BrpA is Involved in Regulation of Cell Envelope Stress Responses in *Streptococcus mutans*

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Abstract

Previous studies have shown that BrpA plays a major role in acid and oxidative stress tolerance and biofilm formation by *S. mutans*. Mutant strains lacking BrpA also display increased autolysis and decreased viability, suggesting a role for BrpA in cell envelope integrity. In this study, we examined the impact of BrpA-deficiency on cell envelope stresses induced by envelope-active antimicrobials. As compared to the wild-type strain UA159, the BrpA-deficient mutant (TW14D) was significantly more susceptible to antimicrobial agents, especially lipid II inhibitors. Several genes involved in peptidoglycan synthesis were identified by DNA microarray analysis as down-regulated in TW14D. Luciferase reporter gene fusion assays also revealed that expression of *brpA* is regulated in response to environmental conditions and stresses induced by exposure to sub-inhibitory concentrations of cell envelope antimicrobials. In a *Galleria mellonella* (wax worm) model, BrpA-deficiency was shown to diminish the virulence of *S. mutans* OMZ175, which unlike *S. mutans* UA159, efficiently kills the worms. Collectively, these results suggest that BrpA plays a role in the regulation of cell envelope integrity and that deficiency of BrpA adversely affects the fitness and diminishes the virulence of OMZ175, a highly invasive strain of *S. mutans*. 
Introduction

The oral cavity is a dynamic environment in which frequent and often rapid fluctuations in pH and the concentrations of antimicrobial agents and other stressors occur. Dental care products, such as toothpastes and mouth rinses, contain a variety of antibacterial compounds, including hydrogen peroxide, sodium lauryl sulfate and chlorohexidine. Many bacteria in the highly complex oral flora can produce hydrogen peroxide and antibacterial peptides, better known as bacteriocins, allowing the producers to ensure their presence in the community by killing competing organisms (24). To survive in the relatively hostile environment of oral biofilms, bacteria must be able to sense, respond to, and cope with these insults. The cell envelope plays a vital role during these processes as it protects the cell from the environment, maintains cell shape, acts as a molecular sieve, and provides a platform for components of the cell involved sensing and transmission of environmental signals. Ensuring envelope integrity is therefore crucial for bacterial cells to survive.

*Streptococcus mutans*, a primary causative agent of human dental caries, lives almost exclusively in biofilms on the tooth surface. This bacterium is known for its ability to survive and adapt to environmental insults, including mounting protective responses in reaction to various stimuli (10, 28). Multiple pathways are utilized by *S. mutans* to modulate its capacity to cope with stresses, but certain two-component signal transduction systems (TCS), including CiaHR, VicRK and LiaSR, play integral roles in survival and adaptation to low pH, reactive oxygen species (ROS) and cell envelope stress induced by antimicrobial agents (5, 6, 11, 38, 40). For example, mutants lacking LiaSR in *S. mutans* displayed increased susceptibility to Lipid II cycle interfering
antibiotics and chemicals that perturb cell membrane integrity (40). In addition, BrpA, for biofilm regulatory protein A, is also involved in acid- and oxidative stress tolerance response and biofilm development by S. mutans (42, 43). Relative to the parent strain, S. mutans strains lacking BrpA had a limited ability to grow and accumulate on a surface and displayed enhanced sensitivity to low pH and hydrogen peroxide.

A predicted surface-associated protein, BrpA contains a region homologous to the LytR-CpsA-Psr (LCP) domain of the LCP family of proteins. The LCP family of proteins is widely distributed among Gram-positive bacteria and its members are generally annotated as cell wall associated transcriptional regulators (17). Originally, the LytR protein of Bacillus subtilis was identified as an autogenous transcriptional attenuator that also regulated the promoter of the divergently transcribed lytABC operon, which encodes a lipoprotein (LytA), an N-acetylmuramoyl-L-alanine amidase (autolysin, LytC), and a modifier protein of LytC (LytB) (26). The LytR parologue CpsA of Streptococcus agalactiae was subsequently shown to function as a transcriptional activator of the capsule operon (14, 16). Recently, LytR in Streptococcus pneumoniae was reported to be essential for normal septum formation (20), with the mutant displaying variability in size and shape. The lytR mutants were also found to form multiple asymmetrical septa. Similar functions were also observed with MsrR, a Psr-like protein in Staphylococcus aureus (36). A mutant lacking MsrR was reported to have a 4-fold decrease in minimal inhibitory concentration (MIC) against oxacillin and a 2-fold reduction against teicoplanin, when compared to the parental strain.

Previously, we showed that BrpA-deficiency in S. mutans causes major defects in biofilm formation and acid- and oxidative stress responses (42, 43). Relative to the parent
strain, the deficient mutant also had an increased rate of autolysis and a decreased viability, suggesting compromise in cell envelope biogenesis/homeostasis. In this study, we used reporter gene fusion and antibacterial susceptibility assays to further characterize S. mutans strains deficient of BrpA. Results showed that S. mutans strains lacking BrpA were more susceptible to cell envelope-targeting antimicrobials and that cell envelope and environmental stresses enhanced the expression of BrpA. In addition, we show that BrpA is required for optimal binding to salivary agglutinin. These results extend previous studies showing that BrpA plays a critical role in cell envelope biogenesis and cell envelope stress responses in S. mutans.

Materials and Methods

Plasmids, bacterial strains, cell lines, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. S. mutans strains were maintained on brain heart infusion (BHI) medium. For biofilm formation, S. mutans was grown in modified biofilm medium (BM) with glucose (18 mM) and sucrose (2 mM) as supplemental carbon and energy sources (BMGS) (29, 42, 43). All solid media were prepared similarly with inclusion of Bacto agar (Difco Laboratories, Franklin Lakes, NJ) at the level of 1.5% (w/v). When needed, erythromycin (10 µg/ml), kanamycin (1 mg/ml), and/or spectinomycin (1 mg/ml) were added. Unless otherwise stated, cells were grown at 37°C in an aerobic environment with 5% CO2. All E. coli strains were grown in Luria Bertani medium at 37°C aerobically, with or without inclusion of kanamycin (40 µg/ml), ampicillin (100 µg/ml), spectinomycin (100 µg/ml), and/or erythromycin (300
µg/ml). Human coronary artery endothelial cells (HCAEC) were grown and maintained in endothelial cell basal medium-2 (EBM-2, Lonza) (2, 31).

DNA manipulation, transcriptional initiation site mapping, and construction of reporter fusions. Standard recombinant DNA procedures were used (12, 37). All restriction and modifying enzymes were purchased from Invitrogen (Carlsbad, CA) or New England Biolabs (Ipswich, MA) and used as recommended by the suppliers. All primers (Table 1) were synthesized by Integrated DNA Technologies, Inc. (Iowa City, IA). RNA Ligase Mediated Rapid Amplification of cDNA Ends (RLM-RACE) (Ambion, Inc., Foster City, CA) was used to map the transcription initiation site (TIS) of brpA. Briefly, total RNA was prepared from early- (OD$_{600nm}$ = 0.2) and late- (OD$_{600nm}$ = 0.8) exponential phase cultures grown in BHI using hot phenol (1, 42). The preparations were then treated with RNase-free DNase I (Ambion, Inc.), and RNA was retrieved using the Qiagen RNeasy purification kit (Qiagen, Inc., Valencia, CA). For cDNA synthesis, total RNA was treated with calf intestinal phosphatase and with Tobacco Acid Pyrophosphatase by following the supplier’s recommendations, and then ligated to the supplied 5’ RACE adapter. cDNA was synthesized using iScript reverse transcriptase III (Invitrogen) and followed by a nested PCR using either the 5’ RACE outer primer or the 5’ RACE inner primer (Ambion, Inc.) and a brpA-specific reverse primer. The TIS was determined by sequencing of the resulting PCR amplicon.

To analyze the regulation of brpA expression, a promoterless luciferase gene (luc) was used as a reporter (23, 35). Briefly, the cognate brpA promoter region was amplified by PCR with primers pbrpA5’NheI and pbrpA3’XhoI. Following proper restriction
digestions, the amplicon was cloned directly in front of the promoterless \textit{luc} gene in integration vector pFW11-\textit{luc} (22), which also contains a Shine-Dalgarno sequence optimized for group A streptococci (35). Following confirmation of the correct sequence of the cloned element, the resulting construct, pFW11::\textit{brpA}::\textit{luc}, was introduced into \textit{S. mutans} UA159 and TW14D and maintained on BHI agar containing 1 mg/mL spectinomycin. The expression of BrpA under different environmental conditions and cell envelope stressors were analyzed using luciferase assay by following the protocol of Podbielski (22, 35).

**DNA microarray and RealTime-PCR analysis.** For DNA microarray analysis, total RNAs were extracted from early-exponential phase (OD$_{600}$nm $\cong$ 0.3) cultures, treated with DNaseI (Ambion, Inc.) to remove all DNA, and then retrieved with the RNeasy purification kit (QIAGEN, Inc.) (42). Array analysis was performed by using the whole-genome \textit{S. mutans} microarrays (version 2) that were obtained from The J. Craig Venter Institute (JCVI, http://pfgrc.jcvi.org) by following the protocols recommended by JCVI as described elsewhere (1, 42). Expression levels of selected genes identified by DNA microarray analysis were confirmed by Real-time PCR procedures detailed elsewhere (Table 1) (5, 42). Microarray data have been deposited in NCBI (accession #GSE35349).

**Cell envelope antimicrobial susceptibility assays.** The susceptibility of \textit{S. mutans} strains to antimicrobial agents was analyzed using microtiter plate-based assays as described previously (30, 40). Cell envelope antimicrobial agents tested included the antibiotics vancomycin (Sigma, St. Louis, MO), bacitracin (Sigma), and $\beta$-lactam
antibiotic pencillin G (Sigma); the bacteriocin nisin (Sigma); and the cell envelope active
compounds sodium dodecyl sulfate (SDS) and chlorhexidine (Sigma). Briefly, 100 µl of
properly diluted mid-exponential phase cultures were added to 96-well plates containing
BHI medium supplemented with two-fold serial dilutions of cell envelope antimicrobial
agents. After 48 hours, bacterial growth was measured spectrophotometrically using a
Synergy 2 plate reader (BioTek, Inc.), and relative cell density percentages (OD₄₉₀nm of
cultures with antimicrobial agents divided by the OD₄₉₀nm of the untreated cultures X
100) were calculated. Minimal inhibitory concentration (MIC) was defined as the lowest
concentration at which the cultures did not grow to over 10% of the relative cell density.
Minimal bactericidal concentration (MBC) assays were carried out using the MIC test
plates. MBC was determined as the lowest concentration where fewer than 5 colony-
forming-units (CFU) were observed after 48 hours when 20 µl of the cultures was plated
on non-selective medium.

Biofilm formation and BIAcore assays. Biofilm formation on 96-well plates pre-
coated with salivary agglutinin was carried out as previously described (4, 42, 43).
Interactions of *S. mutans* whole cells with salivary agglutinin were analyzed using
BIAcore assays in which the receptor was immobilized on Pioneer F1 sensor chips (32).

Preparation of protein fractions and Western blot analysis. Various fractions of
proteins were prepared from BHI-grown early-exponential phase (OD₆₀₀mm = 0.3) cultures
of *S. mutans* (3, 41, 45). Briefly, whole cell lysates were obtained by glass bead-beating
in SDS-boiling buffer (60 mM Tris, pH 6.8, 10% glycerol, and 5% SDS). For surface-
associated fractions, cells from 500 ml cultures were suspended in 25 ml of 0.2% \( N \)-dodecyl-\( N,N \)-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent, Sigma) and incubated at 28°C with shaking at 80 rpm for 1 hour. Following centrifugation, the supernatants were further concentrated using Amicon Ultra centrifugal filters (Millipore, Billerica, MA). In other cases, bacterial cells were suspended in 4% SDS and incubated at room temperature for 30 minutes. For cell-free fractions, cultural supernatants were precipitated by ammonium sulfate. For Western blot analysis, proteins (10 µg total) were separated using 7.5% SDS-PAGE, blotted onto Immobilon-FL membranes, and then probed with anti-P1 monoclonal antibodies (8, 41).

**Bacterial invasion assay.** The impact of BrpA-deficiency on *S. mutans* ability to invade host tissues were analyzed using primary human coronary artery endothelial cells (HCAEC) as described elsewhere (2, 31). Briefly, overnight cultures were harvested by centrifugation at 14,000xg for 5 minutes, and pellets were washed twice with phosphate-buffered saline (pH 7.2) and resuspended in endothelial cell basal medium-2 (EBM-2, Lonza). Aliquots (1 ml) of bacterial cells (with ~5x10^7 cfu/ml) were mixed with HCAEC monolayers in 24-well plates for 2-hours. Following proper washes and additional incubation with gentamicin and penicillin G to eliminate extracellular bacterial cells, HECAE cells were lysed by osmotic shock, and serial dilutions of the lysates and bacterial cells released were plated on BHI agar in triplicate. The percentage of intracellular bacteria relative to the initial inoculum was calculated.

**Wax worm infection model.** *Galleria mellonella* killing assays were performed by
following the procedures described previously (21). Briefly, groups of 20 larvae, ranging
from 200 to 300 mg in weight and with no signs of melanization, were randomly
assigned. A 5 µl aliquot of properly diluted mid-exponential phase (OD$_{600nm}$ = 0.5)
cultures of $S. mutans$ wild-type or the BrpA-deficient mutant were injected into the
hemocoel using a 10 µl Hamilton syringe (Hamilton Co., Reno, NV). Groups receiving
heat-inactivated (10 min at 75°C) $S. mutans$ wild-type or saline were used as controls.
After injection, larvae were incubated at 37°C, and appearance (signs of melanization)
and survival were recorded at selected intervals. Kaplan-Meier killing curves were
plotted and estimates of differences in survival were compared using a log rank test. A $P$
value of $\leq 0.05$ was considered significant. All data were analyzed with GraphPad Prism,
version 4.0.

**Results**

**BrpA-deficiency affects binding to immobilized salivary agglutinin.** Binding to
salivary agglutinin and other glycoproteins, primarily through the multi-functional
adhesin P1 (also called Antigen I/II, PAc, or SpaP), is considered to be a major
mechanism used by $S. mutans$ to colonize the tooth surface (15, 19, 25, 27). On 96-well
plates pre-coated with whole saliva and purified salivary agglutinin (4), $S. mutans$ wild-
type UA159 formed robust biofilms after 24 hours, consistent with previous findings (4).
Relative to UA159, however, biofilm accumulation by the BrpA-deficient mutant,
TW14D was significantly lower ($P<0.05$) (Figure 1A). We also used BIAcore assays to
analyze the impact of BrpA-deficiency on P1-mediated adherence and biofilm formation.
Affinity-purified, high molecular weight salivary glycoprotein agglutinin was
immobilized on a Pioneer F1 sensor chip and interaction of *S. mutans* with immobilized agglutinin was measured by BIAcore, a proven technique for assessment of salivary agglutinin mediated adherence (32). When compared to the wild-type, the capacity of salivary agglutinin-mediated whole cell-receptor interaction in the mutant deficient of BrpA was decreased by more than 57%, with the average resonance signal for the wild-type, UA159 at 938.95(±102.45) versus 413.6(±186.7) (*P*<0.01) for the mutant, TW14D (Figure 1B).

Western blot analysis was then carried out to further examine the levels of P1 in whole cell lysates, cell-free fractions and surface-associated fractions from UA159 and TW14D using monoclonal antibodies (mcAb) against P1 as probes (3, 8, 41, 45). When probed with mcAb 6-8C, which reacts the C-terminus of P1 (8), a single band with molecular weight (MW) around 200 kDa was apparent in the surface-associated fractions of both UA159 and TW14D (Figure 2A). In comparison, however, the density of this reactive band in TW14D was over 2-fold of the one in UA159. A similar band was also detected in whole cell lysate of TW14D, but was not detectable in UA159. When probed with mcAb 4-10A that recognizes the A-P stalk, one major band of MW around 200 kDa was apparent in both TW14 and UA159 (Figure 2B), with the density of the band in TW14D being more than 4-fold higher than that in UA159. Besides, multiple bands with MW around 150 kDa were apparent in the whole cell lysates, but again these bands in TW14D were more than 9-fold denser than those in UA159. With mcAb 3-8D as a probe, which recognizes the A-region of the P1, multiple bands with similar MW around 100 kDa were identified in the whole cell lysates of both UA159 and TW14D (Figure 2C). In comparison, the densities of these bands in the mutant were about 6-fold higher.
than those the wild-type. Multiple bands reactive to mcAb 3-8D were also seen in the surface-associated fractions, but different from the whole cell lysates, these bands were mostly around 75 kDa.

**BrpA-deficient mutants are more sensitive to cell envelope-active antimicrobials.**

Previously, it was shown that BrpA-deficiency in *S. mutans* caused elevations in autolysis and reductions in viability, with more dead cells and cell debris in biofilms relative to the wild-type strain (13, 42). To analyze whether BrpA in *S. mutans* affects cell envelope integrity, MIC and MBC against several cell envelope antibacterial agents were measured using microtiter plate-based assays. As shown in Table 2, the BrpA-deficient mutant had a decreased ability to survive the treatment of several different antimicrobial agents, when compared to the wild-type strain under the same conditions. TW14D had a 1.8- and 2-fold reduction in MIC to nisin and bacitracin, respectively. Similar trends were also detected with vancomycin, penicillin-G, D-cycloserine, SDS and triclosan, although the differences between these two strains were not statistically significant. When MBC’s were analyzed, the deficient mutant had ≥1.5-fold reductions in sensitivity to nisin, chlorhexidine and SDS. Slight, but not statistically significant decreases were also seen with bacitracin, vancomycin and triclosan.

**BrpA-deficiency affects virulence in a *Galleria mellonella* model.** Recent studies have shown that certain strains of *S. mutans*, such as OMZ175 (serotype f), are highly invasive, and consequently may play a significant role in development of certain systemic diseases, such as infective endocarditis (2, 31). In contrast, UA159, a commonly used
laboratory strain, possesses only limited capacity to invade endothelial cell lines (2). To create a BrpA-deficient mutant of OMZ175, PCR reactions were conducted with genomic DNA from TW14D as the template (Table 1) (42). The resulting amplicon containing DNA fragments flanking \textit{brpA} and an erythromycin resistance element (\textit{erm\textsuperscript{r}}) was used to replace the \textit{brpA}-coding sequence in \textit{S. mutans} OMZ175, and mutants were selected on BHI with erythromycin and further confirmed by DNA sequencing. When analyzed by invasion assays using human coronary artery endothelial cells (HCAEC) (2), the BrpA-deficient mutant, TW230 had a slightly reduced invasion efficiency, when compared to OMZ175, with an average invasion rate of 0.18\% for TW230 vs 0.49\% for OMZ175 (\textit{P}=0.089). When tested in the \textit{G. mellonella} (wax worm) virulence model (21), the survival rate of worms receiving the BrpA-deficient mutants was significantly (\textit{P}<0.01) higher than those receiving strain OMZ175 (Figure 3). Not surprisingly, considering the poor virulence of strain UA159 in this model, no major differences (\textit{P}>0.05) were observed when TW14D and UA159 were compared in the wax worms (data not shown).

\textit{brpA} is cotranscribed with SMU.409. The \textit{brpA} gene (SMU.410) in \textit{S. mutans} is flanked by downstream SMU.411 and by upstream SMU.409 (Figure 4), which encodes a streptococcus-specific, hypothetical protein and a putative bacterial ATPase/GTPase (www.oralgen.lanl.gov), respectively. The open reading frames in SMU.409 and \textit{brpA} are arranged in the same orientation, while SMU.411 and \textit{brpA} are transcribed in opposite directions. To map the promoter region of \textit{brpA}, TIS was examined using 5\textsuperscript{\prime}RACE with total RNA extracted from BHI-grown cultures. Results of 5\textsuperscript{\prime} RACE PCR
showed that multiple \textit{brpA} transcripts existed (data not shown). Sequence analysis of the major complementary DNA product revealed that the major TIS of \textit{brpA} was 774 bp upstream of the translational start codon ATG (Figure 4), suggesting that SMU.409 and \textit{brpA} are co-transcribed under the conditions studied. Reverse transcription-PCR with total RNA extracted from BHI-grown planktonic cultures and 3-day biofilms grown on BMGS confirmed that \textit{brpA} was co-transcribed with the upstream gene SMU.409 (Figure 5A) under both of the conditions tested. Insertion of polar kanamycin resistance element, \( \Omega \text{Km} \) (34) at SMU.409 also caused a reduction of more than 25-fold in \textit{brpA} transcription, as shown by RealTime-PCR with total RNA preps of early-exponential (OD\textsubscript{600nm} = 0.25) cultures of the insertional mutant and its parent strain UA159 (data not shown). Similar results were also obtained with Western blot analysis (data not shown).

**Expression of BrpA is regulated in response to environmental conditions.** In cultures grown in BHI broth, luciferase expression from the full \textit{brpA} promoter (a 1119 bp fragment) was measured at its maximum during early-exponential phase (OD\textsubscript{600nm} = 0.3; Figure 5B), consistent with our earlier study by Northern blotting (44). Considering the fact that mutants lacking BrpA had significant defects in their abilities to survive low pH and hydrogen peroxide challenge (42), cells of early-exponential phase cultures carrying the reporter fusion were treated with hydrogen peroxide and methyl viologen (paraquat, Sigma) in the growth medium for 90 min. Results showed that, relative to the un-treated controls, the level of luciferase activity in cells treated with hydrogen peroxide and methyl viologen was increased significantly (Table 3). Such increases appeared to be concentration dependent when the amount used was within a certain threshold (Figure
S1). However, beyond the threshold, luciferase activity was decreased with further increases of hydrogen peroxide and methyl viologen used. Similar results were also obtained with cells treated with various cell envelope antimicrobials at sub-inhibitory concentrations (Tables 2&3), with the most significant impact measured with chlorhexidine, a chemical commonly used in dental care products and in prevention of tooth decay. Efforts were also made to evaluate the impact of pH on BrpA expression by incubating the cells carrying the reporter fusion in BHI broth adjusted to different pH values, but the results were inconclusive, probably due to the impact of low pH on the luciferase enzyme (data not shown). To circumvent this problem, study is underway using chloramphenicol acetyltransferase as a reporter under controlled conditions in a chemostat.

**BrpA-deficiency causes substantial alterations in the transcriptional profiles of the deficient mutant.** In consideration of the fact that BrpA expression is at its maximum during early-exponential phase as shown by Northern blotting (44) and reporter gene fusion assays, we carried out another DNA microarray analysis using total RNA from early exponential phase cultures (OD$_{600nm}$ =0.3). It was found that 92 genes were up-regulated and 90 down-regulated in TW14D by a factor of at least 1.5-fold ($p \leq 0.001$, Tables S1&S2). At a level of $P<0.01$, 176 additional genes were found to be differentially expressed in TW14D, with 77 up- and 90 down-regulated (data not shown). Based on the description and putative functions of the genes identified at the significance level of $P<0.001$, BrpA-deficiency affects almost every aspect of the cellular physiology as well as virulence properties, including amino acid biosynthesis (10), carbohydrates and
energy metabolism (18), nucleic acids and DNA metabolism (10), transcriptional regulation (9), ABC transporters (29), molecular chaperones and other cellular processes (13), and hypothetical and conserved hypothetical proteins (50). The breadth of impact of BrpA-deficiency on the transcriptional profile of the deficient mutant is similar to what was observed previously with mid-exponential phase cells (42). However, comparison of the two transcriptional profiles revealed that only a small number of genes were consistently up- or down-regulated in both early- and mid-exponential phase cultures, which include \textit{recA} (for recombinant protein RecA), \textit{gtfD} (for glucosyltransferase D), \textit{wapA} (for surface-associated protein WapA), \textit{groEL} (for molecular chaperone GroEL) and \textit{sod} (for Mn-dependent superoxide dismutase SOD) (Tables S1&S2) (42). In addition, the magnitude of alterations in gene expression was also more dramatic in cells of the early-exponential phase, when compared to that in the mid-exponential phase cultures.

**Discussions**

The cell envelope is of essential importance for growth, cell division, interaction with the environment, and antimicrobial resistance. Previous studies have shown that BrpA, a paralogue of the LCP family of cell wall-associated transcriptional attenuators, strongly influences \textit{S. mutans} biofilm formation and survival against low pH and reactive oxygen species (42, 43). Strains deficient of BrpA also displayed increased autolysis rates and decreased viability, suggesting a role for BrpA in regulation of cell envelope biogenesis or homeostasis (13, 42, 43). In this study, BrpA-deficiency was shown to significantly weaken the ability of \textit{S. mutans} to survive cell envelope stresses induced by cell
envelope-targeting antimicrobials (Table 2). Among the antimicrobial agents tested, the most significant influences in MIC were measured with nisin and bacitracin, two antibiotics that interfere with Lipid II cycling, blocking peptidoglycan and cell wall biosynthesis (9). The most significant impact in MBC was seen with chlorhexidine and SDS, two chemicals commonly used in oral health care products that compromise membrane integrity. These results provide further support for a role for BrpA in regulation of cell envelope biogenesis or maintenance by S. mutans, consistent with the roles of certain LCP paralogues in other bacterial species (18, 20, 33, 36, 39).

The bacterial cell wall is a repeating, three-dimensional polymer, known as peptidoglycan or murein consisting of a linear, alternating $N$-acetylmuramic acid $\text{(MurNAc)}$ and $N$-acetylglucosamine (GlcNAc) motif, cross-linked via peptides appended to MurNAc. Of the genes altered as a result of BrpA-deficiency in TW14D, several were found to encode proteins with potential roles in peptidoglycan biosynthesis (Table 4). Among them are SMU.246 for a phospho-MurNAc-pentapeptide-transferase (RgpG), SMU.549 for an undecaprenyl-PP-MurNAc-pentapeptide-UDP-GlcNAc transferase (MurG), SMU.599 for a D-alanine-D-alanine ligase (DdlA), and SMU.1677 for a UDP-MurNAc-tripeptide synthetase (MurE). While the exact role of these gene products in S. mutans cellular physiology awaits further investigation, down-regulation of genes involved in peptidoglycan synthesis would have an impact on cell envelope biogenesis, likely leading to defects in wall integrity. Such a defect would be consistent with the weakened resistance to cell envelope antimicrobials, reduced viability and increased autolysis observed for BrpA-deficient mutants (13, 43). In support of a role in cell envelope biogenesis, the expression of a luciferase reporter fusion under the direction of
brpA promoter was also up-regulated in response to cell envelope stresses induced by exposure to sub-inhibitory concentrations of antimicrobial agents that target the cell envelope (Table 3). Defects in cell envelope integrity would likely result in vulnerability of the bacterial cells to environmental insults, and therefore can partly explain the weakened acid- and oxidative stress response of the BrpA-deficient mutants (42).

P1, a cell wall anchored adhesin, is considered a key contributor to S. mutans colonization of the tooth surface (7, 15). P1 mediates the adherence through interactions with high molecular weight salivary agglutinin in the enamel pellicle. Both biofilm formation assays and BIAcore analysis showed that BrpA affects the ability of S. mutans to interact with salivary agglutinin (Figure 1). As shown by Western blotting, however, the level of P1 expression was increased by more than 2-fold as a result of BrpA-deficiency (Figure 2A). When analyzed by DNA microarrays, several genes encoding components of the Sec translocase were also found altered in TW14D. These included secA, secE and secY encoding the ATP-dependent motor of the translocation machinery, SecA, and the translocon pore components, SecE and SecY, respectively. Both secA and secE were down-regulated by more than 2-fold, while secY was up-regulated by more than 2-fold (Table 4). The Sec secretion system participates in translocation of polypeptides across, or integration into, the cytoplasmic membrane (46). Alteration in expression of individual members of the Sec translocon complex, as well as global defects in cell envelope integrity, will likely influence the function of the translocation/secretion machinery. As a result of altered Sec function, the P1 adhesin may be compromised in conformation, stability and/or distribution on the surface. Therefore, the increased expression could be a compensatory response to such a compromise, while
the underlying mechanism awaits further investigation. In addition, the disproportional increases in density of the lower MW bands in TW14D that were reactive to mcAb 3-8D and 4-10A, which are shown to recognize truncated peptides (8), also suggest the stability of P1 may be reduced in the brpA mutant (Figure 2B&C). Therefore, decreased stability and/or mis-folding of P1 may underlie the reduced binding to salivary agglutinin by strain, TW14D. These *in vitro* results also suggest that BrpA-deficiency may affect bacterial adherence and biofilm initiation by *S. mutans* in the oral cavity as well.

Previously, Northern blotting showed that transcription of *brpA* was maximal during early-exponential phase (OD$_{600nm}$ ≈ 0.3) and that deficiency of LuxS dramatically decreased *brpA* transcription, indicating that expression of *brpA* is regulated in response to environmental conditions and by LuxS-mediated quorum sensing (44). In this study, we used luciferase reporter gene fusion assays to show that the expression of BrpA is strongly dependent on growth phase, with maximal activity measured during early-

exponential phase (Figure 5B). These results again suggest that environmental conditions and cell density play an important role in regulation of BrpA expression. Differences in environmental conditions, such as pH and concentration of ROS, and cell density could in part account for some of the discrepancies observed between the two transcriptional profiles between the early- and mid-exponential phase cultures (Tables S1&S2) (42). However, it awaits further investigation whether BrpA affects different group of genes in response to environmental stimuli.

Both 5′RACE and RT-PCR showed that under the conditions studied the major transcript was a product of co-transcription of *brpA* with its upstream locus SMU.409 (Figures 4&5). Consistently, both RealTime-PCR and Western blot analysis (data not
shown) showed that polar insertion at SMU.409 resulted in a dramatic reduction of BrpA expression. However, we have previously showed that possession in trans of the brpA-coding sequence plus a 344 bp fragment upstream of its start codon was able to partially complement the deficient mutant TW14 in acid tolerance response (42). Luciferase reporter fusion with a fragment of 683 bp upstream of brpA also showed promoter activity in this intergenic region, although it is much weaker than the full-length (1119 bp) promoter (data not shown). Computer-based analysis of this intergenic region using BPROM, a bacterial sigma70 promoter recognition program, and Virtual Footprint, a program especially designed to analyze transcription factor binding sites, also revealed putative -10 (TATAAc) and -35 (TTGAgA) sites and regions with high similarity to binding sites for several putative transcriptional regulators (Figure 4). These results further suggest that transcription of brpA may be initiated at different sites under different environmental conditions. Study is currently under way to dissect the underlying mechanisms, including the cis-and trans-acting elements involved in regulation of brpA expression. SMU.409 encodes a putative ATPase/GTPase. While the exact role of SMU.409 in regulation of S. mutans cellular physiology and brpA expression is still under investigation, the close association of this gene with brpA also suggests its likely involvement in BrpA-regulated cell envelope biogenesis/homeostasis.

In summary, results presented here further support that S. mutans BrpA is involved in the regulation of cell envelope biogenesis/maintenance and that deficiency of BrpA affects the fitness of the deficient mutants and decreases the virulence of OMZ175, a highly invasive strain in a wax worm model. Current effort is directed to further investigation of the underlying mechanisms.
Acknowledgement

This work is supported by NIDCR grant DE19452 to Z.T.W. and in part by the South Louisiana Institute of Infectious Disease Research. We would like to thank Dr. Fengxia (Felicia) Qi at the University of Oklahoma Health Sciences Center for her kind gift of the integration vector pFW11-luc, and Mr. James H. Miller for his assistance with invasion and wax worm infection assays.
Figure 1. Biofilm formation (A) and BIAcore assays (B). A: *S. mutans* UA159 and TW14D were grown on 96-well plates that were pre-coated with unstimulated whole saliva (WS) or affinity-purified salivary agglutinin (AG). Data shows the average density (±standard deviation in error bars) of 24-hour biofilms from more than three independent sets of experiments, with a * indicating significant difference between UA159 and TW14D under the conditions specified (P<0.05). B: P1-mediated *S. mutans* whole cell interaction with salivary agglutinin was measured using BIAcore assays. Salivary agglutinin was immobilized on an F1 chip surface. *S. mutans* UA159 and the BrpA-deficient mutant, TW14D were injected for 60 seconds. *S. mutans* UA159 yielded an average resonance signal of 938.95, while TD14D had 413.6. Results indicate that BrpA-deficiency affects P1-mediated whole cell adhesin-receptor interactions. Panel shows representatives of two independent experiments.

Figure 2. Western blot (A-C) and SDS-PAGE (D) analysis of P1 in *S. mutans* wild-type UA159 (lanes 1&3) and the BrpA-deficient mutant TW14D (lanes 2&4). Proteins (10 µg total) of whole cell lysates (lanes 1&2) and surface-associated fractions (lanes 3&4) were separated using 7.5% SDS-PAGE (D), blotted onto a PVDF nitrocellulose membrane, and then probed with anti-P1 monoclonal antibodies (mcAb). Panel A shows results when probed with mcAb 6-8C, which recognizes the C-terminus of the P1. A single band with molecular weight (MW) around 200 kDa was apparent and the density of this band in TW14D was more than 2-fold of the one in UA159. Panels B and C show results when probed with mcAb 4-10A and mcAB 3-8D, which recognizes the A-P stalk and the
alanine-rich region of P1, respectively, but both are shown to react to truncated peptides (8). Multiple bands with MW around 150 (B) and 100 (C) kDa were identified in both UA159 and TW14D, but the densities of these bands in TW14D were significantly higher than those in UA159. M, molecular weight markers.

Figure 3. Killing of G. mellonella larvae infected with S. mutans wild-type and the corresponding BrpA-deficient mutants at 37°C. Figure shows survival (Kaplan-Meier) plots of S. mutans OMZ175 (solid squares) and the BrpA-deficient mutant, TW230 (open cycles) injected at 1x10^7 CFU/larva. There was no killing of larvae injected with saline and minimum killing of larva injected with heat-killed S. mutans UA159 cells (data not shown). The experiments were repeated three times, and the data presented here are results representative of a typical experiment. Compared to the wild-type parent strain, OMZ175, the BrpA-deficient mutant, TW230 showed attenuated virulence (P ≤0.01).

No significant differences were measured between UA159 and TW14D (data not shown).

Figure 4. Schematic diagram of the brpA-flanking region (A) and analysis of the brpA promoter region (B). Panel A shows the genetic organization of the regions flanking brpA, with locus name and size labeled under and above the arrows, respectively. Approximate positions of the primers used for RT-PCR were shown with a solid arrow above SMU.409 and under brpA, respectively. Panel B highlights the promoter region of brpA, which includes the coding sequence of SMU.409, with the translation initiation site indicated by an arrow above the start codon of brpA and SMU.409, respectively. As determined by 5'-RACE, the transcription initiation site (TIS, underlined) of brpA was
774 bp upstream of its translation start site, which overlaps the translation start site of SMU.409 as annotated by NCBI (www.oralgen.lanl.gov). Computer-based analysis using BPROM, a bacterial sigma70 promoter recognition program, revealed two sets of putative -35 and -10 sites (boxed) and binding sites for putative transcriptional regulators (underlined). Further analysis using Virtual Footprint, a program especially designed to analyze transcription factor binding sites, also identified sequences with high similarity to binding sites for transcriptional factors GltC, TnrA, and Fnr (underlined).

Figure 5. RT-PCR analysis (A) and Luciferase activity assays (B). A. RT-PCR analysis was carried out using total RNA isolated from mid-exponential phase (OD_{600nm}=0.5) cultures. Lanes M, 1, 2, and 3 represent DNA markers, RT-PCR products, control with no reverse transcriptase, and PCR products with genomic DNA as the template, respectively. Similar results were obtained with total RNA extracted from 3-day biofilms (data not shown). B. For reporter gene fusion study, the full-length brpA promoter region was cloned in front of a promoterless firefly luciferase gene, and the resulting promoter-reporter fusion was integrated into the S. mutans genome. Cells carrying the reporter fusion were collected at different times during growth and assayed for luciferase activity by mixing 200 µL of whole cells with 50 µL of D-luciferin, 1 mM, pH 6.0. Data shows reporter luciferase activity (open squares) in relative light units (RLU) calibrated with the optical density (OD_{600nm}) of the cultures (solid circles) at each time point.
References


Table 1. Bacterial strains, plasmids and primers used in the study

<table>
<thead>
<tr>
<th>Strains /plasmids</th>
<th>Major properties</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mutans</em> UA159</td>
<td>Wild-type, serotype c</td>
<td></td>
</tr>
<tr>
<td><em>S. mutans</em> OMZ175</td>
<td>Wild-type, serotype c</td>
<td>(2)</td>
</tr>
<tr>
<td><em>S. mutans</em> TW14</td>
<td>UA159 derivative, <em>brpA</em>, erythromycin resistant</td>
<td>(43)</td>
</tr>
<tr>
<td><em>S. mutans</em> TW14D</td>
<td>UA159 derivative, <em>brpA</em>, erythromycin resistant</td>
<td>(42)</td>
</tr>
<tr>
<td><em>S. mutans</em> TW14K</td>
<td>UA159 derivative, <em>brpA</em>, kanamycin resistant</td>
<td>This study</td>
</tr>
<tr>
<td><em>S. mutans</em> TW230</td>
<td>OMZ175 derivative, <em>brpA</em>, erythromycin resistant</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em> DH10B</td>
<td>Cloning host, <em>mcrA</em>, <em>mcrBC</em>, <em>mrr</em>, and <em>hsd</em></td>
<td>Invitrogen, Inc.</td>
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<tr>
<td>pFW5-luc</td>
<td>Integration vector containing promoterless luciferase gene and a spectinomycin resistance marker</td>
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**Primers for RACE**

<table>
<thead>
<tr>
<th>Name</th>
<th>DNA sequence (5’ to 3’)</th>
<th>Applications</th>
</tr>
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<tbody>
<tr>
<td>5’ RACE Adapter</td>
<td>gcugaugcgauguaugaacgtgctcgaaagaa</td>
<td>5’ RACE</td>
</tr>
<tr>
<td>5’ RACE Outer</td>
<td>gcgttgctcgaaagaa</td>
<td>5’ RACE</td>
</tr>
<tr>
<td>5’ RACE Inner</td>
<td>gccggctcgaaagaa</td>
<td>5’ RACE</td>
</tr>
<tr>
<td>RACE brpA Rev</td>
<td>gcgaacctctgttcgag</td>
<td>5’ RACE</td>
</tr>
<tr>
<td>brpA SP1</td>
<td>gcgtttgctgtagtaagccaaagaa</td>
<td>sequencing</td>
</tr>
<tr>
<td>brpA SP2</td>
<td>gcgtttgctgtagtaagccaaagaa</td>
<td>sequencing</td>
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</table>

**Primers for reporter fusion, RT-PCR and qPCR**

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward (5’ to 3’ )</th>
<th>Reverse (5’ to 3’ )</th>
<th>Applications</th>
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</thead>
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<tr>
<td>PbrpA</td>
<td>tttctctgtagttgtaagtg</td>
<td>tcagctcttctcagtaagcatac</td>
<td>promoter-reporter fusion</td>
</tr>
<tr>
<td>SMU.409-brpA</td>
<td>aaggtgctctctgttcagtg</td>
<td>aacgtctctctgttcagtaagcatac</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>brpA:erm</td>
<td>agctctctctctgttcagtg</td>
<td>aacgtctctctgttcagtaagcatac</td>
<td>ΔbrpA:erm amplification</td>
</tr>
<tr>
<td>SMU.409P5</td>
<td>tacaacctctcctgttcagtaagc</td>
<td>attctctctctctgttcagtaagcatac</td>
<td>5’ fragment for polar insertion</td>
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<tr>
<td>SMU.409P3</td>
<td>ccaacctctctcctgttcagtaagc</td>
<td>aacgtctctctctgttcagtaagcatac</td>
<td>3’ fragment for polar insertion</td>
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<tr>
<td>SMU.246</td>
<td>tcggtctctctctgttcagtaagc</td>
<td>tcggtctctctctgttcagtaagcatac</td>
<td>SMU.246 fragment, 135 bp</td>
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<tr>
<td>SMU.549</td>
<td>gcgtctctctgttcagtaagc</td>
<td>gcgtctctctctgttcagtaagcatac</td>
<td>SMU.549 fragment, 84 bp</td>
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<tr>
<td>SMU.599</td>
<td>gcgtctctctctgttcagtaagc</td>
<td>gcgtctctctctgttcagtaagcatac</td>
<td>SMU.599 fragment, 82 bp</td>
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<tr>
<td>SMU.1677</td>
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<td>SMU.1677 fragment, 121 bp</td>
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<td>SecA</td>
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<td>SecY</td>
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<td>gcgtctctctctgttcagtaagcatac</td>
<td>secY fragment, 155 bp</td>
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<td>BtpA</td>
<td>gcgtctctctctgttcagtaagc</td>
<td>gcgtctctctctgttcagtaagcatac</td>
<td>brpA fragment, 148 bp</td>
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*Note: Nucleotides underlined are restriction site engineered for cloning.*
Table 2. Effect of BrpA-deficiency on susceptibility to cell envelope antimicrobials*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lipid II inhibitors</th>
<th>Non-lipid II inhibitors</th>
<th>Cell membrane-disrupting agents</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Van</td>
<td>Nis</td>
<td>Bac</td>
</tr>
<tr>
<td></td>
<td>MIC</td>
<td>MIC</td>
<td>MIC</td>
</tr>
<tr>
<td></td>
<td>MBC</td>
<td>MBC</td>
<td>MBC</td>
</tr>
<tr>
<td>UA159</td>
<td>0.75</td>
<td>1.25</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>TW14D</td>
<td>0.50</td>
<td>1</td>
<td>10^7</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>11000</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Note: *, Van, vancomycin; Nis, nisin; Bac, bacitracin; Pen, penicillin G; D-cyc, D-cycloserine; Chl, chlorhexidine; SDS, sodium dodecyl sulfate; and Tri, triclosan. Superscript a and b represents a reduction in MIC and/or MBC of more than 1.5 and 2-fold, respectively, as compared to UA159. All concentrations are in micrograms per milliliter.
Table 3. Luciferase expression in response to oxidative stress and cell envelope active antimicrobial agents

<table>
<thead>
<tr>
<th>Stimuli and stressor (concentration)</th>
<th>RLU</th>
</tr>
</thead>
<tbody>
<tr>
<td>hydrogen peroxide (0.4 mM)</td>
<td>2.09 ± 0.33c</td>
</tr>
<tr>
<td>methyl viologen (10 mM)</td>
<td>2.07 ± 0.55b</td>
</tr>
<tr>
<td>SDS (8 µg/mL)</td>
<td>2.51 ± 0.50c</td>
</tr>
<tr>
<td>chlorhexidine (1.5 µg/mL)</td>
<td>3.03 ± 0.42c</td>
</tr>
<tr>
<td>vancomycin (0.75 µg/mL)</td>
<td>1.41 ± 0.03a</td>
</tr>
<tr>
<td>bacitracin (20 µg/mL)</td>
<td>2.24 ± 0.13a</td>
</tr>
<tr>
<td>nisin (10 µg/mL)</td>
<td>2.25 ± 0.47b</td>
</tr>
<tr>
<td>D-cycloserine (20 µg/mL)</td>
<td>1.62 ± 0.31b</td>
</tr>
<tr>
<td>penicillin G (0.04 µg/mL)</td>
<td>1.66 ± 0.35a</td>
</tr>
</tbody>
</table>

Note: *, Data were expressed as the ratio (average ±standard deviation) of the luciferase activity in RLU at the conditions specified over controls that received equal amount of solvent instead of the stressor indicated, with a superscript of a, b, and c indicating a difference between the groups at the significance level of P<0.05, 0.01 and 0.001, respectively.
Table 4. Selected genes identified by DNA microarray analysis

<table>
<thead>
<tr>
<th>Unique ID</th>
<th>Description / putative functions*</th>
<th>Array Ratio#</th>
<th>qPCR Ratio@</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMU.246</td>
<td>glycosyl transferase N-acetylglucosaminyltransferase, RgpG</td>
<td>-2.6</td>
<td>-4.0</td>
<td>5.9E-3</td>
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<tr>
<td>SMU.549</td>
<td>undecaprenyl-PP-MurNAc-pentapeptide-UDPGlCNAc GlcNAc transferase, MurG</td>
<td>-2.7</td>
<td>-2.0</td>
<td>3.6E-3</td>
</tr>
<tr>
<td>SMU.599</td>
<td>D-alanine-D-alanine ligase, DdlA</td>
<td>-1.8</td>
<td>-3.2</td>
<td>7.1E-3</td>
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<tr>
<td>SMU.1689</td>
<td>D-alanyl carrier protein, DicC</td>
<td>3.4</td>
<td>nd</td>
<td>6.9E-3</td>
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<tr>
<td>SMU.1691</td>
<td>D-alanine-D-alanyl carrier protein ligase, DltA</td>
<td>3.4</td>
<td>nd</td>
<td>3.7E-5</td>
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<td>SMU.1838</td>
<td>preprotein translocase subunit SecA</td>
<td>-1.9</td>
<td>-2.0</td>
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<td>SMU.1948</td>
<td>preprotein translocase subunit SecE</td>
<td>-2.8</td>
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<td>SMU.2006</td>
<td>preprotein translocase SecY protein</td>
<td>3.9</td>
<td>1.9</td>
<td>1.6E-5</td>
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</table>

Note: *, description and putative function of the identified genes are based upon the published S. mutans database. # and @, defined as levels of expression in the BrpA-deficient mutant relative to those of the wild-type shown by DNA microarray analysis and RealTime-PCR, respectively, with "-" representing down-regulation. nd, not done.
Figure 1
Figure 3
A. SMU.409 brpA (SMU.410) SMU.411 SMU.412

B. SMU.409

TCATTTTTTGAAAATAGTTGGAAGTAATAAAAG
TTTTGATATAATACTTTTA
TGTTTTATAGCCAAAATGAAAATCAGCTAATGGCTC

BrpA

TTGGACAGAGAATTGGTCAAAAACTACAAGCTCAAGACGTTCTTGTTCTGACAGGCGATTTAGGATCTGGGAAAACCACTCTGACAAA
G

OxyR-like

GAATTGCCAAAGGTTTGGAATACAGATTTAAAGTCCAACTACCTAGATTGCTGAAATATAGGGAGGGACTGCCATTTTAT

PhoB

TTGGAGATCTCTTCCAACATTATAATAGCTCAAGTCTCGTATTAATTACCTTGGCTGCTTTTATTGAAAAAAGCTCAATCAGTCAATGAAATTTGTGTGATTGTAAGCTAGTCGGCCAAGTGATAGAGCTTGTGATGATT

Fnr-like

AAAAAGTTATCAGAAAAATTCGGAAAACCGATAGAGATTTTTAATTTGAGATGAAAGTGCCCTCATATATACCTGCTCAAGAAA

Figure 4
Figure 5