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## Characterization of the *Streptococcus sobrinus* acid stress response by interspecies microarrays and proteomics

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### SUMMARY

*Streptococcus mutans* and *Streptococcus sobrinus* are considered the primary organisms responsible for human dental caries. The ability to generate acids and to adapt to low pH conditions is directly associated with the cariogenic potential of these bacteria. To survive acidic conditions, both species have been shown to mount an acid tolerance response (ATR). However, previous characterization of the *S. sobrinus* ATR identified critical differences in the mechanisms of acid adaptation between *S. mutans* and *S. sobrinus*. Here, interspecies microarray and proteomic approaches were used to identify novel, previously unrecognized genes and pathways that participate in the *S. sobrinus* acid stress response. The results revealed that, among other things, metabolic alterations that enhance energy generation and upregulation of the malolactic fermentation enzyme activity constitute important acid resistance properties in *S. sobrinus*. Some of these acid adaptive traits are shared by *S. mutans* and might be considered optimal targets for therapeutic treatments designed to control dental caries.

### Keywords

caries; mutans streptococci; acid stress; *mleS*

### INTRODUCTION

Dental caries is a complex and diverse disease that results from interactions of acidogenic/aciduric bacteria colonizing the tooth surface with the oral environment. Although other species may also be involved, two species of the mutans streptococci (MS) group, *Streptococcus sobrinus* (serotypes *d* and *g*) and *Streptococcus mutans* (serotypes *c*, *e*, *f* and *k*) are considered major etiological agents of human dental caries (Liljemark & Bloomquist, 1996, Lemos & Burne, 2008, Loesche, 1986). The implication of MS in dental caries is grounded on at least four factors; (i) MS are isolated in high numbers from carious lesions, (ii) are absent or found in low numbers in caries-free subjects, (iii) *in vitro* studies demonstrated that MS are highly acidogenic and aciduric, and (iv) experiments with animals confirmed the extreme cariogenic nature of these organisms. It is generally accepted that *S. mutans* can be more frequently isolated from carious lesions than *S. sobrinus* (Loesche, 1986, Carlsson *et al.*, 1987). However, a number of studies suggested that *S. sobrinus* may be more cariogenic than *S. mutans* (de Soet *et al.*, 1991, de Soet *et al.*, 1989, Beighton, 2005), and is more closely associated with smooth surface caries (Lindquist & Emilson,

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1991). In addition, clinical studies have suggested that children harboring both *S. mutans* and *S. sobrinus* had a higher incidence of dental caries than those with *S. mutans* alone (Okada *et al.*, 2005).

The virulence of MS is directly related to properties that enable these organisms to colonize and thrive on the tooth surfaces during acidic conditions. These properties include the production of extracellular polysaccharides such as glucans that allow the bacteria to firmly adhere to the tooth surface in a biofilm, the ability to metabolize sugars obtained from salivary glycoproteins and from the host diet to generate acid end products (acidogenicity), and the ability to tolerate abundant amounts of lactic acid generated by the metabolism of these sugars (aciduricity) (Lemos & Burne, 2008).

In addition to being inherently aciduric, both *S. mutans* and *S. sobrinus* are able to mount an acid tolerance response (ATR). In *S. mutans*, the ATR is characterized by increased acid resistance, enhanced glycolytic capacities, increased activity of the H<sup>+</sup>-translocating F<sub>1</sub>F<sub>0</sub> ATPase enzyme, cell envelope alterations, and possibly, cytoplasmic alkalization (Belli & Marquis, 1991, Fozo & Quivey, 2004, Griswold, *et al.*, 2006, Hamilton & Buckley, 1991, Quivey *et al.*, 2000, Sheng & Marquis, 2007). While the ATR of *S. mutans* have been studied in considerable detail, much less is known about the mechanisms employed by *S. sobrinus* to survive environmental acidification. Factors such as the genetic amenability of *S. mutans* and availability of the *S. mutans* UA159 genome sequence (Ajdic *et al.*, 2002) have clearly contributed to this disparity. Moreover, because of the close relatedness of these two species, the mechanisms for acid adaptation of these bacteria have tacitly been considered to be identical. However, an initial physiologic characterization of the *S. sobrinus* ATR revealed that there are critical differences in the mechanisms of acid adaptation between *S. mutans* and *S. sobrinus* (Nascimento *et al.*, 2004). More specifically, while the ATR of *S. sobrinus* is also characterized by increased acid resistance and enhanced glycolytic capacities, this organism appears to rely on different strategies to adapt to growth at low pH. For example, while the acid adaptation of *S. mutans* is directly correlated to increases in F-ATPase activity (Belli & Marquis, 1991, Hamilton & Buckley, 1991), the F-ATPase enzyme activity showed no significant increases in expression in *S. sobrinus* cells grown at an acidic pH (Nascimento *et al.*, 2004). Instead, expression of the *S. sobrinus* glucose-specific phosphoenolpyruvate sugar:phosphotransferase system (PTS) was enhanced by growth at low pH. Interestingly, the glucose-PTS of *S. mutans* shows the opposite trend and was repressed during growth at low pH (Nascimento *et al.*, 2004, Hamilton & Ellwood, 1978, Vadeboncoeur *et al.*, 1987). In addition to differences in the activities of the F-ATPase during acid adaptation, work from the Quivey lab showed that *S. mutans* UA159 increases the proportions of long-chained monounsaturated membrane fatty acids in response to the acidification of the environment but found only modest changes in the membrane fatty acid composition of *S. sobrinus* 6715 grown under the same conditions (Quivey *et al.*, 2000). Collectively, these results indicate that there are important differences in the mechanisms underlying acid tolerance in these two closely related species. Thus, the continued identification and dissection of the mechanisms associated with the acid stress responses of *S. sobrinus* is central for developing a complete understanding of the pathogenesis of dental caries.

In this report, we further characterized the acid adaptive mechanisms of *S. sobrinus* by using a combination of transcriptomic and proteomic analysis. In particular, we took advantage of the close genetic relatedness between *S. mutans* and *S. sobrinus* and the availability of *S. mutans* UA159 DNA microarrays, and used interspecies microarray hybridizations to identify previously unrecognized genes and pathways that participate in the *S. sobrinus* acid stress responses.

## METHODS

### Growth conditions

*S. sobrinus* 6715 was grown in a BioFlo2000 Fermentor (New Brunswick Scientific, Edison, N.J.) with a working volume of 1 L in TY base medium (3% tryptone and 0.5% yeast extract) containing 1% glucose. The dilution rate of the culture was  $0.24 \text{ h}^{-1}$ , resulting in a generation time of 2.9 h. Initially, the culture was maintained at pH 7 by addition of 2 N KOH. After 10 generations and a cell density of approximately 1.4 at  $\text{OD}_{600}$ , cultures were considered to have achieved steady-state, and 40 ml aliquots were harvested from the vessel by aspiration, centrifuged at 4000 rpm for 12 min at 4°C, pellets were quick-frozen in a dry-ice ethanol bath and stored at  $-80^{\circ}\text{C}$  for later analysis (unadapted cells). Cells growing at pH 7 steady-state were acid shocked to pH 5 using 2 N HCl. Titration time of the vessel from pH 7 to pH 5 was about 2 min. Cells were allowed to recover at pH 5 for another 10 generations, reaching a cell density of approximately 1.1 at  $\text{OD}_{600}$  before samples were harvested (acid-adapted cells). Three additional chemostat runs were obtained for a total of four independent experiments.

To compare the levels of malolactic fermentation (MLF) of cells grown in acidic pH with those cells grown at neutral pH, *S. mutans* UA159 and *S. sobrinus* 6715 were grown for 16 h at 37°C in TY broth containing 25 mM glucose and 25 mM L-malate at pH 7 and pH 5.5. The pH 7 culture was buffered with potassium phosphate (0.2 M) and the acidic TY medium was adjusted to pH 5.5 using 2 N HCl.

### Genomic DNA microarrays

In order to determine whether *S. mutans* UA159 microarray slides were suitable to study the transcriptome of *S. sobrinus* 6715, we hybridized *S. sobrinus* 6715 genomic DNA to the *S. mutans* UA159 slide array. The UA159 microarrays used were obtained from the Pathogen Function Genomics Resource Center (PFGRC) at the J. Craig Venter Institute. Each slide has 1,948 70-mer oligonucleotides printed on the surface of the slide, representing 1,960 open reading frames. Additional information about the UA159 arrays can be found at [http://pfgrc.jcvi.org/index.php/microarray/array\\_description/streptococcus\\_mutans/version1.html](http://pfgrc.jcvi.org/index.php/microarray/array_description/streptococcus_mutans/version1.html). To perform the genomic DNA hybridizations, *S. mutans* UA159 and *S. sobrinus* 6715 genomic DNA were extracted as described elsewhere (Burne *et al.*, 1987) and sheared into fragments of approximately 300 to 600 bp by two sonication steps of 1 min each. The sheared DNA was purified using QIAquick PCR purification kit (Qiagen, Chatsworth, CA) and eluted in sterile water. Then, 1 µg of sheared DNA was treated with Klenow and labeled with Cy3 or Cy5 as detailed in the M009 protocol provided by PFGRC (<http://pfgrc.jcvi.org/index.php/microarray/protocols.html>). The labeled *S. sobrinus* genomic DNA was hybridized to *S. mutans* array slides at 42°C using different concentrations of formamide in the hybridization buffer.

### cDNA Microarrays

RNA was isolated from unadapted (pH 7, steady-state) and acid-adapted (pH 5, steady-state) *S. sobrinus* 6715 chemostat cell pellets as detailed elsewhere (Abranches *et al.*, 2006). A reference RNA was prepared from a large-scale culture of *S. sobrinus* 6715 cells grown in BHI to an optical density of 0.5 at 600 nm. The benefits and analytical methods associated with the utilization of a reference RNA as a normalization tool in microarray experiments are discussed elsewhere (Liu & Slininger, 2007). The *S. sobrinus* 6715 cDNA that was hybridized to the *S. mutans* UA159 arrays was generated according to PFGRC protocol found at <http://pfgrc.jcvi.org/index.php/microarray/protocols.html>, with minor changes. Briefly, 5 µg of RNA was incubated with SuperScriptIII Reverse Transcriptase (Invitrogen, Carlsbad, CA) to increase the amount of cDNA synthesized. The aminoallyl-labeled

reference cDNA was coupled with Cy-5 mono-Reactive Dye (Amersham Biosciences, Piscataway, NJ) and the aminoallyl-labeled chemostat RNA at both pH 5 steady-state and pH 7 steady-state were coupled with Cy-3 mono-Reactive Dye (Amersham Biosciences). The Cy5 and Cy3 probes were dried separately under vacuum. The hybridization of the labeled cDNA probes was carried out according to the PFGRC protocol found at <http://pfgrc.jcvi.org/index.php/microarray/protocols.html> with a single modification: the stringency of the cDNA hybridization buffer was reduced from the recommended 40% formamide to 20% formamide. Each Cy3 and Cy5 labeled probe was resuspended in hybridization buffer and mixed together before heating the mixture at 95°C for 5 min. To obtain statistical power, four probes made from pH 7 steady-state cDNA and four probes made from pH 5 steady-state cDNA were mixed independently with the reference probes and hybridized to 8 independent *S. mutans* UA159 array slides. The slides were hybridized in a MAUI 4-Bay Hybridization System (BioMicro Systems, Salt Lake City, UT) at 42°C for 16 h. The slides were washed according to PFGRC protocols and were scanned by the GenePix scanner (Axon Instruments, Inc., Union City, CA) at 532 nm (Cy3 channel) and 635 nm (Cy5 channel). Slides were pre-scanned, during which the pixel size was set to 40 µm, the lines to average was set to 1, the sensitivity of the photomultiplier tube (PMT) was adjusted to 33% power and the PMT voltages were adjusted to obtain a Cy5: Cy3 ratio of 1:1. Once the 1:1 ratio was achieved during the pre-scan, the PMT power was adjusted to 100% power, lines to average was set to 2, and the pixel size was set to 5 µm for the full scan.

### Microarray data analysis

Microarray data analysis was performed as previously detailed (Abranches et al., 2006). Briefly, single channel images generated from the array slide scans were uploaded into PFGRC Spotfinder software (<http://www.tm4.org/spotfinder.html>) and images were overlaid. A spot grid that was created according to PFGRC specifications was adjusted manually to fit all the spots within the grid. The intensities of the spots were measured and saved into “.mev” and “.tav” files. Data were normalized using LOWESS and iterative log mean centering with default settings, followed by in-slide replicate analysis using microarray analysis software MIDAS (<http://www.tm4.org/midas.html>). Statistical analysis with a cut-off *P*-value of 0.05 was performed using BRB array tools (<http://linus.nci.nih.gov/BRB-ArrayTools.html>).

### Total protein cell lysates for 2D-electrophoresis and Q-TOF MS/MS

Two dimensional (2D) gels were used to compare protein expression profiles from pH 7 and pH 5 steady-state cultures as previously described (Nascimento *et al.*, 2004). For each growth condition, total cell lysates from three independent chemostat runs were analyzed by 2D electrophoresis at Kendrick Labs (Madison, WI), and the gels were stained with a special silver method (O’Connell and Stults, 1997) that omits the glutaraldehyde step and is compatible with downstream identification by mass spectrometry. Selected protein spots with higher intensity at pH 5 that appeared in every gel replicate were excised from one of the gels and sent to the Proteomics and Mass Spectrometry Facility at the Donald Danforth Plant Science Center (St. Louis, MO), where proteins were digested with trypsin, and analyzed by using a quadrupole-time of flight mass analyzer (Q-TOF MS/MS). Searches of the peptide electrospray tandem mass spectra were performed against the *S. mutans* NCBI non-redundant database as well as a bacterial protein database using MASCOT search engine (<http://www.matrixscience.com>). Unambiguous protein identification was judged by: (i) number of peptide sequences, (ii) protein sequence coverage, (iii) total MASCOT score and individual ion score (a probabilistic implementation of the MOWSE score), and (iv) the quality of the MS/MS spectra judged by a full length *y*-ion series of peptides comprising at least six consecutive amino acids and no missed cleavages.

## Malolactic fermentation assay

The MLF assay was performed as previously described (Sheng & Marquis, 2007) with minor modifications. Briefly, cells were harvested by centrifugation at 4000 rpm for 12 min at 4°C, washed with a 50 mM KCl/1 mM MgCl<sub>2</sub> salt solution and starved at 37°C for 1 h. Starved cells were resuspended at a cell density of 1.1 mg cell dry weight ml<sup>-1</sup> in potassium phosphate buffer (20 mM, pH 7), and 4.5 ml of the suspension was added to a glass vial. Based on the optimal pH for MLF activity in *S. mutans* and *S. sobrinus* (Sheng & Marquis, 2007), vials containing *S. mutans* were adjusted to pH 4 and the vials with *S. sobrinus* were adjusted to pH 4.5. Malolactic fermentation was initiated by adding 0.5 ml of 0.5 M L-malate to pre-warmed (37°C) cell suspension. At selected time points, samples were enzymatically assayed for the presence of L-malate in the supernatant fluid by using a L-malic acid detection kit (Boehringer Mannheim, Darmstadt, Germany).

## RESULTS AND DISCUSSION

### Adaptation of the *S. mutans* microarray protocols for interspecies hybridization

Interspecies microarray hybridizations can be used to quickly discover genes that are present in uncharacterized bacterial genomes, providing that the genome sequence of a close relative is available. Because *S. mutans* and *S. sobrinus* are phylogenetically related, we considered the utilization of *S. mutans* microarrays to identify genes associated with acid stress adaptation in *S. sobrinus*. To assess the feasibility of this approach, we hybridized *S. sobrinus* 6715 genomic DNA against the *S. mutans* UA159 slides. By following the conditions recommended by the PFGRC (protocol M008) for hybridization of genomic DNA (i.e., 50% formamide at 42°C for 16 h), 100% of the spots representing coding regions could be detected using *S. mutans* UA159 genomic DNA. Under those conditions, approximately 80% of the spots could be detected using *S. sobrinus* genomic DNA. Lowering the formamide concentration from 50% to 30% for the interspecies hybridizations allowed for detection of approximately 90% of the coding spots without causing increases in the background represented by 500 *Arabidopsis thaliana* 70-mers. Similarly, reducing the concentration of formamide from 40% to 20% in the hybridization buffer for the cDNA microarrays resulted in an increase in the number of spots detected without compromising specificity (fluorescence ratio of 0.99 with only 85 genes without a signal when analyzed with PFGRC Spotfinder software).

### Identification of *S. sobrinus* acid-inducible genes by interspecies microarray

Hybridization of *S. sobrinus* cDNA obtained from RNA extracted from steady-state cultures grown at pH 7 and pH 5 revealed 93 genes with altered expression ( $P$ -value  $\leq 0.05$ ); 54 down-regulated and 39 up-regulated at pH 5 (Table 1). The gene encoding the malolactic enzyme (*mleS*), involved in malolactic fermentation (MLF), displayed the greatest change in the level of transcription in the microarrays (17.9 fold induction) at pH 5. In *S. mutans*, it has been proposed that MLF, which catalyses the conversion of the dicarboxylic L-malate to the monocarboxylic lactic acid and CO<sub>2</sub>, contributes to the ATR by increasing the cytoplasmic pH via production of CO<sub>2</sub> (Sheng & Marquis, 2007). In addition to *mleS*, three other genes (*smu131*, *smu127* and *smu602*) displayed a greater than 7-fold induction at pH 5. These three genes have not been characterized in related streptococci but, based on BLAST searches, are predicted to encode a lipoate-protein ligase (*lplA*, *smu131*), E1 component of the  $\alpha$ -subunit of a pyruvate/acetoin dehydrogenase enzyme (*adhA*, *smu127*), and a bile acid:sodium-symporter (*smu602*). Notably, *adhA* and *lplA* are closely associated (*adhA-adhB-adhC-adhD-lplA*) in the *S. mutans* genome (Ajdic *et al.*, 2002). LplA ligates lipoate, an essential cofactor, to specific metabolic enzyme complexes in *E. coli*, such as pyruvate dehydrogenases (PDH) (Morris *et al.*, 1994). The PDH enzyme oxidizes pyruvate into acetyl-CoA and CO<sub>2</sub>, and acetyl-CoA is further catabolized into formate, acetate, and



ethanol. PDH is involved in the switch from homofermentation to heterofermentation and has been shown to be active only under aerobic conditions (Carlsson *et al.*, 1985). Heterofermentation is believed to play a role in acid tolerance of *S. mutans* because the metabolic end-products yield weaker organic acids, CO<sub>2</sub>, and one additional ATP per pyruvate molecule (Carlsson *et al.*, 1985). In fact, the levels of PDH enzymes were shown to be induced during acid challenge in *S. mutans* (Welin *et al.*, 2003, Len *et al.*, 2004), and inactivation of a second putative PDH operon (*smu1296-smu1299*) resulted in an acid-sensitive phenotype (Korithoski *et al.*, 2008). In addition to being converted into alternative acids, acetyl CoA is an essential precursor in the synthesis of fatty acids, amino acids, and other metabolic intermediates in bacteria, and increased amounts of acetyl CoA may be required for *de novo* synthesis of membrane fatty acids during acid stress.

Proton-extrusion via the H<sup>+</sup>-translocating F-ATPase is common to all streptococci and is considered critical for acid tolerance because it allows the organism to maintain a cytoplasmic pH that is more alkaline than the extracellular environment (Lemos & Burne, 2008, Cotter & Hill, 2003). In *S. mutans*, transcriptional regulation of the *atp* operon correlates well with a significant increase in the enzyme activity during growth at acidic pH (Belli & Marquis, 1991, Gong *et al.*, 2009). Although the *S. sobrinus* F-ATPase activity is constitutively high, the F-ATPase activity of *S. sobrinus* strains grown at pH 5 showed no significant increases as compared to cells grown at pH 7 (Nascimento *et al.*, 2004). Here, we found that transcription of *atpD*, encoding the  $\beta$ -subunit of the F-ATPase enzyme was up-regulated by low pH (Table 1). Clearly, to fully appreciate the role of the F-ATPase in *S. sobrinus* acid tolerance, further studies are warranted.

The syntheses of intracellular polysaccharides (IPS), which are glycogen-like storage polymers, enhance survival during nutrient starvation and have been shown to contribute to caries formation (Busuioc *et al.*, 2009, Spatafora *et al.*, 1995). Previous work in *S. mutans* have identified and characterized the genes responsible for the accumulation of IPS, including *glgD*, encoding a subunit of glucose-1-phosphate pyrophosphorylase (Busuioc *et al.*, 2009, Spatafora *et al.*, 1995). Here, we found that expression of the *S. sobrinus glgD* homologue was induced by 2.4-fold during growth at low pH (Table 1). The importance of the IPS to *S. mutans* virulence has been largely associated with its use as a source of carbohydrate upon nutrient depletion that, as a result, increases survival and extends acid production by the organism. In *S. mutans*, the expression of the *glg* genes is induced by exogenous glucose (Spatafora *et al.*, 1995), but there has been no evidence that this is also the case for low pH. We assessed the ability of batch-grown *S. sobrinus* 6715 and *S. mutans* UA159 cultures to drop the extracellular pH using endogenous sugar and found that the resting pH for *S. sobrinus* 6715 was at least 0.5 units below the resting pH of *S. mutans* UA159 (data not shown), suggesting that *S. sobrinus* 6715 is more acidogenic than *S. mutans* UA159. Interestingly, previous reports have also shown that *S. sobrinus* strains are able to produce acids more rapidly than *S. mutans*, and that fresh isolates of *S. sobrinus* are more cariogenic in rats than fresh isolates of *S. mutans* (de Soet *et al.*, 1991, de Soet *et al.*, 1989).

A number of other genes that have been associated with stress tolerance in *S. mutans* and other related organisms were up-regulated in our microarrays, including genes encoding for the stringent response effector RelA, the MazF toxin of the MazEF toxin-antitoxin system, DNA repair enzymes (SmxA, NrdE, EndIII), and glutathione reductase (Gor), an important antioxidant enzyme involved in oxidative stress and detoxification (Table 1). The up-regulation of these pathways is not surprising since bacteria are known to depend on multiple and often overlapping pathways for dealing with different stresses. In particular, the association between acid stress responses and DNA repair systems has been reported in *S. mutans* and related lactic acid bacteria (Lemos & Burne, 2008, Cotter & Hill, 2003)

In general terms, the interspecies microarray analysis allowed for the rapid identification of several, previously unrecognized genes that participate in the acid stress responses of *S. sobrinus*. However, it is important to consider the limitations inherent to this technology. A clear limitation is that this approach can only identify genes whose sequences are nearly identical to the fully sequenced relative and it fails to identify genes that are not highly conserved or genes that are unique in the species of interest. Thus, the list presented here is likely to represent only a subset of the genes that participate in acid tolerance, and by no means should be viewed as a complete snapshot of the *S. sobrinus* acid stress genes. To further advance the field, the acquisition of a reference genome sequence for *S. sobrinus* is clearly of high priority. This would allow the development of species-specific gene arrays, but more importantly, it would provide a framework for genomic comparisons with *S. mutans* and other related streptococci.

### Proteomic analysis revealed that many glycolytic enzymes are upregulated at low pH

In addition to the interspecies microarray hybridizations, we used a proteomic approach to identify protein spots with higher intensity at pH 5 (cutoff  $\geq 1.5$ -fold change). A 2D gel approach was used to obtain the protein profile of pH 7 and pH 5 steady-state cultures of *S. sobrinus* 6715 (Fig. 1). Because a protein database of *S. sobrinus* is not available, protein identification was obtained by using Q-TOF MS/MS that allowed for the amino acid sequence assignment from the interpretation of the MS/MS spectra. Mass spectra were searched against bacterial protein databases using MASCOT with 11 out of 18 protein spots confidently identified (Table 2). Among the proteins identified, there were two cases of proteins with two isoforms (Dpr and GapC). Six of the identified proteins (Ldh, FbaA, PfkA, PykA, Pkg, and GapC) are involved in glycolysis/ gluconeogenesis. These same proteins were also found to be up-regulated in a proteomic study of acid adapted cells in *S. mutans* (Len *et al.*, 2004), suggesting that enhanced glycolytic activity during low pH conditions may be a common trait in mutans streptococci. To further support these results, the genes encoding lactate dehydrogenase (*ldh*), fructose-1, 6-biphosphate aldolase (*fbaA*), and 6-phosphofructokinase (*pfkA*) were also up-regulated at pH 5 in our *S. sobrinus*-*S. mutans* interspecies microarrays (Table 1). This observation, along with the previous finding that the glucose-PTS was up-regulated at low pH (Nascimento *et al.*, 2004), suggests that glucose transport and glycolysis are enhanced in cells grown at low pH. We speculate that this increase in glycolysis generates higher rates of ATP production that activates ATP-dependent pathways used to enhance the ability of the cells to maintain  $\Delta$ pH across the membrane. However, it should be noted that cell density was lower at pH (OD<sub>600</sub>  $\approx$  1.1) than at pH 7 (OD<sub>600</sub>  $\approx$  1.4). Thus, it is possible that at pH 5 genes encoding glycolytic enzymes could be induced due to an increase in glucose availability, or to cell-density dependent signaling.

In addition to proteins associated with energy metabolism, GdhA, a glutamate dehydrogenase involved in the biosynthesis of glutamate (Cvitkovitch *et al.*, 1997), NrdE, associated with DNA replication and repair (Jordan *et al.*, 1996) and Dpr, an iron-binding protein involved in oxygen tolerance (Yamamoto *et al.*, 2000), were also identified as up-regulated at pH 5. Glutamate serves as a precursor for the synthesis of other amino acids and proteins and has been found to be a required nutrient in both *S. mutans* and *S. sobrinus* (Terleckyj & Shockman, 1975). Moreover, glutamate has been shown to play a role in the ATR of *E. coli* and *Lactococcus lactis* (Castanie-Cornet *et al.*, 1999, Sanders *et al.*, 1998). The NrdE protein corresponds to the  $\alpha$ -subunit of the ribonucleotide reductase enzyme that catalyzes the reduction of ribonucleotide di- or triphosphates providing the molecules necessary for DNA replication and repair (Jordan *et al.*, 1996). Of note, the gene encoding NrdE was also upregulated in our microarray analysis (Table 1). Finally, Dpr is an antioxidant iron-binding protein involved in peroxide resistance by preventing the Fenton

reaction through sequestration of intracellular free iron ions (Yamamoto *et al.*, 2000). Notably, transcription of *dpr* was induced by low pH in *S. mutans* (Gong *et al.*, 2009), and a  $\Delta dpr$  strain of *Streptococcus pyogenes* showed reduced survival rate at low pH when compared to parent strain (Tsou *et al.*, 2008) suggesting that Dpr might be a general stress protein.

The 2D gel approach only reveals a “narrow-slice” of the cell’s total proteome. It is important to recognize the limitations inherent to the 2D gel approach, with limited sample capacity and detection sensitivity that reduces the ability to detect proteins with medium or low abundance, and with membrane proteins being frequently underrepresented because of poor solubility. Regardless of these limitations, the proteomic data showed good correlation with the transcriptomic data with four of the nine identified proteins also identified in the microarray analysis (Ldh, FbaA, PfkA and NrdE), and with a trend suggesting upregulation of metabolic enzymes by low pH observed with both technologies.

### MLF activity is enhanced at low pH

It has been previously demonstrated that the catabolism of L-malate serves to protect *S. mutans* against acid killing *in vitro* (Sheng & Marquis, 2007). It was interesting to note that transcription of the gene encoding the malolactic enzyme (*mleS*) displayed the greatest change in the microarrays (17.8-fold induction). On the other hand, a recent global transcriptomic analysis of acid-inducible genes did not indicate upregulation of the *mle* genes by environmental pH in *S. mutans* (Gong *et al.*, 2009). Therefore, we hypothesized that MLF could play a larger role in the acid tolerance of *S. sobrinus*. To test this hypothesis, we assessed MLF activity in *S. sobrinus* 6715 and *S. mutans* UA159. Because MLF activity is not detected in the absence of malate, we used batch cultures containing L-malate buffered at pH 7 and pH 5.5. When the terminal pH of the pH 7 potassium phosphate buffered and pH 5.5 cultures were measured, the pH was approximately 6 and 4.5, respectively for both *S. mutans* and *S. sobrinus*. When compared to pH 7 grown cells, MLF activity was significantly higher (approximately 2.5-fold induction) in pH 5.5 grown cells in both organisms (Fig. 2) but there was no significant difference between *S. mutans* and *S. sobrinus* MLF activity under the tested conditions. This result indicates that increased MLF activity may be part of a common acid resistance mechanism shared by *S. mutans* and *S. sobrinus*.

### Concluding remarks

In this report, transcriptomic and proteomic approaches have been used to identify novel, previously unrecognized acid tolerance genes and pathways in *S. sobrinus*. Our results indicated that to survive the acid challenge, among other things, *S. sobrinus* relies on the up-regulation of key metabolic activities (PDH and glycolysis) and increased MLF activity. Notably, some of these acid tolerance properties are shared by *S. mutans*, and might be considered ideal targets for the development of new anti-caries strategies. Clearly, the continued investigation of the mechanisms that allow *S. sobrinus* to thrive in dental plaque is central to developing a better understanding of the pathogenesis of dental caries. Unfortunately, progress in this field has been hampered by the lack of a reference genome sequence and by the extremely refractory nature of *S. sobrinus* to genetic manipulations. Work is underway to identify and develop new strategies to enhance transformability of *S. sobrinus*.

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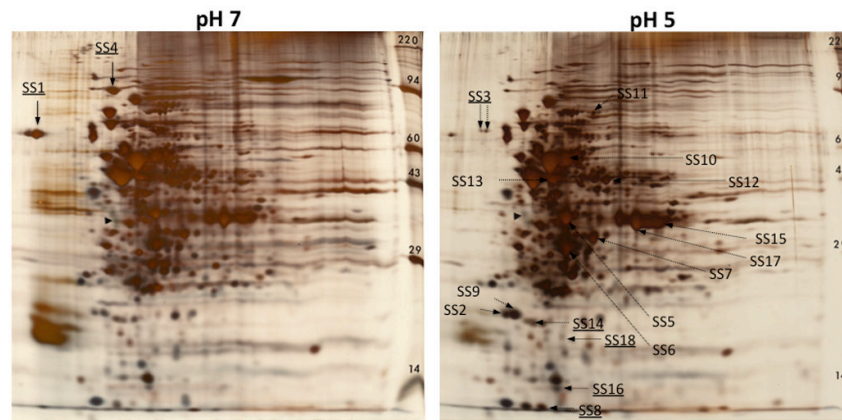
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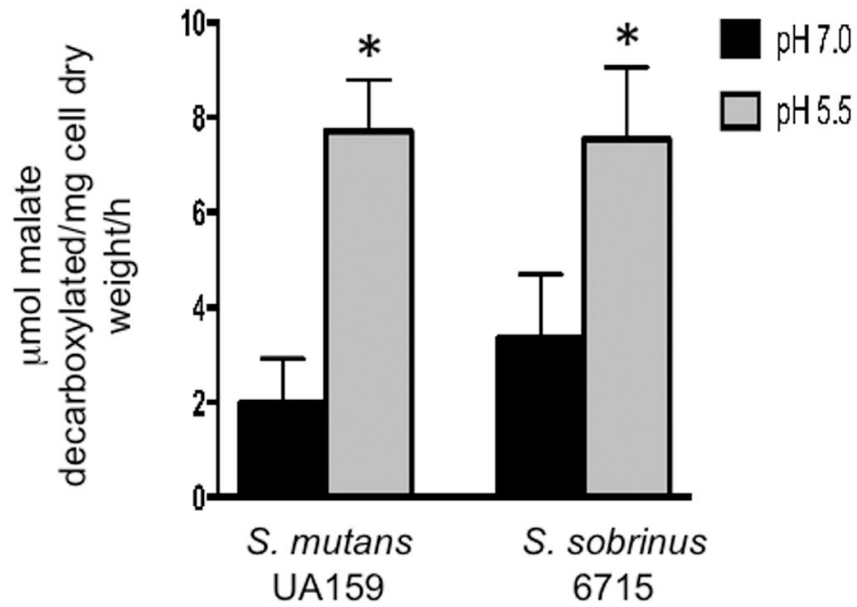
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**Fig. 1.** 2D protein patterns of *S. sobrinus* 6715 grown to pH 7 and pH 5 steady-state levels. The silver-stained proteins with enhanced expression at pH 5 (fold-change  $\geq 1.5$ ) that were identified by Q-TOF MS/MS are indicated in the pH 5 gel image, and proteins that were enhanced at pH 7 (fold change  $\geq 1.5$ ) are indicated in the pH 7 gel image. Underlined spot ID labels indicate proteins that were subjected to Q-TOF MS/MS analysis but the results failed to meet the criteria for confident identification. Refer to Table 2 for name and description of identified proteins. The filled triangle indicates tropomyosin (33 kDa, pI 5.2) loaded as an internal control.



**Fig. 2.** Malolactic fermentation activity of *S. sobrinus* 6715 and *S. mutans* UA159 at pH 5.5 and pH 7. *S. sobrinus* and *S. mutans* cells were grown in TY broth (adjusted with HCl, pH 5.5 or buffered with potassium phosphate, pH 7), supplemented with 25mM glucose and 25mM L-malate until stationary phase. Cells were harvested, washed with salt solution, and resuspended with 50 mM L-malate. The results represent the means  $\pm$  the standard deviation of three independent experiments. (\*)  $P \leq 0.05$ .

Table 1

Genes differentially expressed in *S. sobrinus* 6715 using interspecies microarrays.

Unique ID (GenBank)	Description	Difference (n-fold) of geom. means pH 5/ pH 7	Parametric P value
<b>Up-regulated genes</b>			
<i>Energy metabolism</i>			
SMU.137	malolactic enzyme, <i>mleS</i>	17.893	3.00E-07
SMU.131	putative lipoate-protein ligase, <i>lplA</i>	14.891	3.00E-07
SMU.127	pyruvate/ acetoin dehydrogenase, E1 component $\alpha$ , <i>adhA/acoA</i>	8.646	1.4E-06
SMU.1528	F <sub>0</sub> F <sub>1</sub> membrane-bound proton-translocating ATPase, $\beta$ subunit, <i>atpD</i>	2.742	0.0032706
SMU.1537	glycogen biosynthesis protein, <i>glgD</i>	2.405	0.0081713
SMU.670	aconitate hydratase aconitase, <i>citB/acnA</i>	2.097	0.0146193
SMU.275	L-ribulose 5-phosphate 4-epimerase, <i>rmpF</i>	1.857	0.0287093
SMU.1115	lactate dehydrogenase, <i>ldh/lct</i>	1.850	0.0154089
SMU.99	fructose-1,6-biphosphate adolase, <i>sorG/fbaA</i>	1.660	0.0285881
SMU.1191	6-phosphofructokinase, <i>pfkA</i>	1.593	0.0426727
<i>Transport and binding</i>			
SMU.602	putative sodium-dependent transporter	7.627	0.000666
SMU.1668	putative branched chain amino acid ABC transporter, <i>livH</i>	2.892	0.001657
SMU.248	ABC transporter, membrane protein	1.954	0.0072554
SMU.827	polysaccharide ABC transporter, permease protein, <i>rgpCc</i>	1.953	0.0253459
SMU.1927	ABC transporter, ATP-binding protein, <i>psaA</i>	1.880	0.0126816
SMU.247	putative ABC transporter, ATP-binding protein, <i>yurY</i>	1.758	0.0380766
SMU.1347	ABC transporter permease protein, <i>ylbB</i>	1.734	0.0286651
<i>Amino acid biosynthesis</i>			
SMU.873	putative homocysteine methyltransferase methionine synthase II, <i>metE/metC</i>	1.792	0.035998
<i>Regulatory functions</i>			
SMU.2044	stringent response protein, ppGpp synthetase, <i>relA</i>	2.959	0.0357087
SMU.1515	conserved hypothetical protein CovX (VicX), <i>covX/vicX</i>	2.368	0.0063014
SMU.479	RNA polymerase-associated protein RpoZ, $\Omega$ subunit, <i>rpoZ</i>	2.047	0.0113671
<i>Protein synthesis</i>			
SMU.1311	asparaginyl-tRNA synthetase, <i>asnS</i>	1.829	0.043923
SMU.1272	histidyl-tRNA synthase, <i>hisZ/hisS</i>	1.818	0.0466654



Unique ID (GenBank)	Description	Difference (n-fold) of geom. means pH 5/ pH 7	Parametric P value
<i>Central intermediary metabolism</i>			
SMU.249	putative NifS protein homologue, class-V aminotransferase, <i>nifS</i>	3.007	0.0005185
SMU.1415	putative phosphatases involved in N-acetyl-glucosamine catabolism, <i>nagD/yIfH</i>	2.043	0.0240285
<i>DNA metabolism and repair</i>			
SMU.1649	exodeoxyribonuclease III, <i>exoA/smxA</i>	2.725	0.0006884
SMU.1650	endonuclease III (DNA repair), <i>end3</i>	1.789	0.0359052
SMU.991	similar to ribonucleotide reductase $\alpha$ subunit, <i>nrdE</i>	2.267	0.0059736
<i>Detoxification</i>			
SMU.838	glutathione reductase, <i>gshR/gor</i>	1.791	0.021416
SMU.1451	putative alpha-acetolactate decarboxylase, <i>aldB</i>	2.860	0.0018603
<i>Cell division</i>			
SMU.1713	segregation and condensation protein A, <i>scpA</i>	1.746	0.0334277
SMU.15	cell division protein FtsH, <i>ftsH</i>	1.682	0.0443664
<i>Hypothetical proteins</i>			
SMU.1700	hypothetical protein possible LrgB family protein	3.200	0.0121089
SMU.1755	conserved hypothetical protein	2.202	0.0036035
SMU.573	conserved hypothetical protein	2.127	0.0468976
SMU.564	conserved hypothetical protein	2.125	0.0349508
SMU.591	hypothetical protein	1.850	0.0192613
SMU.748	hypothetical protein	1.781	0.0370629
SMU.173	putative ppGpp-regulated growth inhibitor, <i>mazF</i>	1.695	0.027644
<b>Down-regulated genes</b>			
<i>Energy metabolism</i>			
SMU.671	citrate synthase, <i>citZ</i>	0.291	0.0003693
SMU.1011	putative CitG protein, <i>citG</i>	0.171	0.0003965
<i>Transport and binding</i>			
SMU.998	ABC transporter, periplasmic ferrichrome-binding protein, <i>yclQ/fatB</i>	0.592	0.0415653
SMU.568	amino acid ABC transporter, ATP-binding protein, <i>glnQ</i>	0.538	0.0313816
SMU.2116	osmoprotectant amino acid ABC transporter, ATP-binding, <i>optCA</i>	0.414	0.0034014
SMU.460	putative amino acid ABC transporter, permease	0.374	0.0025901
SMU.461	putative amino acid ABC transporter, ATP-binding protein	0.261	0.0006624
SMU.933	putative amino acid ABC transporter, periplasmic amino acid, <i>atmA</i>	0.147	1.21E-05
SMU.935	putative amino acid ABC transporter	0.135	5.18E-05

Unique ID (GenBank)	Description	Difference (n-fold) of geom. means pH 5/ pH 7	Parametric P value
<i>Amino acid biosynthesis</i>			
SMU.1836	DAHP synthase phosphor-2-dehydro-3-deoxyphosphoheptonate adolase, <i>aroG</i>	0.655	0.0427644
SMU.317	putative tetrahydrodipicolinate succinylase, <i>ykuQ/ dapD</i>	0.569	0.0315775
SMU.335	argininosuccinate lyase, <i>argH</i>	0.563	0.0445401
SMU.1383	3-isopropylmalate dehydrogenase, <i>leuB</i>	0.530	0.0105533
SMU.1382	3-isopropylmalate dehydratase, large subunit, <i>leuC</i>	0.499	0.014424
SMU.784	5-enolpyruvylshikimate-3-phosphate synthase, <i>aroA</i>	0.460	0.0314230
SMU.990	dihydrodipicolinate synthase, <i>dapA</i>	0.385	0.0429038
SMU.1653	D-3-phosphoglycerate dehydrogenase, <i>serA</i>	0.324	0.0001565
<i>Regulatory function</i>			
SMU.1037	Putative histidine kinase, <i>phoR</i>	0.613	0.0425196
SMU.1958	fructose-specific enzyme IIC component, <i>levF</i>	0.149	0.0001353
<i>Biosynthesis of proteins/ purine ribonucleotides/cofactors</i>			
SMU.2003	30S ribosomal protein S13, <i>rs13/rpsM</i>	0.613	0.0482857
SMU.2157	inosine monophosphate dehydrogenase, <i>guaB/impH</i>	0.454	0.0077039
SMU.465	NAD(+) synthetase (nitrogen regulatory protein), <i>nadE</i>	0.386	0.0011386
<i>Central intermediary metabolism</i>			
SMU.376	putative aminotransferase	0.366	0.0406214
SMU.636	N-acetylglucosamine-6-phosphate isomerase, <i>nagB</i>	0.203	0.0026116
<i>DNA metabolism</i>			
SMU.1002	DNA topoisomerase I, <i>topA</i>	0.556	0.0220572
<i>Cell division</i>			
SMU.2165	chromosome segregation protein, ParB-like nuclease domain, <i>smc</i>	0.581	0.0303665
<i>Protein fate/chaperone</i>			
SMU.1929	protease HtpX, heat shock protein, <i>htpX</i>	0.563	0.0345522
SMU.91	peptidyl-prolyl isomerase RopA (trigger factor), <i>ropA</i>	0.560	0.0455648
SMU.539	signal peptidase type IV, <i>hopD</i>	0.439	0.0159205
<i>Hypothetical proteins</i>			
SMU.1151	conserved hypothetical protein	0.626	0.0484003
SMU.607	conserved hypothetical protein	0.608	0.0403010
SMU.1404	conserved hypothetical protein	0.600	0.0351768
SMU.252	hypothetical protein	0.580	0.0251873
SMU.383	conserved hypothetical protein	0.543	0.0491039

Unique ID (GenBank)	Description	Difference (n-fold) of geom. means pH 5/ pH 7	Parametric P value
SMU.1853	conserved hypothetical protein	0.529	0.0357836
SMU.163	hypothetical protein	0.522	0.0464475
SMU.295	hypothetical protein	0.508	0.0096429
SMU.673	conserved hypothetical protein	0.503	0.0269833
SMU.1089	conserved hypothetical protein	0.501	0.0385478
SMU.1884	hypothetical protein	0.472	0.0267431
SMU.462	conserved hypothetical protein	0.442	0.0195586
SMU.1284	conserved hypothetical protein	0.436	0.0146092
SMU.1703	conserved hypothetical protein	0.435	0.0119016
SMU.201	conserved hypothetical protein	0.432	0.0165989
SMU.919	hypothetical protein, putative ATPase, confers aluminum resistance	0.421	0.0229347
SMU.1480	hypothetical protein	0.409	0.0289793
SMU.195	hypothetical protein	0.388	0.0072813
SMU.1956	hypothetical protein	0.383	0.0094685
SMU.862	hypothetical protein	0.354	0.0151455
SMU.213	hypothetical protein	0.327	0.0032760
SMU.1220	conserved hypothetical protein	0.282	0.0013358
SMU.1723	conserved hypothetical protein	0.236	0.0002403
SMU.684	hypothetical protein	0.190	0.0008547
SMU.940	putative hemolysin III, <i>patB</i>	0.382	0.0107347

**Table 2**List of acid-stress proteins of *S. sobrinus* 6715 identified by Q-TOF MS/MS.

Spot ID	Protein	Description	Pathway
SS2 & 9	Dpr	peroxide resistance/iron binding	Oxidative stress
SS5	Ldh	L(+)-lactate dehydrogenase	Glycolysis/ gluconeogenesis
SS6	FbaA	fructose-1,6-biphosphate adolase	Glycolysis
SS7	PfkA	6-phosphofructokinase	Glycolysis/ gluconeogenesis
SS10	PykA	pyruvate kinase	Glycolysis/ gluconeogenesis
SS11	NrdE	ribonucleotide reductase alpha- subunit	DNA replication/ repair
SS12	GdhA	NADP-specific glutamate dehydrogenase	Glutamate metabolism
SS13	Pgk	phosphoglycerate kinase	Glycolysis/ gluconeogenesis
SS15 & 17	GapC	Glycerehyde-3-phosphate dehydrogenase	Glycolysis/ gluconeogenesis