Contribution of different adherent properties of *Granulicatella adiacens* and *Abiotrophia defectiva* to their associations with oral colonization and the risk of infective endocarditis

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(Received January 16, 2019; Accepted March 14, 2019)

Abstract: *Granulicatella adiacens* (G. adiacens) and *Abiotrophia defectiva* (A. defectiva) colonize the oral cavity and form part of the normal flora in the intestinal and genitourinary tracts. As reported previously, the frequency of isolation of *G. adiacens* from the oral cavity was much higher than that of *A. defectiva*. However, it has been reported that compared with *G. adiacens*, *A. defectiva* was isolated at considerably higher frequencies from the blood of patients with infective endocarditis (IE). Hence, in this study, the in vitro interaction of *G. adiacens* and *A. defectiva* strains with host surfaces and biofilm formation was examined to assess whether their different adhesive properties contribute to their associations with oral colonization and IE, respectively. *G. adiacens* exhibited an increased binding ability to saliva-coated hydroxyapatite beads than *A. defectiva* following the addition of CaCl$_2$. Furthermore, biofilm formation was observed only for *G. adiacens* with the use of a polystyrene tube and scanning electron microscopy analysis. Conversely, *A. defectiva* displayed significantly greater adherence to human umbilical vein endothelial cells and immobilized fibronectin than *G. adiacens*. These findings suggest that differences in binding properties to host components imply specific binding mechanisms in *G. adiacens* and *A. defectiva*, which might mediate selective colonization in the oral cavity or are associated with the pathogenicity of endocarditis.

Keywords; *Granulicatella adiacens*, *Abiotrophia defectiva*, salivary pellicle, colonization, HUVECs, endocarditis

Introduction

*G. adiacens* and *A. defectiva* form part of the normal flora in the oral cavity, genitourinary tract, and intestinal tract. These species, so-called nutritionally variant streptococci [1-3], develop as small satellite colonies adjacent with the use of a polystyrene tube and scanning electron microscopy. They are also among the major pathogenic causes of infective endocarditis (IE), accounting for 5-6% of its incidence [4,5]. Although *A. defectiva* was isolated at considerably higher frequencies from the blood of patients with IE [6-8], the isolation frequency of *G. adiacens* from the oral cavity (87.1%) was considerably higher than that of *A. defectiva* (11.8%) [9,10]. Therefore, the adhesive characteristics of *G. adiacens* and *A. defectiva* differ between the oral cavity and host tissues. In the oral cavity, the first step of bacterial adhesion to the primary coat of hard surfaces is binding to a conditioning film primarily composed of l-cysteine and pyridoxal. These properties could play important roles in the formation of plaques and IE, respectively.

Additionally, fibronectin, which is present in the extracellular matrix (ECM) of connective tissues, is considered as an important ligand involved in the binding of bacteria to host tissues [15-18]. Therefore, fibronectin-mediated bacterial adherence to host tissues is a critical process of bacterial infection. The multiplicity of adhesive properties of oral bacteria facilitates colonization in human oral and host cell surfaces, and consequently, these properties could play important roles in the formation of plaques and pathogenesis of infectious diseases [19,20]. In the present study, to elucidate the contribution of the adhesion properties of *G. adiacens* and *A. defectiva* to pathogenesis, in vitro interaction of the bacteria with saliva-coated hydroxyapatite (SHA) along with their biofilm formation capabilities and adhesive properties to cultured endothelial cells or fibronectin were examined.

Materials and Methods

Bacterial strains and culture condition

*G. adiacens* ATCC 49175, *A. defectiva* ATCC 49176, and their two isolates, *G. adiacens* a-1 and a-2 and *A. defectiva* d-1 and d-2, were extracted from the dental plaque of healthy human volunteers and stored at −70°C for use in this study (Tajika S, Dent J Iwate Med Uni, 21, 271-285, 1996). To isolate *G. adiacens* and *A. defectiva* from dental plaque samples, colonies demonstrating bacteriolytic activity on Todd-Hewitt (Difco Laboratories, Detroit, MI, USA) agar plates containing heat-killed *Micrococcus luteus* cells were isolated after incubation at 37°C for 24 h from oral specimens. These isolates were then identified as *G. adiacens* or *A. defectiva* after performing biochemical tests and using the PCR-RFLP method [9]. Bacteria were cultured in Todd-Hewitt broth (THB; Difco Laboratories) supplemented with 10 µg/mL pyridoxal hydrochloride (FujiFilm Wako Pure Chemicals, Tokyo, Japan) and 200 µg/mL 1-cysteine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) and were maintained at 37°C for 24 h. In other experiments, bacteria were cultured with 37 Kbp of [methyl-3H]-thymidine (PerkinElmer Japan, Yokohama, Japan) per mL of THB containing pyridoxal and 1-cysteine for radioactive labeling.

Cultured cells and culture conditions

HUVECs were purchased from the Health Science Research Bank (Osaka, Japan). The cells were maintained in MCDB 131 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal calf serum, 10 ng/mL fibroblast growth factor-basic (Sigma-Aldrich), and penicillin/streptomycin solution (Life Technologies Japan Ltd., Yokohama, Japan) and maintained at 37°C under a 5% CO$_2$ atmosphere.

Saliva

Whole unstimulated saliva was collected from five adult donors into containers chilled in ice. The saliva samples were heated at 60°C for 60 min to inactivate endogenous enzymes. Sodium azide was then added at a final concentration of 0.04%, and the samples were centrifuged at 12,000 ×g for 10 min at 4°C. The supernatant was filtered at 0.45 µm and dialyzed against distilled water. The samples were maintained at −20°C for future utilization.

Preparation of SHA beads

SHA was prepared using the method of Telferseon [21]. Briefly, 10 mg of spheroidal hydroxyapatite beads (Seikagaku, Tokyo, Japan) was added to
polypropylene microfuge tubes and equilibrated in 1 mM phosphate buffer (PB) (pH 7.0) at room temperature for 2 h, after which the supernatant solution was removed. Next, 200 µL of saliva was added to each tube and was coated with hydroxyapatite with overnight rotation at 4°C. The SHA beads were then washed with 1 mM PB to remove non-adsorbed salivary materials.

**Adhesion of bacteria to SHA beads**

The adhesion properties of *G. adiacens* or *A. defectiva* to SHA beads were determined using a modified version of the assay described by Ciardi et al. [22]. Briefly, 1 mL of radioactively labeled [methyl-3H]-thymidine (37 kBq mL⁻¹) labeled *G. adiacens* or *A. defectiva* (10,000 cpm; 1 × 10⁶ CFU) in 20 mM PB (pH 7.0) was added to 10 mg of SHA beads and incubated with mixing at 37°C for 1.5 h. After incubation, the SHA beads were rinsed three times with buffer and transferred to a liquid scintillator (Aquasol 2; New England Nuclear, Boston, MA, USA) and measured using a scintillation counter (Hitachi Appliances, Tokyo, Japan). In some experiments, 20 mM PB with or without 1 mM CaCl₂ or ethylenediaminetetraacetic acid (EDTA) was used.

**Production of EPS biofilm**

*G. adiacens* and *A. defectiva* strains were cultured in sterile polystyrene tubes (Greiner bio-one; Cellstar) with 2 mL of THB or in a 48-well plate (Thermo Fisher Scientific) at 37°C for 24 h. The tubes and plate were emp-tied, and the contents were gently washed twice with phosphate-buffered saline (PBS; Nacalai Tesque, Kyoto, Japan). The tubes were air-dried, and the samples were critical point dried, mounted on aluminum stubs, and examined using an S-4700 scanning electron microscope (Hitachi High-Technologies, Tokyo, Japan).

**Statistical analysis**

All data were analyzed by parametric analysis using Student’s t-test; all values are expressed as mean ± standard error.

**Results**

**Binding of *G. adiacens* and *A. defectiva* to HUVECs or immobilized fibronectin**

In total, 100 µL of radioactively labeled [methyl-3H]-thymidine (37 kBq mL⁻¹) labeled *G. adiacens* or *A. defectiva* (10,000 cpm; 1 × 10⁶ CFU) was added to the wells of a 96-well microtiter plate (Thermo Fisher Scientific) immobilized with 2.0 µg of fibronectin (Sigma-Aldrich Corporation). After incubation at 37°C for 90 min, the microtiter plates were washed with PBS three times to remove unbound bacterial cells. The adherent bacteria were treated with 0.25% trypsin-EDTA solution (Life Technologies Japan Ltd.) at 37°C for 10 min, transferred in a vial in a liquid scintillator, and counted using a scintillation counter [18]. In addition, the detached bacteria (10,000 cpm; 1 × 10⁶ CFU) were then added to a 12-well plate (Thermo Fisher Scientific) containing a semi-confluent culture of HUVECs (10⁶ cells/well) at a multiplicity of infection of 10. After incubation at 37°C for 90 min, the adherent bacteria were removed and collected and counted using a liquid scintillation counter as described previously.

**Production of EPS biofilm**

Following exposure to safranin, the tube containing *G. adiacens* was clearly stained red but that with *A. defectiva* did not (Fig. 2a). Additionally, bacterial cells from walls of the tubes could be recovered. Furthermore,
compared with *A. defectiva*, the values obtained by quantitative analysis of the biofilm formation, stained bacteria, and biofilm of *G. adiacens* were significantly higher (Fig. 2b). In addition, analysis via scanning electron microscopy revealed that *G. adiacens* strains formed dense, continuous networks with adjacent bacteria (Fig. 2c), whereas *A. defectiva* strains had smooth surfaces (Fig. 2d).

**Binding activity of *G. adiacens* and *A. defectiva* to HUVECs and immobilized fibronectin**

The adherence property of *G. adiacens* and *A. defectiva* strains to HUVECs and immobilized fibronectin is presented in Fig. 3a and 3b. The binding percentages of *A. defectiva* and *G. adiacens* strains to HUVECs were 15.7-16.7% and 4.5-6.0%, respectively. In addition, the adherence percentage of *G. adiacens* to fibronectin, an ECM protein, was 7.6-8.6% compared with *A. defectiva* (16.7% and 4.5-6.0%, respectively). In addition, the adherence percentage of *G. adiacens* strains to human umbilical vein endothelial cells (HUVECs) (a) or immobilized fibronectin (b). Values and error bars represent the means and standard errors from three independent experiments, each performed in triplicate.

**Discussion**

*Granulicatella adiacens* and *A. defectiva* were previously reported to colonize the dental plaque of healthy volunteers. In addition, *G. adiacens* (87.1%) was reported to have a much higher isolation frequency than *A. defectiva* (11.8%) [9,10]. However, it was reported that *A. defectiva* was isolated at substantially higher frequencies from the blood of patients with IE than *G. adiacens* [6-8]. Therefore, there could be a difference in the adhesive properties of *G. adiacens* and *A. defectiva* in the oral cavity and host tissues, and these differences might contribute to oral colonization or the pathogenesis of IE.

In this study, to disclose the mechanisms by which *G. adiacens* and *A. defectiva* adhere to oral tooth surfaces, the primary step of bacterial adhesion to hard oral surfaces was first investigated using SHA. The observed differences in the binding properties of *G. adiacens* and *A. defectiva* strains can be assumed to be attributable to surface components. *G. adiacens* possesses multiple cell surface anionic sites featuring phosphate (lipoteichoic and teichoic acids), carboxylate groups (proteins and peptidoglycan) [24], and polysaccharides of biofilm. Ca\(^{2+}\), a cationic ion, is the most major divalent ion in the saliva and can form a bridge between the host anionic sites of salivary components and *G. adiacens* cell surface anionic sites. Therefore, the adhesion of *G. adiacens* to SHA can be significantly enhanced by Ca\(^{2+}\) addition. Conversely, EDTA acts as a chelating agent; therefore, the adhesion of *G. adiacens* to SHA enhanced by Ca\(^{2+}\) was reduced to the initial level. The extracellular polymeric substance matrix comprising polysaccharides is surrounded by a three-dimensional matrix that holds the cells together in a bacterial mass and firmly attaches it to the underlying surface [25,26]. The study results illustrated that *G. adiacens* can firmly attach to tooth surfaces and support the structural integrity of plaque biofilms. Biofilm formation by *Granulicatella* spp. was reported by Karched et al. [23]. In agreement with the results obtained in this study, using plastic plate adherent assay with crystal violet stain, it was revealed that *G. adiacens* possesses the ability to form biofilms. It was also reported that the biofilm formation ability of *Granulicatella* by co-culture with other oral bacteria is much higher than that of *Granulicatella* alone [23]. Therefore, future studies assessing the biofilm formation abilities of *Abiotrophia* and *Granulicatella* cultured with other bacteria and the occurrence of coaggregation are required.

Dental plaques develop in two distinct sequential steps, namely, adhesion of early colonizers to host components such as SHA and time-
dependent accumulation of multi-layered cell clusters embedded in a matrix of bacteria and host constituents. In this study, the adhesion tendencies of the bacteria to SHA in the early stage were similar; however, the adherence property of *G. adiacens* was enhanced by the addition of Ca²⁺, which is physiologically present in saliva. Further, biofilm formation associated with mature dental plaque formation was observed with *G. adiacens* exclusively. These adhesion properties could contribute to the high isolation frequency of *G. adiacens* in the dental plaque.

Fibronectin is a major ECM component, and the ability of bacteria to adhere to ECM is considered as a virulence factor for microorganisms during infection [27-29]. In agreement with previous reports by Tart and van de Rijn [30] and Senn et al. [31], the results obtained in this study revealed that *A. defectiva* ATCC 49176 and its clinical isolates from healthy subjects were more likely to bind to immobilized fibronectin than *G. adiacens* strains. Conversely, Okada et al. reported that *G. adiacens* was more likely to adhere to fibronectin than *A. defectiva* [32]. The difference between these results may be attributable to differences in the experimental pH values and salt concentrations in the utilized buffers [33]. Moreover, because *A. defectiva* more frequently bound to HUVECs, this bacterium might more readily bind to cells via fibronectin than *G. adiacens*. Thus, even if a small number of *A. defectiva* enters the blood, their ability to adhere to endothelial cells via fibronectin could be much higher than that of *G. adiacens*. These factors may explain why *G. adiacens* more frequently colonized the oral cavity than *A. defectiva*; *A. defectiva* is more frequently isolated from the blood of patients with IE. In addition, elucidation of the ligand of *A. defectiva* that binds to fibronectin may help develop preventive measures and remedies against IE caused by *A. defectiva*. Conversely, laminin, an ECM protein, is occasionally the target of pathogenic bacterial ligands for adhesion to host surfaces [34]. To further investigate the adhesion of *A. defectiva* to laminin, the adherence mechanisms of *A. defectiva* to endothelial cells and virulence in IE should be identified.

In conclusion, these results indicate that *G. adiacens* more readily colonized the oral cavity than *A. defectiva* because of its Ca²⁺-dependent adhesion to SHA and subsequent biofilm formation ability. Conversely, the ability of *A. defectiva* to adhere to HUVECs and fibronectin may explain its association with the pathogenicity in IE. Therefore, the different adhesion properties of *G. adiacens* and *A. defectiva* to host surfaces might explain their respective associations with oral colonization and IE pathogenesis.

Acknowledgments

This work was supported, in part, by a Grant-in-Aid for Scientific Research (17K11623 and 17K17286) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Conflict of interest

The authors declare no conflict of interest.

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