Effect of SMU.2055 Gene on Acid Tolerance of Streptococcus mutans UA159: Postprint

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Abstract

Objective: To construct an SMU.2055 gene-deficient strain of Streptococcus mutans (S. mutans) UA159 and investigate the influence of the SMU.2055 gene on the acid tolerance of S. mutans. Methods: The SMU.2055 gene-deficient strain was constructed via homologous recombination. The A values of both the wild-type and gene-deficient strains in various pH environments were continuously monitored to compare their growth capacities under different pH conditions. Both strains were inoculated into a series of BHI media with pH values spanning from 2.5 to 7.0, cultured anaerobically for 3 h, and their lethal pH values were determined. The glycolytic capacity of both strains was measured by adding excess glucose. Permeabilized cells were prepared to measure cell membrane permeability, proton permeability, and H+-ATPase activity of both strains. Biofilm formation capacity of both strains was observed under confocal laser scanning microscopy. Results: Compared with the wild-type strain, the SMU.2055 gene-deficient strain exhibited significantly decreased early growth activity, H+-ATPase activity at pH 5.5, and biofilm formation capacity, while showing significantly increased lethal pH, proton permeability, and cell membrane permeability; no significant change was observed in glycolytic capacity. Conclusion: The SMU.2055 gene is associated with the acid tolerance of S. mutans. The reduced acid tolerance of the SMU.2055 gene-deficient strain is related to its lethal pH, cell membrane permeability, proton permeability, H+-ATPase activity, and biofilm formation, but not related to its glycolytic capacity. This study establishes a foundation for investigating the function of the SMU.2055 gene.

Full Text

Preamble

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Abstract

Objective: To evaluate the effect of SMU.2055 gene on acid resistance of *Streptococcus mutans*. **Methods:** A SMU.2055-deficient mutant strain of *S. mutans* was constructed using homologous recombination technique. The growth of the wild-type and mutant strains was monitored in both normal and acidic conditions. The lethal pH level, glycolysis, proton permeability, cell permeability and biofilm formation of the two strains were compared. **Results:** PCR and sequence analyses verified the successful construction of the SMU.2055-deficient mutant strain. The growth and biofilm formation capacity of the mutant strain were obviously lowered in both normal and acidic conditions. The mutant strain also showed increased lethal pH level, proton permeability, and cell permeability with impaired H+-ATPase activity in acidic conditions, but its minimum glycolytic pH remained unaffected. **Conclusion:** The SMU.2055-deficient *S. mutans* mutant exhibits a lowered acid resistance, which affects the growth, lethal pH, proton permeability, H+-ATPase activity, cell permeability and biofilm formation but not the minimum glycolytic pH of the mutant strain.

Keywords: *Streptococcus mutans*; acetylase; SMU.2055 gene; acid resistance

Introduction

Streptococcus mutans plays a crucial role in the development of dental caries, and its cariogenicity is closely associated not only with its adhesion to tooth surfaces but also with its acidogenicity and acid tolerance. While the molecular mechanisms underlying *S. mutans* adhesion and acid production have been well characterized, research on its acid tolerance mechanisms, particularly at the molecular level, remains limited. Acid tolerance represents a key survival mechanism that enables bacteria to adapt to the dynamically changing acidic environment of dental plaque. With advances in modern molecular biology techniques, an increasing number of genes and related proteins associated with *S. mutans* acid tolerance have been identified.

Acetylation is a crucial type of protein post-translational modification that, like phosphorylation, is important and ubiquitous in biological systems. It participates in various cellular processes including signal transduction, metabolism, protein degradation, and pathogenicity of microorganisms. Many physiological functions, such as cellular responses to environmental stimuli, are mediated through dynamic protein post-translational modifications. Acetylation modification is also implicated in bacterial function, with acetyltransferases playing important roles in bacterial morphology, metabolism, and stress responses.

The SMU.2055 protein in *S. mutans* is considered a putative acetyltransferase belonging to the GNAT family. Bioinformatic analysis predicts a theoretical isoelectric point of 7.74, with 489 base pairs encoding 163 amino acid residues. These enzymes primarily transfer acetyl groups from acetyl-coenzyme A (Ac-CoA) to amino groups on substrates, selectively acetylating one of four to five amino groups. Previous studies have demonstrated that lysine acetylation enables *Escherichia coli* to resist various environmental stresses, and elevated levels of acetyltransferase YfiQ not only increase cell concentration but also enhance resistance to heat and oxidative stress. Research indicates that modulating protein acetylation status can affect cell wall structure and permeability, influencing bacterial morphology and biofilm formation. Our previous work has successfully cloned, purified, crystallized, and determined the three-dimensional crystal structure of SMU.2055 protein, which has been deposited in the Protein Data Bank (PDB) under accession number 3LD2. Subsequently, we employed computer-aided drug design methods to design and screen five small-molecule compounds with high structural compatibility to SMU.2055 protein, establishing a virtual screening model for *S. mutans* UA159 protein inhibitors aimed at altering the cariogenicity of *S. mutans* through small-molecule inhibitors. However, current research on SMU.2055 gene function remains scarce both domestically and internationally. Our preliminary investigations into the effects of this gene on *S. mutans* morphology and cariogenicity-related biological characteristics revealed that after acid adaptation and acid shock treatment, the SMU.2055-deficient mutant exhibited significantly inhibited growth under acidic conditions and reduced acid tolerance. The mutant also showed compromised cell membrane integrity and altered formation of irregular substances. Impaired cell membrane integrity is known to reduce bacterial acid tolerance. We therefore hypothesize that the SMU.2055 gene is closely associated with *S. mutans* acid resistance. This study aims to investigate the role of SMU.2055 gene in *S. mutans* acid tolerance by examining changes in acid resistance and potential influencing factors in the SMU.2055-deficient mutant, which is significant for identifying effective targets for caries prevention and treatment.

1.1 Strains and Culture Conditions

S. mutans UA159 (provided by the Central Laboratory of Peking University School of Stomatology) was cultured in brain heart infusion (BHI) medium (Qingdao Hope Bio-Technology Co., Ltd.). The SMU.2055-deficient mutant strain was cultured in selective BHI medium containing 1 mg/mL spectinomycin (spe) (Sigma, USA). All strains were cultured at 37°C under anaerobic conditions (10% CO , 10% H , 80% N). *Escherichia coli* DH5 [Tiangen Biotech (Beijing) Co., Ltd.] was cultured in Luria-Bertani (LB) medium (Qingdao Hope Bio-Technology Co., Ltd.), with selective LB medium containing 0.1 mg/mL spectinomycin. *E. coli* was cultured aerobically at 37°C with shaking at 200 r/min.

1.2 Construction of SMU.2055-Deficient Mutant Strain

Based on the complete genome sequence of *S. mutans* UA159, upstream and downstream primers for SMU.2055 gene were designed using Primer Premier 5.0 software (Table 1).

Table 1 Sequences of primers for constructing SMU.2055-deficient mutant

Primers	Nucleotide sequence	Purpose
SMU.2055-UP	F: CGGGATCCTTGCTCACCTCGC-CCTTGTR: CCCAAGCTTCCTTCC-TACGCTCTTTCTC	Amplify the upstream fragment of SMU.2055
SMU.2055-DOWN	F: CATGCCATGGATTAG-GTTTTGATTTAGAAGCR: GGAATTCCATATGAGCAAGAC-CAAACCACCT	Amplify the downstream fragment of SMU.2055

Note: Underlined sequences indicate restriction enzyme cutting sites.

The upstream and downstream fragments of SMU.2055 gene were amplified by polymerase chain reaction (PCR), digested with BamHI and HindIII restriction enzymes, and ligated into pFW5 plasmid (kindly provided by Professor Yu Qing from the School of Stomatology, Fourth Military Medical University). The ligation product was transformed into *E. coli* DH5 competent cells. Positive clones were selected on LB agar plates containing spectinomycin, and plasmids were extracted for sequencing verification. The successfully constructed SMU.2055-deficient recombinant plasmid was then transformed into *S. mutans*. Natural transformation was performed by mixing the plasmid with wild-type *S. mutans* UA159 pre-treated with 1 g/mL competence-stimulating peptide (CSP, synthesized by Shanghai Sangon Biotech Co., Ltd.) [16]. Positive clones were selected on BHI agar plates containing spectinomycin, and genomic DNA was extracted for PCR and sequencing verification.

1.3 Growth Ability of SMU.2055-Deficient Mutant Under Different pH Conditions

Overnight cultures of *S. mutans* UA159 and the SMU.2055-deficient mutant were adjusted to A600 0.8 and inoculated at a 1:100 ratio into BHI liquid medium at pH 7.5 or 5.5. The cultures were incubated anaerobically at 37°C for 24 hours, with A600 measurements taken every hour to generate growth curves [17].

1.4 Determination of Lethal pH for SMU.2055-Deficient Mutant

BHI liquid medium was prepared with pH values ranging from 2.5 to 7.0 at 0.5-unit intervals. Logarithmic-phase bacterial cultures were transferred to BHI

medium at different pH values and incubated anaerobically for 3 hours. Samples were then collected, serially diluted, plated, and viable cell counts were determined after 48 hours. The highest pH at which no bacterial survival was observed was defined as the lethal pH [18].

1.5 Detection of Glycolytic Capacity in SMU.2055-Deficient Mutant

S. mutans UA159 and the SMU.2055-deficient mutant were cultured to early stationary phase, harvested, and resuspended in 50 mmol/L KCl, 1 mmol/L MgCl solution. The suspensions were adjusted to the same A600 and titrated with KOH to above pH 7.2. Glucose was added to a final concentration of 1%, and the pH of the reaction system was monitored every 2 minutes for 30 minutes. The lowest pH reached was recorded as the minimum glycolytic pH [19-20].

1.6 Detection of Cell Permeability in SMU.2055-Deficient Mutant

Overnight cultures of *S. mutans* UA159 and the SMU.2055-deficient mutant were harvested, and 25 mL of each culture was resuspended in 2.5 mL Tris-HCl buffer (75 mmol/L, pH 7.0, containing 10 mmol/L MgSO). Toluene (250 L) was added, and the mixtures were incubated at 37°C for 5 minutes, followed by freeze-thaw cycles. Protein concentrations were determined using a BCA protein assay kit according to the manufacturer' s instructions [21].

1.7 Detection of Proton Permeability in SMU.2055-Deficient Mutant

Overnight cultures of *S. mutans* UA159 and the SMU.2055-deficient mutant were harvested and resuspended in PBS buffer for 2 hours. After centrifugation, the pellets were resuspended in 50 mmol/L KCl, 1 mmol/L MgCl solution to a concentration of 20 mg/mL. The pH was adjusted to below 4.7, and measurements were taken every 10 minutes. At 50 minutes, butanol was added to a final concentration of 10% to disrupt cell membranes and allow equilibration of intracellular and extracellular pH. The final pH was recorded at 80 minutes [22].

1.8 Detection of H+-ATPase Activity in SMU.2055-Deficient Mutant Under Different pH Conditions

Prepared permeabilized cells (at pH 7.5 or 5.5) were resuspended in 75 mmol/L Tris-HCl buffer (pH 7.0, containing 10 mmol/L MgSO). Three milliliters of bacterial suspension was measured at A600 using the same buffer as blank control. Bacterial concentration was calculated based on the established relationship between cell dry weight and absorbance. Enzymatic reactions were performed using an H+/K+-ATPase assay kit according to the manufacturer's instructions, with phosphate determination [1]. One unit of ATPase activity was defined as the amount of enzyme that hydrolyzed ATP to produce 1 mol of inorganic phosphate per hour per milligram of dry cell weight.

1.9 Observation of Biofilm Formation by SMU.2055-Deficient Mutant Under Different pH Conditions

Overnight cultures of *S. mutans* UA159 and the SMU.2055-deficient mutant were inoculated at a 1:100 ratio into 5 mL BHI liquid medium (pH 7.5 or 5.5) and transferred to six-well plates containing sterile cover glasses. After 24 hours of incubation, planktonic cells were removed, and the biofilms were washed, dried, and stained with 200 L of SYTO9 working solution for 15 minutes at room temperature in the dark. The stained biofilms were observed using confocal laser scanning microscopy at 488 nm excitation, with five random fields photographed for each strain [17].

Statistical Analysis

All experiments were performed in triplicate. Data were analyzed using SPSS 20.0 software with independent samples t-test. Statistical significance was set at = 0.05.

Results

2.1 Identification of SMU.2055-Deficient Mutant Strain

PCR amplification of the SMU.2055-deficient mutant yielded a fragment size consistent with that amplified from the corresponding recombinant plasmid template and matched the expected size. Sequencing results showed 99% identity when compared with the NCBI database. PCR identification results are shown in Figure 1 [Figure 1: see original paper].

2.2 Growth Ability of SMU.2055-Deficient Mutant Under Different pH Conditions

Compared with the wild-type strain, the SMU.2055-deficient mutant showed significantly impaired early growth in BHI medium at both pH 7.5 and pH 5.5 (Figure 2 [Figure 2: see original paper]). The wild-type strain entered logarithmic phase at 4 hours (pH 7.5) and 6 hours (pH 5.5), reaching stationary phase at approximately 8 hours and 15 hours, respectively. In contrast, the mutant exhibited extended lag phases, entering logarithmic phase at 7 hours (pH 7.5) and 10 hours (pH 5.5), and reaching stationary phase at approximately 11 hours and 21 hours, respectively. Early-stage A600 values were significantly lower at pH 5.5 than at pH 7.5 for both strains, indicating growth inhibition that was more pronounced in the mutant. However, after 20 hours, final cell densities showed no significant differences between the two strains at either pH.

2.3 Lethal pH of SMU.2055-Deficient Mutant

As pH gradually decreased, the viability of both strains diminished progressively. The lethal pH for wild-type *S. mutans* UA159 was 3.5, whereas that for the SMU.2055-deficient mutant was 4.0.

2.4 Glycolytic pH Reduction in SMU.2055-Deficient Mutant

Upon glucose addition, both *S. mutans* UA159 and the SMU.2055-deficient mutant rapidly metabolized the substrate and produced acid, causing a rapid pH decline in the reaction system. At all time points during the first 18 minutes, the pH of the wild-type reaction system was lower than that of the mutant, indicating a significantly higher early glycolytic rate in the wild-type strain ($P < 0.05$). However, no statistically significant difference was observed in the minimum glycolytic pH between the two strains (Figure 3 [Figure 3: see original paper]), suggesting that SMU.2055 gene deletion did not affect bacterial glycolytic capacity.

2.5 Cell Permeability of SMU.2055-Deficient Mutant

The protein concentration of wild-type *S. mutans* UA159 was 1.2768 ± 0.2639 mg/mL, while that of the SMU.2055-deficient mutant was 1.6297 ± 0.1249 mg/mL. Compared with the wild-type, the mutant exhibited significantly enhanced cell permeability (Figure 4 [Figure 4: see original paper]) ($P < 0.001$).

2.6 Proton Permeability of SMU.2055-Deficient Mutant

The slope of the curve for the SMU.2055-deficient mutant was lower than that of the wild-type strain between 50-80 minutes, with the final pH at 80 minutes being lower than that of the wild-type (Figure 5 [Figure 5: see original paper]) ($P < 0.05$), indicating enhanced proton permeability in the mutant.

2.7 H+-ATPase Activity in SMU.2055-Deficient Mutant Under Different pH Conditions

At pH 7.5, H+-ATPase activity in the SMU.2055-deficient mutant was slightly reduced compared with wild-type *S. mutans* UA159 (Figure 6 [Figure 6: see original paper]), but the difference was not statistically significant ($P > 0.05$). At pH 5.5, however, H+-ATPase activity in the mutant was significantly lower than in the wild-type strain ($P < 0.01$).

2.8 Biofilm Formation by SMU.2055-Deficient Mutant Under Different pH Conditions

At both pH 7.5 and pH 5.5, wild-type *S. mutans* UA159 formed relatively dense biofilms with large bacterial aggregates (Figure 7 [Figure 7: see original paper]). In contrast, biofilms formed by the SMU.2055-deficient mutant were sparser than those of the wild-type, with smaller bacterial aggregates displaying a reticular structure containing irregular circular or oval pores of various sizes. The biofilm-forming capacity of the mutant was significantly lower than that of the wild-type at both pH values. Notably, both strains demonstrated enhanced biofilm formation at pH 5.5 compared with pH 7.5, producing denser biofilms with larger bacterial aggregates that nearly covered the entire field of view.

Discussion

SMU.2055 protein is considered a putative acetyltransferase belonging to the GNAT family, which participates in cell growth regulation, gene transcription activation, and DNA damage repair. Acetyltransferase-catalyzed acetylation represents a major mechanism of protein acetylation, which plays important roles in microbial stress responses and pathogenicity control by regulating protein activity, localization, protein-protein interactions, and protein-DNA interactions. Acetylation modification may be involved in carbon metabolism, fatty acid synthesis, and stress responses, and can influence bacterial morphology and biofilm formation by affecting cell wall structure and permeability—factors closely related to *S. mutans* acid tolerance. We therefore hypothesized that SMU.2055 gene might be associated with *S. mutans* acid resistance.

Cell density can regulate *S. mutans* adaptation to acidic environments, with bacteria in logarithmic phase under planktonic conditions and those in biofilms showing better acid tolerance. In this study, the SMU.2055-deficient mutant exhibited significantly extended lag phases at both pH 7.5 and pH 5.5, indicating that SMU.2055 gene plays a role in *S. mutans* growth. This may be because acetylation modification participates in metabolic processes such as the tricarboxylic acid cycle, and disruption of acetylation can affect bacterial glucose utilization. The observation that final cell densities were similar between the mutant and wild-type suggests the existence of compensatory mechanisms to maintain viability. Both strains showed adaptive acid tolerance, with growth inhibition more pronounced at pH 5.5, particularly in the mutant, indicating reduced resistance to low pH environments.

Biofilm formation represents a bacterial strategy to resist environmental interference, with microbial communities, extracellular polysaccharides, water, and metabolites constituting the biofilm matrix. Organisms within biofilms can supplement nutrient deficiencies and buffer pH changes. Impaired biofilm formation contributes to reduced acid tolerance. In this study, the mutant formed significantly weaker biofilms than the wild-type at both pH values, possibly due to reduced formation of irregular substances between bacterial cells—primarily composed of extracellular polysaccharides and proteins—which affects water-insoluble polysaccharide formation and bacterial adhesion. Additionally, reduced acetylation may affect lipid synthesis and consequently biofilm formation. Both strains showed enhanced biofilm formation at pH 5.5 compared with pH 7.5, likely because low pH increases expression of the *gtfBC* gene unique to *S. mutans* in biofilms, promoting water-insoluble polysaccharide formation and bacterial adhesion.

Lethal pH reflects fundamental acid tolerance and serves as an important parameter for adaptive acid resistance. Our study demonstrated that the SMU.2055-deficient mutant had lower resistance to lethal pH environments than the wild-type, possibly because SMU.2055 deletion affected synthesis of various membrane-associated proteins, thereby compromising cell membrane

integrity and acid tolerance. These proteins include general stress proteins and acid-specific proteins.

A lower minimum glycolytic pH indicates stronger capacity for metabolizing sugars and producing acid in acidic environments, reflecting higher acid tolerance. In this study, the early glycolytic rate of the SMU.2055-deficient mutant was significantly lower than that of the wild-type, but no significant difference was observed in minimum glycolytic pH between the two strains. The absence of SMU.2055 did not affect bacterial glycolytic capacity, possibly because the deletion induced other adenosine transferases to maintain glycolytic function. Alternatively, excess glucose may cause non-enzymatic acetylation reactions that can feedback to affect central metabolic pathways including glycolysis.

Under acidic conditions, *S. mutans* maintains intracellular homeostasis through at least two mechanisms: extrusion of acidic products and expulsion of protons via membrane H+-ATPase hydrolyzing ATP to maintain transmembrane pH gradients, thereby preserving a relatively alkaline intracellular environment and protecting acid-sensitive enzymes and proteins. In this study, enhanced cell permeability in the SMU.2055-deficient mutant may have resulted from disrupted acetylation modification, which can alter bacterial cell wall structure and permeability. Increased cell permeability allows free diffusion of H+ into cells down their concentration gradient, affecting synthesis of acid-sensitive enzymes and proteins and potentially influencing H+-ATPase activity and proton permeability, thereby compromising acid tolerance. Alternatively, SMU.2055 deletion may have affected fatty acid synthesis, and altered membrane fatty acid composition—part of the acid tolerance response—may directly affect proton permeability of the lipid bilayer or indirectly influence it through H+-ATPase activity. The enhanced proton permeability and reduced H+-ATPase activity at low pH in the mutant further support this mechanism.

This study investigated the effect of SMU.2055 gene on *S. mutans* acid tolerance. The reduced acid tolerance of the SMU.2055-deficient mutant was associated with altered lethal pH, cell permeability, proton permeability, H+-ATPase activity, and biofilm formation, but not with glycolytic capacity.

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Note: Figure translations are in progress. See original paper for figures.