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Effects of recombinase A deficiency on biofilm formation by *Streptococcus mutans*

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Background/aim: Recombinase A (RecA) is essential for the transformation of both plasmid and chromosomal DNA in *Streptococcus pneumoniae* and is considered to be related to the SOS-response in *Streptococcus mutans*.
Methods: In the present study, a RecA-deficient mutant strain (RAD) was constructed by insertional inactivation of the *recA* gene encoding the RecA protein in strain MT8148 of *S. mutans*, after which the biological functions of acid tolerance and biofilm formation were investigated.
Results: RAD showed reduced acid tolerance and produced lower density biofilm compared with the wild-type strain. In addition, confocal microscopic observation indicated that the biofilm produced by RAD was composed of cells with significantly lower viability compared with that produced by strain MT8148.
Conclusion: These results suggest that RecA has a relationship with biofilm formation.

Key words: biofilm formation; recombinase A; *Streptococcus mutans*

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Streptococcus mutans has been implicated as a primary causative agent of dental caries in humans (6). One of the important virulence properties of the organism is its ability to form biofilm, known as dental plaque, on tooth surfaces. In general, biofilm formation is initiated by interactions between planktonic bacteria and a surface in response to appropriate environmental stress (2, 4, 8, 10, 18). In addition to responses to physical and chemical signals, bacteria regulate diverse physiological processes in a cell density-dependent manner, as well as natural competence and bacteriocin production, and acidogenic and aciduric capabilities (9, 14).

In contrast, recombinase A (RecA) is essential for transformation of both plasmid and chromosomal DNA in *Streptococcus pneumoniae* (16). The *recA* gene, which encodes the RecA protein, has also been proposed to be involved in the late steps of competence development as well as in acid tolerance (22). This gene is required for genetic transformation and is directly regulated by the cell signaling mechanism that induces competence in *S. pneumoniae* (19). In *S. mutans*, several proteins have been shown to play roles in acid survival, including RecA (21). Furthermore, the capacity of bacteria to form biofilms varies in response to environmental factors.

S. mutans is able to tolerate exposure to continual and rapid cycles of acid shock by increasing its expression of a variety of genes, among which several have been demonstrated to be associated with acid tolerance. The *brpA* gene, encoding the biofilm regulatory protein of *S. mutans*, plays a major role in acid tolerance and biofilm formation (26). In addition, the *ffh* gene, which encodes a homolog of the 54-kDa subunit of the signal recognition particle, is involved in the maintenance of a functional membrane protein composition during adaptation of *S. mutans* to changing environmental conditions (5). We performed assays to determine whether the *recA* gene is involved in biofilm formation in association with its role in competence.

Materials and methods

Bacterial strains

S. mutans strain MT8148 (serotype *c*) and its RecA-deficient mutant strain (RAD) were used in the present study. The strains were grown in brain–heart infusion broth (Difco Laboratories, Detroit, MI) or Todd–Hewitt broth (THB) (Difco).

DNA manipulation

DNA isolation, endonuclease restriction, ligation, and transformation of competent *Escherichia coli* were carried out as

described previously (23). Transformation of *S. mutans* was performed using previously reported procedures (15).

Construction of *recA*-defective mutants

The DNA fragment encoding *recA* from *S. mutans* MT8148 was amplified by polymerase chain reaction with AmpliTaq® (Applied Biosystems, Foster City, CA) using the primers RecA-R3: GCT CCT TAT GCT AAG ACA GGC GAG GT, and RecA-F3: GCA ATG GAG AGC CTT AGC ATA GCG G, which were synthesized based on sequences presented in the GenBank database (accession no. AE014133), then ligated into a pGEM-T Easy Vector (Promega, Madison, WI) to generate pSI01. The plasmid pSI01 was digested with *Hin*dIII to become linear at a unique site, then blunted and ligated with an erythromycin-resistant gene (*erm*) from pVA838 (14) to yield pSI02. After being digested with *Spe*I to become linear at a unique restriction site, plasmid pSI02 was introduced into *S. mutans* MT8148 by transformation to allow allelic exchange. The transformants were screened on Mitis-salivarius agar (Difco) plates containing 10 μg/ml erythromycin. Confirmation of plasmid insertions causing gene disruption was performed by polymerase chain reaction (PCR) and sequencing.

Bacterial growth rates

The bacterial growth rates of MT8148 and RAD were measured as described previously (17). Briefly, each strain was grown overnight at 37°C then inoculated into THB at pH 7.0 or 5.0. The pH was adjusted to the desired value before sterilization by addition of HCl. Absorbance at 550 nm was determined at hourly intervals using a spectrophotometer (Shimadzu, Kyoto, Japan).

Assay for *in vitro* biofilm formation

Quantification of biofilm formation

Biofilm formation was assayed using a method described previously (15), with some modifications. Briefly, 96-well polystyrene microtiter plates were prepared by adding 1 μl of a pregrown cell suspension to 100 μl of THB (diluted 1 : 4) in individual wells. The plates were then incubated at 37°C with 5% CO_2 for 48 h, after which liquid medium was removed and the wells were rinsed six times with sterile distilled water. The plates were then air-dried and stained with 1% crystal violet for 5 min. After staining, the plates were rinsed with sterile distilled water to remove excess dye and then air-dried. Stained biofilms were quantified by measuring absorbance at 570 nm with an enzyme-linked immunosorbent assay microplate reader (model 3550; Bio-Rad Laboratories, Richmond, CA). Each assay was performed in triplicate and wells without biofilms were used as blank controls after crystal violet staining.

Viability stain

Determination of viable and non-viable cells was performed using LIVE/DEAD Baclight viability staining (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. The biofilm cells were stained and kept in the dark for 15 min, then examined with a fluorescence microscope, after which photographs were taken with a digital camera.

Real-time quantitative reverse transcription–PCR

Real-time quantitative reverse transcription (RT)–PCR was carried out to determine the expression of the *recA*, *ffh*, and *brpA* genes, as described previously (15). Briefly, total RNA was isolated from 15 ml of log-phase cell cultures. For RT-PCR analysis, RNA samples were treated for 15 min at 37°C with 1.0 U/ml of RNase-free DNase (Amersham Biosciences, Piscataway, NJ) to remove contaminating DNA. Reverse transcription was carried out with SuperScript III (Invitrogen, Carlsbad, CA) according to the instructions of the supplier. Real-time RT-PCR was performed using complementary DNA samples with either 16S ribosomal RNA (rRNA) or specific primers using IQ-Supermix PCR reagent (Bio-Rad) in an iCycler thermal cycler according to the manufacturer's recommendations. Relative expression levels of the target gene transcripts were then calculated by normalizing the levels of the specific RNA of each target gene with the level of 16S rRNA. After normalizing Ct values for the target genes to the total amount of 16S rRNA, all samples were compared and relative fold changes in the samples were calculated using the $-\Delta\Delta$Ct method with a MyIQ real-time PCR detection system (Bio-Rad Laboratories).

Statistical analysis

Intergroup differences of various factors were estimated by a statistical analysis of variance (ANOVA) for factorial models. Fisher's protected least significant difference test was used to compare individual groups. Statistical computations were performed using STAT-VIEW II (HuLinks, Tokyo, Japan).

Results

Effects of environmental stress on *recA* gene expression

There were no obvious differences in growth rate between MT8148 and the RAD mutant grown in THB at pH 7.0 (Fig. 1). In contrast, a significant difference in growth rate was identified between them at pH 5.0. In addition, a real-time RT-PCR assay used to compare the transcriptional level of *recA* in low pH medium showed that *recA* expression was significantly elevated at pH 5.0 (Fig. 2). Further-

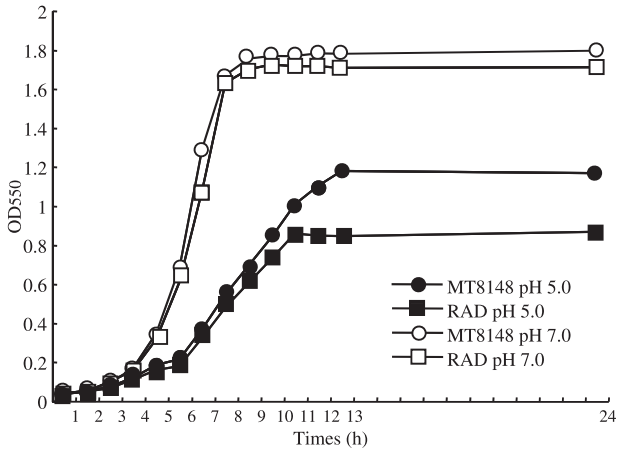

Fig. 1. Bacterial growth rates of MT8148 and RAD mutant strain. The strains were grown in Todd–Hewitt broth at different initial pH levels. ○: MT8148 at initial pH 7.0. □: RAD at initial pH 7.0. ●: MT8148 at initial pH 5.0. ■: RAD at initial pH 5.0.

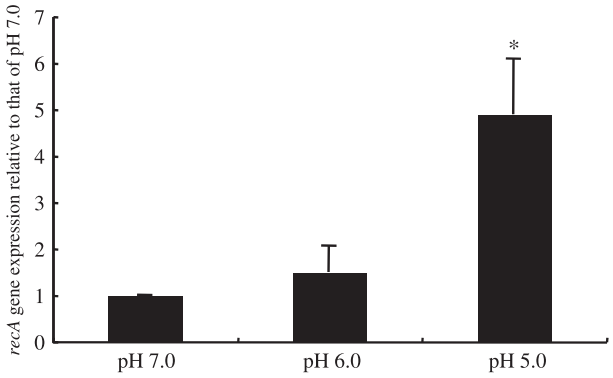

Fig. 2. recA gene expression in MT8148. MT8148 was grown in medium at pH 5.0, 6.0, and 7.0. There were statistically significant differences between the strains grown in pH 7.0 and those grown in pH 6.0 or 5.0 (*$P < 0.01$).

more, expressions of the *brpA* and *ffh* genes, which are related to acid tolerance, were clearly increased in MT8148 but did not change in RAD (Fig. 3).

Analysis of biofilm formation by MT8148 and RAD

The quantity of biofilm formation by RAD was significantly lower than that formed by MT8148 (Fig. 4). In addition, evaluations of viable and non-viable cells in the biofilms using LIVE/DEAD BacLight staining revealed a higher number of non-viable red-stained cells in the biofilms formed by RAD (Fig. 5). Furthermore, the addition of sucrose induced cell aggregation in the biofilms produced by both strains, though that formed by MT8148 had a greater cell density.

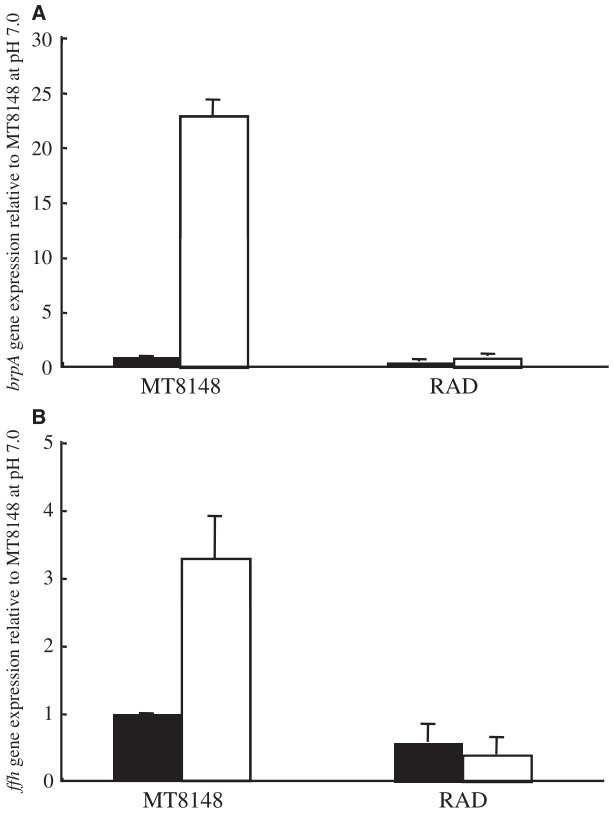

Fig. 3. brpA and *ffh* gene expressions in MT8148 and RAD mutant strain at ■ pH 7.0 and □ pH 5.0. (A) *brpA* gene expression. (B) *ffh* gene expression. RNA was extracted and quantified using real-time reverse transcription–polymerase chain reaction to determine *brpA* and *ffh* gene expression. The quantity of complementary DNA for both genes was determined by real-time reverse transcription polymerase chain reaction, then normalized to the level of 16S rRNA cDNA abundance with each unique reaction.

Discussion

It is becoming increasingly clear that competence-stimulating peptide (CSP) mediates a variety of different physiological responses (3, 15, 28) and functions in the modulation of genetic competence during biofilm development (13). Competence genes such as the *recA* gene have also been reported to be indirectly regulated by CSP in *S. pneumoniae* (11). In the present study, we performed assays to determine if the *recA* gene in *S. mutans* is involved in biofilm formation because of its role in competence. *S. mutans* is able to form biofilm because of the developmental acid adaptation known to be associated with its acid tolerance response (ATR), which is induced during exposure to sublethal pH values to enhance survival at a lower pH (24). It is likely that ATR is acquired via the quorum sensing system to modulate genetic competence (13). However, though a number of the molecular mechanisms of ATR in *S. mutans* have been reported, they have not been fully elucidated.

In the present study, the RAD mutant strain was found to possess a reduced ability to produce biofilm and had a lower rate of growth when cultured in pH 5.0 medium as compared to the parental strain MT8148. In addition, *recA* gene expression was upregulated in acidic conditions (Fig. 2). These results show that exposure to sublethal pH before exposure to a lower pH condition induces ATR, which may enhance bacterial survival. Quivey et al. (21) reported that cell growth at low pH was apparently independent of the RecA protein, suggesting that another gene is functional in cell growth at low pH and that RecA may play an indirect role in acid adaptation. It is also noteworthy that expressions of the *brpA* and *ffh* genes, which are also linked to acid tolerance, were not induced by *recA* deficiency (Fig. 3). BrpA and Ffh are therefore considered to be acid inducible, which is important for the acid tolerance of *S. mutans*. In addition, our results indicate that RecA is an important factor in the regulation of *brpA* and *ffh* gene expression at the transcriptional level, as well as in response to an acidic condition via their genetic competence ability.

The cell density of *S. mutans* biofilm grown under glucose limitation, in terms of total biomass, biofilm thickness, and number of viable cells, is a function of accumulation time. In general, the density of an *S. mutans* biofilm increases with accumulation time and glucose availability,

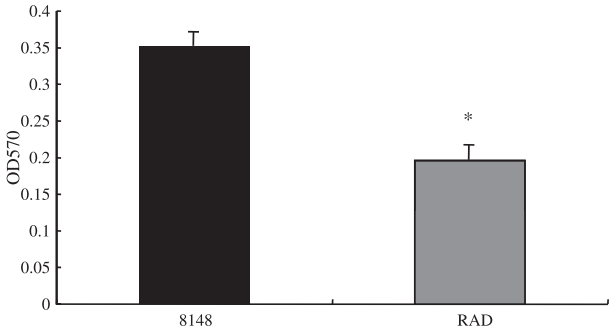

Fig. 4. Assays of biofilm formation in MT8148 and RAD mutant strain. The strains were grown in 1 : 4 diluted Todd–Hewitt broth containing 0.1% sucrose. There were statistically significant differences between the biofilms formed by MT8148 and RAD (*$P < 0.001$).

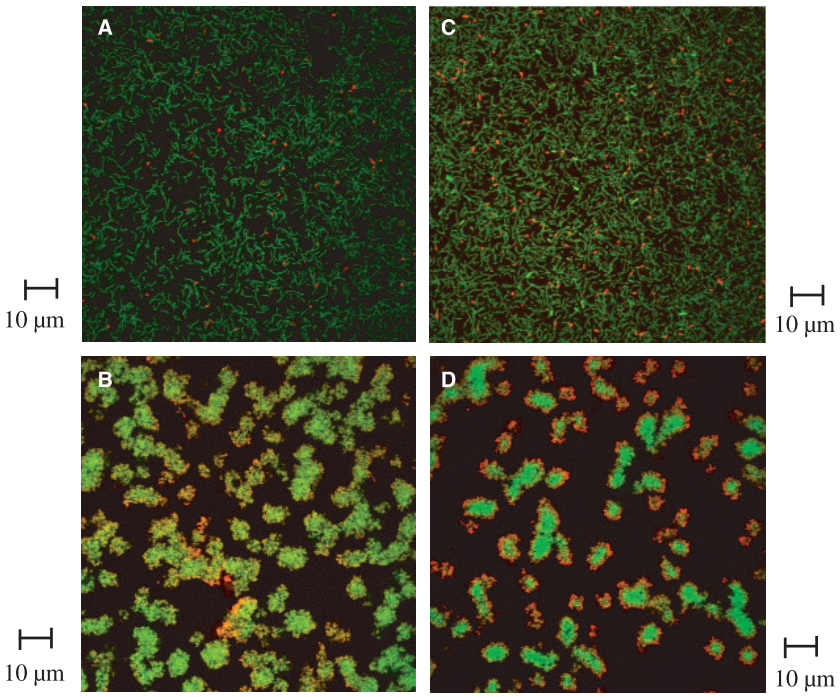

Fig. 5. Confocal microscopic image of LIVE/DEAD staining of biofilm formation by MT8148 and RAD mutant strain. The strains were grown in 1 : 4 diluted Todd–Hewitt broth in the absence (A, C) or presence (B, D) of 0.5% sucrose. (A, B) MT8148; (C, D) RAD. Viable cells were stained green with SYTO9, while cells with damaged membranes were stained red with propidium iodine.

with or without pH control. *S. mutans* relies on a biofilm lifestyle for survival and uses a quorum sensing system to modulate genetic competence via a cell density-dependent mechanism (12). Our biofilm formation assay revealed that the RAD biofilm developed at a lower rate than that formed by MT8148 (Fig. 4). In an attempt to evaluate the viability of the biofilms, we found that the biofilm formed by RAD had a greater number of dead cells scattered throughout (Fig. 5). The decreased viability of RAD biofilm was caused by sensitivity to acid, which may also contribute to defects in its formation. Therefore, our findings confirmed that RecA is a crucial protein for biofilm formation as a sensor for the stress response, such as to low pH.

Sucrose-dependent adherence is known to play a pivotal role in the cariogenicity of *S. mutans* (6), and the *gtf* genes are required for glucan synthesis and biofilm formation (1, 7, 27), while a number of other genes that affect sucrose-dependent biofilm formation have also been recently reported (25). In the present study, we found that biofilm was produced in medium containing limited sucrose, although RecA deficiency diminished the quantity formed (Fig. 4). When the sucrose concentration in the medium was increased, MT8148 formed a higher quality biofilm with a greater cell density. In addition, the structure of the biofilm formed by RAD was also altered when sucrose was added, although it had a lower density than that formed by MT8148 with added sucrose (Fig. 5). Recently, the *ciaH* gene (20), a putative histidine kinase gene, was reported to have a regulatory function that affects sucrose-dependent biofilm formation. We speculated that RecA protein may have an effect on *gtf* gene expression. However, it is difficult to fully explain the regulation of *gtf* expression because of its complex response. In the present study, there was no conspicuous change in the sucrose phenotype in RAD (data not shown), although RecA deficiency led to both diminished biofilm formation and acid tolerance. A possible explanation is that *recA* may function indirectly and be responsible for the regulation of biofilm formation.

In summary, the activities of the RecA protein may be important for the survival of *S. mutans* in specific environments. Additional investigations of the structure and mechanisms of RecA are required to determine the molecular basis for these properties.

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