ROLE OF AN ABC TRANSPORTER COMPLEX IN VIOLOGEN TOLERANCE IN *STREPTOCOCCUS MUTANS*

BY

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ABSTRACT

The gram-positive bacterium *Streptococcus mutans* is the primary causative agent in the formation of dental caries in humans. To persist in the oral cavity, *S. mutans* must be able to tolerate rapid and substantial environmental fluctuations and exposure to various toxic chemicals. However, the mechanisms underlying the ability of this cariogenic pathogen to survive and proliferate under harsh environmental conditions remain largely unknown. In the current study we wanted to understand the mechanisms by which *S. mutans* withstand exposure to various quaternary ammonium compounds (QAC) such as methyl viologen (MV) that also generates superoxide radicals in the cell. To elucidate the genes that are essential for MV tolerance, sensitive mutants of *S. mutans* were generated *via* IS*S1* mutagenesis. Screening of approximately 3,500 mutants revealed fifteen MV sensitive mutants. Among them, five and four independent insertions had occurred in SMU.905 and SMU.906, respectively. These two genes are organized in an operon and encode a putative ABC-transporter complex. Linkage PCR analysis supports the operon organization of these two genes and also indicates that the transcription start site maps further upstream of SMU.905. To confirm our results, SMU.905 was deleted using an antibiotic resistance marker; the SMU.905 deletion mutant was just as sensitive to MV as the IS*S1* insertion mutants. Furthermore, SMU.905 and SMU.906 mutants were sensitive to other viologen compounds such as benzyl- and ethyl- viologen. Sensitivity to various drugs including a wide range of QACs was tested. It appears that a functional SMU.905 is also required for full resistance towards acriflavin, ethidium bromide, and safranin; all are well known QAC. These results indicate that SMU.905/SMU.906 probably constitute a heterodimeric multidrug efflux pump of the ABC family. BLAST-P analysis suggests that this ABC-type efflux pump is widely present in streptococci, enterococci, and clostridia including some important human pathogens.

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ABBREVIATIONS

ABC	ATP binding cassette
AF	Acriflavin
BP	Base pairs
CRISPR	Clustered regularly interspaced short palindromic repeat
Em	Erythromycin
GAS	Group A streptococcus
GBS	Group B streptococcus
IE	Infective endocarditis
IS	Insertion sequence
Km	Kanamycin
LB	Luria-Bertini
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
MV	Methyl viologen
OD	Optical density
ORF	Open reading frame
PMF	Proton motive force
QAC	Quaternary ammonium compounds
RGP	Rhamnose-glucose polysaccharide
RT-PCR	Reverse transcriptase polymerase chain reaction
Sm	Spectinomycin
SMU.	Designation for ORFs of *S. mutans*
THY	Todd-Hewitt with yeast extract broth
Ts	Thermosensitive

CHAPTER 1

INTRODUCTION

1.1. Oral microbial community

Dental plaque is a complex biofilm, which contains diverse bacterial species that are adhered to tooth or other oral tissue surfaces and are embedded in a matrix composed of extracellular DNA or polysaccharides (133, 134). The oral cavity is a dynamic environment that undergoes rapid and substantial changes in pH, nutrient availability, oxygen tension, osmotic stress, and temperature fluctuations (17, 113, 114). The amount of toxic substances that often come from oral care products is also in constant flux in the oral cavity. Despite these harsh environments, over 600 microbial species have been estimated to colonize the oral cavity as complex populations in biofilms (156). About 20% of the oral microorganisms are streptococcal species that are associated with oral health as well as various diseases (99).

Oral bacteria often exhibit distinct tissue-specific tropisms and many of these organisms have been found to colonize different surfaces of the oral cavity, such as the tooth enamel, tongue, and other bacterial lawn (126). Studies focused on examining which species preferentially colonize a particular biological surface in the oral cavity have shown that hard tissue surfaces, such as the teeth, are preferentially colonized by *Actinomyces* species. On the other hand, *Prevotella melaninogenica* and *Veillonella parvula* colonize soft tissue surfaces in higher proportions (126). Furthermore, streptococcal species, such as *S.mitis, S. oralis,* and *S. salivarius* predominantly colonize the soft tissues and are also found in higher proportion in the saliva compared to other organisms (154). However, gram-negative bacteria such as *Capnocytophaga gingivalis* and *V. parvula* primarily colonize the tongue, which is also a soft tissue (126). Since particular species tend to dominate in specific regions of the oral cavity, it suggests that organisms express different cell-surface associated proteins such as adhesins that determine the environmental niche or tissue tropism (126).

The dental plaque initiates with so-called “pioneer” organisms, or early colonizers, which adhere to host salivary components on the tooth enamel (100, 205). Among the early colonizers, about 60-80% are streptococcal species belonging to the viridians group, including *S. mitis, S. oralis* and *S. sanguinis*, and these streptococci form relatively simple biofilm communities (98, 101, 205). Following early colonization, the biofilm microflora becomes more complex as a result of co-aggregation between different bacterial species (98). Adhesins on one species may be capable of recognizing a cell-surface receptor on another organism, thereby allowing diversification of the biofilm community (101). These cell-cell interactions have been extensively studied and various adhesins that promote co-aggregation have been identified. For example, the SsaB protein of *S. sanguinis* mediates co-aggregation with *Actinomyces naeslundii* and *Streptococcus gordonii (101)*. Furthermore, co-aggregation of *A. naeslundii* with various streptococcal species, including *S. gordonii, S. oralis, S. mitis* and *S. sanguinis*, is thought to be mediated by type-II fimbriae on *A. naeslundii* and receptor polysaccharides on the streptococcal species (150, 205). Fusobacteria, on the other hand, are able to co-aggregate with both the early colonizers as well as later colonizers, such as *Actinobacillus* and *Treponema* species, thereby serving as a connector between early and late colonizers (98, 100). These cell-cell interactions lead to the formation of more diverse biofilm involving hundreds of microbial species. As the biofilm matures, more complex interactions begin to occur involving cooperation and competition among various plaque microflora.

A typical characteristic of the community lifestyle of dental plaque is recycling of metabolic byproducts among various bacterial species. The metabolic byproducts of one organism can be potentially used as a source of nutrition by another organism and complex metabolic networks are generated within the biofilm community (105). For example, lactic acid produced by *Streptococcus mutans* can be utilized as an energy source by *Veillonella* species and *Streptococcus oligofermentans* (28, 105). Furthermore, a symbiotic interaction between *S.*

oralis and *A. naeslundii* was observed, when nutrient-limited saliva was provided as the sole energy source in a mixed-species biofilm (153). However, when cultivated independently neither of these two could survive. It was only when these two species were grown together that they were able to flourish in the biofilm. A surprising finding is that during anaerobic growth *S. mutans* requires para-aminobenzoic acid, and *S. sanguinis* is able to provide this important vitamin to *S. mutans* during co-cultivation (28). Although not directly shown for oral streptococci, exogenous quinones produce by host or by bacteria can also promote growth of nearby bacteria. For example, *Streptococcus agalactiae* that colonizes intestine and urogenital tract is thought to capture menaquinone from other bacteria. This acquisition helps the organism to shift from anaerobic metabolism to respiration, and this metabolic shift is crucial for colonization and virulence (78, 202). Bacterial metabolism in dental plaque also results in the establishment of pH, oxygen, nutrient and other physical and chemical conditions that ultimately regulate microbial growth in the biofilm. The heterogeneous environment within the oral biofilm allows typically incompatible organisms to co-exist (134) and facilitates the creation of complex nutritional networks among diverse oral organisms.

Microorganisms in the dental plaque also compete with each other for survival and colonization. They have developed multiple strategies to gain competitive advantages within the biofilm community. Many streptococci produce bacteriocins, which are small peptides with antimicrobial activity against closely related species (6, 148). For example, *S. mutans* produces multiple bacteriocins, called mutacins, which provides *S. mutans* a competitive advantage over other species in the dental palque allowing for enhanced colonization (105, 148). In addition to inhibition of microbial growth, bacteriocins can also act as signaling molecules for cell-to-cell communication. Salivaricin A, a bacteriocin produced by *S. salivarius*, can act as interspecies signaling molecules to regulate gene expression of *Streptococcus pyogenes* (195). Another interesting observation is that the production of mutacins by *S. mutans* in multispecies biofilm

can be inhibited by other oral streptococci such as *S. gordonii*, *S. sanguinis, S. mitis* and *S. oralis* (196). Bacteria also secrete various toxic compounds to inhibit growth of other species (78). For example, *S. sanguinis* and *S. oligofermentans* both generate hydrogen peroxide that inhibits growth of *S. mutans* (103, 192). Thus complex interactions that represent both co-operation and competition among diverse bacteria are found within the dental plaque biofilm.

1.2. Diseases caused by *S. mutans*

S. mutans has been strongly implicated as the principal etiological agent in human dental caries In addition to dental caries, *S. mutans* is also an important agent of infective endocarditis (IE). More than 20% of cases of viridians streptococcus-induced endocarditis are caused by *S. mutans.* The development and progression of these diseases are discussed below.

1.2. a. Dental caries

Dental caries are a dynamic process that involves degradation of the tooth enamel, dentin, and cementum by bacteria present in the dental plaque. The demineralization of the tooth enamel is due to the production of lactic acids during carbohydrate metabolism of acidogenic bacteria (71, 72). Since *S. mutans* is frequently associated with human dental caries, as evident from several studies that found higher levels of *S. mutans* at carious lesions, this organism is attributed as the principal etiological agent (71, 72, 117, 203). Three different hypotheses have been proposed for the development of dental caries: the specific plaque hypothesis, the non-specific plaque hypothesis, and the ecological plaque hypothesis (10, 26, 35, 83). The specific plaque hypothesis, which was first proposed by Clarke in 1924 (35), suggests that only a few species of bacteria, such as *S. mutans*, are involved in caries development. However, this hypothesis remains controversial since there are few reports that indicate many non-mutans bacteria are also capable of producing carius lesions (26, 96, 175). The non-specific plaque hypothesis, which was first presented by Miller in the late 1800s, proposes that all bacteria in

the mouth had the potential to be cariogenic (10). Recent evidence also indicates *S. mutans* are not the only organism that is able to promote caries formation (96). Thus, this particular hypothesis is an attractive and highly accepted alternative explanation for dental caries formation. The ecological plaque hypothesis proposes that plaque-mediated diseases, like dental caries, are due to imbalances in the resident oral microflora. Imbalances may arise from environmental conditions (such as increased acidity) that lead to enrichment for oral pathogens (132). This model suggests that any acidogenic organisms in the mouth can cause caries provided that the local environmental conditions support the overall process of caries formation. In fact the plaque pH is in constant flux, with pH falling due to acid production by acidogenic bacteria following intake of dietary carbohydrate, and then rising due to alkali production by other plaque bacteria (26, 96, 190).

The involvement of *S. mutans* in the initiation and progression of dental caries is due to the organism's ability to metabolize a diverse range of carbohydrates such as fructose, glucose, galactose, and sucrose (3, 4, 140). Fermentation of these carbohydrates via the glycolytic pathway results in the production of lactic acid from pyruvate (71). Lactic acids produced by *S. mutans* and other acidogenic plaque bacteria generate acidification of the local environment below the critical pH 5.5; this pH is needed for remineralisation of tooth enamel (72, 118, 140). Prolonged exposure to lactic acids causes continuous demineralisation of the tooth enamel and ultimately leads to dental caries formation (11).

1.2.b. Infective Endocarditis (IE)

IE is a life-threatening bacterial infection of the endocardium, a smooth layer of tissue that covers the inside the heart to protect the heart muscles (16, 141). While virtually any bacterial organism can cause bacterial endocarditis, the vast majority of infections are caused by gram-positive cocci (141). The viridans group of streptococci and *S. mutans* are the most common

cause of endocarditis involving native heart valves in patients with congenital heart disease (16). Oral streptococci can often cause systemic infections including bacteremia following various dental procedures, including oral surgery, allowing these organisms to gain access and adhere to damaged heart valves, causing IE (143, 145). It is estimated that about 20% of IE cases attributed to viridans streptococci are in fact caused by *S. mutans* (11, 194). A serotype-specific putative adhesin, derived from rhamnose-glucose polysaccharide (RGP), has been identified in *S. mutans* that is thought to be required for attachment to human monocytes, fibroblasts, and platelets ((31, 54). Furthermore, the *S. mutans* adhesin, Antigen I/II, may play a role in IE since this adhesin binds to the extracellular matrix components such as type 1 collagen, fibrinogen, fibronectin, and laminin (15). Although the exact role of *S. mutans* in the pathogenesis of IE is not well understood, the availability of complete genome sequence may suggest various surface adhesins in the pathogenesis.

1.3. Important virulence traits of *S. mutans*

Unlike other infectious diseases caused by various streptococcal species (pneumonia, strep-throat) in which traditional virulence factors (toxins, hemolysins, or proteases) play important roles in the damage to the host tissues, the pathogenesis of dental caries is solely associated with the life-style of bacterium and its metabolic characteristics. The virulence properties of *S. mutans* can be classified in the following core attributes: its adhesion to the tooth surface, abilities to form biofilm, to produce large quantities of organic acids (acidogenecity), to tolerate low pH environment (aciduricity), and production of bacteriocins (mutacins) to suppress the growth of competing organisms. How these core attributes are involved in the *S. mutans* pathogenesis are described below.

1.3.a. Adhesins

Adhesion of oral streptococci to tooth surface is the primary step of the colonization. Salivary proteins and glycoproteins are adsorbed by the oral surfaces, such as enamel, dentin, epithelial cells, and even other bacteria (99). Interaction with these salivary constituents facilitates adhesion to the enamel surfaces. Bacterial interaction with salivary components also promotes removal of the organisms by aggregation or direct killing, or allows the organism to escape recognition by the host's immune system by masking antigenic sites (51, 53). The major salivary component is an agglutinin, ~ 500-kDa oligomeric protein complex composed of the glycoprotein gp340, secretory antibodies (IgA), and an 80-kDa protein; this salivary agglutinin forms a pellicle on the tooth surface (86, 119). Antigen I/II (AgI/II), a major *S. mutans* adhesin binds to the gp340 to initiate the attachment (119).

AgI/II, also known as SpaP and P1 in *S. mutans*, represents a family of polypeptides expressed by many oral streptococci that demonstrate diverse binding specificities and affinities (24, 88, 89, 150). However, the AgI/II family of proteins exhibits highly conserved domain structures that are required for attachment (24, 150). AgI/II is a cell-surface anchored polypeptide whose C-terminal region contains an LPXTG motif that serves as a target for sortase mediated covalent attachment to the cell wall (86). The N-terminal alanine-rich and the C-terminal proline-rich regions are believed to be responsible for interaction with salivary components (150). AgI/II has also been implicated in co-aggregation between different bacterial species in the oral cavity. *S. gordonii* SspB, an AgI/II family protein, was able to mediate co-aggregation with *A. naeslundii* and SspA (also an AgI/II protein) along with SspB were shown to be involved in binding to the periodontal pathogen *Porphyromonas gingivalis* (47, 107, 150). Furthermore, the presence of salivary agglutinin was shown to enhance co-aggregation of *S. mutans* with *S. sanguis* and *Actinomyces viscosus* (106). Because AgI/II is a cell-surface associated protein (150) and because it can elicit immune response (125), it is an important vaccine candidate to control

dental plaque formation by *S. mutans* (86). In fact, active immunization of primates and rodents with AgI/II resulted in protection from dental caries (86, 181) and application of synthetic AgI/II prevented *in vivo* recolonization on human teeth by *S. mutans* (94). Although the development of an AgI/II based anti-caries vaccine seems promising, it is important to note that the peptide components should only confer protection and should not cross-interact with the non-pathogenic oral bacteria so that undesirable changes in the oral ecology are avoided (68).

Another cell-surface associated adhesin, WapA, also known as antigen A or antigen III, is shown to play an important structural role on the cell surface, which ultimately affects cell–cell aggregation (210). However, this adhesin is not very well studied and its function in *S. mutans* pathogenesis is not very clear. Nevertheless, this protein, which is a 29-kd polypeptide, was used as a vaccine candidate in animal studies for immunization against dental caries (173). Interestingly, it has been found that antibodies against WapA do not interfere with cell-cell aggregation (52), or with adherence to saliva-coated hydroxyapatite (53). Thus, the exact role of WapA as an adhesin remains controversial.

1.3.b. Biofilm formation

Dental plaque biofilms are comprised of aggregates of surface-adherent bacteria embedded in an extracellular polysaccharide matrix (134). It is believed that the biofilm mode of growth is the natural state adopted by many bacterial species including pathogens (87, 155). Biofilms are typically comprised of diverse bacterial species that are spatially and functionally organized (75, 155), as it is the case in dental plaque (134). Biofilm lifestyle offers several benefits to bacteria such as defense against shear forces, stresses and antimicrobial agents, colonization of a favorable habitat, and a community lifestyle that promotes genetic transfer and sharing of metabolites (77, 78, 84, 87). Biofilm development can be divided into five major stages. The first stage is the initial attachment to a suitable biotic or abiotic surface. In case of *S. mutans,*

this step is governed by adhesins as described in the previous section. The second stage is irreversible attachment as mediated by the production of extracellular polysaccharide such as glucans. The other stages are early development, maturation of biofilm architecture, and dispersal. Biofilm maturation is associated with the production of a complex architecture, including microcolonies, channels and pores (70, 187). Biofilm dispersal is believed to occur by shedding of daughter cells from actively growing cells, shearing of aggregates due to flow effects or detachment in response to nutrient depletion in the environment. This would allow bacteria to search for new nutrient-rich environments to inhabit (70, 187).

In case of biofilm formation by *S. mutans*, the initial attachment is mediated by interaction between AgI/II adhesin and gp340 salivary protein on the tooth pellicle. However, the second step, which is very crucial, is mediated by glucosyltransferase enzymes (Gtfs), which produce both water-soluble and insoluble polysaccharides, glucans, from sucrose (11, 12). These enzymes cleave disaccharide sucrose into glucose and fructose moeities. Glucose is then added to a growing glucan polymer that is also synthesized by Gtfs (11, 12). *S. mutans* produces three Gtfs: GtfB, GtfC and GtfD. The first two enzymes are cell-associated and are responsible for synthesis of water-insoluble glucans, which predominantly contain α-1,3-glycosidic bonds. On the other hand, GtfD is produced extracellularly and synthesizes water-soluble glucans that predominantly contain α-1,6-glycosidic bond (11, 12). Water-insoluble glucans are major constituents of the plaque biofilm matrix and have been shown to facilitate not only adherence of *S. mutans* to the biofilm, but also to promote cell-cell interactions among oral streptococci (105, 150).

S. mutans also produces four glucan-binding proteins (GBPs): GbpA, GbpB, GbpC and GbpD (11, 12). GBPs are required for the interaction with the extracellular polysaccharides in the biofilm. Among these GBPs, GbpA, GbpB, and GbpD are both secreted and cell-associated,

while GbpC is only found covalently anchored to the cell wall via sortase (177). Various studies have been performed in order to examine the contribution of each of these GBPs to biofilm development. GbpA has been shown to be important for biofilm architecture in sucrose-dependent biofilms (11, 12). GbpB has been shown to be immunologically distinct from the other GBPs produced by *S. mutans* and is believed to be essential for viability with a potential role in cell wall biogenesis (61, 137, 138). Evidence suggests that GbpC is more important for early stages of biofilm formation and is involved in glucan-dependent aggregation of bacteria via binding to the water-soluble glucans produced by GtfD (137). Lastly, the loss of GbpD has been shown to result in extremely fragile biofilms, suggesting that this GBP is important for providing biofilm scaffolding and promoting cohesiveness between glucan and bacteria in the biofilm (11, 12, 124). Based on these studies, it is apparent that each of the GBPs produced by *S. mutans* contributes to the overall biofilm architecture and is therefore important for biofilm formation and maturation.

Besides GTFs and GBPs, early stages of biofilm formation by *S. mutans* involves differential expression of at least 33 proteins, including those involved in carbohydrate metabolism, amino acid biosynthesis, protein folding, and cell division (200). Biofilm formation is also associated with differential expression of various genes compared to planktonic growth. Genes that are differentially expressed in biofilm population include several regulatory proteins, competence genes, the heat shock protein, genes involved in carbohydrate metabolism, and genes involved in cell division (178, 179, 200, 204). The involvement of a number of systems in the control and regulation of biofilm development in *S. mutans* highlights the complexity of the processes and the importance of this property in virulence. As development of a stable biofilm is important for the initiation of dental caries by *S. mutans,* studies examining the genetic involvement in biofilm formation are essential for understanding the process and allow for identification of potential targets to control cariogenesis.

1.3.c. Acidogenicity

S. mutans contains genes necessary for a complete glycolytic pathway and can produce acetate, formate, lactate, and ethanol by fermentation (3, 180). The precise distribution of fermentation products depends on the growth conditions; lactate is the major end product when glucose is abundant (41). Strains deficient in lactate dehydrogenase (LDH), the enzyme that converts pyruvate to lactate, display reduced cariogenicity (59, 90). The frequency with which *S. mutans* produces acid when tested at a pH in the range from 5.0 – 7.0 exceeds that of other oral streptococci in most instances (46). However, the relative acidogenicity of *S. mutans* can vary from one isolate to another, and there are no strict correlations between acidogencity and caries formation (97). Nevertheless, the acidogenicity by *S. mutans* leads to ecological changes in the plaque flora that includes an increase in the proportion of *S. mutans* and other acidogenic and acid-tolerant streptococci. This cariogenic flora further reduces plaque pH to lower levels compared to a healthy plaque flora upon the ingestion of fermentable carbohydrate, and the recovery to a neutral pH will be prolonged (66, 186). Sustained plaque pH values <5.5 leads to the demineralization of enamel and the development of dental caries (118).

1.3.d. Acid tolerance

As mentioned above, the plaque environment is continuously experiencing changes in pH from above pH 7.0 to as low as pH 3.0 due to ingestion of dietary carbohydrates (113, 186). The extreme acidic environments pose a considerable stress on organisms living within the dental plaque. *S. mutans* along with several other oral streptococci posses several acid adaptive strategies, some of which are distributed among all the species and some that are unique to *S. mutans* (113). It has been shown that prior exposure of *S. mutans* to a sub-lethal pH of 5.5 results in enhanced survival at the extremely low pH of 3.0-3.5 (74, 189, 201). This adaptive response, termed as acid tolerance response (ATR), involves changes in the physiology, including alteration in gene and protein expression, increased glycolytic activity, and increased

proton-extrusion via the F1/F0-ATPases, which lead to enhanced survival (11, 113, 201). Glycolytic enzymes in *S. mutans* are maximally active around pH 6.5 for cells growing in acidic conditions, while for other oral bacteria maximum glycolysis is obtained around pH 7.5 (73). However, *S. mutans* can maintain a relatively alkaline intracellular pH and allows glycolysis to occur even when the extracellular pH values are as low as 4.0; thereby, providing a competitive advantage for S*. mutans* over other less acid-tolerant oral bacteria (73, 74). Growth of *S. mutans* below pH5.5 is also accompanied by a decrease in glucose transport by the phosphoenolpyruvate (PEP) phosphotransferase system (PTS), a major glucose transport system (40, 73). Enhancement in the rate of glycolysis at low pH results in increased lactic acid production and further acidification of the environment, which allows *S. mutans* to out-compete other oral bacteria that are unable to tolerate high acidic conditions.

One of the main mechanisms involved in the ATR of *S. mutans* is the maintenance of intracellular pH (ΔpH). This process employs proton extrusion by end-product efflux and membrane-associated F1/F0-ATPases. These systems are responsible for maintaining transmembrane pH gradients with a more neutral intracellular pH and more acidic extracellular pH (40, 74). Protons in the extracellular environment are able to diffuse freely through the cell membrane and acidify the cytoplasm; these protons are constantly extruded by the F1/F0-ATPase. The activity of the F1/F0-ATPases is increased under acidic conditions (74) to facilitate enhanced export of protons at low pH. The pH optimum of F1/F0-ATPases for less acid tolerant bacteria is approximately pH 7.0 (73, 188). On the other hand, *S. mutans* exhibits pH optimum closer to pH 6.0 (188). The ability of F1/F0-ATPases to function at low pH partially explains why aciduric bacteria are able to survive under high acidic condition.

Decreased permeability to extracellular protons as a result of changes in cell membrane composition also contributes to ATP. *S. mutans* cell membrane predominantly composed of

short-chained saturated fatty acids when grown at pH 7.0. However, when grown at pH 5.0, a shift occurs in the fatty acid composition of the membrane with long-chain mono-unsaturated fatty acids become more abundant (60, 168). This shift in membrane composition is not only responsible for reducing the permeability of *S. mutans* to protons, but also indirectly contributes to the increased activity of the membrane-bound F1/F0-ATPases. Furthermore, these mono-unsaturated fatty acids also contribute to increased glycolytic activity, possibly as a result of altered function of membrane-bound proteins.

The ATR of *S. mutans* has been shown to enhance expression of approximately 200 proteins in *S. mutans* (74, 115). These acid-enhanced proteins include enzymes involved in metabolism, including glycolysis and branched-chain amino acid biosynthesis, the various subunits of F1F0-ATPases, general stress proteins such as heat shock proteins, and chaperone proteins (74, 115, 189). Biofilm derived cells are more acid resistant than planktonic cells (116) and this is believed to be due to cell density-dependent regulation of the ATR via the quorum-sensing system in *S. mutans* (39, 116). The ATR is important for survival of *S. mutans* under acid stress. The extent of this response in *S. mutans s*ets it apart from other less acid-tolerant oral bacteria. It provides *S. mutans* w*i*th a competitive advantage, allowing it to dominate over other oral bacteria under the acidic conditions experienced in dental plaque and thus contributes to caries development.

1.3.e. Production of mutacins

The bacteriocins produced by *S. mutans* are termed mutacins, which are mainly bactericidal for bacteria of the same or closely related species as well as for other Gram-positive microorganisms, and are likely to confer an ecological advantage in diverse bacterial communities in the dental biofilm (9). Mutacins can be divided into two groups: (i) the lanthionine-containing (lantibiotic) mutacins (32, 76, 142, 163-165) and (ii) the unmodified

mutacins (8, 166). While most bacteriocin activities characterized to date consist of a single active polypeptide, several two-component lantibiotic and nonlantibiotic bacteriocins have also been described, and these are dependent upon the collaborative activity of two polypeptides to exert their full antimicrobial activity (37, 62, 69).

The relationship between caries activity and the higher synthesis of some virulence factors by different genotypes of *S. mutans* has been demonstrated previously (139, 147). Mutacin production by *S. mutans* may facilitate the transmission of the species between mother and child and increase the ratio of this species in the dental biofilm, contributing to increased risk of caries (67). However, some studies found no association between the inhibitory spectrum of mutacins and infecting levels of mutans streptococci or caries incidence, suggesting that mutacin production may not be relevant in the ability of *S. mutans* to colonize the host and induce disease (5, 120). Kamiya et al. (92) demonstrated distinct mutacin production profiles among *S. mutans* strains isolated from caries-active and caries-free individuals, which can be related to different colonization profiles described in these individuals. Mutacins could play an important biological role in the regulation and composition of dental biofilm due to their synergistic or antagonistic activity, suggesting that wide spectrum mutacins may be more important in the colonization and stabilization of this cariogenic species, mainly in the stable niche of highly complex microbial activity (92).

1.4. Genomic organization and diversity among *S. mutans* clinical isolates

The genomic heterogeneity within a bacterial species governs its lifestyle and niche adaptation. Although organisms belonging to the same genus or species have a common gene set, the core genome of individual organisms may differ in strain-specific gene content that reflects the physiology and virulence properties of an organism. Not all genetic differences among various strains are important for niche adaptation of the pathogen, however some strain-specific genes

are thought to be important for its survival in a chosen niche. The strain-specific gene variation can arise from horizontal gene transfer including indels (108, 109, 184), genetic loss (36), gene duplication (63), and gene modification (56). Horizontal gene transfer provides acquisition of readily available novel pool of genes and a predominant force for exploiting a new niche.

Based on the composition of cell surface RGP*, S. mutans* has been grouped into four serotypes; *c, e, f,* and *k*. Complete genome sequences for two *S. mutans* strains (UA159 and NN2025) belonging to serotype *c* are currently available (3, 136). Both the genomes are composed of a single circular chromosome of ~2.0 Mb in length with an average G+C content of about 36%, a typical pathogens belonging to low G+C gram-positive. Table 1 shows a basic comparative analysis of both the genomes. Analysis of *S. mutans* genome indicates several unique features that support the lifestyle of this organism in the dental plaque. For example, since carbohydrate metabolism is a key survival strategy for this organism, genome data suggest that *S. mutans* is capable of metabolizing a wider variety of carbohydrates than other gram-positive bacteria. Since *S. mutans* is a lactic acid producing bacterium, enzymes responsible for pyruvate metabolism are found in this organism including a newly identified acetoin dehydrogenase (136). However, the genome data suggest that S. mutans lacks genes for a complete tricarboxylic acid (TCA) cycle pathway. It has been suggested that the primary role of the existing TCA enzymes for the synthesis of amino acid precursors (38).

Surprisingly, more than 15% of *S. mutans* genome encodes for transporter proteins (3, 136); among them the most abundant are various ATPases. Besides P-type and F-type ATPases, ABC transporters are present in very high proportion. It has been reported that 10% of the total ORFs encoded by this organism are ABC transporter related genes. About one-third of all ABC transporters are categorized as importers, and the rest are exporters suggesting that this pathogen is capable of actively exporting excess metabolic byproducts and toxic molecules (3).

Another surprising finding is that unlike other streptococci *S. mutans* genome does not contain any temperate prophages. But, the genome contains multiple transposon like sequences and insertion (IS) elements, and the IS3 family is the predominant one. Seven complete IS3 elements and 15 fragmented IS3 elements are found in UA159. Furthermore, one conjugative transposon TnSmu1, and a distinct 40kb genomic island TnSmu2 are present in UA159. The TnSmu2 contains about 29 ORFs predicted to synthesize bacitracin and germicidine. Surprisingly, some of the ORFs encoded within the island are very long including one that is ~ 8kb. This genomic island is also highly variable among clinical isolates (198). In addition to TnSmu1 and TnSmu2, *S. mutans* contains about 12 more isolated genomic islands of unknown origin (3, 136). Both the strains contain clustered regularly interspaced short palindrome repeat (CRISPR) regions; some of the repeat sequences present in CRISPR matches bacterial phage genomes. It seems that *S. mutans*, which is a naturally transformable organism, evades phage infection by Cas protein/CRISPR mediated destruction.

Comparison of the two *S. mutans* genomes suggests that a highly conserved large core-genome. This is somewhat surprising since both *Streptococcus pyogenes* (GAS) and *Streptococcus agalactiae* (GBS) have a shorter core-genome and larger pan-genome (total known genome content). However, multilocus sequence typing (MLST) studies suggest that the natural habitat contains a diverse population of *S. mutans* strains (48, 144). For example, Nakano et. al. (144) showed that 92 sequence types were identified among 102 clinical isolates. Furthermore, MLST analysis also suggests that serotype c is the dominant serotype (about 80%) among *S. mutans* clinical isolates (144). Serotype *c* appears to be the ancestral phenotype and that serotype *e* and *f* strains have evolved by acquiring strain-specific genes. Even though the differences in cell-surface associated antigens suggest an evolutionary trend, differences in cariogenic potential have not been observed among various serotypes. Therefore, the link between serotype and cariogenecity remains unclear.

Table 1. Comparative basic features of *S. mutans* UA159 and NN2025

FEATURES	STRAINS	
	UA159*	NN2025*
Serotype	*c*	*c*
Length of sequence	2,030,921	2,013,587
G+C content	36.83%	36.85%
Open Reading frames		
Percentage of coding	85.82%	85.18%
Protein coding region	1960	1895
Average gene length (bp)	889	903
RNA		
Ribosomal RNA	5	5
Transfer RNA	65	65
Transposon or Insertion sequence (IS)		
Full length	7	6
Partial	15	13

*The GenBank accession numbers for the genomic sequences of *S. mutans* are NC004350 (UA159) and NC013928 (NN2025).

Adapted from Maruyama et. al. (2009).

1.5. Multidrug Resistance in Bacteria

S. mutans, like other bacteria, has incredible ability to develop resistance against toxic compounds and to adapt rapidly to a changing environment (113, 114). A wide range of mechanisms to resist the action of toxic chemicals is present in bacteria (121, 149, 162). These systems can be specific for a drug or closely related compounds such as inactivation of β-lactam ring of antibiotics by β-lactamase enzymes (43). The other mechanism of resistance is by the modification of drug target to reduce the target's affinity for the drug (183). For many pathogens, the frontline defense is to reduce the permeability of the cell envelope. The cytoplasmic membrane acts as a barrier to prevent toxic chemical influx into the cell. Gram-negative bacteria also contain an outer membrane that is less permeable to various toxic compounds. However, gram-positive bacteria, such as streptococci, lack an outer membrane, but are surrounded by thick peptidoglycan cell wall that offers very little resistance to diffusion of toxic chemicals. Probably the most important mechanism of drug resistance is the active efflux of chemicals from the cell (121, 162). These active extrusion mechanisms involve integral membrane proteins that utilize metabolic energy to expel drugs across the membrane against the concentration gradient (121). These effluxes can be specific for a given drug or group-specific effluxes or may have a wide range of structurally and chemically unrelated compounds. The latter efflux system, which is known as multidrug efflux or MDR transporter, is very important for the emergence of antibiotic resistance among pathogens. MDR transporters are also important for bacteria to survive under a complex environment and produce a biofilm (112, 121, 149).

Based on the bioenergetic criteria, MDR transporters can be classified into two major groups, ATP-dependent transporters and protein motive force (PMF) dependent transporter. ATP-dependent transporters are the primary active transporters that belong to ATP binding cassette (ABC) superfamily and utilize the free energy of ATP hydrolysis to extrude the chemicals from

the cells against the concentration gradient. The basic structure of ABC transporters consists of four domains: two integral membrane domains and two ATPase subunits. The ATPase subunits of ABC transporters include a characteristic ABC signature motif (42). Bacterial ABC transporters involved in uptake also require an additional solute binding domain, which provides specificity and maintains the direction of transport into the cell (42). PMF-dependent transporters are secondary transporters that utilizes PMF or sodium motive force for drug expulsion. Secondary MDR transporters can be further classified into several categories on the basis of their predicted secondary structure and amino acid sequence. The major facilitator superfamily (MFS) (130), the small MDR family (SMR) (157), the resistance-nodulation-cell division (RND) family (174), and the multidrug and toxic compound extrusion (MATE) family (174) are the most recognized as well as well characterized PMF-dependent MDR transporters.

1.6. Scope of the study

S. mutans persists in the oral cavity and maintains a biofilm lifestyle in the dental plaque which contains over 600 different microorganisms. The oral cavity is a very dynamic environment that often undergoes rapid changes in pH, nutrient availability, and oxygen tension. The mechanisms by which *S. mutans* cope with these dynamic changes are relatively well studied (26, 113). However, the oral cavity is also exposed to a number of compounds that are toxic to microorganisms including *S. mutans*. Oral healthcare products, tobacco products, and other nutritonal supplements are a significant source of toxic compounds. Furthermore, degradation byproducts of dental composite resins are also a significant source of toxic chemicals. Bacteria in the dental plaque community also generate various toxic compounds, such as methyl mercaptan, dimethyl sulfide that can interfere the growth of other bacteria (85). The acidic environment in the dental plaque not only demineralizes the tooth enamel, but also intensifies the toxicity of various chemical including metal ions. Furthermore, during the growth in the

biofilm, cells also maintain a balance of metabolism that involves production and detoxification of toxic byproducts such that the levels accumulated are well within the capacity of the cell to adapt. Two such examples are the production of methyl glyoxal, a byproduct of glycolysis; and peroxides, a byproduct of redox reaction (49, 50, 57, 65).

How *S. mutans* tolerates exposure to various toxic substances during its growth in the oral cavity is poorly understood. The goal of the project was to understand the mechanism of tolerance to quaternary ammonium compounds (QAC), which are often used in many healthcare products and in antiseptic solutions. The representative QAC that we chose is methyl viologen (MV), also known as paraquat, because this molecule can also generate superoxide stresses in the cell. Our study provides the first evidence of an ABC transporter complex that acts as a major multidrug efflux system in *S. mutans*.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial Strains and Growth Conditions

Escherichia coli strains DH5α[(F- φ80*lac*ZΔM15 Δ (*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-)] (NEB) and Top10 [F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara- leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G (Invitrogen) were used for cloning. *E. coli* TG1Rep+ [(*supE hsd*-5 *thi* Δ(*lac-proAB*) F'(*traD6 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15) (*repA* from pWVO1)] was use for propagation of pGhost9::*IS*S1 at 37°C. These strains were grown in Luria-Bertani medium, and when necessary, ampicillin (100 μg ml^{-1}), kanamycin (100 μg ml^{-1}), and spectinomycin (100 μg ml^{-1}) were included. *S. mutans* UA159 is a standard laboratory strain, which belongs to Bratthall serotype c, was originally isolated by Page Caufield (University of Alabama, Birmingham), and its whole genome has been sequenced recently (3). Twelve other *S. mutans* strains, including several clinical isolates, were also used for genomic analysis. *S. mutans* strains were routinely grown in Todd-Hewitt medium (BBL, Becton Dickinson) supplemented with 0.2% yeast extract (THY). When necessary, kanamycin (300 μg ml^{-1}), erythromycin (10 μg ml^{-1}), and spectinomycin (300 μg ml^{-1}) were included.

2.2. Transposition Assay and Screening

Insertional mutagenesis was performed with the plasmid pGh9:IS*S1,* according to the method of Maguin et al. (18, 128, 191) (Fig. 1). Briefly, *S. mutans* was transformed with pGh9:IS*S1* and transformants were selected on THY agar containing Em, and incubated at 30°C. An overnight-grown liquid culture was made from a single transformed colony. Cultures were diluted 100-fold in the same medium, grown for 2 hrs at 30°C, and then shifted to growth at 37°C for 2.5 hrs to select for transposition events. Cultures were then plated on THY-Em plates to obtain individual mutants, which were then inoculated in 96-well plates containing THY broth supplemented with

Em, and grown overnight. Using a 48-pin replicator (Sigma), the cultures were then spotted on THY agar plates with or without methyl viologen (MV, 5mM), and the plates were incubated at 37°C in a CO2 incubator containing 5% CO2**.** Colonies that grew on THY plates but failed to grow on THY+MV plates, were identified, cultured overnight in THY- Em at 37°C, and processed for analysis (Fig. 2). The transposition frequency was between 10^{-2} to 10^{-3} Em^R cells per total cells plated.

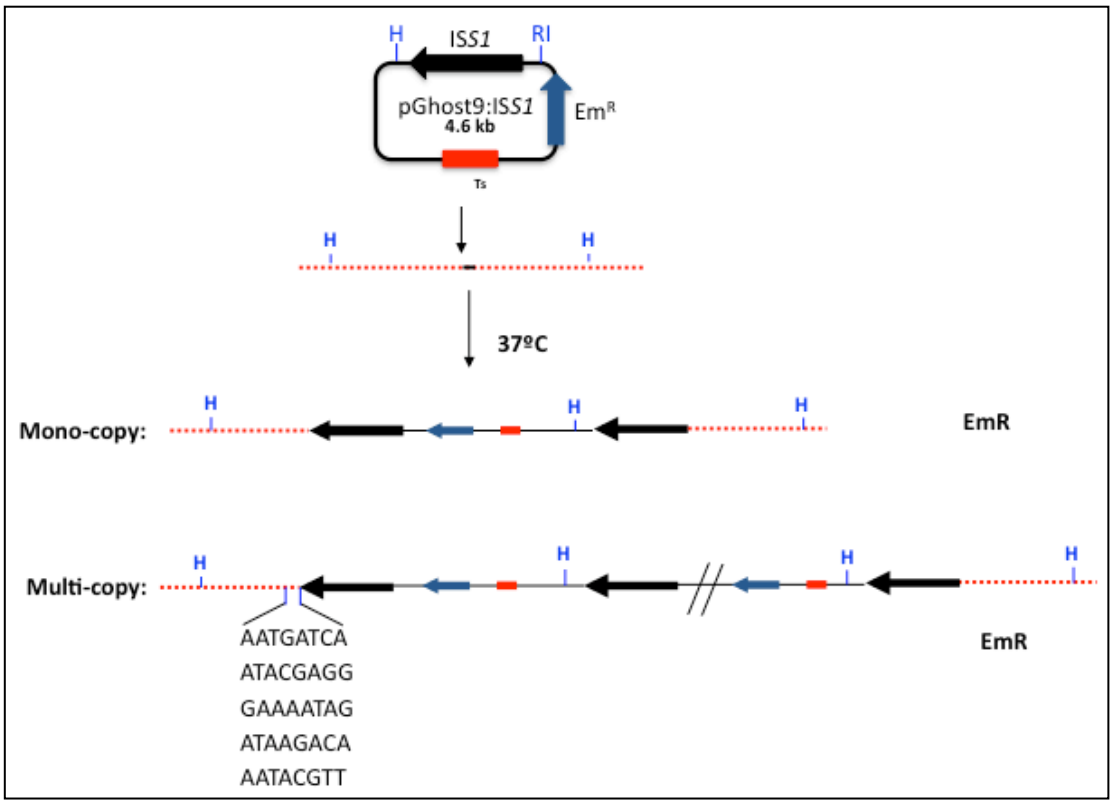


Figure 1: Schematic diagram of the mechanism of integration of IS*S1* in the genomic DNA. IS*S1* replicative transposition is expected to generate mono-copy transposition, i.e., integration of the plasmid vector between duplicated IS*S1* sequences. Transposition may also give rise to a clone containing tandem multi-copies of the transposed structure. Symbols: dotted lines, chromosomal DNA; arrows, IS*S1*; solid line, vector DNA (pGhost9), red box, erythromycin resistance marker. The delivery plasmid also contains unique restriction sites *Hind*III (H) and *Eco*RI (RI) that flank the IS*S1*. These sites were used for mapping the insertion. Examples of sequences (8-bp) at the junction that are duplicated due to replicative integration are shown.

Table 2: List of oligonucleotides in this study

Name	Sequence (5'→3')	Purpose
ISS1-For	CGATGCTCTAGAGCATTCTCTGG	ISS1 probe preparation
ISS1-Rev	GCTTGATGGAGAGAATGGGTTCTGTTGC	ISS1 probe preparation
ISS1-Rout2	AATAGTTCATTGATATATCCTCGCTGTCA	Sequencing PCR products
ISS1Fout1	GCAAGAACCGAAGAAATGGAACG	Inverse PCR
ISS1Rout1	GATTGTAACGTAGATAATAACCAACAGC	Inverse PCR
Lnk905-906F	GCCATGATGAACTCATGAAGTCC	Linkage mapping
Lnk905-906R	GAGCGCCTTGAACTCCGCGAGCC	Linkage mapping
Lnk906-MF	GGGGAACCATCAAAGTCTCTTAGCT	Linkage mapping
Lnk906-MR	CCTCCTTGGTTAAAATCTGCCGCTGC	Linkage mapping
Lnk-905R	CCAAGAATGTCCAATAGTAACCATTTGG	Linkage mapping
Smu.905-6 BamCF	GGCGGATCCTTGAAAAATATGAGCGTTATG	Cloning SMU.905/linkage
Smu.905-6 BamCR	GGCGGATCCTTTCCTGCTTTCTGCTATTATACAC	Cloning SMU.905
Smu.905-CF	CCAAATGGTTACTATTGGACATTCTTGG	Amplification of SMU.905 for disruption
Smu.905-R	CGTTAAACTTTGCTGATTGAGTTGTGAGTTAGC	Amplification of SMU.905 for disruption
Sq902F	TAAGCAAAACAGTTGGCGATAAGACTG	Semiquantitative PCR
Sq902R	TGAGCTGCATTTCCCGCAAA	Semiquantitative PCR
Sq905F	CTATGCTTCTGGCAGATTAACCACG	Semiquantitative PCR
Sq905R	CGCGCAAAGGCACGAATAAC	Semiquantitative PCR
Xho-C905-6R	CGCCTCGAGTCTATGAAAATCAATAGACTT	Cloning SMU.906-SMU.906 region
Smu.CI-2Bam905	CCGGGATCCAATTATGATGAAGCTAATCAGGAGCAATTGGAAAAGG	Cloning SMU.906-SMU.906 region

2.3. Identification of IS*S1* Integration Site

Integration of IS*S1* on *S. mutans* chromosome was first verified by Southern hybridization. Selected MV sensitive mutant clones were grown in THY+Em at 37°C overnight and chromosomal DNAs were isolated using a Gram-positive genomic DNA isolation kit (EpiBio) following manufacturers protocol. Ten micrograms of chromosomal DNA was digested with *Hin*dIII enzyme (an unique site in pGhost9::IS*S1*) and electrophoresed in an 8% TAE-agarose gel. DNA was transferred to Zeta-Probe Nylon membrane (BioRad) following the manufacturers protocol. A ISS1 specific probe (about 900-bp) was amplified by PCR using ISS1-For and ISS1-Rev primer set and pGhost9:: IS*S1* as template. The PCR fragment was radiolabeled with [α-^{32}P]-dATP (3,000 Ci/mmol) (MP Biomedicals) by random priming with a DECAprimeII kit (Ambion), according to the recommendations of the supplier and used as a probe. Unincorporated radionucleotides were removed from the reaction mixture by BioSpin 30 spin column (BioRad). Southern hybridization was carried out using ULTRAhyb hybridization solution (Ambion) at a high stringency (42°C) condition following standard protocol (Maniatis). After overnight hybridization, filters were washed with SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS at 42°C. The membrane was exposed to a phosphorimager plate and developed by Strom instrument (GE Healthcare).

The location of the inserted IS*S1* element was identified by inverse PCR. Chromosomal DNA, isolated from the selected mutants, was digested by *Hin*dIII or *Eco*RI enzymes followed by heat inactivation at 65°C. About 2µg digested DNA was self-ligated by T4 DNA ligase. These ligated samples were subjected to inverse PCR by using primers ISS1Rout1 and ISS1Fout1 (For all the primers, please refer to Table 1. The PCR products were purified from agarose gel and were sequenced with primer ISS1-Rout2. The flanking sequences obtained from sequencing analysis were mapped on the genome of *S. mutans* UA159 by BLAST search.

For some mutants, a cloning method was also used. Total genomic DNA of the pGhost9:IS*S1* mutants was digested with *Eco*RI or *Hin*dIII, ligated, and transformed into *E. coli* TG1Rep+ strain. Erythromycin-resistant *E. coli* clones harboring pGh9:IS*S1* plasmid with genomic streptococcal DNA located adjacent to the insertion site of the plasmid were selected on LB plates supplemented with erythromycin (150 mg/l). Nucleotide sequencing of the inserted genomic DNA was performed with primers ISS1Rout1 or ISS1 Fout1.

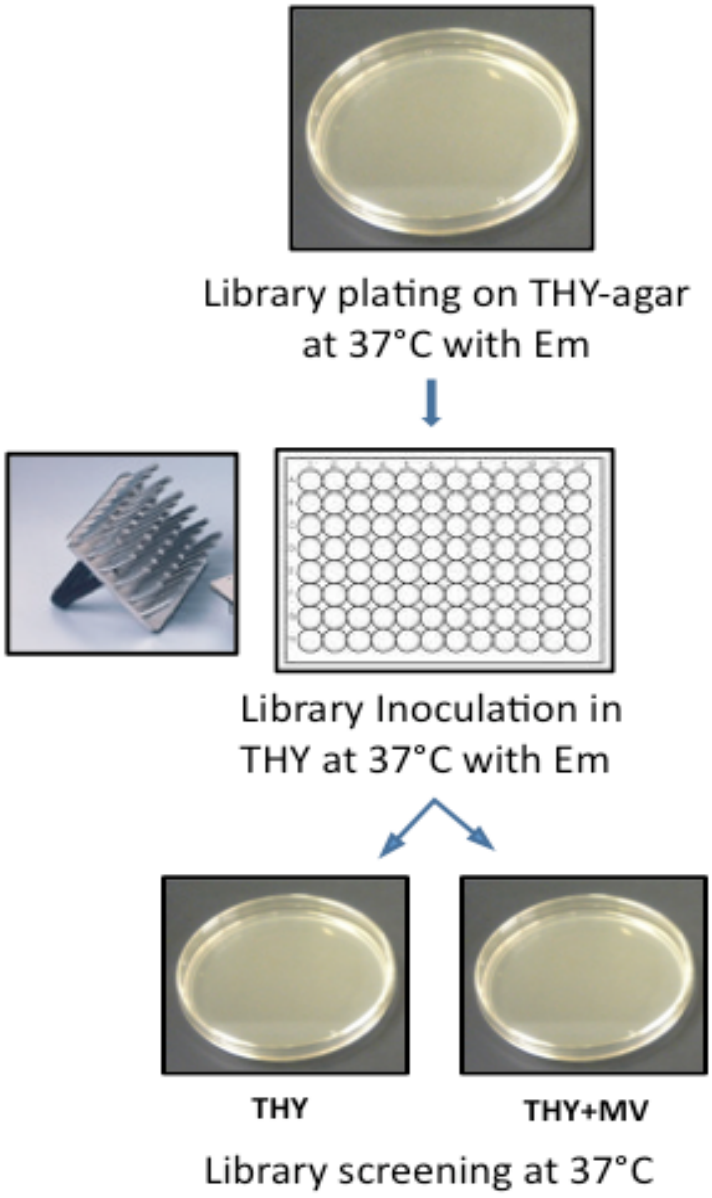


Figure 2: General strategy to isolate viologen sensitive mutants. The transposon library was first selected for the macrolide resistance marker of the transposon delivery plasmid (pGhost9:: IS*S1*). Individual overnight cultures were prepared in 96-well plates in the presence of the antibiotic (Em). The cultures were then replica patched on two plates: one containing 5mM viologen (THY+MV) and the other containing no viologen (THY). The colonies that failed to grow on THY+MV plates, but grew successfully on THY plates are the candidate clones that were analyzed further.

Table 3: List of *S. mutans* strains created in this study

Name	Genotype or relevant description*
UA159	Wild type parental strain, serotype c
IBS-A25	SMU.905 locus deleted by pIB-A19, Km^R
IBS-A26	SMU.905 locus deleted by pIB-A20, Sp^R
IBS-A27	ISS1 insertion in SMU.905 at position 860907 (4D3); Em^R
IBS-A28	ISS1 insertion in SMU.905 at position 861009 (4H4); Em^R
IBS-A29	ISS1 insertion in SMU.283 at position 269349 (5A12); Em^R
IBS-A30	ISS1 insertion site could not be mapped (6D12); Em^R
IBS-A31	ISS1 insertion in SMU.902 at position 858818 (14E4); Em^R
IBS-A32	ISS1 insertion in SMU.906 at position 861579 (15B1); Em^R
IBS-A33	ISS1 insertion site could not be mapped (17D12); Em^R
IBS-A34	ISS1 insertion in SMU.905 at position 859850 (22A9); Em^R
IBS-A35	ISS1 insertion in SMU.905 at position 859515 (23F4); Em^R
IBS-A36	ISS1 insertion in SMU.906 at position 861026 (26G4); Em^R
IBS-A37	ISS1 insertion in SMU.906 at position 862480 (27A12); Em^R
IBS-A38	ISS1 insertion in SMU.906 at position 862730 (28B1); Em^R
IBS-A39	ISS1 insertion in *ciaH* at position 1070008 (32D2); Em^R
IBS-A40	ISS1 insertion in SMU.905 at position 859465 (32D7); Em^R
IBS-A41	ISS1 insertion in *ciaH* at position 1070008 (34G7); Em^R
IBS-A42	Plasmid (pGhost9) cured derivative of IBS-A34 (SMU.905); Em^s
IBS-A43	Plasmid (pGhost9) cured derivative of IBS-A32 (SMU.906); Em^s
IBS-A44	Plasmid (pGhost9) cured derivative of IBS-A31 (SMU.902); Em^s

* Original ISS1 mutant identifiers are indicated in strains IBS-A27 to IBS-A41

2.4. Curing of pGhost9::IS*S1* from the Selected Mutants

IS*S1* is a replicative transpon. Therefore, upon integration the insertion sequence is duplicated and flanks the pGhost9 plasmid. The plasmid vector sequence was excised from the selected mutants by growing the bacteria in THY broth without antibiotics at 30°C, which permits the plasmid replication (128). Broth cultures were diluted, and plated on selective and nonselective THY media at the nonpermissive temperature (37°C) to determine the colony counts. Excision of the vector sequence from the bacterial chromosome was indicated by the loss of the erythromycin resistance marker and stabilized mutant strains (Em^s colonies) were isolated by replica plating at 37°C. Em^s colonies were confirmed for loss of the plasmid sequence by PCR with primers homologous to the flanking regions (Fig. 4).

2.5. Construction of SMU.905 Deletion Mutant

To delete the SMU.905 locus, an1.7-kb fragment spanning the entire SMU.905 region was PCR-amplified from UA159 genomic DNA, using the primers 905CF and 905R. This fragment was cloned into the pGemT-Easy vector (Promega, WI) to create pIBA21. A 0.87-kb spectinomycin-resistance gene (*aad9*) was amplified from pUCSpec (81) using primers Spec-P-For and Spec-Rev (33) . Plasmid pIBA21 was restricted with *XcmI* and blunted by treatment with T4-polymerase. The PCR amplified *add9* was then cloned into this blunted plasmid to generate pIBA20. The orientation of the *aad9* insert in pIBA20 was verified by PCR, and found to be the same direction as the SMU.905 locus. Plasmid pIBA20 was then linearized by *Not*I, and used for transformation of UA159. Spectinomycin-resistant transformants were selected, and the deletion of the SMU.905 locus was verified by PCR. A successful representative transformant was selected and named IBSA26. Similarly, SMU.905 was also deleted using a kanamycin resistant marker amplified from pUC4ΩKm plasmid (158). This KmR resistant mutant strain was named IBSA25.

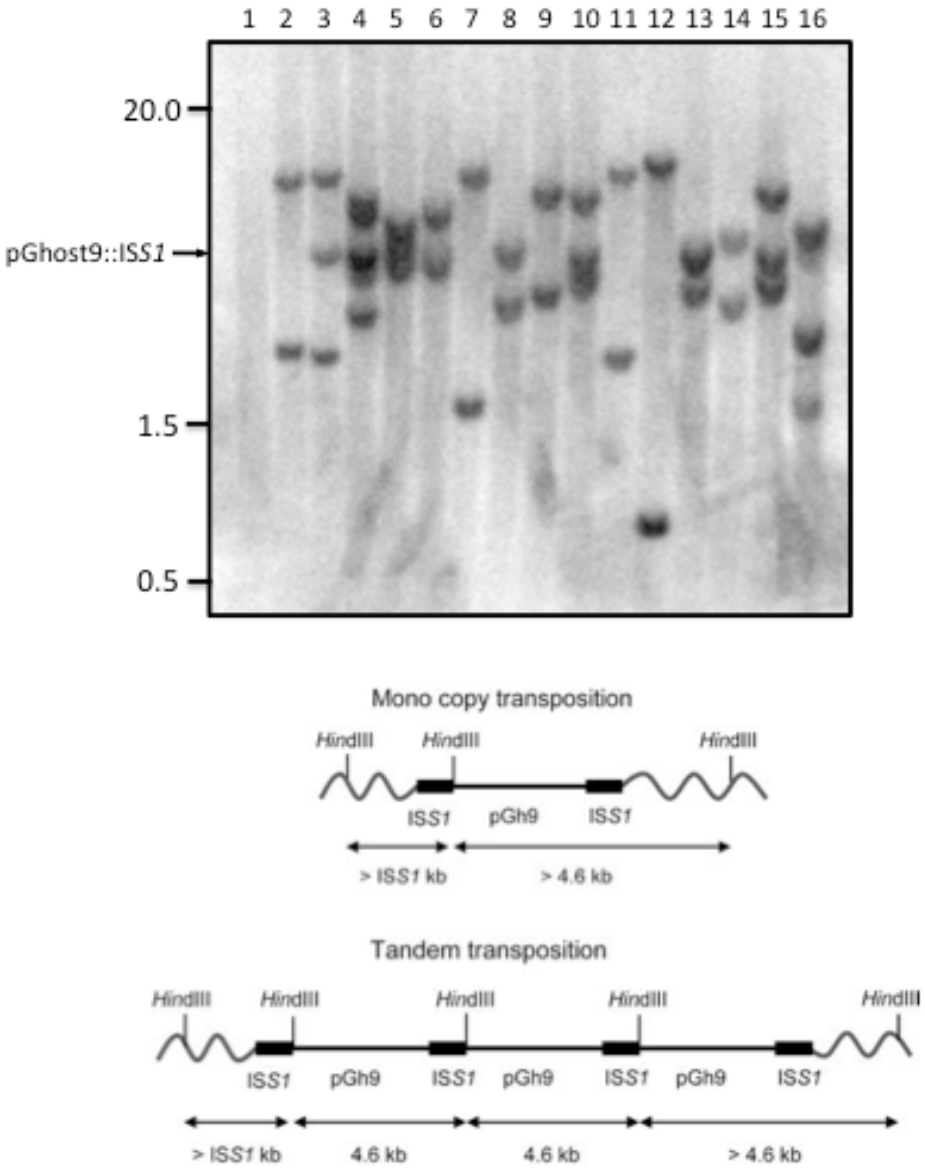


Figure 3: Southern blot analysis of the mutants. Southern blot analysis of the *Hind*III digested DNA of fifteen independent pGhost9:IS*S1* integrants with the labeled IS*S1* probe. Schematic representation of the structure of expected transposition products is shown in the lower panels. In mono copy transposition, the pGhost9 is integrated between duplicated IS*S1*. In tandem transposition, multiple copies of the pGhost9 are integrated at the transposition site. Samples are: lane 1, UA159; lanes 2 to 16, IBSA27 to IBSA41, respectively. *Arrows* DNA fragments, which should hybridize with the IS*S1* probe after *Hin*dIII digestion. *Wavy lines*, *solid lines* and *black rectangles* represent chromosomal DNA, plasmid vector and IS*S1*, respectively.

2.6. Isolation of RNA from Bacterial Cultures

Total RNA was isolated from bacterial cultures according to the protocol described below. *S. mutans* UA159 and its derivatives were grown in THY medium with appropriate antibiotics to mid-exponential phase (70 Klett units), and the cultures were harvested by centrifugation. The cell pellets were then suspended in 10 ml of RNAprotect bacterial reagent (Qiagen) and incubated at room temperature for 10 min. Total RNA was extracted using an RNeasy minikit (Qiagen) according to the manufacturer's instructions, with a modified bacterial-lysis step. Briefly, cells were broken by the addition of an equal volume of 0·10 mm glass beads (MP Biomedicals) and vortexing the suspensions for 45 sec. at speed 6 in a bead beater (Thermo Electron). The supernatants were loaded onto RNeasy mini columns and purified using the manufacturer's protocol. The purified RNA samples were further treated with Turbo DNase (Ambion) according to the manufacturer's instructions to remove residual DNA contamination. The quality and integrity of the purified RNA samples were ascertained on a 1.2% agarose gel electrophoresis. Total RNA was quantitated in a UV spectrophotometer (Shimadzu) according to the optical density at 260 nm (OD_{260}) (1 OD_{260} unit = 40 µg/ml).

2.7. Semiquantitative RT-PCR (sQRT-PCR)

Total RNA (DNA free) was used to prepare cDNA, using Superscript II reverse transcriptase (Invitrogen). Briefly, RNA samples (500 ng) were mixed with random decamer primer (100 ng) and deoxynucleotide triphosphates (dNTP) (0.5 mM), and the cocktail was heated at 65°C for 5 min, followed by quick chilling on ice. First-strand buffer (SuperScript-II reverse transcriptase; Invitrogen), 10 mM dithiothreitol (DTT), and RNase inhibitor (40 U; Roche) were added to the cocktail and incubated at room temperature for 2 min, and the reverse transcriptase was added to the reaction mixture. The reaction mixture was further incubated at room temperature for 10 min, followed by incubation at 42°C for 50 min to synthesize cDNA. The reaction mixture was heat inactivated at 70°C for 15 min. To degrade the DNA-RNA hybrid, RNase H (2 U; Invitrogen)

was added to the reaction mixture and incubated at 37°C for 45 min. Finally, the cDNA was purified using a PCR purification kit (Qiagen), and the cDNA concentration was determined using a UV spectrophotometer (Shimadzu). Five to 20 ng of cDNA was used to carry out the second-step PCR, using ReadyMix *Taq* PCR mix with $MgCl_2$ (Sigma). Twenty to 22 PCR cycles were carried out to amplify the cDNA products of interest. The amplified PCR products were then electrophoresed on a 2% agarose gel. As an internal control, the *gyrA* gene was used to ensure that equal amounts of cDNA were used in each reverse transcription (RT)-PCR.

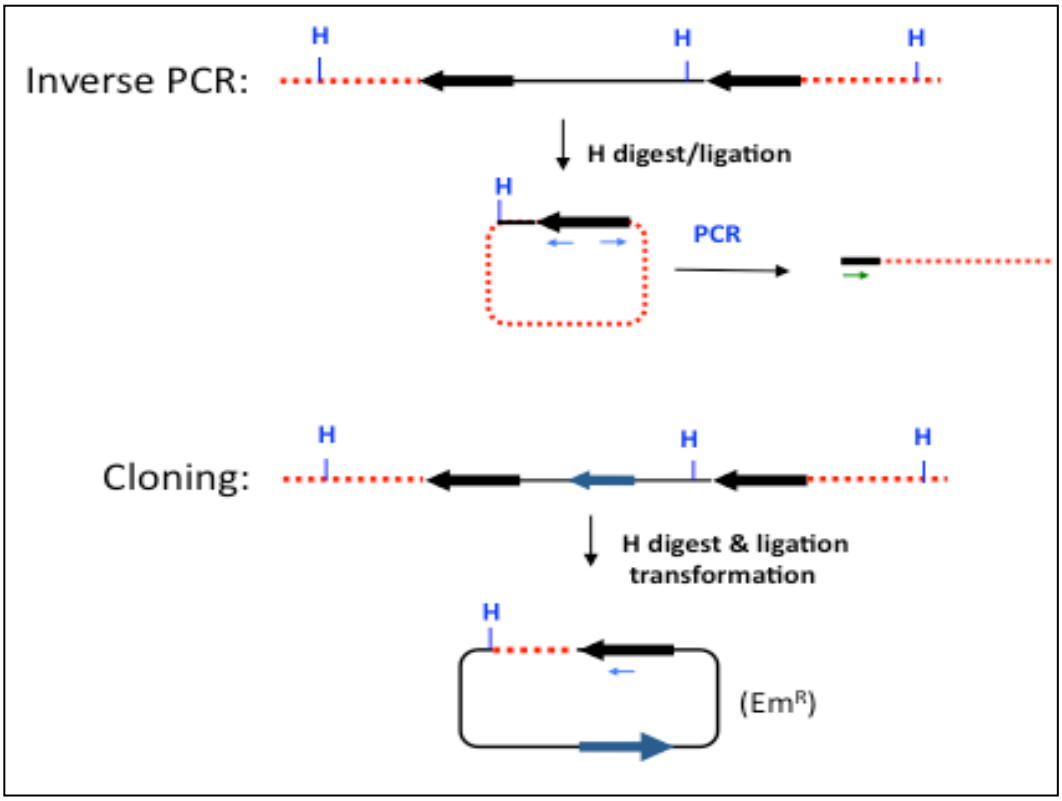


Figure 4: Strategies used to identify IS*S1* insertion sites. Two different strategies were employed, inverse PCR and cloning, to map the insertion sites. Genomic DNAs isolated from the putative clones were digested with restriction enzymes (e.g. *Hind*III (H)) that have a unique site within the plasmid. Restricted DNAs were ligated and used for either inverse PCR (using primer sets shown by blue arrows) or used for *E. coli* transformation with selection for erythromycin (EmR). PCR products or plasmids from *E. coli* clones were sequenced to identify the site of IS*S1* integration.

Table 4: Sequence adjacent to IS*S1* insertion site.

Mutant name (identifier)	Sequence (5'→3')	Position on the genome[$]
IBS-A27 (4D3)	TACTGGAT	860907
IBS-A28 (4H4)	GTTTAACG	861009
IBS-A29 (5A12)	AAAATTCT	269349
IBS-A31 (14E4)	CGCTCAAG	858818
IBS-A32 (15B1)	CCGCTGAC	861579
IBS-A34 (22A9)	CTTGACAA	859850
IBS-A35 (23F4)	GCATAGGC	859515
IBS-A36 (26G4)	TGAAAACC	861076
IBS-A37 (27A12)	AATAATAT	862486
IBS-A38 (28B1)	CCCCTGTT	862730
IBS-A39 (32D2)	GATAATAT	1070008
IBS-A40 (32D7)	TTGGTTTA	859465
IBS-A41 (34G7)*	ATTTACCT	1070497
	GAAGGATC	1088450

[$] Origin is set as position 1
* This mutant contains two ISS1 insertions

2.8. Linkage RT-PCR Analysis

RT-PCR was used to determine the transcriptional organization of the SMU.905-SMU.906 locus, following a protocol described by Chong *et al* (34). Five microgram of DNA-free RNA was used for the synthesis of cDNA using SuperScript II reverse transcriptase (Invitrogen), according to manufacturer's protocol. PCR was then performed on RNA (as negative control), cDNA, and chromosomal DNA (as positive control), using the primer pairs as described in Table 2 and depicted in Fig.7, to determine which of the genes in the SMU.905-SMU906 locus were co-transcribed.

2.9. Sensitivity to Various Reactive Oxygen Species (ROS) Generating Reagents

To evaluate the sensitivity of the *S. mutans* mutants to various ROS producing chemicals, cultures were exposed to reagents either through disk diffusion assays or growth on THY agar plates containing the chemical of interest. For disk diffusion assays, cultures were grown overnight in THY broth (with or without antibiotics as required), and then overlaid on THY agar plates, followed by placement of filter paper disks (6 mm in diameter) containing various chemicals onto the inoculated agar. After overnight incubation at 37ºC, under microaerophilic conditions, the diameters of the zones of bacterial growth inhibition were measured. For growth on THY agar plates containing stress-inducing chemicals, cultures were grown to exponential phase in THY broth with appropriate antibiotics, at 37ºC. Cultures were pelleted via centrifugation, washed twice with 0.85% NaCl, and resuspended in 0.85% NaCl. The cultures were adjusted to an optical density (A_{600}) of 5.0, serially diluted ten-fold, and 7.5 µl of each dilution was spotted onto THY agar containing the oxidative-stress inducing chemicals. The plates were incubated overnight at 37ºC, under microaerophilic conditions, and bacterial growth was evaluated as previously described (20). The following chemicals were used as indicated. Methyl viologen (paraquat, Sigma) was added to THY agar medium to a final concentration of 5mM or 10 mM, while 10 µl from a 1 M stock was added to each disk for the disk diffusion

assay. Ethyl-viologen (1.5M; Sigma), benzyl-viologen (50mg/ml; Sigma), and diquat dibromide (100- or 500mg/ml; Sigma) were added to each disk. Menadione (1.0%; Sigma), pyrogallol (400mg/ml; Sigma) and plumbagin (100mM; Sigma) were used for generating superoxide radicals. Hydrogen peroxide (Sigma) was used for THY plate growth assays (2mM or 4mM) and disk diffusion assays (1.5% w/v). Cumene hydroperoxide (Sigma) and t-butyl-hydroxyperoxide (t-BOOH, Sigma) were used in disk diffusion assays; 10 µl of 10% cumene hydroperoxide or 70% t-BOOH was added to each disk.

2.10. Antibiotic Susceptibility Assay

Disc diffusion assays were performed to evaluate the antibiotic susceptibility of *S.mutans* UA159 and its derivatives as described previously (17). Briefly, antibiotic discs (6mm in diameter; Becton and Dickinson Laboratories) were placed on THY agar plates that were overlaid with 10 ml of THY soft agar, containing 200µl of *S. mutans* strain of choice. The plates were incubated overnight at 37°C under microaerophilic condition and the zones of inhibition were measured. The antibiotics used for this study were listed in Table S1

2.11. Acid Stress Tolerance

To analyze the growth of *S. mutans* cultures at low pH, the initial pH of the THY agar medium was adjusted, prior to sterilization, to pH 5.5 or pH 7.0, with HCl. 50 mM citrate-phosphate buffer of desired pH was added to media after sterilization. Different dilutions of *S. mutans* cultures, prepared as described above, were spotted onto the plates and incubated at 37°C under anaerobic conditions.

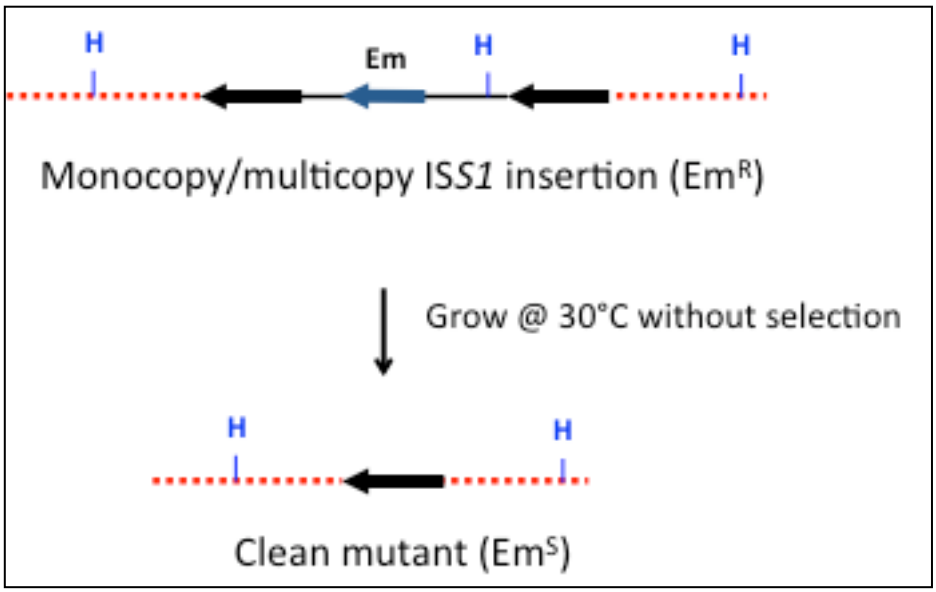


Figure 5: Schematic diagram of generating plasmid cured IS*S1* mutant strains. Since pGhost9 is a thermosensitive plasmid, it can be cured from the strain by stimulating the replication at permissive temperature (30°C). The plasmid-cured strain can be isolated by replica patching and verified by PCR amplification with appropriate primers.

2.12. Sensitivity to Osmotic Stress

Overnight cultures were diluted 20-fold in fresh THY media containing sorbitol (final concentration: 4% or 5%), NaCl (final concentration: 0.5M), or ethanol (final concentration: 4% or 5%), and grown at 37ºC. Growth was monitored using a Klett-Summerson colorimeter with a red filter, as previously described (18).

2.13. Sensitivity to Other Chemical Reagents

Sensitivity of the *S. mutans* mutant strains to various chemicals was evaluated primarily by using the disk diffusion method. THY agar plates inoculated with the wild-type or the mutant cultures were overlaid with disks containing various toxic, QAC, or other stress inducing chemicals. The chemicals (all procured from Sigma) tested were: benzidine (4mM), benzalkonium chloride (10mg/ml), tetraethylammonium bromide (10mg/ml), chlorhexidine gluconate (20%), crystal violet (1.5%), diamide (1.0M), 2'-2'dipyridyl (50mM), 4'-4' dipyridyl (1M), ethidium bromide (1.5%), ethylenediaminetetraacetic acid (EDTA, 0.5M), hexadecyltrimethyammonium bromide (CTAB, 0.5%), hexadecylpyridinium chloride (1%), hydrazine (0.5M), 1, 4' napthaquinone (0.1%), malachite green (1.5%), mitomycin C (12.5 µg/ml), 1,10'–phenanthroline (200mM), potassium telurite (1.0%), puromycin (6.0 µg/ml), pyronin B (5mg/ml), safranin (1.5%), streptonigrin (5mg/ml). A complete list of chemicals used in this study is shown in Table S1.

2.14. SOD Activity Assay

For visualization of SOD activity on non-denaturing polyacrylamide gels, *S. mutans* cells were cultured to stationary phase in THY medium and harvested by centrifugation at 7500 g for 10 min at 4°C. Cells were washed once in 500µl sample buffer (20 mM Tris-Cl, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA) and resuspended in the same buffer with addition of 5µl

Protease Inhibitor Cocktail set III (Calbiochem). Cells were then broken by the addition of an equal volume of 0·10 mm glass beads (MP Biomedicals) and vortexing the suspensions for 45 sec. at speed 6 in a bead beater (Thermo Electron). Cell debris was pelleted by centrifugation at 12000 g for 2 min, and the cell-free supernatant was retained. Protein concentrations were determined by a modified Bradford assay (Bio-Rad), using bovine immunoglobulin as standard. Cell extracts were mixed with equal amounts of 1x sample buffer (62.5 mM Tris.Cl, pH 6.8, 10% glycerol, 0.01% bromophenol blue) and 25 μg of each sample was loaded on a non-denaturing polyacrylamide gel (44) PAGE conditions were a 4.5% stacking gel (pH 8.3) and a 10% separating gel (pH 8.9) with the buffer system of Davis (44), except that the pH of the upper buffer was raised to 8.9 with 10 M NaOH. Gels were electrophoresed at 120V for 120 min and then stained with Coomassie brilliant blue, or SOD activity was detected as described by Beauchamp & Fridovich (13). Briefly, gels were incubated at ambient temperature in 2 mM nitroblue tetrazolium (Fisher BioReagent) solution made with 50 mM KPO_4 (pH 7.8). This solution was replaced with riboflavin buffer (36 mM KPO_4 [pH 7.8] containing 28 mM tetramethylethylenediamine (TEMED, Acros Organics) and 28μM riboflavin (Acros Organics) for 15 min. Gels were then transferred into distilled water and exposed to UV light from a 15 W lamp in a foil-lined box for 15 min. Gel images were digitized using a digital camera and intensities of bands were quantified with Kodak digital science 1D image analysis software.

2.15. Biofilm Formation Assay

UA159 and its derivatives were grown overnight in THY medium at 37°C anaerobically. The culture was diluted 1:10 into fresh THY medium and incubated further for 6 h. The culture was then diluted 1:1,000 with either THY medium containing 1% sucrose. A 0.8-ml volume of this cell suspension was added to each well of a four-well glass chamber slide (Lab-Tek; Nalgen Nunc International) for biofilm formation on glass. For biofilm formation on a polystyrene surface, U-bottom 96-well microtiter plates (Corning Inc.) were used. Biofilms were stained by

0.01% solution of crystal violet, malachite green, or safranin and photographed as described before (19).

2.16. Phenotypic Microarray Analysis

PM analysis was performed using Biolog's PM service facility. A total of twenty 96-well PM plates constituting eight metabolic panels (PM1 to PM8) and 12 sensitivity panels (PM9 to PM20) were used in this study. To assess the altered phenotypes of the *SMU.905* mutant (IBSA26), the growth was compared to its parent *S. mutans* UA159 strain. The basic growth media and the conditions for PM analysis were published previously (20, 22, 209). The inoculating cell densities used in this study were 1:13 dilution of 81% transmittance for both metabolic and sensitivity panels. PM analysis was conducted in duplicate after incubation of the strains at 37°C for 72 hours. An average height difference threshold of 50 for metabolic panels and a difference threshold of 60 for sensitivity panels were used to consider the difference between the two growths significant. The data were further confirmed by Student's *t* test. The growth kinetics for UA159 was displayed as a red tracing, while IBSA26 was displayed as a green tracing. Standard PM testing protocols are described in http://www.biolog.com; the conditions are similar to those used here.

CHAPTER 3

RESULTS

3.1. Isolation of *S. mutans* UA159 methyl viologen sensitive mutants

Red-ox cycling QAC agents such as methyl viologen (MV), ethyl-viologen (EV) are powerful propagators of superoxide radicals inside the cell. The toxicity of red-ox cycling agents may result not only from the toxic effect of ROS but also from the depletion of cellular levels of NADPH. The aim of this study was to understand how *S. mutans* cope with the toxicity and potential damage from QAC compounds that also generate reactive oxygen species. To identify genes that are potentially involved in MV tolerance, we used ISS*1* transposition mutagenesis since ISS*1* appears to integrate randomly into the genome of Gram-positive bacteria, including various streptococci (18, 182, 191). Furthermore, IS*S1* rarely inserts more than once into the genome of the same cell (18, 182, 191). We used plasmid pGhost9::ISS*1*, whose replication is temperature sensitive (127, 128), to introduce the insertion element into wild-type strain UA159. An Em^r transformant containing pGhost9::ISS*1* was isolated and grown overnight at 30°C, and Em^r colonies containing the ISS*1* element were then isolated after incubation at 37°C. The insertion frequency, which was calculated by dividing the number of Em^r colonies by the total number of colonies at 37°C, was found to be below 0.5%, consistent from one independent experiment to the next. This frequency was similar to the frequencies reported for other streptococci (18, 182, 191). Figure 1 shows the mechanism by which ISS1 integrates in the genome and the genomic structure that is generated after the integration of the insertion element.

A schematic diagram for the screening protocol used to identify the MV sensitive mutants is depicted in Figure 2. A collection of approximately 3,500 mutants were grown in 96-well plates and replica-patched onto THY, with or without MV. We obtained 15 mutants that displayed an MV-sensitive growth phenotype. We performed Southern hybridization assay to examine how ISS*1* insertion had occurred in each of the 15 mutants. Genomic DNA was isolated from the mutant clones and UA159, which served as negative control. Genomic DNA samples were

restricted with *Hin*dIII enzyme, which has a unique site in pGhost::IS*S1* plasmid. Restricted DNA samples were separated by agarose gel electrophoresis and blotted to a nylon membrane and probed with only ISS1 fragment, as described in the Materials and Methods. The result of the Southern analysis is shown in Figure 3. We obtained 13 mutants into which IS*S1* was inserted at a single location on the chromosome and two mutants in which ISS1 was integrated at more than one location (possibly at two sites; Fig. 3, lanes 4 and 16). Among the 13 mutants that contained insertion at a single location, four contained multiple ISS1 insertion sequence at the same location (Fig. 3, lanes 3, 5, 10, and 15) and the rest contained a single copy IS*S1* insertion.

3.2. Mapping IS*S1* Insertion Sites on the Chromosome of MV Sensitive Mutants

For a majority of the clones, the site of the IS*S1* insertion was identified by inverse PCR method as described in Chapter 2. For two of the clones, a direct cloning method was also employed. A schematic diagram for inverse PCR and cloning methods is shown in Fig. 4. Out of the 15 MV-sensitive mutants, the location of the insertion sites could be determined unambiguously for 13 mutants. Five independent insertions occurred in SMU.905, which encodes a putative ABC-transporter complex (IBSA27, IBSA28, IBSA34, IBSA35, and IBSA40). Four independent insertions had occurred in SMU.906, which is just downstream of SMU.905 and encodes a putative ABC-transporter (IBSA32). For the remaining four mutants, insertions were mapped to SMU.1128 (encoding the histidine kinase *ciaH;* two insertions), SMU.902 (encoding a putative ABC-transporter), and SMU.283 (encoding a small hypothetical protein). One mutant, IBSA41, had two insertions, one in *ciaH* and one in SMU.1149 that encodes a putative transporter for bacteriocin. Because CiaH was already known to be involved in superoxide stress tolerance in streptococci and other bacteria (167), we did not consider this protein for further characterization. We also excluded the small hypothetical protein encoded by SMU.283 since it shows similarity with bacteriocin like peptide. Our repeated attempt to identify the IS*S1*

insertion sites in two of the mutants (IBSA30 and IBSA33) by inverse PCR and cloning did not generate any fruitful sequence. Since we obtained multiple insertions in SMU.905 and SMU.906 loci, and one insertion in nearby SMU.902 locus, we elected to focus our studies on these loci.

3.3. Characterization of the SMU.905 and SMU.906 Loci

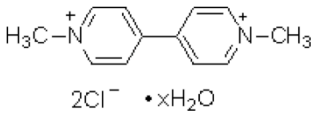
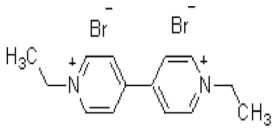
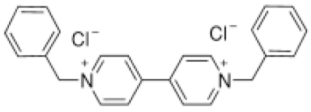
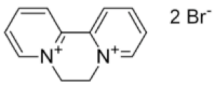
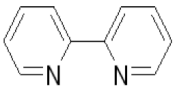
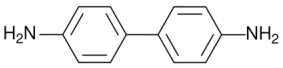
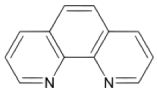
SMU.905 encodes a polypeptide of 579 residues with high homology to an ABC-transporter protein; the ISS*1* insertion occurred at five locations in this gene at codon positions 58 (IBSA40), 75 (IBSA35), 187 (IBSA34), 539 (IBSA27), and 573 (IBA28) (see Fig. 6). SMU.906, which lies just 11-bp downstream of SMU.905, appears to be organized as an operon with SMU.905. SMU.906 encodes a polypeptide of 591 residues, also with high homology to an ABC-transporter protein. The ISS*1* insertion occurred in SMU.906 at positions 13 (IBSA36), 181 (IBSA32), 483 (IBSA37), and 564 (IBSA40) (see Fig. 6). SMU.902 (623 residues) in which a single ISS*1* insertion occurred at codon position 619 is found upstream of SMU.905 (Fig. 6), while SMU.909, which encodes a malate permease, is found downstream of SMU.906. SMU.905 and SMU.906 genes are separated by 11 base pairs. An intergenic region of 456-bp is present between the SMU.902 and SMU.905 loci. *In silico* analysis by BPROM software (www.softberry.com) indicates that this region contains a weak promoter like sequence [-10 Box: TATATT; at position 362], indicating that SMU.905-SMU.906 may be transcribed separately from SMU.902. However, analysis of the same 456-bp intergenic region by FindTerm software (www.softberry.com) failed to find any strong rho-independent terminator. Thus to determine whether SMU.905 and SMU.906 are transcriptionally linked to SMU.902, a linkage PCR analysis was performed using RNA isolated from exponentially grown cultures of UA159. As shown in Fig. 7, it appears that both SMU.905 and SMU.906 are transcriptionally linked to SMU.902, whereas SMU.906 is not linked to the downstream gene SMU.909. A penta-cistronic operon encodes SMU.902 and it appears that SMU.902 is the terminal gene in

that operon. About 3.2-kb upstream of SMU.902 lies another intergenic region of 213-bp that also contains a strong promoter like structure (-10 Box: TGCTATAAT) 37-bp upstream of the first ATG codon. Thus, SMU.902 as well as SMU.905 and SMU.906 may be transcribed from this promoter. Our linkage analysis also indicated that the downstream gene SMU.909 is not transcriptionally linked to SMU.906. The intergenic region (114-bp) between SMU.906 and SMU.909 contains a strong promoter like sequence with a perfect -10 box (TATAAT); SMU.909 is probably transcribed from this promoter sequence independently from the upstream genes.

3.4. SMU.905 is Involved in Viologen Tolerance

To confirm that the observed phenotype of the SMU.905 ISS*1* insertion mutation did not result from additional spontaneous mutations elsewhere in the genome, a deletion mutation of SMU.905 was constructed in strain UA159 by a gene-replacement system using a non-polar antibiotic marker (*aad*) as described previously (19). We first examined the growth of the mutant, IBS A26, in THY broth, which is a rich growth medium. No obvious growth defects were observed in IBSA26, relative to the wild-type UA159 (data not shown). This indicates that SMU.905/SMU.906 loci do not influence overall growth of *S. mutans* cell. IBSA26 was then tested for its ability to withstand exposure to MV. As shown in Fig. 8, IBSA26 displayed the same degree of sensitivity to MV exposure as the original IS*S1* insertion mutant, IBSA34, and its derivative IBSA42, which is cured of the ISS*1* delivery plasmid (Em^S mutant).

Table 5: Sensitivity of wild type and mutant *S. mutans* strains to various chemicals

Compound (conc.)	**Chemical Structure**	**Zone of Inhibition (mm)**		
		UA159	IBS A26	IBSA43
Methyl viologen (1.0M)		NH	20±2	17±1
Ethyl viologen (1.5)		NH	24±2	20±2
Benzyl viologen (50 mg/ml)		.. NH	27±2	23±2
Diquat dibromide (500mg/ml)		33±3	38±3	ND
2, 2'- Dipyridyl (1M)		NH	NH	ND
Benzidine (4mM)		NH	NH	ND
1, 10- phenanthroline (200mM)		15±1	14±1	ND

The mean values from average of three independent experiments are shown

NH: no halo

ND: not done

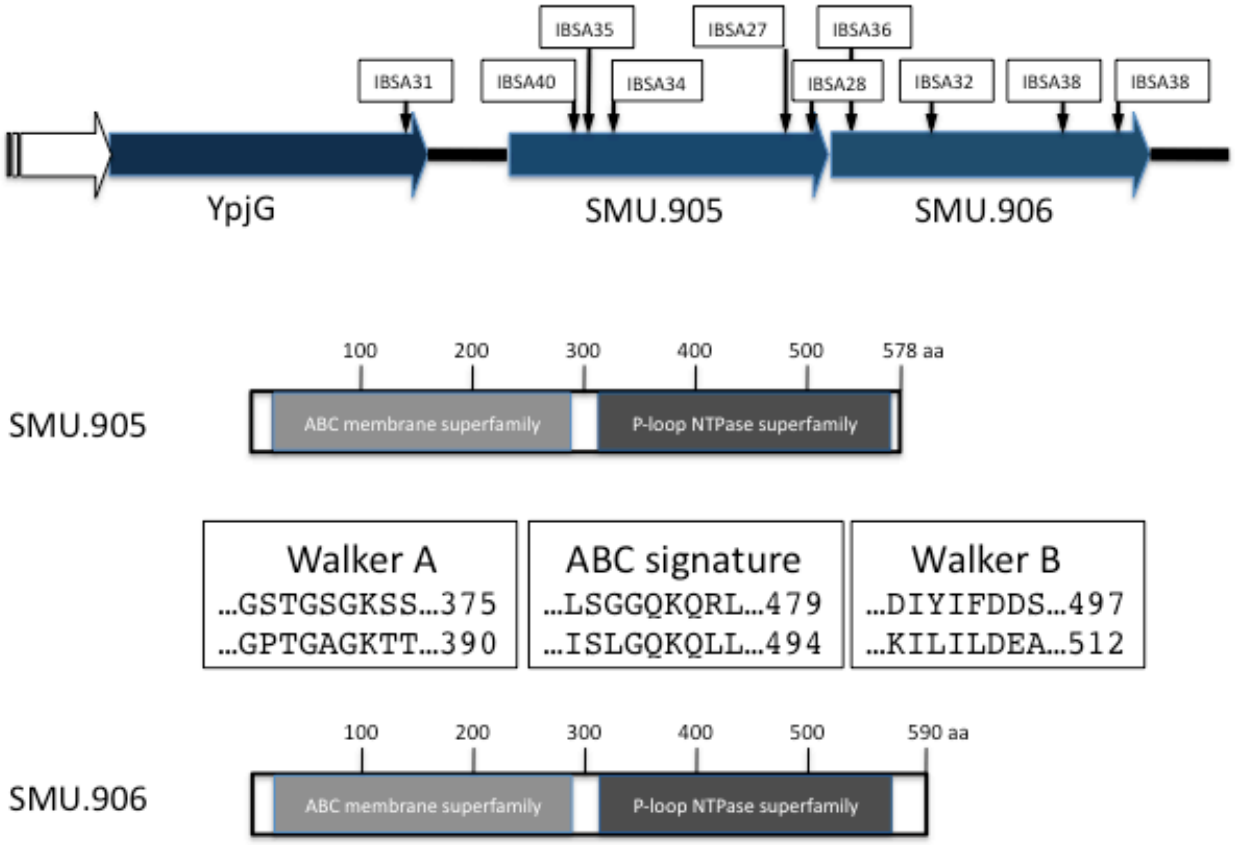


Figure 6: Organization of the SMU.905/SMU.906 loci in *S. mutans*. The predicted topology and the conserved motifs in SMU.905 and SMU.906 are shown. The conserved motifs of the ABC transporter superfamily are indicated for both proteins. The ABC membrane superfamily domain contains six and five predicted α-helices for SMU.905 and SMU.906, respectively. The upstream gene, YpjG, corresponds to SMU.902 in the NCBI database. Boxes above the diagram show the site of IS*S1* insertions and their relative positions. The designation within each box indicates the name of the mutant.

3.5. SMU.905 - SMU.906 Loci are Widely Present in *S. mutans* Strains

A recent microarray based hybridization analysis suggests that as much as 20% of the UA159 genes are absent in several *S. mutans* clinical isolates (198). Furthermore, comparative genome-based analyses of clinical isolates employing PAGE and Southern hybridization suggest that genomic insertions, deletions, and inversions of genomic occur very frequently among the clinical isolates (79, 197, 207). We used PCR analysis to determine whether SMU.905 and SMU.906 loci are present in various *S. mutans* strains. Based on analysis of the UA159 genome sequence, we designed two internal primers for each of the following genes SMU.902, SMU.905, and SMU.906 are used for PCR amplification, using chromosomal DNA isolated from 15 different *S. mutans* clinical isolates as a template. Of the 15 strains chosen for the analysis, 11 belong to serotype c, including three commonly used lab strains (UA159, NG-8, and GS-5), and the remainder taken from serotype e (V100) and serotype f (OMZ175). We found that these three genes were present in all 15 *S. mutans* strains (data not shown). We also tested whether the intergenic region between SMU.902 and SMU.905 loci is conserved among the isolates and found that the length of the intergenic region is also well conserved among various isolates (data not shown).

3.6. SMU.905/SMU.906 is not Involved in Biofilm Formation

In bacteria, many ABC-transporter proteins can positively or negatively influence biofilm formation or maturation. For example, in *Listeria monocytogenes*, an ABC transporter protein (encoded by Lmof2675_1771) negatively affects biofilm formation (211). In other gram-positive bacteria, ABC-transporter proteins are required for successful biofilm development. For example, in *Bacillus subtilis* ABC transporter EcsB is involved in biofilm maturation (25) and *Streptococcus gordonii*, ABC transporter BfrEF is required for biofilm formation (208). To test whether SMU.905/SMU.906 is involved in biofilm formation in *S. mutans*, we used IBS A26 strain and examined its ability to form sucrose dependent biofilms on polystyrene and glass

surfaces using various biological stains. As shown in Fig. 9, we found that the biomass of the biofilm formed by IBSA26 on various abiotic surfaces was similar to that of the wild-type parent UA159 strain. Therefore, we conclude that SMU.905/SMU.906 is not involved in *S. mutans* biofilm formation.

3.7. SMU.905/SMU.906 is not Involved in Superoxide or Oxidative Stress Tolerance

Methyl viologen (MV) generates superoxide that is toxic to cells. However, several other red-ox cycling reagents also generate superoxide but are chemically and structurally distinct from MV. For example, menadione, pryrogallol, and plumbagin are all potent superoxide generators however, unlike MV, these reagents are uncharged and do not contain a dipyridyl ring; rather they contain either benzene (pyrogallol) or napthalene (menadione and plumbagin) rings. To test whether the SMU.905 mutant (IBSA26) is sensitive to these reagents, we used disc diffusion assays as described in Chapter 2. We observed that IBSA26 did not have an increased sensitivity to menadione, pyrogallol, or plumbagin (Table-S1). This suggests that SMU.905 is not involved in general superoxide stress tolerance, rather this ABC transporter is specific for MV mediated toxicity tolerance.

To confirm our hypothesis that SMU.905 is not involved in general superoxide stress tolerance response, we also measured the superoxide dismutase (SOD) activity in IBSA26. *S. mutans,* like other streptococci, encodes a single Mn-type superoxide dismutase (146). SOD activity was measured in the crude cellular extract isolated from mid-exponentially grown cultures on a native polyacrylamide gel by negative staining using nitroblue tetrazolium method (13). As shown in Fig. 10, only a single active band, in approximately equal intensities, was seen in the wild type (UA159), the SMU.905 mutant strains (IBSA26, IBSA25 and IBSA42.), and the SMU.906 ISS1 insertion strain (IBSA43). Thus, SMU.905/SMU.906 is not involved in general superoxide stress response.

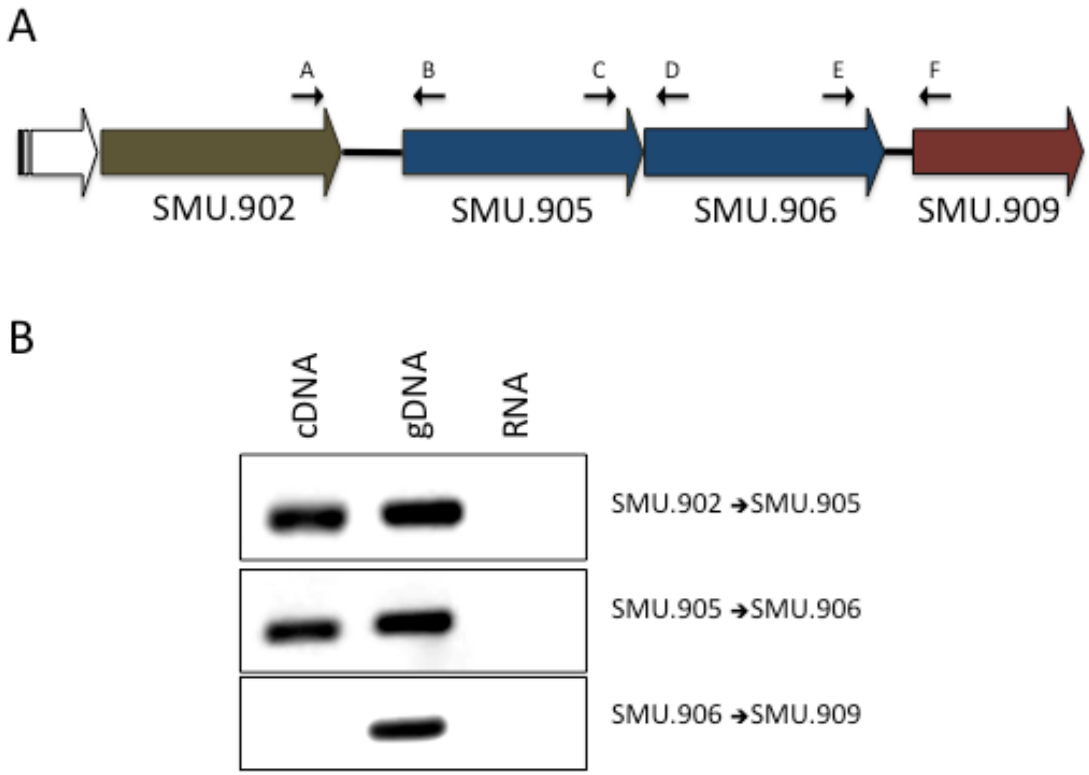


Figure 7: Linage analysis of the SMU.905/SMU.906 loci. (A) Genetic map of the SMU.905/SMU.906 loci in *S. mutans* UA159 with the relative positions of the primers used for the PCR analysis to determine potential linkage of the genes. (B). Results of PCR analysis showing linkages of the various genes that are cotranscribed. RNA was used as template to produce cDNA. PCR was then performed on RNA (control), cDNA, and chromosomal DNA (gDNA), using the primer pairs depicted in panel A, to determine which of the genes are cotranscribed with SMU.905.

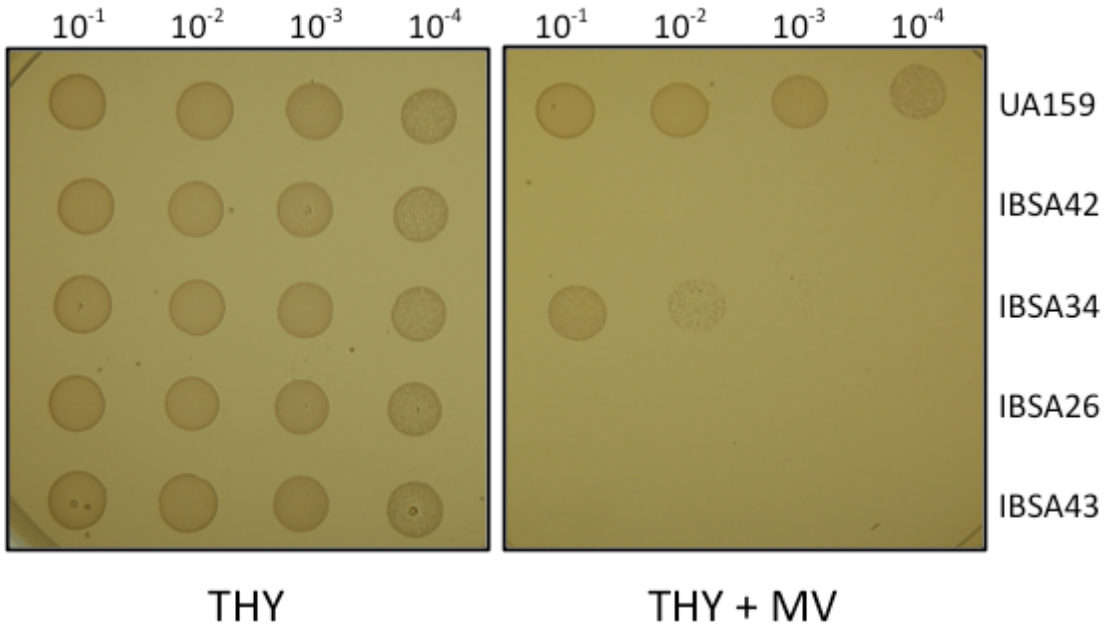


Figure 8: Verification of viologen-sensitive phenotype. ISS*1* transposon mutants that displayed an initial MV-sensitive phenotype were further verified by spotting of 10.0 µl from a 10-fold dilution series, with a starting optical density (A_{600}) of 2.0 made in 0.85% NaCl, onto THY agar plates containing 5 mM MV (THY+MV). As a control, cultures were also spotted on plain THY agar plates with no additions (THY). UA159 is the wild-type strain, while IBSA34 and its plasmid cured derivative IBSA42 are independent MV-sensitive mutants. IBSA43 is a plasmid-cured derivative of IBSA32, another MV-sensitive mutant. IBSA26 is a deletion mutant of SMU.905 locus. Experiments were repeated at least three times, and the relevant areas of the representative plates are shown.

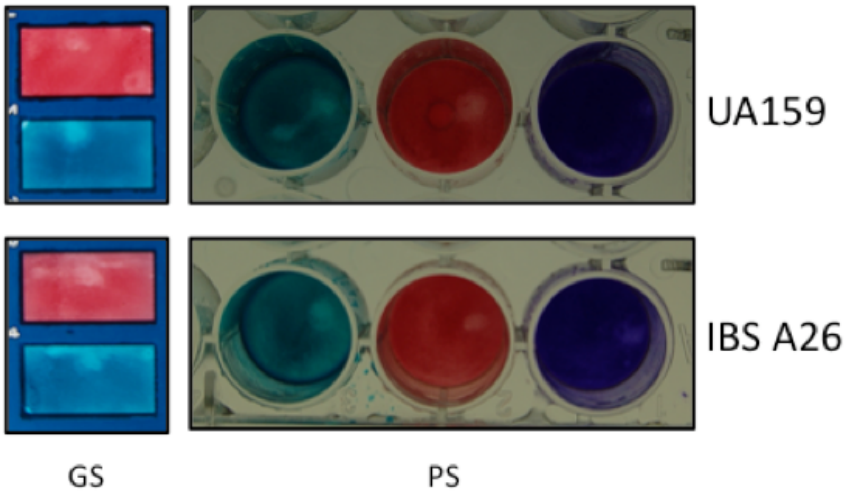


Figure 9: Biofilm formation by UA159 and IBSA26. Cultures were grown in THY medium with 1% sucrose at 37°C for 2 days under microaerophilic conditions. Cells attached to abiotic surfaces were washed and stained with crystal violet (violet), malachite green (green) or safranin (red). Left panel, biofilm on a glass surface (GS; four-chambered glass slide); or, right panel, polystyrene surface (PS; microtiter plate).

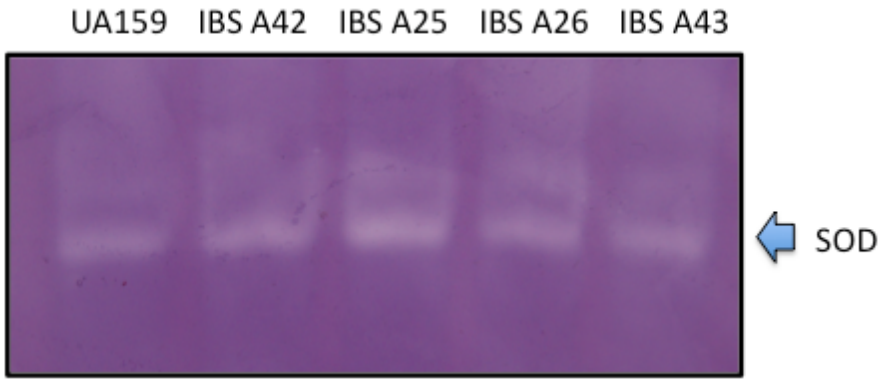


Figure 10: SMU.905 or SMU.906 does not affect SOD activity in *S. mutans*. Wild type (UA159), SMU.905 (IBSA25, IBSA26, and IBSA42), and SMU.906 (IBSA43) mutant cultures were harvested at the stationary growth phase. Total protein (25μg) was applied to a 10% native polyacrylamide gel and electrophoresed. Bands representing SOD activity was visualized by negative staining using nitroblue tetrazolium method as described in Chapter 2.

We also examined the role of SMU.905 in oxidative stress response. Towards this end, we used hydrogen peroxide to generate intracellular oxidative stress. We also included cumene hydroperoxide and *t*-butyl hydroperoxide, which are often used to mimic lipid hydroperoxide stress (7). The *S. mutans* SMU.905 deletion mutant IBSA26 was assayed for sensitivity to these peroxides in a disk diffusion assay. The zones of growth inhibition of IBSA26 by these peroxides were similar to the wild type UA159 strain (Table S1). Thus, SMU.905/SMU.906 does not appear to participate in oxidative stress tolerance response.

3.8. SMU.905/SMU.906 is only Required for Tolerance to Dipyridyl Ring Containing Superoxide Inducing Reagents.

The SMU.905 mutant, IBSA26, while sensitive to MV, did not display an increased sensitivity to the structurally distinct superoxide generators. MV is hydrophilic doubly charged (dication) quaternary ammonium compound (QAC) with a 4,4'-dipyridyl ring structure. Because SMU.905/SMU.906 show high structural similarity with the multidrug efflux pumps, we wanted to examine whether this ABC transporter is also involved in tolerance to other structurally similar compounds. To this end, we tested two additional viologens, ethyl viologen (EV) and benzyl viologen (BV) in disk diffusion assays. We tested the original IS*S1* insertion mutants (IBS A34 and IBSA37) as well as the SMU.905 deletion mutant IBSA26. As shown in Figure 11 (and listed in Table 4), IBSA26 showed enhanced sensitivity to both EV and BV with a zone of inhibition of 24±2 mm and 27±2 mm, respectively. As expected, we observed no obvious growth inhibition in UA159 with EV and BV (Fig. 11). On the other hand, the original IS*S1* mutants of SMU.905 also displayed similar zone of inhibition as the SMU.905 deletion mutant (data not shown). We also tested a plasmid cured IS*S1* mutant of SMU.906 (IBS43) for its sensitivity to EV, BV, and MV. This strain also showed increased sensitivity to these compounds compared to the wild type strain (Table 4). However, the zones of growth inhibition were about 70-80% of the IBSA26 strain.

We then tested the sensitivity of IBSA26 to diquat (DQ) because it represents the 2,2'-dipyridine derivative that is structurally related to viologen, which is 4,4'-dipyridines. However, unlike the viologens, the effect of DQ on the growth inhibition of IBSA26 was not so obvious when fresh overnight cultures were used. Although the diameter of the zone of inhibition for both the wild type and the mutant was similar (~17mm) with DQ (100mg/ml), the wild type produced a cloudy zone of halo with residual bacterial growth present within the inhibited zone while the mutant produced a clear zone of halo. However, when the overlay cultures were 48hrs or older, the zone of inhibition was significantly larger for IBSA26 as compared to UA159, and both the strains produced a clear zone of halo (Table 5).

The viologens and DQ are structurally very similar, charged molecules, and generate superoxide radicals. We wanted to examine whether SMU.905 is also involved in the tolerance to compounds that contain similar dipyridyl structures but do not generate superoxide radicals. Therefore, we tested the sensitivity of the SMU.905 deletion mutant to 4,4'-dipyridyl (4DP), benzidine (BZD), and 1,10'-phenanthroline (PHEN) in disk diffusion assay. We observed no zone of inhibition with 4DP and BZD for both the wild type and the SMU.905 mutant strains while the halo sizes for both the strains were similar when PHEN was used (~22mm, Table S1). Therefore, taken together our results suggest that SMU.905/SMU.906 is specifically involved in the tolerance of viologen and related charged compounds that generate superoxide radicals.

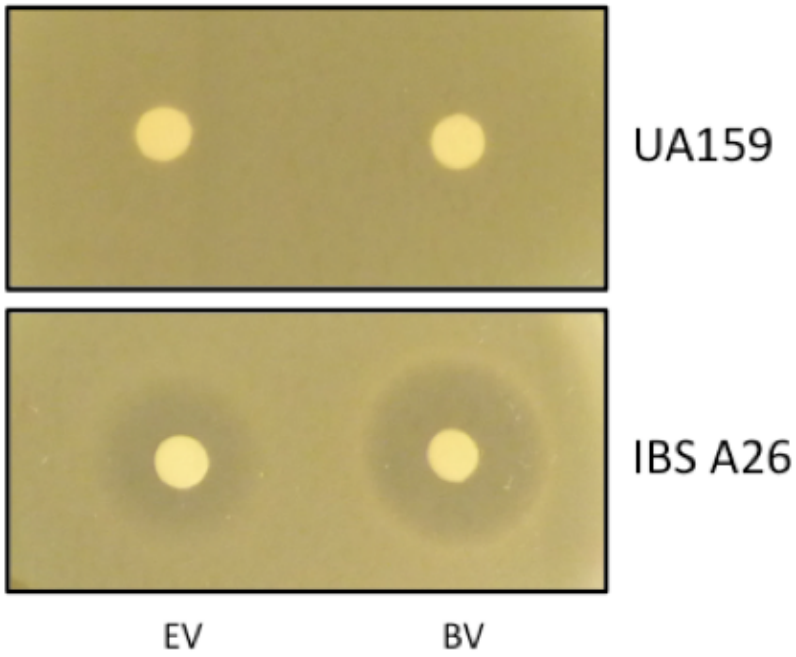


Figure 11: Disk diffusion assay to measure the susceptibility of *S. mutans* to viologen compounds. Lawns of wild type (UA159) and SMU.905 mutant (IBSA26) strains were prepared by overlaying THY agar plates with 10 ml of soft THY agar containing 0.5ml overnight cultures. Ten microliters of ethyl viologen (EV, 1.5M) or benzyl viologen (BV, 50mg/ml) was spotted on the filter paper disk (6mm) and placed on the plates. The plates were then incubated under microaerophilic conditions at 37°C for 16 h. The inhibitory-zone diameters for both cultures were measured and compared.

Table 6: Sensitivity to Diquate (DQ).

DQ conc.	UA159[$]		IBSA26[$]	
	20hr	48hr	20hr	48hr
50mg/ml	NH	12.0±1.0*	NH	16.0±1.0*
100mg/ml	17.0±1.0[@]	16.0±1.0*	17.0±1.0	22.0±1.0*
500mg/ml	22.0±1.0[@]	33.0±2.0*	22.0±1.0	38.0±2.0*

[$] Either 20 hrs old or 48 hrs old cultures was used for the overlay

NH=no distinct halo

[@] Diffused halo

* Significant difference between the wild type and the mutant

values are shown in mm

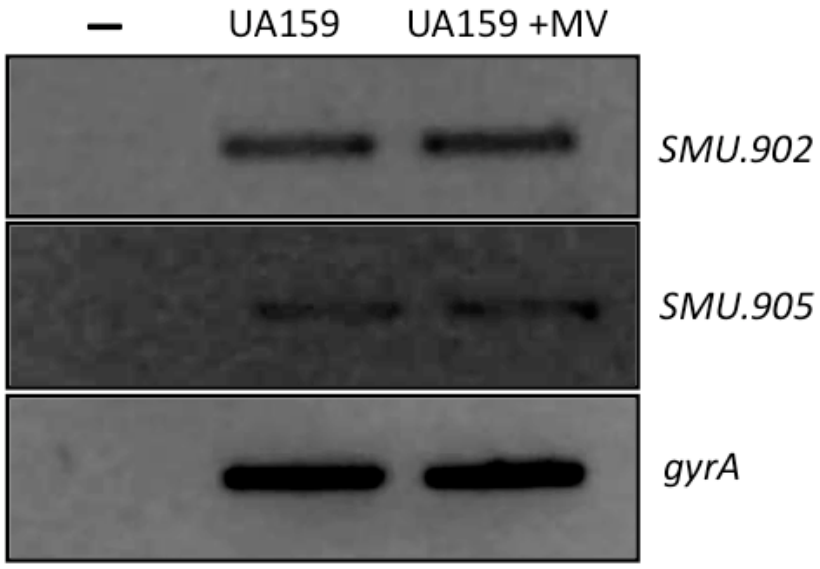


Figure 12: SMU.905 expression is not induced by MV treatment. Semiquantitative RT-PCR was used to study SMU.905 expression. Total RNA was harvested from UA159 culture that was treated with or without MV and subjected to cDNA synthesis. Five nanograms of cDNA from each sample were used for semiquantitative RT-PCR. Two genes (SMU.902 and SMU.905) were subjected to semiquantitative RT-PCR. The *gyrA* gene was included to ensure that equal amounts of RNA were used for all reactions. The data are representative of an RT-PCR analysis resulting from at least two independent RNA isolations.

Compounds that are expelled out from the cell by multidrug resistance family of ABC transporters can often induce the expression of the corresponding ABC transporter (121, 149, 159). We therefore examined whether prior exposure of *S. mutans* cells to sublethal concentration of MV can induce the transcription of SMU.905. For this, we grew UA159 strain to mid-exponential phase and divided the culture into two aliquots. One portion was treated with 5mM MV for 30 min while the other portion was left untreated. RNA was then extracted from these cultures and the amounts SMU.905 transcripts were measured by semi-quantitative RT-PCR as described in Chapter 2 and normalized against *gyrA* transcript. As shown in Fig. 12, prior exposure to MV did not induce SMU.905 transcription suggesting that expression of SMU.905 may not be inducible.

3.9. SMU.905/SMU.906 is also Crucial for Tolerance to Some QACs

QAC are a group of compounds in which a central nitrogen atom is joined to four organic radicals and one hydrophobic alkyl chain. These compounds are widely used as broad-spectrum bactericides in antiseptics and disinfectants and also used as surfactants and dyes. Since viologens belong to the QAC group, and since SMU.905 is required for viologen tolerance, we wanted to know whether SMU.905 is also necessary for resistance to other QAC compounds. We tested the SMU.905 deletion mutant (IBSA26) in disk diffusion assays for sensitivity towards commonly used biological dyes (malachite green, crystal violet, pyronin B, and safranin), compounds commonly used in disinfectants or in mouthwash (acriflavin, benzalkonium chloride, cetrimonium bromide, cetylpyridinium chloride, and tetraethylammonium bromide), and ethidium bromide. As shown in Fig.13 and Fig.14, the SMU.905 mutant revealed an enhanced sensitivity to ethidium bromide and safranin with zones of growth inhibition approximately 130% than those of the wild type UA159 strain. A similar result was also obtained with acriflavin (data not shown). However, there were no significant differences in the zones of growth inhibition between the wild type and the mutant for the other QACs (Table - S1).

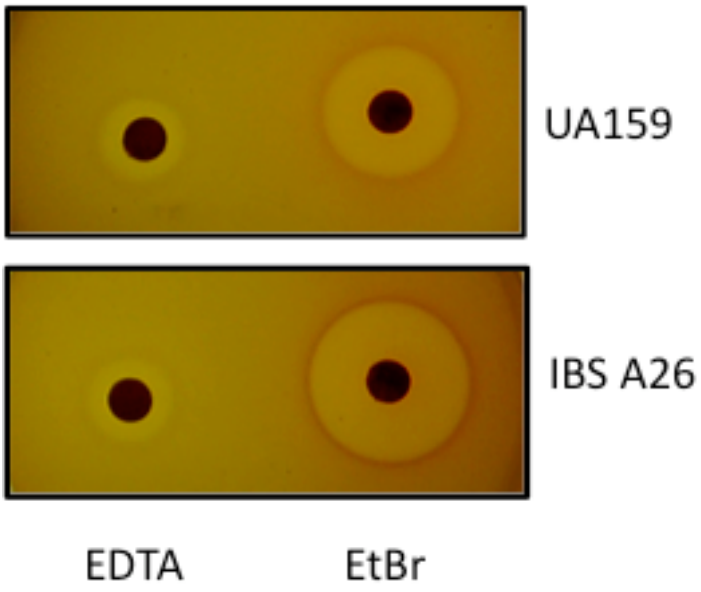


Figure 13: Susceptibility of *S. mutans* to QAC by disk diffusion assay. Plates with overlaid cultures were prepared as described before. Ethidium bromide (10 mg/ml) or EDTA (0.5M) was spotted on paper disk and the plates were incubated overnight under microaerophilic conditions and photographed.

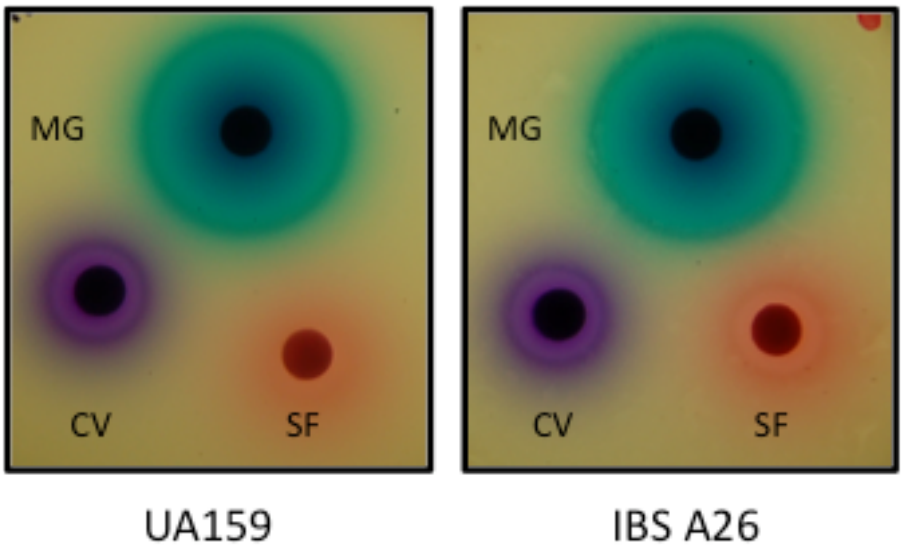


Figure 14: Sensitivity to various biological dyes related to QAC. Disk diffusion assay was conducted as previously described. The samples are: crystal violet (CV, 1.0%), malachite green (MG, 1.0%), and safranin (SF, 1.5%).

To confirm our result that the ABC transporter SMU.905/SMU.906 is indeed involved in the efflux of these the QACs, we tested the plasmid cured IS*S1* insertion mutant of SMU.906 (IBSA43, Table 3) for its sensitivity in disk diffusion assays. To our surprise, IBSA43 did not show increased zones of growth inhibition to these drugs as the SMU.905 mutant strain (data not shown). Thus, while the role of SMU.905 in the tolerance to these QACs is very certain, the role of SMU.906 in the efflux of QACs is not very clear.

3.10. SMU.905/SMU.906 do not Participate in Antibacterial Tolerance or Toxic Chemicals

Many multidrug efflux pumps confer resistance to a variety of structurally unrelated compounds (149, 159, 160, 162). Qunolones are widely used broad-spectrum antibiotics with a one or two heterocylic ring containing nitrogen structure. Since the overall quinolone structures have some similarity with the quat compounds and because many ABC transporters are known to be involved in both QAC and quinolone resistance (152), we examined the role of SMU.905 in the resistance to quinolone antibiotics such as ciprofloxacin, levofloxacin, and nalidixic acid. We also included vancomycin, which contains complex heterocyclic rings; lincosamide drugs such as clindamycin and chloramphenicol; trimethoprim that contains a diamine-pyrimidine group; rifampicin that also contains a complex ring structure similar to quat compound; and bacitracin, a cyclic polypeptide. We included bacitracin in our study because *S. mutans*, unlike other streptococci, are much more resistant to this drug (193) and ABC transporters are known to be involved in the bacitracin resistance (129). All these antibiotics were used in disk diffusion assays with IBSA26 and UA159 strains. As listed in Table S1, we did not find any significant differences in the growth inhibition zones between SMU.905 mutant and the wild type strains.

We also tested sensitivity of IBSA26 to various toxic chemicals such as chlorhexamide, diamide, EDTA, hydrazine, mytomycin C, potassium tellurite, puromycin, and streptonigrin; all these reagents are structurally very different (Table S1). However, we did not observe any significant

difference between the wild type and the mutant strains. Thus, SMU.905/SMU.906 appears to have very restricted substrate specificity.

3.11. Phenotypic Microarray Analysis of the SMU.905 Deletion Mutant (IBSA26)

PM assay is a relatively new method that allows testing for a large number of phenotypes simultaneously for a given strain (21-23). In this assay, bacterial growth in different media is measured with tetrazolium redox dye(22). Respiration by bacteria during growth causes reduction of the dye and produces purple color that accumulates in the well over the incubation period. Total loss of function will result in no growth and therefore no color formation. Thus, colorimetric detection due to respiration can provide a reporter system for phenotypic testing. The growth kinetics of the SMU.905 mutant (IBSA26) under nutrient rich standard growth condition was very similar to the wild type strain (UA159) suggesting that there was no obvious growth defect in the SMU.905 mutant; therefore IBSA26 is a good candidate for PM analysis. PM assays were performed on IBSA26, compared with UA159, in a set of 20 96-well plates containing various nutrients or toxic compounds. This allowed testing of nearly 1,900 cellular phenotypes in a sensitive, highly controlled, and reproducible format.

PM analysis was first performed using metabolic panels (PM1-8). There was no significant difference in the carbon utilization panels (PM1-2, Fig. 15). The signals for the rest of the metabolic panels (PM3-8) were either very low or none. These panels include nitrogen utilization panels (PM3, 6-7), phosphate and sulfate panel (PM4), and nutrient stimulation panel (PM5). The poor growth in these metabolic panels was not surprising since a previous study also demonstrated poor growth of a wild-type NG-8 strain, a different *S. mutans* strain than the one used in this study (20). However, the result also indicated that the mutant (IBSA26) showed no growth defect or advantages in those PM plates. One marginal positive difference was observed for gly-phe-phe (PM8, H8) for the mutant. In the osmotic panel (PM9) there were

no signals detected in about half of the wells. In the remaining wells, there were no significant differences between the wild type and the SMU.905 mutant, except one marginal negative difference in (PM9, B5). PM analysis in the osmotic panel (PM10) also generated no significant differences. Surprisingly, for the toxic chemical panels (PM11- PM20), despite reasonably good growth in most of the conditions, we observed no significant differences between the wild type and the mutant strains. This suggests that SMU.905 has a very restricted substrate specificity that includes viologens and some QACs.

3.12. Role of SMU.902 in Viologen Tolerance

We obtained a single insertion (IBSA31) in SMU.902 locus that lies just upstream of the SMU.905 and SMU.906 loci. SMU.902 encodes a polypeptide of 623 residues that shows high homology with an ABC transporter protein. The insertion that we obtained in SMU.902 was mapped at position 619. To gain a better insight into the function of SMU.902, we used IBSA31 to generate a strain, IBSA44, which is devoid of the delivery plasmid pGhost9. Both IBSA31 and IBSA44 were tested for MV sensitivity. As shown in Fig. 16, both strains showed increased sensitivity towards MV. Furthermore, although both strains were highly sensitive to MV exposure, we observed that IBSA44 (plasmid cured, Table 3) strain was at least 10-fold less resistant to MV as compared to the original insertion mutant IBSA31 that contains the plasmid. We also tested IBSA44 for its sensitivity towards acriflavin and ethidium bromide in disk diffusion assay. As expected, IBSA44 was more sensitive to both these reagents as compared to the wild type strain suggesting that this insertion in SMU.902 somehow disrupted the wild type function (data not shown).

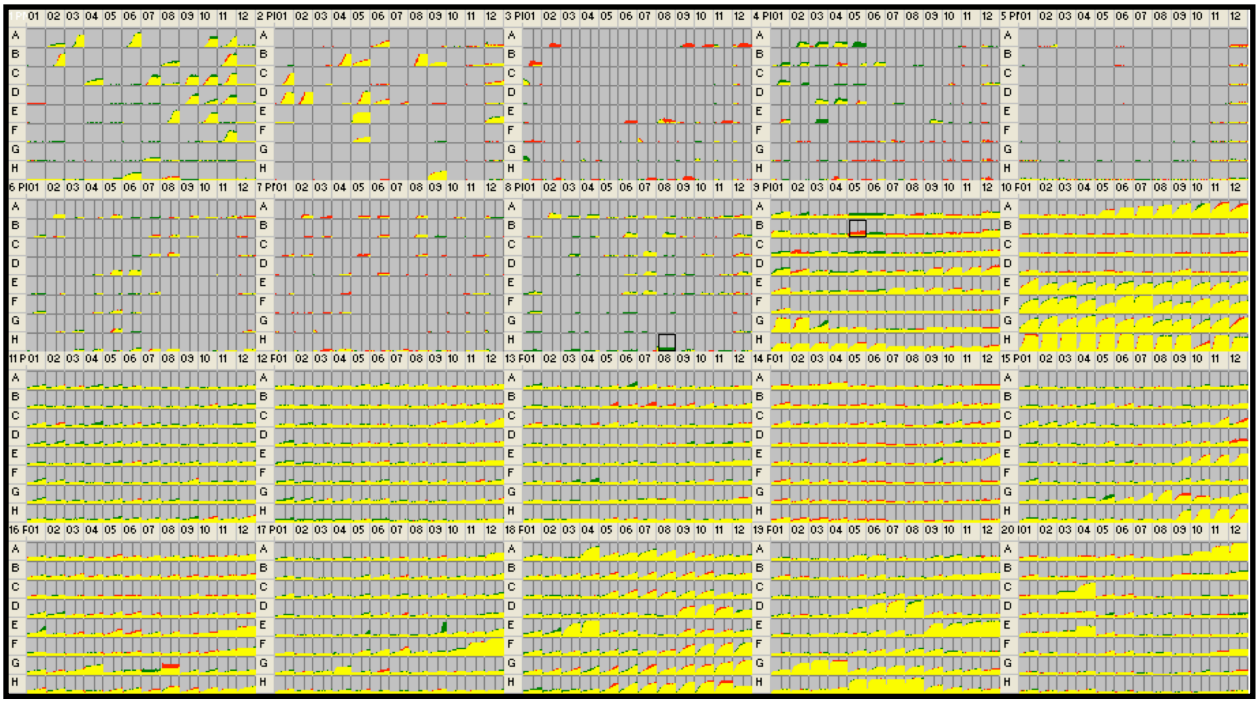


Figure 15: PM analysis for sensitivity to various antibiotics and toxic compounds. A complete catalogue of the reagents used in the sensitivity panel (PM1–PM20) is listed at http://www.biolog.com. The wild type (UA159) and the SMU.905 mutant (IBSA26) were grown in a 96-well plate under different conditions. Growth kinetics were obtained with the OmniLog instrument, a video-based detection system that detects color development of tetrazolium dye due to bacterial respiration. Growth kinetics of wild-type and *SMU.905* mutant are superimposed using OmniLog software. The PM kinetic results show consensus data comparing the mutant (IBSA26, green) and its wild-type parental strain (UA159, red). A growth advantage by the parent is indicated by red, while a growth advantage by the SMU.905 mutant is shown by green. When both strains have equal growth response or metabolisms in a well, the red and green kinetics overlap and produce a yellow color. Two independent growth kinetic experiments were performed. A box around a well indicates a difference in response that was observed in both the experiments.

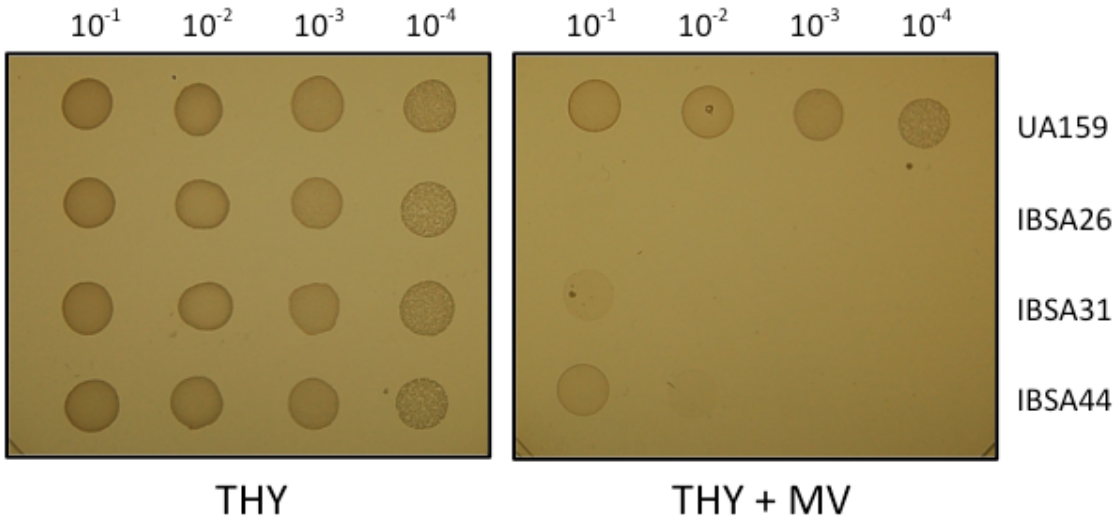


Figure 16: Verification of SMU.902 mutants to methyl viologen sensitivity. Dilutions of fresh overnight cultures were spotted on THY agar plates with or without methyl viologen (5 mM; MV). Plates were incubated at 37°C under microaerophilic conditions. Experiments were repeated no fewer than three times, and relevant areas of representative plates are shown. Strains are: wild type (UA159), SMU.905 mutant (IBSA26) and SMU902 mutants (IBSA31 and IBSA44). Note the slight increase in resistance of IBSA44, a plasmid-cured derivative, compared with IBSA31.

CHAPTER 4

DISCUSSION

In comparison to other pathogenic streptococci such as GAS, GBS, or *S. pneumoniae*, the lifestyle of *S. mutans* is quite different. The natural niche of this pathogen is human oral cavity, which is a very dynamic environment that undergoes rapid changes in temperature, pH, and osmotic and oxygen tension. For successful colonization and maintaining a dominant presence in the oral cavity *S. mutans* has also developed multiple strategies. These strategies also help this organism to grow under nutritional-limiting conditions and to protect from various environmental insults (113). While most of the previous studies were focused on understanding the mechanisms of acid tolerance and oxidative-stress responses, our knowledge of the mechanisms of other stress tolerance response remains limited. To obtain further insight into this process, a collection of random insertion mutants of *S. mutans* UA159 was screened to select clones with high sensitivity to MV, a charged dipyridyl-ring containing QAC that also generates ROS. This approach allowed us to identify genes that may be responsible for defense against QAC, without prior knowledge of the genes' function(s). In this study, we only screened approximately 3,500 such mutants; therefore, the screening process was not particularly exhaustive since *S. mutans* genome encodes about 1900 genes. There were some other drawbacks associated with the approach used for MV sensitive mutant isolation. An insertion in an essential gene would not have been identified in this study. Furthermore, our screening process may overlook mutants with a weak phenotype. Among the five loci that were identified in our analysis at least one gene, *ciaH*, was previously reported as an important player in the oxidative-stress response in streptococcus and other bacteria (2, 151, 167, 206, 212), signifying that the screening method used here is a viable approach. However, we did not identify the *sodA* gene, which encodes the SOD activity, nor did we identify *mreD*, *rodA*, *pbp2b*, or other genes that were isolated by Thibessard et al. (191), who attempted to identify superoxide stress-responsive loci in *S. thermophilus*. Our inability to identify these genes could be either due to the non-exhaustive nature of the screening process or due to the experimental condition used for the screening. In our assay, instead of microaerophilic condition that contains no oxygen, we

used ambient air supplemented with 5% CO2 to intensify the action of viologen. Four unique loci were identified by our search: three ABC transporter-encoding genes (SMU.902, SMU.905, and SMU.906), and a hypothetical protein (SMU.283). In this study, we further characterized the SMU.905 and SMU.906 loci to understand the mechanism and substrate specificity for these ABC transporters.

Sequence analysis and genome organization strongly suggest that SMU.905 and SMU.906 encode a heterodimeric ABC-type exporter pump. Our linkage analysis also demonstrated that these two genes are transcriptionally linked. *S. mutans* genome analysis indicates that this organism encodes several ABC transporters, of which at least 42 are putative exporter pumps (3, 136). Since we specifically obtained multiple insertions in SMU.905 and SMU.906 loci (five in SMU.905 and four in SMU.906), this ABC transporter appears to be the most important for viologen tolerance in *S. mutans*. Analysis of the sequences by a transmembrane (TM) helices prediction program, TMHMM (www.cbs.dtu.dk/services/TMHMM), of both SMU.905 and SMU.906 revealed that these two proteins contain 6 (residues 1-294) or 5 (residues 1-382) TM helices, respectively. Both SMU.905 and SMU.906 also contain putative nucleotide binding domains, Walker A and Walker B motifs, and ABC signature sequences (42, 82) (Figure 6). We also found that both SMU.905 and SMU.906 were necessary for viologen resistance supporting our notion that SMU.905 and SMU.906 is a heterodimeric ABC-type multidrug efflux pump.

Several ABC-type multidrug efflux pumps have been characterized previously in gram-positive bacteria (111, 122, 123, 170, 185). Among them, LmrCD in *L. lactis* has been experimentally demonstrated to be a heterodimeric ABC-type multidrug efflux pump and to contain structurally and functionally distinct nucleotide-binding domains (123). These authors have shown that in LmrD, a canonical glutamate residue following the Walker B motif, which has been postulated to fulfill a critical catalytic role in the ATP hydrolysis is well conserved (29). On the other hand, in

LmrC, this residue is replaced with a noncanonical aspartate residue. In each pair of heterodimeric transporters thus far analyzed, one subunit contains a canonical glutamate residue and the other subunit contains a noncanonical aspartate residue instead of a glutamate residue. This pattern is also found in SMU.905 (contains aspartate) and SMU.906 (contains glutamate) and may be a feature of heterodimeric ABC transporters (Fig. 6).

The ABC transporter that we identified exports, in addition to viologen compounds, some other QACs such as acriflavin, ethidium bromide, and safranin. Analysis of the structures of these compounds failed to identify any common structural moiety that could easily explain the substrate specificity (Table 5). However, all the compounds are charged heterocyclic molecules. Definitely charge plays a role in the substrate recognition since the ABC transporter complex did not recognize dipyridyl and benzidine, which are structurally very similar to viologen but uncharged. Similarly, diquat, which is structurally very similar to phenanthroline but charged, was expelled from the cell by SMU.905, while phenanthroline was not recognized. On the other hand charge alone is not sufficient to explain substrate specificity. This is because we also tested several QACs ranging from compounds that contain simple structure such as tetraethylammonium bromide to compounds that contain heterocyclic rings such as malachite green and crystal violet. None of these QACs were recognized by SMU.905. Thus, in addition to charge, other physical characteristics such as hydrophobicity or amphiphilicity may be important.

Although IBSA43 (SMU.906 mutant) displayed increased sensitivity to viologen compounds, surprisingly this mutant when tested for sensitivity towards acriflavine, ethidium bromide, and safranin did not show any significant difference compared with the wild type. Thus, it appears that SMU.906 is not involved in the resistance of these compounds. Although SMU.905 and SMU.906 are expected to interact with each other to form a functional heterodimeric ABC-transporter, one could envision the possibility that these half transporters may interact with other

ABC transporters to assemble into different heterodimers that differ in their substrate specificity. Such a scenario was proposed for lactococcal transporters involving LmrCD and LmrA (121, 123). Another possibility is that SMU.905 and SMU.906 both can separately form homodimers and these homodimers have different substrate specificity. For example, while SMU.905 homodimer is involved in QAC resistance, SMU.906 homodimer does not take part in QAC resistance. Future studies will address the question whether SMU.905 and SMU.906 can also form homodimers, and, if so, to what extent their substrate specificities differ from the SMU905/SMU.906 heterodimer.

It is possible that SMU.905 or SMU.906 might form heterodimers with SMU.902, which also encodes an ABC-transporter protein. SMU.902 lies just upstream of SMU.905 and SMU.906 loci and is transcriptionally linked. Interestingly, we also obtained an insertion in SMU.902, although the site of insertion was very close to the C-terminus end (at codon position 619). The SMU.902 insertion mutant (IBSA44) also displayed increased sensitivity towards viologens as well as the QACs. Since SMU.902 and SMU.905/SMU.906 were transcriptionally linked, and because the insertion was near the end of the SMU.902 gene, we speculated that the increased sensitivity of IBSA44 to viologens (and other QACs) was due to the polar effect of the insertion and not due to the loss of SMU.902 function. Our speculation was further supported by the observation that the original insertion mutant, IBSA31, showed more sensitivity than the plasmid cured IBSA44 mutant, which is expected to exert less polar effect than the original mutant. However, our results cannot rule out the possibility of heterodimer formation involving SMU.902. Further studies are required to understand the biochemical nature of this ABC-transporter.

BLAST searches using protein sequences as query against the non-redundant database at NCBI show that homologues of SMU.905/SMU.906 are widely present in streptococci, enterococci, and clostridia (Fig. 17A/B). In all cases, two ORFs were located in tandem, and many genes seemed to encode multidrug resistance ABC-type proteins. The closest

homologues (over 90% identity), SAG1338 and SAG1337, are found in GBS; and all the sequenced GBS strains encode these ABC transporter genes. In contrast to other organisms in which SMU.905/SMU.906 homologues are found, the genomic locus for this ABC transporter is somewhat conserved in GBS. Specifically, the upstream four genes are highly conserved including the SMU.902 homologue, SAG1340, which encodes an ABC transporter. On the other hand, two genes immediately downstream of SAG1337 are homologues of SMU.911 and SMU.913. However, homologues of SMU.909 that encodes a malate permease and SMU.910, which encodes a glucosyltransferase, are absent in GBS. Although GBS is an important pathogen, so far no systematic study has been conducted in this organism to understand the role of various ABC transporters in antibiotic or other stress tolerances. While the exact role of SAG1337/SAG1338 remains to be explored, a recent proteomic study demonstrated that SAG1337 might be involved in pathogenesis since this protein is found in greater abundance under highly invasive conditions (with oxygen) compared to poorly invasive conditions (without oxygen) (91).

Our BLAST search also identified two ABC transporter proteins from *Enterococcus faecalis*, EfrA (EF2920) and EfrB (EF2919), which showed over 80% identity with SMU.905 and SMU.906, respectively (111). From genome sequence information, it appears that *E. faecalis* encodes 23 putative multidrug ABC-type transporters. *E. faecalis* shows fairly high levels of resistance to many antimicrobial agents, presumably due to presence of several multidrug efflux pumps. Davis et. al. (45) have created single-gene disruptions in each of these 23 putative ABC-transporters and evaluated susceptibilities of the mutants to a panel 28 structurally diverse antimicrobial agents. Only four out of 23 ABC-transporters were found to be involved in the tolerance of at least one or more compounds with little overlap in the respective substrate spectra (45). Surprisingly, the analysis did not reveal a role for EfrAB ABC-transporter in intrinsic antimicrobial resistance. Lee et. al. (111) have subsequently cloned and expressed

EfrAB in *E. coli*. These authors found that expression of both *efrAB* genes confers resistance to many drugs including acriflavin, ethidium bromide, and safranin (111). Interestingly, neither *efrA* nor *efrB* was able to confer drug resistance when either one of these genes was expressed in *E. coli,* suggesting that EfrAB indeed form a heterodimer to function as a multidrug efflux pump. Unfortunately, methyl viologen or other quat compounds were not tested in this study, thus whether EfrAB is involved in viologen efflux remains to be seen.

Although our BLAST search identified SMU.905/SMU.906 homologues in many streptococci, we could not identify obvious homologous proteins in *S. pneumoniae*. The highest similarity (over 55%) that we obtained was with SP2075/SP2073 (also known as PatA/PatB). This ABC transporter was first described by Marrer et al. (131) as an efflux pump involved in multidrug resistance in a laboratory-selected ciprofloxaxin-resistance mutant. These authors also demonstrated that various antibiotics including quinolones induce expression of *patA* and *patB* genes (131). The genome of *S. pneumoniae* encodes 14 putative ABC-type multidrug efflux systems and Robertson et al (171) carried out a systematic gene inactivation study to identify ABC transporters involved in intrinsic resistance to various antimicrobial agents. Except for one (SP1435), these investigators could disrupt all putative ABC-transporters, and analyzed the resulting mutants for their drug resistance profiles (171). Only the inactivation of the SP2073 or SP7075 genes gave rise to hypersusceptibility to ciprofloxacin and norfloxacin, as well as ethidium bromide and acriflavine. Interestingly, the two mutants were also susceptible to the plant alkaloid berberine, a QAC with heterocyclic moiety. In all cases, the observed susceptibility differences were identical for strains lacking either SP2073 or SP2075, and combining the two mutations were neither additive nor synergistic, which strongly suggests that these two proteins also act as heterodimer (171). Unfortunately, viologen compounds were not evaluated in this study. Interestingly, although SP2075 and SP2073 seem to be the homologue of SMU.905 and SMU.906, respectively, the former two genes do not appear to constitute an

operon, since the middle gene (SP2074, encoding a degenerate transposase) in the three-gene cluster is predicted to be transcribed in the opposite direction of SP2075 and SP2073. Thus, the expression and regulation of SP2075 and SP2073 are different from their counterparts in other organisms.

The ABC transporter complex (SMU.905/SMU.906) that we identified in this study also share high homology with four other putative ABC-type effluxes that are also organized in tandem in the genome. These are SMU.522/SMU.523, SMU.922/SMU.923, SMU1078/SMU.1079, and SMU.1163/SMU.1164 (Table S2). In each of these putative transporter pairs, one subunit contains the canonical glutamate residue and the other subunit contains a noncanonical aspartate residue in the Walker B box and thus expected to form heterodimers (3). We speculate that these heterodimeric ABC transporters are also involved in the efflux of toxic substances including antimicrobials and QACs. A thorough systematic analysis is necessary to determine the substrate specificity for these ABC transporters.

ABC-type transporters are one of the most abundant proteins in *S. mutans*. This bacterium encodes approximately 70 ABC transporters, which accounts for almost 10% of the total number of *S. mutans* ORFs (3, 136). About one-third of all ABC transporters are categorized as importers, whereas the rest are exporters. In contrast, although GBS encodes similar number of ABC transporters (about 70), the majority of them (over 65%) are importers (64). Similarly, GAS encodes about 50 ABC transporters and the ratio of importers to exporters is very similar to that of GBS (58). The presence of numerous ABC-type transporters indicates that *S. mutans* is capable of actively exporting excess and harmful molecules from the cell.

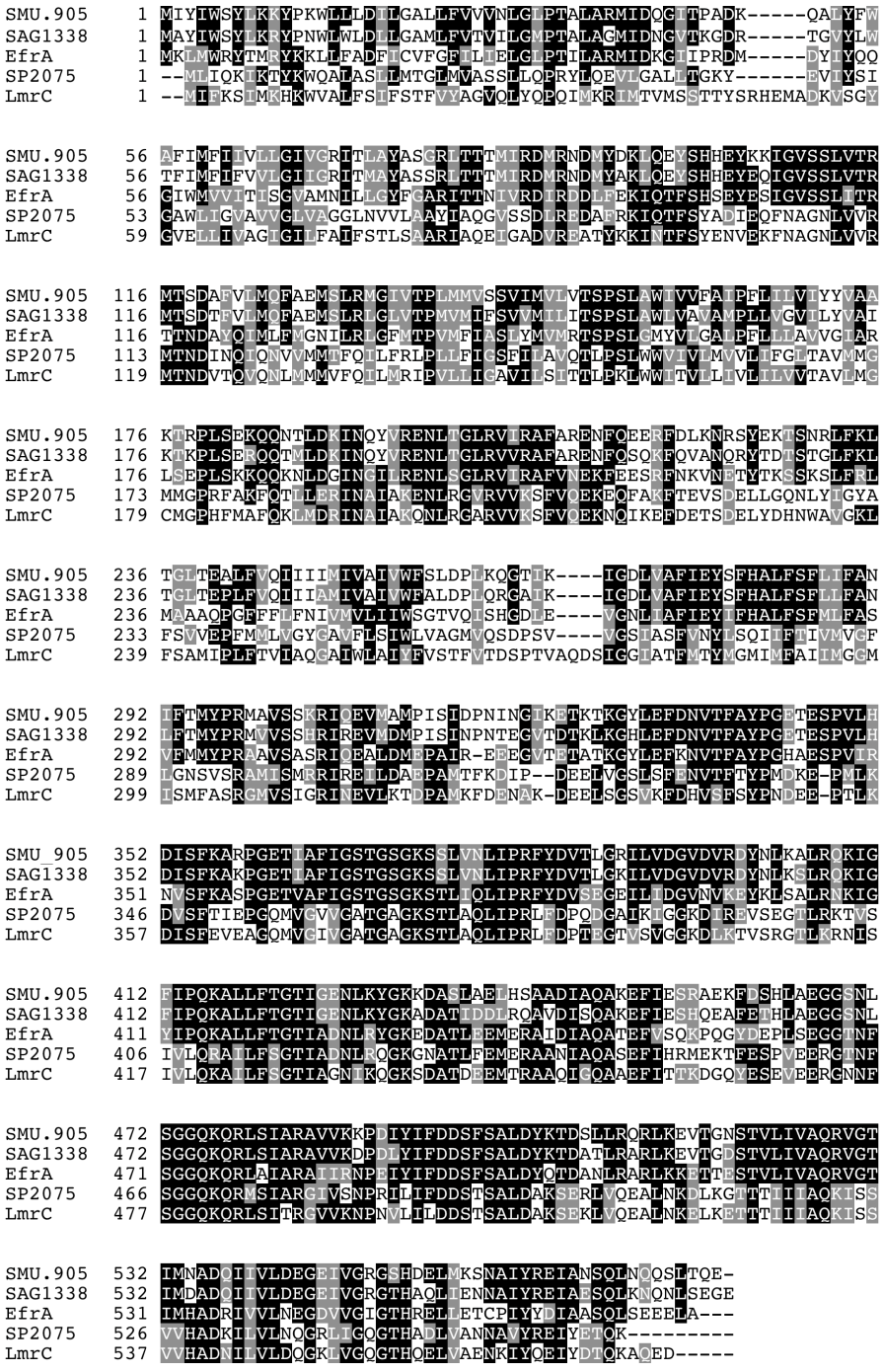


Figure 17 A: Multiple sequence alignment of SMU.905 and its closest homologs.

The sequences shown are (GeneBank accession numbers in parenthesis): *Enterococcus faecalis* EfrA (AAO82608.1), *Lactococcus lactis LmrC (*ABF66006.1), *Streptococcus agalactiae* SAG1338 (AAN00209.1), and *Streptococcus pneumoniae* SP2075 (ABJ55477.1). The alignment was performed using Clustal-W. Degree of shading is indicative of sequence homology and was done using BoxShade.

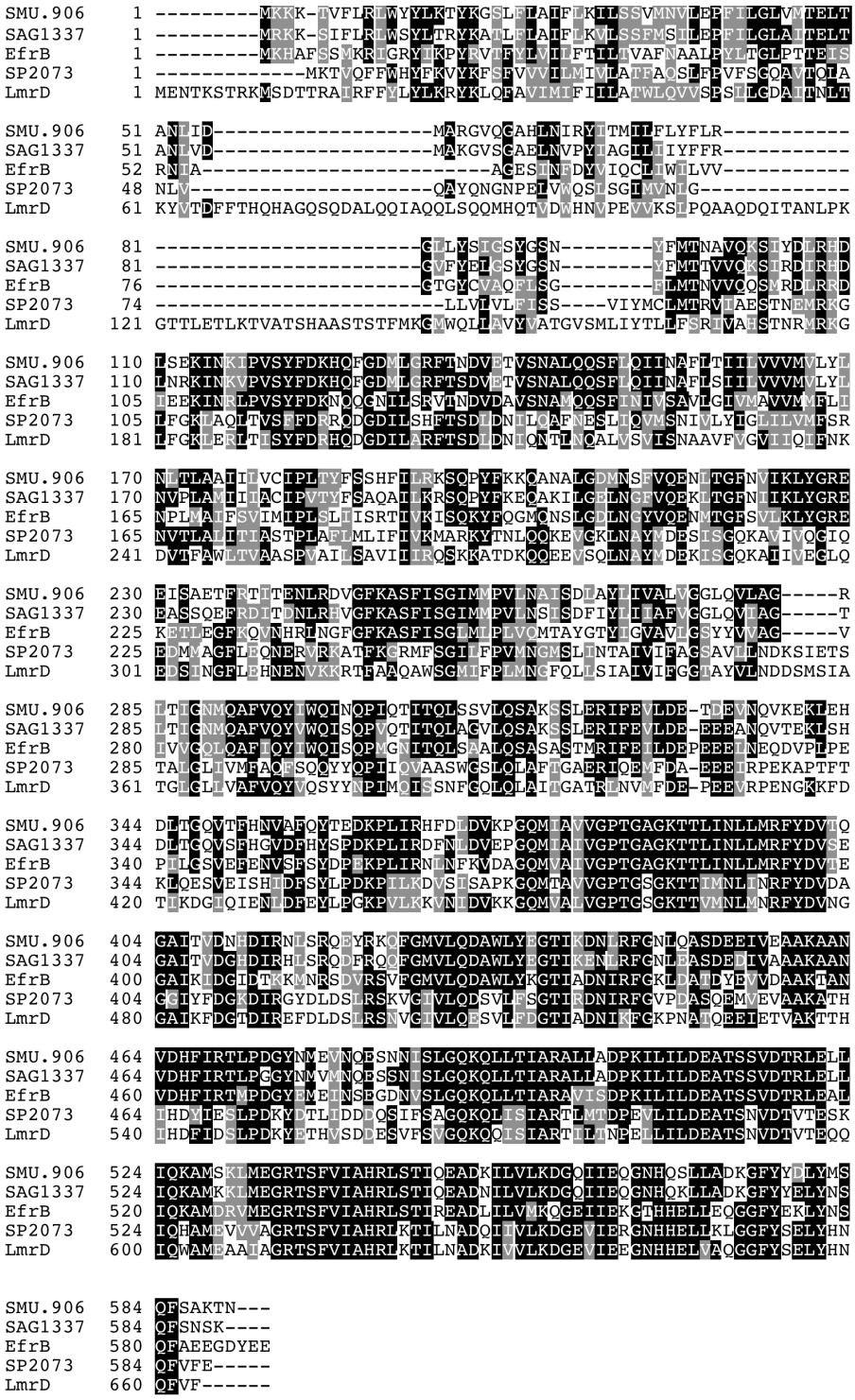


Figure 17 B: Multiple sequence alignment of SMU.906 and its closest homologs.

The sequences shown are: *E. faecalis* EfrB (AAO82607.1), *L. lactis* LmrD (CAL96931.1) *S. agalactiae* SAG1337 (AAN00208.1) and *S. pneumoniae* SP2073 (ABJ53826.1).

However, very little is known about the function of all these transporters. Only three *S. mutans* ABC-type exporters have been studied so far (193, 199). This organism is naturally bacitracin resistant and this property is often exploited in the isolation of this bacterium from the highly heterogeneous oral microflora. Tsuda et al (193) have shown that an ABC transporter complex, consisting of MbrA (SMU.1006) and MbrB (SMU.1007), was involved in the resistance of bacitracin. However, the substrate that is exported out by the ABC transporter has not been identified and the authors proposed that either the transporter exports an unknown molecule that inactivates bacitracin inside the cell or the transporter modulates the movement of bacitracin itself, or both. The other two ABC transporter complexes transport small peptides: NlmT (SMU.286) and NlmE (SMU.287) export mutacins VI and V, whereas CslA (ComA, SMU.1898) and CslB (ComB, SMU.1899) are involved in the secretion of competent simulating peptide (CSP) that is necessary for competence development.

Since violgens are charged compounds, like QACs, they cannot penetrate the cell membrane by simple diffusion; they require an active transport system to enter into the cell. As an example, in *Pseudomonous aeruginosa,* CbcWV, an ABC transporter, is involved in the uptake of many QACs including choline (30). Similarly, *B. subtilis* encodes an ABC transporter, OpuA, which is also involved in the QAC transport (95). Although several ABC-type importers are present in *S. mutans*, the screening method that we used in this study was not designed to identify importers for viologens. In addition to interfering with uptake (93) or enhancing efflux from the cell (161, 176), other poorly studied mechanisms also exist by which bacteria cope with viologen exposure (27, 169). In *B. subtilis*, YqjL protein confers resistance to viologen (27). This protein, which has similarity to members of the α/β hydrolase family protein, may be involved in direct degradation of viologen; although such degradation could not be demonstrated in vitro with purified YgjL (27). In *E. coli*, three cell division associated proteins

are shown to be involved in viologen tolerance. The first two proteins, FtsE and FtsX, form an ABC-transporter complex, while the other protein, SulI, is membrane associated (169)

Though we have identified SMU.905/SMU.906 as an ABC transporter involved in efflux of viologens and QACs, these chemicals may not be the actual ones that the organism encounters during its growth in natural habitat in the dental plaque. Dental plaque is a polymicrobial community that harbors more than 500 species or phylotypes (1, 104, 156) and the cell density can reach as high as 10^{11}CFU/ml (55). The oral biofilm is continuously challenged by changes in the environmental conditions and as a response to such challenges, the bacterial community evolved with individual members with specific functions such as primary or secondary colonizers including ability to metabolize or tolerate toxic excreted products produced by other species (102). About 20% of the oral bacteria are streptococci (134, 135) and these organisms with their specific spatial and temporal distribution determine the development of the biofilms. When present in high numbers, the pioneer colonizers can antagonize *S. mutans* as suggested by clinical studies (14). However, *S. mutans* can become dominant in oral biofilms, which leads to dental caries development. This dominance depends on competition with other organisms and is influenced by various factors. We speculate that the presence of numerous transporters such as SMU.905/SMU.906 allow *S. muatns* to withstand toxic compounds produced by the competing species or present in the plaque environment.

Identification of SMU.905/SMU.906 as a major transporter for toxic substances has an important biotechnological application. *S. mutans* strain lacking SMU.905/SMU.906 will be particularly suitable to screen for MDR transporters from other gram-positive bacteria since this organism is easy to transform. Further strain modification by deleting other MDR transporters, as demonstrated in *Sacchamomyces cereviseae* (172), may lead to hypersusceptibility to a greater number of antimicrobial agents; however, this will first require the identification of other systems that contribute to drug resistance in *S. mutans*. Moreover, a hypersusceptible *S. mutans* strain

could serve as a sensitive indicator for the genome-wide screening of novel MDR determinants involved in resistance development against novel antimicrobials. At present, drug-hypersensitive *E. coli* strains are used as heterologous hosts for screening, expression, and characterization of bacterial MDR systems (80, 110). Because cell envelope contains an additional outer membrane*, E. coli* seems less suited for screening of MDR transporters of gram-positive organisms including many pathogens. Finally, a hypersusceptible *S. mutans* strain would also be beneficial for development of cell-based screening systems for novel antimicrobials against gram-positive pathogens such as GAS, GBS, *E. feacalis* or *S. pneumoniae*.

CHAPTER 5

REFERENCES

1. **Aas, J. A., A. L. Griffen, S. R. Dardis, A. M. Lee, I. Olsen, F. E. Dewhirst, E. J. Leys, and B. J. Paster.** 2008. Bacteria of dental caries in primary and permanent teeth in children and young adults. J Clin Microbiol **46:**1407-17.
2. **Ahn, S. J., Z. T. Wen, and R. A. Burne.** 2006. Multilevel control of competence development and stress tolerance in *Streptococcus mutans* UA159. Infect Immun **74:**1631-42.
3. **Ajdic, D., W. M. McShan, R. E. McLaughlin, G. Savic, J. Chang, M. B. Carson, C. Primeaux, R. Tian, S. Kenton, H. Jia, S. Lin, Y. Qian, S. Li, H. Zhu, F. Najar, H. Lai, J. White, B. A. Roe, and J. J. Ferretti.** 2002. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. Proc Natl Acad Sci U S A **99:**14434-9.
4. **Ajdic, D., I. C. Sutcliffe, R. R. Russell, and J. J. Ferretti.** 1996. Organization and nucleotide sequence of the *Streptococcus mutans* galactose operon. Gene **180:**137-44.
5. **Alaluusua, S., T. Takei, T. Ooshima, and S. Hamada.** 1991. Mutacin activity of strains isolated from children with varying levels of mutants streptococci and caries. Arch Oral Biol **36:**251-5.
6. **Baba, T., and O. Schneewind.** 1998. Instruments of microbial warfare: bacteriocin synthesis, toxicity and immunity. Trends Microbiol **6:**66-71.
7. **Baker, L. M., and L. B. Poole.** 2003. Catalytic mechanism of thiol peroxidase from Escherichia coli. Sulfenic acid formation and overoxidation of essential CYS61. J Biol Chem **278:**9203-11.
8. **Balakrishnan, M., R. S. Simmonds, A. Carne, and J. R. Tagg.** 2000. *Streptococcus mutans* strain N produces a novel low molecular mass non-lantibiotic bacteriocin. FEMS Microbiol Lett **183:**165-9.
9. **Balakrishnan, M., R. S. Simmonds, M. Kilian, and J. R. Tagg.** 2002. Different bacteriocin activities of *Streptococcus mutans* reflect distinct phylogenetic lineages. J Med Microbiol **51:**941-8.
10. **Balakrishnan, M., R. S. Simmonds, and J. R. Tagg.** 2000. Dental caries is a preventable infectious disease. Aust Dent J **45:**235-45.
11. **Banas, J. A.** 2004. Virulence properties of *Streptococcus mutans*. Front Biosci **9:**1267-77.
12. **Banas, J. A., and M. M. Vickerman.** 2003. Glucan-binding proteins of the oral streptococci. Crit Rev Oral Biol Med **14:**89-99.

13. **Beauchamp, C., and I. Fridovich.** 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem **44:**276-87.
14. **Becker, M. R., B. J. Paster, E. J. Leys, M. L. Moeschberger, S. G. Kenyon, J. L. Galvin, S. K. Boches, F. E. Dewhirst, and A. L. Griffen.** 2002. Molecular analysis of bacterial species associated with childhood caries. J Clin Microbiol **40:**1001-9.
15. **Beg, A. M., M. N. Jones, T. Miller-Torbert, and R. G. Holt.** 2002. Binding of *Streptococcus mutans* to extracellular matrix molecules and fibrinogen. Biochem Biophys Res Commun **298:**75-9.
16. **Beynon, R. P., V. K. Bahl, and B. D. Prendergast.** 2006. Infective endocarditis. BMJ **333:**334-9.
17. **Biswas, I., L. Drake, D. Erkina, and S. Biswas.** 2008. Involvement of sensor kinases in the stress tolerance response of *Streptococcus mutans*. J Bacteriol **190:**68-77.
18. **Biswas, I., and J. R. Scott.** 2003. Identification of *rocA,* a Positive Regulator of *covR* Expression in the Group A Streptococcus. J Bacteriol **185:**3081-90.
19. **Biswas, S., and I. Biswas.** 2006. Regulation of the glucosyltransferase (*gtfBC*) operon by CovR in *Streptococcus mutans*. J Bacteriol **188:**988-98.
20. **Biswas, S., and I. Biswas.** 2005. Role of HtrA in Surface Protein Expression and Biofilm Formation by *Streptococcus mutans*. Infect Immun **73:**6923-34.
21. **Bochner, B. R.** 2009. Global phenotypic characterization of bacteria. FEMS Microbiol Rev **33:**191-205.
22. **Bochner, B. R., P. Gadzinski, and E. Panomitros.** 2001. Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. Genome Res **11:**1246-55.
23. **Bochner, B. R., L. Giovannetti, and C. Viti.** 2008. Important discoveries from analysing bacterial phenotypes. Mol Microbiol **70:**274-80.
24. **Brady, L. J., S. E. Maddocks, M. R. Larson, N. Forsgren, K. Persson, C. C. Deivanayagam, and H. F. Jenkinson.** The changing faces of Streptococcus antigen I/II polypeptide family adhesins. Mol Microbiol.
25. **Branda, S. S., J. E. Gonzalez-Pastor, E. Dervyn, S. D. Ehrlich, R. Losick, and R. Kolter.** 2004. Genes involved in formation of structured multicellular communities by Bacillus subtilis. J Bacteriol **186:**3970-9.
26. **Burne, R. A.** 1998. Oral streptococci... products of their environment. J Dent Res **77:**445-52.

27. **Cao, M., C. M. Moore, and J. D. Helmann.** 2005. *Bacillus subtilis* paraquat resistance is directed by sigmaM, an extracytoplasmic function sigma factor, and is conferred by YqjL and BcrC. J Bacteriol **187:**2948-56.

28. **Carlsson, J.** 2000. Growth and nutrition as ecological factors. *In* H. K. a. R. P. Ellen (ed.), Oral Bacterial Ecology: The molecular basis. Horizon Scientific PRess, Wymondham, Norflok, UK.

29. **Carrier, I., M. Julien, and P. Gros.** 2003. Analysis of catalytic carboxylate mutants E552Q and E1197Q suggests asymmetric ATP hydrolysis by the two nucleotide-binding domains of P-glycoprotein. Biochemistry **42:**12875-85.

30. **Chen, C., A. A. Malek, M. J. Wargo, D. A. Hogan, and G. A. Beattie.** The ATP-binding cassette transporter Cbc (choline/betaine/carnitine) recruits multiple substrate-binding proteins with strong specificity for distinct quaternary ammonium compounds. Mol Microbiol **75:**29-45.

31. **Chia, J. S., Y. L. Lin, H. T. Lien, and J. Y. Chen.** 2004. Platelet aggregation induced by serotype polysaccharides from *Streptococcus mutans*. Infect Immun **72:**2605-17.

32. **Chikindas, M. L., J. Novak, A. J. Driessen, W. N. Konings, K. M. Schilling, and P. W. Caufield.** 1995. Mutacin II, a bactericidal antibiotic from *Streptococcus mutans*. Antimicrob Agents Chemother **39:**2656-60.

33. **Chong, P., L. Drake, and I. Biswas.** 2008. LiaS regulates virulence factor expression in *Streptococcus mutans*. Infect Immun **76:**3093-9.

34. **Chong, P., L. Drake, and I. Biswas.** 2008. Modulation of *covR* expression in *Streptococcus mutans* UA159. J Bacteriol **190:**4478-88.

35. **Clarke, J. K.** 1924. On the bacterial factor in the aetiology of dental caries. British Journal of Experimental pathology **5:**141-147.

36. **Cole, S. T., K. Eiglmeier, J. Parkhill, K. D. James, N. R. Thomson, P. R. Wheeler, N. Honore, T. Garnier, C. Churcher, D. Harris, K. Mungall, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. M. Davies, K. Devlin, S. Duthoy, T. Feltwell, A. Fraser, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, C. Lacroix, J. Maclean, S. Moule, L. Murphy, K. Oliver, M. A. Quail, M. A. Rajandream, K. M. Rutherford, S. Rutter, K. Seeger, S. Simon, M. Simmonds, J. Skelton, R. Squares, S. Squares, K. Stevens, K. Taylor, S. Whitehead, J. R. Woodward, and B. G. Barrell.** 2001. Massive gene decay in the leprosy bacillus. Nature **409:**1007-11.

37. **Cuozzo, S. A., P. Castellano, F. J. Sesma, G. M. Vignolo, and R. R. Raya.** 2003. Differential roles of the two-component peptides of lactocin 705 in antimicrobial activity. Curr Microbiol **46:**180-3.

38. **Cvitkovitch, D. G., J. A. Gutierrez, and A. S. Bleiweis.** 1997. Role of the citrate pathway in glutamate biosynthesis by *Streptococcus mutans*. J Bacteriol **179:**650-5.

39. **Cvitkovitch, D. G., Y. H. Li, and R. P. Ellen.** 2003. Quorum sensing and biofilm formation in Streptococcal infections. J Clin Invest **112:**1626-32.

40. **Dashper, S. G., and E. C. Reynolds.** 1992. pH regulation by *Streptococcus mutans*. J Dent Res **71:**1159-65.

41. **Dashper, S. G. a. R., E.C.** 1996. Lactic acid excretion by *Streptococcus mutans.* Microbilogy **142:**33-39.

42. **Davidson, A. L., E. Dassa, C. Orelle, and J. Chen.** 2008. Structure, function, and evolution of bacterial ATP-binding cassette systems. Microbiol Mol Biol Rev **72:**317-64, table of contents.

43. **Davies, J.** 1994. Inactivation of antibiotics and the dissemination of resistance genes. Science **264:**375-82.

44. **Davis, B. J.** 1964. Disc Electrophoresis. Ii. Method and Application to Human Serum Proteins. Ann N Y Acad Sci **121:**404-27.

45. **Davis, D. R., J. B. McAlpine, C. J. Pazoles, M. K. Talbot, E. A. Alder, C. White, B. M. Jonas, B. E. Murray, G. M. Weinstock, and B. L. Rogers.** 2001. *Enterococcus faecalis* multi-drug resistance transporters: application for antibiotic discovery. J Mol Microbiol Biotechnol **3:**179-84.

46. **de Soet, J. J., B. Nyvad, and M. Kilian.** 2000. Strain-related acid production by oral streptococci. Caries Res **34:**486-90.

47. **Demuth, D. R., D. C. Irvine, J. W. Costerton, G. S. Cook, and R. J. Lamont.** 2001. Discrete protein determinant directs the species-specific adherence of Porphyromonas gingivalis to oral streptococci. Infect Immun **69:**5736-41.

48. **Do, T., S. C. Gilbert, D. Clark, F. Ali, C. C. Fatturi Parolo, M. Maltz, R. R. Russell, P. Holbrook, W. G. Wade, and D. Beighton.** Generation of diversity in *Streptococcus mutans* genes demonstrated by MLST. PLoS One **5:**e9073.

49. **Donlan, R. M.** 2002. Biofilms: microbial life on surfaces. Emerg Infect Dis **8:**881-90.

50. **Donlan, R. M., and J. W. Costerton.** 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev **15:**167-93.

51. **Douglas, C. W.** 1994. Bacterial-protein interactions in the oral cavity. Adv Dent Res **8:**254-62.

52. **Douglas, C. W., and R. R. Russell.** 1982. Effect of specific antisera on adherence properties of the oral bacterium *Streptococcus mutans*. Arch Oral Biol **27:**1039-45.

53. **Douglas, C. W., and R. R. Russell.** 1984. The adsorption of human salivary components to strains of the bacterium *Streptococcus mutans*. Arch Oral Biol **29:**751-7.

54. **Engels-Deutsch, M., A. Pini, Y. Yamashita, Y. Shibata, Y. Haikel, M. Scholler-Guinard, and J. P. Klein.** 2003. Insertional inactivation of *pac* and *rmlB* genes reduces the release of tumor necrosis factor alpha, interleukin-6, and interleukin-8 induced by *Streptococcus mutans* in monocytic, dental pulp, and periodontal ligament cells. Infect Immun **71:**5169-77.

55. **Evaldson, G., A. Heimdahl, L. Kager, and C. E. Nord.** 1982. The normal human anaerobic microflora. Scand J Infect Dis Suppl **35:**9-15.

56. **Feldgarden, M., N. Byrd, and F. M. Cohan.** 2003. Gradual evolution in bacteria: evidence from Bacillus systematics. Microbiology **149:**3565-73.

57. **Ferguson, G. P., S. Totemeyer, M. J. MacLean, and I. R. Booth.** 1998. Methylglyoxal production in bacteria: suicide or survival? Arch Microbiol **170:**209-18.

58. **Ferretti, J. J., W. M. McShan, D. Ajdic, D. J. Savic, G. Savic, K. Lyon, C. Primeaux, S. Sezate, A. Suvorov, S. Kenton, H. S. Lai, S. P. Lin, Y. Qian, H. G. Jia, F. Z. Najar, Q. Ren, H. Zhu, L. Song, J. White, X. Yuan, S. W. Clifton, B. A. Roe, and R. McLaughlin.** 2001. Complete genome sequence of an M1 strain of *Streptococcus pyogenes*. Proc Natl Acad Sci U S A **98:**4658-4663.

59. **Fitzgerald, R. J., B. O. Adams, H. J. Sandham, and S. Abhyankar.** 1989. Cariogenicity of a lactate dehydrogenase-deficient mutant of *Streptococcus mutans* serotype c in gnotobiotic rats. Infect Immun **57:**823-6.

60. **Fozo, E. M., and R. G. Quivey, Jr.** 2004. Shifts in the membrane fatty acid profile of *Streptococcus mutans* enhance survival in acidic environments. Appl Environ Microbiol **70:**929-36.

61. **Fujita, K., M. Matsumoto-Nakano, S. Inagaki, and T. Ooshima.** 2007. Biological functions of glucan-binding protein B of *Streptococcus mutans.* Oral Microbiol Immunol **22:**289-92.

62. **Garneau, S., N. I. Martin, and J. C. Vederas.** 2002. Two-peptide bacteriocins produced by lactic acid bacteria. Biochimie **84:**577-92.

63. **Gevers, D., K. Vandepoele, C. Simillon, and Y. Van de Peer.** 2004. Gene duplication and biased functional retention of paralogs in bacterial genomes. Trends Microbiol **12:**148-54.

64. **Glaser, P., C. Rusniok, C. Buchrieser, F. Chevalier, L. Frangeul, T. Msadek, M. Zouine, E. Couve, L. Lalioui, C. Poyart, P. Trieu-Cuot, and F. Kunst.** 2002. Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. Mol Microbiol **45:**1499-513.

65. **Gonzalez-Flecha, B., and B. Demple.** 1997. Homeostatic regulation of intracellular hydrogen peroxide concentration in aerobically growing Escherichia coli. J Bacteriol **179:**382-8.

66. **Graf, H.** 1970. The glycolytic activity of plaque and its relation to hard tissues pathology--recent findings from intraoral pH telemetry research. Int Dent J **20:**426-35.

67. **Gronroos, L., M. Saarela, J. Matto, U. Tanner-Salo, A. Vuorela, and S. Alaluusua.** 1998. Mutacin production by *Streptococcus mutans* may promote transmission of bacteria from mother to child. Infect Immun **66:**2595-600.

68. **Hajishengallis, G., T. Koga, and M. W. Russell.** 1994. Affinity and specificity of the interactions between *Streptococcus mutans* antigen I/II and salivary components. J Dent Res **73:**1493-502.

69. **Hale, J. D., Y. T. Ting, R. W. Jack, J. R. Tagg, and N. C. Heng.** 2005. Bacteriocin (mutacin) production by *Streptococcus mutans* genome sequence reference strain UA159: elucidation of the antimicrobial repertoire by genetic dissection. Appl Environ Microbiol **71:**7613-7.

70. **Hall-Stoodley, L., and P. Stoodley.** 2002. Developmental regulation of microbial biofilms. Curr Opin Biotechnol **13:**228-33.

71. **Hamada, S., and H. D. Slade.** 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol Rev **44:**331-84.

72. **Hamilton, I. R.** 2000. Ecological basis for dental caries., p. 219-266. *In* H. K. a. R. P. Ellen (ed.), Oral bacterial ecology: The molecular basis. Horizon Scientific Press, Wymondham, Norfolk, UK.

73. **Hamilton, I. R., and N. D. Buckley.** 1991. Adaptation by *Streptococcus mutans* to acid tolerance. Oral Microbiol Immunol **6:**65-71.

74. **Hamilton, I. R., and G. Svensater.** 1998. Acid-regulated proteins induced by *Streptococcus mutans* and other oral bacteria during acid shock. Oral Microbiol Immunol **13:**292-300.

75. **Hansen, S. K., P. B. Rainey, J. A. Haagensen, and S. Molin.** 2007. Evolution of species interactions in a biofilm community. Nature **445:**533-6.

76. **Hillman, J. D., J. Novak, E. Sagura, J. A. Gutierrez, T. A. Brooks, P. J. Crowley, M. Hess, A. Azizi, K. Leung, D. Cvitkovitch, and A. S. Bleiweis.** 1998. Genetic and biochemical analysis of mutacin 1140, a lantibiotic from *Streptococcus mutans*. Infect Immun **66:**2743-9.

77. **Hoiby, N., T. Bjarnsholt, M. Givskov, S. Molin, and O. Ciofu.** Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents **35:**322-32.

78. **Hojo, K., S. Nagaoka, T. Ohshima, and N. Maeda.** 2009. Bacterial interactions in dental biofilm development. J Dent Res **88:**982-90.

79. **Huang, W. C., Y. Y. Chen, L. J. Teng, H. T. Lien, J. Y. Chen, and J. S. Chia.** 2008. Chromosomal inversion between *rrn* operons among *Streptococcus mutans* serotype *c* oral and blood isolates. J Med Microbiol **57:**198-206.

80. **Huda, N., E. W. Lee, J. Chen, Y. Morita, T. Kuroda, T. Mizushima, and T. Tsuchiya.** 2003. Molecular cloning and characterization of an ABC multidrug efflux pump, VcaM, in Non-O1 Vibrio cholerae. Antimicrob Agents Chemother **47:**2413-7.

81. **Husmann, L. K., D. L. Yung, S. K. Hollingshead, and J. R. Scott.** 1997. Role of putative virulence factors of *Streptococcus pyogenes* in mouse models of long-term throat colonization and pneumonia. Infection and Immunity **65:**1422-30.

82. **Hyde, S. C., P. Emsley, M. J. Hartshorn, M. M. Mimmack, U. Gileadi, S. R. Pearce, M. P. Gallagher, D. R. Gill, R. E. Hubbard, and C. F. Higgins.** 1990. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature **346:**362-5.

83. **Islam, B., S. N. Khan, and A. U. Khan.** 2007. Dental caries: from infection to prevention. Med Sci Monit **13:**RA196-203.

84. **Jain, A., Y. Gupta, R. Agrawal, P. Khare, and S. K. Jain.** 2007. Biofilms--a microbial life perspective: a critical review. Crit Rev Ther Drug Carrier Syst **24:**393-443.

85. **Jakubovics, N., and P. Kolenbrander.** The road to ruin: the formation of disease-associated oral biofilms. Oral Dis.

86. **Jakubovics, N. S., N. Stromberg, C. J. van Dolleweerd, C. G. Kelly, and H. F. Jenkinson.** 2005. Differential binding specificities of oral streptococcal antigen I/II family adhesins for human or bacterial ligands. Mol Microbiol **55:**1591-605.

87. **Jefferson, K. K.** 2004. What drives bacteria to produce a biofilm? FEMS Microbiol Lett **236:**163-73.

88. **Jenkinson, H. F., and D. R. Demuth.** 1997. Structure, function and immunogenicity of streptococcal antigen I/II polypeptides. Mol Microbiol **23:**183-90.

89. **Jenkinson, H. F., and R. J. Lamont.** 1997. Streptococcal adhesion and colonization. Crit Rev Oral Biol Med **8:**175-200.

90. **Johnson, C. P., S. M. Gross, and J. D. Hillman.** 1980. Cariogenic potential in vitro in man and in vivo in the rat of lactate dehydrogenase mutants of *Streptococcus mutans*. Arch Oral Biol **25:**707-13.

91. **Johri, A. K., I. Margarit, M. Broenstrup, C. Brettoni, L. Hua, S. P. Gygi, J. L. Telford, G. Grandi, and L. C. Paoletti.** 2007. Transcriptional and proteomic profiles of group B Streptococcus type V reveal potential adherence proteins associated with high-level invasion. Infect Immun **75:**1473-83.

92. **Kamiya, R. U., M. H. Napimoga, R. T. Rosa, J. F. Hofling, and R. B. Goncalves.** 2005. Mutacin production in *Streptococcus mutans* genotypes isolated from caries-affected and caries-free individuals. Oral Microbiol Immunol **20:**20-4.

93. **Kao, S. M., and H. M. Hassan.** 1985. Biochemical characterization of a paraquat-tolerant mutant of *Escherichia coli.* J Biol Chem **260:**10478-81.

94. **Kelly, C. G., J. S. Younson, B. Y. Hikmat, S. M. Todryk, M. Czisch, P. I. Haris, I. R. Flindall, C. Newby, A. I. Mallet, J. K. Ma, and T. Lehner.** 1999. A synthetic peptide adhesion epitope as a novel antimicrobial agent. Nat Biotechnol **17:**42-7.

95. **Kempf, B., J. Gade, and E. Bremer.** 1997. Lipoprotein from the osmoregulated ABC transport system OpuA of *Bacillus subtilis:* purification of the glycine betaine binding protein and characterization of a functional lipidless mutant. J Bacteriol **179:**6213-20.

96. **Kleinberg, I.** 2002. A mixed-bacteria ecological approach to understanding the role of the oral bacteria in dental caries causation: an alternative to *Streptococcus mutans* and the specific-plaque hypothesis. Crit Rev Oral Biol Med **13:**108-25.

97. **Kohler, B., D. Birkhed, and S. Olsson.** 1995. Acid production by human strains of *Streptococcus mutans* and *Streptococcus sobrinus*. Caries Res **29:**402-6.

98. **Kolenbrander, P. E.** 1993. Coaggregation of human oral bacteria: potential role in the accretion of dental plaque. J Appl Bacteriol **74 Suppl:**79S-86S.

99. **Kolenbrander, P. E.** 2000. Oral microbial communities: biofilms, interactions, and genetic systems. Annu Rev Microbiol **54:**413-37.

100. **Kolenbrander, P. E., P. G. Egland, P. I. Diaz, and R. J. Palmer, Jr.** 2005. Genome-genome interactions: bacterial communities in initial dental plaque. Trends Microbiol **13:**11-5.

101. **Kolenbrander, P. E., N. Ganeshkumar, F. J. Cassels, and C. V. Hughes.** 1993. Coaggregation: specific adherence among human oral plaque bacteria. FASEB J **7:**406-13.

102. **Kolenbrander, P. E., R. J. Palmer, Jr., A. H. Rickard, N. S. Jakubovics, N. I. Chalmers, and P. I. Diaz.** 2006. Bacterial interactions and successions during plaque development. Periodontol 2000 **42:**47-79.

103. **Kreth, J., Y. Zhang, and M. C. Herzberg.** 2008. Streptococcal antagonism in oral biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* interference with Streptococcus mutans. J Bacteriol **190:**4632-40.

104. **Kroes, I., P. W. Lepp, and D. A. Relman.** 1999. Bacterial diversity within the human subgingival crevice. Proc Natl Acad Sci U S A **96:**14547-52.

105. **Kuramitsu, H. K., X. He, R. Lux, M. H. Anderson, and W. Shi.** 2007. Interspecies interactions within oral microbial communities. Microbiol Mol Biol Rev **71:**653-70.

106. **Lamont, R. J., D. R. Demuth, C. A. Davis, D. Malamud, and B. Rosan.** 1991. Salivary-agglutinin-mediated adherence of *Streptococcus mutans* to early plaque bacteria. Infect Immun **59:**3446-50.

107. **Lamont, R. J., A. El-Sabaeny, Y. Park, G. S. Cook, J. W. Costerton, and D. R. Demuth.** 2002. Role of the *Streptococcus gordonii* SspB protein in the development of *Porphyromonas gingivalis* biofilms on streptococcal substrates. Microbiology **148:**1627-36.

108. **Lawrence, J. G.** 2001. Catalyzing bacterial speciation: correlating lateral transfer with genetic headroom. Syst Biol **50:**479-96.

109. **Lawrence, J. G., and H. Ochman.** 2002. Reconciling the many faces of lateral gene transfer. Trends Microbiol **10:**1-4.

110. **Lee, E. W., J. Chen, M. N. Huda, T. Kuroda, T. Mizushima, and T. Tsuchiya.** 2003. Functional cloning and expression of *emeA*, and characterization of EmeA, a multidrug efflux pump from Enterococcus faecalis. Biol Pharm Bull **26:**266-70.

111. **Lee, E. W., M. N. Huda, T. Kuroda, T. Mizushima, and T. Tsuchiya.** 2003. EfrAB, an ABC multidrug efflux pump in *Enterococcus faecalis*. Antimicrob Agents Chemother **47:**3733-8.

112. **Lee, H. W., Y. M. Koh, J. Kim, J. C. Lee, Y. C. Lee, S. Y. Seol, and D. T. Cho.** 2008. Capacity of multidrug-resistant clinical isolates of *Acinetobacter baumannii* to form biofilm and adhere to epithelial cell surfaces. Clin Microbiol Infect **14:**49-54.

113. **Lemos, J. A., J. Abranches, and R. A. Burne.** 2005. Responses of cariogenic streptococci to environmental stresses. Curr Issues Mol Biol **7:**95-107.

114. **Lemos, J. A., and R. A. Burne.** 2008. A model of efficiency: stress tolerance by *Streptococcus mutans.* Microbiology **154:**3247-55.

115. **Len, A. C., D. W. Harty, and N. A. Jacques.** 2004. Proteome analysis of *Streptococcus mutans* metabolic phenotype during acid tolerance. Microbiology **150:**1353-66.

116. **Li, Y. H., M. N. Hanna, G. Svensater, R. P. Ellen, and D. G. Cvitkovitch.** 2001. Cell density modulates acid adaptation in *Streptococcus mutans*: implications for survival in biofilms. J Bacteriol **183:**6875-84.

117. **Liljemark, W. F., and C. Bloomquist.** 1996. Human oral microbial ecology and dental caries and periodontal diseases. Crit Rev Oral Biol Med **7:**180-98.

118. **Loesche, W. J.** 1986. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev **50:**353-80.

119. **Loimaranta, V., N. S. Jakubovics, J. Hytonen, J. Finne, H. F. Jenkinson, and N. Stromberg.** 2005. Fluid- or surface-phase human salivary scavenger protein gp340 exposes different bacterial recognition properties. Infect Immun **73:**2245-52.

120. **Longo, P. L., R. O. Mattos-Graner, and M. P. Mayer.** 2003. Determination of mutacin activity and detection of *mutA* genes in *Streptococcus mutans* genotypes from caries-free and caries-active children. Oral Microbiol Immunol **18:**144-9.

121. **Lubelski, J., W. N. Konings, and A. J. Driessen.** 2007. Distribution and physiology of ABC-type transporters contributing to multidrug resistance in bacteria. Microbiol Mol Biol Rev **71:**463-76.

122. **Lubelski, J., P. Mazurkiewicz, R. van Merkerk, W. N. Konings, and A. J. Driessen.** 2004. *ydaG* and *ydbA* of *Lactococcus lactis* encode a heterodimeric ATP-binding cassette-type multidrug transporter. J Biol Chem **279:**34449-55.

123. **Lubelski, J., R. van Merkerk, W. N. Konings, and A. J. Driessen.** 2006. Nucleotide-binding sites of the heterodimeric LmrCD ABC-multidrug transporter of *Lactococcus lactis* are asymmetric. Biochemistry **45:**648-56.

124. **Lynch, D. J., T. L. Fountain, J. E. Mazurkiewicz, and J. A. Banas.** 2007. Glucan-binding proteins are essential for shaping *Streptococcus mutans* biofilm architecture. FEMS Microbiol Lett **268:**158-65.

125. **Ma, J. K., C. G. Kelly, G. Munro, R. A. Whiley, and T. Lehner.** 1991. Conservation of the gene encoding streptococcal antigen I/II in oral streptococci. Infect Immun **59:**2686-94.

126. **Mager, D. L., L. A. Ximenez-Fyvie, A. D. Haffajee, and S. S. Socransky.** 2003. Distribution of selected bacterial species on intraoral surfaces. J Clin Periodontol **30:**644-54.

127. **Maguin, E., P. Duwat, T. Hege, D. Ehrlich, and A. Gruss.** 1992. New thermosensitive plasmid for gram-positive bacteria. J Bacteriol **174:**5633-5638.

128. **Maguin, E., H. Prevost, S. D. Ehrlich, and A. Gruss.** 1996. Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. Journal of Bacteriology **178:**931-5.

129. **Manson, J. M., S. Keis, J. M. Smith, and G. M. Cook.** 2004. Acquired bacitracin resistance in *Enterococcus faecalis* is mediated by an ABC transporter and a novel regulatory protein, BcrR. Antimicrob Agents Chemother **48:**3743-8.

130. **Marger, M. D., and M. H. Saier, Jr.** 1993. A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. Trends Biochem Sci **18:**13-20.

131. **Marrer, E., K. Schad, A. T. Satoh, M. G. Page, M. M. Johnson, and L. J. Piddock.** 2006. Involvement of the putative ATP-dependent efflux proteins PatA and PatB in fluoroquinolone resistance of a multidrug-resistant mutant of *Streptococcus pneumoniae.* Antimicrob Agents Chemother **50:**685-93.

132. **Marsh, P. D.** 2003. Are dental diseases examples of ecological catastrophes? Microbiology **149:**279-94.

133. **Marsh, P. D.** 2004. Dental plaque as a microbial biofilm. Caries Res **38:**204-11.

134. **Marsh, P. D.** 2005. Dental plaque: biological significance of a biofilm and community life-style. J Clin Periodontol **32 Suppl 6:**7-15.

135. **Marsh, P. D.** 1999. Microbiologic aspects of dental plaque and dental caries. Dent Clin North Am **43:**599-614, v-vi.

136. **Maruyama, F., M. Kobata, K. Kurokawa, K. Nishida, A. Sakurai, K. Nakano, R. Nomura, S. Kawabata, T. Ooshima, K. Nakai, M. Hattori, S. Hamada, and I. Nakagawa.** 2009. Comparative genomic analyses of *Streptococcus mutans* provide insights into chromosomal shuffling and species-specific content. BMC Genomics **10:**358.

137. **Matsumoto-Nakano, M., K. Fujita, and T. Ooshima.** 2007. Comparison of glucan-binding proteins in cariogenicity of *Streptococcus mutans*. Oral Microbiol Immunol **22:**30-5.

138. **Matsumura, M., T. Izumi, M. Matsumoto, M. Tsuji, T. Fujiwara, and T. Ooshima.** 2003. The role of glucan-binding proteins in the cariogenicity of *Streptococcus mutans*. Microbiol Immunol **47:**213-5.

139. **Mattos-Graner, R. O., D. J. Smith, W. F. King, and M. P. Mayer.** 2000. Water-insoluble glucan synthesis by mutans streptococcal strains correlates with caries incidence in 12- to 30-month-old children. J Dent Res **79:**1371-7.

140. **Mitchell, T. J.** 2003. The pathogenesis of streptococcal infections: from tooth decay to meningitis. Nat Rev Microbiol **1:**219-30.

141. **Moreillon, P., and Y. A. Que.** 2004. Infective endocarditis. Lancet **363:**139-49.

142. **Mota-Meira, M., C. Lacroix, G. LaPointe, and M. C. Lavoie.** 1997. Purification and structure of mutacin B-Ny266: a new lantibiotic produced by *Streptococcus mutans*. FEBS Lett **410:**275-9.

143. **Nagata, E., H. Okayama, H. O. Ito, Y. Yamashita, M. Inoue, and T. Oho.** 2006. Serotype-specific polysaccharide of *Streptococcus mutans* contributes to infectivity in endocarditis. Oral Microbiol Immunol **21:**420-3.

144. **Nakano, K., J. Lapirattanakul, R. Nomura, H. Nemoto, S. Alaluusua, L. Gronroos, M. Vaara, S. Hamada, T. Ooshima, and I. Nakagawa.** 2007. *Streptococcus mutans* clonal variation revealed by multilocus sequence typing. J Clin Microbiol **45:**2616-25.

145. **Nakano, K., R. Nomura, H. Nemoto, T. Mukai, H. Yoshioka, Y. Shudo, H. Hata, K. Toda, K. Taniguchi, A. Amano, and T. Ooshima.** 2007. Detection of novel serotype *k* *Streptococcus mutans* in infective endocarditis patients. J Med Microbiol **56:**1413-5.

146. **Nakayama, K.** 1992. Nucleotide sequence of *Streptococcus mutans* superoxide dismutase gene and isolation of insertion mutants. J Bacteriol **174:**4928-34.

147. **Napimoga, M. H., R. U. Kamiya, R. T. Rosa, E. A. Rosa, J. F. Hofling, R. O. Mattos-Graner, and R. B. Goncalves.** 2004. Genotypic diversity and virulence traits of Streptococcus mutans in caries-free and caries-active individuals. J Med Microbiol **53:**697-703.

148. **Nes, I. F., D. B. Diep, and H. Holo.** 2007. Bacteriocin diversity in Streptococcus and Enterococcus. J Bacteriol **189:**1189-98.

149. **Nikaido, H.** 2009. Multidrug resistance in bacteria. Annu Rev Biochem **78:**119-46.

150. **Nobbs, A. H., R. J. Lamont, and H. F. Jenkinson.** 2009. Streptococcus adherence and colonization. Microbiol Mol Biol Rev **73:**407-50, Table of Contents.

151. **Okano, S., Y. Shibata, T. Shiroza, and Y. Abiko.** 2006. Proteomics-based analysis of a counter-oxidative stress system in *Porphyromonas gingivalis*. Proteomics **6:**251-8.

152. **Oyamada, Y., H. Ito, M. Inoue, and J. Yamagishi.** 2006. Topoisomerase mutations and efflux are associated with fluoroquinolone resistance in *Enterococcus faecalis.* J Med Microbiol **55:**1395-401.

153. **Palmer, R. J., Jr., K. Kazmerzak, M. C. Hansen, and P. E. Kolenbrander.** 2001. Mutualism versus independence: strategies of mixed-species oral biofilms in vitro using saliva as the sole nutrient source. Infect Immun **69:**5794-804.

154. **Papaioannou, W., S. Gizani, A. D. Haffajee, M. Quirynen, E. Mamai-Homata, and L. Papagiannoulis.** 2009. The microbiota on different oral surfaces in healthy children. Oral Microbiol Immunol **24:**183-9.

155. **Parsek, M. R., and P. K. Singh.** 2003. Bacterial biofilms: an emerging link to disease pathogenesis. Annu Rev Microbiol **57:**677-701.

156. **Paster, B. J., S. K. Boches, J. L. Galvin, R. E. Ericson, C. N. Lau, V. A. Levanos, A. Sahasrabudhe, and F. E. Dewhirst.** 2001. Bacterial diversity in human subgingival plaque. J Bacteriol **183:**3770-83.

157. **Paulsen, I. T., R. A. Skurray, R. Tam, M. H. Saier, Jr., R. J. Turner, J. H. Weiner, E. B. Goldberg, and L. L. Grinius.** 1996. The SMR family: a novel family of multidrug efflux proteins involved with the efflux of lipophilic drugs. Mol Microbiol **19:**1167-75.

158. **Perez-Casal, J., M. G. Caparon, and J. R. Scott.** 1991. Mry, a trans-acting positive regulator of the M protein gene of *Streptococcus pyogenes* with similarity to the receptor proteins of two-component regulatory systems. Journal of Bacteriology **173:**2617-2624.

159. **Piddock, L. J.** 2006. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin Microbiol Rev **19:**382-402.

160. **Piddock, L. J.** 2006. Multidrug-resistance efflux pumps - not just for resistance. Nat Rev Microbiol **4:**629-36.

161. **Prosecka, J., A. V. Orlov, Y. S. Fantin, V. V. Zinchenko, M. M. Babykin, and M. Tichy.** 2009. A novel ATP-binding cassette transporter is responsible for resistance to viologen herbicides in the cyanobacterium Synechocystis sp. PCC 6803. FEBS J **276:**4001-11.

162. **Putman, M., H. W. van Veen, and W. N. Konings.** 2000. Molecular properties of bacterial multidrug transporters. Microbiol Mol Biol Rev **64:**672-93.

163. **Qi, F., P. Chen, and P. W. Caufield.** 1999. Functional analyses of the promoters in the lantibiotic mutacin II biosynthetic locus in *Streptococcus mutans.* Appl Environ Microbiol **65:**652-8.

164. **Qi, F., P. Chen, and P. W. Caufield.** 2000. Purification and biochemical characterization of mutacin I from the group I strain of *Streptococcus mutans,* CH43, and genetic analysis of mutacin I biosynthesis genes. Appl Environ Microbiol **66:**3221-9.

165. **Qi, F., P. Chen, and P. W. Caufield.** 1999. Purification of mutacin III from group III *Streptococcus mutans* UA787 and genetic analyses of mutacin III biosynthesis genes. Appl Environ Microbiol **65:**3880-7.

166. **Qi, F., P. Chen, and P. W. Caufield.** 2001. The group I strain of *Streptococcus mutans,* UA140, produces both the lantibiotic mutacin I and a nonlantibiotic bacteriocin, mutacin IV. Appl Environ Microbiol **67:**15-21.

167. **Qi, F., J. Merritt, R. Lux, and W. Shi.** 2004. Inactivation of the *ciaH* gene in *Streptococcus mutans* diminishes mutacin production and competence development, alters sucrose-dependent biofilm formation, and reduces stress tolerance. Infection and Immunity **72:**4895-4899.

168. **Quivey, R. G., Jr., R. Faustoferri, K. Monahan, and R. Marquis.** 2000. Shifts in membrane fatty acid profiles associated with acid adaptation of *Streptococcus mutans*. FEMS Microbiol Lett **189:**89-92.

169. **Reddy, M.** 2007. Role of FtsEX in cell division of *Escherichia coli:* viability of *ftsEX* mutants is dependent on functional SufI or high osmotic strength. J Bacteriol **189:**98-108.

170. **Reuter, G., T. Janvilisri, H. Venter, S. Shahi, L. Balakrishnan, and H. W. van Veen.** 2003. The ATP binding cassette multidrug transporter LmrA and lipid transporter MsbA have overlapping substrate specificities. J Biol Chem **278:**35193-8.

171. **Robertson, G. T., T. B. Doyle, and A. S. Lynch.** 2005. Use of an efflux-deficient *Streptococcus pneumoniae* strain panel to identify ABC-class multidrug transporters involved in intrinsic resistance to antimicrobial agents. Antimicrob Agents Chemother **49:**4781-3.

172. **Rogers, B., A. Decottignies, M. Kolaczkowski, E. Carvajal, E. Balzi, and A. Goffeau.** 2001. The pleitropic drug ABC transporters from *Saccharomyces cerevisiae.* J Mol Microbiol Biotechnol **3:**207-14.

173. **Russell, M. W., D. J. Harrington, and R. R. Russell.** 1995. Identity of *Streptococcus mutans* surface protein antigen III and wall-associated protein antigen A. Infect Immun **63:**733-5.

174. **Saier, M. H., Jr., R. Tam, A. Reizer, and J. Reizer.** 1994. Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. Mol Microbiol **11:**841-7.

175. **Sansone, C., J. Van Houte, K. Joshipura, R. Kent, and H. C. Margolis.** 1993. The association of mutans streptococci and non-mutans streptococci capable of acidogenesis at a low pH with dental caries on enamel and root surfaces. J Dent Res **72:**508-16.

176. **Santiviago, C. A., J. A. Fuentes, S. M. Bueno, A. N. Trombert, A. A. Hildago, L. T. Socias, P. Youderian, and G. C. Mora.** 2002. The *Salmonella enterica* sv. Typhimurium smvA, yddG and *ompD* (porin) genes are required for the efficient efflux of methyl viologen. Mol Microbiol **46:**687-98.

177. **Sato, Y., Y. Yamamoto, and H. Kizaki.** 1997. Cloning and sequence analysis of the gbpC gene encoding a novel glucan-binding protein of *Streptococcus mutans.* Infect Immun **65:**668-75.

178. **Shemesh, M., A. Tam, M. Kott-Gutkowski, M. Feldman, and D. Steinberg.** 2008. DNA-microarrays identification of Streptococcus mutans genes associated with biofilm thickness. BMC Microbiol **8:**236.

179. **Shemesh, M., A. Tam, and D. Steinberg.** 2007. Differential gene expression profiling of *Streptococcus mutans* cultured under biofilm and planktonic conditions. Microbiology **153:**1307-17.

180. **Sheng, J., and R. E. Marquis.** 2007. Malolactic fermentation by *Streptococcus mutans.* FEMS Microbiol Lett **272:**196-201.

181. **Smith, D. J.** 2002. Dental caries vaccines: prospects and concerns. Crit Rev Oral Biol Med **13:**335-49.

182. **Spellerberg, B., B. Pohl, G. Haase, S. Martin, J. Weber-Heynemann, and R. Lutticken.** 1999. Identification of genetic determinants for the hemolytic activity of Streptococcus agalactiae by ISS1 transposition. J Bacteriol **181:**3212-9.

183. **Spratt, B. G.** 1994. Resistance to antibiotics mediated by target alterations. Science **264:**388-93.

184. **Springael, D., and E. M. Top.** 2004. Horizontal gene transfer and microbial adaptation to xenobiotics: new types of mobile genetic elements and lessons from ecological studies. Trends Microbiol **12:**53-8.

185. **Steinfels, E., C. Orelle, J. R. Fantino, O. Dalmas, J. L. Rigaud, F. Denizot, A. Di Pietro, and J. M. Jault.** 2004. Characterization of YvcC (BmrA), a multidrug ABC transporter constitutively expressed in *Bacillus subtilis*. Biochemistry **43:**7491-502.

186. **Stephan, R. M.** 1944. Intra-oral hydrogen-ion concentrations associated with dental caries activity. J Dent Res **23:**257-266.

187. **Stoodley, P., K. Sauer, D. G. Davies, and J. W. Costerton.** 2002. Biofilms as complex differentiated communities. Annu Rev Microbiol **56:**187-209.

188. **Sturr, M. G., and R. E. Marquis.** 1990. Inhibition of proton-translocating ATPases of *Streptococcus mutans* and *Lactobacillus casei* by fluoride and aluminum. Arch Microbiol **155:**22-7.

189. **Svensater, G., U. B. Larsson, E. C. Greif, D. G. Cvitkovitch, and I. R. Hamilton.** 1997. Acid tolerance response and survival by oral bacteria. Oral Microbiol Immunol **12:**266-73.

190. **Takahashi, N., and B. Nyvad.** 2008. Caries ecology revisited: microbial dynamics and the caries process. Caries Res **42:**409-18.

191. **Thibessard, A., F. Borges, A. Fernandez, B. Gintz, B. Decaris, and N. Leblond-Bourget.** 2004. Identification of *Streptococcus thermophilus* CNRZ368 genes involved in defense against superoxide stress. Appl Environ Microbiol **70:**2220-9.

192. **Tong, H., W. Chen, J. Merritt, F. Qi, W. Shi, and X. Dong.** 2007. *Streptococcus oligofermentans* inhibits *Streptococcus mutans* through conversion of lactic acid into inhibitory H2O2: a possible counteroffensive strategy for interspecies competition. Mol Microbiol **63:**872-80.

193. **Tsuda, H., Y. Yamashita, Y. Shibata, Y. Nakano, and T. Koga.** 2002. Genes involved in bacitracin resistance in Streptococcus mutans. Antimicrob Agents Chemother **46:**3756-64.

194. **Ullman, R. F., S. J. Miller, M. J. Strampfer, and B. A. Cunha.** 1988. *Streptococcus mutans* endocarditis: report of three cases and review of the literature. Heart Lung **17:**209-12.

195. **Upton, M., J. R. Tagg, P. Wescombe, and H. F. Jenkinson.** 2001. Intra- and interspecies signaling between *Streptococcus salivarius* and *Streptococcus pyogenes* mediated by SalA and SalA1 lantibiotic peptides. J Bacteriol **183:**3931-8.

196. **Wang, B. Y., and H. K. Kuramitsu.** 2005. Interactions between oral bacteria: inhibition of *Streptococcus mutans* bacteriocin production by *Streptococcus gordonii.* Appl Environ Microbiol **71:**354-62.

197. **Waterhouse, J. C., and R. R. Russell.** 2006. Dispensable genes and foreign DNA in *Streptococcus mutans.* Microbiology **152:**1777-88.

198. **Waterhouse, J. C., D. C. Swan, and R. R. Russell.** 2007. Comparative genome hybridization of *Streptococcus mutans strains.* Oral Microbiol Immunol **22:**103-10.

199. **Webb, A. J., K. A. Homer, and A. H. Hosie.** 2007. Two closely related ABC transporters in *Streptococcus mutans* are involved in di/oligosaccharide uptake. J Bacteriol.

200. **Welin, J., J. C. Wilkins, D. Beighton, and G. Svensater.** 2004. Protein expression by *Streptococcus mutans* during initial stage of biofilm formation. Appl Environ Microbiol **70:**3736-41.

201. **Welin-Neilands, J., and G. Svensater.** 2007. Acid tolerance of biofilm cells of *Streptococcus mutans*. Appl Environ Microbiol **73:**5633-8.

202. **Yamamoto, Y., C. Poyart, P. Trieu-Cuot, G. Lamberet, A. Gruss, and P. Gaudu.** 2005. Respiration metabolism of Group B Streptococcus is activated by environmental haem and quinone and contributes to virulence. Mol Microbiol **56:**525-34.

203. **Yamashita, Y., T. Takehara, and H. K. Kuramitsu.** 1993. Molecular characterization of a *Streptococcus mutans* mutant altered in environmental stress responses. J Bacteriol **175:**6220-8.

204. **Yoshida, A., and H. K. Kuramitsu.** 2002. Multiple *Streptococcus mutans* Genes Are Involved in Biofilm Formation. Appl Environ Microbiol **68:**6283-91.

205. **Yoshida, Y., R. J. Palmer, J. Yang, P. E. Kolenbrander, and J. O. Cisar.** 2006. Streptococcal receptor polysaccharides: recognition molecules for oral biofilm formation. BMC Oral Health **6 Suppl 1:**S12.

206. **Zhang, J., and I. Biswas.** 2009. 3'-Phosphoadenosine-5'-phosphate phosphatase activity is required for superoxide stress tolerance in *Streptococcus mutans*. J Bacteriol **191:**4330-40.

207. **Zhang, L., B. Foxman, D. R. Drake, U. Srinivasan, J. Henderson, B. Olson, C. F. Marrs, J. J. Warren, and M. L. Marazita.** 2009. Comparative whole-genome analysis of *Streptococcus mutans* isolates within and among individuals of different caries status. Oral Microbiol Immunol **24:**197-203.

208. **Zhang, Y., M. Whiteley, J. Kreth, Y. Lei, A. Khammanivong, J. N. Evavold, J. Fan, and M. C. Herzberg.** 2009. The two-component system BfrAB regulates expression of ABC transporters in *Streptococcus gordonii* and Streptococcus sanguinis. Microbiology **155:**165-73.

209. **Zhou, L., X. H. Lei, B. R. Bochner, and B. L. Wanner.** 2003. Phenotype microarray analysis of *Escherichia coli* K-12 mutants with deletions of all two-component systems. J Bacteriol **185:**4956-72.

210. **Zhu, L., J. Kreth, S. E. Cross, J. K. Gimzewski, W. Shi, and F. Qi.** 2006. Functional characterization of cell-wall-associated protein WapA in *Streptococcus mutans*. Microbiology **152:**2395-404.

211. **Zhu, X., F. Long, Y. Chen, S. Knochel, Q. She, and X. Shi.** 2008. A putative ABC transporter is involved in negative regulation of biofilm formation by *Listeria monocytogenes*. Appl Environ Microbiol **74:**7675-83.

212. **Zotta, T., K. Asterinou, R. Rossano, A. Ricciardi, M. Varcamonti, and E. Parente.** 2008. Effect of inactivation of stress response regulators on the growth and survival of *Streptococcus thermophilus* Sfi39. Int J Food Microbiol.

APPENDICES

Table S1: Sensitivity of wild type and mutant *S. mutans* strains to various chemicals

Compound (conc.)	Zone of Inhibition (mm)	
	UA159	IBS A26
Viologen		
Benzyl viologen (50 mg/ml)	0.0	27.0±1.0
Ethyl viologen (1.5 M)	0.0	24.0±1.0
Methyl viologen (1.0 M)	0.0	20.0±2.0
ROS		
Cumene hydroperoxide (10 %)	28.0±2.0	28.0±2.0
Diquat dibromide (500 mg/ml)*	33±2.0	38.0±2.0
Hydrogen peroxide (1.0 %)	22.0±2.0	21.5±0.5
Menadione (0.5 %)	26.0±2.0	24.0±3.0
1,4'-Napthaquinone(0.1 %)	19.0±1.0	20.0±2.0
1,10'-phenanthroline (200 mM)	15.0±1.0	15.0±1.0
Plumbagin (20 mM)	22.0±2.0	21.0±2.0
Pyrogallol (200 mg/ml)	23±1.0	24±2.0
tert-butyl hydroperoxide (70 %)	0.0	0.0
QAC		
Acriflavin (100 mg/ml)	26.0±1.0	31.0±1.0
Benzalkonium chloride (10 mg/ml)	18.0±1.0	18.0±1.0
Crystal violet (10 mg/ml)	12.0±1.0	12.0±1.0
Ethidium bromide (10 mg/ml)	19.0±0.5	24.0±1.0
Hexadecyltrimethylammonium bromide (0.5%)	10.0±0.5	10.0±0.5
Hexadecylpyridinium chloride (1.0 %)	7.0±.05	7.0±0.5
Malachite green (10 mg/ml)	26.0±1.0	26.0±1.0
Pyronin B (5.0 mg/ml)	13.0±1.0	13.0±1.0
Safranin O (15 mg/ml)	10.0±1.0	13.0±1.0
Tetraethylammonium bromide (10 mg/ml)	0.0	0.0

Table: S1 contd…

Compound (conc.)	Zone of Inhibition (mm) UA159	IBS A26
Stressors/Toxic substances		
Benzidine (4 mM)	0.0	0.0
Chlorhexidine gluconate (20 %)	22.0±1.0	23.0±1.0
Diamide (1.0 M)	18.0±1.0	19.0±1.0
Dipyridyl (50 mM)	0.0	0.0
EDTA (0.5 M)	11.0±0.5	11.0±0.5
Mitomycin C1 (1.25 mg/ml)	32.0±2.0	32.0±0.5
Potassium tellurite (1.0 %)	21.0±1.0	21.0±1.0
Puromycin (5.0 mg/ml)	15.0±1.0	15.0±1.0
Reserpine (30 mg/ml)	20.0±1.0	20.0±1.0
Streptonigrin (5.0 mg/ml)	30.0±2.0	30.0±2.0
Antimicrobials[$]		
Amidinocillin (10 mcg) [$]	19.0±1.0	19.0±1.0
Bacitracin (10 units) [$]	10.0±1.0	10.0±0.5
Chlomphenicol (5 mcg) [$]	12.0±1.0	11.5±1.5
Ciprofloxaxin (5 mcg) [$]	13.5±2.0	13.0±1.0
Clindamycin (2 mcg) [$]	22.0±1.0	22.0±1.0
Erythromycin (50 mg/ml)	31.0±1.0	31.0±1.0
Kanamycin (100 mg/ml)	18.0±1.0	18.0±1.0
Levofloxaxin (5 mcg) [$]	15.0±1.0	15.0±2.0
Nalidixic acid (30 mcg) [$]	0.0	0.0
Rifampin (5 mcg) [$]	28.0±2.0	27.0±1.0
Trimethoprime (5 mcg) [$]	13.0±0.5	12.5±1.0
Vancomycin (5 mcg) [$]	15.0±0.5	15.0±1.0

$ per disk
* See text for details

Table S2: Putative ABC Transporters in *Streptococcus mutans* UA159

PROTEIN ABC	Membrane	Binding protein	PUTATIVE SUBSTRATE
SMU.1006	SMU.1007**		Bacitracin
	SMU.28*		Unknown
	SMU.1293		Fe-S assembly/SufBCD system
SMU.247	SMU.248		Fe-S assembly/SufBCD system
	SMU.251		Fe-S assembly/SufBCD system
SMU.1431			Uup homolog/duplicated ATPase
SMU.2159			Uup homolog/duplicated ATPase
SMU.803			Uup homolog/duplicated ATPase
SMU.902			Uup homolog/duplicated ATPase
SMU.1178	SMU.1179	SMU.1177	Amino acid
	SMU.1216**	SMU.1217	Amino acid
	SMU.1941		
	SMU.1942		Amino acid
SMU.461	SMU.460**	SMU.459	Amino acid
	SMU.1347		
	SMU.1348*		Amino acid
	SMU.863*		
	SMU.864*		Antimicrobial peptide
SMU.1148			Bacitracin
SMU.1811			Bacitracin
SMU.1035			Bacitracin
SMU.1445	SMU.1446	SMU.1447	Branched-chain amino acid
SMU.1665	SMU.1667		
SMU.1666	SMU.1668	SMU.1669	Branched-chain amino acid
SMU.1324			
SMU.1325			Cell division
SMU.1934			
SMU.1933			Cobalt ion
SMU.2149	SMU.2148		Cobalt ion
SMU.2150			
SMU.1939	SMU.1938		D-methionine
	SMU.1966		D-ribose

Table S2: cont.

PROTEIN ABC	Membrane	Binding protein	PUTATIVE SUBSTRATE
	SMU.817**	SMU.815	Glutamate/arginine
SMU.1519	SMU.1521** SMU.1522**	SMU.1520**	Glutamine
SMU.241c	SMU.242**		Glutamine
SMU.568	SMU.567**		Glutamine
SMU.805c	SMU.806		Glutamine
SMU.1095 SMU.1096			Glycine betaine/carnitine/choline
SMU.2116	SMU.2117 SMU.2119	SMU.2118	Glycine betaine/carnitine/choline
SMU.1063	SMU.1062		Glycine betaine/L-proline
SMU.936	SMU.934 SMU.935	SMU.933	Histidine/arginine/ornithine
SMU.997	SMU.995 SMU.996	SMU.998	Iron (III)
SMU.654			Lantibiotic export
	SMU.1121		Lipoprotein releasing
SMU.1167c	SMU.1166*		Lipoprotein releasing
	SMU.1365? SMU.1366*		Lipoprotein releasing
	SMU.1412*		Lipoprotein releasing
SMU.1927	SMU.1928		Lipoprotein
SMU.1571	SMU.1569 SMU.1570	SMU.1568	Maltose/maltodextrin
SMU.182	SMU.183	SMU.184	Manganese ion
SMU.1695			Molybdenum
SMU.1068	SMU.1067		Multidrug efflux
	SMU.1163* SMU.1164*		Multidrug efflux
SMU.1194	SMU.1195		Multidrug efflux
SMU.1551c	SMU.1550c*		Multidrug efflux
SMU.238c	SMU.237c		Multidrug efflux
SMU.370	SMU.371		Multidrug efflux
SMU.413	SMU.414		Multidrug efflux

Table S2: cont.

PROTEIN ABC	Membrane	Binding protein	PUTATIVE SUBSTRATE
	SMU.432*		Multidrug efflux
	SMU.524* SMU.525*		Multidrug efflux
SMU.731			Multidrug efflux
	SMU.1078c* SMU.1079c*		Multidrug/protein/lipid
	SMU.905* SMU.906*		Multidrug/protein/lipid
	SMU.922* SMU.923*		Multidrug/protein/lipid
SMU.652c	SMU.653c	SMU.651c	Nitrate/sulfonate/taurine
SMU.258 SMU.259	SMU.256 SMU.257	SMU.255	Oligopeptide
SMU.1134 SMU.1135	SMU.1136 SMU.1137	SMU.1138	Phosphate ion
SMU.828	SMU.827		Polysaccharide
	SMU.1093* SMU.1094*		Polysaccharide/polyol phosphate
SMU.1120	SMU.1118 SMU.1119		Ribose/galactose
SMU.973	SMU.974 SMU.975	SMU.976	Spermidine/putrescine
	SMU.1963c		Sugar
SMU.882	SMU.879 SMU.880	SMU.878	Sugar
SMU.1881			Peptide secretion
SMU.1897	SMU.1898* SMU.1899*		Peptide secretion
SMU.286			Peptide secretion
	SMU.1302		Zinc ion
SMU.1994	SMU.1993		Zinc ion/manganese ion

* contains both an ABC and a membrane domain as one polypeptide

** contains both a membrane domain and a binding protein domain as one polypeptide

Derived from www.membranetransport.org