Jpn. J. Infect. Dis., 70, 399–404, 2017

Original Article

Contribution of *Streptococcus gordonii* Hsa Adhesin to Biofilm Formation

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SUMMARY: Adhesion of oral mitis group streptococci, such as *Streptococcus gordonii*, to acquired pellicle on the tooth surface is the first step in oral biofilm formation. *S. gordonii* strain DL1 possesses an Hsa adhesin, which recognizes the terminal sialic acid of host sialoglycoconjugates. The aim of the present study was to investigate the role of the Hsa adhesin in biofilm formation. The biofilm-forming ability of a *S. gordonii hsa* mutant on microtiter plates pre-coated with saliva, fetuin, or mucin was significantly lower than that of wild-type strain DL1. In contrast, no significant difference in biofilm-forming ability was observed in plates pre-coated with bovine serum albumin, which does not contain sialic acid. The biofilm-forming ability of strain DL1 in saliva-coated microtiter plates was also significantly reduced when the plate was pre-treated with neuraminidase. The sialic acid-dependent biofilm-forming ability of different wild-type *S. gordonii* strains varied. However, Southern and western blot analyses showed that all the tested wild-type strains possessed and expressed *hsa* homologs, respectively. These results indicate that the binding of Hsa adhesin to sialoglycoconjugates is associated with biofilm formation of *S. gordonii* DL1, and imply variation in the contribution of Hsa and its homologs to *S. gordonii* biofilm formation.

INTRODUCTION

Oral mitis group streptococci, such as *Streptococcus gordonii*, primarily colonize the human tooth surface. Thus, these bacteria are members of the biofilm community on teeth known as dental plaque, and are associated with the pathogenesis of dental caries and periodontal disease (1–3). These bacteria are also known to colonize damaged heart valves, and are considered to be an important causative agent of infective endocarditis (4–6).

Adhesion of oral mitis group streptococci to the acquired pellicle on the tooth surface is the initial step in oral biofilm formation, and oral streptococcal adhesins are thought to play an important role in colonization (2). The *S. gordonii* wild-type strain DL1 possesses an Hsa adhesin that recognizes terminal sialic acids in host sialoglycoconjugates (7,8). *S. gordonii* DL1 Hsa contains an amino-terminal non-repetitive region (NR1), a serine-rich region (SR1), another non-repetitive region (NR2), another serine-rich region (SR2), and a carboxyl-terminal cell wall-anchoring domain (CWAD; Fig. 1) (8,9). The NR2 region of Hsa is involved in binding to α2-3-linked sialic acid (9,10). Both SR1 and SR2 are glycosylated with GlcNAc, which can be detected by wheat germ agglutinin (WGA) (7,9). Given the structure of Hsa, it is thought that the amino-terminal NR2 receptor-binding domain attaches to the cell surface via the SR2 as a "molecular stalk" (8,9).

Received October 31, 2016. Accepted December 7, 2016.
J-STAGE Advance Publication December 22, 2016.
DOI: 10.7883/yoken.JJID.2016.492

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Host sialic acid-containing receptors for Hsa adhesin include the salivary mucin MG2 (11,12), leukosialin (CD43) (13), platelet glycoprotein Ibα (14), erythrocyte glycophorin A and band3 (15), and leukocyte CD11b and CD50 (16). The Hsa adhesin and its homologs facilitate attachment of *S. gordonii* to host cells, such as polymorphonuclear leukocytes (13,16), erythrocytes (7,15), platelets (9,14,17,18), macrophages, and monocytes (19). In addition, Hsa adhesin contributes to the pathogenesis of experimental infectious endocarditis caused by *S. gordonii* DL1 (6). Moreover, the results of our recent study suggested that monocytes stimulated with *S. gordonii* DL1 rapidly differentiate into dendritic cells through interactions with Hsa (19).

Hsa and its homologs have been shown to play a role in the binding of *S. gordonii* to saliva-coated hydroxyapatite, which is used as a model of the tooth surface (20), and in biofilm formation on abiotic surfaces (21). However, little is known about its role in oral biofilm formation, especially on saliva-coated surfaces. In the present study, we compared the biofilm-forming ability of wild-type *S. gordonii* DL1 to that of an *hsa* mutant under various conditions. In addition, we also examined several

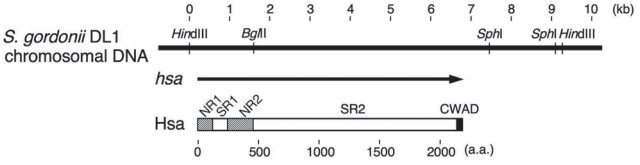

Fig. 1. Physical map of the *S. gordonii* DL1 chromosomal DNA showing the coding region for Hsa. SR1 (amino acid residues 138 to 219) and SR2 (amino acid residues 450 to 2,143) of Hsa are indicated by open bars. CWAD (amino acid residues 2,144 to 2,178) is indicated by a closed bar. NR1 and NR2 are indicated by hatched bars. The consensus sequence SASTSASVSASE is repeated 113 times between amino acid residues 488 and 1,843.

wild-type *S. gordonii* strains for *hsa* homologs and Hsa cell wall expression, as well as their biofilm-forming abilities.

MATERIALS AND METHODS

Bacterial strains and growth conditions: The *S. gordonii* strains used in this study included DL1 (Challis strain, wild type, biovar 2) (12,22) and its *hsa* mutant EM230 (DL1 *hsa*::*ermAM*) (8) as well as several other wild-type *S. gordonii* strains, including 38, SK6 (biovar 1), M5, 10558 (biovar 2), and SK12 (biovar 3) (12,22). All streptococci were cultured overnight in brain-heart infusion broth (Difco, Becton-Dickinson and Company, Sparks, MD, USA) at 37°C. The medium was supplemented with 10 μg/mL of erythromycin (Sigma-Aldrich, St. Louis, MO, USA) as needed. *Escherichia coli* JM109 harboring pMDS8161 or pMDS8741 was cultured overnight in Luria-Bertani medium supplemented with 50 μg/mL of ampicillin (Sigma-Aldrich) and was utilized for propagation of the plasmids used to prepare the DNA probes for Southern blot analysis (8).

Human material: The collection and use of saliva samples in this study was approved by the Research Ethics Committee of Nippon Dental University (NDU-T2012-33). After obtaining written informed consent for using the collected samples in a scientific study, human whole saliva was collected from 6 volunteers by having them chew paraffin (CAT21; Willdent Co, Ltd., Osaka, Japan) that was then spit into an ice-chilled container (23). The collected saliva samples were clarified by centrifugation at 1,500 × *g* for 30 min and at 10,000 × *g* for 20 min, diluted 2-fold with 5 mM phosphate buffer (pH 7.4), filtered using a 0.45-μm filter (Merck Millipore Ltd., Tullagreen, Carrigtwohii, County Cork, Ireland), and stored in 5-mL aliquots at −20°C.

Biofilm assay: Biofilm formation was assessed as previously described (21,24), with some modifications. Biofilm medium (BM) was used in the experiment as the minimal medium, except that $MgSO_4 \bullet 7H_2O$ was used as the source of magnesium ion. Round-bottomed (Corning Inc., Corning, NY, USA) or flat-bottomed (Santa Cruz Biotechnology Inc., Heidelberg, Germany) polystyrene microtiter plates, or flat-bottomed EIA-grade microtiter plates (Corning) containing 200 μL of BM per well were inoculated with an overnight bacterial culture (absorbance at 620 nm [A_{620}] of the final suspension = 0.01). As indicated, each well of the flat-bottomed EIA-grade microtiter plates was pre-coated with 50 μL of 1% w/v bovine serum albumin (Sigma-Aldrich), 50% v/v human saliva, 1% w/v mucin (from bovine submandibular saliva, Sigma-Aldrich), or 1% w/v fetuin (Sigma-Aldrich) for 24 h at room temperature. In addition, as indicated, the human saliva-coated plates were pre-incubated at 37°C for 60 min with 50 μL of phosphate buffered saline (PBS, pH 7.4; untreated), or with PBS containing 1 U/mL neuraminidase (Sigma-Aldrich; neuraminidase-treated) to remove the sialic acid. After aerobic or anaerobic cultivation at 37°C, 25 μL of a 0.2% w/v crystal violet solution was added to each well to stain the biofilm. After 15 min, the wells were rinsed twice with 200 μL of distilled H_2O to remove excess dye and then air-dried. The stained biofilm was photographed, and the dye intensity was measured using ImageJ software (NIH, Bethesda, MD, USA). To confirm that there were no significant differences in bacterial growth among the tested strains, non-stained bacterial cultures in glass tubes were quantified spectrophotometrically by measuring the A_{620}. Each assay was performed in hexaplicate, and biofilm-forming ability was measured as the mean dye intensity. Biofilm-forming abilities were compared using the unpaired t-test.

In the pilot experiment, BM containing 0.8% w/v sucrose, BM containing 0.1 M NaCl, and brain-heart infusion broth containing 1% w/v sucrose were used instead of BM. In addition, the concentration of crystal violet and the staining time were varied to determine the optimal conditions for staining.

Southern blot analysis: Restriction endonucleases were purchased from Takara Bio (Kusatsu, Japan). Chromosomal DNA was prepared from *S. gordonii* strains as previously described (25) and digested with *Hin*dIII and *Bgl*II. Plasmids pMDS8161 and pMDS8741, which contain a 1.6-kb *Hin*dIII-*Bgl*II and 7.4-kb *Hin*dIII-*Sph*I *hsa* insert, respectively (8) (Fig. 1), were prepared as previously described (26) and digested with the appropriate restriction endonucleases to obtain the *hsa* fragment. Preparation of labeled DNA probes obtained from the digested plasmids and Southern hybridization were carried out using the DIG DNA labeling and detection kit (Roche Diagnostics, Indianapolis, IN, USA) as previously described (8).

Western blot and lectin blot analyses: Solubilized bacterial cell wall proteins were prepared as previously described (9) and were boiled for 10 min in reducing sample buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 5% polyacrylamide gels, and electrophoretically transferred to nitrocellulose membranes for western blotting with rabbit antiserum raised against native purified Hsa (anti-Hsa) (7) and for lectin blot analysis with biotinylated wheat germ agglutinin (EY Laboratories, San Mateo, CA, USA) as previously described (9).

RESULTS

Determination of the optimal experimental conditions for detecting biofilm formation: Before comparing the biofilm-forming abilities of the *S. gordonii* wild type and *hsa* mutant strains, pilot biofilm formation experiments were performed under various conditions to determine the optimal experimental conditions. We focused on the medium used for biofilm formation and the concentration of the dye used for staining of the biofilm. The lowest errors and most reproducible results were obtained when BM was used as the growth medium and when the biofilms were stained with 0.2% w/v crystal violet for 15 min staining (data not shown). The *hsa* mutation did not affect the growth of *S. gordonii* DL1 in BM, although the growth was slower under aerobic conditions than under anaerobic conditions (Fig. 2A).

Association of Hsa with biofilm formation on a polystyrene surface: The biofilm-forming abilities of the *S. gordonii* wild type strain DL1 and *hsa* insertional mutant EM230 on the polystyrene surface of a microtiter plate, as measured by the absorbance of crystal violet-stained biofilm, are shown in Fig. 2B. The biofilm-forming ability of the *hsa* mutant on a round-bot-

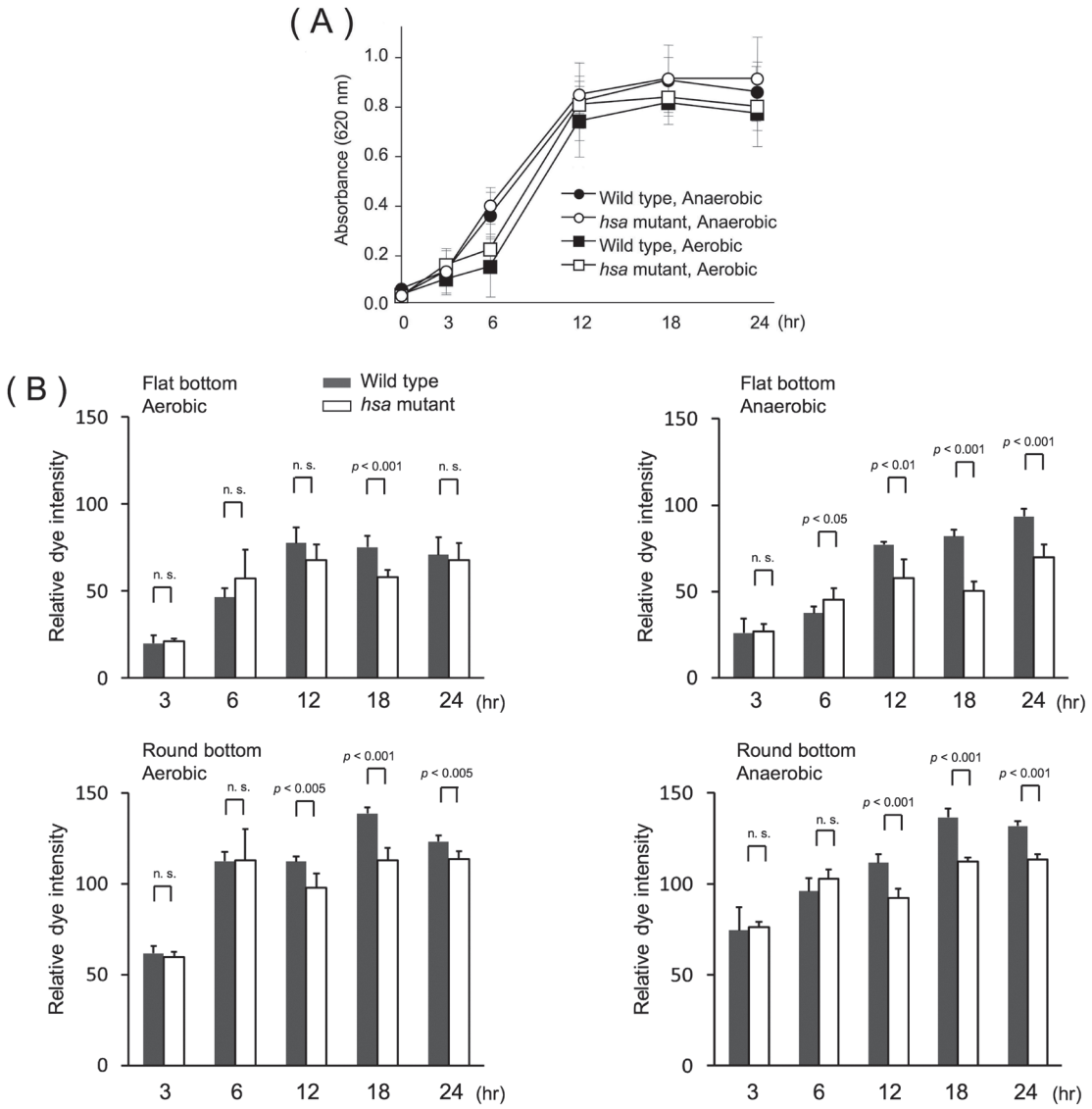

Fig. 2. Comparison of bacterial growth and the biofilm-forming ability on abiotic surfaces between *S. gordonii* wild type strain DL1 and its *hsa* mutant EM230. (A) Bacterial growth of *S. gordonii* wild type strain DL1 and its *hsa* mutant EM230 in BM under aerobic and anaerobic condition. Significant difference of the growth ($p < 0.05$) was observed between aerobic and anaerobic condition for 6 hr cultivation. (B) Comparison of the biofilm-forming ability of *S. gordonii* wild type strain DL1 to that of its *hsa* mutant EM230 under aerobic or anaerobic cultivating conditions in BM on round-bottomed or flat-bottomed microtiter plates. All assays were performed in hexaplicate, and mean and standard deviations of each value are presented. Representative results from 3 independent experiments are shown.

tomed microtiter plate was significantly lower than that of the wild type strain when cultured for more than 12 h. A similar reduction was observed when a flat-bottomed microtiter plate was used, except that no significant difference in biofilm-forming ability was observed at 12 or 24 h. These results indicate that the binding of Hsa to the polystyrene surface contributes to the biofilm-forming ability of *S. gordonii* DL1, especially under anaerobic conditions, as was suggested in previous reports (21). Accordingly, the following experiments were performed under anaerobic conditions in flat-bottomed microtiter plates containing BM for 18 h.

Association of Hsa with biofilm formation on a glycoprotein-coated surface: The tooth surface is usually coated with acquired pellicle, which is mainly derived from the saliva (2). To examine whether Hsa is associated with biofilm formation under conditions similar to those at the tooth surface, the biofilm-forming abilities of the wild type and *hsa* mutant strains were compared on a flat-bottomed microtiter plate pre-coated with saliva or glycoprotein. No significant difference in biofilm-forming ability was observed between the wild-type and *hsa* mutant strains on plates pre-coated with bovine serum albumin, which does not contain sialic acid. In contrast, when the surface was coated with saliva, fetuin, or mucin, a significant reduction in the biofilm-forming ability of the *hsa* mutant was observed when compared to that of the wild-type strain (Fig. 3), indicating that the binding of Hsa to sialoglycoconjugates on the surface is important for *S. gordonii* DL1 biofilm formation.

Association of Hsa with biofilm formation in various *S. gordonii* strains: Previous studies have reported variations in the adhesive properties of different *S. gordonii* strains (12,22). For example, some, but not all, *S.*

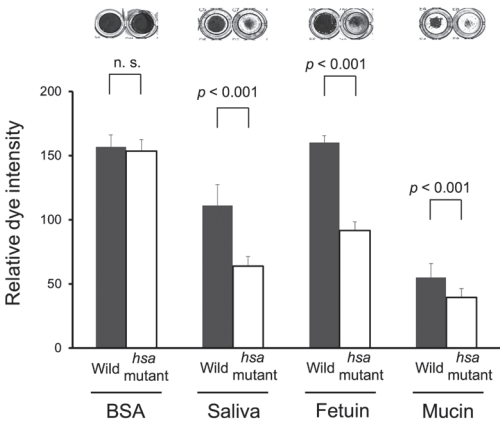

Fig. 3. Comparison of the biofilm-forming ability of *S. gordonii* wild type strain DL1 to that of its *hsa* mutant EM230 on flat-bottomed microtiter plates coated with various proteins or human saliva. All assays were performed in hexaplicate, and mean and standard deviations of each value are presented. Representative results from 3 independent experiments are shown. A representative row of crystal violet-stained microtiter plate wells is shown above the graph. BSA, bovine serum albumin.

gordonii strains bind to the human salivary mucin MG2 in a sialic acid-dependent manner, suggesting that a bacterial adhesin similar to Hsa binds to MG2. As shown in Fig. 4, *hsa* homologs were detected in all *S. gordonii* strains tested by Southern blot analysis when probed with both the 1.6-kb *Hin*dIII-*Bgl*II and 5.8-kb *Bgl*II-*Sph*I DNA fragments (which detected 1.6-kb *Hin*dIII-*Bgl*II and 7.6-kb *Bgl*II-*Hin*dIII fragments of chromosomal DNA, respectively) containing the *hsa* gene encoding the N-terminal region (NR1, SR1, and NR2) and C-terminal region (SR2 and CWAD) of Hsa, respectively (Fig. 1), although some restriction fragment length polymorphism was observed. In addition, expression of the homolog protein in each strain was demonstrated by western blotting using an anti-Hsa antibody and by lectin blot analysis using biotinylated wheat germ agglutinin (Fig. 5). The results indicated that *S. gordonii* strains appear to widely possess *hsa* homologs and express Hsa protein on the bacterial cell surface. To assess whether these *S. gordonii* wild-type strains have biofilm-forming ability similar to that of strain DL1, a biofilm assay was performed on saliva pre-coated surfaces with or

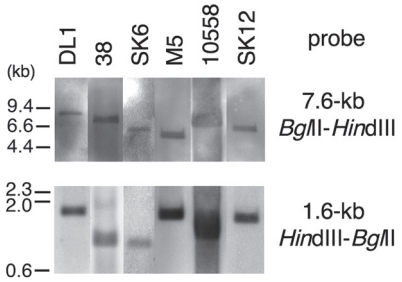

Fig. 4. Southern blot analysis of *Hin*dIII and *Bgl*II-digested chromosomal DNAs from various wild type strains probed with the fragments containing the *hsa* gene. The upper panel and the lower panel show the detection of the 7.6-kb *Bgl*II-*Hin*dIII fragment and the 1.6-kb *Hin*dIII-*Bgl*II fragment of *S. gordonii* DL1 chromosomal DNA containing the *hsa* gene or its homologues, respectively. The positions of the DNA size markers are indicated on the left.

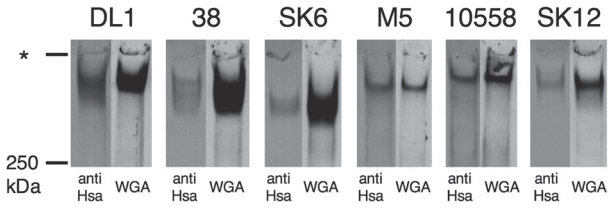

Fig. 5. Expression of Hsa protein and its homologues from various wild type strains. Western blot analysis with anti-Hsa and lectin blot analysis with biotinylated wheat germ agglutinin (WGA) of streptococcal cell wall extracts obtained from 1 mL of bacterial culture for each well are shown. The boundary between the stacking and separating gels (asterisk) and the position of 250 kDa molecular mass marker are indicated on the left.

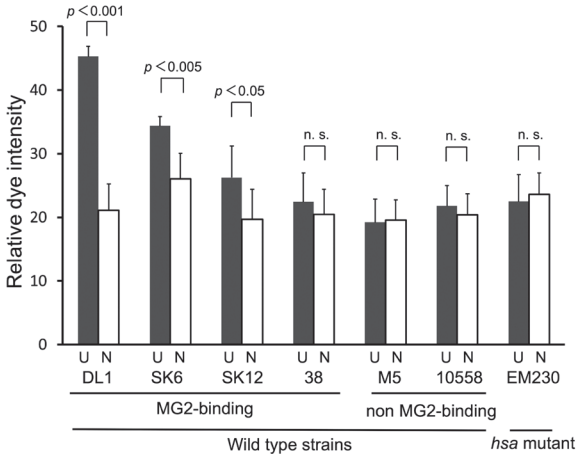

Fig. 6. Biofilm-forming ability of *S. gordonii* wild type strain DL1, its *hsa* mutant EM230, and the other wild type strains in flat-bottom plates which were pre-coated with saliva and untreated (U) or neuraminidase-treated (N). All assays were performed in hexaplicate, and mean and standard deviations of each value are presented. Representative results from 3 independent experiments are shown.

without neuraminidase treatment. As shown in Fig. 6, removal of sialic acid from the saliva-coated surface by treatment with neuraminidase significantly reduced the biofilm-forming ability of the strains that were previously reported as MG2-binding strains (DL1, SK6, and SK12) (12) except strain 38. In contrast, no such reduction was observed for the *hsa* mutant EM230 or for the non-MG2-binding strains M5 and 10558, implying variation in the contribution of Hsa and its homologs to *S. gordonii* biofilm formation.

DISCUSSION

Adhesion of *S. gordonii* DL1 specific to α2-3-linked sialic acid-containing host glycoconjugates was previously reported to be associated with the bacterial cell surface component Hsa adhesin (7,8). However, little is known about the role of Hsa in biofilm formation. The results of the present study suggest that the binding of Hsa adhesin to sialoglycoconjugates is associated with the biofilm-forming ability of *S. gordonii* DL1. The water-insoluble glucans produced by mutans-group streptococci are well-known virulence factors associated with oral biofilm formation (27); however, this is the first report demonstrating that a streptococcal adhesin specific to sialoglycoconjugates is associated with biofilm

formation on a surface coated with acquired pellicle-derived components in a sialic-acid dependent manner.

Various methods and conditions have been described for microtiter plate-based biofilm assays (21,24). These variations prompted us to perform pilot examinations to determine the optimal medium composition, culture conditions, material and shape of the biofilm-forming surface, and procedures to best quantify the biofilm-forming ability. As mitis group streptococci are considered to be the major pioneer colonizers on the human tooth surface, the effect of extracellular glucans is not directly relevant (28). Therefore, we first assessed the effect of sucrose in the medium, and found that it was not essential for *S. gordonii* biofilm formation. This result was consistent with a previous finding that the water-soluble glucans produced by mitis group streptococci are less effective for biofilm formation than other components of the human tooth surface (28). In contrast, assessment of the role of certain components on the human tooth surface, such as acquired pellicle derived from whole saliva, are likely to be more relevant in a sucrose-independent biofilm assay. From this standpoint, we thought that pre-coating of flat-bottomed microtiter plates with glycoconjugates would be a reproducible and comparable method.

Compared with the specificity of interactions between major bacterial adhesins and host cell receptors, biofilm formation appears to be associated with multiple factors. For example, while the *hsa* mutation completely abolished the hemagglutination and platelet aggregation activities of *S. gordonii* DL1 (8,9), it only significantly reduced, but did not completely abolish, biofilm formation. These results suggest that multiple adhesins, such as ScaA (29), a lipoprotein homolog of *Streptococcus parasanguinis* FimA (30); SspA/SspB (31) of the streptococcal antigen I/II family (32); CshA (33); and the amylase-binding proteins AbpA and AbpB (34), may also contribute to biofilm formation.

In this study, both possession of the *hsa* gene and expression of the Hsa proteins were observed in all *S. gordonii* wild-type strains tested. Although *S. gordonii* M5 and 10558 have been reported as non-aggregating strains with anti-Hsa (7), a positive broad band was observed for both strains by western blot and lectin blot analyses. This observation may be due to the high sensitivity of the blotting methods used. In addition, variations in the biofilm-forming ability of the tested *S. gordonii* wild-type strains on surfaces pre-coated with saliva were observed. Interestingly, the biofilm-forming ability of *S. gordonii* 38 was not significantly reduced by neuraminidase treatment of the saliva-coated surface, even though the strain has been reported to bind MG2 in a sialic acid-dependent manner (12). One possible interpretation for this observation is that other terminal saccharides exposed by removal of sialic acid might be recognized by other adhesins in this strain. Taken together, these results indicate the diversity of the Hsa adhesin homologs, as well as the strain specificity of the major adhesive factors contributing to *S. gordonii* biofilm formation.

Hsa adhesin and its homologs, as well as other adhesins or bacterial surface components, have been reported to play important roles in the adhesion and colonization of other oral bacteria, including intrageneric coaggregation (2) and coaggregation with *Porphyromonas gingivalis* (35) or *Veillonella* species (36,37). Further studies are currently underway to determine whether the adhesins in various strains effectively promote biofilm-forming ability. Moreover, the diversity of Hsa homologs, especially within the NR2 domain (38), may be important for facilitating adaptation to specific niches of the human oral environment. Further investigation into these possibilities is expected to provide important insights into the molecular mechanisms contributing to the development of oral microbial flora.

Acknowledgments We thank Drs. Toshiyuki Toen and Tetsuya Fukada for helpful suggestions. This work was supported by Grants-in-Aid for Scientific Research (26861845) from the Japan Society for the Promotion of Science.

Conflict of interest None to declare.

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