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論　文　内　容　の　要　旨

氏　名　（　Arifah Chieko　）	
論文題名	Identification and functional analysis of ammonium transporter in *Streptococcus mutans*（*Streptococcus mutans* におけるアンモニア輸送体の同定と機能解析）

論文内容の要旨

Background:

Streptococcus mutans, a gram-positive bacterium, is considered to be a major etiologic agent of human dental caries and has been reported to form biofilm known as dental plaque on tooth surfaces. This organism is also known to possess a large number of transport proteins in the cell membrane for export and import of molecules, which are considered to be members of the signal transduction system. Among those, the molecule responsible for transport of ammonium across cell membranes is important for nearly all living organism. Most bacteria regulate nitrogen uptake via AmtB, a well-conserved ammonium transport membrane protein. However, the ammonium transporter in *S. mutans* has not been elucidated. Analysis of the complete genome of *S. mutans* UA159 in the database indicated the *nrgA* gene as a possible *S. mutans* ammonium transporter. In addition, the *glnB* gene located upstream of the *nrgA* gene is proposed to be a member of the PII protein superfamily. In this study, I analyzed the function of the *nrgA* gene, which encodes a possible transporter required for transport and utilization of ammonium at a low concentration in *S. mutans*. I also investigated whether *nrgA* gene expression is regulated by the *glnB* gene and essential for export of molecules via the membrane of *S. mutans* cells.

Materials and Methods:

1. Construction of *nrgA*-deficient mutant strains: The DNA fragment encoding *nrgA* was amplified by PCR with the primers Eco-F and Bam-R, which were constructed based on the *nrgA* sequences of UA159, in addition to the restriction enzyme site at the 5' (*Eco*RI) and 3' (*Bam*HI) ends. The amplified fragment was ligated into streptococcal-*E. coli* shuttle vector pSF152 to generate plasmid pCA01. Next, pCA01 was introduced into MT8148, a standard *S. mutans* strain isolated from a Japanese child, and transformants were screened on mitis-salivarius agar plates containing spectinomycin (1 mg/ml). This *nrgA*-deficient mutant was named NRGD.

2. Bacterial growth: Bacterial growth rates of MT8148 and NRGD in the presence of chemical compounds including ammonium chloride, calcium chloride, and manganese sulfate were measured at hourly intervals as changes of absorbance at 550 nm using spectropohotometry.

3. Biofilm formation assay: Biofilm formation was performed by addition of 1 µl of pre-grown MT8148 or NRGD bacterial cells to 100 µl of Todd-Hewitt broth in 96-well polystyrene microtiter plates. The plates were then incubated at 37°C with 5% CO_2 for 48 hours. After staining with 1% crystal violet, the plates were washed, then fixed with 95% ethanol and air-dried. Stained biofilms were quantified by measuring absorbance at 570 nm using a microplate reader.

4. Confocal microscopy analysis: *S. mutans* MT8148 and NRGD were separately cultured at 37°C for 18 hours in chemically defined medium with or without 10 mM glutamine in individual chambers, and stained with hexidium iodide. After washing with PBS, biofilms formed on the cover glasses were observed using a confocal laser scanning microscope with the reflected laser at 543 nm.

5. Fluorescence efflux measurement: A fluorescence probe, 1-(4-trimethylammoniumphenyl)-6- phenyl-1, 3, 5-hexatriene p-toluenesulfonate (TMA-DPH), was initially dissolved in absolute methanol, then fluorescence emission intensity was measured using a fluorometer. The TMA-DPH probe was added to adjusted MT8148 and NRGD cells, then they were plated in 96-well plates, and absorbance at 355/460 nm was measured with a fluorometer.

6. Transcriptional analysis: Northern blotting and PCR analyses were performed to specify the functioning unit or operon of *nrgA* of MT8148. In addition, real-time RT-PCR assay findings were used to determine the expression of the *glnB* gene in MT8148 and NRGD.

Results:

1. Bacterial growth: NRGD grew poorly in the presence of ammonium chloride, calcium chloride, and manganese sulfate as compared to their absence.

2. Biofilm formation: The quantity of biofilm formation by NRGD was significantly lower than that formed by MT8148. In addition, evaluation of confocal microscopic images obtained after hexidium iodide staining revealed greater cell density in MT8148 biofilm. Furthermore, addition of 10 mM glutamine induced alteration of the structure of biofilm produced by NRGD.

3. Extrusion of intracellular compounds: Export activity assay findings obtained with fluorescent probe showed that the fluorescence intensity value for NRGD was higher than that for MT8148.

4. Transcription of *nrgA*: Northern blot analysis for determination of transcriptional organization of the *nrgA* gene locus revealed a positive band, with an estimated size of 1600 bp. The length of the transcription was matched to the entire length of the *nrgA* and *glnB* genes. Moreover, PCR analysis confirmed that the band was located in the intergenic region between the *nrgA* and *glnB* genes.

Discussion:

The present findings suggest that the *nrgA* gene in *S. mutans* is essential for export of molecules and biofilm formation, while the *glnB* gene, which might be related to the function of the nitrogen regulatory protein PII, may activate expression of the *nrgA* gene. Since ammonium transport is structurally related to the membrane transport protein involved in the movement of ions, molecules and macromolecules across biological membranes, these transport proteins including the ammonium transporter may play important roles in biofilm formation by *S. mutans*.

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論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

本研究は、*Streptococcus mutans* のバイオフィルム形成におけるアンモニア輸送体の同定と機能および役割についてアンモニア輸送体関連遺伝子欠失変異株を用いて検討し、そのメカニズムを考察したものである．その結果、*S. mutans* におけるアンモニア輸送体をコードする遺伝子 *nrgA* を新たに特定し、その遺伝子を含むオペロンを明らかにした。さらに、アンモニア輸送体は、アンモニウムイオンをはじめとする物質の排出に関連し、*S. mutans* の生育とバイオフィルム形成に重要な役割を果たすことを明らかにした。

以上の研究結果は *S. mutans* によるバイオフィルム形成のメカニズムの一端を示しており、*S. mutans* の病原性を考察する上で重要な示唆を与えるものであり、博士（歯学）の学位授与に値するものと認める．