Expression of the *Streptococcus mutans* essential two-component regulatory system VicRK is pH and growth-phase dependent and controlled by the LiaFSR three-component regulatory system

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As an inhabitant of the human oral cavity, *Streptococcus mutans* faces frequent environmental changes. Two-component regulatory systems (TCSs) play a critical role in responding to these changes. Recently, an essential TCS, VicRKX, has been identified. The objective of this study was to identify the environmental signal and bacterial factors regulating the expression of the vicRKX operon. The promoter of the vicRKX operon was fused to a promoterless lacZ reporter gene and introduced into *S. mutans* UA159. LacZ plate assay identified pH, vancomycin, ampicillin, penicillin G and polymyxin B, but not carbohydrates, as factors affecting expression. Using RNA dot-blotting, high levels of vicR transcript were observed in cells at the mid- and late-exponential phase of growth and in cells grown in media buffered at pH 7.8. Given that vicR expression was pH-dependent, the genes encoding a putative pH-sensing three-component regulatory system (LiaFSR) were deleted. The liaS mutant exhibited upregulation of vicR regardless of the growth condition. The role of VicK, VicX, and the competence-signal peptide (CSP) was also investigated; the results showed that vicR expression was not autoregulated and was downregulated by the CSP in a ComX-independent manner. In conclusion, the expression of vicRKX is influenced by culture pH, growth phase and antibiotic stress, and is regulated by LiaFRS.

INTRODUCTION

*Streptococcus mutans* is considered to be the major aetiologial agent for dental carries (Banas, 2004; Loesche, 1986). The bacterium possesses several key virulence traits, such as adhesion, acidogenicity and acid tolerance (Banas, 2004). The exact mechanisms regulating the expression of genes contributing to these virulence traits remain unclear, but recent evidence suggests a prominent role for two-component regulatory systems (TCSs). A typical TCS consists of a membrane-associated sensor histidine kinase and a cytoplasmic response regulator. The histidine kinase senses the environmental signal and autophosphorylates at a conserved histidine residue. The phosphate group is then transferred to the response regulator, which in turn induces or represses the expression of target genes. The *S. mutans* genome encodes 14 TCSs (Ajdic et al., 2002; Biswas et al., 2008). Two TCSs, named ComDE and Hk11-Rr11 (recently renamed as LiaFSR; Chong et al., 2008), have been shown to be associated with genetic competence, biofilm formation and adaptation to acidic conditions (Li et al., 2001a, b, 2002a). A third TCS, named VicRRX (Lee et al., 2004; Senadheera et al., 2005), and an orphan response regulator, CovR (SMU.1924c) (Biswas et al., 2007; Biswas & Biswas, 2006), have been characterized as factors controlling the expression of fructosyltransferase, glucosyltransferases and glu- can-binding proteins.

Previous attempts to obtain a VicR-deficient *S. mutans* mutant have failed, suggesting that vicR is an essential gene;
however, the sensor histidine kinase gene vicK can be eliminated from the genome (Bhagwat et al., 2001; Senadheera et al., 2005). In Bacillus subtilis (Fabret & Hoch, 1998), Staphylococcus aureus (Martin et al., 1999) and Streptococcus pneumoniae (Wagner et al., 2002), yycF (or vicR) is an essential gene. In S. pneumoniae, the yycFG (vicRK) systems control several genes encoding proteins involved in cell membrane and cell wall homeostasis. VicR controls the expression of pcsB, which encodes a protein required for growth by S. pneumoniae (Ng et al., 2003); when the expression of yycF or pcsB is decreased, cells become irregular in shape and size. The pcsB homologue is named glucan-binding protein B gene (gbpB); this homologue also appears to be required for viability of S. mutans (Mattos-Graner et al., 2007) but the requirement may vary among strains (Fujita et al., 2006). In addition to being influenced by pcsB, the fatty acid biosynthesis and membrane compositions of S. pneumoniae have also been shown to be modulated by YycF (Mohedano et al., 2005).

Despite the recent interest in VicRXX, the environmental signal and bacterial factors controlling its expression are relatively unknown. The impact of the deletion of VicK or VicX (Senadheera et al., 2007) or the source of carbohydrate (Shemesh et al., 2006) was investigated but none of these factors influenced vicR expression with the exception of xylose. Recently, two potential inducers have been identified: paraquat (Senadheera et al., 2007) and hydrogen peroxide (Deng et al., 2007). However, expression of vicR in S. mutans was marginally lower in bacteria cultured aerobically when compared to cells cultured anaerobically (Ahn & Burne, 2007). In B. subtilis, the expression of YycFG is growth-phase related, with the highest expression during the exponential phase (Fabret & Hoch, 1998; Fukuchi et al., 2000). In this study, we have identified pH, growth phase and antibiotic stress as factors affecting vicR expression. In addition, the expression of vicR is shown to be regulated by the three-component regulatory system LiaFSR.

Table 1. List of bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>E. coli XL1-Blue</td>
<td>Cloning strain</td>
<td>Stratagene</td>
</tr>
<tr>
<td>S. mutans UA159</td>
<td>Sequenced strain</td>
<td>Ajdic et al. (2002)</td>
</tr>
<tr>
<td>HLO001</td>
<td>UA159/pSL</td>
<td>This study</td>
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<tr>
<td>HLO002</td>
<td>UA159/pPvicR-LacZ</td>
<td>This study</td>
</tr>
<tr>
<td>YDNT004</td>
<td>HLO002, vicX::ermAM</td>
<td>This study</td>
</tr>
<tr>
<td>YDNT005</td>
<td>HLO002, liaS::ermAM</td>
<td>This study</td>
</tr>
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<td>YDNT006</td>
<td>HLO002, vicK::ermAM</td>
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<td>YDNT008</td>
<td>HLO002, comX::ermAM</td>
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<td>YDNT009</td>
<td>HLO002, liaR::ermAM</td>
<td>This study</td>
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<td>YDNT010</td>
<td>HLO002, liaF::ermAM</td>
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<td>YDNT016</td>
<td>HLO002, vicXX::ermAM</td>
<td>This study</td>
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<tr>
<td>pPvicR-LacZ</td>
<td>pSL, Kan', PvicR-lacZ promoter fusion</td>
<td>This study</td>
</tr>
<tr>
<td>pMALcLiaR</td>
<td>pMALc, Amp', MBP-LiaR fusion</td>
<td>This study</td>
</tr>
</tbody>
</table>

METHODS

Organisms and growth conditions. S. mutans strains (Table 1) were grown in Todd–Hewitt broth (THB), Brain Heart infusion broth (BHI), or Tryptone-vitamin base medium (TV) (Burne et al., 1999) supplemented with 27.5 mM glucose (TVG) at 37 °C aerobically without shaking. The growth was monitored turbidimetrically at 600 nm (HP8452A, Hewlett-Packard, or UV-1700, Shimadzu). Late-exponential-phase (OD600 ≈ 1.0) cultures grown in TVG were stored frozen at −80 °C as 5 ml aliquots in 15 % (w/v) glycerol. These frozen stocks were used as inocula and gave consistent growth. Escherichia coli was grown in Luria–Bertani (LB) medium at 37 °C with agitation (200 r.p.m.). When required, kanamycin was included in the media at 50 μg ml−1 and 500 μg ml−1 for E. coli and S. mutans, respectively, and erythromycin at 10 μg ml−1 for S. mutans.

Construction of the PvicR-lacZ fusion. The promoter region of the vicRXX operon (PvicR) was amplified by PCR using Taq DNA polymerase (New England Biolabs) and the primer pair SL349/SL350 (Table 2). The 411 bp PCR product was digested with XbaI and KpnI and ligated into the same sites upstream of the promoterless lacZ gene carried on pSL (Syvitski et al., 2007), resulting in plasmid pPvicR-lacZ. The plasmid was confirmed by restriction analysis and PCR and was maintained in E. coli XL1-Blue (Sambrook et al., 1989). S. mutans strain UA159 was transformed with pSL or pPvicR-lacZ using methods described previously (Homonylo-McGavin & Lee, 1996) and the resulting transformants were named HLO001 and HLO002, respectively. The presence of pSL in HLO001 or of pPvicR-lacZ in HLO002 was confirmed by plasmid isolation and restriction analysis.

Construction of deletion mutants. To construct the liaFSR, vicK and vicX mutants, upstream and downstream regions of liaF, vicK and vicX were amplified from the UA159 chromosomal DNA by PCR; the primer pairs used are listed in Table 2. An erythromycin-resistance cassette (ermAM) was PCR amplified from a synthetic
construct template (Claverie et al., 1995) with the primer pair SL550/SL551. The PCR products were extracted with phenol/chloroform, digested with SfiI (50 °C for 16 h) and ligated. The resulting products were amplified by PCR using the respective primer pairs to obtain PCR products containing the ermAM gene flanked by the upstream and downstream sequences of liaF, vicK or vicX. The liaF construct excluded the liaFSR promoter and thus would result in a liaFSR mutant. The constructs were used to transform strain HLO002. Transformants were selected on BHI with 0.3 % (w/v) yeast extract (BHI-YE) containing erythromycin and kanamycin.

To construct the vicK mutant, the upstream vicK region with the ermAM cassette was amplified from the vicK mutant using the primer pair SL571/SL550. The downstream vicX region with the ermAM cassette was amplified from the vicX mutant using the primer pair SL562/SL551. The resulting PCR products were used in overlapping PCR to obtain the ermAM cassette flanked by upstream vicK and downstream vicX regions. The PCR products were subsequently transformed into strain HLO002 to obtain the vicKX double knockout mutant.

To construct the liaS and liaR mutants in UA159, genomic DNA was isolated from the liaS (hkJ1) and liaR (rrl1) mutants of S. mutans strain NG8 (Li et al., 2002a). The regions containing the liaS (2.3 kb) or the liaR (2.4 kb) gene with an ermAM insert were amplified by PCR with the primer pairs SL357/SL358 and SL567/SL568, respectively (Table 2). The PCR products were subsequently transformed into strain HLO002 to obtain the liaS and liaR mutants in the UA159 background.

The comX mutant in UA159 was constructed similarly to the liaS and liaR mutants, using DNA from the NG8 comX mutant as the template (Li et al., 2002b).

The mutants were verified to have the ermAM gene inserted into the chromosomal genes by PCR. A typical 30 μl PCR contained 2 μl template DNA, 0.5 μM upstream primer, 0.5 μM downstream primer, 2 μM dATP, 2 μM dGTP, 2 μM dCTP, 2 μM dTTP, 5 μl 10× ThermoPol reaction buffer and 2.5 U Taq polymerase. The PCR mixture was subjected to 5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 54 °C and 3 min at 72 °C, and a final 5 min at 72 °C, using a thermocycler. The mutants were further verified by sequencing, showing that the ermAM cassette had inserted into the respective genes (McGill University and Genome Québec Innovation Centre, Montreal, Québec, Canada).

**LacZ plate assay.** HLO001 and HLO002 were streaked onto FMC (Terleckyj et al., 1975) or TV agar buffered with 100 mM HEPES and supplemented with 25 mM glucose, 15 mM sucrose or 15 mM maltose. The agar also contained 200 μM X-Gal. When required, the pH of the medium was adjusted with NaOH or HCl prior to autoclaving. Both strains were streaked onto unbuffed FMC or TV agar at pH 7.0, pH 6.0 or pH 5.0. The plates were incubated at 37 °C for 36 h in a candle jar, then examined for X-Gal hydrolysis. The plates could be tested on one plate. Briefly, strains were grown to mid-exponential phase (OD600≈0.5) in TVG or TVG0, harvested by centrifugation (14 000 g, 1 min) and resuspended in Z buffer (600 μl; 60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgCl2 and 2.6 mM β-mercaptoethanol, pH 6.9). The cells were mechanically lysed with glass beads (setting 7, 2 min; Mickle High Speed Vibratory Tissue Disintegrator, Mickle Laboratory Engineering Co.) and cell debris was removed by centrifugation (6000 g, 3 min). The cleared cell lysate (400 μl) was diluted with Z buffer (800 μl). Aliquots (150 μl) of the diluted cell lysate were added to a 96-well microplate and incubated at 37 °C for 30 min. ONPG (2 μM) was then added to the samples and incubated at 37 °C for up to 60 min. The level of ONPG hydrolysis was measured at 405 nm every 15 min, using a microplate reader model 3550 (Bio-Rad) or Synergy HT (Bio-Tek Instruments). The protein concentrations of cell lysates were measured by the method of Bradford as modified by Spector (1978). LacZ activity was calculated as (A600×1000)/[(mg protein×incubation time) and is reported as modified Miller units.

The gene encoding LiaR was PCR amplified with the primer pair SL569/SL570 (Table 2) and ligated into the BamHI and EcoRI sites of the pMALc expression vector (New England Biolabs), creating pMALcLiaR. E. coli BL21 (EMD Chemicals) carrying pMALcLiaR grown in tryptone-salt-glucose medium [TS; 0.8 % (w/v) tryptone, 87 mM NaCl, 27.5 mM glucose] to an OD600 of 0.6 was induced with 1 mM IPTG for 3 h. Following the induction, the MBP-LiaR fusion protein was isolated from the cell lysate by affinity chromatography on an amylose column according to the manufacturer’s instructions (New England Biolabs). The purified MBP-LiaR fusion protein appeared as a single 67 kDa protein by SDS-PAGE analysis (data not shown). BBL/c mice (female, n=5, Charles River Laboratories) were injected subcutaneously with 10 μg purified MBP-LiaR in incomplete Freund’s adjuvant and on days 1, 14 and 21. Mice were euthanized on day 28 to obtain the anti-LiaR antiserum.

Cytoplasmic proteins (10 μg) of S. mutans prepared by mechanical breakage with glass beads were separated on a 12.5 % SDS-polyacrylamide gel and transferred to an Immobilon-P PVDF membrane (Millipore). The membrane was blocked with 5 % (w/v) skim milk in Tris-buffered saline with Tween [TTBS; 145 mM NaCl, 100 mM Tris/HCl pH 7.4, 0.15 % (v/v) Tween 20] and incubated with the anti-MBP-LiaR serum (1:2000) in TTBS containing 1 % (w/v) BSA followed by goat anti-mouse alkaline phosphatase conjugate (1:8000, Sigma-Aldrich). The membrane was developed with the

**LacZ liquid assay.** For the growth experiment, S. mutans was grown in unbuffered TVG at pH 7.8 (TVG6.0), unbuffered TVG at pH 6.0 (TVG6.0) or HEPES-buffered TVG at pH 7.8 (HTVG) at 37 °C. At the indicated time points, cells were harvested by centrifugation (3000 g, 10 min), resuspended in PBS to OD600≈3.0, and stored at −80 °C. A volume (600 μl) of washed cells was centrifuged (14 000 g, 1 min) and resuspended in Z buffer (600 μl; 60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgCl2 and 2.6 mM β-mercaptoethanol, pH 6.9). The cells were mechanically lysed with glass beads (setting 7, 2 min; Mickle High Speed Vibratory Tissue Disintegrator, Mickle Laboratory Engineering Co.) and cell debris was removed by centrifugation (6000 g, 3 min). The cleared cell lysate (400 μl) was diluted with Z buffer (800 μl). Aliquots (150 μl) of the diluted cell lysate were added to a 96-well microplate and incubated at 37 °C for 30 min. ONPG (2 μM) was then added to the samples and incubated at 37 °C for up to 60 min. The level of ONPG hydrolysis was measured at 405 nm every 15 min, using a microplate reader model 3550 (Bio-Rad) or Synergy HT (Bio-Tek Instruments). The protein concentrations of cell lysates were measured by the method of Bradford as modified by Spector (1978). LacZ activity was calculated as (A600×1000)/[(mg protein×incubation time) and is reported as modified Miller units.

**Anti-LiaR antiserum and Western immunoblotting.** The gene encoding LiaR was PCR amplified with the primer pair SL569/SL570 (Table 2) and ligated into the BamHI and EcoRI sites of the pMALc expression vector (New England Biolabs), creating pMALcLiaR. E. coli BL21 (EMD Chemicals) carrying pMALcLiaR grown in tryptone-salt-glucose medium [TS; 0.8 % (w/v) tryptone, 87 mM NaCl, 27.5 mM glucose] to an OD600 of 0.6 was induced with 1 mM IPTG for 3 h. Following the induction, the MBP-LiaR fusion protein was isolated from the cell lysate by affinity chromatography on an amylose column according to the manufacturer’s instructions (New England Biolabs). The purified MBP-LiaR fusion protein appeared as a single 67 kDa protein by SDS-PAGE analysis (data not shown). BBL/c mice (female, n=5, Charles River Laboratories) were injected subcutaneously with 10 μg purified MBP-LiaR in incomplete Freund’s adjuvant and on days 1, 14 and 21. Mice were euthanized on day 28 to obtain the anti-LiaR antiserum.

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Table 2. Primers used in this study

<table>
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<th>Name</th>
<th>Description</th>
<th>Sequence (5′→3′)*</th>
<th>Amplicon (bp)</th>
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<td>SL349</td>
<td>vicR promoter forward, XbaI</td>
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*Restriction sites are underlined.

substrate CSP-Star and images were captured with the VersaDoc MP Imaging system (Bio-Rad).

RNA isolation and dot blotting. RNA was extracted using the method described by Peterson et al. (2000), with modifications. S. mutans strains were grown in TVG6.0 or HTVG. When the cultures reached the early-exponential phase (OD600 ≈ 0.1–0.2), mid-exponential phase (OD600 ≈ 0.5 for the TVG6.0 culture, OD600 ≈ 1.0 for the HTVG culture) or late-exponential phase (OD600 ≈ 2.0 for the HTVG culture), cells were collected by centrifugation (14 000 g, 10 min) and stored at –80 °C. The RNA was further extracted with acidic phenol/chloroform followed by chloroform. The RNA was analysed on formaldehyde-formamide agarose gels to ensure integrity before use in dot-blotting.

In dot-blotting, RNA was denatured with 3 vols RNA-denaturing solution (600 μl deionized formamide, 200 μl 37 % (w/v) formaldehyde, 130 μl MOPS buffer (20 mM MOPS, 2 mM sodium acetate, 1 mM EDTA, pH 7.0) at 65 °C for 5 min. Following the incubation, 20 × SSC (3 M NaCl, 300 mM sodium citrate, pH 7.0) was added immediately to the denatured RNA to a final concentration of 10 × SSC. The RNA was then blotted onto a piece of Hybond H + nylon membrane (GE Healthcare) using a Bio-Dot Microfiltration Apparatus (Bio-Rad) according to the manufacturer's instructions. The blots were blocked and reacted with the digoxigenin (DIG)-labelled vicR or 16S rRNA DNA probes using protocols suggested by Roche Applied Science. We chose 16S rRNA as the control because its expression was relatively constant throughout growth (Stipp et al., 2008). Images of the blots were obtained using a FluorChem SP and the AlphaInnotech. The intensity of RNA dots was measured using the Image J software (National Institutes of Health, Bethesda, MD, USA) and the relative intensity of vicR dots was calculated as (intensity of vicR dot)/(intensity of 16s rRNA dot).

The DIG-labelled DNA probes were synthesized by PCR. Briefly, the PCR mixture (50 μl) was set up as above, with the exception that 2 μM dATP, 2 μM dCTP, 2 μM dGTP and 0.7 μM DIG-dUTP were added. The DIG-labelled vicR and 16S rRNA probes were 412 bp and 350 bp in size, respectively.
**RESULTS**

**Screening of $P_{vicR}$-lacZ activity**

Several environmental factors, such as pH and carbon sources, are known to regulate gene expression in *S. mutans*. To rapidly test if some of these environmental factors influenced the expression of $vicR$, the reporter lacZ gene was fused to the $vicR$ promoter and introduced into *S. mutans*. The strains carrying the fusion construct (HLO002) or the promoterless lacZ gene (HLO001) were tested for lacZ expression on FMC or TV agar containing glucose, sucrose or maltose as the carbon source. In all cases, strain HLO001 showed no X-Gal hydrolysis, confirming that in the absence of a promoter, lacZ was not expressed (data not shown). In addition, HLO001 did not produce any detectable LacZ activity in liquid medium (data not shown). In contrast, HLO002 colonies showed clear X-Gal hydrolysis. The degree of X-Gal hydrolysis was similar ($++$) for the tested carbon sources but was slightly stronger on the defined FMC ($+++)$ than the TV media $(++)$. More interestingly, the LacZ activity was the strongest $(+++)$ on plates at pH 7.0, weaker $(+)$ at pH 6.0, and absent $(-)$ at pH 5.0.

LacZ activity induction by antibacterial agents was also examined using the disc diffusion assay. The results showed that vancomycin, penicillin G, ampicillin and polymyxin B induced the expression of lacZ whereas tetracycline, erythromycin, Triton X-100, bacitracin and H$_2$O$_2$ did not (data not shown).

**The expression of vicR is growth-phase dependent and downregulated at low pH**

To further examine the effect of pH and growth on vicRKX expression, *S. mutans* HLO002 was grown in buffered (HTVG) and unbuffered (TVG$_{6.0}$ and TVG$_{7.8}$) media. HLO002 had a similar generation time when grown in HTVG and TVG$_{7.8}$ (1 h 37 min vs 1 h 34 min) but the bacteria took longer to reach stationary phase in HTVG than in TVG$_{7.8}$ (Fig. 1a). HLO002 had a longer generation time (2 h 13 min) in TVG$_{6.0}$ and did not reach the same growth yield as in the other two media. During growth, the pH of the HTVG culture only dropped by 1 pH unit, to approximately pH 6.8, while the pH of the TVG$_{7.8}$ and TVG$_{6.0}$ cultures dropped rapidly to around pH 4.5 (Fig. 1c).

The LacZ activity of the HTVG culture remained relatively high and stable, correlating with the stable pH (Fig. 1b). In contrast, the LacZ activity of the TVG$_{6.0}$ culture was much lower, while that of the TVG$_{7.8}$ culture decreased rapidly as the culture pH dropped. A correlation between pH and LacZ activity was clearly evident (Fig. 1d).

To verify the results of the LacZ experiments, the levels of $vicR$ transcript in strain UA159 and HLO002 when grown in TVG$_{6.0}$ or HTVG to the early-, mid- or late-exponential phase were analysed by Northern dot-blotting. As shown in Fig. 2, $vicR$ mRNA was present at the highest level in mid- and late-exponential-phase cells grown in HTVG. $vicR$ mRNA was present in only small quantities in early-exponential-phase cells grown in HTVG. Consistent with the LacZ activity results, the level of $vicR$ mRNA was much lower in cells grown in TVG$_{6.0}$ than in HTVG. Similar patterns of $viR$ mRNA levels were observed for strain HLO002. These results further suggest that the vicRKX operon is downregulated at low pH and is growth-phase dependent.

**vicR expression is not autoregulated by VicK and VicX**

TCSs often have the ability to autoregulate their own expression. To examine whether this is the case for VicRKX, mutants with $vicK$, $vicX$ or vicKX deletion were constructed and pP$_{vicR}$-LacZ was introduced into these mutants. All three mutants produced the same level of LacZ activity as the parent strain (data not shown). Furthermore, the $vicK$ and $vicX$ mutants both displayed a similar degree of X-Gal hydrolysis compared to the parent strain. These results suggest that the expression of vicRKX is not autoregulated by VicK and VicX.

**The LiaFSR three-component system regulates vicRKX expression**

Since LiaSR was required for acid adaptation and might act as a pH sensor (Li et al., 2002a) and regulate the cell envelope stress response (Suntharalingam et al., 2009), it was of interest to examine whether it plays a role in vicR expression. Three lia mutants, liaFSR, liaS and liaR, were constructed. The mutants were verified to have the respective genes inactivated by PCR and DNA sequencing (data not shown). Western immunoblotting showed that the liaFSR and liaR mutants lacked the LiaR protein while the liaS mutant continued to produce LiaR, further confirming the mutations (Fig. 3).

The lia mutants were analysed for lacZ expression. When grown in HTVG, the liaS mutant showed the highest level of expression (200–300% of the parent level; $P<0.001$) (Fig. 4). The liaFSR mutant also showed a higher lacZ expression than the parent ($P<0.001$). The liaR mutant produced a similar level of LacZ as the parent. Consistent with earlier results, lacZ expression was much lower when cells were grown in TVG$_{6.0}$, although the liaS mutant still produced significantly more LacZ than the parent. Northern blotting results were consistent with the LacZ results: the liaS mutant produced the highest level of $vicR$ mRNA in HTGV and TVG$_{6.0}$ (Fig. 5). These results strongly suggest that the LiaFSR three-component system is
part of the regulatory network controlling the expression of the \( \text{vicRKX} \) operon.

\textit{vicR} expression is \textit{ComX}-independent but affected by competence-signal peptide (CSP)

Senadheera \textit{et al.} (2005, 2007) previously reported that \( \text{vicK} \) or \( \text{vicX} \) deletion mutants displayed reduced transformation efficiencies in the absence of exogenously supplied CSP, suggesting a role for \( \text{vicRKX} \) in genetic competence. To examine the relationship between CSP, \textit{comX} and \textit{vicR} expression, LacZ activity was assayed in the parent \( \text{S. mutans} \) and a \textit{comX} mutant. The level of LacZ produced by the parent and the \textit{comX} mutant was similar in the absence of exogenously supplied CSP (Fig. 6). Interestingly, in the presence of added CSP, both the parent and the \textit{comX} mutant produced a reduced amount of LacZ when grown in HTVG (Fig. 7). In TVG_{6.0}, the parent strain also showed decreased LacZ activity; however, this decrease was not statistically significant.

\section*{DISCUSSION}

In the present study, using the \textit{lacZ} reporter gene expression system and RNA dot-blotting, we have identified pH as an environmental signal that affects the expression of \( \text{vicRKX} \). The expression was highest at neutral pH but declined at acidic pH. Our RNA data also showed that \( \text{vicRKX} \) expression is growth-phase dependent, with maximal expression at the mid- to late-exponential phase of growth. This finding is consistent with that
Fig. 2. Levels of vicR mRNA produced by S. mutans UA159 and HLO002. (a, b) Representative RNA dot-blot of vicR (V) and 16S rRNA (16S) transcripts from S. mutans UA159 (a) and S. mutans HLO002 (b). (c) Relative intensities of the vicR transcripts normalized against the 16S rRNA transcripts and reported as the mean (±SE) fold changes compared to the early-exponential-phase TVG6.0 sample from three independent experiments. RNA was detected from cells grown to early-exponential phase (E), mid-exponential phase (M) and late-exponential phase (L) in either unbuffered TVG6.0 (6) or buffered HTVG (7.8). *, P<0.05 between the sample and early-exponential-phase sample.

Fig. 3. Western immunoblots showing the production of LiaR by the parent HLO002 and the liaS mutant. Cytoplasmic proteins (10 μg) from the parent and liaS mutants grown in HTGV or TVG6.0 were probed with the anti-LiaR antiserum, A His10-tagged LiaR protein (expressed by pET16b, EMD Chemicals) isolated from E. coli (S. F. Lee & Y. D. N. Tremblay, unpublished) was used as positive control.

Fig. 4. Expression of lacZ under the control of the vicR promoter by the parent HLO002 and the liaFSR, liaS, and liaR mutants grown in HTGV and TVG6.0 to the mid-exponential phase. The results are the means (±SE) of two independent experiments, each consisting of triplicate cultures. *, P<0.001 between the mutant and parent.

Fig. 5. Level of vicR mRNA produced by mid-exponential-phase cells of the parent HLO002 and the liaFSR, liaS, and liaR mutants cultured in HTGV and TVG6.0. (a) Representative RNA dot-blot of vicR and 16S rRNA transcripts. (b) Relative intensities of the vicR transcripts normalized against the 16S rRNA transcripts and reported as the mean (±SE) fold change compared to the parent from three independent experiments. *, P<0.05 between the liaS mutant and parent.
also had no effect; this was surprising, since Senadheera et al. (2007) and Deng et al. (2007) reported that the vicRKX operon was upregulated when S. mutans was stressed with paraquat or H₂O₂. On the other hand, our results are more in line with the observation that an increase in expression of vicR was not observed when S. mutans was grown under aerobic conditions (Ahn & Burne, 2007). The discrepancy may be attributed to the duration of exposure to the stress in these experiments. In the study by Senadheera et al. (2007) cells were exposed to paraquat for 15 min and in that of Deng et al. (2007) for 2 h. A much longer (24 h) exposure time was used in our experiments than in those of Ahn & Burne (2007). It is possible that the effects of paraquat or H₂O₂ are transient.

We believe that the three signals identified in the present study (neutral culture pH, exponential growth and cell-wall-acting antibiotics) induced an envelope stress for the cells. In the case of neutral pH, cells are in an optimal growth condition, and cell wall biosynthesis is rapid, thus generating a stress for cell wall precursors and biosynthetic machineries. Cells in the exponential phase of growth experience a similar stress. In the case of exposure to antibiotics, the cell wall suffers damage leading a demand for repair; thus a stress is generated. Increased expression of vicRKX is a response to the envelope stress. A role for VicRKX in cell wall biosynthesis was implicated in S. pneumoniae, in that when the expression of vicR was decreased, cells became pleomorphic (Ng et al., 2003, 2004).

The novel finding of the present study is that vicRKX expression is regulated by LiaFSR. LiaFSR is known to regulate cell envelope stress in B. subtilis (Jordan et al., 2006), Staph. aureus (Gardete et al., 2006) and S. mutans (Sutharalingam et al., 2009). Thus, it is conceivable that LiaFSR and VicRKX are connected. Our finding that vicR expression is upregulated in the liaS mutant strongly suggests that the LiaFSR system regulates vicR expression. We have attempted to obtain a viable liaFSR clone in E. coli but failed; hence, complementation experiments cannot be performed. Despite this, we are confident that vicR expression is regulated by liaFSR, as the lia mutants were fully validated by PCR analysis, DNA sequencing and Western blotting.

We believe that LiaS regulates vicR expression by sensing envelope stresses resulting from rapid cell growth or antibiotic exposure. Upon sensing the stress, LiaS becomes phosphorylated and in turn phosphorylates LiaR. The phosphorylated LiaR will then activate vicR expression. Under non-stress conditions, LiaS actively dephosphorylates LiaR, which can be phosphorylated by other phospho-donors in the cell. The LiaS homologue (VraS) of Staph. aureus has been shown to possess both kinase and phosphatase activity (Belcheva & Golemi-Kotra, 2008). Thus, under non-stress conditions, vicR expression is not activated. When liaS is inactivated, the dephosphorylation of LiaR does not occur, resulting in a high level of vicR expression.

Fig. 6. Expression of lacZ under the control of the vicR promoter by the parent HLO002 (white bars) and the comX mutant (grey bars) grown in TVG₆₀ and HTVG. (a) Cells in the early-exponential phase. (b) Cells in the mid-exponential phase. The results represent the means (±se) of two independent experiments, each consisting of triplicate cultures.

Fig. 7. Effect of exogenous CSP on lacZ expression under the control of the vicR promoter by the parent HLO002 (a) and the comX mutant (b). The comX mutant was only cultured in HTVG. In both cases, cells were grown to early-exponential phase and CSP (500 ng ml⁻¹) was added (grey bars) or not (white bars). After 1 h, cells were harvested. The results represent the means (±se) of two independent experiments, each consisting of triplicate cultures. *, P<0.05 between CSP addition and no addition.

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expression. When liaR is inactivated, vicR expression cannot be activated. Our results support the above model.

We have analysed the S. mutans vicR promoter for a possible LiaR binding sequence (Martinez et al., 2007) but no match was found. In addition, the recombinant LiaR did not bind to the vicR promoter in a gel mobility shift assay (data not shown). These findings suggest that the regulation of vicR by the LiaR occurs indirectly.

Our results showed that vicR expression is not auto-regulated, as the vicK, vicX and vicKX mutants showed similar levels of LacZ production to the parent strain. This finding is in agreement with that reported by others (Fabret & Hoch, 1998; Ng et al., 2005; Senadheera et al., 2007). Our results further showed that CSP can down-regulate vicR expression. When supplied exogenously, CSP has been associated with a significant reduction in the rate of cell division in a ComX-independent manner (Qi et al., 2005). Thus, it is possible that the effect of CSP on vicR expression is a consequence of alleviation of cell envelope stress from a decrease in cell division.

In conclusion, the expression of the vicRKX operon is induced during exponential growth, at neutral pH and by antibacterial agents, and is regulated by the LiaFSR system.

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REFERENCES


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