Serine-rich Repeat Adhesin Gordonii Surface Protein B Is Important for *Streptococcus gordonii* Biofilm Formation

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Abstract

Introduction: *Streptococcus gordonii* is a predominant member of the oral microflora and has been isolated from root canals of teeth with refractory apical periodontitis. Biofilm formation is important for various dental diseases, and *S. gordonii* is involved in dental biofilm formation as an early colonizer. Although serine-rich repeat (SRR) adhesins of *S. gordonii* such as gordonii surface protein B (GspB) are associated with bacterial colonization, the role of GspB in biofilm formation is not clearly understood. In the present study, we investigated the effect of *S. gordonii* GspB on biofilm formation using wild-type and GspB-deficient mutant *S. gordonii* strains. **Methods:** Confocal microscopy and crystal violet assay were used to determine biofilm formation. Bacterial growth was examined by measuring optical density with spectrometry. Bacterial adherence and biofilm on the culture plate and human dentin slices were visualized with a scanning electron microscope. **Results:** The GspB-deficient *S. gordonii* mutant strain was less potent than the wild-type strain in biofilm formation. Of note, there was no difference in the bacterial growth rate between the mutant and wild-type strains. Differences in biofilm-forming ability between the wild-type and mutant strains were more distinct in the sucrose-supplemented media. Furthermore, the GspB-deficient mutant exhibited attenuated formation of aggregates on the surface of the culture plate and human dentin slices. **Conclusions:** These results suggest that GspB is important for *S. gordonii* biofilm formation, which may contribute to the development of dental biofilm-associated diseases. *(J Endod 2016;■:1–6)*

Key Words

Biofilm formation, gordonii surface protein B, human dentin, serine-rich adhesin, *Streptococcus gordonii*

Significance

Streptococcus gordonii has been isolated from root canals of teeth of patients with refractory apical periodontitis. It is known to play an important role in biofilm formation as an early colonizer. Here, we show that serine-rich repeat adhesin GspB is crucial for *S. gordonii* biofilm formation.

A biofilm is a community of microorganisms that attach to a surface and are embedded in their extracellular polymeric substances (1). Bacteria that can form a biofilm are responsible for approximately 80% of human bacterial infections (2). Bacterial biofilms are associated with various chronic inflammatory diseases such as dental caries, periodontitis, endocarditis, and cystic fibrosis pneumonia (3–6). Bacteria that reside in biofilms are 10 to 1000 times more resistant to antimicrobial agents compared with planktonic bacteria (7, 8) because extracellular polymeric substances act as a physical shield (9). In addition, bacteria are likely to grow slowly in the biofilm, and, thus, they are not efficiently killed by antibiotics that target bacterial growth (10). For these reasons, many researchers have focused on a target molecule involving biofilm formation in order to develop new therapeutics.

Streptococcus gordonii is a gram-positive facultative anaerobic bacterium that is frequently observed as a member of human oral microflora (11). *S. gordonii* is a notorious life-threatening pathogen because it can enter blood vessels and cause infective endocarditis (12). In the oral cavity, it is associated with various oral infectious diseases such as dental caries and periodontitis. It is involved in dental biofilm formation by acting as an early colonizer that facilitates the incorporation of various oral pathogens (13). Moreover, oral streptococcus species, including *S. gordonii*, have the ability to colonize on the dentin surfaces and deeply penetrate dentinal tubules (14). Thus, it is difficult to remove the colonization of *S. gordonii* in the root canal so that *S. gordonii* is often isolated from the root canals with apical periodontitis receiving endodontic treatment (15).

In addition, *S. gordonii* can exchange genes with *Enterococcus faecalis* in root canal biofilms, thereby potentiating its virulence and antibiotic resistance (16). Thus, because *S. gordonii* biofilms are closely related to oral infective diseases, understanding the molecular mechanism of *S. gordonii* biofilm formation is necessary to prevent and treat these diseases.

Serine-rich repeat (SRR) adhesins are cell wall–associated glycoproteins of gram-positive bacteria (17, 18). SRR adhesins have the ability to bind sialic acid on the host

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cells (19). They consist of a signal peptide, a short SRR 1, a binding region, a long SRR 2, and a cell wall–anchoring domain (20). SRR adhesins of *S. gordonii*, such as gordonii surface protein B (GspB), are essential for bacterial binding to sialic acid motifs on platelets (19), which is a crucial component of infective endocarditis (12). Accumulating reports suggest that SRR adhesins mediate the adherence and biofilm of some gram-positive bacteria including *Streptococcus pneumoniae*, *Streptococcus parasanguis*, and *Streptococcus cristatus* (21–24). However, little is known about the role of SRR adhesins of *S. gordonii* in biofilm formation, especially on the human dentin. Therefore, we investigated the role of *S. gordonii* GspB in biofilm formation using a GspB-deficient mutant strain.

Materials and Methods

Bacteria and Reagents

S. gordonii M99 wild-type and GspB-deficient strains (19) were kindly provided by Paul M. Sullam (University of California, San Francisco). *S. gordonii* M99 wild-type and GspB-deficient strains were cultured in Todd-Hewitt broth with 0.5% yeast extract (THY; BD Biosciences, San Jose, CA) at 37°C to the midlog phase. Crystal violet dye and ethanol were purchased from Junsei Chemical Co, Ltd (Tokyo, Japan). Acetic acid was purchased from EMD Chemicals (Gibbstown, NJ). The Live/Dead BacLight Bacterial Viability Kit was purchased from Molecular Probes (Eugene, OR).

Preparation of Human Dentin Slices

The preparation and use of human dentin slices in the experiments were approved by the Institutional Review Board of Seoul National University Dental Hospital, Seoul, Korea (CRI 15007). Single-rooted premolars were obtained from patients undergoing orthodontic extractions in the Department of Oral and Maxillofacial Surgery at Seoul National University Dental Hospital. The root surfaces were cleaned by an ultrasonic scaler and sliced in cross sections to a thickness of 500 μm with an Isomet precision saw (Isomet, Buehler, IL). These dentin slices were treated with 17% EDTA (Sigma-Aldrich, St Louis, MO) for 5 minutes and then treated with 2.5% sodium hypochlorite (Sigma-Aldrich) for 5 minutes. After being neutralized with 5% sodium thiosulfate (Sigma-Aldrich) for 5 minutes, dentin slices were autoclaved for 15 minutes at 121°C. To confirm the sterility, the dentin slices were soaked and incubated in THY broth at 37°C overnight, and the incubation media were plated on THY agar plates and incubated at 37°C for 2 days. No bacterial colonies were observed at the end of the incubation period (data not shown).

Crystal Violet Assay

To compare the biofilm-forming ability of *S. gordonii* M99 wild-type and GspB-deficient strains, 1×10^7, 1×10^8, or 1×10^9 colony-forming units (CFUs)/mL *S. gordonii* strains were cultured in THY media on 96-well plates (Nunc, Roskilde, Denmark) at 37°C for 1, 3, 6, 12, or 24 hours. To examine the biofilm-forming ability in the presence of sucrose, *S. gordonii* M99 wild-type and GspB-deficient strains at 1×10^8 CFU/mL were cultured in THY media containing 0.01%, 0.1%, 1%, or 3% of sucrose on 96-well plates at 37°C for 24 hours. The supernatant was removed, and the wells were rinsed with phosphate-buffered saline (PBS). Then, the biofilm was stained with 0.1% crystal violet for 30 minutes and solubilized in a mixture of 0.2% acetic acid and 95% ethanol. The optical density was measured at 600 nm with the VERSAmax plate reader (Molecular Devices, Sunnyvale, CA).

Confocal Laser Scanning Microscopy

S. gordonii M99 wild-type and GspB-deficient mutant strains (1×10^8 CFU/mL) were cultured on coverglass bottom dishes (SPL, Gyeonggido, Korea) for 24 hours. After the removal of supernatant, the biofilm was stained with SYTO9 and propidium iodide in the Live/Dead BacLight Bacterial Viability Kit to distinguish live and dead bacteria, respectively. After rinsing with PBS, the biofilm was observed using a confocal laser scanning microscope (LSM 5 Pascal; Carl Zeiss MicroImaging GmbH, Thuringen, Germany) at 400× magnification.

Examination of Bacterial Growth

S. gordonii M99 wild-type and GspB-deficient mutant strains were cultured in fresh THY media for 0, 1, 3, 6, 9, 12, 15, 24, 36, and 48 hours. The optical density was measured at 600 nm using a spectrophotometer (GeneSpec III; Hitachi Genetics Corporation, Alameda, CA).

Scanning Electron Microscopic Analysis

S. gordonii M99 wild-type and GspB-deficient mutant strains (1×10^8 CFU/mL) were cultured on 48-well plates (Nunc, Roskilde, Denmark) or human dentin slices for 24 hours. The supernatant was removed, and the biofilm was fixed with a primary fixing solution (2% paraformaldehyde and 2.5% glutaraldehyde in PBS). The biofilm was washed with PBS and fixed again with 1% osmium tetroxide for 1.5 hours (25). Then, the biofilm was rinsed with distilled water and dehydrated in serially graded ethanol solutions (70%, 80%, 90%, and 95% for 15 minutes each and 100% for 15 minutes 3 times). The biofilm was dried using a critical point dryer (HCP-2; Hitachi, Tokyo, Japan), coated with platinum using an ion sputter (Quorum Q150 T S; Quorum Technologies Ltd, East Grinstead, UK), and observed with a scanning electron microscope (S-4700, Hitachi) at 5000 and 10,000× magnifications with an acceleration voltage of 15 kV. The scanning electron microscopic (SEM) images were processed to measure the area of the *S. gordonii* aggregate by using open-source software ImageJ (National Institutes of Health, Bethesda, MD). Then, the area of *S. gordonii* aggregates was converted to the percentage of the total area.

Statistical Analysis

The mean value ± standard deviation was obtained from triplicate samples. Statistical significance was examined with a *t* test. An asterisk in the figures indicates that GspB-deficient *S. gordonii* was significantly different from the wild-type strain at $P < .05$.

Results

GspB Plays an Important Role in *S. gordonii* Biofilm Formation

To examine the role of GspB in the biofilm formation of *S. gordonii*, we compared the biofilm-forming abilities of *S. gordonii* GspB-deficient mutant and wild-type strains. When the bacteria were cultured on the coverglass bottom culture plate followed by confocal microscopic analysis, the GspB-deficient mutant showed decreased biofilm formation compared with the wild-type bacteria (Fig. 1*A*). Quantitative analysis of biofilm formation using crystal violet assay also showed that biofilm formation of the *S. gordonii* GspB-deficient strain was lower than that of the wild-type strain (Fig. 1*B*). These results imply that GspB is important in *S. gordonii* biofilm formation.