Role of ABC Transporter Proteins in Stress Responses of *Streptococcus mutans*

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Abstract

*Streptococcus mutans* possesses a number of ATP-binding cassette (ABC) transporters, which function as multiple sugar metabolism transporters and are promising targets for antimicrobial strategies. In the present study, we performed functional analyses of *Smu0836* and *Smu0837* products, which are possible ABC transporters. Isogenic mutant strains Δ0836 and Δ0837 were generated by insertional inactivation of *Smu0836*, and *Smu0837*, respectively, of strain MT8148 and found to be more sensitive to antibiotics as compared to MT8148. In addition, assays of membrane transport functions using a fluorescent probe showed that export pumps did not function properly in strain Δ0836. Expression of those genes was elevated when strain MT8148 was cultured with a sublethal concentration of tetracycline, as well as when exposed to heat shock, osmotic stress, and oxidative stress conditions. Furthermore, the expressions of other genes including *Smu0374*, *Smu0215*, *Smu0475*, *Smu0986*, and *Smu1051*, which encode possible ABC transporters, were also elevated when strain MT8148 was cultured with a sublethal concentration of tetracycline. Together, these results suggest that the products of *Smu0836* and *Smu0837* are ABC transporters, which may function in stress response.

Key words: *Streptococcus mutans*, ABC transporter, Antibiotic, Biofilm

Introduction

*Streptococcus mutans* is known to be a primary causative agent of dental caries, while its biological properties, such as aciduricity, acidogenicity, biofilm formation, natural competence, and bacteriocin production, are thought to be associated with their onset and development [1-3]. It is generally understood that microorganisms have developed various mechanisms to resist the toxic effects of antibiotics and other chemicals [4,5], including inactivation of drugs by hydrolysis or modification, alteration of the target, creation of alternative pathways, inhibition of drug entry into cells, and active efflux of drugs [6]. On the other hand, the precise mechanism of *S. mutans* antimicrobial resistance remains to be elucidated.

ABC transporters are commonly found in living organisms and comprise one of the largest protein families, and their components are encoded by approximately 5% of the *Escherichia coli* and *Bacillus subtilis* genomes [7,8]. Although these transporters are found in all species and evolutionarily related, they are functionally diverse and participate in a wide range of important cellular functions. In addition, ABC efflux transporters play many roles in bacteria and export a wide range of compounds, including antibiotics, metals, peptides, and lipids [9]. Notably, *S. mutans* produces peptide antibiotics (bacteriocin) and exports those with antimicrobial activities. In our previous studies, bacteriocin immunity proteins were demonstrated to function as transporters of antimicrobial agents and confer protection against certain classes of antimicrobial agents, while they were also shown to often enhance stress tolerance [10]. Genes encoding proteins with these functions are located upstream of the bacteriocin genes. Recently, Seaton et al. [11] reported that products of previously uncharacterized *Smu0835-Smu0836-Smu0837*, which they designated as the rcrRPQ operon for rel-competence-related genes play a major role in regulation of growth, stress tolerance, and competence development, while they also strongly influence the expression of the primary (p)pGpp biosynthetic pathway that is expressed in exponentially growing *S. mutans*. Bioinformatics analysis predicted that *Smu0836* and *Smu0837* encode an ABC transporter and *Smu0835*, a MarR family transcriptional regulator. Furthermore, we identified an RcrRPQ system located near bacteriocin genes as well as other ATP transporters. Therefore, we hypothesized that this operon has a relationship with transport of antibiotics and stress responses. In the present study, we investigated whether their products are involved in membrane transport of several different antibiotics and reactions to various type of stress.

Materials and Methods

Bacterial strains and plasmids

*S. mutans* MT8148 (serotype c) was used in the present study [12] and ABC transporter-defective mutant strains were produced from this wild-type strain. All strains were grown in Brain Heart Infusion (BHI) broth (Becton Dickinson Co., Franklin Lakes, NJ, USA), Todd-Hewitt (TH) broth (Becton Dickinson), or Mitis-Salivarius (MS) agar (Difco), as required, with appropriate antibiotics (erythromycin 10 µg/ml) used for selection.

DNA manipulation

DNA isolation, endonuclease restriction, ligation, and transformation of competent *Escherichia coli* were carried out as previously described, while transformation of *S. mutans*...
was accomplished by procedures routinely carried out in the same laboratory [13]. All primers were constructed using the complete genome of S. mutans UA159 using the Oralgen database (http://oralgen.lanl.gov/oralgen-tng/) using Amplify 3X (Macintosh computer application).

**Construction of ABC transporter-defective mutants**

Analysis of the complete genome of S. mutans UA159 in the Oralgen database indicated that the SMu0836 and SMu0837 genes were possible S. mutans ABC transporters. These 2 genes were inactivated by inserting the *erm* gene into the middle, as follows. First, the SMu0836 and SMu0837 genes were amplified by Polymerase Chain Reaction (PCR) using primers 836f and 836r, and 837f and 837r, respectively (Table 1), then ligated into a pGEM-T Easy vector (Promega, Madison, WI, USA) to generate pNK8360 and pNK8370, respectively. Next, the ending sequences of the restriction enzymes in each primer (Emr-Bam/F and Emr-Bam/R, Emr-Xho/F and Emr-Xho/R, respectively) (Table 1) were added to an *erm* cassette, which was derived from pVA838 [14]. The plasmids pNK8360 and pNK8370 were then digested with *Bam* HI and *Xho* I, respectively, to become linear at a unique site, then blunt and ligated with an *erm* cassette, to yield pNK8361 and pNK8371, respectively. The plasmids were digested at a unique restriction site with *Eco* RI and introduced into S. mutans MT8148 by transformation to allow allelic exchange. Finally, the transformants were screened on MS agar plates containing erythromycin (10 µg/ml). Confirmation of plasmid insertion causing gene disruption was performed using PCR and Southern blotting analyses of each gene.

**Antibiotic susceptibility tests and growth conditions**

The Minimum Inhibitory Concentrations (MICs) of the antimicrobial agents tetracycline, kanamycin, penicillin, and triclosan were determined in TH broth containing those agents at various concentrations using a method previously described [15]. The cells were incubated in test medium at 37°C for 16 hours, and then growth was visually assessed. The MIC values were defined as the lowest concentration of antibiotics that inhibited visible growth. The growth of each *S. mutans* strain in TH broth that contained antibiotics was monitored during incubation at 37°C.

**Extraction of RNA**

Total RNA was extracted using a method previously described [10]. Briefly, total RNA was isolated from 10 ml of log-phase cell cultures. After centrifugation, the cells were suspended in 0.3 ml of diethylpyrocarbonate-treated water, then the samples were transferred to FastRNA tubes with blue caps (Qiagen, Inc., Carlsbad, CA) and 0.9 ml of TRIzol reagent (Invitrogen Co., Carlsbad, CA) was added. The cells were broken using a FastPREP FP120 homogenizer (Qiagen) at a speed setting of 6.5 for 25 seconds. After placing the samples on ice for 2 minutes, 0.2 ml of chloroform was added and the tubes were vortexed for 1 minute. The mixtures were allowed to stand at room temperature for 2 minutes, and then centrifuged at 13,000 x g for 5 minutes at 4°C, after which 0.5 ml of chloroform was added to the supernatant fluids, and the mixtures were vortexed and centrifuged again as described above. Finally, RNA was precipitated from the aqueous phase with isopropanol, and the resulting pellets were dried and resuspended in 20 µl of diethylpyrocarbonate-treated water.

**Table 1. PCR Primers used in this study.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>836f</td>
<td>TAGAAAAGAAGGAGTTACCATGTTAAAAA</td>
<td>Mutant*</td>
</tr>
<tr>
<td>836r</td>
<td>GAAATAGCAAAGCTTGAATATACGTGA</td>
<td>Mutant</td>
</tr>
<tr>
<td>837f</td>
<td>ATGCTAAAATTTGGGATCCAGAAGGAGTG</td>
<td>Mutant</td>
</tr>
<tr>
<td>837r</td>
<td>AAGCGACGGAATATATTTCTCCGGTT</td>
<td>Mutant</td>
</tr>
<tr>
<td>Emr-Bam/F</td>
<td>ATGCTAAAATTTGGGATCCAGAAGGAGTG</td>
<td>Mutant</td>
</tr>
<tr>
<td>Emr-Bam/R</td>
<td>AAGCGACGGAATATATTTCTCCGGTT</td>
<td>Mutant</td>
</tr>
<tr>
<td>Emr-Xho/F</td>
<td>ATGCTCGAGATTTGTAATTAAGGAGTG</td>
<td>Mutant</td>
</tr>
<tr>
<td>Emr-Xho/R</td>
<td>AAGCGACTCGAGGAATTATTCCTCCGGTT</td>
<td>Mutant</td>
</tr>
<tr>
<td>16sF</td>
<td>GTTGGGACGCAAGGAAACACACTGTTGCA</td>
<td>RT-PCRb</td>
</tr>
<tr>
<td>16sR</td>
<td>CGTTCGCTTGGTGAAGCTTACCTTACC</td>
<td>RT-PCRb</td>
</tr>
<tr>
<td>SMu0836/F</td>
<td>GGGGCGATGGTTCCTTCTAGTACTGCG</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>SMu0836/R</td>
<td>CATAGCATCTTCTAGATCCCGATCC</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>SMu0837/F</td>
<td>GCCAATGGTCCTCTCTATGGAAGGC</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>SMu0837/R</td>
<td>GCCAAGCTTGGTCTGTGATGTCGTTGTC</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>SMu0374/F</td>
<td>GACCGGTTGTTCTAGTCTGAATCGA</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>SMu0374/R</td>
<td>GTAAGGCTAGGCTTTCGCTT</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>SMu0215/F</td>
<td>CGTGATATAGGCTTTCGCTT</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>SMu0215/R</td>
<td>GTAATAGGATGACAGCTAAC</td>
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</tr>
<tr>
<td>SMu0475/F</td>
<td>GCCTGTACGTTTCTGATCCTGCT</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>SMu0475/R</td>
<td>GATCAGTCTGACGTTTCTGATCCTGCT</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>SMu0986/F</td>
<td>TATCAGTCTGACGTTTCTGATCCTGCT</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>SMu0986/R</td>
<td>TGTCTGCCGAACTCTGCTGCT</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>SMu1051/F</td>
<td>GAGCTTTGTTCTTGTTGTTGTTGTTTG</td>
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<tr>
<td>SMu1051/R</td>
<td>GGGCTTCACTTTGCTGGTTTACGAC</td>
<td>RT-PCR</td>
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</table>

*Amplification was performed for construction of defective mutant strain.

bInternal control for RT-PCR.
Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed using a previously described method [10]. Briefly, primers for 16SrRNA were also designed and used as internal controls (16sF and 16sR, Table 1). Total RNA was isolated as described above. For Reverse Transcription-PCR (RT-PCR) analysis, RNA samples were treated for 15 minutes at 37°C with 1.0 U of RNase-free DNase (Amersham Biosciences Corp., Piscataway, NJ) per ml to remove contaminating DNA. Reverse transcription was carried out with SuperScript III (Invitrogen) according to the instructions of the supplier. Real-time RT-PCR assays were performed with the cDNA samples using either 16S rRNA or specific primers with IQ-Supermix PCR reagent (Bio-Rad Laboratories, Richmond, CA) in an iCycler thermal cycler (Bio-Rad) according to the manufacturer’s recommendations. Relative expression levels of the target gene transcripts were then calculated by normalizing the levels of each target gene-specific RNA with the level of 16S rRNA. After normalizing the Ct values for the target genes to the total amount of 16S rRNA, all samples were compared and the relative fold change for each sample was calculated using the ΔΔCt method described for the MyIQ real-time PCR detection system (Bio-Rad).

RT-PCR assays were used to determine the expressions of SMu0836 as well as those of other ATP transporters in the presence of tetracycline, 0.5 M NaCl, and heat shock (42°C), under aerobic and anaerobic conditions. In addition, we found other putative ABC transporters in the database, including SMu0475, SMu0986, SMu0215, and SMu0374, from which we selected SMu0836 and SMu0837, which together may comprise an operon. RT-PCR was performed using the following primers: SMu0836/F and SMu0836/R, SMu0374/F and SMu0374/R, SMu0215/F and SMu0215/R, SMu0475/F and SMu0475/R, SMu0986/F and SMu0986/R, and SMu1051/F and SMu1051/R (Table 1).

Preparation of biofilm cells and planktonic cells

S. mutans MT8148 was inoculated into 5-ml quantities of TH broth in 6-well polystyrene microtiter plates (Thermo Fisher Scientific, Rochester, NY) to form biofilms. After 16 hours of incubation, planktonic and biofilm cells were separated for quantitation using a method previously described [16], and then supernatant fluids containing planktonic cells (unattached cells) were transferred into new tubes. The biofilm cells (attached cells) were washed with PBS twice, then detached from the bottoms of the wells, resuspended with PBS, and transferred to new tubes. Total RNA for both types of cells was extracted using the method described above.

Fluorescence efflux measurement

The tested strains were grown to the mid-log phase, then cells were harvested at 800 x g for 10 minutes, washed once with 10 mM NaCl-50 mM sodium phosphate buffer (pH 7.0), and suspended again in the same buffer at an optimal density at 600 nm of 0.2 in the presence of glycerol. Experimental measurements were generally performed within 2 hours after cell preparation. Fluorescence measurements were performed using a method previously described [10], with some modifications. Briefly, the fluorescence probe, 1-(4-trimethylammoniumphenyl)-6-phenyl-1, 3, 5-hexatriene p-toluenesulfonate (TMA-DPH; Life Technologies, Carlsbad, CA, USA), was initially dissolved in absolute methanol, then fluorescence emission intensity was measured using a twinkle LB970 fluorometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). The excitation and emission wavelengths for TMA-DPH were 355 and 460 nm, respectively. Data shown represent the mean ± standard error of 3 separate experiments with each strain.

Statistical analysis

Intergroup differences of various factors were determined using a statistical Analysis of Variance (ANOVA) method for factorial models. Fisher’s protected least-significant difference test was used to compare individual groups. Statistical computations were performed using STAT-VIEW II (Macintosh computer application).

Results

Antimicrobial sensitivity

Inactivation of the SMu0836 and SMu0837 genes resulted in increased sensitivity to aminoglycoside antibiotics, such as kanamycin and tetracycline, and the antiseptic agent triclosan (Figure 1). On the other hand, the sensitivities of these strains for β-lactam antibiotics, such as penicillin, were not significantly different from that of MT8148 (data not shown). We also performed an export activity assay using a fluorescence probe, TMA-DPH, as the intracellular compound, which showed that the fluorescence intensity values for the Δ0836 strains were higher than that for MT8148 (Figure 2).

![Figure 1. Bacterial growth in the presence of various antimicrobial agents.](image)

Bacterial growth was evaluated by measuring OD550 values at 16 hours after starting incubation. (a) Kanamycin, (b) tetracycline, and (c) triclosan were added to the TH broth at the indicated concentrations. There were statistically significant differences in bacterial growth between MT8148 and each of the mutant strains (*P<0.05, **P<0.01, ***P<0.001, Fisher’s PLSD).
The present analysis of exocytosis strains with TMA-DPH showed a decreased amount of molecules released from the transporter in the plasma membrane.

Expressions of ABC transporter genes

The levels of expression of the SMu0836 and SMu0837 genes in planktonic cells were found to be significantly less as compared to those in biofilm cells (Figure 3), while the level of SMu0837 was lower (less than 0.2) than that of SMu0836. In addition, expression of the SMu0836 gene was significantly elevated in the presence of tetracycline (Figure 4). Furthermore, the expression levels of the other putative ABC transporter genes SMu0475, SMu0986, SMu0215, SMu0374, and SMu1051 were also elevated to a much greater degree than that of SMu0836 in the presence of tetracycline at a lethal concentration (0.375 µg/ml) in S. mutans MT8148 (Table 2). On the other hand, the expression of SMu0836 was increased under osmotic stress and oxidative stress, while that was not changed under the heat shock condition (Figure 5).

Discussion

Bacteria with the double-glycine motif are also known to export proteins via ABC transporters [17]. It was previously reported that ABC-type efflux pumps have some roles in addition to drug resistance [18]. Recently, several multidrug transporters have been suggested to be possessed by S. mutans, based on complete genome sequence information [19]. In the present study, we performed functional analysis of 2 putative genes, SMu0836 and SMu0837, encoding the ABC transporter of S. mutans, to determine antibiotic resistance and reaction to various types of stress.

ABC transporters function as export proteins and secrete intracellularly accumulated antimicrobial agents. In the present study, SMu0836- and SMu0837-defective mutant strains showed increased sensitivity to several different antibiotics as compared to the parental strain MT8148, which suggests that the SMu0836 and SMu0837 genes may be correlated with antibiotic transport. Thus, we considered it important to determine if ABC transporter proteins are involved in export of antimicrobial compounds. For this purpose, the fluorescent model export agent TMA-DPH was utilized to compare the export properties of the mutants with those of the parental MT8148 strain, since that agent has been utilized to determine the quantity of drugs exported from bacteria [20]. TMA-DPH is a fluorescence polarization probe known to be sensitive to plasma membrane surfaces [21]. The present results showed that SMu0836 proteins were associated with export of antimicrobial agents, indicating that they are very likely ABC transporters.

We found 6 possible ABC transporter genes in S. mutans, which were expressed at a higher level in the presence of low concentrations of antimicrobial agents as compared to their absence. However, the level of expression varied for each ABC transporter, and the sensitivity to antibiotics by ΔSMu0836, and ΔSMu0837 differed with each antibiotic agent tested. Thus, it is possible to speculate that these genes function together in a dependent manner and each ABC transporter has its own target for antibiotics or stress. In addition, it should also be noted that these ABC transporters...
may function as complementary agents or in combination for antibiotics transport.

Since the present findings suggested a role for ABC transporter proteins in regard to the sensitivity of strain MT8148 to a variety of antimicrobial agents and biofilm cells are more resistant than planktonic cells, we examined the regulation of ABC transporter protein expression during biofilm formation. Bacteria present in biofilm are generally considered to be more resistant to various stress challenges than those in planktonic cells [22–24], while biofilm cells have also been reported to be up to 1000 times more resistant to antibiotics than planktonic cells [25]. Elevated expressions of the genes encoding ABC transporters presumably contribute to the antibiotics resistance of bacteria present in biofilm. Recently, the acid tolerance of biofilm cells was reported to be remarkably greater than that of planktonic cells [26]. Our results indicate that increased expression of these genes during biofilm formation in vitro is compatible with a general role for ATP binding proteins in such resistance.

In the present study, the expression of SMu0836 was increased under osmotic stress and oxidative stress. We speculate that the ABC transporter has a role in response to

<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene</th>
<th>Predicted functions and description</th>
<th>Gene expression (relative to no tetracycline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMu0215</td>
<td>drrB</td>
<td>ABC transporter, multidrug permease protein</td>
<td>61.88 ± 17.88*</td>
</tr>
<tr>
<td>SMu0374</td>
<td>ND†</td>
<td>ABC transporter, ATP-binding protein</td>
<td>10.18 ± 2.06*</td>
</tr>
<tr>
<td>SMu0475</td>
<td>ND†</td>
<td>ABC transporter, ATP-binding / permease protein</td>
<td>17.90 ± 3.68**</td>
</tr>
<tr>
<td>SMu0836</td>
<td>ND†</td>
<td>ATP-binding / permease protein</td>
<td>3.06 ± 1.21*</td>
</tr>
<tr>
<td>SMu0986</td>
<td>ND†</td>
<td>Multidrug ABC transporter, ATP-binding / permease protein</td>
<td>11.24 ± 4.21*</td>
</tr>
<tr>
<td>SMu1051</td>
<td>lctE</td>
<td>ABC transporter, membrane spanning</td>
<td>4.10 ± 0.83*</td>
</tr>
</tbody>
</table>

*a* ND, not designated.

*b* The quantity of cDNA was determined by real-time RT-PCR and standardized by the abundance of cDNA of 16s rRNA within each reaction. There were statistically significant differences of expression for each gene between the presence (0.375 mg/ml) and absence of antibiotics (*P<0.01, **P<0.001).

**Table 2.** Putative genes of *S. mutans* MT8148 that encode ATP-binding proteins.

![Figure 5. SMu0836 gene expression under various stress conditions. (1) Heat shock, (2) osmotic stress, (3) oxidative stress. MT8148 was grown in THB under the various stress conditions. There were statistically significant differences between the presence and absence of stress (*P<0.05, Fisher's PLSD).](image)
oxidative and osmotic environmental stress. Comparisons of the transcriptional profiles of *S. mutans* growing with aeration versus under anaerobic conditions revealed that many genes are regulated by oxygen or redox potential [27]. Thus, the activity of ATP binding proteins may be important for the survival of some bacteria in specific environments.

*S. mutans* genome analysis has shown that this organism encodes several ABC transporters, of which at least 42 are putative exporter pumps [28]. Several recent studies have also suggested that general ABC transporters are involved in transport of substrates across the membranes. Biswas and Biswas [29] reported that *vltA* and *vltB*, genes that appear to be organized in an operon and encode the putative ABC transporter complex VltA/B, constitute a heterodimer multidrug efflux pump. In another study, the ABC transporters MsmEFGK and MalXFGK, which transport disaccharides and/or oligosaccharides, were shown to be principally involved in the uptake of distinct subsets of carbohydrates [30]. In addition, *sloABCR* has been characterized in *S. mutans* and it was proposed that *sloABC* genes encode the ATP-binding protein, integral membrane protein, and solute-binding lipoprotein of a high-affinity manganese transport system [31]. The present results suggest that SMu0836 and SMu0837 may also function as an efflux pump for antimicrobial agents and in response to environmental stress.

In summary, the present findings suggest that ABC transporters of *S. mutans* are correlated with membrane transport of antibiotics and suggest a novel approach for chemotherapy against certain bacteria. Further investigation of the structures and mechanisms of these proteins are needed to clarify their molecular properties.

**Acknowledgements**

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**References**

27. Ahn SJ, Wen ZT, Burne RA. Effects of oxygen on virulence


