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An analysis of the role of glucan-binding proteins in Streptococcus mutans biofilm architecture and caries development

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AN ANALYSIS OF THE ROLE OF GLUCAN-BINDING PROTEINS IN *STREPTOCOCCUS MUTANS* BIOFILM ARCHITECTURE AND CARIES DEVELOPMENT

by

David John Lynch

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Oral Science
in the Graduate College of
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Thesis Supervisor: Professor Jeffrey Banas

ABSTRACT

Tooth decay is a serious health risk and a significant contributor to health care costs in both industrialized and developing nations. Tooth decay is the end result of a change in the balance of plaque ecology towards more acidogenic and aciduric bacterial species. Frequent and prolonged periods of low plaque pH, facilitated by the presence of fermentable simple carbohydrates drive the cycles of enamel homeostasis towards demineralization and ultimately dental caries. *Streptococcus mutans* is the main etiologic agent in the development of dental caries. Their cariogenic potential is based on the ability to produce and tolerate large amounts of acid and to adhere to and accumulate large numbers on the surface of a tooth. They are capable of efficiently fermenting a variety of simple carbohydrates and can produce high concentrations of acid, even in a low pH environment. However, it is the ability of *S. mutans* to rapidly synthesize copious amounts of water-insoluble and water-soluble glucan from dietary sucrose, which allow the bacteria to accumulate large enough numbers to dominate the dental plaque and significantly lower the plaque pH. Synthesis of glucan is mediated by glucosyltransferase enzymes and is crucial to sucrose-dependent adherence and to the cariogenicity of *S. mutans*. *S. mutans* also makes four non-GTF glucan-binding proteins: GbpA and GbpD contain a region that is homologous to the glucan-binding domains of the Gtf enzymes, and GbpC confers the property of dextran-dependent aggregation during stressful conditions, and GbpB whose glucan-binding properties appear secondary to its role in cell-wall metabolism. It was hypothesized that Gbps A, C, and D shape the architecture of *S. mutans* biofilms which in turn affects the cariogenicity of *S. mutans*. To test this hypothesis, a panel of Gbp mutants was constructed from *S. mutans* strain

UA130 that encompasses all deletions of Gbps individually and in combination. Specific pathogen-free rats were infected with the WT *S. mutans* UA130 strain along with each of the Gbp mutants, fed a high sucrose diet, and were then scored for caries. Significant attenuation of caries was observed in some but not all *gbp* mutants. Biofilms were also grown and analyzed via confocal microscopy. Architectural differences were found with all of the *gbp* mutants when compared to the wild-type, most notably the mutant strains lost significant biofilm depth. Several of the architectural parameters correlated with caries attenuation. It was concluded that deletion of one or more Gbps resulted in a partial loss of the cohesive properties of *S. mutans* biofilms and changes in biofilm architecture. In several cases this resulted in significant attenuation of cariogenicity but not a complete loss. The architectural changes that resulted from this loss of biofilm cohesiveness and the specific combinations of Gbp deletions that lead to significant attenuation suggested specific roles for each Gbp in biofilm formation. Furthermore, the attenuation of Gbp mutant strains could not be explained by differences in acidogenicity or aciduricity among the mutants. Therefore, it was concluded that Gbps A, C and D make profound contributions to biofilm architecture and changes in biofilm architecture, as a result of loss of Gbp-mediated cohesion, affects *S. mutans* cariogenicity.

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Graduate College
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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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CHAPTER 1

INTRODUCTION

Tooth decay is one of the most common infectious diseases affecting humans and is a significant health care issue in both modern and developing nations (Mitchell 2003). This decay is the result of the interaction of the oral microflora (plaque), the tooth surface, nutrition, and the oral environment over time and results in a carious lesion of the tooth enamel (Beighton 2005, Takahashi&Nyvad 2008). While in recent years overall incidence of this disease has declined in industrialized nations, caries rates are rising in developing nations (Chu&Lo 2008). Even in the US, some reports show caries incidence among children under 12 to range from about 40 to 50% of children tested, but as many as 70-85% of children have caries by age 17 (Smith 2010, Bagramian *et al.* 2009, Brown&Selwitz 1995, Bowen 2002, Edelstein 2002). Although these figures do represent an overall decline in caries rates in the US over the last 20 years, they still represent a large portion of the population and the rate of decline seems to have reached a plateau (Bagramian *et al.* 2009). Furthermore, caries prevalence is not evenly distributed across the population and communities with the highest incidences are usually those in lower socioeconomic groups that have limited access to sufficient oral health care (Bowen 2002, Featherstone 2000). Despite the fact that studies show a decline in caries in the United States, tens of billions are spent in this country each year on treatment of tooth decay, and this figure represents only the fraction of the population that seeks out and can afford treatment (Loesche 1986, Benjamin 2010). In other industrialized nations such as China and the UK, caries prevalence in the past decade has been over 50% in children. In developing nations, where oral health care is significantly less available, caries rates are rising at an alarming rate. Studies done in the past decade in countries such as the Philippines, Peru, Mexico and Taiwan, revealed caries in 75 to 90% of children (Bagramian *et al.* 2009).

The discovery that demineralization of tooth enamel is caused by acid produced by bacteria in dental plaque occurred as early as the 1890s and coincided with Koch's postulates and the "germ theory" of disease (Russell 2009). Since that time there has been some debate between the two main theories of the role of plaque in caries development. The non-specific plaque theory states that the collective activity of most or all plaque bacteria contributes to the increase of plaque acids that leads to caries. The specific plaque theory states that one or a few specific bacteria are responsible for caries development. An uncertain component of the specific plaque hypothesis was whether the specific cariogenic species represented a classically defined infectious species or the overgrowth of commensal plaque species. These theories are still sometimes debated but most researchers attribute caries to an alteration of plaque ecology most commonly induced by host dietary changes. The predominant species recovered from caries lesions are different than those recovered from healthy sites on a tooth surface (Marsh 2003a). While the contributions of all plaque flora must be taken into account when considering the ecological nature of caries development, certain species of bacteria have had strong associations with caries development (Kleinberg 2002). *Streptococcus mutans* is widely accepted as one of the most important etiologic agents in caries development and has been shown to directly cause caries in germ-free and specific pathogen-free rat models. While incidences of caries have been found without *S. mutans* and high percentages of *S. mutans* have been recovered from non-carious individuals, *S. mutans* remains the most common species associated with caries. Also, in gnotobiotic and specific germ-free rodent models, they have the greatest potential for generating caries (Takahashi&Nyvad 2008, Kleinberg 2002, van Houte 1994, Hamada&Slade 1980, Nyvad&Kilian 1990). Although *S. mutans* possesses several properties that promote its cariogenicity, robust biofilm formation in the presence of dietary sucrose is a critical component in the development of caries. The following work will focus on *S. mutans* biofilms and the function of biofilms in the cariogenicity of *S. mutans*.

Evolution of Biofilm Research

The vast majority and possibly up to 99.9% of the world's bacteria in aquatic environments exist in biofilms on various surfaces (Costerton *et al.* 1995, Donlan&Costerton 2002). This tendency was recognized in the early 20th century when, for example, Claude Zobell observed that in aquatic ecosystems, surface-associated microorganisms greatly outnumbered their presence in the surrounding medium (Zobell 1943). Heukelekian and Heller later demonstrated the "bottle effect" for marine microorganisms which stated that bacterial growth and activity were substantially enhanced by the incorporation of a surface to which they could attach (Heukelekian&Heller 1940). Since the time of those experiments it has been recognized that biofilms are prevalent in non aquatic environments as well. In fact, they are ubiquitous throughout nature and have enormous medical and commercial impacts. These can include infections, contamination of medical devices, corrosion of water pipes and increased drag on ships (Brown *et al.* 1991).

Dental plaque was one of the first biofilms discovered when Antoine van Leeuwenhoek first used his primitive microscope to look at material he collected from between his teeth in 1683 (Nobbs *et al.* 2009). However, it would be centuries before his discovery, which he termed "animalcules," would be realized as a biofilm and even longer for the impact of biofilms to be appreciated (Donlan&Costerton 2002). The study of biofilms began slowly despite a growing acceptance that for most species of bacteria a biofilm was their natural state of existence. For centuries, scientists had investigated planktonic cultures of bacteria and had made many medically important discoveries. However, some of this research was limited in relevance to prominent biofilm infections such as pneumonia in a cystic fibrosis patient or infections caused by medical implants. Often these infections are more highly resistant to antibiotic treatment and the host immune system than would be predicted by examination of planktonic cultures.

Another factor that may have hindered the routine culturing of bacteria in a biofilm is that many lab strains of infectious bacteria that have been continually passaged in broth cultures do not adhere to surfaces or to each other. This may have occurred by selection of planktonic bacteria that had terminated the energy-expensive processes associated with adhesion. This most likely led to misleading conclusions regarding the eradication of many bacterial infections because therapeutic substances were not tested on bacteria in their natural environments (Costerton 1999b). Often laboratory strains are cultured in conditions that favor rapid growth, which is not the case in nature where bacteria face harsh conditions like nutrient deprivation, fluctuations in temperature and pH, competition from other bacteria, and predation from other organisms (Brown *et al.* 1991).

Technological limitations may have also played a role in delaying intense study of bacteria within biofilms. Eventually, however, the use of electron microscopy enabled close-up views of biofilms and revealed much about their general structure. One of the first discoveries was that bacteria stick to surfaces and other bacteria using a mass of tangled polysaccharide fibers that extend from the bacterial surfaces. This "glycocalyx," as it was originally termed, surrounds a cell or group of cells (Costerton *et al.* 1978). It has since become understood that this substance is a major component in what is now referred to as the exopolymeric matrix. The introduction of Confocal Laser Scanning Microscopy enabled live *in situ* visualization of biofilms and led to major advances in understanding biofilm architecture and heterogeneity.

The concept that biofilms are the natural environment for many bacteria is widely recognized, therefore it is important to study these bacteria in this environment. The relevance of the biofilm model has been corroborated by work revealing the extent to which biofilm bacteria behave differently than members of the same species grown planktonically (Beloin&Ghigo 2005). Recent research has uncovered many

physiological qualities of biofilms including altered growth states and differential gene expression of bacteria in biofilms compared to planktonic bacteria (Donlan 2002).

Biofilm Formation

Biofilms generally consist of clusters of bacterial cells (either single species or mixed) irreversibly attached to a surface and embedded in a self-produced exopolymeric matrix. A cluster, or microcolony, is the basic unit of a biofilm and is defined as a discrete group of bacterial cells (from one species or several) enclosed in the exopolymeric matrix (Costerton *et al.* 1994). Microcolonies are arranged and structured differently, depending on the bacterial species, the substrate and the conditions of the surrounding media. Generally speaking, however, the mature biofilm structure is thought to be a heterogeneous structure made up of differing concentrations of bacterial cells and exopolymeric matrix, with water channels running throughout all levels of the biofilm (Costerton 1999a).

The matrix allows the stable juxtaposition of the microcolony and regulates contact with the fluid phase (Costerton *et al.* 1995). There are many traits that are common throughout the vast array of biofilm-forming species. In a natural setting, biofilms often consist of multiple species that contribute to the mature biofilm in unique and sometimes synergistic ways (Donlan 2002). Metabolic by-products from one species may be used as nutrient for others and attachment of one species could provide ligands for attachment of others. Conversely, there is often competition for nutrients among biofilm bacteria. Many produce harmful metabolic by-p roducts, or synthesize antimicrobial agents to prevent colonization and/or growth of certain organisms within the biofilm community (Dunne 2002).

All biofilm formation begins with adhesion of bacteria to a surface. While many species of bacteria can form a biofilm under almost any conditions, there are often unique environmental signals that drive different bacterial species towards biofilm existence.

These signals may involve nutrient concentration, pH, osmolarity, O_2 concentration, and proximity to a surface. Population density can also be a common trigger for biofilm formation (O'Toole *et al.* 2000). It is likely that a species could employ different combinations of the aforementioned cues under varying sets of circumstances.

Adhesion can occur by a variety of mechanisms and generally involves reversible and irreversible stages. The first stage of attachment is reversible and is usually mediated by hydrophobic interactions, electrostatic interactions, or van der Waals forces and can be influenced by temperature or hydrodynamic forces (Dunne 2002). These attractive forces are usually just enough to overcome the natural repulsion of the bacteria and substratum and this stage is usually followed by a more rigorous and irreversible attachment mediated by specific host cell or bacterial cell receptors. Adhesion proteins and receptors are common for clinically relevant biofilm forming bacteria and contribute to this initial attachment, as well as, facilitate co-aggregation of bacteria within a species or of different species. Cell surface proteins like pili, fimbriae or flagella are common adhesion proteins and can bind specific receptors or form hydrophobic bonds with a surface (O'Toole *et al.* 2000, Characklis 1990). Other cell wall structures such as lipopolysaccharide (LPS) can contribute to this process (Donlan 2002). As previously discussed, net charge and hydrophobicity of the substratum affect bacterial adsorption, and these qualities of a bacterial cell also affect adsorption to surfaces. Initial colonizing bacteria can alter substratum properties making colonization favorable, or can provide binding sites for species that would not ordinarily bind to a specific surface. At this stage, it is common for extracellular polysaccharide polymers to contribute to the irreversible nature of the biofilm and the transition to a mature biofilm (Stoodley *et al.* 2002). Microcolonies are formed and the mature biofilm architecture takes shape as the attached bacteria divide and synthesize their exopolysaccharide matrix. The architecture of a mature biofilm varies depending on the species of bacteria present in the biofilm and

environmental conditions (Stickler 1999). This architecture and composition and contribution of the matrix will be discussed at a later point in this thesis.

Many of the differences between planktonically grown bacterial cultures and those that exist in biofilms can be attributed to changes in gene expression. The differential gene expression associated with biofilms has two facets: those genes that are necessary or responsible for biofilm formation, and those that are up- or down-regulated upon biofilm formation (Jefferson 2004a). There are likely as many necessary genetic factors and unique pathways for biofilm formation as there are species of biofilm-forming bacteria. It is also likely that many of these genetic mechanisms are highly influenced by growth conditions and nutrient availability, allowing for multiple pathways within a species (Beloin&Ghigo 2005, O'Toole 2003). For example, there are at least 6 different transcriptional regulators involved in *P. aeruginosa* biofilm formation (O'Toole 2003).

Those genes responsible for biofilm formation or for conversion of a cell from a planktonic to a biofilm state are often driven by signal transduction mechanisms and/or quorum sensing mechanisms – means for detecting a critical mass of bacteria. For example, both the ComCDE system and the VicRKX (CovRS) two-component signaling systems in *S. mutans* have both been shown to be vital for biofilm formation (Senadheera *et al.* 2007, Senadheera *et al.* 2005, Li *et al.* 2002, Tremblay *et al.* 2009, Chong *et al.* 2008). There are ComCDE homologs in other oral streptococcal species such as *S. intermedius, S. gordonii*, and *S. anginosus*, which promote biofilm formation (Senadheera&Cvitkovitch 2008). Other Gram-positive species have similar systems that have been shown to be involved in biofilm development, such as the AgrBCDA system in *S. aureus* which has been shown to be most commonly involved with release of cells from a biofilm (Yarwood *et al.* 2004). Gram-negative organisms also depend on quorum sensing for biofilm formation. The *lasI/lasR* system is one of the quorum sensing systems that exists in *P. aeruginosa,* where the product of *lasI* is a diffusible signaling molecule and *lasR* encodes the transcriptional regulator that activates several virulence

genes upon recognition of sufficient concentrations of N-(3-oxododeconoyl)-L-homoserine lactone. N-(3-oxododeconoyl)-L-homoserine lactone is synthesized by the *lasI* gene product. *lasI* mutants form biofilms that are flatter and more sensitive to SDS treatment than WT biofilms (Davies *et al.* 1998). Interestingly, Gram-negative quorum sensing signals are generally acyl-homoserine lactone whereas Gram-positive quorum sensing systems rely on signal peptides. From unique signals to complex genetic pathways, bacterial species have evolved intricate mechanisms to regulate biofilm formation (O'Toole 2003, Davies *et al.* 1998, Pesci&Iglewski 1997, Swift *et al.* 1994).

The complexity of biofilm formation is revealed in studies that compare gene expression of biofilm grown bacteria with gene expression of bacteria in the planktonic state. Furthermore, even within single species biofilms there is a great deal of heterogeneity of gene expression among the bacteria that populate the biofilm. Biofilm structure causes diffusion limitation leading to variable nutrient, local pH, and oxygen tension levels which in turn leads to a variety of metabolic states among the biofilm cells (Jefferson 2004a). It is thought that quorum sensing signals play an important role in regulating gene expression in biofilms due to the close proximity of the cells. Global gene expression experiments have yielded a wide variety of genes that are up- or down-regulated in biofilm bacteria. These include genes associated with metabolism, adhesion, and stress response, as well as quorum sensing genes (O'Toole *et al.* 2000, Shemesh *et al.* 2008, Motegi 2006). Some of the differential gene expression patterns in mature *S. mutans* biofilms will be discussed later in this thesis.

Attachment of bacteria to a surface and biofilm formation can also be influenced by the physical properties of that surface. Due to increased surface area and decreased sheering forces, a rougher surface generally leads to greater bacterial colonization (Donlan 2002, Characklis 1990). Also, the net charge of a surface as well as the hydrophobicity can influence the composition of adherent bacteria and the efficiency of adsorption (Fletcher&Loeb 1979). In almost all natural aqueous environments solid

surfaces become rapidly coated with a film consisting of adsorbed macromolecules and other hydrophobic molecules from the medium. This conditioning film can alter the surface charge, or hydrophobicity of the substratum. While in some instances the conditioning film can inhibit bacterial adhesion, it can also provide ligands for bacterial adhesins (Donlan 2002, Lejeune 2003, Characklis&Marshall 1990, Davey&O'toole 2000).

One of the most well documented conditioning films is the Acquired Enamel Pellicle (AEP) that forms on the surface of a tooth; this film both lubricates the tooth and forms a protective coating. Upon eruption, the hydroxyl apatite-rich tooth enamel is very porous and needs to mature. Saliva contributes to the maturation of tooth enamel in two ways. The first contribution of saliva is the deposition and incorporation of calcium, phosphates, and fluoride into the maturing hydroxyl apatite (HA), due to the saturation of saliva with these ions. The availability of fluoride is dependent on geography or public health measures but helps form stronger and more acid resistant enamel (Featherstone 1999, Featherstone 2004). Secondly, the tooth is coated with the AEP which is comprised of statherin, proline-rich glycoproteins and mucins, and can include, cystatins, histatins, lysozyme, amylase, lactoferrin, lactoperoxidase, sialic acid, albumin, carbonic anhydrate, sIgA and bacteria-derived glucosyltransferases (Gtfs) and fructosyltransferases (Ftfs) from the surrounding saliva (Bowen 2002, Garcia-Godoy&Hicks 2008, Li *et al.* 2003). The pellicle protects the porous hydroxyl-apatite from demineralization of calcium and phosphates by plaque-generated acids. Many of the proteins, as well as water-insoluble glucan, adsorbed to the tooth surface, are bound by primary colonizing bacteria that initiate plaque development.

Biofilm Architecture

After initial adhesion, biofilms enter a period of growth and accumulation where adherent cells divide exponentially and the mature biofilm architecture starts to take

shape (Characklis 1990). The architecture of a mature biofilm varies depending on the species of bacteria present in the biofilm and environmental conditions (Stickler 1999). The bacterial composition of a community can also be the result of environmental factors such as nutrition, the substrate and cooperative and competitive relationships among bacteria within a biofilm. A number of environmental and bacterial cell factors affect the composition of the bacterial community and the biofilm architecture. These factors, such as the exopolymeric matrix, the characteristics of the aqueous environment, and hydrodynamics will be discussed in greater detail in the following paragraphs (Donlan 2001).

Exopolymeric Matrix and Polysaccharide

The exopolymeric matrix (EPM) of a biofilm serves several important functions in biofilms such as surface attachment, promotion of biofilm structure and protection from environmental factors and dehydration (Vu *et al.* 2009). This matrix usually varies in density which allows for interstitial voids or channels but can account for up to 50-90% of the biofilm mass (Davey&O'toole 2000). The EPM is also a dynamic and heterogeneous component of the biofilm and may be synthesized or degraded depending on the needs of the biofilm bacteria (Flemming&Wingender 2001, Sutherland 2001b).

Interactions between different extracellular polysaccharides can change the physical properties of the polymers and alter the structure of a biofilm (Tait&Sutherland 2002). Some bacterial cells are coated with matrix polymers synthesized by other species and therefore remain in the matrix and either benefit from or produce useful metabolic by-products.

While the primary component of EPM is usually a polysaccharide, it often contains significant quantities of proteins, amino acids, lipids and nucleic acids (Sutherland 2001a). It was previously assumed that large amounts of free DNA in a biofilm matrix were the result of cell death to facilitate gene transfer. However, in many

instances, the presence of DNAse prevented biofilm formation or weakened existing biofilms (Whitchurch *et al.* 2002, Matsukawa&Greenberg 2004, Allesen-Holm *et al.* 2006) leading to the conclusion that DNA also contributes to the structural integrity of the biofilm.

Hydrodynamics

Hydrodynamic forces affect biofilm structure both by presenting a method for nutrient/waste transport and through sheering forces on the cell clusters. Fluid sheer forces can play a role in the removal of reversibly adsorbed bacteria during initial colonization of biofilm cells (Characklis 1990). Biofilms grown in laminar flow often consist of small cell aggregates situated on a substratum while cell aggregates from biofilms grown in turbulent flow will have long streamers of cells and matrix (Davey&O'toole 2000). Considerable evidence exists to show that there is liquid flow in biofilm channels and that this likely has a role in mass transport (de Beer *et al.* 1994a, de Beer *et al.* 1994b). It is probable that the organization of the previously described matrix into channels is affected by hydrodynamic forces and has evolved to take advantage of this flow for mass transport.

Sheering forces also affect detachment and dispersion of biofilm cells, which is important for colonization of new surfaces and can be an active or passive process. There are three modes of dispersal and each can have active and passive elements. Erosion is the continuous detachment of single cells or small biofilm cell clusters. This process can be active or passive and may include an active response to signaling mechanisms, a passive response to sheering forces or a combination of both processes. Sloughing is a similar process that also can be active or passive, but differs from erosion in that it is a sudden detachment of large sections of biofilm. It is believed that large clusters of sloughed fragments may have greater protection from antimicrobials or immune cells than single eroded cells. The third mode of biofilm dispersal is seeding, which, involves

the release of large numbers of cells from a hollow cavity formed inside of microcolonies. The mechanism of this release is not fully understood but it is likely an active process involving death of cells surrounding the cavity and cells that do not aggregate over the top of the cavity (Kaplan 2010). Each of the three methods of dispersal can have active elements involving environmental signals, signal transduction systems and possibly EPM degrading enzymes, but sheering forces are an important part of this process. Dispersal by cell division, or enzymatic degradation of biofilm EPM can be a response to environmental pressure, like starvation, (Allison *et al.* 1998) a change in pH, oxygen or similar stresses, or a response to quorum sensing signals. It should be mentioned that in some cases an abundance of nutrients promotes dispersion rather than starvation. Hydrodynamic or sheering forces also help remove and carry both actively and passively detached cells to new sites and affect binding to new surfaces (Karatan&Watnick 2009).

Characteristics of Aqueous Environment

The aqueous media that surrounds a biofilm provides environmental signals that may be required for biofilm formation, growth and maturation and dispersal. These signals may be a specific nutrient availability or the lack of nutrients. For example, biofilm initiation may require the abundant presence of certain amino acids, as in the case of certain strains of *E. coli* (O'Toole *et al.* 2000).

Certain components of the aqueous environment can be important to the structure of a biofilm. While sucrose is not known to be a signaling factor in *S. mutans* biofilms, it is an important environmental factor in their biofilm formation. *S. mutans* biofilms formed in the absence of sucrose are sparse and vastly different than the robust biofilms formed in the presence of sucrose. Sucrose provides a nutritional source; its metabolic by-product, lactic acid, helps define a low pH niche and the glucose portion of sucrose is utilized to synthesize the polysaccharide portion of the EPM (Bowden&Li 1997).

The Advantages of a Biofilm

The normal environment for many pathogenic bacteria is a biofilm. As described above, biofilm existence is a very coordinated and active process that involves significant resource input from many cooperating individual bacteria. In some ways this appears to go against Darwin's principal of self-preservation, so there must be significant advantages to biofilm existence. These advantages begin with attachment to a surface that allows an organism to remain in an area with an abundant supply of nutrients (Davey&O'toole 2000, Jefferson 2004b). But a biofilm is more than a collection of individually adhered bacteria. The EPM helps stabilize attachment and bacterial co-aggregations.

Another critical advantage of biofilms is their enhanced ability to resist exogenous or host-derived means of clearance or killing. Bacterial cells in biofilms are more resistant to attack from anti-microbial agents such as antibiotics and anti-microbial peptides (defensins). The existence of a cell in a biofilm provides protection from inflammatory cells. In many instances, antibodies generated to antigenic sites of cells in biofilms, and phagocytic cells, are activated but fail to clear a biofilm infection (Costerton *et al.* 1987). There are multiple theories describing how biofilm properties contribute to this resistance. The biofilm matrix is thought to create a barrier that slows diffusion of antimicrobial agents to internal cells and prevents immune cells from phagocytizing embedded cells. Many anti-microbial compounds react with biofilm components such as charged sites on matrix polymers, binding sites on live and dead cells, or anti-microbial degrading enzymes and are thus diluted or quenched before reaching cells in the interior of a microcolony. Reactive oxidants from immune cells are most likely quenched in this way (Mah&O'Toole 2001, Hoiby *et al.* 2010, Brown&Gilbert 1993, Costerton *et al.* 1999). However, there are several anti-microbial agents that are not inhibited by biofilm matrices and biofilm resistance to these agents cannot be explained by retardation of diffusion (Lewis 2001). Under these conditions or

conditions of high anti-microbial concentration, it is more likely that the metabolic heterogeneity contributes to resistance. Populations of biofilm cells usually exist in a low metabolic state and are unaffected by anti-microbials, due to the fact that most anti-microbials are more effective on rapidly growing cells (Donlan&Costerton 2002, Costerton *et al.* 1999). A related school of thought suggests that there is a population of metabolically inactive bacteria located in deep, nutrient devoid, microcolony regions that do not absorb anti-microbial compounds. These "persister" cells exist in a deep state of dormancy and are also common in stationary phase cultures (Lewis 2001, Keren *et al.* 2004). A final mechanism of resistance lies in the fact that many biofilm bacteria are in a state of stress response and can have increased mutations which can lead to selection for resistant genes (Hoiby *et al.* 2010). Biofilms also show increased genetic exchange where antibiotic resistance genes can be transferred to previously non-resistant strains (Li *et al.* 2001). It is likely that the mechanism of resistance will be species and anti-microbial specific and it is likely that many cases will involve a combination of biofilm properties.

Due to the close proximity of biofilm cells, combined with the increased expression of competence genes found in many biofilm bacteria, it is reasonable to assume that there is a considerable amount of genetic exchange occurring among bacteria in biofilms. While several reports show a structural role for extracellular DNA in biofilms (Whitchurch *et al.* 2002, Allesen-Holm *et al.* 2006), genetic exchange is still a common occurrence among biofilm bacteria. Several experiments using antibiotic resistance cassettes on plasmids have shown genetic transfer between different species within a biofilm, (Roberts *et al.* 1999, Lebaron *et al.* 1997, Christensen *et al.* 1998) however some of these transfers were assumed to occur via conjugation. It is likely that the induction of competence among biofilm bacteria may function to take advantage of the large amount of free DNA that has been found in biofilms, increasing the possibility

that multiple beneficial mutations could accumulate in an individual cell (Spoering&Gilmore 2006).

Biofilm matrices also provide nutritional niches within a microcolony that are protected from the environment. Anaerobic bacterial species can often be found in deep regions of microcolonies where the concentration of O_2 is lowest. Acidophilic bacteria are able to create an acidic niche protected from immediate dilution by the surrounding media. The proximity of cells in a biofilm allow for metabolic cooperation where metabolic products of one species are used as nutrients by another. Biofilms of mixed species often show syntrophic relationships in which substrate exchange among two or more species is necessary for certain processes including energy production (Davey&O'toole 2000).

Biofilm existence often results in a reduction in metabolic growth for individual cells. Cells sometimes exhibit altruistic behavior such as autolysis. However, the sacrifices of individual bacteria actually result in a fitter community when the protective and cooperative aspects of a biofilm are factored in (Jefferson 2004a). Because of these biofilm related properties, it is clear that biofilm bacteria present a relevant clinical threat to humans and a tremendous challenge to health care providers.

Caries and Plaque Ecology

Caries Process

Initial colonization of tooth enamel and recolonization after physical debridement of plaque is an ordered and coordinated process where attachment of one bacterial species often presents a target for attachment of others (Kolenbrander *et al.* 2002). Early colonizers bind the AEP, first through reversible interactions between the cell surface and the pellicle and then by irreversible covalent bonds between bacterial adhesive proteins and pellicle receptors (Marsh 2004). Co-adhesion and co-aggregation occur among early and late colonizers and between the various groups of bacteria. These can involve

specific adhesins as well as lectin/carbohydrate polymer binding. Almost all identified plaque bacteria have a co-aggregation partner and some, so called "bridge" organisms, bind several species and can link initial, early and late colonizers (Kolenbrander *et al.* 2010). It is likely that antagonistic and synergistic interactions influence the bacterial composition of the mature biofilm (Marsh 1999).

Initial colonies are dominated by several streptococcal species, namely, *S. sanguinis, S. mitis, S. oralis* and *S. gordonii*. However, it is likely that the composition of initial colonizers is dependent on the host's diet, oral hygiene practices or other factors. For example, individuals with a high propensity for caries, may show a higher percentage of *S. gordonii* in initial colonization (Nyvad&Kilian 1990, Hojo *et al.* 2009).

Caries are an ecological disease where a shift in plaque flora towards more acidogenic and aciduric bacteria leads to more frequent and prolonged periods of low plaque pH. In environments with abundant available carbohydrate these acidic bacteria create an unfavorable niche for non acidic commensals leading to greater accumulation of cariogenic species. Frequent high sucrose meals, combined with factors involving oral hygiene practices, aging, genetic factors, and immune changes, create conditions in the plaque that favor the propagation of the most highly acidogenic and acid-tolerant species such as members of mutans streptococci or lactobacilli (Marsh 2003a, Filoche *et al.* 2010, Mobley 2003, Rethman 2000).

The development of a carious lesion is the result of a gradual, consistent shift in the balance of demineralization and remineralization of tooth enamel that is directly affected by the bacteria colonizing the specific spot of the lesion. Lactic acids, along with other organic acids, produced from the fermentation of dietary carbohydrates diffuse into the HA and promote dissolution of Ca and phosphate ions, which can then diffuse out of the tooth enamel. We have previously described the restorative properties of saliva to the hydroxyl apatite but saliva also offers several protective mechanisms against caries-causing bacteria. It is a buffered solution which helps to raise plaque pH between

meals and it also washes away carbohydrate and acids from the plaque (Featherstone 2000, Featherstone 2004, ten Cate&Featherstone 1991). The Stephan's curve in Figure 1 shows the acidogenic activities of plaques from individuals with different levels of caries. It also shows that cariogenic plaques take longer to recover a neutral pH after a glucose rinse (Stephan 1944). The acid challenge of "cariogenic" plaque overwhelms the ability of saliva, which is supersaturated with Ca and phosphate ions, to raise the plaque pH and drive the ions back into the HA matrix to allow remineralization of the enamel (Featherstone 2004).

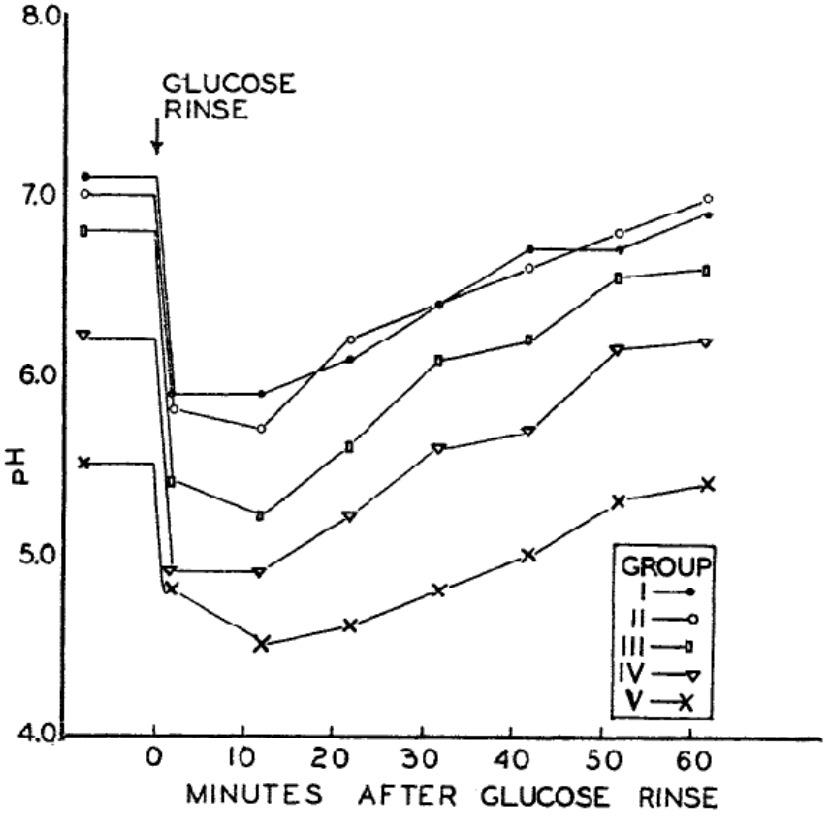


Figure 1 Stephan's curve showing changes in plaque pH on the surfaces of teeth after glucose rinse in different caries activity groups. Groups: I – caries free; II – caries inactive but had previous caries; III – slight caries activity, IV – marked caries activity; V – extreme caries activity (Stephan 1944).

While transient shifts in plaque pH and enamel demineralization alone do not lead to caries, prolonged or more frequent periods of acidification begin to compound over time. As the local pH consistently drops, the balance of HA homeostasis shifts towards demineralization and the HA becomes increasingly weaker. The precursor to a full cavity is a "white spot" lesion where the surface of the enamel remineralizes before the dissolved minerals can travel into the underlying demineralized enamel, forming a weakened region. In severe caries the surface of the enamel is broken exposing the dentin and cementum which are demineralized by a similar process (Featherstone 2000).

Commensal Plaque Microflora

There are at least 800 different species of bacteria that have been identified from the human mouth, and a healthy individual may harbor up to 500 of these species at one time. However, it is widely believed that only a small portion of plaque bacteria are linked to dental caries (Hojo *et al.* 2009, Filoche *et al.* 2010). One early question in caries research was whether the bacteria responsible for caries were infectious species in the classical sense or were the result of an overgrowth of endemic species. Most evidence suggests the presence, in relatively small numbers, of one or more of these species in the absence of disease (Marsh 2003b). In fact, the bacteria that are considered cariogenic make up less than 1% of a healthy biofilm and are of two main types of highly acidogenic bacteria: certain members of oral streptococci, mainly *S. mutans* and *S. sobrinus,* and certain lactobacilli (Featherstone 2004).

In order for the low levels of cariogenic species to outcompete other plaque species and attain cariogenic proportions, environmental changes to the plaque micro-niche are necessary (Marsh 2003b). The ecological shift in plaque bacteria is primarily associated with changes in nutrition, but can be affected by plaque bacterial interactions, oral health care and systemic health of the host, and host genetics (ie. conditions that reduce salivary flow). Host diet consistency and frequency of ingestion play a large role

in determination of bacterial growth and ultimately plaque ecology (Loesche 1986, Mobley 2003, Davey&Costerton 2006, Duarte *et al.* 2008, Loesche 1981).

Since the original discovery by Miller *et al*. in the 1890s that acids generated from plaque were responsible for the enamel erosion, many investigators have examined bacteria cultivated from carious lesions using enzymatic and biochemical analysis to screen cultivated species for cariogenic properties (Kleinberg 2002). The emergence of *S. mutans* as the prevailing etiological agent in dental caries is based on the strong association of *S. mutans* with carious lesions, its ability to induce caries in rodents fed a high sucrose diet, its highly acidogenic and aciduric properties and its ability to convert sucrose into glucan to promote adhesion and accumulation of plaque biofilm (Takahashi&Nyvad 2008, Tanzer *et al.* 2001). More recently, micro-array analysis has been used to confirm the highly cariogenic properties of *S. mutans* which will be described in more detail later (Marsh 2003b).

Taxonomy of *S. mutans* and mutans streptococci

Streptococcus mutans was first isolated from carious lesions by Clarke *et al*. as early as 1924, but it was not until the 1960's that taxonomical advances allowed researchers to match strains of oral streptococci recovered from human carious lesions (and similar strains recovered from rodent caries) with the original *S. mutans* isolate from 1924 (Loesche 1986, Hamada&Slade 1980). But even at that point, the taxonomy of the oral streptococci remained difficult and has remained in a state of flux ever since. *Streptococcus mutans* was the name given to all oral streptococci that were isolated from carious lesions that could ferment mannitol and sorbitol, that produced extracellular glucans and were cariogenic in rodent models of caries. They were called "mutans" due to their appearance on Gram stains where they resembled mutant versions of streptococci, possessing a smaller and more oval appearance (Loesche 1986). The original *S. mutans* strains encompassed a variety of serotypes (a-h). Eventually, as DNA analysis improved,

S. mutans strains were reassigned into several species: *S. mutans, S. sobrinus, S. criceti, S. downei, S. ferus, S. macaccae, S. ratti,* and *S. hyovaginalis* (Facklam 2002, Whiley&Beighton 1998), and collectively designated the mutans streptococci (MS). The most common human species remains *S. mutans; S. sobrinus* is also common in humans, but less so than *S. mutans*. *S. criceti* and *S. ratti* are only rarely recovered from humans.

S. mutans virulence factors

S. mutans possesses a variety of virulence factors that enable it to establish colonization, accumulate large numbers on the tooth surface, utilize a wide array of carbohydrate sources, produce acid and thrive at low pH. Experiments by Harper *et al.* demonstrated that *S. mutans* was more acidogenic than other non-mutans oral streptococci and lactobacilli strains. It also showed the most rapid growth at lower pH than any of these bacteria (van Houte 1994, Harper&Loesche 1984, Takahashi *et al.* 1997). The trait that further separated *S. mutans* from other oral plaque bacteria was the ability to accumulate large numbers in the presence of dietary sucrose. This required both adhesion to the tooth surface or to plaque colonies, and cohesion among the dividing cells (Marsh 2004, Kolenbrander *et al.* 2010).

Adherence and Accumulation

There are two mechanisms by which *S. mutans* adheres to the tooth enamel or plaque surface: sucrose-independent adherence and sucrose-dependent adherence. As previously stated, most people harbor *S. mutans* but this does not always or immediately lead to caries (Gibbons *et al.* 1986). Interactions of *S. mutans* with the enamel pellicle and with primary colonizers enable residence in the plaque biofilm, in small numbers, until conditions are favorable for rapid accumulation, such as host consumption of sucrose (Mitchell 2003).

Although they do not bind to pellicle proteins very efficiently, *S. mutans* possesses multiple surface adhesion proteins. There is a high degree of specificity in the sites that *S. mutans* binds and the primary colonizers that they associate with, which surpasses general ionic, hydrophobic or van der Wall's forces (Gibbons 1984). These adhesion events involve cell surface adhesins of *S. mutans* and interactions with the acquired pellicle and primary colonizing bacteria.

The most important adhesin is the P1 antigen (also known by the following names: antigen I/II, SpaP, Pac, MSL-1, antigen B (Matsumoto-Nakano *et al.* 2008)) which is encoded by the *spaP* gene and binds to salivary glycoproteins (Bowen *et al.* 1991). Cell wall associated protein A (WapA) is a small, immunogenic, cell wall protein that promotes sucrose-independent intercellular aggregation in *S. mutans*. WapA expression is repressed in the presence of sucrose which suggests that it may enable *S. mutans* cells to bind to a biofilm in the absence of sucrose or glucan (Zhu *et al.* 2006). Primary colonizers, like *S. gordonii*, express several proteins that bind *S. mutans.* While these may not directly be considered *S. mutans* virulence proteins, they attach to receptors on the *S. mutans* cell wall and help facilitate initial binding to the biofilm. Sucrose independent adhesion of *S. mutans* allows the species to become part of the plaque biofilm but does not normally allow for the accumulation necessary for caries development.

Glucan production via *S. mutans* glucosyltransferase (GTF) enzymes occurs by splitting sucrose and polymerizing glucose moieties into either water soluble- or water-insoluble glucan extracellular polysaccharide. This is the critical process that enables *S. mutans* to create robust biofilms and become cariogenic (Hamada&Slade 1980). Several studies using glucosyltransferase (*gtf*) mutant strains of *S. mutans* have shown a decrease in the cariogenicity of the bacteria and the ability to adhere to smooth surfaces or form biofilms *in vitro* (Michalek *et al.* 1975b, Tanzer *et al.* 1974, Hirasawa *et al.* 1980a, Hirasawa *et al.* 1980b).

Also, virulence in *S. mutans* mutant strains lacking the ability to produce water-insoluble glucan was partially restored by the addition of exogenous Gtfs (Hirasawa *et al.* 1980a). Wild-type *S. mutans* strains do not accumulate large numbers and are not cariogenic when grown in ample concentrations of sugars other than sucrose (Gibbons 1984). Sucrose-dependent adhesion to glass surfaces has been shown to be mediated by GtfI and GtfSI (Tsumori&Kuramitsu 1997, Ooshima *et al.* 2001). *S. mutans* makes three Gtf enzymes: GtfI, encoded by glucosyltransferase B (*gtfB*); GtfSI, encoded by *gtfC*; and GtfS, encoded by *gtfD*. GtfI and GtfSI primarily synthesize water-insoluble glucan that is comprised mainly of α1-3, glycosidic linkages with varying degrees of branching linkages, while GtfS synthesizes water-soluble glucan that is mostly linear α1-6 linked glucose molecules (Banas&Vickerman 2003).

Gtfs, also called glucan-sucrase enzymes, are found in other oral streptococci, lactococci, lactobacilli, and *Leuconostoc mesenteroides* and have very well conserved sequences and structure. They all contain a signal peptide that is common to all Gram-positive bacteria at the N-terminus. A variable domain of unknown function is located just downstream of the signal peptide. Downstream of the variable domain is the catalytic core, which is highly conserved and contains an active site with 3 necessary Asp residues about 500 amino acids from the N-terminus (Monchois *et al.* 1999, Devulapalle *et al.* 1997). The C-terminal glucan-binding domain is the second functional and highly conserved domain and is responsible for keeping the enzyme attached to the growing glucan polymer. The glucan-binding domain consists of a series of related repeats that form a β-barrel (Banas&Vickerman 2003).

Gtf enzymes are found both extracellularly and cell wall bound, and there is evidence that Gtfs avidly bind the enamel pellicle (Koo *et al.* 2010, Hannig *et al.* 2008, Schilling&Bowen 1988, Vacca-Smith&Bowen 1998). Kuramitsu *et al*. showed that cell wall-associated Gtfs were derived from extracellular Gtfs that became bound to glucan coating the cell wall of *S. mutans* (Kuramitsu&Ingersoll 1978). Germaine *et al.* proposed

that multiple Gtfs binding to cell wall-associated glucan promoted aggregation, while de Stoppelaar *et al.*, using aggregation deficient mutants, showed evidence of a possible protein that could act as a glucan surface receptor (Germaine&Schachtele 1976, de Stoppelaar *et al.* 1971). The idea of a cell surface protein that acted as a glucan receptor grew as McCabe *et al.* also found evidence of a "dextran" receptor in aggregation studies performed in *S. mutans* 6715-49 (now *S. sobrinus*) (McCabe&Hamelik 1978). Using affinity chromatography, several researchers set out to find a possible glucan receptor on the surface. Along with the Gtfs, they discovered non-Gtf glucan-binding proteins (Banas&Vickerman 2003, Kuramitsu&Ingersoll 1978).

The first of these glucan-binding proteins, GbpA, was isolated by Russell in 1979, along with 2 Gtf enzymes, on a dextran affinity column and was resolved at 74 kD on an SDS-PAGE (Russell 1979). Sequence analysis of the GbpA (then known as Gbp) protein revealed that the processed protein was actually 59 kD and contained a glucan-binding domain with repeats that were similar to the Gtfs of *S. mutans* and other oral streptococci (Banas *et al.* 1990). This analysis also showed that the GbpA protein was secreted and released, though Russell *et al.* showed that GbpA is associated with the cell wall through interaction with cell wall-bound glucan. Russell's group also showed a role for GbpA in *S. mutans* adhesion to glass surfaces (Russell *et al.* 1983).

GbpB was isolated by Smith *et al.*, also by affinity chromatography, and was estimated to be 59 kD by SDS-PAGE. This protein was immunologically distinct from GbpA and was shown to be quite antigenic in humans and rodents (Smith *et al.* 1994). Later work by Mattos-Graner *et al*. showed that GbpB's dextran-binding properties may be somewhat weak and showed a possible role for the protein in cell wall synthesis or cell division (Mattos-Graner *et al.* 2006). It should be noted though, that GbpB is an essential gene that is positively regulated by the VicRK system under stress and that the amount of extracellular GbpB production correlated with biofilm growth in a select group of clinical isolates (Mattos-Graner *et al.* 2001).

Dextran-dependent aggregation is a property that *S. mutans* displays under certain stressful conditions, such as sub-inhibitory antibiotic concentrations or the presence of ethanol. GbpC was isolated by Sato *et al.* from a dextran-dependent aggregation (DDAG) deficient mutant strain of *S. mutans*. GbpC was observed to be a cell wall-anchored protein. Among the oral streptococci, proteins capable of promoting dextran-dependent aggregation are also termed glucan-binding lectins (GBL) (Ma *et al.* 1996). Sequence analysis revealed that the GbpC shared some homology with the major streptococcal surface protein P1. P1-like proteins on oral streptococci may possess glucan-binding abilities but confirmatory data does not exist in the literature (Sato *et al.* 1997b).

Recently a fourth Gbp, GbpD, was discovered and isolated based on sequence analysis of the complete, annotated sequence of *S. mutans* UA159 strain. GbpD possesses the amino acid repeats similar to those in the glucan-binding domains of GbpA and the Gtfs (Shah&Russell 2004). Experiments using mutants with an inactivated *gbpD* gene, showed a possible role in aggregation and smooth surface adhesion for GbpD. The GbpD was shown to have lipase activity and binds lipoteichoic acid of *S. sanguinis*. This could have a function in interspecies competition in plaque biofilms (Shah&Russell 2004).

Bacteriocins

The competition among bacteria to establish colonization of a particular niche has been previously described. In biofilm formation, one of the early mechanisms of "biological warfare" utilized by bacteria is the release of anti-microbial peptides called bacteriocins. *S. mutans* produces at least four bacteriocins (called mutacins), three of which (MutI, II and III) are lantibiotics that kill a wide range of Gram-positive bacteria, and one (MutIV) that is a non-lantibiotic that kills closely related streptococcal species such as *S. sanguinis* and *S. gordonii*. (Kreth *et al.* 2006, Hamada&Ooshima 1975)

Several of these mutacin genes are activated by population density through quorum sensing two-component systems. While they may not directly contribute to the virulence or cariogenicity of *S. mutans*, they are an important tool utilized by the bacteria for killing off competing oral streptococcal species when trying to establish colonization of the tooth.

Acidogenicity and Aciduricity

Besides their adhesive and cohesive properties, equally important aspects of *S. mutans* virulence are its abilities to produce and tolerate large quantities of acid. Acidogenicity of *S. mutans* is obtained through the ability of these bacteria to metabolize a wide variety of carbohydrate. Sequence analysis of *S. mutans* UA159 revealed 14 phosphoenolpyruvate-sugar phosphotransferase (PTS) systems, 4 ATP-binding cassette (ABC) transporters, as well as a possible galactoside-pentose hexuronide (GPH) translocator (Ajdic&Pham 2007). PTSs work by transporting and phosphorylating sugars using phosphoenolpyruvate (PEP) and a set of cell wall-bound enzymes. In addition to transport and metabolism of various sugars, some components of PTSs act to regulate other carbohydrate metabolic systems so that the bacteria preferentially select the most rapidly metabolizable sugars (Vadeboncoeur&Pelletier 1997). There is also a multiple sugar metabolism (MSM) system, which most likely uses one of the ABC tranporters that is capable of recognizing and metabolizing several different sugars (Russell *et al.* 1992). Therefore, *S. mutans* is able to rapidly transport, metabolize and possibly regulate metabolism of glucose, fructose, sucrose, lactose, galactose, mannose, cellobiose, β-glucosides, trehalose, maltose/maltodextrin, raffinose, ribulose, melibiose starch, isomaltosaccharides, sorbose mannitol and sorbitol. Through glycolysis, *S. mutans* is able to produce pyruvate from these sugars, and has all necessary enzymes for pyruvate metabolism. Lactic acid is the major by-product of these fermentations, especially when

glucose is abundant but formate, acetate and ethanol are also produced (Ajdic *et al.* 2002).

The acid that *S. mutans* produces is what ultimately leads to dental caries. But the extent to which this species can withstand the low pH it helps create sets it apart from most other plaque species. Acid tolerance in *S. mutans* is due to a robust acid tolerance response (ATR), which includes several genes that are up-regulated in response to low pH. The main contributing factors to this response are the F-ATPase proton pumps that are capable of acting in low pH in *S. mutans.* In fact, *S. mutans* can continue to ferment carbohydrate at pH levels where it has ceased to grow. The continued generation of ATP drives the proton pumps (Quivey *et al.* 2001, Kuhnert *et al.* 2004).

Several oral bacteria utilize an arginine deiminase system (ADS) to produce ammonia from urea and arginine that raises the pH of the surrounding media. *S. mutans* contains genes encoding part of an ADS system but not arginine deiminase. It has been proposed by Ajdic *et al*. that *S. mutans* may use a different enzyme in this pathway (Ajdic *et al.* 2002, Quivey *et al.* 2001).

Other components of the general stress response also contribute to *S. mutans* acid tolerance such as RecA (DNA Recombinase A) and Smn (*S. mutans* Exonuclease) DNA repair enzymes, Ffh (protein secretion and possible assembly), and DagK (fatty acid synthesis), which are all up-regulated under acidic conditions (Quivey *et al.* 2001). The up-regulation of fatty acid synthesis genes agrees with the observation by Fozo *et al.* that *S. mutans* has a greater proportion of long chain mono-unsaturated fatty acids in the cytoplasmic membrane under low pH conditions. The chaperonins DnaK and RopA are up-regulated in *S. mutans* under acidic conditions as is HtrA protease and Clp ATPases (Cotter&Hill 2003, Len *et al.* 2004b). The ATR of *S. mutans* appears to be controlled in part by the ComCDE quorum sensing two-component signaling system. Low pH has been shown to up-regulate a number of other genes, not mentioned here, that have roles

in carbohydrate metabolism, protein folding and fatty acid synthesis (Len *et al.* 2004b, Welin *et al.* 2003).

The properties that make *S. mutans* acidogenic, like the ability to ferment and regulate metabolism of a wide array of carbohydrates, coupled with the aciduric properties, namely the ATR, provide *S. mutans* a selective advantage within dental plaque leading to higher proportions of *S. mutans* and other similarly acidogenic and acid-tolerant species (Kreth *et al.* 2008). They are not only able to out-compete other members of the oral flora, including some oral streptococcal species, for nutrients but the acidic environment is unfavorable to these other species. These properties also lead to demineralization of tooth enamel. At this point the ecology of the plaque is transformed into one that substantially increases the risk of developing dental caries.

S. mutans biofilm architecture

The highly acidogenic and aciduric properties of *S. mutans* must be linked with its ability to adhere to the tooth surface and form a biofilm in order to fully recognize its cariogenicity. The strong association of dietary sucrose with caries highlights the important contribution that Gtf enzymes make to *S. mutans* virulence. There have been many studies linking the loss of sucrose-dependent adhesion or aggregation with a reduction in cariogenicity (de Stoppelaar *et al.* 1971, Van Houte&Upeslacis 1976, Gibbons&Banghart 1967, Willcox *et al.* 1988). Yamashita's *et al. gtf* mutant infections of specific pathogen-free rats suggested that *S. mutans* strains deficient in one or all Gtf enzymes resulted in a marked reduction in cariogenicity (Yamashita *et al.* 1993). However, research in other animal models, such as the gnotobiotic model used by Munro *et al*., showed that while *gtfB* and *gbpC* (GtfI and GtfSI) mutants produced fewer caries, *gtfD* (GtfS) mutants resulted in negligible differences in caries rates (Munro *et al.* 1991). Research by Mattos-Graner *et al.,* using clinical isolates of *S. mutans* from caries-positive and caries-free children, showed a strong correlation between the ability to synthesize

water-insoluble glucan *in vitro* and both colonization on a tooth and caries. (Mattos-Graner *et al.* 2000) Evidence exists which suggests that the two-component system, CovRS, that is responsible for biofilm formation, has been shown to transcriptionally regulate *gtf* genes as well as *gbpC* (Biswas&Biswas 2006, Biswas *et al.* 2007, Idone *et al.* 2003).

Given the role of extracellular glucan in *S. mutans* sucrose-dependent adhesion, biofilm formation and cariogenicity, it is natural to speculate upon the roles of *S. mutans* proteins capable of binding glucan. Since the discovery of Gbps there have been several studies aimed at determining whether they play a role in *S. mutans* cariogenicity. For example, *S. mutans* strain GS-5 was originally isolated from an individual with a high incidence of caries and this strain was once highly cariogenic in rodent models but recently it has been shown to be less cariogenic than other lab strains. Investigators believe that this may coincide with the loss of functional GbpC and P1 proteins stemming from mutations found in the respective genes (Sato *et al.* 2002). Matsumoto *et al.* observed a decrease in adhesiveness to glass in *gbpC* mutants (Matsumoto *et al.* 2006). In an *in vivo* rat model, both *gbpA* mutants and *gbpC* mutants resulted in lower caries scores (Matsumura *et al.* 2003). Work by Douglas and Russell suggested that a *gbpA* mutant has a reduced ability to adhere to glass surfaces but their *gbpA* mutant was chemically generated and may have had more than a single mutation. In experiments done using antibody to GbpA to interfere with its activity, they observed a reduction in the ability of the *S. mutans* to adhere to nichrome wires (Russell *et al.* 1983, Douglas&Russell 1982). Hazlett *et al.* used a *gbpA* knockout strain of *S. mutans* UA130 and showed not only an increase in adherence to hydroxyl-apatite, but an increase in cariogenicity in gnotobiotic rat models. These mutants displayed altered biofilm morphology in which smaller and more numerous microcolonies resulted in a flatter biofilm with an appearance of being more evenly coated (Hazlett *et al.* 1998, Hazlett *et al.* 1999). Shah *et al.* observed an alteration in the morphology of microcolony

aggregates of *gbpD* mutants grown in sucrose, as well as weaker adhesion to nichrome wires.

Experiments involving deletion of genes encoding Gbps have shown that they influence adhesion, aggregation, biofilm architecture and cariogenicity of *S. mutans*, even as multiple studies of *gbpA* mutants have yielded opposing conclusions regarding the nature of that influence. No systematic study using a single, defined model of caries has been carried out to investigate the role of each Gbp. There is a great deal of evidence suggesting a relationship between adhesive and aggregative properties of *S. mutans* and caries. These properties have been shown to alter the architecture of an *in vitro* biofilm in a *gbpA* knockout strain of *S. mutans*. Differences in adhesion and aggregation caused by elimination of Gbps may affect the cariogenicity of *S. mutans*. The goal of this thesis project was to investigate the role of Gbps in *S. mutans* cariogenicity by engineering strains of *S. mutans* with mutations in individual Gbps and combinations of Gbps and examine the effects on biofilm architecture. We propose the following hypothesis:

Hypothesis

The architecture of the biofilm formed by *Streptococcus mutans* depends on glucan-binding proteins and this architecture profoundly affects the cariogenicity of *S. mutans.*

To test this hypothesis, we have proposed three specific aims:

1) Examine the contributions of Gbps to *S. mutans* virulence.
2) Examine the contributions of Gbps to biofilm architecture.
3) Characterize the biofilms formed by Gbp mutants with respect to the properties associated with *S. mutans* virulence.

CHAPTER 2

EXAMINATION OF THE CONTRIBUTIONS OF GBPS TO *S. MUTANS* VIRULENCE

There is extensive research regarding the effect of Gtf enzymes on cariogenicity and individual Gbps have been examined in this regard as well. The development of carious lesions in rodents was crucial in determining that bacteria present in plaque contributed to tooth decay and has been utilized to examine the cariogenic potential of oral streptococci and certain dietary factors for a long time. Animals deemed free of potentially cariogenic strains make it possible to demonstrate the effect that mutations in Gbps have on teeth in animals fed a diet that induces caries in normal animals (Orland *et al.* 1954, Michalek *et al.* 1975a).

For this thesis project a panel of glucan-binding protein mutants was constructed in which each of 3 Gbps (GbpA, GbpC and GbpD) was deleted individually and in combination. Although GbpB may also have an important role in biofilm formation, its glucan-binding capabilities are uncertain. Also, *gbpB* mutations are generally lethal and we are interested in investigating the effects of Gbps on biofilm architecture and cariogenicity without making the fitness of the bacteria another variable. Here we present the first full panel of definitive Gbp mutant *S. mutans* from a cariogenic laboratory strain (UA130) that will be used to infect a specific pathogen-free (SPF) rat model to examine the role of Gbps in cariogenicity.

Materials and Methods

Strains and Culture Conditions

The following strains were used in this study: *E. coli* JM109 (Promega, Madison, WI, USA); *S. mutans* UA130 (provided by Dr. Suzanne Michalek, University of Alabama-Birmingham) was used as the wild-type (WT) and parental strain for generation

of Gbp mutants; and *S. sanguinis* 10556 (provided by Dr. David Drake, University of Iowa) was used in adhesion assays. *E. coli* was cultured in 2xYT broth (Becton, Dickenson and Co., Sparks, MD, USA) at 37°C. *S. mutans* was cultured on Todd Hewitt (TH) (Becton, Dickenson and Co.) plates and in Chemically Defined Media (CDM) (SAFC Biosciences, Inc., Lenexa, KA, USA) and grown at 37°C in an anaerobic chamber (5% CO_2, 10% H_2, 85% N_2). Biofilms were cultured in CDM with 5% sucrose and grown at 37°C in 5% CO_2.

Construction of Glucan-Binding Protein Mutants

A *S. mutans* GbpA mutant UA130/*gbpA::erm* was available from an earlier study (Hazlett *et al.* 1998). Gene sequences for *gbpC* and *gbpD*, based on the *S. mutans* UA159 sequence (primer sequences found in Table 1), were amplified using Eppendorf HotMaster Taq polymerase (Eppendorf AG, Hamburg, Germany) (Ajdic *et al.* 2002). The strategy for engineering the mutations was allelic replacement. For each gene a large portion of the reading frame was deleted and replaced with an antibiotic resistance cassette. To begin this process, PCR products for each gene were cloned into the pGEM^R-T Easy Vector (Promega, Madison, WI, USA) to obtain pGBPC and pGBPD respectively. An internal portion of *gbpC* was removed by first digesting pGBPC with the restriction enzyme *Bsu*361 and then blunting the ends. Since *Bsu*361 cut at a single site, the deletion was completed by digestion with *Bam*H1. The plasmid construct containing the 5′ and 3′ portions of the gene was gel-purified using the GeneClean system (Q-BIOgene, Carlsbad, CA, USA). The spectinomycin (*spec*) gene was amplified from plasmid pDL278 (LeBlanc *et al.* 1992) and ligated into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA, USA). The *spec* gene was digested from the plasmid using *Eco*RV and *Bam*HI and was gel-purified with the GeneClean system. The purified *spec* was inserted into the digested and purified pGBPC using T4 ligase (Promega, Madison, WI, USA) to obtain pGBPCspec. Similarly, the *gbpD* gene was amplified and inserted

into the pGEM^{R}-T Easy Vector, an interior portion was excised using restriction enzymes *Eco*RV and *Mun*I, and replaced with a kanamycin (*kan*) resistance gene cassette from plasmid pDL276 (Dunny *et al.*, 1991) to obtain plasmids pGBPD and pGBPDkan respectively. The *gbpD::kan* sequence was amplified from pGBPDkan and used to transform *S. mutans* WT (UA130), *gbpA::Erm*, *gbpC::Spec*, and *gbpA::Erm/gbpC::Spec*. The resulting strains represented a panel of mutants in which each glucan-binding protein (*gbpA*, *gbpC* and *gbpD*) was knocked out individually and in combination. A map of the mutant constructs can be seen in the top panels of Figure 1A. The plasmids carrying the inactivated *gbp* genes were inactivated in *E. coli* JM109.

Product	**Forward Sequence**	**Reverse Sequence**
spc	ataacgtaacgtgactggcaag	gacgagaaagttatgcaagggttta
gbpC	gataagagaaagcactttgg	ctttttgtcccaacctc
***gbpC* probe**	tggcataaaaatcttgttgt	Use *gbpC* reverse primer
km	gcataggcagcgcgcttatca	ggtcccgagcgcctacgag
gbpD	agtcacacgcatgcataatatagaaaga	tgttattctagacttcgctgaccattta
***gbpA* probe**	tggcagattattgatggta	gagtatgaaatctgctcgtt

Table 1 Oligos used for cloning and screening of Gbp mutant constructs.

Transformation of gene constructs to *S. mutans*

Transformation of *S. mutans* (UA130) with mutant constructs was based on the protocol of Li *et al*. (Li *et al.* 2001). Mutant constructs were isolated by PCR amplification of each gene from plasmid templates using the appropriate primers (Table 1), and DNA of the expected sizes was gel-purified (Qiagen gel purification system). *S. mutans* (UA130) was grown in TH media anaerobically overnight at 37°C. Overnight cultures were then sub-cultured to an OD_{600} of 0.3 and grown for about 3 hours to an OD_{600} of 0.6 (mid-log phase). Synthetic competence stimulating peptide (CSP) was

added at a concentration of 1 μg/ml and incubated for 30 minutes before addition of DNA. Next, at least 2 μg of the isolated mutant DNA was added and incubated for 2 hours, anaerobically at 37°C. The bacteria were pelleted by centrifugation and resuspended in 100 μl of TH buffer and plated on TH plates with the proper antibiotic selection (Li *et al.* 2001). Plates were incubated anaerobically for 48 hours at 37°C. Colonies were screened by PCR amplification to compare the size of the prospective mutant gene with that of the wild-type and confirm the presence of the antibiotic resistance cassettes.

Screening of Mutant Panel

After selecting candidate clones based upon PCR screening, we used Southern hybridization to verify the presence of correctly engineered mutants. Genomic DNA was isolated from *S. mutans* WT and from each mutant using the Gram Positive Genomic DNA Extraction Kit (Epicenter, Madison, WI, USA). Genomic DNA was digested with *Hind*III (for *gbpA* mutations), *Bst*XI (for *gbpC* mutations) or *Mun*I (for *gbpD* mutations). Each digest of the WT and mutant panel was separated on a 0.5X TBE agarose gel and transferred to a nylon membrane (Roche, Indianapolis, IA, USA) by capillary Southern transfer. Probes, all based on the UA159 sequence, were generated using the Roche Dig DNA Labeling Kit (Roche). The 3′ probe for the *gbpA* gene was generated from the PCR product of a portion of a *Hind*III gene fragment that measured 3.5 kb in the WT gene and 3.1 kb in the mutant (Figure 2A (Top Panel)). A PCR product from the 3′ portion of the *gbpC* gene was used to generate a probe that bound to a *Bst*XI gene fragment that measured 3.1 kb in the WT and 1.3 kb in the mutant *gbpC* gene due to a *Bst*XI site within the *spec* gene (Figure 2B (Top)). A 3′ probe for the *gbpD* gene was isolated by restriction digestion from pGBPD using restriction enzymes *Mun*I and *Eco*RI and was gel-purified using the GeneClean Kit. This fragment was used to generate a probe that bound a *Mun*I digested fragment that measured 2.1 kb in the WT gene and 5.9 kb in the

mutant due to the loss of the *Mun*I site from the excised portion of the *gbpD* gene (Figure 2C (Top)). Dig-labeled probe concentrations were measured by dot blot analyses where serial dilutions of labeled probe were blotted and compared to a known standard contained in the Dig DNA Labeling Kit (Roche). Probes were also generated to a λ-DNA/*Sty*I digest that was used as a molecular weight marker.

Membranes were pre-hybridized in Dig Easy Hyb buffer (Roche) for 30 minutes and hybridized in 7 ml (10ng/ml) of the respective probe at 48°C for 16 hours. Hybridized membranes were washed in 2X SSC/0.1% SDS for 5 minutes twice at room temperature and then in 0.5X SSC/0.1% SDS twice for 15 minutes at 65°C. Membranes were rinsed in maleic acid buffer pH 7.5 and blocked for 30 minutes with the blocking reagent supplied with the Roche Dig DNA Labeling Kit (Roche). After washing in maleic acid/0.03% Tween 20 (Fisher, Pittsburgh, PA, USA), the membranes were incubated with alkaline phosphatase (AP) labeled anti-Dig antibody (Roche) (75 mU/ml) for 1 hour. Membranes were washed twice and developed with CSPD Chemiluminescence Substrate (Roche) for 5 minutes and exposed to X-ray film.

Western Blot Verificantion of Mutants

Western blot analysis was performed by Dr. Jeffrey Banas. Overnight cultures were grown in Todd Hewitt broth at 37°C in 5% CO_2. Cultures were centrifuged at 8,000xg and the supernatant was discarded. Cell pellets were resuspended in 150 µl of 2X cracking buffer (0.125M Tris-Cl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) for 2 hours at room temperature, to strip associated proteins from the cells. The suspensions were pelleted at 10,000xg and the protein containing supernatant was resolved via SDS-PAGE. Proteins were transferred to nitrocellulose membrane for Western-blot analysis.

Rabbit-polyclonal antibody to GbpA was commercially generated using the glucan-binding domain portion of the protein, which was isolated and cloned by Haas *et*

al. (Haas&Banas 2000). Rabbit-polyclonal antibody to GbpD was provided by Dr. Roy Russell (University of Newcastle, UK).

Planktonic Growth Rates

Planktonic growth rates were measured for mutants grown in CDM without sucrose by measuring the OD_{540} of each mutant over time after overnight cultures were normalized to an OD_{540} of 0.04. Data points were fit by a least squares method using the nonlinear regression module of Statistica software. The growth rate was determined by using the logistic function OD_{540}=mn+(mx-mn)(1/(1+exp(4S(ht-TIME)/(mx-mn)) where mx is the final or maximum OD, mn is the initial or minimum OD, ht is the time to half max OD and S is the maximal rate of change with respect to time (Feustel 2006).

In vivo Caries Study

In vivo experiments were performed in specific pathogen-free (SPF) rats. Eight groups of five rats each were isolated and each group was infected by the wild-type *S. mutans* UA130 or one of the Gbp mutants. The Fisher 344 rats that were used in this study were derived from a line of germ-free rats in which a few microbial species had been detected by rigorous microbiological analysis. However, no oral streptococcal species were detected in these rats (Michalek 2010).

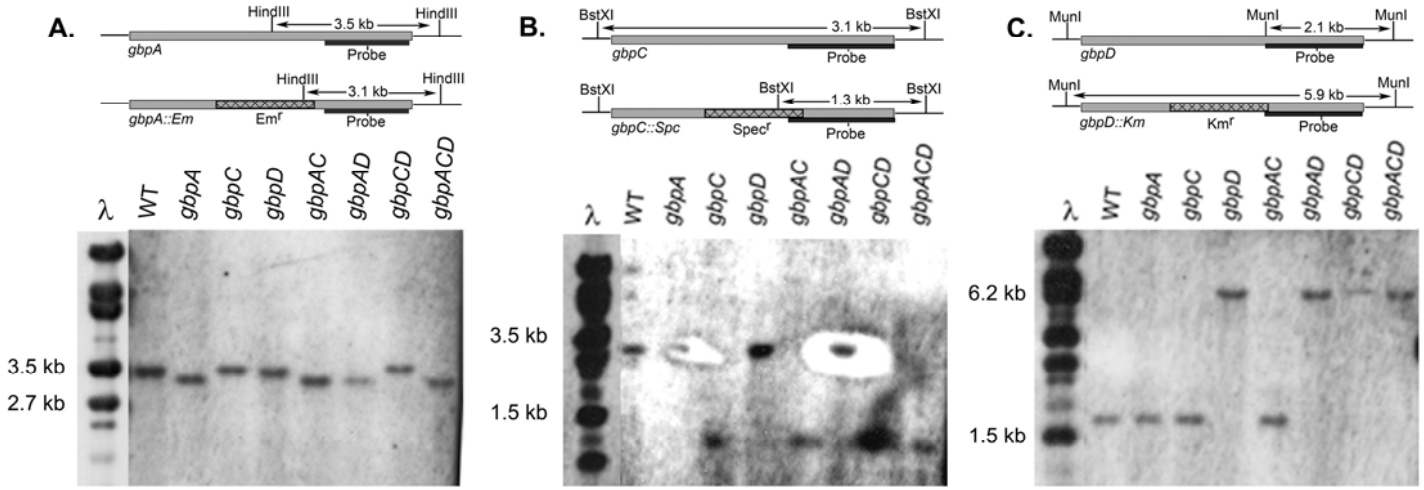


Figure 2 **Design and confirmation of the Gbp mutant panel.** Gene maps (Top) and Southern blots (Bottom) of genomic DNA from the mutant panel digested with (A) *HindIII* and probed with *gbpA*, (B) *BstXI* and probed with *gbpC*, and (C) *MunI* and probed with *gbpD*. The gene maps illustrate the region of DNA replaced by antibiotic resistance cassettes and the differences in the respective restriction fragment sizes caused by the mutations.

Cariogenicity experiments were carried out by Dr. Sue Michalek at the University of Alabama-Birmingham using previously described protocols (Michalek *et al.* 1975a, Barletta *et al.* 1988). At day 17 rats were removed from an isolator and set up in groups of 5 rats per cage (with filter tops). Prior to infection (from day 17 to day 21) rats were provided antibiotic water (sulfamethoxaxole-trimethoprim at 1 ml/47.3 ml H_2O) and food (MIT 305 from Harlan Teklad, Indianapolis, IN) soaked in antibiotic to reduce the oral commensal flora. On days 22-25 rats were infected with *S. mutans* UA130 (WT or one of the Gbp mutants) by oral swabbing a fresh overnight culture of the appropriate strain. Oral swabs were taken and plated on TH plates with appropriate antibiotic concentration and incubated anaerobically at 37°C to confirm the viability of the inocula. Rat food (MIT 305) and water contained 5% sucrose and were provided *ad libitum.* On day 91, rats were sacrificed and the mandibles were removed and cleaned of excess tissue. The right mandible of each rat was placed in a tube containing 3 ml of phosphate buffer which was placed on ice and sonicated to release bacteria from the teeth. Aliquots were serially diluted (10^{-3}, 10^{-4}, 10^{-5}), plated on TH and mitis salivarius/sucrose (MS) plates and incubated anaerobically at 37°C overnight. Right and left mandibles were then placed in 95% ethanol for 24 hours. The mandibles were then cleaned and stained overnight with murexide solution. After drying the mandibles were sent for pathological analysis.

The Keyes method for scoring carious lesions was used to analyze the molars obtained from our specific pathogen-free rats and generate cariogenicity data for the mutant panel (Keyes 1958). Briefly, in this method, caries are scored based on the depth and breadth of the lesion. Each molar surface is broken down into units corresponding to the cusps of the tooth (4 or 6 units). In each section a lesion or lesions are evaluated and graded based on enamel involvement only (E), slight dentinal involvement (D_s; up to 25% of dentin involved), moderate dentinal involvement (D_m; 26 to 75% of dentin involved), or excessive dentinal involvement (D_x; over 75% of dentin involved). Scores

are recorded for the buccal, sulcal and proximal surfaces individually so that differences among the surfaces can be distinguished.

Statistical Analysis

The formula used to determine the growth rate of the WT and *gbp* mutant *S. mutans* was described previously in the "Planktonic Growth Rates" section. Optical density (OD) data as a function of time was fit to a logistic function OD=mn+(mx-mn)(1/(1+exp(4S(ht-TIME)/(mx-mn)) where mx is the final or maximum OD, mn is the initial or minimum OD, th is the time to half-maximal OD and the time to the maximal change in OD with respect to time, and S is the maximal rate of change. For each group, all data was fit by a least squares method using the nonlinear regression module of Statistica software. The S value was shown in the chart in figure 5 (Feustel, 2006).

SPF rats were divided into 7 groups of 5 rats (and 1 group of 7 rats) that would be infected with the WT or one of the *gbp* mutant strains of *S. mutans.* Differences in both raw and weight-adjusted caries scores among the rats in each group were averaged at each level of involvement and were analyzed by analysis of variance (ANOVA). Also, the weights of the rats within each group were averaged and compared via ANOVA. The Tukey post-hoc test was used to compare statistically significant differences between groups.

Results and Discussion

Confirmation of Mutant Panel

The Southern hybridization analyses of genomic DNA from the mutant panel of *S. mutans* UA130 showed that strains representing each Gbp mutation, individually and in combination, were present. Figure 2A (Bottom Panel) shows the altered size of a *HindIII* restriction fragment of *gbpA* mutants. Figure 2B and 2C (Bottom panels) show the altered sizes of *BstXI* and *MunI* restriction fragments in *gbpC* and *gbpD* mutants

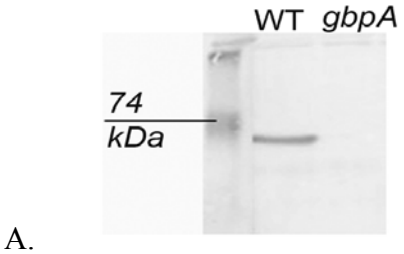
respectively. Figure 3 shows Western blot analysis of GbpA (Figure 3A) and GbpD (Figure 3B) among the mutant panel using antibody to these proteins. GbpA antibody bound to a protein at the predicted size for GbpA. There is no binding of the GbpA antibody to any proteins from the *gbpA* mutant strain (Figure 3A). When WT and *gbpD* mutant protein isolates were incubated with antibody to GbpD, there was no binding to proteins of any *gbpD* mutants. The GbpD antibody did, however, bind to the proper sized fragment in the WT lane and in the lanes from mutants without a *gbpD* mutation (Figure 3B). At the time of this analysis, there was no effective antibody to GbpC so the *gbpC* mutation was confirmed by a dextran-dependent aggregation assay following growth in 4% ethanol. Figure 4 shows that all mutant strains with a deleted *gbpC* gene lost the ability to form aggregates in the presence of dextran.

Growth curves

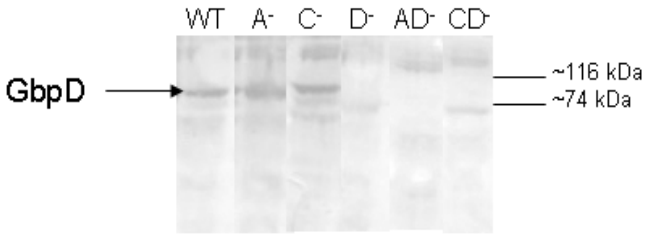
To show that the mutations introduced into *S. mutans* did not interfere with normal growth or metabolism and that the differences in biofilm architecture or cariogenicity were not due to growth abnormalities, we examined the planktonic growth rates for WT and mutant strains. There were no significant differences among the WT or mutants with respect to planktonic growth rates (Figure 5).

While no statistically significant differences in growth rates were determined for any of the mutants, anecdotal lab observations have suggested that the multiple mutant strains often seem to grow at a quicker rate than the wild-type or the single mutants. However these growth patterns are very inconsistent and not entirely reliable. Also, the *gbpC* mutant strain exhibits the most erratic growth patterns often growing slower than other strains. This is also inconsistent and often occurred in the presence of the spectinomycin selection, so there is a possibility that growth rate variability seen in the *gbpC* strain is due to the spectinomycin and not growth defects.

The biofilm cultures that will be described later were inoculated with a high concentration of overnight planktonic cells. Since mature biofilms are considered to be in stationary phase of growth it was assumed that minor differences in planktonic growth rate did not affect biofilm formation. We observed no significant reduction in the number of CFU recovered from the SPF rats and concluded that planktonic growth rates could not explain any possible differences in cariogenicity or biofilm architecture.



A.



B.

Figure 3 Identification of glucan-binding proteins in WT and Gbp mutants by Western blot. (A) Western blot of WT and *gbpA* culture supernatants with antibody to the GbpA glucan-binding domain identifies an approximate 74 kD band present in the WT culture but absent from the *gbpA* strain. Only the original *gbpA* mutant strain is shown because it was generated previously and all combination mutants with a *gbpA* deletion were generated on the original *gbpA* background. (B) Western blot of WT and selected *gbpD* mutants (*gbpACD* mutant is not shown but was generated on *gbpAD* background) using antisera to GbpD (courtesy of Dr. Roy R. B. Russell). Lanes were individually scanned and reassembled into a different order than appeared on the actual blot. The faint bands in the GbpD knockout strains likely represent cross reaction with Gtfs (top) and GbpA (near 74 kD).

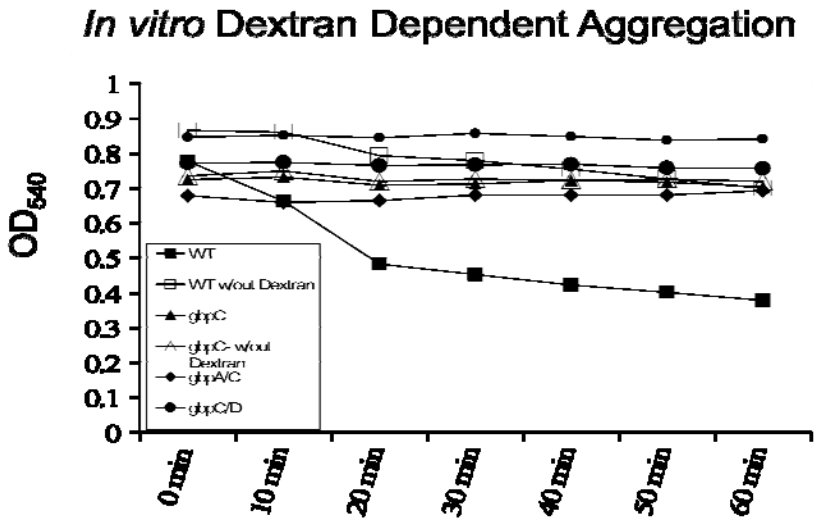


Figure 4 Dextran-dependent aggregation (DDAG) is attributed to the presence of GbpC. *S. mutans* WT or mutant cultures were grown for 2 days in CDM (without sucrose) with 4.0% ethanol. Growth in ethanol is one of the conditions found to be necessary to induce DDAG in *S. mutans*. High molecular weight dextran (T-2000) was added to a final concentration of 100 µg/ml. The cultures were briefly vortexed, placed in cuvettes and the OD_{540} was monitored over time. A drop in OD represents bacteria that form large aggregates and fall to the bottom of the cuvette.

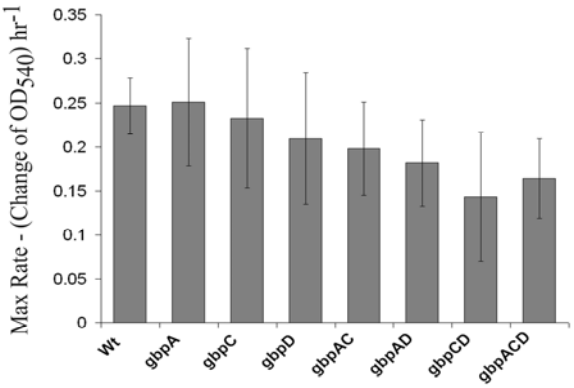


Figure 5 Planktonic growth rates of mutant panel strains. Values represent the slope of a curve fit to plotted points of 3 independent growth trials at the time when the OD_{540} is at ½ max OD (S value of plot equation). This represents the maximum change in OD for each strain which is assumed the max growth rate. Error bars represent the standard error.

Cariogenicity

The *in vivo* data revealed no significant reduction in CFU recovered from the specific pathogen-free (SPF) rats, (Table 2), which would suggest that there were no deficiencies in the abilities of any of the mutants to colonize teeth and establish biofilms in the presence of an existing flora. In fact, the *gbpAD* strain showed a statistically significant increase in colonization but did not exhibit a significant increase in caries. It is unlikely that any differences in caries are due to changes in colonization potential. When analyzed by regression analysis, there was no correlation between the colonization levels of the strains and caries (data not shown).

Examination of the caries scores revealed that the *gbpACD* strain showed significantly reduced caries in both total number and severity (Table 2) across all surfaces of the tooth compared to the WT (Table 3). The *gbpAC* strain showed significant reductions in the number and severity of caries but this was limited to buccal (smooth) surfaces of the tooth (Table 3). The *gbpD* mutant also showed significant attenuation (Table 2), though this was limited to sulcal (fissure) surfaces (Table 3). The *gbpA* and *gbpC* single mutants showed slight, though not significant, increases in enamel (E) caries. There were slight variations in the level of involvement on different surfaces. The *gbpA* strain did not show this increase in excessive dentinal (D_x) caries and the *gbpC* only showed a slight increase in D_x caries on buccal surfaces.

Cariogenicity on all Surfaces								
	WT	***gbpA***	***gbpC***	***gbpD***	***gbpAC***	***gbpAD***	***gbpCD***	***gbpACD***
Total Caries (E)	51.4 ±1.21	53.0 ±2.51	58.2 ±1.77	44.2[b] ±4.12	**40.8[a,b] ±2.04**	55.6[c] ±2.42	46.4[b] ±0.87	**33.9[a,b]±1.77**
Dentinal Caries (D_x)	22.5 ±3.12	19.0 ±2.43	24.8 ±2.84	**8.2[b] ±2.91**	**8.6[b] ±1.60**	25.4[c] ±5.13	18.4 ±0.75	**4.0[a,b]±0.98**
Log CFU Recovered	4.06 ±0.28	4.67 ±0.28	3.89 ±0.11	4.54 ±0.09	4.91 ±0.05	5.23 ±0.23	3.21 ±0.35	3.65±0.13

Table 2 Cariogenicity of the WT and Gbp mutants.

Note: The total scores of all enamel (E) lesions (top row) and excessive dentinal (D_x) lesions (middle row) across all tooth surfaces are shown. The bottom row shows the log value of the recovered *S. mutans* colonies that were counted on mitis salivarius/sucrose plates. Values represent the average caries scores of 5 animals per group (7 in *gbpACD*) ± standard deviation. Mutant values that are significantly different than the wild-type are shown in bold type. Superscript "a" denotes mutant scores that are significantly lower than those for the *gbpA* strain. Superscript "b" denotes mutant caries scores that are significantly lower than those for the *gbpC* strain. Superscript "c" denotes mutant caries scores that are significantly greater than the *gbpD* strain.

Surface Localization of Caries								
	WT	***gbpA***	***gbpC***	***gbpD***	***gbpAC***	***gbpAD***	***gbpCD***	***gbpACD***
Buccal Enamel (E)	20.6 ±0.93	21.0 ±1.73	24.4 ±1.47	16.0[b] ±2.19	**13.2[a,b] ±1.39**	21.4 ±2.68	17.4 ±0.60	**11.9[a,b] ±0.60**
Buccal Dentinal (D_x)	13.0 ±1.00	12.8 ±1.77	17.0 ±0.89	7.4[b] ±2.54	**3.6[a,b] ±0.81**	14.6 ±3.33	9.6 ±0.68	**1.71[a,b] ±0.52**
Sulcal Enamel (E)	23.0 ±0.84	24.0 ±0.89	25.8 ±0.37	20.2[b] ±1.93	19.6[a,b] ±0.75	26.2[c] ±0.97	21.0[b] ±0.32	**18.4[a,b] ±0.57**
Sulcal Dentinal (D_x)	9.8 ±1.53	6.0 ±1.76	7.8 ±2.08	**0.8[b] ±0.37**	5.0 ±0.95	10.8[c] ±1.83	8.8[c] ±0.37	**2.3 ±0.68**
Proximal Enamel (E)	7.8 ±0.20	8.0 ±0.00	8.0 ±0.00	8.0 ±0.00	8.0 ±0.00	8.0 ±0.00	8.0 ±0.00	**3.57[d] ±0.87**
Proximal Dentinal (D_x)	0.0 ±0.00	0.2 ±0.20	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00

Table 3 Caries scores of WT and Gbp mutants for distinct tooth surfaces with enamel (E) and excessive dentinal (D_x) involvement.

Note: The buccal, sulcal and proximal surfaces are shown. Values represent the average caries scores of 5 animals per group (7 in *gbpACD*) ± standard deviation. Mutant values that are significantly different than the WT are shown in bold type. Superscript "a" denotes mutant scores that are significantly lower than those for the *gbpA* strain. Superscript "b" denotes mutant caries scores that are significantly lower than those for the *gbpC* strain. Superscript "c" denotes mutant caries scores that are significantly greater than the *gbpD* strain. Superscript "d" denotes mutant caries score that is significantly lower than scores from all other strains.

While neither the *gbpA* nor *gbpC* mutants differed statistically in cariogenicity compared to the WT, loss of GbpC combined with the loss of GbpA, or loss of all three Gbps resulted in attenuation on buccal surfaces (*gbpAC*) and all surfaces (*gbpACD*) relative to the WT (Table 3). The mutants missing both GbpA and GbpC were also attenuated compared to the *gbpA* and *gbpC* individual mutants on buccal and sulcal surfaces with the exception of sulcal D_x caries. The *gbpD* mutant strain had significantly fewer caries than the *gbpC* mutant on both buccal and sulcal surfaces, but combining the loss of GbpC with the loss of GbpD did not result in significant attenuation from the WT on any surfaces (Table 3). One of the more unexpected results was that the *gbpD* mutant was significantly attenuated compared to the *gbpAD* mutant for total caries.

Deeper investigation into the *in vivo* results revealed that there were significant differences in the average weights of the rat groups inoculated with WT or mutant *S. mutans*. There was a 35% difference between the heaviest group and the lightest group (Figure 6). The average weight of each of the multiple mutants was over 25% more than the average weight of the WT strain, though only the weight variation of the *gbpAD* strain was statistically significant. It is reasonable to assume that the weight of a rat would be directly related to food intake and that this would affect sucrose availability for the *S. mutans* biofilms in the SPF rat model. Caries scores were recalculated after adjusting for the weight of the rats (Tables 4 and 5). This adjustment involved dividing either the total caries score or the caries score on each surface by the weight, in grams, of each particular rat. This allowed for the determination of the average caries score per gram of rat body weight.

Comparison of the enamel (E) caries scores on all surfaces revealed that each of the mutants missing more than one Gbp was significantly attenuated relative to the WT (Table 4). Previously, when using non weight-adjusted data, the *gbpAD* and *gbpCD* strains were not attenuated. Weight adjustment also resulted in the *gbpAC* strain joining the *gbpD* and *gbpACD* strains in being attenuated for sulcal caries relative to the WT

(Table 5). In addition, weight-adjusting the scores altered some of the relationships between different mutants. Notably, the *gbpCD* mutant was attenuated with respect to the *gbpC* strain for buccal caries, but the *gbpD* mutant was no longer attenuated compared to the *gbpAD* mutant (Table 5).

It is peculiar that on sulcal surfaces the *gbpD* strain had a lower weight-adjusted D_x caries score than the *gbpAD* and *gbpCD* strains. It is unclear why the *gbpD* strain behaves differently than strains where *gbpD* is mutated in combination with either *gbpA* or *gbpC*. It is possible the phenotype that results from mutation of *gbpD* affects *S. mutans* accumulation and biofilm formation on sulcal surfaces more than on buccal surfaces. An alternative explanation is an overall slower progression of caries into deeper layers of dentin on sulcal surfaces that reveals attenuation of the progression of caries for the *gbpD* mutant on all surfaces.

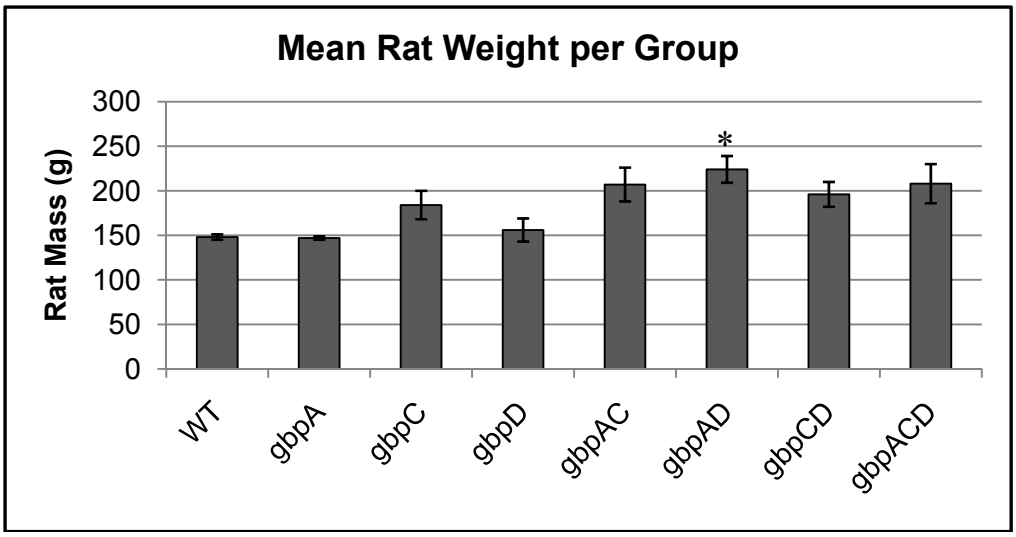


Figure 6 Comparison of mean rat weights among mutant groups. Each strain of *S. mutans* was used to infect a group of specific pathogen-free rats. Each group contained five rats except for the *gbpACD* group, which contained seven rats. The rats were weighed at the time of sacrifice and the mean weight of the groups is represented in the chart. Each value is the average of 5 rats (7 rats in the *gbpACD* group). An asterisk "*" denotes values that are significantly different than the wild-type based on a Tukey post-hoc test.

Whether weight-adjusted or unadjusted, there were more E caries on sulcal surfaces than on buccal surfaces for all strains. However, the progression of caries in all strains was slower on sulcal surfaces than on buccal surfaces. This is based on the percentage of total caries that were D_x at the time of sacrifice (Figure 7). The mean percentage of buccal caries that were D_x was around 50% for all strains while the mean for sulcal surfaces was about 27%. The low number of scoring zones on proximal surfaces do not allow for comparison to the other surfaces in this way. The fact that the *gbpD* mutant has such a low percentage of caries at the D_x stage on sulcal surfaces (~4%) compared to buccal surfaces (~46%), suggests that the impact of a *gbpD* mutation is greater on sulcal surfaces. The *gbpD* mutants had significantly fewer D_x caries on sulcal surfaces compared to the wild-type, but this was not true for buccal surfaces. However, this impact was not reflected in mutants missing GbpD and either GbpA or GbpC. The progression of caries in the *gbpACD* strain was similar for both buccal and sulcal surfaces.

While it appears that the *gbpD* strain is more attenuated on sulcal surfaces, a slower progression of caries on sulcal surfaces might explain the significantly lower level of D_x lesions in the *gbpD* strain. Overall, the difference in the percentage of caries scored as D_x between buccal and sulcal surfaces for all single mutants, including the *gbpD* strain, was about 40%. However, the *gbpAC* and *gbpACD* strains showed no differences in the percentages of caries that scored D_x between buccal and sulcal surfaces, whereas *gbpAD* and *gbpCD* strains showed a 20% difference (Figure 7). Therefore, it is possible that the impact of the loss of GbpD is manifested as a slower progression of caries on the sulcal surfaces.

On buccal surfaces all multiple mutants (when weight-adjusted) were significantly attenuated, while on sulcal surfaces only the *gbpAC* and *gbpACD* strains were significantly attenuated for E lesions. Thus, when considering caries incidence

rather than severity, the loss of GbpD combined with the loss of GbpA or GbpC does not impact sulcal surfaces as much as buccal surfaces.

Other Gbp mutations also had a greater impact on the buccal surface, while the proximal surface was impacted the least. This may be due to the rougher surface in the sulcal environment that offers protection from hydrodynamic and sheering forces. While proximal surfaces provide even more protection from these forces, the lack of differences in proximal caries among mutants on the proximal surface could also be a consequence of the low number of scoring zones compared with buccal and sulcal surfaces.

Interestingly, we found no significant differences in the caries scores between the WT and *gbpA* strain. Hazlett *et al.,* using the same bacterial strain (UA130 *gbpA::erm*), observed a significant increase in *gbpA* caries compared to the WT in a germ-free rat model (Hazlett *et al.* 1998). In that experiment, the rats were sacrificed after a shorter time period (30 days, as opposed to 70 days in the SPF rat experiment). It is possible that since we saw a slight increase in overall caries scores in the *gbpA* strain, the longer timeframe of our experiment may have allowed the WT caries incidence to "catch up" to that measured for *gbpA*-infected rats. However an alternative explanation is that the presence of an established plaque flora mitigated the increased cariogenic effect of a *gbpA* biofilm.

Total Weight Adjusted Caries								
	WT	***gbpA***	***gbpC***	***gbpD***	***gbpAC***	***gbpAD***	***gbpCD***	***gbpACD***
Enamel Caries (E)	0.35 ±0.03	0.36 ±0.05	0.32 ±0.05	0.29 ±0.07	**0.20^{a,b} ±0.02**	**0.25^{a} ±0.03**	**0.24^{a} ±0.03**	**0.17^{a,b,c}±0.05**
Dentinal Caries (D_x)	0.15 ±0.04	0.13 ±0.04	0.13 ±0.01	**0.05^{a,b} ±0.04**	**0.04^{a,b} ±0.01**	0.11 ±0.04	0.10 ±0.01	**0.02^{a,b}±0.02**

Table 4 Rat weight-adjusted caries scores.

Note: The mean caries scores in each group were divided by the mean weight of the rats in each group to compare the caries while taking into account the weight differences between the groups of rats. The total enamel (E) lesions (top row) and excessive dentinal (D_x) lesions (bottom row) scored on all surfaces are represented. Values represent the average caries scores of 5 animals per group (7 in *gbpACD*) ± standard deviation. Mutant values that are significantly different than the wild-type are shown in bold type. Mutant values denoted by a superscript "a" are significantly less that the *gbpA* mutant strain. Mutant values denoted with a superscript "b" are significantly less than the *gbpC* mutant strain. Mutant values denoted with a superscript "c" are significantly less than the *gbpD* mutant strain.

Weight Adjusted Caries on Different Surfaces								
	WT	***gbpA***	***gbpC***	***gbpD***	***gbpAC***	***gbpAD***	***gbpCD***	***gbpACD***
Buccal Enamel (E)	0.14 ±0.02	0.14 ±0.03	0.13 ±0.02	0.10 ±0.03	**0.06**^{a,b,c} ±0.01	**0.09**^{a,b} ±0.02	**0.09**^{a,b} ±0.01	**0.06**^{a,b,c} ±0.02
Buccal Dentinal (D_x)	0.09 ±0.02	0.09 ±0.03	0.09 ±0.01	0.05^{b} ±0.03	**0.02**^{a,b} ±0.01	0.06 ±0.03	0.05^{b} ±0.01	**0.01**^{a,b,c} ±0.01
Sulcal Enamel (E)	0.16 ±0.01	0.16 ±0.02	0.14 ±0.03	0.13 ±0.03	**0.10**^{a,b} ±0.01	0.12 ±0.03	0.11^{a} ±0.01	**0.09**^{a,b} ±0.03
Sulcal Dentinal (D_x)	0.07 ±0.02	0.04 ±0.01	0.04 ±0.02	**0.005**^{a} ±0.00	**0.02** ±0.01	0.05 ±0.01	0.05 ±0.01	**0.01** ±0.01
Proximal Enamel (E)	0.05 ±0.005	0.05 ±0.002	0.04 ±0.009	0.05 ±0.01	0.04 ±0.008	0.04 ±0.006	0.04 ±0.006	**0.02*** ±0.015
Proximal Dentinal (D_x)	0.0 ±0.00	0.001 ±0.003	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00

Table 5 Rat weight-adjusted caries scores for distinct tooth surfaces.

Note: The mean caries scores in each group were divided by the mean weight of the rats in each group to compare caries while adjusting for the weight differences between the groups of rats. The total enamel (E) lesion and excessive dentinal (D_x) lesion scores for the buccal, sulcal and proximal surfaces are represented. Values represent the average caries scores of 5 animals per group (7 in *gbpACD*) ± standard deviation. Mutant values that are significantly different than the wild-type are shown in boldface type. Mutant values denoted by a superscript "a" are significantly less that the *gbpA* mutant strain. Mutant values denoted with a superscript "b" are significantly less than the *gbpC* mutant strain. Mutant values denoted with a superscript "c" are significantly less than the *gbpD* mutant strain. Mutant values denoted with an "*" are significantly less than the scores from all other mutant strains.

Studies by both Nakano *et al.* and Matsumura *et al.* led to some conflicting results regarding the cariogenicity of a *gbpC* mutant despite the fact that the work of both researchers was done in the same lab. Matsumura *et al.* created individual mutants for *gbpA* and *gbpC* on the MT8148 parent *S. mutans* strain. Caries scores in SPF rats showed a significant reduction in caries in both mutant strains compared to the parental strain but no differences in smooth surface (buccal) caries among any of the strains. Based on earlier published protocols from this group, we assume that their rats were infected for 50 days with the *S. mutans* strains before sacrifice making it difficult to directly compare our results. Interestingly, they recovered significantly fewer CFU of the *gbpC* mutant than the other two strains at 10 days, but observed no differences in recovery at later time points. This suggests that Gbps may affect the rate of caries progression, in addition to incidence, and therefore the duration of a cariogenicity study can affect the nature of the outcome (Matsumura *et al.* 2003). It is also possible that the genotype of the parental strain significantly influences the effect of knocking out Gbps. Nakano *et al.* used two blood isolates that had either a mutation in the *gbpC* gene or mutations in both *gbpA* and *gbpC* to examine cariogenicity in a SPF rat model using lab strain MT8148 as a WT control. They observed no differences in cariogenicity of their *gbpC* strain when compared to their WT but the strain lacking both *gbpA* and *gbpC* was significantly attenuated (Nakano *et al.* 2002). Our results are in agreement with those of Nakano *et al*. in that a *gbpC* mutation alone in the UA130 background was not attenuated, but a *gbpC* mutation combined with a *gbpA* mutation resulted in a reduction in caries. It should be noted, though, that the mutant strains used in Nakano's work were human blood isolates that may have possessed other mutations. Both groups observed the expected loss of DDAG in their respective *gbpC* mutants.

In the results presented in this thesis, the weight of the rats had a modest effect on how the caries scores related to each other. Weight adjustment of caries data affected whether or not the *gbpAD* and *gbpCD* could be considered attenuated for total caries and

buccal caries, and whether or not the *gbpAC* strain was attenuated for sulcal caries. Regardless of the weight of the rat population, loss of Gbps A and C resulted in attenuation of the *gbpAC* and *gbpACD* strains for total caries and buccal caries, and loss of GbpD resulted in attenuation of dentinal sulcal caries. The loss of Gbps A and C likely affect some aspect of the plaque biofilm that renders the organism less cariogenic. When the *gbpD* mutation is coupled to the loss of another Gbp, I propose that caries development, at least in the SPF rat model, was linked to food consumption. It is possible that rats from groups with greater weight-adjusted caries scores may have developed caries sooner than mutants with fewer weight-adjusted caries scores. If a particular rat rapidly reached a level of carious lesions that resulted in significant discomfort, perhaps to the point of eating less, then rats infected with attenuated *S. mutans* strains could catch up to those infected with more cariogenic strains. Since none of the mutant strains were completely attenuated, a broader cariogenicity experiment where groups of rats could be sacrificed over several time points could provide clues to the rate of caries development among these mutants. Examination of cariogenicity over time might also provide additional clues to the importance one Gbp over another.

Summary

In this chapter, we conclude that Gbps contribute to the ability of *S. mutans* to promote caries in the presence of sucrose. In the SPF rat model, attenuation was generally dependent on the loss of at least two Gbps, though for particular Gbp combinations attenuation was dependent on adjustment for the weight of the rats. Of the individual Gbp mutants, only the *gbpD* strain was attenuated relative to the parental. We propose that the combined loss of Gbps A and C has the most dominant effect on *S. mutans* cariogenicity, though the additive loss of GbpD extends the magnitude and breadth of the attenuation. While the loss of GbpD alone most affects caries development of sulcal surfaces, double Gbp mutations have the greatest impact on

smooth (buccal) surfaces. Clearly, each Gbp makes a unique contribution to the caries process but the mechanistic contributions of each cannot be explained by the *in vivo* results alone. Therefore, *in vitro* biofilm properties of WT and Gbp mutant *S. mutans* were next examined to investigate the roles of glucan-binding proteins in mature biofilm formation.

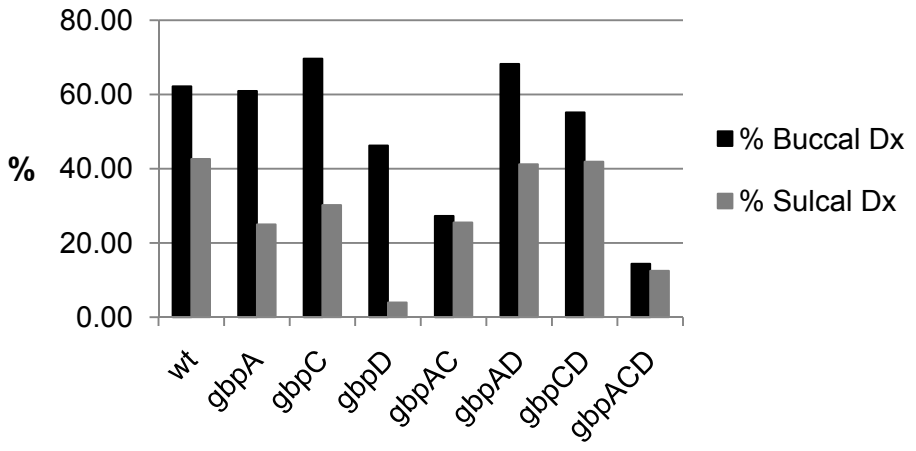


Figure 7 Percentage of total caries that were scored D_x for both buccal and sulcal surfaces in the WT and each Gbp mutant.

CHAPTER 3
EXAMINATION OF THE GBP MUTANT STRAINS TO DETERMINE IF THERE IS AN ALTERATION IN THE BIOFILM ARCHITECTURE AND ABILITY TO FORM A BIOFILM

The ability to form an adherent and cohesive biofilm is a necessary component to the cariogenicity of *S. mutans*. Sheering forces play a role in shaping the architecture of a biofilm. The responses of the Gbp mutant strains to these forces in an *in vitro* biofilm may provide clues to the cohesive properties of a biofilm and highlight contributions of Gbps. The complex architecture of a bacterial biofilm affects processes like nutrient availability and acid diffusion which both have a role in the cariogenicity of *S. mutans*.

It has previously been established that mutation of the *gbpA* gene affected the properties of biofilms formed by *S. mutans in vitro* (Hazlett *et al.* 1999). The complete panel of Gbp mutants will allow comparison of biofilm architecture among these mutants in the same strain background. Confocal microscopy will be used to examine architectural characteristics of WT and mutant *in vitro* biofilms. The development of algorithm-based software that facilitates analysis of confocal image stacks and displays data about a range of architectural parameters has allowed for detailed analysis and comparison of biofilms *in vitro* (Heydorn *et al.* 2000a, Heydorn *et al.* 2000b). The *in vitro* properties of the WT and mutant biofilms will be analyzed and correlated with *in vivo* properties of these strains in the rat caries model in an effort to unravel the mechanisms for how individual Gbps make contributions to *S. mutans* cariogenicity.

Materials and Methods

Confocal Microscopy

Biofilms, for analysis by confocal microscopy were grown on glass coverslips similar to those described in Banas *et al. (Banas et al. 2001)*. Briefly, 1.5 cm diameter

wells containing 1.5 mls of CDM/5% sucrose were inoculated with 100µl (OD_{540} = 1.0) of *S. mutans*. Biofilm cultures were grown in polystyrene chambers with coverglass bottoms at 37°C in 5% CO_2 on a fixed angle rotator at a rotation speed of 20 RPM and an angle of 60° for 12 hours. Chambered coverglass units were assembled by removing the 1.5 cm diameter, round chambers from Sonic Seal slide wells (Nunc, Rochester, NY, USA) and using Krazy Glue (Elmer's, Columbus, OH, USA) to attach a glass coverslip that formed the substratum upon which the biofilm developed.

Twelve hour biofilms were rinsed twice in PBS, stained for 35 minutes with Syto9, a nucleic acid stain (Molecular Probes, Inc., Eugene, OR, USA), and then rinsed twice with PBS. PBS (1 ml) was added to the wells to prevent drying of the biofilm during image collection. Biofilm images were collected using a Zeiss 510 Meta inverted confocal laser scanning microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) with a 40x objective that was zoomed out to 0.7. Five independent biofilm experiments were performed and at least 5 image stacks per experiment were collected.

Image analysis

Image stacks were converted to individual grayscale Tiff images for each slice using the 510 Meta Image Analysis software package (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). COMSTAT was used to analyze the image stacks as described in Heydorn *et al*. (Heydorn *et al.* 2000b). Grey scale images were converted to black/white and compared with the original image to determine a threshold for the images. Each stack of an experiment was examined and the threshold value that best fit all image stacks of a trial was chosen and kept consistent for all stacks within the trial. The image stacks of the WT and each mutant were averaged and compared.

Statistical Analysis

At least five confocal image stacks were collected from each strain per trial. After conversion to TIFF files and thresholding, the COMSTAT measurements for each

image stack were compared. The values for each measurement were averaged within a strain for each trial. A one-way analysis of variance was performed to determine statistically significant differences between strains within a trial. The parameter averages of each trial were then compared among the WT and mutant panel via repeated measures ANOVA (Feustel 2006).

There were some measurements in several of the parameters that seemed to show marked differences from the WT in their absolute values and that would have been expected to be different based on observations of the confocal images, but due to the heterogeneous nature of biofilms and the variability between trials, the large standard deviations prevented statistical significance among these data. In order to detect trends among COMSTAT parameters that might show an association with caries, correlational analyses were performed between COMSTAT measured biofilm parameters and caries scores. The values for each COMSTAT parameter and caries scores were fit into the non-linear regression equation of SPSS statistics software (Qian 2010). The correlation coefficients, or measure of how well the data points fit to the regression line, were used to compare architectural parameters.

Results and Discussion

Confocal Microscopy

Confocal images of WT and Gbp mutant biofilms showed several differences with respect to microcolony structure and arrangement. An overhead view of the first layer of cells, which are in contact with the substratum, revealed that the *gbpA* strain consisted of a great number of smaller microcolonies that seemed to cover more of the substratum than all other strains including the WT (Figure 8). All mutants missing GbpC appeared to consist of less organized microcolonies and contained many more individual cells and chains of cells that were adherent to the substratum. This *gbpC* mutant phenotype seemed to be retained even in the presence of additional Gbp mutations.

WT and mutant biofilms viewed from the side showed that all of the mutant biofilms had a flatter appearance than the WT (Figure 9). The WT *S. mutans* biofilm consisted of very tall microcolonies that extended perpendicularly outward from the substratum. There also appeared to be extensive channeling in the WT biofilms that was absent from the mutant strains.

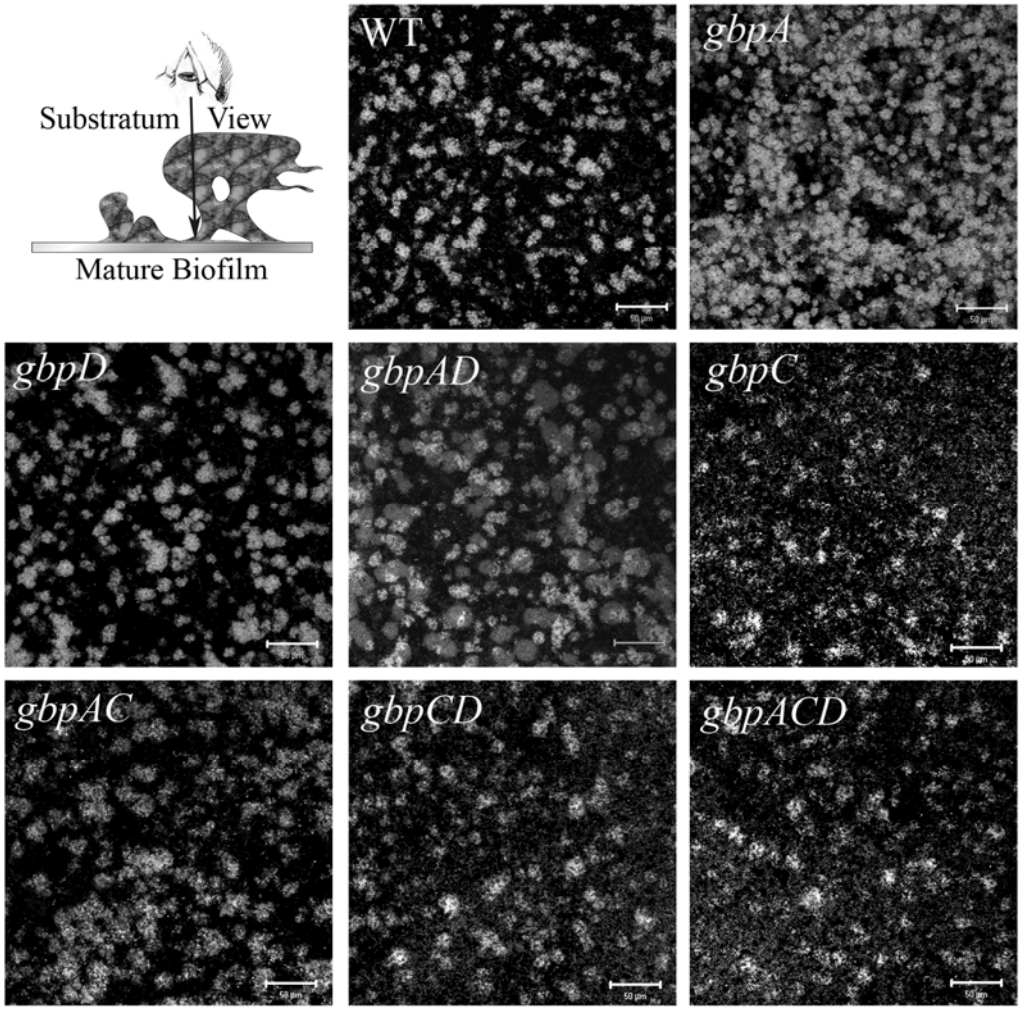


Figure 8 Confocal images of WT and mutant biofilms at the substratum. The top left box shows a schematic diagram of the view. Images are the first slice of a representative image stack of each strain, or the portion of the biofilm adherent to the substratum. White bars are 50μm.

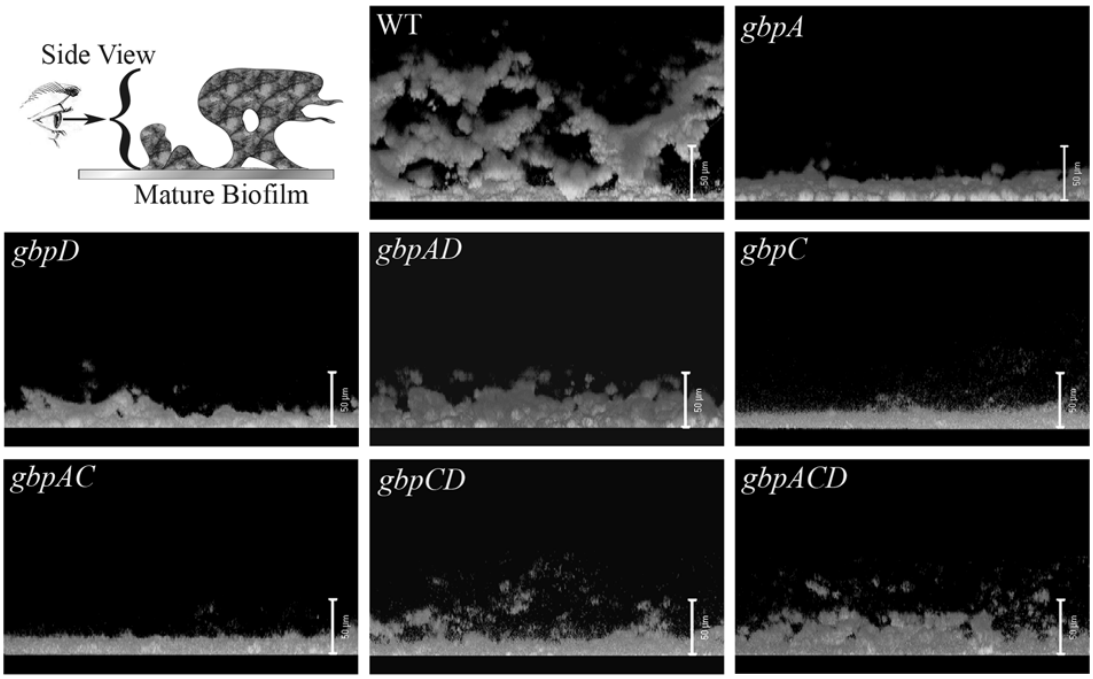


Figure 9 Confocal microscopy side views of WT and mutant biofilms. The top left box shows a schematic diagram of the view. Images are the side view of the maximum density projection from a representative image stack of each mutant. White bars are 50μm.

COMSTAT systematically scans an entire confocal image stack and scores each pixel as positive or negative based on the threshold-defined black and white representations of each slice. Using algorithms to create a 3D mathematical model of an image stack, several architectural parameters can be measured. Parameters that are likely to be important to *S. mutans* cariogenicity were chosen for comparison between strains and included biomass, percent substratum coverage, biofilm thickness surface area and surface area to biovolume ratio.

Biomass (BM) is an estimated value that represents the total biovolume (total positive pixels throughout an image stack) divided by the area of the field that the image stack covers. This measurement provides information about the robustness of the biofilm. It likely serves as an indicator of the ability of a strain to thrive in an *in vivo* biofilm. The Percent Substratum Coverage (%SC) is the percentage of positive pixels in the first optical section which images bacteria attached at the surface of the substratum. This parameter could yield information regarding the adhesion properties of a given strain and the manner in which microcolonies are distributed about the substratum surface. The Average Microcolony Thickness (AMT) is the average of peak heights for all microcolonies within an image stack. The ability of biofilms to produce tall microcolonies could be an indication of biofilm cohesiveness. Surface Area (SA) is the total amount of positive pixels that have at least one neighboring negative (empty space) pixel. Since biofilms are made up of aggregates of cells, many cells rely on diffusion through the matrix for nutrients or waste elimination. Differences in the number of cells exposed to the surrounding media could affect metabolic rates in the biofilm. The Surface Area to Biovolume Ratio (SABV) is the number of positive pixels that have at least one neighboring negative pixel divided by the total number of positive pixels in an image stack. This parameter factors the amount of biomass for a given biofilm into the surface area measurement allowing comparison of diffusion distances and contact with

the surrounding media in biofilms that have different levels of robustness (Heydorn *et al.* 2000b).

The summary of the COMSTAT analyses (Table 6) showed a significant reduction in the average biofilm thickness among all mutant strains. Based on previous results in the *gbpA* mutant and laboratory observations of the mutant panel biofilms, a reduction in microcolony height was expected.

We had also expected to see significant differences between strains in other biofilm parameters such as biomass and substratum coverage. Indeed, values between strains sometimes differed by more than two-fold but did not reach the level of statistical significance (Table 6). This was likely due to the high degree of variability between trials that led to high standard deviations. Based on the high number of individual cells scattered about the substratum in all *gbpC* mutant biofilms, we predicted that there would be significant differences for these strains in the Surface Area and Surface Area to Biovolume ratios. While there was an upward trend in the values of these biofilm parameters for *gbpC* mutants compared to the WT, the differences were not statistically significant (Table 6).

Although the reduction in biofilm thickness certainly should be viewed as an important architectural characteristic, and could likely play a role in *S. mutans* cariogenicity, it did not explain why only certain mutants were significantly attenuated. We also could not dismiss the possibility that strain differences in some of the other parameters played a role in caries development despite the fact that differences did not reach statistical significance. In order to overcome the consequences of the high level of variability in absolute values between independent experimental trials, correlation analyses were performed in an effort to link biofilm properties with cariogenicity.

	WT	*gbpA*	*gbpC*	*gbpD*	*gbpAC*	*gbpAD*	*gbpCD*	*gbpACD*
Biomass ($\mu m^3/\mu m^2$)	7.55 ± 3.10	6.86 ± 4.01	4.64 ± 3.59	5.15 ± 2.56	3.13 ± 1.78	5.31 ± 2.29	4.90 ± 1.99	4.12 ± 2.05
Substratum Coverage (%)	30.91 ± 3.77	43.23 ± 21.85	33.09 ± 21.30	30.89 ± 13.50	22.73 ± 10.80	31.63 ± 11.97	36.55 ± 17.55	29.34 ± 18.47
Average Biofilm Thickness (µm)	32.48 ± 13.68	**10.34** ± 4.58	**12.85** ± 10.68	**10.90** ± 6.95	**7.54** ± 3.55	**11.63** ± 8.84	**10.34** ± 4.94	**10.28** ± 2.05
Surface Area ($10^5 \mu m^2$)	10.80 ± 3.71	5.45 ± 1.54	8.73 ± 5.31	5.30 ± 2.42	6.88 ± 2.60	7.20 ± 4.38	11.40 ± 5.22	9.07 ± 4.75
Surface Area to Biovolume Ratio ($\mu m^2/ \mu m^3$)	1.36 ± 0.44	0.87 ± 0.30	1.96 ± 0.54	1.03 ± 0.27	2.29 ± 0.76	1.31 ± 0.61	2.11 ± 0.30	2.12 ± 0.41

Table 6 Results of the COMSTAT biofilm analyses.

Note: Results are the averaged values of 5 independent trials in which 5 random microscope fields were captured and averaged. Standard deviations of the 5 trials are shown below each value. Values shown in bold type are significantly different than the WT.

Correlation between biofilm properties and cariogenicity

An advantage to correlational analyses is that it can detect trends in data. This is particularly advantageous for analyzing the Gbp mutant panel since identical individual mutations were carried by multiple strains. Each correlational analysis yields a correlation coefficient that can range from 0 (no correlation) to +1 (perfect, positive correlation) or -1 (perfect, negative correlation). We will define a strong correlation as having a correlation coefficient between ±0.70 and ±1.00, a moderate correlation as having a correlation coefficient between ±0.30 and ±0.69, and a weak correlation as having a correlation coefficient between ±0.01 and ±0.29.

A strong statistically significant positive correlation was detected between the percentage of substratum coverage and total enamel (E) unadjusted caries, and for buccal surface enamel unadjusted caries (Tables 7 and 8, Figure 10A&C). The correlations were moderate and were not statistically significant for total D_x unadjusted caries or buccal D_x unadjusted caries (Table 8, Figures 10B&D). Moderate but non-significant correlations were seen for sulcal (Table 8 and Figure 10C) and proximal (data not shown) enamel caries. It was not surprising then, that the three strains (*gbpD, gbpAC* and *gbpACD*) that had lower average substratum coverage than the WT, had significantly reduced raw caries scores. However, when caries scores were adjusted for the weight of the rat, the previously strong correlations were reduced to moderate correlations that were no longer statistically significant (Table 8 and Figure 11 A-F). There were no significant correlations for substratum coverage and raw caries or adjusted caries on proximal surfaces. This was true for all biofilm properties and is probably due to the relative lack of variation, among mutants, for proximal caries scores.

These results are equivocal for whether substratum coverage is an architectural trait that could be associated with virulence. Intuitively, it would make sense that the presence of more metabolically active bacteria in contact with the tooth surface would be

correlated with a higher caries rate. Previous work by Hazlett *et al.* in which a *gbpA* mutant showed an increase in substratum coverage and caries in a germ-free rat model, would support this theory (Hazlett *et al.* 1998). However, the substratum coverage/caries correlations were only moderately strong when the weight of the rat was taken into consideration. Conceptually, normalization of caries data to account for differences in rat weight can be justified based on the possibility that weight differences reflect differences in food consumption and exposure to sucrose. However, it is also possible that the weight-based normalization overcompensates for differences in eating patterns (eg. food intake volume may differ more than frequency of intake and exposure to sucrose) thereby obscuring an authentic correlation between substratum coverage and caries. While the level of substratum coverage shows a promising association with caries, other biofilm properties might have stronger correlational associations.

Correlation Coefficients for Biofilm Parameters (Based on Raw Caries Scores)

	Biofilm Parameters			
	Substratum Coverage	Biomass	Average Thickness	Surface Area to Biovolume Ratio
Total Caries (E)	**0.714**	0.500	**0.719**	-0.524
Total Caries (D_x)	0.548	0.500	**0.707**	-0.357
Buccal Caries (E)	**0.714**	0.500	**0.719**	-0.524
Buccal Caries (D_x)	0.619	0.524	**0.826**	-0.452
Sulcal Caries (E)	0.690	0.571	0.695	-0.571
Sulcal Caries (D_x)	0.476	0.500	0.575	-0.119

Table 7 Correlation matrix showing correlation coefficients between biofilm parameters and the caries scores.

Note: Significant correlations are in bold type.

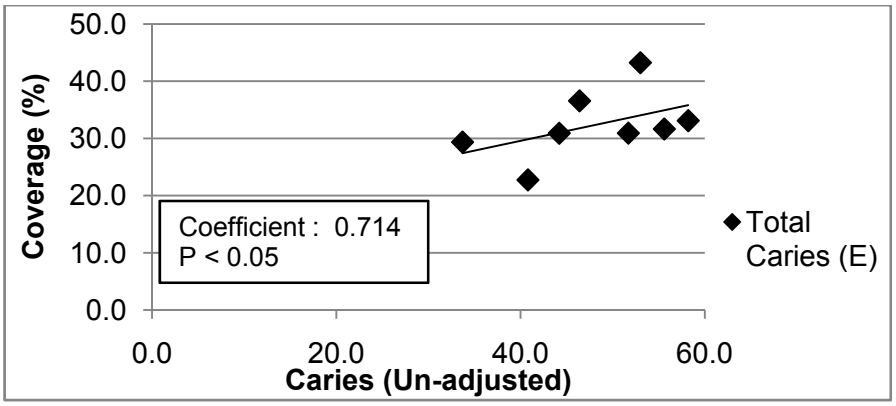
Correlation Coefficients for Biofilm Parameters (Based on Rat Weight-Adjusted Caries Scores)

	Biofilm Parameters			
	Substratum Coverage	Biomass	Average Thickness	Surface Area to Biovolume Ratio
Total Caries (E)	0.619	**0.786**	0.659	**-0.786**
Total Caries (D_x)	0.647	**0.766**	**0.801**	-0.551
Buccal Caries (E)	0.618	**0.800**	**0.707**	**-0.740**
Buccal Caries (D_x)	0.687	**0.724**	**0.772**	-0.638
Sulcal Caries (E)	0.575	**0.802**	**0.717**	**-0.755**
Sulcal Caries (D_x)	0.506	0.578	0.558	-0.108

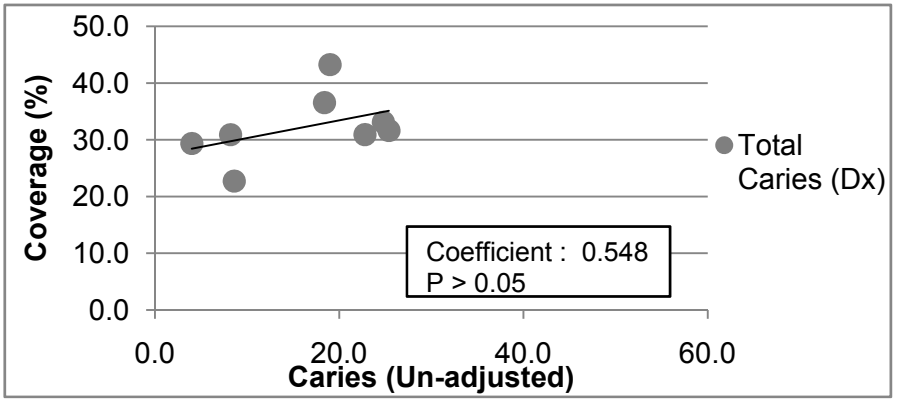
Table 8 Correlation matrix showing correlation coefficients between biofilm parameters and the rat weight-adjusted caries scores.

Note: Significant correlations are in bold type.

A.



B.



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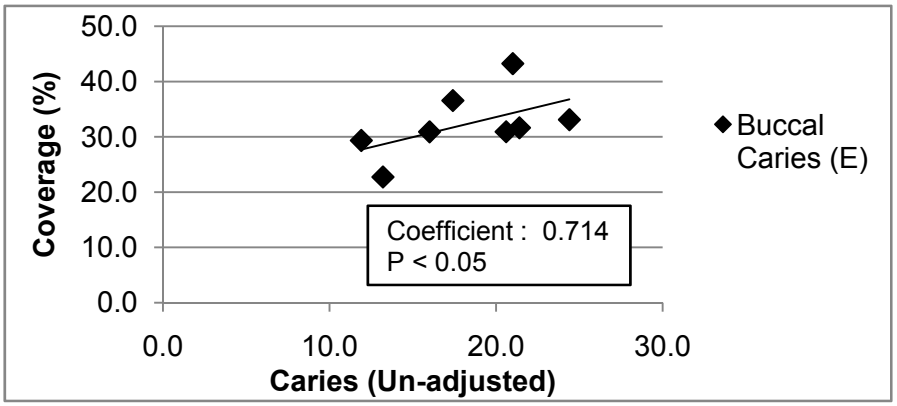
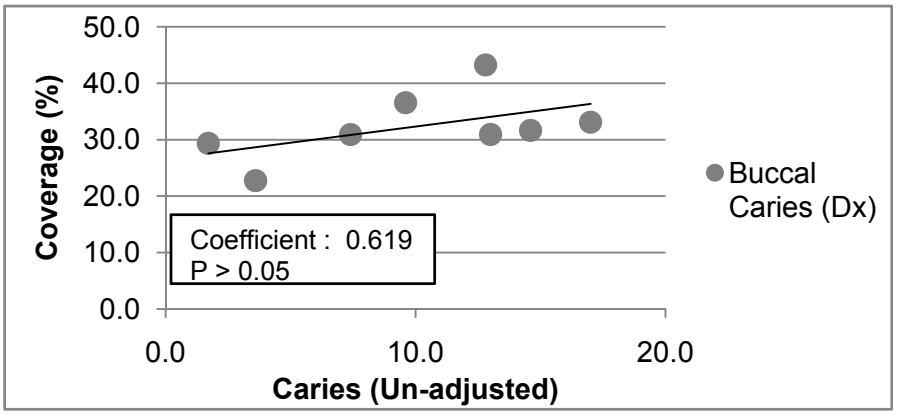


Figure 10 Correlations between Substratum Coverage and Raw Caries. Regression analyses were performed for the percent substratum coverage and (A) total enamel and (B) total excessive dentinal caries, (C) buccal enamel and (D) buccal excessive dentinal caries, (E) sulcal enamel and (F) sulcal excessive dentinal caries. Caries scores were not adjusted for differences in rat weights. The scatter plots, correlation coefficient and P values are shown for each caries score.

D.



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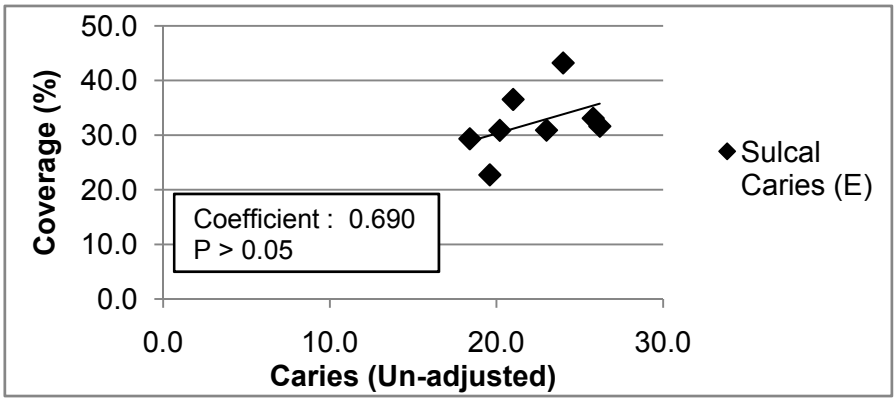

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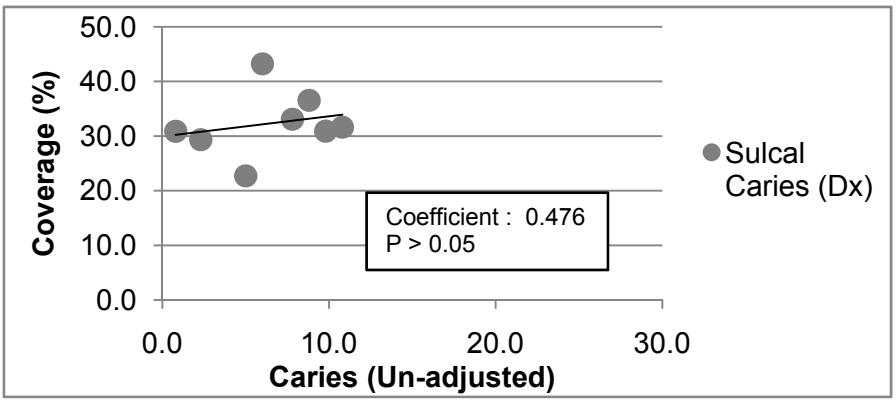
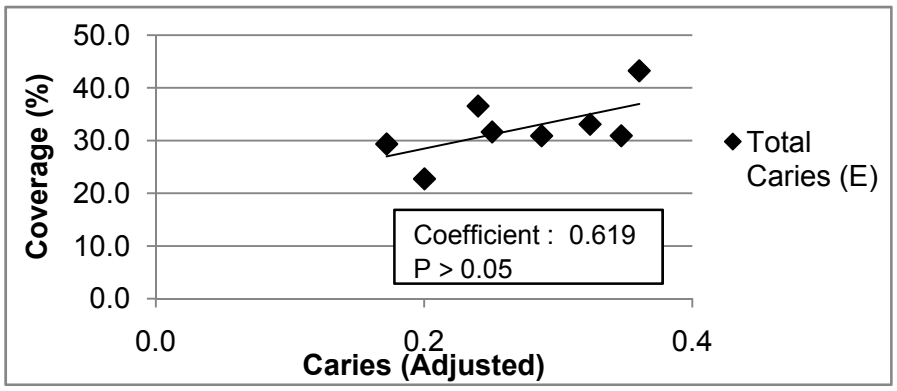


Figure 10 Continued

A.



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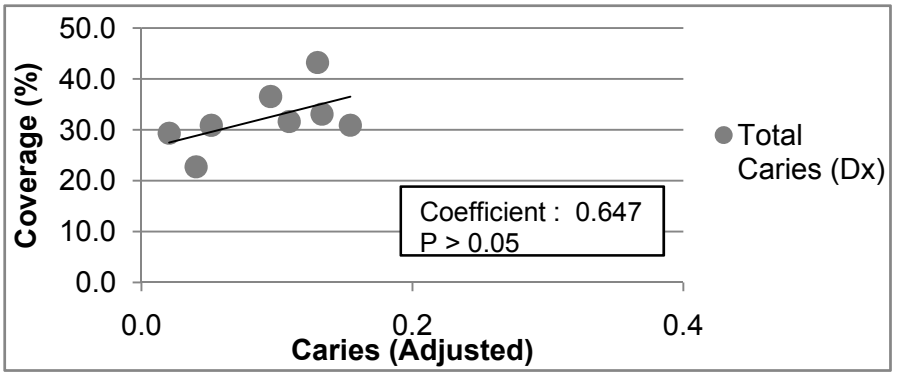

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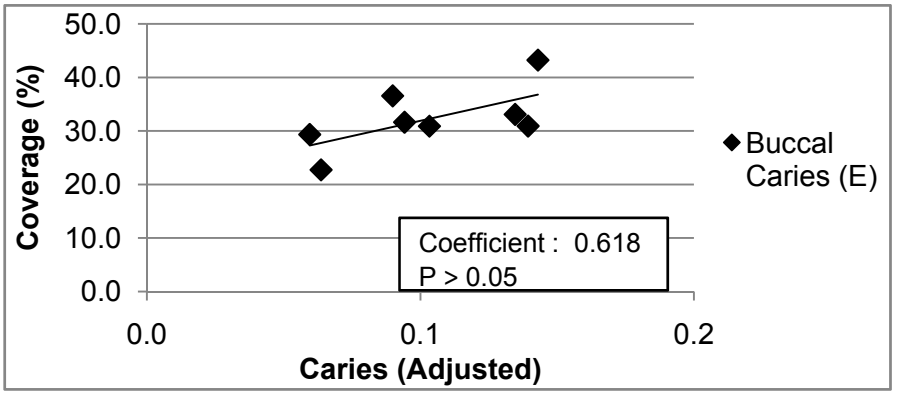
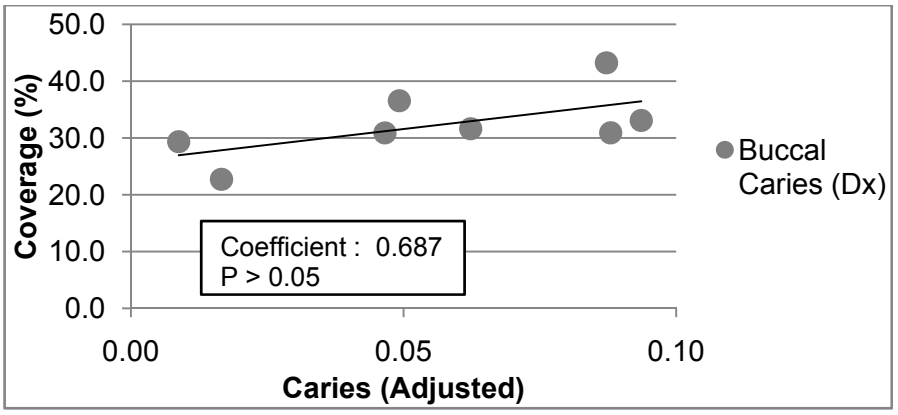


Figure 11 Correlations between Substratum Coverage and Rat Weight Adjusted Caries. Regression analyses for the percent substratum coverage and (A) total E and (B) total D_x caries, (C) buccal E and (D) buccal D_x caries, (F) sulcal E and (G) sulcal D_x caries. Caries scores were adjusted for differences in rat weights. The scatter plots, correlation coefficient and P values are shown for each caries score.

D.



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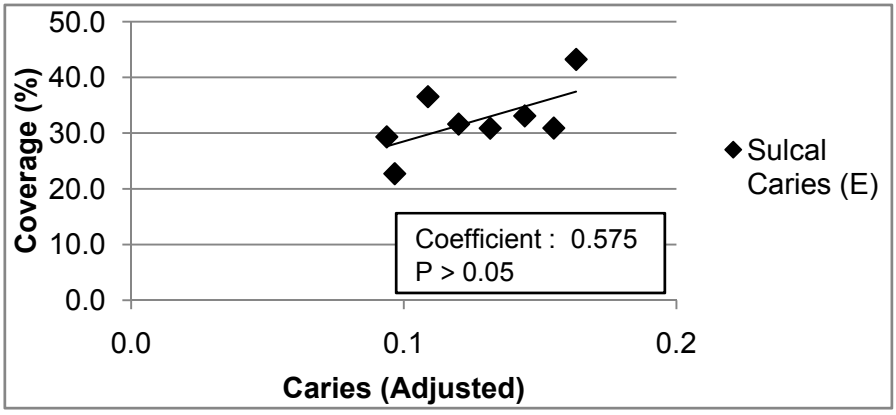

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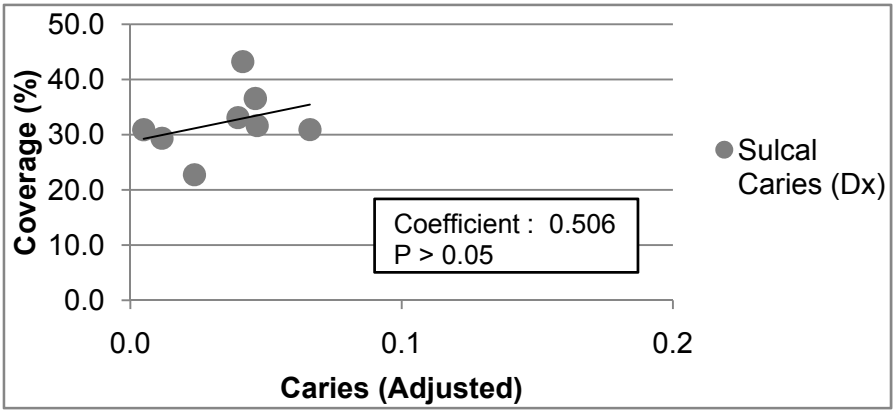
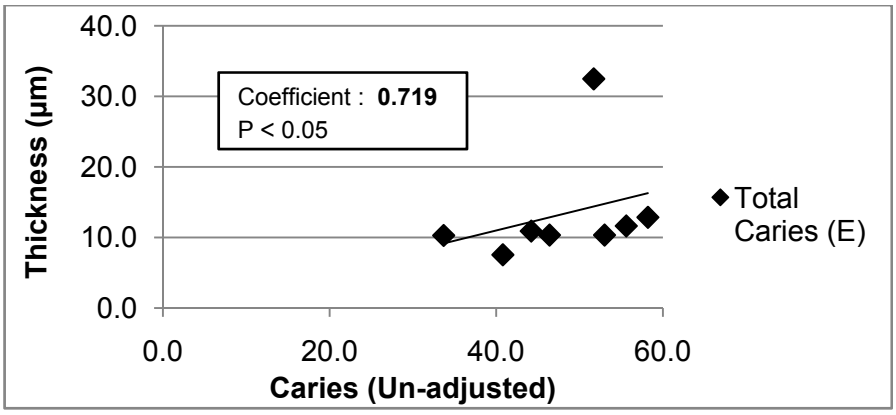


Figure 11 Continued

There were moderate and strong correlations between the average microcolony thickness (AMT) and caries, regardless of whether or not the data were weight-adjusted. This was true for total caries, with the exception for weight-adjusted enamel lesions and for buccal surfaces (Tables 7 and 8, Figure 12A-D, Figure 13C-D). However, the correlations between AMT and sulcal or proximal caries (data not shown) were moderate and weak respectively and were not statistically significant except for the weight-adjusted sulcal enamel lesions (Tables 7 and 8, Figure 12E-F, Figure 13E-F).

The average microcolony thickness had strong significant correlations with both rat weight-adjusted and unadjusted caries scores. It is possible that the extreme difference in AMT measurements, between the WT strain and the Gbp mutants, may have skewed the correlation. When correlation analyses were performed for the AMT and both weight-adjusted and unadjusted caries without the scores from the WT strain present, only the unadjusted caries showed strong significant correlations. The correlations between AMT and adjusted caries, without the WT, were moderate (data not shown). These strong correlations for unadjusted caries scores, in the absence of the WT, affirm that this biofilm property could play a role in caries risk.

A.



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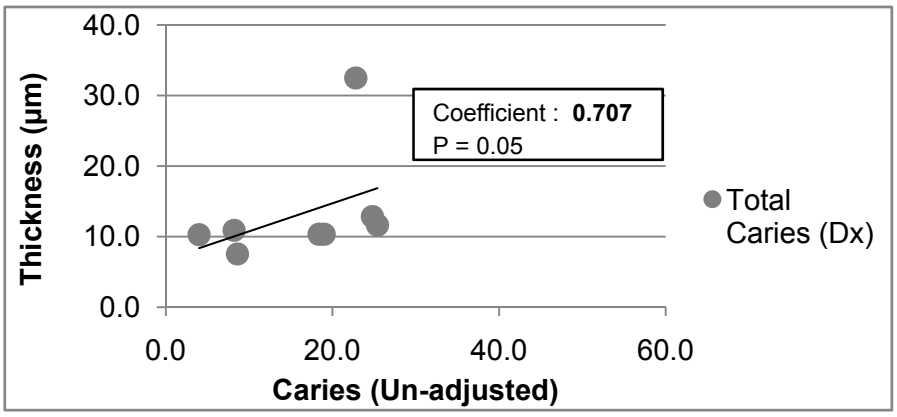

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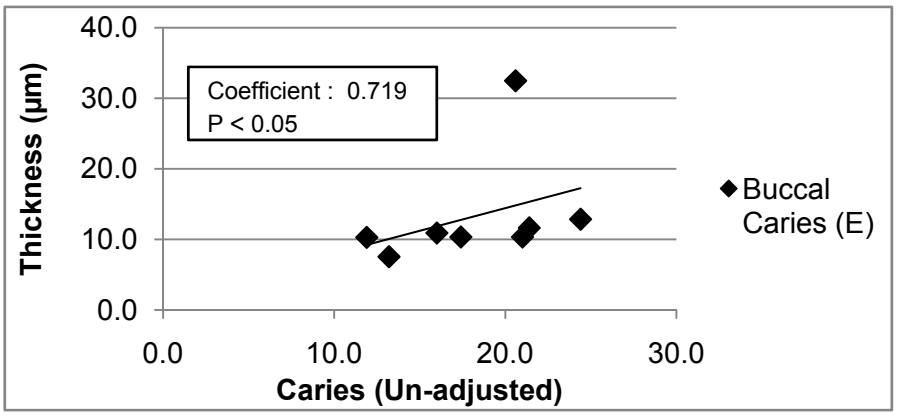
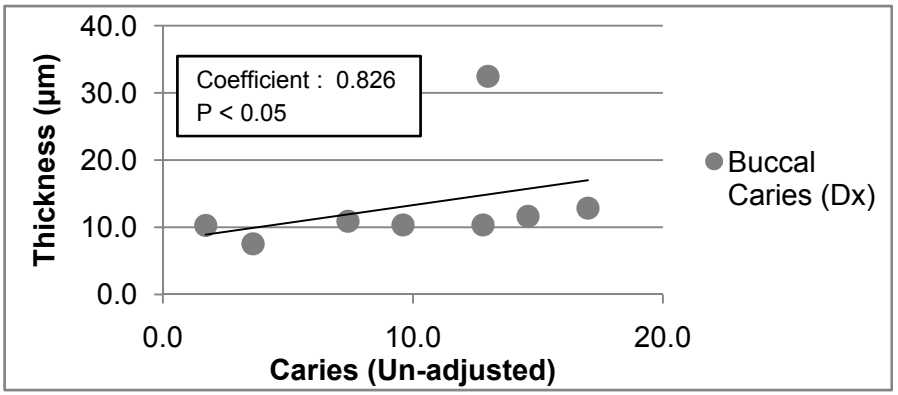


Figure 12 Correlations between Average Microcolony Thickness (AMT) and Raw Caries. The scatter plots, correlation coefficient and P values are shown for the AMT and (A) total E and (B) total D_x caries, (C) buccal E and (D) buccal D_x caries and (F) sulcal E and (G) sulcal D_x caries. The scatter plots, correlation coefficient and P values are shown for each caries score.

D.



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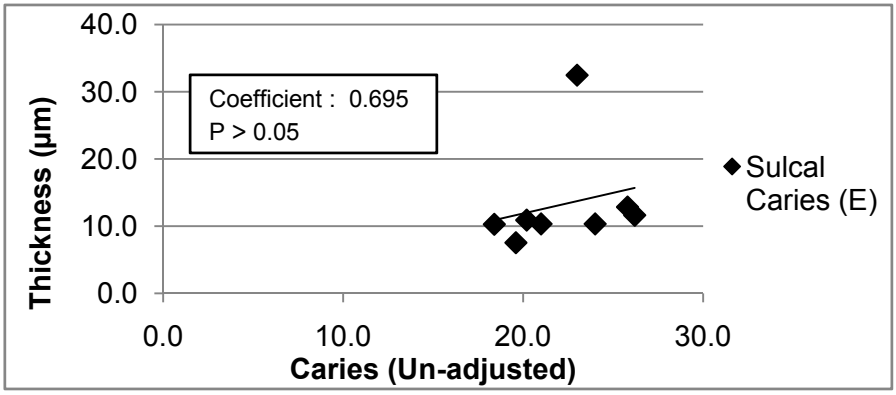

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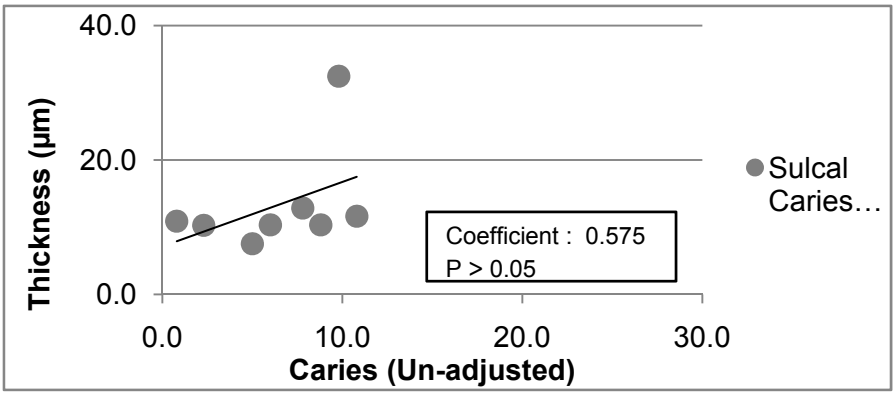
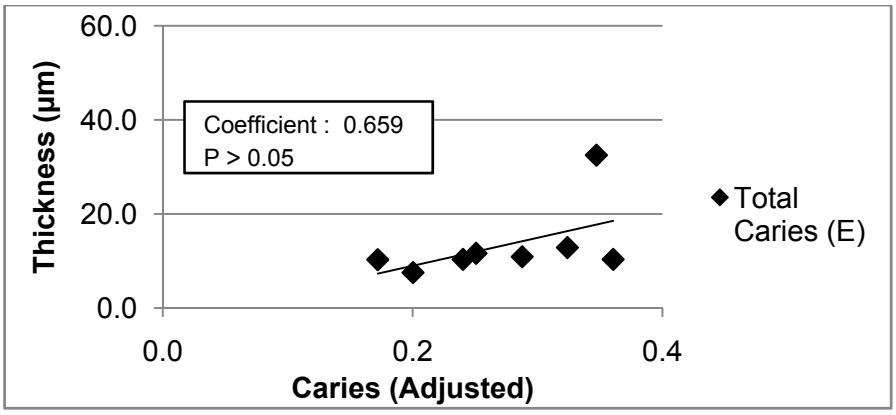


Figure 12 Continued

A.



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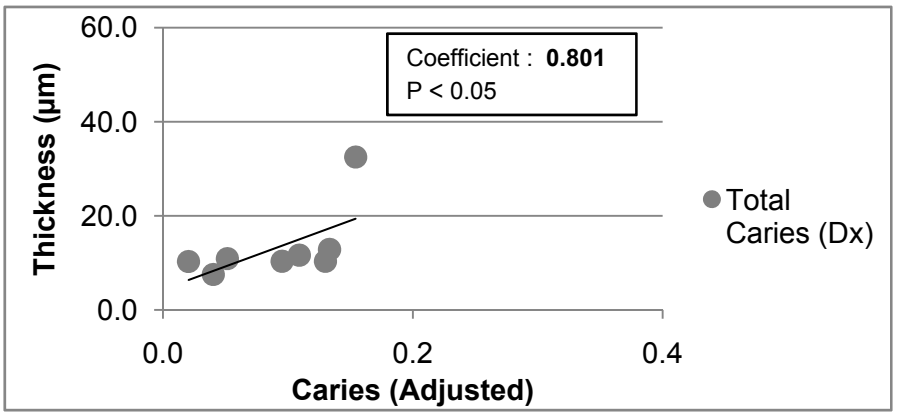

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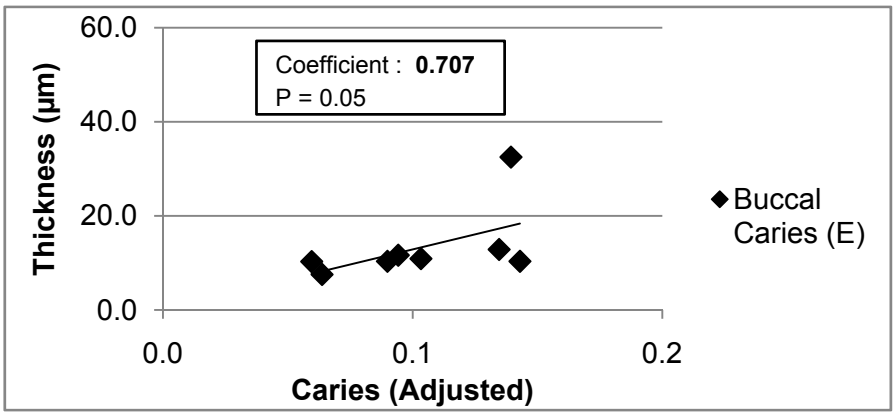
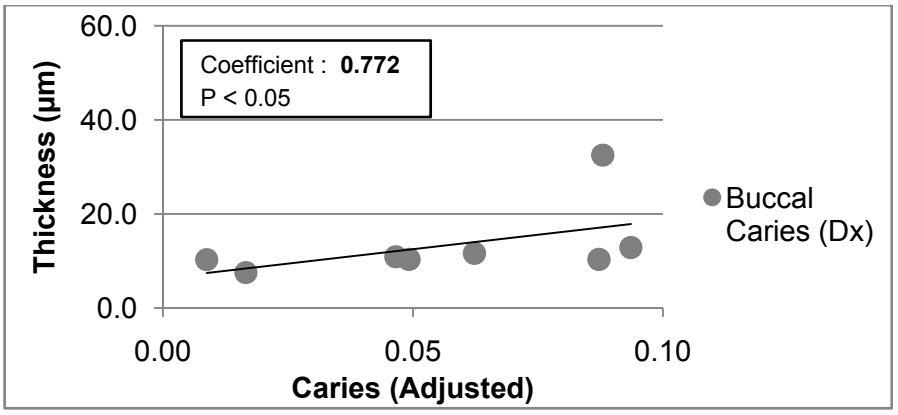


Figure 13 Correlations between average microcolony thickness (AMT) and rat weight-adjusted caries. The scatter plots, correlation coefficient and P values are shown for the AMT and (A) total enamel E and (B) total D_x caries; (C) buccal E and (D) buccal D_x caries and (F) sulcal E and (G) sulcal D_x caries. The scatter plots, correlation coefficient and P values are shown for each caries score.

D.



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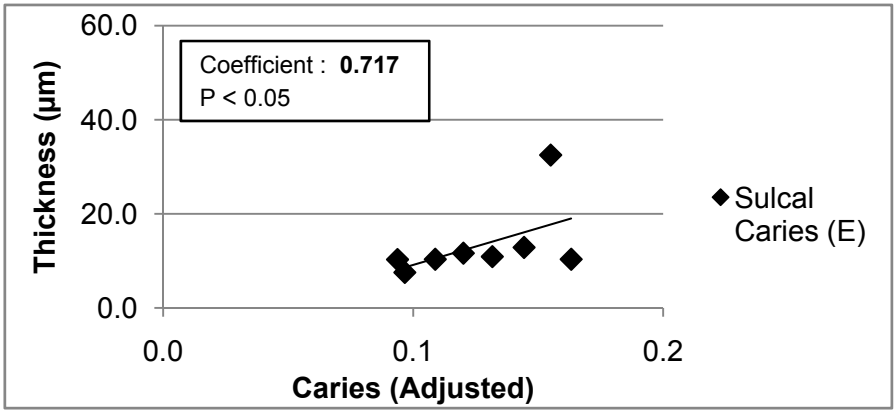


F.

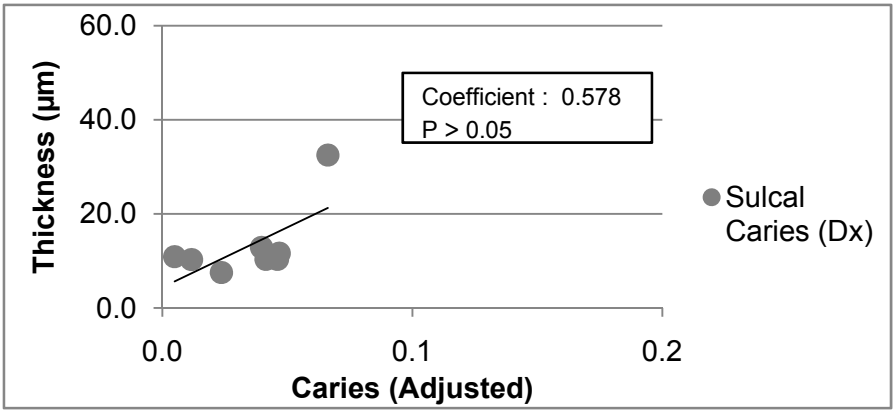


Figure 13 Continued

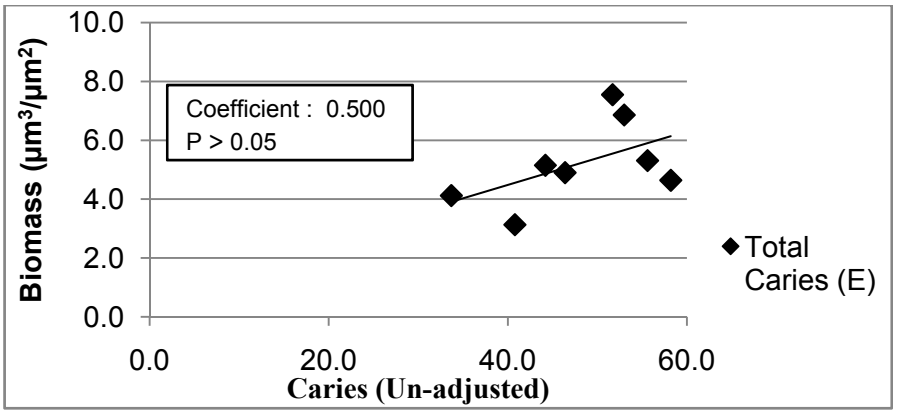
The average microcolony thickness, when measured for *in vitro* biofilms formed in the presence of constant rotation, is likely a good indicator of the cohesive properties of the bacteria within that biofilm. This is supported by the fact that no differences in biofilm architecture were evident between WT and *gbpA* strains when grown under stationary conditions (J. Banas – unpublished lab observation). Strains of *S. mutans* that show a greater level of cohesion in an *in vitro* biofilm may contribute to its ability to form biofilms with taller microcolonies and perhaps confer a selective advantage in the mixed-species plaque environment on smooth, buccal surfaces. The fact that the impact of a reduction in AMT was more pronounced on the buccal surfaces of teeth may also suggest a role in the adhesive properties of the bacteria. Conversely, this trait is less affected by loss of Gbps on fissured or proximal surfaces that are more protected from salivary flow or disruptive mechanical forces.

While there were no significant differences among the mutants with respect to COMSTAT measured biomass, it was noticed that the most attenuated strains had lower biomass measurements than the WT. When the correlational analyses were performed, it was determined that there were strong statistically significant correlations between the *in vitro* biomass measurements and the rat weight-adjusted caries scores (Tables 7 and 8, Figure 14A-E). The only exception was for D_x caries scores on the sulcal surfaces (Tables 7 and 8, Figure 14F). The correlations between the biomass measurements and the unadjusted caries scores were moderate and were not statistically significant (Tables 7 and 8, Figure 15A-F).

Biomass is a parameter that is probably affected by both the adhesive and cohesive properties of a biofilm, as well as growth rate. Since we could not detect any significant differences in the planktonic growth rates and we observed similar biofilm morphologies in older biofilms for each of the strains, we assume that adhesion and cohesion were the largest contributing factors. It should be noted that *in vitro* biomass did not correlate with the recovered CFU from the *in vivo* model (data not shown). There

were large differences in the biomass-related correlation coefficients between the rat weight-adjusted caries and the unadjusted caries. Weight-adjusted caries scores may explain differences in nutrient sources available to *in vivo S. mutans* biofilms. One explanation for the lack of statistically significant correlations in the unadjusted caries scores is that these scores do not reflect the metabolic activity of the strains *in vivo*. A contingency for strains that may lack some of the adhesive and cohesive properties is a higher metabolic state than more cohesive strains. However, rat weight adjustment of caries scores must be considered with caution due to the potential for overcompensation of eating habits as mentioned earlier. Despite the limitations in rat weight adjustment, biofilm biomass is likely reflective of adhesive and cohesive properties important to *S. mutans* biofilm maintenance *in vivo*. Thus, *in vitro* biomass is plausibly linked with the risk of *in vivo* caries, though it may not be a better predictor than substratum coverage or AMT.

A.



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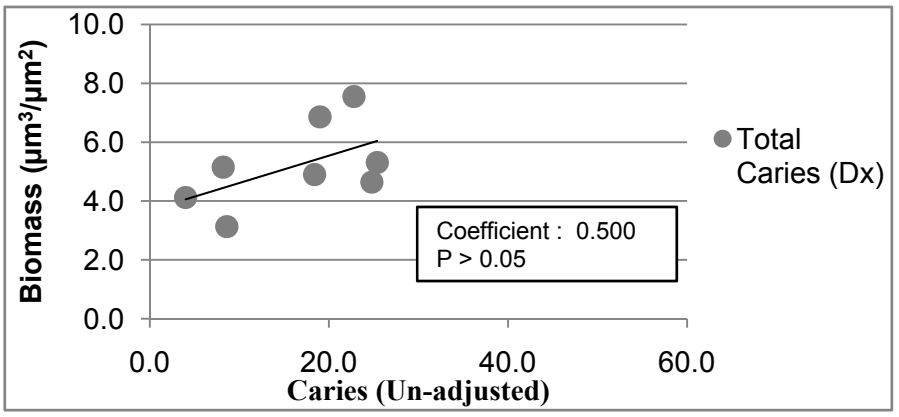

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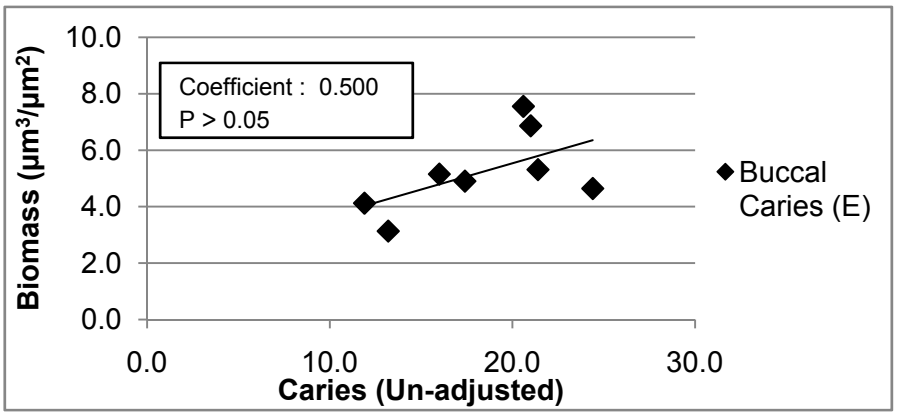
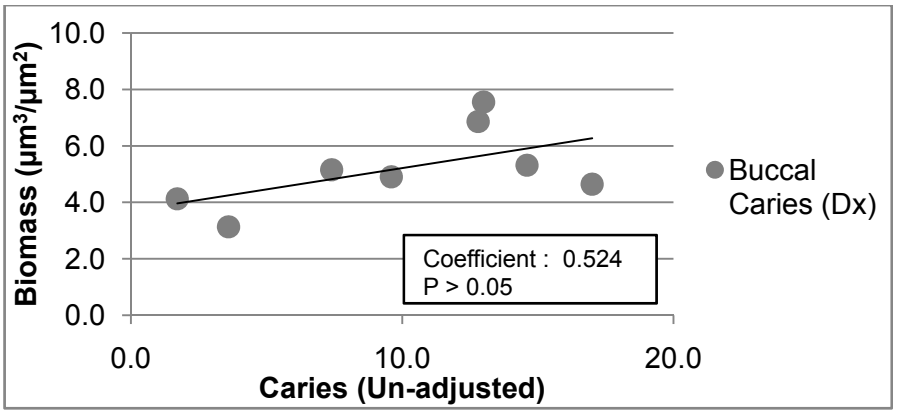


Figure 14 Correlations between *in vitro* Biomass and Raw Caries. Regression analyses between the biomass measurement and the (A) total E caries, (B) the total D_x caries, (C) the buccal E caries, (D) the buccal D_x caries, the (E) sulcal E caries and (F) the sulcal D_x caries. The scatter plots, correlation coefficient and P values are shown for each caries score.

D.



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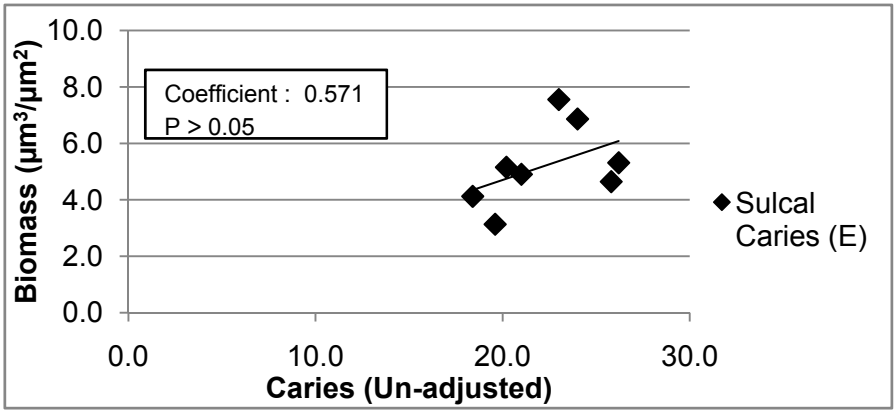

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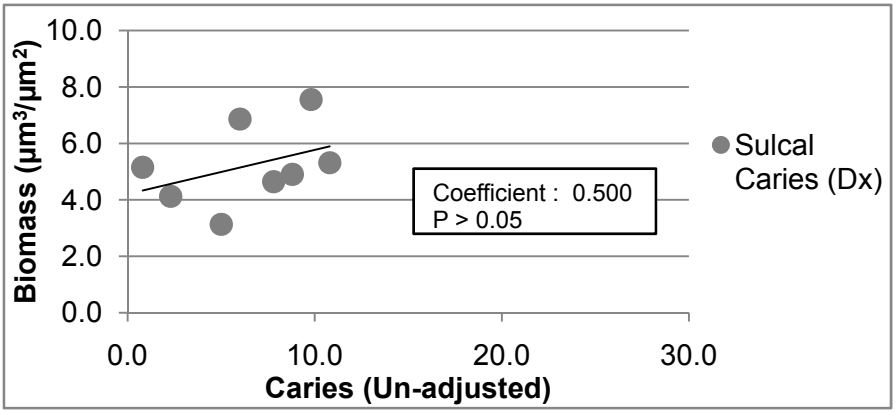
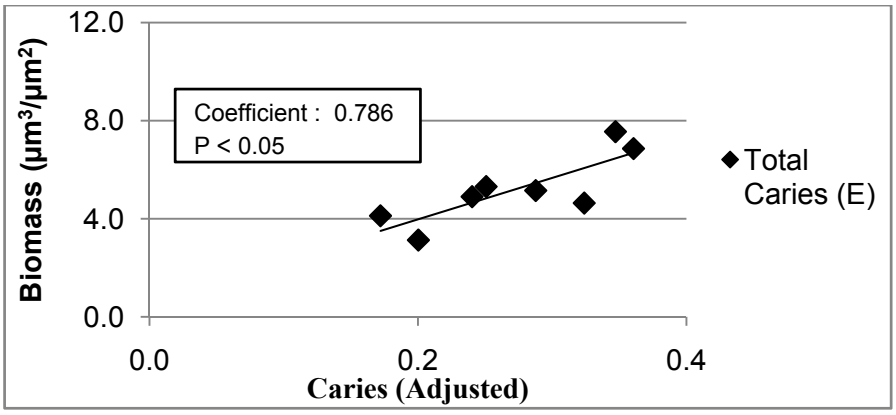


Figure 14 Continued

A.



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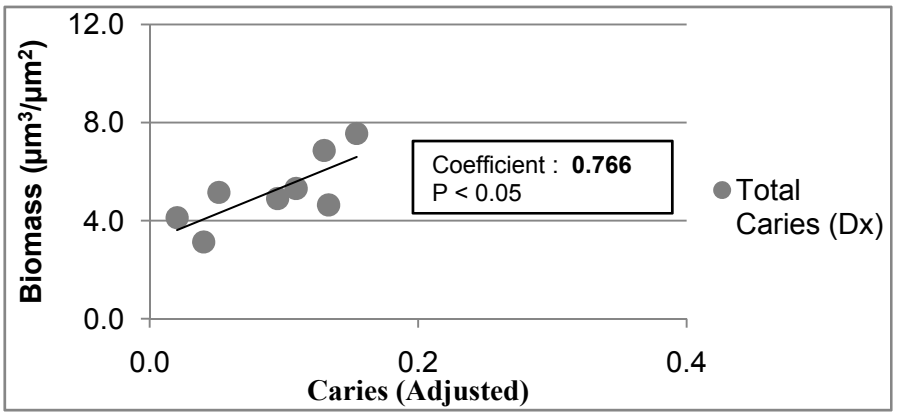

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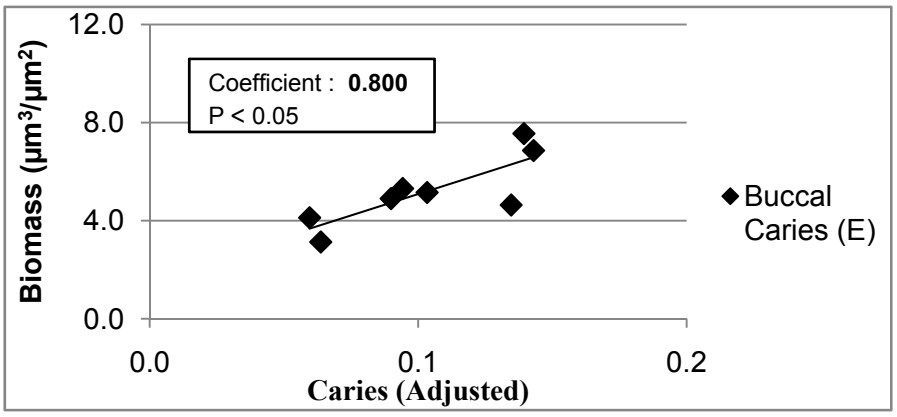
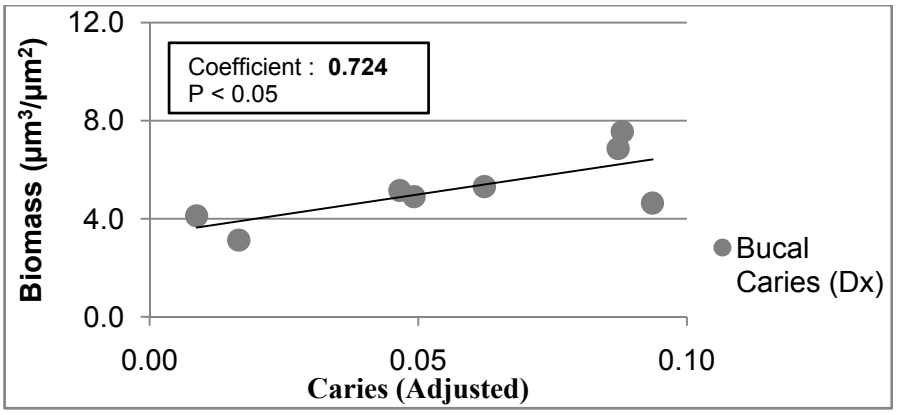


Figure 15 Correlations between *in vitro* Biomass and Rat Weight-Adjusted Caries. Regression analyses between the biomass measurement and (A) the total E caries, (B) the total D_x caries, (C) the buccal E caries, (D) the buccal D_x caries, the (E) sulcal E caries and (F) the sulcal D_x caries. The scatter plots, correlation coefficient and P values are shown for each caries score.

D.



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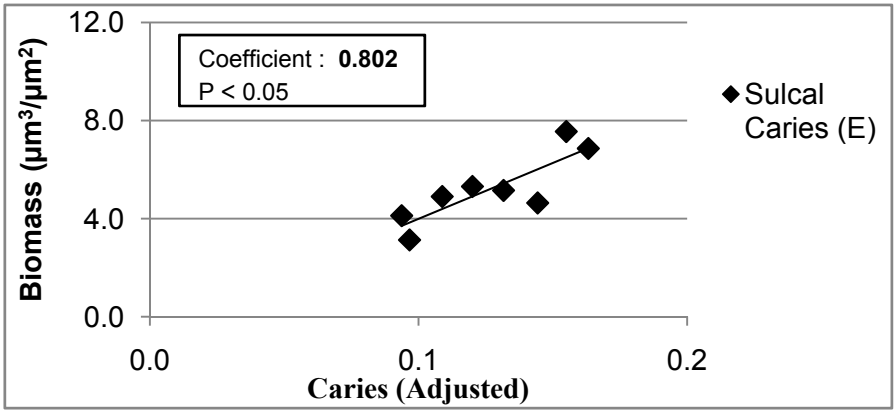

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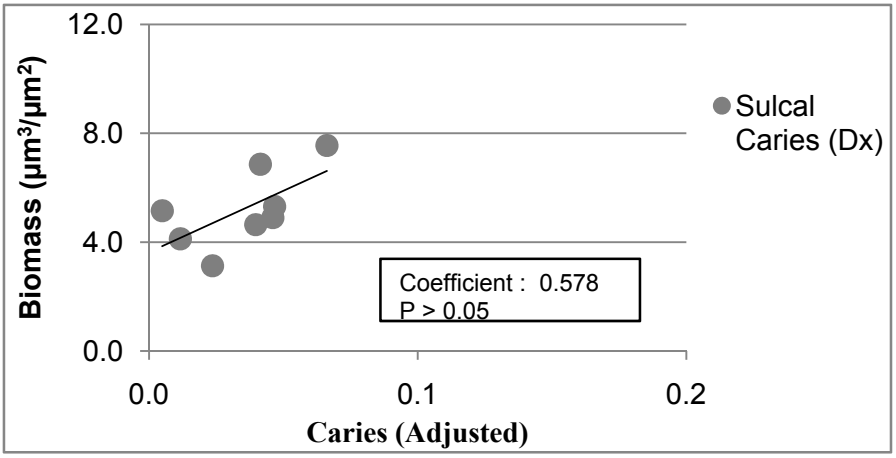
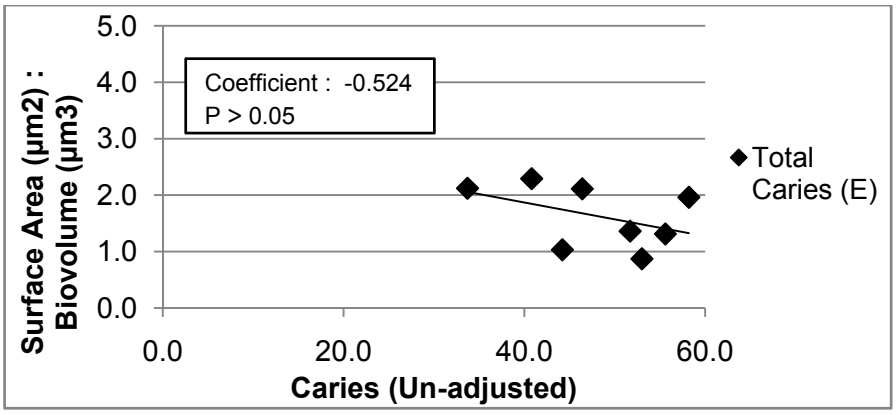


Figure 15 Continued

Another architectural parameter that correlated with caries was the ratio of surface area to biovolume (SABV), only in this instance the correlation was negative. Similar to biomass, statistically significant correlations were limited to the weight-adjusted caries scores (Tables 7 and 8, Figure 17A-F). These included strong negative correlations for total enamel caries (Figure 17A) and enamel caries on the buccal (Figure 17C) and sulcal (Figure 17F) surfaces. The surface area to biovolume parameter is one in which the relationship to *in vivo* caries may depend on the metabolic activity of the bacteria present. If, in fact, the differences in rat weight-adjusted caries and unadjusted caries are due to differences in nutrient intake by the rats, this could explain why the surface area to biovolume ratio only correlates with weight-adjusted caries.

A greater ratio of surface area to biovolume means a greater percentage of bacteria are in contact with the surrounding media and that diffusion distances are most likely shorter. A large surface area suggests that a biofilm has more metabolically active bacteria and is possibly producing more acid. However, it can also mean that this acid is being washed away more rapidly. The flatter biofilms formed by strains such as *gbpA* and *gbpD,* that have not lost as much biomass, have lower ratios of surface area to biovolume. The loss of surface area appears to be the result of a loss of pores and channels within and between the microcolonies (Figures 8 and 9). The biofilm parameters that seem to contribute to the ratio of surface area to biovolume, such as a loss of thickness, loss of substratum coverage and a reduction in biomass, could all be due to a loss of adhesion, cohesion or both. These properties are the same that contribute to biofilm thickness, biomass and substratum coverage, which may correlate with caries more strongly. While the fact that the ratio of surface area to biovolume correlates with weight-adjusted E caries and not with weight-adjusted D_x caries is compelling, and may suggest an association with caries incidence but not caries progression, the basis for the differences in rat weights would have to be more deeply explored before concluding to what extent this parameter is linked to caries incidence.

A.



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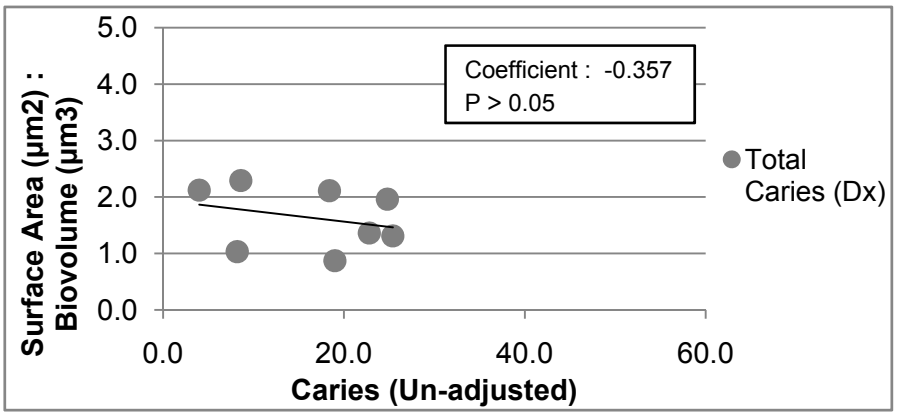

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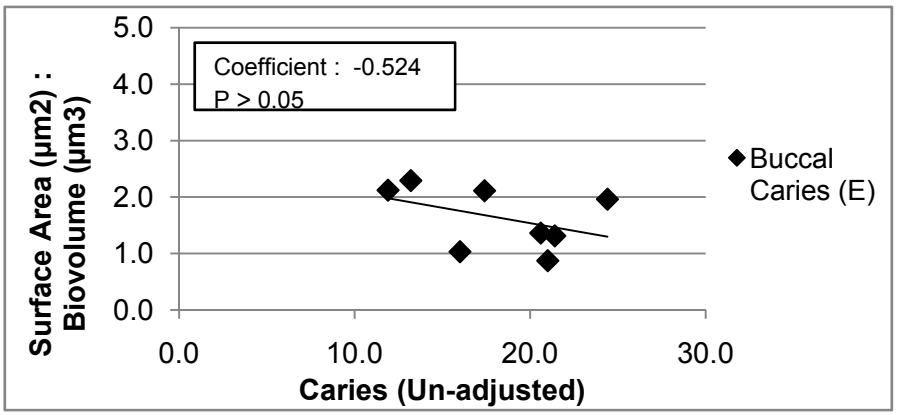
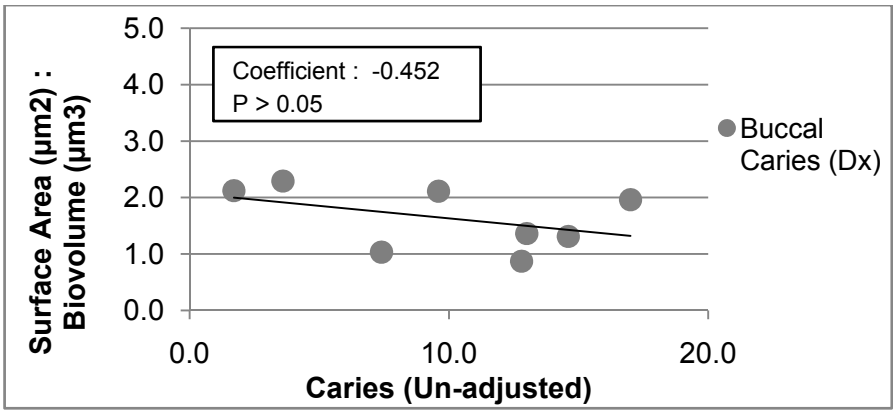


Figure 16 Correlations between Surface Area to Biovolume Ratio and Raw Caries. Regression analyses between the surface area to biovolume measurement and (A) the total E and (B) total D_x caries, (C) the buccal E and (D) buccal D_x caries and (E) the sulcal E and (F) sulcal D_x caries. The scatter plots, correlation coefficient and P values are shown for each caries score.

D.



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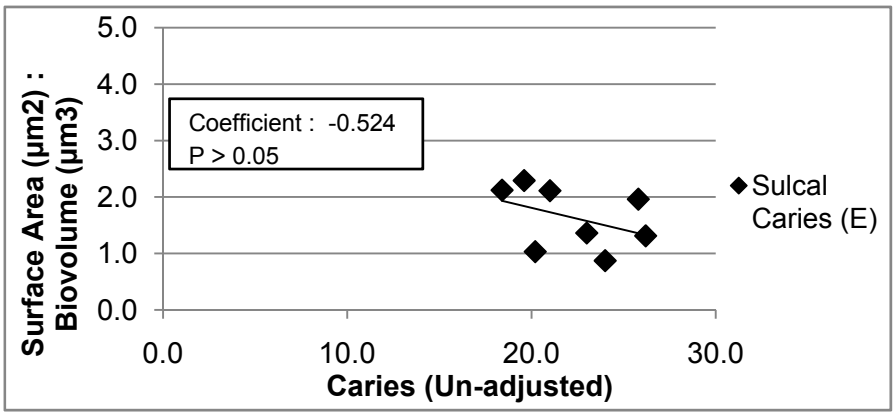


F.

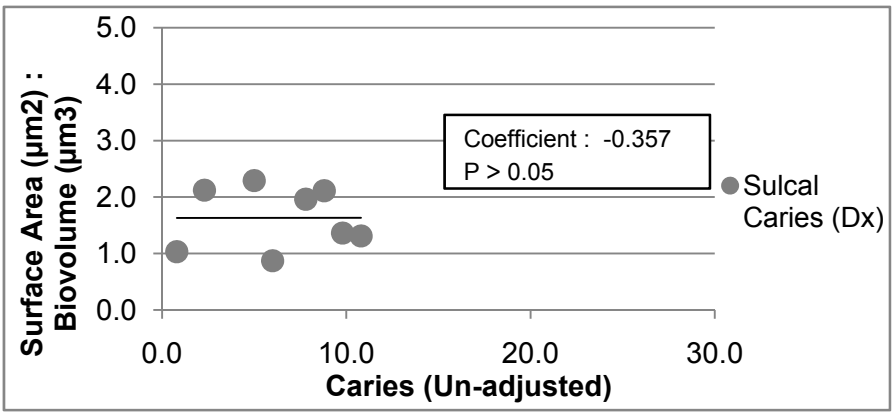
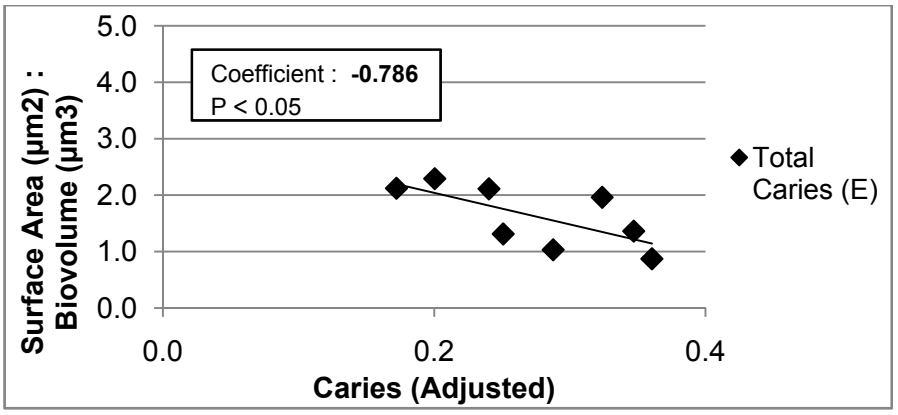


Figure 16 Continued

A.



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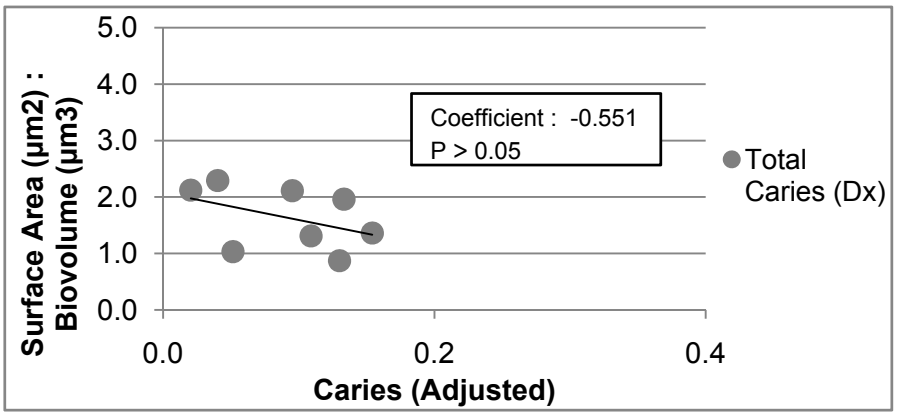

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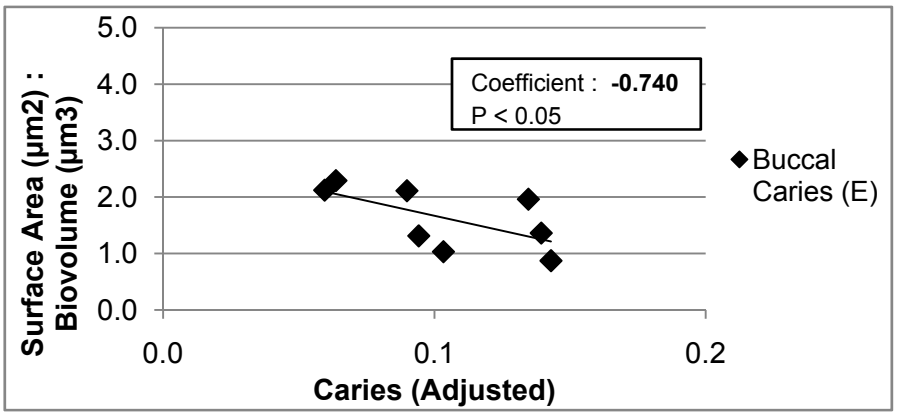
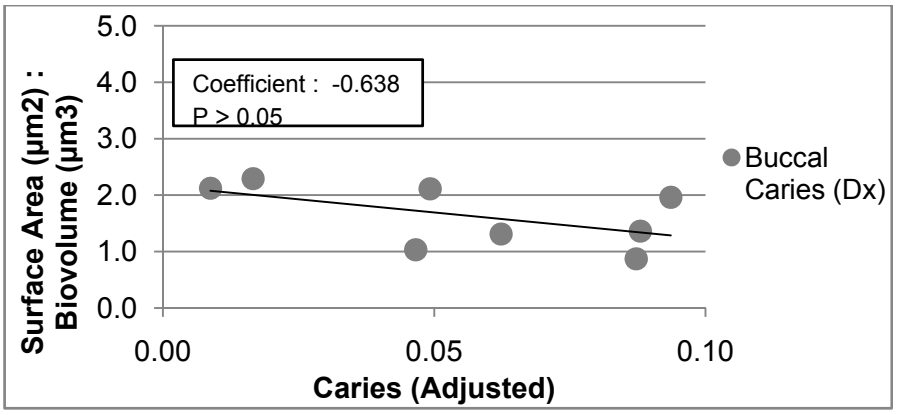
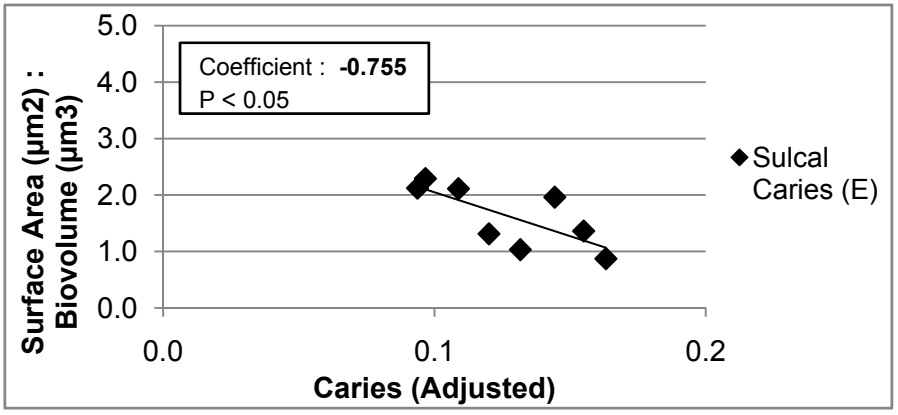


Figure 17 Correlation between Surface Area to Biovolume Ratio and Rat Weight-Adjusted Caries. Regression analyses between the surface area to biovolume measurement and (A) the total E and (B) total D_x caries, (C) the buccal E and (D) buccal D_x caries and (E) the sulcal E and (F) sulcal D_x caries. The scatter plots, correlation coefficient and P values are shown for each caries score.

D.



E.



F.

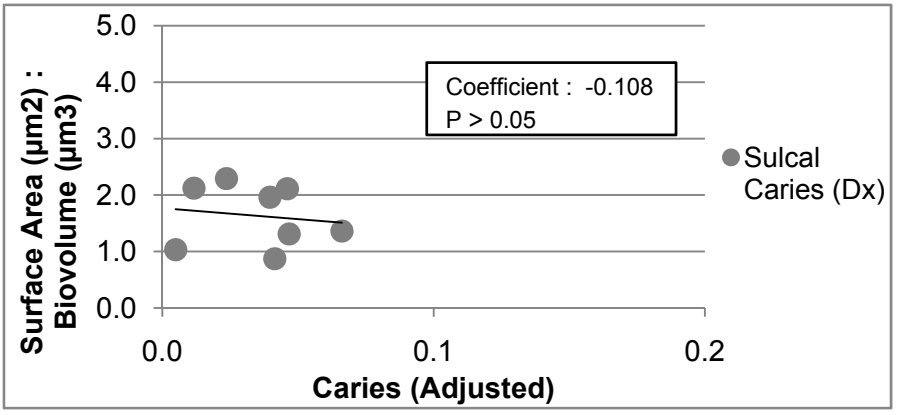


Figure 17 Continued

Biofilm biomass was strongly correlated with both E and D_x caries on multiple surfaces. The average microcolony thickness was better correlated with smooth surface (buccal) caries than sulcal caries. This was also true for substratum coverage measurements and caries, where the strongest correlations were also for buccal surfaces. The surface area to biovolume ratio exhibited weaker correlations than those for biomass and, in some cases, AMT. Given that the biomass measurement is a direct component of the surface area to biovolume ratio, biomass may be a better candidate for correlational analyses than the surface area to biovolume ratio. Correlations were significant for substratum coverage and caries that were not adjusted for differences in rat weights, while correlations for both biomass and surface area to biovolume were only significant for rat weight-adjusted caries. However, the moderate correlations between substratum coverage and weight-adjusted caries were on the high end of the moderate range (Table 8). While weight adjustment of the data may reveal differences in sucrose intake, there is a possibility that the adjustment is overestimating this effect. Additional experiments aimed at determining the nature of the differences in rat weights would need to be performed in order to establish with greater certainty which biofilm properties are most associated with caries risk. The fact that substratum coverage was strongly correlated with caries independent of weight adjustment and on the high side of moderate when caries were weight-adjusted should be taken into consideration and support this parameter as an important factor in cariogenicity. Biofilm thickness (AMT) may also be an important factor given the strong correlations with both weight-adjusted and unadjusted caries. Although the WT may have skewed the AMT correlations, there were still strong correlations with unadjusted caries without the WT values. The biomass-caries correlations were the strongest overall, though these were limited to weight-adjusted caries. Despite the fact that correlations between some biofilm parameters were dependent on whether or not the caries data was weight-adjusted, it was clear that biofilm

architecture impacted caries risk. There is likely some overlap of the contributing biofilm properties, namely adhesion and cohesion, within the preceding parameters.

Overall, correlations of *in vitro* biofilm properties with Gbp mutant caries rates are strongest for buccal surfaces. However, in most instances sulcal surface caries scores were greater than buccal surface scores. It is likely that mutation of one or more Gbps affected the ability to colonize and form large aggregates on smooth surfaces to a greater degree than on other surfaces of the tooth. This theory is supported by studies with Gtf mutant strains of *S. mutans* where buccal cariogenicity was affected to a greater degree than sulcal cariogenicity (Tanzer *et al.* 1974, Yamashita *et al.* 1993, Munro *et al.* 1991). While a single biofilm parameter did not stand out as the definitive *in vitro* property, it was clear that there were reductions in the adhesive and cohesive properties of biofilms that accompanied mutation of Gbps.

For each of the *in vitro* biofilm properties there were statistically significant correlations with caries scores in one form or another. But for each parameter there were also exceptions that did not show statistically significant correlations. Therefore, it is possible that one must simultaneously consider combinations of biofilm parameters in order to best reveal the critical changes in biofilm architecture associated with attenuation of cariogenicity. Unfortunately linear regression using a combination of parameters requires normally distributed data, and our data were not normally distributed. Non-linear regression requires much larger sample sizes than are available. This represents a limitation of our study and our ability to analyze the data.

Nonetheless, the correlational analyses described so far have provided at least some insight into how biofilm properties affect cariogenic potential. What was a bit unexpected was that the data did not reveal an obvious pattern for how each individual Gbp contributed to the biofilm architecture. For example, all the *gbpC* mutants had reductions in biomass compared to the WT. These strains formed very few large microcolony aggregates consisting primarily of single bacteria and chains of bacteria

attached to the substratum. *gbpC* mutants with additional *gbp* mutations were the most attenuated strains, but the strain with only a *gbpC* mutation had the highest raw caries scores. It is possible that a potential loss of cohesion attributed to the loss of GbpC, manifested *in vitro* as a decrease in biomass, also resulted in a decrease in AMT. It was previously suggested that AMT was reflective of biofilm cohesion whereas biomass reflected a combination of cohesion and adhesion. It is also reasonable to expect that cohesion can be manifested in different ways and will be dependent upon the proximity of a Gbp to the cell and the strength of its affinity for glucan.

Since each of the mutants missing a single Gbp formed biofilms with significantly reduced AMT, it can be concluded that Gbps A, C, and D each contribute to cohesion to varying degrees. With recognition that other biofilm parameters did not differ statistically between mutants and WT, we nonetheless can look for clues to Gbp function among the trends that were observed. The decrease in microcolony thickness, the minor decrease in biomass and the increase in substratum coverage in the *gbpA* mutant biofilms may indicate that the contribution of GbpA is mainly to cohesion. The extracellular location of GbpA would suggest that it promotes biofilm thickness by linking microcolonies and stabilizing the structures as they extend outward from the substratum. However, a possible alternative explanation for changes in microcolony thickness in *gbpA* mutants is that the presence of GbpA allows greater expansion of individual microcolonies by increasing cohesive properties or increasing EPM content of the microcolony and reducing erosion. Similar to the *gbpA* mutant, *gbpC* mutant biofilms were decreased in thickness, increased in substratum coverage and were a bit more reduced in biomass, suggesting that GbpC may also be primarily a cohesive factor in *S. mutans* biofilm architecture. However, the location of GbpC on the cell wall may enable bridging of individual bacteria with subsequent formation of large microcolonies. Mutants missing both GbpA and GbpC were the most attenuated of the double mutants.

There also remains a possibility for a temporal relationship among the Gbps and biofilm formation. GbpC may induce dextran-dependent aggregation of *S. mutans* cells early in biofilm development but it may be GbpA and GbpD that maintain microcolony cohesiveness throughout further maturation of the biofilm. Evidence supporting regulated expression of GbpC was provided by Biswas *et al.* where expression of *gbpC* mRNA was observed to peak at mid-log phase and was extremely diminished in stationary phase cultures (Biswas *et al.* 2007). Biofilms are assumed to consist mainly of stationary phase cells, so it is possible that GbpC synthesis is at a low level in mature biofilms. Expression of *gbpC* appears to be tightly regulated and very complex. At least two two-component signaling systems have been associated with regulation of *gbpC* expression, and GbpC mediated DDAG occurs in response to several environmental stress signals (Biswas *et al.* 2007, Merritt *et al.* 2005). The tightly regulated expression coupled with reduced expression in stationary phase cultures both support the possibility that GbpC facilitates initial aggregate formation in response to cell density or environmental stress while GbpA and GbpD provide cohesiveness as microcolonies grow larger. It is also possible for this temporal theory and the previously described differential Gbp distribution theory to mutually co-exist. If the primary role of GbpC is to facilitate cohesion in initial events of biofilm formation, it would not be surprising that the *gbpC* mutants had the lowest biomass measurements but it would also be expected that *gbpC* mutants would be the most attenuated. The *gbpC* mutant *in vivo* behavior would seem to dispute these claims. Perhaps the presence of GbpA and GbpD in *gbpC* mutant biofilms can compensate for the loss of *gbpC* in the SPF rat model.

The biofilm data suggested that the GbpD contribution may be more equitable between cohesion and adhesion. However, the contribution of GbpD is the most enigmatic. The *gbpD* mutant is attenuated for sulcal caries, though glucan-based adhesion and cohesion would be expected to have the greatest effect on smooth surface caries. Combining the loss of GbpD with either GbpA or GbpC yielded mutants at the

borderline of attenuated cariogenicity that was predicated on adjustment for the weight of the animals. The data presented in Aims 1 and 2 were sufficient to confirm a role for Gbps and biofilm architecture in caries development.

Confocal Images and COMSTAT measurements of Non-Sucrose biofilms

Architectural changes in biofilms after deletion of Gbps should be directly caused by alterations in the interactions of *S. mutans* with glucan. In order to ensure that this was the case, sucrose-independent biofilms were formed to determine if the architectures of these biofilms were affected by the loss of Gbps. *S. mutans* biofilms grown in the absence of sucrose generally form sparse colonies consisting of very few cells scattered on the substratum regardless of the presence or absence of Gbps (Figure 18). They do not form the tall microcolonies indicative of sucrose biofilms formed by WT *S. mutans*. In fact, the COMSTAT-measured AMT was equivalent to a few cells (Table 9). It was not surprising that when sucrose-free WT and mutant panel biofilms were analyzed by COMSTAT, we found no significant differences among the strains (Table 9). There were no biofilm parameters measured in the non-sucrose biofilms that correlated with caries attenuation in the SPF rat model (data not shown).

It is also possible that the COMSTAT values are slight overestimates as we observed a fair amount of Brownian motion among the biofilms. This movement may have resulted in cells being represented by additional pixels as they were scanned. This also may have contributed to the variability of the samples. This was seen in all samples regardless of the presence or absence of Gbps. Thus it can be concluded that the architecture of sucrose based biofilms was shaped by the glucan-binding properties of the Gbps.

Complementation of single Gbp mutants

It was expected that mutation of Gbps would not affect the expression of other *S. mutans* genes and that the changes in biofilm architecture were a direct result of deletion of one or more Gbps. Gene complementation, by expressing a formerly mutated gene on a plasmid or reintegrated into the bacterial chromosome, can be used to ensure that a phenotype is directly caused by deletion of the gene of interest. While limitations of available antibiotic resistance markers prevented complementation of all combinations of Gbp mutation, it was decided that complementation of each individual mutant would confirm the monocistronic nature of the Gbp mutations and that addition of Gbp mutations on the same organism would not change this property. Each of the *gbpA*, *gbpC* and *gbpD* genes were individually cloned into the pSF143 integration vector and transformed into the corresponding single Gbp mutant by Dr. Min Zhu. Complementation by expressing the *gbp* genes in trans on a plasmid was not possible due to the fact that these genes are lethal when expressed in *E. coli* and, therefore, could not be cloned into a shuttle vector. Whole well images were examined visually, and it was determined that the WT phenotype was restored (Figure 20). The top panel of figure 20 shows whole well images of the WT, *gbpA* mutant strain and the complemented *gbpA* strain. The microcolonies of the *gbpA* strain are smaller and cover more of the substratum, while the complemented strain has fewer, larger microcolonies. The bottom panel showing the WT, *gbpD* and *gbpD* complemented is similar in that the microcolony size and arrangement of the complemented strain resembles the WT, and the *gbpD* microcolonies are smaller and more numerous. In the middle panel, showing the WT, *gbpC* and *gbpC* complemented strains, the *gbpC* biofilm is somewhat sparse, while the WT and *gbpC* complemented biofilms are more robust. Due to the previously described cloning limitations regarding the complementation of *gbp* mutants, the generation of these complemented strains did not chronologically coincide with the *in vivo* experiments or with the confocal microscopy. Therefore comparison of confocal analysis and *in vivo*

SPF rat analysis was not possible. Since the most marked differences between WT and *gbp* mutants was with biofilm morphology, visual comparison of the colony structure among the WT strains and individual *gbp* mutants was used to verify a reversion to the WT phenotype in complemented *gbp* mutants.

Summary

In conclusion, the deletion of any Gbp affected biofilm architecture. This was most noticeable in the significant loss of biofilm thickness where mutant biofilms were unable to generate tall microcolonies. It is proposed that this loss of biofilm thickness was due to a loss of biofilm cohesiveness. The reduction in microcolony thickness correlated with attenuation of caries in the SPF rat model. Reductions of *in vitro* biomass and substratum coverage also correlated with reductions of caries, while increases in the ratio of surface area to biovolume correlated with reduction in caries. These biofilm parameters are most likely associated with both cohesive and adhesive properties of biofilm cells. Examination of these *in vitro* biofilm parameters and the relationship with *in vivo* cariogenicity has shown a link between biofilm architecture and cariogenicity and allowed for speculation of the function of individual Gbps with regard for biofilm adhesion and cohesion. It was suggested that the cell bound nature of GbpC promotes aggregation of cells into microcolonies and that there may be temporal regulation of GbpC expression that would commit its function to initial microcolony formation. Gbps A and D may provide additional cohesive support in either linking microcolonies or providing structural support as a microcolony expands allowing upward growth. It was further suggested that possible recruitment of glucan by Gbps A and/or D could have a role in preventing erosion or sloughing of the biofilm which would allow for larger and taller microcolonies. What could not be fully explained by the experiments in Aims 1 and 2 was the mechanism for how changes in biofilm architecture lead to caries attenuation. In other words, how *in vitro* biofilm properties relate to *in vivo* biofilms and

changes in the cariogenic potential of a particular strain. For example, it could not be determined if the altered biofilm architecture led to changes in accumulation of bacteria, metabolic activity of biofilm bacteria, access to nutrients or changes in diffusion of fermentable sugars or fermentation by-products (primarily lactic acid). One would expect that Gbp mutants that had diminished cohesive abilities would accumulate fewer bacteria on the tooth surface, but this was not evident when CFU were recovered from WT and *gbp* mutant infected SPF rats. However, the CFU data from the SPF rat experiment in Aim 1 was an end point recovery of bacteria and could not explain differences in accumulation rates. Examination of *S. mutans* biofilm properties that focus on long accepted cariogenic factors may provide clues to these mechanisms and will be the goal of Aim 3. These properties include the acidogenic potential and acid tolerance responses, as well as biofilm adhesion and cohesion capabilities.

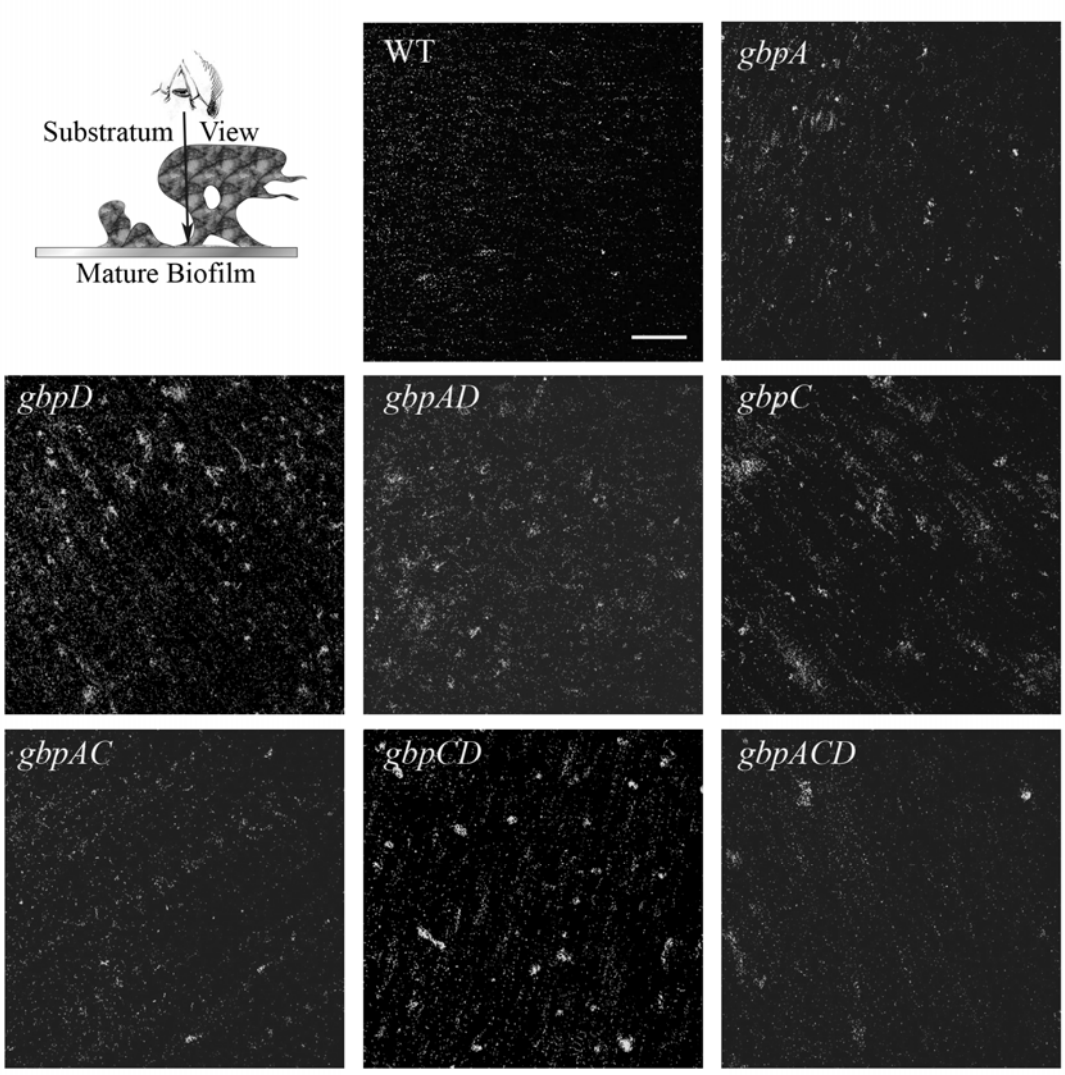


Figure 18 Overhead view of substratum from WT and Gbp mutant biofilms formed in the absence of sucrose. The top left box shows a schematic diagram of the view. Images are the first slice of a representative image stack for each strain, or the portion of the biofilm adherent to the substratum. White bar in WT image is 50µm.

	WT	*gbpA*	*gbpD*	*gbpAD*	*gbpC*	*gbpAC*	*gbpCD*	*gbpACD*
Biomass ($\mu m^3/\mu m^2$)	0.09 ±0.12	0.11 ±0.08	0.30 ±0.51	0.12 ±0.15	0.19 ±0.30	0.10 ±0.04	0.10 ±0.14	0.08 ±0.11
Substraum Coverage (%)	1.56 ±0.96	3.68 ±2.18	5.10 ±5.26	2.44 ±1.66	2.85 ±3.11	2.17 ±0.50	2.27 ±3.23	1.53 ±1.31
Average Thickness (µm)	0.30 ±0.56	0.16 ±0.22	0.62 ±1.18	0.23 ±0.38	0.45 ±0.85	0.18 ±0.15	0.17 ±0.23	0.20 ±0.35
Surface Area (10^4 μm^2)	5.06 ±7.60	5.50 ±4.20	11.71 ±19.06	5.46 ±7.70	7.59 ±12.96	4.16 ±2.31	2.66 ±3.59	3.89 ±5.99
Ratio of Surface Area to Biovolume ($\mu m^2/\mu m^3$)	4.17 ±1.00	4.35 ±0.53	3.98 ±0.53	3.88 ±0.85	3.37 ±0.39	3.99 ±0.36	2.90 ±0.63	3.85 ±0.84

Table 9 COMSTAT biofilm analysis results from non-sucrose biofilms.

Note: Results are the averaged values of 4 independent trials in which 5 random microscope fields were captured and averaged. Standard deviations of the 4 trails are shown below each value. There were no statistically significant differences.

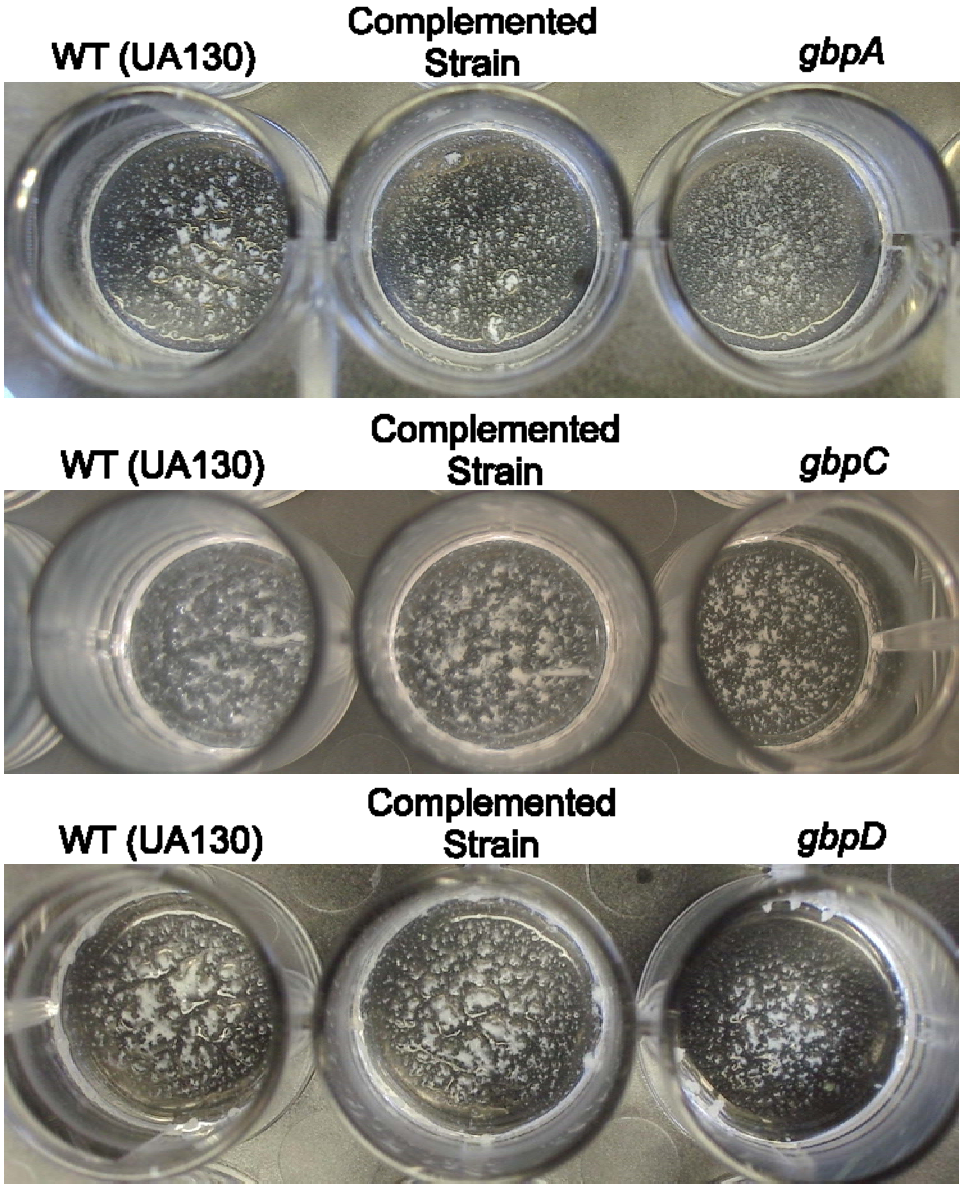


Figure 19 Individual knockouts of *gbpA*, *gbpC* and *gbpD* were complemented with either *gbpA*, *gbpC*, or *gbpD* respectively by reintegration into the *S. mutans* chromosome from plasmid pSF143. Biofilms were grown in 24-well plates overnight, media was aspirated off and whole well photographs were taken. Top) WT (left), *gbpA* (right), *gbpA*-complemented (center); Middle) WT (left), *gbpC* (right), *gbpC*-complimented (center); Bottom) WT (left), *gbpD* (right) and *gbpD*-complimented were photographed to show gross morphological differences between mutant and WT biofilms and the restoration of the WT morphology when the mutation was complemented. Images are courtesy of Zhu, M, University of Iowa, 2006.

CHAPTER 4
CHARACTERIZATION OF THE BIOFILMS FORMED BY GBP MUTANTS WITH RESPECT TO THE PROPERTIES ASSOCIATED WITH *S. MUTANS* VIRULENCE

The main properties associated with virulence in *S. mutans* are acidogenicity, aciduricity, adhesion and cohesion. Acidogenicity of *S. mutans* is recognized through its ability to metabolize a wide array of carbohydrates, with lactic acid being the principle end product. The aciduric properties, due to the robust acid tolerance response (ATR), allow continued metabolism and lactic acid production at low pH. We have shown that there are no statistically significant differences in the growth rates of the Gbp mutant strains, but will further investigate the ability of these strains to produce acid through sugar fermentation.

Acid tolerance in *S. mutans* is well documented (Quivey *et al.* 2001, Len *et al.* 2004b, Welin-Neilands&Svensater 2007, Len *et al.* 2004a, Belli&Marquis 1991). Though it was not expected that any of the Gbp mutations would directly affect expression of ATR genes, it was still necessary to consider that certain Gbp mutations, or combinations thereof, might in some way alter the *S. mutans* ATR thereby explaining attenuation of cariogenicity. For example, both acidogenicity and aciduricity can be affected by biofilm architecture. Differences in diffusion distances or concentration of channels throughout the biofilm may affect localized pH. Hata *et al.* showed that cell pellets with different concentrations of water-insoluble glucan and water-soluble glucan, as well as fructan, affected the rate of hydrogen ion diffusion. However these experiments were performed using centrifuged cell pellets of cultures which would have destroyed the natural architecture of the biofilm (Hata&Mayanagi 2003). The objective of this aim was to determine if deletion of one or more Gbps affects *S. mutans* acid-

related virulence properties directly or indirectly via the changes in biofilm architecture previously documented.

In order for the acidogenic nature of *S. mutans* to induce tooth decay, they must accumulate significant numbers on the tooth surface. Several studies mentioned earlier in this thesis have suggested a link between the aggregative properties of an *in vitro S. mutans* biofilm and cariogenicity (Hamada&Slade 1980, Yamashita *et al.* 1993, Gibbons 1996). Another objective of this aim will be to compare the relative strength of mutant biofilms with that of WT biofilms in order to determine if differences in biofilm adhesion or cohesiveness can explain differences in cariogenicity.

Materials and Methods

Planktonic Glycolytic pH Drop to Measure Acidogenicity

S. mutans UA130 (WT and mutant panel) were grown in 15 ml of Todd Hewitt broth overnight at 37°C anaerobically. The OD_{600} was typically between 1.0 and 1.2 for the overnight cultures and these were normalized to an OD_{600} of 1.0. Cells were pelleted by centrifugation at room temperature at 9000xG for 10 minutes. Cells were rinsed twice by resuspension in 10 ml of a pre-warmed (37°C) 50 mM KCl, 1mM $MgCl_2$ solution that was adjusted to pH 7 just prior to rinsing the cells. After pelleting cells following the second rinse, the cells were resuspended in 9 ml of the 50 mM KCl, 1mM $MgCl_2$ solution and the pH was adjusted to 7.2. After the pH stabilized, 1 ml of 10% glucose was added and the pH was measured and recorded every 30 seconds until it reached a plateau. A 50 mM KCl, 1mM $MgCl_2$ solution without bacteria served as a negative control by recording the pH after addition of glucose for the same time as the experimental samples (Belli&Marquis 1991).

Planktonic Acid Tolerance

This procedure was adapted from Ma *et al*. (Ma *et al.* 1997). Cultures of *S. mutans* were grown overnight in 15 ml of Todd Hewitt broth at 37°C anaerobically. These overnight cultures were diluted 1:2 and incubated at 37°C until the OD_{600} reached 0.7. At this point 2.5 ml of each sample culture was added to 6 ml each of 37°C pH 7.0 and pH 5.0 TYG broth.

The TYG broth was made by adding 0.6% glucose to 2xYT (tryptone, yeast extract) and mixing 1:1 with 100 mM potassium phosphate buffer. For pH 7.0 TYG the above was mixed with pH 7.0 phosphate buffer, and to make the pH 5.0 TYG it was mixed with pH 4.5 phosphate buffer. The final concentration of the TYG was 0.3% glucose and 50mM potassium phosphate.

Cultures were grown in TYG at their respective pH for one OD_{600} doubling. The cultures were then diluted 1:100 into 1% tryptone broth at pH 3.0 or pH 7.0 (control) and incubated for 2.5, 5 and 10 minutes. At the respective time points, aliquots were removed and diluted 1:100 in pH 7.4 PBS buffer. The diluted aliquots were plated on TH agar and incubated overnight anaerobically at 37°C. Acid tolerance data was expressed as a percentage of CFU from pH 3-incubated biofilms relative to pH 7-incubated biofilms.

Biofilm Acid Tolerance

S. mutans cultures were grown overnight in CDM to an OD_{600} of 1.0. 100 µl of the overnight culture for the wild-type and each mutant were sub-cultured into 1.5 ml of CDM with 5% sucrose in a 24-well plate as in previous biofilm experiments. Biofilms were grown overnight at 37°C in 5% CO_2 with rotation (20 rpm). The biofilms were rinsed with PBS and 1 ml of 1% peptone (pH 7.0) was added to control wells for each strain. To four additional wells for each corresponding strain, 1 ml of 1% peptone (pH 3.0) was added and incubated for 0, 15, 30, and 45 minutes. At the specific time points

the wells were rinsed 1x with PBS (pH 7.4) and then 1 ml of PBS (pH 7.4) was added to the wells. The wells were sonicated to disrupt the biofilms and then the sample was diluted and plated on TH plates. After 48 hours incubation in an anaerobic chamber at 37°C, the CFU were recorded. Biofilm acid tolerance data was expressed as a percentage of CFU from pH 3-incubated biofilms relative to pH 7-incubated biofilms.

Biofilm SDS Tolerance

To measure tolerance of biofilm bacteria to killing by SDS, overnight *S. mutans* biofilms were prepared in the same way as described for the experiments that measured biofilm acid tolerance. Biofilms were then washed 2x with room temperature PBS (pH 7.4). One ml of PBS was added to control wells, and then experimental wells were incubated with PBS and either 0.01% SDS or 0.1% SDS for 10 minutes. The ten minute time point was chosen because it was determined that substantial killing occurred after this amount of time at the two concentrations of SDS chosen. After 10 minutes, the PBS/SDS mixture was aspirated, the wells were rinsed 1x with PBS and then 1 ml of PBS was added to the wells. The wells were sonicated to disrupt the biofilms and then the samples were diluted and plated. CFU were counted after 2 days incubation in an anaerobic chamber at 37°C and data were expressed as a percentage of CFU from SDS-incubated biofilms relative to PBS-incubated biofilms.

Measuring the Capacity of *S. mutans* Culture to Buffer
Changes in the pH of Growth Medium

In order to determine if Gbps played any role in buffering the surrounding media, we first attempted to determine if an *in vitro*, planktonic culture of *S. mutans* would buffer changes in the pH of the growth medium and if there were differences based on the presence or absence of Gbps. Cultures of *S. mutans* (UA130), UA130-*gbpA*, U130-*gbpC,* UA130-*gbpD,* and UA130-*gbpACD*, were grown overnight in 75 ml of Todd Hewitt (TH) broth anaerobically at 37°C. 75 ml of uninoculated TH broth was used as a

negative control. Each culture was inactivated with NaN_3 (Sigma-Aldrich, Milwaukee, WI) at a final concentration of 0.1 mg/ml. The optical density was measured and when necessary, cultures were normalized to an OD_{600} of 1.00 (±0.04). Each culture was divided into two 30 ml aliquots to ensure reproducibility and consistency of the pH probe. The pH of each culture was measured and all were at an identical pH (± 0.01). However, the media-only (negative control) had a pH close to neutral (7.0) so it was adjusted to 4.8 with 1N HCl (Fisher Scientific, Pittsburgh, PA) to give it the same starting pH as the culture samples.

To each culture, 1N NaOH (Fisher Scientific, Pittsburgh, PA) was added in 100 µl increments and the pH was read using an Accumet AB 15 pH meter and probe (Fisher Scientific, Pittsburgh, PA) after each increment. The pH and amount of NaOH was recorded for comparison among the mutants. Two repetitions were performed for each O/N sample and the negative control. Two independent trials were performed for this study. The experiments were then repeated using additional *gbp* mutants: UA130-*gbpAC*, UA130-*gbpAD*, and UA130-*gbpCD*.

Mechanical Stress Response

In order to determine the ability of a biofilm to withstand mechanical stress, we directed a stream of water at experimental biofilms with sufficient force to cause disruption. Wild-type and mutant *S. mutans* biofilms were grown in 24-well culture plates as previously described. Duplicate plates were grown; one plate acted as a set of control biofilms that were not subjected to the water stream, while the experimental plate was subjected to the water stream. After aspiration of the growth media (CDM-5% sucrose), the experimental plate was held vertically. The nozzle from a MiliQ water purification system was held in a perpendicular orientation at the exact top of the well for each sample. The water was jetted into the well for exactly 2 seconds and then stopped. After all wells were jetted, the plate was washed by gently submerging it in sterile PBS to

remove dislodged, non-adherent bacteria. The control plate was washed in a similar manner. Additional experiments were done in which the control plate was not washed and the overnight media was simply aspirated and 1 ml of PBS was added to the wells. After washing, the plates were patted dry and 1 ml of PBS was added to each well. Each sample was sonicated, diluted and plated. The results were expressed as a percentage of CFU recovered from jetted wells relative to CFU from non-jetted wells.

Adhesion to a *S. sanguinis* Biofilm

As *S. mutans* is not considered a primary plaque colonizer, it was proposed that one role of Gbps could be to aid in adherence to glucan from an existing biofilm. To examine this possibility, we examined the initial adhesion events of *S. mutans* wild-type and Gbp mutant strains to a pre-formed *S. sanguinis* biofilm. *S. sanguinis* is a primary plaque colonizer.

Flat bottom, 96-well culture plates were used for adhesion experiments. Planktonic overnight cultures of *S. sanguinis* were grown in TY broth (3% tryptone, 0.06% yeast extract) anaerobically at 37°C. On day 2, plates were incubated in 50 µg/ml BSA (Fisher Scientific, Pittsburgh, PA) in $NaHCO_3$ (Fisher Scientific, Pittsburgh, PA) for 1 hour at 37°C. While the plate was incubating in BSA solution the overnight culture of *S. sanguinis* was diluted into 20 ml of pre-warmed 2xTY broth to an OD_{600} of 0.08. This OD provided an inoculum of $1.0x10^6$ CFU that was critical for forming a stable *S. sanguinis* biofilm. 75 µl of a 4%-sucrose/2%-glucose solution (control wells have only 2%-glucose) was added to sample wells and 75 µl of the *S. sanguinis* in 2xTY broth was added to each well (except the media-only control wells). The biofilm was grown without rotation overnight at 37°C in 5% CO_2. On day 3, wells were aspirated and rinsed once with PBS and then incubated for 30 minutes with 0.1% BSA in PBS. Frozen stocks of test bacteria were thawed, diluted to working concentration (10^7 bacteria) and added to the *S. sanguinis* biofilms along with 10,000 units/ml of catalase to prevent killing of the

S. mutans by hydrogen peroxide generated by *S. sanguinis*. An aliquot of each stock sample was plated to determine the CFU of each experimental inoculum. 50 µl of 0.2% BSA in PBS was added to each well followed by 50 µl of the specific sample bacterial preparation. The samples were incubated for 1, 2 and 3 hours at 37°C in 5% CO_2 with rotation (20 RPM). At each time point, samples were washed 5x with PBS and then incubated for 2 additional hours at 37°C in 100 µl TY supplemented with 1% glucose. After incubation in the TYG, the biofilms were disrupted by vigorous pipetting and transferred to 900 µl of PBS. The samples were then sonicated to break up aggregates, diluted and plated on either TH plates for total biofilm counts or MSKB plates selective for *S. mutans*.

Statistical Analysis

The curves in the planktonic acidogenicity assay were generated by calculating the mean values from 3 independent trials at each time point. One-way analysis of variance was used to examine the values at each time point for statistically significant differences. The values presented in the acid-tolerance assays are the means of the values from three independent trials. One way ANOVA and the Tukey post-hoc test were used to determine statistically significant differences from the WT values. Examination of the buffering capacities of the Gbps involved the same statistical treatment as the acid tolerance assays.

The mechanical stress tests to determine the strength of a biofilm were performed three times. Values expressed are the means of the values from the three experiments. One way ANOVA and the Tukey post-hoc test were used to determine statistically significant differences from the WT values. Examination of *S. mutans* binding to *S. sanguinis* biofilms experiments were performed three times. The values shown in the binding curves are the means of the three values. One-way ANOVA and the Tukey post-hoc test were used to find possible significant differences among the tested strains.

Results and Discussion

Glycolytic pH Drop

In order to examine the role that acid and acid diffusion plays in the biology of a *S. mutans* biofilm, we must first determine that the mutant strains are capable of producing acid at the same level as WT strains. Essentially, it is a test of the metabolic rates of acid production by the mutant strains to see if the Gbp mutations had any adverse effects on the organisms' ability to metabolize sugar. To do this we observed the rate at which WT and mutant cultures, when resuspended in a solution, lowered the pH of this solution following the addition of glucose. Our results indicated that all of the single mutants reduced pH at the same rate as the WT (Figure 20). Based on these results, we concluded that the Gbp mutations had no negative effect on the ability of *S. mutans* to metabolize sugar and produce acid. This result was expected due to the fact that we saw no significant differences in growth rates among the WT and mutant strains. This supports the fact that biomass and substratum coverage or biomass and thickness are important cariogenicity predictors. The more metabolically active bacteria in a microcolony, the lower the local pH would be.

Measurement of the rate of glycolytic pH drop also provides clues to the acid tolerance mechanism of the bacterial cells. A deficiency in the ATR could cause a decrease in F-ATPase activity and increased pH within the cell (Murata *et al.* 2008). An increase in the internal pH of the cell would decrease the efficiency of the glycolytic enzymes and glycolysis would slow as the pH decreased. While the results of the above acidogenicity tests suggest a functioning ATR in all the single Gbp mutants, we further investigated the tolerance of the mutant panel with a direct acid challenge.

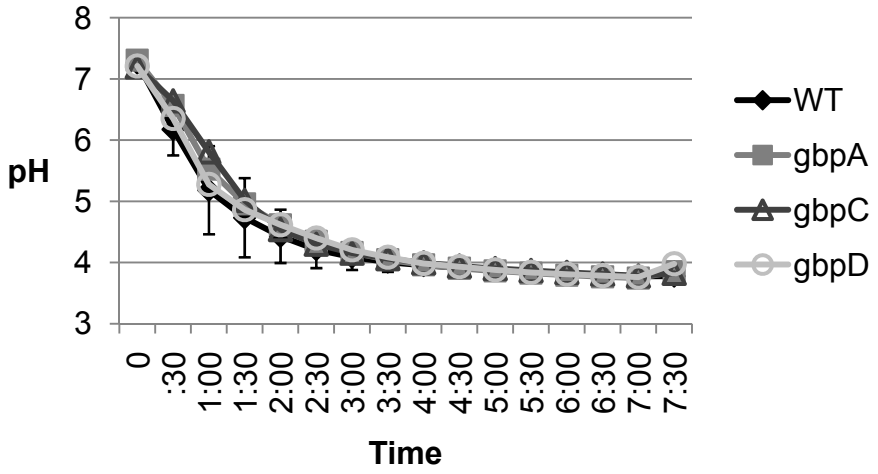


Figure 20 Planktonic acidogenicity test. Measurements of the drops in pH of cultures suspended in an un-buffered solution, normalized to pH 7.2, were made after the addition of 1% glucose. WT and all single *gbp* mutants were tested. Plotted points are the mean values of three independent trials. Error bars are shown for the WT and represent one standard deviation from the WT.

Measurement of acidogenicity in biofilm cultures consistent with the planktonic measurements was not feasible. It would have been nearly impossible to determine the concentrations of biofilm bacteria and to normalize these cultures to ensure identical numbers of bacteria at the start of the experiment. This prevented measurement of the rate of glycolytic pH drop in biofilm cultures. However, *in vitro* biofilm cultures of WT and Gbp mutants had a similar terminal pH of 4.5 (data not shown). This suggested that, similar to the planktonic acidogenicity studies, the *gbp* mutations did not negatively alter the acid producing potential of *S. mutans* cells in biofilms.

Planktonic Acid Tolerance

Having confirmed that the WT and Gbp mutant strains all had the same potential for acidogenicity, we measured the planktonic acid tolerance to determine if the Gbp mutations had any effect on the ability of the bacteria to undergo an acid tolerance response. One possibility that was considered was that a Gbp mutation might alter cell

wall structure making the bacteria more acid sensitive. In contrast, the results in Table 10 show that not only were there no significant decreases in acid tolerance, the *gbpACD* mutant actually showed a significant increase in acid tolerance in unadapted culture conditions. This increase was seen at the earliest time point (2.5 minutes) and was not observed at later time points. Since this reduced sensitivity to acid killing, albeit limited to an early time point, could be considered an advantage over the wild-type, it was determined that there was no diminished level of acid tolerance in the Gbp mutant strains that could account for attenuation of cariogenicity. However, earlier initiation of acid tolerance suggests the possibility that the most severely attenuated strain has a mechanism for early induction of the acid tolerance response.

Biofilm Acid Tolerance

The acid tolerance experiments with planktonic cultures determined that Gbp mutations had no negative effect on the ability of *S. mutans* to tolerate acidic environments, and for the *gbpACD* mutant may have resulted in premature induction of the ATR. As previously discussed, biofilm architecture can affect acid diffusion. Therefore, we next investigated the acid tolerance of WT and Gbp mutant strains in biofilm cultures. Table 11 shows that there were no decreases in acid tolerance among any of the mutant biofilms when compared to the WT biofilm. As with planktonic cultures, acid tolerance differences found among some mutants were in the direction of improved acid tolerance. Increased survival of each of the mutants examined at the first time point was observed, though the increases were only significant for the *gbpA* and *gbpACD* strains. Mutant survival rates matched that of the WT in later time points similar to the pattern observed for the *gbpACD* mutant in planktonic culture.

Biofilm SDS Tolerance

The results of the acid killing experiments suggest that there is an earlier ATR or a higher basal level of ATR gene expression in some or all of the Gbp mutants.

However, enhanced survival at early time points for the single knockout Gbp mutants, though not statistically significant, was only observed in biofilm cultures. Therefore, it was important to determine the extent to which changes in biofilm architecture may have altered acid diffusion and been responsible for early resistance to acid killing. In order to test this, another biofilm survival experiment was performed using sodium dodecyl sulfate (SDS) instead of acid. This eliminated the ATR as a variable. Figure 21 shows that there were no significant differences in SDS killing among the WT and the Gbp mutant strains.

The SDS tolerance data would seem to eliminate hindrance of diffusion, secondary to changes in biofilm architecture, as an explanation for increases in acid tolerance at early time points. This does not discount the possibility that differences in localized pH exist within biofilms or that the distribution of high or low pH regions could vary among WT and Gbp mutant strains. However, if these differences affect cariogenicity, it is not due to a negative effect on acid tolerance. The possibility remains that localized alterations in acid distribution initiate acid tolerance more quickly in certain mutants by affecting gene expression of one or more ATR genes or other stress response genes.

Planktonic Acid Challenge – Percent Survival				
Unadapted Cultures	**T = 0**	**T = 2.5 minutes**	**T = 5 minutes**	**T = 10 minutes**
WT	134.03 ± 28.50	3.96 ± 3.94	0.36 ± 0.38	0.02 ± 0.03
gbpA	107.68 ± 41.86	0.31 ± 0.38	0.09 ± 0.10	0.01 ± 0.02
gbpC	105.16 ± 25.82	0.41 ± 0.50	0.08 ± 0.03	3.34 ± 6.66
gbpD	100.23 ± 11.70	0.69 ± 0.55	0.17 ± 0.08	0.04 ± 0.03
gbpACD	103.27 ± 25.66	**34.85 ±** 24.70	4.27 ± 5.26	0.32 ± 0.53
Acid-Adapted Cultures				
WT	102.84 ± 26.16	88.12 ± 14.72	78.83 ± 20.98	56.24 ± 17.41
gbpA	109.56 ± 25.08	89.48 ± 15.85	77.40 ± 12.65	34.89 ± 23.86
gbpC	120.02 ± 30.48	92.51 ± 17.50	60.18 ± 43.54	26.24 ± 19.57
gbpD	129.53 ± 16.52	75.26 ± 13.46	70.66 ± 7.50	25.07 ± 12.07
gbpACD	127.81 ± 28.06	89.92 ± 8.20	75.78 ± 20.59	54.80 ± 28.68

Table 10 Planktonic acid tolerance.

Note: Shown are the percentages of surviving cells in planktonic cultures after acid challenge compared with survival in unchallenged cultures. Values represent the average of three independent trials +/- standard deviation. **Bold** values are significantly different than the WT as determined by ANOVA using the Tukey post-hoc test.

Biofilm Acid Challenge – Percent Survival				
	Time = 0	**Time = 15 minutes**	**Time = 30 minutes**	**Time = 45 minutes**
WT	100.00 ± 0.00	42.59 ± 15.39	27.87 ± 14.76	19.99 ± 18.08
gbpA	100.00 ± 0.00	**72.56** ± 11.22	35.50 ± 9.64	23.70 ± 15.17
gbpC	100.00 ± 0.00	69.79 ± 22.74	38.37 ± 23.25	21.26 ± 13.25
gbpD	100.00 ± 0.00	64.26 ± 12.01	44.90 ± 23.32	31.10 ± 17.00
gbpACD	100.00 ± 0.00	**68.33** ± 12.67	28.46 ± 4.70	19.27 ± 7.75

Table 11 Biofilm acid tolerance.

Note: Shown are the percentages of surviving cells in biofilms after acid challenge compared with unchallenged cultures. Values represent the average of three independent trials +/- standard deviation. **Bold** values are significantly different than the WT as determined by ANOVA using the Tukey post-hoc test.

There are no published reports showing increases in ATR or stress response genes in any of the Gbp mutants. However, in work by Hazlett *et al.* in a germ-free rat study where WT and *gbpA* cariogenicity was examined, a much higher proportion of *gtfBC* recombinant organisms were recovered from rats infected with the *gbpA* strain (Hazlett *et al.* 1998). This recombination was determined to be RecA-dependent as 2-D gel electrophoresis revealed increased intensities of 2 spots that were identified as RecA isoforms. However, real time (RT)-PCR analysis of WT and *gbpA* biofilms showed no increase in *recA* expression. Further analysis suggested that this increased recovery of recombinant organisms was the result of natural selection and not an increase in recombination events. However, a transient increase in *recA* expression at an earlier point in biofilm formation or maturation could not be ruled out (Banas *et al.* 2007).

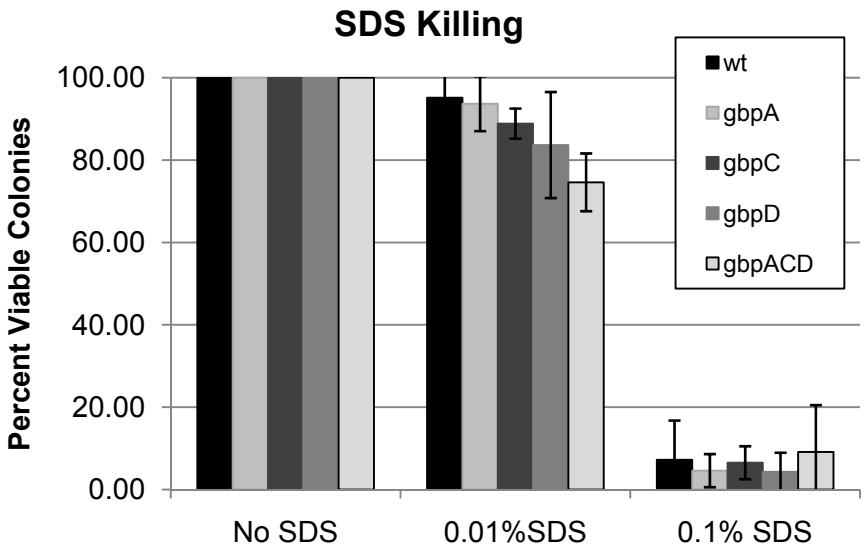


Figure 21 The effect of SDS on bacterial survival in biofilms. Shown are the percentages of surviving cells from overnight biofilms challenged with 0.1% or 0.01% SDS compared with recovered cells from unchallenged biofilms. Each value is the mean of 3 replicate biofilms. Error bars represent one standard deviation.

An alternative explanation for why some Gbp mutants were more acid resistant at early time points is that differences in growth phase or pH of the initial overnight inoculums could have been a contributing factor. However, in planktonic experiments the pH of the overnight cultures was measured to ensure that cultures started from identical conditions. The diluted cultures were also grown for at least one population doubling and carefully monitored to ensure similar optical densities throughout the experiment. We performed this experiment under the assumption that ATR gene expression would revert to that of an unadapted culture after a population doubling at a high pH (7.4). It is possible that this gene expression was not shut off or there was a delay in the signal to down-regulate these genes in the mutant strains.

It may be worthwhile to examine the possibility that differences in the expression levels of stress response genes accompany mutations in particular *gbp* genes or occur as a consequence of unique biofilm architecture. Testing the ability of bacteria to undergo DNA repair after exposure to UV irradiation or H_2O_2 treatment at various points in planktonic and biofilm growth could test levels of stress response gene expression without having to account for the pH of the media (Quivey *et al.* 2001, Quivey *et al.* 1995). Early time points in the acid tolerance response or relevant population densities could be used for microarray analyses or real-time PCR of specific ATR or stress response genes. However this is a particularly large undertaking that was beyond the scope of this aim.

All of the mutant strains that were examined for biofilm acid tolerance showed enhanced survival of about the same magnitude. Only the values for the *gbpA* and *gbpACD* mutants were statistically significant, presumably because the others had larger standard deviations. An important point is that some of these strains were among those that showed significant attenuation in caries. The most attenuated strain, *gbpACD*, showed a transient increase in planktonic acid tolerance. This makes differences in acid

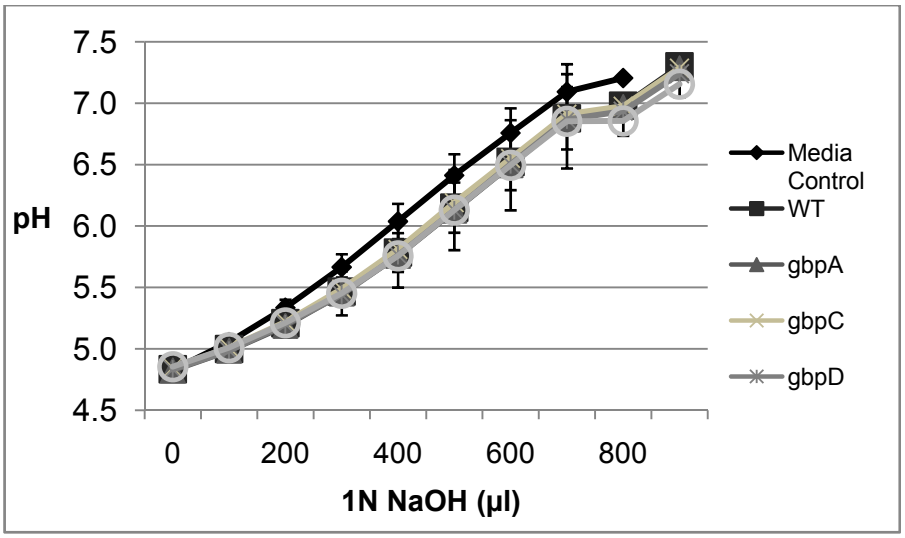
tolerance, on the scale that we measured, an unlikely explanation for biofilm architecture-based caries attenuation.

If differences in acid production, distribution and diffusion contribute to the different levels of cariogenicity among the WT and Gbp mutants, then it must occur in a localized manner that is beyond our technical capabilities of measuring.

Gbp buffering capacity

We also examined the possibility that glucan-binding proteins could provide a direct buffering ability to the media adjacent to the cell or bind glucan that may provide this buffering effect. In order to determine the buffering capacity of Gbps we examined the ability of an overnight culture to resist increases in pH caused by the addition of base (Brogden 2009). Figure 22A shows that the pH of WT and mutant cultures increased in an identical fashion with the addition of equal concentrations of base. Dibdin *et al*. demonstrated a buffering effect of both *S. mutans* cells as well as EPM, which we also see in the examples in Figure 22A-C (Dibdin&Shellis 1988). Both the cell pellets and supernatants were examined after incubation with sucrose to determine if Gbps affected distribution of glucan and if this in turn affected the buffering capacity of the cells. There were no differences among the WT or mutant strains when the cultures were incubated overnight with sucrose (Figure 22B) and this held true when we observed cell pellets and supernatants from this incubation (Figure 22C).

A.



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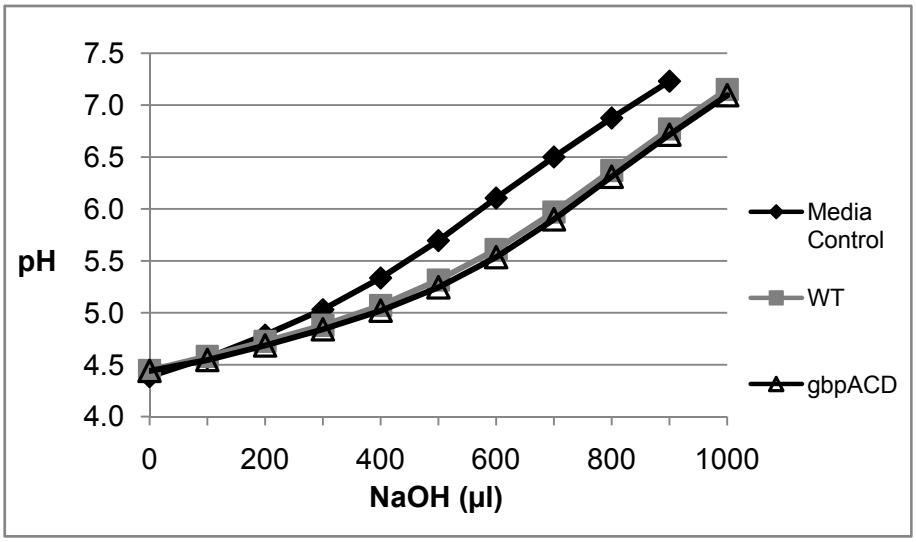


Figure 22 Buffering capacity of *S. mutans* and Gbp mutant strains. (A) The amount of NaOH (µl) needed to raise the pH of overnight cultures of WT and select mutant strains. Values represent the mean of 3 independent trials and error bars represent one standard deviation from the mean. (B) WT and *gbpACD* cultures were incubated overnight with sucrose and the amount of NaOH needed to raise the pH to 7.0 was measured. A representative of 2 independent trails is shown. (C) The amount of NaOH needed to raise the pH of cell pellets and supernatants of overnight WT and *gbpACD* cultures incubated with sucrose to pH 7.0 was measured. A representative of 2 independent trials is shown.

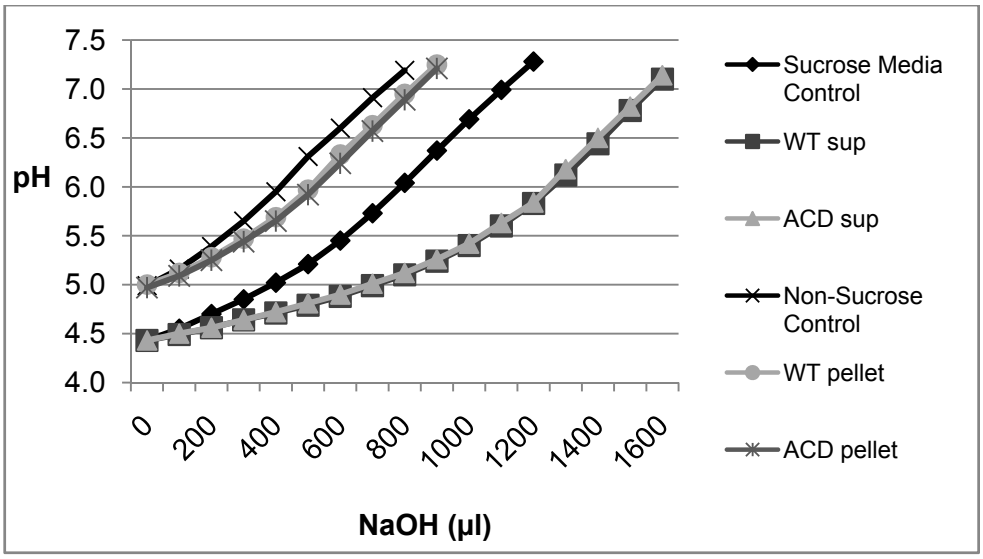
C. 

Figure 22 Continued

While the differences that were observed in aciduric properties did not provide an explanation for the specific differences in cariogenicity among the mutant panel, correlations between COMSTAT measured biofilm architectural parameters and caries suggested that differences in the adhesive and cohesive properties of mutant biofilms may explain the attenuation of certain strains. Glucan has been cited as the primary component in the ability of *S. mutans* to accumulate large enough numbers to be cariogenic, and the role of Gtf enzymes in catalyzing the synthesis of glucan has been well documented (Yamashita *et al.* 1993, Mattos-Graner *et al.* 2004). There have been studies done that show a reduction in the adhesive and cohesive properties of individual Gbp mutants. Matsumura *et al*. demonstrated a decrease in the ability of mutants that did not express GbpA or GbpC to adhere to glass surfaces in the presence of sucrose (Matsumura *et al.* 2003). However, Hazlett *et al*. observed an increase in sucrose-dependent adherence to glass surfaces in *gbpA* mutants (Hazlett *et al.* 1998). Inactivation of *gbpD* was shown by Shah *et al.* to reduce adherence of *S. mutans* to nichrome wires (Shah&Russell 2004). Multiple investigators have demonstrated reductions in dextran-dependent aggregation in *gbpC* mutant strains (Lynch *et al.* 2007, Sato *et al.* 2000). Changes in the ability of *S. mutans* to adhere to smooth surfaces (eg. glass) or to form cohesive aggregates in the presence of dextran, are key factors that contribute to alterations of biofilm architecture and ultimately changes in cariogenic potential. In this section of aim 3 we addressed the roles of specific adhesion and cohesion factors thought to contribute to biofilm architecture and cariogenicity.

Mechanical Stress Response

By observing the ability of WT and Gbp mutant biofilms to withstand the mechanical stress of a jetted stream of water, it was possible to quantitatively measure the overall strength of a typical *in vitro* biofilm formed by each strain. The results of this assay (Figure 23) showed that while the percent retention among the WT and mutant

strains was quite variable, there were no significant differences when the control wells were gently rinsed. It was speculated that this rinse might remove more than just non-adherent cells and that this could affect the outcome. When the control wells were not rinsed, three strains, *gbpC, gbpAC* and the *gbpCD*, showed significantly greater retention than the WT. The strains that showed significantly greater retention compared to the WT when measured against unwashed controls were also the strains that had lower COMSTAT biomass measurements than the WT. The *gbpAD* and *gbpACD* strains followed a similar pattern but the increase in retention was not statistically significant. The *gbpD* strain had a lower biomass measurement than the WT but did not follow the trend with respect to the percent retention. However, because the strains were quite variable for cariogenic potential, this method of measuring biofilm strength may not be the best means of linking cohesion with caries.

On the surface these results seem to be the opposite of expectations. But an alternative interpretation can be derived by focusing on the differences in percent retentions between experiments with washed and unwashed controls. We propose that only the WT, *gbpA*, and *gbpD* strains were capable of building a biofilm under the conditions of constant rotation that included bacteria that were susceptible to removal by washing. In other words, these strains had the capacity to build a mature biofilm that included more tenuous associations among some proportion of the bacteria. The associations were strong enough to form despite the mechanical stress that accompanies constant rotation but could not withstand the forces associated with washing. Thus, it appears that the loss of GbpC, or both extracellular Gbps (A & D), changes the nature of the bacterial associations within the biofilm. These changes may contribute to attenuation of the strains, though the attenuation may at times be mitigated by other biofilm changes, for example the increased substratum coverage by the *gbpC* mutant.

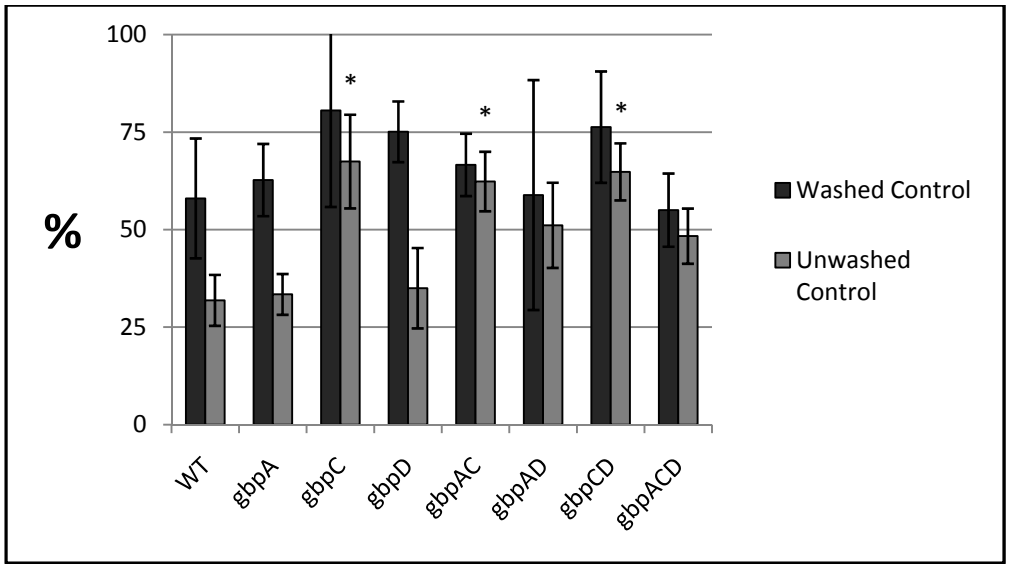


Figure 23 The percentages of CFU retained on a plate that has been subjected to a water stream directed from a nozzle at the biofilm, when compared to biofilms that were gently rinsed (dark bars) or not rinsed at all (lighter bars). An asterisk "*" indicates strains where the percent retentions compared to unwashed biofilm controls were significantly greater than the WT ($p < 0.01$)

Adhesion to *S. sanguinis* Pre-Formed Biofilm

Elimination of Gbps has a definite effect on biofilm architecture and certain of these changes in architecture are correlated with caries reduction. We also entertained the possibility of a role for Gbps in initial attachment to a pre-formed biofilm. *S. mutans* is generally considered a late colonizer to the tooth surface and therefore would most likely colonize an existing biofilm. Glucan-producing oral streptococcal species, such as *S. sanguinis* or *S. salivarius*, are considered early colonizers of the tooth pellicle. Although not as prolific glucan producers as *S. mutans*, the EPM they synthesize may represent possible adhesion targets for *S. mutans*. We grew *S. sanguinis* biofilms in the presence of sucrose to create an existing glucan-rich biofilm to examine binding of *S. mutans* WT and Gbp mutants (Drake 2009). Based on the results in Figure 24, there was no loss of binding detected for either the *gbpACD* or the *gbpA* mutants compared to the

WT strain. The *gbpACD* mutant was examined because it lacked all Gbps and was the most seriously attenuated in the rat model. We predicted that if Gbps were critical for binding to glucan in a pre-existing biofilm, then the *gbpACD* would show the largest loss of binding ability. Surprisingly, the *gbpACD* showed no loss of binding to the *S. sanguinis* biofilm and in most cases even showed an increase in binding, though the increases were not statistically significant (Figure 24A). Despite our attempts to block non-specific binding sites on the *S. sanguinis* biofilm, we could not discount the fact that non-specific binding may have played a role in the increased recovery of the *gbpACD* strain since this strain showed the highest binding to non-biofilm control wells. We subtracted out the CFU from non-biofilm control wells to account for adhesion to the plastic surface but the *gbpACD* still showed higher (albeit not statistically significant) levels of adhesion. It is possible that the *gbpACD* does possess some adhesion advantage over the WT strain but due to potential non-specific binding, we cannot fully assume that this binding increase is sucrose-dependent.

We also examined the *gbpA* strain because this strain showed the highest degree of substratum coverage *in vitro* and also showed a slight increase in cariogenicity *in vivo*. The binding of the *gbpA* strain was very similar to the WT strain. The results of the three strains remained unchanged even after the results were normalized to the initial inocula to account for slight variations in inoculum size (Figure 24C).

Because neither mutant strain showed a diminished ability to adhere to an established, glucan-containing biofilm, nor a significant increase in binding, we conclude that Gbps are not major contributors to initial attachment to a pre-formed biofilm.

Summary

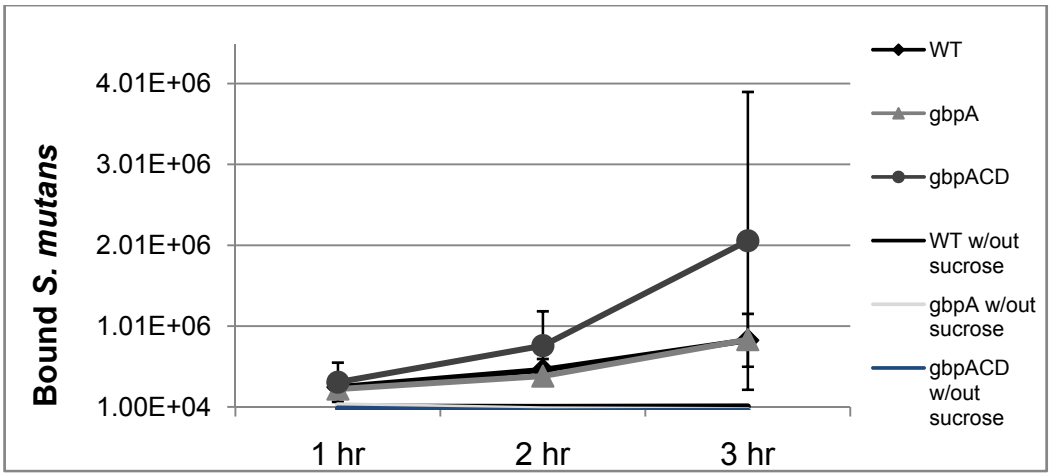
The results of Aim 3 show that while there was a possibility that Gbp mutants initiate an acid tolerance response or stress response earlier than the WT strain, there is no diminishment of their ability to produce acid or tolerate acidic environments. It was

anticipated that one of the explanations for attenuation of Gbp mutants would be that altered biofilm architecture would affect the rates of acid diffusion through the biofilm. The biofilm acid tolerance and SDS tolerance experiments showed no evidence of differences in the ability of acid or detergent to penetrate a WT or Gbp mutant biofilm. Diffusion distances can also impact nutrient availability to deeper regions of the biofilm and create localized areas of higher or lower metabolic activity. However, the experiments in this aim measured the global tolerance of the biofilm to an environmental stress and there may be subtle pH or nutrient gradients that were not accounted for. Investigation of the breadth or number of low pH zones may provide a more clear understanding of the impact of altered biofilm architecture on acid/nutrient diffusion. However, the measurement of pH in the minute localizations that would be necessary to establish a pH gradient or regions of differential pH would require nanometer-sized pH probes and are therefore beyond the technical capabilities of this lab. It is possible that mutant strains may differ in the distribution and number of low pH zones from the WT. Based on the fact that all of the Gbp mutants that were examined showed an increased acid tolerance at the earliest time point, one could state that there were higher percentages of metabolically active bacteria in biofilms formed by these strains. However, it is possible that the loss of biofilm cohesion in Gbp mutants prevented accumulation of bacteria in large structures to form concentrated zones of low pH that would promote caries development. Therefore, examination of global biofilm acid tolerance, coupled with planktonic acid tolerance and acidogenicity, suggests that the ability of *S. mutans* to form large structures and accumulate in large numbers is a key to cariogenicity and that Gbps facilitate cariogenicity by allowing accumulation of large structures.

The response of *S. mutans* WT and Gbp mutant biofilms to mechanical stress suggest that the loss of Gbps impact the cohesive abilities of the biofilm to a greater degree than the adhesive abilities. The strains that showed the greatest retention of biofilm cells and that did not demonstrate loss of cells during gentle washing, were also

strains that were the least able to form large microcolonies *in vitro*. There was also a possible improvement in the ability of the *gbpACD*, the most attenuated Gbp mutant, to bind to a pre-formed biofilm. These data suggest that changes in *S. mutans* Gbp mutant *in vitro* biofilm architecture, facilitated by the loss of Gbp-mediated cohesion, result in an *in vivo* biofilm in which certain mutant strains lack the ability to accumulate sufficient numbers in optimally arranged large structures to promote enamel demineralization.

A..



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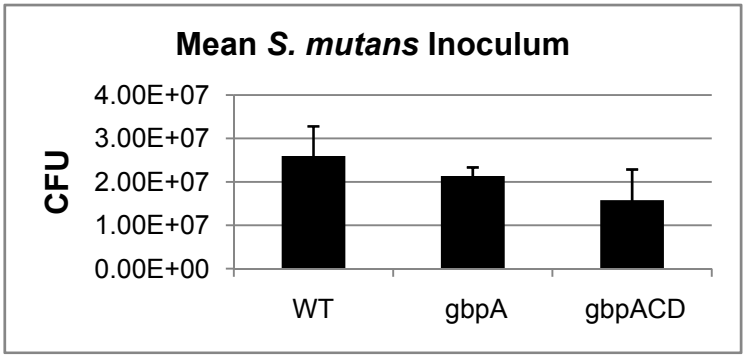

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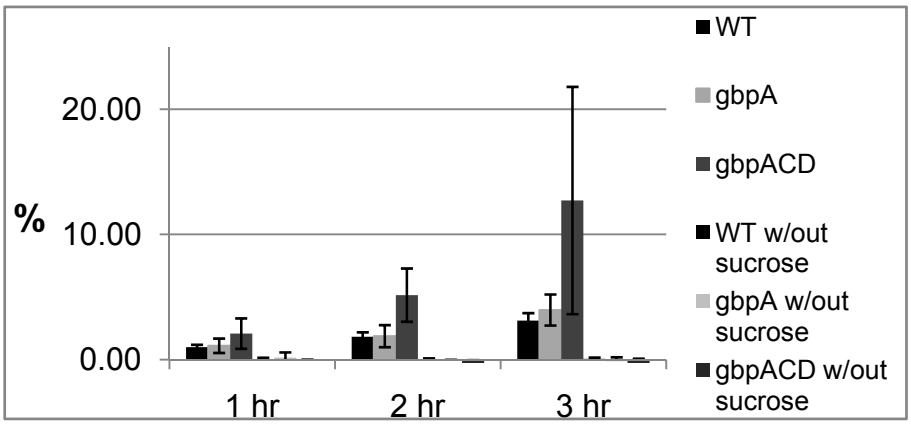

Figure 24 ***S. mutans*** **binding to established** ***S. sanguinis*** **biofilms.** (A) Curves showing bound WT (♦), gbpA (▲) and gbpACD (●) to *S. sanquinis* biofilms for 3 hours. Also shown is binding to *S. sanguinis* biofilms without sucrose (lines without markers). (B) Concentration of *S. mutans* cells used to initiate binding to *S. sanguinis* biofilms. (C) Percent of bound cells normalized to the initial inoculum.

CHAPTER 5

FINAL DISCUSSION AND CONCLUSIONS

The mature plaque biofilm takes on many classic biofilm architectural and behavioral characteristics such as abundant matrix with extensive channeling, intercellular communication and signaling, pH and O_2 gradients and detachment of cells and cell clusters. *In vitro*, *S. mutans* biofilms also demonstrate a distinct three-dimensional architecture when grown in the presence of sucrose. Glucan synthesis is essential to the cariogenicity of *S. mutans* by promoting adhesive and cohesive properties of *S. mutans* biofilms. Glucan-binding proteins have also been found to contribute to the adhesive and cohesive properties of *S. mutans* biofilms and affect the architecture of these biofilms *in vitro* (Matsumura *et al.* 2003, Hazlett *et al.* 1999, Nakano *et al.* 2002).

In this study, the deletion of each of three Gbps (GbpA, GbpC and GbpD) in all combinations allowed the examination of Gbp mutant strains with respect to cariogenicity in a rat model, biofilm architecture and *S. mutans* properties long recognized to be associated with cariogenic potential. Architecturally, the greatest *in vitro* effect was seen when GbpC was deleted. These biofilms lost biomass and showed a drastic and significant loss of biofilm depth. Consistent with previous research regarding GbpC (Sato *et al.* 2000), *gbpC* mutants lost aggregative properties which resulted in greater numbers of individual bacteria distributed about the substratum. This phenotype was dominant even in the presence of other Gbp mutations. However, in the SPF rat model the loss of GbpC led to attenuation only when combined with the loss of other Gbps. It is possible that adhesive or additional cohesive contributions by other Gbps compensated for *gbpC* mutant-mediated loss of biofilm cohesion so that the strain retained full cariogenic potential. Once another Gbp was lost, however, the combination of effects on the biofilm was sufficient to bring about attenuation of cariogenicity.

GbpA and GbpD are both secreted Gbps and could possibly share some overlapping functions. The loss of GbpA resulted in a loss of biofilm depth, a minor reduction in biomass, and an increase in substratum coverage. It is likely that the reduction in height was compensated by a spreading of the biofilm on the horizontal plane. This would support the increased adhesion results that were demonstrated by Hazlett *et al* (Hazlett *et al.* 1998). The loss of GbpD also resulted in a loss of biofilm depth but was accompanied by a reduction in biomass rather than an increase in substratum coverage. The *gbpD* mutant did not show a reduced ability to form a biofilm in the SPF rat as there was not a significant reduction in CFU recovered from rats infected with this strain, though this was measured only at the termination of the experiment. The *gbpA* strain showed no attenuation in the SPF rat model while the *gbpD* strain showed some reduction in caries. The loss of GbpA and GbpD together did not compound the effects of loss of one Gbp individually arguing against an overlap of function between the two proteins. The loss of GbpC combined with the loss of GbpA, GbpD or both, seemed to have more influence over both the *in vitro* and *in vivo* properties of *S. mutans*.

It has been reported that both GbpA and GbpC bind with greater affinity to α1-6 linked water-soluble glucan produced by GtfS (Matsumoto *et al.* 2006, Haas&Banas 2000). It is possible that these proteins provide a link between the water-insoluble glucan synthesized by the GtfI and GtfSI and the water-soluble glucan synthesized by the GtfS. It has been proposed that the role of GbpC is a cell-bound glucan receptor that facilitates coating of the *S. mutans* cell with glucan (Germaine&Schachtele 1976, de Stoppelaar *et al.* 1971). This is still a plausible explanation for the function of GbpC. GbpC has homology to the GBL described by Ma *et al.,* which was also considered a glucan receptor (Ma *et al.* 1996). GbpC and possibly other Gbps may serve to recruit water-soluble glucan to the cell wall and allow for binding of Gtfs and synthesis of both water-insoluble glucan and water-soluble glucan (Kuramitsu&Ingersoll 1978). While there

have been no studies investigating the binding affinities of GbpD to either water-soluble or water-insoluble glucan, it is likely that since it shares homology in its glucan-binding domain with GbpA and Gtfs that, like those proteins, GbpD would bind water-soluble glucan with higher affinity than water-insoluble glucan. However, experimental confirmation of the glucan-binding affinities of GbpD would be necessary before these comparisons can be made between GbpD and the other Gbps of *S. mutans*.

The COMSTAT measured biofilms of the *gbpD* mutants, as described in Aim 2, suggest that the primary role of GbpD is in both biofilm cohesiveness and possibly adhesion to the substratum. It is expected that loss of adhesion or cohesion would affect buccal surfaces more than sulcal surfaces. It was previously proposed that caries progress was slower on sulcal surfaces in general, and this may have been enhanced in the *gbpD* mutant, though it was only statistically significant on sulcal surfaces. A possibility exists that the attenuation observed in the *gbpD* mutant was the result of altered biofilm architecture that affected the rate of caries progression. However, this was not observed for *gbpD* mutants with additional mutations in *gbpA* or *gbpC*. Alternatively, the contribution of GbpD may have less to do with promoting biofilm structure than with its lipase activity which is directed against competing plaque species. This activity could impact the rate of accumulation of *S. mutans* in an *in vivo* plaque and affect caries progression and could have been overshadowed by additional Gbp deletions.

Ooshima *et al.* demonstrated, using Gtf knockout *S. mutans* and recombinant Gtf complementation, that a GtfB:GtfC:GtfD ratio of 5:0.25:1, respectively, results in the greatest level of adherence of *S. mutans* to a glass surface (Ooshima *et al.* 2001). This study suggests that a specific ratio of Gtfs could have evolved as a need to synthesize a specific ratio of water-insoluble and water-soluble glucans. Gbps may play a role in the maintenance of this ratio of water-soluble and –insoluble glucans. While the experiments described by Ooshima *et al.* focused on the impact of a specific Gtf ratio on adhesion to glass surfaces, it is not unreasonable to assume that this ratio may affect cohesiveness of

a biofilm as well. The presence of large amounts of Gbp-bound glucan in the EPM could be a mechanism for joining of several smaller microcolonies or growth and expansion of individual microcolonies.

While it is important to be careful when extrapolating *in vitro* biofilm properties to *in vivo* cariogenicity (Yamashita *et al.* 1993), the combination of *in vitro* biofilm analyses with an experimental *in vivo* modeling of cariogenicity that employed genetically defined strains, allowed the most comprehensive investigation of the roles of *S. mutans* Gbps ever undertaken. There are other factors in the rat model that are not represented in the *in vitro* biofilm, such as the contribution or interference of commensal bacteria to *S. mutans* biofilm formation. The majority of glucan-binding protein related cariogenicity studies were performed using gnotobiotic rats (Matsumura *et al.* 2003, Hazlett *et al.* 1998, Nakano *et al.* 2002, Matsumoto-Nakano *et al.* 2007). While germ-free rat cariogenicity studies provide a nice model to observe the cariogenic properties of an *S. mutans* biofilm, results from these studies do not consider the ecological nature of *S. mutans* cariogenicity. By using the SPF rat model, the results presented in this thesis show not only the effect that a single mutation or set of mutations has on the ability of *S. mutans* to form an *in vivo* biofilm but also shows its ability to compete with the other flora and establish a cariogenic biofilm in response to high concentrations of sucrose. It is possible that this approach mitigated some of the effects of the mutations. For example, Hazlett *et al*. observed a statistically significant increase in cariogenicity for the *gbpA* mutant strain in the gnotobiotic rat model while the *gbpA* mutant in the SPF model did not show a significant increase in caries (Hazlett *et al.* 1998). However, in the SPF model, the *gbpA* mutant did not show a decrease in caries. One possible explanation for this is that the increase in substratum coverage that was observed in the *gbpA* mutant *in vitro* biofilm was at least partially prevented from forming *in vivo* by the existing oral flora. If spreading out across the substratum is prevented, the cohesive properties that allowed for tall microcolonies, *in vitro*, may allow accumulation of *S. mutans*

perpendicular to the substratum. Without these cohesive properties sheering forces might prevent accumulation of numbers necessary for sufficient acid production to force an ecological change and induce caries.

Another important factor that must be taken into account in the SPF rat model was the response of the host to the different mutant strains and abilities of those strains to overcome host immune defenses. In humans, several defensins are associated with saliva and are thought to be synthesized in salivary ducts or oral keratinocytes. Three α-defensins (HNPs 1-3) and 3 β-defensins (HBDs 1-3) have been detected in saliva. While it is speculated that these defensins protect crevicular junctions and salivary glands from invasion by microbes, there is a good possibility that they could affect biofilm cells as well. Defensins and other antimicrobial peptides are part of both the innate and adaptive immune responses with some defensins, such as HBD2 and HBD3, induced by inflammatory cytokines in response to an infection or cancer (Abiko *et al.* 2003). Defensins are also known to trigger production of antibodies, interferon and other cytokines (Brogden *et al.* 2003).

Rats, like humans, form an immune response to colonization by *S. mutans*. There have been several studies reporting the immunogenicity of Gbps and Gtfs, as well as the immune response to *S. mutans* colonization (Smith *et al.* 2003, Zhao *et al.* 2006, Jespersgaard *et al.* 1999, Nogueira *et al.* 2008, Nogueira *et al.* 2005)(Mattos-Graner *et al.* 2006, Zhao *et al.* 2006). Of the glucan-binding proteins examined in this thesis project, only GbpD has been shown to induce an immune response in rodents (Zhao *et al.* 2006). However Gtfs and the glucan-binding domain of Gtfs are immunogenic (Nogueira *et al.* 2008) and it is possible that loss of Gbps could expose other antigenic sites on the *S. mutans* cell wall. It would be of interest to examine the ability of WT and Gbp mutant biofilms to survive challenge from host defenses. While biofilm bacteria benefit from enhanced protection from host defenses such as defensins and immune cells, bacteria that

have lost the ability to aggregate may be at a greater risk. This may affect the cariogenicity of the strain.

The correlations of COMSTAT measurements and the SPF rat results show that there is a relationship between alteration of *S. mutans* biofilm architecture and the cariogenicity of the bacteria. What was surprising, though, was the subtlety of both the *in vitro* and *in vivo* consequences of *gbp* mutation. Although knocking out all three Gbps greatly reduced caries in the rat model, none of the mutants were completely attenuated. Also, changes in biofilm architecture among the mutant panel were only significantly different from the WT with respect to the average microcolony thickness. The lack of statistical significance for other biofilm properties could be attributed, in part, to the heterogeneity and inherent variability within biofilm samples. It is likely that other glucan-binding proteins, like Gtfs or other adhesive proteins, are capable of providing at least a minimal amount adhesion and cohesiveness that allow *S. mutans* to survive and cause caries in our model.

It is reasonable to assume that *S. mutans* has inherent redundancy in its sucrose-dependent adhesion mechanisms. There is well documented redundancy in sugar metabolism, which includes multiple PTS and other transport systems for a wide variety of dietary carbohydrates (Ajdic&Pham 2007). *S. mutans* colonizes oral hard surfaces and is not found in appreciable numbers on other surfaces in the oral cavity. Given that the ability of *S. mutans* to adhere to the surface of the tooth is critical to its retention and propagation in the oral cavity, and given that sucrose provides an enormous boost to *S. mutans* adhesion and aggregation it is reasonable to expect that the organism has evolved layers of redundancy in sucrose dependent colonization. Glucosyltransferase enzymes share similarity in their glucan-binding domains with GbpA and GbpD and likely provide similar structural contributions to glucan-based biofilms (Michalek *et al.* 1975b, Tanzer *et al.* 1974, Kuramitsu&Ingersoll 1978, Hamada *et al.* 1981).

While the P1 adhesin is most often associated with sucrose-independent adherence, it has been shown to be an important factor in cariogenicity of *S. mutans* in the presence of sucrose. In a gnotobiotic rat model, infection with a *spaP* mutant resulted in a decrease in caries when compared with the WT, which was not accompanied by a reduction in colonization levels (Crowley *et al.* 1999). More recently, when *irvA*, which encodes a putative transcriptional regulator that is repressed by the product of the *irvR* gene, was over-expressed in *gbpC* mutant strains dextran-dependent aggregation was partially restored. This phenotype was accompanied by an increase in *spaP* transcription (Zhu *et al.* 2009). There is some sequence homology between the P1 protein and GbpC (Sato *et al.* 1997a). WapA is another cell surface adhesin protein that has been shown to bind dextran (Han&Dao 2005). It is possible that these adhesins and possibly other cell surface proteins can contribute to biofilm cohesion through glucan-binding properties.

It is now well established that extracellular DNA has a structural role in both Gram-positive and Gram-negative biofilms (Whitchurch *et al.* 2002, Moscoso *et al.* 2006). Petersen *et al*. demonstrated that extracellular DNA was important for *S. mutans* biofilm formation. Although some of this DNA was bound and internalized by the DNA-uptake mechanism, it is possible that a significant amount of DNA could remain extracellular (Petersen *et al.* 2005). The DNA-uptake system of *S. mutans* consists of ComA-E and ComG which are an NTPase, a polytopic membrane protein, a major pseudopilin homologue, and 3 minor pseudopilins respectively. The DNA-take system shares some homology with type IV pili, which in *P. aeruginosa* are known to bind DNA but not internalize it (Petersen *et al.* 2005, van Schaik *et al.* 2005). It is possible that in some instances the DNA-uptake system binds DNA but does not induce DNA uptake. Alternatively, other proteins could participate in binding extracellular DNA and contribute to biofilm architecture.

While the possibility exists for redundancy of adhesion and aggregation systems, the deletion of Gbps had profound effects on the *in vitro* biofilm architecture of *S.*

mutans. Multiple Gbp mutations resulted in partial attenuation of bacteria in a specific pathogen-free rat caries model. It should be noted that the *in vivo* model presents optimal conditions for *S. mutans* survival, such as a large inoculum to establish colonization, antibiotic treatment before inoculation to reduce numbers of established flora and a higher than normal level of sucrose in the rat diet. Despite conditions that favored caries development several strains were significantly attenuated for caries.

Deletion of any of the Gbps resulted in marked changes in biofilm architecture. The statistically significant reduction in the average microcolony thickness in all Gbp mutants compared to the WT strain correlated with attenuation of raw caries and caries that were adjusted for differences in rat weights. While the average microcolony thickness of the WT skewed the correlation a bit, there was still a correlation between the thickness and rat weight-adjusted caries in the absence of the WT scores. Other changes in biofilm architecture occurred but were not statistically significant based on the COMSTAT parameters that were used to measure these differences. These parameters did, however, correlate with caries scores in the SPF rat model. The biomass and the surface area to biovolume ratio both correlated with rat weight-adjusted caries. The substratum coverage had strong statistically significant correlations with unadjusted caries. While the differences in rat weight-adjusted caries scores and unadjusted scores point to a potential limitation in the SPF rat model, it is clear that changes in several biofilm architectural parameters correlate with caries attenuation.

The COMSTAT-measured biofilm parameters and correlational analyses also provide clues to the function of individual Gbps. Adhesion and cohesion are properties that contribute to average microcolony thickness, biomass, substratum coverage, and surface area to biovolume ratio. While it is possible that each Gbp contributes, in differing proportions, to both biofilm cohesion and adhesion, figure 25 illustrates the roles that we propose for each Gbp based on the results of experiments performed for this thesis project.

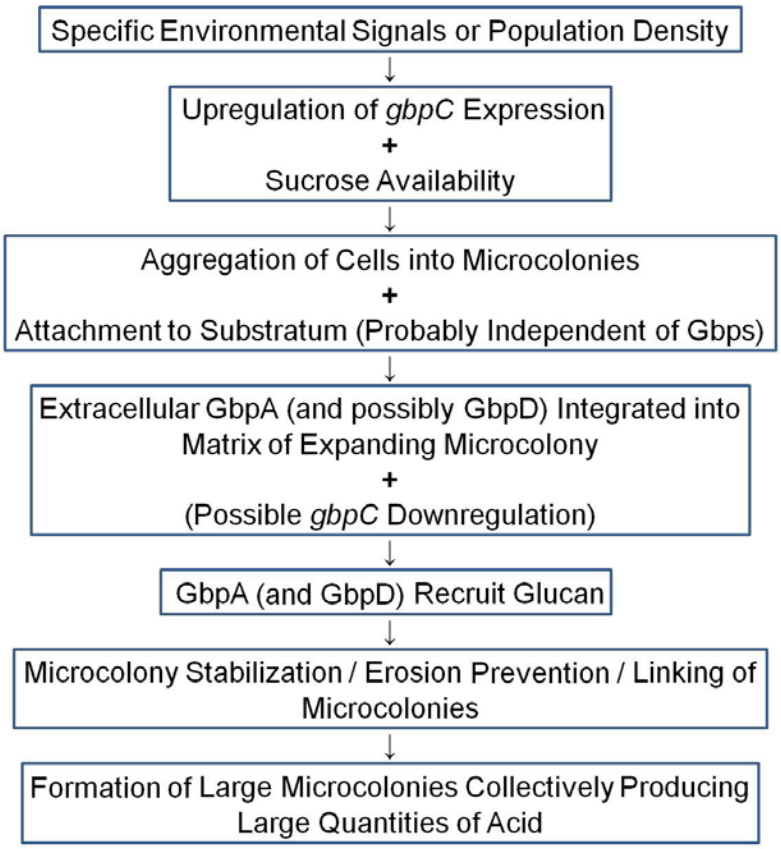

Figure 25 Proposed model for Gbp function in *S. mutans* caries development.

Figure 25 speculates that expression of *gbpC* is upregulated under certain conditions such as an environmental stress or at a given population density. The presence of sucrose allows for the synthesis of glucans by the Gtf enzymes allowing cell-wall bound GbpC to facilitate aggregation of cells into microcolonies. Based on the adhesion and biofilm strength experiments shown previously, cells adhere to the substratum or existing biofilm independently of the presence of Gbps. Extracellular Gbps (GbpA and GbpD) are released and integrated into the matrix. This allows for expansion of the microcolony by several potential mechanisms. These steps allow for accumulation of cell numbers sufficient enough to lower the plaque pH enough for the generation of caries.

The results presented in this thesis have shown that glucan-binding proteins indeed contribute to biofilm architecture. Alteration of the *in vitro* biofilm architecture through deletion of Gbps was linked to attenuation of cariogenicity, though in the SPF rat this generally required deletion of multiple Gbps. Attenuation of cariogenicity for mutant strains could not be explained by changes in metabolism, acid tolerance, or initial adhesion. Thus, altered biofilm architecture, most notably a reduced ability to form large cell-rich microcolonies secondary to loss of Gbp-mediated biofilm cohesion, led to the attenuation of *S. mutans* cariogenicity.

Future Directions

As discussed in chapter 2, further investigation into cariogenicity in SPF rats is likely warranted. Based on the results presented in this thesis and the possible limitations of the long infection time combined with a possible effect of uneven weight distribution among the rats, it was suggested that several intermediate time increments be observed, as well as a controlled feeding and watering schedule, to ensure similar sucrose intake among all rats. In addition, it would also be helpful to examine colonization levels throughout the infection period in order to determine whether rates of accumulation are affected by *gbp* mutations. These conditions may show additional differences among mutants with respect to the rate of caries development. Controlled feeding should eliminate the need for weight adjustment of the caries data. This deeper *in vivo* investigation can also be accompanied by analysis of host antibody responses in both blood and saliva, throughout the infection time. These experiments could provide clues to the immunogenicity of Gbps and could also reveal the exposure of potential immunogenic proteins on the *S. mutans* cell surface, in the absence of Gbps. These experiments, however, would be an enormous undertaking and would require the services of a lab with established SPF rat protocols, such as that of Dr. Michalek at the University of Alabama-Birmingham. Although this would provide important follow up results to

our initial SPF rat data, due to the great amount of work that would need to be done by an outside lab, this study would probably not be of the highest priority in the immediate future.

Comparison of data between the *in vitro* biofilm analysis and the *in vivo* results presents questions about the role of the host response to *S. mutans* colonization and the potential differences in susceptibility of *gbp* mutants to these responses. Changes in biofilm architecture may render strains more susceptible to killing by host defensins, immune cells or immune effector-cell products. Experiments designed to expose WT and *gbp* mutant biofilms to either defensins or even co-colonization with immune effector cells may reveal differences in susceptibility to host immune responses.

GbpD presents multiple avenues for follow up study. It was previously stated that, although it shares a similar glucan-binding domain sequence as GbpA, the glucan-binding affinity for GbpD remains unknown. Experiments designed to determine whether the glucan-binding domain of GbpD binds more avidly to α1-6 or α1-3-linked glucans could involve measuring protein retention in either dextran (α1-6) or mutan (α1-3) affinity columns or measuring retardation of flow through dextran or mutan containing native PAGE gels vs. control gels (Haas&Banas 2000). The potential lipase activity of GbpD was also discussed. It was shown by Shah *et al.* that the lipoteichoic acid (LTA) from *S. sanguinis* competed with biotin dextran for binding to GbpD and could release free fatty acids (FFAs) from this and other triglycerides (Shah&Russell 2004). It may be useful to examine the ability of GbpD to bind and release FFAs from LTAs of other oral streptococci. Also, competitive colonization assays between *S. sanguinis* (and possibly other oral streptococci) and either WT *S. mutans* or each *gbpD* mutant could show a role for GbpD in interspecies antimicrobial competition.

The possibility of an increased basal level of stress response or acid tolerance response genes was previously discussed as a possible explanation for elevated acid survival at early time points in the acid tolerance experiments. As previously suggested

in chapter 4, treatment of both planktonic cultures and biofilm cultures with either UV irradiation or H_2O_2 could provide clues to increased expression of DNA repair enzymes that are often upregulated in both the acid tolerance responses and general stress responses (Quivey *et al.* 1995). It is likely that several time increments and several planktonic growth phases would need to be examined in order to determine whether these genes are expressed at a higher level in the *gbp* mutants than in the WT *S. mutans* strains. However this method would still be more economical and potentially less labor intensive than micro array or real-time PCR, though those techniques may be used as a follow-up if specific time points showed differential survival due to increased presence of DNA repair enzymes.

Examination of cell hydrophobicity could further explore the adhesive capacities of the organisms and possibly reveal differences in the cell wall structure among WT and *gbp* mutant strains. Differences in hydrophobicity could indicate differences in the ability of *S. mutans* to bind to the acquired enamel pellicle or may in part explain differences in biofilm structure. A lack of differences would reinforce the idea that the interactions of Gbps and glucan are solely responsible for architectural changes in *gbp* mutants.

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