A phenotypic microarray analysis of a Streptococcus mutans liaS mutant

Jiaquin Zhang1,2 and Indranil Biswas1

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

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). Streptococcus mutans is also an inducer of infective endocarditis, with more than 14% of viridans streptococci-induced endocarditis caused by Streptococcus mutans (Hamada & Slade, 1980; Loesche, 1986; Ullman et al., 1988). Streptococcus 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, Streptococcus 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 Streptococcus 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 content that resides in
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., 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 (5, 300 μg ml⁻¹) was included in the growth medium. For some confirmatory growth experiments, *Strep. mutans* cultures were grown in THY containing 1 % NaCl, 3 % NaSO₄ 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 anaad 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
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.

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).

PM analysis in the pH panel (PM9) there were no signals detected in about half the wells (data not shown). In the majority of the 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-

Table 1. Growth advantages in the liaS mutant

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

*IBS148 contains a spectinomycin-resistant gene.
†Mode of action includes anti-capsule, antimicrobial, fatty-acid synthesis, histidine biosynthesis, tyrosine phosphatase.
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.

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 chemicals 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-
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 \( \mu \)g ml\(^{-1}\)) compared to UA159 (MIC 0.75 \( \mu \)g ml\(^{-1}\), data not shown). No difference in vancomycin resistance was observed between the UA159 and IBS148 strains (MIC 2.0 \( \mu \)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 \( \beta \)-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 \( \beta \)-lactams and vancomycin (Kuroda et al.,...
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 (Martínez 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-sigma 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


Edited by: M. Kilian