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Fimbriae-associated Genes are Biofilm-forming Factors in
Aggregatibacter actinomycetemcomitans Strains

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Abstract

Aggregatibacter actinomycetemcomitans colonizes human periodontal lesions and is implicated in both aggressive periodontitis and chronic periodontitis. Clinical isolated colonies of A. actinomycetemcomitans were rough type. The rough type has a remarkable ability to adhere tenaciously to solid surfaces and colonize firmly. Rough type colonies change into smooth type colonies during the course of repeated inoculation and biofilm-forming activity ceases. Adherence by A. actinomycetemcomitans is mediated by the tight-adherence (tad) gene locus, which includes flp, rcpA and rcpB. In this study, we investigated the relationship between its biofilm-forming ability and expression of the flp, rcpA and rcpB genes associated with fimbriae protein production. First, we changed rough type strain organized biofilm on glass into smooth type and confirmed it by observation of biofilm on glass surfaces. Then, we carried out Real-Time PCR and found that expression of the rcpA and rcpB genes was clearly reduced in smooth type colonies. This suggests that expression of rcpA and rcpB plays a key role in biofilm formation by A. actinomycetemcomitans strains and the establishment of persistent infections in periodontal lesions.

Key words: Biofilm formation—Aggregatibacter actinomycetemcomitans—Fimbriae—Gene expression

Introduction

Some microorganisms can attach to solid surfaces and aggregate in a hydrated polymeric matrix of their own synthesis to form a slimy and slippery coat called biofilm. Such biofilms constitute a protected mode of growth that allows survival in a hostile environment, and contain channels for circulation of nutrients. More than 500 taxa of bacteria have been reported to organize into unique niches in the human oral cavity. In the gingival crevice, many species of microorganism form a biofilm called dental plaque. One of the most profound differences between biofilm bacteria and their planktonic counterparts is
the critical matter of resistance to antibacterial agents. In addition, biofilm-forming bacteria such as Pseudomonas aeruginosa and Staphylococcus species are resistant to host defense mechanisms and are able to create persistent infections. The adherence activity directed by structures such as the fimbriae of these bacteria plays an important role in establishing the biofilm on the tooth surface.

Aggregatibacter actinomycetemcomitans, a Gram-negative anaerobic rod, is associated with localized aggressive periodontitis and chronic periodontitis. This microorganism has several pathogenic factors, including attachment activity, leukotoxin, cytolethal distending toxin, and lipopolysaccharide. In periodontitis lesions, clinical strains of A. actinomycetemcomitans form rough type colonies, with a star-like structure in the center of each colony. These colonies are known to manufacture fimbriae proteins RcpA and RcpB, which have been implicated in the formation of rough type colonies.

Bacterial gene expression shows different patterns in different regions of the biofilm. The gene associated with adherence by A. actinomycetemcomitans is mediated by the tight-adherence (tad) gene locus, which consists of 14 genes (flp-1-flp-2-tadV-rcpCAB-tadZABCDEFG). The genes for rcpA and rcpB, are tandemly located downstream of the fimbriae genes fa and fbp. The roles of these genes remain to be fully clarified.

In the present study, we investigated the relationships among biofilm-forming activity, colony type and expression of fimbriae-associated genes in various A. actinomycetemcomitans strains.

Materials and Methods

1. Bacterial strains

The A. actinomycetemcomitans AB55-r used in this study was isolated from the subgingival plaque of patients with chronic periodontitis. Subgingival plaque samples were collected from the patients after obtaining informed consent. The samples were transferred to 100μl reduced transport fluid (RTF), dispersed by mixing with 0.8 mm-diameter glass beads using a Vortex mixer for 15 sec and serially diluted. Part of each suspension was inoculated onto TSBV plates and incubated for 7 days for isolation of A. actinomycetemcomitans AB55-r. The rest of the microorganisms were centrifuged at 15,000 × g at 4°C for 10 min. Detection and confirmation of A. actinomycetemcomitans species in these samples was performed by polymerase chain reaction (PCR) using a species specific primers according to the method of Ashimoto et al.

2. Observation of biofilm formation by confocal laser scanning microscopy

Clinical isolates of A. actinomycetemcomitans AB55-r were maintained on TSBV plates in 10% CO₂ in air at 37°C as described above. Smooth type colonies obtained after several passages were also maintained under the same conditions. Rough and smooth colonies were inoculated into Tryptic soy broth containing 0.4% yeast extract in glass bottom dishes (Matsunami Glass, Osaka, Japan) under microaerophilic conditions using the Anaeropack system (Mitsubishi Gas Chemical, Tokyo, Japan) and grown for 3 days. The biofilm that organized on the glass surface was stained with a Live/Dead BacLight Bacterial Viability Kit (Molecular Probe, Eugene, Oregon) and observed by confocal laser scanning microscopy (MRC-1024UV: Bio-Rad Co., Hercules, CA).

3. Evaluation of gene expression

The clinical isolates of A. actinomycetemcomitans AB55-r were rough type. The smooth type phenotype AB55-s was obtained by repeated inoculation. All strains were incubated in Trypticase soy broth (Bacto, Franklin Lakes, NJ) containing 0.4% yeast extract under 10% CO₂ in air at 37°C. One ml cells grown overnight was harvested by centrifugation at 15,000 × g for 10 min. Total RNA from each A. actinomycetemcomitans strain was isolated using the Purescript RNA Isolation Kit (Gentra, Minneapolis, MN). Obtained RNA was treated with RNase free DNase (Qiagen
Inc., Valencia, CA) and isolated using the RNeasy Miniprep Kit.

Expression of the $fap$, $rcpA$ and $rcpB$ genes was evaluated using the TaqMan one-step RT-PCR master mix reagent and SD7700 (Applied Biosystems, Foster City, CA). Briefly, total RNA samples (1 μg) were mixed with 25 μl 2× MasterMix (Applied Biosystems), 1.25 μl 40× MultiScribe and RNase Inhibitor Mix (Applied Biosystems), 1 μl forward primer, 1 μl reverse primer and 1 μl Taqman probe; the reaction mixture was then adjusted to 50 μl with RNase free water. The sequences of specific sets of forward primer, reverse primer and Taqman probe are listed in Table 1. Real-Time PCR was performed under the following conditions: an initial amplification cycle of 42°C for 30 min and 94°C for 10 min, followed by 40 cycles of 94°C for 15 s and 60°C for 1 min using SD7700 (Applied Biosystems). Relative mRNA expression was evaluated as $[\text{mRNA quantity of } fap, rcpA \text{ and } rcpB]/[\text{16S rRNA quantity}]^{12}$. 

### Results

Phenotypic differences between the rough and smooth type colonies were investigated (Fig. 1). Rough type strain AB-55r attached to the glass surface firmly and was sedimented by autoaggregation. In contrast, smooth type strain AB-55s showed turbid growth and did not attach to the glass surface. Analysis by confocal microscopy confirmed that the rough type strain formed a biofilm on the glass surface, but the smooth type strain did not (Fig. 2). The side view of the rough type strain revealed biofilm formation. These results demonstrated that the rough type strain had strong biofilm forming activity.

Next, relative mRNA expression, evaluated as $[\text{mRNA quantity of } fap, rcpA \text{ and } rcpB]/[\text{16S rRNA quantity}]$, was carried out. $[\text{mRNA quantity of } rcpA]/[\text{16S rRNA quantity}]$ was 0.61 in the rough type and 0.0033 in the smooth type. $[\text{mRNA quantity of } rcpB]/[16S$
rRNA quantity] was 0.1 in the rough type and 0.006 in the smooth type. These results suggest that expression of the *rcpA* and *rcpB* genes significantly reduces in smooth type strains (Fig. 3). However, expression of *fap* was too low to allow evaluation with Real-Time PCR (data not shown).

**Fig. 2** Comparison of colony phenotypes between rough type (A, C) and smooth type (B, D). Figs. 2A and B are bird’s-eye views of biofilm. Figs. 2C and D are side views of biofilm.

**Fig. 3** Difference in gene expression between rough type (white bar) and smooth type (black bar). Gene expression was evaluated by ratio ([mRNA quantity of fimbriae-associated-genes]/[16S rRNA quantity]).

**Discussion**

Clinical isolates of *A. actinomyctemcomitans* AB55-r, which formed rough type colonies with star-like structures in the centers of those colonies, were examined. The fresh clinical isolates of *A. actinomyctemcomitans* from local-
ized aggressive periodontitis (LAP) patients exhibited a non-specific tight adherence phenotype. In addition, many groups have reported a close relationship between this rough type colony morphology and possession of fimbriae. Kachlany et al. demonstrated that the tad locus contained 14 genes (flp-1-flp-2-tadV-rcpCAB-tadZABCDEFG). The genes for rcpA and rcpB were found tandemly located downstream of the fimbriae genes fap and flp.

To investigate the roles of RcpA and RcpB in biofilm formation by these microorganisms, we studied a rough type strain called AB-55r. Rough type colonies of A. actinomycetemcomitans strains change into smooth type colonies during the course of repeated serial inoculations. One such a clone, AB-55s, a smooth colony derived from AB-55r, was therefore used as a comparison. In this study, we found phenotypic differences between the rough and smooth type clones on a glass surface. Observation of biofilm formation also revealed strong biofilm forming activity in the rough type colony. Haase et al. reported that more than 16 times the amount of chlorhexidine was needed to inhibit growth of the rough type than the smooth. Their results showed that rough type A. actinomycetemcomitans had strong biofilm-forming activity, and that it acts as an evading factor from host defense mechanisms and antimicrobial reagents.

The activity of genes fap, rcpA and rcpB has been reported to correlate with rough type colony formation. Expression of rcpA and rcpB reduced in the smooth type strains. Expression of fap was too low to allow evaluation with Real-Time PCR. Our results agree with those of Haase et al. Although expression of fap was too low to allow evaluation by Real-Time PCR in this study, we have reported reduction of its expression by Real-Time PCR in a previous report. Kachlany et al. have also reported that fimbrial protein is important in the non-specific adherence of this microorganism. Together, these results suggest that expression of fap, rcpA, and rcpB plays an important role not only in rough type colony formation, but also in biofilm-forming activity.

Microorganisms in dental plaque have been reported to communicate with each other. Several reports have indicated that gene expression pattern differs between microorganisms in biofilm and planktonic cells. Further analysis is required to understand changes in the expression of fap, rcpA and rcpB during the biofilm forming process in order to clarify the roles of these genes. Taken together, fap, rcpA and rcpB appear to play an important role in colonization by A. actinomycetemcomitans and in the formation of biofilms that create persistent infections in periodontal regions.

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