The SloR/Dlg Metalloregulator Modulates *Streptococcus mutans* Virulence Gene Expression

Elizabeth Rolerson, Adam Swick, Lindsay Newlon, Cameron Palmer, Yong Pan, Britton Keeshan, and Grace Spatafora*

Department of Biology, Middlebury College, 276 Bicentennial Way, MBH354, Middlebury, Vermont 05753

Received 27 January 2006/Accepted 17 April 2006

Metal ion availability in the human oral cavity plays a putative role in *Streptococcus mutans* virulence gene expression and in appropriate formation of the plaque biofilm. In this report, we present evidence that supports such a role for the DtxR-like SloR metalloregulator (called Dlg in our previous publications) in this oral pathogen. Specifically, the results of gel mobility shift assays revealed the *sloABC*, *sloR*, *comDE*, *ropA*, *sod*, and *spaP* promoters as targets of SloR binding. We confirmed differential expression of these genes in a GMS584 SloR-deficient mutant versus the UA159 wild-type progenitor by real-time semiquantitative reverse transcriptase PCR experiments. The results of additional expression studies support a role for SloR in *S. mutans* control of glucosyltransferases, glucan binding proteins, and genes relevant to antibiotic resistance. Phenotypic analysis of GMS584 revealed that it forms aberrant biofilms on an abiotic surface, is compromised for genetic competence, and demonstrates heightened incorporation of iron and manganese as well as resistance to oxidative stress compared to the wild type. Taken together, these findings support a role for SloR in *S. mutans* adherence, biofilm formation, genetic competence, metal ion homeostasis, oxidative stress tolerance, and antibiotic gene regulation, all of which contribute to *S. mutans*-induced disease.

*Streptococcus mutans*, the principal causative agent of dental caries, is a successful oral pathogen owing to its ability to adhere to host tissues, form biofilms, and adapt to conditions of oxidative stress and low pH. Adherence of *S. mutans* to the tooth surface facilitates the colonization of other oral pathogens and hence the formation of a mixed-species biofilm known as dental plaque. In the plaque environment, *S. mutans* generates lactic acid as a by-product of sugar metabolism, resulting in a localized drop in pH at the tooth surface and the demineralization of tooth enamel. In addition to direct damage to the dentition, *S. mutans* can also cause endocarditis, a life-threatening valvular inflammation of the heart (8). The present study centers on the investigation of a putative role for the SloR metalloregulator in *Streptococcus mutans* virulence gene expression.

Metal ions such as iron and manganese are essential micronutrients, and their incorporation has been implicated in the pathogenesis of many bacteria, including *Legionella pneumophila*, *Mycobacterium tuberculosis*, *Staphylococcus epidermidis*, and *Streptococcus gordonii* (2, 9, 16, 24, 30, 33, 34, 35). Specifically, iron is an important enzyme cofactor in the respiratory pathways of many aerobic and anaerobic microorganisms (26). However, the accumulation of iron in bacteria can lead to the production of toxic oxygen radicals via Fenton chemistry. Thus, while sufficient levels of iron are necessary to promote bacterial survival and growth, its intracellular transport must be tightly controlled.

Iron availability is also restricted in the mammalian host, where it is sequestered to host proteins such as transferrin and lactoferrin (42, 46). Such an iron-withholding system protects the host from the harmful effects of reactive oxygen species while restricting the availability of this essential micronutrient to invading pathogens. In response, microorganisms have evolved specialized mechanisms for robbing iron from host proteins, some of which depend on small iron chelating molecules called siderophores. In previous work, we and others confirmed a siderophore-independent mechanism for iron transport in *S. mutans* (12, 38).

Manganese is another essential micronutrient of particular importance to the oral streptococci (39). In fact, high concentrations of manganese have been strongly correlated with increased prevalence of dental caries (1, 5). This is not surprising given that sucrose-dependent adherence and glucan binding by the mutans group streptococci both require manganese (4, 25). Unlike iron, however, manganese does not promote Fenton chemistry but rather plays a crucial role in bacterial defense against oxidative stress (2, 49). This is so, in part, because manganese serves as a cofactor for superoxide dismutase, which facilitates the conversion of damaging oxygen radicals into harmless by-products.

Metalloregulatory proteins have been implicated in virulence gene control for a variety of gram-positive pathogens, including *Mycobacterium tuberculosis* (IdeR), *Staphylococcus epidermidis* (SirR), and *Corynebacterium diphtheriae* (DtxR) (3, 16, 27). These bacterial metalloregulators repress the transcription of downstream genes upon binding to palindromic consensus sequences when free iron or manganese is available. However, in the human host, where metal ions are limiting, these consensus sequences remain unoccupied, and the transcription of genes, some of which may contribute to virulence, is derepressed. A report in the literature describes inactivation of the IdeR metalloregulator in *M. tuberculosis* which attenuates virulence in vivo (27), and others describe targets of the
DtxR transcriptional regulator in Corynebacterium diphtheriae, which include the disease-causing diphtheria toxin (tox) gene (21, 37, 40).

Numerous SloR homologs have also been identified for streptococci, including the recently reported MtsR metalloregulator in Streptococcus pyogenes, which has been implicated in iron homeostasis and oxidative stress tolerance (3). Other SloR homologs that likely contribute to virulence include the ScaR and AdcR metalloregulators in S. gordonii, which modulate transcription of the scaCBA and adcCBA operons, respectively. Both of these operons encode manganese transport systems that are required for genetic competence and biofilm formation (17, 24).

Previously, we described the cariogenic potential of an S. mutans UA130 sloR-deficient mutant (GMS800), as attenuated in a germ-free rat model, thereby implicating SloR as a metalloregulator in S. mutans-induced disease (38). In addition, work conducted in our laboratory and by others revealed that the expression of sloC, an LraI lipoprotein adhesin and metal ion transporter, contributes to S. mutans-induced disease and is subject to repression by SloR when free iron or manganese is plentiful. In contrast, sloC expression is derepressed when the availability of these metal ions becomes limiting (18, 38). Taken together, these findings implicate metal ions in the regulation of S. mutans virulence gene expression.

In the present study, we hypothesize a role for the S. mutans SloR metalloregulator similar to that of its DtxR homolog in C. diphtheriae and propose that either iron or manganese can associate with the metalloregulator to modulate virulence gene expression. This suggests the presence of a SloR regulon in this oral pathogen that promotes the expression of virulence genes in a host organism where metal ions are limiting. Herein, we describe the characterization of the S. mutans UA159 sloR-deficient mutant (GMS84) and identify multiple virulence attributes that are targeted by the SloR metalloregulator. SloR modulation of S. mutans virulence gene expression in response to metal ion availability is likely a significant contributor to caries formation, and its study could reveal novel candidates for therapeutic intervention.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and primers.** Bacterial strains and plasmids used in the present study are described in Table 1. Oligonucleotide primers designed using MacVector 7.0 software and purchased from Sigma Genosys (St. Louis, Mo.) are presented in Table 2.

**Bacteriological media and reagents.** Escherichia coli DH5α and TB1 cells were grown at 37°C in L broth with gentle aeration. E. coli transformants were also grown in this manner, with the addition of 100 μg/ml ampicillin or 100 μg/ml spectinomycin to the medium. S. mutans cultures for RNA isolation, biofilm formation, and Western blot analysis were grown at 37°C and 5% CO2 in a semidefined medium (SDM) that contained 0.38 μM Fe and 1.75 μM Mn, as determined by inductively coupled argon plasma analysis. For metal ion incorporation assays, S. mutans was grown in Todd-Hewitt broth (THB) at 37°C and 5% CO2. For all other assays, S. mutans was grown at 37°C and 5% CO2 in THB supplemented with 0.3% yeast extract (THYE). Spectinomycin (1,200 μg/ml) or erythromycin (10 μg/ml) was added to THYE to select for transformants. All buffers and reagents for purification of the SloR and maltose binding proteins (MBP) were prepared according to the pMal protein and purification system instruction manual and in accordance with the recommendations of the supplier (New England BioLabs, Beverly, MA).

**Cloning of the S. mutans sloR gene.** A 1,063-bp ampiclon that harbors the wild-type sloR coding sequence and promoter region, amplified with primers dlgL.R.BamH1 and dlgL.R.BamH1R (Table 2), was cloned into the BamHI site of the replicative shuttle plasmid pDL277. The resulting recombinant was introduced into E. coli DH5α by electroporation (36), and transformants were selected on L agar supplemented with spectinomycin. Plasmid DNA was isolated from selected transformants on minispin columns according to the recommendations of the supplier (Qiagen) and subsequently mapped with restriction enzymes to confirm the presence of the pER4 recombinant construct.

**Construction of S. mutans GMS854, a sloR-deficient mutant.** An established PCR ligation mutagenesis approach (20) was used to inactivate the sloR gene on the S. mutans chromosome. Primers dlgL.R.P1, dlgL.R.P2, dlgL.R.P3, and dlgL.R.P4 (Table 2) were used to amplify the 5’ and 3’ regions of the sloR coding sequence from the S. mutans UA159 chromosone by use of a thermal cycler (Hybaid, Ltd., Ashford, United Kingdom) for 94°C for 10 min, 35 cycles at 94°C for 1 min, optimal annealing temperature (Table 2) for 2 min, 72°C for 2 min, and 72°C for 10 min. The resulting 5’ and 3’ sloR amplicons were ligated with pME and Fsel and ligated to an ermAM amplicon with compatible Asel and Fsel overhangs. The ligation mixture was then used as a template for PCR amplification with primers dlgL.R.P1 and dlgL.R.P4. The resulting amplification products, including a 1,784-bp sloR-ermAM-sloR linear construct (Fig. 1a), were used to transform S. mutans UA159 in the presence of 150 μg competence-stimulating peptide (CSP) (22). Transformants were selected on THYE agar plates supplemented with erythromycin. Chromosomal DNA was isolated from selected transformants according to established protocols (36), and PCR and nucleotide sequencing with primers dlgL.R.P1 and dlgL.R.P4 were used to confirm sloR disruption by allelic exchange (Fig. 1b and c). The resulting sloR mutant was named GMS854.

**Complementation of the sloR mutation in S. mutans GMS854.** Plasmid pER4 was used to complement the sloR mutation in S. mutans GMS854 in trans. The plasmid was introduced into S. mutans by Csp-induced transformation as described previously (22), and transformants resistant to spectinomycin were selected on THYE agar plates. Complementation of the sloR-specific mutation in the resulting strain, GMS855, was confirmed with real-time PCR experiments by monitoring sloR- and sloC-specific expression.

**Isolation and purification of the S. mutans SloR protein.** The sloR gene and flanking DNA sequences were PCR amplified from the S. mutans UA159 chromosome with sloR-specific primers Dlg_c Forward and Dlg_c Reverse (Table 2) and in the presence of Platinum Taq DNA high-fidelity polymerase (Invitrogen). The cycling conditions consisted of a 94°C denaturation step for 2 min, followed by 35 cycles at 94°C for 30 s, 50°C for 2 min, and 68°C for 1 min, followed by a 10-min extension period at 68°C. The resulting 756-bp ampiclon was PCR purified (Qiagen), digested with the restriction enzyme XbaI, and ligated into the XbaI and XmnI cloning sites on the pMal-c2x vector (New England Biolabs) with T4 DNA ligase (Promega) at 16°C overnight. The ligation mixture was then

<table>
<thead>
<tr>
<th>Strains or plasmid</th>
<th>Genotype or phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus mutans</td>
<td>Wild type, serotype c UA159 derived, sloR deficient, Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>ATCC 700610 This work</td>
</tr>
<tr>
<td>GMS855</td>
<td>GMS84 transformed with plasmid pER4 UA159 derived, Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>DH5α F&lt;sup&gt;−&lt;/sup&gt; supE44 lacZ&lt;sup&gt;−&lt;/sup&gt;/169 8800lacZAM&lt;sup&gt;15&lt;/sup&gt; hsdR&lt;sup&gt;R&lt;/sup&gt;17 recA1 endA1 gyrA&lt;sup&gt;496&lt;/sup&gt; thi&lt;sup&gt;−1&lt;/sup&gt; rel&lt;sup&gt;A&lt;/sup&gt; 496 thi&lt;sup&gt;R&lt;/sup&gt; hsdR&lt;sup&gt;3&lt;/sup&gt; New England BioLabs</td>
<td></td>
</tr>
<tr>
<td>PB277</td>
<td>E. coli-streptococcal shuttle vector, Sp&lt;sup&gt;R&lt;/sup&gt; pDL277 derived, harbors sloR gene, Sp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pMal-c2x</td>
<td>E. coli expression vector with MBP, Ap&lt;sup&gt;R&lt;/sup&gt; containing SloR, Ap&lt;sup&gt;R&lt;/sup&gt; This work</td>
<td></td>
</tr>
</tbody>
</table>
used to electroporate *E. coli* DH5α (36), and transformants resistant to ampi-
cillin were selected on X-Gal (5-bromo-4-chloro-3-indolyl-D-galactopyrano-
side)- and IPTG (isopropyl-D-thiogalactopyranoside)-containing L ampicillin 
plates. Plasmid DNA was isolated from selected colonies on minispin columns 
(QIAGEN) and used as a template for PCR amplification with vector-specific 
primers 

dlg.LR.P1

dlg.LR.P2

dlg.LR.P3

derm.Ascl.F

derm.Fse1.R

Primers

<table>
<thead>
<tr>
<th>Primer use</th>
<th>Primer name</th>
<th>Nucleotide sequence (5’ to 3’)</th>
<th>Annealing temp (°C)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR ligation mutagenesisa</td>
<td>dlg.LR.P1</td>
<td>GGCGGGCCGAAAAGGTTCCGCTACTCCACGC</td>
<td>52.3</td>
<td>441</td>
</tr>
<tr>
<td>PCR ligation mutagenesisa</td>
<td>dlg.LR.P2</td>
<td>GGCGGGCCGATGTCCTGCGATAAAGAGAT</td>
<td>GACG</td>
<td>53.4</td>
</tr>
<tr>
<td>PCR ligation mutagenesisa</td>
<td>dlg.LR.P3</td>
<td>GGCCGGCCGGCATGGAGGAACCCATTC</td>
<td>53.4</td>
<td>513</td>
</tr>
<tr>
<td>PCR ligation mutagenesisa</td>
<td>erm.Ascl.F</td>
<td>GGCGGGCCGGGGGCCCCAAAAATTGTTGGAT</td>
<td>52.3</td>
<td>860</td>
</tr>
</tbody>
</table>

**Real-time PCR**

dlg.intern.LR.F

dlg.intern.LR.R

**Cloningb**

dlg.LR.BamHIF

dlg.LR.BamHIR

**Gel mobility shift**

preSloABCFor

recA.GelLN.F

preSlo-P.CAS-For

presol-PCR-PAS-For

preSlo-P.CAS-R

preSlo-P.CAS-Rev

preSlo-P.CAS-R

spapB.Bk.Forward

spapB.Bk.Reverse

Annealing temperatures and amplicon sizes are relevant to the primer pair.

a AscI restriction sites appear in boldface type, whereas FseI restriction sites are underlined.
b BamHI cut sites are underlined and appear in boldface type.

c In silico analysis. The PredictRegulon search engine (47) was used to search the *S. mutans* UA159 genome for sequence homology to a 22-bp SloR consensus sequence (AAATTAACTTGACTTAATTTTT) (18), as well as to 24-bp (AAA AATTATACTGTTACTATATATTT) and 38-bp (CTAATAAAAATATATACTG TACTATATATATATATATATA) versions of the consensus sequence. The search variables for upstream and downstream positioning of this sequence were set at 300 bp and 20 bp, respectively. Targets identified by PredictRegulon were subsequently screened for characteristic stem-loop secondary structure by use of Mfold algorithms, with conditions for folding set at 37°C, 300 mM sodium, and 0 mM magnesium (50).

**Gel mobility shift assays.** Gel mobility shift assays were performed using a protocol developed by John Murphy at Boston University (31), with some mod-

**TABLE 2. List of primers used in this study**
FIG. 1. Construction of an S. mutans sloR-deficient mutant (GMS584) by PCR ligation mutagenesis. (a) We generated a 139-bp deletion in the sloR coding sequence, into which an 860-bp ermAM cassette was ligated. The resulting product was introduced into the UA159 genome by allelic exchange. (b) Amplions derived by PCR confirm disruption of the sloR coding sequence by ermAM. A 1,063-bp wild-type fragment is increased by the size of ermAM, minus the 139-bp deletion, to yield a 1,784-bp product in the mutant. (c) Nucleotide sequence across the sloR-ermAM cassette junction further confirms disruption of the sloR coding sequence.
SloR/Dlg MODULATES S. MUTANS VIRULENCE GENE EXPRESSION

Confirmation of the knockout mutation in S. mutans GMS884. PCR analysis with primers dgl.LR.P1 and dgl.LR.P4 (Table 2) was used to confirm disruption of the sloR locus on the S. mutans GMS884 chromosome (Fig. 1). Nucleotide sequence analysis of GMS884 chromosomal DNA with dgl.LR.P1, dgl.LR.P2, dgl.LR.P3, and dgl.LR.P4 primers (Table 2) also confirmed incorporation of the 860-bp erythromycin cassette into the sloR coding sequence by allelic exchange (Fig. 1). Moreover, the expression of sloR in GMS884 was 230-fold less than in the wild type, as determined by real-time qRT-PCR experiments (Table 3), consistent with sloR inactivation in GMS884. In addition, the C\textsubscript{T} values we noted for the mutant and the no-template control were comparable (29.26 and 29.5, respectively), indicating that little if any sloR-specific mRNA is present in GMS884.

Complementation of GMS884. We confirmed the sloR-specific mutation in GMS884 by complementation in trans in pER4-containing transformants. The expression of sloR was restored to near-wild-type levels in these transformants, as revealed by real-time qRT-PCR. Specifically, sloR-specific expression in the GMS885 complemented strain was amplified in real time at nearly the same cycle number as for the wild type (C\textsubscript{T} = 22.74 and 21.28, respectively), compared to the GMS884 mutant strain (C\textsubscript{T} = 29.26), in which sloR expression is diminished. In addition, sloC-specific expression was restored to near-wild-type levels in the complemented GMS885 strain, (C\textsubscript{T} = 23.04 and 22.28 in GMS885 and UA159, respectively), with sloC amplification occurring considerably earlier (C\textsubscript{T} = 16.97) in GMS884, where sloC expression is heightened. Taken collectively, these findings support complementation of the GMS884 sloR-specific mutation in trans.

Identification of candidate genes subject to SloR control in silico. In silico analysis of the S. mutans genome revealed palindromic SloR consensus sequences in the promoter regions of multiple genes, including sloABC, ropA, and spaP. Genes whose levels of expression were differentially expressed in real-time qRT-PCR experiments and which shared sequence identity with putative metal ion transporters and mediators of S. mutans virulence were selected for further analysis by gel mobility shift assays.

Gel mobility shift assays. To confirm that disruption of the sloR coding sequence in GMS884 rendered the resulting SloR incorporation of \(^{55}\text{Fe}\) and \(^{54}\text{Mn}\) was determined by dividing the radioactivity associated with the cell pellet (in counts per minute) by the total radioactivity associated with the bacterial cell pellet plus the culture supernatant/washes (in counts per minute).

Assay for oxidative stress tolerance. S. mutans overnight cultures grown in THYE with appropriate antibiotic selection were diluted 1:15 in more of the same fresh medium. Mid-logarithmic-phase cells (\(\text{OD}_{490} = 0.4\) to 0.6) were harvested by centrifugation and washed in 0.1 M glycine (pH 7.0), from which 100-\(\mu\)l aliquots were further diluted 1:10 in 0.1 M glycine and stored on ice. The cell suspensions were exposed to oxidative stress upon addition of 30\% \(\text{H}_2\text{O}_2\) (final concentration, 58.8 mM) and mixed by inversion. At 20, 40, 60, and 80 min, 100 \(\mu\)l of cells was removed and diluted 1:10 in glycine buffer containing 5 mg/ml catalase (Sigma-Aldrich). Cells from UA159 and GMS884 suspensions were subsequently plated on THYE agar and THYE agar supplemented with erythromycin, respectively, and grown at 37\°C and 5\% \(\text{CO}_2\) for 36 to 48 h. Colonies were counted and percent survivorship quantified as the number of CFU that survived exposure to \(\text{H}_2\text{O}_2\) per number of CFU that were not exposed to the stressor.

RESULTS

Confirmation of the knockout mutation in S. mutans GMS884. PCR analysis with primers dgl.LR.P1 and dgl.LR.P4 (Table 2) was used to confirm disruption of the sloR locus on the S. mutans GMS884 chromosome (Fig. 1). Nucleotide sequence analysis of GMS884 chromosomal DNA with dgl.LR.P1, dgl.LR.P2, dgl.LR.P3, and dgl.LR.P4 primers (Table 2) also confirmed incorporation of the 860-bp erythromycin cassette into the sloR coding sequence by allelic exchange (Fig. 1). Moreover, the expression of sloR in GMS884 was 230-fold less than in the wild type, as determined by real-time qRT-PCR experiments (Table 3), consistent with sloR inactivation in GMS884. In addition, the C\textsubscript{T} values we noted for the mutant and the no-template control were comparable (29.26 and 29.5, respectively), indicating that little if any sloR-specific mRNA is present in GMS884.

Complementation of GMS884. We confirmed the sloR-specific mutation in GMS884 by complementation in trans in pER4-containing transformants. The expression of sloR was restored to near-wild-type levels in these transformants, as revealed by real-time qRT-PCR. Specifically, sloR-specific expression in the GMS885 complemented strain was amplified in real time at nearly the same cycle number as for the wild type (C\textsubscript{T} = 22.74 and 21.28, respectively), compared to the GMS884 mutant strain (C\textsubscript{T} = 29.26), in which sloR expression is diminished. In addition, sloC-specific expression was restored to near-wild-type levels in the complemented GMS885 strain, (C\textsubscript{T} = 23.04 and 22.28 in GMS885 and UA159, respectively), with sloC amplification occurring considerably earlier (C\textsubscript{T} = 16.97) in GMS884, where sloC expression is heightened. Taken collectively, these findings support complementation of the GMS884 sloR-specific mutation in trans.

Identification of candidate genes subject to SloR control in silico. In silico analysis of the S. mutans genome revealed palindromic SloR consensus sequences in the promoter regions of multiple genes, including sloABC, ropA, and spaP. Genes whose levels of expression were differentially expressed in real-time qRT-PCR experiments and which shared sequence identity with putative metal ion transporters and mediators of S. mutans virulence were selected for further analysis by gel mobility shift assays.

Gel mobility shift assays. To confirm that disruption of the sloR coding sequence in GMS884 rendered the resulting SloR...
protein dysfunctional, we conducted gel mobility shift experiments with S. mutans GMS584 and UA159 whole-cell lysates. The results of these studies revealed a shift for the sloABC promoter region when reacted with the wild-type repertoire of proteins but no shift when proteins from the mutant were present in the reaction mixture (data not shown). In separate assays, the presence of increasing concentrations of the SloR fusion protein increased the magnitude of the band shift, consistent with SloR oligomerization (Fig. 2). This supports the work of others, who described the oligomerization of the DtxR metallocregulator in Corynebacterium diphtheriae (31).

The results of gel shift assays performed with a SloR fusion protein that we purified from S. mutans UA159 revealed SloR binding to the promoter regions of the sloABC, sloR, comDE, ropA, sod (superoxide dismutase), and spaP genes, the products of which likely contribute to S. mutans virulence (Fig. 3). Binding of the SloR metallocregulator to the sloR-specific promoter sequence was also noted, although no palindromic consensus sequence was identified in this region. Addition of EDTA to the gel shift reaction mixtures abrogated the band shift, and substitution of the SloR-MBP fusion protein with purified MBP yielded no shift. Binding assays performed with up to 10-fold excess cold noncompeting recA DNA confirmed the specificity of SloR binding to the test promoter regions (Fig. 3).

**Real-time PCR.** The results of real-time qRT-PCR experiments revealed differential expression levels in GMS584 and UA159 for several genes identified in silico and for other genes identified as possible targets of SloR control by gel shift assays. For instance, the expression levels of ropA, spaP, comDE, sod, sko6, and sko9 were down-regulated in GMS584 relative to wild-type levels (Table 3). Importantly, reverse transcriptase negative-control reactions confirmed the absence of contaminating DNA from the RNA templates. Moreover, analysis of melting curves revealed specific primer annealing and lack of primer secondary structure, and relative standard curves showed acceptable primer pair efficiency.

**Western blotting.** Immunoblotting of S. mutans UA159, GMS584, and GMS585 whole-cell lysates reacted with a polyclonal antiserum directed against the S. mutans SloC protein revealed derepression of a 34-kDa band in GMS584 relative to wild-type levels (Fig. 4). This is consistent with compromised binding of an altered SloR protein to the SloR consensus sequence located upstream of the sloABC operon on the S. mutans chromosome. Furthermore, these results corroborate successful complementation of the sloR mutation in GMS585 transformants.

**Characterization of S. mutans biofilms.** To define a putative role for SloR in S. mutans biofilm formation, crystal violet release assays and growth determination experiments were performed. The results of these studies revealed similar doubling times and biofilm biomasses (data not shown) for S. mutans UA159 and GMS584. Despite these similarities, these strains demonstrated notably different biofilm architectures on scanning electron micrographs (Fig. 5). For S. mutans biofilms grown in the presence of dextrose, GMS584 behaved much like an aggregation mutant, with cells demonstrating considerably more clumping than wild-type cells. However, morphometric analysis of GMS584 biofilms grown in the presence of sucrose revealed significantly larger channels in the mutant (on average, 57.7 μm in GMS584 versus 36.6 μm in UA159, independent-samples t test, P < 0.01).

**Genetic competence in GMS584.** To determine if altered biofilm formation in GMS584 has any impact on genetic competence, we determined the transformation efficiencies of the mutant and wild-type strains upon transformation with 1 μg of DNA. The results of these studies revealed specific primer annealing and lack of primer secondary structure, and relative standard curves showed acceptable primer pair efficiency.

**TABLE 3. Impact of SloR inactivation on S. mutans gene expression**

<table>
<thead>
<tr>
<th>Gene(s)</th>
<th>Gene product(s)</th>
<th>Type of change in expression in GMS584</th>
<th>Fold changea</th>
</tr>
</thead>
<tbody>
<tr>
<td>sloR</td>
<td>Metal ion-dependent transcriptional regulator</td>
<td>Decrease</td>
<td>230</td>
</tr>
<tr>
<td>sloABC</td>
<td>Metal ion transporter, surface adhesion protein</td>
<td>Increase</td>
<td>29</td>
</tr>
<tr>
<td>comD</td>
<td>Histidine kinase sensor protein</td>
<td>Decrease</td>
<td>1.6</td>
</tr>
<tr>
<td>comE</td>
<td>Cognate response regulator</td>
<td>Decrease</td>
<td>1.7</td>
</tr>
<tr>
<td>ropA</td>
<td>Trigger factor, surface protein biogenesis, chaperone protein</td>
<td>Decrease</td>
<td>6.0</td>
</tr>
<tr>
<td>sod</td>
<td>Superoxide dismutase (oxidative stress defense)</td>
<td>Decrease</td>
<td>2.2</td>
</tr>
<tr>
<td>spaP</td>
<td>Cell surface antigen, saliva-interacting protein</td>
<td>Decrease</td>
<td>6.0</td>
</tr>
<tr>
<td>gbpB</td>
<td>Glucan binding protein</td>
<td>Decrease</td>
<td>1.5</td>
</tr>
<tr>
<td>gfpB</td>
<td>Glucosyltransferase</td>
<td>Decrease</td>
<td>2.6</td>
</tr>
<tr>
<td>sko6</td>
<td>Hypothetical putative grammicidin synthetase enzyme</td>
<td>Decrease</td>
<td>3.4</td>
</tr>
<tr>
<td>sko9</td>
<td>Hypothetical putative integral membrane protein</td>
<td>Decrease</td>
<td>2.9</td>
</tr>
</tbody>
</table>

* Expression relative to that in UA159 and normalized to an S. mutans kk11 endogenous control.
plasmid pDL277 in the presence of CSP. The results of these experiments (n = 9) confirm that genetic competence is compromised nearly threefold in GMS584 relative to the wild type (data not shown). Consistent with these findings are the results of real-time qRT-PCR experiments, which revealed a 1.7-fold decrease in expression for the \textit{comE} response regulator and its cognate \textit{comD} histidine kinase in GMS584 compared to levels for the wild type.

**Metal ion incorporation by GMS584.** To determine whether metal ion transport is compromised in GMS584, we monitored the accumulation of $^{55}$FeCl$_3$ and $^{54}$MnCl$_2$ in \textit{S. mutans} GMS584 and UA159 cultures grown in THYE (Fig. 6). We noted significantly increased $^{55}$Fe incorporation in the mutant (5.56% in GMS584 and 1.92% in UA159, independent-samples t test, $P = 0.050$). $^{54}$Mn incorporation in the mutant was also increased, although this difference was only trending toward significance (35.53% in GMS584 and 20.5% in UA159, independent-samples t test, $P = 0.127$).

**Resistance to oxidative stress in GMS584.** To determine the sensitivity of GMS584 to oxidative stress and to discover whether SloR might be involved in the \textit{S. mutans} oxidative stress response, we compared the levels of resistance of the GMS584 and UA159 strains to challenge with sublethal concentrations of H$_2$O$_2$ [58.8 mM]. Surprisingly, survivorship of the mutant was nearly threefold greater than that of the wild type after a 20-min exposure to the hydrogen peroxide stressor (data not shown). This finding is consistent with the results of preliminary experiments performed in our laboratory which used paraquat as the stressor, for which the mutant also demonstrated a heightened tolerance to oxidative stress (data not shown).

**DISCUSSION**

In the present study, we characterized an \textit{S. mutans} sloR-deficient mutant (GMS584) that was compromised for genetic competence relative to the UA159 wild type but that demonstrated increased incorporation of iron and manganese and heightened resistance to oxidative stress. GMS584 also formed altered biofilms, with pronounced cell aggregation for cultures grown in the presence of dextrose and enlarged water channels for cultures supplemented with sucrose. These phenotypic assays were performed in rich THB or THYE media, however, which we propose might have an inhibitory effect on bacterial gene expression and cell signaling. Specifically, the results of real-time qRT-PCR experiments revealed differences in \textit{S. mutans} gene expression levels that were less pronounced when cells were grown in THYE than when cells were grown in SDM.
FIG. 4. Immunoblots with an anti-SloC antibody support disruption of SloR in GMS584. Western blotting of S. mutans UA159, GMS584, and GMS585 protein extracts reveals the presence of a 34-kDa SloC protein in all strains, the expression of which is up-regulated considerably in GMS584, consistent with disruption of the sloR gene in this strain. Conversely, down-regulation of SloC in the pER4-transformed GMS585 strain supports reversion of this mutant to the wild type, owing to complementation of the sloR defect in this strain in trans. Partial repression of sloC in the wild-type UA159 strain is likely due to the presence of exogenous Mn (1.75 μM), which we confirmed in SDM by inductively coupled argon plasma analysis.

(unfinished observations). This is consistent with work conducted by Merritt et al., who reported inhibition of the S. mutans luxS gene expression for cells grown in rich media containing glucose and sucrose (29). To address this putative medium effect, we performed all of our real-time qRT-PCR experiments, as well as scanning electron microscopy biofilm visualization, in an SDM that contains iron and manganese concentrations commensurate with those we noted in human saliva (0.1 to 10 μM [unfinished observations]).

The incorporation of 55Fe was increased in S. mutans GMS584 (Fig. 6a), indicating that SloR functions as a repressor of S. mutans iron transporters under the wild-type condition. This is consistent with real-time qRT-PCR experiments that support derepression of the sloC metal ion transporter in GMS584. We believe 54Mn transport is also subject to repression by SloR, since incorporation of this metal ion in GMS584 was consistently greater than that of the wild type across biological replicates. However, considerable variation in 54Mn transport, recorded in counts per minute, across replicate experiments gave rise to a data set with high standard error (Fig. 6b), which is the reason these data were only trending toward significance.

The results of gel mobility shift assays confirmed the metal ion-dependent binding of SloR to DNA containing a conserved 32-bp SloR palindrome (CTAATATAAAAATTAACGTGCTTTTATTTTATTTAG) in the sloABC promoter region, as well as to a 184-bp intervening region that is devoid of this sequence and located immediately downstream of sloC and upstream of the sloR gene on the S. mutans chromosome. This finding supports autoregulation of SloR. The absence of a conventional SloR consensus sequence from the sloR promoter region, however, suggests that SloR may also bind to unique sequences to regulate downstream genes. Alternatively, the consensus sequence for SloR binding may be degenerate in this intergenic region, further confounding the identification of an AT-rich consensus sequence in an S. mutans genome with less than 40% GC content. Abrogation of the band shifts in the presence of EDTA supports a SloR-DNA interaction that is metal ion dependent. Additionally, substitution of the SloR:MBP fusion protein with purified MBP in the gel shift reaction mixture gave rise to no band shift, indicating that the noted protein-DNA interaction is mediated by the SloR (and not the MBP) portion of the fusion protein. We did not use a pure SloR protein in our gel shift experiments because repeated attempts to cleave the MBP tag from the fusion construct were unsuccessful, even when performed under a variety of test conditions. In summary, our findings support dual control of the sloR gene from the sloABC promoter as well as from an independent promoter and suggest an essential role for SloR in S. mutans.

The results of gel mobility shift assays also revealed binding of the SloR:MBP fusion protein to the promoter regions of the S. mutans sloABC, comDE, ropA, sod, and spaP genes (Fig. 3). SloC is an LraI lipoprotein adhesin that comprises part of an ABC-type operon on the S. mutans chromosome (14) and functions as a transporter of iron and manganese (10, 19). Previous work conducted in our laboratory revealed sloC derepression in a UA130 sloR-deficient mutant, called GMS800 (38). The results of real-time qRT-PCR performed in the present study confirmed derepression of sloC expression in the UA159-derived GMS584 mutant (Table 3), thereby supporting SloR as a repressor of S. mutans sloC expression. Indeed, our results are consistent with reports in the literature that describe a repressor role for SloR homologues in metalloregulation (16, 40). In contrast to the repressor effect of SloR on sloC, the results of real-time qRT-PCR studies implicate SloR metalloregulation in maintaining expression of comDE, ropA, sod, spaP, gbpB, gfpB, sko6, and sko9 (Table 3). This is consistent with the reported role for the IdeR metalloregulator in Mycobacterium tuberculosis, which likewise promotes the expression of some genes, while repressing the expression of others (34).

The comC and comDE genes encode a competence-stimulating peptide and a histidine kinase sensor protein and its cognate response regulator, respectively, and act in concert to regulate competence in S. mutans (23). Previous studies report that S. mutans strains deficient in any component of the comCDE pathway are significantly compromised for genetic competence (22). Moreover, S. mutans strains with defects in comD or comE were unable to produce bacteriocin, a class of molecules involved in DNA release from neighboring bacteria, and formed thin biofilms with reduced biomass compared to those of the wild type. Since the phenotype of GMS584 is consistent with that of a comCDE mutant, we decided to investigate the regulation of the com genes in this mutant. The results of qRT-PCR experiments revealed repression of comDE expres-
FIG. 5. Scanning electron micrographs of *S. mutans* UA159 and GMS584 biofilms formed on polystyrene coverslips. Shown are representative regions of UA159 and GMS584 biofilms. GMS584 biofilms grown in the presence of dextrose demonstrate pronounced cellular aggregation (white arrows) relative to wild-type biofilms. Morphometric analysis of GMS584 biofilms grown in the presence of sucrose reveals significantly larger water channels (white dotted lines) than those present in UA159 biofilms (mean diameter, 57.7 μm for GMS584 versus 36.6 μm for UA159, independent-samples t test, *P* < 0.01).
decreased GMS584 and UA159 biofilms were similar, indicating that expression by SloR in the wild type. However, the biomasses of S. mutans comDE competence in the mutant and with maintenance of tance (independent-samples wild type, although this difference only approaches statistical signifi-
P/H11005 by 100. (a) 55Fe incorporation was significantly greater in GMS584 assays are represented as counts per minute associated with the bac-
terial cell pellet plus the culture supernatant/washes multiplied by 100. (a) 55Fe incorporation was significantly greater in GMS584 than in the UA159 wild-type progenitor (independent-samples t test, P = 0.05). (b) 54Mn incorporation was greater in GMS584 than in the wild type, although this difference only approaches statistical significance (independent-samples t test, P = 0.127).

FIG. 6. 55Fe and 54Mn incorporation is greater in GMS584 than in the wild-type progenitor. The results of 55Fe and 54Mn incorporation assays are represented as counts per minute associated with the bacterial cell pellet divided by the total counts per minute associated with the bacterial cell pellet plus the culture supernatant/washes multiplied by 100. (a) 55Fe incorporation was significantly greater in GMS584 than in the UA159 wild-type progenitor (independent-samples t test, P = 0.05). (b) 54Mn incorporation was greater in GMS584 than in the wild type, although this difference only approaches statistical significance (independent-samples t test, P = 0.127).

sion in GMS584, consistent with the noted decrease in genetic competence in the mutant and with maintenance of comDE expression by SloR in the wild type. However, the biomasses of GMS584 and UA159 biofilms were similar, indicating that decreased comDE expression in GMS584 is not sufficient to impact accumulation of the biofilm on an abiotic surface.

The ropA gene product is a trigger factor and critical stress response element in S. mutans (45). Reports in the literature describe an S. mutans ropA-deficient mutant, TW90, that demonstrating altered biofilm architecture on scanning electron micrographs and increased sensitivity to acid and oxidative stress in vitro (45). The altered biofilm architecture and down-regulation of ropA we observed for GMS584 in this study reinforce the reported role for ropA in mediating appropriate formation of the S. mutans biofilm. Taken collectively, these findings implicate the ropA gene product in S. mutans disease, although to our knowledge in vivo studies have not yet been performed to define a specific role for ropA in cariesogenesis.

The superoxide dismutase gene (sod) encodes an enzyme that plays a major role in bacterial defense against oxidative stress. The sod gene product promotes the conversion of damaging superoxides into harmless by-products. S. mutans harbors a single manganese-dependent superoxide dismutase, and mutants deficient in this activity grow more slowly under aerobic conditions than the wild type (32). In the present study, we noted sod expression that was down-regulated twofold in GMS584 compared to expression in UA159. However, the results of hydrogen peroxide challenge assays revealed that the mutant has increased tolerance to oxidative stress. It is possible that the pronounced aggregation we noted in GMS584 biofilms, while having an as-yet-undetermined effect in vivo, may serve to protect S. mutans from the deleterious effects of radical oxygen species. Wen and Burne (44) describe a similar phenotype for a luxS knockout mutant that demonstrates heightened resistance to oxidative stress. It is also possible that other S. mutans oxidative stress genes are up-regulated in the mutant and thereby compensate for sod repression. There is precedent in the literature for this, in light of redundant oxidative stress response genes, the expression levels of which can vary widely (11). In summary, these findings underscore the fact that oxidative stress defense is a very high priority for the bacteria.

The spaP gene encodes an antigen I/II polypeptide called P1 that is involved in sucrose-independent adherence (7). Mutants deficient in spaP are nonadherent in vitro and give rise to significantly fewer carious lesions on the molar surfaces of germ-free rats (7). In fact, the spaP gene product contributes to S. mutans virulence by facilitating the formation of the plaque biofilm, which creates a microenvironment suitable for the progression of carious lesions.

Among the genes we identified as targets of SloR control by real-time qRT-PCR are gtfB and ghpB, which encode a glucosyltransferase and a glucan binding protein, respectively. Glucosyltransferase B synthesizes insoluble glucans from sucrose, which, in turn, associate with glucan binding proteins such as ghpB in sucrose-dependent adherence. It has been shown that gtfB expression is up-regulated in S. mutans cells grown as biofilms but not when grown planktonically. Moreover, the production of GbpB protein has been positively correlated with the ability of S. mutans to grow in biofilms. Taken collectively, these genes are among the most significant contributors to S. mutans colonization and subsequent disease (28, 48).

A hypothetical gene that we call sko6 is as yet uncharacterized in S. mutans but shares amino acid identity with gramici-
din synthetase enzymes from Bacillus subtilis and Bacillus brevis. Another hypothetical gene, sko9, encodes a putative integral membrane protein containing domains that have been implicated in membrane transport. A putative Sko9 homologue in Pyrococcus abyssi has been implicated in glucose-dependent multidrug resistance through efflux transport. The S. mutans sko6 and sko9 genes are located within a 13-gene cluster flanked by putative transposons, the organization of which is consistent with a polycistrionic operon. At least five additional genes within this cluster also encode proteins putatively associated with antibiotic regulation. Further characterization of the regulation and function of this group of putative antibiotic regulatory genes is ongoing in our laboratory.

Taken collectively, the SloR metalloregulator functions in S. mutans as a positive regulator of virulence gene expression, including comDE, ropA, sod, spaP, sko6, sko9, ghpB, and gtfB expression, and a negative regulator of metal ion transporters, such as sloC. Such control is consistent with the principles of evolution and bacterial virulence, that is, intermediate virulence is often the preferred strategy for successful pathogens, since it serves to sustain both host and microbe over time (13).
Ultimately, such an evolutionary détente can promote bacterial persistence within a host, as well as dissemination of a pathogen from one host to another. *S. mutans* is a prime example of this infection model, given that it persists in the transient conditions of the human oral cavity over the lifetime of the host and is transmitted from the dentition of one host to another almost ubiquitously. To strike a delicate balance with its host, we believe S. mutans depends on metalloregulation to fine-tune the expression of its virulence attributes. Specifically, when metal ions are limiting in the oral cavity (such as between mealtimes), the SloR metalloregulator cannot readily bind to DNA, and hence the expression of *S. mutans* virulence genes that are otherwise "off" is derepressed. When metal ions become plentiful (such as during or shortly after mealtimes), the SloR metalloregulator cannot readily bind to metal ions for incorporation. Ultimately, intermediate pathogenicity is achieved; the payoff is a dentition that can sustain late cariogenesis as transport proteins readily bind metal ions so as to protect the microbe from metal ion toxicity. The result is to promote *S. mutans*-induced cariogenesis as the microbe actively scavenges essential micronutrients and to down-regulate cariogenesis as transport proteins readily bind metal ions for incorporation. Ultimately, intermediate pathogenicity is achieved; the payoff is a dentition that can sustain *S. mutans* for a lifetime and a pathogen that can successfully promote its transmission to new hosts.

In summary, the importance of metal ions in modulating a putative *S. mutans* regulon that contributes to virulence is highlighted by the increased incorporation of metal ions noted for the SloR-deficient GMS584 mutant in this study, together with the multiple *S. mutans* virulence attributes we report as subject to SloR control. We expect microarray studies under way in our laboratory will support the putative relationships we observed in this study between SloR, metal ion availability, and *S. mutans* virulence and will identify other targets of SloR control. We acknowledge that the SloR effects reported herein may be the result of direct or indirect impact on gene expression. Continued investigations will address probable cross talk between and among members of the SloR regulon.

**REFERENCES**


are regulated by the diphtheria toxin repressor (DtxR) and iron. J. Bacteriol. 176:1141–1149.


