2013

SYSTEMATIC ANALYSIS OF ABC TRANSPORTERS IN STREPTOCOCCUS SANGUINIS

Sawsan Atia
Virginia Commonwealth University

Follow this and additional works at: https://scholarscompass.vcu.edu/etd
Part of the Medicine and Health Sciences Commons

© The Author

Downloaded from
https://scholarscompass.vcu.edu/etd/3054
SYSTEMATIC ANALYSIS OF ABC TRANSPORTERS IN
STREPTOCOCCUS SANGUINIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

By
SAWSAN SALMAN ATIA MUBARAK
M.S., Baghdad University, Iraq, 2000
B.S., Baghdad University, Iraq, 1991

Director: DR. PING XU
Associate Professor of Oral & Craniofacial Molecular Biology and Microbiology & Immunology

Virginia Commonwealth University
Richmond Virginia
April 2013
Acknowledgements

It is a great pleasure to give respect to those who made this thesis possible. I owe sincere thankfulness to my research advisor, Ping Xu, who allowed me the opportunity to work on a project and guided me throughout my journey in lab. I would also like to show my gratitude to all my colleagues in the lab: Dr. Xiuchun Ge, Dr. Lei Chen, Victoria Stone, Alleson Dobson, Tara Jeneille Nulton, Karra Evens, Gregory Mayes, and Jenishkumar Patel, for all of their assistance and encouragement with this project. I would especially like to thank Victoria for her help and moral support, understanding and encouragement I felt when working on my project. I would like to thank Dr. Greg Buck and Dr. Daniel Conrad with their lab members for their assistance and for allowing me to use their spectrofluorescence reader for a long time.

I am sure that my journey would not have been possible without family support that always believed in me. I could not also have accomplished this project without their love, prayers and continuous support. I would especially like to thank my mother for encouraging and inspiring me to continue my education in the sciences.

I would like to show my gratitude and my respect to the J. William Fulbright Foreign Scholarship Board (FSB) for awarding me this grant. My selection for a Fulbright award is in itself, an achievement for which I can be proud. Lastly, I would like to express my gratitude to Virginia Commonwealth University for supporting and assisting me to complete my degree.
# Table of Contents

List of Tables...............................................................................................................III

List of Figures............................................................................................................IV

List of abbreviations.................................................................................................V

Abstract.....................................................................................................................VI

1. Introduction...........................................................................................................1

2. Materials and Methods.......................................................................................14

   Bacterial strains, media, and growth conditions.................................................25

   Determination of pH range for *S. sanguinis* growth.........................................25

   Acid sensitivity assay.........................................................................................26

   Carbohydrate fermentation assay......................................................................26

   Growth on carbohydrate substrates..................................................................27

   Examination of intracellular pH by fluorescence probe.................................27

   Toxicity of BCECF.............................................................................................28

3. Results..................................................................................................................29

4. Discussion............................................................................................................62

5. References............................................................................................................71
List of Tables

Page

Table 1: Bacterial strains and ABC mutant genes used in this study..................................15

Table 2: Carbon source utilization by S. sanguinis and eight ABC transporter mutants.......40
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>The structure of ABC transporters</td>
<td>8</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Construction of single gene deletion mutants into SK36 chromosome</td>
<td>23</td>
</tr>
<tr>
<td>Figure 3</td>
<td>The growth rate characteristics for wild type and control strains</td>
<td>30</td>
</tr>
<tr>
<td>Figure 4</td>
<td>The growth rate characteristics of selected ABC transporter mutants</td>
<td>33</td>
</tr>
<tr>
<td>Figure 5</td>
<td>The gene locus encoding the acid sensitive mutants</td>
<td>35</td>
</tr>
<tr>
<td>Figure 6</td>
<td>The growth rate characteristics for the rest of the mutants in the operon</td>
<td>37</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Growth profiles of acid sensitive ABC transporter mutants on different carbon sources</td>
<td>42</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Growth profile of the neighboring gene mutants in the operon on different carbon sources</td>
<td>46</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Comparison of intracellular pH and extracellular pH of <em>S. sanguinis</em></td>
<td>55</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Comparison of the intracellular pH of the two mutants</td>
<td>57</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Statistical comparison of intracellular pH for the wild type and two mutants</td>
<td>60</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

% Percentage

ABC ATP-binding cassette

BHI Brain heart infusion

BLAST Basic local alignment search tool

CFU colony forming unit

DNA deoxyribonucleic acid

hr hour

min minute

ml milliliter

O. D. Optical density

O/N overnight

°C degress Celsius

PBS phosphate buffered saline

PCR polymerase chain reaction

TCS Two Component System

Wt/Vol Weight/ volume

µg microgram

µl microliter
ABSTRACT

SYSTEMATIC ANALYSIS OF ABC TRANSPORTERS IN
STREPTOCOCCUS SANGUINIS

By Sawsan S. Atia, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University.

Virginia Commonwealth University,
April 2013

Major Director: Dr. Ping Xu
Associate Professor of Oral & Craniofacial Molecular Biology and
Microbiology & Immunology

The bacterium *Streptococcus sanguinis* is a primary member of the human oral
microflora and also has been recognized as a key player in the bacterial colonization of the
mouth. It is considered the most common viridians streptococcal species implicated in infective
endocarditis. In all kingdoms of life, ATP binding cassette (ABC) transporters are essential to
many cellular functions. Sequencing of the SK36 genome provided the opportunity to study
ABC transporter mutants and their relationship with acidity of the oral environment. Despite
numerous studies that have focused on carbohydrate uptake systems in closely related
streptococcal species such as *S. mutans*, *S. pneumonia* and *S. pyogenes*, the mechanism of the
response of these ABC transporters to acidic conditions in *S. sanguinis* is still unknown. The capability of *S. sanguinis* to adapt in these harsh environments suggests this bacterium is capable of responding to various environmental stimuli. The purpose of this study was to examine ABC mutants to identify functions that contribute to acid tolerance in *S. sanguinis*.

This study demonstrates that two acid-sensitive mutant genes, SSA_1507 and SSA_1508, identify genes involved in acid tolerance. The two mutants grew on different sugars and none of them showed a defect in sugar utilization at acid pH. We couldn’t recognize any significant differences in sugar uptake for the two acid sensitive mutants or in mutants of their neighboring genes. Thus, the observed acid sensitivity is not due to a failure to take up any of the common sugars tested. The cytoplasmic pH of *S. sanguinis* was studied with the fluorescent pH indicator (BCECF) and SK36 was observed to have a wider pH range than either of the two acid-sensitive mutants SSA_1507 or SSA_1508. In these two mutants, intracellular pH was not as well maintained. At all pH values tested, the mutants displayed a lower intracellular pH than the wild type. These observations indicate that the cell membrane of these two mutants is unable to protect the interior components from adverse effects of higher pH values and lower pH values, and prove that these two mutant genes SSA_1507 and SSA_1508 are unable to grow in lower pH values. These results support a role for these ABC transporters in proton pump or export and indicate that the mutants are less able to pump out protons.
1. Introduction

*Streptococcus sanguinis* is a gram-positive facultative anaerobe bacterium and is one of the pioneer colonizers of the human oral cavity (1, 2). In most cases, these oral streptococci produce green pigment or alpha-hemolysis on blood agar and are often considered as one of the viridans group streptococci that have the ability to oxidize hemoglobin in erythrocytes by secretion of H$_2$O$_2$ (2,3,4). The viridans streptococci are the most common cause of native-valve infective endocarditis. Among those bacteria isolated from patients with endocarditis, *S. bovis, S. mutans* and *S. sanguinis* are the most common (1, 5).

In the oral streptococci, the mechanisms by which different sugars are being transported are of specific interest because of the contribution of fermentable carbohydrates to the acid in dental plaque. This process produces enamel demineralization and can result in dental caries. Many studies have noted that bacterial cells use a variety of different mechanisms for the uptake of sugars, and cells have the capacity to use various carbohydrates as carbon and energy sources employing various transport proteins and catabolic enzymes for the metabolism of the different carbohydrates. A specific species may exhibit several different processes acting on the same or different sugars (6, 7, 8 ). Earlier studies have shown that viridans streptococci, such as *S. gordonii, S. mitis* and *S. sanguinis*, and some rods such as *Actinomyces* spp., serve as precursor bacteria that initiate the formation of dental plaque. These early colonizers generate a scaffold for the extension of secondary colonizers that give rise to a diverse biofilm community (9). These viridans streptococci, referred to as the non-mutans streptococci, are distinct from the mutans streptococci (MS).

The streptococci have been forced to develop different mechanisms to cope with the stress induced by declining pH values in their environment. One of streptococci species is *S. mutans*. *S. mutans* is considered to be the most important pathogen in dental caries. An important
mechanism in its cariogenicity is the ability to tolerate acid stress and the production of acid from carbohydrates. While *S. sanguinis* compete with *S. mutans* for survival in the oral cavity, *S. mutans* is more acid resistant than *S. sanguinis*. Since acid tolerance contributes to the ability of *S. sanguinis* cells to adapt and to compete with *S. mutans* in the plaque environment, we were interested in the role of ABC transporters in responding to environmental stimuli and in finding more acid tolerant mutants than the wild type strain in order to antagonize *S. mutans* and reduce caries.

**Acid Tolerance:**

Adaptation of oral bacteria to low pH is a strategy employed by streptococcal species to resist the inimical influences of acidification of the human mouth. Many researchers have suggested that oral streptococci retain several acid adaptive strategies, some of which are distributed among all of the species and some that are unique. In order to exist in the oral cavity, bacterial cells must be able to tolerate rapid and large environmental fluctuation in pH, nutrient source and availability (10). Also, there are some protective mechanisms that appear to be specific to certain species, such as ammonia production from urea by *S. salivarius* and arginine by *S. sanguinis*, *S. gordonii* and *S. rattus* (10). Additionally, malolactic fermentation (MLF) was recently identified as a major system for alkali production by *Streptococcus mutans* (11).

Other studies have identified another protective mechanism which is called the acid tolerance response (ATR). It has been proposed that the survival rate of bacteria is increased after challenge at lethal acid pH if the bacteria were first subjected to a period of mild sublethal acidic conditions (12, 13, 14, 15). It was noticed in these studies that the range of pH and lethal
pH that trigger the ATR are characteristic for each species and are indicative of their acid resistance capacity. It has been suggested that the F0 F1 ATPase is one of the systems used by bacteria to respond to acidic pH. ATP is hydrolyzed to generate a proton gradient for a variety of transport processes and adjusts the intracellular pH via proton extrusion. In other studies dealing with the regulation of *S. mutans* acid tolerance by GcrR, a response regulator, it was proposed that a drop in dental plaque pH stimulates an adaptive acid-tolerance response in *S. mutans* that allows it to survive acid challenge at pH 3.0. These studies also concluded that GcrR plays a vital role in *S. mutans* colonization of the tooth surface and later on pathogenesis of this bacterium, which is considered a primary causative agent of human dental caries (16, 17). In many streptococci species, the intracellular pH varies in response to extracellular pH, with the organisms working to maintain a ΔpH of 0.5 to 1 unit above the external environment. For instance, in the highly acid-tolerant *S. mutans*, the optimal pH for the F-ATPase enzyme is 6.0, while in the lesser acid tolerant *S. salivarius* and *S. sanguinis*, the pH optima of the ATPase enzymes are 7.0 and 7.5, respectively (9, 18, 19).

The bacterial cells of non-mutans streptococci (*Streptococcus sanguis* [now *S. sanguinis*, *Streptococcus gordonii*, *Streptococcus oralis* and *Streptococcus mitis*) that grown at pH 7.0 showed 71% viability after acidification at pH 4.0 for 60 min, while the cells of mutans streptococci (*S. mutans*) were not killed by acidification (20). It has also been noticed that non-mutans bacteria were capable of increasing their acid tolerance and acidogenicity in response to environmental acidification and moreover, it has been proposed that one of mechanisms in the acid adaptation observed in non-mutans streptococci could involve the induction of H⁺ -ATPase. H⁺ ATPase is recognized to maintain intracellular pH suitable for metabolic reactions by expelling protons across the cell membrane against environmental acidification. Other
mechanisms in acid adaptation could also include arginine deiminase or synthesis of stress proteins, as have been observed in non-mutans streptococci. Ultimately, these adaptive responses were similar to those found in the strains of mutans streptococci and Enterococcus hirae, although the cells of non-mutans streptococci were less acidogenic and acid-tolerant than mutans streptococci.

**Systems of sugar uptake transporters**

Earlier studies identified a high number of carbohydrate transport systems, which are generally in the ATP-binding cassette (ABC) superfamily or the phosphotransfer-driven group translocators (PTS, Phosphoenolpyruvate: sugar phosphotransferase system) (21, 22, 23). Previous studies have shown that the PTS system is composed of four proteins, which transfer a phosphoryl group from PEP to an entering sugar parallel with membrane translocation (24, 25). They have also noted that utilization of ABC transporter carbohydrates requires more energy than those that are substrates for PTS, both for transportation and for modification of carbohydrates once they are intracellular. Although PTS substrates are known to be size-restricted, the ABC transporters are able to import longer and more varied carbohydrate linkages. The same studies demonstrated that growth on eight carbohydrates *in vitro* is affected by mutation of more than one transporter system (24, 25).

It is generally agreed that ABC importers and PTS are particularly attractive drug targets, since both mechanisms are unique to prokaryotes. Also, it has been proposed that PTS and ABC importers might be used as novel drug delivery systems. Many publications suggest that ABC sugar transporters are necessary for full virulence for most bacteria— for example, Streptococcus pneumoniae in mouse models of infection (26, 27, 28). However, their influence might be
defeated by the significant potential for redundancy in sugar uptake. Additionally, *S. mutans* also has a non-PTS sugar uptake system involving a multiple sugar metabolism (msm) operon. This operon is responsible for the uptake and metabolism of a variety of sugars (such as melibiose, raffinose, and isomaltosaccharides) and is unique because it is the first binding protein-dependent sugar uptake system described to be involved in the uptake and metabolism of multiple sugars. In addition, it represents the first model of this kind of transporter in Gram-positive bacteria (29).

It is crucial to mention that the profusion of sugar transport systems places the streptococci, together with clostridia and enterococci, among the species with the highest numbers of carbohydrate uptake systems (30). In addition to the systems have been mentions above, sugar substrates can also be taken up by galactoside-pentose hexuronide (GPH) translocators. Typically, GPH translocators are electrochemical transporters; they catalyze the uptake of sugars in symport with monovalent cations (H\(^+\), Na\(^+\)). This system is also involved in the regulation of a variety of metabolic and transcriptional processes (31). It also has been determined that galactose is not transported through the PTS transport system in *S. mutans*. This sugar might be transported via an ABC or a GPH transporter.

**ABC transporters:**

In all kingdoms of life, ATP binding cassette (ABC) transporters are essential to many cellular functions. It is believed that the ABC transporter superfamily is more prevalent in bacteria than other types of transporters. Its structure comprises one of the largest protein group families and displays a shared global organization (31, 32, 33, 34). The common feature of all
ABC transporters is that they consist of two distinct domains, a nucleotide binding domain that binds ATP, and a transmembrane domain (TMD). The alpha-helical TMD is embedded in the membrane, where it recognizes various substrates and transports them across the membrane. This domain has variable sequence and structure, depending on the substrate to be translocated. The ATP binding cassette (ABC domain) is located on the cytoplasmic side of the membrane and is highly conserved. Most exporters consist of dimers of polypeptides in which the TMD and ABC domains are fused as a single polypeptide chain (Figure 1). Additional features of ABC transporters may be present, depending on whether the transporter functions as an importer or an exporter. Importers have an extra-cytoplasmic solute-binding receptor that can be a lipoprotein or oligoprotein assembly anchored to the external surface of the cytoplasmic membrane that interacts with a specific substrate molecule in the periplasm for delivery to the appropriate ABC transporter. This provides specificity and maintains the direction of transport into the cell (35). Exporters, on the other hand, generally have an extra intracellular domain that connects the membrane-spanning domain to the ABC domain. These protein subunits combine in order to form an active ABC transporter. Studies in *S. mutans* based on the genome sequence identified sixty apparent ABC transporter systems, but only three of these are involved in carbohydrate uptake (32). In contrast, there are more than 150 genes related to ABC transporters in *S. sanguinis*. This means a wider range of substrates might be transported through them and potentially gives them the ability to withstand various stress conditions.

It has been noted that ABC transporters are the only class of transporters with specificity for larger substrates like oligosaccharides, and they also display higher expression under several tested conditions. This suggests that they might be involved in transporting multiple substrates. The availability of bioinformatics genome data allowed demonstration that these transport
systems and their operons were components of the adaptable part of the bacterial genome (21). These data also revealed that bacterial carbohydrate uptake operons are functional units and include genes for glycosyl-hydrolases for generation of mono- or disaccharides in addition to the genes for their transporters. These enzymes are useful for the steps of metabolism linking the sugar to glycolysis and a regulator. It has been proposed that these bacteria that exploit ATP as a sole energy source have ATP pumps but lack channels and electrochemical driven transport. It has also been noted that genes for both PTS systems and ABC transporters are high in terms of the genome size and that there is a significant effect of many of these carbohydrate uptake systems in virulence. These systems have been shown to have a role in bacterial physiology during carriage and the regulation of invasive disease (21).

Transporters involved in carbohydrate uptake within the ABC superfamily can be divided into two families, the carbohydrate uptake transporter-1 and -2 (CUT1 and CUT2) families. While CUT1 systems are largely concerned with oligosaccharide uptake, CUT2 systems frequently reveal specificity for monosaccharides (such as ribose, galactose, xylose, and rhamnose) and ribonucleosides that contain 2-deoxyribonucleosides, but not ribose or nucleobases. In the high G+C Gram-positive bacteria, most of the sugar transporters are either secondary carriers (48%) or ABC-type transporters (50%). An ABC transporter that contributes to the uptake of ribonucleosides has also been described, thus extending the range of substrates transported by members of the ABC transporter superfamily (36, 35).
Figure 1: The structure of ABC transporters. Shown is the structure of a typical ABC importer, BtuCD, in complex with its periplasmic binding protein BtuF (PDB 2QJ9). The gray box indicates the cytoplasmic membrane. The Transmembrane domain (TMD), Nucleotide-binding domain (ABC) and periplasmic binding protein are indicated.
Figure 1.
Further details of the physiological roles of bacterial ABC transporters can be found in recent reviews. It is proposed that ABC transporters have a varied range of functions that are essential in response to the environment. First, they import a variety of substrates, including sugars, amino acids, peptides, metal ions, sulfate, iron, and molybdate. Additionally, they are responsible for the targeted export of other substrates across the cytoplasmic membrane, for the production of antibiotics in some antibiotic-producing bacteria, and for the export of toxins (33). It has been proposed that bacterial species that live in different environments and need to adapt to diverse conditions might require numerous ABC systems. ABC transporters are attractive targets for antimicrobial approaches, and components of the transporters have been proposed as appropriate targets for mutation in order to develop live attenuated antibacterial vaccines. Thus, a wide-ranging characterization of those transporters in any species can afford a valuable insight into the standard of living of an organism. On the other hand, different approaches are exploiting ABC transporters as a system for the carriage of antimicrobials into the cell rather than as the target for antimicrobial compounds.

**Maintenance of the intracellular pH of bacterial cells:**

The capacity of oral bacteria to endure acidic environments is of critical importance in the ecology of dental plaque and in the pathogenesis process of dental caries (37, 38, 39, 40). It is necessary to investigate the ability of *S. sanguinis* to grow, survive and maintain their intracellular pH when subjected to the acidic environment in the absence of an extracellular energy source.

One of the systems that might be involved in response to acidity is the H\_glucose symport system. This system could be the initial transporter for glucose at low pH. Although the role of
ABC transporters in the response to acidity is not established yet. ABC transporters are proposed to be energetic for high-affinity and high-capacity transport of carbohydrates and a key contributor to acidogenesis (37, 41, 42). Work on a wide range of microorganisms has led to a number of conclusions about bacterial pH homeostasis. For example, there is no magic value of intracellular pH which all organisms must achieve, organisms exhibit different abilities to regulate their cytoplasmic pH, and anaerobic organisms exhibit a greater range of value of intracellular pH over which growth will occur than do aerobic organisms. For neither class of these organisms do we have reasonable description of the mechanism of control of cytoplasmic pH (43, 44, 45). Streptococci metabolize dietary sugars to create ATP and yield organic acids and then maintain an intracellular pH by through a proton-translocating ATPase. Acidic extracellular pH moderates the physiological activity of oral microorganisms, but in the absence of dietary sugars between meals the relationship between intracellular pH and extracellular pH is unclear (46).

A variety of methods have been developed to measure intracellular pH in bacteria. These include nuclear magnetic resonance techniques, pH-sensitive fluorescent probes, and analysis of the distribution of (radiolabeled) weak acids or weak bases. Another method that has been introduced to allow fast and noninvasive determination of intracellular pH is suited for direct analysis of individual bacterial cells present in a complex environment. Noninvasive measurement of intracellular pH on a single-cell level with green fluorescent protein was used to measure intracellular pH in both Gram-positive and Gram-negative bacterial cells (47). This has been done by introducing specific amino acid substitutions to the chromophore, causing the resulting protein to alter its excitation spectrum according to the pH of the surrounding environment.
In other studies, efflux of the pH-sensitive fluorescent dye 2′, 7′-bis-(2-carboxyethyl)-5 (and-6) carboxyfluorescein (BCECF) has been used to measure intracellular pH in eukaryotic and bacterial cells (46). This highly sensitive probe is introduced into the bacteria using the membrane-permeable acetoxymethyl ester BCECF-AM. In streptococcal cells, it was found that in the absence of glucose, the background fluorescence of *S. mutans*, *S. sanguinis*, *S. salivarius* and *S. sobrinus* was low after incubation with BCECF-AM for 20 min. at 35 degrees Celsius (°C) (46). This raised the possibility of using BCECF for determination of the intracellular pH of oral streptococci strains. The intracellular pH of the bacteria is determined by creating a relationship between the fluorescence ratio 505/450 and pH using the ionophore nigericin (48, 49, 50, 51).

**Purpose of Study**

ABC transporter function might affect the lifestyle of *S. sanguinis* by increasing the capacity of this organism to live in the acidity of oral cavity. ABC transporters have previously been observed to affect virulence and might affect the degree of pathogenesis of *S. sanguinis*. Previous work in our lab investigated the roles of two-component systems (TCS) in acid stress. TCS serve as a response coupling mechanism that allows organisms to sense a wide range of signals and respond to stress conditions. It has been demonstrated previously that mutants affecting two response regulators in *S. sanguinis*, SSA_0204 and SSA_0217, are more resistant than wild type to acid stress. Microarray analysis of the genes affected by the SSX_0204 mutation showed that gene SSA_0260 was regulated by this response regulator and plays a role in acid tolerance (52). The SSA_0260 gene product is annotated as an ABC type metal transporter. This led to an interest in a comprehensive survey of all ABC transporter genes in *S. sanguinis* and determining whether there was a relationship with the acid tolerance.
The complete genome sequence of *S. sanguinis* SK36 strain was determined at VCU (4). The genome sequence provides an opportunity to greatly advance our understanding of this organism by enabling the construction of a comprehensive set of genome-wide mutants. The genome encodes 2,274 predicted proteins, sixty-one tRNAs, and four rRNA operons, from which one hundred and fifty genes have been identified that encode putative ABC transporters (4). Each of the genes was systematically inactivated and replaced by an antibiotic resistance gene cassette, and all of these mutants were confirmed by sequencing. This comprehensive mutant library provides a unique resource to perform a systematic analysis in order to investigate the effect of genetic mutations on ABC transporter function and the behavior of *S. sanguinis* SK36 in the acid environment.

This study consisted of the screening of 150 ABC transporter mutants of *S. sanguinis* SK36 for acid tolerance. Growth of the mutants was assessed along with the different types of carbon sources that might be related to acid tolerance. Determination of intracellular pH in *S. sanguinis* mutants that were found to be involved or correlated with acid tolerance in *S. sanguinis* was also examined.
2. Materials and Methods

Bacterial strains and mutants

SK36 used in this study was isolated from human dental plaque (53) and originally acquired from Drs. Kilian & Holmgren of Arhus University, Denmark. Single gene deletion mutants for the SK36 strain of *S. sanguinis* were created previously using a PCR-based recombinant method employing linear DNA for gene deletion *in vitro* (4). For each target gene three sets of primers (F1/R1, F2/R2, and F3/R3) were designed to amplify a linear DNA fragment containing a kanamycin resistance cassette (54) flanked by upstream and downstream sequences of the targeted gene (Figure 2). The 5’ ends of the F2 and R2 primers of the kanamycin cassette were designed to complement the sequences of DNA that flank the target gene. The linear recombinant PCR amplicons containing the kanamycin cassette flanked by *S. sanguinis* DNA were introduced into competent *S. sanguinis* cells by transformation and integrated into the *S. sanguinis* genome via double crossover recombination (1). A genome-wide mutant library containing 2,048 deletion mutants was constructed using a 96 well high-throughput format (4, 7). Genes of SK36 are designated as “SSA” followed by the corresponding gene number while mutants are indicated by “SSX” followed by the gene number. The 150 mutants in genes identified as ABC transporters investigated in this study are shown in Table 1, along with control strains.
Table 1. Bacterial Strains Used in This Study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Putative function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. sanguinis</em> SK36</td>
<td>Human dental plaque isolate</td>
<td>(2,3)</td>
</tr>
<tr>
<td>Non-ABC transporter mutants</td>
<td></td>
<td>(4,7)</td>
</tr>
<tr>
<td>SSX_0260</td>
<td>Manganese/Zinc ABC transporter</td>
<td></td>
</tr>
<tr>
<td>SSX_0204</td>
<td>Nisin biosynthesis two-component response transcriptional regulator nisR</td>
<td></td>
</tr>
<tr>
<td>SSX_1506</td>
<td>Lipopolysaccharide biosynthesis protein</td>
<td></td>
</tr>
<tr>
<td>SSX_1509</td>
<td>Polysaccharide biosynthesis protein</td>
<td></td>
</tr>
<tr>
<td>SSX_1510</td>
<td>Rhamnosesyltransferase</td>
<td></td>
</tr>
<tr>
<td>SSX_1511</td>
<td>Glycosyltransferase</td>
<td></td>
</tr>
<tr>
<td>SSX_1512</td>
<td>Predicted membrane protein</td>
<td></td>
</tr>
<tr>
<td>SSX_1513</td>
<td>Glycosyltransferase</td>
<td></td>
</tr>
<tr>
<td>SSX_1514</td>
<td>Glycosyltransferase</td>
<td></td>
</tr>
<tr>
<td>ABC transporter mutants</td>
<td></td>
<td>(4, 7)</td>
</tr>
</tbody>
</table>

SSX_0072   ABC sugar transporter, MsmK-like protein
SSX_0074   ABC transporter substrate-binding protein-sugar transport
SSX_0075  ABC sugar transporter, permease protein
SSX_0076  ABC sugar transporter, permease protein
SSX_0136  ABC transporter, Zn porter
SSX_0137  ABC transporter (permease), Zn porter
SSX_0148  Sugar ABC transporter, ATP-binding protein
SSX_0201  ABC multidrug transporter (3-component subtilin immunity exporter)
SSX_0261  ABC-type Mn2+/Zn2+ transport systems, permease component
SSX_0262  ABC-type Mn/Zn transporter, ATP-ase component
SSX_0375  D-methionine-binding lipoprotein (ABC-type transporter)
SSX_0376  ABC-type methionine transporter, ATPase component
SSX_0377  ABC-type methionine transporter, permease component
SSX_0385  ABC transporter, glycine-betaine/proline permease protein
SSX_0386  Glycine-betaine ABC transporter, ATPase component
SSX_0393  Bacteriocin ABC-type exporter, ATP binding/permease protein
SSX_0407  ABC-type multidrug transport system (3-component subtilin immunity exporter)
SSX_0409  ABC-type multidrug transport system, ATPase component
SSX_0412  ABC-type multidrug transport system (3-component subtilin immunity exporter)
SSX_0422  ABC-type multidrug transport system, ATPase component
SSX_0443  Possible ABC-type efflux pump permease component
SSX_0461  ABC-type multidrug transport system, ATPase and permease components
SSX_0462  ABC-type multidrug transport system (phospholipid, LPS, lipid A and drug exporter)
SSX_0480  Cobalt ABC transporter, ATP-binding protein, putative
SSX_0493  ABC-type dipeptide/nickel transport system, periplasmic component
SSX_0494 ABC-type dipeptide/oligopeptide/nickel transport system, ATPase component
SSX_0495 ABC-type oligopeptide/nickel transport system, ATPase component
SSX_0497 Nickel ABC transporter
SSX_0498 ABC-type dipeptide/oligopeptide/nickel transport systems, permease components
SSX_0499 ABC-type dipeptide transport system, periplasmic component
SSX_0500 Peptide ABC transporter, permease protein
SSX_0502 Peptide ABC transporter, permease protein
SSX_0503 Peptide ABC transporter, ATP-binding protein
SSX_0504 Peptide ABC transporter, ATP-binding protein
SSX_0588 L-cystine ABC transporter, substrate-binding component
SSX_0602 ABC-type cobalt transport system, ATPase component
SSX_0606 ABC-type antimicrobial peptide transport system, ATPase component
SSX_0607 Probable ABC transporter, permease component
SSX_0724 ABC-type multidrug/protein/lipid transport system (pediocin PA-1 exporter), ATPase
SSX_0796 ABC-NBD transporters with duplicated ATPase domains
SSX_0798 ABC transporter membrane-spanning permease-macrolide efflux
SSX_0845 ABC-type multidrug transport system, ATPase component
SSX_0908 ABC-type uncharacterized transport system, periplasmic component
SSX_0910 ABC-type multidrug transporter, ATPase component
SSX_0924 ABC-type antimicrobial peptide transport system, permease component
SSX_0925 ABC-type antimicrobial peptide transport system, ATPase component
SSX_0928 ABC-type multidrug transporter, ATPase and permease components
SSX_0929 ABC-type multidrug transporter, ATPase and permease components
SSX_0941  ABC-type phosphate transport system, periplasmic component
SSX_0942  ABC transporter membrane-spanning permease-phosphate transport
SSX_0943  ABC transporter membrane-spanning permease-phosphate transport
SSX_0987  ABC-type choline transporter, membrane-spanning permease
SSX_1003  ABC transporter substrate-binding protein-multiple sugars
SSX_1004  ABC transporter membrane-spanning permease-multiple sugars
SSX_1005  Sugar ABC transporter, permease protein
SSX_1007  ABC transporter ATP-binding protein-multiple sugar transport
SSX_1026  ABC-type multidrug transporter, ATPase component
SSX_1039  Sugar ABC transporter, ATP-binding protein
SSX_1040  Sugar ABC transporter, permease protein
SSX_1041  Sugar ABC transporter, permease protein
SSX_1048  ABC transporter ATP-binding protein-spermidine/putrescine transport
SSX_1049  ABC transporter membrane-spanning permease-spermidine/putrescine transport
SSX_1050  ABC transporter membrane-spanning permease-spermidine/putrescine transport
SSX_1051  Spermidine/putrescine ABC transporter, spermidine/putrescine-binding protein
SSX_1066  ABC-type oligopeptide transport system
SSX_1087  ABC-type transporter (antibiotic resistance protein), ATPase component
SSX_1109  ABC transporter ATP binding/permease protein
SSX_1298  Putative maltose/maltodextrin ABC transporter, sugar-binding protein MalX
SSX_1299  Putative maltose/maltodextrin ABC transport system
SSX_1300  Maltose ABC transporter, permease protein
SSX_1340  Zn/Mn ABC-type porter lipoprotein
SSX_1359  Arginine/histidine ABC transporter, permease component
SSX_1360  Arginine/histidine ABC transporter, ATPase component
SSX_1373  ATPase components of ABC transporters with duplicated ATPase domains
SSX_1374  Multidrug ABC transporter, ATPase and permease components
SSX_1375  Multidrug ABC transporter, ATPase and permease components
SSX_1402  ABC-type multidrug transport system, ATPase and permease components, highly conserved
SSX_1403  Multidrug ABC transporter, ATPase and permease components
SSX_1507  ABC-type lipopolysaccharide transport system, ATPase component
SSX_1508  ABC-type lipopolysaccharide transport system, permease component
SSX_1530  ABC-type antimicrobial peptide transport system, permease component
SSX_1531  ABC-type antimicrobial peptide transporter, ATPase component, highly conserved
SSX_1566  Polar amino acid ABC transporter, ATP-binding protein
SSX_1567  Polar amino acid ABC transporter, amino acid-binding protein
SSX_1568  ABC transporter membrane-spanning permease, arginine/histidine transport
SSX_1569  ABC transporter membrane-spanning permease, arginine/histidine transport
SSX_1578  ABC-type Fe3+-siderophore transport system, permease component
SSX_1579  ABC-type Fe3+-siderophore transport system, ATPase component
SSX_1581  Metal-binding ABC transporter (probably hemin)
SSX_1588  Conserved ABC-type antimicrobial permease-like protein
SSX_1589  ABC-type antimicrobial peptide transport system, ATPase component
SSX_1636  Possible ABC transporter (possible antibiotic exporter), ATPase component
SSX_1659  ABC-type antimicrobial peptide transport system, permease component
SSX_1660  ABC-type antimicrobial peptide transport system, ATPase component
SSX_1678  ABC-type multidrug transport system, permease component
SSX_1679  ABC-type multidrug transport system, ATPase component
SSX_1680  ABC-type multidrug transport system (bacitracin-resistance related protein A)
SSX_1681  ABC-type multidrug transport system (bacitracin-resistance related protein A)
SSX_1725  Branched-chain amino acid ABC transporter, ATP-binding protein
SSX_1726  ABC transporter ATP-binding protein-branched chain amino acid transport
SSX_1727  ABC transporter membrane-spanning permease-branched chain amino acid transport
SSX_1728  ABC transporter membrane-spanning permease-branched chain amino acid transport
SSX_1729  ABC transporter substrate-binding protein-branched chain amino acid transport
SSX_1741  ABC-type Fe3+-siderophores transporter, ATPase component
SSX_1743  ABC-type Fe3+-siderophore transport system, permease component
SSX_1744  Iron compound ABC transporter, permease protein
SSX_1763  ABC-type molybdenum transport system, ATPase component
SSX_1766  Bacitracin ABC transporter, permease protein
SSX_1767  Bacitracin ABC transporter, ATP-binding protein
SSX_1867  ABC-type polar amino acid transport system, ATPase component
SSX_1868  ABC-type arginine/histidine transport system, permease component
SSX_1904  ABC-type multidrug transport system, permease component
SSX_1905  ABC-type multidrug transport system, ATPase component
SSX_1950  ABC-type oligopeptide transport system, periplasmic component
SSX_1952  ABC-type transporter (Fe-S cluster assembly), permease component, highly conserved
SSX_1955  ABC-type transport system involved in Fe-S cluster assembly, permease component
SSX_1956  ABC-type transport system involved in Fe-S cluster assembly, ATPase component
SSX_1961  Amino acid ABC transporter, amino acid-binding protein/permease protein
SSX_1962  Amino acid ABC transporter, ATP-binding protein
SSX_1974  ABC-type multidrug transport system, permease component
SSX_1975  ABC-type multidrug transport system, ATPase component
SSX_1987  ABC transporter permease protein
SSX_1988  ABC-type transport system (uncharacterized), permease component
SSX_1989  ABC-type transport system (uncharacterized), ATPase component
SSX_2010  ABC-type multidrug transport system, permease component
SSX_2011  ABC-type multidrug transport system, ATPase component
SSX_2040  ABC transporter ATP-binding protein-multiple sugar transport
SSX_2098  ABC-type arginine/histidine transporter, permease protein
SSX_2099  ABC-type arginine/histidine transporter, permease protein
SSX_2101  Amino acid ABC transporter, periplasmic amino acid-binding protein
SSX_2152  ABC-type transporter (uncharacterized), ATPase component
SSX_2153  ABC-type transporter (uncharacterized), permease component
SSX_2165  ABC-type oligopeptide transporter, periplasmic component
SSX_2166  ABC-type multidrug transporter, ATPase and permease components
SSX_2167  ABC-type multidrug transporter, ATPase and permease components
SSX_2249  ABC-type antimicrobial peptide transporter, ATPase component
SSX_2250  ABC-type antimicrobial peptide transporter, permease component
SSX_2351  ABC-type nitrate/sulfonate/bicarbonate transport system, ATPase component
SSX_2352  ABC-type nitrate/sulfonate/bicarbonate transporter, periplasmic component
SSX_2353  ABC-type nitrate/sulfonate/bicarbonate transport system, permease component
SSX_2366  ABC-type cobalt transport system, ATPase component
SSX_2367  ABC-type cobalt transport system, ATPase component
SSX_2376  ABC transporter with duplicated ATPase domains
SSX_2377  ABC transporter, permease component (possible copper exporter)
Figure 2: Construction of single gene deletion mutants. Illustrated below is a schematic representation of the method for replacing a target gene with the insertion of a kanamycin (KM) resistance gene cassette into the SK36 chromosome (4).
Figure 2.
**Bacterial growth conditions**

Each of the SK36 derivatives was transferred from the stock freezer tubes to a culture tube and cultured in Brain Heart Infusion (BHI) broth (Difco Inc., Detroit, MI) supplemented with kanamycin (500µg/ml) as appropriate. Briefly, the mutant strains from -80°C were inoculated into 2 ml of BHI medium supplemented with antibiotic and incubated overnight at 37°C under microaerophilic conditions in an Anoxomat jar (Spiral Biotech) placed in a microaerophilic atmosphere (10% H₂, 10%CO₂, and 80% N₂) generated by the Anoxomat system (Mart). As a control, SK36 was cultured along with each group of mutants as above but without the addition of kanamycin.

**Determination of pH range for S. sanguinis growth**

Initially, we needed to determine the pH range for the S. sanguinis growth. Overnight cultures for the wild type and two control mutants (ΔSSA_0204 and ΔSSA_0260) from previous studies in our lab were sub-cultured 1:100 into fresh medium, grown to mid-exponential phase (OD₆₀₀=0.5), and diluted 1:10000 into fresh growth media with different pH values, from pH 8.0 to pH 4.0. Media was titrated to the acidic values with HCl. Growth was then monitored by dispensing 200µl of the diluted cultures in triplicate into wells of a black plate with a sterile mineral oil overlay to reduce exposure to oxygen, unless otherwise indicated. Plates were incubated at 37°C for 24 h in a Fluostar plate reader (BMG Labtechnologies, Offenburg, Germany) and the OD 595 nm was read every 10 min for 18 h.

**Acid sensitivity assay**

To determine the ability to survive an acid challenge, cells from an overnight culture were diluted 1:100 into BHI broth and incubated to OD₆₀₀=0.3. They were then diluted into BHI at
two different pH values, either a normal pH around pH 7.6 or BHI broth that had been acidified with HCL to pH 5.8. Growth was then monitored by dispensing 200µl of the diluted cultures in triplicate into wells of a black plate with a sterile mineral oil overlay to reduce exposure to oxygen. Plates were incubated at 37°C for 18 h in a Fluostar plate reader (BMG labtechnologies, Offenburg, Germany) with reading every 10 min at OD 595 nm.

**Carbohydrate fermentation assay**

To evaluate sugar uptake and fermentation, the production of acid from sugar was determined using different carbohydrates. The bacteria were cultured in BHI pH 7.6 as described previously to mid-exponential phase. The cells were harvested by centrifugation at 4,000 x g at 4°C, washed twice with sterile saline, resuspended in 3M phenol red broth (0.1 mg/ml) (BBL™, Becton, Dickinson company, Sparks, MD, USA) and aliquots (0.5ml) were distributed into microcentrifuge tubes. Sugars were added to a final concentration of 0.5% (wt/vol) and then incubated for 2 h at 37°C. Fermentation was scored positive if phenol red changed to orange or yellow, this would be indicative of pH lower than 6.8. All the carbohydrates used in fermentation and growth assays were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA) or Sigma-Aldrich Co. (St. Louis, MO, USA).

**Growth on carbohydrate substrates**

For monitoring growth analysis on different sugars, the control strain and mutants were grown in BHI pH 7.6 as described previously to mid-exponential phase. The cells were harvested by centrifugation at 4,000 x g at 4°C, washed twice with sterile saline, and resuspended in 600µl semi defined growth media, CDM, adjusted with HCl to two different pH values; pH 7.6 and pH 6.3 (52). In this medium we had to use pH 6.3 for acidic conditions because the cells
wouldn’t grow in this medium at pH 5.8. CDM is comprised of the following biochemically defined low molecular weight constituents: L- Glutamic acid (2 g), L-Cysteine (0.2 g), L-Leucine (0.9 g), NH₄Cl (1 g), K₂HPO₄ (2.5 g), KH₂PO₄ (2.5 g), NaHCO₃ (4 g), MgSO₄ (1.2 g), MnCl₂ (0.02 g), FeSO₄ (0.02 g), Na-pyruvate (0.6 g), Riboflavin (1.0 mg), thiamine (0.5 mg), D-Biotin (0.1 mg), Nicotinic acid (1.0 mg), P-amino-benzoic acid (0.1 mg), Ca-pantothenate (0.5 mg), Pyridoxal (1.0 mg), and Folic acid (0.1 mg). Different carbohydrates were added from a 20% stock to a final concentration of 0.5% and growth was then monitored by dispensing 200µl of the two different CDM cultures in triplicate into wells of a black plate with a sterile mineral oil overlay to reduce exposure to oxygen. Plates were incubated at 37ºC for 18 h in a reader. OD 600nm was measured every 10 minutes.

Examination of intracellular pH by fluorescence probe

Cell suspensions of S. sanguinis (5×10⁸ cells/ml) were prepared using 100mM of 2-(N-morpholino) ethane sulfonic acid (MES) pH 5.0-6.5 and HEPES pH 7.5-8.0. The buffers were titrated to the desired pH using 3M NaOH. The cell suspensions were incubated with 0.5µM BECEF-AM (stock solution 1 mM in DMSO, Molecular Probes, Inc., Eugene, Ore.) at 37ºC for 15 min prior to examination. The suspension was then centrifuged at 4000 rpm for 10 min at 4ºC. The pellet was resuspended and washed three times with sterile saline before final resuspension in buffers (4ml) with the desired pH.

The fluorescence excitation spectra of BCECF were recorded using a spectrophotometer with double monochromators and automatic correction for variations in the excitation source. Excitation was performed at the wavelengths 440-505 nm and the emission wavelength was set at 535 nm. The ratio of fluorescence of BCECF at 505-440 nm is a function of pH. The slit width for the excitation and emission monochromators was 5 nm and10 nm, respectively. To
relate the fluorescence ratio 505/440 to pH, the ionophore nigericin (final concentration 5 µM, Sigma-Aldrich, Co., St. Louis, MO, USA) and KCl (final concentration 100mM) were added to the suspension, which eliminates the pH gradient across the bacterial cell membrane. The intracellular pH was then fixed at the extracellular pH; the fluorescence ratio at different pH values was obtained. A calibration curve of fluorescence ratio as a function of pH was then constructed and prepared for individual experiments using the method of least squares from which the intracellular pH of the sample was determined.

**BCECF toxicity testing**

The possible toxicity of BCECF was tested by comparing the surviving fractions of both wild type and mutant bacteria incubated with and without BCECF-AM. A cell suspension was prepared as described above using 100 mM MES pH 6.5. The suspension was divided into two equal fractions, one of which was incubated with 0.5µM BCECF-AM. The two suspensions were kept at 37°C for 15 min. Samples of 100µl were removed from the suspensions and diluted in buffer to obtain a cell density of $2 \times 10^4$ cells/ml. Twenty microliters of the diluted suspensions was seeded on BHI agar. After 24 h of microaerophilic incubation, the colonies formed on the dishes were counted.
3. RESULTS

**Determination of pH range for *S. sanguinis* growth**

The growth rates of SK36 and two control strains were examined during incubation at a full range of pH values from pH 8.0 to pH 4.0 in BHI broth at 37°C. Strain ΔSSX_0204 is a TCS mutant that was previously shown to be more acid resistant, while strain ΔSSA_0260 is acid-sensitive and lies in a gene that encodes an ABC type metal transporter (52). The cell density was monitored by spectrophotometer at 595 nm. Figure 3 shows the growth rate results at the two specific pH values that were chosen for the acid sensitivity assay because there was no growth at pH values outside of this range in either direction. The wild type and ΔSSX_0204 strains had similar cell densities and grew better than the ΔSSX_0260 even at normal pH, and this latter strain showed a more severe growth defect at acidic pH than either wild type or ΔSSX_0204 (Figure 3).

**Acid sensitivity screen**

The growth rates of the 150 mutants in genes previously identified in our laboratory as putative ABC transporters were compared to the wild type SK36 and the two control mutants described above, ΔSSX_0204 and ΔSSX_0260. Bacterial growth was examined during incubation at two different pH values in BHI broth (pH 7.6 and pH 5.8) at 37°C. The cell density was monitored by spectrophotometer at 595 nm. Out of the 150 putative ABC transporter mutants, we found only two, ΔSSX_1507 and ΔSSX_1508, that showed increased sensitivity to acidic stress. The results in Figure 4 show the growth results for these two ABC transporter mutants as well as the control strains and two other representative mutants from the collection.
**Figure 3:** Growth rate characteristics for wild type strain *S. sanguinis* SK36 and two control strains. Growth in BHI at normal pH (A; pH 7.6) and the acid limit for the wild type strain (B; pH 5.8) is shown for SK56 and mutants ΔSSX_0204 and ΔSSX_0260). Results shown are the average of three individual repeats for each strain.
Figure 3.

A.

![Growth at pH 7.6](image)

B.

![Growth at pH 5.8](image)
Strain ΔSSX_1507 and ΔSSX_1508 grew slower than the controls, even at normal pH, and showed a severe growth defect at acidic pH. The genes deleted in these two mutants, SSA_1507 and SSA_1508, are adjacent genes in the same operon, predicted to encode for the cytoplasmic and membrane components of an ABC type lipopolysaccharide transporter. A BLAST search reveals that SSA_1507 belongs to the ABC-ATPase superfamily of proteins and encodes a protein with characteristic motifs including a predicted ATP-binding site, ABC transporter signature motif, walker A/P-loop, Q-loop/lid, walker B, D-loop, and H-loop/switch region. SSA_1508 encodes a protein that belongs to the ABC_2membrane superfamily, which plays a role in capsular polysaccharide export. Both have numerous close homologs in other streptococcal species. They lie within an operon of genes with putative functions involved in polysaccharide biosynthesis and transport. This locus is illustrated in Figure 5. We tested the other available mutants in this gene cluster; none of them exhibited an acid sensitive phenotype (Figure 6).
**Figure 4:** The growth rate characteristics of selected ABC transporter mutant strains. The two acid sensitive mutants and two representative nonsensitive mutants are shown in comparison with the wild type strain *S. sanguinis* SK36 and control strains (0204 and 0260) at A: pH 7.6, B: pH 5.8 in BHI medium. Results were obtained from average of three individual repeats for each strain.
Figure 4:

A.

B.
**Figure 5.** The gene locus encoding the acid-sensitive. The arrows indicate the direction of the genes from 5' to 3'. Gene numbers are indicated above the arrows. The red arrows represent the candidate genes SSA_1507, SSA_1508, which were found to be acid sensitive; the grey arrows represent genes for which mutants were not available for testing.
Figure 5:
Figure 6: The growth rate characteristics are shown for the rest of the ABC transporter mutant genes in the operon. Comparison with the wild type strain *S. sanguinis* SK36 and control strain (0204 and 0260) at A: pH 7.6, B: pH 5.8 in BHI medium. Results were obtained from average of three individual repeats for each mutant strain.
Figure 6:

A.

Growth at normal pH (7.6)

B.

Growth at acidic pH (5.8)
Carbohydrate fermentation assay

To evaluate differences in metabolic substrates and the effect of mutations on substrate utilization, we examined the mutants ΔSSX_1507 and ΔSSX_1508, as well as those in the neighboring genes of the operon, for their ability to utilize various sugars. A simple method for the determination of the carbohydrate substrate utilization profile of *S. sanguinis* mutants is the measurement of acid generation (fermentation) during growth on single carbon sources. As an initial step, carbohydrate fermentation was assayed using phenol red. This colorimetric assay measures acid production so could only be used for bacteria growing at neutral pH. Eleven sugars were chosen because of their availability in our dietary food. The wild type was found to metabolize ten of these carbohydrates at two different pH values. None of the strains fermented stachyose. 1507 showed a defect in glucose fermentation; 1514 showed a defect in lactose fermentation. Also 1506, 0260, and 1511 showed a defect in trahalose fermentation (Table 2).

Growth on carbohydrate substrates

The phenol red assay did not reveal major differences in carbohydrate fermentation. However, this assay is not quantitative. Furthermore this assay does not allow determination of carbohydrate utilization under acidic conditions because that leads to a color change independent of fermentation. To further investigate carbohydrate utilization, a comparison of mutant growth rates on different carbohydrates at normal and acid pH was performed. To compare neighboring gene differences in metabolic substrates and the effect of mutations on substrate utilization, we used again ΔSSX_1507 and ΔSSX_1508 plus their neighboring genes in the operon. Because these strains would not grow at pH 5.8 in the defined media, this assay was performed at pH 6.3 rather than pH 5.8 in order to obtain a suitable growth curve under acidic conditions for these mutants. All mutants showed much reduced growth at lower pH, and some small changes in
Table 2: Carbon source utilization by *S. sanguinis*.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>SK36</th>
<th>0204</th>
<th>0260</th>
<th>1507</th>
<th>1508</th>
<th>1506</th>
<th>1510</th>
<th>1511</th>
<th>1512</th>
<th>1513</th>
<th>1514</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Stachyose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Fermentation was scored (+) if phenol red changed to yellow or (+/-) if it changed to orange.

No change in color (red) is considered (-).
preferred sugars were seen between the wild type and the mutants. Comparison of growth rates showed that for the wild type (SK36) glucose was the sugar supporting growth best at normal pH. At acid pH, growth was reduced in all carbohydrates and galactose showed the highest growth rate. For SSX_1507, at normal pH maltose and galactose supported the highest growth rates, while maltotriose and galactose supported the higher growth rates at acidic pH. For SSX_1508, galactose supported the highest growth rate at normal pH (Figure 7), while maltotriose was the preferred sugar at acidic pH. For the rest of the mutants in the region e.g. SSX_1506, SSX_1510, SSX_1511, SSX_1512, SSX_1513, and SSX_1514, galactose was the most preferred sugar at normal pH and maltotriose showed higher utilization at acidic pH (Figure 8).
**Figure 7:** Growth profiles of acid sensitive ABC transporter mutants on different carbon sources. (A) The wild type and mutant ΔSSX_1507 (B) and ΔSSX_1508 (C) were grown in CDM medium containing 0.5% of the sugar indicated in the panel and OD 600 nm values were recorded at 10 minute intervals automatically in a thermostatic 96 well microplate reader. Results are shown here with (1) pH 7.6 and (2) pH 6.3. The experiments were repeated three times and mean values from triplicate wells are shown. Consistent results were obtained.
Figure 7:

A. 1

Wild type pH 7.6

A. 2

Wild type pH 6.3
Figure 7:

B. 1

![Graph 1507 in pH 7.6]

B. 2

![Graph 1507 pH 6.3]
Figure 7:

C. 1

![Graph showing OD600nm over time for pH 7.6]

C. 2

![Graph showing OD600nm over time for pH 6.3]
Figure 8: Growth profiles of the neighboring gene mutants on different carbon sources. Growth was monitored in microplate reader at pH 7.6 and pH 6.3. OD_{600} was measured every 10min. Mean values from three replicate wells are shown. A1 to A6 at pH 7.6 and B1 to B6 at pH 6.3.
Figure 8:

A. 1

B. 1

1506 in pH 7.6

1506 pH 6.3
Figure 8:

A.2

![Graph 1: 1510 in pH 7.6](image1)

B.2

![Graph 2: 1510 pH 6.3](image2)
Figure 8:

A.3

![Graph of 1511 in pH 7.6](image)

B.3

![Graph of 1511 pH 6.3](image)
Figure 8:

A.4

1512 in pH 7.6

B.4

1512 pH 6.3
Figure 8:

A.5

1513 in pH 7.6

B.5

1513 pH 6.3
Figure 8:

A.6

B.6
Examination of intracellular pH by fluorescence probe

In the absence of an obvious difference in sugar utilization, we wished to investigate the ability of the acid-sensitive mutants to maintain their intracellular pH when subjected to the acidic environment in the absence of an extracellular energy source. Before examination of intracellular pH, we determined the toxicity of BCECF. Bacteria were treated with BCECF and survival was measured by determination of *S. sanguinis* colony-forming units (CFU) on agar plates.

It was found that no significant differences were obtained between the surviving fractions of bacterial cells (wild type and mutants) incubated for up to 30 minutes with or without BCECF-AM. Thus, we concluded that BCECF did not appear toxic at least for the course of the 30 minute treatment used in the fluorescence assay. In our assay, the BCECF treated samples were examined immediately after resuspension such that any leaked BCECF was determined.

Next, we determined whether BCECF can be used to measure the intracellular pH of *S. sanguinis*. Cell suspensions were prepared in buffer with a pH varying between 4.0 and 8.0. The intracellular pH at various extracellular pH values was then determined. During the incubation in BCECF-AM, BCECF accumulated in the bacteria, leaving them suitably fluorescent after 15 minutes. Calibration of the intracellular pH of each sample was performed in the presence of the ionophore nigericin and KCl. Addition of nigericin and KCl led to a rapid change in the fluorescence intensity at the pH-dependent wavelengths of BCECF. The magnitude of this change was proportional to the difference between the pH of the external medium and the intracellular pH. After changing the pH of the sample, a calibration curve was constructed that, in all cases, had a correlation coefficient \( R \geq 0.9 \) (Figure 9B). This allowed a determination of the intracellular pH of *S. sanguinis* at different extracellular pH values (Figure 9B). A similar
analysis was carried out on the two acid-sensitive mutants 1507 and 1508 (Figure 10). These results are summarized in Figure 11. As can be seen in Figure 9A, a good correlation was obtained between the intracellular pH and extracellular pH determined using BCECF. This indicates that the fluorescent probe BCECF along with nigericin calibration can be used to measure the intracellular pH of these bacteria. In the case of wild type bacteria over all extracellular pH range from pH 4.0-8.0, the intracellular pH, they were maintained bacteria between pH 5.5 and 7.2. The internal pH increased with increasing extracellular pH except when the extracellular pH reached 7.0, where the intracellular pH dropped to 6.0. This was because the pH gradient under the zero around extracellular pH 7.0 (48). At an extracellular pH of 8.0, the pH values were inverted with the interior being close to neutral (pH 7.2). The results for the two mutants can be seen in Figure 10. Over the extracellular pH range from 4.0-8.0, both ΔSSA_1507 and ΔSSA_1508 exhibited a more acidic internal pH than the wild type. The ΔSSA_1507 mutant maintained pH range of an internal pH 5.1 - 6.5, ΔSSA_1508 maintained an internal pH of 5.1-6.8. As we seen for the wild type, at an extracellular pH of 7.0 the pH values inverted. In this case, however, the interior was acidic with an intracellular pH of 5.6. In summary, at all pH values tested the mutant gene strains maintained a lower intracellular pH than the wild type. These results are summarized in Figure 11. The differences in the intracellular pH values for wild type and two mutants were found statistically significant. These results suggest the presence of a proton pump and indicate that the mutants are less able to pump out protons.
**Figure 9:** The intracellular pH compared with the extracellular pH of wild type *S. sanguinis*. (A) Calibration curve. The intracellular pH was obtained using BCECF as a pH sensitive probe. (B) The comparison between the Fluorescence intensity ratio 505/440 for bacterial cells and the calibration curve reading. Correlation coefficient R= 0.9, from which the intracellular pH was derived from the calibration of extracellular pH.
Figure 9:

A.1

\[ y = 0.0167x^2 + 0.189x + 4.4291 \]

\[ R^2 = 0.5751 \]

B.2

Comparison of internal pH of Bacteria with calibrated pH

Fluorescence ratio (505/440nm) vs pH external
**Figure 10:** Comparison of the intracellular pH of the two mutants. (A) ΔSSA_1507; (B) ΔSSA_1508 (1) calibration curve, (2) comparison of intracellular vs extracellular
Figure 10:

A.1

![A.1](image1.png)

\[ y = 0.0743x^2 - 0.5175x + 5.906 \]

\[ R^2 = 0.7789 \]

A.2

![A.2](image2.png)

Comparison of internal pH of 1507 mutant with calibrated pH.

Fluorescence ratio (505/440nm) vs pH External.
Figure 10:

B.1

```
Figure 10: B.1

y = 0.103x^2 - 0.839x + 6.847
R^2 = 0.8208
```

B.2

```
Comparison of internal pH of mutant(1508) with calibrated pH
```

```
Fluorescence ratio (505/440nm)
```

```
pH External
```

```
Fluorescence ratio (505/440nm)
```

```
pH External
```

```
Fluorescence ratio (505/440nm)
```

```
pH External
```

```
Fluorescence ratio (505/440nm)
```

```
pH External
```

```
Fluorescence ratio (505/440nm)
```

```
pH External
```

```
Fluorescence ratio (505/440nm)
```

```
pH External
```
Figure 11: Comparison of intracellular pH for the wild type and the mutants. The experiments were conducted with three individual repeats for each strain. Standard deviations were indicated. The p-values of statistical significant differences were determined for comparing the wild type with the mutants.
Figure 11.

<table>
<thead>
<tr>
<th>pH</th>
<th>1507/SK36</th>
<th>1508/SK36</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.002447</td>
<td>0.002654</td>
</tr>
<tr>
<td>5</td>
<td>0.000267</td>
<td>0.000811</td>
</tr>
<tr>
<td>6</td>
<td>4.65E-05</td>
<td>0.000147</td>
</tr>
<tr>
<td>7</td>
<td>0.000185</td>
<td>0.000263</td>
</tr>
<tr>
<td>8</td>
<td>0.000361</td>
<td>0.003211</td>
</tr>
</tbody>
</table>
4. DISCUSSION

The gram-positive bacterium *S. sanguinis*, a member of the human pioneer oral microflora, has been recognized as a key player in bacterial colonization of the mouth. It has been reported that non-mutans streptococci were dominant in dental plaque (55). It is also the most common viridians streptococcal species implicated in infective endocarditis (5). *S. sanguinis* has been shown to compete against the major cariogenic species, *S. mutans*, that is well-adapted to the various hostile environmental conditions of the mouth. It has been thought that the molecular mechanisms that allow these two bacterial species to resist these conditions are important characteristics, but the underlying mechanisms are poorly understood (56). These organisms likely exploit a variety of physiological features for survival in the mouth. These bacteria are known to be voracious in their ability to consume dietary sugars, so they must be able to cope with the acid production that is the outcome of their metabolism (57). It has been noticed that an antagonistic relationship between *S. sanguinis* and *S. mutans* was an important feature in this environmental niche. Since the acid tolerance contributes to the ability of *S. sanguinis* cells to adapt and to compete with *S. mutans* in the plaque environment, it is important to figure out this mechanism and identify genes involved in acid tolerance in *S. sanguinis*. Understanding its regulation could enhance the process by which development of therapeutics targets the streptococcal caries-forming process.

One of the processes important for these bacteria is acid resistance. Acid tolerance in plaque bacteria is considered necessary for the formation of caries. Several studies have emphasized that organisms with higher pH limits for growth are unable to respond to acidic environments in order to survive, whereas the more aciduric organisms would possess or acquire acid tolerance (58, 59). Many early colonizing streptococcal species in plaque, such as *S.
sanguinis, S. gordonii and S. oralis are able to ferment carbohydrates into metabolic acids, ultimately lowering the plaque pH (60). Also, a recent review mentions that non-mutans streptococci are considered a major bacterial group in plaque associated with white spots on teeth, and the dissolution of enamel can be traced to the members of these early microfloras (55). This review emphasized an extended ecological plaque hypothesis in which the entire consortium of acidogenic bacteria contributes to the caries process. The hostile environment inside the mouth (pH 3.0 to 4.0) requires that S. sanguinis adapt and thrive under these conditions, suggesting that this microorganism is capable of sensing and responding to different environmental stimuli through specific mechanisms (55).

Various mechanisms are likely to participate in the bacterial resistance response to the acidic stress of the oral environmental niche. The main mechanism by which the streptococcal species are considered to survive acid stress is their ability to remove protons from the cytoplasm by a membrane-bound enzyme, ATPase or F-ATPase. It has been proven that proton translocation by F₁F₀ ATPase is of the one key mechanisms for regulating intracellular pH; this enzyme pumps protons back out of the cell in order to protect the interior from adverse influences of acidification (57). Similarly, it has been determined that the F₁F₀ ATPase plays a major role in H. pylori survival in a wide pH gradient from two to seven (61). Some earlier studies showed that the Mg2 (+)-dependent proton-translocating ATPase was also found to play an important role in acid tolerance in different strains, e.g. Salmonella typhimurium (62).

Ultimately, it has been determined that there are multiple mechanisms exploited by different organisms for survival in acid. For example, S. salivarius degrades urea to ammonia and S. sanguinis produces ammonia by arginolysis, and many more examples can be found in the literature (10).
One of the mechanisms known to play a role in environmental stress response is the TCS (a signal transduction system). It has been proposed that this system regulates gene expression and thus allows the adaptation to a changing environment (63). Previous studies in our lab carried out by J. Patel et al. (52) examined 29 genes involved in TCS including 15 putative response regulators and 14 putative sensor kinases. These studies identified one TCS response regulator gene, SSA_0204, involved in an acid stress response. One of the genes regulated by this response regulator was SSA_0260, a component of an ABC type metal transporter. Mutation of this gene resulted in a strain that was sensitive to acidic stress. This suggested a role for ABC transporters in acid tolerance. Further interest in ABC transporters was spurred by a recent study with a Brucella abortus ABC transporter ATPase gene, in which it was shown that inactivation of this gene generated a novel rough mutant strain with attenuated virulence, suggesting the potential of at least some ABC transporters as future vaccine candidates (64).

The principal goal of our study was to expand our screening and look at all ABC transporter genes in the single gene mutant library that was constructed in our laboratory, in order to determine whether there were additional mutants in ABC transporter genes that were more sensitive to acidic conditions and to determine the contribution of each gene identified in this screen to the acid stress response in S. sanguinis. In our screening, the 150 annotated ABC transporter mutants were assessed for their ability to withstand acidic environmental stress. We found that only two ABC mutants (SSX_1507& SSX1508) were more sensitive to acidic conditions than the wild type S. sanguinis SK36 cells. These two adjacent genes were annotated as components of a lipopolysaccharide ABC transporter system, the ATPase and a permease. We were therefore encouraged to include these mutants in further studies.

In this study we focused on the sugar uptake mechanism in relationship to acidity and
acid tolerance and examined the growth rate on eleven different sugars for the wild type strain and each of the genes in the operon that included the two acid sensitive ABC transporter mutants. The ability of oral streptococci to transport and metabolize a wide variety of sugars, especially at low pH, allows these bacteria to grow in the oral cavity (65). A complication in analysis of sugar uptake systems is that many transporters have multiple substrates, and many sugars are transported by more than one system. Many studies have addressed this question regarding diversity of transporter systems in the uptake of different substrates in a wide range of microorganisms. For example, in *Streptococcus pneumoniae* a genomic survey allowed recognition of seven carbohydrate ABC transporters in this particular bacterium, and this might serve as a reference for related species (21). It has also been confirmed that the number of substrates is much higher than the number of carbohydrate transporters, indicating that each transporter can be used for multiple substrates (65). In *Streptomyces coelicolor*, transcriptional analysis revealed expression of a large number of solute binding proteins in response to environmental stress, which confers specificity to a small number of ABC transporters (34). The ATPase component, which is required to energize the transport of substrate, does not contribute to solute specificity (35).

One of the main features in *S. mutans* is their ability to transport sugars by more than one system e.g. the phosphoenolpyruvate- phosphotransferase system (PTS) and ATP-binding cassette (ABC) transporters. The ABC transporters are believed to play a more important role in sugar transport under stress conditions because they showed increased transcription levels under several tested conditions (31). Our results in growth rate analysis with 11 different sugars showed that *S. sanguinis* is able to utilize at least ten of these as substrates at two different pH values (Figure 7). This is a hallmark of bacteria adapted to ecological niches providing a wide
variety of carbon sources, such as the oral cavity, which contain food and host-derived sugars. Another interesting observation is that no significant differences were observed between the mutants and the wild type in their utilization of different sugars at two different pH values except that maltotriose showed a higher utilization in the mutants compared with wild type. In other words, none of the mutants showed a specific defect in sugar utilization at two different pH values. Therefore, the observed acid sensitivity is not due to a failure to take up any of the common sugars tested. We also identified some sugars that are weakly metabolized by *S. sanguinis*. Slow acid production from trehalose and raffinose was demonstrated for mutants in this study and low but detectable growth could also be detected.

It must be noted that assays used in this work are metabolic assays and do not detect carbohydrate uptake rate directly. Our assays thus miss any sugar uptake that would not feed into carbon metabolism. An example of this is stachyose, which cannot be metabolized, since genes required for stachyose catabolism might be missing. Construction of mutants in these two ABC transporter genes does not permit identification of the substrate specificity for these two mutant genes. For those sugars which were inefficiently metabolized, fermentation by phenol red assay proved to be suitable for detection but it is considered not quantitative and did not allow determination of utilization under acidic conditions. For those substrates more efficiently metabolized, analysis of growth kinetics was the most sensitive assay method and allowed a comparison of growth rates on different carbohydrates under two conditions. No distinct phenotype was observed in our mutants for this ABC transporter system possibly due to the presence of other redundant sugar transporter systems in the bacterial genome. Construction of double mutants might be useful, due to uptake of the same substrate by different transporters, which could compensate for growth in single mutants. Our observations based on analysis of
growth rate for the two acid-sensitive mutants showed that this defect might not be related directly with ABC sugar uptake systems but it might be related with sugar export permeases, which is more consistent with the results of the BLAST search. For future plans, understanding *S. sanguinis* growth on different natural substrates may help in finding a correlation between carbon source availability and the acidic tolerance.

Our observations are consistent with studies by Alexander on two closely related ABC transporters in *S. mutans* (35). It has been proposed that the ATPase genes of these ABC transporters can interact with either their own or an alternative transporter complex. They also observed that no significant differences were seen in the transport of different sugars by mutants in these genes.

Many investigators have proposed other mechanisms involved in the acid stress response. Non-mutans streptococci have been shown to be capable of increasing their acid tolerance in response to environmental acidification and this acid adaptation can involve the induction of H\(^+\)-ATPase, arginine deiminase and synthesis of stress proteins (20). One model proposed for the regulation of streptococcal cytoplasmic pH is by changes in the amount and activity of the H\(^+\)-ATPase; this occurs when the enzyme biosynthesis is stimulated due to a down shift in cytoplasmic pH. A further explanation regarding the regulation of cytoplasmic pH proposes that bacteria change their fermentation to the pathway producing lactate in order to maintain intracellular pH near neutral (43). The question still rises in our study is how does this ABC transporter mutant gene get its energy and support the low level growth observed for substrates like mannose or trehalose under acidic conditions. One of the possibilities is that it may get energy from another ATPase gene associated with a different ABC transporter to which the strain can respond weakly. Another possibility is that the formation of specific acid-regulated
proteins is required for survival in acid environments, and thus these might be lacking for our two acid sensitive mutants, but the mechanism behind such a process is still unclear. We did not recognize any relationship between a defect in sugar fermentation and response to acidity in these two specific mutants.

Bacteria need to maintain their intracellular pH against extracellular pH extremes for survival. Regulation of pH regulated genes has been studied primarily in *S. typhimurium* and *Escherichia coli*, where *lacZ* fusions to pH-regulated genes have demonstrated that maximal induction for each gene occurs in an internal the pH range of 5.5 to 6.0 (62). Previous investigations of the intracellular pH of Gram-positive bacteria (*P. acnes*) have been conducted using $^{31}$P-NMR spectroscopy (48, 66) but the high cell densities required proved to be inconvenient. Accordingly, an alternative was sought and the probe BCECF was chosen as a suitable probe for measuring the intracellular pH of *S. sanguinis*. It well known that streptococci have been forced to develop mechanisms to cope with the stress induced by declining pH values in their environments. The purpose of this part of the study was to determine a possible relationship between ABC transporter genes and maintenance of the intracellular pH. Specifically, measurement of the intracellular pH of *S. sanguinis* was used to determine the role of the two acid sensitive mutants in regulating the cytoplasmic pH of this bacterium. The assay of BCECF as a suitable intracellular pH probe for *S. sanguinis* was determined. Initially, it was confirmed that BCECF was not toxic (48, this study) at least for a time period required for pH determination. Also, it was important to demonstrate that the intracellular pH in these bacteria could be determined from the ratio of the fluorescence signal at the pH-sensitive wavelength (505) and the fluorescence signal at the pH-insensitive wavelength (440). This ratio method reduces problems due to the efflux of the fluorescent probe from the cells during the
measurement (67). In our experiments, good correlation was obtained. In this assay we had some difficulties in the measurements. The ratio after the equilibration of intracellular pH and extracellular pH could be fitted with a four parameter sigmoid function, \( y = a + \frac{b}{1 + \exp \left[-\frac{(x-c)}{d}\right]} \), as previously reported by Breeuwer et. al. (67). It has been reported that reliable results are obtained using BCECF when nigercin is utilized to calibrate the sample and extracellular BCECF is removed. The variation of the intracellular pH of *S. sanguinis* as a function of extracellular pH could was deduced from some of the experiments discussed here. The pH of the natural environment of *S. sanguinis* lies between pH 5.0 and 9.0 (68) and the optimal growth occurs at approximately pH 7.0. However, *S. sanguinis* can survive at a pH of up to 9.0 (68), indicating that intracellular pH regulation over a wide pH range should be possible. It has been noticed that *S. sanguinis* maintains a pH gradient, the inside being higher than surrounding, in the range pH 5.5 to 7.2 also, the pH gradient is lower than zero at approximately pH 7.0. At an extracellular pH greater than 7.0, this gradient inverts, the inside being more acidic, as has been reported for other bacteria (43). Our observations have indicated that intracellular pH for the wild type when we tested with BCECF probe begins around 5.5 and wild type SK36 cells showed a suitable intracellular pH range in comparison with larger pH range outside the cells. The mutant strains of SSX_1507 and SSX_1508 showed less ability to maintain their intracellular pH range. In addition, at all pH values tested, the mutants maintained a lower intracellular pH than the wild type.

Many streptococcal researchers have proposed the role for H^+-ATPase in maintaining the appropriate intracellular pH for metabolic reactions by expelling protons across the cell membrane against environmental acidification (69). Hence, we investigated here the role of the ATPase domain in one ABC transporter gene in regulating the intracellular pH and we have
showed that this ABC transporter contributes to the ability of these bacteria to maintain their internal pH. It has been suggested that the capacity to maintain intracellular pH by ATPase genes could also protect the cells from intracellular acidification and increase the acid tolerance.

In summary, the present study demonstrates that two ABC transporter mutants in *S. sanguinis* are sensitive to acidic stress, and suggested that these transporters are related to the export of protons. The mechanism form this remains to be determined. Since ABC transporter genes are involved in the regulation of intracellular pH of this bacterium, a complete picture of the regulatory network of pH-regulated transporter genes may reveal novel signaling pathways. Furthermore, since these particular ABC transporter genes are predicted to be related to sugar transport related, a complete transcription profile analysis of *S. sanguinis* genes involved in sugar transport, metabolism and regulation during growth with different sugars under different stress conditions may also provide an opportunity to advance our understanding of this bacterium.
5. REFERENCES


16. Dunning DW, McCall LW, Powell WF Jr. Arscott WT, McConocha EM, McClurg Cd, Goodman SD, Sptafora GA. SIoR modulation of the Streptococcus mutans acid...


24. Postma PW, Lengler JW, Jacobson GR. Phosphoenolpyruvate carbohydrate phosphotransferase systems. In: Escherichia coli and Salmonella: cellular and


32. Webb AJ, Hosie AH. A member of the second carbohydrate uptake subfamily of 
ATP-binding cassette transporters is responsible for ribonucleoside uptake in 

33. Garmory HS, Titball RW. ATP-binding cassette transporters are targets for the 
development of antibacterial vaccines and therapies. Infect Immun. 2004 Dec;
72(12):6757-63.

and transcriptional analysis of carbohydrate uptake systems of Streptomyces 

35. Webb AJ, Homer KA, Hosie AH. Two closely related ABC transporters in 
Streptococcus mutans are involved in disaccharide and/or oligosaccharide uptake. J 

36. Lorca GL, Barabote RD, ZIolpolski V, Tran C, Winnen B, Hvorup RN, Stonestrom 
AJ, Nguyen E, Huang LW, Kim DS, Saier MH JR. Transport capabilities of eleven 
gram-positive bacteria: comparative genomic analysis. Biochim Biophys Acta. 2007 

90.

38. Kitten T, Munro CL, Michalek SM, Macrina FL. Genetics characterization of a 


47. Olsen KN, Budde BB, Siegumfeldt H, Rechinger KB, Jakobsen M, Ingmer H. 
Noninvasive measurement of bacterial intracellular pH on a single-cell level with 
green fluorescent protein and fluorescence ratio imaging microscopy. Appl Environ 

48. Futsaether CM, Kjeldstad B, Johnsson A. Measurement of the intracellular pH of 
Propionibacterium acnes: comparison between the fluorescent probe BCECF and 31P-


51. Grant RL, Acosta D. Ratiometric measurement of intracellular pH of cultured cells 

Streptococcus sanguinis. Master thesis. Virginia Commonwealth University. USA.


54. Turner LS, Das S, Kanamoto T, Munro CL, Kitten T. Development of genetic tools 

55. Takahashi N, Nyvad B. The role of bacteria in the caries process: ecological 


