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Acid tolerance mechanisms utilized by *Streptococcus mutans*

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Abstract

Since its discovery in 1924 by J Clarke, *Streptococcus mutans* has been the focus of rigorous research efforts due to its involvement in caries initiation and progression. Its ability to ferment a range of dietary carbohydrates can rapidly drop the external environmental pH, thereby making dental plaque inhabitable to many competing species and can ultimately lead to tooth decay. Acid production by this oral pathogen would prove suicidal if not for its remarkable ability to withstand the acid onslaught by utilizing a wide variety of highly evolved acid-tolerance mechanisms. The elucidation of these mechanisms will be discussed, serving as the focus of this review.

Keywords

acid tolerance; caries; *Streptococcus mutans*

Impact of dental caries

Dental caries has been recognized as one of the most common types of bacterial infections in humans; often a recurring infection that most individuals have to contend with throughout the duration of their life [1]. As such, carious lesions have received much attention due to the social and economic impact that they have on a population [2]. Along with periodontal disease, dental caries is considered the most significant global oral health burden, consequentially industrialized countries spend 5–10% of public health expenditures on related care [3]. Furthermore, caries affects the vast majority of adults and is diagnosed in 60–90% of school-aged children in industrialized nations [3]. This equates to billions of dollars spent every year in the treatment of tooth decay alone [4,5] and this amount is predicted to increase in response to an aging demographic [6]. That said, it has been of great interest to the scientific community to examine the ecological basis of dental caries with regards to the dynamic changes in the composition of the oral flora and how these changes can cause the transition from health to disease.

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Caries etiology

Relevant to the discussion on the etiology of caries is the effect of varying sucrose concentrations on the metabolism of oral bacteria and the changes in environmental conditions that result in a lowering of the local pH [7]. Studies conducted by Stephan in the 1940s provided definitive evidence linking bacterial metabolism to the acidification of plaque [8,9], which could drop to values as low as pH 3.0 following continuous sugar exposure [10]. These observations supported earlier findings in the late 19th Century where Miller had linked microbial acid production by dental plaque bacteria to the presence of dietary substrates [11]. While he could not identify the species responsible for the acid produced, his ideas and findings helped researchers later determine that oral bacteria metabolize dietary carbohydrates and produce acid, mainly lactate, as an end product [12]. Essentially, the production of lactate by the acidogenic oral microflora causes demineralization of calcium and phosphate present in the crystal form of hydroxyapatite that comprises the enamel of the teeth [13]. When the frequency and rate of acid production exceeds the natural remineralization activity of the teeth [14], demineralization occurs and results in the subsequent progression of cavitations, provided the pH remains below a 'critical' value of approximately 5.5–5.3 for a sufficient amount of time [15,16].

***Streptococcus mutans*: a role in dental decay**

Many of the organisms responsible for acid production in plaque primarily belong to the streptococci and members of the genus *Lactobacillus* [12]. Of the streptococci, particular interest is attributed to the mutans streptococci, which consists of the species *Streptococcus sobrinus*, *Streptococcus rattus*, *Streptococcus cricetus*, *Streptococcus downei*, *Streptococcus ferus*, *Streptococcus macacae* and *Streptococcus mutans*, each identified based on their genetic and antigenic properties [17,18]. Concentrated studies on this pathogen began in the mid-1950s when Orland *et al.* established that rodents inoculated with mutans streptococci could induce caries formation [19]. Similar results were also obtained by Zinner *et al.* in 1965 [20], and findings by Fitzgerald and Keyes would provide a definitive link between *S. mutans* and caries in gnotobiotic rats and hamsters proving that it was an infectious, transmissible disease [21, 22]. Primary focus was given to *S. mutans* in the mid 1980s, when microbiologist Walter Loesche, in conjunction with many other research contributions (see [65]), provided compelling results that identified this species as the principal causative agent of tooth decay; prevalent in 70–100% of human caries cases [12].

Virulence properties of *Streptococcus mutans*

Streptococcus mutans is a Gram-positive bacterium that is first acquired by infants soon after their first tooth emerges, with the mother being identified as the major source since similar or identical genotypic profiles of the isolated strains are shared between mother and child [23]. Once *S. mutans* has been acquired, selection for a cariogenic flora has been attributed to alterations in the dental plaque ecology, driven by a drastic drop in pH [10] due to the exogenous consumption of fermentable dietary carbohydrates and the acidic metabolic end products that result [12]. Examination and characterization of the virulence properties expressed by *S. mutans* reveals how this change in the environment can favor its propagation and explain its ability to thrive in a place that many competing oral bacteria find lethal [24]. In general, three major cariogenic factors associated with *S. mutans* include adhesion, acidogenicity (the ability to produce acid) and aciduricity (the ability to tolerate acid) [25]. The acid tolerance mechanisms utilized by this organism will be the major focus of this review.

Acid tolerance

The capacity of *S. mutans* to initiate caries via acid production from the metabolism of dietary carbohydrates [12] would be suicidal if not for its remarkable ability to tolerate acid; signifying a crucial aspect of its virulence [26]. Inhabitants of dental plaque experience rapid, dynamic pH fluctuations that are greatly influenced by carbohydrate intake resulting in pH levels that can drop from neutral pH 7.0 to acidic values below pH 3.0 in less than 20 min [10,27]. In order to withstand these continual cycles of acid shock, *S. mutans* has evolved a repertoire of mechanisms that fall under two distinct categories; constitutive mechanisms and acid-induced mechanisms [28], also referred to as the acid tolerance response (ATR) [24]. More specifically, the ATR of this microorganism is defined as the ability to adapt to acid stress by prior exposure to a low, sub-lethal pH of approximately 5.5, resulting in the induction of a stimulon (expression of certain genes) that enhances survival at a pH as low as pH 3.0 [26,29]. Rather than describing the constitutive and inducible mechanisms separately, both will be described together in an attempt to highlight the collective benefits of these systems, focusing primarily on their contributions towards: the protection and repair of macromolecules; alterations of metabolic pathways; secondary metabolism; cell density, biofilm formation and regulatory systems; and intracellular pH homeostasis (see Figure 1) [30].

Protection & repair of macromolecules

A major problem of bacteria that live in acidic environments is the potential of these surroundings to acidify the intracellular cytoplasm. Negative consequences of this include loss of glycolytic enzyme activity and structural damage to the cell membrane, proteins and DNA [31]. With regards to the latter, acid resistance is optimal when an organism has the ability to protect DNA or repair DNA damage from the harmful effects of intracellular acidification [30]. To date we know of multiple proteins that support these functions, including RecA; a recombinase protein that is conserved throughout all kingdoms of life [32–34]. Furthermore, RecA operates as a moderator of homologous recombination and serves a housekeeping role that repairs and restarts stalled DNA replication forks, which are linked to genome instability [34]. Recombination as a form of DNA repair has been extensively studied in *Escherichia coli* and involves the mechanisms broken fork repair, double-stranded break repair and recombinational gap-filling repair; all of which are particularly important during DNA replication [32,35].

With regard to *S. mutans*, initial studies with RecA-deficient mutants demonstrated increased susceptibility to a killing pH of 2.5 relative to the parent strain [36]. Incredibly, this deficiency could be abolished when the mutant was permitted to elicit an acid-adaptive response, directly suggesting the presence of a DNA repair system independent of RecA [36]. This notion was further supported by an observed increase in resistance to hydrogen peroxide and UV radiation following acid adaptation [36], which involves an apurinic-apyrimidinic (AP) endonuclease [37] and a UV repair excinuclease (*uvrA*) [38], described below.

At the molecular level, DNA damage is often manifested as a loss of purines and pyrimidines [31], which results in the formation of an abasic or AP site [39]. This event occurs more rapidly at an acidic pH where protonation of the nitrogenous base consequentially leads to cleavage of the glycosyl bond [31]. Recognition of the AP site in duplex DNA and initiation of its repair is attributed to AP endonucleases that effectively cleave the phosphodiester bond located directly 5' or 3' proximal to the damaged site, depending on the class of endonuclease involved [37,40]. This form of base excision repair [39] is a multistep process that involves different classes of nucleases, including exonuclease III, which accounts for 90% of abasic site repair and is induced as a stress-response mechanism [40]. Studies conducted by Hahn *et al.* demonstrated that under acid conditions, *S. mutans* upregulates the expression of an AP

endonuclease that has a similar activity and regulation of exonuclease III of *E. coli*, therefore implicating its involvement in acid adaptation [37].

Further research in the area of DNA protection and repair in *S. mutans* includes the characterization of *uvrA* by Hanna *et al.*, who used differential display PCR to identify genes with increased expression at pH 5.0 compared with cells grown under neutral conditions (pH 7.5) [38]. The deletion of *uvrA* resulted in a mutant with increased sensitivity to UV irradiation and increased sensitivity to killing at pH 3.0, regardless of previous acid exposure at a sublethal pH of 5.0 [38]. In *Bacillus subtilis*, the *uvrA*-UV excinuclease gene is involved in the nucleotide excision repair (NER) pathway, which locates and excises bulky DNA lesions [38,39]. Moreover, the mechanism of NER is characterized by dual incisions on either side of the damaged DNA region, followed by the replacement of bound repair enzymes with replication proteins that fill the excised site and are ligated upon completion [39]. The *uvrA* gene of *S. mutans* shares 67% identity with *uvrA*-UV of *B. subtilis*, which suggests that *S. mutans* can successfully adapt to low pH through the contributions of the NER pathway and its ability to repair acid-induced DNA damage [38].

With respect to DNA repair mechanisms, *S. mutans* is well equipped to deal with genetic corruption via acid stress, which can be attributed to the collective induction of the AP endonuclease in concert with *uvrA* to further enhance its acid-tolerant phenotype. Additionally, these mechanisms have been suggested to enable *S. mutans* to respond to minor DNA damage through base excision repair (via AP endonuclease activity), as well as larger DNA lesions through the *uvrA* pathway [38].

Our discussion will now focus on the relationship between chaperonins and their impact on the acid tolerance of *S. mutans* through protein interactions. The biological role of these molecules has been recognized in a variety of different stress responses including protein folding, protection of denatured proteins and removal of damaged proteins [41]. More specifically, *S. mutans* can upregulate the expression of DnaK and GroEL under acid shock; however, only elevated levels of DnaK are maintained during adaptation to acid [42,43]. Changes in the levels of these proteins profoundly affected acid tolerance and also decreased the ability of the mutant strain (which expressed elevated levels of GroEL and reduced levels of DnaK) to lower the external pH relative to the parent [43].

DnaK and GroEL are part of the controlling inverted repeat of chaperone expression regulon [44], which is negatively regulated by HrcA and constitutes the HrcA-controlling inverted repeat of chaperone expression system [43]. Although the precise role of these gene products and their involvement in acid adaptation in *S. mutans* is poorly understood, it has been postulated that DnaK assists in the biogenesis of the F₁-F₀-ATPase, which would enhance the cell's ability to maintain a functional intracellular pH through removal of protons from the cytoplasm [43]. Interestingly, studies in *E. coli* have demonstrated that DnaK can increase the stability of *uvrA*, therefore suggesting a secondary target that can further promote the acid tolerance of organisms expressing this protein by ensuring the proper function of DNA repair systems [45]. Recent attempts to further characterize DnaK and GroEL were achieved by the development of knockdown strains which had the levels of these chaperonins reduced by 95 and 80%, respectively [46]. These strains exhibited multiple phenotypic changes including a slower growth rate, impaired biofilm formation in glucose and major proteomic changes when compared with the wild-type [46]. Notably, the DnaK knockdown strain was acid sensitive [46], again demonstrating the involvement of this protein in acid adaptation as well as the expression of other key virulence properties of *S. mutans*.

Additionally, proteomic analysis of *S. mutans* grown in continuous culture under neutral and acidic conditions identified 30 different proteins with altered levels of expression at pH 5.0

relative to pH 7.0, representing cellular and extracellular gene products associated with stress-response pathways [47]. Of these, 25 proteins were upregulated or uniquely expressed at pH 5.0 and were involved in DNA replication, transcription, translation, protein folding and proteolysis [47]. More notably, five of these proteins (Ssb, GreA, PnpA, ClpL and PepD) were isolated based on the fact that they have never been associated with acid tolerance and/or have never been studied in oral streptococci in detail [47]. Interestingly, recent studies involving a ClpL-deficient mutant demonstrated decreased viability under acid stress compared with the parent [48–50]. Moreover, the ClpL protein functions as a chaperonin that is associated with the ClpXP proteolytic complex, which is involved in the remodeling and reactivation activities of proteins [50]. Similarly, strains deficient in HtrA, a surface protease, also involved in the degradation of aberrant proteins, also reduced the ability of the mutant strain to endure acidic conditions compared with the wild-type [48,51]. Although further studies are required to examine the specific roles that each of these identified proteins play in acid adaptation, these results demonstrate the seemingly vast number of molecules available for combating acid stress that are incorporated by *S. mutans*.

In summary, this growing arsenal of induced macromolecule protection and repair mechanisms exhibited by *S. mutans* provides a strong advantage in an acidic environment over competitors not harboring these crucial systems. Collectively, these pertinent repair pathways facilitate the proper function of the essential machinery involved in transcription, translation and enzymatic activity in an acidic environment.

Alteration of metabolism

Central to caries formation is the prevalence of organic acids in the dental plaque and the decalcification of enamel that results. *S. mutans* is extremely competent at metabolizing different sugars into lactic acid even when the external pH of its environment is lowered [26]. Glycolytic enzymes involved in these pathways express a wide functional pH range, demonstrating activity at pH values as low as 4.0 [26,52], which is well below the critical pH value for caries initiation. Furthermore, it has been demonstrated that in most instances, the speed of acid production tested in a pH range of 7.0–5.0 by *S. mutans* surpasses the rates of acid production by other oral streptococci [53]. This helps explain the rapid pH drop of dental plaque harboring *S. mutans* in the presence of carbohydrates [10].

Continuous culture studies involving *S. mutans* cells grown at pH 5.5 in complex media, as compared with cells grown at pH 6.5, demonstrated a threefold increase in glycolytic activity of the lower pH-adapted cells [54]. Since no difference in overall cell yield was observed in the lower pH-adapted cultures, these results demonstrated the ability of *S. mutans* to alter its metabolism in response to a changing external pH [54]. In addition, it was shown that both the pH optimum of glucose uptake and glycolysis would shift to lower levels in accordance with lower extracellular pH values, further supporting the ability of *S. mutans* to alter its metabolic activities as an acid-adaptive response [26].

In 1992, Dashper and Reynolds determined that the extracellular pH optimum of glycolysis exhibited a much broader profile than the intracellular pH (pH_i) optimum, which decreased to zero as the pH_i dropped from pH 7.0 to 5.0 [55]. In both studies conducted by Hamilton *et al.* [26] and Dashper and Reynolds [55], *S. mutans* was able to maintain a transmembrane pH gradient that was alkaline inside relative to external pH. Specific mechanisms involved with the regulation of cytoplasmic pH values will be described later.

In 2004, Len *et al.* conducted a second proteome analysis of *S. mutans* that studied the metabolic phenotype associated with acid tolerance during steady-state continuous culture. This enabled cells to be sampled under strictly defined conditions [56]. Interestingly, 70 of the 155 upregulated protein spots detected at pH 5.0 relative to pH 7.0 were involved in metabolism,

the majority of them being associated with glycolysis, alternative acid production and branched-chain amino acid synthesis [56]. With regard to the latter, it has been postulated that *S. mutans* may be able to utilize amino acid biosynthesis to reduce H⁺ accumulation through the buffering capacity of NH₃, which is formed by glutamine synthetase as a result of this pathway [56,57]. Furthermore, synthesis of branched amino acids could also reduce H⁺ concentrations in the cytoplasm through the consumption of nicotinamide adenine dinucleotide phosphate (an intermediate step in this anabolic pathway) and by removing reducing equivalents in the form of pyruvate and 2-oxobutanoate [56].

The ability of *S. mutans* to alter its metabolic activity in response to external pH fluctuations reveals another acid tolerance mechanism that promotes its selection over competing species in an acidic environment. However, this ability is not restricted to the metabolic pathways described above. Recent studies from our laboratory have demonstrated the involvement of secondary metabolism in acid tolerance phenomena of this cariogenic pathogen and this topic will be the focus of the next section.

Secondary metabolism

Citric acid is ubiquitous in nature and is present in fruit juices and teeth, which are comprised of 0.3% citric acid by weight [58]. In lactic acid bacteria, citrate uptake via the citrate/lactate antiport system leads to increased acidurance through the generation of a proton motive force (PMF) [59]. Although *S. mutans* is unable to survive when citrate is the only carbon source, it has evolved a citrate lyase pathway similar to the oxaloacetate decarboxylase pathway of *Klebsiella pneumoniae* [60,61]. In this pathway, citrate uptake can contribute to acid tolerance via the conversion of citrate to oxaloacetate, which is then converted to pyruvate by oxaloacetate decarboxylase – a process that consumes an intracellular proton [60]. Genomic analysis of the *S. mutans* strain UA159 chromosome suggests that multiple genes are involved in citrate transport (*citM*) and metabolism (citrate lyase: *citCDEFGX* and oxaloacetate decarboxylase: *bbc*, *oad* and *pycB*) [61,62]. When citrate is present in the growth medium at neutral and acidic pH, *S. mutans* exhibits increased acid tolerance [61]. Although the precise relationship between the observed phenotype and citrate transport remains unclear, it has been suggested that *S. mutans* may exploit citrate uptake to acquire ferric iron into the cell as ferric citrate, as it is the preferred substrate of the system [61]. Further studies regarding this phenomenon are warranted to conclusively determine the involvement of citrate transport in acid tolerance.

The previously described metabolic proteomic analysis by Len *et al.* identified lactoylglutathione lyase (LGL) as one of the upregulated genes in *S. mutans* under pH 5.0, suggesting its involvement in acid tolerance [47]. LGL is essential for cell survival, due to its detoxification of methylglyoxal, a glycolytic by-product that inactivates cytoplasmic macromolecules such as proteins and nucleic acids [63]. Methylglyoxal is formed via enzymatic production from the fragmentation of triose-phosphates during glycolysis [64] and can accumulate under conditions of uncontrolled carbohydrate metabolism [65]. In general, LGL activity contributes to the acid tolerance of an organism via detoxification of increased methylglyoxal concentrations that result from highly glycolytically active cells [65]. In *S. mutans*, quantitative real-time PCR was used to examine the expression of LGL under acidic growth and acid adaptation, revealing increased expression in response to low pH in both parameters tested [63]. These findings were further supported by delayed generation times of the LGL-deficient mutant compared with the wild-type UA159 strain under low pH and decreased survival rates at pH 3.0 [63]. These studies confirmed the ability of LGL in *S. mutans* to detoxify methylglyoxal and support its role in acid tolerance mechanisms.

The aforementioned fate of pyruvate via homofermentation (lactic acid production) and heterofermentation (via the pyruvate–formate lyase pathway) is dictated by the extracellular

concentration of glucose [66]. Alternatively, pyruvate catabolism can occur via a third heterofermentive pathway designated the pyruvate dehydrogenase complex (PDH) pathway [67]. This pathway oxidizes pyruvate into CO₂ and acetyl-CoA, which can be further broken down into acetate or ethanol via acetate kinase or alcohol dehydrogenase, respectively. In *S. mutans*, the E1 α subunit of the PDH complex is encoded by the gene *pdhA* and has been shown to be strongly upregulated in response to acid stress and acid adaptation [68]. Moreover, loss of *pdhA* via mutagenesis demonstrated an acid-sensitive phenotype and expression of this protein resembled metabolic activity consistent with heterofermentive growth (i.e., decreased activity when glucose was in excess) [68]. These observations link *pdhA* to the acid-tolerant phenotype and add to the repertoire of mechanisms employed by *S. mutans* in response to acid challenges.

Cell density, regulatory systems & biofilm formation

Cell density has been shown to modulate the ability of *S. mutans* to adapt to acid challenge and its effect is dictated by whether the samples have been derived from log-phase grown planktonic or biofilm cultures [69]. Preadapted biofilm cells at low cell density exhibited decreased resistance to the killing pH (3.0) when compared with cells extracted at high cell density [69]. Further research in this area has shown that biofilm cells, specifically those that have been newly adhered to a surface, have increased acid resistance compared with their planktonic counterparts [70]. Similar results were obtained for the ATR of biofilm cells grown in a chemostat for 2 and 5 days expressing significantly higher resistances to a killing pH of 3.5 than planktonic cells [71]. Additionally, fasting biofilm cells were more resistant to acid shock by lactic acid at pH 3.8 compared with planktonic cells and both cultures exhibited increased acid resistance when they were starved compared with sugar metabolizing cells [72]. Protein analysis of planktonic and biofilm cells of *S. mutans* grown under neutral conditions, via extraction and purification by 2D polyacrylamide electrophoresis and mass spectrometry, identified 57 proteins that were upregulated 1.3-fold in biofilm cultures compared with planktonic cells [73]. Many of these proteins had unknown function; however, the results confirm the existence of a biofilm phenotype that results in the expression of certain genes not induced in the planktonic phase of existence [73].

In *S. mutans*, cell density can be monitored via a quorum sensing system encoded by the *comCDE* operon, which is similar to the Com system in *Streptococcus pneumoniae* [74,75]. Briefly, *comC* encodes a competence-stimulating peptide (CSP), while *comD* and *comE* encode a histidine kinase and response regulator, respectively [75]. The histidine kinase and response regulator comprise a two-component signal transduction system (TCSTS) that detects external CSP. Also relevant to the *comC* system is the *comAB* operon, which encodes an ABC-type transporter responsible for the expulsion of CSP out of the cell [76]. Once a density-dependent threshold has been achieved, CSP binds to ComD, which results in the autophosphorylation of ComE, which ultimately leads to the transcription and expression of competence genes and consequent regulation of physiological activities [77]. These activities are directly involved in competence development – a process that introduces novel genes to the chromosome through the acquisition and integration of exogenous DNA [75] – as well as acid tolerance and biofilm formation in particular strains. In addition to being deficient in genetic competence, *S. mutans* NG8 strains deficient in the *comC*, *-D* or *-E* genes exhibited a diminished log-phase ATR [69]. Notably, addition of exogenous CSP to the *comC* mutant restored the reduced ATR and demonstrated the importance of cell density and cell–cell communication in acid-adaptive mechanisms [69].

As previously mentioned, the ComCDE system has also been directly linked to biofilm formation in *S. mutans* strain NG8 [77]. Mutants deficient in any of the genes comprising the *comCDE* operon demonstrated an abnormal biofilm, further demonstrating the critical role that

this system plays in this process [77]. Results from these studies demonstrate the intimate relationship between different cellular functions (competence development, acid tolerance and biofilm formation), a relationship that is just beginning to be understood.

It should be noted that the role of the *comCDE* operon in biological functions other than genetic competence are different depending on the *S. mutans* isolate under scrutiny. Ahn *et al.* reported that the ComCDE system in *S. mutans* UA159 [78] did not exhibit the same role in biofilm formation and acid tolerance as was observed in *S. mutans* NG8 [69,78]. More specifically, deletion mutations in ComD or ComE did not have a significant effect on the aforementioned phenotypes, suggesting that although the role of the ComCDE system has been conserved with respect to genetic competence amongst isolates of *S. mutans*, substantial divergence has occurred amongst the streptococci [78]. In addition to these results, Ahn *et al.* also demonstrated the complexity of the TCSTS network present in *S. mutans* by examining the relationship between the ComCDE and CiaRH two-component systems and their involvement in regulating genetic competence [78]. Interestingly, mutations in CiaR and CiaH (response regulator and histidine kinase, respectively) elicited different phenotypic variations, including biofilm formation at low pH (see [78]), and varying effects on gene expression. Moreover, strains with a mutation in CiaH behaved significantly differently from CiaR, suggesting that CiaH can cross-talk with another response regulator from a different system, further demonstrating the complex nature of signaling mechanisms that exist within the cell [78].

To shed further light on the complexity of the TCSTS present in *S. mutans*, recent studies have elucidated yet another alternate role for CSP, whereby CSP can act as an ‘alarmone’ that functions to trigger autolysis in a portion of the population under stress [79]. Immunity to this programmed termination is conferred by an immunity protein designated CipI and protects against the self-produced bacteriocin CipB, which is accumulated intracellularly [79]. Furthermore, it has been demonstrated that CSP production and function as an alarmone may enhance biofilm formation by inducing cell death, which leads to the release of chromosomal DNA into the extracellular matrix as well as increased nutrients that can enhance the overall survival capabilities of the biofilm [80].

As mentioned, the ComCDE system is not the only TCSTS in the *S. mutans* genome [62]. In fact, 14 histidine kinases and 15 response regulators have been identified [62,81,82], some of which have already been characterized. In an attempt to understand the physiological role of these two-component systems and the genes they regulate, knockout mutations of the various response regulators and histidine kinases have been constructed and the mutants’ phenotypes studied. In particular, the *hk11/rr11* (also known as LiaFSR) TCSTS in *S. mutans* NG8 was shown to be important in stress-response mechanisms and deletion of the *hk11* gene resulted in a mutant with increased sensitivity to low pH [83]. The same effect was not observed in the *rr11*-deficient strain, suggesting that the histidine kinase may function as a pH sensor with the ability to interact with response regulators from different systems [83]. Notably, deletion mutants in regulatory genes separate from TCSTS also demonstrated acid-sensitive phenotypes, suggesting that other loci are involved in the ATR of *S. mutans*. These gene products include GcR, a response regulator of *ffh* and *atpA/E* (genes that encode subunits of the F₁-F₀-ATPase) [84], and BrpA, a transcriptional regulator also involved in biofilm formation, autolysis and cell division [85].

Recent studies conducted by Kawada-Matsuo *et al.* analyzed a mutant library of histidine kinase and response regulator deficient strains in *S. mutans* and their respective double-mutants for their ability to grow under neutral and acidic conditions [86]. In this study, the LytR, CiaH, ComC and an uncharacterized putative TCSTS had decreased doubling times and a diminished ATR which supported similar previous studies by Levesque *et al.* and Biswas *et al.* [81,87].

Gong *et al.* recently demonstrated that a number of TCSTS are upregulated under acid stress, including CiaHR, LevSR, LiaSR, ScnKR, HK/RR1037/1038 and ComDE [82].

Further research involving the inactivation of both the ComCDE and *hk11/rr11* TCSTS in *S. mutans* demonstrated attenuated virulence, defects in genetic competence, reduced acid production and an inability to grow at pH 5.0 [88]. In animal models, the mutant harboring this deficiency displayed a reduced ability to colonize, propagate in the oral cavity and, more importantly, initiate caries [88]. In addition, studies have demonstrated a regulatory role for *hk11/rr11* (LiaFSR) in acid-tolerance mechanics and has been recently shown to regulate the cell envelope stress response [89]. Deletions in these genes resulted in sensitivity to antibiotics that perturb the cell membrane [89], again demonstrating the multiple roles that such systems have in dealing with multiple external stresses.

Intracellular pH homeostasis

Discussion of the acid-tolerance capabilities of *S. mutans* would be incomplete without commentary on the ability of this pathogen to maintain an intracellular pH that is more alkaline relative to its surrounding medium [55,90]. This is accomplished by *S. mutans* through the prevention of proton influx by altering its membrane composition and by increasing proton extrusion via end-product efflux and F₁-F₀-ATPase activity. These mechanisms allow *S. mutans* to cope with the constantly changing pH levels in plaque and reduce the denaturing effects of an acidic cytoplasm as previously described. Furthermore, these functions allow sustained growth and permit the proper functioning of enzymes and other cellular processes that would otherwise be inhibited by an acidic intracellular compartment [91].

Generally, studies have shown that acid tolerance exhibited by oral lactic acid bacteria corresponds to their relative permeabilities to protons [30]. Studies involving increased proton permeability in *S. mutans* via the antibiotic gramicidin affected glycolytic activity and cell viability at pH 5.5, demonstrating the effect of a compromised membrane and an acidified cytoplasm [92]. In order to prevent the passive inflow of H⁺ ions, *S. mutans* can alter the fatty acid composition of its membrane as an acid-adaptive mechanism [93]. More specifically, increased levels of monounsaturated and longer chain fatty acids (C_{18:1} and C_{20:1}) were observed in membranes from cells grown at pH 5, when compared with cells grown at pH 7 [93]. Furthermore, the unsaturated to saturated fatty acid ratios were four-times higher in pH 5 cells when compared with pH 7 cells, indicating the importance of these ratios in acid tolerance, which was supported by the lack of such a response in the less aciduric *S. sobrinus* [93]. Fozo *et al.* demonstrated that *fabM* is the sole gene responsible for the production of monounsaturated fatty acids in *S. mutans* and deletion of this gene resulted in a mutant that exhibited distinct acid-sensitive attributes when compared with the wild-type strain [94]. These differences included reduced acidogenicity (1.5 log units less), sensitivity to extreme acid (3.5 log units more sensitive) and an inability to maintain a transmembrane ΔpH that was equal to the parent (reduced by half) [94]. These observations encouraged this group to assess the ability of the *fabM*-deficient strain to cause caries in a rodent model, and determined the cariogenicity and transmission efficacy from host to host *in vivo* [95]. The *fabM* mutant strain was poorly transmissible compared with the wild-type and produced fewer and less severe cavitations in animals that were directly infected, linking the virulence of this pathogen to a single gene product in this example [95].

In terms of acid tolerance, it has been proposed that membrane composition can affect proton permeability either directly or indirectly [93]. Direct effects involve the base permeability of the lipid bilayer to H⁺ ions [93], whereas indirect effects have been described in studies that demonstrated that changes in membrane lipid composition affect the optimal activity of F₁-F₀-ATPase proton pumps [96]. The relationship of these ATPases to pH homeostasis will be described later.

From a genetic standpoint, disruptions in the genes responsible for the biogenesis, assembly and maintenance of the cell membrane resulted in acid-sensitive phenotypes compared with parent strains of *S. mutans*, further implicating the importance of membrane architecture and composition to acid adaptation [30]. These acid-sensitive phenotypes were seen with: the inactivation of *dltC*, which encodes Dcp (a D-alanyl carrier protein involved in the synthesis of D-alanyl-lipoteichoic acid) [97]; deletion of Ffh (a 54-kDa subunit homologue of the signal recognition particle of *E. coli* involved in membrane biogenesis) [98,99]; and a deficiency in Dgk (a diacylglycerol kinase linked to phospholipid metabolism) [100].

More specifically, Shibata *et al.* have recently implicated the importance of Dgk in the virulence of *S. mutans*, showing that the degree of C-terminal deletion of this protein is proportional to the acid-tolerance properties of the mutants [100]. Furthermore, when three or more amino acid residue deletions were made, acidurance was diminished in an increasing fashion up until the eighth deletion, at which point no growth was observed at pH 5.5 [100]. Dgk generates phosphatidic acid and catalyses the ATP-dependent phosphorylation of sn-1,2-diacylglycerols, both of which are second messengers in eukaryotic cellular signal transduction pathways [100]. Infection of gnotobiotic rats with the *dgk*-deficient strain in *S. mutans* resulted in reduced levels of smooth surface caries compared with the wild-type. These results, in conjunction with similar findings with the *fabM*-deficient strain (previously described), demonstrate the importance of maintaining membrane integrity in *S. mutans*' ability to cause carious lesions *in vivo*.

As sugar levels in the oral cavity increase due to ingestion by the host, fermentation products change from mixed acid end products to lactic acid [101]. Thus, continued carbohydrate metabolism and fermentation through lactate dehydrogenase causes accumulation of this end product within the cytoplasm [102]. Studies have demonstrated that lactate build-up adversely affects the cells ability to maintain an elevated intracellular pH relative to the extracellular environment, resulting in the inhibition of glycolysis at pH 5.0 [102]. In order to prevent this inhibitory effect, *S. mutans* can excrete lactate in an electroneutral process in the form of lactic acid, which dissociates in aqueous solution (pK_a value of 3.86) and significantly contributes to the acidification of the external environment [101,102]. This event is independent of metabolic energy and suggests the presence of a membrane carrier specific for lactic acid extrusion [103]. Therefore, *S. mutans* possesses the ability to remove potentially harmful metabolic end products concomitantly with H^+ ions [103], contributing to pH homeostasis and providing yet another advantage over competing species under acidic conditions.

Central to the ability of *S. mutans* to maintain pH homeostasis are constitutive F_1F_0 -ATPase proton pumps that can become further expressed at low pH [104]. Induction of F_1F_0 -ATPases under acidic environments and consequent expulsion of protons from the cell helps maintain an elevated cytoplasmic pH in relation to its surroundings [104,105]. The activity of these enzymes is paramount to acid tolerance in a variety of species, such that the pH optimum of the F_1F_0 -ATPases has been directly linked to an organism's ability to survive in acidic conditions [104,105].

In addition to the F_1F_0 -ATPases, recent studies have shown that other enzymatic mechanisms can help maintain a neutral cytosolic pH during growth [106]. Specifically, these results identified a 100-kDa membrane protein that could maintain an intracellular pH that was one pH unit above that of the external media despite inhibition of the F_1F_0 -ATPases, with the uncoupler dicyclohexylcarbodiimide. This group postulated that this protein functions as a P-type ATPase due to its sensitivity to orthovanadate and lansoprazole, with H^+ -ATPase or H^+ , ion ATPase activity [106]. Furthermore, these studies demonstrated that *S. mutans* is not solely dependent on the activity of the F_1F_0 -ATPases for intracellular pH regulation. Sequence analysis of the *S. mutans* UA159 genome supports these findings, identifying

multiple diverse transport mechanisms with putative function for inorganic ions and other essential nutrient transport [62]. Further studies in these areas may reveal H⁺ antiport activity, which would further enhance the acid response capabilities of *S. mutans*.

A major consequence of proton efflux is the establishment of a PMF across the plasma membrane, which can be defined as the sum of the pH difference (ΔpH) and the transmembrane electrical potential ($\Delta\psi$, internally negative) [91,107]. The PMF acts as a major driving force that ultimately synthesizes ATP and facilitates transport of other solutes into the cell depending on their extracellular availability [108]. As such, the PMF plays an important role in dictating the intracellular conditions of the cell and it may seem intuitive to assume that this force can determine the degree to which energy production can successfully occur in acidic conditions. However, studies have shown that this is not always the case and the observed PMF under low pH values (5.0) is insufficient for ATP production in *S. mutans* [55,108]. This implies that the PMF established from the extrusion of protons from the cytoplasm is primarily used to raise the internal pH rather than to manufacture energy [55].

The connection between the PMF and its involvement in the acid-tolerance strategies of bacteria can be seen more clearly when observing the relationship that exists between the PMF and cation uptake. More specifically, regulation of the PMF can be manipulated accordingly by the fine balancing of its components $\Delta\psi$ and ΔpH [90]. Accumulating evidence has demonstrated that bacteria can adjust the PMF to their advantage under low pH conditions through the interconversion of $\Delta\psi$ via the uptake of cations, specifically potassium [30,90, 91,107,109]. Under relatively neutral pH, the initial generation of the pH gradient can be attributed to the constitutive proton pumps (i.e., F₁-F₀-ATPase) that eject H⁺ outside of the cell, thereby acidifying the external environment and further developing a transmembrane potential that is inside negative [90]. When the cell is introduced to an acidic environment, the pH is further decreased, effectively increasing the magnitude of ΔpH which would promote the re-entry of H⁺ into the cell [90]. In order to prevent this influx of protons from occurring, bacteria can compensate by increasing their uptake of potassium [90]. The uptake of potassium results in the depolarization of the membrane, and if potassium uptake continues in this manner, dissipation of $\Delta\psi$ occurs and results in the transition from a membrane potential that was initially negative to one that is positive [90,91]. Essentially, the uptake of potassium at low pH serves to invert and maintain a positive membrane potential inside the cell and ultimately functions to prevent the influx of protons and thus contributes to the acid tolerance capabilities of the organism [91].

Of the bacterial species studied utilizing these mechanisms of pH regulation, *Enterococcus faecalis* and *E. coli* have been researched extensively and have provided much insight into the current understanding of the affect that potassium has on the PMF [110,111]. Although research in this field with regards to *S. mutans* is very limited, preliminary investigation into this phenomenon by Dashper and Reynolds demonstrated a potential link to this cation and pH regulation. Described in two separate publications, they demonstrated that glycolyzing cells of *S. mutans* Ingbritt rapidly take up potassium under neutral (pH 7.0) conditions [55] and cells were also able to increase their glycolytic activity with the addition of KCl under all the concentrations tested in an acidic (pH 5.0) environment [102]. More importantly, their work in 1992 showed that glycolysis was optimal only when the cell was able to maintain a transmembrane pH gradient that was more alkaline than the external environment under low pH [55]. Previous work by Sato *et al.* regarding this relationship demonstrated that the development of a large ΔpH was dependent on the concentration of potassium in the media [112]. Since intracellular pH was directly influenced by the concentration of potassium in the extracellular medium, Dashper and Reynolds postulated that pH homeostasis near neutrality could be established through the exchange of K⁺ ions for H⁺ ions [55]. More specifically, the magnitude of the $\Delta\psi$ established via F₁-F₀-ATPase proton extrusion could be dissipated by

the electrogenic uptake of potassium while still maintaining a large ΔpH across the cell membrane [55]. Notably, these observations were dependent on the existence of a PMF and the presence of ATP [55].

In the Gram-negative bacterium *E. coli*, potassium transport has been extensively studied and over the years, multiple potassium systems in this microorganism have been characterized consisting primarily of the Trk, Kdp and Kup systems [111]. Interestingly, Gong *et al.* recently demonstrated that multiple putative potassium uptake proteins designated *trkA*, *trkH* and *trkB* were significantly upregulated under acid stress, suggesting their involvement in acid-tolerance strategies of *S. mutans* [82]. These critical observations provide the bulk of knowledge concerning the impact of K^+ uptake on intracellular pH regulation in *S. mutans*. To date, the particular system(s) involved in potassium uptake in *S. mutans* have yet to be determined. Investigation into this area can provide further insight into the already diverse mechanisms of acid tolerance employed by this dental pathogen.

The maintenance of intracellular pH via alkali production has been understudied in *S. mutans* due to the absence of genes encoding urease and arginine deiminase [62]. Both of these systems have been characterized as alkali-producing mechanisms linked to acid tolerance [30]. *S. mutans* does, however, possess an agmatine deiminase system, which closely resembles the arginine deiminase system, and is encoded by the *aguBDAC* operon [113]. Agmatine is a decarboxylated derivative of arginine and can be acquired by the cell via an agmatine-putrescine antiporter [114]. Agmatine is then hydrolyzed by agmatine deiminase to produce ammonia (NH_3) and *N*-carbamoylputrescine, which can be further catabolized to CO_2 , NH_3 and putrescine through a series of intermediate steps that also yields an ATP [113]. The putrescine formed via this pathway can then be exchanged for agmatine, which effectively restarts the cycle. Notably, this system is thought to be induced under low pH and contributes to the ATR of *S. mutans* by providing energy in the form of ATP and buffering capacity through ammonia production [113]. Research conducted by Griswold *et al.* examining the particular role of this system has demonstrated the induction of agmatine deiminase under low pH and heat, which suggests the involvement of this pathway in response to certain environmental stresses [114]. Further studies are required to concretely implicate the agmatine deiminase system as an acid-tolerance mechanism of *S. mutans*.

Another promising area of research regarding the ability of *S. mutans* to inhibit the toxic effects of acidification is focused on malolactic fermentation. In this process, decarboxylation of L-malate, an acid commonly found in fruits, can contribute to the alkalinization of the cytoplasm relative to the environment through the production of CO_2 and possible diffusion of CO_2 itself and H_2CO_3 out of the cytoplasm [115]. Interestingly, this fermentation process can also lead to ATP synthesis through the reversible action of the $\text{F}_1\text{-F}_0\text{-ATPase}$, thus contributing not only to the acid-tolerance capabilities of the organism, but also to the preservation of energy sources during times of starvation [115].

Future perspective

With such a large repertoire of mechanisms available to combat the destructive nature of an acidified environment, it is not surprising that *Streptococcus mutans* can thrive and propagate in the dynamic and often harsh conditions of the oral cavity. The ability to maintain a cytoplasmic pH within the functional range of its cellular machinery and capacity to prevent, repair and regulate protonation of its intracellular compartment allow this pathogen to outcompete other inhabitants when the conditions select for organisms with more favorable aciduric characteristics. Over the years, our understanding of these particular traits have become much more advanced; however, many questions still remain as to the extent of these abilities as a concerted operational unit and their involvement in a mixed-species community

such as that represented by dental plaque. Technological advancements will continue to develop and improve and should provide the means necessary to carry out further research efforts that will open new doorways to ideas on how we view and interpret molecular systems and stress-response pathways. Microarray analyses, proteomic and bioinformatic databases as well as genetic expression assays have already provided a multitude of new areas of interest that further expand the broad spectrum of gene products that are associated with acid tolerance. With data constantly being submitted to the literature, this consortium of information will only continue to grow and careful management and organization of these findings will be of paramount importance to understanding the intimate relationships and coordination that is involved in the regulation and expression of said systems in *S. mutans*. This daunting task can be approached through our own intricate systems that will undoubtedly include and encourage meticulous communication amongst laboratories in an attempt to better understand and identify not only the systems and proteins responsible for the stress responses observed, but also the manner in which these different systems interact with one another from a global perspective. This will provide a new insight into these systems and identify novel targets for therapeutic and preventive applications. Furthermore, increasing knowledge in these fields may eventually replace the traditional ideology of gene deletion as a method to elicit a particular phenotypic response. Instead, understanding the specific role that a gene plays in a pathway and how it is regulated will permit the manipulation, rather than complete disruption, of the components involved. As these new advancements continue to be incorporated into the laboratory setting, we will develop more precise approaches in order to unravel the mysteries behind the cariogenic properties of *S. mutans*, the primary etiological agent of dental caries.

Executive summary

Impact of dental caries

- Carious lesions are a worldwide disease that represents a severe social and economic burden.

Caries etiology

- Caries initiation and progression can be dictated by the host diet and the ability of the oral flora to metabolize nutrients. Fermentation of carbohydrates leads to acid production, thereby selecting for pathogenic species with aciduric and acidogenic properties, which further decrease the pH of the oral cavity and ultimately promote tooth decay via demineralization of the enamel.

***Streptococcus mutans*: a role in dental decay**

- The composition of the oral microflora has a significant impact on the progression of caries, often associated with the dental pathogen *S. mutans*.

Virulence properties of *S. mutans*

- Three of the most characterized virulence properties exhibited by this organism consist of adhesion, acidogenicity and aciduricity.

Acid tolerance

- Defined as the ability to tolerate acid, a property that is heavily utilized by *S. mutans* and is expressed as both constitutive and acid-inducible mechanisms.

Protection & repair of macromolecules

- DNA repair occurs through the concerted actions of a RecA-dependent and RecA-independent pathway, the latter of which utilizes *uvrA* and an apurinic-aprimidinic endonuclease, which are upregulated under acid conditions along

with a number of other uncharacterized proteins. Protein repair occurs through the chaperonins DnaK, GroEL, ClpL and HtrA.

Alteration of metabolism

- *S. mutans* can alter the rate of metabolic activity by lowering the pH optimum of glucose uptake and glycolysis in accordance with an acidified environment. Numerous uncharacterized genes involved in metabolic processes are also upregulated under acid when compared with neutral conditions.

Secondary metabolism

- Increased expression of lactoylglutathione lyase effectively detoxifies methylglyoxal (an end product of glycolysis). Upregulation of *pdhA*, an enzyme involved in pyruvate metabolism that consumes an intracellular proton.

Cell density, regulatory systems & biofilm formation

- Biofilm cells exhibit increased acid-tolerant characteristics when compared with their planktonic counterparts. Cell density is monitored via quorum sensing, which utilizes a two-component regulatory systems. Many of these systems, including the ComCDE, *hk11/rr11* and CiaH/K are involved in the acid tolerance of this organism, whereby deletion of these systems results in acid-sensitive phenotypes and decreased cariogenic potential.

Intracellular pH homeostasis

- *Streptococcus mutans* maintains an alkalized intracellular compartment relative to the external environment by altering the composition of its membrane fatty acids, end product efflux via lactic acid and increasing the expression of proton pumps.

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Figure 1. Acid tolerance mechanisms utilized by *Streptococcus mutans*

Roman numerals denote the particular aspect of acid tolerance each component is involved in:

i) the protection and repair of macromolecules; ii) alterations of metabolic pathways; iii) secondary metabolism; iv) cell density, biofilm formation and regulatory systems; and v) intracellular pH homeostasis. AP: Apurinic-apyrimidinic; LGL: Lactoylglutathione lyase; pH_i : Intracellular pH; pH_o : External pH; RR: Response regulator.