Responses of Cariogenic Streptococci to Environmental Stresses

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Abstract

To persist in the oral cavity, bacteria must be able to tolerate rapid and substantial environmental fluctuations, particularly in pH and nutrient source and availability. Various species of *Streptococcus*, one of the most abundant genera in the mouth, are associated with oral health, as well as with dental caries. Cariogenic streptococci depend on a biofilm lifestyle for survival and persistence in the oral cavity and have developed sophisticated mechanisms to cope with environmental stresses. Here, we analyze the primary factors that allow these bacteria to emerge as significant members of tooth biofilms during adverse conditions. Our focus is on the molecular mechanisms of biofilm formation, stress tolerance and sugar metabolism by pathogenic oral streptococci, mainly *Streptococcus mutans*. Overlaps in the roles and regulation of these virulence attributes are highlighted and areas of research that deserve further investigation are proposed.

Introduction

The oral cavity is a dynamic environment that undergoes rapid and often substantial changes in pH, nutrient availability, carbohydrate source and oxygen tension. Despite constant environmental fluctuations, it has been estimated that over 500 different bacterial species colonize oral tissues, co-existing as complex populations in biofilms. Among the most abundant microorganisms in the mouth, the oral streptococci are represented by species that are associated with oral health, as well as by species that are primarily associated with disease. Streptococci are widely distributed in the oral cavity, and include soft tissue colonizers, such as *Streptococcus salivarius, S. oralis* and *S. mitis*, as well as hard surface colonizers, such as *S. mutans*, *S. sobrinus* and *S. gordonii*.

Bacteria colonizing the teeth form dental plaque, a multispecies biofilm that is normally beneficial to the host since it prevents colonization by exogenous species. Usually, oral biofilms exist in equilibrium with the host, with no detrimental impact on the tissues of the mouth (Bradshaw and Marsh, 1998; Marsh, 1994; Marsh, 2003). In some cases though, environmental perturbations can change the composition and metabolic activities of biofilm communities, leading to an increase in the proportions of pathogenic species. In the case of dental caries, prolonged periods of plaque acidification allow for the emergence of an aciduric flora and demineralization of the tooth enamel. Two species of mutans streptococci, *S. mutans* and *S. sobrinus*, are believed to be primary etiological agents of human dental caries because numerous studies have correlated the presence of caries with elevated numbers of these organisms (Hamada and Slade, 1980; Liljemark and Bloomquist, 1996; Loesche, 1986). Also, studies with experimental animals have revealed the extreme cariogenic nature of these species (Madison et al., 1990; Tanzer, 1995; Yamashita et al., 1993a). Although both species are believed to be highly cariogenic, the majority of genetic studies have been carried out with *S. mutans*, primarily because of the strong association of *S. mutans* with caries in developed nations, but also because of the relative ease of genetic manipulation of *S. mutans* compared to *S. sobrinus*. In particular, *S. mutans* is readily transformable through natural competence (Perry and Kuramitsu, 1981) and more recently, the complete sequence of the genome of *S. mutans* UA159 became available (Ajdic et al., 2002). Of note, the *S. sobrinus* 6715 sequencing project is currently underway at The Institute for Genomic Research (TIGR) and promises to stimulate research that contrasts these two pathogens at the molecular level.

Of the many environmental stresses to which oral biofilms are exposed, low pH and profound changes in carbohydrate source and availability appear to have the greatest impact on supragingival plaque ecology and the development of dental caries. In the mouth, carbohydrate concentrations can rapidly increase from around 10 μM during fasting periods to well in excess of 10 mM during intake of heavily sweetened foodstuffs. Concurrent with the influx of dietary carbohydrates is a dramatic decrease in pH to values of 4 and below. This so-called “feast or famine” lifestyle (Carlsson, 1983) demands that organisms such as mutans streptococci, which take advantage of periods of high carbohydrate and low pH to emerge as numerically significant members of cariogenic plaque, compete effectively when environmental conditions are less favorable for other species in the biofilms. Thus, stable biofilm formation, stress tolerance and efficient acid production from carbohydrates are key virulence attributes of this organism. In this review, we analyze the primary factors that allow cariogenic streptococci to thrive in tooth biofilms during stressful conditions. In particular, we focus on the molecular mechanisms of biofilm formation, stress tolerance and sugar metabolism of pathogenic oral streptococci, mainly in *S. mutans*.

Biofilm Formation

Formation of oral biofilms on the hard tissues of the mouth is initiated by specific interactions between adhesins on the surface of oral bacteria and receptors of host and bacterial origin coating the surfaces of the teeth. Viridans streptococci, such as *S. gordonii, S. mitis* and *S.*

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sanguinis, and gram-positive rods such as *Actinomyces* spp., serve as pioneer bacteria that initiate the formation of dental plaque. These early colonizers create a scaffold for the attachment of secondary colonizers that give rise to a diverse biofilm community. The co-adhesion events promoting oral biofilm formation have been the subject of detailed reviews by Kolenbrander and colleagues (Kolenbrander, 2000; Kolenbrander et al., 2002; Rickard et al., 2003) and are not discussed in detail here.

Mutans streptococci colonize the tooth surface via sucrose-independent and sucrose-dependent mechanisms. In the presence of sucrose, the glucosyltranferase (GTF) enzymes, *gtfB*, *C* and *D*, produce water-insoluble glucan polymers that, in concert with specific glucan binding proteins (GBPs), play key roles in adhesion and accumulation of biofilms (Banas and Vickerman, 2003; Burne et al., 1997; Hudson and Curtiss, 1990; Yamashita et al., 1993a). In addition to GTFs, *S. mutans* makes a fructosyltransferase (FTF) enzyme that also participates in the production of extracellular polymers (Birkhed et al., 1979). It is generally believed that the fructan polymers produced by FTF function mainly as nutrient reserves and do not appear to be a major route for colonization of oral streptococci (Burne et al., 1996). However, there is preliminary evidence of a role for fructans in adhesion and colonization (Rozen et al., 2001). At least two independent studies, using rat model systems, have demonstrated the importance of GTFs and FTF in the development of dental caries (Munro et al., 1991; Yamashita et al., 1993a).

The surface-associated protein P1 (SpaP), also known as antigen I/II or Pac, was one of the first gene products linked to adherence of *S. mutans* to saliva-coated surfaces. SpaP is a multi-functional adhesin, facilitating binding of the bacteria to components of the enamel pellicle (Curtiss, 1985; Lee et al., 1989), as well as to collagen and other host proteins (Demuth and Irvine, 2002; Love et al., 1997). Mutants lacking SpaP showed an impaired capacity to attach to saliva-coated hydroxylapatite. However, when this mutant was implanted in rats that were fed a diet rich in sucrose, the strain was as cariogenic as the parental strain (Bowen et al., 1991), presumably because adherence through glucans could compensate for loss of SpaP. In fact, when a different animal model was used in which the amount of dietary sucrose was reduced, the impact of loss of P1 on caries became evident (Crowley et al., 1999). More recently, efforts have been devoted to identifying new genes of *S. mutans* that are involved in adherence and biofilm development. With the completion of the *S. mutans* genome sequencing project, many groups have begun to use “functional genomics” approaches to identify genes that are necessary for stable biofilm formation (Bhagwat et al., 2001; Lemos et al., 2004; Lemos and Burne, 2002; Li et al., 2002a; Merritt et al., 2003; Wen and Burne, 2002; Wen and Burne, 2004), while others have used transposon mutagenesis to isolate biofilm-defective mutants (Cvitkovitch et al., 2000; Idone et al., 2003; Yamashita et al., 1993b; Yoshida and Kuramitsu, 2002). The functional genomics approach has been driven largely by the rationale that a primary requirement for biofilm formation involves integration of genetic regulatory circuits with environmental sensing to alter the expression of factors required for adhesion, intercellular interactions, and growth in response to the evolving environmental conditions encountered during biofilm formation. Recent studies have implicated the *comABCDE* genes, associated with genetic competence (Li et al., 2002b; Yoshida and Kuramitsu, 2002), global regulators such as *ccpA*, *luxS* and *relA* (Lemos et al., 2004; Merritt et al., 2003; Wen and Burne, 2002; Wen and Burne, 2004), and two-component response regulators (Bhagwat et al., 2001; Li et al., 2002a) as key factors enabling biofilm formation in *S. mutans*. Moreover, defects in stress-responsive genes have been also associated with biofilm-impaired phenotypes (Baev et al., 1999; Lemos and Burne, 2002; Lemos et al., 2001; Lis and Kuramitsu, 2003).

Environmental Stresses

Acid Stress

In the mouth, salivary secretions constitute the main source of carbon and nitrogen for the resident oral microflora. Saliva keeps oral biofilms warm and moist. The pH of saliva is around neutrality, which is optimal for the growth of the majority of oral microorganisms (Marsh, 2003). After intake of heavily sweetened foodstuffs, sugar levels can increase up to 1,000-fold and a dramatic decrease in pH in oral biofilms to values around 4 is common. In general, organisms like the mutans streptococci, which can grow and carry out glycolysis at low plaque pH values, gain a selective advantage over less aciduric species when oral biofilms become acidified. Sustained acidification of plaque can lead to significant increases in the proportions of acid tolerant and strongly acidogenic species, which drive the development of caries. Therefore, the abilities to survive extreme and rapid changes in pH, as well as sustained acidification, are arguably the most important attributes of cariogenic bacteria.

Oral streptococci possess several acid adaptive strategies, some of which are distributed among all of the species and some that are unique. Proton-extrusion via the F-ATPase is common to all oral streptococci and is the major mechanism for maintaining internal pH that is more alkaline than that of the environment; conferring protection to acid-sensitive glycolytic enzymes and maintaining ΔpH for bioenergetic processes. There are additional protective mechanisms that appear to be peculiar to certain species, such as ammonia production from urea by *S. salivarius* and from arginine by *S. sanguinis*, *S. gordonii* and *S. rattus*.

Proton Pumps

In contrast to enteric bacteria, which usually maintain internal pH (pH_i) fairly constant at 7.6 - 7.8 through the use of antiporters, pH_i in streptococci fluctuates in response to extracellular pH, with the organisms working to maintain a ΔpH of 0.5 to 1 unit above the external environment. In *S. mutans*, the membrane-bound F_1F_0-ATPase is the primary mechanism of proton extrusion to maintain pH homeostasis. In oral streptococci, acid tolerance correlates well with the pH optimum of the F-ATPase enzyme (Sturr and Marquis, 1992). For example,

in the highly acid-tolerant *S. mutans*, the optimal pH for the F-ATPase enzyme is 6.0, whereas in less acid-tolerant *S. salivarius* and *S. sanguinis*, the pH optima of the ATPase enzymes are 7.0 and 7.5, respectively. Many oral streptococci, including *S. mutans*, are also capable of mounting an acid tolerance response (ATR) (Belli and Marquis, 1991; Hamilton and Buckley, 1991; Quivey et al., 2000b). The ATR, also known as acid adaptation or acid habituation, is characterized by an increased resistance to acid killing in cells grown at an acidic pH compared with cells grown at a neutral pH. In *S. mutans*, increased acid tolerance has been correlated with an increase in the activity of the F-ATPase (Belli and Marquis, 1991; Hamilton and Buckley, 1991), but the basis for the increased activity is not definitively established. It has been demonstrated that increases in F-ATPase activity correlate with increased transcription of the F-ATPase operon (Quivey et al., 2000b) and that the *atp* operon of *S. mutans* can be transcriptionally regulated in response to growth pH (Smith et al., 1996). Transcriptional fusions to the *atp* operon promoter revealed more reporter gene activity in cells grown at pH 5.0 compared to pH 7.0 (Quivey et al., 2000b). However, it cannot yet be excluded that allosteric regulation of the activity of the enzyme at low pH is responsible for the enhanced ATPase activity in cells that have undergone adaptation to growth in acidic conditions (ATR).

Membrane Composition

A few independent studies have demonstrated the importance of the cell membrane of *S. mutans* in acid tolerance, especially in relation to proton permeability and F-ATPase activity. Quivey and co-workers showed that *S. mutans* strains grown at pH 5.0 had increased levels of mono-unsaturated fatty acids and longer chain fatty acids than cells grown at pH 7.0 (Quivey et al., 2000a). More recently, Fozo and Quivey showed that as the growth pH is lowered there is an incremental change from short-chained saturated fatty acids to long-chained, mono-unsaturated fatty acids (Fozo and Quivey, 2004). The authors also showed that cells treated with cerulenin, a fatty acid biosynthesis inhibitor, were unable to survive severe acidification. It was speculated that the shift in lipid composition could affect the permeability of the cells to protons or by affecting the F-ATPase activity. One of the main mechanisms by which changes in membrane composition are thought to affect acid tolerance is through decreased proton permeability, although an equally important potential mechanism is that the altered lipid environment strongly influences the activity of the F-ATPase and transport proteins in a manner that enhanced acid resistance.

The importance of membrane protein biogenesis has been emphasized by the finding that a strain lacking the *ffh* gene, which encodes the highly conserved 54-kDa subunit ortholog of the eukaryotic signal recognition particle (SRP) complex, was incapable of mounting an ATR (Gutierrez et al., 1999). It was speculated that Ffh, which chaperones newly synthesized proteins into the membrane, might be required for proper assembly or insertion of the F_o portion of the F-ATPase enzyme in the membrane. Similar results were obtained with a strain defective in D-alanyl-lipoteichoic acid (LTA) synthesis (Boyd et al., 2000). In this case, increased proton permeability was directly implicated with the failure to induce an ATR. Another *S. mutans* mutant deficient in diacylglycerol kinase (*dagK*) demonstrated an acid-sensitive phenotype (Yamashita et al., 1993b). DagK was shown to function in fatty acid synthesis, so the mutation was inferred to affect membrane architecture and composition.

DNA Repair

Previously, Quivey and co-workers showed that growth of *S. mutans* at pH 5.0 confers enhanced resistance to multiple stresses, increasing the survival rates of cells exposed to acid, hydrogen peroxide and UV radiation (Quivey et al., 1995). Therefore, there appears to be substantial overlap in the stress response pathways, as has been observed in a variety of other bacteria. Many of the stresses that oral biofilm bacteria encounter induce DNA damage. The role of oxidative stress in DNA damage is well documented. The glycosyl bond of deoxyribonucleotides is unstable at low pH, so intracellular acidification can enhance loss of purines and pyrimidines from DNA due to protonation of the base followed by cleavage of the glycosyl bond (Lindahl and Nyberg, 1972). The remaining residues at the site of cleavage are referred to as abasic sites or AP sites (for apurinic and apyrimidinic). Repair of AP sites is initiated by AP endonucleases that specifically recognize the damage in duplex DNA and cleave the phosphodiester bond immediately 5' or 3' to the AP site. Hahn and co-workers identified an AP endonuclease in *S. mutans* that showed higher levels of activity in cells grown at low pH compared with cells grown at pH 7.0 (Hahn et al., 1999), providing the first evidence linking DNA repair enzymes with acid adaptation in oral streptococci. In another study, differential display PCR identified an *S. mutans* gene, with similarity to the UV repair excinuclease UvrA of *B. subtilis*, which showed increased expression in response to low pH (Hanna et al., 2001). In *E. coli*, UvrA participates in the nucleotide excision repair pathway, which is responsible for excising larger DNA lesions caused by acid and other DNA-damaging agents. An *S. mutans uvrA* mutant was extremely sensitive to UV irradiation and had enhanced sensitivity to acid killing when compared to the parent strain (Hanna et al., 2001).

Stress Proteins

The production of stress proteins, including the molecular chaperones GroEL and DnaK, is central to the tolerance of environmental insults by microorganisms. Chaperones prevent aggregation and misfolding of proteins, and can refold acid-damaged proteins or present irreparably damaged polypeptides to the protein degradation machinery. In *S. mutans*, *groEL* and *dnaK* are rapidly induced by acid shock, with elevated levels of DnaK expression maintained under acidic conditions (Jayaraman et al., 1997; Lemos et al., 2001). A strain lacking the major repressor of the *dnaK* and *groEL* operons, HrcA, was constructed with a non-polar insertion in *hrcA*, the first gene in the *dnaK* operon. This strain had constitutively high levels of GroEL and diminished levels

of DnaK, the former because of the loss of the repressor and the latter because the insertion did not allow for full expression of the downstream genes. Interestingly, this strain was substantially more sensitive to acid killing than the wild-type strain (Lemos et al., 2001). The acid-sensitive phenotype of the HrcA-mutant strain was shown to be attributable, at least in part, to a failure to up-regulate F-ATPase activity when the cells were grown at pH 5.0. It was proposed that DnaK participates in the biogenesis of the F-ATPase complex, either at the folding and/or assembly stages, and that insufficient DnaK was present to achieve the levels of enzyme needed at low pH, although this remains an untested hypothesis. Another stress protein that has been implicated in tolerance by *S. mutans* is the ClpP peptidase, since a strain lacking the *clpP* gene showed impaired growth at low pH (Lemos and Burne, 2002). ClpP, when associated with members of the Clp ATPase family, acts as a serine protease and prevents accumulation of improperly folded proteins that may be toxic for the bacteria (Maurizi et al., 1990). Another explanation that is not mutually exclusive is that ClpP participates in acid tolerance by modulating the stability of transcriptional regulators of the ATR. In *E. coli*, transcription factors governing stress responses are in fact substrates for ClpP (Flynn et al., 2003), so this latter hypothesis has merit.

Global Regulators and Two-component Signal Transduction Systems

Alternative sigma factors, transcriptional regulators and two-component regulatory systems participate in the coordinate regulation of gene expression in response to environmental changes. More than 100 transcriptional regulators and thirteen two-component regulatory systems, but only three sigma factors, were identified in the *S. mutans* UA159 genome (Ajdic et al., 2002). The comparatively small number of sigma factors, as compared with *E. coli* or *B subtilis*, is a characteristic of other streptococci and suggests that response regulators and other mechanisms have evolved to take the place of alternative sigma factors in adaptation to environmental flux.

Global regulators modulate the expression of multiple genes, and therefore the inactivation of a global regulator generally results in pleiotropic effects on the cell. Recently, we demonstrated that the stringent response effector, RelA, which encodes a (p)ppGpp synthetase/ hydrolase, is involved in acid tolerance and inactivation of *relA* resulted in impaired biofilm formation (Lemos et al., 2004). Intriguingly, there were no differences in the sensitivity of the parent and *relA* strains to acid killing when cells were grown in planktonic cultures. However, when cells were grown in biofilms, *relA* mutants became more acid resistant than the parent. The inactivation of two other putative global regulators, which share homology with the *S. pyogenes* RopA (trigger factor) and Rgg (or RopB) proteins, also induced an acid sensitive phenotype (Z. T. Wen and R. A. Burne, submitted for publication). Similar to the *relA* mutant, the RopA- and Rgg-deficient strains also had impaired biofilm formation when grown in glucose-containing media. RopA, or trigger factor, associates with ribosomes, binds to nascent polypeptides, and has peptidyl-prolyl *cis-trans* isomerase activity (Lill et al., 1988). The *rgg* gene is a transcriptional regulator shown to regulate transcription of *gtfG* in *S. gordonii* (Sulavik and Clewell, 1996), and to regulate expression of several exo-proteins that are preferentially expressed during stationary phase growth in *S. pyogenes* (Chaussee et al., 2001).

In gram-positive bacteria, the operons containing the *dnaK* and *groEL* stress genes are negatively regulated by HrcA (Hecker et al., 1996; Schulz and Schumann, 1996). HrcA binds to a DNA element called CIRCE (for controlling inverted repeat of chaperone expression) that is located in the regulatory region of these operons (Schulz and Schumann, 1996). As previously mentioned, an *S. mutans* HrcA mutant strain, which had constitutively high levels of GroEL but low levels of DnaK, was more sensitive to acid killing than the parent strain (Lemos et al., 2001). Interestingly, protein profiles obtained by two-dimensional gel analysis indicated that several other proteins had altered expression on the HrcA-mutant strain, suggesting that HrcA may exert, directly or indirectly, pleiotropic effects (Lemos et al., 2001).

Two-component systems play important roles in bacterial adaptation, survival and virulence by sensing changes in the environment and altering expression of specific sets of genes. Two-component systems consist of a sensor, which is a membrane-associated histidine kinase, and a response regulator, which is a DNA binding protein that modulates expression of target genes when phosphorylated by the kinase. Investigating the role of five different two-component systems in *S. mutans* UA159 biofilm formation, Bhagwat and co-workers demonstrated that disruption of one particular response regulator resulted in a substantial decrease in biofilm formation (Bhagwat et al., 2001). Later, Li and co-workers confirmed the role of this response regulator (designated *rr11*) in biofilm formation and showed that deletion of the putative histidine kinase (*hk11*), but not of *rr11* resulted in diminished resistance to low pH (Li et al., 2002a).

Quorum Sensing

It has been demonstrated that bacterial populations can coordinate gene expression in a cell-dependent manner through a process known as quorum sensing (QS). Quorum sensing bacteria convey their presence to one another by releasing and responding to the accumulation of chemical signal molecules called autoinducers. Concomitant with increases in bacterial cell density, the levels of extracellular autoinducer increases until there are sufficient amounts of the compound(s) to trigger a signal transduction cascade that governs expression of many genes. In the oral cavity, *S. mutans* lives almost exclusively in biofilms that form on the tooth surface. The structure and composition of these densely populated and diverse biofilms are strongly influenced by source and availability of nutrients, pH and the capacity of its constituents to adapt to environmental fluctuations. Because QS systems coordinate gene expression in a cell density-dependent manner, QS genes are thought to participate in regulation of several physiological activities in oral biofilms, including biofilm differentiation and stress management. In fact, there appear to be at least two QS

systems in *S. mutans* and related oral streptococci, and both of these are tied intimately in tolerance of acid and other stresses and in biofilm formation. The first is the competence regulon, which relies on a low molecular mass peptide as the signalling molecule that is detected by a two-component regulatory system. Inactivation of *comD* and *comE* conferred an acid sensitive phenotype to *S. mutans* and resulted in the formation of biofilms with reduced biomass (Li et al., 2001; Li et al., 2002b). The second QS in *S. mutans* is the widely distributed *luxS* system, which produces AI-2, a furanosyl borate diester (Chen et al., 2002) that regulates a large panel of genes in a variety of microorganisms (Surette et al., 1999). A strain of *S. mutans* lacking LuxS was shown to have altered biofilm architecture and to have increased sensitivity to acid killing (Wen and Burne, 2004).

Alkali Production

In less acid-tolerant oral streptococci, alkali generation involving the urease or the arginine deiminase system (ADS) appears to be an important mechanism for acid resistance (Burne and Marquis, 2000). Organisms carrying these enzymes can convert urea or arginine to CO_2 and ammonia, which results in alkalinization of the cytoplasm and environment, preventing acid killing and allowing the organisms to multiply when acid production might otherwise inhibit growth. Neither urease nor ADS are present in *S. mutans* (Ajdic et al., 2002). However, a cluster of genes with similarity to ADS genes was identified in the UA159 genome (Ajdic et al., 2002). Subsequently, it was shown that this gene cluster is an operon that encodes an agmatine deiminase system (AgDS) (Griswold *et al.*, 2004), a system that is analogous to the ADS but involved in the catabolism of agmatine, a decarboxylated derivative of arginine (Simon and Stalon, 1982). It was demonstrated that ammonia is produced from agamatine at low pH, suggesting that the AgDS may contribute to acid tolerance (Griswold et al., 2004).

Oxidative Stress

Although oxidative stress has relevance in plaque ecology (Marquis, 1995), few detailed studies of the molecular mechanisms regulating oxidative stress responses in oral bacteria are available. Yet, these organisms are constantly exposed to oxidative agents from either intracellular or extracellular origin. External sources of reactive oxygen species (ROS) include host defenses, such as the lactoperoxidase system, as well as peroxide-containing oral hygiene products and Fenton chemistry. The production of H_2O_2 by some oral bacteria, such as *S. sanguinis*, *S. gordonii* and *S.oralis*, is also believed to add to the oxidative stress burden of oral biofilm bacteria. During respiration, ROS are generated inside the cell from single electron reductions of oxygen and the cells must cope with oxygen metabolites in order to survive. Even *Treponema denticola* and *Porphyromonas gingivalis*, two anaerobic oral bacteria, have a fairly high capacity to metabolize oxygen (Caldwell and Marquis, 1999; Diaz et al., 2004), although these organisms lack adequate levels of protective enzymes.

As would be expected, oral bacteria do possess multiple, and often overlapping, pathways for dealing with oxidative stresses in the cell and environment (Marquis, 1995; Mongkolsuk and Helmann, 2002). The levels of respiratory/oxidative stress enzymes reflect the respiratory capacity of the organism. Streptococci do not possess cytochromes and therefore do not carry out oxidative phosphorylation. Instead, the bulk of the respiration activity in these organisms is due to NADH oxidases (Marquis, 1995). Oral streptococci also lack catalase, a major protective enzyme against oxidative insults, but harbor superoxide dismutase (SOD), NADH peroxidase, glutathione reductase and alkyl hydroperoxide reductase (Marquis, 1995). The growth mode of bacteria, i.e., planktonic or as biofilms, largely influences the respiratory rates and the levels of activity of protective enzymes like SOD and NADH oxidase. In a study comparing the levels of NADH-oxidase and SOD in planktonic and biofilm populations of several oral streptococci species, Nguyen and co-authors demonstrated that both enzymes were repressed in the biofilm state (Nguyen et al., 2002). It was suggested that oxidative stress appears to be minimized in streptococcal biofilms because of reduced oxygen metabolism by the bacteria. Reducing oxygen metabolism and consequently the production of ROS could be an important strategy to live in a slow growth mode and crowded environment such as a biofilm.

Aside from the information on the physiology of oxidative stress in oral streptococci (Marquis, 1995), there is very limited information on the oxidative stress response in these organisms. Several genes, including members of the *oxyR*, *perR* and *ohrR* families that are involved in sensing and responding to oxidative insults in other bacteria, are present in the *S. mutans* genome. However, functional studies are still needed in order to assess the roles of these gene products and better understand the coordination of oxidative stress tolerance. Betzenhauser and Quivey showed that a strain lacking a *perR* homologue gene was hypersensitive to hydrogen peroxide, although this strain showed little difference when compared to the wild-type strain with respect to acid resistance (Betzenhauser and Quivey, 2002). Recently, Wen and Burne showed that a *luxS* mutant strain of *S. mutans* was more resistant to hydrogen peroxide when compared to the wild type strain (Wen and Burne, 2004). Somewhat surprisingly, the *luxS* strain displayed an acid-sensitive phenotype, suggesting that even though there are some common features between stress responses, acid and oxidative stress responses may also involve separate pathways. To support this theory, Svensater and co-workers compared the two-dimensional electrophoretic protein profile of exponentially growing cells of *S. mutans* grown under different stress conditions (Svensater et al., 2000) to identify several stress-specific proteins and proteins that are commonly elevated in response to a variety of stresses. These results are similar to those observed in *B. subtilis* in which the specific responses to oxidative damaging agents could be classified into two groups; i) stimuli that activate the same pool of genes, and ii) stimuli that activate a specific response that is not shared with other oxidative damaging agents (Mostertz et al., 2004).

Osmotic Stress

Dental plaque is a densely-packed environment and infusion of high concentrations of nutrients in the diet can conceivably stress oral biofilm populations. In the crowded plaque environment, accumulation of solutes in the extracellular matrix might increase osmolality, forcing the cells to alter gene expression and physiology to maintain internal water balance. Intake of foods can lead to high concentrations of nutrients in plaque and production of organic acids by plaque bacteria leads to tooth demineralization, which can increase the concentration of dissolved enamel, mostly calcium salts, in the plaque fluid (Gao et al., 2001). The ionic strength of plaque fluid was reported by Margolis and co-workers to be roughly 150 mM (Margolis et al., 1988) and after sugar challenge it can increase by approximately 2-fold (Gao et al., 2001). Thus, the organisms in plaque may need mechanisms to cope with elevated osmolality, which can be inhibitory to growth. Most bacteria do not possess active water transport mechanisms to help to maintain cell turgor, which is essential for survival (Kempf and Bremer, 1998). Instead, the pooling of "compatible solutes" is a strategy for dealing with hyperosmotic conditions or low water activity. Compatible solutes are soluble organic compounds that can reach high intracellular concentrations without disturbing vital cellular functions (Kempf and Bremer, 1998; Sleator and Hill, 2002). Such osmoprotectants are common among most microorganisms and include neutral sugars, polyols, free amino acids and their derivatives, and quaternary amines (Kempf and Bremer, 1998), although glycine betaine, carnitine and proline are the three major compatible solutes used for bacterial osmoadaptation (Sleator and Hill, 2002). Some compatible solutes, such as glycine betaine and carnitine, can be synthesized and accumulated intracellularly during osmotic stress, but their synthesis can be repressed if exogenous sources of these solutes are present (Kempf and Bremer, 1998; Sleator and Hill, 2002). For compounds that cannot be synthesized, or when compatible solutes are present in the environment, the cells use efficient transport systems. It is not known to what extent oral bacteria encounter osmotic stress, but several genes that share homology to osmotic stress genes of other microorganisms are present in the genome of *S. mutans* UA159 (Ajdic et al., 2002). Increases in the intracellular levels of K^+ and accumulation of compatible solutes are common responses of many microorganisms to an increase in osmolality (Kempf and Bremer, 1998; Sleator and Hill, 2002; Weber and Jung, 2002). Three genes sharing homology with *trkA, trkB* and *trkH*, which are involved in the transport of K^+ into *E. coli* (Dosch et al., 1991; Epstein, 2003; Kempf and Bremer, 1998) and open reading frames sharing homology with the Opu family of ABC transporters that internalize compatible solutes were identified in the *S. mutans* genome (Ajdic et al., 2002). For the most part though, the mechanisms of osmoregulation remain an open field to be explored in oral streptococci.

Living the Feast-or-famine Existence: Carbohydrate Source and Availability

Access to adequate nutrients is a key determinant for bacterial survival in dental plaque. In the oral cavity, bacteria live a "feast-or-famine" lifestyle (Carlsson, 1983), coping with long periods where saliva is the primary nutrient and times when nutrients from the diet are abundant. Although the ability to survive long periods of carbohydrate starvation is considered crucial for oral bacteria, sudden exposure to an excess amount of sugar is also a major challenge for bacteria. The rapid entry and degradation of sugars in cells can result in an accumulation of toxic levels of glycolytic intermediates, resulting in cell death. In order to survive such conditions, oral streptococci have developed sophisticated physiologic and genetic mechanisms to regulate sugar metabolism according to source and availability.

At low sugar concentrations, the phosphoenolpyruvate (PEP) sugar:phosphotransferase system (PTS) is the major system for internalizing sugar into the cell. The PTS consists of two proteins that are common to all PTS substrates, Enzyme I (EI) and HPr, the heat stable phosphocarrier protein, as well as sugar-specific permeases known as Enzyme II (EII) complexes. The EII complexes usually consist of three domains, A, B and C, but a fourth domain may be required. The cytoplasmic A and B domains are directly responsible for the phosphorylation of the incoming sugar, whereas the C and D domains comprise the membrane permeases. The EII domains can either be individual polypeptides or be covalently linked in a variety of combinations (Postma et al., 1993; Vadeboncoeur and Pelletier, 1997). In order for an incoming sugar to be phosphorylated by the PTS, a phosphate group from a PEP molecule is transferred to EI, which phosphorylates HPr at His-15. Then, the phosphate group is transferred to the sugar-specific EII complex A and B, and finally to the incoming sugar. The EII C and D membrane do not receive the phosphate group (Postma et al., 1993; Vadeboncoeur and Pelletier, 1997). The PTS is widely distributed in eubacteria, including gram-negative organisms such as *E. coli* and gram-positive organisms, including *Streptococcus*, *Staphylococcus* and *Bacillus* spp. The PTS internalizes a wide variety of sugars, including glucose, fructose and mannose, and disaccharides such as sucrose and lactose (Postma et al., 1993).

The PTS is also a major determinant in carbohydrate catabolite repression (CCR). In gram-positive bacteria, the metabolism of a non-preferred sugar source is repressed by CCR in the presence of a preferred sugar, usually a PTS-sugar, but not only glucose. In this case, CCR is exerted through HPr and the global regulatory protein catabolite control protein A (CcpA). The accumulation of glycolytic intermediates, reflecting the carbon flow through the glycolytic pathway, activates an HPr kinase, which phosphorylates HPr at Ser-46. The Ser-46 phosphate group can not be transferred to EII enzymes, allowing Hpr (Ser-P) to accumulate in the cell and interact with CcpA to form a complex that is competent to bind to catabolite response elements (CRE) located in the promoter region of genes under the control of CCR, blocking their transcription (Bruckner and

Titgemeyer, 2002; Hueck and Hillen, 1995; Saier, 1996). Inactivation of an apparent *ccpA* homologue of *S. mutans* did not relieve CCR of some genes and diauxic growth was not affected, indicating that CCR in this organism is substantively different and perhaps more complex than in many other gram-positive organisms (Burne et al., 1999). This observation was particularly surprising since CRE sequences are highly conserved in the regulatory regions of CCR-sensitive genes and deletion of CREs alleviates CCR in *S. mutans*. Also, CcpA is active in CCR in other streptococci. In particular, in *S. gordonii,* CcpA was shown to be the primary regulator for CCR of the arginine deiminase operon (Dong et al., 2004) and of the gene for amylase binding protein (Rogers and Scannapieco, 2001). In spite of an inability to disclose a role of CcpA in CCR, this protein does appear to play roles in global control of gene expression. Strains of *S. mutans* that lack CcpA formed 60% less biofilm than the parental strain (Wen and Burne, 2002) and other members of our lab group have documented an impact on virulence gene expression in CcpA-deficient strains (Browngardt et al., in press).

In oral streptococci, some attention has been devoted to the mannose-PTS in relation to sugar transport and regulation of gene expression. Initially, Vadeboncouer and colleagues demonstrated that mutations in the EII^{man} complex of *S. salivarius* caused aberrant regulation of sugar catabolism. Using 2D gels, many alterations in expression of cytoplasmic and membrane proteins were observed in these mutants. More recently, it was shown that $EIIAB^{Man}$ plays a role in CCR by regulating diauxic growth in *S. mutans* and *S. salivarius* (Abranches et al., 2003; Pelletier et al., 1998; Vadeboncoeur and Pelletier, 1997). In *S. mutans,* $EIIAB^{Man}$ can also regulate the activity of other sugar-specific EII enzymes, as well as the expression of important virulence genes, including *gtfBC* and *ftf* (Abranches et al., 2003). The mechanism by which $EIIAB^{Man}$ regulates these virulence genes remains unknown, but it is becoming evident that the PTS plays a central role in gene regulation in oral streptococci (Abranches et al., 2003). Other PTS enzymes of *S. mutans*, including a fructose PTS enzyme, have been shown to regulate expression of catabolism of polysaccharides (Wen et al., 2001). Of note, inactivation of the inducible fructose-PTS of *S. gordonii* resulted in a biofilm-defective phenotype (Mitrakul et al., 2003), again suggesting a global regulatory function for the PTS.

When *S. mutans* cells are grown in an excess of carbohydrate, lactic acid is by far the dominant glycolytic end product. The ability of *S. mutans* to open the "lactate" gate is believed to protect the organisms from sugar killing, perhaps through more rapid movement of carbohydrate through glycolysis, more efficient movement of lactic acid out of the cell compared with other organic acids, and maintenance of $NAD/NADH^+$ balances. The shift to homolactic fermentation is rapid and involves the activation by fructose 1,6-diphosphate, a glycolytic intermediate, of lactate dehydrogenase (LDH). In reducing pyruvate to L-lactate, $NADH^+$ is oxidized to NAD and the redox potential of the cells is preserved (Len et al., 2004a). Mutations in the *ldh* gene appear to be lethal in *S. mutans*, suggesting that $NAD/NADH^+$ imbalance and/or accumulation of glycolytic intermediates in the cell have a toxic effect (Chen et al., 1994; Hillman et al., 1996).

In addition to regulating sugar metabolism, bacteria have also developed sophisticated methods to survive nutrient deprivation and to cope with the detrimental effects of an excess of glycolytic intermediates. Oral streptococci, like many other bacteria, accumulate intracellular and extracellular polysaccharides in the presence of sufficient exogenous sources of sugar. The accumulation of sucrose-derived extracellular polymers and of intracellular polysaccharides (IPS), which are glycogen-like storage polymers, enhances survival during nutrient starvation and has been shown to contribute to caries formation (Burne et al., 1996; Spatafora et al., 1995). Glucans and fructans are the major extracellular polysaccharides (EPS) produced by oral streptococci, serving as a reserve of energy and helping the cells to adhere to the tooth surface. The ability to synthesize and catabolize glucan and fructan exopolysaccharides is an established virulence attribute of these organisms. Glucosyltransferases (Gtfs) catalyze the production of glucans from sucrose that are involved in the colonization of the tooth surface. Fructosyltransferase (Ftf) catalyzes the production of fructans, also from sucrose, but it is believed that fructans serve primarily as extracellular carbohydrate reserves. *S. mutans* can rapidly synthesize EPS when sucrose is introduced in the diet, shunting a small diffusible dissacharride into a non-diffusing form. When exogenous carbohydrates are depleted, the organisms can use hydrolases to access these polysaccharides for energy and growth. One enzyme, fructanase is responsible for the degradation of fructans; liberating fructose that will be immediately consumed for energy generation. *S. mutans* also produces an extracellular dextranase that attacks α1,6-linkages in the glucan products of the GTFs. The regulation of *gtf* and *ftf* gene, as well their importance in the virulence of *S. mutans*, has been well studied (Hudson and Curtiss, 1990; Li and Burne, 2001; Ooshima et al., 2001; Rozen et al., 2001; Sato and Kuramitsu, 1986; Shiroza and Kuramitsu, 1988; Steinberg et al., 2002). Also, GTFs are considered as primary candidates for a human caries vaccine (Childers et al., 2002; Jespersgaard et al., 1999; Michalek et al., 2001; Smith et al., 2001; Taubman et al., 2000). An excess of sugar also leads to accumulation of IPS, which can be used as an energy source when nutrients are not available, presumably between meals. Spatafora and colleagues demonstrated that a mutant strain with elevated levels of IPS was hypercariogenic (Spatafora *et al.*, 1995) and another mutant that was IPS-deficient was significantly less cariogenic than the parental strain (Spatafora-Harris *et al.*, 1992).

During periods of starvation, bacteria undergo a "stringent response" that is characterized by the inhibition of stable rRNA synthesis, the induction or repression of metabolic pathways according to physiological needs, and the induction of stationary phase survival genes (Cashel, 1996). Current evidence supports that many of the regulatory effects of the stringent response are related to variations in the concentrations of the chemical messengers guanosine tetraphosphate and guanosine

pentaphosphate, (p)ppGpp, that allow bacteria to adjust their metabolism from a growth mode to a survival mode. In gram-positive bacteria, RelA is a bifunctional enzyme with (p)ppGpp-synthetic and -degradative activities (Mechold et al., 1996). Recently, we have identified a RelA homolog in *S. mutans* and demonstrated that the lack of RelA affects acid tolerance and biofilm formation (Lemos et al., 2004), highlighting again an overlap between circuits that govern nutrient starvation, acid tolerance and biofilm formation.

Proteomics of *S. mutans*

The completion of the *S. mutans* UA159 genome (Ajdic et al., 2002) has allowed for the identification of proteins in two-dimensional gels by matrix-assisted laser desorption ionization-time-of flight (MALDI-TOF). Previously, by comparing the two-dimensional eletrophoretic protein profiles of cells subjected to acid stress, Svensäter and co-workers showed that expression of several proteins was altered (64 enhanced, 49 diminished), although the identities of these proteins were not established (Svensater et al., 2000). More recently, a study by Beighton and co-workers identified thirty proteins (18 up-regulated, 12 down-regulated) that showed altered expression at pH 5.2 when compared to pH 7.0-grown cells (Wilkins et al., 2002). The stress protein GroEL, cell division proteins such as FtsZ and FtsA, as well as key glycolytic enzymes were shown to be up-regulated under acidic pH. Conversely, small- and large-subunit ribosomal proteins, components of the PTS, protein translation elongation factors and, surprisingly, DnaK, were down-regulated. The down-regulation of DnaK during growth at low pH is opposite to that of a previous report, which examined *dnaK* mRNA and DnaK protein levels in chemostat-grown cells, that showed increased synthesis of *dnaK* in acid shocked and acid-adapted cells (Jayaraman et al., 1997). It is also unusual that GroEL and DnaK, which are regulated by the same repressor, HrcA, showed opposite regulation during growth at low pH in the former study. The discrepancies between the two reports are likely due to differences in growth conditions, in particular growth rate. In the work by Jayaraman and colleagues (Jayaraman et al., 1997), cells were grown in continuous chemostat culture to pH 7.0 or 5.0 steady-state, whereas in the work by Wilkins and colleagues (Wilkins et al., 2002), cells were grown in batch culture using media buffered at pH 7.0 or 5.2. In the latter study, the growth rate of the organisms at low pH was nearly 6-times longer than the cells growing at neutral pH.

A more recent, comprehensive proteomic study identified a total of 167 protein spots differentially expressed following steady-state growth at pH 5.0 (Len et al., 2004b). Of these, the majority (106 protein spots) was found to be proteins involved in metabolism. The other 61 protein spots were associated with stress response pathways (Len et al., 2004b) and corresponded to isoforms or cleavage products of 30 different proteins. Among the proteins found to be up-regulated at pH 5.0 were gene products involved in DNA replication, transcription, translation, protein folding and proteolysis. Several proteins identified in this study were previously shown to play roles in acid stress survival, including RecA (Quivey et al., 1995), the molecular chaperones GroEL, DnaK and trigger factor (RopA) (Jayaraman et al., 1997; Lemos et al., 2001) and components of the F-ATPase enzyme AtpA and AtpC (Quivey et al., 2000b). In addition, five proteins that have not been associated with acid tolerance were identified, including a single-stranded DNA-binding protein, Ssb; two proteinases; the ATP-binding ClpL of the Clp family of proteinases; and a dipeptidase that belongs to the *pep* gene family (Len et al., 2004b).

When biofilm cells of *S. mutans* were subjected to a sudden pH decrease, from 7.5 to 5.5, 86 protein spots were enhanced and 40 spots diminished in the acid-shocked biofilms (Welin et al., 2003). In this particular study, glycolytic enzymes were up-regulated in acid-adapted biofilms, but were down-regulated in the planktonic phase, indicating key metabolic differences between planktonic and biofilm cells and/or in the conditions to which the cells were exposed in the two environs. In general, the use of proteomics to address stress tolerance in oral streptococci is increasing and has been used very effectively of late to demonstrate the effects on protein expression of mutating transcriptional repressors, response regulators and proteases of *S. mutans,* that are known to regulate key virulence properties of the organism (Abranches et al., 2003; Lemos and Burne, 2002; Lemos et al., 2001; Wen and Burne, 2004).

When comparing the findings obtained from these proteomic studies (Len et al., 2004b; Wilkins et al., 2002), one can observe that these reports contain striking discrepancies. As noted above, differences in growth conditions are likely to influence the outcomes of these types of studies, which highlights a problem confronting molecular biologists that are taking advantage of microarray and proteomic technologies. As these studies have evolved with a variety of bacteria, the recognition that continuous chemostat culture is particularly advantageous for physiologic studies has emerged specifically because steady-state cultures that are grown under different conditions differ only in the variable being examined, and do not have different growth rates, nutrient availability and so forth. By using chemostats to tightly control the physiology of the organisms, one can effectively rule out the possibility that variables other than pH are influencing the phenotypic properties. In future studies where microarray or proteomic technologies are used to profile cells, chemostats are likely to become the “gold standard” for preparing homogeneous populations of cells.

Links Between Biofilm Formation and Stress Response

It is noteworthy that many of the gene products addressed in this review are required both for an appropriate response by organisms to environmental stress and to form biofilms (Table 1), establishing a molecular link between two critical virulence attributes of oral streptococci. Perhaps it is not surprising that stress tolerance pathways, which are intimately involved in growth and homeostasis of the organisms, influence the capacity of the bacteria to form stable biofilms. However, this potentially simplistic view

Table I. *S. mutans* genes involved in biofilm formation/ maturation and acid tolerance.

Strain	Homolog/ Function	Acid Tolerance	Biofilm Formation	Other Relevant Phenotypes
SM11 (*hrcA*$^-$)	*hrcA* – regulates Class I heat shock gene expression	Sensitive	Severely impaired	Lower F-ATPase activity, poor pH drop. Altered proteome
SMHK11 (*hk11*$^-$)	*hk11* – histidine kinase 2-component response regulator	Sensitive	Impaired	N.A.
SMCC1 (comC$^-$*)*	*comC* – competence peptide	Sensitive	Altered architecture	Lack of competence
SMCD1 (comD$^-$*)*	*comD* – competence factor	Sensitive	Impaired	Lack of competence
JL-*clpP*	*clpP* – serine protease	Poor growth at low pH	Impaired	Form long chains, multiple stress sensitivity. Altered proteome
JL-Δ*relA*	*relA* – stringent response effector	Enhanced in biofilms Planktonic- Like parent	Impaired	Enhanced pH drop, higher PTS activity. Altered proteome
TW26 (*luxS*$^-$)	*luxS* – AI-2 synthase	Sensitive	Impaired	H_2O_2 resistant, reduced *fruA* expression. Altered proteome
TW14 (*brpA*$^-$)	*brpA* – biofilm regulatory protein	Sensitive	Impaired	Form long chains
TW90 (*ropA*$^-$)	*ropA* – trigger factor	Sensitive	Impaired	Form long chains
TW91 (*rgg*$^-$)	*rgg* – transcriptional regulator	Sensitive	Impaired	N.A.
N. A. Not available.				

of the molecular basis for these observations ignores the fundamental concept that biofilm maturation, by virtue of the imposition of mass transport limitations, imposes stresses on the cells that they may not otherwise encounter during growth in planktonic cultures. The gradients that develop during biofilm maturation allow for accumulation of end products, concentration of diffusible molecules, generation of heterogeneity in growth domain within the populations, and reduced access to nutrients. Thus, an appropriately regulated response, both temporally and spatially, by the microorganisms to the environmental stresses they encounter within a maturing biofilm may have a profound influence on biofilm structure or whether the organisms will form biofilms at all. Viewed in this light, the intimate linkage of biofilm formation and stress tolerance is logical, and understanding more about the homeostatic mechanisms of adherent populations is clearly needed to better control oral biofilms in health and diseases.

It is also interesting to speculate that the stress response pathways, which are highly conserved in oral streptococci, may have evolved additional roles as the organisms evolved in this highly specialized environment. Oral streptococci do not have a free-living lifestyle and the only natural habitat for these organisms is in biofilms. These bacteria have small genomes and lack many of the complex genetic circuits that other bacteria possess for regulating and coping with stress, such as the alternative sigma factors σ^S and σ^B, and some of their target genes. Could the core stress regulon of *S. mutans* govern a broader set of biological functions than it may in organisms with more complex genomes? Clearly, there is a need to address these types of questions to come to terms with how oral streptococci thrive in a continuously changing environment.

Future Directions

In the oral cavity, biofilms are the natural habitat for cariogenic streptococci. Recent progress on understanding the genetics and physiology of adherent oral streptococci has reinforced that the organisms can acquire a “biofilm phenotype”. Still, relatively little information is available on the behavior of these organisms in complex populations such as dental biofilms. There is a major need to maintain the inertia that the research community has generated by initiating the study of surface-bound bacteria and to contrast the behavior of sessile bacteria with their planktonic counterparts. Even then, a major challenge remains in the study of *S. mutans* and other oral biofilm bacteria. In particular, *S. mutans* has not evolved in isolation from other bacteria - the organisms have evolved as members of highly dynamic and complex adherent communities. How intercellular contact with other organisms and antagonistic and synergistic interactions within biofilms shape the phenotype of these organisms is not at all understood. The study of oral species, including *S. mutans*, with well-developed genetic systems, excellent *in vitro* and animal models, and ready access to samples from human volunteers provides an exciting and unique opportunity to gain insight to the genetics, physiology and pathogenesis of organisms that share a life-long journey with their host – from birth to grave. Today, the tools of molecular biology have made conducting these studies in a meaningful way a reality.

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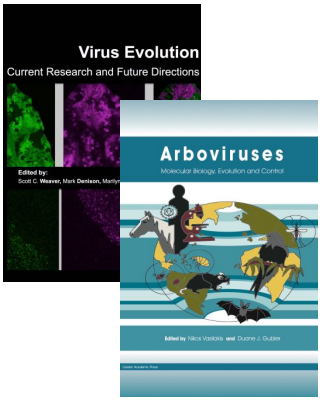

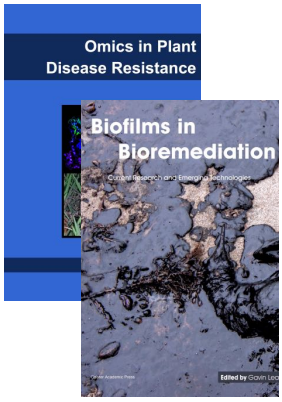

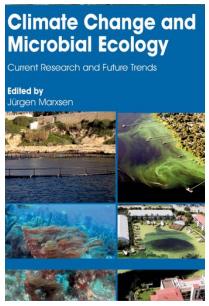

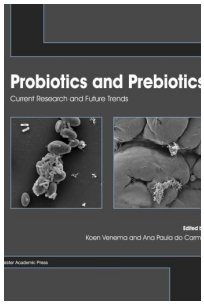

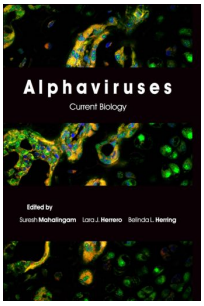

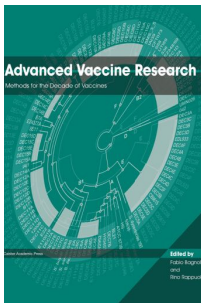

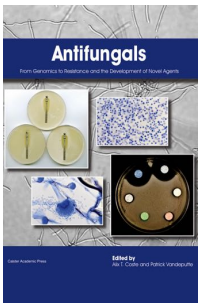

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