Global transcriptional analysis of acid-inducible genes in *Streptococcus mutans*: multiple two-component systems involved in acid adaptation

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Streptococcus mutans in dental biofilms is regularly exposed to cycles of acidic pH during the ingestion of fermentable dietary carbohydrates. The ability of *S. mutans* to tolerate low pH is crucial for its virulence and pathogenesis in dental caries. To better understand its acid tolerance mechanisms, we performed genome-wide transcriptional analysis of *S. mutans* in response to an acidic pH signal. The preliminary results showed that adaptation of *S. mutans* to pH 5.5 induced differential expression of nearly 14 % of the genes in the genome, including 169 upregulated genes and 108 downregulated genes, largely categorized into nine functional groups. One of the most interesting findings was that the genes encoding multiple two-component systems (TCSs), including CiaHR, LevSR, LiaSR, ScnKR, Hk/Rr1037/1038 and ComDE, were upregulated during acid adaptation. Real-time qRT-PCR confirmed the same trend in the expression profiles of these genes at pH 5.5. To determine the roles of these transduction systems in acid adaptation, mutants with a deletion of the histidine-kinase-encoding genes were constructed and assayed for the acid tolerance response (ATR). The results revealed that inactivation of each of these systems resulted in a mutant that was impaired in ATR, since pre-exposure of these mutants to pH 5.5 did not induce the same level of protection against lethal pH levels as the parent did. A competitive fitness assay showed that all the mutants were unable to compete with the parent strain for persistence in dual-strain mixed cultures at acidic pH, although, with the exception of the mutant in *liaS*, little effect was observed at neutral pH. The evidence from this study suggests that the multiple TCSs are required for *S. mutans* to orchestrate its signal transduction networks for optimal adaptation to acidic pH.

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INTRODUCTION

The human oral cavity is a highly dynamic environment that undergoes rapid and often substantial changes in nutrient availability, nutrient type, pH, oxygen tension and interbacterial interactions involving either competition or cooperation (Burne *et al.*, 1999; Kuramitsu *et al.*, 2007; Lemos *et al.*, 2005; Marsh, 1994). Bacteria, such as

streptococci, living in dental biofilms are frequently exposed to cycles of such environmental challenges (Burne *et al.*, 1999; Lemos *et al.*, 2005). During intake of sweetened foodstuff, for example, carbohydrate concentrations in the oral cavity can rapidly increase from around 10 µM to well over 10 mM (Lemos *et al.*, 2005; Lemos & Burne, 2008). Concurrent with the influx of dietary carbohydrates is a dramatic decrease in pH to values of 4 or lower in dental biofilms (Quivey *et al.*, 2001). This so-called 'feast-and-famine' lifestyle provides some organisms, such as *Streptococcus mutans*, with a significant advantage in competition with other species in dental biofilms, resulting in an emergence of numerically significant members in dental plaque that may cause caries (Marsh, 1994). These environmental variations are known to have profound impacts on bacterial gene expression and have been unequivocally shown to be the factors that influence the microbial composition and biological activities of

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Abbreviations: ATR, acid tolerance response; CI, competitive index; CSP, competence signalling peptide; TCS, two-component system.

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A figure describing the strategy for construction and confirmation of gene deletion mutants by PCR ligation mutagenesis, and tables of primers used for qRT-PCR and construction of the mutants in this study, and of genes differentially expressed in *S. mutans* during acid adaptation are available with the online version of this paper.

dental plaque biofilms (Lemos *et al.*, 2005; Marsh, 1994; Quivey *et al.*, 2001). To persist in dental biofilms, oral bacteria must be able to sense, respond and adapt to such rapid and unexpected environmental changes.

S. mutans is a Gram-positive bacterium that depends on a 'biofilm life-style' for survival and persistence in its natural ecosystem, dental plaque (Burne *et al.*, 1999). Upon ingestion of dietary fermentable carbohydrates, *S. mutans* can rapidly produce acids and lower the plaque pH, initiating the demineralization of the tooth surface. *S. mutans* is therefore considered as a primary aetiological agent of dental caries (Ajdic *et al.*, 2002). The ability of *S. mutans* to produce and tolerate acids is crucial to its virulence and cariogenicity (Quivey *et al.*, 2001; Li *et al.*, 2008). Acid tolerance by *S. mutans* has been studied in some detail. It is well known that this organism can grow and carry out glycolysis at pH values below 5.0 and can drive the pH to values well below 4.0 (Quivey *et al.*, 2001). The aciduricity of this organism has been attributed largely to the proton-extruding F₁F₀-ATPase that functions well at pH 5.0 and below, allowing the organism to maintain adequate Δ pH when the external pH falls to 4.0 and lower (Bender *et al.*, 1986; Cotter & Hill, 2003). Other mechanisms of acid resistance in *S. mutans* include induction of stress proteins (Hamilton & Svensäter, 1998; Len *et al.*, 2004; Svensäter *et al.*, 1997; Wilkins *et al.*, 2002), changes in membrane-associated proteins and fatty acid composition (Fozo *et al.*, 2007; Hasona *et al.*, 2007), DNA repair enzymes (Hahn *et al.*, 1999; Hanna *et al.*, 2001) and increase in alkali production through several metabolic pathways (Burne *et al.*, 1999; Griswold *et al.*, 2006). Moreover, the ComCDE quorum-sensing system has been found to play a role in cell-density-dependent acid tolerance by *S. mutans* (Li *et al.*, 2001b). *S. mutans* is also capable of mounting an adaptive acid tolerance response (ATR), since pre-exposure of *S. mutans* to pH 5.0–6.0 for 1–2 h (adaptation) renders the organism less susceptible to lethal acidification, as opposed to a quick pH drop by adding HCl to growing cultures (Nascimento *et al.*, 2004;

Svensäter *et al.*, 1997). This pH-inducible ATR has been well characterized in a number of bacteria, in which a signal pH that results in a sublethal effect on the cells for sufficient time appears to be important for induction of ATR (Cotter & Hill, 2003; Foster, 1995). However, unlike other bacteria, such as *Bacillus subtilis* and *Escherichia coli* that often use alternative sigma factors to coordinate their gene expression in response to environmental stresses (Haldenwang, 1995; Helmann, 2002), the *S. mutans* genome contains only two sigma factors, σ^{70} and σ^X , and their roles in regulating acid tolerance are unclear (Ajdic *et al.*, 2002; Lemos & Burne, 2008; Quivey *et al.*, 2001). How *S. mutans* senses and integrates pH signals for acid adaptation remains unknown. In this study, we provide the evidence that multiple two-component systems (TCS) are involved in acid adaptation by *S. mutans*. The results from this study provide new insights into the molecular mechanisms by which *S. mutans* senses and adapts to acidic pH in dental biofilms.

METHODS

Bacterial strains, media and growth conditions. All strains used in this study and their relevant characteristics are listed in Table 1. *S. mutans* wild-type strains were grown on Todd-Hewitt (3%) medium plus 0.3% yeast extract (THYE), while the mutants were maintained on THYE containing 10 μ g erythromycin ml⁻¹. The medium used for acid adaptation and RNA isolation was TYG (tryptone, 3%; yeast extract, 0.3%; glucose, 20 mM) at a desirable pH buffered with 40 mM potassium phosphate/citric acid. The strains were grown at 37 °C in an incubator with an atmosphere of 5% CO₂.

Acid adaptation and RNA isolation. To identify acid-inducible genes, we grew exponential-phase cells of *S. mutans* UA159 for 2 h in TYG broth at pH 5.5, a signal pH that has been demonstrated to induce acid adaptation of *S. mutans* effectively (Li *et al.*, 2001b; Svensäter *et al.*, 1997); cells grown at pH 7.5 were used as a control. Following acid adaptation, the cell pellets were collected by centrifugation at 8000 g for 15 min at 4 °C and resuspended in 1 ml cool Trizol (Invitrogen). Total RNA was extracted by a FastPrep method as described by Hanna *et al.* (2001). The extracted RNAs were precipitated with ethanol and resuspended in diethyl pyrocarbon-

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source or reference
<i>S. mutans</i>		
UA159	Wild-type, Erm ^s	Ajdic <i>et al.</i> (2002)
NG8	Wild-type, Erm ^s	Li <i>et al.</i> (2001a)
SmciaH	UA159 Δ ciaH::erm, Erm ^r	This study
SmlevS	UA159 Δ levS::erm, Erm ^r	This study
SmliaS	UA159 Δ liaS::erm, Erm ^r	This study
SmscnK	UA159 Δ scnK::erm, Erm ^r	This study
Smhk1037	UA159 Δ hk1037::erm, Erm ^r	This study
Amplicon		
Erm	erm cassette (860 bp)	This study
Plasmid		
pVA-gtfA	pVA891 harbouring the <i>S. mutans</i> gtfA gene, Erm ^r	Li <i>et al.</i> (2001a)

treated (DEPC) water. The RNA was treated with RNase-free DNase I to remove genomic DNA and purified by passing through Qiagen RNeasy mini columns (Qiagen). Purified RNA was dissolved in 20 µl RNase-free water and stored at -80°C until required for cDNA labelling. The concentration and quality of RNA samples were confirmed by spectrophotometer and gel electrophoresis.

cDNA synthesis, dye coupling and array hybridization. An indirect labelling method using SuperScript plus indirect cDNA labelling kit (Invitrogen) was used to make labelled cDNA probes. The labelling process included two steps: the first step involved the synthesis of first-strand cDNA with aminoallyl-dUTP in a reverse transcription reaction; and the second step involved labelling of aminoallyl-modified cDNA with AlexaFluor 555 (green) or AlexaFluor 647 (red). The labelled cDNA probes were purified and assessed for dye coupling efficiency and concentration using the procedures recommended by the manufacturer (Invitrogen). The labelled probes were then used for array hybridization or stored at -20°C until required.

S. mutans microarray slides used in this study were obtained from the Pathogen Functional Genomics Resource Center (PFGRC) at the J. Craig Venter Institute. Each array slide consisted of 1948 70-mer oligonucleotides representing 99% of the ORFs of the *S. mutans* UA159 genome. Array hybridization was conducted using a PFGRC protocol (<http://pfgrc.tigr.org/protocols.shtml>) with minor modification. After hybridization, the microarray slides were subjected to a series of washings in a buffer of $1\times$ SSC plus 0.1% SDS, dried in high-pressure air and scanned by a ScanArray 5000XL Reader (Perkin Elmer).

Array data analysis. After array slides were scanned, the resulting TIFF images were loaded into TIGR Spotfinder software (www.tigr.org/software/) and overlaid. A spot grid was created according to TIGR specifications and manually adjusted to fit all spots within the grid, and the intensity values of each spot were determined. Signal intensities of individual channels from an array slide were averaged and normalized using microarray data analysis software (MIDAS) by using LOWESS and iterative log mean centring with default settings, followed by in-slide replicate analysis. A *t*-test was used to determine the consistency of ratios or fold changes across replicate hybridizations. Only genes whose ratios were $\geq$ twofold changes (either increase or decrease) with 99% confidence ($P\leq 0.01$) were considered statistically significant.

Real-time quantitative RT-PCR. To validate the microarray data, we performed real-time qRT-PCR to examine the expression of a subset of the differentially expressed genes identified by the microarray analysis. qRT-PCR was carried out using a Cepheid Smart Cycler System and the QuantiTech SYBR green PCR kit (Qiagen). All primers designed for qRT-PCR analysis are listed in Supplementary Table S1. The expression levels of target genes were normalized using the *gyrA* gene of *S. mutans* as an internal standard, since it produced little variation of expression (Ajdic & Pham, 2007; Hasona *et al.*, 2007). A standard curve was plotted with cycle threshold (C_T) values obtained from amplification of known quantities of cDNAs and also used to determine the efficiency of amplification. A non-template control was included to confirm the absence of primer dimer formation. Each assay was performed with RNA samples in triplicate, and fold changes of the expression levels were calculated by using the following equations: $\Delta C_T = C_T$ (target gene) $- C_T$ (*gyrA*); $\Delta\Delta C_T = \Delta C_T$ (reference cDNA) $- \Delta C_T$ (test cDNA), so that the fold (ratio) $= 2^{-\Delta\Delta C_T}$.

Construction of gene deletion mutants. To determine the effect of inactivation of each TCS on acid adaptation, we constructed gene deletion mutants defective in the histidine kinase sensor proteins in *S.*

mutans UA159 by an allelic replacement strategy involving PCR-ligation mutagenesis as described by Lau *et al.* (2002). All the primers used to construct and confirm these mutants are presented in Supplementary Table S1. To construct the *ciaH* mutant, for example, an 821 bp fragment 5' from the *ciaH* start codon (*ciaH*-up) was amplified from *S. mutans* UA159 genomic DNA by using primers *ciaH*-P1 and *ciaH*-P2 (containing an *Ascl* site at its 5' end). Another amplicon, designated *ciaH*-dw, was 625 bp 3' from *ciaH* and was amplified with *ciaH*-P3 (with an *FseI* site at the 5' end) and *ciaH*-P4 primers. An 860 bp erythromycin resistance cassette (Em-860) was amplified from a mutant (Li *et al.*, 2002) by using primers Em-P1 and Em-P2. These amplicons were digested, purified and ligated to produce a *ciaH*-up::Em::*ciaH*-dw fragment. The ligation product was directly used to transform *S. mutans* UA159. Following double-crossover recombination, the internal region of the *ciaH* gene was completely replaced by the erythromycin cassette. The newly constructed mutants were genetically confirmed by a PCR strategy and sequencing (Supplementary Fig. S1). The same strategy was used to construct and confirm the rest of the mutants. The confirmed mutants were named *SmciaH* (Δ *ciaH*::Em), *SmlevS* (Δ *levS*::Em), *SmliaS* (Δ *liAS*::Em), *SmscnK* (Δ *scnK*::Em) and *Smhk1037* (Δ *hk1037*::Em). All the mutants were then assayed for their growth at pH 7.5 and 5.5. Their growth curves were obtained by OD₆₀₀ readings using a spectrophotometer.

Assay for ATR. To determine the effect of inactivation of the histidine kinase sensor protein on acid adaptation by *S. mutans*, all the mutants and the parent were assayed for their survival at pH 3.5 with or without pre-exposure to pH 5.5 for 2 h using a protocol described by Li *et al.* (2001b). Briefly, exponential-phase cells of each strain were divided into two groups: one resuspended in fresh TYG broth at pH 5.5 and another resuspended in TYG broth at pH 7.5. The cultures were grown at 37°C for 2 h and the cell pellets were collected by centrifugation at 8000 g for 15 min. Both adapted (pH 5.5) and non-adapted cells (pH 7.5) were immediately resuspended in 5 ml fresh TYG (pH 3.5) at a cell density of 0.6 at OD₆₀₀. The cultures were incubated at 37°C for 2 h and an aliquot of cell suspension was taken from each sample to assay viable cell counts. All the samples were serially diluted in 10 mM potassium phosphate buffer (pH 7.2) and plated on THYE agar plates. The numbers of surviving cells were determined by viable cell counts after the plates were incubated at 37°C for 2 days. The percentage survival was calculated from the numbers of viable surviving cells and total viable counts at time zero. All samples were taken in duplicate from two independent experiments.

Glycolytic pH drop assay. A glycolytic pH drop assay was conducted to determine acid production by the mutants using the method described by Li *et al.* (2008). Briefly, stationary-phase cells (overnight culture) were harvested and resuspended in a salt solution (50 mM KCl and 1 mM MgCl₂) to a final cell density of 1.0 at OD₆₀₀. The cell suspensions were adjusted to pH 7.5 and glucose was then added to a final concentration of 56 mM. Changes of pH dropping profile were recorded for 2 h using a digital pH meter (Fisher) at room temperature.

Competence assay. To determine the effect of acidic pH on competence development, we assayed genetic transformation of two naturally transformable strains, *S. mutans* UA159 and NG8, grown at pH 5.5 or 7.5 using a modified protocol (Syvitski *et al.*, 2007). Briefly, a 1:20 diluted overnight culture was grown in THYE at 37°C for 2 h. When the culture reached early to mid-exponential phase, the cell suspension was transferred into fresh pre-warmed THYE broth (1:10 dilution) in four test tubes, two at pH 7.5 and two at pH 5.5. The cultures were incubated at 37°C until the cell density of each culture reached 0.25 at OD₆₀₀. An aliquot ($1\text{ }\mu\text{g ml}^{-1}$) of competence signalling peptide (CSP) was added into one culture in each group.

The cultures were incubated at 37 °C for 20 min and transforming DNA was added into each culture at a final concentration of 1 µg ml⁻¹. The transforming DNA was an integration plasmid, pVA-gtfA, harbouring a 2.4 kb fragment of the *S. mutans* *gtfA* gene and an erythromycin resistance marker. After incubation for an additional 2 h, the cell suspension was taken from each culture, diluted and spread on THYE and THYE plus erythromycin (10 µg ml⁻¹) plates to determine the numbers of transformants and total recipient cells, respectively. Transformation frequency was expressed as the number of transformants over the total number of recipient cells (ml culture)⁻¹.

Competitive fitness assay. To further determine the effects of inactivation of each TCS on acid adaptation, we established dual-strain mixed cultures in a chemostat for competitive fitness analysis of the mutants in terms of their ability to compete with the parent for persistence under acidic pH culture conditions. Dual-strain mixed cultures were initiated in the chemostat by inoculating a 1:1 ratio of a mutant and the parent UA159. The chemostat and its operation were previously described in detail (Li *et al.*, 2001a, b). The temperature in the chemostat was maintained at 37±0.2 °C by a temperature controller (model R-600F; Cole Parmer), while culture pH was controlled by a pH meter via a digital pH controller (NV Multi-parameter Analyser R735; Consort). Four-times-diluted THYE medium plus 10 mM glucose was used for continuous cultures. The steady-state culture of each pair of strains was first established in the chemostat at pH 7.5±0.2 at dilution rate of 0.1 h⁻¹. After sampling at pH 7.5, the pH controller was disconnected to allow the culture pH to drop through glycolytic acid production. When the culture reached steady-state, samples were taken again to determine viable cell counts and the pH profile of the cultures was recorded. The mutants were distinguished from the parent by growth on THYE agar plates with or without the addition of erythromycin (10 µg ml⁻¹). A competitive index (CI) analysis was performed by determining the ratio of a mutant population to the parent population in the output samples divided by the ratio of these two populations in the input samples. CI values were analysed by Student's *t*-test, with *P*<0.05 considered statistically significant.

RESULTS AND DISCUSSION

Overview of the effects of signal pH on the transcriptome

To gain an insight into the molecular basis of acid adaptation by *S. mutans*, we used DNA microarrays to analyse genome-wide transcriptional profiles of *S. mutans* during adaptation to pH 5.5. The microarray data revealed that approximately 14% of the genes in the *S. mutans* genome displayed differential expression with a *P*-value ≤0.01 during acid adaptation (Supplementary Table S2). Notably, more genes (*n*=169) were upregulated than downregulated (*n*=108) in response to acidic pH. To validate the microarray data, we used real-time qRT-PCR to further examine the expression of a subset of the genes (*n*=26), including six genes of unknown function and three genes, *levR*, *liaR* and *hk1037*, that did not show selectable levels of expression in microarray analysis (Table 2). With the exclusion of these three genes, and *levS* that showed a sevenfold higher qRT-PCR value than the microarray analysis, we were able to measure the correlation in the expression ratios of 22 genes between

the microarray and qRT-PCR. The results showed a fairly good correlation (*r*²=0.67) in gene expression ratios between the two methods (Fig. 1). Despite some variations, the qRT-PCR results clearly revealed the same trend in the expression of all the genes tested, thus validating the microarray data.

Upregulated genes

The majority of upregulated genes were categorized into nine functional groups (Fig. 2), (i) transport and ATP-binding proteins (*n*=28), (ii) energy metabolism (*n*=14), (iii) cellular processes (*n*=13), (iv) two-component signal transduction (*n*=9), (v) transcriptional regulators (*n*=15), (vi) cation and ion uptake and binding proteins (*n*=6), (vii) biosynthesis of proteins, amino acids and co-factors (*n*=9), (viii) metabolism of nucleic acids (*n*=16), and (ix) unknown or unassigned hypothetical proteins (*n*=48). Many encode ATP-dependent transporters, including F-type, P-type and ABC-type ATPases (Supplementary Table S2). F-type ATPases that use an electrochemical gradient of H⁺ or Na⁺ to synthesize ATP, or hydrolyse ATP to reverse an electrochemical gradient, are known to play important roles in maintaining internal pH (pH_i) homeostasis (Cotter & Hill, 2003; Quivey *et al.*, 2001). For example, the proton-translocation F₁F₀-H/ATPase operon in *S. mutans* consists of eight genes, *atpEBF* and *atpHAGDC*, which respectively encode the c, a and b subunits of the membrane-embedded F₀ complex and the δ, α, γ, β, and ε subunits of the peripherally bound F₁ protein (Kuhnert *et al.*, 2004). The F₀ complex has proton-translocating activity, while the F₁ complex has ATPase activity. The F₁F₀-ATPase largely contributes to the aciduricity of *S. mutans*, since it functions well below pH 5.0 and allows the organism to maintain an adequate ΔpH when the external pH falls to 4.0 and lower (Bender *et al.*, 1986; Quivey *et al.*, 2001). Our microarray work revealed that all the genes from SMU.1527 to SMU.1534, encoding the eight subunits of the F₁F₀-ATPase, were upregulated (2.3–3.8-fold) during acid adaptation. To confirm the array data, we used real-time qRT-PCR to assay the expression of two of the subunit-encoding genes, *atpD* and *atpF*, in the cells grown at pH 5.5. The results revealed that adaptation of *S. mutans* to pH 5.5 indeed induced 2.26–3.87-fold increases in the expression of these genes (Table 2), thus validating the microarray data. Previous studies suggested that the F₁F₀-ATPase in *S. mutans* might be constitutively expressed, since the promoter analysis of the F₁F₀-ATPase operon showed a putative Pribnow box (TAAACT) and a clear -35 sequence of TTGACA, homologous with the canonical σ⁷⁰ promoter in *E. coli*, suggesting a housekeeping function for F₁F₀-ATPase synthesis (Quivey *et al.*, 2001). However, using transcriptional reporter gene fusions to the promoter of the *atp* operon, Quivey and colleagues found that the increase in F₁F₀-H/ATPase activity was correlated with increased transcription of the *atp* operon of *S. mutans* grown at low pH (Kuhnert *et al.*, 2004). Using 2-DGE and mass spectroscopic analysis, Len *et al.* (2004) have found

Table 2. A comparison of gene expression between microarray and qRT-PCR

Microarray fold changes were based on the means ($\geq$ or $\leq$ twofold with $P \leq 0.01$) of triplicate reactions. qRT-PCR values indicate fold changes in relative gene expression levels (C_T) of cells grown under two pH conditions. The fold differences (ΔC_T) in expression levels of the genes tested were calculated from triplicate reactions against the C_T value of constitutively expressed *gyrA* of *S. mutans* UA159 using the equations described in the text. –, Not applicable.

Locus ID	Gene name	Functional description	Fold difference (pH 5.5/7.5)	
			Microarray	qRT-PCR
Transporters				
SMU.1528	<i>atpD</i>	F ₀ F ₁ ATPase, beta subunit	3.01	2.26
SMU.1532	<i>atpF</i>	F ₀ F ₁ ATPase, b subunit	3.23	3.87
SMU.1093	<i>vex3</i>	Putative ABC transporter	6.74	3.48
TCS				
SMU.1128	<i>ciaH</i>	Histidine kinase protein, CiaH	2.19	3.46
SMU.1129	<i>ciaR</i>	Response regulator, CiaR	2.96	8.39
SMU.1964	<i>levR</i>	Response regulator, LevR	–	8.56
SMU.1965	<i>levS</i>	Histidine kinase protein, LevS	2.74	18.2
SMU.486	<i>liaS</i>	Histidine kinase protein, LiaS	2.05	3.18
SMU.487	<i>liaR</i>	Response regulator, LiaR	–	4.25
SMU.1814	<i>scnK</i>	Histidine kinase protein, ScnK	2.15	2.74
SMU.1815	<i>scnR</i>	Response regulator, ScnR	2.05	2.68
SMU.1037	<i>hk1037</i>	Histidine kinase protein, Hk1037	–	3.65
SMU.1038	<i>rr1038</i>	Response regulator, Rr1038	2.19	4.66
SMU.1916	<i>comD</i>	Histidine kinase protein, ComD	2.11	2.84
SMU.1917	<i>comE</i>	Response regulator, ComE	2.26	3.25
Competence				
SMU.1997	<i>comX</i>	Competence-specific sigma factor	0.36	0.50
SMU.1987	<i>comYA</i>	Competence, DNA transport	0.28	0.22
Bacteriocins				
SMU.1913	<i>immA</i>	Putative immunity protein, Blp-like	2.38	5.82
SMU.1914	<i>nlmC</i>	Non-lantibiotic mutacin V	2.92	7.07
Transcriptional factor				
SMU.1805	–	Transcriptional regulator	3.20	5.04
Unknown function				
SMU.239	–	Hypothetical protein	3.74	4.27
SMU.635	–	Hypothetical protein	4.13	5.96
SMU.1243	–	Hypothetical protein	5.87	3.65
SMU.673	–	Hypothetical protein	0.28	0.43
SMU.1642	–	Hypothetical protein	0.31	0.35
SMU.1803	–	Hypothetical protein	0.26	0.29

that pH 5.0 induces significantly higher levels of expression of at least two subunit proteins, α and ϵ , of the F₁F₀-H/ATPase. Similar results have also been reported showing pH-dependent increases in the transcription of the *atp* operon in both *Streptococcus pneumoniae* and *Streptococcus bovis* (Martin-Galiano *et al.*, 2001). Clearly, the results from our study support the observations that acidic pH induces the expression of the genes encoding F₁F₀-ATPase subunits at a transcriptional level in *S. mutans*.

In addition to F-type transporters, a large number of genes encoding P-type and ABC-type ATPase transporters were upregulated, including SMU.426 (*copA*), SMU.1563 (*pacL*), SMU.2057 (*cdaA*), SMU.1093/1094 (*vex3/vex2*), SMU.653 (*tauC*), SMU.879–882 (*msmFGK*) and SMU.1412 (*yhcA*). The P-type ATPases are responsible for the transport of

various inorganic ions, such as calcium, cadmium, copper and potassium (Kakinuma, 1998; Magalhaes *et al.*, 2005). For instance, the *copYAZ* operon in *S. mutans* encodes a P-type ATPase responsible for transport of copper, allowing this organism to tolerate the high concentrations of copper potentially released from dental amalgam (Vats & Lee, 2001). *pacL* (SMU.1563), together with two other genes, SMU.1561 and SMU.1562, at the same locus, encodes a P-type potassium transporter system. These genes, along with two other genes, SMU.1708 and SMU.1709, which also encode a putative potassium uptake system, were upregulated at pH 5.5, suggesting that potassium might play an important role in regulating pH homeostasis during acid adaptation in *S. mutans*. Although little is known about potassium transport in acid adaptation of *S. mutans*, the mechanism involving H⁺ extrusion/K⁺ influx through

Fig. 1. Correlation of the expression ratios of 22 genes at pH 5.5 versus 7.5 using data from microarray and real-time qRT-PCR. $r^2=0.67$.

F_1F_0 -ATPase and potassium uptake systems at acidic pH has been reported in *E. coli* and *Enterococcus hirae* (Kakinuma, 1998). ABC-type ATPases are the most abundant transporters in *S. mutans* and exhibit specificity for different substrates, amino acids, carbohydrates, oligopeptides, osmoprotectants (proline/glycine betaine, choline), inorganic ions, such as Fe^{3+} , Co^{2+} , Mn^{2+} and Zn^{2+} , phosphate, nitrate, sulfate and molybdenum (Ajdic & Pham, 2007; Cotter & Hill, 2003; Kakinuma, 1998). These gene products may function to maintain cellular

Fig. 2. The distribution of differentially expressed genes and their functional categories in *S. mutans* UA159 during acid adaptation. The genes were grouped according to the Los Alamos *S. mutans* genome database (www.oralgen.lanl.gov/). ■, Upregulated genes; □, downregulated genes.

processes, metabolism and ion balance during acid adaptation. However, whether these gene products play direct roles in the ATR of *S. mutans* remains to be studied. Together, all three types of ATP-dependent ATPases may coordinately play their roles in the regulation and maintenance of cellular processes, ion balance and pH homeostasis in *S. mutans* during acid adaptation.

One of the most interesting findings from the microarray analysis was that genes encoding multiple TCSs, including CiaHR, LevSR, LiaSR, ScnKR, Hk1037/Rr1038 and ComDE, were upregulated during acid adaptation of *S. mutans*. Using real-time qRT-PCR, we confirmed the same trend in the expression of these genes, including the genes encoding their cognate response regulators, in response to acidic pH (Table 2). In the *S. mutans* genome, there are 14 TCSs, and at least one orphan response regulator has been identified (Ajdic *et al.*, 2002; Biswas *et al.*, 2008; Idone *et al.*, 2003). Some of these systems, such as LiaSR (formerly HK/RR11), CiaHR, ComDE and CovR, have been found to play a role in acid resistance and considered as a major attribute to the virulence of *S. mutans* (Ahn *et al.*, 2006; Idone *et al.*, 2003; Li *et al.*, 2001b, 2002; Lévesque *et al.*, 2007; Kawada-Matsuo *et al.*, 2009). However, little is known of the actual mechanisms of how these TCSs sense and integrate pH signals to regulate gene expression for acid adaptation. In *B. subtilis* and *E. coli*, many alternative sigma factors have been found to play major roles in regulating gene expression in response to various stresses, including acidic pH (Foster, 1995; Helmann, 2002). For example, at least 17 sigma factors have been identified in *B. subtilis*, seven of which are extracytoplasmic function sigma factors that play central regulatory roles in gene expression in response to stress (Haldenwang, 1995; Helmann, 2002). In the *S. mutans* genome, however, only two sigma factors, σ^{70} and σ^X , are identified and their roles in regulating acid adaptation is unclear (Ajdic *et al.*, 2002). Thus, TCSs in *S. mutans* may play major roles in coordinating regulatory networks and gene expression in response to acid stress.

In addition to multiple TCSs, several genes encoding putative transcriptional regulators were upregulated, including *sloR* (SMU.186), *rgg* (SMU.1509), *lacI* (SMU.105), *copY* (SMU.424) and *recX* (SMU.1780). Their roles in acid adaptation are unclear. However, *sloR* is known to encode a metal-dependent transcriptional regulator involved in regulating the *sloABCR* operon for Mn and Fe uptake (Dunning *et al.*, 2008). A recent study has linked the expression of the *sloABCR* operon to the acid stress response in *S. mutans* (Dunning *et al.*, 2008; Idone *et al.*, 2003). It is also known that *rgg* encodes a transcriptional regulator in many Gram-positive bacteria, including *S. mutans* (Ajdic *et al.*, 2002). Rgg coordinately regulates amino acid catabolism and virulence expression in *Streptococcus pyogenes* (Chaussee *et al.*, 2003) and plays a role in the regulation of *gtfG* that encodes glucosyltransferase in *Streptococcus gordonii* (Vickerman & Minick, 2002). Presumably, the Rgg regulator in *S. mutans* may play a

similar role in regulating an extracellular polysaccharide during acid adaptation.

Downregulated genes

The majority of the downregulated genes belong to six groups, including (i) transport and binding proteins ($n=19$), (ii) energy metabolism ($n=18$), (iii) cellular processes ($n=8$), (iv) biosynthesis of proteins, amino acids and co-factors ($n=17$), (v) nucleic acid metabolism ($n=6$) and (vi) unknown or undesigned hypothetical proteins ($n=31$). The general trend observed in the downregulated genes was that genes encoding products for transport, cellular processes and biosynthesis requiring higher energy levels were repressed at acidic pH (Supplementary Table S2). Possibly, *S. mutans* cells under acidic conditions might largely use energy to maintain internal pH homeostasis rather than for active energy-consuming processes and biosynthesis. For example, several genes, including SMU.670 (*citB*), SMU.671 (*citZ*) and SMU.672 (*citC*) that encode enzymes required for citrate metabolism in the Krebs cycle, were downregulated (Supplementary Table S2). Similarly, many genes encoding enzymes and transporters involved in the biosynthesis and transport of sugar were downregulated, including *ptsG* that encodes the glucose-specific phosphoenolpyruvate-phosphotransferase system (PEP-PTS) (IIABC component). It is well known that bacterial PTSs are responsible for binding, transport and phosphorylation of numerous sugar substrates (Ajdic & Pham, 2007; Ajdic *et al.*, 2002). They play important roles in transporting specific sugars under sugar substrate limitation. These systems are also involved in the regulation of various metabolic and transcriptional processes. The downregulation of these genes might be a reflection of the cells' efforts to preserve energy at low pH, particularly when a high concentration of sugar is still available in the culture.

Another interesting finding was that several late competence genes, *comYA* (SMU.1987), *comYB* (SMU.1985), *comYD* (SMU.1983), *comEC* (SMU.626) and *comX* (SMU.1997), were downregulated. To confirm these results, we examined two late competence genes, *comYA* and *comX* (Merritt *et al.*, 2005), an early competence gene, *comE*, and its controlled bacteriocin-producing and immunity genes, *nlnC* (SMU.1914) and *immA* (SMU.1913), by real-time qRT-PCR. The results showed that, similar to the microarray data, *comYA* and *comX* were indeed downregulated at pH 5.5, but *comE* and the two bacteriocin-related genes were upregulated at this pH (Table 2). These results are surprising, since the ComCDE quorum sensing system in *S. mutans* is known to control both genetic competence and bacteriocin production (Li *et al.*, 2001a; van der Ploeg, 2005). One may expect the same impact when the ComCDE signal pathway is activated. This is true when CSP is added into a culture at neutral pH (Li *et al.*, 2001a; Syvitski *et al.*, 2007). In this case, however, the early and late competence genes were

divergently regulated at low pH. The mechanism involved in such divergent regulation is currently unclear. To further confirm the results, we assayed competence development for genetic transformation in two naturally transformable strains of *S. mutans*, UA159 and NG8, grown at either pH 5.5 or 7.5. The results showed almost no detectable transformation for the cells grown at pH 5.5, regardless of use of CSP (Fig. 3). In contrast, the cells grown at pH 7.5 had a normal transformation frequency [10^3 – 10^4 transformants ($\mu\text{g DNA}^{-1} \text{ ml}^{-1}$), similar to that reported by Li *et al.* (2001a) and Syvitski *et al.* (2007). Addition of CSP at neutral pH could further increase the transformation frequencies of these two strains. The data suggest that acidic pH indeed inhibits competence development for genetic transformation. Since the early competence gene *comE* and its controlled bacteriocin-related genes were not affected at pH 5.5, we assume that acidic pH most probably inhibits competence development by suppressing the late competence genes, such as *comX* and *comYA*. It is well known that ComE is a key regulator in coupling CSP-dependent genetic competence and bacteriocin production in *S. mutans* (Li *et al.*, 2001a; Kreth *et al.*, 2007). However, there appears to be a lack of evidence that ComE in *S. mutans* directly interacts with *comX*, which directs transcription of late competence genes, as there is no ComE binding site at the promoter site of *comX* (Martin *et al.*, 2006). Thus, our data appear to favour the model of Burne and colleagues, who have proposed that competence development is hierarchically regulated both via CSP-dependent and CSP-independent circuits involving two signalling systems, ComCDE and CiaHR (Ahn *et al.*, 2006). The evidence from our study suggests that *S. mutans* may divergently regulate the early and late competence genes through different pathways in response to different signals.

Fig. 3. Development of genetic competence in *S. mutans* UA159 at pH 7.5 and 5.5 with (■) or without (□) the addition of CSP.

Acidic pH is a significant factor that may directly or indirectly affect one of these signal transduction pathways. Clearly, further work is required to study the mechanism of how these genes are divergently regulated.

Effect of inactivation of the histidine kinases (HK) on ATR

To determine if TCSs might play a role in ATR of *S. mutans*, we constructed gene deletion mutants defective in histidine kinase sensor proteins CiaH, LevS, LiaS, ScnK and HK1037 of individual systems by using an allelic replacement strategy involving PCR-ligation mutagenesis (Lau *et al.*, 2002). All the mutants were genetically confirmed (Supplementary Fig. S1) and examined for their growth kinetics in TYG broth at pH 5.5 and 7.5. The results showed that all the mutants grew similarly to the parent UA159 at pH 7.5 (data not shown). The growth yields of these mutants, except SmlevS that had slightly higher yield, were almost the same as that of the parent after 16 h growth. However, all the mutants, particularly SmliaS, SmciaH and Sm1037, at pH 5.5 were retarded in their growth compared to the parent UA159 (Fig. 4). These results suggest that inactivation of each of these systems results in a mutant that has a reduced growth rate at acidic pH. Recent studies have shown that several TCS have been involved in acid resistance in *S. mutans* (Ahn *et al.*, 2006; Biswas *et al.*, 2008; Li *et al.*, 2002; Lévesque *et al.*, 2007; Kawada-Matsuo *et al.*, 2009). Some of the TCS identified to be involved in acid tolerance in these studies appear to be different from those in our study, probably owing to the differences in the strains, mutant construction, growth

Fig. 4. Growth kinetics of the *S. mutans* mutants grown at pH 5.5 and the parent UA159 grown at pH 5.5 and 7.5. ●, UA159 (pH 7.5); ○, UA159 (pH 5.5); ▲, SmciaH (pH 5.5); △, SmlevS (pH 5.5); ◆, SmliaS; (pH 5.5); ◇, SmcnK (pH 5.5); ×, Smhk1037 (pH 5.5).

conditions and assay methods. To further determine the roles of these TCS in inducible ATR, in which pre-exposure of *S. mutans* to pH 5.5 for 2 h (adaptation) would render the organism less susceptible to a subsequent lethal acid exposure (Svensäter *et al.*, 1997), we examined inducible ATR of these mutants. The results showed that all the mutants were also impaired in exponential-phase ATR, since pre-exposure of these mutants to pH 5.5 for 2 h did not significantly enhance the protection of the strains from being killed at pH 3.5 (Fig. 5a and b). In contrast, the parent strain showed a 30-fold higher level of survival at pH 3.5 after the cells were adapted to pH 5.5. The evidence from this study clearly shows that these TCSs are required

Fig. 5. ATR of *S. mutans* strains. (a) Survival kinetics of acid-adapted (■, ●) or non-adapted (□, ○) cells of *S. mutans* UA159 (■, □) and SmciaH (●, ○) following exposure to pH 3.5. (b) Percentage survival of acid-adapted (■) or non-adapted (□) cells of all the mutants and parent strain UA159 following exposure to pH 3.5 for 2 h.

for *S. mutans* to sense and integrate acidic pH signals for optimal induction of acid adaptation.

Effect of inactivation of histidine sensor kinases (HK) on acid production

In addition to ATR, we examined acid production of these mutants and found that all the mutants, particularly SmliaS and SmciaH, had reduced glycolytic pH drop rates than the parent strain (Fig. 6). The differences, albeit subtle, could be observed between the first 10–20 min immediately following the glucose pulse. Also, the final pH levels of all the mutants were slightly higher than the parent strain. If the acid was neutralized, glycolysis in the cultures took place again, indicating that glycolytic activity had ceased due to inhibition by the lower pH, and not by glucose depletion. The data further supported that these mutants were more sensitive to acidic pH than the parent.

Competitive fitness analysis

To assess the fitness of the mutants grown at acidic pH, we established dual-strain mixed cultures in a chemostat for CI analysis by inoculating a 1 : 1 ratio of a mutant and the parent strain UA159. Continuous chemostat culture is considered to be particularly advantageous for studying the effects of environmental factors on bacterial growth, because a chemostat culture allows good control of the growth-limiting substrate, growth rate, pH and other growth parameters (Li *et al.*, 2001a, b). By controlling these parameters, one can effectively minimize the variations often encountered in batch cultures and reproduce the data

Fig. 6. Glycolytic pH drop assay of the *S. mutans* histidine sensor kinase mutants. All the mutants showed slower glycolytic pH drop rates than UA159 during the first half hour following the glucose pulse. ●, UA159; △, SmciaH; □, SmlevS; ○, SmliaS; ×, SmscnK; ◇, Smhk1037.

observed in the chemostat. Our results revealed that at neutral pH ($\text{pH } 7.5 \pm 0.2$), all the mutants, except SmliaS, maintained a stable co-culture with the parent population under steady-state chemostat conditions (Fig. 7a, b). There was no significant difference in the CI values between the samples at the end of day 5 and the initial inocula. The results suggested that all the mutants, except SmliaS, grew equally well in the chemostat at neutral pH at a low dilution rate ($D=0.1 \text{ h}^{-1}$). In contrast, the numbers of the mutant cells dramatically decreased in the co-cultures without pH control (pH ranging from 5.0–4.6), resulting in a 1.5–2.0 magnitude decrease in the CI values ($P < 0.01$ or

Fig. 7. A CI analysis of the *S. mutans* mutants versus parent UA159 in dual-strain mixed cultures. Each culture was initiated by inoculating a 1 : 1 ratio of a mutant and the parent. (a) An example of the time-course CI analysis of SmciaH versus UA159 grown in a chemostat at either pH 7.5 or at an acidic pH ranging from 4.6 to 5.2. ○, pH control; ●, no pH control. (b) Final CI values of five pairs of dual-strain cultures grown at either pH 7.5 or at acidic pH 4.6–5.2 after 5 days of continuous culture. *, $P \leq 0.05$; **, $P \leq 0.01$ in the CI values between pH control (□) and no pH control (■).

0.05) between the samples at the end of day 5 and the time when the pH controller was disconnected. Clearly, these mutants were less competitive than the parent for persistence in co-cultures at acidic pH, suggesting that the mutants had reduced fitness to maintain their population sizes at lower pH levels. Taken together, the data from this study support the notion that multiple TCSs in *S. mutans* play regulatory roles in acid production, sensing and adaptation. Further study is necessary to characterize how these TCSs sense acidic pH signals to activate the regulatory networks for acid adaptation.

Concluding remarks

Acidic pH represents a critical environmental constraint for all macromolecular structures, physiological activities and energy conversions in bacteria. Accordingly, the ability to sense, respond and adapt to pH changes represents a critical regulatory module that interconnects with many components of cellular processes and is essential for all organisms. *S. mutans* in dental biofilms is exposed to cycles of pH fluctuation during carbohydrate consumption. The gene expression and physiological activities of this organism can be profoundly influenced by its capacity to adapt to dynamic environmental changes. The results presented herein indicate that pH is an important environmental signal that induces genome-wide transcriptional changes in *S. mutans*. Considering the large number of genes discovered to be pH-regulated in bacteria, the actual mechanism responsible for sensing and integrating H⁺ signals for acid adaptation is poorly understood. The evidence from this study suggests that multiple TCSs are involved in sensing and transducing the pH signal by *S. mutans* to coordinate its regulatory networks and gene expression for enhanced adaptation to acidic pH. Analysis of these signal transduction systems in pH sensing, response and regulation of specific gene products will shed light on the molecular mechanism by which *S. mutans* adapts to acidic pH in dental biofilms. In addition, large numbers of genes encoding hypothetical proteins of unknown function, which display either increased or decreased expression during acid adaptation, appear to be interesting, as they may be important in ATR of *S. mutans*. Thus, continued analysis of these gene products in acid adaptation and their regulatory mechanisms is clearly needed.

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