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Author(s)	Arifah, Chieko
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# **Thesis**

## **Identification and Functional Analysis of Ammonium Transporter in *Streptococcus mutans***

Arifah Chieko

Department of Pediatric Dentistry

Course for Molecular Oral Biology and Dentistry

Osaka University Graduate School of Dentistry,  
Osaka, Japan

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## Introduction

Membrane transporters are commonly found in living organisms and comprise one of the largest protein families, while their components are encoded by approximately 5% of the *Escherichia coli* and *Bacillus subtilis* genomes (Linton and Higgins, 1998; Young and Holland, 1999). 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. Bacterial transport systems enable bacteria to accumulate needed nutrients and extrude unwanted products, thus allowing bacteria to survive stress and create conditions conducive for growth and development (Padan, 2009). Merrick *et al.* (2006) noted that transport of ammonia across biological membranes is a key physiological process found in all domains of life. In addition, ammonium transporters have been described as important in supporting optimal growth rates for cells for ammonium uptake, especially when the concentration of  $\text{NH}_3$  is quite low (Marini *et al.*, 1997 and Soupene *et al.*, 1998).

*Streptococcus mutans*, a Gram-positive facultative anaerobic bacterium, is considered to be a major etiologic agent of human dental caries and reported to form biofilms known as dental plaque on tooth surfaces (Loesche, 1986). This organism also possesses a large number of transport proteins in the cell membrane for export and import of molecules (Ajdić *et al.*, 2002). Nitrogen is an essential nutrient for Gram-positive bacteria, though alternative nitrogen sources such as ammonium can also be utilized (Kleiner, 1985). Therefore, organisms have evolved highly effective systems for nitrogen acquisition and efficient utilization of scarce resources is ensured by a system of selective use of nitrogen sources (Detsch and Stülke, 2003). In order to obtain

nitrogen for macromolecular synthesis, nitrogen-containing compounds must be transported into the cell and, if necessary, degraded to either  $\text{NH}_4^+$  or glutamate. The expression of enzymes required for the utilization of nitrogen-containing compounds is generally induced by their substrates. In addition, the expression of many degradative and transport systems is regulated in response to nitrogen availability in the growth medium (Reitzer and Magasanik, 1987). Nitrogen metabolism in Gram-positive bacteria has been reported in a variety of studies (Merrick and Edwards, 1995; Leigh and Dodsworth, 2007 and Amon *et al.*, 2010) and demonstrated to be linked to virulence in *Staphylococcus aureus* (Pohl *et al.*, 2009). However, the ammonium transporter (Amt) in *S. mutans* remains to be characterized.

Ammonium transport linked to nitrogen uptake is regulated via AmtB, a well-conserved ammonium transport membrane protein present in many bacterial species (Merrick and Edwards, 1995). In *B. subtilis*, the NrgA protein encoded by the *nrgA* gene expresses the ammonium transporter, which is required for transport and utilization of ammonium at low concentrations (Detsch and Stülke, 2003). Analysis of the complete genome of *S. mutans* strain UA159 in the Oralgen database (<http://oralgen.lanl.gov/oralgen-tng/>) indicates that the *SMu1510* gene corresponds to *nrgA* in *S. mutans*, functioning as the ammonium transporter, since it is homologous to the ammonium transporter gene in *B. subtilis* (Fig. 1). In addition, regulation of nitrogen metabolism is coordinated by the PII type signal transduction proteins GlnB and GlnK, which control the activities of the membrane transport proteins and a transcription factor (Arcondéguy *et al.*, 2001). The PII protein family is composed of proteins that regulate enzyme activity, gene expression and are involved in nitrogen regulation, as well as glutamine synthesis activities in bacterial species (Hsieh *et al.*, 1998 and Coutts

*et al.*, 2002). GlnK is homologous to GlnB and can substitute for GlnB to some degree (Van Heeswijk *et al.*, 1996). In *S. mutans* UA159, *SMu1509* is located upstream of the *nrgA* gene and predicted to be *glnB*, a member of the PII protein family. The nitrogen regulatory proteins PII and AmtB are often paired and found in most bacteria (Wolfe *et al.*, 2007).

For most bacteria, glutamine is an optimal source of nitrogen (Hu *et al.*, 1999). It is synthesized from ammonium, which is a major pathway for cells to assimilate the nitrogen required for biosynthesis of all amino acids, thus affecting protein synthesis and the structural and functional integrity of the cell (Van Heeswijk *et al.*, 1996). *B. subtilis* uses alternative nitrogen sources such as ammonium, in the absence of glutamine. Ammonium utilization involves the uptake of the gas or the ammonium ion, the synthesis of glutamine by the glutamine synthetase and the recycling of the glutamate by the glutamate synthase (Detsch and Stülke, 2003).

*S. mutans* metabolizes carbohydrates to adhere to and form biofilm on tooth surfaces thus allowing the pathogen to tolerate rapid and frequent environmental fluctuations (Lemos and Burne, 2008). Oral biofilms are especially subject to a number of environmental fluctuations, such as nutrient availability, aerobic-to-anaerobic transitions, and pH changes (Yoshida and Kuramitsu, 2002a). Therefore, it is essential to study ammonium transporters, which play a crucial role in the uptake of nutrients by *S. mutans* in biofilm.

The present study focused on characterizing the ammonium transporter gene of *S. mutans* and its operon and regulatory genes were also analyzed. In addition, the influence of several inorganic carbons on gene expression was examined.

## Materials and methods

### 1. Bacterial strains and culture conditions

*Streptococcus mutans* strain MT8148 (serotype *c*) isolated from a Japanese child was used in the present study (Ooshima, *et al.*, 1983). *S. mutans* was grown in Brain Heart Infusion (BHI) medium (Becton Dickinson and Company (BDC), Franklin Lakes, NJ, USA) or Todd-Hewitt (TH) medium (BDC) as well as on Mitis-salivarius (MS) agar (BDC) at 37°C. When required, spectinomycin (SP; 1 mg/ml; Wako Pure Chemical Industries, Osaka, Japan) was supplemented.

*E. coli* XL-2 strain (Agilent Technologies, Santa Clara, CA, USA) and *E. coli* DH5 $\alpha$  strain (Nippon Gene, Tokyo, Japan) were used as host strains for transformation of plasmid DNA. *E. coli* strains were grown in Luria-Bertani (LB; 1% tryptone, 0.5% yeast extract, 0.5% NaCl) medium while LB agar was prepared by the addition of 1.5% agar. When necessary, SP (100  $\mu$ g/ml), Ampicillin sodium (AM; 100  $\mu$ g/ml) and Tetracycline Hydrochloride (TC; 7.5  $\mu$ g/ml) were added to the medium. All manufactures except as noted were commercially available from Wako Pure Chemical Industries, Osaka, Japan.

### 2. Molecular biology techniques

#### 1) Transformation of *E. coli* strains

The construction of competent cell was performed as described by Inoue *et al.* (1990). After the competent cells were thawed on ice, 100  $\mu$ l of the competent cells was mixed with 10  $\mu$ l of DNA and incubated on ice for 30 minutes. After incubation on ice, a mixture of chemically competent cells and DNA was placed at 42°C for 40

seconds and then placed back on ice for 2 minutes. The SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, 20 mM sucrose) was added and the transformed cells were incubated at 37°C for one hour with agitation. Next, the transformation mixtures were plated on LB agar medium supplemented with antibiotic and incubated overnight at 37°C.

## 2) Polymerase Chain Reaction (PCR)

PCR was performed using AmpliTaq Gold<sup>®</sup> (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instruction. The iCycler thermal cycler (BioRad Laboratories, Hercules, CA, USA) was used for PCR. Designed primer names and locations in this study are listed in Table 1 and Fig. 2. After PCR, electrophoresis was performed on a 0.7% or 1.5% gel in the presence of TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA, pH8.0) at 100V. The 100 bp or 1 kb DNA Ladders (New England Biolabs, Ipswich, MA, USA) were used as DNA size markers. DNA fragments were stained with Ethidium Bromide (EB; 1 µg/ml) for 15 minutes and visualized with an ultraviolet transilluminator at a wavelength of 312 nm.

## 3) Preparation of chromosomal DNA

Chromosomal DNA of *S. mutans* was extracted using Puregene Yeast/Bact. Kit B (QIAGEN Sciences, Hilden, Germany). Grown cells were collected by centrifugation and suspended in 250 µl of Glu-TE Buffer (1 M glucose, 10 mM 2-amino-2-hydroxymethyl-1, 3-propanediol, 1 mM ethylnediamine tetraacetic acid; Wako Pure Chemical Industries), then digested with 62.5 µl of N-acetylmuramidase SG (2.0 mg/ml; Seikagaku corporation, Tokyo, Japan) and 0.25 µl of lysozyme hydrochloride from egg white (10 mg/ml) for 90 minutes at 37°C. The resulting samples were then added with 600 µl of cell lysis solution (QIAGEN) to lyse the cells for 5 minutes at

80°C. Lysed cells were treated with 3 µl of RNase (10 mg/ml; QIAGEN) for 30 minutes at 37°C and 200 µl of protein precipitation solution (QIAGEN) was added. The purified DNA was treated with 600 µl isopropanol and centrifugation. The pellet was then washed with 70% ethanol, air dried and dissolved in 100 µl of DNA Hydration Solution (QIAGEN).

### **3. Construction of a NrgA-deficient mutant**

Figure 3 illustrates the procedure for generating the plasmid for construction of a NrgA-deficient mutant. First, the DNA fragment encoding *nrgA* was amplified by PCR with AmpliTaq® (Life Technologies) using primer sets nrgAs-Eco-F and nrgAs-Bam-R (Table 1). The amplified DNA fragment was purified with phenol chloroform and precipitated with ethanol. This fragment was then ligated into a pGEM-T Easy Vector (Promega Co., Madison, WI, USA). The resultant plasmid was digested with *Eco*RI and *Bam*HI, and cloned into a streptococcal-*E. coli* shuttle vector which encodes for SP resistance (pSF152) to generate pCA01.

Transformation to *S. mutans* MT8148 was carried out with the protocol of Lindler and Macrina (1986). Overnight cultures of *S. mutans* MT8148 were inoculated into TH medium supplemented with 10% heat-inactivated horse serum (Invitrogen, Carlsbad, CA, USA) and incubated for 2 hours. About 200 µg of pCA01 plasmid was added to grow liquid cultures and incubated for 2 hours at 37°C. The cells were then collected by centrifugation, plated on MS agar containing SP (1 mg/ml) and incubated anaerobically at 37°C for 48 hours. One positive transformant, NRGD, was selected and confirmed. Appropriate introduction of pCA01 into strain NRGD was confirmed by primer extension analysis. Following chromosomal DNA extraction of the



transformants, primer extension analysis was used to determine the *nrgA* transcription sites in MT8148 with primers listed in Table 1 (*nrgAs-Eco-F*, *nrgAs-Bam-R*, *nrgA-F* and *nrgA-R*). Agarose gel electrophoresis of the PCR product showed an amplified band of approximately 500bp (Fig. 4A). However, no extension product for NRGD was observed with the 1200 bp of *nrgA-F* and *nrgA-R* primers (Fig. 4B).

#### **4. Bacterial growth rates**

MT8148 and NRGD organisms were grown overnight at 37°C, then inoculated into TH medium or TH medium containing 20 or 40 mM ammonium. Growth curves were determined by measuring changes in optical density at 550 nm at 1-hour intervals using a spectrophotometer (GE Healthcare, Fairfield, CT, USA). Furthermore, bacterial growth rates of NRGD were evaluated in the presence of 10 mM calcium chloride and 5 mM manganese sulfate.

#### **5. Biofilm assay**

The ability of *S. mutans* strains to form biofilms was assessed by growing cells in wells of 96-well polystyrene microtiter plates (BDC). TH medium (diluted 1:4) containing 0.1% sucrose was added with 1 µl of pregrown cell suspension, and then 100 µl of the prepared samples were inoculated into the individual wells, using ten wells per strain. The plates were incubated at 37°C with 5% CO<sub>2</sub> for 48 hours. After incubation, formed biofilms were stained with 1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes at room temperature. The plate was next rinsed 6 times with sterile distilled water to remove loosely bound bacteria and any crystal violet that was not specifically staining the adherent bacteria. Washed plate was inverted several times on a

Kimtowel paper towel (Kimberly-Clark, Irving, TX, USA) to dry, then fixed with 95% ethanol. Finally, the plate was air dried and biofilms solubilized with MilliQ. Stained biofilms were quantified by measuring the absorbance at 570 nm with an enzyme-linked immunosorbent assay microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

## **6. Confocal laser scanning microscope (CLSM) observation of biofilms**

Quantitative and structural analysis of biofilms by confocal laser scanning microscopy was assayed according to the method described by Kuboniwa *et al.* (2006). MT8148 and NRGD were cultured in THB and 10 mM glutamine overnight. Following incubation, strains were centrifuged and washed with distilled water. Next, the bacterial cells were labeled with 5 µl of 10 mM hexidium iodide (Invitrogen) and incubated in the dark for 15 minutes at room temperature. Each cell suspension was adjusted to 0.1 at an optical density of 600 in a chemically defined medium supplemented with 0.5% sucrose (sCDM) (Van de Rijn and Kessler, 1980). Saliva was collected from two healthy male adults and diluted 1:4 with MilliQ to produce 25% saliva. Biofilms were formed in Lab-Tek® Chambered #1.0 Borosilicate Coverglass System 8 chamber (Nunc, Rochester, NY, USA) that were coated with filtered 25% human saliva. The chamber was then incubated at 37°C with light shielding in an anaerobic chamber for 24 hours. At the end of the experimental period, the sCDM was removed and PBS was added.

Imaging was performed using confocal laser scanning microscopy LSM 510 (Version 4.2, Carl Zeiss MicroImaging Co., Ltd., Jena, Germany) with a laser wavelength of 543 nm and the biofilm images of each sample were acquired from random positions. The confocal images were analyzed by Image J for Macintosh (Version 10.2, Bethesda, MD, USA).

## 7. Fluorescence efflux measurement

The MT8148 and NRGD strains were grown until 0.4 at an optical density of 550 nm and pelleted by centrifugation at 2400 g for 10 minutes at 4°C. The cells were then washed with 10 mM NaCl-50 mM NaPB (pH 7.0) and suspended again in the same buffer. Prior to fluorescence probe labeling, cultures were adjusted to an optical density of 0.2 at 600 nm and 1 ml of the adjusted samples was transferred to 13x100 mm test tubes (IWAKI, Shizuoka, Japan).

Fluorescence measurement was performed by a modification of methods described by Ocaktan *et al.* (1997). The adjusted samples were then labeled with fluorescence probe (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate), (TMA-DPH; Invitrogen), reacted at a final concentration of 1 µg/ml and 2 µg/ml and incubated with light shielding for 30 minutes. Following incubation, labeled cultures was centrifuged at 2400 g for 10 minutes at 4°C and the resulting pellets were washed twice with 500 µl of 10 mM NaCl-50 mM NaPB (pH 7.0). Thereafter, 100 µl samples were plated in 96 well plates (Nunc<sup>TM</sup>, Roskilde, Denmark) and absorbance was measured with a Twinkle LB970 fluorometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany) with wavelengths at 355/460 nm.

## 8. Northern blot hybridization

### 1) RNA isolation and electrophoresis

Overnight culture of MT8148 were added to TH medium and grown to late exponential phase at an optical density of 600 nm. The cells were collected by centrifugation at 2400 g for 15 minutes at 4°C and suspended in diethyl pyrocarbonate (DEPC-treated water). The mixtures were transferred to Lysing matrix B (MP

Biomedicals, Santa Anna, CA, USA) and isolated with TRI reagents (Sigma-Aldrich). Chloroform (200 µl) was then added to the RNA and vortexed. Then, RNA was resuspended in 500 µl chloroform, precipitated with isopropanol and washed two times with 75% ethanol. The resulting pellets were dried and suspended in DEPC treated water. RNA samples were treated for 15 minutes at 37°C with RNase-free DNase (Promega). RNA (5 µg/ml) was then added to a loading buffer (50% formamide, 6.142% formaldehyde, 20 mM MOPS, 5 mM sodium acetate, 20 mM EDTA, 10% glycerol, 0.05% bromophenolblue and DEPC treated water), incubated at 65° C for 10 minutes and immediately placed on ice for 1 minutes. Then, RNA was loaded onto a formaldehyde gel (1.2% agarose, 20 mM MOPS, 5 mM sodium acetate, 2 mM EDTA and 2.0% formaldehyde) and run with 1 x MOPS running buffer (20 mM MOPS, 5 mM sodium acetate, 20 mM EDTA).

## 2) RNA transfer and fixation

Capillary transfer was performed using positively charged nylon membranes (GE Healthcare) with 20x SSPE (3M NaCl, 173 mM NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 25 mM EDTA; pH 7.4). After overnight of RNA transfer, RNA was fixed to the membrane with 0.01 N NaOH alkali fixation for 5 minutes and washed two times with 2x SSC (diluted from 20 x SSC, Nippon Gene). After that, the membrane was baked at 80° C for 2 hours and fixed with UV cross-linking at 312 nm.

## 3) Hybridization and detection

To prepare the probe, the 200 bp fragments of the *nrgA* gene were PCR amplified using primer nrgART-F and nrgART-R (Table 1) and labeled according to the DIG high prime DNA northern labeling kit (Roche).

RNA fixed to the membrane was pre-hybridized with DIG easy hybridization solution (Roche) at 50° C for 30 minutes, followed by DNA probe hybridization with rotation and gentle agitation overnight. The next day, the membrane was stringently washed with 2x SSC, 0.1% SDS twice for 15 minutes and 0.1x SSC and 0.1% SDS at 68° C, twice for 15 minute. The membrane was then exposed by the methods described above.

Subsequently, the membrane was incubated for 30 minutes in blocking solution (10% blocking solution, 90 mM maleic acid, 135 mM NaCl; NaOH; pH 7.5) and 30 minutes in antibody solution (75 mU/ml antidigoxigenin-AP, 10% blocking solution, 90 mM maleic acid, 135 mM NaCl; NaOH; pH 7.5). Following incubation, the membrane was rinsed 2 times with washing buffer for 15 minutes, equilibrated with detection buffer (0.1 M Tris-HCl, 0.1M NaCl; pH 9.5) for 3 minutes, and finally the membrane was applied incubated with CDP-*star* (Roche) and exposed to x-ray film for 20 minutes.

## **9. PCR analysis of the *nrgA* operon and adjacent genes**

To characterize the *nrgA* operon, RNA was extracted from cells grown to late exponential phase as described above. The RNA samples were treated for 15 minutes at 37°C with RNase-free DNase (Promega). SuperScript III<sup>®</sup> Reverse transcriptase (Invitrogen) and random primers (Promega) were used to obtain complementary DNA (cDNA) from DNA-free RNA. PCR was then performed on DNA (as a positive control), cDNA and MilliQ (as a negative control), with specific primers that span *serC* and *glnB* (SMu1508F and SMu1509R), *glnB* and *nrgA* (SMu1509F and SMu1510R2), and *nrgA* and *SMu 1511* (SMu1510F and SMu1511R) (Table 1).

## 10. Quantitative real-time PCR

The quantitative real-time reverse transcription-PCR (qRT-PCR) was performed to evaluate the expression of the *nrgA* and *glnB* genes. Real-time RT-PCR was performed using complementary DNA samples with either 16S ribosomal RNA (rRNA) or specific primers. The expression of *glnB* gene with SMu1657RT-F and SMu1657RT-R primers was determined in MT8148 and NRGD (Table 1). Moreover, primers *nrgART-F* and *nrgART-R* and SMu1657RT-F and SMu1657RT-R were used to monitor *nrgA* and *glnB* gene expression under the influence of 2 mM glutamine (Table 1). The qRT-PCR reaction was conducted with SYBR<sup>®</sup> green (Biorad) and run in an iCycler thermal cycler (Biorad), according to the manufacturer's instructions.

## 11. Statistical analysis

All quantitative data are expressed as means  $\pm$  SD of at least three independent experiments. Statistical analysis of variance (ANOVA) was employed to compare mean values, and *P* values  $< 0.05$  indicate statistically significant differences.

## **Results**

### **1. Bacterial growth rates**

Figure 5A shows the bacterial growth curves of MT8148 and NRGD when cultured in THB alone. There were no significant differences in growth rates between these strains. On the other hand, the NRGD strain grown in the presence of 20 mM ammonium chloride showed significantly decreased growth as compared to MT8148 from 5 to 10 hours (Fig. 5B). In the presence of 40 mM ammonium chloride, NRGD also grew poorly and a significant difference was identified after 5 hours (Fig. 5C). In the presence of 10 mM calcium chloride and 5 mM manganese sulfate, the growth rate of NRGD was also delayed, and significant differences were identified between the strains in the periods from 7-9 hours and 5-11 hours respectively (Fig. 5D and E).

### **2. Biomass assay**

Biofilms formed by NRGD displayed significantly lower quantity than those formed by MT8148 (Fig. 6). According to evaluation with CLSM images, biofilms formed by MT8148 had greater thickness than those formed by NRGD, while NRGD biofilms showed both small and large amorphous micro-colonies (Fig. 7).

Three-dimensional images of the biofilms revealed that addition of 10 mM glutamine gave rise to thicker biofilms formed by MT8148 (Fig. 8). In contrast, such addition resulted in a significant loss of micro-colony formation and more coarse structure in NRGD biofilms as compared to those formed by MT8148. Finally, the biofilm mass formed in the presence of 10 mM glutamine by NRGD was drastically decreased as compared to that formed by MT8148 (Fig. 9).

### 3. Analysis of exocytosis

Addition of 1 and 2 µg/ml of TMA-DPH led to an increase in the fluorescence intensity of MT8148 and NRGD at 355/460 nm (Fig. 10). However, the intensity of NRGD was significantly greater as compared to that of MT8148, suggesting a decrease in the amount of molecules released.

### 4. Evaluation of *nrgA* and its operon

Northern blot analysis of the transcriptional organization of the *nrgA* gene locus detected a band specific to the *nrgA* and *glnB* genes, which was estimated to be approximately 1600 bp (Fig. 11A). The molecular size of the band was found to be consistent with the 1578-bp band that spans the *nrgA* and *glnB* genes determined from the nucleotide sequence of this region.

Transcriptional analysis using cDNA with specific primers showed that primer extension yielded an amplified band indicating that *glnB* and *nrgA* constitute the operon. On the other hand, no amplified bands were detected in the A and C regions with use of cDNA, suggesting that *serC* and *SMu1511* are not part of the same operon *glnB* and *nrgA*, respectively (Fig. 11B).

Real-time RT-PCR demonstrated that *glnB* gene expression in NRGD was drastically decreased as compared with that in MT8148 (Fig. 12). In the presence of 2 mM glutamine, real-time RT-PCR assays showed that the *nrgA* and *glnB* genes were elevated as compared to in its absence (Fig. 13).



## Discussion

For successful colonization and biofilm formation, *S. mutans* has developed multiple strategies (Biswas and Biswas, 2011), which help the pathogen to grow under nutrition-limiting conditions and protect it from various environmental insults (Lemos *et al.*, 2005). The transport systems are one of the methods used for survival and *S. mutans* contains more than 280 genes associated with various transport systems involved in uptake of ions, molecules and carbohydrates (Ajdić *et al.*, 2002). The physical and chemical properties of ammonium determine its interactions with transporters that are specific for it, as well as other channels that transport ions and molecules. The K<sup>+</sup> and H<sub>2</sub>O channels have been suggested to have a relationship in transport of ammonium in *Arabidopsis thaliana* and *Saccharomyces cerevisiae* (Howitt and Udvardi, 1999). It has also been speculated that if other channels are involved with the transport of ammonium, then the presence of an ammonium transporter may be relevant not only to the transport of ammonium, but also other inorganic metabolites which are important for uptake of essential nutrients and maintenance of cellular ionic homeostasis as well as playing a role as growth factors. In the present study, it was speculated that the ammonium transporter in *S. mutans* is important for not only uptake of ammonium but also other inorganic metabolites as nutrients. The PII protein, a product of the *glnB* gene, plays a central role in signal transduction of nitrogen-regulatory systems in prokaryotes and control transcriptional under conditions of nitrogen limitation (Bueno *et al.*, 1985; Holtel and Merrick, 1988). In addition, the present results also demonstrated that the PII protein encoded by *glnB* regulates the ammonium transporter.

Genes encoding ammonium transporters have been reported for several bacterial

species including *Azopirillum brasilense* (Van Dommelen *et al.*, 1998), *Corynebacterium glutamicum* (Siewe *et al.*, 1996), and *Lactococcus lactis* (Larsen *et al.*, 2006). Enteric bacteria such as *E. coli* express a specific ammonium or methylammonium ion transport system under nitrogen limiting conditions (Jayakumar *et al.*, 1989). In *B. subtilis*, ammonium utilization is characterized by the *nrgAB* operon (Wray *et al.*, 1994). NrgA is a membrane protein, whereas NrgB is a member of the PII family. The *nrgB* gene closely resembles the *glnB* gene in *S. mutans*. In the present study, the *nrgA* gene of *S. mutans* MT8148 (serotype *c*) was characterized based on a search for a homologue of the ammonium transporter gene in other bacteria at the initial stages of a published database. The amino acid alignment of NrgA encoded by the *S. mutans nrgA* gene suggests that those of *L. lactis* and *B. subtilis* are closely related with 70% and 50% identities, respectively. The similarity between the genes is 60%. This finding suggests that the *nrgA* gene could act as an ammonium transporter in *S. mutans*.

Next, functional analysis of the ammonium transporter in *S. mutans* was performed. When the Amt proteins from *S. cerevisiae* (Marini *et al.*, 1994) and *A. thaliana* (Ninnemann *et al.*, 1994) were first described, it was confirmed that the ability of those bacterial cells to take up ammonium may not be a consequence of simple diffusion across the cell membrane, but may also be mediated by an integral membrane protein under some conditions. NrgA deficiency in *S. mutans* was found to result in a delay in growth in medium supplemented with ammonium chloride, calcium chloride, and manganese sulfate. In addition, growth rate data demonstrated that NrgA is required for transport and utilization of ammonium in *S. mutans*, which is involved in transport of other inorganic metabolites used as nutrient sources.

Adherence to abiotic surfaces and subsequent biofilm formation are two primary steps in the etiology of caries formation by *S. mutans* strains (Yoshida and Kuramitsu, 2002b). This process is mediated by glucan and promotes tight adherence and coherence of bacterial cells bound to each other and abiotic surfaces, leading to formation of microcolonies of *S. mutans* and thereby modulating the initial steps of cariogenic biofilm development (Koo *et al.*, 2010). In the present study, the NrgA-deficient isogenic mutant strain NRGD produced a lower quantity of biofilm as compared to that formed by the parent strain MT8148 under a sucrose-limited condition. Confocal laser scanning microscopy with hexidium iodide staining was also performed to examine *S. mutans* biofilms attached to the wells of polystyrene plates. The advantage of using a nucleic acid stain such as hexidium iodide in biofilm studies is because of sufficient intensity for visualization with confocal microscopy with minimum toxicity as well as loss of cell viability (Peyalla *et al.*, 2011). Confocal laser microscopy observations showed that NrgA deficiency causes major alterations in biofilm formation as compared to MT8148, which was supported by the results of quantitative assays. When 10 mM glutamine was added, biofilms formed by NRGD had a coarse structure with reduced biofilm mass. Therefore, it is speculated that inactivation of the ammonium transporter gene is a main factor for the non-responsiveness of NRGD to the presence of glutamine, which resulted in a reduction in biofilm formation. Together, these results indicate that *nrgA* gene products play important roles in *S. mutans* biofilm formation, possibly by modulating *nrgA* expression in response to specific environmental conditions.

Fluorescent probes are suitable for uptake experiments because they are non-fluorescent in aqueous environments, while they become strongly fluorescent in nonpolar or hydrophobic environments (Ocaktan *et al.*, 1997). TMA-DPH is a

fluorescence polarization probe known to be sensitive to plasma membrane surfaces (Kuhry *et al.*, 1983). The present analysis of exocytosis strains with TMA-DPH showed a decreased amount of molecules released from the ammonium transporter in the plasma membrane. Based on the permease type and energy source, Saier (1999) characterized the ammonium transporter as a carrier type that functions in the efflux of ions, molecules, and toxic substances. This consistent with the present findings, as it was found that inactivation of the *nrgA* gene blocked the export functions of *S. mutans*. Therefore, it seems that the *nrgA* gene has a function related to export of molecules, which may be one of the strategies used by *S. mutans* to respond to changes in its environment.

It has been reported that bacterial ammonium transporter activity is regulated by a second gene (Merrick *et al.*, 2006), with the PII proteins GlnB and GlnK the most common, and their expression is usually regulated by nitrogen (Tremblay and Hallenbeck, 2009). In the present study, two lines of evidence suggest that *nrgA* and *glnB* are co-transcribed as a single operon. First, Northern blotting to characterize the *nrgA* operon identified a band estimated to be approximately 1600 bp, consistent with the length of the *nrgA* and *glnB* genes. Second, a band amplified with cDNA was detected by PCR using primers spanning the *nrgA* and *glnB* genes, whereas no bands were detected with two primers spanning the upstream and downstream locations of the *glnB* and *nrgA* genes. These results indicate that *nrgA* and *glnB* are co-transcribed as a single operon.

In the present study, the *glnB* gene was found to act as a regulator of the *nrgA* gene. Based on real-time RT-PCR assay, observations suggest that interaction between those genes is dependent on the presence of functional NrgA. Deletion of the *nrgA* gene

significantly reduced NRGD expression. Therefore, the *glnB* portion of the *nrgA* operon may function as a regulatory gene. Furthermore, the *glnB* gene may act as a receptor and transfer the signal to the *nrgA* gene, suggesting a direct physical interaction between these proteins. Indeed, the location of PII could play a crucial role in regulation of the expression or activity of glutamine synthase (GS) and nitrogenase (Leigh and Dodsworth, 2007). Glutamine is synthesized from glutamate and ammonium, a major means for cells to assimilate nitrogen required for biosynthesis (Krastel *et al.*, 2010). Growth rate data with ammonium chloride in the present study showed decreased growth of NRGD. Therefore, glutamine that is derived ultimately from ammonium was selected as a nutrient for the growth medium in the confocal biofilm and real-time RT-PCR assays. Based on observations of formed biofilms using confocal laser microscopy and a real-time RT-PCR assay, it was concluded that glutamine is a potential inducer of the *nrgA* operon.

The present findings suggest that the *nrgA* gene of *S. mutans* is essential for biofilm formation and export of molecules by the organisms, while the *glnB* gene may regulate *nrgA* expression. In addition, it was shown that biofilm formation occurs in response to the availability of nutrients supplied by the ammonium transporter. Since ammonium transporters are responsible for the movement of ammonium ions across cell membranes and fundamental for nitrogen metabolism in bacteria (Kleiner, 1985; Khademi *et al.*, 2004; McDonald *et al.*, 2012), elucidation of how these transporters are regulated at the genetic level may provide substantial insight into their metabolic pathways. Clearly, further studies are required to provide more insight into the role of the ammonium transporter in *S. mutans* in relation to the virulence of this pathogen.

## Conclusions

This study provided evidence related to identification and functional analysis of the ammonium transporter in *S. mutans*.

1. NrgA is required for transport of ammonium and other inorganic metabolites in *S. mutans* cells.
2. NrgA plays an important role in biofilm formation.
3. NrgA has a function related to the export of molecules.
4. Expression of the *nrgA* gene is likely regulated by the *glnB* gene.

Based on these results, NrgA is considered to be essential for biofilm formation and plays an important role in the export of molecules in *S. mutans*, which is possibly regulated by GlnB. Altogether, the ammonium transporter plays an important role in the physiology and pathogenesis of *S. mutans*.

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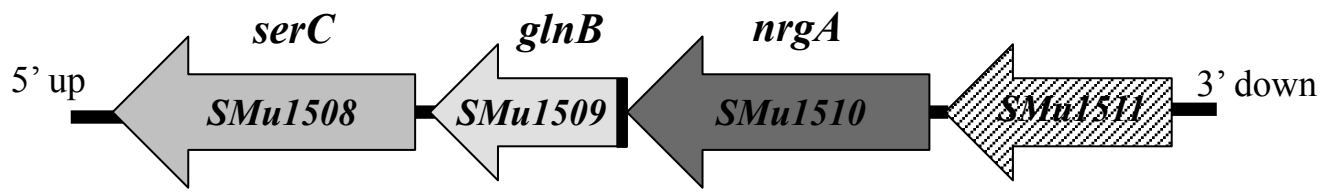
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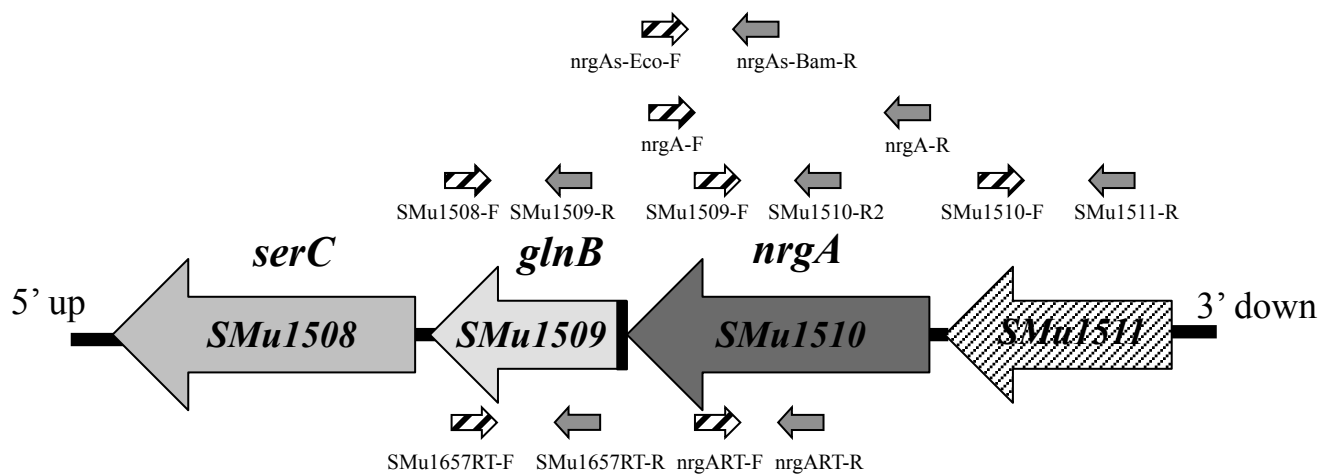
Table 1. Primers used in this study

Names	Sequence (5' to 3')
nrgAs-Eco-F	GAA TCC GAG CTG ACC AAA TAA TCG T
nrgAs-Bam-R	GGA TCC GGC CTA CTC TGG TTT GGT T
nrgA-F	ATG GAT TCA GGA TCT ATA GCA TTT A
nrgA-R	CTA AGA ATC AAG TCC CAT AAA GGT T
nrgART-F	ATG GAT TCA GGA TCT ATA GC
nrgART-R	CCA ACA CCA CTA AAA GAT AAG G
SMu1508-F	GGC AAG ACT GCA GGA CCT GCA G
SMu1509-R	ACG GAG AAC GTG ATG GTG ATG CC
SMu1509-F	GAG CGT TGG AGT GAT CTT TTG ACC ACG G
SMu1510-R2	GAG AAG AAG CTA CTG GAC TTG ACG
SMu1510-F	CTT GCG GCG TCC CAA GCC TCC ATA G
SMu1511-R	GGG AGA ATG CCT CTT ACT GGT ATC TGG
SMu1657RT-F	GGC ATC ACC ATC ACG TTC TCC TGT ACG
SMu1657RT-R	CCG TGG TCA AAA GAT CAC TCC AAC GCT C



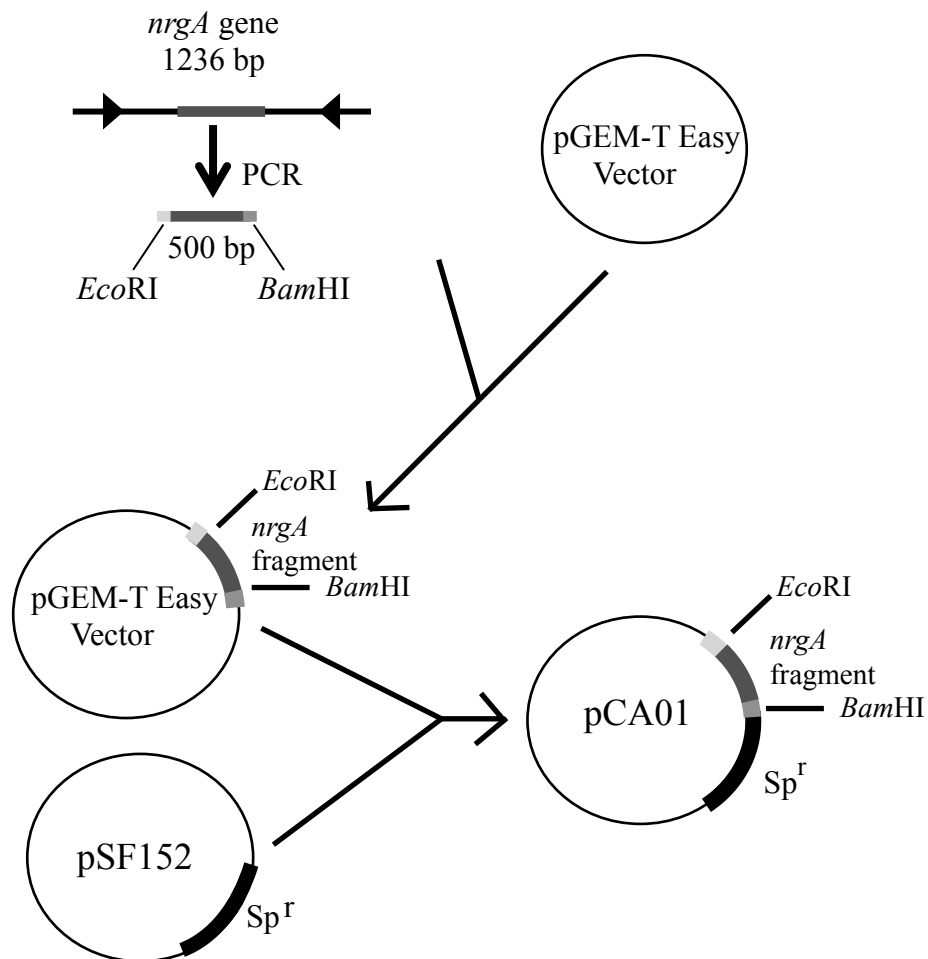
Gene	Predicted name	Predicted function	Molecular weight (bp)
<i>SMu1508</i>	<i>serC</i>	Phosphoserine amino transferase	1091
<i>SMu1509</i>	<i>glnB</i>	Nitrogen regulatory protein PII	342
<i>SMu1510</i>	<i>nrgA</i>	Ammonium transporter	1236
<i>SMu1511</i>	-	Unknown	866

**Fig. 1.** Illustration of *SMu1510* and adjacent genes in UA159.

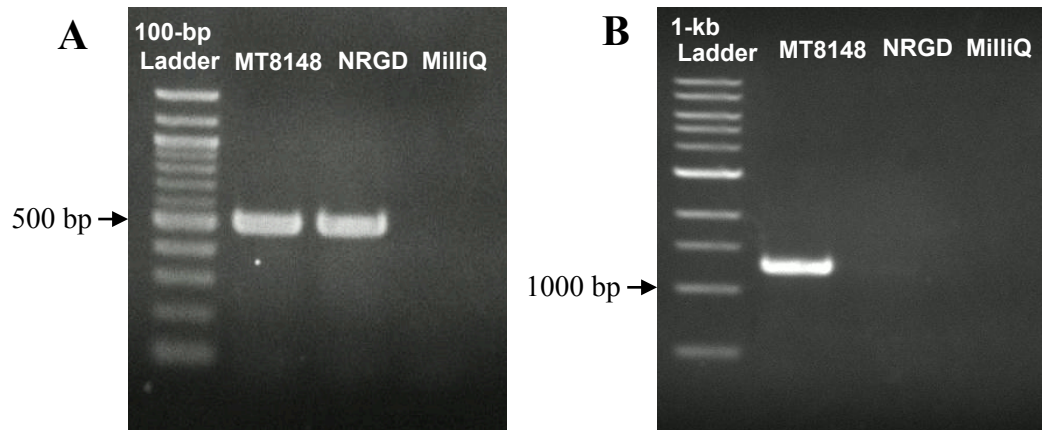


**Fig. 2.** Illustration showing position of each primer designed for the present study. The primers are listed in Table 1.

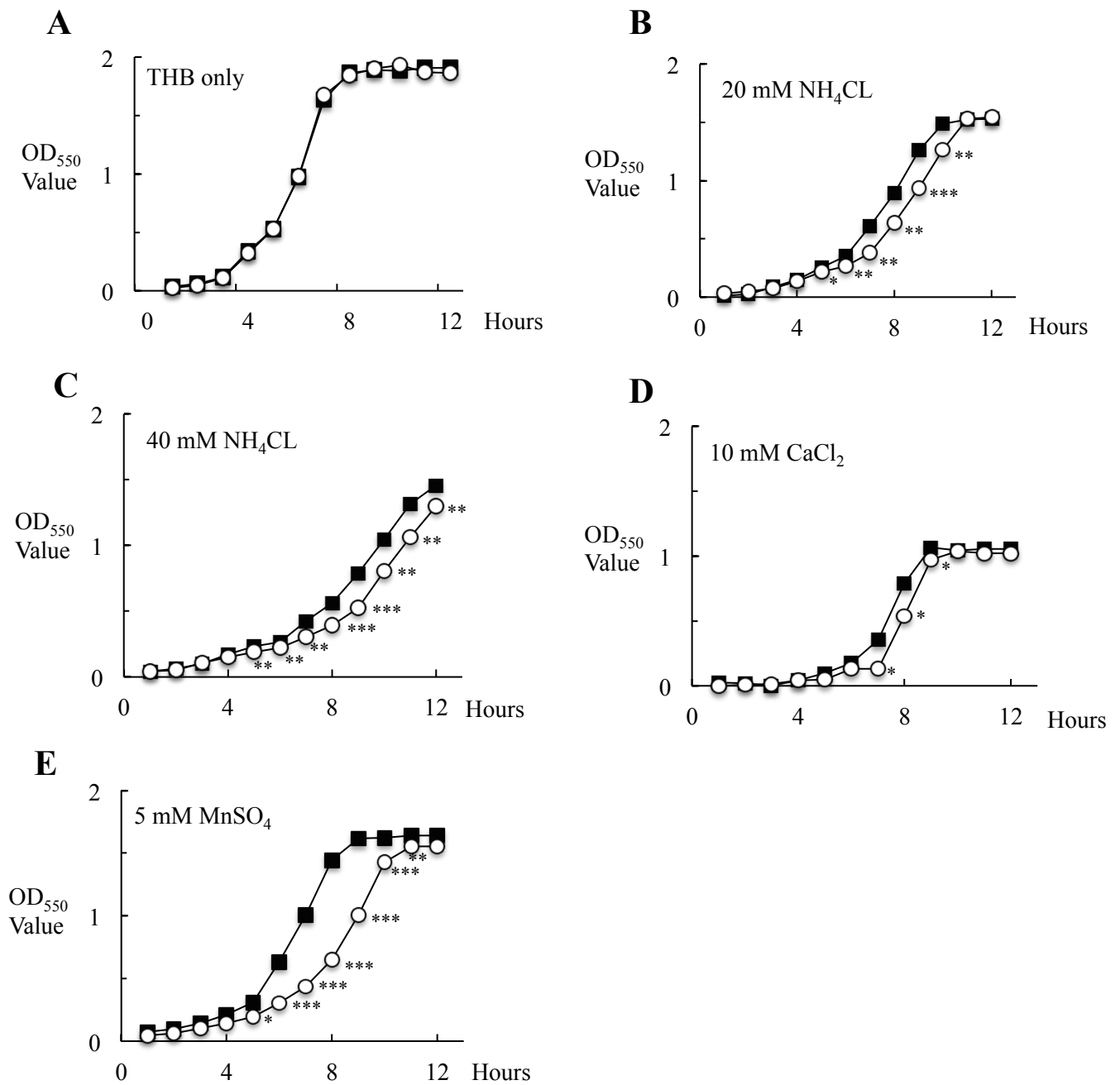




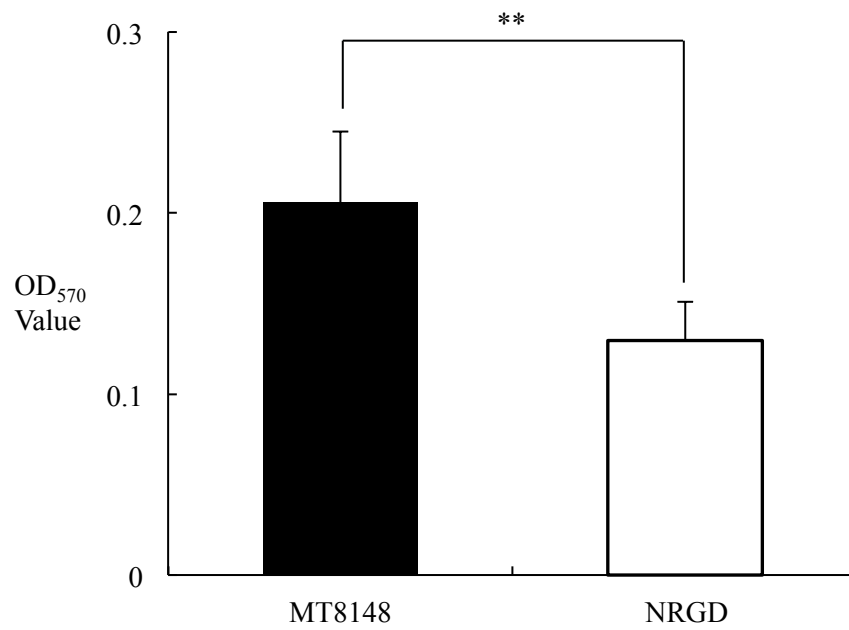
**Fig. 3.** Construction of plasmid pCA01 for generation of NrgA-deficient mutant strain NRGD.



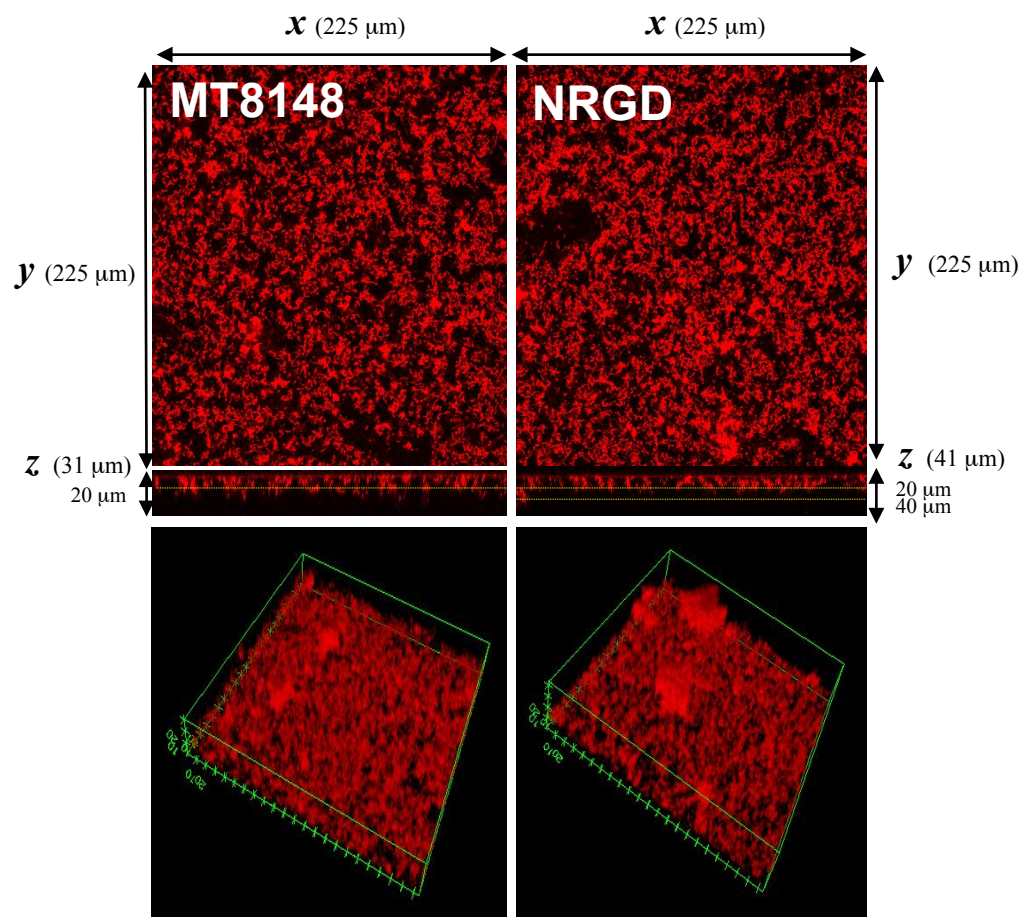
**Fig. 4.** Confirmation of appropriate generation of NRGD by a agarose gel electrophoresis of the PCR product. (A) PCR with primer set *nrgAs-Eco-F* and *nrgAs-Bam-R*. (B) PCR with primer set *nrgA-F* and *nrgA-R*.



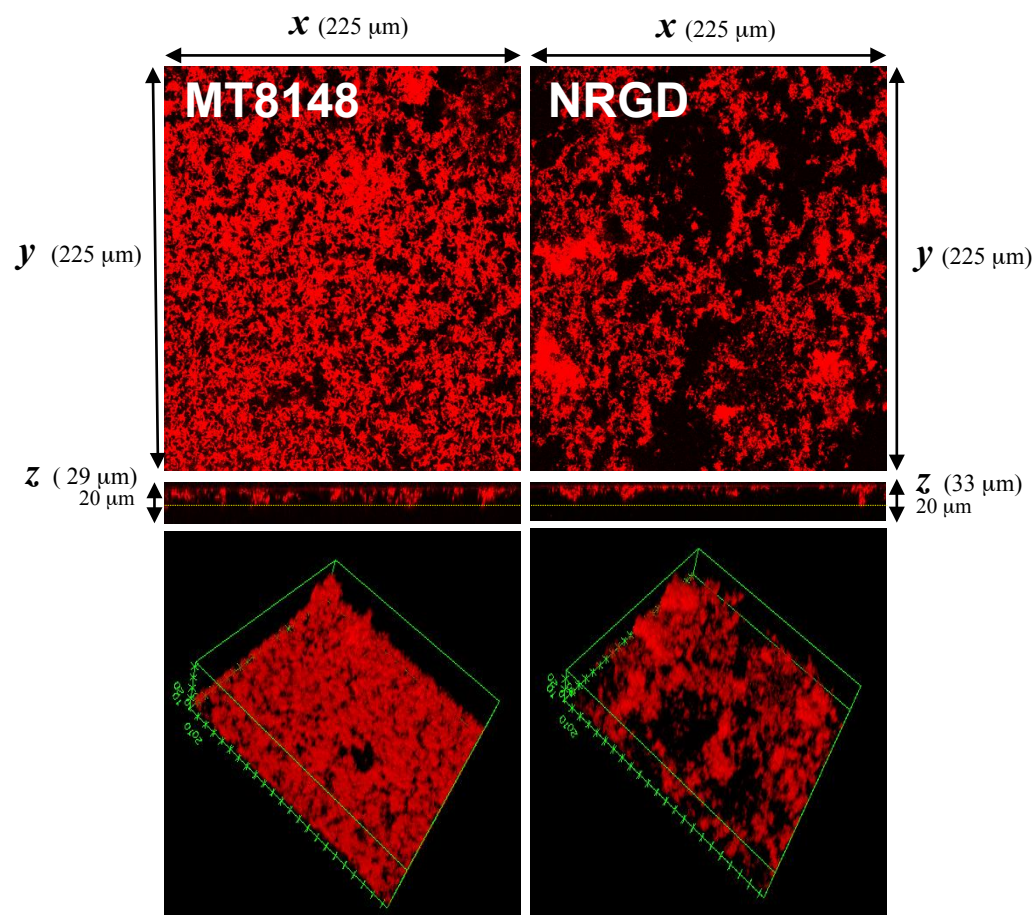
**Fig. 5.** Bacterial growth rates of MT8148 and NRGD. (A) THB only. (B) THB with 20 mM ammonium chloride. (C) THB with 40 mM ammonium chloride. (D) THB with 10 mM calcium chloride. (E) THB with 5 mM manganese sulfate. ■ MT8148, ○ NRGD. There were significant differences in the values between MT8148 and NRGD (\* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ , ANOVA).



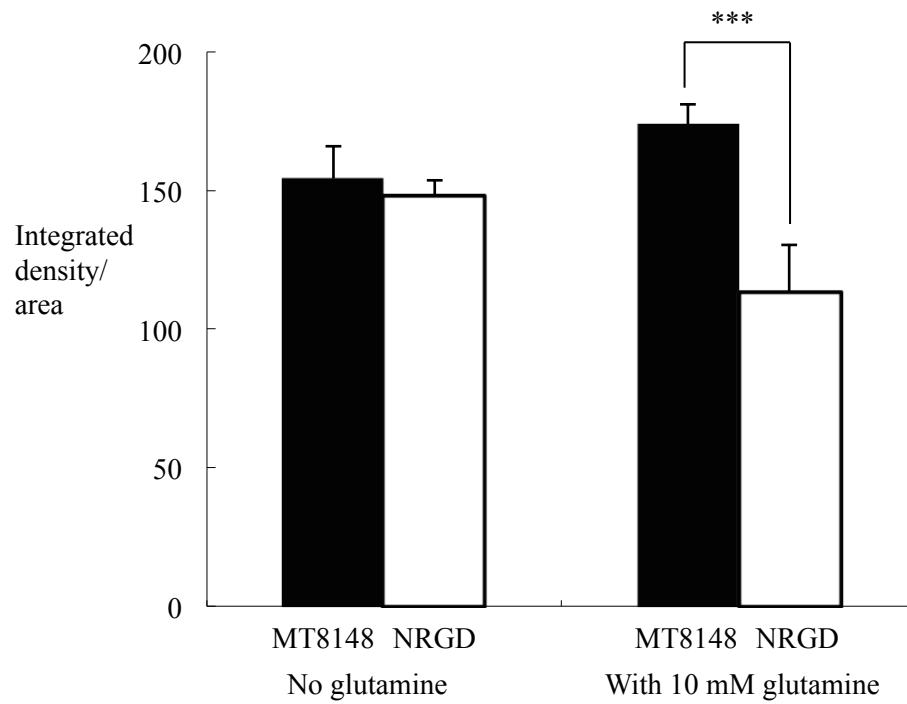
**Fig. 6.** Quantity of biofilm formed by MT8148 and NRGD in 1:4 diluted THB containing 0.1% sucrose. There were statistically significant differences in the quantity of biofilm formed between MT8148 and NRGD (\*\* $P < 0.01$ , ANOVA).



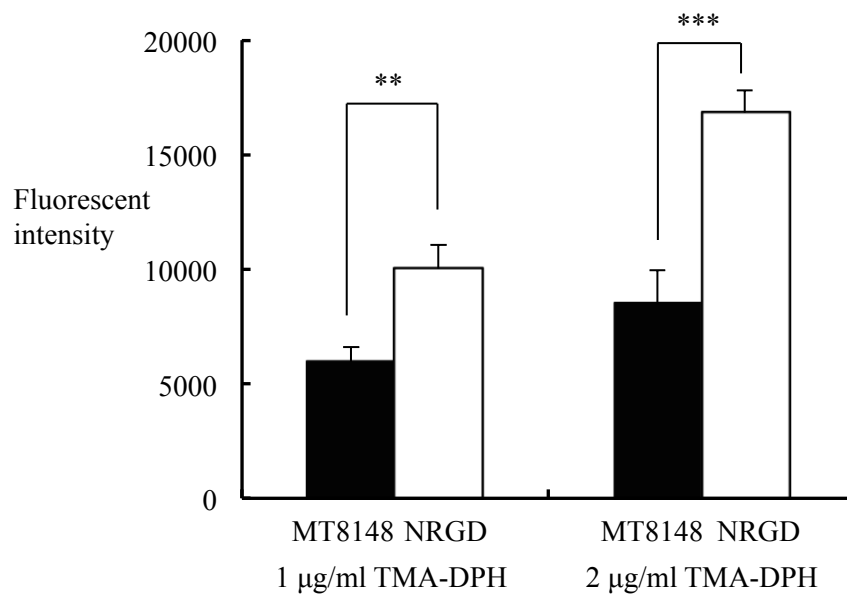
**Fig. 7.** CLSM images of MT8148 and NRGD.



**Fig. 8.** CLSM images of MT8148 and NRGD grown in medium supplemented with 10 mM glutamine.

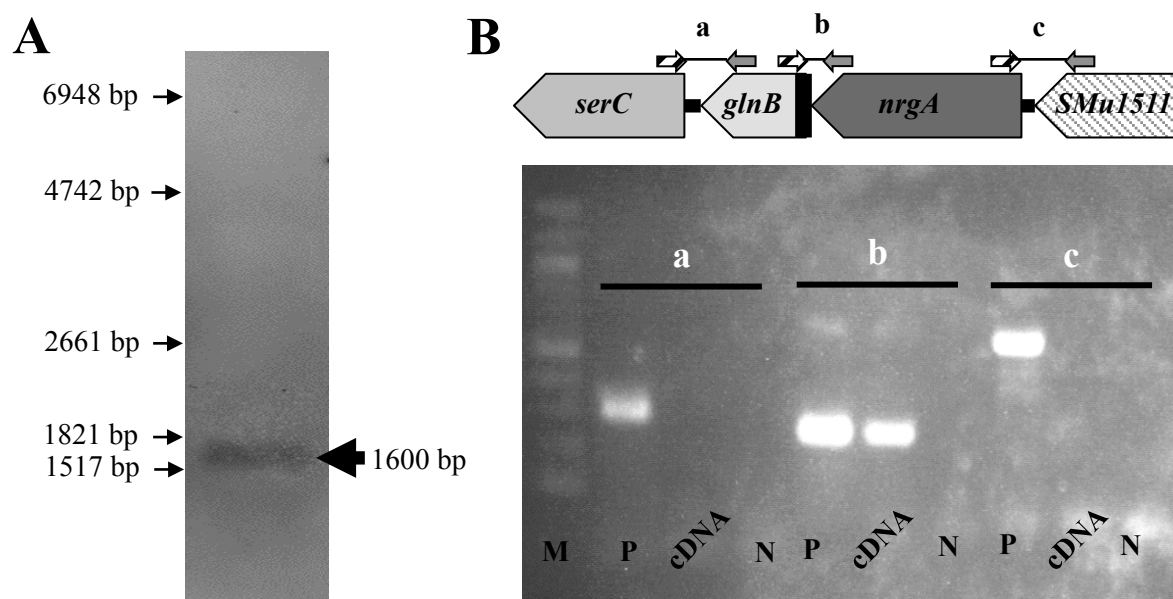


**Fig. 9.** Analysis of density of biofilm generated by MT8148 and NRGD with or without glutamine. There was a statistically significant difference between MT1848 and NRGD (\*\*\*)  $P < 0.001$ , ANOVA).

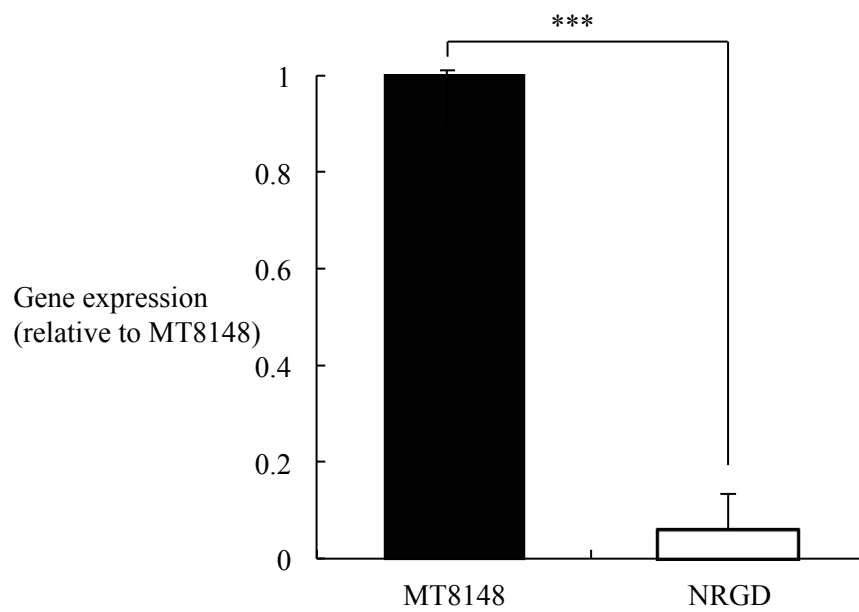


**Fig. 10.** Analysis of exocytosis of strains with TMA-DPH. The intensity of NRGD was greater as compared to that of MT8148 (\*\* $P < 0.01$  and \*\*\* $P < 0.001$ , ANOVA).





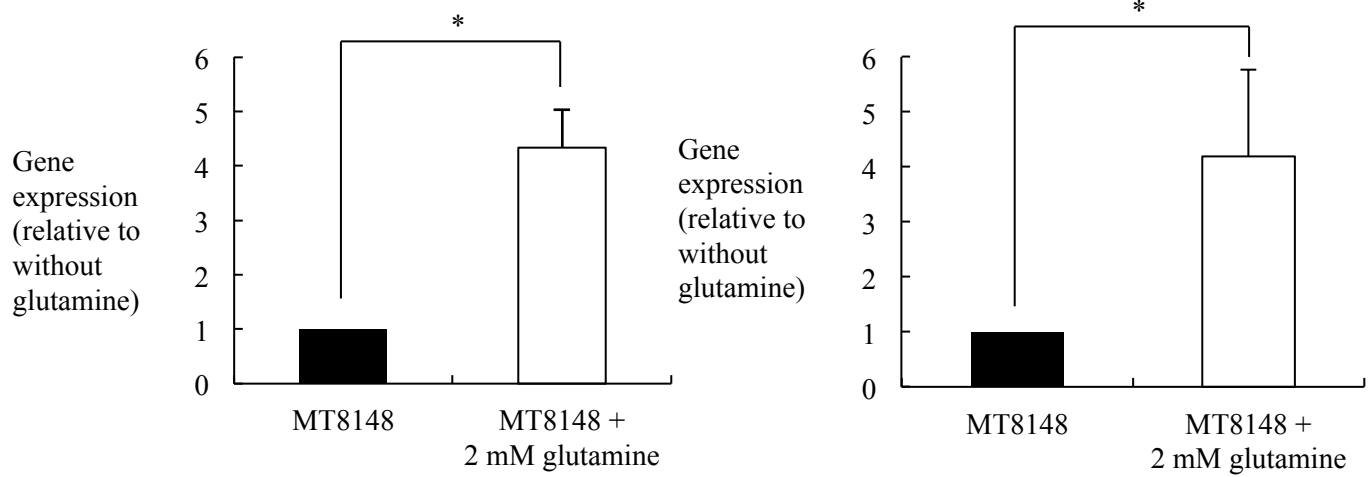
**Fig. 11.** Evaluation of *nrgA* and its operon. (A) Northern blot analysis of *nrgA* transcripts. (B) PCR analysis of organization of the *nrgA* operon and adjacent genes by using cDNA. The letters a, b, and c correspond to the amplified regions illustrated above the electrophoresis gel. Lanes: M, 100 bp DNA Ladder; P, chromosomal DNA of MT8148; cDNA, cDNA of MT8148, N, MilliQ.



**Fig. 12.** Expression level of *glnB* in MT8148 and NRGD. Transcript levels were measured using real-time PCR with 16SrRNA as the control. There was a statistically significant difference between MT8148 and NRGD (\*\* $P < 0.001$ , ANOVA).

**A. *nrgA* gene**

**B. *glnB* gene**



**Fig. 13.** Real-time quantitative RT-PCR analysis of influence of glutamine on *nrgA* and *glnB* gene expression. (A) *nrgA* gene. (B) *glnB* gene. There were statistically significant differences in the expressions of the *nrgA* and *glnB* genes between with and without glutamine (\* $P < 0.05$ , ANOVA).