

# Acid-adaptive mechanisms of *Streptococcus mutans*—the more we know, the more we don't

J.L. Baker<sup>1</sup>, R.C. Faustoferri<sup>2</sup> and R.G. Quivey Jr<sup>1,2</sup>

<sup>1</sup> Department of Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA

<sup>2</sup> Center for Oral Biology, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA

**Correspondence:** Robert G. Quivey, Jr, Department of Microbiology & Immunology and Center for Oral Biology, University of Rochester School of Medicine & Dentistry, Rochester, NY 14642, USA Tel.: + 1 585 275 0382; fax; + 1 585 276 0190; E-mail: Robert\_Quivey@urmc.rochester.edu

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## INTRODUCTION

Bacterial stress can be defined as any change in conditions resulting in a deviation from the ideal growth rate of a bacterium. Hence, there is a continuum of stresses ranging in severity from a modest reduction in availability of a critical metabolite to rapid exposure to lethal, damaging environmental conditions. The consequences of such stresses also range from no change in growth rate, due to rapid adaptation by the bacteria, to irreparable damage or cell lysis of an entire population. A stress response, therefore, is any adaptation by the species that results in a greater abundance of viable organisms following exposure to a stressful condition than would have occurred without adaptation. Environmental stress exists in every ecological niche, and the microbiome of the human tooth surface is certainly no exception.

Despite widespread and improving education regarding oral hygiene, dental caries remains a prevalent disease associated with a tremendous economic burden (Bowen & Tabak, 1993; Bowen, 2002, 2015). Caries is a multifactorial disease, with host factors such as diet and hygiene playing a critical role in disease development, in addition to interspecies interactions and the battle for dominance inside the dental plaque biofilm (Bowen & Tabak, 1993; Bowen, 2002, 2015). Despite the complexity of disease initiation and progression, *Streptococcus mutans* is nevertheless the organism most frequently associated with the development of caries, and a reduction of its population in the oral cavity remains the primary goal in

research aimed at reducing or eliminating the disease (Bowen, 2015).

In the presence of generous host sugar intake and the absence of scrupulous oral hygiene, *S. mutans* is able to strongly adhere to teeth via synthesis of a glucan matrix, and to rapidly dominate dental plaque by using glycolytic end-products to acidify the microenvironment and kill competitors (Carlsson *et al.*, 1997; Bowen & Koo, 2011). As this rise to prominence is highly dependent on the ability of *S. mutans* to survive the acidic environment that it has created, the ability of *S. mutans* to respond to acid stress is paramount (Tong *et al.*, 2007; Lemos & Burne, 2008). Upon exposure to acid, *S. mutans* elicits many changes at the transcriptional and physiologic levels to respond to the threat of acid-damage to sensitive molecules such as DNA and the metabolic machinery. The collective response of *S. mutans* has been termed the acid tolerance response (ATR) (Quivey *et al.*, 2001; Lemos & Burne, 2008; Matsui & Cvitkovich, 2010). In this review, the known physiological, metabolic and regulatory aspects of the ATR will be discussed, as well as components that have been implicated by recent studies, but are not yet well understood.

The primary defense of most bacteria against acid is the plasma membrane, which under typical conditions is relatively impermeable to protons. This barrier allows organisms to maintain a more neutral cytoplasm relative to the extracellular space when the environment becomes acidic (a difference referred to

as  $\Delta\text{pH}$ ). Although streptococci do not maintain a fixed-point intracellular pH as Gram-negative organisms commonly do, *S. mutans* does attempt to maintain an intracellular pH roughly one pH unit higher than that of the extracellular environment, to prevent damage to acid-sensitive DNA and enzymes (Bender *et al.*, 1986). *Streptococcus mutans* accomplishes this by both actively extruding protons from the cytoplasm, as well as generating basic molecular species to neutralize protons (Lemos & Burne, 2008). *Streptococcus mutans* can continue to grow in continuous cultures at pH values of 4.5–5.0; however, the organism can survive brief periods of extreme acidification (pH 2.5), and can continue to perform glycolysis and exhibit ATPase-associated proton transport at pH 2.5–3.0 (Bender *et al.*, 1986; Belli & Marquis, 1991; Sheng & Marquis, 2006). A significant proportion of the other bacterial species inhabiting dental plaque, including the mitis group of streptococci, are unable to grow or metabolize in acidic pH ranges, giving *S. mutans* a significant competitive edge against these species in acidic conditions (Bender *et al.*, 1986; Sheng & Marquis, 2006). Additionally, *S. mutans* alters its exported fermentative end-products based upon environmental conditions, including pH, which is thought to prevent cytoplasmic acidification by re-entry of weak acids (Iwami *et al.*, 1992). *Streptococcus mutans* also encodes acid-inducible machinery to repair degraded DNA and proteins damaged by acidic conditions (Hahn *et al.*, 1999; Ajdic *et al.*, 2002; Faustoferri *et al.*, 2005; Matsui & Cvitkovitch, 2010). Several recent reports have shown that a large component of the *S. mutans* genome is required for acid-tolerance, and that several pathways appear to be induced by the ATR that have not previously been described (Baker *et al.*, 2015; Quivey *et al.*, 2015). The putative role of these pathways is further discussed below.

## THE PLASMA MEMBRANE AND CELL WALL AS A BARRIER AGAINST ACIDIFICATION

### Unsaturated fatty acids

*Streptococcus mutans*, along with several other oral species including *Streptococcus gordonii*, *Streptococcus salivarius*, and *Lactobacillus casei* have been shown to significantly increase the proportion of monounsaturated fatty acids in their plasma

membrane in response to environmental acidification (Fozo *et al.*, 2004). This change is also accompanied by a shift to longer carbon chains in the membrane fatty acids. At pH 7, saturated fatty acids with 14-carbon and 16-carbon chains are the dominant fatty acid species (~60% of the total membrane); whereas at pH 5, unsaturated fatty acids (UFAs) with 18-carbon and 20-carbon chains are predominant (~60% of the total membrane) (Fozo *et al.*, 2004). These UFAs appear to be critical in the aciduricity of *S. mutans*. Severe defects in the ability to withstand acid challenge and maintain  $\Delta\text{pH}$  occurred when the transition to a UFA-dominant membrane was blocked, either through inhibitors or deletion of biosynthetic machinery (Fozo & Quivey, 2004a,b). Additionally, a mutant strain, unable synthesize UFAs *de novo* because of a lack of a functional FabM enzyme, was significantly reduced in its ability to cause caries (Fozo *et al.*, 2007). Cardiolipin, in particular, appears to be a critical phospholipid species in acid tolerance and, in *S. mutans*, typically harbors UFA chains. Deletion of the *S. mutans* cardiolipin synthase produced a more acid-sensitive strain with a lower percentage of UFAs (MacGilvray *et al.*, 2012).

*Streptococcus mutans* is able to incorporate exogenous fatty acids into its plasma membrane, and provision of exogenous UFAs was able to partially restore the defect in aciduricity displayed by the *fabM* mutant strain (Fozo & Quivey, 2004a). Although the mechanisms of exogenous fatty acid incorporation in *S. mutans* remain unclear, *S. mutans* does encode several putative fatty acid kinases (Ajdic *et al.*, 2002), orthologs of which were recently reported to be responsible for incorporation of exogenous fatty acids in *Staphylococcus aureus* (Parsons *et al.*, 2014a,b). Another Gram-positive organism, *Enterococcus faecalis*, uses the incorporation of host fatty acids into its membrane to increase resistance to several environmental factors (Saito *et al.*, 2014). The concept that *S. mutans* may similarly use host fatty acids as part of the ATR or other stress responses remains an exciting avenue for future study.

Although clearly important for survival in an acidic environment, it remains unclear how the shift to a UFA-dominant membrane is activated and how exactly it contributes to aciduricity. Several lines of evidence suggest that the shift in membrane saturation index is controlled at the post-translational level, including the fact that the shift still occurs in the

presence of the protein synthesis inhibitor chloramphenicol (Fozo & Quivey, 2004b). Mechanistically, the UFAs themselves may directly change the permeability of the membrane to protons. Alternatively, changes in the physical properties of the membrane due to the increase in UFAs may alter the activity of transmembrane proteins important in maintaining  $\Delta\text{pH}$ , such as the ATPases.

### Glucan matrix

Production of an insoluble glucan capsule has long been considered an important virulence factor of *S. mutans* due to its role in mediating firm attachment to the tooth surface (Hamada *et al.*, 1984; Bowen & Koo, 2011). Recent work has expanded upon the idea that the extracellular, insoluble glucans also play a role in induction of the ATR by trapping and concentrating protons, preconditioning the cells against future acid stress by activating ATR pathways (Hata & Mayanagi, 2003; Guo *et al.*, 2015). Evidence for this mechanism was provided by a *gtfBC* mutant strain that was unable to produce a glucan matrix and was impaired in its ability to adapt to, and survive, acidic conditions. Additionally, transcriptomics showed that without the glucan matrix, the ATR, at the level of transcription, was impaired, as canonical players of the ATR were not upregulated during acid exposure in the *gtfBC* mutant, as they were in the parent strain (Guo *et al.*, 2015).

## MAINTENANCE OF AN ALKALINE CYTOPLASM

### ATPase activity

In addition to the barrier function provided by the cell wall and plasma membrane,  $\Delta\text{pH}$  is also maintained through alkalization of the cytoplasm. In *S. mutans*, this is accomplished by several mechanisms that either pump protons out of the cell or generate basic species to consume the intracellular protons. The F-type ATPase has been extensively studied as the primary generator of ATP in many organisms. Its role in the *S. mutans* ATR is disparate, however, because it operates in 'reverse' to its canonical function – it hydrolyzes ATP to actively pump protons out of the cytosol (Kuhnert & Quivey, 2003; Sheng & Marquis, 2006). The F-ATPase of *S. mutans* has a lower optimal pH than that of many other oral microbes and is

also significantly upregulated during growth in acidic conditions, contributing to the relative aciduricity of the organism (Kuhnert *et al.*, 2004; Len *et al.*, 2004a; Sheng & Marquis, 2006).

Since the F-ATPase is traditionally considered one of the most important components of the ATR, it was surprising that several studies have suggested that *S. mutans* can still survive in acid and excrete protons despite the deletion of several F-ATPase subunits (Suzuki *et al.*, 2000; Quivey *et al.*, 2015). In addition to the F-ATPase, *S. mutans* encodes two putative P-type ATPases, which likely contribute to the ATR as well (Magalhaes *et al.*, 2003, 2005). Interestingly, one P-type ATPase (SMU.1563) exhibited an increase in transcription shortly after glucose-shock and the associated reduction of culture pH, while the transcription of the subunits of the F-ATPase was not upregulated until the cells had been acid-adapted for some time, indicating that this P-type ATPase is likely important early in the ATR, whereas the F-type ATPase is important for long-term survival at low pH (Baker *et al.*, 2015). Deletion mutants of both putative P-type ATPases were viable, and exhibited either no increase in acid sensitivity (SMU.1563), or a modest increase in acid sensitivity (SMU.723), indicating redundant function of the F-type and P-type ATPases in the ATR of *S. mutans* (Quivey *et al.*, 2015). The regulator CcpA has been implicated in the regulation of the F-type ATPase (Abranches *et al.*, 2008); however, regulation of the ATPase enzymes at the transcriptional level is not well understood. Although it has been hypothesized that the shifts in membrane unsaturation may affect activity of the *S. mutans* F-ATPase (Fozo & Quivey, 2004a; MacGilvray *et al.*, 2012), measuring ATPase activity in live, intact cells presents a technical challenge (Thedei *et al.*, 2008); and, so a link between ATPase activity and the UFA : saturated fatty acids ratio in the *S. mutans* membrane has yet to be directly confirmed. In *E. faecalis*, F-type ATPase activity is regulated at the level of assembly, where a reduction in intracellular pH halts degradation of the complex subunits and allows association of the active enzyme in the membrane. It is possible that the *S. mutans* F-ATPase is regulated in a similar manner in addition to its regulation at the transcriptional level (Kobayashi *et al.*, 1984; Kuhnert *et al.*, 2004). Future research elucidating the specific role of the various ATPases, as well as how each enzyme is regulated

both at the level of transcription and at the level of assembly and activity, will provide a much greater understanding of the coordination of the *S. mutans* ATR.

### Agmatine deiminase system

An additional mechanism employed by *S. mutans* to maintain an alkaline cytoplasm is the agmatine deiminase system (AgDS). The AgDS gene cluster consists of the *aguBDAC* genes plus the regulator encoded by *aguR* (Griswold *et al.*, 2004, 2006). The AgDS catabolizes agmatine, a decarboxylated form of arginine, and generates putrescine, as well as CO<sub>2</sub>, NH<sub>3</sub>, and ATP, all three of which contribute to the maintenance of  $\Delta$ pH (Griswold *et al.*, 2004, 2006). Ammonia, as a basic molecule, serves to alkalize the cytoplasm. The ATP generated by the AgDS may be used for proton extrusion via ATPases. *Streptococcus mutans* appears to encode at least two carbonic anhydrases, which can use the CO<sub>2</sub> generated by the AgDS to form bicarbonate, another alkaline molecule (Ajdic *et al.*, 2002). Interestingly, deletion of either of the two putative carbonic anhydrases encoded by *S. mutans* resulted in a significantly more acid-sensitive mutant strain (Quivey *et al.*, 2015). Transcription of the AgDS was sensitive to both acid and the presence of agmatine, and is regulated by CcpA, VicRK, ComDE and CiaRH, in addition to AguR (Liu & Burne, 2009). Although it is thought that activity of the *S. mutans* AgDS is too low to substantially alkalize the extracellular environment, its activity is likely sufficient to play a role in maintaining  $\Delta$ pH (Liu & Burne, 2009).

### Malolactic fermentation

*Streptococcus mutans* encodes machinery to perform malolactic fermentation (MLF), in which malate is fermented to the less acidic lactate, while generating ATP for use in proton extrusion (Sheng & Marquis, 2007; Sheng *et al.*, 2010). The genes encoding the malolactic enzyme and permease have acid-inducible promoters and are regulated by the local regulator, MleR (Lemme *et al.*, 2010), and the global modulator, RpoE (the  $\delta$  subunit of the RNA polymerase) (Xue *et al.*, 2010). Additionally, the genes involved in MLF were downregulated in an SpxA1/A2 double mutant, indicating that they may be subject to Spx-mediated

regulation as well (Kajfasz *et al.*, 2011). This was supported by the fact that MLF activity was significantly increased in the absence of ClpXP proteolysis, which normally degrades the Spx proteins (Kajfasz *et al.*, 2011).

### Branched-chain amino acid biosynthesis

Several studies have confirmed the upregulation of the machinery to perform branched-chain amino acid (BCAA) biosynthesis during growth at low pH, at both the transcriptional and protein levels. Specifically, synthesis of the IlvE aminotransferase protein and *ilvE* mRNA was upregulated during growth in steady-state conditions at pH 5, compared with steady-state growth at pH 7 (Len *et al.*, 2004a; Baker *et al.*, 2015). BCAA biosynthesis may serve to alleviate acid stress through several mechanisms. An upregulation of BCAA biosynthesis leads to a re-routing of carbon away from pyruvate, and, therefore acidic end-products of carbon metabolism. Additionally, the reaction performed by IlvE produces ammonia, which, as stated above, is an alkaline molecule. Deletion of *ilvE*, the *S. mutans* gene encoding the BCAA aminotransferase, rendered the strain significantly more sensitive to acid (Santiago *et al.*, 2012). Transcription of *ilvE* is modulated by the global regulators CcpA and CodY (Santiago *et al.*, 2013). A recent study also indicated that the putative amino acid transporter encoded by SMU.745/SMU.746 was important for growth of *S. mutans* in acidified media, further indicating a role for amino acid metabolism in acid tolerance (Krol *et al.*, 2014).

### ION TRANSPORT

Extrusion of protons via ATPase activity generates an electric potential that is thought to be offset by the influx of potassium ions. Indeed, two studies showed that the *trk* potassium uptake system was upregulated following acid shock or during steady-state growth at pH 5 (Gong *et al.*, 2009; Baker *et al.*, 2015). A recent study confirmed the role of the *trk* system in potassium uptake and maintenance of balance between  $\Delta$ pH and the electrochemical gradient in *S. mutans* (Binopal *et al.*, 2016).

In addition to potassium transport, the genes encoding the copper transport machinery (*copYAZ*) in *S. mutans* are upregulated during acid stress (Gong

*et al.*, 2009; Baker *et al.*, 2015). In this case, however, copper is being extruded from the cells. Copper impairs F-ATPase activity under acidic conditions, affecting the organism's ability to maintain a  $\Delta\text{pH}$  (Dunning *et al.*, 1998). Recent work has confirmed that the *copYAZ* operon modulates the ATR and helps to maintain membrane potential in *S. mutans* (Singh *et al.*, 2015).

### ALTERATION OF SUGAR IMPORT

*Streptococcus mutans* is capable of using a staggering array of carbohydrate sources for energy metabolism. To this end, *S. mutans* encodes at least 14 phosphoenolpyruvate phosphotransferase systems (PTS), as well as at least four ABC-family transport systems, including the multiple sugar metabolism (Msm) transporter, for import of carbohydrates (Ajdic *et al.*, 2002). *Streptococcus mutans* relies on different systems of sugar import at neutral versus acidic pH. Multiple studies have shown the downregulation of a number of PTS during growth at low pH (Len *et al.*, 2004a; Gong *et al.*, 2009; Baker *et al.*, 2015). This has led to the hypothesis that since the PTS require ATP hydrolysis for sugar import, these systems are shut off during growth at low pH to conserve ATP for ATPase-based proton extrusion. It has been proposed that other transporters taking advantage of the  $\Delta\text{pH}$  are used during growth at low pH. The presence of an  $\text{H}^+$ /glucose symporter has long been suspected, but such an enzyme remains unidentified in *S. mutans*. Several studies have shown an upregulation of the Msm transporter during growth at low pH and deletion of *msmG* resulted in a significant increase in acid sensitivity (Gong *et al.*, 2009; Baker *et al.*, 2015; Guo *et al.*, 2015; Quivey *et al.*, 2015). As the Msm transporter is traditionally thought of as a translocator of larger, complex polysaccharides (Russell *et al.*, 1992; Tao *et al.*, 1993), and has been recently shown to play only a minor role in sucrose transport (Zeng & Burne, 2013), it remains unknown why this transporter is upregulated at low pH during growth in glucose.

### ALTERATION OF GLYCOLYTIC END-PRODUCTS

As mentioned above, the acidic environment inhabited by *S. mutans* is created by the fermentative end-products of *S. mutans* itself and neighboring bacteria in the dental plaque, suggesting that *S. mutans* has

some level of control in the amount of acid stress it experiences. Oxygen concentration, pH, and the type and availability of carbohydrates are all variables that determine the fermentative end-products produced by *S. mutans*. Mixed acid fermentation, via pyruvate-formate lyase and lactate dehydrogenase, produces lactate, acetate, formate, and ethanol, and occurs during growth in anaerobic, carbohydrate-limited conditions (Abbe *et al.*, 1982). Mixed acid fermentation via pyruvate dehydrogenase, acetoin dehydrogenase, and lactate dehydrogenase occurs during aerobic, carbohydrate-limited conditions, and produces lactate, acetate, ethanol, and acetoin (Carlsson *et al.*, 1985). Growth at low pH or in excess of simple sugars (i.e. glucose) favors production of lactate by lactate dehydrogenase almost exclusively (Iwami *et al.*, 1992). Differential expression of the various fermentative enzymes during growth at acidic versus neutral pH has been demonstrated by several studies, as has the relative amounts of glycolytic and fermentative intermediates (Crowley *et al.*, 2004; Len *et al.*, 2004a; Gong *et al.*, 2009; Baker *et al.*, 2015).

Formate, acetate, and lactate are acidic end-products, whereas ethanol and acetoin are not. *Streptococcus mutans* must balance production of acidic products to outcompete less aciduric competitors while avoiding the generation of an environment toxic to itself. Although the plasma membrane can keep charged molecules and protons out of the cytosol, weak acids pose a significant threat to the integrity of the *S. mutans*  $\Delta\text{pH}$ . These molecules, in their un-dissociated, uncharged state, can cross the hydrophobic membrane and then dissociate in the more neutral cytosol, lowering the internal pH. Therefore, despite the fact that lactate is a stronger acid than acetate, acetate may be more damaging to *S. mutans* because it is more likely to remain protonated in the extracellular space and retain its ability to diffuse through the plasma membrane. This reasoning offers an explanation to the seemingly counterintuitive fact that *S. mutans* generates almost exclusively lactate during growth at low pH.

### DNA REPAIR/PROTEIN FATE

As referred to above, *S. mutans* encodes a number of DNA/protein repair enzymes, as well as proteases and chaperones, that are able to repair damage caused by acid. In addition to the ubiquitous *recA*

DNA repair enzyme and the UV-inducible *uvrA* DNA repair enzyme, *S. mutans* also encodes an acid-inducible base excision repair system involving the *smx*, *smn*, *fpg*, and *mutY* genes (Hahn *et al.*, 1999; Hanna *et al.*, 2001; Faustoferri *et al.*, 2005; Gonzalez *et al.*, 2012). *Streptococcus mutans* also encodes the DnaK and GroES/GroEL chaperone systems, which are both regulated by the HrcA repressor (Laport *et al.*, 2001; Lemos *et al.*, 2001). These systems appear to be important for acid tolerance; however, their exact mechanism of action remains unclear. Although *S. mutans* lacks the well-studied Lon and ClpYQ proteases that degrade damaged proteins, it does encode the homologs of ClpB, ClpC, ClpE, ClpL, ClpX, and ClpP, of which ClpC, ClpL, and ClpXP have been studied in-depth (Ajdic *et al.*, 2002; Lemos & Burne, 2002; Kajfasz *et al.*, 2009, 2011). Deletion of ClpP or ClpX led to increased aciduricity, possibly due to accumulation of the Spx family regulators, which control a number of genes involved in the ATR and oxidative stress (as described above, in the case of MLF) (Kajfasz *et al.*, 2009, 2010, 2011).

## REGULATION OF THE ATR

Signaling in *S. mutans* is highly complex, with at least 14 two-component systems and 27 transcriptional regulators identified. These regulatory networks also exhibit a large amount of overlap and redundancy. Among two-component systems, LevRS, VicRK, CiaHR, LiaRS, SpaKR, ScnRK, Hk03/Rr03, and the orphan regulator GcrR (also called TarC and CovR) have been shown to play some role in acid tolerance (Idone *et al.*, 2003; Qi *et al.*, 2004; Chen *et al.*, 2008; Gong *et al.*, 2009; Dmitriev *et al.*, 2011; Zeng *et al.*, 2011; Smith & Spatafora, 2012; Stipp *et al.*, 2013; Downey *et al.*, 2014; Shankar *et al.*, 2015). These signaling networks are exquisitely labyrinthine and have been recently reviewed in depth (Smith & Spatafora, 2012). A large number of other transcriptional regulators have either been implicated in controlling the expression of genes involved in acid tolerance, or are differentially expressed during acid-shock or acid-adaptation. These include the regulators encoded by *recX*, *lacl*, *rgg*, *sloR*, *glnR*, *copY*, *furR*, *malR*, *ccpA*, *brpA*, *fruR*, *pyrR*, *hrcA*, and *cpsY*; however, a regulator (or regulators) that specifically sense low pH have yet to be identified (Lemos *et al.*, 2001; Wang & Kuramitsu, 2003; Wen *et al.*, 2006; Abranches *et al.*,

2008; Dunning *et al.*, 2008; Gong *et al.*, 2009; Chen *et al.*, 2010; Baker *et al.*, 2015; Singh *et al.*, 2015). Recent work has greatly improved our knowledge of the signaling networks used by *S. mutans* during stress, but there is still much that is poorly understood about the precise mechanism for what molecules are being sensed and how.

## THE ATR ENCOMPASSES A LARGE PROPORTION OF GENES

Collectively, studies suggest that the ATR encompasses a wide diversity of genes and pathways, some not previously associated with acid tolerance. *Streptococcus mutans* growing at steady-state pH 5 displayed 182 differentially expressed genes, compared to growth at pH 7, representing approximately 10% of the genome (Baker *et al.*, 2015). Proteomic studies using similar experimental conditions had shown that 123 proteins were differentially regulated at pH 5 compared with pH 7 (Len *et al.*, 2004a,b). These changes in expression represent long-term adaptations to acidic conditions. Amid a transition from neutral pH to acidic pH following addition of a bolus of glucose, there were 424 genes differentially expressed, compared with expression levels in steady-state growth at neutral pH, representing roughly 20% of the genome (Baker *et al.*, 2015).

Further evidence for the ATR encompassing 10–20% of the genome comes from another recent study that constructed a genome-wide deletion library containing 1112 single-gene deletion mutants in *S. mutans* UA159, representing 57% of the genome (Quivey *et al.*, 2015). When the mutants were screened for defects in aciduricity, 199 mutants (19% of the genes tested or ~10% of the genome) were more acid-sensitive than the parent strain when grown at pH 5.4 (Quivey *et al.*, 2015). The same study revealed that, of the genes in which construction of a deletion was attempted, roughly 20% did not yield a mutant strain, implying that a deletion of that gene results in a lethal phenotype (Quivey *et al.*, 2015). It is likely that a large number of these indispensable genes contribute to aciduricity as well (i.e. *ldh*, ATPase subunits, etc.).

In addition to confirming at the transcriptional level a number of well-described ATR pathways, the Baker *et al.* transcriptomic study also implicated several other pathways whose role in the ATR was not as

**Table 1** A summary of the acid tolerance response pathways and their associated knowledge gaps

Well studied/ understood	Acid tolerance response component	Description	Remaining questions
↑ ↓ Novel/ poorly understood	Agmatine deiminase system	Catalyzes conversion of agmatine to putrescine, generating CO <sub>2</sub> , NH <sub>3</sub> , and ATP, which are all used for cytoplasmic alkalinization. Is regulated by AguR, CcpA, VicRK, ComDE, and CiaRH.	How significantly does this system contribute to ΔpH <i>in vivo</i> ?
	Malolactic fermentation	Converts malate to less acidic lactate and generates ATP for proton extrusion. Is regulated by MleR, RpoE, and possibly SpxA1/A2.	How significantly does this system contribute to ΔpH <i>in vivo</i> ?
	Repair enzymes	DNA and protein repair enzymes are up-regulated during growth at low pH to repair acid damage.	How the DnaK and GroES/GroEL systems function is poorly understood.
	ATPase activity	Both F-type and P-type ATPases contribute to acid tolerance by using ATP hydrolysis to extrude protons.	How are the ATPases regulated?
	Fermentation products	Lactate becomes the dominant end-product during growth at low pH.	How is this metabolic shift regulated and executed?
	Membrane UFAs	A shift to a membrane dominated by unsaturated fatty acids is critical to aciduricity.	How is the shift initiated and controlled? How specifically do the UFAs contribute to acid tolerance?
	Branched-chain amino acid biosynthesis	IleE aminotransferase is up regulated at low pH and is critical for acid tolerance. IleE is regulated by CcpA and CodY.	How much does flux through this pathway actually change during acid adaptation? Is ammonia generation the contributing factor?
	Regulation/Signaling	Numerous two-component systems and transcriptional regulators are involved in regulating and controlling the ATR, and there is a large amount of redundancy and cross-talk between these complex signaling pathways.	How specifically is a decrease in pH sensed by the organism?
	Trk potassium transport system	Potassium influx is crucial for maintaining electrochemical balance to counter proton extrusion during acid adaptation.	How is this system regulated?
	CopYAZ copper transport system	Critical for exporting copper, which is toxic to the F-ATPase	How is this system regulated?
	Sugar Import	PTS systems are down-regulated at low pH and the Msm ABC sugar transporter is up-regulated.	Why is the Msm transport system important at low pH? Proton motive force powered transporters have yet to be identified.
	Glucan matrix	Proton trapping by the glucan matrix preconditions cells for acid tolerance.	What is the biochemistry of the proton trapping? What are the implications of the proton trapping in a multispecies biofilm?
	SloABC iron/manganese transport system	This system is down-regulated during growth at low pH.	Very little is known about why expression of this system is reduced during growth at low pH.
	Partial TCA cycle	The three TCA cycle enzymes encoded by <i>S. mutans</i> are down-regulated during growth at low pH.	Very little is known about why expression of this system is reduced during growth at low pH.
Purine biosynthesis	This pathway is up-regulated during growth at low pH.	Very little is known about why expression of this system is increased during growth at low pH.	

well-described (Baker *et al.*, 2015). These included upregulation of the purine biosynthetic machinery, as well as downregulation of the *sloABC* manganese/iron transport system and the enzymes involved in the partial tricarboxylic acid (TCA) cycle encoded by

*S. mutans* (Baker *et al.*, 2015). The importance of purines to acid tolerance has been previously shown, as a deletion in the *fts* gene, involved in purine synthesis, rendered *S. mutans* significantly more sensitive to acid (Crowley *et al.*, 1997). A tempting

hypothesis is that purine synthesis would be particularly important during growth in acidic conditions because synthesis of adenine to generate ATP for proton extrusion may be limiting. The same study showed, however, that ATP concentrations were similar in the *fhs* mutant and the parent strain during growth in acidic conditions; therefore, the reasons for upregulation of the *pur* operon during growth in acidic conditions remain unknown (Crowley *et al.*, 1997). Despite being associated primarily with oxidative stress, the differential regulation of the *sloABC* transporter in response to acid stress is not particularly surprising, given that interplay between acid and oxidative stress responses through the *sloABC*-associated SloR and GcrR regulators has been established (Dunning *et al.*, 2008; Crepps *et al.*, 2015). The downregulation of *sloABC* suggests that either manganese or iron is not limiting during growth at acidic pH, or that there may be an increased need to prevent iron uptake and resulting Fenton chemistry at low pH. Downregulation of the partial TCA cycle enzymes during growth in acidic conditions had been previously observed (Gong *et al.*, 2009). This could possibly indicate a reduced need for amino acids of the glutamate family, however the role that the downregulation of the partial TCA cycle enzymes plays in acid tolerance remains unclear. These three pathways clearly deserve further study into their role in the *S. mutans* ATR.

## CONCLUSION

In order to outcompete other members of the dental plaque microflora, *S. mutans* generates acid to lower the local pH to a level it can tolerate, but competing microorganisms cannot. This involves a balance of acid output with mechanisms to tolerate acid, so preventing *S. mutans* from becoming a victim of its own metabolism. When the extracellular environment becomes acidic, *S. mutans* protects its acid-sensitive intracellular machinery by maintaining a  $\Delta$ pH of approximately one pH unit. Extrusion of protons, influx of other cations, and generation of intracellular basic molecular species all directly contribute to upkeep of this  $\Delta$ pH. Other contributors to the  $\Delta$ pH and ATR include modification of the plasma membrane and extracellular matrix, as well as management of sugar import and output of fermentation end-products. *S. mutans* also encodes a large repertoire of enzymes

to repair acid damage to proteins and DNA that may occur. While knowledge of the activation and regulation of the ATR has increased significantly in recent years, the pathways of its regulation are intensely complex and much additional research will be needed to bring sufficient clarity to the field. In Table 1, we summarize the ATR pathways discussed in this review and their associated knowledge gaps. The ATR of *S. mutans* represents a critical virulence factor of this ubiquitous disease-causing organism and a more complete understanding of how the ATR functions is likely to lead to novel therapeutics to prevent and treat dental caries.

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