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Expression of BrpA in *Streptococcus mutans* Is Regulated By FNR-box Mediated Repression

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Abstract

Our previous studies showed that *brpA* in *S. mutans*, which encodes a member of the LytR-CpsA-Psr family of proteins, can be cotranscribed with *brpB* upstream as a bicistronic operon, while the intergenic region also has strong promoter activity. To elucidate how *brpA* expression is regulated, the promoter regions were analyzed using PCR-based deletions and site-directed mutagenesis and a promoterless luciferase gene as a reporter. Allelic exchange mutagenesis was also used to examine genes encoding putative *trans*-acting factors, and the impact of such mutations on *brpA* expression was analyzed by reporter assays. Multiple elements in the short *brpA* promoter (nucleotide –1 to –344 relative to start codon ATG) were shown to have a major impact on *brpA* expression, including an FNR-box, for putative binding site of an FNR-type of transcriptional regulator. When compared to the intact *brpA* promoter, mutations of the highly conserved nucleotides in FNR-box from TTGATgtttAcCtt to **gcacag**tttAcCtt resulted in 769-fold increases of luciferase activity ($P<0.001$), indicative of the FNR-box mediated repression as a major mechanism in regulation of *brpA* expression. When luciferase reporter was fused to the upstream *brpBA* promoter (nt –784 to –1144), luciferase activity was decreased by 4.5-fold ($P<0.001$) in the *brpA* mutant, TW14D, and by 67.7-fold ($P<0.001$) in the *brpB* mutant, JB409, as compared to the wild-type, UA159. However, no such effects were observed when the reporter gene was fused to

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Conflicts of interest

The authors declare no conflict of interest.

the short *brpA* promoter and its derivatives. These results also suggest that *brpA* expression in *S. mutans* is auto-regulated through the upstream *brpBA* promoter.

Introduction

Streptococcus mutans, a key etiological agent of human dental caries, lives primarily in the plaque biofilms on the tooth surface, where more than 700 different phylogenetic groups exist^{1,2}. This bacterium possesses multiple mechanisms to colonize the tooth surfaces, including high affinity adhesin P1 (also SpaP) that mediates binding to the tooth via interactions with the salivary pellicle and amyloid formation as well^{3,4}, and three glucosyltransferases that convert sucrose into high molecular weight, α 1, 3-linkage rich, adhesive water-insoluble glucans⁵. In addition, this bacterium has also recently been shown to actively release extracellular deoxyribonucleic acids and membrane vesicles, which form major constituents of the extracellular matrix contributing to both biofilm formation and structure integrity, although the mechanisms that underlie the respective process remain unclear (Liao et al., 2014). When grown in the mixed species model, *S. mutans* can enhance bacterial adherence and accumulation on various surfaces tested by several other prominent groups, including cariogenic lactobacilli⁶. In addition, *S. mutans* is also well known for its ability to survive and effectively respond to various environmental insults, such as low pH and reactive oxygen species (ROS), antimicrobial agents such as hydrogen peroxide of the bacterial origin and from oral care products^{7,8}.

The cell envelope plays an essential role in maintenance of bacterial cell shape, cell growth and cell division, and is fundamental in protection against various environmental insults, antimicrobials and host immune defense. In addition, the cell envelope is directly involved in bacterial cell-environment, cell-surface and cell-cell interactions, and thus, influencing bacterial adherence and biofilm formation. The cell envelope of Gram-positive bacteria is featured with the presence of a thick layer of peptidoglycan (PG) and PG attached anionic cell wall polymers, including teichoic acids (TA) and acidic capsular polysaccharides^{9,10}. *S. mutans* is not known to have copious capsules, but does produce rhamnose-glucose polysaccharides (RGP) and cell wall TA, mostly poly(glycerol phosphate)^{11–15}.

BrpA is a polypeptide of 406 amino acid residues. It is predicted to contain a short cytoplasmic domain, a short transmembrane domain, and an extracellular C-terminal region with homology to the LytR-CpsA-Psr (LCP) family of proteins^{16,17}. *S. mutans* also contains a Psr homologue of the LCP family proteins^{17,18}. Unlike Psr and other members of the LCP family proteins in other bacteria, BrpA also possesses a seemingly unique region at the C-terminus of c.a. 100 residues rich in serine and threonine^{16,18,19}. While some redundancy in functionality exists between these paralogues, BrpA is shown to be a predominant player in *S. mutans* pathophysiology¹⁸. Deficiency of BrpA drastically weakens the ability of the deficient mutants to survive low pH, oxidative challenge, and cell envelope stress¹⁸. *In vitro*, the BrpA-deficient mutant can still bind and establish on a surface, but its ability to accumulate and develop into mature biofilms decreases drastically^{16,19,20}. Besides, as revealed by DNA microarray analysis, BrpA-deficiency also causes alteration of expression

of large groups of genes, including those involved in cell envelope homeostasis, stress tolerance response and biofilms formation^{18–20}.

Our recent studies have shown that *brpA* can be co-transcribed with its upstream locus, *brpB*, as a two-gene operon^{19,20}, while the intergenic region of 344 basepairs also possesses promoter activity^{19,20}. Under regular growth conditions, BrpA expression is extremely low even when it is at its maximum during early-exponential phase, but is highly regulated in response to various environmental cues^{19,21}. In agreement with a role of BrpA in environmental and cell envelope stress responses, the level of luciferase reporter activity under the direction of *brpA* promoter also significantly increases in cells following treatment with hydrogen peroxide, methyl viologen, SDS, chlorhexidine, and various cell envelope antimicrobials at their respective sub-inhibitory concentrations¹⁹.

In this study, we used polymerase chain reaction (PCR)-based deletions and reporter gene fusions and functional genomics approach to further dissect the *cis*- and *trans*-acting elements that are involved in regulation of *brpA* expression. Our results have shown that *brpA* expression is drastically increased upon deletion of a region with similarity to the binding site for a FNR (fumarate-nitrate reductase regulator)-like regulator. Reporter fusion analyses also showed that *brpA* expression is autoregulated, and such autoregulation is exerted through the upstream *brpBA* promoter.

Materials and Methods

Bacterial strains and growth conditions

All bacterial strains used in this study are listed in Table 1. Unless otherwise specified, *S. mutans* strains were grown in Brain Heart Infusion (BHI, Becton, Dickinson and Company, MD) at 37°C in an aerobic chamber with inclusion of 5% CO₂²². All solid media were prepared similarly with inclusion of Bacto agar (Difco Laboratories) at the level of 1.5% (w/v). When needed, erythromycin (Erm, Sigma) (10 µg/mL), spectinomycin (Spc, Sigma) (1 mg/mL), and/or kanamycin (Kan, Sigma) (1 mg/mL) were added to the growth medium.

DNA manipulations and strategy for dissecting *cis*-acting factors

Unless otherwise stated, standard recombinant DNA techniques were performed²³. All restriction and modifying enzymes and reagents were purchased from New England Biolabs (Beverly, MA) and/or InVitrogen (Life Technologies, Grand Island, NY), and used as recommended by the suppliers. To analyze the regulation of *brpA* expression, a promoterless luciferase gene (*luc*) was used as a reporter^{24,25}. The cognate *brpA* promoter and its derivatives with targeted deletions and/or mutations were generated by regular PCR and recombinant PCR (17). All primers (Table 2) were designed in such a way as to introduce an *NheI* site at the 5' end and a *XhoI* site at the 3' end of the PCR products for subsequent cloning purpose. Following proper restriction digestions, the resulting PCR products were cloned directly in front of the promoterless *luc* gene in integration vector pFW11-*luc*²⁶, which also contains a Shine-Dalgarno sequence optimized for group A streptococci²⁵. Following confirmation of the correct sequence of the cloned elements by Sanger's sequencing, the resulting promoter-*luc* fusion constructs were introduced into *S. mutans*

UA159 and TW14D and maintained on BHI agar containing Spc. The expression of *brpA* in response to the targeted deletions and /or mutations were analyzed using luciferase assays ^{19,25,26}, with the intact *brpA* promoter-reporter fusion serving as a control.

Functional genomics approach to identification of *trans*-acting factors

BLAST was used to search the genome database of *S. mutans* with the amino acid sequence of known regulatory proteins as a query (www.theseed.org). Once genes encoding putative regulatory proteins were identified, deficient mutants of the target genes were constructed with a non-polar kanamycin resistance marker replacing the coding region by following the PCR-Ligation-mutation strategy (Table 2) ^{21,27}. Following verification of sequence accuracy by Sanger's sequencing, the resulting mutants were transformed with the promoter-reporter fusions of interest, and the impact of the respective deficiency on *brpA* expression was analyzed by luciferase assays with the wild-type as a control.

Luciferase assay

Luciferase assay was carried out by following the protocol of Podbielski ^{19,25,26}. Briefly, for luciferase assay, bacterial cells were collected at early- (optical density, OD_{600nm}≈0.3), middle- (OD_{600nm}≈0.5 and late- (OD_{600nm}≈0.7) exponential phase during growth and then assayed for luciferase activity by adding 200 µL whole cells into a well on a 96-well plate, followed by the addition of 25 µL 1 mM D-luciferin, pH 6.0. The relative light units (RLU) were normalized to the OD_{600nm} at each time point.

mRNA stability assay

Briefly, *S. mutans* strains carrying the *brpA* promoter-luciferase reporter fusions with deletions of the untranslated 5' region were grown in BHI-Spc until early exponential phase (OD_{600nm}≈0.3) when rifampicin (Sigma) was added at the level of 300µg/ml ²⁸. Total RNA was extracted from cells immediately prior to and 5-, 10-, 20-, and 30-minutes post-rifampicin treatment using hot phenol ²⁰. It was then treated with DNase I (Ambion) and retrieved using RNeasy purification kit (Qiagen, Inc.) ²⁰. Reverse-transcription and Real Time-PCR were then used to evaluate the impact of the deletions on the stability of the luciferase reporter transcripts as described previously ²⁰.

Statistic analysis

All experiments were repeated at least three independent times. The results were analyzed using student *t*-test, and a difference of *P*<0.05 is considered as statistically significant.

Results

Deletion and site-directed mutations of the FNR-box caused drastic increases of luciferase reporter activity

Previously, we showed that *brpA* can be co-transcribed with upstream *brpB* as an operon highly conserved throughout oral streptococci ¹⁹, while the untranslated, intergenic region (−1 to −344 relative to translational initiation site, ATG) also possesses promoter activity ^{19,20}. Computer-based analysis of the short promoter (SP, −1 to −344) revealed several

regions with similarity to binding sites of known transcription regulators (Fig. S1 and Table S1). Among the putative transcriptional regulator binding sites is the FNR-box, for a putative binding site of an FNR (fumarate-nitrate reductase regulator)-like regulator^{29,30}. It is located at nucleotides (nt) –30 to –43 and is highly similar to the consensus sequence, (TTGATgtttAcCtt) (low case letters representing mis-matches comparing to the consensus), of the binding sites of the FNR regulators^{29,31}. Consistent with our previous studies^{19,20}, luciferase activities were low when the luciferase reporter gene was fused to the full-length intact promoter (data not shown). Similar results were also obtained when the luciferase reporter was fused to the promoter without its cognate Shine-Dalgarno sequence (SP, with deletion of nt –1 to –28). However, when the reporter gene was fused to the promoter construct with the FNR-box (nt –1 to –42) deleted (dF), luciferase activity was increased by 3166-fold ($P<0.001$) (Fig. 1), as compared to the intact promoter reporter fusion SP. Similar results were also obtained when the reporter was fused to promoter derivatives with internal deletion of the FNR-box (dFi, nt –30 to –41) (Table S1, Data not shown). When the FNR-box was mutated using site-directed mutagenesis from TTGATgtttAcCtt to **gcacagtttAcCtt** (dFm, Fig. 1), the luciferase reporter activity was elevated by 769-fold ($P<0.001$), as compared to SP. These results further suggest that the FNR-box is a repressor and likely as a binding site for an FNR-like transcriptional regulator. Deletion of the FNR-box led to the complete loss of the binding site, resulting in de-repression of luciferase reporter. On the other hand, site-directed mutagenesis in dFm likely reduced its binding capacity.

Deletion of a DegU-like binding site reduces luciferase reporter activity

DegU is the response regulator of the two component signal transduction system, DegSU of *B. subtilis*³². Two regions, termed DegU1 and DegU2, with similarity to the binding site of DegU were also identified in the short promoter region, with DegU1 partly overlapping the FNR-box at nt –42 to –62. To examine the role of the two putative DegU sites, deletions were made using the FNR-box deletion (dF) construct (Fig. S1 and Table S1), and the impact of such deletions were analyzed with dF serving as a control. When both FNR-box and DegU1 region (nt –59 to –33) were deleted (dFU1) and compared to the FNR-box deletion alone (dF), a 3.27-fold reduction in luciferase activity was observed ($P<0.01$) (Fig. 1), suggesting a role of the DegU1 region as a putative binding site for a DegU-like positive regulator in regulation of *brpA* expression. Deletion of putative DegU2 site did not show any major effect under the conditions studied (data not shown). By BLAST search of *S. mutans*' genome, LevR of the LevQRST four-component system³³ and LiaR of the LiaFSR three-component system³⁴ were found to possess some similarities with DegU of *B. subtilis* with an identity of 35% and 28% and a similarity of 57% and 49%, respectively. To test if LevR and LiaR are involved in *brpA* expression, reporter fusions with the intact promoter (SP) and the FNR-box deletion (dF) were transformed into the respective mutant, TW178 (*levR*) and TW42 (*liaR*)^{21,33}, and the reporter activities were analyzed together with the wild-type, UA159 as a control. The results have shown no significant differences in reporter activities between the mutants and their parent strain, UA159.

Deletion of stem-loop SL1 reduced mRNA stability and luciferase reporter activity

Three stem-loops (SL) and one dyadic sequence (DS1) were also identified in the 5' untranslated intergenic region (Fig. S1 and Table S1). SL1 (nt –73 to –190) and SL2 (nt

–117 to –181) are partly overlapping with each other (Fig. S1). Overlapping SL1&2 is also DS1 at nt –147 to –174. SL3 is position at nt –277 to –328. PCR-based deletions of these regions were made using the dF construct. No significant differences in luciferase reporter activity were identified when half of the SL2 sequence (nt –117 to –140) in the promoter region was deleted (data not shown). Similar results were observed with deletion of part of SL3 (nt –269 to –334). However, deletion of half of the stem of SL1 (nt –73 to –96) (dFS1) was found to lead to a 1.9-fold reduction in luciferase activity when compared to FNR-box deletion alone (dF) ($P<0.05$). Interestingly, when both SL1&2 and DegU1 were deleted (dFUS, nt –1 to –167), the reporter activity was further reduced by 9.62-fold ($P<0.001$) (Fig. 1), as compared to reporter fusion dF. This can be in part attributed to the additive effects of SL1 and DegU1 deletions. However, deletion of nt –1 to –167 also dismantled the DS1 sequence. To examine if DS1 plays a role in the reduced luciferase expression, a fragment of nt –142 to 157, constituting half of the DS1 element, was deleted. The results showed that when compared to the reporter fusions with the DS1 intact, there were no significant differences in reporter activity, indicative of no significant role for DS1 in *brpA* expression under the conditions studied.

Effort was also made to check if deletion of SL1 (nt –73 to –96) affected mRNA stability. It was done using Real Time-PCR with total RNA extracted from cells carrying reporter fusion dF and cells with dFS1 following treatment with rifampicin, which inhibits transcription via interactions with RNA polymerase. No significant differences in luciferase mRNA copy number were observed between cells carrying dF, which has deletion of the FNR-box alone and those carrying additional SL1 deletion in dFS1 immediately prior to and 5, 10 minutes after rifampicin treatment (Data not shown). However, relative to dF cells, cells carrying dFS1 displayed a 5.3-fold ($P<0.001$) reduction in copy number of the luciferase transcripts 20 minutes after rifampicin treatment, with an average of $2.3E+07$ ($\pm 1.3E+07$) copies / μ g total RNA for cells with dF vs $4.3E+06$ ($\pm 1.2E+06$) copies / μ g total RNA for cells with dFS1, respectively. These results further suggest that deletion of SL1 affects the stability of luciferase reporter mRNA, which can be at least partly attributed to the reduction of luciferase activity observed with the reporter fusions with SL1 deletion.

Deficiency of CRP, an FNR-like protein led to reduction of luciferase reporter activity

By BLAST searches, two genes, *arcR* (SMU.2094c or SMU_1901) and *crp* (SMU.144c or SMU_129), were identified to encode proteins with homology to FNR, with ArcR showing an identity of 25% and a similarity of 48% and CRP with an identity of 28% and a similarity of 55 %, respectively. Annotated as a hypothetical protein with roles in DNA interactions, *arcR* is linked to genes of arginine metabolism³⁵. Annotated as a CRP-like transcriptional regulator, the gene product is predicted to play a role in NAD and NADP cofactor biosynthesis (www.theseed.org). To analyze the role of ArcR and CRP on *brpA* expression, *S. mutans* mutants with deficiency of CRP and ArcR were constructed by allelic exchange with the respective coding sequence replaced by a non-polar kanamycin resistance element^{21,33} (see Table 2 for primers used). Following verification of sequence accuracy by Sangers sequencing (Table 2), the resulting mutants were transformed with the above reporter fusions, and the impact of *arcR* and *crp* deficiency on *brpA* expression was analyzed by luciferase assays. Relative to UA159, the CRP-deficient mutant, TW83, had a reduced

luciferase activity by 2-fold ($P<0.01$). Such effect was seen with reporter fusions to the short promoter with and without the presence of FNR-box (data not shown). No major differences were observed between UA159 and the ArcR-deficient mutant, TW357, in luciferase activity of the different promoter constructs tested (data not shown).

Growth in amino sugar increased luciferase reporter activity, but no effect was observed with the deficiency of NagR

Amino sugars *N*-acetyl-glucose (GlcN) and *N*-acetyl-D-glucosamine (GlcNAc) are ubiquitous in the natural environment and major building blocks of the cell envelope. In *S. mutans*, NagR is a repressor/activator of genes required for metabolism of amino sugars, such as GlcN and GlcNAc^{36,37}. Considering the critical roles of BrpA in cell envelope biogenesis^{19,20} and the presence of putative NagR binding sites (Fig. S1), the impact of amino sugars on *brpA* expression was analyzed by reporter fusion assays with cultures grown in a tryptone-vitamin based (TV) medium²² with either glucose (Glu) or GlcNAc as the supplemental carbon source. The results showed that growth in GlcNAc as carbon source led to increases of luciferase activity by more than 1.8-fold ($P<0.05$) (Fig. 2). However, such inductions were only observed when the reporter gene was under the direction of the intact promoter SP. In contrast, as many as 2.5-fold ($P<0.05$) reductions were measured when the reporter gene was fused to promoter constructs with the FNR-box deleted (Fig. 2). A NagR-deficient mutant, TW414, was also constructed by allelic exchange similarly as described above²¹ (Tables 1&2), and was transformed with the reporter fusions described above. Relative to the wild-type, UA159, slight reductions in luciferase activity were measured with the mutant, TW414, when grown in TV with GlcNAc when the luciferase reporter was expressed under the direction of the intact promoter, although such reductions were not statistically significant. No differences were identified with the other constructs analyzed between UA159 and TW414. These results further suggest that amino sugar GlcNAc induces *brpA* expression and such induction is also mediated through FNR-box.

The *brpA* gene is auto-regulated

Considering the fact that *brpA* can be co-transcribed with upstream *brpB*¹⁹, an effort was made to examine if BrpA and BrpB influences the expression of *brpA* and/or *brpB*. The above reporter fusions with and without the FNR-box were transformed to the *brpA* mutant, TW14D and the *brpB* mutant, JB409³⁸. In addition, a *brpBA* promoter (nt -784 to -1144)-reporter fusion was also constructed similarly as described above (Tables 2 and S1) and introduced into *S. mutans* UA159, JB409 and TW14D, respectively. When compared to UA159, no significant differences in reporter activity were observed with TW14D and JB409 with the reporter fusion SP and its derivatives when grown in BHI. Interestingly, when the reporter gene was fused to the upstream *brpBA* promoter, decreases of 67.7- and 4.5-fold in luciferase activity were measured in JB409 and TW14D, respectively ($P<0.001$) (Fig. 3). When the intact promoter-reporter fusion SP was expressed in JB409, 1.7-fold higher luciferase reporter activity was measured in cells grown in glucose than those in GlcNAc ($P<0.05$), which is contrary to the results of the wild-type, UA159 (Fig. 2). Similar trends were also observed when the reporter fusion dF, but the differences were not statistically significant. These results suggest that BrpA as well as BrpB is subjected to auto-regulation under the conditions studied, and such autoregulation is exerted primarily through

the upstream *PbrpBA* promoter. BrpB is also involved in carbohydrate source mediated regulation of *brpA* expression.

Discussion

Accumulating data suggest that the LCP proteins play important roles in cell envelope biogenesis^{39–41}. However, there is currently no information available concerning how they are regulated in any other bacterium. Previously, we showed by reporter fusions that expression of *brpA* is regulated in response to environmental conditions, such as acid and oxidative stress and cell envelope stresses¹⁹. By using similar strategies, these studies have shown that carbohydrate sources can also significantly influence the level of *brpA* expression. Of particular significance is the ability of amino sugars, such as GlcNAc, a major building block and a naturally occurring sugar, to significantly induce *brpA* expression. These results again reflect the important roles of BrpA in regulation of cell envelope biogenesis¹⁹, which itself is central in environmental protection, signaling transduction, and adaptation of the bacterial cells.

Consistent with computer-based analyses, multiple transcriptional regulatory sequences are shown by reporter fusion assays to play important roles in regulation of BrpA expression, including stem-loop SL1, putative transcriptional regulator binding sites DegU1 and the FNR-box. As shown by the mRNA stability assays, SL1 deletion resulted in significant reduction of the reporter transcripts 20 minutes post-rifampicin treatment, indicative of a role for SL1 in mRNA stability. The importance of 5' untranslated regions in regulation of gene expression has been well documented in eukaryotes, although similar functions have been identified in prokaryotes, including regulatory sRNA, riboswitch, and SL structures^{42–44}. The SL1 region has the potential to form a hairpin-like structure, but does not have the poly-U stretches surrounding it (Fig. S1). It is therefore unlikely to function as a *Rho*-independent transcriptional terminator⁴⁵. The exact underlying mechanism how SL1 modulates *brpA* expression awaits further investigation.

S. mutans is not known to possess a DegSU-like two component signal transduction system, which plays a key role in regulation of post-exponential phase processes in *B. subtilis*, including competence development and biofilm formation⁴⁶. While both LevR and LiaR possesses some similarities to DegU, the results that neither LevR nor LiaR showed any major effects on luciferase reporter activity under the conditions tested are not totally surprising. It awaits further investigation as to how the DegU1 element functions in regulation of *brpA* expression.

It is apparent that the FNR-box is a major transcriptional repressor, whose deletion and/or mutation confers drastic de-repression consistently. The identification of such a highly conserved FNR-binding sequence in the *brpA* promoter region as well as its major effects as shown by reporter fusion assays suggests the presence of a FNR-like regulator in *brpA* expression. Of the two proteins identified to share homology to FNR, only CRP showed some significant effects on luciferase reporter expression. CRP for cAMP receptor protein or CAP for catabolite activator protein was the first member of the CRP-FNR super-family of transcriptional regulators⁴⁷. Along with cAMP, CRP plays a central role in regulation of a

plethora of biological processes in response to the energy status of the cell, including colonization and virulence. However, *S. mutans* is not known to possess cAMP, and the negative effect of CRP deficiency on luciferase reporter activity is contrary to the positive effects of FNR-box deletion. In addition, no significant differences in reporter activities were observed when the *S. mutans* strains carrying the reporter fusions were grown in an anaerobic box, as compared to those grown in an aerobic chamber with 5% CO₂. These results further suggest that CRP plays a role in *brpA* expression but likely not via interactions with the FNR-box and that a new type of FNR-like regulator may function in regulation of *S. mutans*' pathophysiology. When BLAST searched, both LevR of the LevQRST four-component system³³ and LiaR of the LiaFSR three-component system⁴⁰ showed some similarities with DegU of *B. subtilis*. To test if LevR and LiaR are involved in *brpA* expression, reporter fusions with intact promoter and FNR-box deletion were also transformed into the respective mutant {Zeng, 2006 #403; Wen, 2004 #233}, and analyzed together with the wild-type strains as controls. The results have shown no significant differences in reporter activities between the mutants and their parent strain, UA159.

In group B streptococci, such as *Streptococcus agalactiae*, LCP protein CpsA is known to be involved in regulation of capsule biosynthesis³⁹. By gel mobility shift assay, Hanson *et al.* recently also showed that recombinant protein CpsA was able to bind to the promoter regions of the target genes^{39,48}. Like CpsA in *S. agalactiae*, BrpA in *S. mutans* also significantly influences the expression of large groups of genes, including those known to be involved in cell envelope biogenesis, stress tolerance and biofilm formation^{18–20}. Consistently, data presented here showed that BrpA as well as BrpB can significantly influence the expression of the *brpBA* operon¹⁹. BrpB is a new type of ATPase homologous to *E. coli*'s TseA (formerly YjeE), part of a three protein complex required for biosynthesis of threonylcarbamoyl adenosine (t⁶A), a modified tRNA nucleoside involved in codon decoding^{38,49}. As we previously showed, the tight genetic linkage of a *lcp* gene with a gene encoding an enzyme of tRNA modification is highly conserved in oral streptococci³⁸. To the best of our knowledge, the finding that a LCP protein plays a significant role in regulation of such an important enzyme is also novel. Studies are underway using *in vitro* transcription assay and others to examine if BrpA can directly interact with the promoter region modulating transcription of the *brpBA* operon. However, it is also possible that BrpA modulates gene transcription via interacting with a transcriptional regulator. We also hypothesize that BrpA and BrpB function in concert and probably other proteins, such as Psr, are also involved in that process¹⁸. As suggested by their auto-regulation, proper stoichiometry of BrpB and BrpA in *S. mutans* may be crucial to a functional Brp system.

In summary, the results suggest that consistent with the broad role of BrpA in *S. mutans* pathophysiology, multiple factors, including BrpA itself and BrpB, are involved in optimal expression of *brpA* and that under the conditions studied, *brpA* expression is mostly repressed via a FNR-like transcriptional regulator. Studies are underway to identify the proteins that interact with FNR-box and the other *cis* elements and elucidate how the *brpBA* promoter regulates *brpA* expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The *brpA* short promoter and its derivatives (left panel) and luciferase activities (right panel). Bar SP shows the intact short *brpA* promoter and the putative *cis* elements (see Figure S1 and Table S1 for more details). All constructs, SP and its derivatives generated from deletions and/or site directed mutagenesis, were fused to a promoterless luciferase gene (*luc*) that has its own ribosomal binding site. The transcriptional fusions were then integrated into *S. mutans* chromosome. SP, intact short *brpA* promoter fusion; dF, SP with FNR-box deleted; dFU, SP with DegU1 and FNR-box deleted; dFS1, SP with FNR-box and SL1 deleted; dFUS, SP with SL1&2, DegU1 and FNR-box deleted; and dFm, with 5 of the conserved nucleotides TTGAT of the FNR-box mutated to GCACA. See Table S1 for more details on the different constructs. Luciferase activity is expressed as relative light units normalized with optical density of the cultures at 600nm, and the values represent the average ($\pm$ standard deviation in error bars) from at least three separate experiments.

Figure 2.

Effects of sugars on the luciferase activity expressed from the *brpA* promoter. *S. mutans* strains harboring luciferase reporter fusions to the intact promoter (SP) and promoter with FNR-box deletion (dF) were grown in a tryptone-vitamin based (TV) medium either with glucose (Glu, 10 mM) or *N*-acetyl glucosamine (GlcNAc, 10 mM) as the carbohydrate source, and reporter activity in the wild-type, UA159 (WT) and mutant JB409 and TW414, with deficiency of *brpB* and *nagR*, respectively, was analyzed by luciferase assays. Data presented here represent the average ratio ($\pm$ standard deviation in error bars) of reporter activities in Glu-grown cultures over GlcNAc-grown cultures. *, $P < 0.05$, Glu vs GlcNAc; @ $P < 0.05$, UA159 vs JB409; # $P < 0.05$, SP vs dF.

Figure 3.

Effects of BrpA and BrpB on luciferase activity expressed from *brpBA* promoter. The *brpBA* promoter region was fused to the promoterless luciferase gene, and resulting construct was integrated into the chromosome of *S. mutans* UA159 and its *brpB* (JB409) and *brpA* (TW14D) mutant, respectively. Data is expressed as the average ratio ($\pm$ standard deviation in error bars) of normalized luciferase activity in the specified mutant over the wild-type, UA159, when grown to different optical density (OD_{600nm}) of 0.3, 0.5 and >0.7, respectively.

Table 1

Bacterial strains and plasmids used in this study

Strains /Plasmid	Relevant characteristics	References
<i>S. mutans</i> UA159	wild-type	35
<i>S. mutans</i> TW14D	UA159/ <i>brpA</i> , <i>Erm</i> ^r	16,20
<i>S. mutans</i> JB409	UA159/ <i>brpB</i> , <i>Kan</i> ^r	38
<i>S. mutans</i> TW357	UA159/ <i>arcR</i> , <i>Kan</i> ^r	This study
<i>S. mutans</i> TW83	UA159/ <i>crp</i> , <i>Erm</i> ^r	This study
<i>S. mutans</i> TW414	UA159/ <i>nagR</i> , <i>Kan</i> ^r	This study
<i>S. mutans</i> TW178	UA159/ <i>levR</i> , <i>Kan</i> ^r	33
<i>S. mutans</i> TW42	UA159/ <i>liaR</i> , <i>Erm</i> ^r	21
<i>E. coli</i> DH10B	Cloning host, <i>mcrA</i> , <i>mcrBC</i> , <i>mrr</i> , and <i>hsd</i>	Invitrogen, Inc.
pFW5-luc	Integration vector containing a promoterless luciferase gene and a <i>Spc</i> ^r marker	26

Note: *Kan*^r, *Erm*^r and *Spc*^r for kanamycin, erythromycin and spectinomycin resistance, respectively.

Table 2

Primers used in this study

Names	Forward (5' to 3')	Reverse (5' to 3')	Application
arcR 5F	55- tcaattaattctgcctccttatgtc	53-t atctatagacgaattctgaaaatcc	5' <i>arcR</i> for mutation
arcR 3F	35- aaggttcacgaattccgtttaag	33- tttcaacagcttgaaaagcttg	3' <i>arcR</i> for mutation
nagR 5F	tctaccaccaacatttcataacc	actgaattcgttctgccaatcac	5' <i>nagR</i> allelic exchange
nagR 3F	tttgaattcggaaaaagtcagcaaac	aaaagcaaagctatctcaacaaag	3' <i>nagR</i> allelic exchange
nagRC	tggtaaggagagacgatgttac	tgcggacatattcaaaaggcatacc	<i>nagR</i> sequencing confirmation
crp 5F	aacccaaaacgaatacaactag	ttttctcgagcttttgaatccattc	5' <i>crp</i> for allelic exchange
crp 3F	tgctcttgatccattggtaagca	acagtttgctactcttgctgac	3' <i>crp</i> for allelic exchange
crpC	acaactccttaaacacaaaacttg	agttagaggaaattagccttattc	<i>crp</i> sequencing confirmation
pbrpB Rev	ttatgatgtagcaagtctcaagaca	tatcaaaatcctcgagtactccaact	brpBA promoter
PbrpA	aagctagcgagcatgactgagcaggaagt	tcagtttctcgagaggtaaacac	short intact brpA promoter SP
FS3		aactcgaatttcattgactgattgag	SL1&2 and their 3' deletions
3brpA		tgtttttctactcgagcagtttcc	3' primer, short promoter SPi
dFnr3		taaacctcgagaataactccatct	FNR-box and its 3' deletions
DegU1 5SP	aagctagcgagcatgactgagcaggaag	tcctaagtgttttgagccgttc	5' fragment, DegU1 deletion
DegU1 3F	tcaaaaacacttaggaggtcctgatag	accaactgaaccagcccttc	3' fragment, DegU1 deletion
dSL1	taatgtcgaccggctcaaaaacaaaagat	taatgtcgaccgctatgtgctaaaatcat	deletion of SL1
dSL2	taatgtcgacgatgattttgacacatagcgaa	taatgtcgacagcttacaatcacacaaatttca	deletion of SL2
dSL3	atcagctagctcgaaaaaacgattaagag		5' primer, deletion of SL3
NagR/DegU2	taatgtcgacttaatttgatgaagagtgccct	taatgtcgactctgataaactttttctgtatcttc	deletion of NagR/DegU2
FNRS5	aagctagcgagcatgactgagcaggaag	gagtaaaactgtcgaaatactccatcttttg	5', FNR-box mutation
FNRS3	ttcgacagtttactcgaggaggaac	accaactgaaccagcccttc	3', FNR-box mutation
Luc	taatggtaccatggaagacgcaaaaacata	aggaaaccaggcgctatctct	qPCR, mRNA stability
dFnr1	taatgtcgacaggaggaaactgatagattag	taatgtcgacaagaataactccatctttgttttg	internal FNR-box deletion

Note: Sequences underlined are restriction sites engineered for cloning.