SloR modulation of the *Streptococcus mutans* acid tolerance response involves the GcrR response regulator as an essential intermediary

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Streptococcus mutans, the primary causative agent of human dental caries, grows as a biofilm on the tooth surface, where it metabolizes dietary carbohydrates and generates acid byproducts that demineralize tooth enamel. A drop in plaque pH stimulates an adaptive acid-tolerance response (ATR) in this oral pathogen that allows it to survive acid challenge at pHs as low as 3.0. In the present study, we describe the growth of an *S. mutans* mutant, GMS901, that harbours an insertion–deletion mutation in *gcrR*, a gene that encodes a transcriptional regulatory protein. The mutant is acid-sensitive and significantly compromised in its ATR relative to the UA159 wild-type progenitor strain. Consistent with these findings are the results of real-time quantitative RT-PCR (qRT-PCR) experiments that support the GcrR-regulated expression of known ATR genes, including *atpA/E* and *ffh*. Although we observed *gcrR* transcription that was not responsive to acidic pH, we did note a significant increase in *gcrR* expression when *S. mutans* cells were grown in a manganese-restricted medium. Interestingly, the results of gel mobility shift assays indicate that the *S. mutans* SloR metalloregulatory protein is a potential regulator of *gcrR* by virtue of its manganese-dependent binding to the *gcrR* promoter region, and expression studies support the hypothesis that *sloR* transcription is responsive to manganese deprivation and acidic pH. Taking these results together, we propose that SloR–Mn modulates *S. mutans gcrR* expression as part of a general stress response, and that GcrR acts downstream of SloR to control the ATR.

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INTRODUCTION

Streptococcus mutans is the principal causative agent of human dental caries, affecting populations in both developed and developing nations worldwide (Smith, 2001). It colonizes the oral cavity upon adhering to the dentition, where it forms biofilm communities (Cotter & Hill, 2003). Within the plaque biofilm, *S. mutans* metabolizes dietary carbohydrates and generates lactic acid as a byproduct. Plaque and its associated diffusion barrier promote the accumulation of organic acids at the tooth surface that, in turn, demineralize tooth enamel and mark the onset of dental decay (Loesche, 1986). Despite improvements in oral hygiene, the implementation of

education programmes, water fluoridation and commercialized sugar substitutes, the caries pandemic persists. In the United States alone, the cost of dental care continues to exceed \$70 billion a year, and adults lose more than 164 million work hours annually to complications associated with active carious lesions (US Department of Health and Human Services, 2003).

S. mutans is aciduric in the plaque environment owing to the induction of its acid-tolerance response (ATR). The ATR allows cells that are exposed to a sublethal drop in pH to modulate genes whose products allow *S. mutans* to survive in acidic conditions at a pH as low as 3.0–4.0 (Cotter & Hill, 2003; Foster & Hall, 1990; Jensen *et al.*, 1982; Len *et al.*, 2004a; Nascimento *et al.*, 2004). To date, a number of genes that contribute to the *S. mutans* ATR have been identified, including those involved in membrane composition, proton extrusion and DNA repair (Fozo & Quivey, 2004; Griswold *et al.*, 2006; Hamilton & Svensater, 1998; Hanna *et al.*, 2001; Jayaraman *et al.*, 1997; Kuhnert

Abbreviations: ATR, acid-tolerance response; Ct, threshold cycle; MBP, maltose binding protein; qRT-PCR, quantitative RT-PCR.

The array data discussed in this paper have been deposited in the NCBI Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO series accession number GSE10215.

et al., 2004; Lemos *et al.*, 2001; Len *et al.*, 2004b; Li *et al.*, 2001a, b). Since the ATR contributes to the ability of *S. mutans* to adapt to and compete in the plaque environment, an improved understanding of this response and its regulation could prove paramount to the development of therapeutics that target the *S. mutans* caries-forming process.

Previously, we reported on a GcrR (also designated TarC) orphan response regulator in *S. mutans* that promotes sucrose-dependent adherence and cariogenesis (Idone *et al.*, 2003). Specifically, we demonstrated aberrant biofilm formation for a UA130-derived *gcrR* mutant (GMS900) and confirmed a decreased incidence of dental caries when this mutant was used to mono-infect a germ-free rat model. We also observed that the expression of *gtfD* and *gbpC*, known to be involved in *S. mutans* sucrose-dependent adherence, was derepressed in GMS900 relative to the UA130 progenitor strain. From these studies, we concluded that GcrR plays a significant role in *S. mutans* colonization of the tooth surface and subsequent pathogenesis.

In the present study, *in silico* analysis of the *S. mutans* GcrR sequence revealed that it shares significant amino acid identity with response regulators from *Streptococcus pyogenes* (CovRS), *Enterococcus faecalis* (EtaRS), and *Listeria monocytogenes* (LisRK). Like *S. mutans*, these bacterial pathogens can tolerate exposure to acidic conditions during the progression of host infection by mounting an appropriate ATR (Dalton & Scott, 2004; Ferreira *et al.*, 2001; Flahaut *et al.*, 1996a), and reports have described mutants with lesions in *covRS*, *etaRS* or *lisRK* as more sensitive to acid than their wild-type progenitors (Cotter *et al.*, 1999; Dalton & Scott, 2004; Teng *et al.*, 2002). In light of these studies and given the association of *S. mutans* acid production with carbohydrate metabolism, we propose a putative role for the GcrR response regulator in *S. mutans* aciduricity and acid tolerance. To this end, we generated a *gcrR* insertion-deletion mutation in the *S. mutans* UA159 chromosome, for which the genome

sequence is known, and compared the acid sensitivity and ATR of the resulting GMS901 mutant with that of its UA159 progenitor and the previously described isogenic SloR-knockout mutant, GMS584 (Rolerson *et al.*, 2006). SloR is an iron- and manganese-dependent metalloregulatory protein in *S. mutans* that modulates the expression of multiple genes, some of which contribute to virulence (Rolerson *et al.*, 2006). Herein, we present evidence to support SloR regulation of the *S. mutans* ATR that involves GcrR as an essential intermediary.

METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown as overnight cultures at 37 °C in Luria broth (Difco) with gentle aeration, or, when appropriate, on Luria agar plates with erythromycin or chloramphenicol at final concentrations of 300 and 25 µg ml⁻¹, respectively. *S. mutans* UA159 was grown routinely as an overnight standing culture at 37 °C and 5 % CO₂ in Todd-Hewitt broth supplemented with 0.3 % yeast extract (THYE). Erythromycin (10 µg ml⁻¹) was added to the growth medium to maintain selective pressure for the *S. mutans* GMS584, GMS901, GMS902-3 and *citM*⁻ strains. To assess *S. mutans* acid sensitivity in growth determination assays, cells were grown in THYE adjusted to pH 7.5 or pH 5.0 with 2 M dibasic potassium phosphate and 1 M potassium citrate, as previously described (Hanna *et al.*, 2001). The THYE pH 7.5 medium was further supplemented with 0.1 M morpholinepropanesulfonic acid to maintain neutral pH conditions during *S. mutans* growth and acidogenesis. To monitor *S. mutans* gene expression at physiological and acid pH in real-time quantitative RT-PCR (qRT-PCR) experiments, cultures grown overnight in THYE pH 7.5 were pelleted at 7000 r.p.m. in a Sorvall SS-34 rotor for 10 min at 4 °C and then exposed to either THYE pH 7.5 (unadapted) or pH 5.0 (adapted) for 2 h prior to RNA isolation. For expression profiling under manganese-depleted versus -replete conditions, *S. mutans* cells were grown to mid-exponential phase in a semi-defined minimal medium (SDM) (Li *et al.*, 2002) that had been Chelex-treated and supplemented with ferric citrate, magnesium chloride and 0.1 or 10 µM manganese chloride, as described elsewhere (Rolerson *et al.*, 2006), for which the metal ion content was confirmed by inductively coupled argon plasma analysis.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source or reference
<i>S. mutans</i> strains		
UA159	Wild-type, serotype <i>c</i>	ATTC 700610
GMS584	UA159-derived, <i>sloR</i> -deficient, Em ^R	Rolerson <i>et al.</i> (2006)
GMS901	UA159-derived, <i>gcrR</i> -deficient, Em ^R	This work
GMS902-3	GMS901-derived, Em ^R , <i>gcrR</i> mutation complemented <i>in trans</i> with cloned <i>gcrR</i> on plasmid pDD-1	This work
<i>citM</i> ⁻	UA159-derived, Em ^R , source of <i>ermAM</i> cassette	Korithoski <i>et al.</i> (2005)
<i>E. coli</i> strains		
DH5α	GC5 chemically competent cells F ⁻ ϕ 80lacZAM15 Δ [lacZYA-argF]U169 <i>recA1 endA1 hsdR17, phoA supE44 thi-1 gyrA96 relA1 λ^-, tonA</i>	Gene Choice
Plasmids		
pVA838	<i>S. mutans</i> shuttle plasmid, Em ^R , Cm ^R	Macrina <i>et al.</i> (1982)
pDD-1	pVA838 with cloned 1.4 kb <i>gcrR</i> gene and promoter region	This work

Construction of an *S. mutans gcrR* knockout mutant. PCR ligation mutagenesis (Lau *et al.*, 2002) was used to disrupt the *gcrR* coding sequence of the *S. mutans* UA159 chromosome, as illustrated in Fig. 1. Primers were designed using MacVector (v. 7.2.2) software and purchased from Sigma Genosys. PCR amplicons were generated in a 40 µl final reaction volume using primers *gcrR*_P1 and *gcrR*_P2, *gcrR*_P3 and *gcrR*_P4, and *erm*.AscI.F and *erm*.FseI.R (Table 2). The reaction mixtures were placed in an automated thermal cycler (Hybaid) that was programmed to run one 10 min cycle of denaturation at 94 °C followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing at the optimal temperature (Table 2) for 2 min, and extension at 72 °C for 3 min, followed by a final extension at 72 °C for 10 min. The resulting P1/P2 and P3/P4 PCR amplicons were purified with a PCR Purification Kit (Qiagen), digested with AscI and/or FseI (New England Biolabs), and ligated to the 860 bp *ermAM* cassette for 18 h at 16 °C in each of two separate reactions and in the presence of T4 DNA ligase. The resulting ligation mixture was used as a template in another round of amplification with the P1 and P4 primer set to generate a 1295 bp tripartite ligation end product. *S. mutans* UA159 cells were transformed with the final ligation mixture in the presence of competence-stimulating peptide, as described elsewhere (Lau *et al.*, 2002; Li *et al.*, 2001b). Transformed colonies were selected on THYE-agar plates with 10 µg erythromycin ml⁻¹ after overnight incubation at 37 °C and 5% CO₂. Chromosomal DNA was isolated from selected transformants and the UA159 progenitor according to established protocols (Sambrook *et al.*, 1989) so that subsequent PCR and nucleotide sequencing could confirm insertion of the 860 bp *ermAM* cassette at the desired *gcrR* locus. Purified amplicons were sequenced across the *gcrR-ermAM* junctions with the existing *gcrR*_P1 and *gcrR*_P4 primer set on a Perkin-Elmer ABI Prism 377 DNA sequencer using dye-terminator chemistry.

Monitoring *S. mutans* aciduricity in growth determination assays. To determine the sensitivity of *S. mutans* cells to growth at pH 5.0, overnight cultures of *S. mutans* UA159, GMS901, GMS902-3 and GMS584 were diluted 1:10 in fresh THYE pH 7.5 medium and monitored for growth with a Spectronic Genesys 2 spectrophotometer. The cells were harvested at mid-exponential phase (OD₆₀₀=0.4–0.6) and the culture densities were standardized to within 0.05 OD units with fresh THYE. The resulting cell suspensions

were diluted 1:20 into fresh THYE pH 7.5 or pH 5.0 and distributed in 420 µl aliquots into the wells of a 100-well honeycomb microtitre plate (Growth Curves USA) in triplicate. The cells were incubated for 48 h at 37 °C in a Bioscreen C Microbiology Reader (LabSystems), and OD₆₀₀ measurements were obtained every 15 min with gentle agitation preceding each reading. Doubling times from each of three independent experiments were determined for cells in exponential phase and at points corresponding to when the rate of growth was fastest. The viability of *S. mutans* stationary-phase cells at pH 5.0 was determined by plating cells from the *t*=24 h time point onto THYE agar so that c.f.u. could be enumerated.

Monitoring *S. mutans* acid tolerance in acid-challenge experiments. To assess the *S. mutans* ATR, the established protocol of Hanna *et al.* (2001) was followed. Briefly, UA159, GMS901, GMS902-3 and GMS584 mid-exponential-phase cells were grown in THYE and harvested by centrifugation as described above, followed by resuspension in THYE pH 7.5 (unadapted) or pH 5.0 (adapted) and cellular de-aggregation by successive passages through a 26 gauge, 5/8 inches (15.9 mm) length needle. The cell suspensions were then incubated for 2 h at 37 °C and 5% CO₂, during which no appreciable growth occurred as determined by spectrophotometry. Following incubation, the cells were pelleted and resuspended in THYE pH 3.5 for 45, 90 or 180 min before being passed once again through a fine-gauge needle and spread-plated onto THYE agar. The ATR was assessed for both adapted and unadapted cultures by viable plate counting, with survival at each time point expressed as a percentage of viable cells (in c.f.u. ml⁻¹) that were present in the original pH 5.0 cell suspension immediately after exposure to the lethal pH of 3.5 (*t*=0).

Complementation experiments. A 1.4 kb amplicon containing the wild-type *gcrR* gene and promoter region was PCR-amplified with primers *gcrR*.*PvuII*.F and *gcrR*.*EcoRI*.R (Table 2), digested with *EcoRI* and *PvuII* according to the recommendations of the supplier (Promega), and cloned into the *EcoRI/PvuII* site of the replicative shuttle plasmid pVA838 (Table 1). The resulting recombinant was introduced into chemically competent *E. coli* DH5α (Gene Choice), after which transformants were screened for erythromycin resistance and chloramphenicol sensitivity, and processed for plasmid DNA isolation on miniprep spin columns (Qiagen). Recombinant plasmids

Fig. 1. Organization of the *gcrR* locus in *S. mutans* UA159 and GMS901. Shown is a map of the *gcrR* locus on the *S. mutans* UA159 and GMS901 chromosomes. The *gcrR* gene is flanked by ~350 bp of non-coding DNA upstream and downstream, and is not co-transcribed with a cognate histidine kinase. A search of the Los Alamos Oral Pathogen Sequence database (<http://www.oralgen.lanl.gov>) revealed the gene products of SMU.1747 and SMU.1749 to be conserved hypothetical proteins of unknown function. We used a 1295 bp PCR construct to disrupt the *S. mutans gcrR* coding sequence on the UA159 chromosome. Details of the mutagenesis procedure are described in Methods. The resulting allelic replacement was confirmed by nucleotide sequencing with primers P1 and P4 (indicated by arrowheads), and the mutant was designated GMS901.

Table 2. Primers used in this study

Primer use	Primer name	Nucleotide sequence (5'–3')*	Annealing temperature (°C)	Amplicon size (bp)
Mutagenesis	<i>erm</i> .Asc1.F	GGCGCGCCCCGGGCCCAAAATTTGTTTGAT	52.3	860
	<i>erm</i> .Fse1.R	ATTCTATGAGTCGCTGCCGACTGGCCGGCC		
	<i>gcrR</i> _P1	GTTAGAGTTTTGTTGAGGAGT	47.7	219
	<i>gcrR</i> _P2	GGCGCGCCGGAATCACTTCATACCCGTC		
	<i>gcrR</i> _P3	GGCCGGCCGTGGGAAGATTGATGTTC	48	216
Complementation*	<i>gcrR</i> .PvuII.F	CCAGCTGGAAAGGAAAGAAAGAAAAGAGCAGTCC	52.8	1387
	<i>gcrR</i> .EcoRI.R	GGAATTCCTGACCGAGGTGAGAAAACGTAGC		
Real-time PCR	<i>atpA</i> _RT.F	TATTGCTCGTGCTTGCGGAC	54.6	251
	<i>atpA</i> _RT.R	TTTCAACCAGACCATCAACAGG		
	<i>atpE</i> _RT.F	TTGCTGTTTTAGGCGTTAG	46.7	88
	<i>atpE</i> _RT.R	TTACCATACATTTCAAGGCTG		
	<i>ffh</i> _RT.F	GCTCTTGCCAATGTCAAAGTTG	51.7	105
	<i>ffh</i> _RT.R	CGGAGTTAGGAGGTCAGGATTC		
	<i>gcrR</i> _RT.F	ACCAGAGATGGACGGGTATG	51.3	242
	<i>gcrR</i> _RT.R	CACGATAGGTAGTGTCAATTTTAGAAG		
	<i>gyrA</i> -RT-F	ATTGTTGCTCGGGCTCTCCAG	52.5	105
	<i>gyrA</i> -RT-R	ATGCGGCTTCTCAGGAGTAACC		
	<i>hk11</i> _RT.F	GCTGGCTAATAATGTCATCAAGC	50.8	88
	<i>hk11</i> _RT.R	CTCAACAGTTACTTCAATCTCCTCC		
Gel mobility shift	<i>gcrR</i> _GS.F	TAATAGATTTTGTTTTTCCTTAGTAAC	48.3	308
	<i>gcrR</i> _GS.R	TCAAGTGCTCTTCCAGGC		
	<i>recA</i> _GS.F	CGGTTATCCAAAAGGGCGTATC	55.3	212
	<i>recA</i> _GS.R	CCTGTTCTCCTGAATCTGGTTGTG		
	<i>sloA</i> _GS.F	ATCGGTGAATCGCACTGTCTG	51.1	310
	<i>sloA</i> _GS.R	TAAGGTTGACTTGCCCCGAC		

*Restriction sites are shown in bold type.

containing the 1.4 kb insert were confirmed by *Hind*III restriction mapping and PCR amplification with the *gcrR*_RT.F and *gcrR*_RT.R primer set (Table 2). The confirmed *gcrR*-containing plasmid, designated pDD1, was introduced into *S. mutans* GMS901 by CSP-induced transformation, as described previously (Li *et al.*, 2001b), and erythromycin-resistant transformants were screened for 'loss' of the GMS901 aggregation phenotype upon overnight growth in THYE broth. Selected strains were processed for plasmid DNA isolation according to the rapid screening procedure of Leblanc & Lee (1979), and *S. mutans* clones that tested positive for pDD-1 were designated GMS902-3 and analysed for complementation of the ATR defect in growth-determination and acid-challenge experiments.

RNA isolation. Mid-exponential-phase cultures ($OD_{600}=0.5$) of *S. mutans* wild-type and mutant strains were harvested by centrifugation as described above and resuspended in 3 ml TRI Reagent (Ambion). The cell slurry was mechanically disrupted for 40 s at 4 °C in a Bio 101 FastPrep machine in the presence of zirconium beads, and the aqueous phase, containing nucleic acid, was ethanol-precipitated. The DNA/RNA pellets were washed in 70 % ethanol and finally resuspended in nuclease-free water prior to DNase I treatment on an RNeasy column (Qiagen) according to the recommendations of the supplier. RNA integrity was assessed by ethidium bromide staining of the 23S and 16S rRNA subunits on a 1 % agarose gel, and quantified spectrophotometrically at A_{260} for concentration and at A_{260}/A_{280} for purity.

Monitoring gene expression in real-time qRT-PCR experiments. Total intact RNA obtained from *S. mutans* UA159,

GMS584 and GMS901 was reverse-transcribed into cDNA using a First Strand cDNA Synthesis kit (MBI Fermentas), according to the manufacturer's instructions. Reactions containing no RNA template (NTC) or no reverse transcriptase (RT–) served as controls. The resulting cDNA (10 ng) was used as a template along with 150 nM primers (Table 2) for amplification in a Cepheid Smart Cycler using SYBR Green I as the intercalating dye (Invitrogen). The RT– controls were used as a template with the same primer sets to confirm the absence of contaminating chromosomal DNA in the RNA preparations. The cDNAs were denatured at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 15 s, primer annealing at optimal temperature (Table 2) for 30 s, and primer extension at 72 °C for 30 s. The expression of *gcrR* in UA159 versus GMS584 was normalized to that of *hk11*, which we determined did not change in these strains when grown under the experimental test conditions. For UA159 and GMS901 expression profiling at different pH and manganese concentrations, we used *gyrA* for normalization, the expression of which we determined was invariant under the test conditions. Standard curves for each primer set were derived from an *S. mutans* genomic DNA dilution series and subsequently used to calculate DNA copy number. Threshold cycle (Ct) values were assigned a cycle number at which the second derivative of the fluorescence curve was at its maximum.

Gel mobility shift assays. Gel mobility shift assays were performed according to a modification of the protocol of Tao *et al.* (1992) with a SloR–MBP (maltose binding protein) fusion protein that we had isolated previously (Rolerson *et al.*, 2006). The primers used to

PCR-amplify the promoter regions of the *S. mutans gcrR*, *recA* and *sloABC* genes are listed in Table 2. The purified PCR products (Qiagen) were quantified spectrophotometrically before end-labelling in separate reaction mixtures containing 10 µCi (370 kBq) [γ - 32 P]ATP (Perkin Elmer) and 10 U T4 polynucleotide kinase (New England Biolabs). Reaction mixtures were incubated for 30 min at 37 °C and then for 20 min at 70 °C before the final sample volume was adjusted to 50 µl with sterile distilled H₂O. Unincorporated radiolabel was removed by passage through a G-25 Sepharose column (Roche Applied Science). Binding reactions were prepared in total volumes of 16 µl containing 1 µl (~17 ng) end-labelled DNA, up to 8.5 µM SloR-MBP fusion protein or MBP, and 3.2 µl 5× binding buffer (42 mM NaH₂PO₄, 58 mM Na₂HPO₄, 250 mM NaCl, 25 mM MgCl₂, 50 µg BSA ml⁻¹, 1 mg sonicated salmon sperm DNA and 50%, v/v, sterile glycerol). The 5× binding buffer was supplemented, when appropriate, with MnCl₂ to a final concentration of 150 mM to facilitate SloR-DNA binding. To determine the metal ion dependency of SloR binding, an EDTA metal ion chelator was added to the reaction mixtures at a final concentration of 15 mM. In an attempt to restore the SloR-DNA binding phenotype, 14 µl 0.1M MnCl₂ stock solution was added to EDTA-containing samples. A 310 bp amplicon that harbours the *sloABC* promoter region was used as a positive control in these experiments, and a 212 bp amplicon that harbours the *S. mutans recA* promoter region was used as a negative control. Cold *gcrR* amplicon (308 bp) was included as competitor DNA in certain reaction mixtures to confirm specificity of SloR-MBP binding to the *gcrR* promoter region. All protein-DNA reaction mixtures were incubated for 20 min at room temperature before they were resolved on 18% non-denaturing polyacrylamide gels for 1 h at 300 V. The gels were processed for autoradiography and exposed to Kodak BIOMAX film for 24 h at -80 °C in the presence of an intensifying screen.

RESULTS

In silico analysis of the *S. mutans gcrR* gene

GcrR, first described by Sato *et al.* (2000) and later renamed TarC (Idone *et al.*, 2003) is an orthologue of the CovR regulator of *S. pyogenes* (Graham *et al.*, 2002) and *Streptococcus agalactiae* (Jiang *et al.*, 2005; Lamy *et al.*, 2004), where it is found in association with its cognate CovS histidine kinase. In contrast, the *S. mutans gcrR* gene, not to be confused with *vicR* of the *S. mutans vicRKX* locus (Senadheera *et al.*, 2005), for which orthologues have been characterized in *S. pneumoniae* (Wagner *et al.*, 2002) and *Bacillus subtilis* (Szurmant *et al.*, 2005), encodes a response regulator that is organized on the *S. mutans* chromosome as a monocistron, and as we demonstrated previously, is not cotranscribed with a cognate histidine kinase (Idone *et al.*, 2003). Interestingly, a query of the Los Alamos Oral Pathogen database (<http://www.oralgen.lanl.gov>) with the *S. mutans* GcrR amino acid sequence revealed homologues in other bacterial pathogens that survive acid stress in the host environment. Specifically, the results of a BLASTP search demonstrated that GcrR shares 75% amino acid identity with the CovR response regulator of *S. pyogenes*, 64% identity with the EtaR response regulator of *Ent. faecalis*, and 57% identity with the LisR response regulator in *L. monocytogenes*. An analysis of the *S. mutans gcrR* promoter region with the MFold search engine (Workman *et al.*, 2002) revealed stem-loop structures, to which SloR

could potentially bind, although a recognizable consensus palindrome (AAATTAACCTTGACTTAATTTTT) defined previously by Kitten *et al.* (2000) could not be confirmed given the low G+C content of the *S. mutans* genome.

Confirmation of *gcrR* disruption in *S. mutans* GMS901

Erythromycin-resistant transformants were screened for deletion of the *gcrR* locus and replacement with *ermAM* as a result of double-crossover recombination. Genomic DNA was isolated from selected transformants and used as a template for PCR amplification with primers *gcrR*_P1 and *gcrR*_P4 (Fig. 1, Table 2). The resulting PCR amplicons were analysed by agarose gel electrophoresis, and their sizes were compared with those predicted following a successful allelic exchange event. Nucleotide sequencing of the purified amplicons with the same primer set confirmed disruption of the *gcrR* coding sequence, and the resulting mutant was designated GMS901. Finally, total RNA from GMS901 was reverse-transcribed and quantified for *gcrR* transcription in real-time qRT-PCR experiments, the results of which revealed *gcrR* expression that was like that of the RT-control ($C_t = 31.11 \pm 0.3$ and 29.18 ± 0.67 , respectively).

S. mutans GMS901 is acid sensitive

We conducted *S. mutans* growth determination experiments to monitor the aciduricity of the *S. mutans sloR*- (GMS584) and *gcrR*- (GMS901) deficient mutants relative to their UA159 wild-type progenitor at physiological and acidic pH. Results representative of six independent experiments are shown in Fig. 2. Importantly, we observed equal survivorship for all *S. mutans* strains following 24 h of growth at pH 5.0 (data not shown). The growth rate of GMS901 at pH 7.5 was comparable to that of the wild-type during mid-exponential phase (doubling times ~60 min), but was significantly compromised relative to the wild-type at pH 5.0 (Student's *t* test; $P=0.024$). Specifically, the exponential-phase doubling time for GMS901 at pH 5.0 was ~30 min longer than that of UA159, and GMS901 ultimately attained a lower final resting culture density than wild-type (OD_{600} ~0.7 and ~1.0, respectively). GMS901 reached a lower resting culture density at pH 7.5 as well, a result that we attribute to the clumping phenotype of the mutant when grown in broth culture. Finally, growth of *S. mutans* GMS584 at acidic pH was not significantly different from that of the wild-type, with doubling times of 146 and 135 min, respectively. Taken together, these findings support the involvement of GcrR in the ability of *S. mutans* to withstand acid stress and indicate that defects in acid tolerance arise from *gcrR* deficiency.

The ATR is significantly impaired in *S. mutans* GMS901

We examined the kinetics of acid killing for *S. mutans* UA159 and GMS901 adapted and unadapted cells that had

Fig. 2. *S. mutans* GMS901 is significantly more acid-sensitive than GMS584 and the UA159 wild-type progenitor. *S. mutans* UA159 (solid lines), GMS584 (dashed lines) and GMS901 (dotted lines) were grown in a rich THYE medium adjusted to pH 7.5 or pH 5.0, as described in Methods. Plate counts confirmed the viability of all strains during the stationary phase of growth at both pHs. *S. mutans* UA159, GMS584 and GMS901 demonstrate similar growth rates at pH 7.5 with doubling times ~60 min. The lower final culture density for GMS901 at physiological pH may be attributed to the clumping phenotype of this mutant when grown in liquid culture. At pH 5.0, the growth of GMS901 was significantly compromised relative to both GMS584 and the wild-type ($P=0.002$ and 0.024 , respectively). Specifically, the pH shift from 7.5 to 5.0 increased the doubling times for UA159, GMS584 and GMS901 by 77, 85 and 100 min, respectively. Results representative of six independent experiments are shown.

been exposed to the killing pH of 3.5 for up to 3 h. The results of a single representative experiment from a total of at least five independent experiments are expressed as percentage survival in Fig. 3(a). From these experiments we noted that 90 min of incubation at the killing pH was sufficient to reduce the number of unadapted wild-type and mutant survivors by 2–3 log units (Fig. 3a, filled symbols). In addition, we observed similar survivorship for GMS901 and UA159 unadapted cells after 3 h of exposure to pH 3.5 (differing by only 0.05 %), indicating that the clumping phenotype of GMS901 has little if any impact on acid sensitivity following cellular deaggregation through a fine-gauge needle. Importantly, the survivorship we noted for adapted GMS901 cells differed from that of unadapted cells by less than 0.5 log units after 3 h of exposure to the killing pH, whereas adapted and unadapted wild-type cells differed by more than 2 log units, with adapted cells demonstrating significantly greater survivorship (Student's *t* test, $P<0.05$, Table 3). Taken together, these findings

underscore the importance of GcrR as a modulator of the *S. mutans* ATR.

Disruption of the *sloR* ORF in *S. mutans* GMS584 did not significantly affect the viability of adapted cells after 3 h at the killing pH (Table 3), although taking the large variations between experiments into consideration, the results do show a trend that is consistent with a role for SloR in the *S. mutans* response to acid stress.

Complementation analyses

Complementation of the *sloR* mutation in *S. mutans* GMS584 was reported previously by Rolerson *et al.* (2006). In the present study, *in silico* analysis of the *S. mutans* UA159 genome revealed no ORFs within 350 bp of the *gcrR* coding sequence on either DNA strand, making polar effects resulting from the insertion–deletion mutation in GMS901 unlikely. Nevertheless, we complemented the *gcrR* mutation on the GMS901 chromosome *in trans* to confirm specific involvement of GcrR in *S. mutans* acid tolerance. To this end, we performed growth determination assays as described in Methods with *S. mutans* UA159, GMS901 and the pDD1-containing complemented mutant GMS902-3. We observed GMS902-3 exponential-phase growth that was intermediate between that of the GMS901 mutant and the UA159 wild-type strain when grown in THYE pH 5.0 (data not shown). In addition, the results of acid-challenge experiments (Fig. 3b) reveal that 26 % of adapted GMS902-3 cells survived a 3 h exposure to the killing pH, whereas only 2.25 % of unadapted GMS902-3 cells survived this acid challenge. These findings support rescue of the ATR defect in the GMS902-3 complemented strain (Fig. 3c). While the clumping phenotype of GMS902-3 was alleviated somewhat by complementation, it was not ‘fixed’, as determined by phase-contrast microscopy (data not shown). The persistence of cell clumps combined with restoration of the ATR in GMS902-3 supports a complementation effect that derives from *gcrR* expression *in trans* rather than from cell dispersal. Finally, we observed unadapted GMS902-3 cells that were considerably more acid-resistant than unadapted wild-type cells (Fig. 3b), a result that we attribute to the presence of multiple copies of the *gcrR* transcript in the complemented strain.

Expression of *gcrR* is not responsive to acidic pH

To determine whether *S. mutans* *gcrR* expression is acid-responsive, we performed three independent real-time qRT-PCR experiments with cDNAs that we isolated from *S. mutans* UA159 mid-exponential phase cells exposed to physiological (pH 7.5) or acidic (pH 5.0) conditions for 2 h. The results of these experiments, each performed in triplicate, revealed no significant differences in *gcrR* expression at either pH, indicating that *gcrR* expression is not acid-responsive at the level of transcription (mean DNA copy number = 2.4×10^7 and 1.8×10^7 at pH 7.5 and 5.0, respectively).

Fig. 3. The *S. mutans* ATR is significantly compromised in GMS901 relative to the wild-type UA159 progenitor strain. *S. mutans* UA159 and GMS901 cells were grown to mid-exponential phase in THYE, as described in Methods, and harvested by centrifugation, and this was followed by resuspension and de-aggregation in fresh THYE pH 7.5 (unadapted) or pH 5.0 (adapted). The cells were incubated under these conditions at 37 °C and 5% CO₂ for 2 h before exposure to the killing pH of 3.5 for up to 3 h. Importantly, there was no change in cell density (OD₆₀₀) for any of the *S. mutans* cultures during the 2 h incubation period. Following acid challenge at pH 3.5 for 0, 45, 90 and 180 min, the cells were plated onto THYE agar and incubated at 37 °C and 5% CO₂ for 48 h. (a) The results of viable plate counting are expressed as the percentage of viable cells (in c.f.u. ml⁻¹) at each time point relative to viable cells (in c.f.u. ml⁻¹) present at *t*=0. The survival of GMS901 adapted cells (Δ) after 3 h at the killing pH differed from that of unadapted cells (▲) by 0.5 log units, whereas that of adapted and unadapted wild-type cells (○ and ●, respectively) differed by at least two orders of magnitude, suggesting a defective ATR in GMS901. The results of a single representative experiment from a total of at least five independent experiments are shown. (b) Percentage survivorship for adapted (A, hatched bars) and unadapted (UA, black bars) UA159, GMS901 and complemented GMS902-3 cells after a 3 h exposure to the killing pH. The ATR defect was rescued in GMS902-3 by at least 1 log unit, despite the persistence of clumping in this strain. (c) The efficiency of the ATR in each strain is expressed as the difference in survivorship between adapted and unadapted cells. The complemented mutant demonstrates an ATR that is intermediate between that of the wild-type UA159 strain and the GMS901 *gcrR*-deficient mutant, supporting *gcrR* complementation of the ATR defect *in trans*.

GcrR is subject to SloR metalloregulation in *S. mutans*

Transcription of the *S. mutans gcrR* gene was unaffected by pH, despite our hypothesis that GcrR is involved in modulating the *S. mutans* ATR. This led us to investigate

other potential mechanisms of *gcrR* control that may affect, but otherwise be unrelated to, the ATR. Interestingly, microarray experiments that we had performed previously with RNAs isolated from *S. mutans* UA159 and the GMS584 *sloR*-deficient mutant indicated that *gcrR* expression was down-regulated in the mutant by more than

Table 3. Acid-adaptive response for *S. mutans* UA159 and its isogenic derivatives

<i>S. mutans</i> strain	Growth pH	Survival (%) after 3 h*	Student's <i>t</i> test†
UA159	7.5	7.3 × 10 ⁻² (± 1.1 × 10 ⁻¹)	<i>P</i> <0.05
	5.0	260 × 10 ⁻² (± 41 × 10 ⁻¹)	
GMS901	7.5	1.2 × 10 ⁻¹ (± 0.5 × 10 ⁻¹)	NS
	5.0	5.3 × 10 ⁻¹ (± 1.6 × 10 ⁻¹)	
GMS584	7.5	1.1 × 10 ⁻¹ (± 0.1 × 10 ⁻¹)	NS
	5.0	7.6 × 10 ⁻¹ (± 2.7 × 10 ⁻¹)	

*Mean (± SD).
†NS, Non-significant.

twofold when cells were grown in a nutrient-replete SDM (data not shown). These results are consistent with *gcrR* expression that is subject to SloR control. We validated SloR modulation of *gcrR* expression in real-time qRT-PCR experiments, where we observed fourfold less *gcrR* mRNA in GMS584 relative to UA159 (DNA copy number = 3.4×10^6 and 1.5×10^7 , respectively) for cells grown in nutrient-replete SDM. Taken collectively, these findings led us to suspect that *S. mutans gcrR* expression might be responsive to manganese. Additional microarray experiments that we had performed with RNAs isolated from UA159 cells grown in a manganese-depleted ($0.1 \mu\text{M}$ MnCl_2) versus a manganese-replete ($10 \mu\text{M}$ MnCl_2) SDM supported *gcrR* expression being manganese-responsive, and validation of these results in real-time qRT-PCR experiments revealed that *gcrR* transcription was upregulated more than threefold at the lower manganese concentration (DNA copy number = 1.7×10^6 at $0.1 \mu\text{M}$ and 4.9×10^5 at $10 \mu\text{M}$). These findings support induction of *S. mutans gcrR* transcription under conditions of micronutrient deprivation, and are consistent with *gcrR* regulation that is stress-responsive and subject to SloR and/or manganese control.

S. mutans sloR expression is acid-responsive

To better describe SloR metalloregulation and its impact on GcrR as part of a general stress response in *S. mutans*,

we set out to determine whether *sloR* expression, already known to be metal ion-responsive (Kitten *et al.*, 2000; Paik *et al.*, 2003), might also be sensitive to acidic pH. To this end, we monitored *sloR* transcription in real-time qRT-PCR experiments with cDNAs derived from *S. mutans* UA159 cells grown in THYE pH 7.5 or pH 5.0. The results of these studies support *sloR* transcription that is increased sixfold at acidic pH (DNA copy number = 1.2×10^8 at pH 7.5 and 6.8×10^8 at pH 5.0), indicating that SloR is indeed responsive to acid stress.

SloR binds upstream of the *S. mutans gcrR* gene

The results of gel mobility shift assays support direct SloR binding to the *gcrR* promoter region. That is, migration of the *gcrR* promoter fragment was hindered in the presence of the SloR-MBP fusion protein, but not when the EDTA metal ion chelator was added to the reaction mixture. The band shift was rescued when MnCl_2 was added to the EDTA-containing reaction mixture, however, supporting the hypothesis that SloR binding at this region is manganese-dependent (Fig. 4). Abrogation of the band shift with increasing amounts of cold *gcrR* promoter DNA supports the suggestion that SloR-MBP binding is specific to the *gcrR* promoter region, and the absence of a shift when pure MBP was present in the reaction mixture supports the involvement of the SloR portion of the fusion

Fig. 4. The *S. mutans* SloR protein binds directly to the *gcrR* promoter region. The results of gel mobility shift assays confirm direct binding of SloR to the *gcrR* promoter region. Specifically, the *S. mutans sloABC* (310 bp), *recA* (212 bp) and *gcrR* (308 bp) promoter regions were PCR-amplified as described in Methods and end-labelled with [γ - ^{32}P]ATP in the presence of T4 polynucleotide kinase. The resulting radiolabelled amplicons (~17 ng) were incorporated into separate reaction mixtures containing $8.5 \mu\text{M}$ purified MBP or SloR-MBP fusion protein. Competition assays with excess cold *gcrR* DNA were also performed to address the binding specificity of SloR to the *gcrR* promoter fragment. Other controls included reaction mixtures without any added protein (-), supplemented with EDTA, or supplemented with both EDTA and MnCl_2 . Collectively, the results indicate that the SloR portion of the fusion protein binds directly and specifically to the *sloABC* and *gcrR* promoter regions, and that the SloR-DNA binding interaction is manganese-dependent. The *recA* promoter region, devoid of recognizable SloR binding palindromes, was included in these studies as a negative control.

protein in binding to DNA. An upward band shift was similarly noted for the *sloABC* promoter region, which harbours a 'classical' SloR-binding palindrome, whereas no shift was evident for the *recA* promoter region, which is devoid of recognizable SloR palindromes. The affinity of the fusion protein for *gcrR* DNA binding appears to be even greater than that of the *sloABC* DNA control, based on the relatively robust intensity of the *gcrR* promoter fragment on the autoradiogram and comparable specific activities for the end-labelling reactions.

GcrR modulates expression of known contributors to the *S. mutans* ATR

To validate the importance of GcrR as a regulator of the *S. mutans* ATR, we compared the expression of several known ATR genes in UA159 and GMS901 cells that had been resuspended in THYE pH 5.0 for 2 h. The results of these real-time qRT-PCR experiments support GcrR as a regulator of *ffh*, which encodes a bacterial signal recognition particle that is linked to the ATR (Gutierrez *et al.*, 1999), and of *atpA/E*, which encode the functional and membrane-spanning subunits of the *S. mutans* proton-translocating F_0 -ATPase. Specifically, the transcription of *ffh* was down-regulated in GMS901 relative to UA159 by 1.6-fold (DNA copy number = 5.5×10^6 and 8.5×10^6 , respectively), and *atpE* expression was likewise repressed in the *gcrR*-deficient mutant by 2.5-fold (DNA copy number = 2.4×10^8 in GMS901 and 5.7×10^8 in UA159). These findings support a direct relationship between GcrR and the activation of genes whose products contribute to mechanisms that maintain internal pH homeostasis in *S. mutans*.

S. mutans ATR genes are SloR- and manganese-responsive

To confirm a role for GcrR as a regulator of the *S. mutans* ATR that acts downstream of SloR metalloregulation, we monitored *atpA/E* expression for SloR- and manganese-responsiveness in real-time qRT-PCR studies. Specifically, we observed *atpA/E* expression that was upregulated 3.6-fold in an SDM containing 0.1 μ M manganese (DNA copy number = 8.7×10^6) as compared to the same medium containing 10 μ M manganese (DNA copy number = 2.4×10^6). Moreover, the expression of *atpA/E* was decreased threefold in GMS584 relative to UA159 (DNA copy number = 1.2×10^8 and 3.1×10^8 , respectively). Taken together, these findings support an *S. mutans* ATR that is SloR-modulated and affected by growth-limiting manganese.

DISCUSSION

This study presents evidence to support GcrR modulation of the *S. mutans* ATR that expands on its functional role during cariogenesis. Our previous report (Idone *et al.*, 2003) described the involvement of GcrR in *S. mutans*

sucrose-dependent adherence owing to its effect on expression of the glucosyltransferase-D (*gtfD*) and glucan binding protein C (*gbpC*) genes. In those studies we described a UA130-derived *gcrR*-deficient mutant, designated GMS900, which formed aberrant biofilms and was hypocariogenic in a germ-free rat model. These findings implicate GcrR as a major modulator of *S. mutans* virulence, yet the molecular mechanisms that regulate its expression are poorly understood. In this study, we describe the impact of GcrR on *S. mutans* acid tolerance and investigate its regulation as part of a stress response involving SloR metalloregulation.

The byproducts, especially sucrose, of dietary carbohydrate metabolism by *S. mutans* and other acidogenic constituents of the oral microflora can yield a plaque pH as low as 3.0 (Jensen *et al.*, 1982; Loesche, 1986). *S. mutans* can adapt to this killing pH by modulating an arsenal of genes that contribute to its ATR (Cotter & Hill, 2003). Specifically, when conditions of acid stress (pH ~5.0) prevail in the plaque environment, genes whose products facilitate DNA repair (*uvrA*), modulate cytoplasmic pH (*atpE*) and foster protein secretion (*ffh*) are induced (Fozo & Quivey, 2004; Hanna *et al.*, 2001; Kuhnert *et al.*, 2004). Collectively, these and other constituents of the ATR promote *S. mutans* survival at acidic pH, and their regulation is likely to be complex, involving mechanisms of control at the transcriptional and post-transcriptional levels.

Other pathogens that confront acid conditions in the host environment during infection include *S. pyogenes*, *Ent. faecalis* and *L. monocytogenes*, all of which harbour homologues of the *S. mutans* GcrR protein (Arikado *et al.*, 1999; Farber & Peterkin, 1991; Flahaut *et al.*, 1996b; Mead *et al.*, 1999). Namely, CovR, EtaR and LisR each form part of a two-component signal transduction system in their respective micro-organisms, in which they have been shown to promote an ATR (Cotter *et al.*, 1999; Foza *et al.*, 2007; Teng *et al.*, 2002). To reveal a putative role for the GcrR response regulator in the ability of *S. mutans* to adapt to and survive acid stress, we generated an insertion-deletion mutation in the *S. mutans gcrR* gene on the fully sequenced *S. mutans* UA159 chromosome and analysed the resulting mutant, GMS901, in acid-challenge experiments. From these experiments we noted growth rates for UA159 and GMS901 that were comparable in a rich THYE pH 7.5 medium but that differed considerably at pH 5.0 (Fig. 2). At the latter pH, GMS901 displayed a prolonged lag phase and a decreased final resting culture density relative to the wild-type, consistent with GMS901 growth that is acid-sensitive, and implicating GcrR in *S. mutans* aciduricity.

We successfully induced an *S. mutans* ATR in the wild-type UA159 strain, since the survival of unadapted and adapted cells differed by more than 2 log units after a 3 h exposure to the killing pH (Fig. 3a). In contrast, the survivorship of GMS901 unadapted and adapted cells differed by 0.5 log units, thereby implicating GcrR in the *S. mutans* ATR. Importantly, GMS901 and UA159 cells appeared to be

equally sensitive to acid after 3 h at the killing pH (Fig. 3a, closed symbols), despite the propensity of GMS901 to aggregate in liquid culture. We therefore attribute attenuation of the ATR in GMS901 to the *gcrR* defect in this strain and not to its clumping behaviour that could otherwise protect cells from the effects of low-pH environments. Lending further support to GcrR involvement in the *S. mutans* ATR is the increased survivorship we noted for the adapted GMS902-3 complemented strain after 3 h at the killing pH. Specifically, the survival of these cells was more than 10-fold greater than that of unadapted GMS902-3 cells (Fig. 3b), despite the persistence of clumping in this strain (i.e. the clumping phenotype of GMS902-3 was alleviated, but not completely 'fixed' by complementation). Collectively, these findings support rescue of the ATR defect by *gcrR* complementation *in trans*, and substantiate the relationship between GcrR and regulation of the *S. mutans* ATR.

A response as complex as the ATR likely involves a repertoire of *S. mutans* genes that are GcrR-responsive. In this report we describe a significant effect of GcrR on *S. mutans ffh* and *atpA/E* transcription, the products of which contribute significantly to the *S. mutans* ATR (Gutierrez *et al.*, 1999; Crowley *et al.*, 2004). Specifically, the *ffh* gene, which is part of the *sat* operon on the *S. mutans* chromosome, encodes a eukaryotic signal-recognition particle homologue that has been implicated in acid tolerance. A mutation in the *ffh* coding sequence gives rise to mutants that are strongly acid-sensitive (Gutierrez *et al.*, 1999) and severely compromised in F₀-ATPase activity at physiological and acidic pH (Crowley *et al.*, 2004). Previous work has correlated *S. mutans* acid adaptation with increased proton-extruding F₀-ATPase activity, and the latter correlates with increased transcription of the F₀-ATPase operon (Hamilton & Buckley, 1991). The F₀-ATPase operon, which comprises the *atpEBFHAGDC* genes, is cotranscribed to give rise to the functional and membrane-spanning domains of the proton-translocating F₀-ATPase. In the present study we describe a decrease in GMS901 *ffh* transcription that coordinates with a more than twofold decrease in *atpA/E* expression, supporting an *S. mutans* ATR that is subject to GcrR control.

The results of transcription profiling experiments support *atpA/E* and *gcrR* expression that is both SloR-dependent and affected by growth-limiting manganese, consistent with a model that implicates GcrR control as part of a general stress response in *S. mutans*. Constitutive expression of the *S. mutans gcrR* gene under both physiological (pH 7.5) and acidic (pH 5.0) conditions further supports multiple mechanisms of *gcrR* control that can affect the ATR. Taken together, our experimental findings support a direct interaction between SloR and GcrR that is stress-responsive but not directly related to the ATR. Specifically, GcrR and the ATR genes that are subject to its control are de-repressed under conditions of manganese deprivation when formation of SloR–Mn repressor complexes is restricted. In contrast, *gcrR* transcription is repressed by

SloR–Mn in manganese-replete environments that are less stressful for the bacteria. These findings demonstrate a role for manganese as a general stress mediator of *S. mutans gcrR* expression. Lending further support to *gcrR* regulation as part of a general stress response is the induction of *gcrR* expression upon exposure of *S. mutans* to serine hydroxymate, an amino acid analogue that induces growth arrest (Robert Burne, Department of Oral Biology, University of Florida, Gainesville, personal communication). In summary, these findings support the existence of a link between SloR–Mn and GcrR in the *S. mutans* response to acid stress.

Although this report presents evidence to support direct regulation of GcrR by SloR, cross-communication between GcrR and contributors to *S. mutans* acid tolerance is likely to be more complex. Future experiments will investigate the mode of action of manganese in *gcrR* repression and reveal downstream target genes of GcrR so that the mechanisms that control *S. mutans* adaptation to acidic pH can be revealed. An improved understanding of this and other gene networks in this oral pathogen could give rise to novel therapies aimed at alleviating caries and their complications.

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