Downregulation of GbpB, a Component of the VicRK Regulon, Affects Biofilm Formation and Cell Surface Characteristics of *Streptococcus mutans*^{▽†}

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The virulence of the dental caries pathogen *Streptococcus mutans* relies in part on the sucrose-dependent synthesis of and interaction with glucan, a major component of the extracellular matrix of tooth biofilms. However, the mechanisms by which secreted and/or cell-associated glucan-binding proteins (Gbps) produced by *S. mutans* participate in biofilm growth remain to be elucidated. In this study, we further investigate GbpB, an essential immunodominant protein with similarity to murein hydrolases. A conditional knockdown mutant that expressed *gbpB* antisense RNA under the control of a tetracycline-inducible promoter was constructed in strain UA159 (UACA2) and used to investigate the effects of GbpB depletion on biofilm formation and cell surface-associated characteristics. Additionally, regulation of *gbpB* by the two-component system VicRK was investigated, and phenotypic analysis of a *vicK* mutant (UAvicK) was performed. GbpB was directly regulated by VicR, and several phenotypic changes were comparable between UACA2 and UAvicK, although differences between these strains existed. It was established that GbpB depletion impaired initial phases of sucrose-dependent biofilm formation, while exogenous native GbpB partially restored the biofilm phenotype. Several cellular traits were significantly affected by GbpB depletion, including altered cell shape, decreased autolysis, increased cell hydrophobicity, and sensitivity to antibiotics and osmotic and oxidative stresses. These data provide the first experimental evidence for GbpB participation in sucrose-dependent biofilm formation and in cell surface properties.

The virulence of *Streptococcus mutans*, the major pathogen of dental caries, depends in part on the expression of surface proteins involved in the synthesis of and interaction with an extracellular glucan matrix. This process might influence several factors that modulate biofilm ecology, such as population density, nutrient availability, diffusion of metabolites, and evasion of host immune components. Among these proteins, glucosyltransferases (GtfB, GtfC, and GtfD) synthesize glucan from sucrose. Additionally, it has been suggested that other surface proteins with affinity for glucan, i.e., glucan-binding proteins (Gbps), contribute to *S. mutans* biofilm growth, and hence virulence, by mediating bacterial interaction with extracellular glucan (reviewed in reference 4). *S. mutans* expresses at least four Gbps (GbpA, GbpB, GbpC, and GbpD), but apart

from their affinity for glucan, these proteins differ in structure, function, and immunological properties (4, 24).

GbpB has unique immunodominant properties in children and adults (45, 46) and induces protective antibody responses to experimental caries in animal models (48). In addition, strong natural salivary IgA responses to GbpB during the initial phases of *S. mutans* challenge were associated with low susceptibility to *S. mutans* colonization in young children (34, 35). For a large number of strains with different biofilm phenotypes, production of GbpB was associated positively with the amounts of biofilms formed *in vitro* (28, 29). Formally, GbpB expression appeared essential for viability in several *S. mutans* strains, since bona fide *gbpB* mutants could not be isolated (30), except for a mutant with limited viability recovered for strain GS5 (12), a strain with several mutations in its genome (18, 41).

GbpB orthologues have been found in several Gram-positive species (28), including proteins, designated PcsB (protein required for cell separation of GBS), expressed by *Streptococcus agalactiae* (group B streptococci [GBS]) (38) and *Streptococcus pneumoniae* (32). PcsB/GbpB proteins differ in a central variable domain (120 amino acid residues), and there is evidence that PcsB/GbpB-like proteins have species-specific functions (31). For GBS, *pcsB* could be deleted in one strain under conditions of osmotic protection (38), while for *S. pneumoniae* strains R6 and D29, it was not possible to isolate *pcsB* null mutants (5, 31). Downregulation of *pcsB* in *S. pneumoniae*

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TABLE 1. Strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristics or sequence (5' to 3') ^a	Reference, source, 5' position, product size, or restriction site
Strains		
<i>S. mutans</i> strains		
UA159	Erm ^s	ATCC
UACA2	UA159/p _{tet} ASgbbpB; Erm ^r	This study
UA223	UA159/pMM223; Erm ^r	This study
UAvicK	UA159 ΔvicK::Erm ^r	This study
<i>S. gordonii</i> strains		
Challis	Erm ^s	H. K. Kuramitsu
Challis CA2	Propagation of p _{tet} ASgbbpB; erm ^r	This study
<i>E. coli</i> strain		
DH5α	General cloning and plasmid propagation	Invitrogen
Plasmids		
pVA838	Erm ^r cassette source	26
pMM223	8 kb; Erm ^r ; ori of <i>E. coli</i> , <i>Streptococcus</i>	H. K. Kuramitsu (52)
pALC2073	6.41 kb, with TetO/TetR sequence	Ambrose L. Cheung (6)
p _{tet} ASgbbpB	8.8 kb; Erm ^r ; pMM223 with a 220-bp sequence of <i>gbpB</i> (positions 19 to 260) in the antisense orientation under the control of the TetO/TetR promoter	This study
Primers		
Ptet-5	GGAATTCAAGCTTGCATCCCTGCAG	EcoRI (53)
Ptet-6	GAAGATCTATTCGAGCTCGGTACCC	BglII (53)
TetF	TGT TTT TCT GTA GGC CGT GT	Position 19 of TetO/TetR promoter
ASGbpBF	TCAGCAGTTTTAGTGAGTGGTG	Position 18 of <i>gbpB</i>
ASGbpBR	TTGGTACCCCCAAAGTAGCAGACTGAGCTT	Position 259 of <i>gbpB</i> ; KpnI
CGbpBF	CAACAGAAGCACAAACCATCA	Position 920 of <i>gbpB</i>
CGbpBR	TGTCCACCATTACCCAGT	Position 1070 of <i>gbpB</i>
16SRNA-F	CGGCAAGCTAATCTCTGAAA	
16SRNA-R	GCCCCTAAAAGGTTACCTCA	49
E1-AscI	TGGCGCGCCTGGCGGAACGTAAAGAAG	Erm ^r cassette from pVA838
E2-XhoI	TTCTCGAGGGCTCCTTGAAAGCTGTCACT	
vicKP1	TTACCAGATGCTTTTGTGCT	Position 268 upstream of <i>vicK</i> to bp 288 of <i>vicK</i>
vicKP2-AscI	TTGGCGCGCTACAGACGGTTTTTCTCCTGTG	Position 268 upstream of <i>vicK</i> to bp 288 of <i>vicK</i>
vicKP3-XhoI	TTCTCGAGGTGACCGTTTTTATCGTGTG	Position 1156 of <i>vicK</i> to bp 421 downstream of <i>vicK</i>
vicKP4	CTCTTGCCGCTTTTCATCAG	Position 1156 of <i>vicK</i> to bp 421 downstream of <i>vicK</i>
vicRHisF-NcoI	AACCATGGAGAAAATTCTAATCGTTGACGA	717-bp <i>vicR</i> ORF for His tag fusion
vicRHisR-XhoI	AACTCGAGGTCATATGATTTTCATGTAATAAC	717-bp <i>vicR</i> ORF for His tag fusion
SMU.22 (<i>gbpB</i>)	TTGACAGCTTATCCTTTAAATG	223 bp upstream to position 87
	TTTACAGCTGATAATGTTGTCG	223 bp upstream to position 87
SMU.1005 (<i>gtfC</i>)	GATGCTAACTCTGGAGAACGA	231 bp upstream to position 99
	TCCTGAAAGAGAGGTCAAAGTC	231 bp upstream to position 99
SMU.1924 (<i>covR</i>)	AGATGTCTCTACCCATTGAAAAATGG	269 bp upstream to position 87 (9)
	AACCTCATATCCTTCATGTTGTAATTCTAAAG	269 bp upstream to position 87 (9)

^a Restriction sites are underlined. Erm, erythromycin.

strain R6 provided evidence that PcsB functions in cell wall growth and septum division (31). For *S. mutans* GS5, the *gbpB* null mutant exhibited aberrant cell shape and slow growth (12), but because of its limited viability, further investigation of the role of GbpB in biofilm growth could not be performed. However, there is no experimental evidence that GbpB functions directly in cell wall biogenesis or division. Therefore, in the present study, we used an antisense RNA strategy to down-regulate *gbpB* to explore protein function in *S. mutans*. We analyzed the effects of *gbpB* downregulation on the biofilm phenotype and on several traits associated with a role in cell surface properties. Because of evidence suggesting that *gbpB* is regulated by the two-component system (TCS) VicRK (43), we also investigated whether *gbpB* is directly regulated by this system and compared the phenotypes of *gbpB* knockdown and *vicK* mutant strains.

MATERIALS AND METHODS

Strains, plasmids, growth conditions, and reagents. Strains, plasmids, and oligonucleotides used in this study are shown in Table 1. All reagents were purchased from Sigma-Aldrich unless specified otherwise. A *vicK* mutant strain of UA159 (UAvicK) was obtained by double-crossover recombination with a null allele constructed by PCR-ligation (22), using the primers listed in Table 1, such that *vicK* was replaced by an erythromycin resistance gene. *Streptococcus gordonii* strain Challis and *Escherichia coli* DH5α were used for plasmid propagation. Streptococcal strains were cultivated in brain heart infusion broth (BHI; Difco) or tryptic soy agar (TSA) at 37°C and 10% CO₂. Erythromycin (5 μg/ml) was added to media for selection and maintenance of conditional mutant or plasmid-containing strains. The Tet promoter was induced by doxycycline (dox) at 50, 100, 150, and 200 ng/ml. Purified native GbpB (pGbpB) was obtained by high-pressure liquid chromatography from *S. mutans* culture fluids as described elsewhere (45). Purified protein was stored in aliquots at -70°C in sodium phosphate buffer (pH 6.8).

Construction of inducible *gbpB* RNA antisense system. A shuttle vector was constructed to express the *gbpB* antisense sequence under the control of the inducible TetO/TetR promoter, as previously described (53). The promoter

sequence was obtained by PCR from plasmid pACL2073, kindly provided by Ambrose L. Cheung (Dartmouth Medical School, Hanover, NH), by use of primers pTet5 and pTet6 (53). This sequence was cloned into pMM223 at EcoRI and BglII sites, generating plasmid pTet. The TetO/TetR fragment obtained from digestion of pTet with EcoRI and BglII was cloned into pMM223 at EcoRI-BglII sites, resulting in pTetE. A 242-bp amplicon corresponding to the N-terminal sequence of GbpB (*ASGbpB* fragment) was obtained by PCR with primers ASGbpBF and -R. The amplicon was digested with KpnI and cloned in the antisense orientation into pTetE, generating plasmid p_{tet}ASGbpB, which was transformed into *S. gordonii* strain Challis. This vector was amplified in one of the transformed Challis strains (CA2) selected in TSA with erythromycin. The correct insertion of the antisense *gbpB* sequence in relation to the TetO/TetR promoter was verified by PCR using primers TetF and ASGbpBF, and amplicons of the expected sizes were validated by sequencing. Plasmid p_{tet}ASGbpB was purified from CA2 by alkaline lysis and transformed into *S. mutans* UA159, as described elsewhere (36). Transformants were selected and maintained in BHI with erythromycin. The clone carrying the correct p_{tet}ASGbpB plasmid was designated UACA2. A UA159 strain containing the pMM223 empty plasmid was designated UA223. Strains UA159 and UA223 were used as controls.

Induction of antisense *gbpB* RNA. Optimal conditions for *gbpB* downregulation were determined by quantifying cell-associated and secreted GbpB in strains UACA2, UA223, and UA159 by Western blotting. Cultures of each strain (A_{550} , 0.4) were treated with 0, 100, 150, and 200 ng/ml dox for 1, 2, 3, 4, 5, and 8 h of antisense induction at 37°C (10% CO₂, 90% air). Reductions in growth yield were observed previously for 18-h cultures of UA159 in BHI with 200 ng/ml dox (mean A_{550} , 0.82; standard deviation [SD], 0.03) compared to cultures in BHI only (mean A_{550} , 0.93; SD, 0.06); these results were similar in extent to those reported in a previous study with *S. mutans* LT11 (53). The greatest reductions in cell-associated and secreted GbpB were obtained after 2 h of induction of *ASGbpB* with 200 ng/ml dox. These reductions were sustained for up to 3 to 4 h of dox exposure, but some variability was observed at later time points. Thus, in all further experiments, conditional mutant strain UACA2 was grown in BHI with erythromycin for 18 h, and then cultures were diluted in fresh medium (1:100) and incubated to an A_{550} of 0.4, at which point dox (200 ng/ml) was added and incubation was continued for 2 h. Samples of 1.5 to 3.0 ml of culture suspension were centrifuged (10,000 × g), and cell pellets as well as culture fluids were collected. Protein and RNA were extracted to quantify cell-associated GbpB and *gbpB* transcripts, respectively. Secreted GbpB was quantified in culture fluids. Cultures of control strains (UA159 and UA223) were also exposed to dox under the same conditions, and cells and culture fluids were collected and analyzed similarly.

Analysis of *gbpB* regulation by the VicRK TCS. Electrophoretic mobility shift assays (EMSAs) to investigate direct regulation of *gbpB* by VicRK were performed as described previously (43), with some modifications. Briefly, to obtain a recombinant VicR (rVicR)-His fusion protein, the *vicR* open reading frame (ORF) was amplified with primers vicRHisF-NcoI and vicRHisR-XhoI (Table 1). The PCR fragment was digested with NcoI and XhoI, purified, and cloned into NcoI-XhoI-digested pET-22b(+) (Novagen) to yield pET-rVicR. The plasmid was transformed into *E. coli* BL21, and recombinant protein was isolated from 1 liter of culture (optical density at 550 nm [OD₅₅₀], 0.8) after 3 h of induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After cell lysis, recombinant protein was purified by affinity chromatography on Ni²⁺-nitrilotriacetic acid agarose (Qiagen), eluted rVicR was dialyzed, and its purity and integrity were visualized by Coomassie blue staining. Purified protein was stored at -20°C. For EMSA, PCR amplicons obtained with the primers listed in Table 1 included putative promoter regions of *gbpB* and positive- and negative-control genes (*gtfC* and *covR*, respectively) end labeled with a DIG Gel Shift kit (Roche) and stored at -20°C. Promoter fragments (0.12 ng; ~0.6 fmol) and increasing amounts of rVicR (0, 30, 60, 90, and 120 pmol) were incubated in reaction volumes of 25 μl (60 min, 25°C) in binding buffer [20 mM HEPES, 1 mM EDTA, 10 mM (NH₄)₂SO₄, 1 mM dithiothreitol (DTT), 0.2% Tween 20 (wt/vol), 30 mM KCl, pH 7.6] (Roche). As nonspecific competitors, 1 μg poly(dC-dG), 1 μg poly(dA-dT), and 0.1 μg poly-L-lysine were included in each reaction mix, as recommended by the manufacturer. Protein-DNA complexes were separated by nondenaturing gel electrophoresis on 6% DNA retardation gels (Invitrogen) at 70 V for 3 h. Gels were electrotransferred to positively charged nylon membranes, and probes were cross-linked under UV light. Chemiluminescent signals were detected using digoxigenin (DIG) wash and block buffers according to the manufacturer's protocol (Roche). Specificity of binding was evaluated in competition assays with a 200-fold excess of the respective unlabeled probe.

Because there is a conserved VicR-binding sequence among Gram-positive species (TGTWAHNNNNNTGTWAH, where W is A or T and H is A, T, or C)

(16), we examined *gbpB* promoter regions to identify a putative VicR box, with the help of Gibbs Motif Sampler (50) and AlignACE (21, 40).

Protein and RNA extraction. Protein extracts were prepared from cells previously exposed to dox as described above. After being harvested (10,000 × g, 5 min), cells were washed twice in cold saline, suspended in 220 μl of 10 mM Tris, 1 mM EDTA, pH 8.0 (TE), and stored at -70°C until use. Mechanical disruption of frozen cells (in 350 μl of water) was carried out in a Mini-Bead Beater apparatus (Biospec) at maximum power (4 cycles of 60 s, with 1-min intervals on ice). Extracts were centrifuged (7,000 × g, 30 s), and supernatants containing cell proteins were stored at -70°C until use. Samples of culture fluids (1.0 ml) were also collected after centrifugation of cultures (10,000 × g, 4°C, 5 min), treated with 10 μM phenylmethylsulfonyl fluoride (PMSF), and frozen at -70°C. Protein concentrations were determined using Bradford assay reagents (Bio-Rad) according to the manufacturer's protocol. RNA extraction was carried out using an RNeasy Mini kit (Qiagen), and samples were stored at -70°C until use. Purity and integrity of the RNA samples were determined by spectroscopy ($A_{260/280}$), and the samples were analyzed in formaldehyde agarose gels stained with ethidium bromide.

Determination of GbpB production. The effects of *ASGbpB* RNA induction on the expression of cell-associated and secreted GbpB were analyzed using Western blot assays. Proteins from cell extracts (8 μg) or culture fluids (15 μl) were resolved in 6% SDS-PAGE gels and transferred to nitrocellulose membranes as previously described (35). The amounts of GbpB in the volumes of culture fluids were within the linear range for GbpB detection by Western blotting, determined as described elsewhere (28). Membranes were washed, blocked overnight at 4°C in TBST, pH 7.6 (100 mM Tris-HCl, 0.25% Tween, 5.0% nonfat milk [Nestlé, Brazil]), and probed with rat antisera specific to GbpB or with sham-immunized sera (48), diluted (1:4,000) in the same buffer. After several washes in TBST, membranes were incubated with mouse anti-rat IgG conjugated with peroxidase (1:2,000). GbpB was detected using an enhanced chemiluminescence (ECL) system (GE, Amersham Bioscience, United Kingdom). Digital images of the autographs were analyzed with a densitometer (Bio-Rad GS-700 imaging densitometer), and relative amounts of GbpB are expressed as arbitrary units for band intensities detected within a linear range of GbpB concentrations (28).

Transcriptional analysis. Quantitative reverse transcriptase PCR (RT-PCR) was used to quantify *gbpB* transcripts, expressed either as antisense RNA (*ASGbpB*) or as a sequence corresponding to the 3' end of the ORF (*CgbpB*), i.e., transcribed from the chromosomal copy of the gene. GbpB transcripts were also determined for UAvicK by use of primers CGbpBF and -R (Table 1). Reverse transcription of experimental samples together with negative controls was carried out with 1 μg RNA, using Superscript III RT (Invitrogen) and a pool of cDNA obtained with random primers as described elsewhere (49). SYBR green PCR assays were performed with an iCycler system (Bio-Rad). Standard amplification and melting point product curves were obtained for each set of primers (*ASGbpB*, *CgbpB*, and 16S rRNA primers). Expression levels of the genes tested were normalized to expression of the 16S rRNA gene of *S. mutans* (49). Assays were performed in duplicate with at least three independent RNA samples.

Electron microscopic analysis of cell morphology and biofilm formation phenotypes. The effects of *gbpB* downregulation on cell morphology and the initial phases of biofilm formation were investigated by scanning electron microscopy (SEM). Briefly, overnight cultures of mutant and control strains were cultured in BHI with or without erythromycin, diluted 100-fold in fresh medium, and incubated to an A_{550} of 0.4. All cultures were treated with 200 ng/ml dox for 2 h (37°C, 10% CO₂). Cells were harvested by centrifugation as described above, washed three times with phosphate-buffered saline (PBS), and processed in microcentrifuge tubes for SEM analysis. To analyze cells in biofilms, 1.0-ml cultures at an A_{550} of 0.4 were transferred to 24-well culture plates containing sterile glass slides, followed by addition of 200 ng/ml dox. Plates were gently mixed and incubated under the same conditions for 2 h for biofilm formation during downregulation of *gbpB*. Biofilms were washed three times with PBS to remove nonadherent cells and then processed for SEM analysis. Similar experiments were performed with medium containing 1.0% sucrose. For SEM processing, bacteria adhering to slides were fixed with paraformaldehyde (3% in PBS, 1 h, 4°C). After three PBS washes, samples were incubated with 1% osmium tetroxide at room temperature (1 h) and then washed and dehydrated in ethanol. Dried samples were sputter coated with gold and analyzed with a scanning electron microscope (JSM 5600LV; JEOL, Japan).

Complementation of biofilm formation phenotypes with purified native GbpB. Biofilm assays were performed during GbpB downregulation in medium supplemented with native GbpB purified from *S. mutans* culture fluids (pGbpB). The amount of exogenously added GbpB (20 μg/ml) was comparable to that secreted by wild-type strain UA159 grown to an approximately equivalent cell density under similar conditions. Thus, after UACA2 was grown in BHI with erythro-

mycin for 18 h, cultures were diluted (1:100) in fresh medium containing erythromycin and dox (200 ng/ml), with or without pGbpB, and incubated for 2 h (37°C, 10% CO₂) for bacterial adherence and biofilm growth. Control cells harvested from 3.0-ml planktonic cultures and biofilm cells were processed for SEM. Nonadherent cells from biofilm culture fluids were also processed and analyzed by SEM.

Cell hydrophobicity assays. Cell surface hydrophobicities were determined using a previously described assay (19). Briefly, cultures of UA159, UA223, UACA2, and UAvicK were grown to an A_{550} of 0.4, exposed to dox as previously described, harvested (10,000 × g, 5 min), washed twice with PUM buffer (22.2 g K₂HPO₄ · 3H₂O, 7.26 g KH₂PO₄, 1.8 g urea, 0.2 g MgSO₄ · 7H₂O per liter, pH 7.1), and resuspended in the same buffer to an A_{550} of 0.900. Bacterial suspensions (3 ml) were mixed with 400 µl of hexadecane and incubated at 30°C for 30 min. Suspensions were mixed twice for 30 s by vortexing, with intervals of 5 s, and allowed to stand for complete separation of inferior aqueous from hexadecane phases. Aqueous phases were removed for determination of the A_{550} . Percentages of cells retained in the aqueous phase (in relation to the A_{550} values of respective suspensions without hexadecane, but treated similarly) were subtracted from 100% to express the percentage of hydrophobic cells. Three independent experiments were performed in triplicate.

Autolysis assay. The autolytic activities of strains were analyzed using a previously described assay (2), with some modifications. Briefly, cultures of strains UA159, UA223, UACA2, and UAvicK were grown to an A_{550} of 0.4 and exposed to dox for 2 h, as described above. Cells were centrifuged (10,000 × g, 5 min) and washed twice with PBS. Cell pellets were resuspended in 20 mM phosphate buffer (pH 6.5, with 1 M KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 0.4% sodium azide) to an A_{550} of 0.4. Cell suspensions were incubated at 44°C to activate autolysins expressed by UA159, and autolysis was monitored spectrophotometrically (A_{550}) for 12, 24, and 48 h. Because of the variability observed in these assays, especially with UAvicK, at least 5 independent experiments were performed for strain comparisons.

Sensitivity to antibiotics. We investigated the sensitivity of UACA2 to antibiotics that affect cell wall synthesis (penicillin G), protein synthesis (thiostrepton and streptomycin), and DNA replication (ciprofloxacin) during *gbpB* downregulation in comparison with strains UA159, UA223, and UAvicK. The MIC of each antibiotic was measured for the parent strain UA159 grown on BHI agar with 0.0004 µg/ml to 1 mg/ml of each antibiotic after 48 h of incubation. MICs of penicillin G, thiostrepton, streptomycin, and ciprofloxacin were 0.035, 0.04, 60, and 1.4 µg/ml, respectively. Respective sublethal concentrations used for these antibiotics (0.02, 0.02, 40, and 1.2 µg/ml) were defined as the maximum concentrations below the MICs that supported growth of wild-type strain UA159 and were used to test the sensitivities of strains UACA2, UA159, UA223, and UAvicK. Overnight cultures in BHI or BHI-erythromycin were diluted 100-fold in fresh medium and incubated to an A_{550} of 0.4 before the addition of dox (200 ng/ml) and a sublethal concentration of each antibiotic. Incubation continued for an additional 2 h, and serial dilutions of cultures were plated on BHI agar containing the respective antibiotics at the same concentration as that of dox (200 ng/ml). Plates were incubated for 48 h (37°C, 10% CO₂) to determine the CFU/ml. Cultures not exposed to antibiotics were used as controls.

Viability under osmotic and oxidative stress. Osmotic stress conditions previously shown to induce *gbpB* expression in GS5 and UA159 (13, 30) were used to investigate the effects of *gbpB* downregulation on cell viability. Similar experiments were performed with UAvicK. Briefly, cultures of test and control strains were exposed to dox for 2 h, followed by addition of NaCl (0.5 M) and further incubation for 30 min. Serial dilutions of cultures were plated on BHI agar supplemented with and without dox to determine cell viability.

Because the VicRK system has been implicated in oxidative stress responses in *S. mutans* (43), the sensitivities of the conditional mutant UACA2, UAvicK, and control strains to hydrogen peroxide were tested as described elsewhere (20), with some modifications. Briefly, strains were grown and exposed to dox for 2 h, as described above, before addition of H₂O₂ (10 µM) and incubation for 1 h. A lethal dose of H₂O₂ (100 µM) was then added, and incubation was continued for another 30 min. Serial dilutions of cells were plated on BHI agar with 200 ng/ml of dox for determination of the CFU/ml. At least three independent experiments were performed.

Data analysis. Cell morphologies were analyzed visually in SEM digital images obtained at magnifications of ×800, ×9,000, and ×13,000. Biofilm growth in the presence of sucrose was analyzed in SEM digital images at a magnification of ×1,300 for 32 predetermined areas (63 to 97 µm) equally distributed on each glass slide. Using ImageJ image processing and analysis software in Java (NIH [http://rsbweb.nih.gov/ij/index.html]), cell lengths were determined by linear measurement of cells from isolated chains in which division was at the stage

where equatorial width was equal to or greater than cell length. Numbers of cells and/or microcolonies per field were also determined for biofilm images. Parametric analysis of variance (ANOVA) followed by pairwise multiple comparisons (Tukey test) was used to compare *gbpB* expression levels (transcript levels and densitometric measurements of GbpB levels in Western blots). Biofilm formation, cell length, cell hydrophobicity, autolysis, and stress sensitivity were compared between UACA2, control strains, and/or strains complemented with pGbpB, using a nonparametric Kruskal-Wallis or Mann-Whitney test.

RESULTS

Induction of *gbpB* antisense RNA leads to reduced expression of GbpB. Induction of the TetO/TetR promoter in UACA2 grown with increasing concentrations of dox (0, 150, and 200 ng/ml) led to significant dose-dependent increases in the levels of *ASgpbB* transcripts. A 4.9-fold relative increase in *ASgpbB* was observed in conditional mutant strain UACA2 after a 2-h exposure to 200 ng/ml dox (UACA2-dox) compared to the same mutant (UACA2) grown in the absence of dox (Fig. 1A). Amounts of *ASgpbB* transcripts were significantly higher in UACA2-dox than in the UA159-dox or UA223-dox negative control (ANOVA) ($P < 0.01$). To confirm that these increases were not due to increased transcription of the chromosomal gene, we performed RT-PCR assays with the same samples, using primers for *CgpbB*, from the 3' terminus of *gbpB*. Levels of *CgpbB* did not change significantly in UACA2-dox (Fig. 1B), confirming that high levels of *ASgpbB* transcripts in UACA2-dox resulted from induction of the TetO/TetR promoter in vector CA2. In addition, levels of *ASgpbB* transcripts in UA159-dox and UA223-dox were similar to those detected in UACA2 grown in the absence of dox (Fig. 1A).

Induction of *ASgpbB* transcription in UACA2-dox led to consistent reductions in the amounts of GbpB produced. Maximum reductions in cell-associated and secreted GbpB were obtained after a 2-h induction of UACA2 with 200 ng/ml of dox (Fig. 1C). Densitometric determination of the amount of GbpB revealed 5.5- to 3.2-fold reductions in the mean levels of cell-associated and secreted protein, respectively, in UACA2 compared to the parental strain (Fig. 1C, D, and E). In addition, total amounts of GbpB (the sum of densitometric measurements of cell-associated and secreted protein) in UACA2 were significantly lower than those produced by UA159 or UA223 (ANOVA and Tukey test; $P < 0.05$). GbpB production remained low after 3 and 4 h of dox exposure but increased progressively after 5 to 8 h of incubation in the presence of dox (data not shown).

GbpB is regulated directly by the VicRK TCS. Previously, it was shown that VicR binds to *gtfB*, *gtfC*, and *ftf* promoters *in vitro* and that expression of these genes and *gbpB* was reduced in a *vicK* mutant (43). Direct binding of VicR to the promoter of *gbpB* was not previously shown; by using EMSA (Fig. 2A), we established that recombinant VicR binds to *gbpB* promoter regions, leading to a shift in migration of similar extent to that observed for *gtfC*, a gene previously shown to bind VicR (43). Consistently, transcript levels of *gbpB* in UAvicK were approximately 60% lower than that in UA159 at the same growth phase (Fig. 2B), and amounts of cell surface and secreted GbpB were approximately 10-fold lower in UAvicK than in the wild-type strain (Fig. 2C), as revealed by the densitometry of GbpB bands detected by Western blotting. Thus, our data show that *gbpB* is regulated directly and positively by the

FIG. 1. Effects of expression of *gbpB* antisense RNA (*ASgbpB*) on suppression of GbpB production. (A) Relative expression of *ASgbpB* in conditional mutant UACA2 in response to 2 h of dox induction, determined by quantitative RT-PCR. Controls included wild-type strain UA159 and UA159 carrying an empty plasmid (UA223). **, $P < 0.01$ (ANOVA). (B) Relative levels of *gbpB* transcripts of chromosomal origin (*CgbpB*) after treatment with or without dox. (C) Representative Western blot analysis of levels of cell-associated and secreted GbpB in strains treated with increasing concentrations of dox. (D and E) Quantitative analysis of GbpB production in response to dox. Columns indicate means for three independent experiments, and error bars represent standard deviations. *, $P < 0.05$ (ANOVA).

VicRK TCS and that *UAvicK* expresses low levels of GbpB. Additionally, a putative VicR-binding sequence was identified in the *gbpB* promoter region (TGTAATAATGAcGTAAT [the lowercase letter indicates a mismatch]), which is located 7 bp upstream from the transcriptional start site.

Different cell morphologies result from GbpB depletion and *vicK* mutation. We examined the morphology of mid-log-phase cells (A_{550} , 0.4) after exposure to dox (2 h) in BHI without sucrose. As depicted in Fig. 3B, downregulation of GbpB in UACA2 promoted alterations in cell shape compared to control strains (Fig. 3A). These morphological analyses were repeated several times, and the results presented are representative of six independent experiments. Typically, the wild-type strain showed chains of cocci with a rod-like shape (Fig. 3A), while UACA2 formed chains of shorter cocci (Fig. 3B). To substantiate this qualitative analysis, we measured cell lengths of 200 cells from randomly selected isolated chains, determining mean cell lengths of 0.75 μm (SD, 0.11 μm) and 0.62 μm (SD, 0.14 μm) for strains UA159 and UACA2, respectively, following dox exposure (Fig. 3C); the median cell length of UA159-dox (0.75 μm ; lower and upper quartiles, 0.67 and 0.81 μm , respectively) was significantly longer than the median cell

length of UACA2-dox (0.63 μm ; lower and upper quartiles, 0.52 and 0.70 μm , respectively) (Mann-Whitney test; $P < 0.01$). The differences in these two-dimensional measures between strains, although small, were observed consistently. No significant differences were observed when UACA2 grown in the absence of dox was compared with the wild-type strain (data not shown). Although the coccoid morphology of UACA2-dox resembled that of the *UAvicK* mutant (Fig. 3D), the latter strain also formed extremely long chains (Fig. 3D) that were not observed in UACA2. These results indicated that depletion of GbpB expression (about 5-fold) influenced cellular morphology but did not affect chain formation as in *UAvicK*. Additionally, medium supplementation with exogenous pGbpB did not significantly restore the wild-type phenotype (data not shown), suggesting that GbpB may have to be cell associated in order to participate in cell wall morphogenesis.

Downregulation of *gbpB* impairs sucrose-dependent biofilm formation. Visual inspection of initial stages of biofilm growth in BHI containing 1.0% sucrose revealed that UA159-dox and UA223-dox or UACA2 grown in the absence of dox clearly formed homogeneous biofilm layers. In contrast, UACA2-dox

FIG. 2. (A) EMSA. Twenty-five-microliter DNA-binding reaction mixtures were prepared with end-labeled DNA fragments (~ 0.6 fmol) from the 5'-proximal region of *gbpB*, *gtfC*, or *covR* and with purified His-tagged VicR. Lanes 1, no protein; lanes 2, 30 pmol of VicR; lanes 3, 60 pmol; lanes 4, 90 pmol; lanes 5, 120 pmol. Specific competitions were performed with 120 pmol VicR and a 200-fold excess of unlabeled DNA (lanes 6). The promoters of *gtfC* (known target of VicR) and *covR* (not regulated by VicR) were used as positive and negative controls, respectively. (B) Reduction in *gbpB* transcript level in UA*vicK* relative to that in the wild-type strain. (C) Reductions in production of cell-associated and secreted GbpB in UA*vicK* relative to that in the wild-type strain. Amounts of protein are expressed as densitometric units of GbpB bands detected in Western blot assays. Columns represent means for three independent experiments, and error bars indicate standard deviations. *, $P < 0.01$ (ANOVA).

and UA*vicK* were not able to cover slides but formed sparse compacted microcolonies that detached from the slides during rinsing. Analysis of SEM digital images of the initial stages of biofilm formation confirmed these visual observations; the levels of adhered cells or microcolonies determined for 32 pre-defined areas of each slide could be compared consistently between strains (total of 128 images analyzed per experiment). As depicted in Fig. 4A, UA159 formed a homogeneous layer of primarily diplococcal chains or microcolonies on the glass surfaces (mean, 129.94 per area; SD, 30.4; median, 133), in that a continuous extracellular matrix could clearly be detected in intimate contact with cells. With a contrasting phenotype, UACA2 formed few microcolonies (mean, 33.75 per area; SD, 16.21; median, 32) that were distributed heterogeneously on the slides (Fig. 4B). These microcolonies contained an extracellular matrix but were of atypical structure. To confirm the effects of GbpB depletion on the biofilm growth phenotype, we performed experiments in which *gbpB* was downregulated in medium supplemented with native GbpB purified from *S. mutans* culture fluids (pGbpB). Addition of pGbpB to the medium partially restored biofilm formation (Fig. 4C), and biofilms acquired a wild-type phenotype. The number of cells or microcolonies per area for UACA2-dox with pGbpB (mean, 59.00; SD, 22.12; median, 67) was significantly higher than that observed for UACA2-dox (Kruskal-Wallis test; $P < 0.01$). In the UA159 strain background, inactivation of *vicK* altered the structure of 18-h mature biofilms in *S. mutans* (43), so we examined the initial phases of UA*vicK* biofilm formation in the presence of sucrose. Few microcolonies of UA*vicK* were observed per area (mean, 4.97; SD, 3.51; median, 4) compared to

the other strains, but these were distinct from the UACA2-dox microcolonies in that they were formed by extremely long chains and were apparently devoid of extracellular matrix (Fig. 4D). Addition of exogenous pGbpB promoted a 2.1-fold increase in the number of microcolonies (mean, 10.44; SD, 5.80; median, 9) formed by UA*vicK* in comparison to UA*vicK* without pGbpB (Mann-Whitney U test; $P < 0.01$), and the size of microcolonies was apparently larger (Fig. 4E).

GbpB downregulation affects *S. mutans* autolytic activity and hydrophobicity. GbpB shares extensive similarity with murein hydrolases, including a CHAP domain commonly identified in autolysins which are important for cell surface biogenesis and adaptation during growth and, possibly, biofilm formation (2, 10). Therefore, we investigated the effects of *gbpB* downregulation on cell autolysis at high temperature and on cell hydrophobicity. The autolytic activities of strains, expressed as percentages of final A_{550} values with respect to initial A_{550} values, are shown in Fig. 5A. Following incubation at 44°C, mean percentages of cells resistant to autolysis were 62.0, 54.4, 44.3, and 40.4% for strains UA*vicK*, UACA2-dox, UA159-dox, and UA223-dox, respectively. No significant difference in susceptibility to autolysis was observed between control strains UA159 and UA223 (Mann-Whitney test; $P > 0.05$). UA*vicK* and UACA2-dox were significantly more resistant to autolysis than the wild-type strain (Mann-Whitney test; $P < 0.05$). UA*vicK* was the most resistant to autolysis, although no significant difference in autolytic activities was observed between UA*vicK* and UACA2-dox (Mann-Whitney test; $P > 0.05$). Thus, inactivation of *vicK* and downregulation of *gbpB* increase resistance to autolysis.

FIG. 3. Scanning electron microscopic analysis of cell morphology. Wild-type strain UA159 and UACA2 at mid-log phase (A_{550} , 0.4) were exposed to dox (200 ng/ml) for 2 h before being processed. UA*vicK* was not exposed to dox. (A) Wild-type UA159. (B) UACA2. (C) Comparison of mean cell lengths. *, $P < 0.01$ (Mann-Whitney test). (D) UA*vicK*.

The results of hydrophobicity assays (Fig. 5B) indicated that depletion of GbpB caused a significant increase in hydrophobicity and that UA*vicK* was the most hydrophobic strain. After dox exposure, mean percentages of hydrophobic cells were 35.6% (SD, 0.07%) and 38.4% (SD, 0.09%) for UA159 and UA223, respectively; significantly higher percentages of UACA2 (mean, 67.8%; SD, 0.07%) and UA*vicK* (mean, 93.4%; SD, 0.02%) cells were retained in the hydrophobic phases.

GbpB depletion and *vicK* inactivation result in different sensitivities to antibiotics and osmotic and oxidative stresses. The *pcsB* mutant of *S. agalactiae* is highly sensitive to antibiotics that affect different cellular functions (37, 38). Thus, the sensitivities of UACA2, UA223, UA159, and UA*vicK* to several antimicrobial agents were determined (see Table S2 in the supplemental material). Reductions in the numbers of viable UACA2-dox cells exposed to drugs that target intracellular components (ciprofloxacin, streptomycin, and thiostrepton) were low and of similar magnitudes (1.18 to 1.53 log CFU/ml) to those for untreated controls (Mann-Whitney test; $P < 0.001$ to 0.05). A reduction of only 0.67 log CFU/ml was observed for UACA2 exposed to penicillin relative to the control level, and this difference did not achieve statistical significance. UA*vicK* was significantly more sensitive to ciprofloxacin (a reduction of 5.32 log CFU/ml of viable cells), and modest reductions in cell viability were observed for streptomycin, thiostrepton, and penicillin compared to the control levels (reductions of 0.42 and 0.71 log CFU/ml) (see Table S2). UA159 and UA223 also showed small reductions in cell viability in response to sublethal levels of each antibiotic.

Previously, *gbpB* was identified in a screen for genes that were upregulated in response to osmotic and acidic stresses (13); however, we did not observe consistent *gbpB* upregulation in response to these conditions in three strains tested (30). VicK has a conserved PAS domain that is typically involved in oxidative stress responses (43); therefore, we compared the sensitivities of UACA2, UA*vicK*, and control strains to osmotic and oxidative stresses. A 1.1-log reduction in the number of viable cells of UACA2 compared to control cells was observed after a 30-min exposure to 0.5 M NaCl (see Table S2 in the supplemental material). The reduction in the number of viable cells of UACA2-dox following treatment with H_2O_2 (10 to 100 μ M) was of a greater magnitude (2.72-log reduction in induced UACA2 exposed to oxidative stress compared to the control strain; $P < 0.05$ [Mann-Whitney test]). In general, UACA2 that was not stressed behaved similarly to control strains UA159 and UA223. Although UA*vicK* was sensitive to oxidative stress (reduction of 1.95 log CFU/ml compared to the control), the numbers were not statistically significant (Mann-Whitney test; $P = 0.080$) due to great variability between independent experiments. This variability might be explained in part by the formation of long chains by UA*vicK*, because fewer CFU were recovered from UA*vicK* cultures than from similar cultures of UACA2, which makes shorter chains. UA*vicK* showed a 1.09-log reduction in CFU/ml compared to the control after exposure to osmotic stress, and this difference was statistically significant (Mann-Whitney test; $P < 0.01$). Under the same experimental conditions, UA159-dox and UA223-dox were essentially unaffected by osmotic or oxidative stress.

FIG. 4. Scanning electron microscopic analysis of initial stages of biofilm formation (2 h) on the surfaces of glass slides in BHI with 1.0% sucrose. (A) Strain UA159 under exposure to dox. (B) Conditional mutant UACA2 under exposure to dox. (C) Same as in panel B, but in medium supplemented with native pGbpB. (D) Representative microcolony formed by UA*vicK*. (E) Representative microcolony formed by UA*vicK* in the presence of native pGbpB, showing the tendency of increasing sizes. This experiment is representative of at least four independent experiments.

DISCUSSION

Based on its affinity for polysaccharides, it was hypothesized that GbpB plays a role in virulence through biofilm development (28, 48), although the affinity of GbpB for glucans with alpha-1,6 glucosidic linkages is modest (47). *gbpB* is essential in several strains (30), and a *gbpB* mutant obtained from *S. mutans* strain GS5 was too fragile for further cultivation and study (J. S. Chia, personal communication), so the function of GbpB in *S. mutans* biofilm growth and/or virulence remains unexplored. A viable *gbpB* knockout mutant (designated BD1) was reported for strain MT8148 (17). However, Western blot analysis of strain BD1 and the parent strain

MT8148 (kindly provided by Takashi Ooshima, Osaka University Graduate School of Dentistry, Osaka, Japan) revealed that expression of GbpB in BD1 was similar to that of MT8148 (data not shown). Furthermore, the gene product knocked out in BD1 was 100 kDa (27), while GbpB is 59 kDa (28, 45). Thus, we concluded that BD1 is not a bona fide *gbpB* knockout mutant.

In this study, we showed that downregulation of *gbpB* clearly altered the initial steps of sucrose-dependent biofilm formation (Fig. 4). These steps involve cell division and other physiological processes required for the transition from planktonic to biofilm growth (54). In *S. mutans*, extracellular polysaccha-

FIG. 5. (A) Autolysis of strains after 2 h of exposure to dox (200 ng/ml). Values represent means for five independent experiments, and error bars represent standard deviations. At 48 h of incubation, UACA2 and UAvicK showed lower reductions in initial absorbances than UA159. No significant difference was observed between UAvicK and UACA2. *, $P < 0.05$ (Mann-Whitney test). (B) Percentages of cells retained in the hydrophobic hexadecane phase in strains after exposure to dox. Columns represent means for three independent experiments, and error bars indicate standard deviations. *, $P < 0.05$; **, $P < 0.001$ (Mann-Whitney test).

rides derived from sucrose are essential for development and structure of microcolonies during the initial steps (2 to 8 h) of biofilm growth *in vitro* (55). The biofilm phenotype of the knockdown mutant UACA2 was partially restored by exogenous pGbpB (Fig. 4), suggesting that GbpB plays an extracellular function in biofilm formation. This limited restoration of biofilm growth by pGbpB may reflect reduced protein function under experimental conditions. For example, exogenous protein likely functions only during the initial steps of biofilm growth, because preformed polysaccharides might prevent pGbpB from gaining access to bacterial cell surfaces. While GbpB and PcsB are expressed in secreted and cell-associated forms (5, 28, 38), the functional relevance of expression in both forms is still unclear. The GbpB precursor protein has a 21-amino-acid signal peptide, but it does not have the LPTXG cell wall anchor motif (28). GbpB does not contain glucan-binding domains typical of Gtfs, GbpA, or GbpD and is not homologous to GbpC, a cell wall protein with glucan-binding properties (4, 41). However, GbpB has a conserved N-terminal domain containing a

leucine zipper typically involved in protein-protein interactions and a C-terminal domain containing a CHAP motif associated with murein hydrolases (23, 28, 31).

The production of GbpB correlated positively with sucrose-dependent biofilm growth *in vitro* at 18 h of growth (28); similar associations were also observed for GtfB and GtfC but not for GtfD or GbpA (28, 29). In this study, we could not verify the effects of GbpB downregulation on mature 18-h biofilms, since *gbpB* downregulation could not be sustained for more than 5 to 8 h. Because of the apparently essential function of *gbpB*, unknown transcriptional and/or posttranscriptional regulatory mechanisms might be activated to restore GbpB expression in the knockdown mutant (37). GbpB expression may also be controlled by posttranscriptional events, since increased expression of GbpB and other proteins involved in glucan-dependent virulence, e.g., GtfB, was observed in a mutant defective in HtrA protease (8), a protein which modulates adaptation to environmental stresses and expression of virulence factors (25, 39).

In the present study, we established that *gbpB* is part of the regulon of the VicRK TCS; VicR binds to the *gbpB* promoter region, possibly in the VicR box (5'-TGTAATAATGACGTAAT-3'), and deletion of *vicK* impairs GbpB expression at the RNA and protein levels (Fig. 2). Previously, it was shown that VicR directly regulates *gtfB*, *gtfC*, and *ftf* genes, implying the importance of this TCS in sucrose-dependent biofilm growth (43). VicR is an essential response regulator in several Gram-positive bacteria, including *S. mutans* (15, 43), and thus it is expected that *vicR* is still expressed in *vicK* mutants. Using Western blot assays, we observed that amounts of VicR produced by UAvicK were only about 10% of that produced by the wild-type strain UA159 (data not shown); thus, the role of VicK in the expression of its own cognate response regulator warrants further investigation. Here we observed that inactivation of *vicK* in UA159 impaired GbpB expression and altered biofilm formation, cell morphology, and different cell surface properties in a manner comparable to that observed when *gbpB* was downregulated, apart from some differences discussed below. UAvicK showed a poor ability to form biofilms on glass surfaces in the presence of sucrose, but the biofilm phenotype was different from that of UACA2-dox (Fig. 4). It was previously reported for an *S. mutans vicK* mutant that mature biofilms (grown for 16 h in sucrose medium) comprised long cell chains that detached at cellular junctions (43). Interestingly, in this study, we observed that addition of native pGbpB to the medium of UAvicK increased the number and size of microcolonies (Fig. 4), suggesting that extracellular GbpB may participate in cell-to-cell interactions.

In *S. pneumoniae* strain R6, 3- to 4-fold downregulation of *pcsB* transcripts promoted severe alterations in morphology and formation of long chains of ovoid cells (31), a phenotype that resembled the phenotype of the *vicRKX* conditional mutant, and it was proposed that the essentiality of the VicRK system was due to its role as a positive regulator of PcsB (31–33). In this study, we observed that UAvicK typically forms long chains of short spherical cells (Fig. 3D), as also observed for an *S. mutans vicK* mutant grown in biofilms (43). Although downregulation of *gbpB* promoted cells of spherical shape (Fig. 3B), long chains were not observed. Interestingly, the chain length phenotype associated with *pcsB* downregulation is strain

dependent in *S. pneumoniae* (5), and *pcsB* depletion in encapsulated strain D39 leads to shortening of chains and spherical cells (5), a phenotype that resembles that of UACA2-dox. The effects of PcsB on *S. pneumoniae* morphogenesis were dependent on expression of capsular polysaccharide, controlled by the *cps* locus, since downregulation of *pcsB* in D39Δ*cps* promoted a long-chain phenotype similar to that of the unencapsulated strain R6 (5). The influence of GbpB or PcsB depletion on phenotypes that are dependent on glucan or capsular polysaccharides might be reconciled by a model whereby synthesis of extracellular polysaccharides is coordinated with cell growth and cell surface biogenesis. Our working hypothesis is that GbpB participates in a glucan-dependent process of biofilm formation in which cell surface biogenesis, cell growth, and interaction with glucan are coordinated.

Despite a high similarity to murein hydrolases, such activity has not been detected with GbpB (28) or with purified PcsB proteins (5, 38). Murein hydrolases are an extensive group of enzymes with different specificities and redundant or complementary activities that frequently have more than one function (reviewed in reference 51). Most bacteria express murein hydrolases which participate in physiological processes that include cell wall turnover, cell separation, autolysis, competence, assembly of cell surface structures, and biofilm formation (51). In several bacterial species, VicRK regulates functions implicated in cell wall metabolism and synthesis of autolysins (15), and we are currently characterizing new genes directly regulated by the VicRK system whose products have putative roles in cell wall biogenesis in *S. mutans* (R. N. Stipp et al., unpublished data). The changes in autolytic activity and hydrophobicity observed in the *gbpB* knockdown mutant are further compatible with its protein homology with murein hydrolases and occurred in a mode similar to that of UAvicK, although to a lesser extent. *S. mutans* produces autolysins, which include AtlA and SmaA (SMU.609) (1, 11). AtlA also appears to be regulated by VicRK (3) and is involved in cell surface biogenesis and biofilm formation (1, 10). Thus, VicRK apparently regulates functions implicated in cell surface biogenesis in *S. mutans*, which may explain, in part, the more severe alterations in UAvicK phenotypes than in *gbpB* knockdown mutant phenotypes. The large increase in cell hydrophobicity promoted by *vicK* inactivation might account for the formation of cell aggregates previously reported for biofilms and broth cultures of a *vicK* mutant of UA159 (43). The aggregation of long chains likely accounts for the poor ability of UAvicK to initiate biofilms (Fig. 4). Although hydrophobicity was also promoted in UACA2-dox, this occurred to a lesser extent than that with UAvicK, and no aggregation of UACA2-dox short chains was detected (data not shown).

In GBS, inactivation of *pcsB* increases sensitivity to antibiotics which affect cytoplasmic and extracytoplasmic functions (37, 38), suggesting an increase in cell permeability. In *S. pneumoniae*, *pcsB* downregulation does not affect sensitivity to penicillin (5). GbpB downregulation for 2 h slightly increased the sensitivity of *S. mutans* to different antibiotics, promoting reductions in numbers of viable cells of 1 to 1.5 log. Differences exist in antibiotic and stress sensitivity phenotypes between UACA-dox and UAvicK. Unlike UACA2, UAvicK was clearly sensitive to ciprofloxacin. On the other hand, UACA2-dox was sensitive to oxidative stress, while reductions in cell viability

under this stress were less evident for UAvicK. Antibiotic and oxidative stress sensitivity phenotypes of *S. mutans vicK* mutants are not consistent among studies, likely due to different experimental conditions (7, 14, 42–44). Given the essential function of GbpB, we cannot exclude the possibility that downregulation of GbpB in UACA2 and UAvicK promotes indirect effects on *S. mutans* physiology, thus accounting for the observed phenotypic changes.

In this study, we present experimental evidence that depletion of GbpB affects glucan-dependent biofilm formation and several cell surface properties. Some of these changes are similar to those observed in UAvicK, in accord with the role of VicRK as a direct regulator of *gbpB*. Further studies will be necessary to define the functional domains of GbpB and their role(s) in cell surface biogenesis and biofilm growth.

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REFERENCES

- Ahn, S. J., and R. A. Burne. 2006. The *atlA* operon of *Streptococcus mutans*: role in autolysin maturation and cell surface biogenesis. *J. Bacteriol.* **188**: 6877–6888.
- Ahn, S. J., and R. A. Burne. 2007. Effects of oxygen on biofilm formation and the *AtlA* autolysin of *Streptococcus mutans*. *J. Bacteriol.* **189**:6293–6302.
- Ahn, S. J., Z. T. Wen, and R. A. Burne. 2007. Effects of oxygen on virulence traits of *Streptococcus mutans*. *J. Bacteriol.* **189**:8519–8527.
- Banas, J. A., and M. M. Vickerman. 2003. Glucan-binding proteins of the oral streptococci. *Crit. Rev. Oral Biol. Med.* **14**:89–99.
- Barendt, S. M., et al. 2009. Influences of capsule on cell shape and chain formation of wild-type and *pcsB* mutants of serotype 2 *Streptococcus pneumoniae*. *J. Bacteriol.* **191**:3024–3040.
- Bateman, B. T., N. P. Donegan, T. M. Jarry, M. Palma, and A. L. Cheung. 2001. Evaluation of a tetracycline-inducible promoter in *Staphylococcus aureus* *in vitro* and *in vivo* and its application in demonstrating the role of *sigB* in microcolony formation. *Infect. Immun.* **69**:7851–7857.
- Biswas, I., L. Drake, D. Erkina, and S. Biswas. 2008. Involvement of sensor kinases in the stress tolerance response of *Streptococcus mutans*. *J. Bacteriol.* **190**:68–77.
- Biswas, S., and I. Biswas. 2005. Role of HtrA in surface protein expression and biofilm formation by *Streptococcus mutans*. *Infect. Immun.* **73**:6923–6934.
- Biswas, S., and I. Biswas. 2006. Regulation of the glucosyltransferase (*gtfBC*) operon by CovR in *Streptococcus mutans*. *J. Bacteriol.* **188**:988–998.
- Brown, T. A., Jr., et al. 2005. A hypothetical protein of *Streptococcus mutans* is critical for biofilm formation. *Infect. Immun.* **73**:3147–3151.
- Catt, D. M., and R. L. Gregory. 2005. *Streptococcus mutans* murein hydrolase. *J. Bacteriol.* **187**:7863–7865.
- Chia, J. S., et al. 2001. A 60-kilodalton immunodominant glycoprotein is essential for cell wall integrity and the maintenance of cell shape in *Streptococcus mutans*. *Infect. Immun.* **69**:6987–6998.
- Chia, J. S., Y. Y. Lee, P. T. Huang, and J. Y. Chen. 2001. Identification of stress-responsive genes in *Streptococcus mutans* by differential display reverse transcription-PCR. *Infect. Immun.* **69**:2493–2501.
- Deng, D. M., M. J. Liu, J. M. Ten Cate, and W. Crielaard. 2007. The VicRK system of *Streptococcus mutans* responds to oxidative stress. *J. Dent. Res.* **86**:606–610.
- Dubrac, S., P. Bisicchia, K. M. Devine, and T. Msadek. 2008. A matter of life and death: cell wall homeostasis and the WalKR (YycGF) essential signal transduction pathway. *Mol. Microbiol.* **70**:1307–1322.
- Dubrac, S., and T. Msadek. 2004. Identification of genes controlled by the essential YycG/YycF two-component system of *Staphylococcus aureus*. *J. Bacteriol.* **186**:1175–1181.
- Fujita, K., M. Matsumoto-Nakano, S. Inagaki, and T. Ooshima. 2007. Biological functions of glucan-binding protein B of *Streptococcus mutans*. *Oral Microbiol. Immunol.* **22**:289–292.
- Fujiwara, T., et al. 1998. Molecular analyses of glucosyltransferase genes among strains of *Streptococcus mutans*. *FEMS Microbiol. Lett.* **161**:331–336.
- Gibbons, R. J., and I. Etherden. 1983. Comparative hydrophobicities of oral

- bacteria and their adherence to salivary pellicles. *Infect. Immun.* **41**:1190–1196.
20. Higuchi, M., et al. 1999. Functions of two types of NADH oxidases in energy metabolism and oxidative stress of *Streptococcus mutans*. *J. Bacteriol.* **181**: 5940–5947.
  21. Hughes, J. D., P. W. Estep, S. Tavazoie, and G. M. Church. 2000. Computational identification of cis-regulatory elements associated with groups of functionally related genes in *Saccharomyces cerevisiae*. *J. Mol. Biol.* **296**: 1205–1214.
  22. Lau, P. C., C. K. Sung, J. H. Lee, D. A. Morrison, and D. G. Cvitkovich. 2002. PCR ligation mutagenesis in transformable streptococci: application and efficiency. *J. Microbiol. Methods* **49**:193–205.
  23. Layec, S., B. Decaris, and N. Leblond-Bourget. 2008. Characterization of proteins belonging to the CHAP-related superfamily within the Firmicutes. *J. Mol. Microbiol. Biotechnol.* **14**:31–40.
  24. Lynch, D. J., T. L. Fountain, J. E. Mazurkiewicz, and J. A. Banas. 2007. Glucan-binding proteins are essential for shaping *Streptococcus mutans* biofilm architecture. *FEMS Microbiol. Lett.* **268**:158–165.
  25. Lyon, W. R., and M. G. Caparon. 2004. Role for serine protease HtrA (DegP) of *Streptococcus pyogenes* in the biogenesis of virulence factors SpeB and the hemolysin streptolysin S. *Infect. Immun.* **72**:1618–1625.
  26. Macrina, F. L., J. A. Tobian, K. R. Jones, R. P. Evans, and D. B. Clewell. 1982. A cloning vector able to replicate in *Escherichia coli* and *Streptococcus sanguis*. *Gene* **19**:345–353.
  27. Matsumoto-Nakano, M., K. Fujita, and T. Ooshima. 2007. Comparison of glucan-binding proteins in cariogenicity of *Streptococcus mutans*. *Oral Microbiol. Immunol.* **22**:30–35.
  28. Mattos-Graner, R. O., et al. 2001. Cloning of the *Streptococcus mutans* gene encoding glucan binding protein B and analysis of genetic diversity and protein production in clinical isolates. *Infect. Immun.* **69**:6931–6941.
  29. Mattos-Graner, R. O., M. H. Napimoga, K. Fukushima, M. J. Duncan, and D. J. Smith. 2004. Comparative analysis of Gtf isozyme production and diversity in isolates of *Streptococcus mutans* with different biofilm growth phenotypes. *J. Clin. Microbiol.* **42**:4586–4592.
  30. Mattos-Graner, R. O., K. A. Porter, D. J. Smith, Y. Hosogi, and M. J. Duncan. 2006. Functional analysis of glucan binding protein B from *Streptococcus mutans*. *J. Bacteriol.* **188**:3813–3825.
  31. Ng, W. L., K. M. Kazmierczak, and M. E. Winkler. 2004. Defective cell wall synthesis in *Streptococcus pneumoniae* R6 depleted for the essential PcsB putative murein hydrolase or the VicR (YycF) response regulator. *Mol. Microbiol.* **53**:1161–1175.
  32. Ng, W. L., et al. 2003. Constitutive expression of PcsB suppresses the requirement for the essential VicR (YycF) response regulator in *Streptococcus pneumoniae* R6. *Mol. Microbiol.* **50**:1647–1663.
  33. Ng, W. L., H. C. Tsui, and M. E. Winkler. 2005. Regulation of the *pspA* virulence factor and essential *pcsB* murein biosynthetic genes by the phosphorylated VicR (YycF) response regulator in *Streptococcus pneumoniae*. *J. Bacteriol.* **187**:7444–7459.
  34. Nogueira, R. D., et al. 2007. Age-specific salivary immunoglobulin A response to *Streptococcus mutans* GbpB. *Clin. Vaccine Immunol.* **14**:804–807.
  35. Nogueira, R. D., A. C. Alves, M. H. Napimoga, D. J. Smith, and R. O. Mattos-Graner. 2005. Characterization of salivary immunoglobulin A responses in children heavily exposed to the oral bacterium *Streptococcus mutans*: influence of specific antigen recognition in infection. *Infect. Immun.* **73**:5675–5684.
  36. Perry, D., and H. K. Kuramitsu. 1981. Genetic transformation of *Streptococcus mutans*. *Infect. Immun.* **32**:1295–1297.
  37. Reinscheid, D. J., K. Ehlert, G. S. Chhatwal, and B. J. Eikmanns. 2003. Functional analysis of a PcsB-deficient mutant of group B streptococcus. *FEMS Microbiol. Lett.* **221**:73–79.
  38. Reinscheid, D. J., B. Gottschalk, A. Schubert, B. J. Eikmanns, and G. S. Chhatwal. 2001. Identification and molecular analysis of PcsB, a protein required for cell wall separation of group B streptococcus. *J. Bacteriol.* **183**:1175–1183.
  39. Rigoulay, C., et al. 2005. Comparative analysis of the roles of HtrA-like surface proteases in two virulent *Staphylococcus aureus* strains. *Infect. Immun.* **73**:563–572.
  40. Roth, F. P., J. D. Hughes, P. W. Estep, and G. M. Church. 1998. Finding DNA regulatory motifs within unaligned noncoding sequences clustered by whole-genome mRNA quantitation. *Nat. Biotechnol.* **16**:939–945.
  41. Sato, Y., Y. Yamamoto, and H. Kizaki. 1997. Cloning and sequence analysis of the *gbpC* gene encoding a novel glucan-binding protein of *Streptococcus mutans*. *Infect. Immun.* **65**:668–675.
  42. Senadheera, D., et al. 2009. Inactivation of VicK affects acid production and acid survival of *Streptococcus mutans*. *J. Bacteriol.* **191**:6415–6424.
  43. Senadheera, M. D., et al. 2005. A VicRK signal transduction system in *Streptococcus mutans* affects *gtfBCD*, *gbpB*, and *fif* expression, biofilm formation, and genetic competence development. *J. Bacteriol.* **187**:4064–4076.
  44. Senadheera, M. D., et al. 2007. The *Streptococcus mutans* *vicX* gene product modulates *gtfB/C* expression, biofilm formation, genetic competence, and oxidative stress tolerance. *J. Bacteriol.* **189**:1451–1458.
  45. Smith, D. J., H. Akita, W. F. King, and M. A. Taubman. 1994. Purification and antigenicity of a novel glucan-binding protein of *Streptococcus mutans*. *Infect. Immun.* **62**:2545–2552.
  46. Smith, D. J., W. F. King, H. Akita, and M. A. Taubman. 1998. Association of salivary immunoglobulin A antibody and initial mutans streptococcal infection. *Oral Microbiol. Immunol.* **13**:278–285.
  47. Smith, D. J., W. F. King, and M. A. Taubman. 1995. Synthesis, cellular distribution and immunogenicity of *S. mutans* glucan-binding protein (GbpB₅₉). *J. Dent. Res.* **74**:123.
  48. Smith, D. J., and M. A. Taubman. 1996. Experimental immunization of rats with a *Streptococcus mutans* 59-kilodalton glucan-binding protein protects against dental caries. *Infect. Immun.* **64**:3069–3073.
  49. Stipp, R. N., R. B. Goncalves, J. F. Hofling, D. J. Smith, and R. O. Mattos-Graner. 2008. Transcriptional analysis of *gtfB*, *gtfC*, and *gbpB* and their putative response regulators in several isolates of *Streptococcus mutans*. *Oral Microbiol. Immunol.* **23**:466–473.
  50. Thompson, W., E. C. Rouchka, and C. E. Lawrence. 2003. Gibbs Recursive Sampler: finding transcription factor binding sites. *Nucleic Acids Res.* **31**: 3580–3585.
  51. Vollmer, W., B. Joris, P. Charlier, and S. Foster. 2008. Bacterial peptidoglycan (murein) hydrolases. *FEMS Microbiol. Rev.* **32**:259–286.
  52. Vriesema, A. J., R. Brinkman, J. Kok, J. Dankert, and S. A. Zaat. 2000. Broad-host-range shuttle vectors for screening of regulated promoter activity in viridans group streptococci: isolation of a pH-regulated promoter. *Appl. Environ. Microbiol.* **66**:535–542.
  53. Wang, B., and H. K. Kuramitsu. 2005. Inducible antisense RNA expression in the characterization of gene functions in *Streptococcus mutans*. *Infect. Immun.* **73**:3568–3576.
  54. Welin, J., J. C. Wilkins, D. Beighton, and G. Svensater. 2004. Protein expression by *Streptococcus mutans* during initial stage of biofilm formation. *Appl. Environ. Microbiol.* **70**:3736–3741.
  55. Xiao, J., and H. Koo. 2010. Structural organization and dynamics of exopolysaccharide matrix and microcolonies formation by *Streptococcus mutans* in biofilms. *J. Appl. Microbiol.* **108**:2103–2113.