ* genes are upregulated in response to mupirocin treatment in a (p)ppGpp-dependent manner (Nascimento and others 2008). Thus, the *rcrRPQ* operon could provide the missing molecular connection between competence development and stress tolerance by engaging in cross-regulation with the *relPRS* system. For example, RelP-dependent (p)ppGpp accumulation could enhance *rcrRPQ* expression, resulting in increased stress tolerance and decreased competence. Subsequently, the accumulation of RcrR associated with derepression of the *rcrRPQ* operon could lead to down-regulation of *relPRS* expression and diminished (p)ppGpp production. Such interactions could allow the cells to delay the decision to commit to competence, and thus avoid expending considerable energy generating the machinery for DNA uptake and internalizing DNA, when (p)ppGpp levels are

elevated due to stress or some other signal for activation of the RelRS pathway. By establishing the binding capacities and targets for RcrR, this study has helped to begin to unravel some of the complexities of how RcrRPQ coordinately influence cellular physiology and competence. Studies are ongoing to dissect the signals modulating RcrR binding activity, to identify the substrates for the RcrPQ exporters and to explore whether there are other inputs regulating *rcrRPQ* operon expression to provide a more comprehensive picture of the role of RcrRPQ in physiologic homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Identification of RcrR binding sites

(A) RcrR binds the *rcrRPQ* promoter region in gel mobility shift assays. Purified rRcrR 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. (B) Schematic map of the promoter region of the *rcrRPQ* operon in *S. mutans* UA159. The ATG start codon of the *rcrR* gene is highlighted and in italics. An 18-bp binding site located 34-bp upstream of the start codon (RS1, gray) was identified via RegPrecise (http://regprecise.lbl.gov/RegPrecise/browse_regulogs.jsp) software prediction tool. The IR motif, typical of MarR-type protein, is marked by arrows. The secondary predicted site RS2 (15-bp, also in gray) is located 7-bp upstream of RS1. The -10 region (TATAA) is located between RS1 and RS2, and the -35 region (TTGACA) is located immediately upstream of RS2 (highlighted and underlined). Note that a negative control showing that RcrR does not bind to an irrelevant promoter region, i.e. a promoter for a gene that is not affected by mutations in *rcrR*, can be found in Figure 7 and competition experiments with unlabeled probes are shown in the supplementary material.

Fig 2. Gel mobility shift assays of rRcrR binding with different regions of P_{rCrR} DNA
 (A) Purified (10 pmoles) rRcrR protein was added to 5 fmoles of biotinylated P_{rCrR} PCR products (RS1) and (RS1+RS2) in a binding reaction for 40 min. (B) Purified rRcrR protein of various amounts (0, 0.15, 0.25, 0.75, 1, 1.5, 2.5, 5 pmol) were 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 percent of DNA shifted was calculated based on IDV (Integrated Density Value).

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Fig. 3. Gel mobility shift assays of rRcrR binding with mutated P_{rcrR} DNA

(A) Lanes 1-3 contain 5 fmoles of biotinylated P_{rcrR} WT DNA with 0, 2.5, 5 pmoles of RcrR protein respectively. Lanes 4-6 contain 5 fmoles of biotinylated P_{rcrR} NBS1 DNA with 0, 2.5, 5 pmoles of RcrR protein respectively. (B) Lanes 1-4 contain 5 fmoles of biotinylated P_{rcrR} WT DNA with 0, 1.5, 2.5, 5 pmoles of RcrR protein respectively. Lanes 5-8 contain 5 fmoles of biotinylated P_{rcrR} BBS1 DNA with 0, 1.5, 2.5, 5 pmoles of RcrR protein respectively. The reactions were run on a nondenaturing polyacrylamide gel and the signal observed via chemiluminescence. The percent of DNA shifted was calculated based on IDV.

Fig. 4. Effect of the RcrR binding mutations on promoter activity of the *rcrRPQ* operon

The promoter regions of *rcrRPQ* with the various mutations were fused to a promoterless *lacZ* gene and integrated into the wild-type chromosome. In all cases cells were grown to mid-exponential phase ($OD_{600} = 0.5$) and β -galactosidase activity was measured as described in the methods section. The results are representative of three independent experiments performed in at least triplicate. *, Differs from the PWT activity at $P < 0.05$ (Student's *t*-Test). **, Differs from the PWT activity at $P < 0.005$. **, Differs from the PNBS1 activity at $P < 0.005$ (Student's *t*-Test).

Fig 5. Effect of the RcrR binding mutations on CSP-induced growth inhibition

The binding mutant strains were grown in triplicate to mid-exponential phase in BHI broth, then diluted 1:100 and transferred to fresh BHI broth that was supplemented with 2 μ M CSP, covered with sterile mineral oil and placed in a Bioscreen C at 37°C to monitor growth. WT, diamonds; *NBS1*, squares; *BBS*, triangles, *rcrR*-NPXs; *rcrR*-P, stars. The results are representative of three independent experiments

Fig. 6. Effect of the RcrR binding mutations on *comYA* mRNA level

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 copy number of *comYA* mRNA per µg of input RNA was quantified then log transformed. *, Differs from the wild-type at $P < 0.01$ (Student *t*-Test).

Fig. 7. Gel mobility shift assays of rRcrR binding to P_{relP} and P_{comX}

Purified RcrR protein of various amounts was added to 5 fmol of biotinylated P_{relP} (A) or P_{comX} (B) DNA in a binding reaction for 40 min. As a negative and positive control, 0 and 100 pmoles of RcrR protein was added to 10 fmoles of P_{fruA} and P_{rcrR} , respectively. The reactions were run on a non-denaturing polyacrylamide gel and the signal observed via chemiluminescence.

TABLE 1

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

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

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