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Molecules and Cells

The Influence of *Htr*A Expression on the Growth of *Streptococcus mutans* during Acid Stress

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When proteins are damaged under stresses conditions, these proteins are either refolded or degraded by a quality control system of molecular chaperones and protease. High-temperature requirement A (*htr*A) is of particular interest because it can perform the roles of both a protease and a chaperone. *Htr*A plays an important role in maintaining the physiological homeostasis of bacteria against environmental stress such as elevated temperature, oxidative and osmotic stress. Inactivation of *htr*A genes can thus restrict the survival ability of bacteria. These observations suggested that *htr*A might be responsible for acid tolerance of S*treptococcus mutans*. In this study, we have generated an *htrA* mutant and an *htrA*-complemented strain of *S. mutans* K7 isolated from a Korean in order to investigate the role of *htrA* in growth under acidic conditions. In terms of growth under acidic conditions, the *htrA* mutant exhibited 20% to 23% lower growth than the control group. In addition, *glucosyltransferase*B and *glucosyltransferase*C expression levels significantly decreased. When the *htrA* expression level was restored by adding the *htrA* gene to the *htrA* mutant strain, the normal growth phenotype was restored under acid stress. Further, similar results were obtained for *S. mutans* UA159. Thus, *htrA* in *S. mutans* K7, as well as *S. mutans* UA159, can be concluded to play an important role during acid stress.

INTRODUCTION

Periodontal disease and dental caries are typically caused by bacterial infection (Marsh, 1992). *Streptococcus mutans* is believed to be a principal pathogen responsible for dental caries (Mcneill and Hamilton, 2003). *S. mutans* is a bacterium commonly found in the human oral cavity. It adheres to the tooth-surface membrane and uses sucrose as a substrate and produces lactic acid as an end-product of glycolysis. It also produces insoluble glucan, a glucose polymer, after secreting glucosyltransferase. This insoluble, mucous glucan promotes the adherence of oral bacteria to the teeth and accelerates tooth demineralization by allowing the organic acid to accumulate. Consequently, *S. mutans* causes dental caries by producing a very concentrated acid via carbohydrate metabolism (Trahan, 1995). In many studies, *S. mutans* has been considered to be an acid-resistant bacterium that can efficiently metabolize carbohydrates, even under low pH conditions (Len et al., 2004). In *S. mutans*, it appears that acid stress-related mechanisms confer virulence, which is critical for causing dental caries (Mcneill and Hamilton, 2003).

With regards to stress, gram-negative and gram-positive bacteria both have quality-control systems that function even under adverse environmental conditions. When proteins are damaged and unfolded under stress conditions, they are either folded back into their functional states by molecular chaperones or degraded into smaller peptides by proteases (Gottesman, 2003; Yu et al., 2005). It has been reported that high-temperature requirement A (*htr*A) performs the roles of proteases and chaperones and plays an important role in maintaining physiological homeostasis in organisms under environmental stress. A major role of this protein is to help organisms survive environmental stresses such as elevated temperature, or oxidative or osmotic stress. Inactivation of the *htrA* gene can thus decrease the survival of bacteria; this fact has been exploited to generate attenuated mutants of a number of pathogens (Stack et al., 2005; Wilson et al., 2006).

The *htr*A family of serine proteases is widely distributed, and homologues have been found in bacteria, yeast, plants, and humans (Pallen and Wren, 1997). The proteins characteristically possess an amino-terminal hydrophobic region; a trypsin-like catalytic domain with conserved His, Ser, and Asp residues; and a PDZ domain thought to be involved in the formation of multimeric enzyme complexes and targeting. Gram-positive cocci, including *S. mutans*, each have a single *htrA* gene that includes a single PDZ domain at the carboxyl-terminus (Sassoon et al., 1999).

Interestingly, *htrA* has been reported to have diverse functions in different types of bacteria. The *htrA* genes in *Brucella abortus*, *Lactococcus lactis*, and *S. pyogenes* protect against heat and oxidative stress; whereas the *htrA* genes in *Escherichia coli* and *Yersinia pestis* are related only to heat stress (Elzer et al., 1996; Johnson et al., 1991; Lipinska et al., 1989; Skorko-Glonek et al., 1995; Tatum et al., 1994).

*Htr*A sensitivity to thermal and oxidative stress varies, even within a single species; the *htrA* mutant of *S. pneumoniae* strain

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Table 1. Bacterial strains and plasmids in this study

Strain or plasmid	Relevant characteristic (s)	Source or reference
S. mutans strains		
UA159	Wild type	ACTT, USA
K7	Clinically isolated type	KCTC, Korea
SAB2-13	Δ*htr*A::NPKm^r	R. A. Burne, Univ. of Florida
SKHM	Δh*tr*A::NPKm^r	This study
SKHMC	*htrA*-complemented strain, Em^r	This study
Plasmids		
pLAH124	Vector harboring an NPKm^r cassette	R. A. Burne, Univ. of Florida
pMSP3535	*E. coli-Streptococcus* shuttle vector, Em^r	R. A. Burne, Univ. of Florida

D39 exhibited sensitivity to both kinds of stresses (Ibrahim et al., 2004), but the *htrA* mutant of strain TIGR4 did not (Ahn et al., 2005). In the case of *S. mutans*, Ahn et al. (2005) reported that the *htrA* gene is related to heat stress only in *S. mutans* UA 159 and has little involvement in protection against acid and oxidative stress. In contrast, Banerjee and Biswas (Banerjee and Biswas, 2008) reported that the *htrA* gene in UA 159 plays an important role in protection against heat, oxidative and acid stresses. Similarly, Biswas and Biswas (Biswas and Biswas, 2005) reported that *htrA* is involved in protection against acid, oxidative, and salt stresses in NG-8.

These observations suggest that *htrA* might be responsible for acid tolerance in *S. mutans*. Studies investigating the adaptive and defensive mechanisms against acid in acid-tolerant bacteria have revealed that these mechanisms have a critical impact on pathogenicity (Lee et al., 1994). In *S. mutans*, as well, the virulence is closely related to its acid tolerance (Mcneill and Hamilton, 2003). To date, most of the studies investigating the physiological, functional, and genetic characteristics of acid tolerance in *S. mutans* have been conducted using Caucasian subjects (Len et al., 2004; Skorko-Glonek et al., 1995; Trahan, 1995). It is very difficult to find a study on the acid tolerance of *S. mutans* conducted on Asians. No such study has been carried out with Korean subjects.

Therefore, this study aims to investigate the acid stress-induced changes in *htrA* expression in *S. mutans* that had been isolated from a Korean child with caries, and to investigate the influence of *htrA* expression on the growth of *S. mutans* during acid stress. We found that *htrA* plays a central role in biofilm formation, as well as acid-stress survival, in *S. mutans*. Further, the growth of *S. mutans* under acidic conditions was significantly influenced by *htrA* expression.

MATERIALS AND METHODS

Bacterial strains

S. mutans K7 (KCTC 13616) (Kang et al., 2007) isolated from a Korean child with caries was used. *S. mutans* UA159 (ATCC 71610) obtained from the American Type Culture Collection was used as a control. The bacterial strains and plasmids used in the present study are listed in Table 1. *S. mutans* UA159 and K7 were grown in brain heart infusion (BHI) broth (Difco) (Ahn et al., 2005). To select antibiotic-resistant colonies following genetic transformation, erythromycin (10 μg/ml) or kanamycin (1,000 μg/ml) was added to the medium.

Growth conditions

S. mutans was grown in BHI broth at 37°C for 12 h. Then, 1% of the cultured *S. mutans* was inoculated into fresh BHI broth and grown at 37°C. The optical density (OD) was measured at 600 nm by using a spectrophotometer (Ultrospec 2100 Pro, Amersham Bioscience, USA). For acid-stress treatments, lactic acid was added to BHI broth, depending on the concentration level required.

To evaluate the capacity of the strains to form colonies at low pH, cells were grown in BHI medium to an OD_{600} of 0.5; 5 μl of the cell suspension was spotted on BHI agar plates containing either 0, 4, 8, 12, 16, 20, or 24 mM lactic acid. All plates were incubated at 37°C for 72 h.

Identification of *htr*A from *S. mutans* K7 by polymerase chain reaction

To isolate chromosomal DNA, *S. mutans* was incubated in BHI broth at 37°C for 72 h. Genomic DNA was obtained by using a genomic DNA isolation kit (Intron, Korea).

The *htrA* gene region was amplified from the genomic DNA of *S. mutans* K7 by using forward (5′-ACAAGCAAATAAGCG-ATTTATC-3′) and reverse (5′-CTATTGAAGAAGCCAAAGC-TT-3′) primers designed on the basis of the *S. mutans* UA159 genome (GenBank accession no. AE014133). The polymerase chain reaction (PCR) product was purified with a QIAquick PCR purification kit (Qiagen, Germany). The sequence was then analyzed by Macrogen (Korea).

Real-time PCR

Total RNA was extracted using TRIreagent (Molecular Research Center Inc., USA), according to the manufacturer's instructions. RNA purity and concentration were measured by using the NanoDrop ND-1000 (NanoDrop Technologies, USA).

Total RNA (1 μg) was subjected to reverse transcription to synthesize cDNA using prime script cDNA Synthesis (Takara Shuzo Co., Japan). The experiment was performed in accordance with the manufacturer's instructions.

All primers used in real-time PCR were produced using Primer Express V1.5 software (Applied Biosystems, USA), as shown in Table 2. SYBR Green PCR Master Mix (Qiagen GmbH, Germany) and an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) were used for real-time PCR. The PCR protocol was as follows: pre-denaturation at 50°C for 2 min, dissociation at 95°C for 10 min, and polymerization at 95°C for 15 s and at 60°C for 1 min. The process was repeated for 40 cycles. For internal calibration of gene expression level, 16S rRNA was been used. The relative gene expression level was analyzed using the ΔΔCt method (Lee et al., 2008; Livak and Schmitteng, 2001).

Construction of *htr*A mutant strain

To construct *htrA* mutant strains, the entire *htrA* gene in *S. mutans* K7 was replaced by a nonpolar kanamycin resistance

Table 2. Primers used for in this study

Primer	Nucleotide sequences (5′ → 3′)	Amplicon
Primers for construction of mutant strain		
HtrA-A	TCCCTCCAATAACGAAGGTACA	5′ end
HtrA-BamHI-B	AAAGGGTGAGGATCCGTATTATTCA	5′ end
HtrA-BamHI-C	TCTATCGCGGGATCCATAAACAAAC	3′ end
HtrA-D	TTGCAGTCGAGGTTGATAGGG	3′ end
Primers for construction of complemented strain		
C-*Htr*A-BamHI-FW	CTTAGACTAGGATCCATAAATTTTG	
C-*Htr*A-XhoI-RV	TTTATAAAGCTCGAGTGTTTTATCT	
Primers for real-time PCR		
*Htr*A-FW	GCCCATTCCTTCAACAGCAAC	
*Htr*A-Rv	CAGACGCAGCTATTAACCCTG	
*Gtf*B-FW	AGCAATGCAGCCAATCTACAAAT	
*Gtf*B-RV	ACGAACTTTGCCGTTATTGTCA	
*Gtf*C-FW	GGTTTAACGTCAAAATTAGCTGTATTAGC	
*GtfF*C-RV	CTCAACCAACCGCCACTGTT	

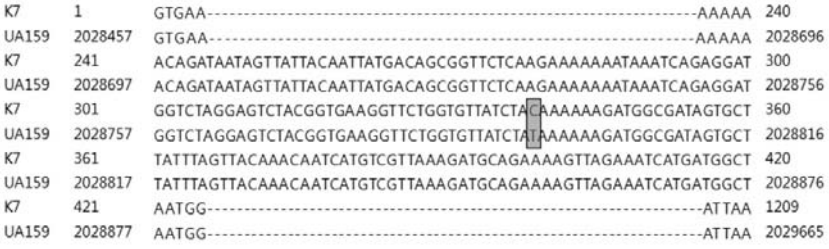

Fig. 1. Alignment of the *htr*A gene from *S. mutans* K7 and UA159. The *htrA* gene is from nucleotide 2028457 to 2029665 on the completed *S. mutans* UA159 genome. A gray box represents a portion with a different base pair. K7: *S. mutans* K7; UA159: *S. mutans* UA159.

cassette (NPKm^r) released from pALH124 (Ahn et al., 2006) by using a PCR ligation-mutagenesis approach (Lau et al., 2002). The NPKm^r was obtained as a *Bam*HI fragment and was ligated to two PCR fragments flanking *htrA*, both of which had also been digested with *Bam*HI. The PCR fragments flanking *htrA* were obtained by using four primers–HtrA-A, HtrA-*Bam*HI-B, HtrA-*Bam*HI-C, and HtrA-D–as listed in Table 2. A ligation mix was used to transform *S. mutans* K7. Transformants were selected on BHI agar containing kanamycin, and double-crossover *htrA* mutants were confirmed by PCR amplification and sequencing.

Construction of an *htrA* complemented strain

To ensure that the *htrA* mutant phenotypes were due solely to the absence of *htrA*, we established an *htrA*-complemented strain on the base of the method proposed by Ahn et al. (Ahn et al., 2005). The entire *htrA* gene was amplified from *S. mutans* K7 genomic DNA by using the primers C-HtrA-*Bam*HI-FW and C-HtrA-*Xho*I-RV (Table 2). The amplicon was digested with *Xho*I and *Bam*HI, ligated into the same restriction sites in plasmid pMSP3535 (Bryan et al., 2000), and transformed into the *htrA* mutant strain. Transformants were selected on BHI agar containing erythromycin, and gene integration was confirmed by PCR amplification and sequencing.

RESULTS AND DISCUSSION

Identification of the *htrA* gene in *S. mutans* isolated from a Korean child

PCR was performed on the basis of the chromosomal DNA in *S. mutans* UA159, for which a genome database had been established in 2002 (Ajdic et al., 2002); PCR was performed after isolating chromosomal DNA from *S. mutans* K7. The PCR product was subjected to direct sequencing. The *htrA* gene sequence was then confirmed. Homolog searches were performed using the BLAST programs provided by the National Center for Biotechnology Information.

S. mutans UA159 and K7 *htrA* genes exhibited 99.9% homology, with a difference of one base pair (Fig. 1). Base pair analysis of the *S. mutans* K7 *htrA* gene showed a high degree of homology with the *S. mutans* UA159 *htrA* gene. Starting at nucleotide 2028457 on the completed *S. mutans* UA159 genome, a difference was detected only in the 339th nucleotide. Thymine, the 339th base, has been converted into cytosine. However, there has been no change in the amino acid sequence. Compared with the *htr*A amino acid sequence of *S. mutans* UA159, that of *S. mutans* k7 was completely (100%) matched. *HtrA* was identified in the complete genome of *S. mutans* UA159, from nucleotide 2028457 through to 2029665. The *S. mutans htrA* gene is a 1,209-bp open reading frame encoding 402 amino-acid residues. The *htr*A protein showed high levels of similarity with htrA proteins of *S. pneumoniae* (50% identity), *L. lactis* (49%), and *E. coli* (37%) (Ahn et al., 2005). Jones et al. (2001) also reported that *htr*A is a highly conserved periplasmic protease that is detected in most bacteria, and that htr*A* homologs influenced stress responses by degrading abnormal proteins.

Growth under acidic conditions

To determine the growth phenotype in an acidic environment, *S. mutans* K7 was incubated in BHI broth containing 10 mM (pH 6.8), 15 mM (pH 6.0), 20 mM (pH 5.8), or 25 mM (pH 5.4) lactic acid at 37°C. Observations were made hourly at OD_{600}

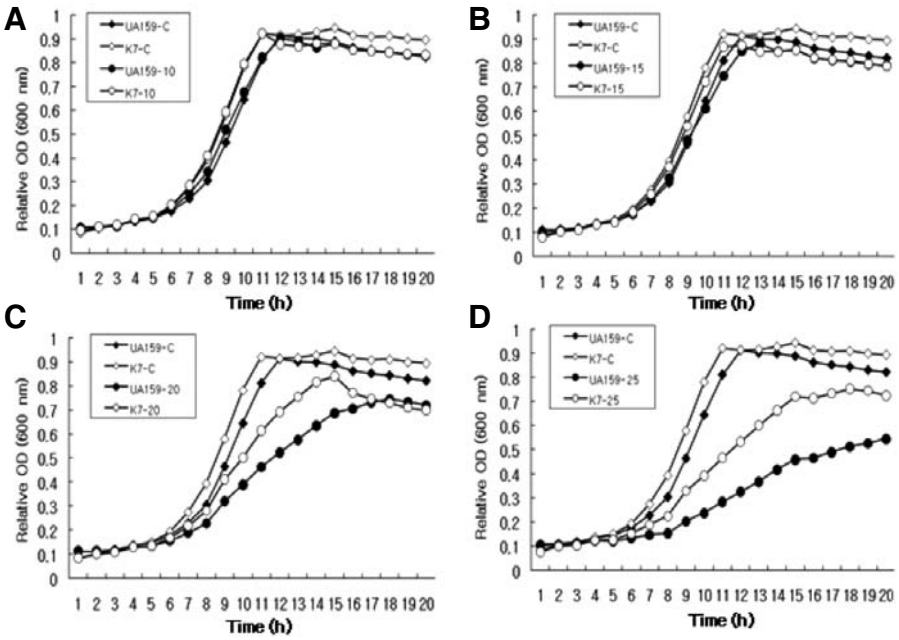

Fig. 2. Growth curve of *S. mutans* under acidic conditions. Lactic acid was added to BHI media (pH 7.2) at different concentrations: 10 mM (pH 6.8), 15 mM (pH 6.0), 20 mM (pH 5.8), or 25 mM (pH 5.4). Cells were grown for 20 h at 37°C. And the growth level was determined by optical density at 600 nm. Cells were treated with 0 mM (-C) and 10 mM lactic acid (-10) (A), 0 mM (-C) and 15 mM lactic acid (-15) (B), 0 mM (-C) and 20 mM lactic acid (-20) (C) and 0 mM (-C) and 25 mM lactic acid (-25) (D). UA159: *S. mutans* UA159; K7: *S. mutans* K7.

(Fig. 2). The control strains were *S. mutans* K7 isolated from a Korean child with caries and wild-type *S. mutans* UA159; these strains had been grown in lactic acid-free broth.

In the broths containing 10 and 15 mM lactic acid, the growth of the wild-type and K7 strains was similar to that of the control group (Figs. 2A and 2B). In the broth containing 20 mM lactic acid, growth rate of K7 decreased by 12% and that of the wild type decreased by 19%, as compared to the control group (Fig. 2C). The growth rate of K7 and the wild type decreased by 18% and 43% respectively in the broth containing 25 mM lactic acid, as compared to the control group (Fig. 2D). Furthermore, in the broth containing 25 mM lactic acid, wild type showed a considerable decrease in growth rate, which was decreased by 43%, compared to the K7 strain. Therefore, it was confirmed that the K7 strain is more acid-tolerant than the wild-type strain.

HtrA expression under acid stress

To determine the influence of the *htrA* on acid tolerance in *S. mutans* K7, lactic acid was added to the BHI broth when the OD_{600} was 0.5; this was followed by incubation at 37°C for 1 h. Real-time PCR was then performed after extracting RNA. *S. mutans* K7 and UA 159 that had been grown in a lactic acid-free broth were used as controls.

The comparison of *htrA* mRNA expression levels by real-time PCR revealed a significant increase in the experimental groups grown in acidic conditions. When lactic acid was added, the *htrA* expression level increased 6.1-fold in K7; in UA159, it increased 4.2-fold (Fig. 3). When K7, in which acid tolerance was stronger, was compared to UA159, the former was found to have the higher *htrA* mRNA expression level under acid stress. During acid stress, *htr*A was overexpressed in *S. mutans* K7 and UA159. This acid stress induction in *S. mutans* suggests that *htr*A plays an important role in acid tolerance of *S. mutans* K7 as well as UA159.

Acid stress-sensitive growth phenotype of *htrA* mutant strain

To verify the role of *htrA* in *S. mutans* during acid stress, an *S. mutans* K7 *htrA* mutant (SKHM) was created. The *S. mutans* UA159 *htrA* mutant (SAB2-13) was provided by Dr. R.A. Burne

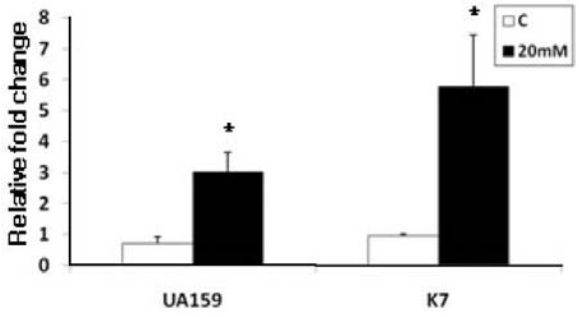

Fig. 3. Real-time PCR quantification of *htrA* mRNA under acidic conditions. Real-time PCR was used to measure *htrA* mRNA in *S. mutans* UA159 and *S. mutans* K7 which had treated for 1 h with 20 mM lactic acid at the exponential growth phase. Data are expressed as mean ± SEM values. *P < 0.05 vs. control. UA159: *S. mutans* UA159; K7: *S. mutans* K7; C: control, not treated with lactic acid; 20: treated with 20 mM lactic acid at the exponential growth phase.

of the University of Florida.

SKHM was generated by replacing the entire *htrA* gene with an nonpolor kanamycin resistance cassette (Figs. 4A and 4B). Transformants were selected on BHI agar containing kanamycin, and the double-crossover *htrA* mutant was confirmed by PCR amplification and sequencing. The forward (HtrA-A) and reverse (HtrA-D) primers (Table 2) used in the PCR verification of *htrA* deficiency. The primers generated a 2.2-kb fragment from the parental strain and a 1.8-kb fragment from the *htrA* mutant strain (Fig. 4C). Sequence analysis of the PCR product, a 1.8-kb fragment, confirmed that an $NPKm^r$ was inserted to the *htrA* gene area (data not shown). It was also confirmed via real-time PCR that *htrA* expression had not been detected in SKHM (Fig. 5C).

To examine the growth phenotypes of SKHM in an acidic environment, 10 or 15 mM lactic acid was added to the BHI broths, followed by incubation at 37°C. Observations were made hourly at OD_{600}. The parental strains, *S. mutans* K7 and UA159, were

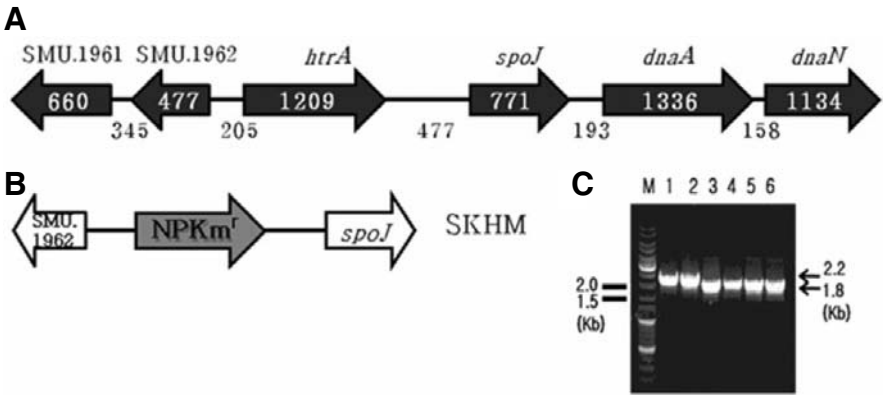

Fig. 4. Schematic representation of the *htr*A region and construction of *htr*A mutant strain. The size of each region is indicated by numbers (bp) based on the genomic sequence information of *S. mutans* UA159. Arrows indicate the directions of transcription (A). The entire *htrA* gene in *S. mutans* K7 was replaced by a nonpolar kanamycin resistance cassette (NPKm^r) (B). The primer pairs used for PCR verification of *htr*A mutant generate a 2.2-kb fragment from the parental strain, *S. mutans* K7, and 1.8-kb fragment from the *htr*A mutant strain (C). SKHM: *htrA* mutant strain of *S. mutans* K7.

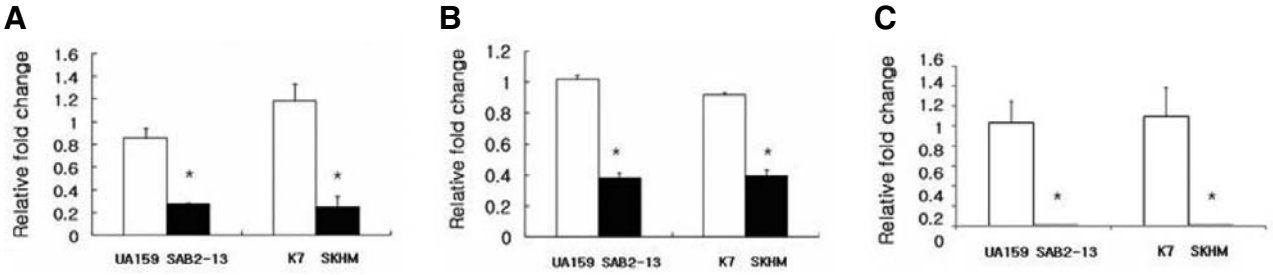

Fig. 5. Real-time PCR quantification of *gtf*s and *htrA* mRNA. In parental strains (*S. mutans* UA159 and K7) and *htrA* mutant strains (SBB2-13 and SKHM), real-time PCR was used to measure gtfB mRNA (A), and gtfC mRNA (B), *htrA* mRNA (C). Data are expressed as mean ± SEM values. *P < 0.05 vs. control. UA159: *S. mutans* UA159; K7: *S. mutans* K7; SAB2-13: *htrA* mutant strain of *S. mutans* UA159; SKHM: *htrA* mutant strain of *S. mutans* K7.

used as controls.

When lactic acid (10 or 15 mM) was added, a significant decrease in growth rate was detected between SKHM and its parental strain, unlike the results shown in Fig. 2. Specifically, decreases of 20% (Fig. 6A) and 23% (Fig. 6B) in growth rate were observed between SKHM and its parental strain, respectively. In SAB2-13 as well, the growth decreased in a manner similar to the decrease in growth rate in SKHM (Figs. 6A and 6B).

To determine the sensitivity of mutant strains to acid, SKHM and SAB2-13 were treated with various concentrations of lactic acid ranging from 0 to 24 mM. In terms of survival, significant differences were detected between SKHM and SAB2-13 and their respective parental strains (Fig. 7). The results indicate that strains lacking a functional *htrA* were more sensitive to an acidic environment. This means that *htrA* is essential for the survival of *S. mutans* K7, as well as *S. mutans* UA159, under acid stress. To understand how *htr*A participates in acid stress responses requires further investigation.

Effect of *htr*A deficiency on the expression of glucosyltransferase enzymes

S. mutans produces glucan from sucrose via glucosyltransferase (*gtf*) enzymes. This insoluble, mucous glucan becomes the framework of biofilm formation and accelerates decalcification of the tooth structure by allowing germ adherence to the tooth surface and the local accumulation of organic acids. To determine the influence of *htr*A deficiency on *gtf*s expression, *gtf*B mRNA and *gtf*C mRNA expression levels in SKHM were compared to a control group by using real-time PCR.

The data showed differential expressions of *gtf*B mRNA and *gtf*C mRNA in SKHM and control strain. Compared to the control group, *gtf*B and *gtf*C expressions had decreased 4.7-fold (Fig. 5A) and 2.3-fold (Fig. 5B), respectively. In SAB2-13 as well, the *gtf*B and *gtf*C expression levels decreased in a manner similar to the decrease in *gtf*B and *gtf*C expression levels in SKHM (Figs. 5A and 5B).

The enzymes *gtf*B and *gtf*C are involved in the formation of α1,3-linked adhesive glucan. Glucans are of central importance in dental plaque formation and in the pathogenesis of dental caries because they are primarily responsible for establishing the extracellular polysaccharide matrix and facilitate adhesion and accumulation of microorganisms to the tooth surface (Ooshima et al., 2001). Ahn et al. (2005) reported that *gtf*B mRNA and *gtf*C mRNA expression levels decrease in the *S. mutans* UA159 *htrA* mutant; whereas Biswas and Biswas (2005) assert that *gtf*B mRNA expression levels increase in the *htrA* mutant strain of *S. mutans* NG-8. In this study, it has been confirmed that *gtf*B and *gtf*C mRNA expression levels decreased in SKHM, as well as in SAB2-13. The results suggested that *S. mutans htr*A impacts biofilm formation mediated by water-insoluble glucans synthesized by *gtf*B and *gtf*C; however, the coordination mechanism among these genes is unknown. Further study of this mechanism is required.

Complementation of the *htrA* mutation

To verify whether the different phenotypes observed in the *htrA* mutant were only caused by an *htr*A deficiency, we integrated the *htrA* gene into SKHM by using pMSP3535. Transformants were selected on BHI agar containing erythromycin, and the integration of the gene was confirmed by PCR amplification and sequencing. PCR was conducted using a forward primer (5′-AGGTGCATCACCACGCATTA-3′) and a reverse primer

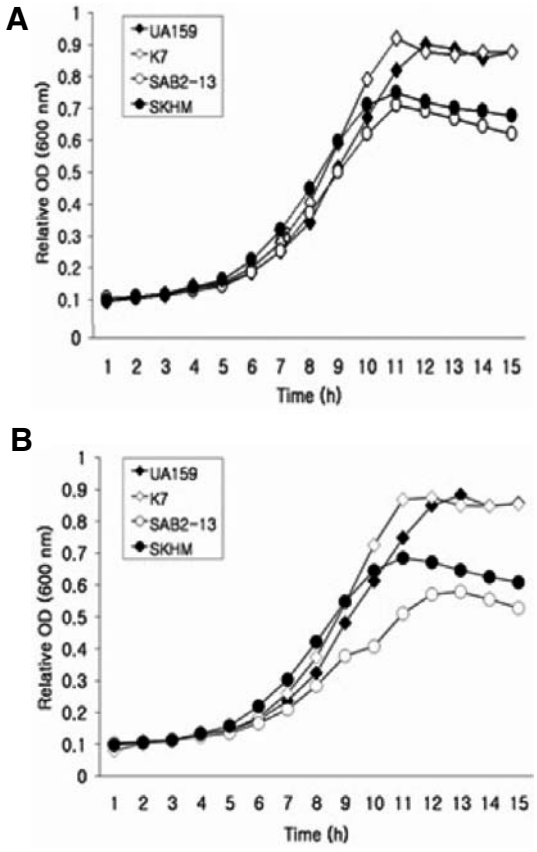

Fig. 6. Growth phenotypes of *htrA* mutant strains under acidic conditions. Lactic acid was added to BHI media at different concentrations: 10 (A) and 15 mM (B). Cells were grown for 15 h at 37°C. And the growth level was determined by optical density at 600 nm. UA159: *S. mutans* UA159; K7: *S. mutans* K7; SAB2-13: *htrA* mutant strain of *S. mutans* UA159; SKHM: *htrA* mutant strain of *S. mutans* K7.

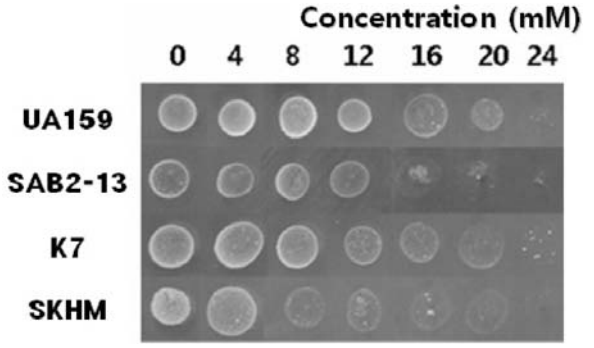

Fig. 7. Sensitivity test of *htr*A mutant strains against acid. Strains were grown in BHI medium to an OD_{600} of 0.5; 5 μl of the cell suspension was spotted on BHI agar plates containing either 0, 4, 8, 12, 16, 20, or 24 mM lactic acid. All plates were incubated at 37°C for 72 h. UA159: *S. mutans* UA159; K7: *S. mutans* K7; SAB2-13: *htrA* mutant strain of *S. mutans* UA159; SKHM: *htrA* mutant strain of *S. mutans* K7.

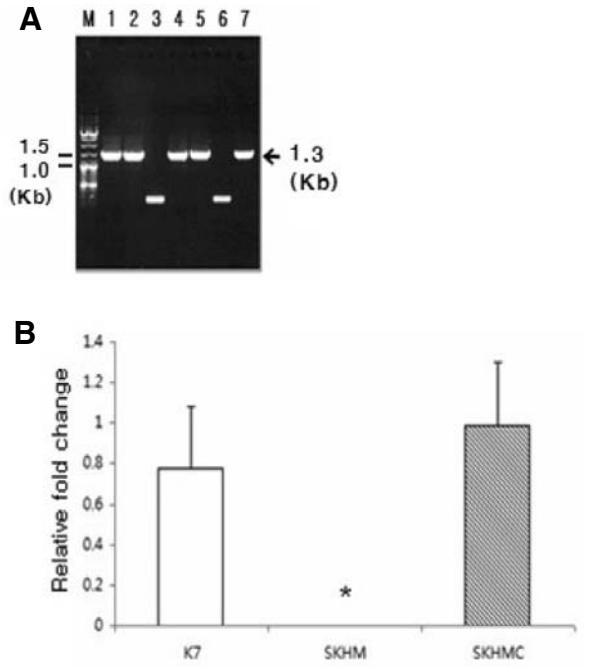

Fig. 8. PCR verification of *htr*A-complemented strain and Real-time PCR quantification of *htr*A m-RNA. After integrating the *htr*A gene into *htr*A mutant strain using pMSP3535, the integration was confirmed by PCR. The primer pairs used for this amplification generated a 1.3-kb fragment from *htr*A-complemented strain (A). Line 1, 2, 4, 5, 7: Successful insertion of htrA gene to *htr*A mutant strain; Line 3, 7: Unsuccessful insertion of htrA gene to *htr*A mutant strain. In *htr*A mutant strain and *htr*A-complemented strain, real-time PCR was used to measure *htr*A mRNA expression (B). Date are expressed as the mean ± SEM. *P < 0.05 vs, control. K7: *S. mutans* K7; SKHM: *htr*A mutant strain of *S. mutans* K7; SKHMC: *htr*A-complemented strain, treated with 40 ng of nisin.

(5′-CAATAC GCAAACCGCCTCTC-3′) that had been designed on the basis of the plasmid pMSP3535. The primer pairs used for this amplification generated a 1.3-kb fragment from the *htrA*-complemented strain (SKHMC), which had been created by integrating the *htrA* gene into the SKHM using pMSP3535 (Fig. 8A). Sequence analysis of the PCR product, the 1.3-kb fragment, confirmed that the *htrA* gene had been added (data not shown).

pMSP3535 is a vector including a nisin-inducible promoter. When 40 ng of nisin was added to BHI broth, the *htrA* mRNA expression level in SKHM was almost similar to that in the control group; this indicated that the levels of *htrA* mRNA in the SKHMC were restored to those found in the control (Fig. 8B). Furthermore, when 40 ng of nisin was added to the broth, the normal growth phenotype was restored in SKHMC under acid stress (Fig. 9). This suggest that *htrA* is essential for survival of *S. mutans* K7 in an acidic environment.

HtrA, a multifunctional chaperone and protease, has been shown to contribute in large part to protein quality control in the periplasmic spaces of bacteria. Streptococci possess a single *htrA* homolog. Bacterial *htrA* has a known housekeeping function in the turnover of damaged or malfolded proteins that may accumulate, particularly under stress conditions such as salt, heat, oxidative, acid, or penicillin G-induced stresses (Wilson et al., 2006). *HtrA* is a member of a regulon that has a promoter sequence utilized by RNA polymerase containing σ^E, the stress response sigma factor. Also, *htrA* is regulated by the CpxAR two-component regulatory system, which senses and responds

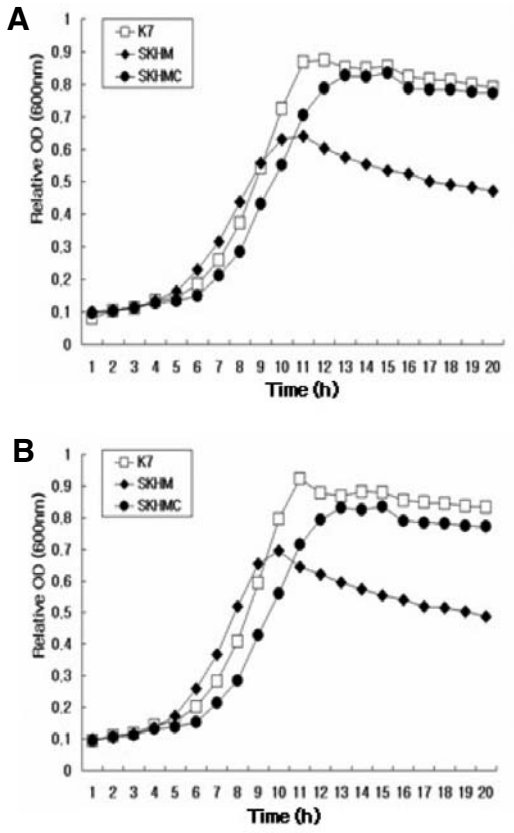

Fig. 9. Growth phenotypes of *htr*A-complemented strain under acidic environment. Lactic acid was added to BHI media at different concentrations: 10 (A) and 15 mM (B). Cells were grown for 20 h at 37°C. And the growth level was determined by optical density at 600 nm. K7: *S. mutans* K7; SKHM: *htr*A mutant strain of *S. mutans* K7; SKHMC: *htr*A-complemented strain, treated with 40 ng of nisin.

to periplasmic stress, such as protein misfolding and aggregation (Jones et al., 2001). *Htr*A comprises the N-terminal protease domain and one or two PDZ domains at the carboxyl terminus. The protease domain of *htr*A shares the same fold with the chymotrypsin-like serine proteases. The PDZ domains appear to be involved in the modulation of protease activity or in the recognition of stress signals (Kim et al., 2008). Recently, it was reported that the inactivation of surface-associated *htr*A protease generated multiple stress-sensitive phenotypes in *S. mutans*; it was also linked to altered biofilm formation and reduced genetic competence (Biswas and Biswas, 2005; Deng et al., 2007).

In the present study, we identified the *htrA* gene in *S. mutans* K7 isolated from a Korean child with caries; we revealed that *htr*A mRNA expression level significantly increased in the experimental groups grown in acidic condition. Further, we also generated an *htrA* mutant and an *htrA*-complemented strain to study the influence of *htrA* under acidic conditions. SKHM showed significant differences in growth and phenotypic characteristics compared to the control strain. These observations are consistent with *htrA* being involved in stress response. In terms of growth under acidic conditions, the *htrA* mutant exhibited lower growth than the control group. In addition, *gtf*B and *gtf*C expression levels, both of which are typically involved in biofilm formation, significantly decreased. When the *htrA* expression level was restored by adding the *htrA* gene to the *htrA* mutant strain SKHM, the normal growth phenotype was restored under acid stress. Such results suggest that *htrA* plays an important role during acid stress in *S. mutans* K7. Furthermore, similar results were obtained for *S. mutans* UA159. Thus, *htrA* in *S. mutans* K7, as well as *S. mutans* UA159, can be concluded to influence acid tolerance.

Notably, the close relationship between stress responses and biofilm formation suggests that the stress regulon of *S. mutans* may be responsible for controlling a broader set of biological functions. The results of this study will provide the basic data required for understanding the acid-tolerance mechanism of *S. mutans*, obtained from the oral cavity of a Korean. Furthermore, the data obtained in this study could serve as useful information in future acid stress-related studies. *HtrA* appears to be part of a regulatory network that coordinates cellular growth, stress tolerance and biofilm formation in *S. mutans*. Therefore, further studies need to be conducted on the role of *htrA* in growth, stress tolerance, and biofilm formation.

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