JOURNAL OF BACTERIOLOGY, May 2009, p. 2973–2984 Vol. 191, No. 9
0021-9193/09/$08.00+0 doi:10.1128/JB.01563-08

The LiaFSR System Regulates the Cell Envelope Stress Response in *Streptococcus mutans*▽†

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Received 4 November 2008/Accepted 13 February 2009

Maintaining cell envelope integrity is critical for bacterial survival, including bacteria living in a complex and dynamic environment such as the human oral cavity. *Streptococcus mutans*, a major etiological agent of dental caries, uses two-component signal transduction systems (TCSTSs) to monitor and respond to various environmental stimuli. Previous studies have shown that the LiaSR TCSTS in *S. mutans* regulates virulence traits such as acid tolerance and biofilm formation. Although not examined in streptococci, homologs of LiaSR are widely disseminated in *Firmicutes* and function as part of the cell envelope stress response network. We describe here *liaSR* and its upstream *liaF* gene in the cell envelope stress tolerance of *S. mutans* strain UA159. Transcriptional analysis established *liaSR* as part of the pentacistronic *liaFSR-ppiB-pnpB* operon. A survey of cell envelope antimicrobials revealed that mutants deficient in one or all of the *liaFSR* genes were susceptible to Lipid II cycle interfering antibiotics and to chemicals that perturbed the cell membrane integrity. These compounds induced *liaR* transcription in a concentration-dependent manner. Notably, under bacitracin stress conditions, the LiaFSR signaling system was shown to induce transcription of several genes involved in membrane protein synthesis, peptidoglycan biosynthesis, envelope chaperone/proteases, and transcriptional regulators. In the absence of an inducer such as bacitracin, LiaF repressed LiaR-regulated expression, whereas supplementing cultures with bacitracin resulted in derepression of *liaSR*. While LiaF appears to be an integral component of the LiaSR signaling cascade, taken collectively, we report a novel role for LiaFSR in sensing cell envelope stress and preserving envelope integrity in *S. mutans*.

In any microorganism, the first and major cellular structure to be impacted by threats from the environment is the cell envelope. This structure is vital for survival since it protects the cell from the environment, counteracts the inner high turgor pressure, acts as a permeability barrier, and provides a sensory platform that transmits information from the cell's surroundings ultimately to its genome (15). Hence, maintaining cell envelope integrity in the face of environmental insults by responding to cell envelope stress is critical for bacterial survival. Two-component signal transduction systems (TCSTSs) are among the primary sensory-regulatory mechanisms that mediate bacterial adaptation processes (e.g., countering envelope stress) in response to environmental perturbations (5, 21). These systems modulate the expression of genes, encoding products crucial to cell survival, via a cytoplasmic response regulator (RR), upon receipt of an external stimulus detected by a membrane-bound histidine kinase (HK) sensor (21). Signal transduction is mediated through a phosphorelay cascade from an autophosphorylated His residue located in the activated HK sensor to a conserved Asp residue in the cognate RR, altering the RR's affinity to bind to promoter regions of target genes and regulating their expression (21).

A prototypical gram-positive TCSTS that orchestrates cell envelope stress response is the *Bacillus subtilis* LiaRS system (24). This system is transcriptionally activated by exposure to alkaline shock, organic solvents, detergents, secretion stress, and notably lipid II cycle inhibitors such as the antibiotics vancomycin and bacitracin, the bacteriocin nisin, and cationic antimicrobial peptides (39, 51); hence, its cogname, lipid II-interacting antibiotics LiaRS. Lipid II contains the complete peptidoglycan (PG) subunit linked to the membrane-embedded lipid carrier C_{55}-isoprenyl phosphate (36, 60). The molecule "flips" between the cytoplasmic and extracellular faces of the cell membrane in a dynamic process (referred to as the lipid II cycle) essential for translocating PG precursors for cell wall biosynthesis (36, 60). The Lipid II cycle is considered the rate-limiting step of PG polymer biosynthesis and, consequently, the subject of intense scrutiny in the development of novel inhibitors that target or exploit this process (9).

B. subtilis LiaRS is widely disseminated in *Firmicutes* (low G+C gram-positive) bacteria, and homologs have been characterized in *Lactococcus lactis* and *Staphylococcus aureus* as part of the complex regulatory network that counteracts cell envelope stress (24, 29, 37). However, the nature of the envelope stress signal and the regulon genes controlled by this system diverges based on the organism. While *liaRS* homologs in both *L. lactis* (*cesSR*) and *S. aureus* (*vraSR*) are transcriptionally induced by lipid II cycle inhibitors, the latter *S. aureus* system is unique in responding to a wider array of cell envelope antibiotics including teicoplanin, β-lactams and D-cycloserine (29, 37, 70). Moreover, in *B. subtilis*, LiaR regulates the transcription of its own *liaIHGFSR* operon and another operon encoding a second TCSTS (24). In contrast, recent transcrip-

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† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 27 February 2009.

tome profiling of *S. aureus* and *L. lactis* exposed to lipid II cycle inhibitors identified 46 VraSR-dependent and 23 CesSR-dependent genes (29, 37), many of which are presumably involved in cell envelope biogenesis or stress-related functions. The physiological role (especially the envelope stress response function) of *B. subtilis* LiaRS homologs in streptococci is, however, poorly understood.

Streptococcus mutans is considered to be one of the major pathogens associated with human dental caries. Life in the oral cavity is typically characterized by fluctuating environmental or physiochemical factors that include changes in the availability of nutrients, pH, oxygen, the presence of bacteriocins, and antimicrobial compounds; all of which strongly influence the survival of *S. mutans* within the plaque ecosystem. Hence, among 13 TCSTSs identified in the *S. mutans* UA159 genome, four (ComDE, CiaRH, VicRK, and LiaSR) have to some extent been characterized and shown to play a prominent role in regulating environmental stress tolerance and other diverse phenotypes conducive to persistence (3, 4, 7, 32, 33). The present study describes the *S. mutans* cell envelope stress response via LiaSR TCSTS, a system previously shown to be involved in tolerating acidic pH and biofilm formation (32). This TCSTS was originally referred to as HK11/RR11 by Li et al. (32) and was recently renamed LiaSR by Chong et al. (12), owing to its close homology to the *B. subtilis* LiaRS TCSTS (24). A recent transcriptome comparison by Perry et al. (49) between a *liaR* mutant and its UA159 progenitor strain identified 174 LiaR-dependent genes in *S. mutans* biofilm versus planktonic growth, including many genes with functions in protein translation, energy metabolism, transport, and stress tolerance. These authors also reported several LiaR-dependent gene products involved in cell envelope functions and cells derived from *liaR*-deficient biofilms revealed a distinctive round morphology, in contrast to the rod-shaped cells typical of the wild-type UA159 (49), thus implying a role for LiaR in the cell envelope and/or cell shape of *S. mutans*.

Herein, we define the transcriptional organization of the LiaSR TCSTS in *S. mutans* strain UA159 as part of a pentacistronic *liaFSR-ppiB-pnpB* operon. We show that *liaSR* and the 5′ proximally encoded *liaF* assist in the tolerance of *S. mutans* to a variety of environmental threats, including stressors that specifically target the cell envelope. Under noninducing conditions, *liaF* was shown to have a negative role on *liaRS* transcription, whereas expression of *liaR* was induced by inhibitors that compromised cell membrane integrity or hindered lipid II-mediated cell wall biosynthesis. Moreover, the *liaFSR* system was shown to upregulate gene products involved in cell wall PG matrix biosynthesis and membrane protein biogenesis, thus expanding our understanding of how *S. mutans* can respond to cell envelop stress and elucidating a novel role for the LiaFSR system in responding to cell envelope stress in *S. mutans*.

MATERIALS AND METHODS

***S. mutans* strains and growth conditions.** The *S. mutans* strains used in the present study are listed in Table 1. *S. mutans* UA159 and its mutant derivates were routinely grown in solid/liquid Todd-Hewitt-yeast extract (THYE) medium and incubated as standing cultures at 37°C in air with 5% CO_2. When required erythromycin (10 μg/ml) was supplemented in the medium for the selection and growth of mutant strains. Insertional deletion mutants of the *liaFSR* genes in *S. mutans* UA159 background were constructed by PCR ligation mutagenesis and allelic replacement as previously described (30). Primers used to construct and confirm the gene deletions are listed in Table S1 in the supplemental material. DNA sequencing was also performed to further confirm correct in-frame insertion of the antibiotic cassette into the target gene. Quantitative real-time reverse transcription-PCR (qRT-PCR) experiments showed no *lia*-specific gene expression in these mutants. Relative expression analysis of mutant and wild-type cDNAs using primers specific for downstream genes ruled out negative polar effects during mutagenesis of the *lia* genes (data not shown). Growth kinetic analysis were performed using a microbiology workstation (Bioscreen C Labsystems, Helsinki, Finland) equipped with BioLink software to monitor the turbidity (i.e., the optical density at 600 nm [OD_{600}]) readings translated to growth curves (26). The following stressors affecting growth rates were tested as previously described (54): acid (pH 5.5), 5% (vol/vol) ethanol, 0.4 M NaCl, and 0.006% (vol/vol) H_2O_2.

TABLE 1. *S. mutans* strains used in this study

Strain	Characteristics[a]	Source or reference
UA159	Wild-type strain; Erm^s	J. Ferretti, University of Oklahoma
SMULiaF	UA159 Δ*liaF*::*erm*; Erm^r	This study
SMULiaS	UA159 Δ*liaS*::*erm*; Erm^r	This study
SMULiaR	UA159 Δ*liaR*::*erm*; Erm^r	This study
SMULiaSR	UA159 Δ*liaSR*::*erm*; Erm^r	This study
SMULiaFSR	UA159 Δ*liaFSR*::*erm*; Erm^r	This study

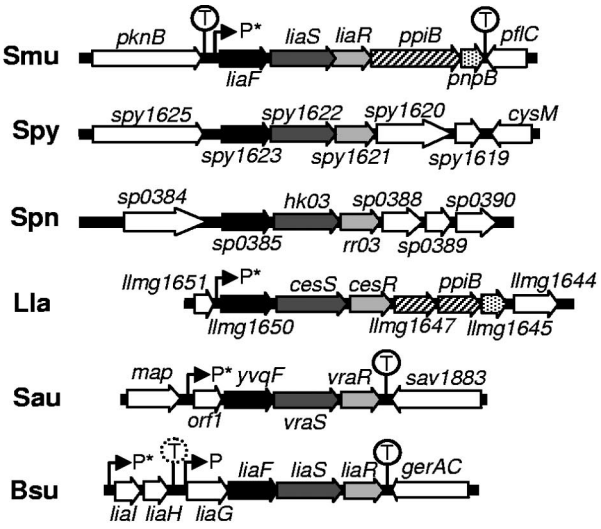
[a] Erm^s, erythromycin sensitivity.

Cell envelope antimicrobial susceptibility assays. The MIC and minimum bactericidal concentration (MBC) of cell envelope inhibitors were examined in *S. mutans* parent UA159 and mutant strains. Cell envelope inhibitors tested included the antibiotics vancomycin, D-cycloserine, bacitracin, and β-lactam antibiotics (i.e., penicillin G and oxacillin); the bacteriocin nisin; and cell membrane-interfering compounds chlorhexidine and sodium dodecyl sulfate (SDS) (all obtained from Sigma-Aldrich Canada, Ltd., Oakville, Ontario, Canada). The methodology was based on McBain et al. (40), with the following modifications: (i) 100 μl of mid-log phase bacterial cells adjusted to an OD_{600} of ~0.01 (~10^5 CFU/ml) was added to a 96-well microtiter plate containing THYE medium supplemented with twofold serial dilutions of cell envelope inhibitors; (ii) bacterial growth after 48 h was spectrophotometrically measured by using an ELISA microtiter plate reader (model 3550; Bio-Rad Laboratories, Richmond, CA) at an absorbance of 490 nm (OD_{490}) and; (iii) relative cell density percentages were calculated by using the following equation: (OD_{490} of culture in the presence of each concentration of antibiotics)/(OD_{490} of culture in the absence of antibiotics) × 100. The MIC was determined as the lowest product concentration needed to ensure that culture did not grow to over 10% of the relative cell density. MBC testing was carried out using MIC microtiter plates. Briefly, aliquots of 20 μl were taken from each well (including the MIC endpoint) and spot plated onto THYE agar and incubated for 48 h. MBCs were determined as the lowest concentration of biocide at which <5 CFU were observed after 48 h of incubation at 37°C and 5% CO_2. Two independent MIC or MBC determinations were carried out in duplicate for each trial. Susceptible strains were defined as strains with at least twofold lower MIC or MBC levels than the wild-type UA159 control.

Cell preparation for gene expression analysis. To study gene expression of *S. mutans* cells treated with cell envelope inhibitors, total RNA was isolated from cultures grown in THYE medium supplemented with various envelope antimicrobials. Briefly, overnight cultures were diluted 1:20, grown to mid-log phase ($OD_{600} \approx 0.4$), and treated with or without different concentrations of antimicrobials for 10, 30, or 60 min. RNA was extracted from treated or untreated cells and converted to cDNA as previously described by Senadheera et al. (54). To determine gene expression levels at different growth stages, total RNA was harvested from *S. mutans* cultures grown in THYE to early-log ($OD_{600} \approx 0.15$), mid-log ($OD_{600} \approx 0.4$), and stationary ($OD_{600} \geq 1.0$) phases.

qRT-PCR analysis was performed by using the Mx3000P QPCR system (Stratagene, La Jolla, CA) and Quantitect SYBR-Green PCR kit (Qiagen, Mississauga, Ontario, Canada) as previously described (54). qRT-PCR primers specific for target genes are listed in Table S2 in the supplemental material. A relative expression ratio (i.e., the fold induction) was derived from the primer efficiency (Eff) and cycle threshold (C_T) values of the target gene in the experimental condition (C_T-experimental) versus control samples (C_T-control) according to

(a)



(b)

Protein (size aa)	Putative function and conserved domain(s)[a]	Homolog/ortholog (NCBI accession no.; % protein identity/% protein similarity)
LiaF (231 aa)	Ortholog in *B. subtilis* inhibits LiaRS TCSTS (24). N-in C-in membrane protein, 2 TM[b]. Unknown COG4758.	*B. subtilis* LiaF (NP_391190.1; 21/43)[c].
LiaS (334 aa)	Intramembrane-sensing histidine kinase (37, 38). N-in C-in membrane protein, 2 TM. Cytoplasmic C-terminal HisKA-3 (dimerization/phosphoacceptor) motif and HATPase-C (ATPase) domain.	*L. lactis* CesS (YP_001032931; 38/59)[c].
LiaR (215 aa)	Transcriptional response regulator (37). N-terminal CheY-like signal receiver domain and C-terminus HTH[b]-LuxR DNA-binding motif.	*L. lactis* CesR (YP_808935.1; 56/75)[c].
PpiB (471 aa)	Cytoplasmic hydrolase-foldase enzyme. N-terminus haloacid dehalogenase (HAD)-like hydrolase domain and C-terminal cyclophilin-type peptidyl-prolyl *cis/trans* isomerase (PPIase) domain.	*S. thermophilus* PpiB (YP_808935.1; 69/81)[d].
PnpB (130 aa)	Exoribonuclease involved in mRNA processing and turnover (46) and orthologous to *S. mutans* stress-responsive PnpA protein (31). N-terminal ribosomal protein S1-like RNA-binding motif and polyribonucleotide nucleotidyltransferase (polynucleotide phosphorylase, PNPase) domain.	*S. mutans* PnpA (NP_720625; 31/52)[c].

[a] Databases to determine conserved domains/motifs: NCBI BLASTP, Pfam, COG, SMART and TMHMM.
[b] TM, transmembrane helices; HTH, Helix-Turn-Helix.
[c] Closest characterized protein match.
[d] Best protein match.

FIG. 1. Organization and properties of *liaFSR-ppiB-pnpB* locus genes. (a) Genetic map of *S. mutans liaFSR-ppiB-pnpB* operon and homologs in representative streptococci and characterized *Firmicutes* bacteria. Arrows labeled "P" represent identified or putative promoter regions ("*" indicates LiaR-like autoregulated promoters), while circles labeled "T" represent putative transcriptional terminators or a weak terminator (dashed circle) (24, 37, 39, 70). Homologs and orthologs to the *liaFSR-ppiB-pnpB* genes are shown as black, dark gray, light gray, hatched black lines, and spotted locus boxes, respectively. Genes flanking the loci of interest are represented as white boxes. For clarity the loci boxes are not drawn to scale. Abbreviations of bacterial species: Smu, *S. mutans* strain UA159; Spy, *S. pyogenes* strain M1 GAS; Spn, *S. pneumoniae* strain TIGR4; Lla, *L. lactis* subsp. *cremoris* strain MG1363; Sau, *S. aureus* subsp. *aureus* strain Mu50; and Bsu, *B. subtilis* subsp. *subtilis* strain 168. The gene names in the presented bacterial species are according to GenBank entries of the published genomes. (b) Conserved domains and (putative) functions of *liaFSR-ppiB-pnpB* operon genes.

the $2^{-\Delta\Delta C_T}$ method (35). The fold expression change was calculated according to the method of Pfaffl et al. (50) using the following formula: fold change = $[\mathrm{Eff}^{\text{target gene } (C_T\text{-control} - C_T\text{-experimental})}]/[\mathrm{Eff}^{\text{16S rRNA } (C_T\text{-control} - C_T\text{-experimental})}]$. Expression results were normalized relative to the *S. mutans* internal standard 16S rRNA gene whose expression was invariant under the experimental assay conditions tested. Relative gene expression was calculated in triplicate from at least three independent experiments ($n \geq 3$). Statistical analysis was performed by using Student *t* test. A gene was considered significantly altered in expression when $P \leq 0.05$ and expression was up- or down-regulated ≥2.0-fold.

Reverse transcription-PCR. To detect polycistronic transcripts, total RNA was isolated from mid-log-phase *S. mutans* UA159 cultures, DNase treated, and converted to cDNA (54). PCR was performed with 700 ng of cDNAs as described by the manufacturer (MBI Fermentas, Canada). Primers for the amplification of cDNAs are listed in Table S3 in the supplemental material. Controls without RT enzyme were included in all experiments. The same reverse transcription-PCR primers were used for colony PCR as a positive control to directly amplify chromosomal DNA from single colonies of *S. mutans* UA159 as described by Poyart et al. (52). Reverse transcription and colony PCR products were resolved on a 1.2% agarose gel.

5′-RACE PCR analysis. To detect the *S. mutans liaF* transcriptional start site (TSS), 5′-RACE (rapid amplification of cDNA ends) PCR was performed essentially as described by Sambrook and Russell (53) using two primer pairs (Table S3 in the supplemental material and see Fig. 3a). Total RNA (10 μg) was harvested from mid-log-phase ($OD_{600} \approx 0.4$) *S. mutans* UA159 cultures, treated with RQ1 DNase I (Promega, Madison, WI), and then used to synthesize cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and the RACE outer primer as recommended by the supplier. The double-stranded cDNA-RNA complex was treated with RNase H and RNase T_1 (Roche Molecular Biochemicals, Indianapolis, IN), purified, and eluted by using a Qiagen PCR purification kit to a final volume of 30 μl. cDNA (12.5 μl) was used for poly(dG) and poly(dT) 3′-end tailing with terminal deoxyribonucleotidyltransferase, as recommended by the manufacturer (Amersham Pharmacia Biotech). Tailed DNA (1 μl) was used for PCR amplification with poly(dC) or poly(dA) tail primer combined with the RACE PCR inner primer to generate a PCR product. This PCR amplicon was purified and sent for nucleotide sequence analysis (AGTC Corp., Toronto, Ontario, Canada).

RESULTS AND DISCUSSION

***liaFSR* forms part of a pentacistronic operon in *S. mutans*.** The *S. mutans* SMu485 (*liaF*)-SMu486 (*liaS*)-SMu487 (*liaR*)-SMu488-SMu489 locus spans 4,282 bp in size (position 454530 to 458812 bp; National Center for Biotechnology Information [NCBI]). LiaFSR orientation, conserved topological organization, domains, and putative functions are shown in Fig. 1. Although not explored in the present study, the two remaining genes downstream of *liaR* (i.e., SMu488 and SMu489), have been renamed *ppiB* and *pnpB*, respectively, reflecting their presumed enzymatic peptidyl-prolyl *cis*/*trans* isomerase and polynucleotide phosphorylase properties.

To determine whether the *liaFSR-ppiB-pnpB* genes constitute a pentacistronic operon, we used reverse transcriptase PCR and further analyzed the nucleotide sequence to identify potential transcriptional terminators. Sequence analysis suggested a distinct operon encoding a eukaryotic-type serine/threonine protein kinase (PknB), its phosphatase counterpart (PppL), and a transcriptional terminator located ~318 bp 5′ of *liaF* (22). Reverse transcriptase PCR analysis utilizing primers specific for the *pknB* and *liaF* coding regions indicated that *liaF* was not transcriptionally linked with the upstream *pppL-pknB* operon (data not shown). Eleven base pairs downstream of the *pnpB* open reading frame (ORF), a 59-bp inverted repeat ($\Delta G = -29.4$ kcal/mol) was followed closely by a stretch of 12 A-T-rich nucleotides, indicating good potential for this area to form a rho-independent transcriptional terminator (14) and suggesting that *liaFSR-ppiB-pnpB* is a transcriptionally discrete

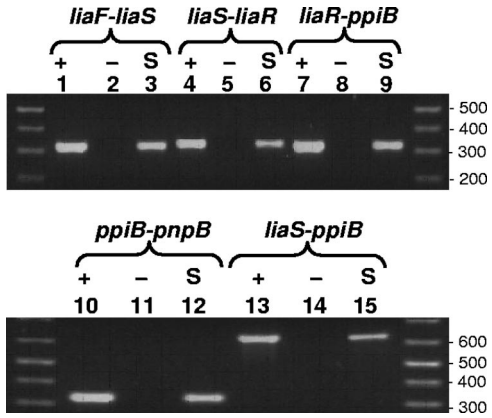
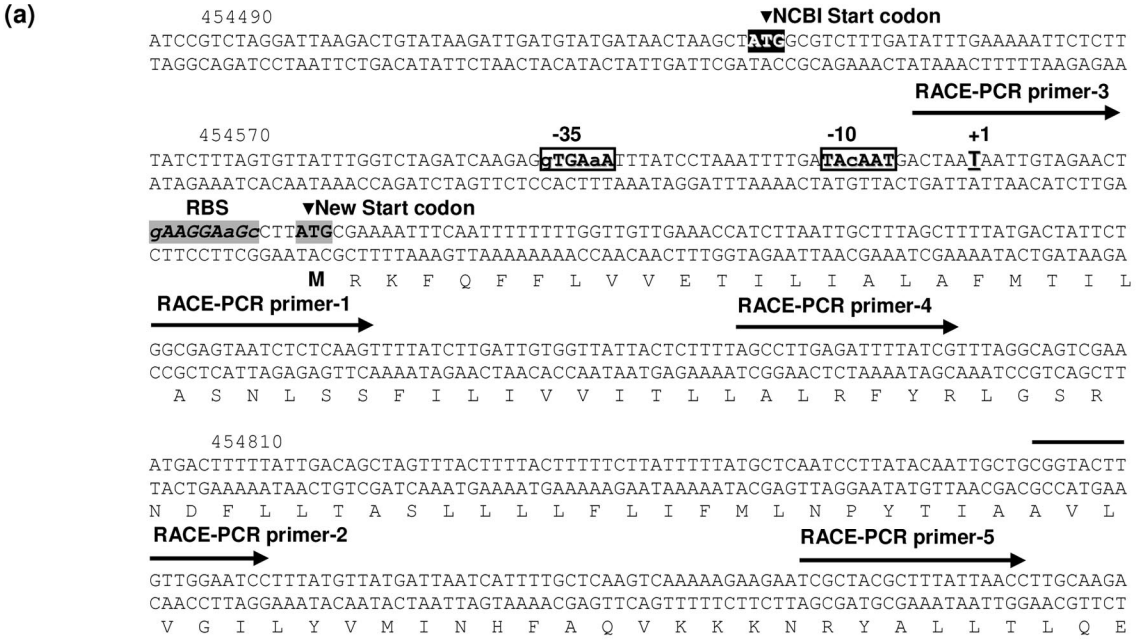


FIG. 2. Reverse transcriptase and colony PCR products to detect transcripts from the *liaF-pnpB* operon. Lanes 1, 4, 7, 10, and 13 represent colony PCR products of UA159 wild type (positive controls ["+"]). Lanes 2, 5, 8, 11, and 14 represent reverse transcriptase PCR products using no reverse transcription (negative control ["–"]) samples of UA159 cDNA. Lanes 3, 6, 9, 12, and 15 represent reverse transcriptase PCR products using UA159 cDNA. Regions of the *liaF-pnpB* operon detected by the reverse transcriptase PCR primers are shown.

locus. Reverse transcription-PCR was again utilized to confirm the presence of a five-gene operon. Analysis of the *lia* transcripts using this method indicated that a pentacistronic transcript encompassing the *liaFSR-ppiB-pnpB* genes was generated (Fig. 2). Moreover, Northern blot experiments using *liaF* or *liaS* gene specific probes further established the presence of an ~4.3-kb pentacistronic mRNA (data not shown).

To precisely map the promoter region upstream of *liaF*, the TSS was determined by using 5′ RACE PCR. The +1 TSS was identified 95 bp downstream of the NCBI annotated ATG translational start site (Fig. 3a). No RACE PCR product implicating additional TSSs upstream of the currently identified +1 start point was obtained. A *B. subtilis* σ^A-type putative −10 promoter box (TAcAAT) was located 7 bp upstream from the +1 TSS, and a weakly conserved −35 promoter motif (gTGAaA) separated by 17 bp was identified (Fig. 3a). Since our results were inconsistent with the NCBI annotated *liaF* translational start site, we determined the optimal ATG start codon of *liaF* ORF to be located 24 bp 3′ from the TSS (position 454653 bp) with a putative ribosomal binding site (RBS) motif 5 bp 5′ from the initiation codon (Fig. 3a). The identified TSS reduced the predicted size of the *liaF* ORF by 123 bp. Moreover, the new *liaF* start codon aligns with the annotated start codons of some its closest homologs/orthologues in *Streptococcus* species and *B. subtilis liaF*.

Recently, Martínez et al. (37) identified a *L. lactis* CesR binding motif and showed that streptococcal, lactococcal, and staphylococcal LiaR/CesR/VraR proteins phylogenetically cluster together. The CesR motif appears to be conserved in the genomes of *S. aureus* and *S. pneumoniae* and is notably present upstream of the *liaSR* homologs in these species (37). A CesR-like binding motif was also detected upstream of the *S. mutans liaF* and other LiaR-regulated genes, although the *pseudo*-CesR box is located at positions −82 to −62 compared to −46/−72 of *L. lactis* CesR-dependent genes (Fig. 3b) (37).



(b)

Gene	Sequence	Position	Similarity
CesR-binding motif	*TCAGHCTnnAGDCTGA*	-72/-46	
llmg1650	AAAAATTaAGTCTTAAGTCcGATAAAAATTACTATATTTTTTGATACAAT	-46	12/14 (86%)
spyM3_1368	AATAGTtAGTCTCAtGTtTtACTAGCATGCCACTAACTATTCTTTTTaACt	-75	10/14 (71%)
sp0385	AAGGATCAGAtgAAAAAGGCgtAACCAGCGCCTTTTTTCCATcctC	-71	10/14 (71%)
liaF	TTATCTTtAGTgTTATTTgGTCTagATCAAGAGgTGAaA	-62	9/14 (64%)
spxA	ACAATTgAGCtTGAAGAtTaAATAAAAATAGGTCCCAAGACCGACTCTTTATTTaAtA	-82	10/14 (71%)
smu.1727	CTAGCTCAGTCaGATAAtGCTGAAAAACTAGCTAAGTTTATTCAAGAAATTcgCA	-80	12/14 (86%)
*smu.751**	TTGAGTtAtAaTAAAGAtTtACAAAGTATAATTAATCAGATGcTtACA	-72	9/14 (64%)

FIG. 3. TSS and sequence analysis of *liaF* gene. (a) DNA sequence surrounding the NCBI predicted LiaF translational start codon (highlighted in black). The identified TSS is marked (+1 and underlined) and the putative −10 and −35 promoter motifs are boxed. Predicted RBS and translational initiation codon are in bold and highlighted in gray. It should be noted that there is no typical RBS motif located upstream of the NCBI annotated translational start codon. Lowercase nucleotides are mismatches to established RBS and *B. subtilis* σ^A-type promoter boxes consensus sequences (11, 42). 5′ RACE-PCR primers used for mapping the TSS are represented by arrows. (b) Identification of the *L. lactis* CesR-like IR binding motifs (boxed) (37) in the putative promoter regions of *S. mutans* strain UA159 *liaF*, *spxA*, SMu1727, and SMu751 genes together with *liaF* homologs in *L. lactis* subsp. *cremoris* strain MG1363 (llmg1650), *S. pyogenes* strain MGAS315 (spyM3_1368), and *S. pneumoniae* strain TIGR4 (sp0385). The position of CesR-like binding motif relative to (putative) promoter regions and the nucleotide similarity to CesR consensus are shown. Conserved residues are in boldface; mismatches to consensus are in lowercase. Also shown is the putative −10 promoter (double underlined) or −35 promoter motifs (single underlined). H = A+T+C; D = A+T+G. Bioinformatic sequence analysis was carried out using the MacVector 7.2 software (Oxford Molecular). *, Although no CesR-like binding motif was detected immediately preceding SMu753 gene, a CesR sequence was detected upstream of the (presumably cotranscribed) SMu751 gene.

The existence of this potential *cis* element suggests positive autoregulation, as with other LiaSR homologs (24, 37, 70).

Growth kinetics under stress. To examine the importance of the *lia* gene products in *S. mutans*' stress tolerance, we constructed isogenic in-frame deletions of the *liaF*, *liaS*, and *liaR* genes. The mutants were designated SMULiaF, SMULiaS, and SMULiaR, respectively (Table 1), and used for growth kinetic measurements to expand upon previous studies that focused exclusively on *liaSR* (7, 32, 65). While overnight cultures of UA159, SMULiaS, and SMULiaR strains grew as uniformly buoyant and turbid solutions, SMULiaF cells consistently aggregated and settled at the bottom of the test tube (data not shown). Although the *liaS* and *liaR* mutants displayed similar growth rates relative to wild type in THYE at pH 7.5, the growth rate and yield of SMULiaF was markedly impaired in this medium, as well as under a number of other

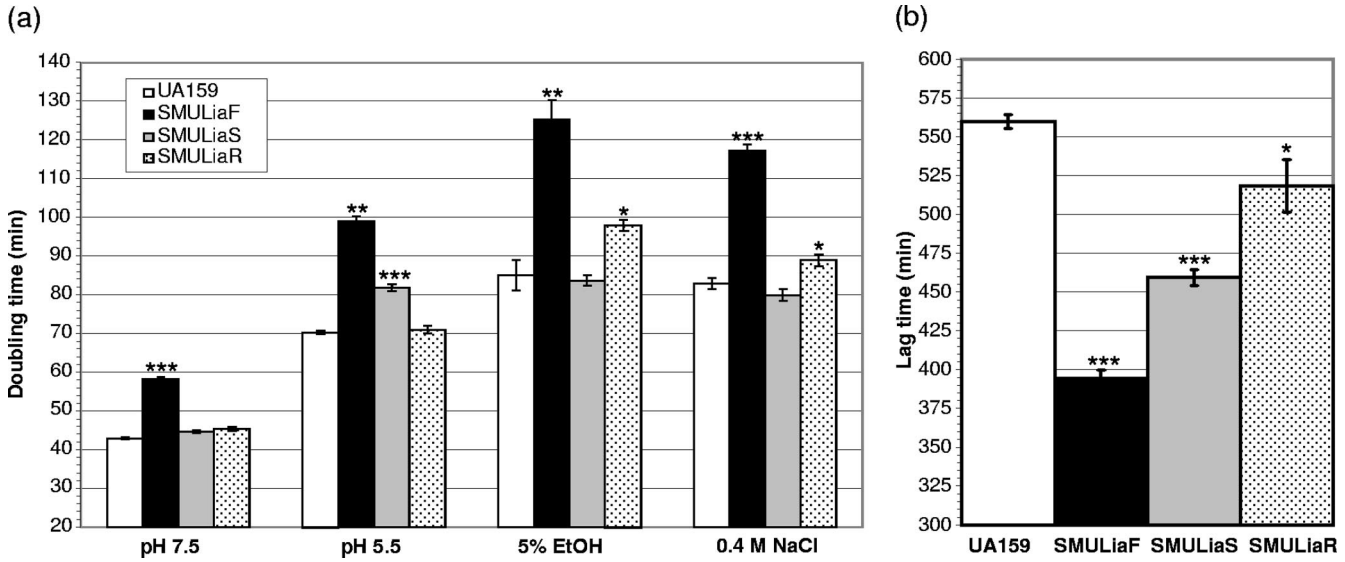


FIG. 4. Growth rates and lag times of *S. mutans* strains exposed to stress conditions. (a) Growth rates of *S. mutans* strains exposed to specific stressors are presented as doubling times (min). Stressors: THYE medium supplemented with neutral (pH 7.5), acid (pH 5.5), 5% (vol/vol) ethanol (EtOH), and 0.4 M NaCl. (b) Lag times for *S. mutans* strains to reach an OD_{600} of ~0.1 when exposed to 0.006% (vol/vol) hydrogen peroxide (H_2O_2)-mediated oxidative stress. The results were obtained from three independent experiments conducted with replicates of five for each strain. Significant differences in growth rates or lag times compared to UA159 parent are indicated as calculated by using the Student *t* test (***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$). Error bars represent the standard errors.

stressors, including acidity at pH 5.5, 5% ethanol and 0.4 M sodium chloride (Fig. 4a, growth yield data not shown). Interestingly, under H_2O_2-induced oxidative stress, the growth of all *lia* mutants was enhanced relative to the wild-type UA159 strain, as judged by the decreased lag time (Fig. 4b).

Since SMULiaR did not share the same level of sensitivity to acid, ethanol, and high osmolarity stressors as that seen with SMULiaS, the possibility of in vivo cross talk between LiaS and other noncognate RR(s) or between LiaR and other phosphodonors is likely (63). Li et al. (32) and Chong et al. (12) have previously suggested the possibility of cross talk between the LiaS sensor with one or more RRs by comparing phenotypic variations (e.g., acidic pH resistance and bacteriocin mutacin IV production) between the *liaS* and *liaR* mutants.

***liaFSR* mutants are sensitive to cell wall lipid II inhibitors and cell membrane-disrupting agents.** *S. mutans* LiaSR homologs in *L. lactis* (CesSR), *S. aureus* (VraSR), and *B. subtilis* (LiaRS), respond to stress elicited by cell envelope interfering antibiotics, primarily lipid II-interacting inhibitors such as vancomycin and bacitracin (18, 37, 39). To determine whether the *S. mutans* LiaSR system is part of the cell envelope stress response, the tolerance of wild-type UA159 and *liaFSR* mutants to cell envelope biosynthesis inhibitors was assessed by a microtiter plate-based MIC and MBC assays. Table 2 displays antibiotic susceptibility profiles of UA159 parent and *liaF*, *liaS*, and *liaR* mutants tested with a panel of eight cell envelope inhibitors, each active at different steps in PG biosynthesis. Similar to antibiotic resistance studies of *L. lactis cesR* and *S. aureus vraSR* mutants (18, 29, 37), *S. mutans liaFSR*-deficient mutants were approximately two- to eightfold more sensitive than the parent strain UA159 to vancomycin and bacitracin, antibiotics that interfere with Lipid II recycling. These mutants also had at least a two- to fourfold reduction in MICs and MBCs relative to UA159 when exposed to compounds that compromise the integrity of the cell membrane (i.e., the pore-forming chlorhexidine and the detergent SDS). On the other hand, the *lia* mutants were not sensitive to nisin, a lantibiotic that both disrupts bacterial membranes through pore forma-

TABLE 2. Susceptibility profiles of *S. mutans* strains exposed to cell envelope-interfering antimicrobials[a]

Strain	Lipid II inhibitor						Non-lipid II inhibitor						Cell membrane-disrupting agents			
	VAN		BAC		NIS		DCS		PENG		OXA		% SDS		CHX (μg/ml)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
UA159	1.25	10	500	2,000	0.5	0.5	200	>3,200	0.0625	0.0625	0.125	0.125	0.01953	0.01953	1	8
SMULiaF	1.25	1.25**	250*	1,000*	0.5	0.5	200	3,200	0.0625	0.0625	0.125	0.125	0.00977*	0.00977*	0.5*	4*
SMULiaS	1.25	5*	500	2,000	0.5	0.5	200	3,200	0.0625	0.0625	0.125	0.125	0.00977*	0.00977*	1	8
SMULiaR	1.25	2.5**	250*	1,000*	0.5	0.5	200	3,200	0.0625	0.0625	0.125	0.125	0.01953	0.01953	0.5*	2**
SMULiaSR	1.25	2.5**	250*	1,000*	0.5	0.5	200	3,200	0.0625	0.0625	0.125	0.125	0.00977*	0.00977*	0.5*	2**

[a] VAN, vancomycin; DCS, D-cycloserine; BAC, bacitracin; NIS, nisin; CHX, chlorhexidine; PENG, penicillin G; OXA, oxacillin. *, Reductions (2-fold [*] or ≥4-fold [**]) in MIC/MBC compared to UA159 parent are indicated. All concentrations are in micrograms per milliliter.

tion and blocks the lipid II moiety of PG biosynthesis (67). It has been shown that exposure of an *L. lactis cesR* mutant to nisin resulted in a twofold decrease in the 50% lethal dose relative to its parent strain (37). In *L. lactis*, the organism that synthesizes nisin, the CesSR system may well be part of the self-protection resistance mechanism to this lantibiotic.

MIC/MBC assays conducted with non-lipid II interacting β-lactams (penicillin G and oxacillin) that prevent the later transpeptidation step of PG biosynthesis, as well as D-cycloserine, which competitively inhibits the completion of the PG subunit pentapeptide side chain, revealed no effect on the sensitivities of *lia* mutants relative to parent (Table 2) (10). Unlike *S. mutans* LiaSR, resistance to these antibiotics in *S. aureus* partly involves the homologous VraSR system (8, 18, 29). Indeed, oxacillin showed drastic (at most 64-fold) reduction in the MICs of a *vraSR* mutant compared to the parent methicillin-resistant *S. aureus* strain (8).

Similar to results from growth kinetics under environmental stress, the antibiotic-mediated stress response of isogenic *liaF* and *liaR* mutants exhibited enhanced sensitivity to vancomycin, bacitracin, and chlorhexidine that was noticeably different from that of the *liaS* HK mutant. One explanation for the greater *liaR* deficiency relative to the *liaS* mutant strain may be that the LiaR signaling cascade is activated independently of phosphorylation by its cognate LiaS sensor (64). In fact, the LiaR homolog in *S. aureus* (VraR) is capable of undergoing in vitro phosphorylation by acetyl phosphate, although the rate is much slower than direct phosphorylation by it cognate sensor VraS (6). Another route of LiaR phosphorylation could result from cross talk with other sensor HK(s) such as CiaH or VicK, both of which are implicated in cell envelope stress tolerance (7).

Biswas et al. (7) recently demonstrated tolerance of *S. mutans liaS* mutants to vancomycin, bacitracin, and other β-lactam cell envelope antibiotics. Although our findings pertaining to bacitracin and β-lactam susceptibility of the *liaS* mutant agreed with those of Biswas et al. (7), *lia* mutants exposed to vancomycin in our study displayed at least a twofold decrease in MBC sensitivity relative to the wild type. This inconsistency may be due to the disk diffusion assay with measurement of zones of inhibition used by the former group (7) compared to our microdilution method to establish MIC/MBC and the growth stage of tested strains. In addition, it should be noted that disparities between disk diffusion methodology and the determination of MIC have been reported in the clinical literature for several antibiotics (28, 41).

LiaR responds to lipid II-interfering and cell membrane-perturbing antimicrobials. Since *liaFSR* mutant strains exhibited increased susceptibility to lipid II-interfering antibiotics, the role of these inhibitors in inducing transcription of the *lia* operon was investigated by qRT-PCR. Accordingly, *liaR* expression was examined using mid-log-phase UA159 cultures treated with or without inhibitory and subinhibitory concentrations of several cell envelope antimicrobials for 10 min. Of the antimicrobial agents tested, vancomycin, bacitracin, nisin, and chlorhexidine acted as *liaR* inducers in a concentration-dependent manner (Fig. 5). All of these compounds interfere with the lipid II-cycle except chlorhexidine (a cell membrane pore-forming antiseptic) whose molecular effect on the lipid II cycle is currently unknown. While the expression levels of *liaR* remained elevated at high concentrations of vancomycin and nisin, its transcription was decreased down to slightly induced levels (~1.7-fold) or repressed levels (~0.5-fold) under the highest concentrations of bacitracin and chlorhexidine (Fig. 5). Since exposure of UA159 cells to the latter compounds present at 10× the MIC, revealed only 2 and 12% decreases in cell counts, respectively (data not shown), it is likely that the reduction in *liaR* transcripts was due to an active mechanism rather than biocide-mediated cell death.

Exposure to subinhibitory and inhibitory concentrations of bacitracin for up to 60 min suggested that peak induction of *liaR* expression occurred between 10 and 30 min after bacitracin treatment (Fig. 5). This result was consistent with the temporal response of *liaSR* homologs in *L. lactis* and *S. aureus* exposed to related lipid II cycle inhibitors (29, 37). Other cell envelope antimicrobials, such as the β-lactams (penicillin G and oxacillin) or D-cycloserine, were unable to significantly stimulate *liaR* expression in the concentration ranges of 0.5× to 10× the MIC, respectively (data not shown). Similar lipid II inhibitor concentration-dependent induction profiles were illustrated in the homologous *B. subtilis* LiaRS and *L. lactis* CesSR systems (37, 39). Relative to other inducers, bacitracin was utilized in further qRT-PCR experiments due to its high induction potential at subinhibitory concentrations. In addition, UA159 cells treated with different concentrations of bacitracin displayed nearly equivalent expression levels for the *liaF* and *liaS* genes as *liaR*, supporting coinduction and cotranscription of all *lia* genes (data not shown).

***liaFSR* transcription is growth phase-dependent.** In *B. subtilis*, the LiaR-dependent *liaIHGFSR* promoter (P_{liaI}) was shown to be induced at the onset of the stationary phase in the absence of an external stress stimulus and appeared to be tightly controlled by at least five regulators involved in the transition into stationary phase (25), a growth state typified by a nutrient-depleted and hostile environment (43). To investigate whether *S. mutans* LiaSR is also induced by the transition into stationary-phase *liaF*, *liaS*, and *liaR* expression levels at the mid-log and stationary growth phases were compared to that of early-log-growth-state cells. Notably, *lia* gene expression levels were repressed at least 10-fold in stationary phase (*liaF* = 0.1 ± 0.02, *liaS* = 0.1 ± 0.03, and *liaR* = 0.1 ± 0.02) and 2-fold in mid-log phase (*liaF* = 0.5 ± 0.02, *liaS* = 0.5 ± 0.01, and *liaR* = 0.4 ± 0.001) relative to early-log growth state (mean fold increase ± the standard error; $n = 3$). While these data suggested growth phase-dependent regulation of the *lia* genes, they also emphasized the involvement of LiaSR in early-log-phase growth marked by high growth rates, as well as increased cell division, cell separation, and PG biosynthesis.

LiaFSR positively regulates cell envelope biogenesis, chaperone/proteases, and transcription factors. *B. subtilis* LiaRS TCSTS is genetically and functionally linked to the upstream *liaF* gene product to constitute a three-component signaling system (24). We envisioned a similar role with *S. mutans* LiaFSR, since this gene cluster is conserved, coexpressed, and transcriptionally inducible by envelope-specific antimicrobials, resembling the *B. subtilis* LiaFRS model. To elucidate the underlying regulon components involved in *S. mutans* LiaFSR-mediated cell envelope stress tolerance, cDNAs derived from UA159 and SMULiaFSR cells were used to study the expression levels of several genes encoding the following products:

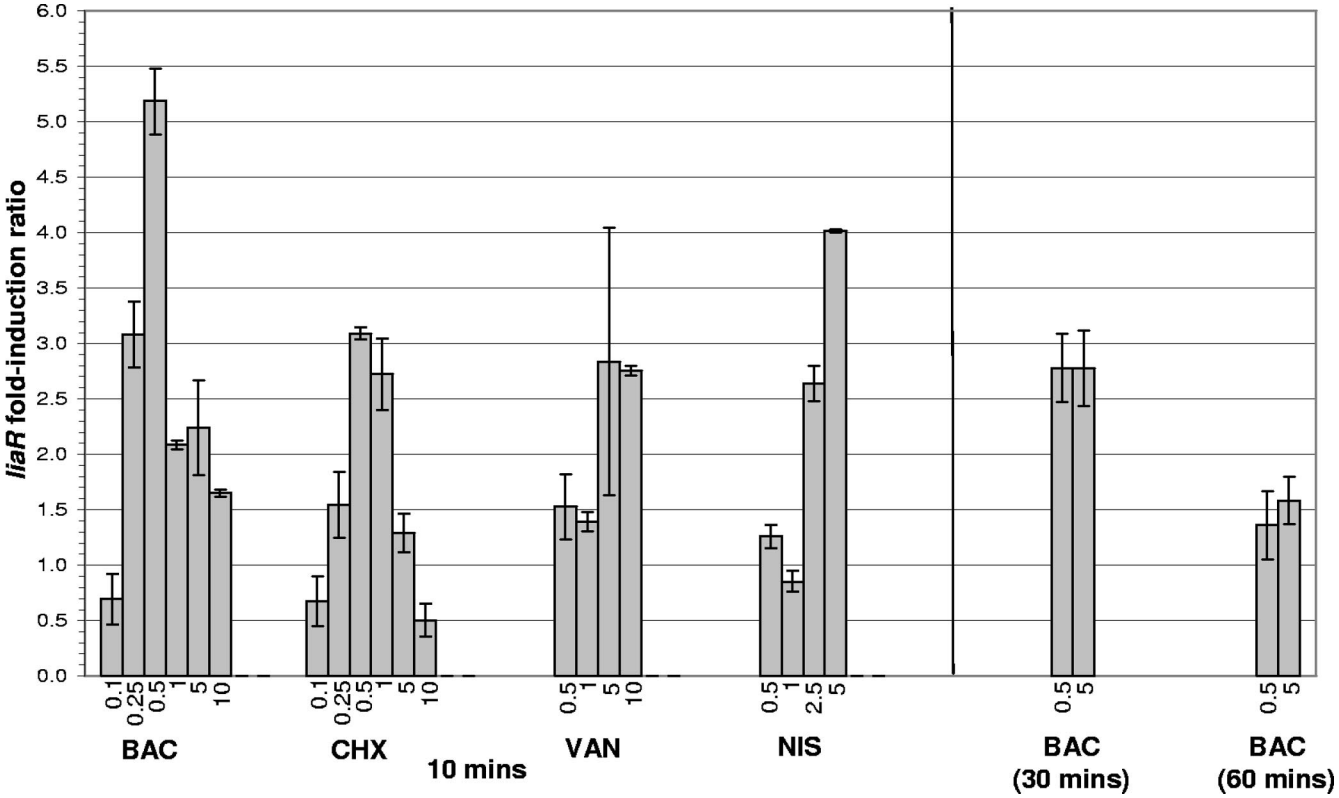


FIG. 5. Fold expression of *liaR* gene expression in *S. mutans* UA159 wild-type strain exposed to cell envelope biosynthesis inhibitors. Concentration-dependent induction of *liaR* expression exposed to cell envelope inhibitors for 10, 30, and 60 min. The inhibitor and concentrations utilized (in × the MIC) are shown on the *x* axis. VAN, vancomycin; BAC, bacitracin; NIS, nisin; and CHX, chlorhexidine. The results were from at least three independent experiments, and error bars represent the standard errors.

membrane-targeted or secreted proteins; PG biosynthesis, remodeling, and modification enzymes; cell envelope chaperone/proteases; and transcriptional attenuators. The functions of these selected genes and their fold expression in UA159 and SMULiaFSR cells are listed in Table 3. The target genes were based either on previously identified LiaR-regulated genes (49) or on homologs and/or orthologs to *S. aureus* VraR or *L. lactis* CesR-upregulated genes stimulated by cell envelope inhibitors (29, 37). Moreover, putative *L. lactis* CesR-like binding motifs were detected 5′ proximal to most of the predicted TSSs of these genes (Fig. 3b and data not shown for other genes), except for *htrA* and *ftsH*, where the promoter boxes or pseudo-CesR binding sequence were difficult to define.

Treatment of UA159 cultures with 0.5× the MIC bacitracin concentration increased all target gene expression by 2- to 10-fold relative to control cultures, whereas expression remained uninduced in the *liaFSR*-deficient mutants (Table 3). The result above confirmed LiaFSR-dependent upregulation of the tested genes, suggesting a multifaceted response by this system to bacitracin inhibition of PG synthesis. The induction of *murB*, *dagK*, and SMu707c genes can be interpreted as an attempt by *S. mutans* cells to boost the rate of PG synthesis and murein remodeling to restore stress-induced damaged or missing cell wall material. In *S. aureus*, Utaida et al. (59) reported that bacitracin-challenged cells induced *pbpB*, *sgtB*, *murA*, and *bacA* gene expression to increase the rate of PG synthesis. Our observed induction of *rgpG* may also suggest increased levels of cell wall polysaccharide modification and resistance to bacitracin. Tsuda et al. (57) had previously shown that the presence of rhamnose-glucose polysaccharide in the cell wall confers resistance to bacitracin by *S. mutans* cells, although the precise mechanism is unknown.

An elevated expression level of the envelope chaperone/proteases *ftsH* and *htrA* implies that damaged, misfolded, or aggregated proteins accumulate in the cell envelope, triggering the activation of genes encoding these compensatory proteins. In fact, several genes belonging to the cell envelope protein biogenesis/repair, chaperonin, or protease functional categories (e.g., *htrA*, *ftsH*, *prsA*, *mrsA1*, and *hslO*) were noted to be upregulated in *S. aureus*, *S. pneumoniae*, and *L. lactis* exposed to cell-wall-active antibiotics (19, 37, 47, 59). Pechous et al. (47) have proposed that, in *S. aureus*, the inhibition of PG synthesis by cell wall antibiotics interferes with the incorporation of integral proteins covalently attached to PG, which are linked to PG at the lipid II stage (48). These authors speculated that the resulting accumulation of unincorporated cell wall proteins in the cell membrane and the subsequent disruption of normal envelope functions and protein translocation may lead to the activation of repair, chaperonin, and protease genes (47). A similar process could be operating in *S. mutans* cells exposed to bacitracin stress with the induction of *htrA* and *ftsH* chaperone/proteases.

Likewise, the upregulation of trigger factor (*ropA*), *ftsY*, and SMu1727 (*oxaA*-like) genes involved in the biogenesis, trans-

TABLE 3. qrt-PCR expression ratios and function of selected envelope-related genes in *S. mutans* strains

Category	Gene	Mean qRT-PCR fold expression ± SE[a]		(Putative) function	Database or reference
		UA159	SMULiaFSR		
Synthesis of membrane/secreted proteins	Trigger factor (*ropA*)	1.7 ± 0.1	0.8 ± 0.01	Ribosome-associated peptidyl-prolyl *cis/trans* isomerase (PPIase) foldase with chaperone and acid stress resistance functions	66
	ftsY	2.1 ± 0.2	1.0 ± 0.1	Membrane-associated receptor component of the signal recognition particle system responsible for targeting nascent peptides to the cell membrane	20, 44
	SMu1727	3.2 ± 0.4	0.7 ± 0.1	OxaA-like precursor protein required for the insertion of integral membrane proteins into cell membranes	pFam02096 (37)
Cell wall PG biosynthesis, remodeling, and modification enzymes	*murB*	2.4 ± 0.2	0.8 ± 0.03	Essential UDP-*N*-acetylenolpyruvoylglucosamine reductase enzyme involved in the second step of the "sugar building block" necessary for PG synthesis	56
	dagK (*dgk*)	3.3 ± 0.3	0.9 ± 0.1	Diacylglycerol kinase catalyzes the formation of phophatidic acid by phosphorylating diacylglycerol; also involved in stress resistance and presumed to have C_{55}-isoprenyl kinase activity involved in PG synthesis	34, 69
	SMu707c	2.3 ± 0.2	1.3 ± 0.1	Endolysin containing a β-1,4-*N*-acetylmuramidase domain, possibly involved in diverse cell wall functions, including PG restructuring and turnover, cell separation, and autolysis	62
	rgpG	2.0 ± 0.1	0.9 ± 0.03	Involved in the first step of *S. mutans* cell wall rhamnose-glucose polysaccharide synthesis by transferring *N*-acetylglucosamine-1-phosphate to a lipid carrier	58, 68
Cell envelope chaperone/proteases	*ftsH*	2.5 ± 0.04	0.9 ± 0.1	Universally conserved membrane bound metalloprotease and chaperone; in *E. coli*, FtsH maintains inner membrane stability by processing/degrading specific membrane proteins and transcription factors	23
	htrA (*degP*)	2.9 ± 0.6	0.8 ± 0.003	Highly conserved cell wall-associated serine protease and chaperone; *S. mutans* HtrA is involved in stress resistance, maturation of extracellular and surface attached proteins, biofilm formation, and genetic competence	2, 16, 27
Transcriptional regulators	SMu753	10.6 ± 1.4	0.5 ± 0.1	Hypothetical membrane protein that contains *E. coli* phage shock protein C (PspC) domain; *E. coli* PspC is a transcriptional regulator via protein-protein interactions with other phage shock proteins	pFam04024 (13)
	spxA (SMu2084c)	6.8 ± 0.9	0.7 ± 0.1	Conserved regulator in gram-positive bacteria that interacts with RNA polymerase haloenzyme; in *B. subtilis*, Spx is a regulator of the disulfide stress response to alleviate damage caused by thiol oxidation	45

[a] qRT-PCR expression ratios of selected genes in mid-log-phase *S. mutans* UA159 parent and SMULiaFSR strains, treated for 10 min with 0.5× the MIC of bacitracin versus untreated control cells. Results were obtained from three independent experiments.

location, and correct insertion of integral membrane or secreted proteins into the cell membrane may be viewed as an attempt by *S. mutans* cells to maintain the integrity of membrane-associated or secreted polypeptides. This could be accomplished by replacing damaged or denatured peptides and/or increasing the synthesis and deployment of envelope stress-related repair enzymes, chaperones, and proteases. In *S. mutans*, both FtsY and trigger factor have been demonstrated to play important roles in stress response such as acid and high salt-osmolarity tolerance (20, 66). Martínez et al. (37) also established a link between envelope integrity and the *L. lactis* CesR-mediated induction of membrane protein biogenesis or protein secretion genes (e.g., *ppiB* and *oxaA2*) in their study of lactococcal cells exposed to a lipid II inhibitor.

Lastly, the transcriptional modulators SMu753 and SpxA (SMu2084c) are highly induced by the LiaFSR system, and homologs/orthologs of these genes play a role in envelope stress tolerance in other bacteria (Table 3). SMu753 encodes a

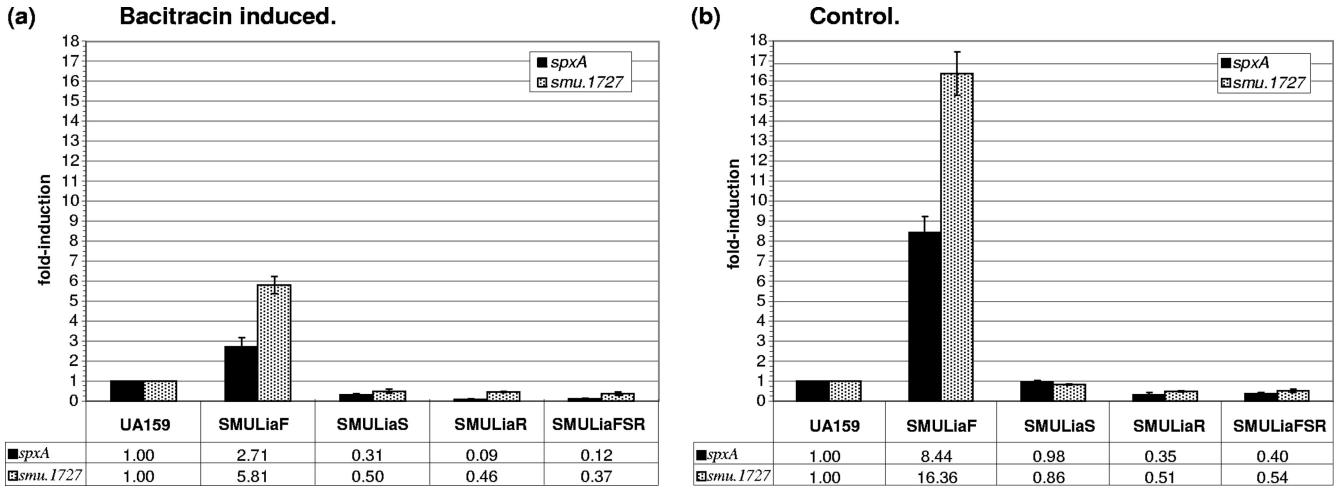


FIG. 6. Fold expression of *spxA* and SMu1727 genes in *S. mutans lia* mutant strains normalized to wild-type UA159 response under 0.5× the MIC bacitracin inducing (a) and noninducing (b) control conditions. In panels a and b, the fold expression ratios of *spxA* and SMu1727 genes in UA159 are set at 1.0 under both inducing and control conditions. The results were obtained from three independent experiments, and error bars represent standard error.

membrane protein with a conserved phage shock protein C (PspC) domain, a major component of the *E. coli* PspABC DEF envelope stress response system (13). The Psp system is induced in response to ethanol, heat, osmotic shock, and bacteriophage infection, with PspC playing a role in modulating expression of its own phage shock operon and other stress resistance genes (1, 13). Whether the SMu753 gene product has a similar function in *S. mutans* remains to be determined. The transcriptional factor SpxA is orthologous to the *L. lactis* SpxB and *B. subtilis* Spx regulators, both of which are inducible by cell-wall-active inhibitors and involved in envelope stress tolerance (17, 61). Although the *S. mutans spxA* gene is clearly LiaFSR dependent, its role in the resistance to envelope stress warrants further investigation.

LiaF is integral to and represses LiaSR-mediated signal transduction. The *B. subtilis* transmembrane protein LiaF, part of the LiaFRS three-component signaling system, acts as a potent inhibitor of LiaR-dependent gene expression in the absence of inducing conditions (24). To determine whether the *S. mutans* LiaF played a similar role on LiaSR signal transduction, we studied the role of each *lia* component in regulating LiaSR-dependent *spxA* and SMu1727 transcription under bacitracin-induced and control conditions. While the absence of either *liaR* or *liaS* resulted in a 2- to 10-fold decrease in *spxA* and SMu1727 expression under inducing conditions, deficiency in *liaF* drastically increased their transcription with or without an inducer (Fig. 6). Specifically in strain SMULiaF, *spxA* and SMu1727 expression was upregulated 2.5- and 5.5-fold, respectively, under inducing conditions, as well as 8- and 16-fold under noninducing conditions (Fig. 6). These results suggest a functional role for LiaF in repressing LiaSR-dependent induction of *spxA* and SMu1727 in the absence of an inducer. Since a lower inhibitory effect is apparent under inducing conditions, it is likely that the presence of bacitracin alleviated LiaF repression of *spxA* and SMu1727 by enhancing the transcription of *liaR*.

Under uninduced conditions, the lack of *liaF* caused 13- and 15-fold increases in *liaR* and *liaS* expression relative to wild-type levels, respectively (data not shown). Since antibiotic resistance cassettes (e.g., erythromycin resistance [Erm^r]) inserted into the chromosome of insertion-deletion mutant strains can exhibit downstream polar effects, we measured the downstream gene expression in strains SMULiaS and SMULiaR versus the wild type. In SMULiaR, *ppiB* transcription was increased by nearly 3.4-fold, whereas strain SMULiaS showed approximate 3.7- and 3.6-fold increases in *liaR* and *ppiB* expression compared to UA159 parent (data not shown). Notably this ~4-fold increase in expression of downstream genes (due to insertion of the Erm^r cassette) is still drastically lower than the 13- to 15-fold increase of *liaS* and *liaR* expression in the *liaF* mutant, suggesting a high likelihood for *liaF*-mediated repression of *liaSR*. Although the above results may not directly demonstrate LiaF's negative effect on *liaSR* transcription, more studies are warranted to examine its precise interaction with LiaS and/or LiaR components to confer repression of *liaSR* transcription. Interestingly, both an overexpression of *liaSR* (as possibly witnessed in a *liaF*-deletion strain) and diminished *liaR* expression (as observed in a *liaR* mutant) exhibited growth defects in the presence of a number of environmental stressors, including envelope-specific antimicrobials. Hence, we speculate that *S. mutans liaFSR* transcription is tightly regulated and expresses its locus and regulon genes at the optimal time and transcript level to secure envelope integrity.

Conclusion. Sensing and responding to physical and chemical threats that may compromise the functional integrity of the bacterial cell envelope are vital to the survival of a bacterium. The present study describes a role for the LiaFSR system in responding to cell envelope stress in *S. mutans*. Although this system is involved in tolerance to a variety of stressors including pH, NaCl, SDS, H_2O_2, etc., the LiaFSR appears to be a cell envelope damage-sensing system rather than a direct stress-sensing mechanism. In the former case, it is possible that these environmental threats could have deleterious effects on enve-

lope macromolecular structures (55), compromising envelope integrity and activating the LiaFSR system, as well as regulon genes involved in combating stress-related damage. While LiaFSR in *S. mutans* responded to a variety of cell envelope active antimicrobials (e.g., bacitracin, vancomycin, nisin, and chlorhexidine), we also showed its regulatory role in inducing genes encoding PG synthesis and remodeling enzymes in addition to membrane protein synthesis and envelope chaperone/proteases, a finding consistent with a pathway that responds to PG or envelope protein damage. Moreover, our study has highlighted similarities (e.g., lipid II inhibitors as inducers) and major differences (e.g., growth phase regulation, mutant phenotypes displayed, and regulon genes) between the *S. mutans* LiaFSR and its analogous system in *B. subtilis*. Overall, this variance in the *S. mutans* LiaFSR attributes may reflect its adaptation to its particular niche in the oral cavity, compared to the soil-inhabiting *B. subtilis*. The results from the present study enhance our understanding of how *S. mutans* can sense and respond to environmental threats that cause cell envelope stress. Such progress in elucidating the components underlying mechanism can eventually lead to the design and synthesis of drug targets to efficiently control bacterial pathogenesis.

ACKNOWLEDGMENTS

This study was supported by NIH grant 5R01DE013230-08 and CIHR grant MT-15431 to D.G.C. D.G.C. is a recipient of a Canada Research Chair position. P.S. is a CIHR Strategic Training Fellow supported by training grant STP-53877 and a Harron Scholar.

We thank Elena Voronejskaia for technical assistance with the Northern blot analysis.

REFERENCES

1. **Adams, H., W. Teertstra, J. Demmers, R. Boesten, and J. Tommassen.** 2003. Interactions between phage-shock proteins in *Escherichia coli*. J. Bacteriol. **185:**1174–1180.
2. **Ahn, S. J., J. A. Lemos, and R. A. Burne.** 2005. Role of HtrA in growth and competence of *Streptococcus mutans* UA159. J. Bacteriol. **187:**3028–3038.
3. **Ahn, S. J., Z. T. Wen, and R. A. Burne.** 2006. Multilevel control of competence development and stress tolerance in *Streptococcus mutans* UA159. Infect. Immun. **74:**1631–1642.
4. **Ajdic, D., W. M. McShan, R. E. McLaughlin, G. Savic, J. Chang, M. B. Carson, C. Primeaux, R. Tian, S. Kenton, H. Jia, S. Lin, Y. Qian, S. Li, H. Zhu, F. Najar, H. Lai, J. White, B. A. Roe, and J. J. Ferretti.** 2002. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. Proc. Natl. Acad. Sci. USA **99:**14434–14439.
5. **Beier, D., and R. Gross.** 2006. Regulation of bacterial virulence by two-component systems. Curr. Opin. Microbiol. **9:**143–152.
6. **Belcheva, A., and D. Golemi-Kotra.** 2008. A close-up view of the VraSR two-component system: a mediator of *Staphylococcus aureus* response to cell wall damage. J. Biol. Chem. **283:**12354–12364.
7. **Biswas, I., L. Drake, D. Erkina, and S. Biswas.** 2008. Involvement of sensor kinases in the stress tolerance response of *Streptococcus mutans*. J. Bacteriol. **190:**68–77.
8. **Boyle-Vavra, S., S. Yin, and R. S. Daum.** 2006. The VraS/VraR two-component regulatory system required for oxacillin resistance in community-acquired methicillin-resistant *Staphylococcus aureus*. FEMS Microbiol. Lett. **262:**163–171.
9. **Breukink, E., and B. de Kruijff.** 2006. Lipid II as a target for antibiotics. Nat. Rev. Drug Discov. **5:**321–332.
10. **Bryskier, A.** 2005. Antimicrobial agents: antibacterials and antifungals. ASM Press, Washington, DC.
11. **Chen, H., M. Bjerknes, R. Kumar, and E. Jay.** 1994. Determination of the optimal aligned spacing between the Shine-Dalgarno sequence and the translation initiation codon of *Escherichia coli* mRNAs. Nucleic Acids Res. **22:**4953–4957.
12. **Chong, P., L. Drake, and I. Biswas.** 2008. LiaS regulates virulence factor expression in *Streptococcus mutans*. Infect. Immun. **76:**3093–3099.
13. **Darwin, A. J.** 2005. The phage-shock-protein response. Mol. Microbiol. **57:**621–628.
14. **de Hoon, M. J., Y. Makita, K. Nakai, and S. Miyano.** 2005. Prediction of transcriptional terminators in *Bacillus subtilis* and related species. PLoS Comput. Biol. **1:**e25.
15. **Delcour, J., T. Ferain, M. Deghorain, E. Palumbo, and P. Hols.** 1999. The biosynthesis and functionality of the cell-wall of lactic acid bacteria. Antonie van Leeuwenhoek **76:**159–184.
16. **Diaz-Torres, M. L., and R. R. Russell.** 2001. HtrA protease and processing of extracellular proteins of *Streptococcus mutans*. FEMS Microbiol. Lett. **204:**23–28.
17. **Eiamphungporn, W., and J. D. Helmann.** 2008. The *Bacillus subtilis* sigma(M) regulon and its contribution to cell envelope stress responses. Mol. Microbiol. **67:**830–848.
18. **Gardete, S., S. W. Wu, S. Gill, and A. Tomasz.** 2006. Role of VraSR in antibiotic resistance and antibiotic-induced stress response in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **50:**3424–3434.
19. **Haas, W., D. Kaushal, J. Sublett, C. Obert, and E. I. Tuomanen.** 2005. Vancomycin stress response in a sensitive and a tolerant strain of *Streptococcus pneumoniae*. J. Bacteriol. **187:**8205–8210.
20. **Hasona, A., P. J. Crowley, C. M. Levesque, R. W. Mair, D. G. Cvitkovitch, A. S. Bleiweis, and L. J. Brady.** 2005. Streptococcal viability and diminished stress tolerance in mutants lacking the signal recognition particle pathway or YidC2. Proc. Natl. Acad. Sci. USA **102:**17466–17471.
21. **Hoch, J. A.** 2000. Two-component and phosphorelay signal transduction. Curr. Opin. Microbiol. **3:**165–170.
22. **Hussain, H., P. Branny, and E. Allan.** 2006. A eukaryotic-type serine/threonine protein kinase is required for biofilm formation, genetic competence, and acid resistance in *Streptococcus mutans*. J. Bacteriol. **188:**1628–1632.
23. **Ito, K., and Y. Akiyama.** 2005. Cellular functions, mechanism of action, and regulation of FtsH protease. Annu. Rev. Microbiol. **59:**211–231.
24. **Jordan, S., A. Junker, J. D. Helmann, and T. Mascher.** 2006. Regulation of LiaRS-dependent gene expression in *Bacillus subtilis*: identification of inhibitor proteins, regulator binding sites, and target genes of a conserved cell envelope stress-sensing two-component system. J. Bacteriol. **188:**5153–5166.
25. **Jordan, S., E. Rietkotter, M. A. Strauch, F. Kalamorz, B. G. Butcher, J. D. Helmann, and T. Mascher.** 2007. LiaRS-dependent gene expression is embedded in transition state regulation in *Bacillus subtilis*. Microbiology **153:**2530–2540.
26. **Khalichi, P., D. G. Cvitkovitch, and J. P. Santerre.** 2004. Effect of composite resin biodegradation products on oral streptococcal growth. Biomaterials **25:**5467–5472.
27. **Kim, D. Y., and K. K. Kim.** 2005. Structure and function of HtrA family proteins, the key players in protein quality control. J. Biochem. Mol. Biol. **38:**266–274.
28. **Klement, E., M. Chaffer, G. Leitner, A. Shwimmer, S. Friedman, A. Saran, and N. Shpigel.** 2005. Assessment of accuracy of disk diffusion tests for the determination of antimicrobial susceptibility of common bovine mastitis pathogens: a novel approach. Microb. Drug Resist. **11:**342–350.
29. **Kuroda, M., H. Kuroda, T. Oshima, F. Takeuchi, H. Mori, and K. Hiramatsu.** 2003. Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. Mol. Microbiol. **49:**807–821.
30. **Lau, P. C., C. K. Sung, J. H. Lee, D. A. Morrison, and D. G. Cvitkovitch.** 2002. PCR ligation mutagenesis in transformable streptococci: application and efficiency. J. Microbiol. Methods **49:**193–205.
31. **Len, A. C., D. W. Harty, and N. A. Jacques.** 2004. Stress-responsive proteins are upregulated in *Streptococcus mutans* during acid tolerance. Microbiology **150:**1339–1351.
32. **Li, Y. H., P. C. Lau, N. Tang, G. Svensater, R. P. Ellen, and D. G. Cvitkovitch.** 2002. Novel two-component regulatory system involved in biofilm formation and acid resistance in *Streptococcus mutans*. J. Bacteriol. **184:**6333–6342.
33. **Li, Y. H., N. Tang, M. B. Aspiras, P. C. Lau, J. H. Lee, R. P. Ellen, and D. G. Cvitkovitch.** 2002. A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation. J. Bacteriol. **184:**2699–2708.
34. **Lis, M., and H. K. Kuramitsu.** 2003. The stress-responsive *dgk* gene from *Streptococcus mutans* encodes a putative undecaprenol kinase activity. Infect. Immun. **71:**1938–1943.
35. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}CT$ method. Methods **25:**402–408.
36. **Martin, N. I., and E. Breukink.** 2007. Expanding role of lipid II as a target for lantibiotics. Future Microbiol. **2:**513–525.
37. **Martinez, B., A. L. Zomer, A. Rodriguez, J. Kok, and O. P. Kuipers.** 2007. Cell envelope stress induced by the bacteriocin Lcn972 is sensed by the lactococcal two-component system CesSR. Mol. Microbiol. **64:**473–486.
38. **Mascher, T.** 2006. Intramembrane-sensing histidine kinases: a new family of cell envelope stress sensors in *Firmicutes* bacteria. FEMS Microbiol. Lett. **264:**133–144.
39. **Mascher, T., S. L. Zimmer, T. A. Smith, and J. D. Helmann.** 2004. Antibiotic-inducible promoter regulated by the cell envelope stress-sensing two-component system LiaRS of *Bacillus subtilis*. Antimicrob. Agents Chemother. **48:**2888–2896.
40. **McBain, A. J., R. G. Ledder, P. Sreenivasan, and P. Gilbert.** 2004. Selection

for high-level resistance by chronic triclosan exposure is not universal. J. Antimicrob. Chemother. **53:**772–777.
41. **Montanari, M. P., L. Ferrante, E. Tili, I. Cochetti, V. Rossi, and P. E. Varaldo.** 2005. Interpretive criteria for disk diffusion susceptibility testing of ulifloxacin, the active metabolite of prulifloxacin. J. Chemother. **17:**138–142.
42. **Moran, C. P., Jr.** 1993. RNA polymerase and Transcription factors, p. 653–667. *In* M. M. Zukowski, A. T. Ganesan, and J. A. Hoch (ed.), Genetics and biotechnology of bacilli, vol. 3. Academic Press, Inc., New York, NY.
43. **Msadek, T.** 1999. When the going gets tough: survival strategies and environmental signaling networks in *Bacillus subtilis*. Trends Microbiol. **7:**201–207.
44. **Nagai, K., C. Oubridge, A. Kuglstatter, E. Menichelli, C. Isel, and L. Jovine.** 2003. Structure, function, and evolution of the signal recognition particle. EMBO J. **22:**3479–3485.
45. **Nakano, S., E. Kuster-Schock, A. D. Grossman, and P. Zuber.** 2003. Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **100:**13603–13608.
46. **Oussenko, I. A., T. Abe, H. Ujiie, A. Muto, and D. H. Bechhofer.** 2005. Participation of 3′-to-5′ exoribonucleases in the turnover of *Bacillus subtilis* mRNA. J. Bacteriol. **187:**2758–2767.
47. **Pechous, R., N. Ledala, B. J. Wilkinson, and R. K. Jayaswal.** 2004. Regulation of the expression of cell wall stress stimulon member gene *msrA1* in methicillin-susceptible or -resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **48:**3057–3063.
48. **Perry, A. M., H. Ton-That, S. K. Mazmanian, and O. Schneewind.** 2002. Anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. III. Lipid II is an in vivo peptidoglycan substrate for sortase-catalyzed surface protein anchoring. J. Biol. Chem. **277:**16241–16248.
49. **Perry, J. A., C. M. Levesque, P. Suntharalingam, R. W. Mair, M. Bu, R. T. Cline, S. N. Peterson, and D. G. Cvitkovitch.** 2008. Involvement of *Streptococcus mutans* regulator RR11 in oxidative stress response during biofilm growth and in the development of genetic competence. Lett. Appl. Microbiol. **47:**439–444.
50. **Pfaffl, M. W.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29:**e45.
51. **Pietiainen, M., M. Gardemeister, M. Mecklin, S. Leskela, M. Sarvas, and V. P. Kontinen.** 2005. Cationic antimicrobial peptides elicit a complex stress response in *Bacillus subtilis* that involves ECF-type sigma factors and two-component signal transduction systems. Microbiology **151:**1577–1592.
52. **Poyart, C., M. C. Lamy, C. Boumaila, F. Fiedler, and P. Trieu-Cuot.** 2001. Regulation of D-alanyl-lipoteichoic acid biosynthesis in *Streptococcus agalactiae* involves a novel two-component regulatory system. J. Bacteriol. **183:**6324–6334.
53. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
54. **Senadheera, M. D., A. W. Lee, D. C. Hung, G. A. Spatafora, S. D. Goodman, and D. G. Cvitkovitch.** 2007. The *Streptococcus mutans vicX* gene product modulates *gtfB/C* expression, biofilm formation, genetic competence, and oxidative stress tolerance. J. Bacteriol. **189:**1451–1458.
55. **Svensater, G., B. Sjogreen, and I. R. Hamilton.** 2000. Multiple stress responses in *Streptococcus mutans* and the induction of general and stress-specific proteins. Microbiology **146**(Pt. 1)**:**107–117.
56. **Sylvester, D. R., E. Alvarez, A. Patel, K. Ratnam, H. Kallender, and N. G. Wallis.** 2001. Identification and characterization of UDP-*N*-acetylenolpyruvylglucosamine reductase (MurB) from the gram-positive pathogen *Streptococcus pneumoniae*. Biochem. J. **355:**431–435.
57. **Tsuda, H., Y. Yamashita, Y. Shibata, Y. Nakano, and T. Koga.** 2002. Genes involved in bacitracin resistance in *Streptococcus mutans*. Antimicrob. Agents Chemother. **46:**3756–3764.
58. **Tsukioka, Y., Y. Yamashita, T. Oho, Y. Nakano, and T. Koga.** 1997. Biological function of the dTDP-rhamnose synthesis pathway in *Streptococcus mutans*. J. Bacteriol. **179:**1126–1134.
59. **Utaida, S., P. M. Dunman, D. Macapagal, E. Murphy, S. J. Projan, V. K. Singh, R. K. Jayaswal, and B. J. Wilkinson.** 2003. Genome-wide transcriptional profiling of the response of *Staphylococcus aureus* to cell-wall-active antibiotics reveals a cell-wall-stress stimulon. Microbiology **149:**2719–2732.
60. **van Heijenoort, J.** 2007. Lipid intermediates in the biosynthesis of bacterial peptidoglycan. Microbiol. Mol. Biol. Rev. **71:**620–635.
61. **Veiga, P., C. Bulbarela-Sampieri, S. Furlan, A. Maisons, M. P. Chapot-Chartier, M. Erkelenz, P. Mervelet, P. Noirot, D. Frees, O. P. Kuipers, J. Kok, A. Gruss, G. Buist, and S. Kulakauskas.** 2007. SpxB regulates O-acetylation-dependent resistance of *Lactococcus lactis* peptidoglycan to hydrolysis. J. Biol. Chem. **282:**19342–19354.
62. **Vollmer, W., B. Joris, P. Charlier, and S. Foster.** 2008. Bacterial peptidoglycan (murein) hydrolases. FEMS Microbiol. Rev. **32:**259–286.
63. **Wanner, B. L.** 1992. Is cross regulation by phosphorylation of two-component response regulator proteins important in bacteria? J. Bacteriol. **174:**2053–2058.
64. **Wanner, B. L., and M. R. Wilmes-Riesenberg.** 1992. Involvement of phosphotransacetylase, acetate kinase, and acetyl phosphate synthesis in control of the phosphate regulon in *Escherichia coli*. J. Bacteriol. **174:**2124–2130.
65. **Wen, Z. T., and R. A. Burne.** 2002. Functional genomics approach to identifying genes required for biofilm development by *Streptococcus mutans*. Appl. Environ. Microbiol. **68:**1196–1203.
66. **Wen, Z. T., P. Suntharaligham, D. G. Cvitkovitch, and R. A. Burne.** 2005. Trigger factor in *Streptococcus mutans* is involved in stress tolerance, competence development, and biofilm formation. Infect. Immun. **73:**219–225.
67. **Wiedemann, I., E. Breukink, C. van Kraaij, O. P. Kuipers, G. Bierbaum, B. de Kruijff, and H. G. Sahl.** 2001. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J. Biol. Chem. **276:**1772–1779.
68. **Yamashita, Y., Y. Shibata, Y. Nakano, H. Tsuda, N. Kido, M. Ohta, and T. Koga.** 1999. A novel gene required for rhamnose-glucose polysaccharide synthesis in *Streptococcus mutans*. J. Bacteriol. **181:**6556–6559.
69. **Yamashita, Y., T. Takehara, and H. K. Kuramitsu.** 1993. Molecular characterization of a *Streptococcus mutans* mutant altered in environmental stress responses. J. Bacteriol. **175:**6220–6228.
70. **Yin, S., R. S. Daum, and S. Boyle-Vavra.** 2006. VraSR two-component regulatory system and its role in induction of *pbp2* and *vraSR* expression by cell wall antimicrobials in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **50:**336–343.