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## Transcriptional profile of glucose-shocked and acid-adapted strains of *Streptococcus mutans*

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### Summary

The aciduricity of *Streptococcus mutans* is an important virulence factor of the organism, required to both out-compete commensal oral microorganisms and cause dental caries. In this study, we monitored transcriptional changes that occurred as a continuous culture of either an acid-tolerant strain (UA159) or an acid-sensitive strain (*fabM::Erm*) moved from steady-state growth at neutral pH, experienced glucose-shock and acidification of the culture, and transitioned to steady-state growth at low pH. Thus, the timing of elements of the acid tolerance response (ATR) could be observed and categorized as acute vs. adaptive ATR mechanisms. Modulation of BCAA biosynthesis, DNA/protein repair mechanisms, ROS metabolizers, and PTS occurred in the initial acute phase, immediately following glucose-shock, while up-regulation of F<sub>1</sub>F<sub>0</sub>-ATPase did not occur until the adaptive phase, after steady-state growth had been re-established. In addition to the archetypal ATR pathways mentioned above, glucose-shock led to differential expression of genes suggesting a re-routing of resources away from synthesis of fatty acids and proteins, and towards synthesis of purines, pyrimidines and amino acids. These adjustments were largely transient, as upon establishment of steady-state growth at acidic pH, transcripts returned to basal expression levels. During growth at steady-state pH 7, *fabM::Erm* had a transcriptional profile analogous to that of UA159 during glucose-shock, indicating that even during growth in rich media at neutral pH, the cells were stressed. These results, coupled with a recently established collection of deletion strains (Quivey *et al.*, 2015), provide a starting point for elucidation of the acid tolerance response in *S. mutans*.

### Keywords

Oral streptococci; acid adaptation; stress response; transcriptome; genomic characterization

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## Introduction

The oral pathogen *Streptococcus mutans*, an acidogenic and aciduric Gram-positive organism, is the chief causative agent of the disease dental caries (De Stoppelaar *et al.*, 1969, Loesche, 1986, Nyvad *et al.*, 2013). Dental caries has a high prevalence in nations where refined sugar consumption is a large part of the diet, with a strong trend for occurrence of the disease in segments of the population that can least afford treatment (Dye *et al.*, 2007, Dye & Thornton-Evans, 2010). *S. mutans* is found exclusively on the surfaces of teeth in dental plaque, a matrix composed of bacteria, bacterially-produced polysaccharides, mainly water-insoluble glucans, salivary proteins, and food particles (Bowen & Koo, 2011). An important virulence trait of *S. mutans* is its ability to metabolize a wide array of human dietary carbohydrates from which it produces a large amount of organic acids through fermentation, which acidify the dental plaque (Carlsson *et al.*, 1997). Demineralization of the tooth surface occurs from these transient drops in pH, but is typically repaired due to the immersion of the tooth in mineral-rich saliva. In the absence of scrupulous oral hygiene, plaque buildup can prevent salivary minerals from re-crystallizing the tooth surface at a rate that can keep pace with the demineralization, allowing progression to a disease state (Koo *et al.*, 2002).

As *S. mutans* is more aciduric than many organisms with which it frequently competes, namely the *mitis* group of Streptococci and other early colonizers of the tooth surface, it was unsurprising that metagenomic studies identified *S. mutans* as a more dominant member of supragingival flora as plaque matures and becomes more acidic (Gross *et al.*, 2010, Gross *et al.*, 2012). The collective adjustments to low pH made by *S. mutans* have been termed the acid-adaptive response (ATR) and have been reviewed in depth (Lemos & Burne, 2008, Matsui & Cvitkovitch, 2010). The ATR of *S. mutans* is dependent on a number of traits and systems including the ability to carry out glycolysis at a lower pH than many other oral microbes (Belli & Marquis, 1991, Svensater *et al.*, 1997). Importantly, *S. mutans* also maintains a cytosolic pH that is significantly higher than the extracellular pH (Quivey *et al.*, 2000, Lemos *et al.*, 2005, Lemos & Burne, 2008). This is accomplished by several mechanisms, most notably the up-regulation of the F<sub>1</sub>F<sub>0</sub> ATPase, which, in this case, uses hydrolysis of ATP to pump protons out of the cytosol (Kuhnert & Quivey Jr, 2003, Sheng & Marquis, 2006). The *S. mutans* F<sub>1</sub>F<sub>0</sub> ATPase can operate at low pH much more efficiently than the ATPase of several other competing oral bacterial species such as *S. salivarius* and *S. sanguinis* (Bender *et al.*, 1986). Other important systems for cytosolic alkalization include up-regulation of branched-chain amino acid (BCAA) biosynthesis (Len *et al.*, 2004a, Santiago *et al.*, 2012) and the agmatine deiminase system (Griswold *et al.*, 2006, Lemos & Burne, 2008) as well as induction of malolactic acid fermentation (Sheng & Marquis, 2007). It was also shown that during growth at low pH, *S. mutans* incorporates a larger percentage of unsaturated fatty acids (UFAs) into the plasma membrane (Fozo *et al.*, 2004, Fozo & Quivey, 2004b). Production of *de novo* UFAs requires the *fabM* gene, encoding a *trans*-2-*cis*-3-decenoyl-ACP isomerase (Fozo & Quivey, 2004a). The loss of the ability to synthesize UFAs in a *fabM* mutant strain, *fabM::Erm*, resulted in a significantly smaller pH across the organism's membrane compared to the parental strain (Fozo & Quivey, 2004a), suggesting alterations in the ability of the mutant strain to maintain normal membrane permeability for

protons, or in its ability to pump protons out of the cell. The *fabM::Erm* strain also exhibited a reduced ability to survive acid challenge, and was considerably less virulent in a rat model for dental caries (Fozo & Quivey, 2004a, Fozo *et al.*, 2007). *S. mutans* encodes several DNA repair enzymes which are also induced in response to acid (Hanna *et al.*, 2001, Faustoferri *et al.*, 2005, Gonzalez *et al.*, 2012b), supporting the concept that the response of *S. mutans* to acidification includes both defensive and repair capabilities.

While the ATR pathways discussed above are well known, the mechanisms by which these pathways are induced, and the timing of their induction on a large scale, are not fully understood. Many previous studies in *S. mutans* have used cDNA microarray analysis or RNA-seq to elucidate the regulon of a particular gene or condition on a global scale. These have included the studies of the oxidative-stress regulon and redox control through aeration (Ahn *et al.*, 2007, Baker *et al.*, 2014), the Nox regulon (Baker *et al.*, 2014), the Rex regulon (Bitoun *et al.*, 2011), and the SpxA1 and SpxA2 regulons (Kajfasz *et al.*, 2010). Studies have also been conducted on the global response to nutrients through growth in various sugars (Zeng *et al.*, 2013, Moye *et al.*, 2014) and elucidation of the CodY regulon (Lemos *et al.*, 2008), the CcpA regulon (Zeng *et al.*, 2013), the ManL regulon (Abranches *et al.*, 2006, Moye *et al.*, 2014), and the CcpA-regulated FabT regulon (Faustoferri *et al.*, 2014).

Although the changes in the *S. mutans* proteome in response to growth at steady-state pH 5 versus steady-state pH 7 and the transcriptomic effects of growth in acidic buffered batch cultures have been described (Wilkins *et al.*, 2002, Len *et al.*, 2004a, Len *et al.*, 2004b, Crowley *et al.*, 2004, Gong *et al.*, 2009, Krastel *et al.*, 2010), global transcriptional changes in response to glucose-shock (which leads to a rapid acidification of the environment through the production of lactic acid) and steady-state growth at acidic pH have not been elucidated in *S. mutans*.

In this study, cDNA microarray analysis was used to examine the global transcriptional changes occurring when a continuous culture of *S. mutans* grown to steady-state at pH 7 was administered a glucose-shock, which resulted in rapid acidification of the culture, and then transitioned to steady-state growth at pH 5. The conditions described here represent an examination of glucose and/or acid-inducible genes in *S. mutans* using cells harvested from tightly controlled growth conditions. This is in contrast to previous transcriptomic studies describing acid-inducible genes in *S. mutans* that utilized buffered media in batch cultures (Gong *et al.*, 2009, Krastel *et al.*, 2010). To take a step further, the transcriptional profile from both an acid-tolerant strain, UA159, and an acid-sensitive strain, *fabM::Erm*, was obtained. Based on the timing of transcriptomic changes observed, we divided several of the major trends into three groups: the acute ATR, the adaptive ATR, and the transient ATR/glucose-shock response. This study demonstrates that during steady-state growth at pH 7, *fabM::Erm* had an elevated basal level of stress responses compared to UA159, suggesting that even during favorable growth conditions, the homeostasis of this mutant is under duress.

## Materials and Methods

### Bacterial strains and growth conditions

The *S. mutans* UA159 (parent strain) and UR117 ("*fabM::Erm*", (Murchison *et al.*, 1986, Fozo & Quivey, 2004a) strains were maintained on brain heart infusion agar (BHI; BD/

Difco, Franklin Lakes, NJ) at 37°C in a 5% (vol/vol) CO<sub>2</sub>/95% air environment. Erythromycin was added to a final concentration of 5 µg ml<sup>-1</sup>, where necessary. Organisms were cultured in TY medium (3% tryptone, 0.1% yeast extract, 0.5% KOH, 1 mM H<sub>3</sub>PO<sub>4</sub>) + 1% (w/v) glucose and were grown in liquid culture or in continuous culture in a Sixfors fermenter (Infors, Laurel, MD) as described previously (Quivey *et al.*, 1995, Fozo & Quivey, 2004b). Continuous cultures of UA159 or *fabM::Erm* were grown at a dilution rate of 0.24 h<sup>-1</sup> under glucose-limiting conditions (2.3 mM), with a continuous impeller speed of 200 rpm. Steady-state pH levels of 7.0 or 5.0 (5.5 for *fabM::Erm*) were maintained by the addition of 2 N KOH. The culture pH was continuously monitored throughout the experiment by using an in-dwelling pH probe (Mettler Toledo, Columbus, OH). After continuous culture at pH 7 had been maintained for a minimum of 10 generations, aliquots of the culture were removed and cells were collected by centrifugation (referred to as steady-state pH 7). Glucose was added to 200mM and the pH control was turned off to allow acidification of the culture. Upon reaching pH 5.5, aliquots were removed and cells harvested. These aliquots were referred to as “glucose-shock.” At this point, the pH control was set to 5.0 for UA159, or 5.5 for *fabM::Erm* and the culture was maintained for 10 generations, at which time the final aliquots were removed (referred to as “steady-state pH 5” or “steady-state pH 5.5”). All cell pellets were washed and stored frozen at -80°C. Five steady-state cultures were grown for each strain.

### cDNA microarray analysis

*S. mutans* UA159 microarray slides were provided by The Institute for Genomic Research (TIGR) through a cooperative agreement with the National Institutes for Allergy and Infectious Disease (NIAID) and Dental and Craniofacial Research (NIDCR). Reference RNA was isolated from UA159 cells grown in BHI medium to an optical density at 600nm of 0.5 and purified as previously described (Abranches *et al.*, 2006). Total RNA from the experimental and reference conditions was isolated and purified as previously described (Abranches *et al.*, 2006, Kajfasz *et al.*, 2010). cDNA was synthesized and labeled using Cy3-dUTP or Cy5-dUTP (GE Healthcare, Piscataway, NJ) as previously outlined (Abranches *et al.*, 2006). Cy3-dUTP-labeled test cDNA and Cy5-dUTP-labeled reference cDNA were hybridized overnight at 42°C. Hybridization was carried out using a MAUI Hybridization system (BioMicro Systems, Inc., Salt Lake City, UT), washed according to protocols provided by J. Craig Venter Institute (JCVI), and scanned using a GenePix 4000b Microarray Scanner (Molecular Devices, Inc., Sunnyvale, CA). After scanning, the images were analyzed using TIGR Spotfinder, and normalized, as previously described (Abranches *et al.*, 2006). Four replicate slides were used for each sample and statistical analysis was carried out using BRB array tools (<http://linus.ncin.nih.gov/BRB-ArrayTools.html>). Microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo>) with accession number GSE64236.

## Results

### Experimental Design

Although the *S. mutans* adaptive acid tolerance response (ATR) has been thoroughly characterized, how the response itself is activated and controlled remains less clear. Two

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previous studies have explored acid-inducible genes in *S. mutans* via microarray analysis, however, these studies were done by suspending batch cultures in acidic media (Gong et al., 2009, Krastel et al., 2010). In contrast, this study used continuous cultures in which the pH was lowered by the acidic end products of carbon metabolism by *S. mutans*; no exogenous acid was added. The scheme described here is more analogous to what happens in the oral cavity, where the pH in dental plaque is lowered not by exogenous acid, but by bacterial fermentation products. The UA159 and *fabM::Erm* strains were grown in continuous culture at pH 7 for ten generations. At this point, samples were taken for downstream microarray analysis, and referred to as “steady-state pH 7,” (Figure 1A) which was used as a baseline for expression levels. Upon sampling of the pH 7 steady-state cells, glucose was added to the culture to a final concentration of 200mM and pH control via KOH was turned off, allowing for acidification of the culture, as previously described (Fozo & Quivey, 2004b). When culture pH values had fallen to pH 5.5, samples were harvested for microarray analysis. This second time point was referred to as “glucose-shock” (Figure 1A). After the glucose-shock samples had been harvested, the pH control was turned on, such that the culture was not permitted to acidify below pH 5.0 for UA159 or pH 5.5 for *fabM::Erm*, the lowest achievable steady-state pH value for this strain (Fozo & Quivey, 2004a). The final set of samples, referred to as “steady-state pH 5” (or “steady-state 5.5”, for *fabM::Erm*), were harvested after an additional ten generations of growth in the chemostat (Figure 1A). Microarray analysis was performed on all the samples and seven comparisons were made between the strains and time points, as shown in Figure 1B. Figure 1B also displays the number of genes that were up- or down-regulated in each comparison. In this manuscript, we cannot address all of the differences that were observed, but we present here a synopsis of the major trends. We implemented a p-value cutoff of 0.01 and a 2-fold change cutoff for the data detailed below. Table 1 displays notable genes of interest, many of which are specifically discussed in the text below, and shows the fold-change in the comparisons in which they appeared. The full list of genes appearing in the comparisons using our parameters are shown in Supplemental Tables S1–S7. The complete raw data from all the comparisons is available in the GEO Database under accession number GSE64236.

### Differences in the *fabM::Erm* transcriptome compared to UA159 during growth at steady-state pH 7 (Comparison 1, Table S1)

We compared the transcriptomes of *fabM::Erm* and UA159 during growth of each strain to steady-state at pH 7. This comparison allowed us to estimate the effects of the loss of the ability to synthesize UFAs on gene expression in the *fabM::Erm* strain and, importantly, to establish a baseline for comparisons involving the *fabM::Erm* strain during glucose-shock and growth at acidic pH. Comparison 1 revealed 196 genes up-regulated and 130 genes down-regulated in *fabM::Erm*; of these, about one third of the genes (n=107) encoded hypothetical proteins or proteins of unknown function (Figure 1B).

Genes encoding proteins involved in detoxification, particularly those involved in redox/oxidative stress-sensing including alkyl hydroperoxidase (*ahpC*, SMU.764), thiol peroxidase (*tpx*, SMU.924), and glutathione oxidoreducase (*gor*, SMU.838), were up-regulated in *fabM::Erm*. Several well-established pathways of the ATR were also up-regulated in the *fabM::Erm* strain even though the strain was growing at neutral pH. The genes encoding the

malolactic fermentation (MLF) enzymes *mleS* (SMU.137) and *mleP* (SMU.138) were up-regulated 5.8-fold and 12.9-fold, respectively. Additionally, *ilvE* (SMU.1203), encoding an aminotransferase required for branched-chain amino acid (BCAA) synthesis was up-regulated by 2.3-fold. Both MLF and BCAA synthesis are mechanisms employed to alkalinize the cytoplasm as part of the *S. mutans* ATR (Sheng & Marquis, 2006, Sheng & Marquis, 2007, Sheng *et al.*, 2010, Lemme *et al.*, 2010). The gene encoding acetolactate synthase, *alsS* (SMU.1452), was also up-regulated (2.5-fold). Not only is acetolactate a precursor for BCAA biosynthesis, production of acetolactate leads to formation of acetoin as a fermentation end-product, routing carbon away from production of the acidic end-products lactate and acetate. Moreover, the glycolytic enzymes *pfk* (SMU.113) and *gapN* (SMU.676) also exhibited elevated expression in *fabM::Erm*, possibly reflecting increased need of NADPH for the elevated BCAA biosynthesis (Len *et al.*, 2004a). These observations are consistent with the concept that the *fabM::Erm* strain experiences elevated levels of acid stress, and with a need to protect stress-sensitive glycolytic enzymes (Baldeck & Marquis, 2008), carbon is routed away from the production of lactic acid. Reduced lactic acid production by the *fabM::Erm* strain is consistent with our previously reported data showing that the *fabM::Erm* strain cannot lower the environmental pH below 4.2 compared to a glycolytic minima of 3.3 for UA159 (Fozo & Quivey, 2004a).

Transcription of *trkA* (SMU.1708) and *trkH* (SMU.1709), involved in potassium uptake, was up-regulated, consistent with the role of potassium as the major counter-ion for outward-driven proton pumping via F-ATPases (Sato *et al.*, 1989, Dashper & Reynolds, 1992, Iwami *et al.*, 1997, Kashket & DePaola, 2002, Sheng & Marquis, 2006, Gong *et al.*, 2009, Krastel *et al.*, 2010). Regulation of PTS genes was complex, with the genes encoding the HPr kinase (SMU.754) and components of the  $\beta$ -glucoside (*bglP*, SMU.980) and cellobiose (*ptcC*, SMU.1596) PTS up-regulated, and *manL* (SMU.1877) and *manM* (SMU.1878) components of the glucose/mannose PTS down-regulated. Collectively, the data indicate an up-regulation of alternative carbon transport mechanisms, and a reduction in the reliance for glucose uptake via PTS, possibly indicating a shift in carbon metabolism in the *fabM::Erm* strain.

We also observed a down-regulation of genes encoding ribosomal subunits, suggesting a rerouting of resources away from protein production, or a reduction in the relative abundance of proteins. Metabolically, *S. mutans* does not code for a complete TCA cycle, only *citB* (SMU.670), *citZ* (SMU.671), and *idh* (SMU.672) are predicted in the genome (Ajdic *et al.*, 2002). All three of these genes encoding TCA cycle enzymes were down-regulated, which may suggest a response to reduced need for amino acids in the glutamate family. This agrees with the observation that the *argJ* (SMU.664) gene, also involved in glutamate family biosynthesis was down-regulated in this comparison. Overall, there was a general up-regulation of genes known to be involved in stress responsiveness, suggesting that even during growth at neutral pH, the *fabM::Erm* strain experiences a higher degree of stress than the parent strain.

## Transcriptomic changes in *S. mutans* UA159 following glucose-shock, compared to steady-state growth at pH 7 (Comparison 2, Table S2)

The removal of pH control and addition of a bolus of glucose to chemostat cultures resulted in rapid acidification of the cultures (Belli & Marquis, 1991, Fozo & Quivey, 2004b, Baker *et al.*, 2014). Similarly, it has been known for many years that dental plaque samples from humans are also fully capable of rapid metabolism of glucose to organic acids (Kleinberg, 2002). Here, our goal was to establish the effects of such a “glucose-shock”, mimicking sugar consumption by the human host, on the transcriptome of the parent strain UA159, with the express purpose of identifying, if possible, genes that might be uniquely involved with a rapid acid-response. Following addition of glucose, we observed that 219 genes were up-regulated and 205 genes were down-regulated, compared to UA159 samples from steady-state pH 7 growth (Comparison 2, Figure 1B). Broadly, the changes in the transcriptome of cells subjected to glucose-shock acidification indicate several hallmarks of the acid stress response, as well as a shift away from protein synthesis and replication, and towards production of nucleotides and amino acids. Five genes encoding proteins involved in detoxification were up-regulated, including *sod* (SMU.629), *ahpC*, *ahpF*, *tpx*, *gor*, and the peroxide resistance protein (*dpr* (SMU.540)) which acts to sequester iron in order prevent damaging Fenton chemistry (Yamamoto *et al.*, 2000). Eight amino acid biosynthesis genes were up-regulated, including those involved in glutamate, histidine, cysteine, and BCAA biosynthesis. Nine genes predicted to encode proteins involved in toxin production or resistance were up-regulated. In particular, SMU.1914c, SMU.1906c and SMU.423 were highly up-regulated, at 118-fold, 75-fold and 47-fold, respectively. Genes involved in nucleotide and nucleoside synthesis displayed increased expression, including the fifteen genes listed in Table 1, representing the majority of both the *pur* and *pyr* operons, as well as *fhs*, shown to be important in purine biosynthesis (Crowley *et al.*, 1997). The genes encoding the DNA repair enzymes Smm (SMU.1650) and MutY (SMU.1865) (Gonzalez *et al.*, 2012) were up regulated by 6.8-fold and 3.4-fold, respectively. Six genes encoding two-component systems were up-regulated, including the *comDE* system (SMU.1916 and SMU.1917) involved in regulation of competence. Thirty genes involved in transport and binding were up-regulated, with 11 of those genes encoding iron and cation carrying proteins. Among these were genes involved in potassium uptake (*trk* (SMU.1562), *trkA*, and *trkB* (SMU.1561)), copper transport (*copA* (SMU.426) and *copZ* (SMU.427)). Ten genes encoding known and putative transcription factors and nine genes encoding proteins involved in repair and degradation of proteins were up-regulated. Transcription was also elevated in 19 genes involved in energy and central intermediary metabolism. These included the genes encoding the glycolytic enzymes Pfk, AlsS and GapN, discussed in the previous paragraph, as well as the acetoin dehydrogenase complex (AdhABCD (SMU.127-SMU.130) and acetolactate decarboxylase (AldB (SMU.1451)). As mentioned previously, up-regulation of these enzymes serves to re-route carbon metabolism away from production of acidic end products and towards production of acetoin.

Twenty-five genes encoding proteins involved in cell envelope maintenance and eight genes involved in DNA replication, transcription and cell division were down-regulated. The fatty acid biosynthesis pathway was also broadly down-regulated. Twenty-three genes involved in protein synthesis were down-regulated, including several encoding tRNA synthetases and

the majority of the genes encoding ribosomal subunits. Five genes encoding transcription factors were down-regulated, notably including the gene encoding the carbon catabolite control protein, CcpA (SMU.1591c). Genes encoding PTS systems were generally down-regulated, including the general Enzyme I (EI) and histidine protein HPr, as well as components of the  $\beta$ -glucoside (*bglP*), sucrose (*scrA*, SMU.1841), glucose/mannose (*manL* and *manM*), trehalose (*pttB*, SMU.2038), cellobiose (*ptcC*), and glucose (*ptsG*, SMU.2047) PTS. This is likely due to the abundance of available glucose, and may suggest a switch from PTS transport to permease sugar transport systems, as external acidic pH values would create an environment that would greatly facilitate proton-motive force-driven uptake of glucose via permease activity, thus conserving ATP (Buckley & Hamilton, 1994). *Ffh*, the gene encoding the signal recognition particle protein (SMU.1060) (Gutierrez *et al.*, 1999), saw a 0.3-fold decrease in expression, correlating with reduced membrane biosynthetic genes. Likewise, twenty-three genes encoding transport and binding proteins were also down-regulated, including the maltose permease system. Fifteen genes involved in energy metabolism were down-regulated, including the NADH oxidase (*nox*, SMU.1117), the glycolytic enzymes *glk* (SMU.542), *eno* (SMU.1247), and *pfkB* (SMU.871), alcohol/ acetaldehyde dehydrogenase (*adhE*, SMU.148), acetate kinase (*ackA*, SMU.1978), sucrose and trehalose hydrolases (*scrB*, SMU.1843 and *treA*, SMU.2037), and the glycogen biosynthesis operon (*glgBCD*, SMU.1537-SMU.1539).

As a group, these results indicate that during glucose-shock, transcriptional changes are made to induce several well-characterized facets of the ATR including: up-regulation of ROS defense, DNA repair, BCAA biosynthesis, down-regulation of PTS, and re-routing of fermentation away from acidic end products. In addition, it appears that the cells are shifting metabolism away from production of new proteins and cell envelope components, and towards repair of existing macromolecules and synthesis of amino acids and nucleotides.

### **Changes in the transcriptome of the *fabM::Erm* strain following glucose-shock, as compared to steady-state pH 7 (Comparison 3, Table S3)**

cDNA microarray analysis revealed 111 genes up-regulated and 179 genes down-regulated in the *fabM::Erm* strain following glucose-shock compared to the transcriptome derived from the strain at steady-state pH 7 (Figure 1B). Interestingly, when making the same comparison of growth conditions for UA159 (Comparison 2), we observed an additional 134 genes that were differentially regulated in the UA159 strain, indicating that the loss of *fabM* results in either a reduced response to glucose-shock, or that stress responses are already engaged to some extent during growth at steady-state pH 7. The data above comparing *fabM::Erm* to UA159 at steady-state pH 7 (Comparison 1) would suggest the latter, as many of the changes in expression seen in Comparison 2 are similar to those observed in Comparison 1. Similar to Comparison 2, genes involved in oxidative stress, amino acid biosynthesis, toxin production and resistance, acetolactate fermentation, copper transport, DNA repair, acetoin production and protein repair and degradation were up-regulated in Comparison 3. Additionally, similar to Comparison 2, genes involved in glycolysis, the cell envelope, fatty acid biosynthesis, cellobiose PTS, and maltose transport were down-regulated. Although these general trends were similar to those seen in Comparison 2, there were fewer genes differentially regulated in many functional classes and many of the genes



that were differentially regulated had a less sizable fold-change in Comparison 3 than in Comparison 2. For example, in Comparison 3, genes encoding two-component systems, potassium uptake, purine and pyrimidine synthesis, Zn/Mn transport, PTS and ethanol fermentation were not differentially regulated to the same extent as they had been in Comparison 2. Reiteratively, this reduced response may be because *fabM::Erm* struggles to maintain cell homeostasis, even at neutral pH. Interestingly, the genes encoding ribosomal subunits were down-regulated to a greater extent (in both number of genes and fold-change of the genes) in Comparison 3 vs. Comparison 1. Taken together, these data show that following glucose-shock, the *fabM::Erm* strain exhibits many transcriptional changes; however, the changes are more modest compared to the changes made by UA159 during the same transition. This suggests that the acid-sensitive *fabM::Erm* strain, already in an elevated state of stress responsiveness, does not require as robust of a transcriptional response to fully adapt to a glucose-shock.

### **Genes differentially regulated in UA159 after transition from glucose-shock to steady-state pH 5 (Comparison 4, Table S4)**

Next, we compared the transcriptome of cells grown to steady-state at pH 5 versus cells experiencing glucose-shock. In this category, there were 119 genes up-regulated and 150 genes down-regulated (Figure 1B). We observed the reversal of several trends in expression seen in Comparison 2. Seventeen genes encoding proteins involved in cell envelope maintenance were up-regulated, many of which were reduced in expression in Comparison 2, in accord with the well-established thickening of peptidoglycan in mutants streptococci during growth in acidic conditions (van Houte & Saxton, 1971). Several genes involved in energy metabolism that were reduced in expression levels in Comparison 2 also exhibited elevated expression levels after the transition from glucose-shock to steady-state pH 5. These included: *glgBCD*, *scrB*, *treA*, and *pfkB*, which suggested mobilization of the glycogen formation/utilization pathway and involvement of the trehalose pathway.

Several genes involved in energy metabolism were up-regulated in Comparison 4 that were not differentially regulated in Comparison 2. These included the genes encoding the subunits of the F<sub>1</sub>F<sub>0</sub> ATPase (SMU.1527-SMU.1534), in order to preserve membrane pH (Dashper & Reynolds, 1992, Sheng & Marquis, 2006). The reduced expression of the PTS enzymes and the ribosomal subunits observed during glucose-shock were partially reversed, with six genes encoding ribosomal proteins and the glucose/mannose (*manLM*) and β-glucoside (*bglP*) PTS genes displaying an increase in expression. The genes encoding the multiple sugar-binding ABC transporter complex (*msmEFGK*, SMU.878-SMU.882) were up-regulated at steady-state pH 5, but had not been differentially regulated during glucose-shock conditions in the parent strain (Comparison 2), likely reflecting the conditions that excluded sugars other than glucose. Altogether, 22 genes involved in transport and binding were up-regulated in Comparison 4.

As with the genes up-regulated in this comparison, many of the genes down-regulated exhibited a reversal of expression patterns observed in Comparison 2, including decreased expression of many of the genes involved in detoxification, such as *sod*, *ahpCF*, *tpx*, *dpr*, and *gor*. Expression of the pyrimidine biosynthesis operon was decreased, while expression

of the purine biosynthesis operon was not significantly altered, compared to glucose-shock. Eighteen genes involved in solute binding and transport were down-regulated in Comparison 4, including the *sloABC* Mn/Zn transporter operon (SMU.182-SMU.184) (Rolerson *et al.*, 2006). Finally, many of the genes involved in energy metabolism that exhibited elevated expression during glucose-shock were reduced in expression levels in Comparison 4, including the *adhABCD* operon, *aldB*, and *alsS*. The genes encoding the incomplete TCA cycle (*citB*, *citZ*, and *idh*), were all significantly down-regulated in Comparison 4, and had not been differentially expressed following glucose-shock (Comparison 2), possibly indicating carbon flow away from the production of intermediates that result in the production of lactic acid.

The data obtained from Comparison 4 indicates that while some transcriptomic adjustments made following glucose-shock remain in effect during growth at steady-state pH 5, a large number of the transcriptional changes observed following glucose-shock were transient. The expression levels for many genes observed during growth at steady-state pH 5 were comparable to those seen during growth at steady-state pH 7, demonstrating that the cells were adapted to the acidic environment, and that steady-state metabolism has resumed (i.e. the excess glucose has been consumed). Thus, the genes which experienced transient differential regulation, were either required for metabolism of the excess glucose or were needed to adapt the cells to an acidic environment.

#### Changes in the *fabM::Erm* transcriptome at steady-state pH 5.5 compared to glucose-shock (Comparison 5, Table S5)

We compared the transcriptome of the *fabM::Erm* strain grown to steady-state at pH 5.5 to the transcriptome following glucose-shock (Comparison 5). In Comparison 5, 211 genes were up-regulated (92 more genes than Comparison 4) and 141 (9 fewer than the Comparison 4) genes were down-regulated. As with the parent strain UA159 grown to steady-state at low pH, the transcriptome of the *fabM::Erm* strain revealed a reversal of many of the trends seen immediately following glucose-shock. However, we observed a broader up-regulation of genes involved in central metabolic functions, including those of glycolysis, fatty acid biosynthesis, and protein synthesis, coupled with down-regulation of amino acid synthesis. Down-regulation of genes encoding the incomplete TCA cycle, acetoin production enzymes, pyrimidine synthesis enzymes, DNA repair enzymes, and oxidative stress enzymes did not appear in Comparison 5. With the exception of *citB*, *citZ*, and *idh*, we interpreted these results in the context of the inability of *fabM::Erm* to cope with acidic stress to the same extent as the parent strain (Fozo & Quivey, 2004a). In the case of *citB*, *citZ*, and *idh*, this may be because they were already down-regulated in *fabM::Erm* at steady-state pH 7 (Comparison 1). The *spxA2* gene (formerly designated *spxB*), encoding a redox-sensing transcriptional regulator (Kajfasz *et al.*, 2010), was up-regulated 5.5-fold in this comparison, supporting previously published observations from our group indicating an overlap in the oxidative stress response with the acid stress response (Derr *et al.*, 2012, Baker *et al.*, 2014). Importantly, the *atp* operon, encoding the membrane-bound F<sub>1</sub>F<sub>0</sub> ATPase was up-regulated in this comparison to a similar extent as seen in the same comparison in UA159 (Comparison 4), indicating that the acid sensitivity exhibited by *fabM::Erm* was not directly due to dysregulation of the *atp* operon.

### Steady-state pH 5 compared to steady-state pH 7 in UA159 (Comparison 6, Table S6)

Here, we used cDNA microarray analysis to examine gene expression levels in cells grown at low pH in a tightly-controlled environment. We observed 104 genes up-regulated and 78 genes down-regulated in cells grown to steady-state pH 5 compared to steady-state pH 7. There were 36 genes appearing in Comparison 6 that did not appear in Comparison 2 or Comparison 4. This is most likely due to the fold-change or *p*-value of these 36 genes in Comparisons 2 and 4 being outside the cutoff applied here (2-fold and *p* = 0.01). The  $F_1F_0$  ATPase encoding genes were up-regulated in Comparison 6. While the *atp* operon had not changed expression in Comparison 2, it had been up-regulated in Comparison 4. Comparison 6 also displayed an up-regulation of genes encoding enzymes involved in BCAA synthesis, oxidative stress, purine and pyrimidine synthesis, copper transport, potassium uptake, and a down-regulation in genes involved with alcohol fermentation, Zn/Mn transport, maltose transport, TCA cycle and several PTS systems.

Comparison 6 also served as an internal control for Comparisons 2 and 4. The multiplicative product of the fold-changes from Comparisons 2 and 4 should be similar to the fold-change observed in the direct comparison (Comparison 6). For example, one would predict that by calculating the product of the fold-change in *ahpC* expression in Comparisons 2 and 4, that up-regulation of *ahpC* in Comparison 6 should be approximately 2.8-fold. Indeed, the direct comparison (Comparison 6) showed that *ahpC* was up-regulated 2.84-fold. Overall, in Comparison 6, transcriptional changes in UA159 indicate an elevated stress response compared to growth at neutral pH; however, the organism does not appear to be experiencing the same level of stress as it was immediately following glucose-shock due to acid-adaptation via the ATR.

### The transcriptome of the *fabM::Erm* strain at steady-state pH 5.5 compared to steady-state pH 7 (Comparison 7, Table S7)

Our final comparison was that of *fabM::Erm* grown to steady-state pH 5.5 versus steady-state pH 7. As described above for Comparison 6, Comparison 7 served to validate both Comparisons 3 and 5. In Comparison 7, 71 genes were up-regulated and 79 genes were down-regulated during growth at low pH. The data from this analysis agreed with Comparisons 3 and 5, corroborating transcriptional shifts seen in genes from samples examined following glucose-shock.

Sixty-five genes appeared in Comparison 7 that did not appear in either Comparison 3 or Comparison 5. Again, the changes in expression that occurred in these genes in Comparisons 3 and 5 were outside of our pre-determined cutoff values. In Comparison 7, similar to Comparison 6 in UA159, genes involved in potassium uptake and the  $F_1F_0$  ATPase were up-regulated, and the Zn/Mn transport *slo* operon was down-regulated. Unlike UA159 in Comparison 6, *fabM::Erm* in Comparison 7 did not show any differential regulation of BCAA biosynthesis, oxidative stress, copper transport, alcohol fermentation or maltose transport. Several genes involved in purine and pyrimidine biosynthesis, toxin production and resistance, the cell envelope, and PTS exhibited differential regulation in the same direction as Comparison 6, but to a lesser extent. Interestingly, of all the analyses, Comparison 7 had the fewest number of differentially regulated genes. As we hypothesized

with the results from Comparison 3, we attribute the smaller number of changes in transcription in Comparison 7 to a higher basal level of gene expression in *fabM::Erm* involved in response to stress, even during growth at steady-state pH 7, the baseline for our comparisons.

## Discussion

In this study, we show that as the environmental pH decreases due to the rapid accumulation of lactic acid, as a byproduct of glucose fermentation, many changes occur at the transcriptional level in *S. mutans*. In addition, upon adaptation to the low pH environment, many, but not all, of these changes are reversed. The transcriptional changes outlined above can be grouped into several categories, depicted in Figure 2. The differences in transcription observed in *fabM::Erm* in Comparison 1 are similar to those observed in UA159 in Comparison 2, when the homeostasis of steady-state growth is disrupted by the glucose-shock. Therefore, Comparison 1 defines the heightened basal stress level experienced by the *fabM::Erm* strain at pH 7. The genes that are differentially regulated promptly following glucose-shock, which remain differentially regulated even after the transition to steady-state acidic pH, represent the immediate or acute ATR. The late or adaptive ATR consists of the genes that are differentially regulated between glucose-shock and steady-state acidic pH, but were not differentially regulated between steady-state pH 7 and glucose-shock conditions. These mechanisms of the ATR appear to require the cells to become adapted to growth at low pH for some time. Finally, a large amount of genes were up- or down- regulated following glucose-shock, but transcripts returned to basal (steady-state pH 7) levels once the cells were acid-adapted (steady-state low pH). These transcriptional changes represent either a response to the glucose bolus, which is metabolized by the time steady-state low pH is reached, or may indicate portions of the ATR that have functioned to allow cells to acid-adapt and whose expression is not required after adaptation has occurred (transient ATR/ glucose-shock response).

Our results uphold several observations from three previous omics studies of the *S. mutans* ATR, although it should be noted that only our study allows temporal separation of the ATR (Crowley *et al.*, 2004, Len *et al.*, 2004a, Gong *et al.*, 2009). Specifically, both the Gong *et al.* report and our report demonstrate up-regulation of *copY*, *copA*, *comDE*, the  $F_1F_0$  ATPase, and the *trk* potassium transport system, and down-regulation of *citB*, *citZ*, *idh*, and *ptsG* during growth in an acidic environment. Proteomic and enzymatic data from the Crowley *et al.* study showed that growth at steady-state pH 5 compared to steady-state pH 7 caused an increase in ATPase activity and expression of the genes encoding AhpCF and FabK, and a decrease in PTS activity and expression of the genes encoding Eno, AckA (Crowley *et al.*, 2004). These results were in agreement with the transcriptomic data provided in this study. Our comparisons also support results from the Len *et al.* proteomic study, that illustrated up-regulation of ATPase, GapN, Pfk, GlnA, IivE and down-regulation of ManL, AdhE, AckA, and Eno during growth at steady-state pH 5, compared to steady-state pH 7 (Len *et al.*, 2004a). Len *et al.* hypothesized that the glycolytic adjustments indicated a decrease in the phosphoenolpyruvate (PEP) pool consistent with reduced need for PEP in PTS, specifically  $EII^{man}$  (encoded by *manLMN*), whose protein levels were also reduced (Len *et al.*, 2004a). Our transcriptomic analysis showed that the adjustments to

glycolysis and EII<sup>man</sup> occurred immediately following glucose-shock (acute ATR), in accordance with the idea that as culture pH falls and glucose becomes readily available, energy, in the form of ATP, no longer needs to be expended to power the PTS, as glucose uptake can be powered by H<sup>+</sup> symport via the permease system. Transcription of *ilvE* and *glnA* were also elevated following glucose-shock, suggesting that BCAA biosynthesis is also a portion of the acute ATR. BCAA biosynthesis serves to both re-route carbon towards less-acidic end products and alkalize the cytoplasm through generation of NH<sub>3</sub> (Len *et al.*, 2004a). The prompt increase in *gapN* transcription is conceivably to generate the additional NADPH needed to support an increase in BCAA biosynthesis, as well as an increase in nucleotide synthesis suggested by the sweeping up-regulation of the *pur* and *pyr* operons, and *fhs*, following glucose-shock. Genes involved in potassium transport were also part of the acute ATR, likely in an effort to maintain the electrochemical gradient across the organism's membrane.

Up-regulation of the *atp* operon and down-regulation of genes encoding metal transporters (*slo*) and *citB*, *citZ*, and *idh* did not occur until cells were acid-adapted (steady-state acidic pH, adaptive ATR), suggesting that these arms of the ATR are not activated until either low pH has been maintained for a considerable amount of time, or steady-state growth at acidic pH has been reached. The *atp* operon, encoding F<sub>1</sub>F<sub>0</sub> ATPase, is utilized by *S. mutans* to extrude protons from the cell to maintain a more alkaline cytoplasm (Kuhnert & Quivey Jr, 2003, Sheng & Marquis, 2006). It is possible that following glucose-shock, the acid stress is not yet severe enough to warrant ATP usage, and more energy-efficient pathways, such as the early ATR pathways listed above (and in Figure 2), are used to maintain a more neutral intracellular space. Down-regulation of the *slo* operon suggests that either iron and/or manganese are not limiting at steady-state pH 5, or that iron uptake needs to be limited to prevent damaging Fenton chemistry from occurring. This is likely to be mediated in part by the metalloregulator of the *slo* operon, *sloR*, which has been implicated in control of certain aspects of the ATR, including the genes encoding the partial TCA cycle, also down-regulated at steady-state pH 5 (O'Rourke *et al.*, 2010). Down-regulation of the genes encoding the partial TCA cycle may signify a reduced need for amino acids in the glutamate family, due to either lack of necessity, or because glutamate is highly acidic. An increase in transcription of the multiple sugar metabolism system (*msm*) operon also appeared to be induced with the adaptive ATR. This is presumably because under the glucose-limited conditions in a chemostat culture, the cells are searching for alternate carbohydrate sources to generate ATP for proton extrusion. Interestingly, the agmatine deiminase system, and the enzymes responsible for malolactic fermentation, both recognized components of the *S. mutans* ATR (Griswold *et al.*, 2006, Lemos & Burne, 2008, Sheng *et al.*, 2010, Lemme *et al.*, 2010) did not appear to be differentially regulated as either strain went through transitions in pH (although *fabM::Erm* had a higher level of transcription of *mleS* and *mleP*), suggesting that the activity of these two pathways are regulated at the post-transcriptional level during the ATR.

Genes involved in ROS stress response were highly up-regulated following glucose-shock, then subsequently down-regulated following the transition from glucose-shock to steady-state at low pH. However transcription of these genes at steady-state low pH was still

moderately up-regulated compared to levels observed at steady-state pH 7. These results indicate that the ROS defense genes are part of both the acute ATR and the transient ATR/glucose-shock response. This likely reflects the need to prevent and respond to higher levels of ROS formed following glucose-shock, due to increased metabolic rate and the concomitant drop in culture pH. After acid-adaptation has occurred, the expression levels presumably reflect induction of the acute ATR only, as excess glucose has been exhausted and metabolism has returned to steady-state rates. As stated above, following glucose-shock, both the *pur* and *pyr* operons were highly up-regulated. The *pyr* genes followed a similar pattern to the ROS stress response genes, and were down-regulated at steady-state pH 5 compared to glucose-shock, but their expression was not significantly altered between steady-state pH 5 versus steady-state pH 7, indicating that their induction was part of the transient ATR/glucose-shock response. The *pur* genes, on the other hand, remained up-regulated at steady-state pH 5, suggesting that they are, in fact, important for long-term acid tolerance. Purine biosynthesis has been shown previously to be critical for acid-tolerance (Crowley *et al.*, 1997). These observations are consistent with a need to synthesize adenine to generate ATP for ATPase-mediated proton extrusion (Belli and Marquis, 1991).

Genes involved in cell wall maintenance, replication and division, fatty acid biosynthesis, and genes encoding the ribosomal proteins had an opposite pattern of expression to the ROS stress response and nucleotide synthesis genes, in that they were down-regulated following glucose-shock compared to steady-state pH 7, but not differentially regulated at steady-state low pH versus steady-state pH 7. Again, this suggests that these pathways were part of the transient ATR/glucose-shock response and that their repression was no longer needed once acid adaptation had occurred and glucose was once again limiting. While glucose is in excess, it appears that the priority is shifted away from proteins, DNA, and cell-wall macromolecules, and towards production of small molecules: amino acids and nucleotides.

Previous work has elucidated that an increase in the percentage of UFAs in the UA159 plasma membrane occurs immediately upon a decrease in culture pH (acute ATR) and does not reverse once the cells are acid-adapted (Fozo & Quivey, 2004a). It is noteworthy, however, that in the current study, no difference in expression of the *fab* gene cluster between steady-state pH 7 and steady-state pH 5 was observed. Therefore, this current study does not provide any novel clues as to how this shift in membrane composition occurs. Since the *fabM::Erm* strain cannot synthesize UFAs and is much less aciduric than UA159, we hypothesized that either due to, or in addition to, the absence of UFAs, there would be differences in the transcriptome between *fabM::Erm* and UA159 that would explain the apparent malfunction of the ATR in *fabM::Erm*. Expression levels in the *fabM::Erm* strain displayed similar patterns to UA159 throughout the transition from steady-state pH 7 to glucose-shock to steady-state low pH, but the transcriptional response was less pronounced, particularly following the transition from steady-state pH 7 to glucose-shock. In fact, expression levels of many genes in the *fabM::Erm* strain at steady-state pH 7 are more similar to those observed in UA159 following glucose-shock, rather than the transcript levels measured in UA159 at steady-state pH 7. These results indicate that even at neutral pH, the *fabM::Erm* strain experiences a heightened basal level of stress. Further, the magnitude of the response of *fabM::Erm* to the bolus of glucose and subsequent pH drop

suggests that the *fabM::Erm* stress response, at the transcriptional level, either cannot be elevated beyond basal levels, or is already sufficiently heightened, so that the organism can cope with the changes in the growth environment. The results of a previous study indicated that ATPase activity in *fabM::Erm* is altered with respect to UA159 (Fozo & Quivey, 2004a). An attractive hypothesis to then explain the acid-sensitivity of the *fabM::Erm* strain is dysfunction of transmembrane proteins, notably the F<sub>1</sub>F<sub>0</sub> ATPase, due to the absence of UFAs in the plasma membrane. The data presented here suggests that the cause of aberrant ATPase activity in *fabM::Erm* is post-transcriptional, as the levels of the F<sub>1</sub>F<sub>0</sub> ATPase transcript were not disparate between UA159 and *fabM::Erm* at any of the three conditions in our experimental design. Further studies are, therefore, required to elucidate specifically why *fabM::Erm* is exceptionally acid-sensitive.

As the ability of *S. mutans* to cause disease is intimately tied to its ability to thrive and out-compete other oral microbes in an acidic feast-or-famine environment, understanding the interplay between metabolism and the ATR is crucial. We envision that the extensive data set presented here, coupled with information derived from a genome-wide collection of genetic deletion strains (Quivey *et al.*, 2015), will provide a platform to analyze how the ATR in *Streptococcus mutans* is activated and controlled. Further endeavors coupling this data to promoter analysis and construction of the transcriptional regulatory response network of the *S. mutans* ATR and oxidative stress response are currently in progress.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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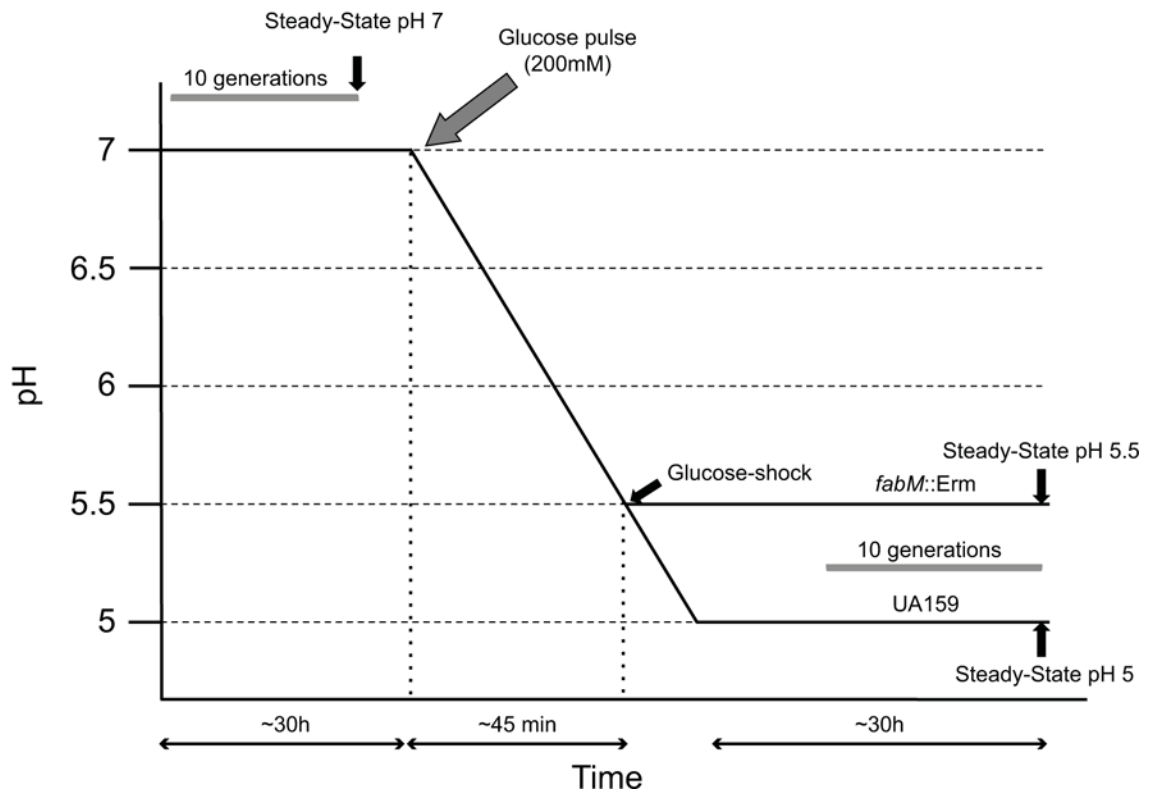
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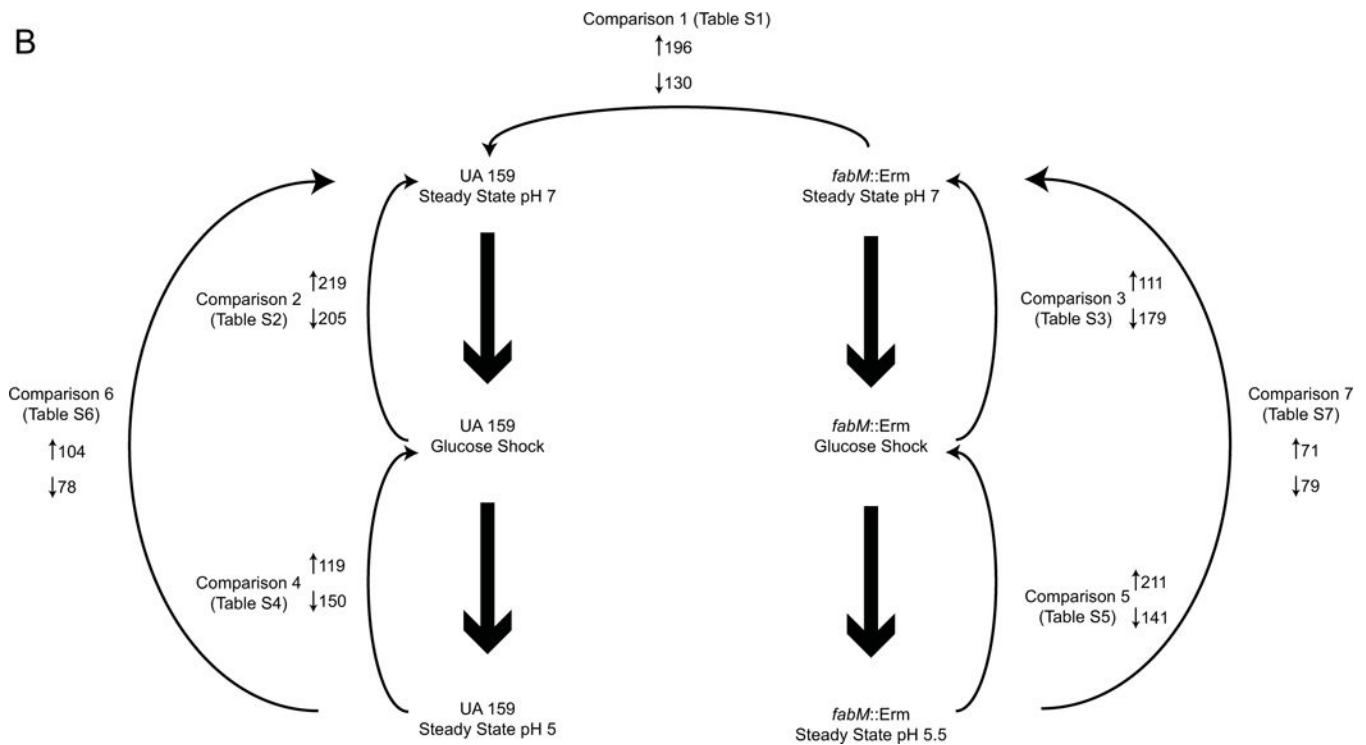


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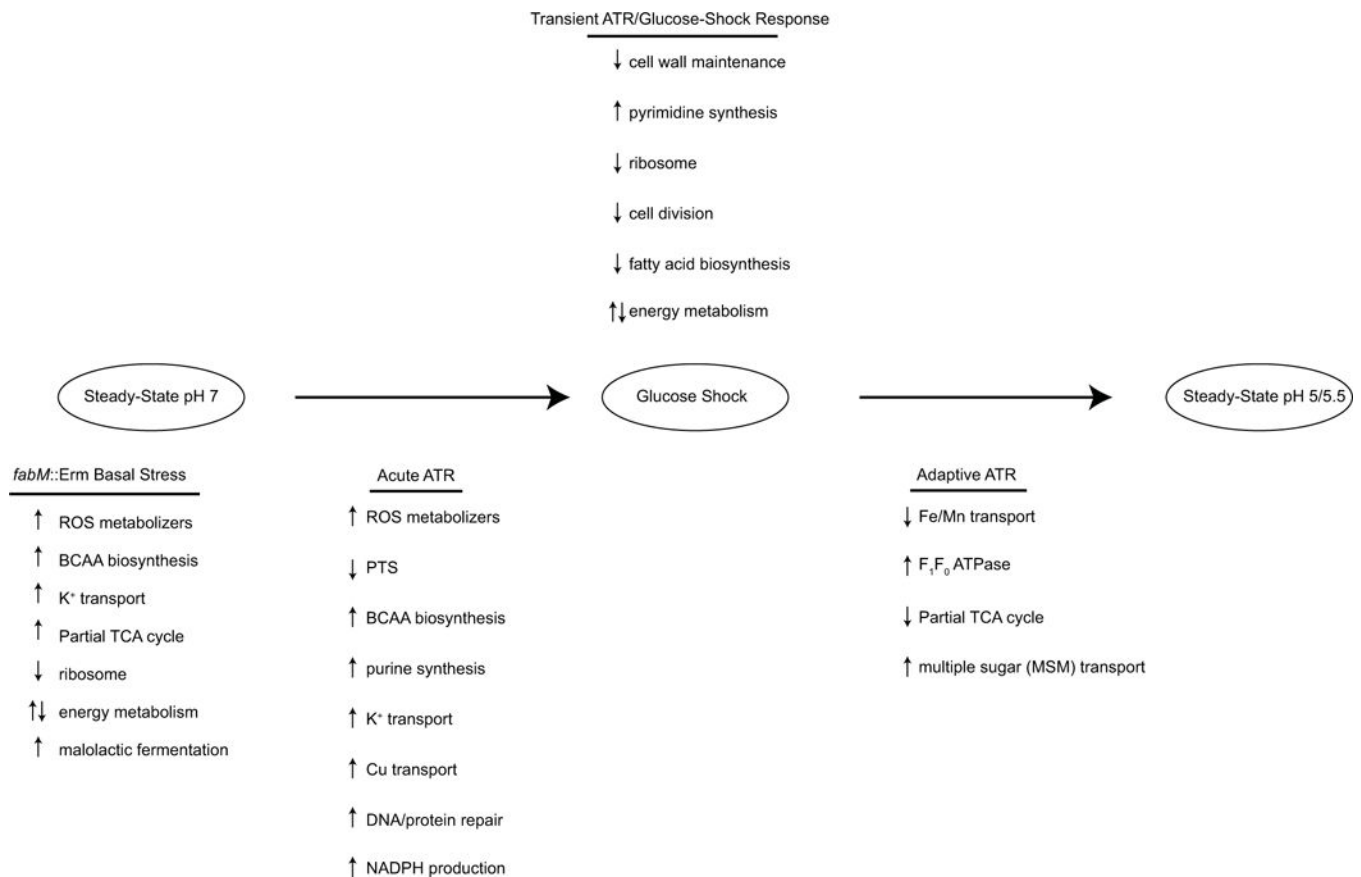
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**Figure 1. Overview of experimental design and comparisons used for data analysis**

(Panel A) Overview of experimental design. Black arrows denote time points where samples (50ml) were taken from the culture vessel to prepare RNA for cDNA microarray analysis. “Steady-State pH 7” sample was harvested after 10 generations (~30 hours) of growth in the chemostat at pH 7. Glucose was added to the culture to a final concentration of 200mM (grey arrow) and the pH was allowed to fall. When the pH reached 5.5, the “Glucose-shock” sample was taken (typically occurred about 45 minutes after addition of glucose). pH control was re-established at pH 5 for UA159 and pH 5.5 for *fabM::Erm*. Cultures were allowed to continue growth for another 10 generations (~30 hours) and the final time point, “Steady-State pH 5/5.5,” was harvested. (Panel B) Overview of comparisons used for analysis of cDNA microarray data. Numbers next to upward pointing arrows denote number of genes up-regulated in that comparison and numbers next to downward pointing arrows denote number of genes down-regulated in that comparison. Table designations reflect supplemental tables where complete lists of data analysis for that particular comparison may be found, using cutoffs for a *p*-value of 0.01 and a 2-fold change in gene expression.



**Figure 2. Summary of trends observed during glucose-shock and steady-state growth at pH 5 (or 5.5)**

A diagram summarizing observed responses as UA159 and *fabM::Erm* move from steady-state pH 7 through glucose-shock to steady-state pH 5 (or 5.5). The responses were divided into categories to represent their respective contribution to the ATR of *S. mutans*.

“*fabM::Erm* basal stress” indicates the differences discovered in gene expression in the *fabM::Erm* strain at steady-state pH 7 compared to those in UA159 at steady-state pH 7.

“Acute ATR” was defined as genes/pathways that were differentially regulated at the glucose-shock time point and remained differentially regulated through steady-state growth at low pH. The “adaptive ATR” was defined as genes/pathways that were differentially regulated between glucose-shock and growth to steady-state at low pH that were not differentially regulated between steady-state pH 7 and glucose-shock. The “transient ATR/glucose-shock” response was defined as those genes/pathways that were differentially regulated at the glucose-shock time point, but were not differentially regulated between steady-state pH 7 and steady-state pH 5/5.5. ‘ROS’ = reactive oxygen species; ‘BCAA’ = branched-chain amino acid; ‘TCA’ = tricarboxylic acid; ‘PTS’ = phosphoenolpyruvate:phosphotransferase system.

Genes up- and down-regulated in UA159 and *fabM::Erm* during glucose-shock and steady-state growth. This table includes genes commented on in the text. The complete dataset is available in Supplementary Tables S1–S7. Statistically significant changes in expression are given as a ratio of the first strain/condition listed versus the 2<sup>nd</sup> strain/condition listed. Data is grouped according to functional class. Number in parenthesis (where applicable) indicates value for comparison calculated from other available comparisons. ‘SS’ = steady-state; ‘GS’ = glucose-shock.

Table 1

Gene	Locus	Description	<i>fabM::Erm</i> SS 7 vs UA159 SS 7	UA159 GS vs. SS 7	UA159 SS 5 vs. GS	UA159 SS 5 vs. SS 7	<i>fabM::Erm</i> GS vs. SS 7	<i>fabM::Erm</i> SS 5.5 vs. GS	<i>fabM::Erm</i> SS 5.5 vs. SS 7
<b>Oxidative Stress</b>									
<i>sod</i>	SMU.629	superoxide dismutase		8.809	0.256	(2.25)	2.891		
<i>ahpC</i>	SMU.764	alkyl hydroperoxide reductase	2.41	13.46	0.211	2.84 (2.84)			
<i>ahpF</i>	SMU.765	H <sub>2</sub> O <sub>2</sub> -forming NADH oxidase		12.397	0.242	3.00 (3.00)	3.058		
<i>tpx</i>	SMU.924	thiol peroxidase	3.141	9.811	0.307	(3.01)	2.983		
<i>gor</i>	SMU.838	glutathione oxidoreductase	3.982	8.894	0.295	(2.62)	4.481		
<i>dpr</i>	SMU.540	peroxide resistance protein		2.429	0.375	(0.91)	3.442		
<b>Amino Acid Biosynthesis</b>									
<i>glnA</i>	SMU.364	glutamine synthase		2.857	0.345	(0.99)			
<i>proC</i>	SMU.1974	pyrroline carboxylate reductase		2.2			2.01	0.382	
<i>hisA</i>	SMU.1265	ProFAR		4.025					
<i>hisB</i>	SMU.1268	IGPD		2.843				0.250	
<i>ilvE</i>	SMU.1203	BCAA aminotransferase	2.391	4.571		3.521	2.19	0.417	
<i>ilvB</i>	SMU.231	acetolactate synthase, large subunit		2.718				0.480	
<i>ilvC</i>	SMU.233	ketol-acid reductoisomerase							
<i>nifS</i>	SMU.249	class-V aminotransferase		3.677	0.381	(1.40)	2.255		
<i>cysK</i>	SMU.496	cysteine synthetase	2.848	3.035				0.313	
<i>cysD</i>	SMU.1173	O-acetylhomoserine sulphydrylase					1.894		1.877
<i>hisF</i>	SMU.1264	imidazoleglycerol-phosphate synthase					2.542		(0.895)
<i>argJ</i>	SMU.664	ornithine acetyltransferase	0.44						
<b>Toxin Production and Resistance</b>									
<i>bip</i>	SMU.1914c		12.916	74.647		117	4.454		7.97
	SMU.1906c		12.204	118.112		169.786	7.016		7.93

Comparison #	1	2	4	6	3	5	7
<b>Gene</b>	<i>fabM::Erm SS 7 vs UA159 SS 7</i>	UA159 GS vs. SS 7	UA159 SS 5 vs. GS	UA159 SS 5 vs. SS 7	<i>fabM::Erm GS vs. SS 7</i>	<i>fabM::Erm SS 5.5 vs. GS</i>	<i>fabM::Erm SS 5.5 vs. SS 7</i>
<b>Locus</b>	SMU.423	46.787		48.724			6.809
<b>DNA Repair</b>							
<i>mutY</i>	SMU.1865	3.4	0.312	(1.06)	3.374		
<i>smn (end3)</i>	SMU.1650	6.755	0.304	(2.06)	3.214		
<b>Nucleotide Synthesis</b>							
<i>purB</i>	SMU.59					0.220	0.36
<i>purL</i>	SMU.30	2.44			6.342		
<i>purD</i>	SMU.48	2.672					
<i>purF</i>	SMU.32	6.066			4.659		
<i>purM</i>	SMU.34	8.39			4.632	0.290	
<i>purN</i>	SMU.35	5.714				0.258	
<i>pyrB</i>	SMU.858	6.68	0.150	(1.00)			
<i>pyrA</i>	SMU.859	14.654	0.104	(1.52)			
<i>pyrAB</i>	SMU.860	9.405	0.115	(1.09)			
<i>pyrD</i>	SMU.595	5.206			3.691		
<i>pyrE</i>	SMU.1221	5.128	0.117	(0.60)			
<i>pyrF</i>	SMU.1222	5.711	0.144	(0.82)			
<i>pyrDB</i>	SMU.1223	5.877	0.089	(0.53)		0.409	
<i>pyrK</i>	SMU.1224	37.417	0.029	(1.10)			
<i>fhs</i>	SMU.1073	4.277			1.963		
<b>Two-Component Systems</b>							
<i>comD</i>	SMU.1916	7.47					3.341
<i>comE</i>	SMU.1917	8.176					
<i>ciaR</i>	SMU.1129					6.733	
<b>PTS</b>							
<i>EI</i>	SMU.675	0.341					
<i>ptsH</i>	SMU.674	0.363					
<i>bgIP</i>	SMU.980	0.11	10.841	(1.193)			0.356
<i>scrA</i>	SMU.1841	0.188					0.491

Comparison #	1	2	3	4	5	6	7
<b>Gene</b>	<b>Locus</b>	<b>Description</b>	<b><i>fabM::Erm SS 7 vs UA159 SS 7</i></b>	<b>UA159 GS vs. SS 7</b>	<b>UA159 SS 5 vs. SS 7</b>	<b><i>fabM::Erm GS vs. SS 7</i></b>	<b><i>fabM::Erm SS 5.5 vs. SS 7</i></b>
<i>manL</i>	SMU.1877	mannose-specific component IIAB	0.353	0.147			2.969
<i>manM</i>	SMU.1878	mannose-specific component IIC	0.387	0.162	(0.464)		
<i>pttB</i>	SMU.2038	trehalose-specific IIABC component		0.149	0.236		
<i>ptcC</i>	SMU.1596	cellobiose-specific IIC component	6.058	0.435	0.265		
<i>ptxG</i>	SMU.2047	glucose-specific IIABC component		0.353			0.214
<i>pthP</i>	SMU.754	HP $\alpha$ kinase/phosphatase	2.807				
<b>Transport &amp; Binding</b>							
<i>trk</i>	SMU.1562	potassium uptake protein		4.655	3.075		3.63
<i>trkA</i>	SMU.1708	potassium uptake protein	2.585	2.661	3.212		
<i>trkB</i>	SMU.1561	potassium uptake protein		3.447	2.486		3.678
<i>trkH</i>	SMU.1709	potassium uptake protein	2.914				
<i>copA</i>	SMU.426	copper transporting ATPase		8.104	3.125	5.023	
<i>copZ</i>	SMU.427	copper chaperone		8.271	3.085	2.804	
<i>sloA</i>	SMU.182	ABC transporter		0.157	0.192		0.190
<i>sloB</i>	SMU.183	Mn/Zn ABC transporter		0.203	0.288		0.107
<i>sloC</i>	SMU.184	metal-binding ABC transporter	0.345	0.147	0.324 (0.32)		0.313
<i>malF</i>	SMU.1569	maltose/maltodextrin ABC transporter		0.421	0.385		0.223
<i>malX</i>	SMU.1568	maltose/maltodextrin ABC transporters		0.162		0.317	0.15
<i>malG</i>	SMU.1570	maltose/maltodextrin ABC transporters		0.389			0.361
<i>msmE</i>	SMU.878	MSM sugar-binding protein precursor			6.615		
<i>msmF</i>	SMU.879	MSM permease protein		6.351	3.714		2.026
<i>msmG</i>	SMU.880	MSM permease protein		4.270	3.595		
<i>msmK</i>	SMU.882	MSM ATP-binding protein		3.852	3.326		
<b>Replication and Cell Division</b>							
<i>ssb</i>	SMU.1859	single-stranded DNA-binding protein	0.23	0.357		0.166	(0.626)
<i>dnaI</i>	SMU.1921	DNA replication protein primosome	0.502	0.568			
<i>holB</i>	SMU.1662	DNA polymerase III, delta subunit		0.348			
<i>recJ</i>	SMU.1472	single-stranded DNA exonuclease		0.268	(0.788)	0.175	(0.587)
<i>rpoA</i>	SMU.2001	RNA polymerase, alpha subunit	0.245	0.199		0.141	(1.69)



Gene	Locus	Description	Comparison #							
			1 <i>fabM::Erm SS 7 vs UA159 SS 7</i>	2 UA159 GS vs. SS 7	4 UA159 SS 5 vs. GS	6 UA159 SS 5 vs. SS 7	3 <i>fabM::Erm GS vs. SS 7</i>	5 <i>fabM::Erm SS 5.5 vs. GS</i>	7 <i>fabM::Erm SS 5.5 vs. SS 7</i>	
<i>ftsH</i>	SMU.15	cell division protein	0.332						6.449	
<i>ftsX</i>	SMU.1324	cell division protein	2.261							0.35
<i>ftsQ</i>	SMU.550	cell division protein								
<i>ftsW</i>	SMU.713	cell division protein	2.063	2.876	2.224					
<b>Protein Repair &amp; Degradation</b>										
<i>clpE</i>	SMU.562	ATP-dependent protease		2.821						
<i>thdF</i>	SMU.1235	thioprene & furan degradation protein		4.456			3.647			
<i>grpE</i>	SMU.81	heat shock protein (HSP-70) co-factor	0.387	2.123	0.292	(0.620)	2.554			
<i>clpB</i>	SMU.1425	Clp proteinase, ATP-binding subunit				4.922				
<b>Energy Metabolism</b>										
<i>pfk</i>	SMU.1191	phosphofructokinase	2.977	1.806						
<i>gapN</i>	SMU.676	GAD-3-P dehydrogenase	1.759	2.152						
<i>adhA</i>	SMU.127	acetoin dehydrogenase		5.114	0.252	(1.29)	2.648			4.328
<i>adhB</i>	SMU.128	acetoin dehydrogenase		6.335	0.246	(1.56)	7.386			
<i>adhC</i>	SMU.129	dihydroliipoamide acetyltransferase		7.334	0.230	(1.69)	8.811			
<i>adhD</i>	SMU.130	dihydroliipoamide dehydrogenase		11.891	0.222	(2.64)	9.068			4.05
<i>alsS</i>	SMU.1452	$\alpha$ -acetolactate synthase	2.518	10.263	0.198	(2.03)	4.21		0.281	(1.18)
<i>aldB</i>	SMU.1451	$\alpha$ -acetolactate decarboxylase		8.274			2.549			
<i>citB</i>	SMU.670	aconitate hydratase aconitase	0.149		0.055	0.025			0.338	0.333
<i>citZ</i>	SMU.671	citrate synthase	0.134		0.083	0.041				
<i>idh</i>	SMU.672	isocitrate dehydrogenase	0.156		0.075	0.036				0.426
<i>nox</i>	SMU.1117	H <sub>2</sub> O <sub>2</sub> -forming NADH oxidase		0.182			0.319		2.615	(0.834)
<i>glk</i>	SMU.542	glucose kinase		0.411						
<i>eno</i>	SMU.1247	enolase		0.507			0.453		2.816	(1.28)
<i>pfkB</i>	SMU.871	fructose-1-P kinase		0.167	4.048	(0.676)			2.269	
<i>adhE</i>	SMU.148	alcohol/acetaldehyde dehydrogenase		0.186		0.142				
<i>ackA</i>	SMU.1978	acetate kinase		0.382						
<i>scrB</i>	SMU.1843	sucrose-6-P hydrolase		0.181	5.082	(0.920)				(0.598)
<i>treA</i>	SMU.2037	trehalose-6-P hydrolase		0.161	2.704	(0.435)	0.093		6.423	

Comparison #	1	2	4	6	3	5	7
Gene	<i>fabM::Erm SS 7 vs UA159 SS 7</i>	UA159 GS vs. SS 7	UA159 SS 5 vs. GS	UA159 SS 5 vs. SS 7	<i>fabM::Erm GS vs. SS 7</i>	<i>fabM::Erm SS 5.5 vs. GS</i>	<i>fabM::Erm SS 5.5 vs. SS 7</i>
Locus	Description						
<i>dex A</i>							
	dextranase precursor						
<i>dex B</i>			3.689				
	dextran glucosidase						
<i>gtfA</i>			3.974				
	sucrose phosphorylase						
<i>pykF</i>						2.507	2.182
	pyruvate kinase						
<i>pgk</i>						3.318	
	phosphoglycerate kinase						
<i>capP</i>						3.046	
	phosphoenolpyruvate carboxylase						
<i>gtfC</i>					0.386	6.047	2.205
	glucosyltransferase-SI						
<i>pgm B</i>					0.29		
	beta-phosphoglucosaminidase						
<i>citE</i>		2.26					
	citrate lyase						
<i>ldh</i>							
	lactate dehydrogenase						
<i>mleS</i>		12.874					
	malolactic enzyme						
<i>mleP</i>		5.778					
	malate permease						
<i>pfl</i>							
	pyruvate formate-lyase						
<i>g/gD</i>		0.194	4.567	(0.886)			
	glycogen biosynthesis protein						
<i>g/gC</i>		0.31	2.942	(0.912)			
	glucose-1-P adenyllyltransferase						
<i>g/gB</i>		0.177	3.066	(0.543)			
	1,4-alpha-glucan branching enzyme						
<i>g/gP</i>		0.357		0.387			
	glycogen phosphorylase						
<b>Cell Envelope</b>							
<i>spaP</i>		6.012				2.704	
	cell surface antigen						
<i>mreC</i>		0.104	7.711	(0.802)			
	cell shape-determining protein						
<i>mreD</i>		0.287	3.438	(0.987)	0.34	3.459	(1.18)
	cell shape-determining protein						
<i>dltC</i>		0.228	3.405	(0.776)	0.279		
	putative D-alanyl carrier protein						
<i>dltD</i>		0.302	4.720	(1.425)			
	putative extramembranal protein						
<i>murC2</i>		0.35					
	UDP-N-acetylmuramyl tripeptide synthetase						
<i>bbp2a</i>		0.366				5.880	
	carboxypeptidase						
<i>bbp2x</i>		0.32	2.572	(0.823)	0.422	2.644	(1.12)
	penicillin-binding protein 2X						
<i>pgm A</i>		0.247	4.449	(1.099)			
	alpha phosphoglucosaminidase						
<i>mraY</i>		0.378	2.487	(0.940)			
	penicillin-N-acetyltransferase						
<i>fff</i>		0.355					
	signal recognition particle protein						

Comparison #	1	2	4	6	3	5	7			
<b>Gene</b>	<b>Locus</b>	<b>Description</b>	<b><i>fabM::Erm</i> SS 7 vs <i>UA159</i> SS 7</b>	<b><i>UA159</i> GS vs. SS 7</b>	<b><i>UA159</i> SS 5 vs. GS</b>	<b><i>UA159</i> SS 5 vs. SS 7</b>	<b><i>fabM::Erm</i> GS vs. SS 7</b>	<b><i>fabM::Erm</i> SS 5.5 vs. GS</b>	<b><i>fabM::Erm</i> SS 5.5 vs. SS 7</b>	
<i>pbp1b</i>	SMU.1991	penicillin-binding protein 1 b		4.511			0.417	2.929	(1.22)	
<i>thiD</i>	SMU.85	phosphomethylpyrimidine kinase					2.362	0.288	(0.68)	
<b>Fatty Acid Biosynthesis</b>										
<i>accA</i>	SMU.1734	acetyl-CoA carboxylase, alpha subunit		0.324						
<i>accD</i>	SMU.1735	acetyl-CoA carboxylase, beta subunit	0.381	0.371			0.263	4.759	(1.25)	
<i>fabZ</i>	SMU.1737	3-hydroxymyristoyl-ACP dehydratase		0.395				2.303		
<i>fabG</i>	SMU.1740	3-oxoacyl-ACP reductase		0.331						
<i>fabD</i>	SMU.1741	malonyl-CoA (ACP) transacylase		0.338				2.562		
<i>fabK</i>	SMU.1742c	trans-2-enoyl-ACP reductase		0.384				3.029		
<i>fabH</i>	SMU.1744	3-oxoacyl-ACP synthase III		0.314			0.351	3.400	(1.19)	
<i>fabF</i>	SMU.1739	3-oxylacyl-ACP-synthase					0.481	2.097	(1.01)	
<i>acp</i>	SMU.1743	acyl carrier protein			4.262		0.21			
<i>fabK2</i>	SMU.1335c	enoyl-ACP-reductase				0.258				
<i>cls</i>	SMU.988	cardiolipin synthase	0.421				0.249	2.911	(0.725)	
<b>Ribosome</b>										
<i>rs1</i>	SMU.1200	putative ribosomal protein S1		0.275			0.409			
<i>r11</i>	SMU.1626	50S ribosomal protein L1		0.341		(0.97)	0.363			
<i>r111</i>	SMU.1627	50S ribosomal L11 protein			2.831		0.366			
<i>rs11</i>	SMU.2002	30S ribosomal protein S11	0.328	0.364	2.171		0.283	6.347	(1.80)	
<i>rs13</i>	SMU.2003	30S ribosomal protein S13		0.23			0.265	11.605	(3.08)	
<i>rs5</i>	SMU.2009	30S ribosomal protein S5	0.489	0.38	2.699	(1.03)	0.265	3.500	(0.928)	
<i>r118</i>	SMU.2010	50S ribosomal protein L18	0.39		2.189		0.366	3.994	(1.46)	
<i>r16</i>	SMU.2011	50S ribosomal protein L6 (BL10)	0.415	0.38			0.418	5.677	(2.37)	
<i>rs8</i>	SMU.2012	30S ribosomal protein S8	0.276	0.403			0.149	10.983	(1.64)	
<i>rs14</i>	SMU.2014	30S ribosomal protein S14		0.471			0.428	5.719	(2.45)	
<i>r15</i>	SMU.2015	50S ribosomal protein L5	0.348	0.464			0.412	5.117	(2.11)	
<i>r124</i>	SMU.2016	50S ribosomal protein L24	0.331	0.303	3.662	(1.11)	0.252	10.765	(2.71)	
<i>r114</i>	SMU.2017	50S ribosomal protein L14		0.398			0.428	5.719	(2.48)	
<i>r129</i>	SMU.2019	50s ribosomal protein L29	0.335	0.274			0.242	8.009	(1.94)	

Comparison #	1	2	4	6	3	5	7
<b>Gene</b>	<i>fabM::Erm SS 7 vs UA159 SS 7</i>	<i>UA159 GS vs. SS 7</i>	<i>UA159 SS 5 vs. GS</i>	<i>UA159 SS 5 vs. SS 7</i>	<i>fabM::Erm GS vs. SS 7</i>	<i>fabM::Erm SS 5.5 vs. GS</i>	<i>fabM::Erm SS 5.5 vs. SS 7</i>
<b>Locus</b>							
<b>Description</b>							
<i>r16</i>	0.473	0.493		0.45	0.412	4.477	(1.84)
<i>rs2</i>	0.512						
<b>F<sub>1</sub>F<sub>0</sub> ATPase</b>							
<i>atpA</i>			2.468	2.538			2.609
<i>atpB</i>			2.968	3.327			2.456
<i>atpC</i>			2.343			2.386	2.937
<i>atpD</i>			2.488	2.779		2.121	2.825
<i>atpE</i>						2.271	
<i>atpF</i>			3.050	2.626		3.20	
<i>atpG</i>			3.109	2.452		3.555	2.942
<i>atpH</i>			3.228	2.978		3.909	2.954
<b>Transcriptional Regulators</b>							
<i>sloR</i>			0.150				0.537
<i>gluR</i>		2.879	0.218	(0.628)			
<i>copY</i>		15.129		4.779	3.545		
<i>furR</i>		2.853					
<i>malR</i>		2.841					
<i>fabT</i>		0.365					
<i>ccpA</i>		0.319					
<i>brpA</i>		0.412			0.445		
<i>treR</i>				0.19			
<i>spxA1</i>							0.527
<i>spxA2</i>						5.546	
<i>codY</i>					0.372		
<i>fruR</i>		0.244					
<i>pyrR</i>		7.095	0.075	(0.532)		3.085	
<i>cysR</i>	6.849						
<i>cpsY</i>		2.249					0.343
<i>yhcF</i>			5.510				

Comparison #	1	2	4	6	3	5	7
<b>Gene</b>	<i>fabM::Erm SS 7 vs UA159 SS 7</i>						
<b>Locus</b>		UA159 GS vs. SS 7	UA159 SS 5 vs. GS	UA159 SS 5 vs. SS 7	<i>fabM::Erm GS vs. SS 7</i>	<i>fabM::Erm SS 5.5 vs. GS</i>	<i>fabM::Erm SS 5.5 vs. SS 7</i>
<b>Description</b>							
<i>hrcA</i>			0.165	0.254	2.841		5.492
<i>dnaA</i>							
<i>rggD</i>					3.524		