The two-component system BfrAB regulates expression of ABC transporters in *Streptococcus gordonii* and *Streptococcus sanguinis*

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The putative two-component system BfrAB is involved in *Streptococcus gordonii* biofilm development. Here, we provide evidence that BfrAB regulates the expression of *bfrCD* and *bfrEFG*, which encode two ATP-binding cassette (ABC) transporters, and *bfrH*, which encodes a CAAX amino-terminal protease family protein. BfrC and BfrE are ATP-binding proteins, and BfrD, BfrF and BfrG are homologous membrane-spanning polypeptides. Similarly, BfrAB_{ss}, the BfrAB homologous system in *Streptococcus sanguinis*, controls the expression of two *bfrCD*-homologous operons (*bfrCD*_{ss} and *bfrXY*_{ss}), a *bfrH*-homologous gene (*bfrH1*_{ss}) and another CAAX amino-terminal protease family protein gene (*bfrH2*_{ss}). Furthermore, we demonstrate that the purified BfrA DNA-binding domain from *S. gordonii* binds to the promoter regions of *bfrCD*, *bfrEFG*, *bfrH*, *bfrCD*_{ss}, *bfrXY*_{ss} and *bfrH1*_{ss} *in vitro*. Finally, we show that the BfrA DNA-binding domain recognizes a conserved DNA motif with a consensus sequence of TTCTTTAGAAATATTTAGAATT. These data suggest, therefore, that *S. gordonii* BfrAB controls biofilm formation by regulating multiple ABC-transporter systems.

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INTRODUCTION

Dental plaque is a polymicrobial biofilm on the saliva-coated tooth surface that can cause caries, gingivitis and periodontal diseases (Rosan & Lamont, 2000). *Streptococcus gordonii* is a pioneer colonizer of dental plaque, preferentially adhering to cleaned saliva-coated tooth surfaces (Nyvad & Kilian, 1987, 1990). *S. gordonii* also colonizes other oral sites and is not normally harmful in the human oral cavity (Frandsen *et al.*, 1991; Socransky *et al.*, 1998). When introduced into the blood, however, *S. gordonii*, like other oral streptococci, can be involved in the development of infective endocarditis (Bayliss *et al.*, 1983; Herzberg, 1996; Manford *et al.*, 1992).

Abbreviations: ABC, ATP-binding cassette; EMSA, electrophoretic mobility shift assay; TCS, two-component system.

The array data discussed in this paper have been deposited in MIAMEXPRESS (<http://www.ebi.ac.uk/microarray>) and are accessible through accession number E-MEXP-1712.

A supplementary table listing primers used in this work is available with the online version of this paper.

To colonize several intraoral sites or to enter the circulating blood and infect heart valves, oral streptococci must adapt to environmental changes. Gene regulation by two-component systems (TCSs) is a common mechanism used by bacteria to modulate cell behaviour in response to environmental changes (Stock *et al.*, 2000). TCSs are involved in the regulation of biofilm development in several species, including *Escherichia coli* (Dorel *et al.*, 1999; Otto & Silhavy, 2002; Prigent-Combaret *et al.*, 1999), *Pseudomonas aeruginosa* (Parkins *et al.*, 2001), *Staphylococcus aureus* (Fournier & Hooper, 2000; Toledo-Arana *et al.*, 2005), *Streptococcus mutans* (Bhagwat *et al.*, 2001; Li *et al.*, 2002; Shemesh *et al.*, 2007) and *Enterococcus faecalis* (Hancock & Perego, 2004). A TCS is typically composed of a histidine kinase and a response regulator. The response regulator generally acts to regulate transcription. After detection of the environmental signal, the histidine kinase undergoes autophosphorylation of a conserved histidine residue. The phosphoryl group is subsequently transferred to a conserved aspartate residue of a cognate response regulator. Aspartate phosphorylation

of the response regulator alters the binding affinity for target sites and leads to changes in gene expression.

In *S. gordonii*, the TCS ComDE is involved in competence, regulating DNA uptake (Havarstein *et al.*, 1996; Lunsford, 1998), and is required for biofilm formation *in vitro* (Loo *et al.*, 2000). The signal is a secreted competence-stimulating peptide (CSP), encoded by *comC*, which is a member of a three-gene operon *comCDE*. The extracellular CSP binds the histidine kinase ComD, which subsequently phosphorylates the response regulator ComE. ComE transcriptionally regulates expression of over 100 genes (Vickerman *et al.*, 2007).

We previously characterized the role of a TCS, BfrAB, in *S. gordonii* biofilm development (Zhang *et al.*, 2004). In the present study we identify genes regulated by the BfrAB TCS in *S. gordonii*. Using DNA microarray analysis, RT-PCR and electrophoretic mobility shift assays (EMSAs), we show that under the tested conditions, BfrAB regulates the expression of *bfrCD* and *bfrEFG* operons, which encode two ATP-binding cassette (ABC) transporters, and one additional gene *bfrH*, which encodes a homologue to the CAAX amino-terminal protease family. We also provide evidence that *bfrAB* and *bfrAB*-homologous systems share common roles as regulators of *bfrCD* and *bfrCD*-homologous operons in *S. gordonii* and *Streptococcus sanguinis*. These results suggest that BfrAB controls biofilm formation by regulating multiple ABC-transporter systems.

METHODS

Bacterial strains and culture conditions. Strains of *S. gordonii* V288 and *S. sanguinis* SK36 (listed in Table 1) were routinely grown in chemically defined synthetic medium (FMC) (Terleckyj &

Shockman, 1975; Terleckyj *et al.*, 1975) at 37 °C in 5% CO₂. *E. coli* DH5 α cells were grown aerobically at 37 °C in Luria–Bertani (LB) medium. When required, antibiotics were added to the medium at the following concentrations: erythromycin (Em), 10 μ g ml⁻¹ (*S. gordonii* and *S. sanguinis*); kanamycin (Km), 50 μ g ml⁻¹ (*E. coli*), 250 μ g ml⁻¹ (*S. gordonii* and *S. sanguinis*); and tetracycline (Tc) 10 μ g ml⁻¹ (*S. gordonii*).

Genetic manipulations. Standard recombinant DNA techniques were employed as described by Sambrook *et al.* (1989). Plasmids (listed in Table 1) were purified from *E. coli* cells using the QIAquick Spin Miniprep Purification kit (Qiagen). Chromosomal DNA was prepared as described previously (Zhang *et al.*, 2004). Oligonucleotides (see Supplementary Table S1) were synthesized by Integrated DNA Technologies. PCR products were purified using the High Pure PCR Product Purification kit (Roche). DNA restriction and modification enzymes were used as specified by the manufacturer (Promega).

The *bfrAB* operons of *S. gordonii* V288 and *S. sanguinis* SK36 were inactivated by allelic exchange with the erythromycin-resistance determinant *ermAM*. *ermAM* was amplified from plasmid pVA891 (Macrina *et al.*, 1983) and cloned into pPCR-Amp SK (+) (Stratagene). Two DNA fragments constituting the flanking sequences of the *bfr* operons were then amplified and fused with the *ermAM* genes sequentially (Nobbs *et al.*, 2007). The fused construct was then PCR-amplified, purified and transformed into *S. gordonii* V288 or *S. sanguinis* SK36 as described previously (Tao & Herzberg, 1999), generating the deletion mutants *S. gordonii* V288*bfr*⁻ and *S. sanguinis* SK36*bfr*⁻. The insertions were confirmed by PCR amplification and sequencing.

To complement the *bfrAB* deletion mutant, a DNA fragment encompassing the entire *bfrAB* operon was amplified by PCR and cloned into the *E. coli*–streptococcal shuttle vector pDL276 (Dunny *et al.*, 1991). The construct was amplified in *E. coli*, purified, and used to transform the *S. gordonii* V288*bfr*⁻ mutant to obtain the complemented strain V288*bfr*⁺. Predicted insertions were confirmed by PCR amplification and sequencing. Complementation was confirmed by detection of the *bfr* RNA transcript. The complemented strain of *S. sanguinis*, SK36*bfr*⁺, was constructed using the same method.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Source or reference
<i>E. coli</i> strain		
DH5 α F'I ^q	F' <i>proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^q Δ (<i>lacZ</i>)M15 <i>zzf</i> ::Tn10 (Tet ^R)/ <i>fluA2</i> Δ (<i>argF-lacZ</i>)U169 <i>phoA glnV44 ϕ80 Δ(<i>lacZ</i>)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	New England Biolabs
<i>S. gordonii</i> strains		
V288	Wild-type	G. Dunny, University of Minnesota
MG288-1015	<i>bfrAB</i> :: <i>tet</i> (Tc ^R)	Zhang <i>et al.</i> (2004)
V288 <i>bfr</i> ⁻	Δ (<i>bfrAB</i>):: <i>ermAM</i> (Em ^R)	This study
V288 <i>bfr</i> ⁺	Δ (<i>bfrAB</i>):: <i>ermAM</i> (pDL276 <i>bfrAB</i>) (Em ^R ,Km ^R)	This study
<i>S. sanguinis</i> strains		
SK36	Wild-type	Kilian & Holmgren (1981)
SK36 <i>bfr</i> ⁻	Δ (<i>ssA_0401–0402</i>):: <i>ermAM</i> (Em ^R)	This study
SK36 <i>bfr</i> ⁺	Δ (<i>ssA_0401–0402</i>):: <i>ermAM</i> (pDL276 <i>ssA_0401–0402</i>) (Em ^R ,Km ^R)	This study
Plasmids		
pPCR-Amp SK (+)	3.0 kb; Ap ^R ; pUCori	Stratagene
pDL276	6.9 kb; Km ^R ; ColE1ori, <i>E. coli</i> –streptococcal shuttle vector	Dunny <i>et al.</i> (1991)
pVA891	5.4 kb; Em ^R , Cm ^R ; pACYC <i>ori</i> , <i>E. coli</i> –streptococcal shuttle vector	Macrina <i>et al.</i> (1983)
pQE80L	4.7 kb; Ap ^R ; ColE1ori, <i>E. coli</i> expression vector	Qiagen

RNA extraction and cDNA synthesis. RNA extraction from bacterial cultures, confirmation of RNA integrity and estimation of purity on agarose gels, and cDNA synthesis were performed as described previously (Zhang *et al.*, 2004, 2005). For each RNA sample, a control cDNA reaction in the absence of reverse transcriptase was performed to check for DNA contamination.

DNA microarray design, cDNA labelling and hybridization. Fresh FMC medium containing appropriate antibiotics was inoculated with 10% of the overnight cultures of *S. gordonii* strains and incubated at 37 °C in 5% CO₂. After 6 h, bacterial cells reached early stationary phase (OD₆₂₀ ~1.8). RNA was then isolated. DNA contamination was assessed by PCR amplification of the *clpX* gene, and RNA integrity was monitored by agarose gel electrophoresis of glyoxylated samples (Ambion). RNA was prepared for hybridization to a custom *S. gordonii* Affymetrix GeneChip microarray as previously described (Brown & Whiteley, 2007; Palmer *et al.*, 2005). Probes for the GeneChip were designed for each ORF using the *S. gordonii* genome sequence available at <http://www.ncbi.nlm.nih.gov> (probe sequences available from the authors). Washing, staining and scanning of the GeneChips was performed at the University of Iowa DNA Core Facility using an Affymetrix Fluidics Station. GeneChips were hybridized in duplicate for each condition. Data analysis was performed using GeneChip Operating Software version 1.4 to compare between two conditions, and genes were reported as differentially regulated ($P \leq 0.05$, $n=2$) based on pairwise statistical analysis as described previously (Brown & Whiteley, 2007).

Semiquantitative PCR. To compare the specific gene expression levels among the wild-type *S. gordonii* V288, V288*bfr*⁻ and V288*bfr*⁺, and among the wild-type *S. sanguinis* SK36, SK36*bfr*⁻ and SK36*bfr*⁺, semiquantitative RT-PCR (Nambu *et al.*, 2003) was used. Fourfold serial dilutions (1:4, 1:16, 1:64 and 1:256) of cDNA were prepared in deionized water. Each PCR was performed in a total volume of 25 µl containing 1 × Green GoTaq Flexi Buffer (Promega), 0.2 mM of each dNTP, 1.0 µM of each upstream primer and downstream primer, GoTaq DNA polymerase (Promega) and 3 µl diluted cDNA. The 16S rRNA gene was used as an internal control for RT-PCR as reported previously (Zhang *et al.*, 2005). The PCR conditions for protein-encoding genes were (i) 95 °C for 5 min; (ii) 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and (iii) 72 °C for 4 min. Sixteen amplification cycles were performed for the PCR of the 16S rRNA gene. The PCR products (5 µl) were analysed on 1.7% agarose gels and stained with ethidium bromide.

Expression and purification of the DNA-binding domain of BfrA. The DNA fragment (327 bp) encoding the DNA-binding domain of BfrA was amplified by PCR. The PCR products were digested with *Bam*HI and *Sal*I, ligated into the *Bam*HI/*Sal*I sites within the expression vector pQE80L (Qiagen) in the reading frame of the 5' (His)₆-encoding sequence, and transformed into NEB DH5α F'1^q competent *E. coli* cells (New England Biolabs). The construct was confirmed by DNA sequencing. The resulting cells were cultured in LB medium until growth reached OD₆₀₀ ~0.5–0.6. In these cultures, expression of the His-tagged DNA-binding domain of BfrA (His₆DBBfrA) was induced by incubation with 1 mM IPTG for 4 h at 37 °C. Cells were lysed and inclusion bodies were isolated using B-PER Bacterial Protein Extraction Reagent (Pierce) following the manufacturer's instructions. The isolated inclusion bodies were dissolved in 6 M guanidine hydrochloride and purified using a Ni-NTA Superflow column (Qiagen) under denaturing conditions, as described in The QIAexpressionist, fifth edition (Qiagen). The peak fractions were adjusted to pH 8.0 by 1 M Tris base. The refolding buffers were first optimized using the Protein Refolding kit (Athena Environmental Sciences). The refolding was then performed in the selected buffer (50 mM Tris/HCl, pH 8.5, 9.6 mM NaCl, 0.4 mM

KCl, 1 mM EDTA, 0.5% Triton X-100 and 1 mM DTT) following the manufacturer's instructions.

EMSA. A non-isotopic EMSA kit (Molecular Probes) was used to test the interactions of the His₆DBBfrA and the upstream promoter-containing regions of the target genes. The upstream intergenic regions of the target genes were amplified by PCR. The purified PCR product (40 ng) was then mixed with the refolded His₆DBBfrA in 1 × binding buffer (10 mM Tris/HCl, pH 8.0, 50 mM KCl, 0.5 mM DTT, 0.05 mM EDTA, 1 mM MgCl₂ and 5%, v/v, glycerol) in a total reaction volume of 20 µl. The mixture was incubated for 30 min at room temperature and then mixed with 4 µl 6 × loading buffer. The DNA–protein complexes (20 µl per lane) were then examined by electrophoresis using 5% nondenaturing polyacrylamide gels stained with SYBR Green and visualized using a 314 nm UV transilluminator following the manufacturer's protocol.

An isotopic EMSA was used to test for interactions of His₆DBBfrA with the synthesized double-strand DNA probes. The probes were 5' end-labelled with [γ -³²P]ATP by T4 polynucleotide kinase (Promega) and purified using Mini Quick Spin Oligo Spin Columns (Roche). ³²P-labelled probes (10 000 c.p.m.) were mixed with the refolded His₆DBBfrA in 1 × binding buffer (10 mM Tris/HCl, pH 8.0, 50 mM KCl, 0.5 mM DTT, 0.05 mM EDTA, 1 mM MgCl₂, 5%, v/v, glycerol, 0.025 mg poly(dI-dG) ml⁻¹ and 0.5 mg BSA ml⁻¹) in a total reaction volume of 20 µl. In a competition binding assay, 6 pmol of the unlabelled competitor DNA probe or noncompetitor probe was added. The mixture was incubated for 30 min at room temperature and then mixed with 4 µl 6 × loading buffer. The DNA–protein complexes (20 µl per lane) were then examined by electrophoresis using 8% nondenaturing polyacrylamide gels and visualized by autoradiography.

Gene sequences. The annotated genomes of *S. gordonii* (NC_009785) and *S. sanguinis* (NC_009009) are available at <http://www.ncbi.nlm.nih.gov>.

RESULTS

Identification of genes regulated by the BfrAB TCS in *S. gordonii*

To identify BfrAB-regulated genes in *S. gordonii*, a transcriptome analysis was performed for *S. gordonii* V288 wild-type and two *bfr*-negative mutants using a custom Affymetrix GeneChip microarray. Two *bfrAB* mutants, an insertional mutant (MG288-1015) (Zhang *et al.*, 2004) and a deletion mutant (V288*bfr*⁻) constructed for this study, were utilized to ensure that any genes identified as differentially regulated were not specific to either mutant. For DNA microarray assays, RNA was extracted from early stationary phase cells, because at this phase the *bfrA* promoter exhibits higher activity than during exponential phase (Zhang *et al.*, 2004). Seven putative genes (designated *bfrC*, *bfrD*, *bfrE*, *bfrF*, *bfrG*, *bfrH* and *bfrX*; Table 2) were expressed at lower levels in both mutants compared with the wild-type *S. gordonii* V288. Since the altered expression of these genes was not specific to either *bfr* mutant, their reduced expression levels more probably resulted from the impaired functions of *bfrAB*. These genes, therefore, were selected for further characterization in this study.

Table 2. Genes differentially expressed in the *S. gordonii* V288 wild-type and the Bfr[−] mutants

Gene name: function (GenBank accession no.; microarray probe ID)	Fold-change in gene expression relative to V288 wild-type	
	V288bfr [−]	MG288-1015
<i>bfrC</i> : ABC transporter, ATP-binding protein (ABV10088; SG1561)	−8.9	−5.5
<i>bfrD</i> : unknown (ABV09959; SG1560)	−8.7	−6.7
<i>bfrE</i> : ABC transporter, ATP-binding protein (ABV11086; SG1564)	−11.7	−6.3
<i>bfrF</i> : unknown (ABV10645; SG1563)	−12.2	−8.9
<i>bfrG</i> : unknown (ABV10185; SG1562)	−12.6	−9.8
<i>bfrH</i> : CAAX amino-terminal protease family protein (ABV10380; SG0950)	−3.4	−4.9
<i>bfrX</i> : ABC transporter, ATP-binding protein (ABV09881; SG1558)	−3.0	−4.4

The chromosomal location of the *bfrH* gene, which encodes a CAAX amino-terminal protease family protein, is remote from the *bfr* locus (Fig. 1). The other six genes are located immediately downstream of *bfrAB* (Fig. 1) and are transcribed in the direction opposite to *bfrAB*. The *bfrC* and *bfrD* sequences are separated by a 12 bp intergenic sequence, which suggested that *bfrC* and *bfrD* were co-transcribed as an operon. The co-transcription of *bfrC* and *bfrD* was confirmed by RT-PCR (data not shown). The *bfrE* and *bfrF* sequences are separated by a 16 bp intergenic sequence, and *bfrF* and *bfrG* are separated by a 37 bp intergenic sequence. The co-transcription of *bfrEF* and *bfrFG* was also confirmed by RT-PCR (data not shown). Thus, *bfrEFG* forms a three-gene operon. Similarly, *bfrX* and its immediate downstream gene, *bfrY*, are separated by a 6 bp intergenic sequence, and are co-transcribed (data not shown). Protein sequence analysis suggested that *bfrXY*, *bfrCD* and *bfrEFG* encoded three putative ABC transporters. Among these genes, *bfrX*, *bfrC* and *bfrE* encode three Walker motif-containing ATP-binding proteins with high similarities; *bfrY*, *bfrD*, *bfrF* and *bfrG* encode four homologous transmembrane polypeptides. Bioinformatic analysis using the SOSUI server (<http://bp.nuap.nagoya-u.ac.jp/sosui/>) predicts that BfrH, BfrY, BfrD, BfrF and BfrG each contain six transmembrane helices.

S. gordonii

S. sanguinis

Fig. 1. Genetic loci regulated by BfrAB and BfrAB_{ss}. Grey arrows, genes regulated by BfrAB/BfrAB_{ss} systems based on data from this study; white arrows, genes not regulated by BfrAB/BfrAB_{ss} systems based on data from this study.

To validate the data from DNA microarray experiments, semiquantitative RT-PCR experiments were performed. In agreement with the data from the DNA microarray results, the RT-PCR experiments showed that the *S. gordonii* V288bfr[−] strain expressed lower levels of *bfrCD*, *bfrEFG* and *bfrH* than the wild-type (Fig. 2a). However, the decreased expression of *bfrX* in *S. gordonii* V288bfr[−] seen in DNA microarray analysis was not observed using semiquantitative RT-PCR (Fig. 2a).

To determine whether the altered expression of *bfrCD*, *bfrEFG* and *bfrH* was directly associated with the inactivation of the BfrAB TCS, a strain complemented for the deletion of *bfr* was constructed by transforming the V288bfr[−] strain with the shuttle vector pDL276 harbouring the *bfrAB* operon. The expression levels of *bfrCD*, *bfrEFG* and *bfrH* were largely restored in the complementary strain V288bfr⁺ (Fig. 2a), indicating that BfrAB is involved in the regulation of these genes.

Identification of BfrAB-homologous TCSs in *S. sanguinis*

Using the BLASTP program (Altschul *et al.*, 1997; Schaffer *et al.*, 2001), we searched the nonredundant sequence database (<http://www.ncbi.nlm.nih.gov>) using BfrA and BfrB protein sequences to find Bfr-homologous TCSs in other bacterial species. The *S. sanguinis* TCS of SSA_0401-0402 (BfrAB_{ss}) showed the highest similarity (~90 %) to BfrAB. In addition, the *S. sanguinis* genome contains genes homologous to BfrC, BfrD, BfrE, BfrF, BfrG, BfrH, BfrX and BfrY.

As in *S. gordonii*, the *bfrH*-homologous gene *ssA_0253* (*bfrH1_{ss}*) in *S. sanguinis* was outside the *bfr*-homologous locus (Fig. 1). However, *bfrAB_{ss}* is immediately followed by *ssA_0403* (*bfrH2_{ss}*), which encodes another CAAX amino-terminal protease family protein, and *ssA_0405*, which encodes a xenobiotic response element (XRE) family transcriptional regulator (Fig. 1). The XRE family proteins interact with cognate DNA sequences through their helix–turn–helix (HTH) motifs. Both *bfrH2_{ss}* and *ssA_0405* have their own putative promoters, which were identified using the program ‘Neural Network Promoter Prediction’

Fig. 2. Semiquantitative RT-PCR of selected genes. (a) Expression of selected genes in *S. gordonii* V288 wild-type, V288bfr⁻ and V288bfr⁺. (b) Expression of selected genes in *S. sanguinis* SK36 wild-type, SK36bfr⁻ and SK36bfr⁺. The 16S rRNA gene was used as an internal control for RT-PCR. Fourfold serial dilutions (represented by the declining slopes of the triangles) of cDNAs were used as PCR templates to amplify the indicated transcripts. All data shown are representative of two independent experiments.

(http://www.fruitfly.org/seq_tools/promoter.html), and are transcribed in the same direction as the *bfrAB_{ss}* operon. Three *bfrCD/bfrEFG/bfrXY*-homologous operons, *ssA_0407–0406* (*bfrXY_{ss}*), *ssA_0409–0408* (*bfrCD_{ss}*) and *ssA_0412–0411–0410* (*bfrEFG_{ss}*), are located in close proximity downstream of *ssA_0405* and transcribed in the opposite direction (Fig. 1). The co-transcription of *bfrX_{ss}/bfrY_{ss}*, *bfrC_{ss}/bfrD_{ss}* and *bfrE_{ss}/bfrF_{ss}/bfrG_{ss}* was confirmed by RT-PCR (data not shown).

Identification of genes regulated by TCS BfrAB_{ss} in *S. sanguinis*

The high similarity of the protein sequences between BfrAB in *S. gordonii* and BfrAB_{ss} in *S. sanguinis* suggests that the functions of the TCSs might be conserved in the two species. To identify genes regulated by BfrAB_{ss} in *S. sanguinis*, we constructed a deletion mutant by allelic exchange. The expression of a set of selected genes was first compared between the *bfrAB_{ss}* deletion mutant strain (SK36bfr⁻) and the *S. sanguinis* wild-type. SK36bfr⁻

expressed *bfrH1_{ss}*, *bfrH2_{ss}* and the two operons *bfrXY_{ss}* and *bfrCD_{ss}* at substantially lower levels than the wild-type (Fig. 2b). However, the expression levels of *ssA_0405* and the operon *bfrEFG_{ss}* were similar between SK36bfr⁻ and the parent wild-type strain (data not shown). Furthermore, we constructed a complemented strain for SK36bfr⁻ to confirm that the altered expression of these genes was the direct result of inactivation of the BfrAB_{ss} TCS. The expression levels of *bfrH1_{ss}*, *bfrH2_{ss}* and of the operons *bfrXY_{ss}* and *bfrCD_{ss}* were restored in the complementary strain SK36bfr⁺ (Fig. 2b), indicating that the BfrAB_{ss} TCS is involved in the regulation of these genes in *S. sanguinis*.

Binding of the BfrA DNA-binding domain to the upstream promoter-containing regions of BfrAB-regulated genes *in vitro*

The response regulator BfrA is comprised of two conserved domains, an N-terminal signal receiver domain (amino acid residues 5–116) and a C-terminal DNA-binding domain (residues 129–223). Our results indicated that BfrAB is involved in the regulation of multiple genes in *S. gordonii*. We then examined whether BfrA could bind to the upstream promoter-containing regions of these genes, thus directly controlling their transcription activities. First, a fusion protein (His₆DBBfrA), including a C-terminal fragment of the BfrA protein (DBBfrA, amino acids 118–226) and an N-terminal 6× histidine tag, was over-expressed in *E. coli* and purified. The interactions of the purified His₆DBBfrA with the upstream promoter-containing regions of selected genes were then tested by EMSA.

Purified His₆DBBfrA bound to the upstream promoter-containing regions of *bfrCD*, *bfrEFG* and *bfrH* as shown by EMSA (Fig. 3). In contrast, His₆DBBfrA protein could not bind to the upstream promoter-containing regions of the *S. gordonii* *sthAB* operon (Fig. 3) or *comCDE* operon (data not shown). Since BfrA and its *S. sanguinis* counterpart BfrA_{ss} share high similarity (Fig. 4), we tested the interactions of His₆DBBfrA with the upstream promoter-containing regions of BfrAB_{ss}-regulated genes in *S. sanguinis*. His₆DBBfrA bound to the upstream promoter-containing regions of *bfrXY_{ss}*, *bfrCD_{ss}* and *bfrH1_{ss}* (Fig. 3). His₆DBBfrA, however, did not bind to the upstream promoter-containing regions of *bfrH2_{ss}* (Fig. 3). In addition, binding to His₆DBBfrA by the promoter-containing regions of *bfrCD*, *bfrEFG*, *bfrH*, *bfrXY_{ss}*, *bfrCD_{ss}* and *bfrH1_{ss}* was His₆DBBfrA dose-dependent in all cases (Fig. 3), which also suggested that His₆DBBfrA interacted selectively with these six promoter-containing regions.

Identification of the DNA motif recognized by BfrA

To identify the potential DNA motif recognized by BfrA, we aligned the upstream promoter-containing regions of *bfrH*, *bfrC*, *bfrE*, *bfrXY_{ss}*, *bfrCD_{ss}* and *bfrH1_{ss}* (Fig. 5). A 24 bp conserved DNA motif with a consensus sequence of

Fig. 3. Non-isotopic EMSA of binding of His₆DBBfrA to the upstream promoter-containing regions of selected genes. The upstream promoter-containing regions of the selected genes were amplified by PCR, purified and used to test the interactions with varying amounts of purified His₆DBBfrA. The reaction mixtures were separated on 5% non-denaturing polyacrylamide gels, stained with SYBR Green and visualized using a 314 nm UV transilluminator. Control, His₆DBBfrA protein-only control.

TTTCTTTAGAAATATTTTAGAATT was identified. This conserved motif contains two direct repeats of TTTAGAA, which are separated by a 4 bp intervening sequence. This 24 bp conserved motif is not found in the upstream promoter-containing regions of *bfrH2_{ss}*, or the *S. gordonii* *sthAB* and *comCDE* operons. As described above, the upstream promoter-containing regions of *bfrH2_{ss}*, and the *S. gordonii* *sthAB* and *comCDE* operons were not recognized by His₆DBBfrA.

Purified His₆DBBfrA protein could bind to and retard the mobility of the 24 bp conserved DNA motif found in *bfrCD* (Fig. 6a, lane 2). In contrast, an 18 bp DNA fragment, TTTAGAAATATTTTAGAA, which is located

in the 24 bp conserved motif and includes the two direct repeats of TTTAGAA and a 4 bp intervening sequence, could not complex with His₆DBBfrA (Fig. 6b, lane 2). The presence of excess unlabelled 24 bp conserved DNA motif could effectively inhibit the His₆DBBfrA-mediated gel shift (Fig. 6a, lane 3). However, the presence of the excess unlabelled 18 bp DNA fragment had no effect on the complex formation of His₆DBBfrA and the 24 bp DNA motif (Fig. 6a, lane 4). Similarly, purified His₆DBBfrA protein could interact with the 24 bp conserved DNA motifs found in *bfrEFG* (Fig. 6c) and *bfrH* (Fig. 6d). These results suggested that His₆DBBfrA could specifically recognize this 24 bp conserved DNA motif.

BfrA	MARILVIEDNNDIHAILTSLFEKEHEVYSAYSGTEGLAVFEREKPDVLVLLDIMLPKGNGD	60
BfrAss	MAHILLIEDNSDIHEILKDLFQKEHTVYSAYSGTEGISLFEEREIDVLVLLDIMLPKGNGD	60
SMU.1038c	MARILQIEDNNDIQKILRDFEFEEHEVIQAYSGETGLRLFESENIDVLVLLDIMLPKGSGE	60
	: ***:***: ** *:***:*** ***:***:*** ***:***:*** ***:***:*** ***:***:***	
BfrA	QVLKEIRRTNRQVPFILMLTALGEKSLVSEYLLNGANDYIVKPFNLDEVFARVTVQLR---	117
BfrAss	EVLKEIRSSNATVPVVMLTALGEKALVSQYLLNGANDYIVKPFNLDEVFARITVQLR---	117
SMU.1038c	QVLQEIRKVS-SVPIIMLTALSDKQMSIQYLLAGANDYIVKPFNLEEVYARVTVQLRST	119
	:***:*** . ***:***:*** ***:***:*** ***:***:*** ***:***:*** ***:***:***	
BfrA	-SQHASSAEESLCKFKNIQLLPATFQAVCGELEVRLSKKEFQIFQLLLRHPKKIFTKEAL	176
BfrAss	-SQQALSGEIEGQSFKNIRLLKATFEAVCGEKSIVRLSKKEYHILQLLLRHPKKIYTKEEL	176
SMU.1038c	KSHNPATTKQTALSINKNITVNPDTFEISYLEKSVRLGKKEFQILHALQYPRKIFTKEDL	179
	*::: .:: .:::***: ***: ***: ***:***:*** ***:***:*** ***:***:***	
BfrA	FESVWEEAYLPGDNTLNLTHLSNLRKKLAQLDPSEYIETVWGLGVRLK-ED	226
BfrAss	FEQVWEEAYLPGDNTLNLTHLSNLRKKLAQLDPTEYIETVWGLGVRLK-ED	226
SMU.1038c	YESVWKEPYLPGDNTLNAQLSNLRKKLAQVDDSTDYIETIWGLGVRLKGED	230
	:***	

Fig. 4. Comparison of the primary sequences of BfrA, BfrAss and SMU.1038c. *S. gordonii* BfrA and homologous genes in *S. sanguinis* (BfrAss) and *S. mutans* (SMU.1038c) were aligned using CLUSTALW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). '*', ':' and '.' represent identical residues, highly conserved residues and weakly conserved residues, respectively.

P_{bfrC}	ATATACAGAGTTAGTCCCTGACTAGCTCTTTT-TGTTTGTCTTTGTATTTC-G--TATTT 56
P_{bfrC_{ss}}	ATTTAAGCAGTCAGCCCTTTGGCTGCTTTTATTGCTTTTGGATTGATTCTG--CATT 58
P_{bfrH}	GGCAAGAAAATGGAAGCAGTAAAAATCTTGGGTTT-TATACCTTTAAAAATCTC-TTTTT 58
P_{bfrH_{1ss}}	AGCAACAAGTTGAAGTGGCGATAATCCTTGATTGTTTATGTTTATAAATTTCTTTTCATT 60
P_{bfrX_{ss}}	----AAACAATGAGCTGGTCTTGACTGGCTCATTTTAATCTTTCAATTTT--AAATTT 54
P_{bfrE}	ACACAAGCTAGATATAACTGAAAAATTTCTGCTATTTCAGGAATTTTGTCTAAAAA 60
P_{bfrC}	CTTTAGAAATACITTTAAAAAGACTTGAGAAATATTTTAAATTTAGTTTGTATACTT-TAG 115
P_{bfrC_{ss}}	CTTTAGAAATATTTTAGAATTCTTGAGAAATATTTGAAATCTAGCTTGTATACTA-AAA 117
P_{bfrH}	CTTTAGAAACATTTTAGAATTACTTAATAAATAATTGAAGTTTGCTTTATATAATG-ATG 117
P_{bfrH_{1ss}}	CTTTAGAAATATTTTAGAATTACTTTATAAATCTTGAAAGTTTGCTTTGTATAATC-ATA 119
P_{bfrX_{ss}}	CTTTAGAAATATTTTAGAATTCTTGAGAAATATTTGAAATTTAGTTTGTATACTA-AAA 113
P_{bfrE}	CTTTAGAAATATTTTAGAATGCTTGAGAAATATTTGAAAGATTGGTACTATACTGGATA 120
P_{bfrC}	TTA--AACCTAA--GAAAGCGAGAA-TATGCT-----ATG 145
P_{bfrC_{ss}}	TCA--AACCTAG--GAAAGCGAGAA-TATACC-----ATG 147
P_{bfrH}	GTA--AAGAAAT--GAGAGAAAGGAG-TTCTA-----ATG 148
P_{bfrH_{1ss}}	TTA--AAGAAA-----AGAAAGGAG-TCTCTTATC-----ATG 149
P_{bfrX_{ss}}	GTATAACAAAG-TAATTAAAGGAGAAACATC-----ATG 147
P_{bfrE}	GTGAAAAGAGAGCTTCCTCTCCCTATCTATACAGGAGAACTCATCATG 169

Fig. 5. Alignment of the His₆DBBfrA-recognized promoter sequences. His₆DBBfrA binds to the upstream regulatory regions of *bfrC*, *bfrE*, *bfrH*, *bfrC_{ss}*, *bfrH_{1ss}* and *bfrX_{ss}*. To identify the potential common motif recognized by His₆DBBfrA, the upstream regulatory regions of *bfrC*, *bfrE*, *bfrH*, *bfrC_{ss}*, *bfrH_{1ss}* and *bfrX_{ss}* were aligned using CLUSTALW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). The conserved 24 bp DNA motif among these regulatory regions is underlined. The specific interaction between His₆DBBfrA and this conserved 24 bp DNA motif was then confirmed by EMSA (Fig. 6). ATG in bold type indicates the start codon.

DISCUSSION

We have previously identified a putative TCS, BfrAB, which is involved in the development of oral biofilms. To further characterize the functions of this system, efforts were made in

Fig. 6. EMSA to identify the DNA motif recognized by His₆DBBfrA. ³²P-labelled probes were mixed with purified His₆DBBfrA, incubated and separated on 8% non-denaturing polyacrylamide gels. The gel was visualized by autoradiography. (a) ³²P-labelled 24 bp conserved DNA motif of *bfrCD*. (b) ³²P-labelled 18 bp DNA fragment containing two direct repeats of TTTAGAA. (c) ³²P-labelled 24 bp conserved DNA motif of *bfrEFG*. (d) ³²P-labelled 24 bp conserved DNA motif of *bfrH*. Lanes: 1, reaction mixtures containing ³²P-labelled probe alone; 2, ³²P-labelled probe with purified His₆DBBfrA protein; 3, ³²P-labelled probe with purified His₆DBBfrA protein and excess unlabelled probe; 4, ³²P-labelled probe with purified His₆DBBfrA protein and excess of unlabelled 18 bp DNA fragment containing two direct repeats of TTTAGAA.

this study to identify the genes regulated by BfrAB in *S. gordonii*. As shown by DNA microarray analysis and confirmed by RT-PCR, six genes were downregulated in two *bfrAB* mutants when compared with *S. gordonii* wild-type.

Among the genes regulated by *bfrAB* in *S. gordonii*, *bfrCD* and *bfrEFG* encode two homologous ABC transporters. BfrC and BfrE are putative ATP-binding proteins, and BfrD, BfrF and BfrG are putative transmembrane proteins. ABC transporters exist in all species (Decottignies & Goffeau, 1997; Holland & Blight, 1999), and serve to transport substrates across the cell membrane, driven by energy from ATP hydrolysis. The typical ABC transporter comprises two hydrophilic domains, which bind and hydrolyse ATP to facilitate the transportation process, and two hydrophobic domains, which translocate the substrate across the membrane and may determine substrate specificity (Higgins, 1992; Jones & George, 2004). An ABC transporter can be composed of homo- or heterodimers of ATP-binding domains and membrane-spanning domains. In bacteria, these domains are often produced as separate polypeptides (Higgins, 1992). BfrD, BfrF and BfrG all contain six membrane-spanning segments, the structure that typically characterizes these transmembrane domains/polypeptides. Another gene regulated by BfrAB, *bfrH*, encodes a CAAX amino-terminal protease family protein. Members of this protein family are grouped based on the sequence alignment, and are putative membrane-bound metalloproteases (Pei & Grishin, 2001).

BfrC and BfrE share a high degree of homology with the members of the BcrA subfamily ABC transporters. The

ABC transporter BcrABC is responsible for the bacitracin resistance of *Bacillus licheniformis* (Podlesek *et al.*, 1995). BcrA is an ATP-binding protein, and BcrB and BcrC are integral membrane proteins. BfrD, BfrF and BfrG, however, do not share high homology with BcrB and BcrC or other proteins with known functions. Since BfrCD, BfrEFG and BfrH, a putative membrane protease, are all regulated by BfrAB, we speculate that these membrane components are involved in the transport of some proteins/peptides, which may be required by *S. gordonii* for biofilm development. BfrH may process the protein/peptides, while BfrCD and BfrEFG may be responsible for the transport.

The TCS with the highest similarity to *S. gordonii* BfrAB was identified in *S. sanguinis*. As reported previously (Zhang *et al.*, 2004), another TCS (SMU.1038c–1037c) with significant similarity to BfrAB has been identified in another oral streptococcus, *S. mutans*. In *S. mutans*, the counterpart of BfrAB, SMU.1038c–1037c, is involved in the regulation of a *bfrCD*-homologous operon, which is located directly downstream of *smu.1038c–1037c* (J. Kreth and others, unpublished data). The *S. sanguinis* chromosome encodes three operons for putative ABC transporters homologous to *bfrCD* and *bfrEFG*, *bfrCD_{ss}*, *bfrEFG_{ss}* and *bfrXY_{ss}*. Among them, *bfrCD_{ss}* and *bfrXY_{ss}* are regulated by *S. sanguinis* BfrAB_{ss}. These results suggest that the BfrAB-homologous systems are organized similarly in oral streptococci to regulate the conserved ABC transporters. We have previously reported that *S. gordonii* biofilm-associated cells express higher levels of *bfrAB* than free-growing cells (Zhang *et al.*, 2004). Furthermore, *S. mutans* sessile cells also express higher levels of *bfrAB*-homologous *smu.1038c–1037c* than free-growing cells (Shemesh *et al.*, 2007). BfrAB-homologous systems, therefore, may be commonly involved in biofilm development by oral streptococci.

Two genes, *bfrH1_{ss}* and *bfrH2_{ss}*, encoding CAAX amino-terminal protease family proteins are regulated by BfrAB_{ss} in *S. sanguinis*. Differing from *bfrH1_{ss}*, the upstream promoter-containing region of *bfrH2_{ss}* cannot be recognized by BfrA, suggesting that BfrAB_{ss} does not directly regulate *bfrH2_{ss}* transcription. On the other hand, the reduced expression level of *bfrH2_{ss}* in the SK36*bfr*[−] strain is restored in the complemented strain SK36*bfr*⁺, in which *bfrAB_{ss}* is expressed in a shuttle vector. Hence, altered expression of *bfrH2_{ss}* in the SK36*bfr*[−] strain is unlikely to be caused by a polar effect of the disruption of BfrAB_{ss}. Further studies will clarify the mechanism by which BfrAB_{ss} is involved in the regulation of *bfrH2_{ss}*.

Dental plaque is initiated by the adherence of pioneer colonizers to saliva-coated tooth surfaces. Pioneer colonizers in the early plaque interact with and facilitate incorporation of later colonizers. Among the later colonizers, *Porphyromonas gingivalis* is implicated as a pathogen in periodontal infections, and can interact with *S. gordonii* (Brooks *et al.*, 1997; Chung *et al.*, 2000; Kuboniwa *et al.*, 2006; Lamont *et al.*, 2002; McNab *et al.*, 2003; Park *et al.*, 2005; Simionato *et al.*, 2006). Interestingly, *S. gordonii* *bfrC*

and *bfrG* appear to be required for the maturation of dual-species biofilms with *P. gingivalis* (Kuboniwa *et al.*, 2006; McNab *et al.*, 2003). We now plan to define the role of BfrAB in the development of *S. gordonii*–*P. gingivalis* biofilms.

In conclusion, we provide evidence that *bfrAB* and *bfrAB*-homologous systems share common roles as regulators of the ABC transporters in *S. gordonii* and *S. sanguinis*. Studies are ongoing to identify the substrates transported by the BfrAB-regulated ABC transporters and to explore potential signals sensed by the BfrAB TCS. These data will provide valuable information for the molecular dissection of the regulatory pathways for development of oral streptococcal biofilms.

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