The Sialic Acid Binding Protein, Hsa, in *Streptococcus gordonii* DL1 also Mediates Intergeneric Coaggregation with *Veillonella* Species

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Abstract

Dental biofilm development involves initial colonization of the tooth’s surface by pioneer colonizers, followed by cell-cell coaggregation between the pioneer and later colonizers. *Streptococcus gordonii* is one of the pioneer colonizers. In addition to its role in oral biofilm development, *S. gordonii* also is a pathogen in infective endocarditis in susceptible humans. A surface adhesin, Hsa, has been shown to play a critical role in colonization of *S. gordonii* on the heart tissue; however, its role in oral biofilm development has not been reported. In this study we demonstrate that Hsa is essential for coaggregation between *S. gordonii* and *Veillonella* sp., which are bridging species connecting the pioneer colonizers to the late colonizers. Interestingly, the same domains shown to be required for Hsa binding to sialic acid on the human cell surface are also required for coaggregation with *Veillonella* sp. However, sialic acid appeared not to be required for this intergeneric coaggregation. This result suggests that although the same domains of Hsa are involved in binding to eukaryotic as well as *Veillonella* cells, the binding mechanism is different. The gene expression pattern of *hsa* was also studied and shown not to be induced by coaggregation with *Veillonella* sp.

Introduction

It is believed that more than 700 bacterial species colonize the human oral cavity, and each human mouth may harbor as many as 120 species [1, 2]. The formation of this multispecies community involves a sequential process, with pioneer colonizers adhering to the tooth’s surface, followed by early/middle colonizers adhering to the pioneer colonizers, and finally by the late colonizers adhering to the early/middle colonizers [3, 4]. The mitis streptococci (*S. gordonii*, *S. oralis* and *S. sanguinis* etc.) are considered pioneer colonizers, which can comprise as
much as 80% of the early dental biofilm population [5]. The Veillonella species are one of early/middle colonizers. They are closely associated with the streptococci via cell-cell coaggregation as well as metabolic complementation—lactic acid produced by the streptococci serves as major carbon source for veillonellae, and the consequential lactate removal raises local pH, thus relieving the streptococci from toxicity of their own metabolic waste. This mutualistic relationship helps Veillonella sp to colonize and grow in the early biofilm community, which then provides attachment sites and possibly different kinds of metabolic complementation for the late colonizers such as the periodontopathogen Porphyromonas gingivalis [6, 7]. Given the importance of early/middle colonizers such as veillonellae in biofilm development and dysbiosis, understanding the mechanism of coaggregation between Veillonella species and streptococci would provide an important knowledge base for developing strategies of disease prevention.

Our previous studies have identified a surface protein named Hag1 in V. atypica strain OK5, which is responsible for Veillonella binding to S. gordonii [8]. We further demonstrated that the binding partner for Hag1 is likely a protein, as proteinase treatment of S. gordonii completely abolished coaggregation [8]. In this study, we report the identification of the corresponding adhesin from S. gordonii. We show that a previously characterized sialic acid binding adhesin, Hsa, is required for coaggregation with V. atypica as well as other Veillonella species. We further demonstrate that the same functional domains of Hsa responsible for binding to sialic acid on mammalian cells also are required for coaggregation with V. atypica; however, a different mechanism seems to be utilized in Hsa binding to mammalian cells rather than to veillonellae cells. Finally, reporter expression analyses showed that hsa gene expression is not induced or enhanced by coaggregation with V. atypica.

Materials and Methods

Bacterial strains and culture conditions

Bacterial strains and plasmids used or constructed in this study are listed in Table 1. Streptococcus gordonii DL1 was grown in brain heart infusion broth (BHI; Difco), or on BHI agar plates. For transformant selection, cells were grown in Todd–Hewitt broth (Difco) with 0.3% yeast extract (Difco) (THYE) plus erythromycin (12.5 µg ml⁻¹) or spectinomycin (500 µg ml⁻¹). For selection of genetically complemented mutant strains, THYE broth supplemented with 250 µg ml⁻¹ spectinomycin was utilized. Veillonella atypica OK5 and other veillonella strains were grown in brain heart infusion broth (BHI; Difco) supplemented with 0.6% sodium lactate (BHIL), or on BHIL agar plates. All bacterial strains were grown anaerobically (85% N₂, 10% CO₂, 5% H₂) at 37°C. Escherichia coli DH5α cells (Thermo Fisher Scientific) were grown in Luria-Bertani (LB; Difco) medium with aeration at 37°C. E. coli strains carrying plasmids were grown in LB medium containing spectinomycin (250 µg/ml) or tetracycline (10 µg/ml).

Construction of S. gordonii surface protein mutants

PCR primers used in this study are listed in Table 2. The hsa mutant, srtA mutant, and sspa/B double mutant were constructed by overlapping PCR. Briefly, the upstream and downstream fragments of the target genes were generated by PCR using the specific primer pairs (Table 2). The ermAM fragment was generated by PCR using primer pair ermAM-F/ermAM-R. The three fragments were mixed and used as template for overlapping PCR without primers. The final amplicons were transformed into S. gordonii DL1 and mutants were selected for erythromycin resistance.
Coaggregation assay

Co-aggregation assays were performed as previously described [13] with minor modifications: Mid-log cells were harvested and washed twice with coaggregation buffer (1 mM Tris buffer [pH 8.0], 0.1 mM CaCl₂, 0.1 mM MgCl₂, 150 mM NaCl) at room temperature. Then, cells were resuspended in coaggregation buffer and normalized to OD₆₀₀ = 1.2. Equal volumes (0.1 ml) of each cell suspension were mixed in a micro-centrifuge tube and vortexed briefly. The tubes were left standing at room temperature until aggregates appeared, usually 15–30 min. Tubes containing each cell suspension alone (0.2 ml) were included as controls. All assays were repeated at least 3 times.

Construction of hsa reporter strain

The hsa-luc reporter was constructed as follows: A ~ 600 bp fragment of the hsa promoter region was PCR amplified using primer pairs hsa₀-luc-F/hsa₀-luc-R (Table 2). The product

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Table 1. Bacterial strains and plasmids used in this study.

| Strains and plasmids | Characteristics | Reference |
|----------------------|----------------|-----------|
| **Strains**          |                |           |
| *E. coli* DH5a       | Cloning strain |           |
| *V. atypica* OK5     | Wild type     | [9]       |
| *V. parvula* PK1910  | Wild type     | This work |
| *V. parvula* OK1     | Wild type     | This work |
| *V. atypica* OK2     | Wild type     | This work |
| *V. rogosae* OK3     | Wild type     | This work |
| *V. dispar* OK4      | Wild type     | This work |
| *V. atypica* OK6     | Wild type     | This work |
| *V. atypica* OK7-1   | Wild type     | This work |
| *V. dispar* OK7-2    | Wild type     | This work |
| *V. dispar* OK9      | Wild type     | This work |
| *V. dispar* OK9      | Wild type     | This work |
| *V. rogosae* OK10    | Wild type     | This work |
| *V. atypica* OK11    | Wild type     | This work |
| **S. gordontii** DL1 | Wild type     | [10]      |
| Δhsa                  | DL1 hsa deletion mutant | This work |
| ΔsrtA                 | DL1 srtA deletion mutant | [11] |
| ΔsspA/B               | DL1 sspA/B deletion mutant | This work |
| **Plasmids**         |                |           |
| pFW5-hsa₀-luc        | Suicide vector pFW5::luc of S. gordontii with hsa promoter region; Spec<sup>+</sup> | This work |
| pAS8741              | Plasmid containing the complete hsa gene | [12] |
| pAS8748              | Deletion of SR1 and NR2 regions in Hsa | [12] |
| pAS8744              | Deletion of SR1 region in Hsa | [12] |
| pAS8746              | Deletion of NR2 region in Hsa | [12] |
| pAS8749              | Deletion of part of SR2 region in Hsa | [12] |
| pAS8375              | Deletion of part of SR2 region in Hsa | [12] |
| pAS8165              | Deletion of SR2 region in Hsa | [12] |
| pAS8164              | Deletion of SR1 and SR2 regions in Hsa | [12] |

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was digested with *Sal*I and *Bam*HI and cloned into the suicide vector pFW5-luc digested with the same restriction enzymes to create the fusion plasmid pFW5-hsa*p-luc*. The confirmed plasmid was transformed into *S. gordonii* DL1 and transformants were selected on BHI plates supplemented with spectinomycin (500 μg mL⁻¹). Transformants were further confirmed by PCR.

**Luciferase assays**

Overnight culture of *S. gordonii* hsa-luc reporter strains were spun down and re-suspended with fresh BHI media to OD₆₀₀ ~1.0. Suspended cultures then were 1:20 diluted into fresh BHI media. For co-culture test, overnight culture of *V. atypica* OK5 was centrifuged and re-suspended with fresh BHI to OD₆₀₀ ~1.0, and then was diluted into BHI media with *S. gordonii*/*V. atypica* ratio 5:1. All cultures were grown in anaerobic condition for 24 hours, and the data were measured every 2 hours. Luciferase assays were performed by adding 25 μL of 1 mM D-luciferin (Sigma) solution (suspended in 0.1 M citrate buffer, pH 6.0) into 100 μL samples, and luciferase activities were measured using a TD 20/20 luminometer (Turner Biosystems, Sunnyvale, CA).

For single species cultures, the optical densities at 600 nm were measured with a spectrophotometer (Bio-Rad, smartspec 3000). For mixed species cultures, colony-forming units (CFU/ml) were obtained by plate counting. Luciferase activity was expressed either as relative light unit (RLU)/OD₆₀₀ (for single-species culture), or as RLU/CFUx1000 (for mixed species culture).

**Results**

**Coaggregation between *S. gordonii* DL1 and veillonellae**

Coaggregation between oral streptococci especially *S. gordonii* and veillonellae has been studied previously [8, 14]. To gain a general picture of the coaggregation pattern between our model strain *S. gordonii* DL1 and our clinical veillonellae isolates, we used the modified *in vitro* coaggregation assay [13] to qualitatively assess the coaggregation ability between *S. gordonii* DL1 and the veillonellae strains. As shown in Table 3, *S. gordonii* DL1 coaggregated with two of four *V. atypica* strains, all two *V. parvula* strains, and one of two *V. rogosae* strains. Interestingly, none of the five *V. dispar* strains coaggregated with *S. gordonii* DL1.
Identification of the adhesin in *S. gordonii* DL1 responsible for coaggregation with veillonellae

In our previous study, we demonstrated that proteinase K treatments of *S. gordonii* abolished co-aggregation with *V. atypica*, suggesting that a surface protein/adhesin is probably involved [8]. Four types of surface adhesins have been identified in *S. gordonii*: cell-surface fibrillar proteins (CshA and CshB), sialic-acid binding protein (Hsa/GspB), amylase-binding proteins (AbpA and AbpB), and antigen I/II (AgI/II) family proteins (SspA and SspB) [15–19]. To have a quick survey of the known surface adhesins in *S. gordonii*, we initially constructed mutant strains with deletion of surface adhesin genes sspA/B, hsa, and srtA. As both Hsa and SspA/B belong to LPXTG-motif-containing surface proteins, SrtA was used as a positive control because its deletion would eliminate all surface proteins with a LPXTG-containing cell wall anchoring domain [11]. In vitro coaggregation assay was used to assess the effect of these mutations on coaggregation with *V. atypica*. As shown in Fig 1, the sspA/B double mutation did not have any effect on coaggregation with *V. atypica*, while the hsa and srtA mutations completely abolished coaggregation. This result suggested that Hsa is the protein responsible for coaggregation with *V. atypica*. To see if the hsa mutation had the same effect on other *Veillonella* strains that coaggregated with *S. gordonii*, the same coaggregation assay was performed. As shown in Table 3, the mutation abolished coaggregation with all but one (*V. parvula* PK1910) coaggregation partner. This result indicates that Hsa is the adhesin mediating coaggregation with *V. atypica* strains OK2 and OK5, *V. parvula* strain OK1, and *V. rogosae* OK3, and that coaggregation with *V. parvula* PK1910 (previously *V. atypica* PK1910) is probably mediated by a completely different mechanism.

### Identification of Hsa domains that are responsible for coaggregation between *S. gordonii* DL1 and *V. atypica* OK5

The surface adhesin Hsa/GspB has been characterized extensively [16, 19]. The protein has been shown to bind to sialic acid on the surface of different types of mammalian cells [20–22]. In *S. gordonii*, the protein has 2178 amino acids, which can be divided into 5 domains: the N-
terminal non-repeat region (NR1), followed by serine-rich repeat region 1 (SR1), non-repeat region 2 (NR2), serine-rich repeat region 2 (SR2), and the C-terminal wall-anchor domain (CWAD) [Fig 2, [12]]. A series of plasmids containing deletions of different domains has been previously constructed by Dr. Takahashi’s group, and all plasmids were shown by Western blot.

![Fig 1. Coaggregation of S. gordonii DL1 (wt) and surface protein mutants with V. atypica OK5.](image)

Coaggregation-positive pairs form aggregates that precipitate at the bottom of the tube (arrowhead), while cells of the single species cultures or mixed cultures with the hsa and srtA mutants remain in suspension. Sg: S. gordonii DL1; Va: V. atypica OK5.

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![Fig 2. Graphic illustration of the various regions deleted in the complementing plasmids. The basic region of Hsa was located on amino acid position 228–449. pAS8741 carries an intact hsa gene; pAS8748 encodes a Hsa protein lacking the SR1 and NR2 regions (HsaΔ105–446); pAS8744 encodes a Hsa protein lacking the SR1 region (HsaΔ105–245); pAS8746 encodes a Hsa protein lacking the NR2 region (HsaΔ246–446); pAS8749 encodes a Hsa protein lacking the N-terminal part of the SR2 region (HsaΔ448–1459); pAS875 encodes a Hsa protein lacking the C-terminal part of the SR2 region (HsaΔ1092–2143); pAS8165 encodes a Hsa protein lacking the entire SR2 region (HsaΔ448–2143); and pAS8164 encodes a Hsa protein lacking both SR1 and SR2 regions (HsaΔ105–245, Δ448–2143). The dotted lines indicate the regions deleted. BR: basic region; NR: non-repeat region; SR: serine-rich repeat region; CWAD: cell wall anchoring domain.](image)

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to express truncated Hsa proteins corresponding to the respective hsa deletions they carry in the mutant strains [12]. To determine which domain is responsible for binding to V. atypica, we used these plasmids to complement the hsa deletion mutation strain. A total of 8 complement strains were thus constructed and tested in an in vitro coaggregation assay with V. atypica. As shown in Fig 3, five strains containing plasmids pAS8748, pAS8744, pAS8746, pAS8165, and pAS8164 failed to restore coaggregation with V. atypica, suggesting that the deleted regions of Hsa encoded by the DNA fragments in these plasmids are required for coaggregation. As illustrated in Fig 2, plasmid pAS8165 contains a complete deletion of the SR2 region, suggesting that the SR2 region is required for coaggregation. However, since plasmids pAS8749 contains only the C-terminal part of SR2, and plasmid pAS8375 contains only the N-terminal part of SR2, and both restored coaggregation with V. atypica, it appears that the N- and C-terminal region of SR2 is redundant for Hsa function. In addition to the SR2 fragment, both SR1 and NR2 domains are also required for coaggregation, because plasmids containing SR1 (pAS8746) or NR2 (pAS8744) deletions did not complement the coaggregation deficiency of the hsa deletion. Taken together, we conclude that the intact domains of SR1 and NR2 plus either the N- or C-terminal domain of SR2 are required for Hsa binding to V. atypica.

Sialic acid is not involved in Hsa binding to V. atypica OK5

It was well known that sialic acid is the binding partner for Hsa adhesin, and the basic region (BR) of Hsa mediates its adhesion to sialic acid[23, 24]. The BR of Hsa is located on amino acid position 228–449, encompassing the C-terminal portion of SR1 and the entire region of NR2 (Fig 2). Results presented above demonstrated that both SR1 and NR2 regions are also required for coaggregation with V. atypica OK5, suggesting that sialic acid or similar molecules may exist on the veillonellae cell surface[20, 25]. To see whether V. atypica could potentially synthesize sialic acids, we searched the genome sequence of V. atypica strains in the HOMD database for putative sialic acid biosynthesis genes, and found none. The draft sequence of strain V. atypica OK5 (P. Zhou and F. Qi, unpublished data) used in this study was also searched, and no sialic acid biosynthesis genes were found. To further confirm that no sialic acid from the V. atypica cell surface were involved, then adding fetuin would inhibit coaggregation. It turned out that fetuin had no effect on coaggregation either (Fig 4B). Similarly, adding fetuin to the buccal cells nearly completely abolished attachment by S. gordonii DL1 (Fig 4A). Next, we added fetuin to the coaggregation assay. Fetuin is a group of molecules with sialylated carbohydrate structures that are similar to the sialoproteins on mammalian cell surface [23]. We reasoned that if a sialic acid-like structure on V. atypica cell surface were involved, then adding fetuin would inhibit coaggregation. It turned out that fetuin had no effect on coaggregation either (Fig 4B). Similarly, adding fetuin to the buccal cells nearly completely abolished attachment by S. gordonii DL1 cells (Fig 4). Taken together, we concluded that sialic acid is not involved in Hsa-mediated coaggregation with V. atypica OK5, although it is required for S. gordonii attachment to human buccal cells.

The expression of hsa gene is not regulated by coaggregation with V. atypica

To see whether the expression of hsa gene is regulated by coaggregation between S. gordonii and V. atypica, we constructed a firefly luciferase reporter in the S. gordonii DL1 wild type as well as in the Δhsa mutant strain. The expression of hsa-luc reporter was first measured over the growth curve in the wild type and the mutant single species cultures. As shown in Fig 5A, the hsa gene expression started at early log phase, peaked at mid-log phase, and rapidly turned down at early stationary phase. There is no difference in the level or the pattern of gene
expression in the wild-type and the hsa mutant strains, suggesting no feedback regulation. Next, we measured hsa-luc expression of the two strains in mixed cultures with V. atypica OK5 (Fig 5B). No difference was observed between single and mixed species cultures, or between coaggregated (S. gordonii wild-type + OK5) and non-coaggregated (S. gordonii Δhsa + OK5) mixed cultures, suggesting that coaggregation does not affect gene expression of hsa.

Fig 3. Coaggregation of S. gordonii DL1 (wt), hsa mutant (Δhsa), and the eight complemented strains with V. atypica OK5. S. gordonii DL1 and complemented strains containing pAS8741, pAS8749 and pAS8375 formed aggregates with V. atypica OK5 and precipitated at the bottom of the tube (arrowhead), while cells of the single species cultures or mixed cultures with the Δhsa mutant or complemented strains containing pAS8748, pAS8744, pAS8746, pAS8165 and pAS8164 remained in solution.

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Fig 4. Coaggregation of S. gordonii DL1 with V. atypica OK5 treated with neuraminidase (A) or fetuin (B). Note that none of the treatments affected coaggregation between the two species, and S. gordonii cannot adhere to buccal cells treated by neuraminidase or fetuin. The arrows indicate the aggregates at the bottom of the tube. Sg: S. gordonii DL1; Va: V. atypica OK5.

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Discussion

In the oral biofilm, cell-cell coaggregation plays a critical role in biofilm development. Of particular importance is the coaggregation between pioneer colonizers and the early/middle colonizers. As the latter are bridging species for colonization of late colonizers many of which are periodontopathogens, understanding the mechanisms of coaggregation between the pioneer colonizers and the bridging species can lead to development of knowledge-based strategy for prevention of periodontal diseases.

The mitis streptococci are major pioneer colonizers, and species of the *Veillonella* genus are one of the most prevalent and numerically dominant bridging species. The two groups of bacteria physically coaggregate with each other especially amongst strains isolated from the same anatomical sites [14, 26]. This coaggregation enables the *Veillonella* species to join the early biofilm community, for the veillonellae bacteria on their own have very weak (if any) attachment ability to the salivary pellicles on the tooth's surface [1, 27, 28].

A number of early studies attempted to identify surface adhesins mediating coaggregation between streptococci and veillonellae [29, 30]. Weerkamp and McBride analyzed the surface component that is responsible for *Streptococcus salivarius* HB binding to *V. alcalescens* V1 (now *V. parvula* V1), and they found that a fraction adhered to *V. alcalescens* V1 was released upon lysozyme treatment of the latter [29]. Using chemical mutagenesis and natural selection, Handley et al. selected four mutants of *S. salivarius* HB that abolished the ability to coaggregate with *V. parvula* V1. However, the nature of these mutants has not been determined [29, 30].

Thus, until now no adhesin has been definitively identified in streptococci that is responsible for coaggregation with veillonellae. In this study, we identified Hsa from *S. gordonii* as the adhesin mediating coaggregation with *V. atypica* strains OK2 and OK5, *V. rogosae* strain OK3, and *V. parvula* strain OK1, but not with *V. parvula* strain PK1910 (Table 3). Additionally, not all strains in *V. atypica* and *V. rogosae* coaggregated with *S. gordonii*, suggesting that this intergeneric coaggregation may be strain specific, which is also consistent with previous reports [26].

The finding that Hsa being the adhesin mediating coaggregation of *S. gordonii* with some *Veillonella* species/strains is very interesting, because Hsa has been well characterized as a sialic acid binding adhesin mediating attachment of *S. gordonii* to human cells including heart tissues.
Thus, Hsa has been considered an important virulence factor in infective endocarditis [32, 33]. Our finding demonstrates, for the first time, that Hsa is perhaps a multivalent adhesin mediating not only attachment to mammalian cells but also to prokaryotic cells. Interestingly, in our recent study, we identified a surface adhesin, Hag1, in \textit{V. atypica}, which mediates coaggregation not only with several bacterial species including \textit{S. gordonii}, but also with human buccal cells [8]. Whether it is common for oral microbial species to produce adhesins with binding capacity to multiple partners awaits further investigation.

Perhaps the most exciting finding from this study is that the same functional domains of Hsa required for binding to sialic acid on mammalian cells are also required for binding to \textit{Veillonella} cells. However, genomic analysis, combined with neuraminidase treatment of \textit{V. atypica} cells and fetuin inhibition assay, failed to provide evidence for the existence of sialic acids or similarly structured molecules on the \textit{Veillonella} cell surface. Thus, there exists a possibility that subdomains may exist in the binding domain of Hsa, which could be responsible for binding to different target molecules on different cell surfaces. Detailed genetic mutagenesis and identification of the amino acid residues involved in binding are needed to answer this question.

**Author Contributions**

Conceived and designed the experiments: FQ. Performed the experiments: PZ JL XL. Analyzed the data: PZ. Contributed reagents/materials/analysis tools: YT. Wrote the paper: PZ FQ.

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