SECTION 16

Adaptation to acid stress

CHAPTER 16.1

Acid-adaptive responses of *Streptococcus mutans*, and mechanisms of integration with oxidative stress

Robert G. Quivey Jr., Roberta C. Faustoferri, Brendaliz Santiago, Jonathon Baker, Benjamin Cross, and Jin Xiao

Center for Oral Biology, Eastman Institute for Oral Health, Rochester, New York, USA
School of Medicine and Dentistry, University of Rochester, Rochester, New York, USA

16.1.1 Introduction

In this chapter, we describe facets of the adaptive stress response systems of the oral bacterium *Streptococcus mutans*. The organism referred to as *S. mutans* is actually a group of oral streptococci, referred to as the *mutans* group, found almost exclusively on the surfaces of human teeth and in saliva (Nyvad *et al.*, 2013) (see Chapter 11.3). Historically, the group has been differentiated by serotyping of rhamnose–glucose polymers on the cell surface into the *c*, *e*, *f*, and *k* serotypes. More recently, polymerase chain reaction–amplification of the genes encoding the rhamnose–glucose polymerizing proteins has been used to distinguish each of the serotypes (Nakano and Ooshima, 2009). Approximately 70–80% of *S. mutans* found in the oral cavity of humans are serotype *c*, followed by serotype *e* (approx. 20%), with serotypes *f* plus *k* comprising less than 5% (Nakano and Ooshima, 2009). The bacteria that constitute this group of alpha–gamma-hemolytic microorganisms are homofermentative microbes that produce copious amounts of lactic acid from the sugars consumed in the human diet. Sucrose, the most prominent of refined sugars found in the diets of people living in industrialized nations, is a disaccharide composed of glucose and fructose. Extracellular enzymes produced and secreted by the *mutans* streptococci have the capability of splitting sucrose into its component monosaccharides and using the resulting glucose moiety to build long-chain glucans. These reactions are catalyzed by secreted glucosyltransferases. Typically, known *mutans* genomes contain genes encoding multiple isoforms of the basic enzyme. The glucosyltransferases produce several products, including polymers of glucose strands (glucans) composed of the following: α(1→3) linked glucose, which are water-insoluble glucans; α(1→3) glucans with α(1→6) cross-links, which are water-soluble; and α(1→6) glucans that are also water-soluble (Bowen and Koo, 2011). The extracellular, water-insoluble glucans are highly adherent to surfaces and other bacteria and, therefore, provide the primary mechanism by which *mutans* streptococci are bound, irreversibly, to teeth (Yamashita *et al.*, 1993). Moreover, studies have shown that glucosyltransferases can be active in saliva samples and also when bound to other oral organisms, further advancing the accumulation of bacteria on tooth surfaces (Schilling and Bowen, 1992; Steinberg *et al.*, 1996). As glucans, bacteria, food particles, and salivary products all accrue on teeth, the complex microbial biofilm referred to as *dental plaque* (or *matrix*) forms and accumulates (Bowen and Koo, 2011) (see Chapter 11.3).

As the plaque matrix accrues, *mutans* streptococci face multiple environmental stresses, including the presence of the peroxigenic streptococci and other organisms (described in further detail in this chapter). Accumulation of organic acids from the metabolism of sugars also occurs, which can reduce local pH values to 4.0 and lower (Jensen *et al.*, 1982; Schachtele and Jensen, 1982). The organism has evolved numerous mechanisms to cope with each of these challenges. As research continues on adaptive stress responses in *S. mutans* and in related organisms, the community of oral microbiologists is learning how *S. mutans* responds to these stresses and survives.

16.1.2 A brief history of stress survival in *Streptococcus mutans*

The ability to survive acidic conditions not only is a hallmark of *S. mutans* physiology, but also is directly connected with the organism's ability to grow on the surfaces of teeth and to

Stress and Environmental Regulation of Gene Expression and Adaptation in Bacteria, First Edition. Edited by Frans J. de Bruijn.
© 2016 John Wiley & Sons, Inc. Published 2016 by John Wiley & Sons, Inc.

cause disease. The study of oxygen-metabolite-mediated stress recognition emerged, subsequently, during investigation of the interactions of peroxigenic streptococci with the *mutans* group organisms.

Decades of inquiry into the relationship between *mutans* bacteria and dental caries were firmly established by studies conducted by De Stoppelaar and colleagues, who reported the relationship between extracellular glucans formed by the *mutans* organisms, and the development and progression of dental caries (De Stoppelaar *et al.*, 1969). The association of *mutans* streptococci with dental disease then led to studies concerning the mechanisms by which the bacteria survived low pH values and the role that low pH played in the competition between the hundreds of microorganisms that we now know are present in the microbiome of dental plaque (Gross *et al.*, 2012).

Initial studies on survival at low pH were focused on mechanisms by which oral streptococci respond to acidification of their environment. It was found that the organisms, as a genus, are capable of maintaining a ΔpH across the membrane of approximately 2 pH units, although generally, 0.5–1 pH unit is more commonly measured (Dashper and Reynolds, 1992; Kashket and Barker, 1977). It is important to consider that 2 pH units is essentially a 100-fold difference in acidification. In addition, the movement of two log orders of protons across a bacterial membrane would be an electrogenic event, such that the studies also involved the possibility that counterion movement could be occurring simultaneously with transmembrane proton movement. Results from multiple laboratories showed that potassium transport, flowing outward from cells at acidic pH values, accounted for maintaining ΔΨ (the electrical gradient) in the face of descending pH values (Belli and Marquis, 1991; Dashper and Reynolds, 1993; Kashket and Barker, 1977).

Subsequent work focused on the mechanisms by which *S. mutans* maintained their membrane ΔpH and the role it may have in the relationship between the *mutans* group of organisms and other oral streptococci. It became clear that what differentiated the *mutans* bacteria from other species of oral streptococci was their ability to adapt to low pH values, and to survive in greater abundance than competing organisms (Belli and Marquis, 1991, 1994; Bender *et al.*, 1986; Sturr and Marquis, 1992).

Research conducted during subsequent years has demonstrated that the *mutans* group utilizes a variety of metabolic activities to neutralize its internal pH (pH_i) and employ mechanisms to resist and/or repair damage to the cells caused by acidification (Belli and Marquis, 1991; Bowden and Hamilton, 1987; Dashper and Reynolds, 1992, 1993; Griswold *et al.*, 2006, 2009; Santiago *et al.*, 2012; Sheng and Marquis, 2006). More recently, it has also become clear that the mechanisms used to repair acid-mediated damage include mechanisms thought to be responsive to oxidative stress (Faustoferri *et al.*, 2014; Kajfasz *et al.*, 2010) (see Section 10). In the following sections of this chapter, we will review current facets of *S. mutans* biology that provide insights into the microbe's survival in the presence of stress conditions.

16.1.3 Barrier functions in stress adaptation: membrane fatty acid composition reflects the presence of stress in *Streptococcus mutans*

A variety of bacterial species, including Gram-positive and Gram-negative organisms, are now known to modulate the composition of their membranes in response to environmental stresses (see Section 18). For example, *Escherichia coli* will elevate its proportion of membrane cyclopropane fatty acids in response to external acidification (Brown *et al.*, 1997; Chang and Cronan, 1999). *Bacillus subtilis* produces elevated levels of *anteiso* branched-chain fatty acids to increase membrane fluidity in response to a drop in temperature (Mansilla *et al.*, 2004). Our previous reports have shown that *S. mutans* will alter its membrane fatty acid composition during growth at low pH values, compared to growth at neutral pH (Quivey *et al.*, 2000a,b). When growing in the steady state at pH 7, the membrane of the UA159 genomic type strain of *S. mutans* (Ajdic *et al.*, 2002; Murchison *et al.*, 1986) contains approximately 60% saturated fatty acids (SFAs) and approximately 40% unsaturated fatty acids (UFAs) (Fozo and Quivey, 2004b). However, during growth in steady state at pH 5, the ratio becomes inverted, and approximately 60% of the membrane fatty acids are unsaturated, and 40% are saturated (Fozo and Quivey, 2004b). This change is also accompanied by a shift to longer carbon chains in the membrane fatty acids. At pH 7, 14, and 16, carbon chains are dominant; while at pH 5, 18, and 20, carbon chains are predominant (Fozo and Quivey, 2004b). Along with *S. mutans*, the oral species *Streptococcus gordonii*, *Streptococcus salivarius*, and *Lactobacillus casei* also produce elevated proportions of UFAs in the membrane in response to acid stress (Fozo *et al.*, 2004). The inversion of the normal UFA–SFA ratio occurs rapidly and in a pH-dependent manner, based on the magnitude of the drop in pH (Fozo and Quivey, 2004b). Reversion to steady-state growth at pH 7 leads to reestablishment of the original ratio; however, the UFA–SFA transition in this case is much slower, on the order of hours rather than minutes. The shift in the UFA–SFA ratio does not require new growth, as the transition can occur in the presence of chloramphenicol. However, a block in the activity of the elongation enzyme FabF, by the compound cerulenin, is able to prevent a rise in UFAs from occurring (Fozo and Quivey, 2004b). *S. mutans* synthesizes UFAs *de novo* through the action of a *trans*-2-*cis*-3-decenoyl-ACP isomerase, referred to as FabM (Fozo and Quivey, 2004a). The *S. mutans* genome does not contain homologous open-reading frames coding for fatty acid desaturases (Ajdic *et al.*, 2002), and deletion or disruption of the *fabM* gene (SMU.1746c) completely eliminates UFAs from the plasma membrane (Fozo and Quivey, 2004a; Fozo

et al., 2007). Another Gram-positive organism, *S. aureus*, has been recently shown to incorporate exogenous fatty acids into its membrane through the action of fatty acid kinases (Faks) (Parsons *et al.*, 2014a,b). In some species, incorporation of host fatty acids is critical to causing disease and may provide mechanisms of drug resistance. For example, incorporation of certain exogenous fatty acids was recently shown to confer resistance to both bile shock and daptomycin in *Enterococcus faecalis* (Saito *et al.*, 2014). *S. mutans* is also able to incorporate exogenous fatty acids into the plasma membrane and appears to encode several Fak homologs. However, the precise mechanisms of exogenous fatty acid incorporation in *S. mutans* remain to be elucidated.

Blockage of the shift to a UFA-dominant membrane, via either cerulenin treatment or deletion of *fabM*, leads to a phenotype with severely compromised aciduricity (Fozo and Quivey, 2004a; Fozo *et al.*, 2007). Addition of exogenous UFAs partially rescues this phenotype in the *fabM* mutant strain, but fails to do so in cerulenin-treated cells (Fozo and Quivey, 2004a). This is likely because cerulenin blocks the entire fatty acid biosynthetic pathway, whereas the *fabM* mutant is still able to generate SFAs *de novo*. In a rat model of dental caries (Bowen, 2013), the *fabM* mutant is greatly reduced in its capacity to cause severe disease, indicating a substantial role for UFAs in the organism's virulence potential (Fozo and Quivey, 2007).

Among the Streptococcaceae, the most completely understood fatty acid biosynthesis pathway in terms of regulation is that of *S. pneumoniae* (Jerga and Rock, 2009; Lu and Rock, 2006). Like *S. mutans, S. pneumoniae* generates UFAs *de novo* through a FabM isomerase (Marrakchi *et al.*, 2002; Pesakhov *et al.*, 2007). Control over the UFA–SFA ratio in *S. pneumoniae* is thought to occur through the relative activities of the FabK and FabF enzymes, immediately downstream of FabM in the fatty acid biosynthesis pathway. FabK activity pulls the *trans* enoyl-ACP intermediates toward SFA production, while FabF activity pulls the *cis* enoyl-ACP intermediate toward UFA production (Lu and Rock, 2006). Transcription of *fabK* in *S. pneumoniae* is controlled in part by the local regulator FabT, which is responsive to cytoplasmic levels of $C_{18:1}$ fatty acids (Jerga and Rock, 2009; Lu and Rock, 2006). *S. mutans* also encodes a FabT regulator, which appears to repress *fabM* and its own expression (Faustoferri *et al.*, 2014). The global regulator, CcpA (Henkin, 1996), was shown to regulate *fabM* and *fabT* in *S. mutans*, and it appears to do so in a pH-dependent manner (Faustoferri *et al.*, 2014). Despite increasing knowledge of the complexities surrounding transcriptional regulation of the *fab* operon, the mechanisms by which the UFA–SFA ratio is controlled in *S. mutans* remain incompletely understood. The involvement of FabT and CcpA strongly argues for a balance between the local regulator (FabT), possibly fine-tuning fatty acid biosynthesis, and the global regulator (CcpA) moderating carbon metabolism. It is clearly in the interests of the organism to balance formation of new cells, via membrane biosynthesis, with carbon availability. Thus, the coordination of these central metabolic functions might be a productive target for therapeutic intervention (Heath *et al.*, 2001; Parsons and Rock, 2011).

16.1.4 Oxidative stress response in *S. mutans*

Oxidative damage is a significant concern for *S. mutans*. Bacteriocidal levels of H_2O_2 are generated by the peroxigenic oral streptococci, a group of organisms, including *S. gordonii, Streptococcus sanguinis,* and *Streptococcus oligofermentans*, that are early colonizers of dental plaque and produce H_2O_2 through enzymes such as pyruvate oxidase and lactate oxidase (both lacking in *S. mutans*) (Kreth *et al.*, 2005, 2008; Liu *et al.*, 2012; Tong *et al.*, 2007) (see Section 10). In addition to H_2O_2, oxygen gas is present in the oral cavity, and its metabolism by metal-binding enzymes can lead to formation of the toxic superoxide radical (Imlay, 2003; Marquis, 1995). Even more reactive and damaging are hydroxyl radicals, formed from H_2O_2 in the presence of a metal ion via the Fenton reaction; and the perhydroxyl radical, which can be formed through protonation of the superoxide radical in acidic conditions (Bielski *et al.*, 1983). *S. mutans* must be able to limit the formation of damaging reactive oxygen species (ROS), safely eliminate ROS that do form, and repair damage caused by ROS. *S. mutans* encodes several enzymes that accomplish these tasks, including two NADH oxidases (*nox* and *ahpF*), NADH peroxidase (*ahpC*), superoxide dismutase (*sod*), peroxide resistance protein (*dpr*), and glutathione oxidoreductase (*gor*) (Ajdic *et al.*, 2002; Derr *et al.*, 2012; Higuchi *et al.*, 1999, 2000; Poole *et al.*, 2000; Yamamoto *et al.*, 1999). Transcription of all six of the aforementioned genes, and the enzymatic activities of Gor and Sod, has been shown to be elevated under conditions of oxidative stress (Baker *et al.*, 2014; Derr *et al.*, 2012). Sod catalyzes the reduction of superoxide to H_2O_2, and the protein Dpr is thought to sequester iron, preventing the Fenton reaction from occurring (Higuchi *et al.*, 1999, 2000). Both *dpr* and *sod* are required for optimal growth of *S. mutans* in aerobic conditions, and their deletion reduces the ability of *S. mutans* to compete with the peroxigenic oral streptococci (Fujishima *et al.*, 2013). Gor has been shown to protect *S. mutans* from diamide, a thiol-specific oxidant (Yamamoto *et al.*, 1999). The enzyme encoded by *nox* reduces oxygen to water with the concomitant oxidation of NADH to NAD^+ (Higuchi *et al.*, 2000). This critical reaction serves two important functions: eliminating oxygen, which may otherwise end up as a damaging ROS, and regeneration of NAD^+, critical for glycolysis (Derr *et al.*, 2012). AhpC and AhpF together perform the same reaction as Nox, with formation of H_2O_2 as an intermediate; however, disruption of the genes encoding the AhpCF enzymes shows insignificant effects on both NADH oxidation and resistance to oxidative stress (Derr *et al.*, 2012; Fujishima *et al.*, 2013; Higuchi *et al.*, 2000). It is possible that in *S. mutans,* AhpC and AhpF serve as a "backup" to Nox or are important in alternate growth conditions. It has also been shown that during oxidative stress, in the context of aeration, the ability of *S. mutans* to form a biofilm is inhibited, and

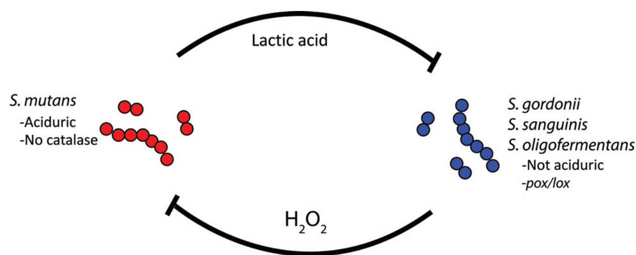

Figure 16.1.1 Competition between *S. mutans* and the perioxigenic oral streptococci. *S. mutans* generates lactic acid, thereby generating an acidic microenvironment that the organism is able to tolerate, but the peroxigenic oral streptococci (*S. gordonii*, *S. sanguinis*, and *S. oligofermentans*) cannot. Perioxigenic oral streptococci generate bactericidal levels of H_2O_2 through lactate oxidase (Lox) or pyruvate oxidase (Pox) in an effort to kill *S. mutans*.

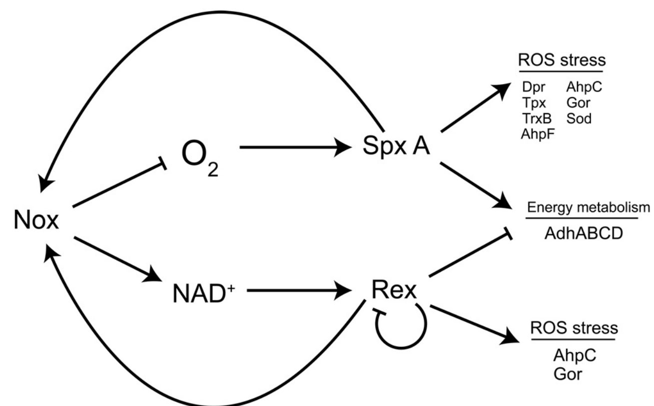

Figure 16.1.2 Proposed model for the role of Nox in the Spx and Rex regulons. Nox generates NAD^+, which, when bound, activates the transcriptional regulatory activity of Rex. Rex positively regulates ROS stress response genes *nox, ahpC,* and *gor* (indicated by an arrow), while downregulating itself and *adhABCD* (indicated by a blunt-ended line). Nox metabolizes oxygen that, when in abundance, leads to oxidation of SpxA, activating its transcriptional regulatory activity. When active, SpxA positively regulates ROS stress genes *nox, dpr, tpx, trxB, ahpCF, gor,* and *sod* as well as the *adhABCD* complex. Adapted from Baker *et al.* (2014).

this result has been attributed to a reduction of expression in *gtfB,* the enzyme responsible for making extracellular glucans (Ahn *et al.*, 2007, 2009).

In addition to upregulation of ROS detoxification genes and elevated activity of the enzymes they encode, *S. mutans* makes changes to its membrane composition in response to oxidative stress. Similar to the changes seen during acid stress, the percentage of UFAs in the plasma membrane has been found to increase substantially during oxidative stress, generated through deletion of *nox* or sparging cultures with oxygen (Derr *et al.*, 2012). Once again, the shift to higher percentages of membrane UFAs is accompanied by a shift to longer, 18- and 20-carbon chains (Derr *et al.*, 2012). Baker *et al.* also show that during this type of oxidative stress, there are genome-wide changes to the *S. mutans* transcriptome. In addition to an upregulation of the genes encoding ROS-detoxifying enzymes, changes have been observed in several important genes involved in carbon metabolism (Baker *et al.*, 2014) (see Chapter 10.1).

The transcriptional changes seen during oxidative stress are likely accomplished by proteins that sense redox state or oxidative stress and either positively or negatively affect transcription of target genes. Three well-characterized redox sensors in *S. mutans* are the SpxA, SpxB, and Rex proteins (Bitoun *et al.*, 2011, 2012; Kajfasz *et al.*, 2009, 2010). SpxA and SpxB belong to the Spx family of regulators, which upon oxidation of a CXXC motif interact with the αC-terminal domain of the RNA polymerase (αCTD) and regulate transcription of target genes (Kajfasz *et al.*, 2010; Zuber, 2004). Rex in *S. mutans* is generally bound to either NAD^+ or NADH and binds DNA, acting as either a repressor or an activator, when NAD^+ is bound (Bitoun *et al.*, 2012; Brekasis and Paget, 2003). Thus, the NAD^+–NADH ratio determines Rex activity. Both SpxA and Rex are positive regulators of the ROS-detoxifying enzymes in *S. mutans* (Baker *et al.*, 2014; Bitoun *et al.*, 2011, 2012; Kajfasz *et al.*, 2010). SpxB appears to play a more minor role as a global regulator, compared to SpxA, but seems to be involved with controlling a large number of genes related to membrane and cell wall homeostasis (Kajfasz *et al.*, 2010). Whether the changes in UFA–SFA ratio in *S. mutans* are regulated, in part, by the Spx proteins during either acid stress or oxidative stress remains unclear. However, the CXXC motif in the regulator could be protonated at low pH, or oxidized during oxidative stress conditions, suggesting that Spx proteins are key regulators that are responsive to both oxidative and acidic conditions (Baker *et al.*, 2014; Kajfasz *et al.*, 2010).

16.1.5 Overlap of acid and oxidative stress responses

There are several pieces of evidence indicating overlaps between the acid tolerance response and the oxidative stress response in *S. mutans*. The shift to a membrane dominated by UFAs in both cases is perhaps the most obvious. Indeed, the combination of acid stress (pH 5.0) and oxidative stress (in the form of 8.4% dissolved oxygen), at least, results in a greater increase in the abundance of unsaturated membrane fatty acids than observed with either stressor alone (Baker *et al.*, 2014; Derr *et al.*, 2012) (see Section 10, 18). Cells grown at pH 5 in the presence of exogenously added oxygen had membranes composed of nearly 75% UFAs. While sensors of oxidative stress have been identified (as discussed in Section 16.1.4), proteins "sensing" acidic conditions have not yet been assigned specific mechanistic roles. The two-component regulator, VicRK, like the global regulators of metabolism CcpA and CodY, has a large and overlapping regulon that includes genes involved in the acid tolerance response, although its signaling molecule has yet to be identified. It is also likely that the UFA–SFA ratio is not controlled solely at the transcriptional level, and that rapid metabolic changes in response to acid or oxidative stress promote the change in the unsaturation index.

In addition to overlap in the responses in terms of the membrane changes, ROS-metabolizing enzymes have been found to be elevated in *S. mutans* grown at low pH, and in the *fabM* mutant, suggesting further interaction between the two stress responses. The reason why the organism would elevate its membrane UFA content in response to oxidative stress remains to be elucidated.

16.1.6 Physiological control of intracellular pH homeostasis

Maintaining an intracellular environment that is alkaline, with respect to the external environment, in an organism typically exposed to an acidic extracellular environment requires coordination of physiological processes that necessarily work together (Quivey *et al.*, 2000a,b). The importance of maintaining a pH gradient between the external and internal environments may be driven by the acid and oxidative sensitivity of glycolytic enzymes (Thibodeau and Marquis, 1983), as well as the physiological damage that may occur to critical components such as the membrane (see Section 18), DNA, and proteins during exposure to an acidic pH (Fozo *et al.*, 2004; Gonzalez *et al.*, 2012). Several mechanisms have been studied in detail for their contribution to the maintenance of the intracellular pH (pH_i), the most significant being the F_1F_0 ATPase (Bender *et al.*, 1986; Hsu *et al.*, 1995; Kuhnert *et al.*, 2004). This multi-subunit, proton-pumping enzyme, embedded within the membrane, extrudes protons from the intracellular milieu at the expense of adenosine triphosphate (ATP), aiding in their removal, especially during acidic pH growth conditions. The F-ATPase proton pump is a complex of eight proteins, encoded in a single *atp* operon, whose transcriptional regulation is strongly tied to external pH conditions encountered by the organism under conditions of acidic pH (Kuhnert and Quivey, 2003; Kuhnert *et al.*, 2004; Smith *et al.*, 1996).

16.1.7 Mechanisms of proton consumption to maintain intracellular pH

In addition to the role of active transport of protons out of the cell, protons can also be “consumed” through metabolic enzyme reactions. Upon exposure to an acidic environment, many bacterial species rely on regulation of physiological means of proton consumption, specifically changes in metabolism that would result in upregulation of enzymes whose activity leads to the consumption of protons. These changes would be necessary for the maintenance of a stable intracellular pH that is more alkaline than the external environment (Krulwich *et al.*, 2011).

Decarboxylases contribute to this alkalinization process through the utilization of protons in the reactions they catalyze. These reactions play a role in pH homeostasis via the internalization of an amino acid, which is subsequently decarboxylated. Decarboxylation results in the generation of an intracellular product, carbon dioxide, that can be metabolized to the more alkaline bicarbonate via the action of carbonic anhydrase, with the concomitant consumption of a proton and, as a consequence, the elevation of internal pH (Cotter and Hill, 2003). Several examples exist for various substrates, including amino acids such as lysine and glutamate, which are transported into the organism and aid in the process of maintaining an alkaline pH. The glutamate decarboxylase (GAD) has been widely studied in bacterial species such as *Clostridium*, *Mycobacteria*, *Listeria*, and *Lactococcus lactis*, which all use this alkalization system, also known as *indirect proton extrusion*, as a way to control intracellular pH (Cotter *et al.*, 2001; Cotter and Hill, 2003; van de Guchte *et al.*, 2002).

An additional mechanism employed by *S. mutans* to maintain an alkaline cytoplasm is the agmatine deiminase system (AgDS). The AgDS catabolizes agmatine, a decarboxylated form of arginine, and generates putrescine, CO_2, ammonia, and ATP (Griswold *et al.*, 2004, 2006). As indicated here, the CO_2 and ammonia both serve to alkalinize the cytoplasm, and the ATP generated can be used by the F_1F_0 ATPase for proton extrusion. The AgDS exhibits elevated transcription during growth at low pH, through regulation by the two-component systems VicRK, ComDE, and CiaRH, further supporting its role in the acid tolerance response (Liu and Burne, 2009a,b).

Comparison of the metabolites and proteins of *S. mutans* cultures grown under steady-state conditions (pH 7 vs. pH 5) shows a significant upregulation of several metabolic pathways, indicating that the organism alters its metabolic profile during growth at acidic pH values (Len *et al.*, 2004a). The Len study shows that *S. mutans* consistently altered mechanisms that aid in the minimization of free protons, such as the rerouting of acidic intermediates toward production of less acidic end products as a way of modulating acid production and, hence, elevating external pH values.

For example, *S. mutans* encodes machinery to perform malolactic fermentation, in which malate is fermented to the less acidic lactate, while generating ATP for use in proton extrusion (Sheng *et al.*, 2010; Sheng and Marquis, 2007). 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) (see Chapter 4.7).

In addition to alteration of fermentation end products, the Len study also revealed that *S. mutans* elevates amino acid biosynthesis at low pH. One pathway, in particular, that is significantly tied to exposure to an acidic environment is branched-chain amino acid biosynthesis (Len *et al.*, 2004a,b).

16.1.8 Branched-chain amino acid metabolism and acid adaptation

Branched-chain amino acid metabolism contributes to intracellular pH regulation in *S. mutans* in several ways. *S. mutans*

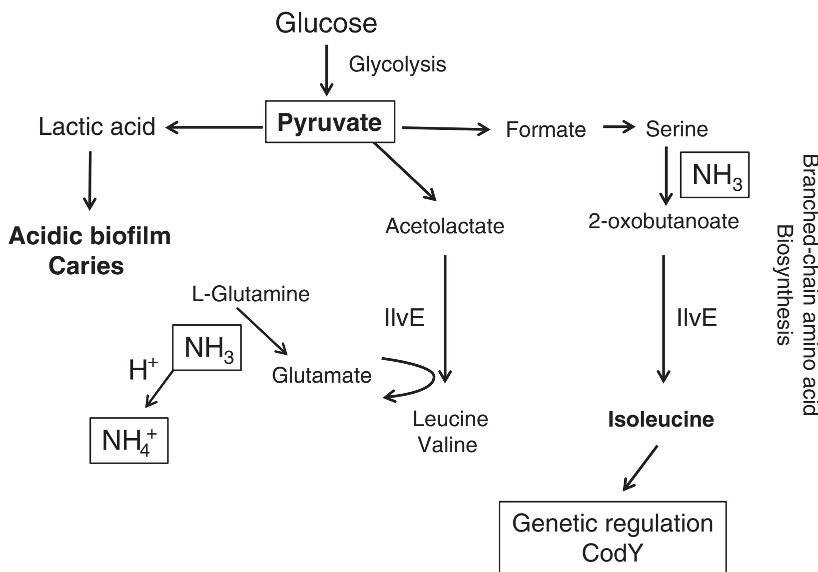

Figure 16.1.3 *S. mutans* internal pH modulation through branched-chain amino acid metabolism. Branched-chain amino acid metabolic pathways can contribute to pH modulation via several mechanisms. Deviation of pyruvate away from lactic acid production toward amino acid biosynthesis is effective toward acid end product modulation. Indirectly, during the synthesis of branched-chain amino acids, glutamine synthetase generates NH_3, which can then react with protons to produce ammonia (NH_4^+), a buffering compound, to maintain an alkaline cytoplasm. Branched-chain amino acid synthesis also contributes to the genetic regulation machinery necessary during acid adaptation, as the function of CodY, the global regulator, is dependent on intracellular isoleucine levels in *S. mutans*. Adapted from the data obtained by Len *et al.* (2004) and Santiago *et al.* (2012).

rapidly metabolizes available carbohydrates through glycolysis, generating pyruvate. Pyruvate, the acidic end product of glycolysis, is rapidly converted to lactic acid by the action of lactate dehydrogenase, typically while growing aerobically (Hillman *et al.*, 1987). This process results in the generation and excretion of an easily deprotonated organic acid that is responsible for the acidic environment in the biofilm in which *S. mutans* resides within the oral cavity (Mitchell, 2003). However, synthesis of branched-chain amino acids alleviates the accumulation of lactic acid by redirecting glycolysis toward the formation of branched-chain amino acids (Santiago *et al.*, 2012).

Another aspect of branched-chain amino acid biosynthesis that was supported by the Len *et al.* study is the contribution to acid modulation by *S. mutans* via the buffering capacity of ammonia (Len *et al.*, 2004a,b). The process of synthesizing one branched-chain amino acid involves the degradation of another, wherein during the deamination reaction, glutamine synthase generates NH_3^+. Protons that are present within the cytoplasm can then react with free ammonia to create ammonium ion, leading to alkalinization of the cytoplasm (Len *et al.*, 2006b). Differentially expressed genes observed during growth of the organism in acidic pH conditions also support the results from other studies indicating that *S. mutans* relies, in part, on branched-chain amino acid metabolism to modulate pH (Chia *et al.*, 2001; Len *et al.*, 2003). Proteins whose expression was elevated during growth at pH 5.0 include members of the branched-chain amino acid biosynthetic enzymes: IlvC, a keto-acid reducto-isomerase, and IlvE, a branched-chain amino acid aminotransferase. Similar results have also been reported in an independent study (Chia *et al.*, 2001).

16.1.9 Aminotransferases and acid tolerance

Aminotransferases are both catabolic and biosynthetic enzymes, whose dual enzymatic capability can support pH homeostasis. Aminotransferases catalyze the final step in amino acid biosynthesis, and are therefore directly linked in the pathway for rerouting pyruvate away from lactic acid production. In their catabolic function of degrading amino acids, they generate α-keto-acid intermediates, which in turn can be utilized as precursors for branched-chain fatty acid biosynthesis (Beck, 2005; Yvon *et al.*, 2000). In certain bacterial species, branched-chain fatty acids are directly linked to adaptation to important environmental stresses such as acidic pH and cold temperatures (see Section 15), since their incorporation into membranes has a similar effect on membrane fluidity as that observed with UFAs (Giotis *et al.*, 2007; Kaneda, 1963).

Due to its high degree of predicted homology with known enzymes, the SMU.1203c locus in *S. mutans* has been annotated as *ilvE*, which encodes a branched-chain amino acid aminotransferase. In *S. mutans,* the gene product is directly involved with acid adaptation, due to its link with acid end-product modulation identified during growth at acidic pH values (Santiago *et al.*, 2012).

Studies performed by Santiago *et al.* have shown the significance of IlvE in *S. mutans* physiology and the contribution of branched-chain amino acid biosynthesis in the organism's acid-adaptive capability (Santiago *et al.*, 2012). Growth in media titrated to pH 5.0 revealed that, in the absence of *ilvE*, *S. mutans* has a substantial growth defect, suggesting that the acid tolerance response is reduced in a strain lacking the enzyme (Δ*ilvE*).

In minimal medium lacking the branched-chain amino acids isoleucine, leucine, and valine, the Δ*ilvE* strain also displays a defect in growth when compared to the parent strain, UA159 (Santiago *et al.*, 2012). A significant reduction in the degradation of isoleucine and valine also indicates that the catabolism of these amino acids is defective in the absence of *ilvE.* The most significant effect with regard to acid tolerance has been revealed in the inability of the Δ*ilvE* strain to mount an acid-adaptive response. Cultures of Δ*ilvE* grown under steady-state conditions of pH 7 versus pH 5 showed that, at low-pH values, the organism was unable to survive an acidic challenge. Typically, when a culture of *S. mutans* is grown to steady state at pH 5, the cells are better able to withstand an acid challenge, compared to cells grown to steady state at pH 7 (Quivey *et al.*, 1995; Santiago *et al.*, 2012).

The importance of branched-chain amino acid metabolism in acid tolerance has been further demonstrated in strains already shown to be defective in their adaptive ability. The *fabM* mutant, a strain whose acid tolerance is severely impaired, displays a dramatic increase in branched-chain amino acid aminotransferase activity (Santiago and Quivey, unpublished results), suggesting that amino acid metabolism has a considerable dynamic range and can be activated as a mechanism for coping with elevated acidic stress.

Transcriptional studies also support the role of aminotransferases in acid tolerance since their expression has been shown to be regulated by acidic pH (Liu *et al.*, 2012; Tong *et al.*, 2007). Elevated transcription under these growth conditions reveals the significance of these genes in contributing to the modulation of acid production.

16.1.10 IlvE aminotransferase and virulence

The virulence properties of the Δ*ilvE* mutant strain have been assessed in the widely used *in vivo* rat caries model (Bowen, 2013; Fozo *et al.*, 2007). Rats infected with the Δ*ilvE* strain exhibited decreased virulence, as determined by caries scoring severity (Quivey *et al.*, unpublished data). Extensive smooth surface caries were significantly reduced in rats colonized by Δ*ilvE* in comparison to those carrying the fully virulent parent strain. These studies demonstrated that deletion of genes involved in the branched-chain amino acid pathway can lead to reduction in the cariogenic capacity of *S. mutans*, and solidifies the importance of metabolic changes to adaptation and virulence.

16.1.11 Branched-chain amino acid biosynthesis and genetic regulation

The role of branched-chain amino acid biosynthesis in acid adaptation extends beyond its metabolic role and encompasses genetic regulation. Branched-chain amino acids are important signaling molecules for bacterial homeostasis, and therefore make excellent co-effectors for genetic regulation (Sonenshein, 2007). Bacteria utilize precursors for branched-chain amino acids for numerous pathways, and hence make these molecules, and their fluctuations, important gauges for the overall "state" of a cell (Villapakkam *et al.*, 2009). Branched-chain amino acids contribute to genetic regulation in their role as co-effectors for CodY, a global regulator involved in virulence of several bacterial pathogens, including *S. mutans* (Lemos *et al.*, 2008; Sonenshein, 2005, 2007; Stenz *et al.*, 2011). More importantly, it has been shown that the function of CodY is tightly regulated by changes in the intracellular pools of branched-chain amino acids, which are modulated by branched-chain amino acid aminotransferases like IlvE (Chambellon and Yvon, 2003; Guedon *et al.*, 2001).

CodY is predicted to control a wide range of genes in the *Streptococcus mutans* genome, based on the presence of the CodY signature binding sequence (Cod-box) within the promoter region of these genes (Lemos *et al.*, 2008; Novichkov *et al.*, 2010a,b). Microarray analysis performed by Lemos *et al.* (2008) show that, in the absence of CodY, the majority of the genes that are upregulated are annotated as being involved with amino acid synthesis, amino acid transport, as well as transcriptional regulation (Lemos *et al.*, 2008).

Among the genes involved in acid adaptation and regulated by CodY were those associated with branched-chain amino acid biosynthesis: *ilvC*, *ilvB*, and *ilvE*. In a strain lacking CodY, intracellular levels of branched-chain amino acids are elevated, indicating that in the absence of the regulator, branched-chain amino acid biosynthesis may be elevated as a consequence of *ilvE* disregulation (Santiago and Quivey, unpublished observations). In *S. mutans*, the *codY* deletion strain also displays decreased acid tolerance and defects in biofilm formation (Lemos *et al.*, 2008). These two properties, which are key to the cariogenic potential of *S. mutans*, make CodY and its regulation important components of acid adaptation.

16.1.12 Acid-adaptive responses of *S. mutans* in model biofilms

S. mutans is particularly effective at forming biofilms (see Section 22) on the hard tissues of the human oral cavity. Adherence of *S. mutans* to dental surfaces is the first step in the formation of biofilms by this organism and is mediated by sucrose-dependent and sucrose-independent mechanisms (Cvitkovitch *et al.*, 1995; Koga *et al.*, 1986). *S. mutans* expresses several surface adhesins that can bind to salivary pellicles formed on the teeth (Mitchell, 2003), whereas sucrose-dependent adherence is mediated by glucan-binding proteins and water-insoluble glucans produced from sucrose by glucosyltransferase (GTF) enzymes (Bowen and Koo, 2011; Koga *et al.*, 1986).

Confocal microscopy studies involving single- and mixed-species model biofilm cultures have revealed pH heterogeneity within the biofilms (Xiao *et al.*, 2012). The more acidic

regions of the biofilm have been identified in the center of microcolonies, and at the interface between biofilms and attached surfaces (Xiao *et al.*, 2012). These observations provide insights into the relationship of dental plaque structure on tooth surfaces and the initiation of dental caries. Furthermore, studies focusing on the acid tolerance of *S. mutans* biofilm cultures, compared to planktonic cultures, show that biofilms are highly resistant to acid killing (pH 3.5), whereas planktonic and dispersed biofilm cells are acid sensitive (McNeill and Hamilton, 2003).

The type of carbohydrate substrate used in *S. mutans* biofilm cultures also affected the organism's aciduric capability. Biofilms formed in medium containing starch plus sucrose are more resistant to acid killing (pH 2.5) than biofilms grown in the presence of sucrose alone (Xiao and Koo, 2010).

A mixed-species biofilm model, containing *S. mutans*, *S. oralis*, and *A. naeslundii*, has been used to investigate the proteomic profile of *S. mutans* grown in the presence of other commensal oral microorganisms (Klein *et al.*, 2012). The mixed-species model was designed to mimic the ecological and biochemical changes associated with cariogenic biofilm assembly (Koo *et al.*, 2010). Klein *et al.* (2012) found that the F_1F_0 ATPase (particularly AtpD), membrane fatty acid biosynthetic proteins, and BCAA metabolic proteins play major roles in acid tolerance, particularly when *S. mutans* shifts from a minor constituent of plaque to become a major resident within an increasingly acidic milieu found in the interior of microcolonies. The expression of AtpD is elevated during the shift in population dominance between *S. mutans* and other organisms, while FabM is detected in high levels in the middle stage of mixed-species biofilm development.

Proteins directly responsible for cytoplasmic alkalinization have also been detected in elevated amounts during biofilm development, including proteins involved in BCAA metabolism (Lemos and Burne, 2008; Santiago *et al.*, 2012), the malolactate fermentation system (MLF) (Lemme *et al.*, 2010; Sheng and Marquis, 2007), and the AgDS (Griswold *et al.*, 2004, 2006, 2009). The results strongly indicate that the acid-adaptive response elicited in planktonic cells is very similar to the acid-adaptive response of *S. mutans* growing in the low-pH environment of biofilms.

In a dual-species biofilm model using *S. mutans* and *C. albicans*, both *fabM* and *atpD* are transcriptionally upregulated when *C. albicans* was present, as compared to biofilm cultures of *S. mutans* alone (Falsetta *et al.*, 2014). This result indicates that the ability of *S. mutans* to cope and thrive in an acidified environment may be enhanced in co-species biofilms, a trait that is critical for the bacterium to persist within biofilms and cause disease.

16.1.13 Summary

Oral microbes are subjected to rapid changes in their environment. We have briefly summarized in this chapter the contribution of specific genes to the acid and oxidative stress-adaptive response systems of *Streptococcus mutans*. It is clear that *S. mutans* faces many environmental challenges, including changes in sugar availability and abundance, changes in environmental pH values, the presence of oxidizing agents, and the presence of competing microorganisms. A number of gene products are now known to directly affect the ability of *S. mutans* to compete with non-*mutans* species, or the ability to productively infect the oral cavity of the rat model of dental caries. All available evidence indicates that stress management is a key element of the *S. mutans* virulence potential and that the organism elicits a multilayered, coordinately deployed system of overlapping mechanisms to prevent cellular damage or to repair it. Some components of adaptive responses are already available in the cell, such as ROS detoxification proteins, whereas other elements of the adaptive response are inducible (via pH, ROS, oxygen, or metabolites) at the level of gene transcription. Continued research will likely focus on the mechanisms used by *S. mutans* to compete with other, less virulent oral bacteria, and the possibility of using *S. mutans*–specific metabolic pathways as targets for new therapeutic approaches to prevent dental caries.

Dedication

The authors dedicate this chapter to our deceased colleague, Dr Robert E. Marquis, for his generosity of time, breadth of knowledge, commitment to excellence, and mentorship of students and faculty over five decades.

References

Ahn SJ, Ahn SJ, Browngardt CM, Burne RA. 2009. Changes in biochemical and phenotypic properties of *Streptococcus mutans* during growth with aeration. *Applied and Environmental Microbiology* 75(8): 2517–2527.

Ahn SJ, Wen ZT, Burne RA. 2007. Effects of oxygen on virulence traits of *Streptococcus mutans*. *Journal of Bacteriology* 189(23): 8519–8527.

Ajdic D, McShan WM, McLaughlin RE, Savic G, Chang J, *et al.* 2002. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. *Proceedings of the National Academy of Sciences USA* 99(22): 14434–14439.

Baker JL, Derr AD, Karuppaiah K, Macgilvray ME, Kajfasz JK, *et al.* 2014. *Streptococcus mutans* NADH oxidase lies at the intersection of overlapping regulons controlled by oxygen and NAD^+ levels. *Journal of Bacteriology* 196(12): 2166–2177.

Beck HC. 2005. Branched-chain fatty acid biosynthesis in a branched-chain amino acid aminotransferase mutant of *Staphylococcus carnosus*. *FEMS Microbiology Letters* 243(1): 37–44.

Belli WA, Marquis RE. 1991. Adaptation of *Streptococcus mutans* and *Enterococcus hirae* to acid stress in continuous culture. *Applied and Environmental Microbiology* 57(4): 1134–1138.

Belli WA, Marquis RE. 1994. Catabolite modification of acid tolerance of *Streptococcus mutans* GS-5. *Oral Microbiology and Immunology* 9(1): 29–34.

Bender GR, Sutton SV, Marquis RE. 1986. Acid tolerance, proton permeabilities, and membrane ATPases of oral streptococci. *Infection and Immunity* 53(2): 331–338.

Bielski BH, Arudi RL, Sutherland MW. 1983. A study of the reactivity of HO_2/O_2- with unsaturated fatty acids. *Journal of Biological Chemistry* 258(8): 4759–4761.

Bitoun JP, Liao S, Yao X, Xie GG, Wen ZT. 2012. The redox-sensing regulator Rex modulates central carbon metabolism, stress tolerance response and biofilm formation by *Streptococcus mutans*. *PLoS One* 7(9): e44766.

Bitoun JP, Nguyen AH, Fan Y, Burne RA, Wen ZT. 2011. Transcriptional repressor Rex is involved in regulation of oxidative stress response and biofilm formation by *Streptococcus mutans*. *FEMS Microbiology Letters* 320(2): 110–117.

Bowden GH, Hamilton IR. 1987. Environmental pH as a factor in the competition between strains of the oral streptococci *Streptococcus mutans*, *S. sanguis*, and "*S. mitior*" growing in continuous culture. *Canadian Journal of Microbiology* 33(9): 824–827.

Bowen WH. 2013. Rodent model in caries research. *Odontology* 101(1): 9–14.

Bowen WH, Koo H. 2011. Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. *Caries Research* 45(1): 69–86.

Brekasis D, Paget MS. 2003. A novel sensor of $NADH/NAD^+$ redox poise in *Streptomyces coelicolor* A3(2). *EMBO Journal* 22(18): 4856–4865.

Brown JL, Ross T, McMeekin TA, Nichols PD. 1997. Acid habituation of *Escherichia coli* and the potential role of cyclopropane fatty acids in low pH tolerance. *International Journal of Food Microbiology* 37: 163–173.

Chambellon E, Yvon M. 2003. CodY-regulated aminotransferases AraT and BcaT play a major role in the growth of *Lactococcus lactis* in milk by regulating the intracellular pool of amino acids. *Applied Environmental Microbiology* 69(6): 3061–3068.

Chang YY, Cronan JE Jr. 1999. Membrane cyclopropane fatty acid content is a major factor in acid resistance of *Escherichia coli*. *Molecular Microbiology* 33(2): 249–259.

Chia JS, Lee YY, Huang PT Chen JY. 2001. Identification of stress-responsive genes in *Streptococcus mutans* by differential display reverse transcription-PCR. *Infection and Immunity* 69(4): 2493–2501.

Cotter PD, Gahan CG, Hill C. 2001. A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. *Molecular Microbiology* 40(2): 465–475.

Cotter PD, Hill C. 2003. Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiology Molecular Biology Reviews* 67(3): 429–453.

Cvitkovitch DG, Boyd DA, Hamilton IR. 1995. Regulation of sugar transport via the multiple sugar metabolism operon of *Streptococcus mutans* by the phosphoenolpyruvate phosphotransferase system. *Journal of Bacteriology* 177(19): 5704–5706.

Dashper SG, Reynolds EC. 1992. pH regulation by *Streptococcus mutans*. *Journal of Dental Research* 71(5): 1159–1165.

Dashper SG, Reynolds EC. 1993. Branched-chain amino acid transport in *Streptococcus mutans* Ingbritt. *Oral Microbiology and Immunology* 8(3): 167–171.

De Stoppelaar J, Van Houte J, Backer DO. 1969. The relationship between extracellular polysaccharide-producing streptococci and smooth surface caries in 13-year-old children. *Caries Research* 3(2): 190–199.

Derr AM, Faustoferri RC, Betzenhauser MJ, Gonzalez K, Marquis RE, Quivey RG Jr. 2012. Mutation of the NADH oxidase gene (*nox*) reveals an overlap of the oxygen- and acid-mediated stress responses in *Streptococcus mutans*. *Applied and Environmental Microbiology* 78(4): 1215–1227.

Falsetta ML, Klein MI, Colonne PM, Scott-Anne K, Gregoire S, *et al.* 2014. Symbiotic relationship between *Streptococcus mutans* and *Candida albicans* synergizes virulence of plaque biofilms *in vivo*. *Infection and Immunity* 82(5): 1968–1981.

Faustoferri RC, Hubbard CJ, Santiago B, Buckley AA, Seifert TB, Quivey RG Jr. 2014. Regulation of fatty acid biosynthesis by the global regulator CcpA and the local regulator FabT in *Streptococcus mutans*. *Molecular Oral Microbiology* 30(2): 128–146.

Fozo EM, Kajfasz JK, Quivey RG Jr. 2004. Low pH-induced membrane fatty acid alterations in oral bacteria. *FEMS Microbiology Letters* 238(2): 291–295.

Fozo EM, Quivey RG Jr. 2004a. The *fabM* gene product of *Streptococcus mutans* is responsible for the synthesis of monounsaturated fatty acids and is necessary for survival at low pH. *Journal of Bacteriology* 186(13): 4152–4158.

Fozo EM, Quivey RG Jr. 2004b. Shifts in the membrane fatty acid profile of *Streptococcus mutans* enhance survival in acidic environments. *Applied and Environmental Microbiology* 70(2): 929–936.

Fozo EM, Scott-Anne K, Koo HK, Quivey RG Jr. 2007. Role of unsaturated fatty acid biosynthesis in virulence of *Streptococcus mutans*. *Infection and Immunity* 75: 1537–1539.

Fujishima K, Kawada-Matsuo M, Oogai Y, Tokuda M, Torii M, Komatsuzawa H. 2013. *dpr* and *sod* in *Streptococcus mutans* are involved in coexistence with *S. sanguinis*, and PerR is associated with resistance to H_2O_2. *Applied and Environmental Microbiology* 79: 1436–1443.

Giotis ES, McDowell DA, Blair IS, Wilkinson BJ. 2007. Role of branched-chain fatty acids in pH stress tolerance in *Listeria monocytogenes*. *Applied and Environmental Microbiology* 73: 997–1001.

Gonzalez K, Faustoferri RC, Quivey RG Jr. 2012. Role of DNA base excision repair in the mutability and virulence of *Streptococcus mutans*. *Molecular Microbiology* 85: 361–377.

Griswold AR, Chen YY, Burne RA. 2004. Analysis of an agmatine deiminase gene cluster in *Streptococcus mutans* UA159. *Journal of Bacteriology* 186: 1902–1904.

Griswold AR, Jameson-Lee M, Burne RA. 2006. Regulation and physiologic significance of the agmatine deiminase system of *Streptococcus mutans* UA159. *Journal of Bacteriology* 188: 834–841.

Griswold AR, Nascimento MM, Burne RA. 2009. Distribution, regulation and role of the agmatine deiminase system in mutans streptococci. *Oral Microbiology and Immunology* 24: 79–82.

Gross EL, Beall CJ, Kutsch SR, Firestone ND, Leys EJ, Griffen AL. 2012. Beyond *Streptococcus mutans*: dental caries onset linked to multiple species by 16S rRNA community analysis. *PLoS One* 7: e47722.

Guedon E, Serror P, Ehrlich SD, Renault P, Delorme C. 2001. Pleiotropic transcriptional repressor CodY senses the intracellular pool of branched-chain amino acids in *Lactococcus lactis*. *Molecular Microbiology* 40: 1227–1239.

Heath RJ, White SW, Rock CO. 2001. Lipid biosynthesis as a target for antibacterial agents. *Progress in Lipid Research* 40: 467–497.

Henkin TM. 1996. The role of CcpA transcriptional regulator in carbon metabolism in *Bacillus subtilis*. *FEMS Microbiology Letters* 135: 9–15.

Higuchi M, Yamamoto Y, Kamio Y. 2000. Molecular biology of oxygen tolerance in lactic acid bacteria: functions of NADH oxidases and Dpr in oxidative stress. *Journal of Bioscience and Bioengineering* 90: 484–493.

Higuchi M, Yamamoto Y, Poole LB, Shimada M, Sato Y, *et al.* 1999. Functions of two types of NADH oxidases in energy metabolism and oxidative stress of *Streptococcus mutans*. *Journal of Bacteriology* 181: 5940–5947.

Hillman JD, Andrews SW, Dzuback AL. 1987. Acetoin production by wild-type strains and a lactate dehydrogenase-deficient mutant of *Streptococcus mutans*. *Infection and Immunity* 55: 1399–1402.

Hsu MT, Sturr G, Curran TM, Marquis RE. 1995. Inhibition of streptococcal growth, F-ATPase and pyrophosphatase by diphosphonates. *Oral Microbiology and Immunology* 10: 47–53.

Imlay JA. 2003. Pathways of oxidative damage. *Annual Reviews of Microbiology* 57: 395–418.

Jensen ME, Polansky PJ, Schachtele CF. 1982. Plaque sampling and telemetry for monitoring acid production on human buccal tooth surfaces. *Archives of Oral Biology* 27: 21–31.

Jerga A, Rock CO. 2009. Acyl-Acyl carrier protein regulates transcription of fatty acid biosynthetic genes via the FabT repressor in *Streptococcus pneumoniae*. *Journal of Biological Chemistry* 284: 15364–15368.

Kajfasz JK, Martinez AR, Rivera-Ramos I, Abranches J, Koo H, *et al.* 2009. Role of Clp proteins in expression of virulence properties of *Streptococcus mutans*. *Journal of Bacteriology* 191: 2060–2068.

Kajfasz JK, Rivera-Ramos I, Abranches J, Martinez AR, Rosalen PL, *et al.* 2010. Two Spx proteins modulate stress tolerance, survival, and virulence in *Streptococcus mutans*. *Journal of Bacteriology* 192: 2546–2456.

Kaneda T. 1963. Biosynthesis of branched chain fatty acids. I. Isolation and identification of fatty acids from *Bacillus subtilis* (ATCC 7059). *Journal of Biological Chemistry* 238: 1222–1228.

Kashket ER, Barker SL. 1977. Effects of potassium ions on the electrical and pH gradients across the membrane of *Streptococcus lactis* cells. *Journal of Bacteriology* 130: 1017–1023.

Klein MI, Xiao J, Lu B, Delahunty CM, Yates JR 3rd, Koo H. 2012. *Streptococcus mutans* protein synthesis during mixed-species biofilm development by high-throughput quantitative proteomics. *PLoS One* 7: e45795.

Koga T, Asakawa H, Okahashi N, Hamada N. 1986. Sucrose-dependent cell adherence and cariogenicity of serotype *c Streptococcus mutans*. *Journal of General Microbiology* 132: 2873–2883.

Koo H, Xiao J, Klein MI, Jeon JG. 2010. Exopolysaccharides produced by *Streptococcus mutans* glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms. *Journal of Bacteriology* 192: 3024–3032.

Kreth J, Merritt J, Shi W, Qi F. 2005. Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm. *Journal of Bacteriology* 187: 7193–7203.

Kreth J, Zhang Y, Herzberg MC. 2008. Streptococcal antagonism in oral biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* interference with *Streptococcus mutans*. *Journal of Bacteriology* 190: 4632–4640.

Krulwich TA, Sachs G, Padan E. 2011. Molecular aspects of bacterial pH sensing and homeostasis. *Nature Reviews Microbiology* 9: 330–343.

Kuhnert WL, Quivey RG Jr. 2003. Genetic and biochemical characterization of the F-ATPase operon from *Streptococcus sanguis* 10904. *Journal of Bacteriology* 185: 1525–1533.

Kuhnert WL, Zheng G, Faustoferri RC, Quivey RG Jr. 2004. The F-ATPase operon promoter of *Streptococcus mutans* is transcriptionally regulated in response to external pH. *Journal of Bacteriology* 186: 8524–8528.

Lemme A, Sztajer H, Wagner-Dobler I. 2010. Characterization of *mleR*, a positive regulator of malolactic fermentation and part of the acid tolerance response in *Streptococcus mutans*. *BMC Microbiology* 10: 58.

Lemos JA, Burne RA. 2008. A model of efficiency: stress tolerance by *Streptococcus mutans*. *Microbiology* 154: 3247–3255.

Lemos JA, Nascimento MM, Lin VK, Abranches J, Burne RA. 2008. Global regulation by (p)ppGpp and CodY in *Streptococcus mutans*. *Journal of Bacteriology* 190: 5291–5299.

Len AC, Cordwell SJ, Harty DW, Jacques NA. 2003. Cellular and extracellular proteome analysis of *Streptococcus mutans* grown in a chemostat. *Proteomics* 3: 627–646.

Len AC, Harty DW, Jacques NA. 2004a. Proteome analysis of *Streptococcus mutans* metabolic phenotype during acid tolerance. *Microbiology* 150: 1353–1366.

Len AC, Harty DW, Jacques NA. 2004b. Stress-responsive proteins are upregulated in *Streptococcus mutans* during acid tolerance. *Microbiology* 150: 1339–1351.

Liu L, Tong H, Dong X. 2012. Function of the pyruvate oxidase-lactate oxidase cascade in interspecies competition between *Streptococcus oligofermentans* and *Streptococcus mutans*. *Applied and Environmental Microbiology* 78: 2120–2127.

Liu Y, Burne RA. 2009a. Multiple two-component systems modulate alkali generation in *Streptococcus gordonii* in response to environmental stresses. *Journal of Bacteriology* 191: 7353–7362.

Liu Y, Burne RA. 2009b. Multiple two-component systems of *Streptococcus mutans* regulate agmatine deiminase gene expression and stress tolerance. *Journal of Bacteriology* 191: 7363–7366.

Lu YJ, Rock CO. 2006. Transcriptional regulation of fatty acid biosynthesis in *Streptococcus pneumoniae*. *Molecular Microbiology* 59: 551–566.

Mansilla MC, Cybulski LE, Albanesi D, de Mendoza D. 2004. Control of membrane lipid fluidity by molecular thermosensors. *Journal of Bacteriology* 186: 6681–6688.

Marquis RE. 1995. Oxygen metabolism, oxidative stress and acid-base physiology of dental plaque biofilms. *Journal of Industrial Microbiology* 15: 198–207.

Marrakchi H, Choi KH, Rock CO. 2002. A new mechanism for anaerobic unsaturated fatty acid formation in *Streptococcus pneumoniae*. *Journal of Biological Chemistry* 277: 44809–44816.

McNeill K, Hamilton IR. 2003. Acid tolerance response of biofilm cells of *Streptococcus mutans*. *FEMS Microbiology Letters* 221: 25–30.

Mettraux GR, Gusberti FA, Graf H. 1984. Oxygen tension (pO_2) in untreated human periodontal pockets. *Journal of Periodontology* 55: 516–521.

Mitchell TJ. 2003. The pathogenesis of streptococcal infections: from tooth decay to meningitis. *Nature Reviews Microbiology* 1: 219–230.

Murchison HH, Barrett JF, Cardineau GA, Curtiss R 3rd. 1986. Transformation of *Streptococcus mutans* with chromosomal and shuttle plasmid (pYA629) DNAs. *Infection and Immunity* 54: 273–282.

Nakano K, Ooshima T. 2009. Serotype classification of *Streptococcus mutans* and its detection outside the oral cavity. *Future Microbiology* 4: 891–902.

Novichkov PS, Laikova ON, Novichkova ES, Gelfand MS, Arkin AP, *et al.* 2010a. RegPrecise: a database of curated genomic inferences of transcriptional regulatory interactions in prokaryotes. *Nucleic Acids Research* 38: D111–D118.

Novichkov PS, Rodionov DA, Stavrovskaya ED, Novichkova ES, Kazakov AE, *et al.* 2010b. RegPredict: an integrated system for regulon inference in prokaryotes by comparative genomics approach. *Nucleic Acids Research* 38: W299–307.

Nyvad B, Crielaard W, Mira A, Takahashi N, Beighton D. 2013. Dental caries from a molecular microbiological perspective. *Caries Research* 47: 89–102.

Parsons JB, Broussard TC, Bose JL, Rosch JW, Jackson P, *et al.* 2014a. Identification of a two-component fatty acid kinase responsible for host fatty acid incorporation by *Staphylococcus aureus*. *Proceedings of the National Academy of Sciences of the USA* 111: 10532–10537.

Parsons JB., Frank MW, Jackson P, Subramanian C, Rock CO. 2014b. Incorporation of extracellular fatty acids by a fatty acid kinase-dependent pathway in *Staphylococcus aureus*. *Molecular Microbiology* 92: 234–245.

Parsons JB, Rock CO. 2011. Is bacterial fatty acid synthesis a valid target for antibacterial drug discovery? *Current Opinion in Microbiology* 14: 544–549.

Pesakhov S, Benisty R, Sikron N, Cohen Z, Gomelsky P, *et al.* 2007. Effect of hydrogen peroxide production and the Fenton reaction on membrane composition of *Streptococcus pneumoniae*. *Biochimica et Biophysica Acta* 1768: 590–597.

Poole LB, Higuchi M, Shimada M, Calzi ML, Kamio Y. 2000. *Streptococcus mutans* H_2O_2-forming NADH oxidase is an alkyl hydroperoxide reductase protein. *Free Radical Biology and Medicine* 28: 108–120.

Quivey RG Jr, Faustoferri RC, Clancy KA, Marquis RE. 1995. Acid adaptation in *Streptococcus mutans* UA159 alleviates sensitization to environmental stress due to RecA deficiency. *FEMS Microbiology Letters* 126: 257–261.

Quivey RG Jr, Faustoferri RC, Monahan K, Marquis R. 2000a. Shifts in membrane fatty acid profiles associated with acid adaptation of *Streptococcus mutans*. *FEMS Microbiology Letters* 189: 89–92.

Quivey RG Jr, Kuhnert WL, Hahn K. 2000b. Adaptation of oral streptococci to low pH. *Advances in Microbial Physiology* 42: 239–274.

Saito HE, Harp JR, Fozo EM. 2014. Incorporation of exogenous fatty acids protects *Enterococcus faecalis* from membrane-damaging agents. *Applied and Environmental Microbiology* 80: 6527–6538.

Santiago B, MacGilvray M, Faustoferri RC, Quivey RG Jr. 2012. The branched-chain amino acid aminotransferase encoded by *ilvE* is involved in acid tolerance in *Streptococcus mutans*. *Journal of Bacteriology* 194: 2010–2019.

Schachtele CF, Jensen ME. 1982. Comparison of methods for monitoring changes in the pH of human dental plaque. *Journal of Dental Research* 61: 1117–1125.

Schilling KM, Bowen WH. 1992. Glucans synthesized in situ in experimental salivary pellicle function as specific binding sites for *Streptococcus mutans*. *Infection and Immunity* 60: 284–295.

Sheng J, Baldeck JD, Nguyen PT, Quivey RG Jr, Marquis RE. 2010. Alkali production associated with malolactic fermentation by oral streptococci and protection against acid, oxidative, or starvation damage. *Canadian Journal of Microbiology* 56: 539–547.

Sheng J, Marquis RE. 2006. Enhanced acid resistance of oral streptococci at lethal pH values associated with acid-tolerant catabolism and with ATP synthase activity. *FEMS Microbiology Letters* 262: 93–98.

Sheng J, Marquis RE. 2007. Malolactic fermentation by *Streptococcus mutans*. *FEMS Microbiology Letters* 272: 196–201.

Smith AJ, Quivey RG Jr, Faustoferri RC. 1996. Cloning and nucleotide sequence analysis of the *Streptococcus mutans* membrane-bound, proton-translocating ATPase operon. *Gene* 183: 87–96.

Sonenshein AL. 2005. CodY, a global regulator of stationary phase and virulence in Gram-positive bacteria. *Current Opinion in Microbiology* 8: 203–207.

Sonenshein AL. 2007. Control of key metabolic intersections in *Bacillus subtilis*. *Nature Reviews Microbiology* 5: 917–927.

Steinberg D, Beeman D, Bowen WH. 1996. Kinetic properties of glucosyltransferase adsorbed onto saliva-coated hydroxyapatite. *Artificial Cells Blood Substitutes and Immobilization Biotechnology* 24: 553–566.

Stenz L, Francois P, Whiteson K, Wolz C, Linder P, Schrenzel J. 2011. The CodY pleiotropic repressor controls virulence in gram-positive pathogens. *FEMS Immunology Medicine Microbiology* 62: 123–139.

Sturr MG, Marquis RE. 1992. Comparative acid tolerances and inhibitor sensitivities of isolated F-ATPases of oral lactic acid bacteria. *Applied and Environmental Microbiology* 58: 2287–2291.

Thibodeau EA, Marquis RE. 1983. Acid sensitivity of glycolysis in normal and proton-permeable cells of *Streptococcus mutans* GS-5. *Journal of Dental Research* 62: 1174–1178.

Tong H, Chen W, Merritt J, Qi F, Shi W, Dong X. 2007. *Streptococcus oligofermentans* inhibits *Streptococcus mutans* through conversion of lactic acid into inhibitory H_2O_2: a possible counteroffensive strategy for interspecies competition. *Molecular Microbiology* 63: 872–880.

van de Guchte M, Serror P, Chervaux C, Smokvina T, Ehrlich SD, Maguin E. 2002. Stress responses in lactic acid bacteria. *Antonie Van Leeuwenhoek* 82: 187–216.

Villapakkam AC., Handke LD, Belitsky BR, Levdikov VM, Wilkinson AJ, Sonenshein AL. 2009. Genetic and biochemical analysis of the interaction of *Bacillus subtilis* CodY with branched-chain amino acids. *Journal of Bacteriology* 191: 6865–6876.

Xiao J, Klein MI, Falsetta ML, Lu B, Delahunty CM, Yates JR 3rd, Heydorn A, Koo H. 2012. The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. *PLoS Pathogens* 8: e1002623.

Xiao J, Koo H. 2010. Structural organization and dynamics of exopolysaccharide matrix and microcolonies formation by *Streptococcus mutans* in biofilms. *Journal of Applied Microbiology* 108: 2103–2113.

Xue X, Tomasch J, Sztajer H, Wagner-Dobler I. 2010. The delta subunit of RNA polymerase, RpoE, is a global modulator of *Streptococcus mutans* environmental adaptation. *Journal of Bacteriology* 192: 5081–5092.

Yamamoto Y, Kamio Y, Higuchi M. 1999. Cloning, nucleotide sequence, and disruption of *Streptococcus mutans* glutathione reductase gene (*gor*). *Bioscience Biotechnology and Biochemstry* 63: 1056–1062.

Yamashita Y, Bowen WH, Burne RA, Kuramitsu HK. 1993. Role of the *Streptococcus mutans gtf* genes in caries induction in the specific-pathogen-free rat model. *Infection and Immunity* 61: 3811–3817.

Yvon M, Chambellon M, Bolotin A, Roudot-Algaron F. 2000. Characterization and role of the branched-chain aminotransferase (BcaT) isolated from *Lactococcus lactis* subsp. *cremoris* NCDO 763. *Applied and Environmental Microbiology* 66: 571–577.

Zuber P. 2004. Spx-RNA polymerase interaction and global transcriptional control during oxidative stress. *Journal of Bacteriology* 186: 1911–1918.