JOURNAL OF BACTERIOLOGY, Sept. 2007, p. 6521–6531 Vol. 189, No. 18
0021-9193/07/$08.00+0 doi:10.1128/JB.00825-07

Regulation of *gbpC* Expression in *Streptococcus mutans*▽

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Received 25 May 2007/Accepted 2 July 2007

***Streptococcus mutans*, the principal causative agent of dental caries, produces four glucan-binding proteins (Gbp) that play major roles in bacterial adherence and pathogenesis. One of these proteins, GbpC, is an important cell surface protein involved in biofilm formation. GbpC is also important for cariogenesis, bacteremia, and infective endocarditis. In this study, we examined the regulation of *gbpC* expression in *S. mutans* strain UA159. We found that *gbpC* expression attains the maximum level at mid-exponential growth phase, and the half-life of the transcript is less than 2 min. Expression from P*gbpC* was measured using a P*gbpC-gusA* transcriptional fusion reporter and was analyzed under various stress conditions, including thermal, osmotic, and acid stresses. Expression of *gbpC* is induced under conditions of thermal stress but is repressed during growth at low pH, whereas osmotic stress had no effect on expression from P*gbpC*. The results from the expression analyses were further confirmed using semiquantitative reverse transcription-PCR analysis. Our results also reveal that CovR, a global response regulator in many *Streptococcus* spp., represses *gbpC* expression at the transcriptional level. We demonstrated that purified CovR protein binds directly to the promoter region of P*gbpC* to repress *gbpC* expression. Using a DNase I protection assay, we showed that CovR binds to DNA sequences surrounding P*gbpC* from bases −68 to 28 (where base 1 is the start of transcription). In summary, our results indicate that various stress conditions modulate the expression of *gbpC* and that CovR negatively regulates the expression of the *gbpC* gene by directly binding to the promoter region.**

Streptococcus mutans, an important etiological agent of dental caries, has developed multiple mechanisms for successful survival, colonization, and continual presence in the human oral cavity. *S. mutans* produces acids using the dietary carbohydrates ingested by its human host (8). In the dental plaque, where the pH can vary from above 7.0 to as low as 3.0 after exposure to carbohydrates (10), *S. mutans* induces an acid tolerance response that enables this pathogen to survive and grow in low-pH environments (20). Low pH also initiates demineralization of the tooth enamel, leading to the formation of dental caries. The ability of *S. mutans* to produce acid also provides a selective advantage to this microorganism by inhibiting the growth of other oral bacteria (30). In addition, *S. mutans* also synthesizes extracellular polysaccharides using its hosts' dietary carbohydrates to promote plaque biofilm formation (20).

Glucans constitute the majority of the various extracellular polysaccharides produced by *S. mutans*. Glucans are generally synthesized from sucrose by the activities of at least three different glucosyltransferases (GTFs) (for a recent review, see reference 2). The glucans produced by *S. mutans* contain polymers of glucose moieties connected by α-(1-3) and α-(1-6) glucosidic linkages. GTF-I and GTF-SI (encoded by the *gtfB* and *gtfC* genes, respectively) synthesize α-(1-3)-rich glucans that are water insoluble, whereas GTF-S (encoded by *gtfD*) is responsible for the synthesis of water-soluble α-(1-6)-rich glucan. Water-insoluble glucans are a significant component of plaque biofilm that facilitates cell adherence and accumulation of stable biofilms, mediated by glucan-binding proteins. All of the above GTFs have some degree of glucan-binding capacity (2). In addition, there are cell surface-associated proteins that do not have any identifiable GTF activity but are able to bind glucan with very high affinity.

S. mutans produces at least four glucan-binding proteins (Gbp), designated GbpA, GbpB, GbpC, and GbpD (2). The genes encoding these proteins are not clustered in an operon but are distributed along the *S. mutans* chromosome (1). GbpC is believed to be the most important Gbp since it directly contributes to the cariogenicity of *S. mutans* (28, 33). Moreover, *S. mutans* strains expressing low levels of GbpC are more virulent for bacteremia, possibly due to their lower susceptibility to phagocytosis by polymorphonuclear leukocytes (34). Strains defective in *gbpC* expression exhibit a drastic reduction in sucrose-dependent adherence to glass surfaces, as well as sucrose-independent adherence to saliva-coated hydroxyapatites (28). GbpC-defective *S. mutans* strains also show significant deficiency in biofilm formation, as the structure of the biofilm formed is markedly different than that of the wild-type strain (24). GbpC is a cell wall-associated protein that contains an LPXTG motif in the C-terminal sequence; the membrane-localized sortase A mediates the cell wall anchoring of GbpC utilizing the LPXTG motif. Homologs of GbpC have been identified in many mutans group streptococci, including *S. cricetii*, *S. downii*, *S. ferus*, *S. macacae*, and *S. ratii* (35), many of which are also involved in plaque biofilm formation and infective endocarditis.

GbpC has been shown to be involved in rapid, dextran (glucan)-dependent aggregation (ddag) of bacteria, a phenomenon in which cells grown in liquid cultures autoaggregate upon exposure to exogenously added dextran, a polysaccharide related to glucan (25, 41, 42). This ddag phenotype is growth phase independent but depends on the growth conditions (40).

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▽ Published ahead of print on 6 July 2007.

TABLE 1. List of oligonucleotides used in this study

Primer	Sequence (5′ to 3′)[a]	Purpose
Bam-GbpC-F2	GCGCGGATCC**GGGGATGCAGAAGTGGAGAAGTTGAC**	P*gbpC-gusA* fusion
Xho-GbpC-R1	CGCGCTCGAG**CCGCCAAAGCAAGGCTGCTTAGCA**	P*gbpC-gusA* fusion
GbpC-F4	**GAGAAAGCACTTTTGGTTTCAATTGGAAC**	EMSA
GbpC-Fout2	**GGCTGCTTAGCAAAGTAATTTTAGCAGTTTTCG**	EMSA, 5′ RACE, and primer extension
GbpC-F5	**GCTAAGCAGCCTTGCTTTGGCG**	DNA probe, RT-PCR
GbpC-R2	**CTGCAGCTTCTACCGAAGGCTG**	DNA probe, RT-PCR
GyrA-For	**GACTATGCTATGAGTGTTATTGTTGCTCGGGC**	RT-PCR
GyrA-Rev	**GGCCATTCCAACAGCAATACCTGTCGCTCC**	RT-PCR
GbpC-Fout1	**CTTCATCTGCAAAAACATTCGTTGCTCC**	5′ RACE
5′-RACE outer	GCTGATGGCGATGAATGAACACTG	5′ RACE
5′-RACE inner	CCGCGGATCCGAATTAATACGACTCACTATAGG	5′ RACE
5′RACE adapter	GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA	5′ RACE
Bam-pASK-F1	GCCGGATCCCTTTAAGAAGGAGGATATACAAATGGCTAGCAGAGGA TCGC	CovR expression
pASK-IBA-Rev	CGCAGTAGCGGTAAACG	CovR expression
Eco-CovR-F2	GCGCGAATTC**ATGGCTAAGGACATTTTAATTATTGAAGATG**	CovR expression
Bam-CovR-R2	GCGCGGATCC**TTAATTATTTTCGCGAATGAATACCCCATGCC**	CovR expression

[a] Sequences homologous to the *S. mutans* genome sequence are in bold type. Added restriction sites used for cloning are underlined.

The ddag phenotype was not observed under standard laboratory growth conditions but was induced in cells grown under a variety of stress conditions (40).

Little is known about the regulation of *gbpC* expression. It was proposed that the expression of *gbpC* may be affected by the growth conditions, as well as various cellular stresses (42). However, it has recently been shown that CovR (also known as GcrR [41] or TarC [18]), a global response regulator in many streptococci, regulates *gbpC* expression (18, 41). Response regulators typically function as the cytoplasmic portion of two-component signal transduction systems that work with membrane-associated sensor kinases and modulate gene expression in response to changes to the external environment.

In this communication, we report the transcriptional regulation of *gbpC*. We show that *gbpC* expression reaches maximum levels at mid-exponential phases of growth and that the *gbpC* transcript is very unstable. We also demonstrate that different carbohydrates have little or no effect on *gbpC* transcription, whereas environmental stresses, such as temperature and osmolarity, can influence *gbpC* transcription. In addition, we show that CovR represses *gbpC* expression by directly binding to the promoter region of this gene.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* strain DH5α was grown in Luria-Bertani medium that was supplemented with (when necessary) ampicillin (100 μg/ml), erythromycin (300 μg/ml), kanamycin (100 μg/ml), and/or spectinomycin (100 μg/ml). For all of the genetic experiments, *S. mutans* UA159 and its derivatives were used. Except where noted below, *S. mutans* strains were routinely grown in Todd-Hewitt medium (BBL, Benton Dickson) supplemented with 0.2% yeast extract (THY medium). The medium was supplemented, when necessary, with the following antibiotics: erythromycin (10 μg/ml), kanamycin (300 μg/ml), and/or spectinomycin (300 μg/ml).

The pH of THY medium was adjusted to the desired value prior to sterilization via addition of HCl. Thereafter, 50 mM potassium phosphate-citric acid buffer (22) at desired pH values was added to the sterilized media. To determine the effect of sugar alcohol on the growth of *S. mutans*, sterilized sugar alcohol solutions were added to sterile THY media at a final concentration of 0.5%. Sterile THY medium was supplemented with NaCl or ethanol at the amount indicated below for osmotic stress experiments.

For some *gbpC* expression experiments, *S. mutans* was grown in chemically defined medium (CDM) consisting of 58 mM K_2HPO_4, 15 mM KH_2PO_4, 10 mM $(NH_4)_2SO_4$, 35 mM NaCl, 0.1 mM $MnCl_2$, 2 mM $MgSO_4 \cdot 7H_2O$, and 0.2% (wt/vol) casein hydrolysate as previously described (22, 23). CDM was supplemented with filter-sterilized vitamins (catalog no. R7256; Sigma Aldrich), amino acids (1 mM L-arginine HCl, 1.3 mM L-cysteine HCl, 4 mM L-glutamic acid, and 0.1 mM L-tryptophan), and different sugars at a concentration of 0.5%.

For all *S. mutans* cultures, growth was monitored using a Klett-Summerson colorimeter with a red filter (3).

Isolation of RNA from bacterial cultures. *S. mutans* cultures were grown until the desired optical density was reached, and then the cultures were harvested by centrifugation. Each cell pellet was resuspended in an amount of RNAprotect bacterial reagents (QIAGEN) equivalent to 1 culture volume and incubated for 10 min at room temperature. Total RNA was isolated using an RNeasy minikit (QIAGEN) according to the manufacturer's instructions, with a modified bacterial lysis step. The cells were lysed with glass beads (diameter, 0.1 mm) in a high-speed homogenizer (Thermo Electron Corporation). The supernatant was loaded onto an RNeasy minicolumn, and DNA contaminants were removed by on-column DNase I treatment by following the manufacturer's instructions (QIAGEN). The quality and quantity of the RNA samples were verified using an RNA 6000 Nano chip with an Agilent 2100 bioanalyzer (Agilent Technologies) according to the manufacturer's protocol.

Stability of mRNA. Aliquots of *S. mutans* were grown to mid-exponential phase (70 Klett units [KU]). Rifampin, which inhibits bacterial RNA polymerase, was added to all but one of the cultures at a final concentration of 300 mg/ml. At nine time intervals during a total incubation period of 60 min at 37°C, metabolic processes were terminated by addition of 100 mM sodium azide, followed by freezing of the cultures on dry ice. The rifampin-free aliquot was used as a control to demonstrate that levels of *gbpC* mRNA were in a steady state during the period of study. Total RNA was isolated as described above, and the quality of the RNA was evaluated using a bioanalyzer.

Northern blotting. Total RNA (4 μg) isolated from *S. mutans* was denatured, separated in a 1.0% agarose gel by electrophoresis, and transferred to positively charged Zeta-probe nylon membranes (Bio-Rad) by following the NorthernMax-Gly protocol (Ambion). DNA probes were prepared by PCR amplification using primers GbpC-F5 and GbpC-R2 (Table 1) and *S. mutans* strain UA159 chromosomal DNA as the template. PCR fragments were labeled with [α-^{32}P]dATP by random priming using a DECAprimeII kit (Ambion). Blots were hybridized using a radiolabeled *gbpC* probe in ULTRAhyb buffer (Ambion) and were washed according to the manufacturer's protocol. RNA blots were analyzed using a phosphorimager (Molecular Dynamics).

Determination of initiation of transcription. FirstChoice RLM random amplification of cDNA ends (RACE) (Ambion) was used to determine the initiation of *gbpC* transcription by following the manufacturer's procedure, with some modifications. Total RNA (4 μg) isolated from *S. mutans* grown in THY medium was used for 5′ RACE adapter ligation. Ligated samples were subjected to reverse transcription (RT) using random decamers and Moloney murine leukemia virus reverse transcriptase, followed by nested PCR. For the first-round PCR the 5′ RACE outer primer and the GbpC-Fout1 primer were used along with the

RT reaction products as the template. Samples from the first-round PCR were then subjected to second-round PCR amplification using the 5′ RACE inner primer and the GbpC-Fout2 primer. Final PCR products were separated by electrophoresis on a 1% agarose gel and purified using a QIAGEN gel extraction kit before cloning into the pCR2.0 TOPO linearized vector (Invitrogen) for sequencing. The sequences were determined using the universal M13 reverse primer. Primer extension reactions were performed as previously described (4), with the following modification. Briefly, RNA samples isolated from mid-exponential-phase cultures were incubated with 0.5 pmol of 5′-end-labeled GbpC-Fout2 primer for 5 min at 70°C, followed by cooling to 4°C. Primer extension was accomplished using SuperscriptII RNase H reverse transcriptase (Invitrogen) by following the manufacturer's recommended protocol. Samples were analyzed on a 8% sequencing gel using a sequencing reaction mixture (SequiTherm Excel II; Epicenter) as markers, followed by phosphorimaging.

Semiquantitative RT-PCR. RNA samples were isolated as described above from cultures of *S. mutans* incubated under different growth conditions, as indicated below. The concentration of RNA was determined using UV spectrophotometry as well as a bioanalyzer. Semiquantitative analyses of transcript levels of *gbpC* and *gyrA* (as a control) were performed using the Titan one-tube RT-PCR system (Roche) by following the manufacturer's instructions. The primers specific for the *gbpC* transcript (GbpC-F5 and GbpC-R2) produce a 308-bp PCR product, while the primers specific for the *gyrA* transcript (GyrA For and GyrA Rev) generate a 470-bp PCR product. Fifty nanograms of RNA was used for each RT-PCR, which was followed by electrophoresis of the PCR products on a 1% agarose gel and quantification using Doc-It-LS (UVP) software. Expression of the *gyrA* gene served as an internal control to ensure that equal amounts of RNA were used in all of the RT-PCRs.

Construction of P*gbpC-gusA* reporter strains. To construct a reporter strain, we chose plasmid pIB107, which contains a *gusA* gene and can be used for integration at the Smu1405 locus for single-copy reporter fusion as previously described (4). The *gbpC* promoter region (476 bp) was amplified from *S. mutans* chromosomal DNA using primers Bam-GbpC-F2 and Xho-GbpC-R1 (Table 1) and cloned into BamHI-XhoI-restricted pIB107 to create pIB121. Plasmid pIB121 was linearized with BglI and transferred to UA159 by natural transformation to create strain IBS131. Linearized pIB121 was also used to transform strain IBS10 (4), a *covR* mutant strain, to create IBS132.

GusA assays. β-Glucuronidase (Gus) assays were performed after the *S. mutans* cultures reached mid-exponential phase (70 KU), as described by Biswas and Biswas (4). The Gus activities of the culture lysates were standardized by comparison with the corresponding activities of known concentrations of glucuronidase (Sigma Aldrich). The protein concentrations of the lysates were determined using a Micro BCA protein assay kit (Pierce) standardized with bovine serum albumin (Sigma Aldrich).

Cloning and expression of CovR. In order to produce a His-tagged CovR protein suitable for binding studies, *covR* was PCR amplified from UA159 chromosomal DNA using primers Eco-CovR-F2 and Bam-CovR-R2 (Table 1). The amplified fragment was then cloned into the pASK-IBA43plus vector (IBA, Germany) using EcoRI and XhoI restriction sites, creating pIB81,which contains the *covR* coding region fused to six histidine residues. A DNA sequence containing the entire *covR* coding region along with the six histidine residues was PCR amplified from pIB81 using primers Bam-pASK-33-F1 and pASK-IBA-Rev and then cloned into pJRS1315 (36) using BamHI and HindIII, creating plasmid pIB132.

His-tagged CovR was expressed in *E. coli* by growing the cells containing pIB132 overnight at 37°C. The cells were then collected, resuspended in binding buffer (20 mM Tris-Cl [pH 7.6], 0.5 mM imidazole, 0.5 M NaCl), and lysed using a sonicator. The cleared lysate was loaded onto a Ni-nitrilotriacetic acid column (Novagen) and washed with 6 column volumes of wash buffer (20 mM Tris-Cl [pH 7.6], 60.0 mM imidazole, 0.5 M NaCl). His-CovR was eluted from the column with 2 volumes of elution buffer (wash buffer with 1.0 M imidazole). The protein was dialyzed overnight (20 mM Tris-Cl [pH 7.6], 100 mM NaCl, 2 mM EDTA, 10% [vol/vol] glycerol); following dialysis, the protein samples were stored at −20°C. The protein was purified to over 95% homogeneity as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. The concentration of CovR was estimated using a Bradford protein assay kit (Bio-Rad) with bovine serum albumin as the standard.

EMSA and DNase I protection assay. DNA binding and an electrophoretic mobility shift assay (EMSA) were carried out essentially as previously described (4). Briefly, radiolabeled PCR-amplified DNA fragments were incubated with CovR (4) in DNA-binding buffer [50 mM $NaPO_4$ (pH 6.5), 50 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol, 2 μg/ml poly(dI-dC), 10% glycerol; final volume, 40 μl] for 40 min at room temperature. After incubation, the samples were loaded onto a 4.8% native acrylamide gel containing 50 mM $NaPO_4$ buffer (pH 6.5). Gels were electrophoresed in 50 mM $NaPO_4$ (pH 6.5) at room temperature at 120 V, dried, and exposed to a phosphorimager plate. DNase I protection assays were performed as previously described. Briefly, DNase I (2 μl of a 0.02-U/ml preparation; Epicenter) was added to CovR-bound radiolabeled DNA fragments (as described above) and incubated for 2 min at room temperature. The DNase I-CovR-bound radiolabeled DNA mixture, along with the DNA sequencing reaction mixtures for the DNA fragment, was electrophoresed on an 8% denaturing sequencing gel and analyzed by autoradiography on a phosphorimager plate.

RESULTS

Genetic characterization of the *gbpC* transcript. A genomic map of *gbpC* and the genes in its vicinity is shown in Fig. 1A. Sequence analysis of the upstream region of the *gbpC* gene revealed many −10-like elements located within the 212-bp intergenic region. In order to study the regulation of *gbpC* expression, it was necessary to accurately map the transcriptional start site within the upstream region of *gbpC*. Two separate methods were used to identify the transcriptional start site.

The first method used was the 5′ RACE-PCR assay using RNA extracted from exponentially growing *S. mutans* UA159 cells to map the transcriptional start site. The resulting electropherogram obtained from the 5′ RACE-PCR analysis (Fig. 1B) localized the transcriptional start point (position 1) of the *gbpC* gene to a site 37 bp upstream of the putative start codon. A perfect pribnow box (−10 box; TATAAT) was found 8 bases upstream of the transcription start site, and a putative −35 box (TTTGAA) was separated by 19 bp from the pribnow box. A potential ribosome-binding site (GGATGG) was also identified 5 bp upstream of the start codon. Second, primer extension assays were also used to identify the transcriptional start site, using the same RNA sample that was used for the 5′ RACE-PCR assay. Using this method, the transcriptional start site was mapped 10 bp downstream of the site originally identified via 5′ RACE-PCR analysis (Fig. 1C). The primer extension analyses were repeated with several RNA samples extracted from UA159 at two different growth phases (mid- and late-exponential phases), using various reverse transcriptase enzymes; the second transcriptional start site was observed in both cases (data not shown). While these two methods did not generate the same results, it is possible that the second transcriptional start site resulted from the processing of the transcript generated from the first transcriptional start site.

In order to obtain insight into the transcriptional organization of the *gbpC* locus, Northern blot hybridization of RNA extracted from UA159 was performed as described in Materials and Methods. Northern blot analysis revealed the presence of two distinct transcripts (Fig. 1D). The major transcript (~88% of the total) corresponds to a length of approximately 3.7 kb, suggesting that the two genes immediately downstream of *gbpC*, a small open reading frame and the *lepA* gene, are cotranscribed with *gbpC* (Fig. 1A). In addition, a low-abundance transcript (~12% of the total) about 5.3 kb long was also observed, indicating that the three genes immediately downstream of *lepA* are also cotranscribed with *gbpC* (Fig. 1A). Thus, *gbpC* expression is apparently linked to *lepA* and possibly to other downstream genes.

Expression of *gbpC* during different growth phases and stability of the *gbpC* transcript. A semiquantitative RT-PCR assay

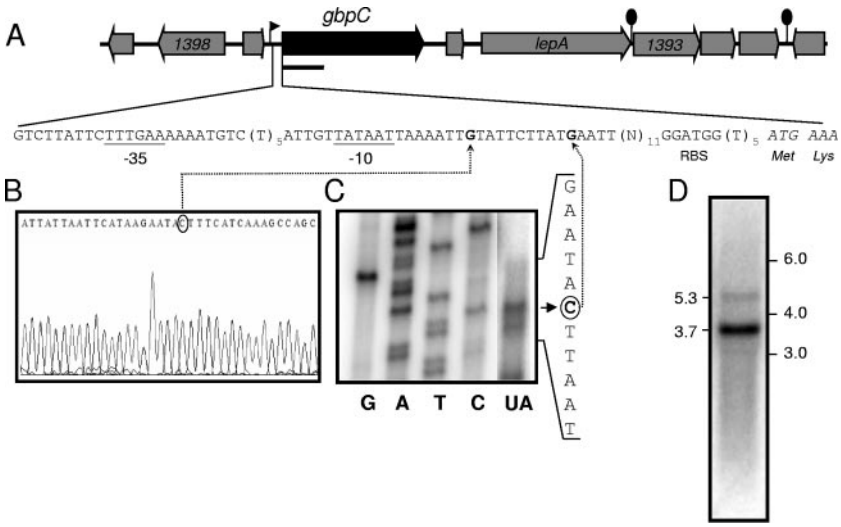

FIG. 1. Mapping of the transcriptional initiation site of *gbpC*. (A) Schematic representation of the *gbpC* locus. Open reading frames are represented by block arrows, and their orientations indicate the transcriptional direction. The upstream sequence of the *gbpC* open reading frame is shown below the diagram. (B) Transcription initiation site as determined using 5′ RACE-PCR. (C) Transcription initiation site as determined using primer extension. (D) Northern blot analysis of total RNA isolated from *S. mutans* UA159 after growth in THY medium. The predicted −35 and −10 sites are underlined, and the ribosome-binding site (RBS) is indicated. The bent arrow indicates the transcription start site, and the lollipop symbols indicate the location of putative transcriptional termination.

was used to measure the amount of *gbpC* transcript produced during various phases of growth. For this, we used strain IBS131, a wild-type derivative of UA159. The *gyrA* transcript level was also measured to ensure that equal amounts of RNA were used in the RT-PCR analysis. As shown in Fig. 2, *gbpC* expression was found to be higher at mid-exponential growth phase than at other stages of growth. The stability of the *gbpC* transcript produced by IBS131 was measured using RNA extracted at the mid-exponential growth phase. Just prior to RNA extraction, rifampin was added to the cell culture to prevent de novo synthesis of mRNA. RNA was then extracted from IBS131 at 0, 1, 2, 4, 6, 10, 15, and 20 min, and the stability of the *gbpC* transcript was determined using Northern blot analysis (Fig. 3A). The decay kinetics of the *gbpC* transcript were measured, and the chemical half-life of the *gbpC* tran-

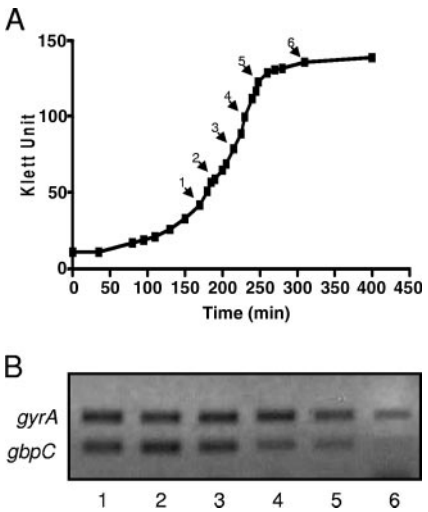

FIG. 2. Expression of *gbpC* at different stages of growth. (A) The total RNA was extracted at the indicated time points from IBS131 cells grown in THY broth. (B) RNA samples (50 ng) were subjected to semiquantitative RT-PCR analysis with primers specific for the *gbpC* gene or the *gyrA* gene as described in Materials and Methods. The experiments were repeated at least twice with independent RNA isolations. The growth points are as follows: 1, 42 KU; 2, 59 KU; 3, 79 KU; 4, 100 KU; 5, 123 KU; and 6, 136 KU. The *gyrA* gene was included to ensure that equal amounts of RNA were used for all reactions.

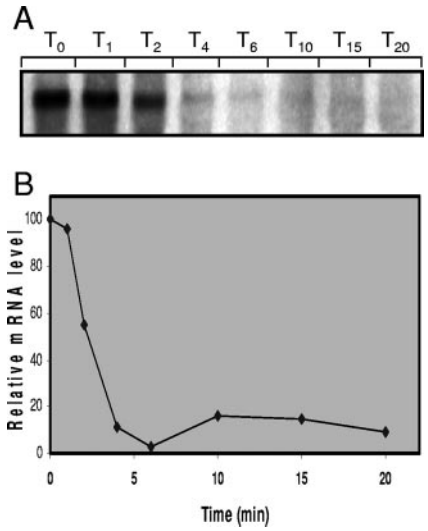

FIG. 3. Stability of *gbpC* transcript. The stability of the *gbpC* transcript was measured using cultures at mid-exponential growth phase (70 KU). RNA was extracted from IBS131 at the times (in minutes) indicated above the lanes following the addition of rifampin to block the synthesis of new mRNA. Northern blotting (A) was performed with 4 μg of total RNA, using the *gbpC* sequence as a probe. The decay curve calculation (B) was performed as described in the text. Experiments were repeated at least twice, and a representative Northern blot and representative graph are shown.

script was determined to be less than 2 min, indicating that the *gbpC* transcripts were short-lived (Fig. 3B).

Expression of *gbpC* in the presence of various carbohydrates. To further study the regulation of *gbpC* expression, a transcriptional reporter strain was constructed using the information obtained from the transcriptional start site mapping experiments described above. In this reporter construct, the promoter region of *gbpC*, along with the sequence encoding first 18 amino acids of GbpC, was fused to the 5′ end of the *gusA* reporter gene in pIB107, generating pIB121. The P*gbpC-gusA* reporter construct (pIB121) was inserted into the UA159 chromosome at the Smu1405 locus (which is linked neither to the *gbpC* locus nor to the *covR* locus [see below]) to produce IBS131. This strain and its derivatives were used for subsequent gene expression analysis. Transcription from P*gbpC* was quantified by measuring the activity of GusA produced from P*gbpC-gusA* strains as described previously (3).

Expression of many enzymes, such as GTF and fructosyltransferase, which produce and bind to extracellular polysaccharides (i.e., glucan), is affected by the presence of various carbohydrates in the growth medium (5, 6, 21). Therefore, to determine if expression of *gbpC* is regulated by the presence of carbohydrates, strain IBS131 was cultured in CDM supplemented with various carbohydrates, including glucose, sucrose, fructose, galactose, lactose, and maltose. Gus activity expressed from P*gbpC-gusA* was measured at mid-exponential phase, since *gbpC* expression is optimal in this growth phase. No significant differences in P*gbpC-gusA* expression were observed in response to the presence of various carbohydrates (data not shown). However, the expression of P*gbpC-gusA* in the presence of sucrose was about 0.7-fold the expression in the presence of glucose. Thus, while other sugars have little or no effect on *gbpC* expression, sucrose appears to repress *gbpC* expression, although the repression is marginal.

Expression of *gbpC* is affected by environmental stresses. Expression of P*gbpC-gusA* was measured under various stress conditions, as it has been suggested that *gbpC* may be induced under various stress conditions, including thermal and osmotic stresses, based on the appearance of the ddag$^+$ phenotype (40). To observe the effect of thermal stress on expression from P*gbpC*, IBS131 was grown in THY medium at various temperatures (Fig. 4A). Gus activity was measured when the cultures reached mid-exponential phase. The growth of *S. mutans* at various temperatures was not significantly different. As shown in Fig. 4A, expression from P*gbpC* was about 1.7-fold lower at 28°C than at 37°C and 1.6-fold higher when the culture was grown at 42.5°C. These results indicate that *gbpC* expression is likely temperature dependent and the expression is induced during growth at high temperature.

Since *gbpC* expression is thought to be induced under osmotic stress conditions (42), P*gbpC-gusA* expression was also examined in the presence of various osmotic stress-inducing agents. First, IBS131 was grown in THY medium supplemented with two different concentrations of NaCl (0.5 and 1.0%) or ethanol (2.0 and 4.0%), and the Gus activity was compared with the activity in a culture grown in THY medium in the absence of any stressors. As shown in Fig. 4B, there was no significant difference in the levels of P*gbpC-gusA* expression with and without osmotic stressors.

Sugar alcohols, also known as polyols, are also able to generate osmotic stress in bacteria, although the genes induced are distinct from those induced under salt stress conditions (22, 40). In *S. mutans*, 0.5% (vol/vol) sugar alcohols are sufficient to induce osmotic stress (40, 42). Induction of P*gbpC-gusA* expression was studied with *S. mutans* cultures grown in THY medium supplemented with xylitol, sorbitol, or mannitol (0.5%, vol/vol). As shown in Fig. 4C, the P*gbpC-gusA* expression in the presence of various sugar alcohols was not significantly different than the expression under the normal growth conditions. Thus, taken together, the results suggest that *gbpC* expression in *S. mutans* is not significantly altered under osmotic stress conditions.

The effect of acidic pH on *gbpC* expression was examined next. Strain IBS131 was grown in pH-buffered THY medium with potassium-citrate buffer as described in Materials and Methods, and Gus activity was measured when the culture reached mid-exponential phase. As shown in Fig. 4D, the Gus activity at pH 5.75 was 2.5-fold lower than the activity in the THY medium without potassium-citrate buffer (the pH of this culture was 7.2 at the time of sampling), indicating that an acidic pH represses expression of *gbpC*. This effect was also observed at pH 6.56 (~2.0-fold lower). Thus, *gbpC* expression appears to be strongly dependent on the pH of the culture media.

CovR regulates *gbpC* expression. Many genes that encode glucan-binding proteins are regulated by two-component regulatory systems. For example, *gbpB* has been shown to be regulated by VicRK in *S. mutans* UA159 (43). Similarly, regulation of *gbpC* was shown to be modulated by CovR in strains UA130 and 109c (18, 41). However, in both of the previous studies, the degree and the mechanism by which CovR regulates this gene were not addressed. CovR regulates expression of *gtfB* and *gtfC* by directly binding to the promoter region of these genes in *S. mutans* strain UA159. It was of great interest to determine whether CovR indeed regulates *gbpC* expression by directly binding to the promoter region in strain UA159.

In order to study the effect of CovR on *gbpC* expression, the *covR*-null strain IBS10 (4) was transformed with pIB121, which contains the P*gbpC-gusA* reporter construct, and the transformant was designated IBS132. Transcription from P*gbpC* was measured in both the wild-type strain (IBS131) and the *covR* mutant (IBS132) by measuring the activity of GusA produced from P*gbpC-gusA* strains as described previously (3). As shown in Fig. 5A, the *covR* mutant strain showed 2.5-fold more P*gbpC-gusA* expression than its *covR*$^+$ parent (IBS131). To verify that this effect was the result of the *covR* inactivation, Gus activity was also measured from P*gbpC-gusA* in IBS132 transformed with pIB30, a plasmid containing full-length *covR* with its potential promoter region (4), and with IBS132 containing the vector pOri23. The latter strain had the same GusA activity as strain IBS132, while the complemented strain, IBS132/pIB30, demonstrated slightly lower activity than the wild-type parental *covR*$^+$ strain (IBS131), probably due to higher expression of *covR* from the multicopy plasmid.

To confirm the transcriptional fusion data described above, *gbpC* transcription was measured directly using semiquantitative RT-PCR analysis. RNA was isolated at two different growth phases, mid-exponential and stationary, from strains IBS131, IBS132, and IBS132/pIB30. Semiquantitative RT-PCR was performed using *gbpC*-specific primers to measure

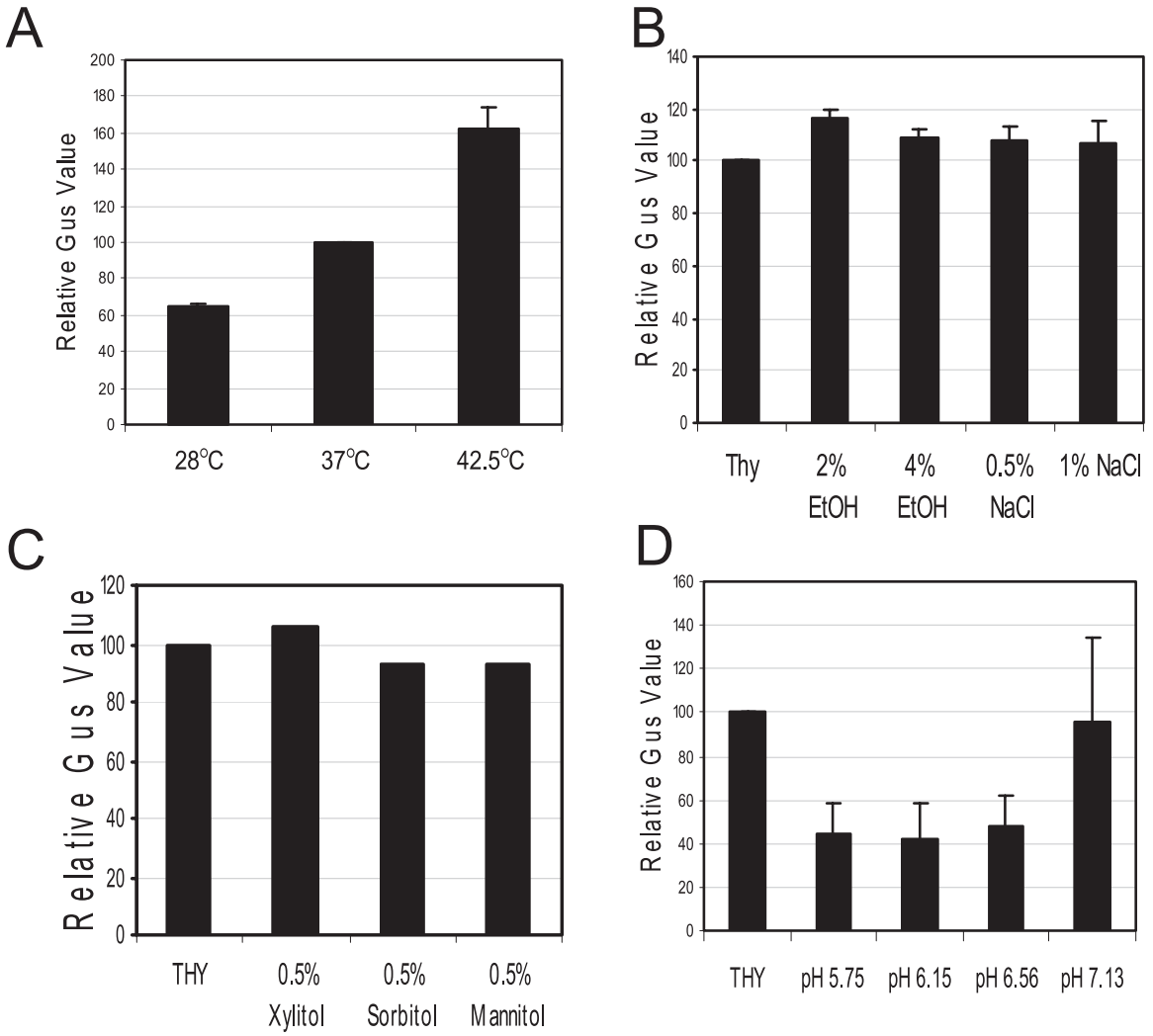

FIG. 4. Expression of P*gbpC* under different stress conditions. The reporter strain IBS131 contains the promoter region of *gbpC* (P*gbpC*) along with the sequence encoding the first 18 amino acids of GbpC fused to the *gusA* gene. IBS131 was grown in THY medium under different stress conditions, as indicated. Expression from P*gbpC* at mid-exponential phase was quantified by determining the Gus activity as described in the text. The values were normalized with the Gus activity obtained with IBS131 grown in THY medium at 37°C. Experiments were performed at least three times, and the means and standard deviations are shown.

the level of the *gbpC* transcript; the level of the *gyrA* transcript produced was also measured to ensure that equal amounts of RNA were used in the RT-PCR assay. As expected, the *gbpC* transcript level at the mid-exponential phase was 2.2-fold higher in strain IBS132, while the complemented strain, IBS132/pIB30, produced 2.5-fold less *gbpC* transcript than the wild-type IBS131 strain. The relative level of the *gbpC* transcript at the stationary phase followed the same pattern as the level in the mid-exponential phase (Fig. 5B). However, the overall level of the *gbpC* transcript was lower in the stationary phase than in the mid-exponential phase, which is consistent with the results of the growth phase analysis, indicating that *gbpC* expression is highest in mid-exponential phase (Fig. 2). Taken together, the results indicate that higher expression from P*gbpC* is due to the inactivation of *covR*, consistent with the previous reports (18, 41).

CovR directly binds to the promoter region of the *gbpC* gene. Since *gbpC* is regulated by CovR, it would be expected that CovR regulates by directly binding to the promoter region. An EMSA was used to verify the binding of CovR to the promoter region of *gbpC*. A 204-bp fragment (from position −126 to position 78), which includes the putative promoter P*gbpC*, was used for the EMSA, along with purified CovR protein. As shown in Fig. 6, lanes 1 to 8, addition of CovR caused a shift in the mobility of the DNA fragment, indicating that CovR does indeed bind to P*gbpC*. To demonstrate the specificity of CovR for P*gbpC*, competition assays were performed using unlabeled DNA fragments. Addition of a 2- to 10-fold molar excess of the unlabeled 500-bp P*rpsL* region had no effect on CovR binding, whereas addition of a 2- to 10-fold molar excess of unlabeled P*gbpC* reduced binding to the labeled P*gbpC* fragment (Fig. 6, lanes 9 to 12). Thus, it appears that CovR specifically binds to the P*gbpC* promoter to repress P*gbpC* expression.

CovR binds to a large region covering the P*gbpC* promoter. DNase I protection assays were used to identify the region

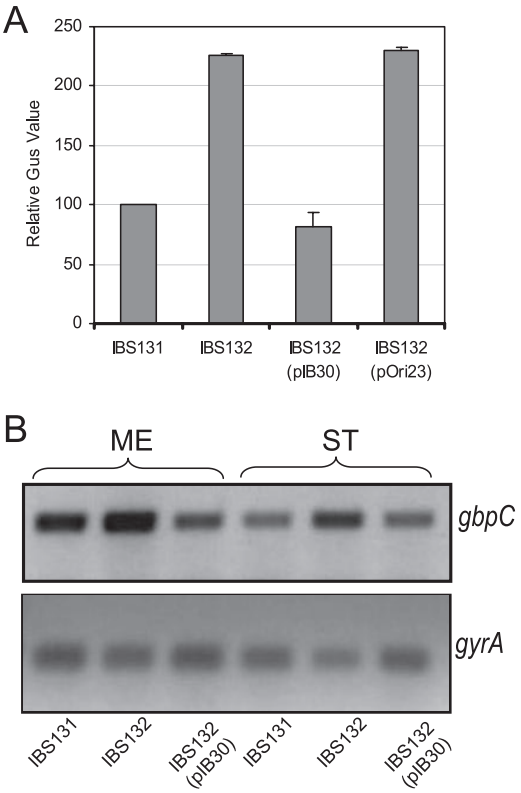

FIG. 5. GbpC expression is regulated by CovR. (A) Expression of P*gbpC* in the wild-type and *covR* mutant strains. Strains were grown in THY broth at 37°C and harvested at mid-exponential phase, and Gus activity was measured as described in the text. The data are the means and standard deviations of at least three experiments that were normalized with wild-type (IBS131) values. (B) Semiquantitative RT-PCR analysis of *gbpC* transcription. RNA was harvested at mid-exponential (ME) and stationary (ST) phases of growth and subjected to RT-PCR analysis using primer pairs specific for *gbpC* or *gyrA*, as indicated. The data are representative of an RT-PCR analysis resulting from at least two independent RNA isolations.

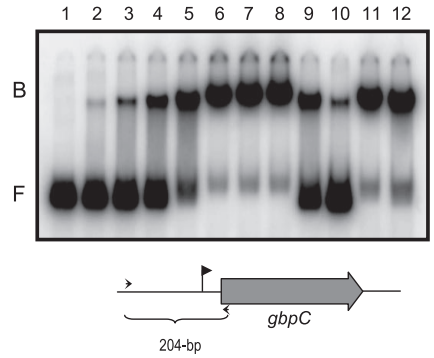

FIG. 6. Binding of CovR to the P*gbpC* promoter. A 204-bp P*gbpC* DNA fragment was radiolabeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, and 0.52 pmol of labeled DNA was used for binding with various amounts of CovR. CovR-P*gbpC* DNA reaction mixtures were incubated at room temperature for 40 min, which was followed by electrophoresis in a 50 mM $NaPO_4$-buffered 5% acrylamide gel (pH 6.5). Gels were electrophoresed at 100 V for 90 min at room temperature, dried, and exposed to a phosphorimager screen. Lane 1 contained no CovR; lanes 2 to 8 contained 1.15, 2.3, 4.6, 9.2, 18.4, 23.0, and 27.6 pmol of CovR, respectively; and lanes 9 to 12 contained 18.4 pmol of CovR along with 1.0 or 5.4 pmol of unlabeled P*gbpC* DNA (lanes 9 and 10, respectively) or 1.0 or 5.1 pmol of unlabeled P*rpsL* (lanes 11 and 12, respectively). F, free probe; B, bound probe.

within P*gbpC* essential for CovR binding. The 204-bp P*gbpC* fragment used for EMSA was also used for the DNase I protection assay, except that the DNA was end labeled on only one strand. As shown in Fig. 7, the binding of CovR generated a large footprint on the P*gbpC* promoter at the −35 and −10 positions, with concomitant increases in the size and/or intensity of the footprint with increases in the CovR concentration (Fig. 7, lanes 2 to 5). Comparisons of the sequence of the P*gbpC* fragment with the footprint generated during the DNase I protection assay indicate that the total region protected by CovR spans approximately 100 bp, between positions −68 and 28 (position 1 is the transcriptional start site) on the P*gbpC* promoter fragment. Thus, CovR binds to and protects a large region on the P*gbpC* promoter, covering both −35 and −10 sequences.

Stress mediated *gbpC* expression is CovR independent. While stress-dependent (i.e., temperature- and pH-dependent) modulation of expression from P*gbpC* was observed, it was not clear whether this regulation was dependent on the presence of CovR. In order to elucidate whether CovR has any role in stress-dependent *gbpC* expression, IBS131 and IBS132 were cultured under different stress conditions as indicated in Fig. 8, and RNA was isolated from mid-exponential and stationary phases of growth from the strains. Semiquantitative RT-PCRs were then performed to measure the levels of *gbpC* transcript produced in IBS131 and IBS132. As shown in Fig. 8, the ratio of *gbpC* expression at 28, 37, and 42°C in strain IBS131 was 0.5:1.0:2.2, while the ratio in strain IBS132 was 0.6:1.0:1.8. This strongly suggests that temperature-induced *gbpC* expression is CovR independent, since similar transcription ratios were observed in both the wild-type and *covR* mutant strains.

Earlier results indicated that growth at low pH inhibited P*gbpC-gusA* expression, but it was not known whether CovR might have a role in pH-dependent repression. To determine whether CovR had a role in pH-dependent repression of expression from P*gbpC*, RNA was isolated from cultures of IBS131 and IBS132 grown in medium at pH 5.75 or 7.13. As expected, the level of the *gbpC* transcript measured in IBS131 was 2.3-fold lower at pH 5.75 than at pH 7.13; similarly, the level of the *gbpC* transcript in strain IBS132 was 1.8-fold lower at pH 5.75 than at pH 7.13. In both cases, the levels of the *gbpC* transcript were similar in the unbuffered and pH 7.13 media, implying that CovR does not have a role in the pH-dependent repression of *gbpC* expression Taken together, the data strongly suggest that CovR does not play a role in stress-dependent expression of *gbpC*.

DISCUSSION

Although the role of GbpC in the pathogenesis of *S. mutans* has been studied in detail, very little is known about its transcriptional regulation. To gain a better understanding of the regulation of *gbpC* expression, detailed transcriptional analysis of the *gbpC* locus was carried out, and a number of novel findings emerged from our study. The first finding is that *gbpC* is coexpressed with the downstream *lepA* gene that encodes a

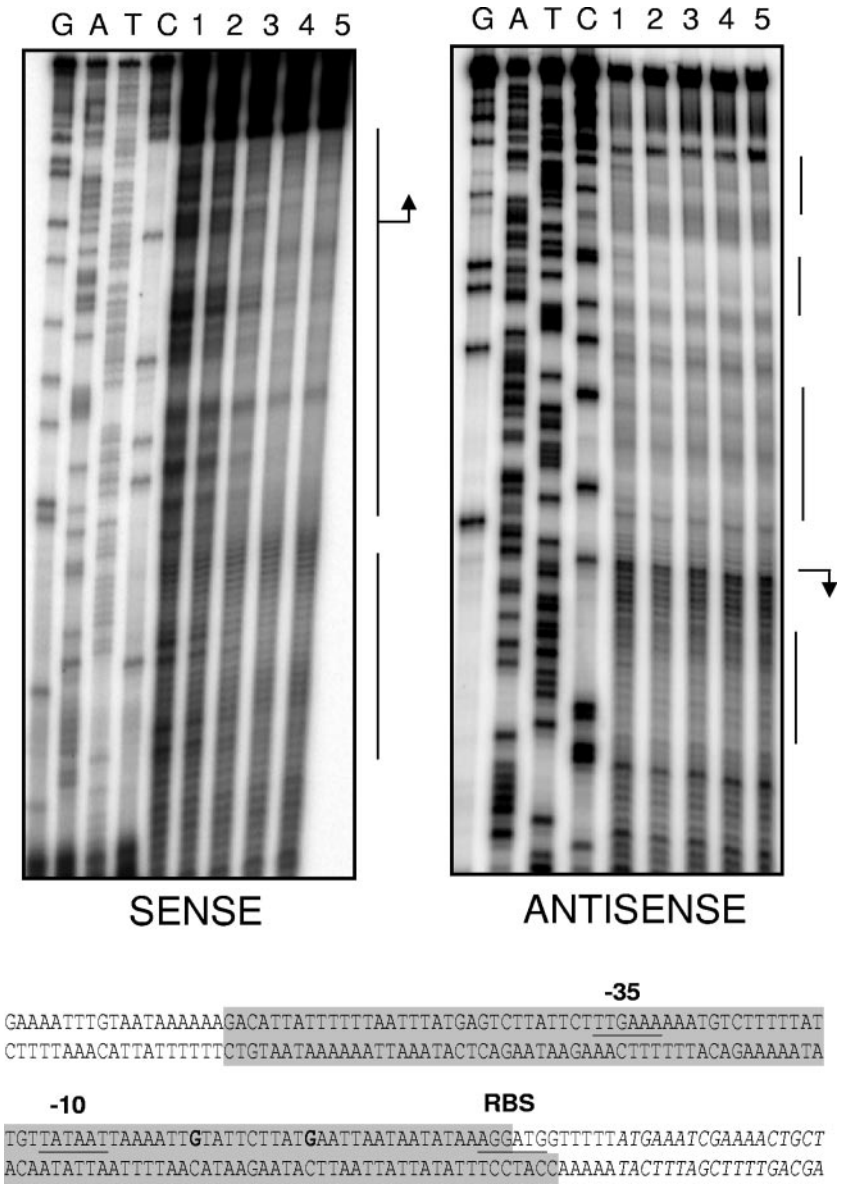

FIG. 7. DNase I protection assay of the *gbpC* promoter. EMSA was performed with CovR and a 204-bp P*gbpC* DNA sequence, as described in the legend to Fig. 6. The amounts of CovR added to 0.52 pmol of the P*gbpC* were 0, 4.6, 9.2, 18.4, and 27.6 pmol (lanes 1 to 5, respectively). DNase I protection assays were carried out as described in Materials and Methods. Samples were electrophoresed on an 8.0% sequencing gel adjacent to P*gbpC* sequencing reaction mixtures (lanes G, A, T, and C). Binding regions are indicated by vertical lines, and the transcription start site is indicated by a bent arrow. The region of P*gbpC* protected from DNase I digestion is distinguished by gray shading. RBS, ribosome-binding site.

GTPase protein similar to EF-G (7). LepA-encoding genes are universally present in all bacterial genomes; however, the locus that encodes *lepA* is very diverse. Although the physiological role of LepA in the cell is unclear, a recent study indicates that LepA is involved in the so-called back-translocation of ribosomes to provide EF-G an opportunity to replace tRNA correctly during translation (37). Since there is no apparent functional similarity between GbpC and LepA, it is surprising that the genes are apparently cotranscribed. Second, it appears that *gbpC* expression is growth phase regulated and that expression is optimal at mid-exponential phase and then gradually decreases; extremely low levels of *gbpC* expression were observed after 24 h of growth (data not shown). We were surprised to find that *gbpC* expression was so greatly diminished in the stationary phase, since GbpC is required to develop proper biofilm structure (24). Our studies also indicate that the half-life of the *gbpC* transcript is very short, less than 2 min. Based on these findings, we speculate that while *gpbC* is only transiently expressed, with high turnover of the *gbpC* transcript, GbpC itself may be a fairly stable protein; Western blot analyses of proteins derived from stationary-phase cultures of various *S. mutans* strains indicated the presence of considerable levels of GbpC protein (27, 33). Therefore, one possible explanation is that the transcriptional regulation observed may ensure that sufficient quantities of GbpC are available during the early stages of biofilm formation. Once a biofilm is estab-

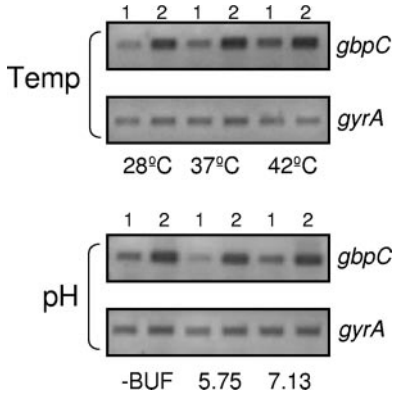

FIG. 8. Stress-induced *gbpC* expression is CovR independent. RNA was isolated from wild-type (IBS131) (lanes 1) and *covR* mutant (IBS132) (lanes 2) strains grown under the indicated growth conditions. RNA was subjected to semiquantitative RT-PCR analysis using either *gbpC*- or *gyrA*-specific primers as previously described. The data are representative of an RT-PCR analysis resulting from at least two independent experiments. −BUF, no buffer.

lished, GbpC would no longer be required, and other glucan-binding proteins, such as GbpA and GbpD, may be responsible for maintenance of the biofilm.

The expression of *gbpC* is very dependent on the growth medium. The ddag$^+$ phenotype was observed only in a semi-rich medium (BTR broth) containing glucose and was absent in cultures grown in rich media, such as Todd-Hewitt or brain heart infusion broth (40). It has been reported that the source and concentration of carbohydrate added to the medium also influence the transcription of genes associated with glucan and other polysaccharide biosynthesis and binding (6, 21, 44). Genes such as *gtfB*, *gtfC*, and *ftf* are induced at different levels in the presence of different sugars (19, 44). We did not observe any significant difference in expression from P*gbpC* during analyses of *S. mutans* cultures grown in CDM supplemented with assorted sugars. This implies that unlike the effect on *gtf* and *ftf* expression, various carbohydrate sources have very little effect on *gbpC* expression.

It is believed that *gbpC* expression is induced by various environmental stresses, due to observation of the ddag$^+$ phenotype under such conditions. Our results show that high temperature, which has been shown to induce the ddag$^+$ phenotype, also stimulates *gbpC* expression. However, we did not observe any induction of *gbpC* expression under osmotic stress conditions generated by the addition of a high level of salt or ethanol to the growth medium (Fig. 4). Similarly, osmotic stress due to sugar alcohols, including xylitol, did not have a stimulating effect on *gbpC* expression. This is in contrast to an earlier report which suggested that *gbpC* may be induced when *S. mutans* is grown in semirich medium (BTR) supplemented with xylitol. There are many differences between our controlled expression study and the study conducted by Sato et al. (42). In the latter study, prolonged growth (over 13 passages) in xylitol-supplemented medium led to a slight increase in expression from P*gbpC*. However, Sato et al. (42) employed a transcriptional fusion of P*gbpC-lacZ* that was inserted into the chromosome via single-crossover recombination; in this case, the reporter fusion could potentially be excised from the chromosome under stress conditions. Furthermore, expression of P*gbpC-lacZ* was measured using a stationary-phase (overnight) culture, when *gbpC* expression would be very low. Sato et al. (42) proposed that accumulation of spontaneous, stress-induced mutations in some regulatory loci may have been responsible for the observed *gbpC* induction. As discussed below, one such locus could be *covR*, which was shown in this study to directly repress *gbpC* expression.

Interestingly, we also found that the pH of the medium has an inhibitory effect on expression from P*gbpC*. Changes in the pH of the growth medium are known to induce complex regulatory networks, as well as differential expression of multiple genes, including those involved with metabolism of sugars (26). It was previously shown that expression of *gbpB*, which encodes glucan-binding protein B, is altered under low-pH conditions (29). Expression of *gbpC* was reduced at low pH, comparable to *gbpB* expression in strain UA159. However, these two proteins have different cellular functions, apart from a common capacity to bind glucan. GbpB is an essential protein responsible for maintenance of cell shape and may also possess murein hydrolase activity (29). In contrast, GbpC is not essential for growth of *S. mutans*, and the physiological function of this protein has not been elucidated (40).

It is important to point out that low pH in the environment is detected by various two-component signal transduction systems (12, 17, 32). Interestingly, we found that a two-component system in *S. mutans* that senses acidity in the environment (22) also regulates *gbpC* expression (I. Biswas, unpublished observation). This indicates that during conditions of cellular stress, two-component regulatory systems may also play a role in *gbpC* expression.

In this study, we clearly demonstrated that another two-component response regulator, CovR, plays a role in *gbpC* expression. CovR is a very important response regulator in many pathogenic streptococci; in group A streptococci (GAS), CovR generally acts as a repressor by directly binding to the promoter region of various genes (4, 11, 16). However, CovR repression at some promoters is indirect and mediated by other transcriptional regulators, such as RivR (38). CovR also acts as a transcriptional activator via direct interaction with the promoters, although direct activation by CovR has been demonstrated only for the *dppA* promoter of GAS (14). We have previously shown that in *S. mutans*, CovR acts as repressor by directly binding to the promoter regions of at least two genes, *gtfB* and *gtfC* (4). In the current study, we showed that CovR directly represses *gbpC* expression by binding to the *gbpC* promoter. Our DNase I protection analysis indicates that CovR binds to a large region (about 100 bp) on the P*gbpC* promoter. The regions protected from DNase I digestion were not continuous, indicating that CovR may bind to multiple sites on the *gbpC* promoter. The DNase I-protected regions include the transcriptional start site, suggesting that binding of CovR to the P*gbpC* promoter excludes binding of RNA polymerase, thus repressing transcription *of gbpC*.

A comparison of the DNase I-protected regions of P*gbpC*, P*gtfB*, and P*gtfC* did not reveal the presence of a unique sequence motif. However, several small (6- to 8-bp) AT-rich motifs were observed in the *gbpC* promoter region that are also present in the *gtfB* and *gtfC* promoters. While we cannot yet speculate on the significance of these small AT-rich motifs in

CovR binding, experiments are currently under way to determine their role in sequence recognition by CovR. While the complex molecular mechanism of *gbpC* expression requires further characterization, it is sufficient at this point to conclude that CovR directly regulates transcription of the *gbpC* gene.

In GAS, the CovR/S system is involved in bacterial stress response and adaptation (9). However, unlike GAS, *S. mutans* does not encode CovS, which is expected to be involved in dephosphorylation of CovR, a requirement for derepression of gene expression under stress conditions. Our results suggest that CovR does not play a role in the regulation of *gbpC* expression under cellular stress conditions, at least under the conditions tested. Therefore, it is possible that the mechanisms of CovR-mediated gene regulation in GAS are distinct from those in *S. mutans*. To this end, our recent study using DNA microarray analysis suggests that CovR predominantly acts as a transcriptional activator in *S. mutans* (I. Biswas, unpublished), while it acts as a repressor in GAS (13). We have previously shown that phosphorylation of CovR does not affect its affinity for the promoter regions of *gftB* or *gtfC* (4); in contrast, phosphorylation of CovR in GAS stimulated binding to the promoter region of the *has* gene (15). Thus, it is possible that CovR is always present in an activated state in *S. mutans* and its activity is regulated solely by the amount of CovR molecules present in the cell. The activity of many response regulators is regulated by proteolytic degradation; for example, the CtrA response regulator, which is essential for cell cycle progression in *Caulobacter crescentus*, is regulated by ClpXP protease (39). It is not known if the activity of CovR in *S. mutans* is regulated in a similar manner by proteolysis, and we are currently studying this phenomenon in the laboratory.

S. mutans requires GbpC to adhere to tooth enamel and to form dental plaque. It appears that the level of *gbpC* expression by *S. mutans* is very tightly regulated. A recent report indicates that *gbpC* expression is also controlled by LuxS-mediated quorum sensing (31). Moreover, the oral environment induces transient stress, such as acid or thermal stress, and *gbpC* expression is modulated under such conditions. A fine level of regulation is required by the *S. mutans* cells, since elevated levels of GbpC may not be beneficial. Planktonic cells overexpressing *gbpC* autoaggregate in the presence of glucan before they can adhere to plaque. Since CovR also regulates genes (*gtfB*/*C*) that encode GTFs required for glucan biosynthesis, we speculate that CovR coordinates glucan production and *gbpC* expression so that the appropriate proportion of glucans and GbpC is maintained under ever-changing growth conditions.

ACKNOWLEDGMENTS

We thank Anirban Banerjee and Patrick Chong for critically reading the manuscript.

This publication was made possible in part by NCRR grant 2 P20 RR016479 from the SD-INBRE program and by NIDCR grants DE016056 and DE016686 to I.B.

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