A phenotypic microarray analysis of a *Streptococcus mutans* *liaS* mutant

Jiaqin Zhang^{1,2} and Indranil Biswas¹

Correspondence

Indranil Biswas
ibiswas@kumc.edu

¹Department of Microbiology, University of Kansas Medical Center, Kansas City, KS 66160, USA

²Department of Parasitology, Shandong University School of Medicine, 44# Wenhua Xi Road, Jinan, Shandong 250012, PR China

Received 7 August 2008
Revised 22 September 2008
Accepted 27 September 2008

Streptococcus mutans, a biofilm-forming Gram-positive bacterium that resides in the human oral cavity, is considered to be the primary aetiological agent of human dental caries. A cell-envelope stress-sensing histidine kinase, LiaS, is considered to be important for expression of virulence factors such as glucan-binding protein C and mutacin production. In this study, a *liaS* mutant was subjected to phenotypic microarray (PM) analysis of about 2000 phenotypes, including utilization of various carbon, nitrogen, phosphate and sulfur sources; osmolytes; metabolic inhibitors; and susceptibility to toxic compounds, including several types of antibiotics. Compared to the parental strain UA159, the *liaS* mutant strain (IBS148) was more tolerant to various inhibitors that target protein synthesis, DNA synthesis and cell-wall biosynthesis. Some of the key findings of the PM analysis were confirmed in independent growth studies and by using antibiotic discs and E-test strips for susceptibility testing.

INTRODUCTION

The human pathogen *Streptococcus mutans* is a Gram-positive bacterium with low G+C content that resides in the oral cavity, and is considered to be the primary aetiological agent in the formation of dental caries (Loesche, 1986). This pathogen has evolved a biofilm lifestyle for survival in the dental plaque formed on the tooth surface (Hamada & Slade, 1980). *Strep. mutans* is also an inducer of infective endocarditis, with more than 14% of viridians streptococcus-induced endocarditis caused by *Strep. mutans* (Hamada & Slade, 1980; Loesche, 1986; Ullman *et al.*, 1988). *Strep. mutans* uses sucrose, which is often present in the dietary carbohydrates, and converts it to sticky polysaccharides known as glucans (Banas & Vickerman, 2003; Kuramitsu, 1993). With the help of surface-associated proteins that bind to glucan, *Strep. mutans* colonizes in the oral cavity through the formation of diverse, multispecies biofilms on the tooth surface, commonly known as dental plaque. This organism has developed several mechanisms to maintain its presence in the oral cavity (Carlsson & Hamilton, 1994) and to withstand drastic environmental changes (Ahn *et al.*, 2006; Lemos *et al.*, 2005).

Adaptive responses of bacteria to environmental changes, such as nutrient limitation, oxygen deprivation, antibiotic

stress and osmotic shock, are regulated by the so-called two-component signal transduction system (TCS) pathways (Dalton & Scott, 2004; El-Sharoud, 2005; Mascher *et al.*, 2006; Verneuil *et al.*, 2004). TCSs typically consist of a membrane-bound sensor histidine kinase and a cytoplasmic response regulator, with a common biochemical mechanism involving phosphoryl-group transfer between two distinct protein components. The sensor histidine kinase is composed of two components: an amino-terminal sensor/input domain that detects specific stimuli from the extracellular environment, and a cytoplasmic transmitter/histidine phosphotransferase domain that autophosphorylates at a specific histidine residue in response to stimulation of the sensor domain (Fabret *et al.*, 1999; Mascher, 2006). The genome of *Strep. mutans* UA159 encodes a total of 14 TCSs (Biswas *et al.*, 2008), in addition to an orphan response regulator, CovR (Ajdic *et al.*, 2002; Biswas & Biswas, 2006). These TCSs are critical for survival under adverse conditions, as well as for regulation of virulence-associated factors of this pathogen (Biswas *et al.*, 2007, 2008; Biswas & Biswas, 2006; Idone *et al.*, 2003).

LiaSR is a TCS that is believed to be a part of a complex regulatory network that monitors and responds to cell-envelope stress in *Bacillus subtilis* (Gardete *et al.*, 2006; Jordan *et al.*, 2006). Homologous systems such as VraSR in *Staphylococcus aureus* (Gardete *et al.*, 2006; Jordan *et al.*, 2006) and CesSR in *Lactococcus lactis* (Martinez *et al.*, 2007) have been shown to function in a similar manner. LiaS and VraS both belong to a subclass of intramembrane-sensing histidine kinases (IM-HKs) that are found exclusively in Gram-positive bacteria with a low G+C

Abbreviations: IM-HK, intramembrane-sensing histidine kinase; PM, phenotypic microarray; TCS, two-component signal transduction system.

A supplementary table with full data from the PM analysis is available with the online version of this paper.

content (*Firmicutes*) (Mascher, 2006). Most IM-HKs sense various stresses to the cell envelope, and the gene targets of these TCSs are those that are involved in the maintenance of cell-envelope integrity, mediation of antibiotic resistance, or detoxification processes (Mascher, 2006). In *Streptococcus pneumoniae*, a LiaSR homologue, TCS03 (SP0386 and SP0387), was expressed as a part of the vancomycin stress response of this micro-organism (Haas *et al.*, 2005).

LiaSR and VraSR are induced by various inhibitors of cell-wall synthesis, but only VraSR has been shown to have a direct effect on the expression of genes related to peptidoglycan synthesis and antibiotic resistance (Butcher *et al.*, 2007; Gardete *et al.*, 2006). LiaSR is activated in the presence of sublethal concentrations of lipid II-interacting antibiotics, including bacitracin, vancomycin, ramoplanin and nicin, and strongly induces expression of its own locus (Mascher *et al.*, 2003, 2004). LiaSR is also activated in the presence of cationic antimicrobial peptides, and, to a lesser extent, alkaline shock, detergents, ethanol, exposure to organic solvents, and secretion stress (Mascher, 2006). Additionally, it has been reported that LiaSR regulates the expression of the *lia* operon during the transition from exponential growth phase to stationary phase, in the absence of exogenous cell-wall inhibitors and in the presence of an as-yet-unidentified stimulus (Jordan *et al.*, 2007). VraSR of *Staph. aureus* is also activated in the presence of cell-wall inhibiting antibiotics, such as β -lactams (meticillin, oxacillin, etc.) or glycopeptides (i.e. vancomycin); however, in contrast to LiaSR, activation of VraSR leads to the overexpression of a number of genes, including those associated with cell-wall biosynthesis and β -lactam antibiotic resistance (Gardete *et al.*, 2006; Kuroda *et al.*, 2003; Yin *et al.*, 2006).

In *Strep. mutans*, the SMU.486/SMU.487 locus, which was formerly known as RR11/HK11 (Li *et al.*, 2002), encodes a TCS that is homologous to LiaSR (Biswas *et al.*, 2008). A recent study in our laboratory with the sensor kinase LiaS of *Strep. mutans* UA159 revealed several novel findings. It was found that LiaS negatively regulates expression of *gbpC*, which encodes a glucan-binding protein essential for biofilm formation and cariogenicity (Biswas *et al.*, 2008; Chong *et al.*, 2008). LiaS was also shown to regulate expression and secretion of mutacin, a bacteriocin produced by *Strep. mutans* to suppress the growth of other competitor bacteria present in the dental plaque (Chong *et al.*, 2008). In another study, Li *et al.* (2002) showed that mutations in LiaS in *Strep. mutans* NG8 resulted in the formation of aberrant biofilms and an acid-sensitive phenotype. Therefore, LiaS appears to be very important for the pathogenesis of *Strep. mutans*.

To gain a complete understanding of the role of LiaS in *Strep. mutans* biology, physiological and phenotypic changes of a *liaS* mutant were compared to its isogenic parental strain using phenotypic microarray (PM) technology (Biswas & Biswas, 2005; Bochner *et al.*, 2001; Zhou

et al., 2003). PM is an integrated system of cellular assays for the simultaneous, high-throughput screening of a large number of phenotypes. Here we report the results of PM analysis performed with a *liaS* mutant in which we examined nearly 2000 cellular phenotypes.

METHODS

Bacterial strains and growth conditions. *Strep. mutans* strains UA159 and IBS148 (Chong *et al.*, 2008) were routinely grown in Todd–Hewitt medium (BBL; Becton Dickinson) supplemented with 0.2 % yeast extract (THY) at 37 °C. When necessary, spectinomycin (Sp, 300 $\mu\text{g ml}^{-1}$) was included in the growth medium. For some confirmatory growth experiments, *Strep. mutans* cultures were grown in THY containing 1 % NaCl, 3 % Na_2SO_4 or other chemicals as indicated.

PM analysis. PM analysis was performed using Biolog's PM service facility. A total of 20 96-well PM plates constituting eight metabolic panels (PM1 to PM8) and 12 sensitivity panels (PM9 to PM20) were used in this study. To assess the altered phenotypes of the *liaS* mutant (IBS148), the growth was compared to its parent *Strep. mutans* UA159 strain. The basic growth media and the conditions for PM analysis were published previously (Biswas & Biswas, 2005; Bochner *et al.*, 2001; Zhou *et al.*, 2003). The inoculating cell densities used in this study were 1 : 13 dilution of 81 % transmittance for both metabolic and sensitivity panels. PM analysis was conducted in duplicate after incubation of the strains at 37 °C for 72 h. A mean height difference threshold of 50 for metabolic panels and a difference threshold of 60 for sensitivity panels were used to consider the difference between the two growths significant. The data were further confirmed by Student's *t* test. The growth kinetics for UA159 was displayed as a red tracing, while IBS148 was displayed as a green tracing. The phenotypic changes listed in Table 1 were the changes detected in both PM runs (for a complete analysis, see Supplementary Table S1, available with the online version of this paper). Standard PM testing protocols are described in <http://www.biolog.com>; the conditions are similar to those used here.

Antibiotic susceptibility stress. Disc inhibition assays were performed as described previously to evaluate antibiotic susceptibility of the *Strep. mutans liaS* mutant (Biswas *et al.*, 2008). Antibiotic discs (6 mm in diameter; Becton Dickinson) were placed on THY agar plates inoculated with the wild-type or the *liaS* mutant strains. Following overnight incubation under microaerophilic conditions, the zones of inhibition were measured. In addition, susceptibilities to selected antimicrobial agents were tested using E-test strips (AB-Biodisk) on THY agar plates.

RESULTS

The LiaSR family of TCSs is known to be involved in sensing cell-envelope stress. However, the nature of the cell-envelope stress and the genes under the control of the LiaSR regulon vary greatly depending on the organism. To better understand the role of LiaS in the cell-envelope stress response in *Strep. mutans*, we performed a complete PM analysis using a previously constructed *liaS* mutant strain, IBS148 (Chong *et al.*, 2008). This strain was generated by replacing an internal part of the *liaS* ORF with an *aad* gene encoding spectinomycin resistance (Chong *et al.*, 2008).

To ensure that the expression of the downstream *liaR* gene was unaffected in *liaS* mutant IBS148, we performed a

Table 1. Growth advantages in the *liaS* mutant

Mode of action	Compounds
Cell wall synthesis	Phosphomycin, glycine, cefsulodin, cetoperazone, cephalosporin, ceftioxin, moxalactam, piperacillin, D-cycloserine, ceftriaxone
Membrane and ion channel	4-Aminopyridine, polymyxin B, colistin
DNA damage	Semicarbazide hydrochloride, hydroxylamine, phleomycin
DNA synthesis	Nitrofurantoin, bleomycin
DNA unwinding	Norfloxacin, ciprofloxacin, oxolinic acid, lomefloxacin, ofloxacin, enoxacin, nalidixic acid
DNA intercalator	4-Hydroxycoumarin
DNA methyltransferase	5-Azacytidine
Nucleic acid analogues	Cytosine arabinoside, 5-fluorouracil, 5-fluoro-5'-deoxyuridine
Folate antagonist	Sulfachloropyridazine, sulfanilamide, sulfamethazine, sulfamethoxazole, trimethoprim, sulfisoxazole
Ribonucleotide reductase	Hydroxyurea
Thymidylate synthetase	Trifluorothymidine
t-RNA synthetase	L-Glutamic- γ -hydroxamate, glycine hydroxamate
Protein synthesis	Cinoxacin, capreomycin, chloramphenicol, spectinomycin*, streptomycin, hygromycin, geneticin (G418), tetracycline, gentamicin, amikacin, kanamycin, minocycline
Osmotic sensitivity	1 % NaCl, 3 % sodium sulfate
Oxidizing agents	DL-Thioctic acid
Respiration	Pentachlorophenol (PCP), sorbic acid, sodium caprylate, cinnamic acid, sodium azide
Toxic anion	Sodium nitrite, sodium metaborate, sodium tungstate, sodium arsenate, sodium dichromate
Toxic cation	Caesium chloride, thallium(I) acetate, chromium chloride, ferric chloride, aluminium sulfate, boric acid, potassium tellurite, sodium periodate, potassium chromate, sodium metasilicate
Chelating agents	Sodium pyrophosphate decahydrate, EGTA, EDTA, fusaric acid
Others†	5-Fluoroorotic acid, chlorambucil, thiosalicylate, ethionamide, patulin, apramycin, triclosan, chloroxylenol, aminotriazole, PMSF, phenylarsine oxide

*IBS148 contains a spectinomycin-resistant gene.

†Mode of action includes anti-capsule, antimicrobial, fatty-acid synthesis, histidine biosynthesis, tyrosine phosphatase.

semiquantitative RT-PCR analysis. RNA was extracted from strains UA159 and IBS148 after growth reached mid-exponential phase. Semiquantitative RT-PCR was performed using *liaR*-specific primers (Chong *et al.*, 2008) to measure the level of *liaR* expression; the level of *gyrA* transcript was also measured to ensure that equal amounts of RNA were being used in the RT-PCR assay. As expected, the level of *liaR* transcript in IBS148 was equivalent to that of the wild-type strain UA159 (Fig. 1b), indicating that the insertion of the *aad* gene in *liaS* has little or no effect on the transcription of the downstream genes.

Since PM analysis relies on bacterial growth in media containing various growth inhibitors or toxic compounds, we wanted to measure the growth kinetics of IBS148 under nutrient-rich standard growth conditions. Both IBS148 and wild-type UA159 were grown in THY medium at 37 °C under static (microaerophilic) conditions. As shown in Fig. 1(c), the growth kinetics for both UA159 and IBS148 were very similar, indicating that there was no obvious growth defect in the *liaS* mutant.

PM analysis was first performed using the metabolic panels (PM1–8). There was no significant difference in the carbon utilization panels (PM1–2, data not shown). This was expected, since LiaS is not involved in sensing nutritional signals (Mascher, 2006; Mascher *et al.*, 2006). The signals

for the rest of the metabolic panels (PM3–8) were very low, which made the comparison difficult (data not shown). These panels include the nitrogen utilization panels (PM3, 6–7), phosphate and sulfate panel (PM4), and nutrient stimulation panel (PM5). The poor growth in these metabolic panels was not surprising since a previous study also demonstrated poor growth of a wild-type NG-8 strain, a different *Strep. mutans* strain from the one used in this study (Biswas & Biswas, 2005).

PM analysis in the osmotic panel (PM10) generated mixed results (data not shown). In the majority of the wells, there were no growth advantages for either of the strains. However, UA159 produced stronger signals in media containing 4 % urea and 0.2 M sodium benzoate. In contrast, IBS148 generated stronger signals in media containing 1 % NaCl or 3 % sodium sulfate. However, this growth advantage of IBS148 may not be significant since the growth kinetics of both IBS148 and UA159 were similar in THY medium supplemented with 1 % NaCl or 3 % sodium sulfate (data not shown).

In the pH panel (PM9) there were no signals detected in about half the wells (data not shown). In the remaining wells, there were no significant differences between the wild-type and the *liaS* mutant strains. However, it was previously reported that a *liaS* mutant derivative of an NG-

Fig. 1. (a) Schematic diagram of the *lia* locus in the wild-type UA159 and *liaS* mutant strains. Directions of transcription from the open reading frames are depicted by block arrows, with the bent arrow and the lollipop showing putative promoter and rho-independent terminator, respectively. A part of the *liaS* ORF is deleted by *XhoI* (Xh) and *XbaI* (Xb) digestion and replaced with a spectinomycin-resistant gene *aad* (Sp). (b) Level of downstream *liaR* transcription measured by semiquantitative RT-PCR in the wild-type (UA159) and *liaS* mutant (IBS148). (c) Growth kinetics of UA159 and IBS148 in rich medium (THY). (d) Growth kinetics of UA159 and IBS148 in THY medium buffered with citrate/phosphate at the indicated pHs. Cultures were grown at 37 °C under microaerophilic conditions. Growth experiments were repeated at least three times and a representative growth curve is shown.

8 strain displayed a growth defect at pH 5.0 (Li *et al.*, 2002). Under the PM condition tested, both UA159 and IBS148 grew equally well at pH 5.0, but they both failed to grow below pH 5.0 in PM plates. Failure to grow below pH 5.0 under the PM condition tested is not a strain-specific phenomenon, since a similar growth defect was previously reported for *Strep. mutans* NG-8 strain (Biswas & Biswas, 2005). We have studied further the pH sensitivity of our IBS148 strain by growing the strain in THY medium buffered with citrate-phosphate buffer (Biswas *et al.*, 2007). As shown in Fig. 1(d), IBS148 grew poorly compared to the wild-type UA159 in media with pH 6.0. At pH 5.5, both the strains grew poorly, whereas at pH 7.0 both the strains grew equally well. Thus, taken together, our results show that LiaS is indeed involved in the acid-tolerance response of *Strep. mutans*. However, the apparent discrepancy between the growth of the cultures in the PM plate (at pH 5.0) and in the THY-buffer broths remain to be examined.

Surprisingly, PM analyses for the chemical sensitivity panels generated many positive differences for the IBS148 strains (Fig. 2). A list of the various inhibitors and toxic substances that produced a growth advantage for IBS148 is compiled in Table 1. IBS148 showed better growth than the wild-type UA159 in the presence of many antibiotics that target cell-wall biosynthesis, such as phosphomycin, D-cycloserine and cefoxitin (Fig. 2). A growth advantage was also observed for IBS148 in the presence of polymyxin B and colistin; both these chemicals target the cell membrane.

Compounds that also produced positive phenotypes for strain IBS148, but not the wild-type strain, include chemicals that block nucleotide biosynthesis, DNA replication or DNA unwinding, or induce DNA damage. Other chemicals such as folate antagonist, inhibitor of ribonucleotide reductase, and nucleotide analogues inhibited the growth of the wild-type UA159, but not the growth of IBS148. Similarly, IBS148 was resistant to many protein-synthesis inhibitors, including chloramphenicol, gentamicin and tetracycline. Resistance to spectinomycin was also detected in IBS148 (Table 1), but this was presumably due to the presence of the *aad* gene used for the disruption of *liaS*. Resistance was also noted with chemicals that interfere with t-RNA synthesis, such as glycine hydroxamate and L-glutamic- γ -hydroxamate.

IBS148 displayed growth advantages in the presence of many toxic anions and cations, such as sodium nitrate, sodium arsenate and potassium tellurite, to name a few (Table 1). Several chelating agents, such as EGTA and EDTA, also generated positive results for IBS148. In addition, growth of IBS148 was better in the presence of chlorambucil, thiosalicylate, ethionamide, patulin, apramycin, triclosan, PMSF and phenylarsine oxide. Cellular targets for some of these compounds such as triclosan (fatty-acid biosynthesis) and PMSF (protease inhibitor) are known, while cellular targets of the other chemicals in *Strep. mutans* are currently unknown.

To confirm the PM sensitivity results, some of the key findings were investigated further by performing inde-

Fig. 2. PM analysis for sensitivity to various antibiotics and toxic compounds. A complete catalogue of the reagents used in the sensitivity panel (PM11–PM20) is listed at <http://www.biolog.com>. The wild-type and the *liaS* mutant were grown in a 96-well plate under different conditions. Growth kinetics were obtained with the OmniLog instrument, a video-based detection system that detects colour development of tetrazolium dye due to bacterial respiration. Growth kinetics of wild-type and *liaS* mutant are superimposed using OmniLog software. The PM kinetic results show consensus data comparing the *liaS* mutant (IBS148, green) and its wild-type parental strain (UA159, red). A growth advantage by the parent is indicated by red, while a growth advantage by the *liaS* mutant is shown by green. When both strains have equal growth response or metabolisms in a well, the red and green kinetics overlap and produce a yellow colour. Two independent growth kinetic experiments were performed. A box around a well indicates a difference in response that was observed in both the experiments. The phenotypic changes are listed in Table 1; for a complete list, please refer to Supplementary Table S1.

pendent studies (Fig. 3). Susceptibilities to some antibiotics were verified using antibiotic disc diffusion assays and MICs were determined using E-tests. As shown in Fig. 3, treatment with phosphomycin (cell-wall inhibitor) or trimethoprim (folate antagonist) resulted in a visible difference in growth for IBS148 when compared with UA159. The MIC for ciprofloxacin (DNA gyrase inhibitor) was also twofold higher in IBS148 (MIC $2.0 \mu\text{g ml}^{-1}$) compared to UA159 (MIC $0.75 \mu\text{g ml}^{-1}$, data not shown). No difference in vancomycin resistance was observed between the UA159 and IBS148 strains (MIC $2.0 \mu\text{g ml}^{-1}$). Taken together, our results demonstrate that IBS148 is more tolerant to several antibiotics that interfere with cell-wall biosynthesis and to compounds that interfere with the overall DNA replication process.

DISCUSSION

In *B. subtilis* and *Staph. aureus*, IM-HKs such as LiaS and other sensor kinases are involved in sensing cell-envelope stresses (Jordan *et al.*, 2008; Mascher, 2006). Although the term ‘cell-envelope stress’ is not well defined, it generally indicates a condition that affects the composition and

integrity of the cell membrane. These IM-HKs function as a sentinel system to detect conditions that presumably damage the integrity of the envelope or interfere with cell-wall synthesis. However, the nature of the signal that is detected by LiaS or its homologues can vary depending on the organisms. In *B. subtilis*, LiaS generally detects damage caused by antibiotics that interfere with lipid II recycling during cell-wall biosynthesis, such as bacitracin or vancomycin (Mascher *et al.*, 2003, 2004); in *Staph. aureus*, VraS responds to an even broader spectrum of antibiotics, such as glycopeptides and β -lactams (Kuroda *et al.*, 2003). In contrast to the wealth of knowledge on the role of LiaS in sensing cell-envelope stress in the above-mentioned organisms, little is known about its function in other Gram-positive pathogens, including *Strep. mutans*. In this study, we report a comprehensive phenotypic analysis of a *liaS* mutant of *Strep. mutans* where we document several unexpected findings.

Unlike its *B. subtilis* and *Staph. aureus* counterparts, inactivation of *liaS* in *Strep. mutans* produced gain-in-function phenotypes. For example, inactivation of *vraS* in *Staph. aureus* made the bacterium more susceptible to treatment with β -lactams and vancomycin (Kuroda *et al.*,

Fig. 3. Sensitivity to various antibiotics. E-strip tests were performed using either strain UA159 or strain IBS148 on THY agar plates. Plates were incubated at 37 °C under micro-aerophilic conditions for 20 h.

2003). In contrast, we observed that deletion of the *liaS* gene in *Strep. mutans* resulted in increased tolerance, and not increased sensitivity, to cell-wall-damaging antibiotics such as phosphomycin and other β -lactams (Table 1). This is quite surprising since LiaS and VraS are positively involved in sensing cell-envelope stresses in *B. subtilis* and *Staph. aureus*, respectively (Mascher, 2006). One explanation could be that LiaS in *Strep. mutans* works differently than its counterpart in other bacteria. In *Strep. mutans*, it is possible that the primary function of LiaS is to inhibit the activity of LiaR, the cognate response regulator. In the absence of LiaS, LiaR might become constitutively activated (due to a change in phosphorylation status), allowing the gene expression necessary to survive cell-envelope stresses. The number and nature of the genes regulated by LiaR and its homologues differ greatly. In *B. subtilis*, LiaR regulates the expression of only two operons, including its own expression. On the other hand, there are approximately 46 genes in *Staph. aureus* and about 21 genes in *L. lactis* that are part of the LiaR regulon (Martinez *et al.*, 2007). Negative regulation of LiaR by LiaS is rather possible in *Strep. mutans*, since a recent study shows that a *liaR* mutant is phenotypically quite similar to its isogenic wild-type parent, while a *liaS* mutant is not (Chong *et al.*, 2008).

We also observed that two other antibiotics, vancomycin and bacitracin, which are known to be involved in LiaS-dependent signalling, have no noticeable effect on the *liaS* mutant. In *B. subtilis*, there are at least three different IM-HKs (LiaS, BceS and YvcQ) that are responsible for sensing different types of cell-envelope stresses. While LiaS can sense many different types of cell-envelope stresses, BceS only responds to bacitracin in *B. subtilis* (Jordan *et al.*, 2008; Mascher, 2006). In *Strep. mutans*, MbrD

(SMU.1009), an IM-HK similar to LiaS, is involved in sensing cell-envelope stress generated by bacitracin (Tsuda *et al.*, 2002). In addition, another sensor kinase, SMU.1965, also plays a role in bacitracin sensitivity (Biswas *et al.*, 2008). Therefore, it is possible that either of these two sensor kinases, or both, may be involved in sensing cell-envelope stress generated by vancomycin, while LiaS takes part in detecting other cell-envelope stresses. However, based on the sequence homology and the genomic context, neither MbrD nor SMU.1965 appears to be the true homologue of LiaS.

We observed that the *liaS* mutant was more tolerant to compounds that interfere with nucleotide synthesis, DNA replication and DNA repair. This is rather surprising, since TCSs are not commonly involved in sensing DNA replication status or damage to DNA. It is possible that changes in the cell-envelope structure in the mutant strain may prevent these chemical agents from penetrating into the cell, or the efflux systems in the mutant are derepressed and the toxic chemicals are pumped out from the cell more efficiently, thereby making the *liaS* mutant more resistant to the action of these chemicals. On the other hand, there are a few examples in which TCSs have been shown to be involved in sensing DNA replication blockages or DNA damages. For example, in *Caulobacter crescentus*, a multi-component signal transduction system involving three sensor kinases (CckA, PleC and DivJ), along with the master response regulator CtrA and another response regulator, DivK, respond to cell-cycle signals and other developmental cues (Jacobs-Wagner, 2004; Jenal, 2000). Moreover, in *Escherichia coli*, the ArcAB system seems to control initiation of DNA replication (Lee *et al.*, 2001). Thus it is also possible that LiaS either directly or indirectly senses DNA replication blockage or DNA damage.

In bacteria, the process of cell division and DNA replication is highly coordinated (Huisman & D'Ari, 1981; Liu *et al.*, 2001). Interruption of DNA replication or chromosomal segregation interferes with proper cell division, which ultimately leads to elongation of cells in rod-shaped bacteria such as *E. coli* and *B. subtilis*. However, in the case of *Enterococcus faecalis*, a spherical-shaped bacterium similar to *Strep. mutans*, inhibition of DNA synthesis by mitomycin C (Higgins *et al.*, 1974) and nalidixic acid (Patel & Weaver, 2006) leads to the inhibition of cell division, and an increase in cell-surface area. We speculate that treatment of *Strep. mutans* with chemicals that block DNA synthesis also leads to improper cell division that causes enlargement of the cell. This cell enlargement disrupts normal cell-envelope integrity, which is recognized by LiaS. Interestingly, a recent report indicates that in *B. subtilis*, a sensor kinase, YycG, coordinates cell-wall architecture during cell division by co-localizing with FtsZ at the cell-division septum and perceiving the signal (Fukushima *et al.*, 2008). *Strep. mutans* also encodes a homologue of YycG, known as VicK; inactivation of *vicK* makes the *Strep. mutans* susceptible to many cell-wall-targeted antibiotics including β -lactams (Biswas *et al.*, 2008). Whether LiaS or VicK also localize at a particular site on the cell surface for detecting cell-envelope stress signals remains to be examined in *Strep. mutans*.

The bacterial cell envelope is one of the crucial cellular structures whose integrity needs to be maintained at all times. Various environmental insults such as acidic pH, high osmotic pressure and toxic chemicals, including antibiotics, can damage the integrity of the cell envelope. To cope with the cell-envelope damage, bacteria have evolved various mechanisms to detect perturbations to the envelope. There are at least four different types of signal transduction systems that sense and respond to cell-envelope stresses in Gram-positive bacteria: TCSs; membrane-anchored anti- σ factor and its corresponding ECF (extracytoplasmic function) σ factor; transmembrane sensory protein BlaR1/MecR1; and a membrane-attached hybrid sensor/transcriptional regulator (Jordan *et al.*, 2008). However, *Strep. mutans* does not encode any alternative σ factors, and no BlaR1/MecR1 system has been identified in its genome. Therefore, we speculate that *Strep. mutans* primarily relies on TCSs to recognize cell-envelope stress, and LiaS is one of the most important IM-HKs that plays a significant role in recognizing cell-envelope stress in this pathogen.

ACKNOWLEDGEMENTS

We thank members of the Biswas laboratory for critically reading this manuscript. This work was supported in part by funding from NIDCR (DE016686) to I.B.

REFERENCES

Ahn, S. J., Wen, Z. T. & Burne, R. A. (2006). Multilevel control of competence development and stress tolerance in *Streptococcus mutans* UA159. *Infect Immun* **74**, 1631–1642.

Ajdic, D., McShan, W. M., McLaughlin, R. E., Savić, G., Chang, J., Carson, M. B., Primeaux, C., Tian, R., Kenton, S. & other authors (2002). Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. *Proc Natl Acad Sci U S A* **99**, 14434–14439.

Banas, J. A. & Vickerman, M. M. (2003). Glucan-binding proteins of the oral streptococci. *Crit Rev Oral Biol Med* **14**, 89–99.

Biswas, S. & Biswas, I. (2005). Role of HtrA in surface protein expression and biofilm formation by *Streptococcus mutans*. *Infect Immun* **73**, 6923–6934.

Biswas, S. & Biswas, I. (2006). Regulation of the glucosyltransferase (*gtfBC*) operon by CovR in *Streptococcus mutans*. *J Bacteriol* **188**, 988–998.

Biswas, I., Drake, L. & Biswas, S. (2007). Regulation of *gpbC* expression in *Streptococcus mutans*. *J Bacteriol* **189**, 6521–6531.

Biswas, I., Drake, L., Erkina, D. & Biswas, S. (2008). Involvement of sensor kinases in the stress tolerance response of *Streptococcus mutans*. *J Bacteriol* **190**, 68–77.

Bochner, B. R., Gadzinski, P. & Panomitros, E. (2001). Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. *Genome Res* **11**, 1246–1255.

Butcher, B. G., Lin, Y. P. & Helmann, J. D. (2007). The *yydFGHIJ* operon of *Bacillus subtilis* encodes a peptide that induces the LiaRS two-component system. *J Bacteriol* **189**, 8616–8625.

Carlsson, J. & Hamilton, I. R. (1994). *Metabolic Activity of Oral Bacteria*, 2nd edn. Copenhagen: Munksgaard.

Chong, P., Drake, L. & Biswas, I. (2008). LiaS regulates virulence factor expression in *Streptococcus mutans*. *Infect Immun* **76**, 3093–3099.

Dalton, T. L. & Scott, J. R. (2004). CovS inactivates CovR and is required for growth under conditions of general stress in *Streptococcus pyogenes*. *J Bacteriol* **186**, 3928–3937.

El-Sharoud, W. M. (2005). Two-component signal transduction systems as key players in stress responses of lactic acid bacteria. *Sci Prog* **88**, 203–228.

Fabret, C., Feher, V. A. & Hoch, J. A. (1999). Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world. *J Bacteriol* **181**, 1975–1983.

Fukushima, T., Szurmant, H., Kim, E. J., Perego, M. & Hoch, J. A. (2008). A sensor histidine kinase co-ordinates cell wall architecture with cell division in *Bacillus subtilis*. *Mol Microbiol* **69**, 621–632.

Gardete, S., Wu, S. W., Gill, S. & Tomasz, A. (2006). Role of VraSR in antibiotic resistance and antibiotic-induced stress response in *Staphylococcus aureus*. *Antimicrob Agents Chemother* **50**, 3424–3434.

Haas, W., Kaushal, D., Sublett, J., Obert, C. & Tuomanen, E. I. (2005). Vancomycin stress response in a sensitive and a tolerant strain of *Streptococcus pneumoniae*. *J Bacteriol* **187**, 8205–8210.

Hamada, S. & Slade, H. D. (1980). Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol Rev* **44**, 331–384.

Higgins, M. L., Daneo-Moore, L., Boothby, D. & Shockman, G. D. (1974). Effect of inhibition of deoxyribonucleic acid and protein synthesis on the direction of cell wall growth in *Streptococcus faecalis*. *J Bacteriol* **118**, 681–692.

Huisman, O. & D'Ari, R. (1981). An inducible DNA replication-cell division coupling mechanism in *E. coli*. *Nature* **290**, 797–799.

Idone, V., Brendtro, S., Gillespie, R., Kocaj, S., Peterson, E., Rendi, M., Warren, W., Michalek, S., Krastel, K. & other authors (2003). Effect of an orphan response regulator on *Streptococcus mutans* sucrose-dependent adherence and cariogenesis. *Infect Immun* **71**, 4351–4360.

- Jacobs-Wagner, C. (2004).** Regulatory proteins with a sense of direction: cell cycle signalling network in *Caulobacter*. *Mol Microbiol* **51**, 7–13.
- Jenal, U. (2000).** Signal transduction mechanisms in *Caulobacter crescentus* development and cell cycle control. *FEMS Microbiol Rev* **24**, 177–191.
- Jordan, S., Junker, A., Helmann, J. D. & Mascher, T. (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.
- Jordan, S., Rietkotter, E., Strauch, M. A., Kalamorz, F., Butcher, B. G., Helmann, J. D. & Mascher, T. (2007).** LiaRS-dependent gene expression is embedded in transition state regulation in *Bacillus subtilis*. *Microbiology* **153**, 2530–2540.
- Jordan, S., Hutchings, M. I. & Mascher, T. (2008).** Cell envelope stress response in Gram-positive bacteria. *FEMS Microbiol Rev* **32**, 107–146.
- Kuramitsu, H. K. (1993).** Virulence factors of mutans streptococci: role of molecular genetics. *Crit Rev Oral Biol Med* **4**, 159–176.
- Kuroda, M., Kuroda, H., Oshima, T., Takeuchi, F., Mori, H. & Hiramatsu, K. (2003).** Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. *Mol Microbiol* **49**, 807–821.
- Lee, Y. S., Han, J. S., Jeon, Y. & Hwang, D. S. (2001).** The arc two-component signal transduction system inhibits in vitro *Escherichia coli* chromosomal initiation. *J Biol Chem* **276**, 9917–9923.
- Lemos, J. A., Abranches, J. & Burne, R. A. (2005).** Responses of cariogenic streptococci to environmental stresses. *Curr Issues Mol Biol* **7**, 95–107.
- Li, Y. H., Lau, P. C., Tang, N., Svensater, G., Ellen, R. P. & Cvitkovitch, D. G. (2002).** Novel two-component regulatory system involved in biofilm formation and acid resistance in *Streptococcus mutans*. *J Bacteriol* **184**, 6333–6342.
- Liu, G., Begg, K., Geddes, A. & Donachie, W. D. (2001).** Transcription of essential cell division genes is linked to chromosome replication in *Escherichia coli*. *Mol Microbiol* **40**, 909–916.
- Loesche, W. J. (1986).** Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev* **50**, 353–380.
- Martinez, B., Zomer, A. L., Rodriguez, A., Kok, J. & Kuipers, O. P. (2007).** Cell envelope stress induced by the bacteriocin Lcn972 is sensed by the lactococcal two-component system CesSR. *Mol Microbiol* **64**, 473–486.
- Mascher, T. (2006).** Intramembrane-sensing histidine kinases: a new family of cell envelope stress sensors in *Firmicutes* bacteria. *FEMS Microbiol Lett* **264**, 133–144.
- Mascher, T., Margulis, N. G., Wang, T., Ye, R. W. & Helmann, J. D. (2003).** Cell wall stress responses in *Bacillus subtilis*: the regulatory network of the bacitracin stimulon. *Mol Microbiol* **50**, 1591–1604.
- Mascher, T., Zimmer, S. L., Smith, T. A. & Helmann, J. D. (2004).** Antibiotic-inducible promoter regulated by the cell envelope stress-sensing two-component system LiaRS of *Bacillus subtilis*. *Antimicrob Agents Chemother* **48**, 2888–2896.
- Mascher, T., Helmann, J. D. & Udden, G. (2006).** Stimulus perception in bacterial signal-transducing histidine kinases. *Microbiol Mol Biol Rev* **70**, 910–938.
- Patel, S. & Weaver, K. E. (2006).** Addiction toxin Fst has unique effects on chromosome segregation and cell division in *Enterococcus faecalis* and *Bacillus subtilis*. *J Bacteriol* **188**, 5374–5384.
- Tsuda, H., Yamashita, Y., Shibata, Y., Nakano, Y. & Koga, T. (2002).** Genes involved in bacitracin resistance in *Streptococcus mutans*. *Antimicrob Agents Chemother* **46**, 3756–3764.
- Ullman, R. F., Miller, S. J., Strampfer, M. J. & Cunha, B. A. (1988).** *Streptococcus mutans* endocarditis: report of three cases and review of the literature. *Heart Lung* **17**, 209–212.
- Verneuil, N., Sanguinetti, M., Le Breton, Y., Posteraro, B., Fadda, G., Auffray, Y., Hartke, A. & Giard, J. C. (2004).** Effects of the *Enterococcus faecalis* *hypR* gene encoding a new transcriptional regulator on oxidative stress response and intracellular survival within macrophages. *Infect Immun* **72**, 4424–4431.
- Yin, S., Daum, R. S. & Boyle-Vavra, S. (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.
- Zhou, L., Lei, X. H., Bochner, B. R. & Wanner, B. L. (2003).** Phenotype microarray analysis of *Escherichia coli* K-12 mutants with deletions of all two-component systems. *J Bacteriol* **185**, 4956–4972.

Edited by: M. Kilian