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A YadA-like autotransporter, Hag 1, in *Veillonella atypica* is a Multivalent Hemagglutinin Involved in Adherence to Oral Streptococci, *Porphyromonas gingivalis*, and Human Oral Buccal Cells

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Abstract

Dental biofilm development is a sequential process, and adherence between microbes and the salivary pellicle (adhesion) as well as among different microbes (co-adhesion or coaggregation) plays a critical role in building a biofilm community. The *Veillonella* species are among the most predominant species in the oral cavity and coaggregate with many initial, early, middle and late colonizers. Similar to oral fusobacteria, they are also considered bridging species in biofilm development. However, the mechanism of this ability has yet to be reported, due to the previous lack of a genetic transformation system in the entire genus. In this study, we used our recently discovered transformable *Veillonella* strain, *V. atypica* OK5, to probe the mechanism of coaggregation between *Veillonella* species and other oral bacteria. By insertional inactivation of all 8 putative hemagglutinin genes, we identified one gene, *hag1*, which is involved in *V. atypica* coaggregation with the initial colonizers *Streptococcus gordonii*, *Streptococcus oralis* and *Streptococcus cristatus*, and the periodontal pathogen *Porphyromonas gingivalis*. The *hag1* mutant also abolished adherence to human buccal cells. Inhibition assays using various chemical or physiological treatments suggest different mechanisms being involved in coaggregation with different partners. The entire *hag1* gene was sequenced and shown to be the largest known bacterial hemagglutinin gene.

INTRODUCTION

The human oral biofilm is a multispecies community. The development of this community is a sequential process with pioneer colonizers attaching to the tooth surface via adherence to the salivary pellicle, followed by adherence of early/middle colonizers to the initial

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colonizers, and finally by adherence of late colonizers to the early/middle colonizers (Kolenbrander, *et al.*, 2006, Kolenbrander, *et al.*, 2010). Due to constant clearance forces in the oral cavity (saliva flow, swallowing, mastication, oral hygiene etc.), adherence to the tooth's surface by the pioneer colonizers and subsequent cell-cell coadhesion/coaggregation among microorganisms are essential for the formation of the oral biofilm (Kolenbrander, 1988, Kolenbrander, 2000).

The mitis streptococci (*S. mitis*, *S. sanguinis*, *S. gordonii*, *S. oralis*, *S. cristatus* etc) are considered “pioneer colonizers”, comprising >80% of the early biofilm population on a newly emerged or professionally cleaned tooth surface (Nyvad & Kilian, 1987, Kolenbrander, *et al.*, 2010). Growth and metabolic activity of these pioneer colonizers provide nutrients (metabolic products/waste) and sites for attachment (via coadhesion or coaggregation) by early/middle colonizers, such as the *Veillonella* species.

Veillonellae are Gram-negative cocci, although taxonomically belonging to the Firmicutes phylum. There are currently 12 species in the *Veillonella* genus (Gronow, *et al.*, 2010, Mashima, *et al.*, 2012), and five of which (*V. parvula*, *V. atypica*, *V. dispar*, *V. rogosae*, *V. denticariosi*) are routinely isolated from the human oral cavity. As a group, the *Veillonella* species are one of the most prevalent and numerically dominant bacteria in the oral cavity (Becker, *et al.*, 2002, Aas, *et al.*, 2005, Aas, *et al.*, 2008). A unique characteristic, shared by all species in the *Veillonella* genus, is their use of lactic acid as major carbon source. As the streptococci are major producers of lactate in the early biofilm community, it is not surprising that *veillonellae* were often found to associate with streptococci in epidemiological studies (Bradshaw & Marsh, 1998, Gross, *et al.*, 2012). In vitro, *Veillonella* species are found to physically coaggregate with streptococci, particularly amongst strains isolated from the same anatomical site (Hughes, *et al.*, 1988, Hughes, *et al.*, 1992).

In addition to streptococci, in vivo and in vitro studies also found *veillonellae* to coaggregate with middle and late colonizers including the periodontopathogen *Porphyromonas gingivalis* (Kolenbrander, 2011, Valm, *et al.*, 2011). Despite these initial studies, the mechanism of coaggregation between *veillonellae* and other oral bacteria is largely unknown, due to the lack of a genetic transformation system within the *Veillonella* genus. Recently, our laboratory successfully developed the first and only tractable genetic system in a clinical isolate of *V. atypica* (OK5) (Liu, *et al.*, 2012). In this study, we used this system to identify *veillonellae* surface proteins (hemagglutinins) responsible for coaggregation with oral bacteria. We show that a Yad A-like autotransporter family protein named Hag1 is required for coaggregation with streptococcal species, *P. gingivalis*, and human buccal cells. The complete gene sequence of *hag1* was assembled and shown to be the largest known bacterial hemagglutinin gene.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *V. atypica* OK5 and its derivatives were grown in BHI broth (Difco) with 0.6% sodium lactate (BHIL) or on BHIL agar plates. For transformation, cells were grown in Todd–Hewitt broth (Difco) with

0.6% sodium lactate (THL). For the selection of transformants, cultures were supplemented with tetracycline at $2.5 \mu\text{g mL}^{-1}$ (Sigma). The streptococcal species were grown in BHI broth (Difco) or on BHI agar plates. *P. gingivalis* strain ATCC 33277 was grown in Columbia Broth (CB; Difco) supplemented with vitamin K ($1.2 \mu\text{M}$) and hemin ($7.7 \mu\text{M}$). All bacterial strains were grown anaerobically (85% N_2 , 10% CO_2 , 5% H_2) at 37°C . *E. coli* cells were grown in Luria–Bertani (LB; Difco) broth with aeration at 37°C . *E. coli* strains carrying plasmids were grown in LB containing $100 \mu\text{g mL}^{-1}$ ampicillin (Fluka), or $10 \mu\text{g mL}^{-1}$ tetracycline.

Construction of insertional mutations

PCR primers used in this study are listed in Table S1. All plasmids were constructed by using a restriction enzyme-free and ligase-free method (You, *et al.*, 2012) except for pBST-hag7, which used regular restriction-ligation-cloning method. To construct the suicide vector pBST, the *tetM* cassette was PCR amplified with the primer pair tetMF/R using *V. parvula* PK1910 genomic DNA as the template as described previously (Liu, *et al.*, 2012). The pBluescript II KS (+) backbone excluding the beta-lactamase gene was PCR amplified with the primer pair pBSF/R, then the two amplicons were mixed and a DNA concatemer was obtained by prolonged overlapping extension PCR (POE-PCR) without primers, using the following parameters: 1 cycle of 98°C for 30 s; 28 cycles of 98°C for 10 s, 55°C for 10 s, and 72°C for 1 min; 1 cycle of 72°C for 5 min. POE-PCR was performed using Phusion DNA polymerase. The resulting DNA concatemer was transformed into *E. coli*, and transformants were selected on tetracycline plates. To construct insertional inactivation plasmids for the hemagglutinin (*hag*) genes, the same restriction enzyme-free and ligase-free method was used. Briefly, a target gene fragment was amplified by PCR using genomic DNA of *V. atypica* OK5 as template and a primer set with each primer 40–44 nt long, containing 20–22 nt 5' half homologous to the vector backbone and 20–22 nt 3' half homologous to the target gene. The plasmid backbone (pBST) was also amplified by PCR using a second set of primers, each containing the same length of 5' half homologous to the target gene and the same length of 3' half homologous to the vector sequence. After PCR, the two fragments were mixed in a 1:1 ratio and amplified by a third PCR reaction (POE-PCR) without primers. This mixture was then transformed into *E. coli* and transformants selected on tetracycline plates. Plasmids were isolated from the transformants and sequenced to ensure the correct construction. The confirmed plasmid was then transformed into *V. atypica* OK5 using the established protocol (Liu, *et al.*, 2012). The resulting transformants were selected on tetracycline plates and confirmed by PCR.

Coaggregation assay

In vitro coaggregation assays were performed as previously described with minor modifications (Cisar, *et al.*, 1979). Mid-log cells were harvested and washed twice with coaggregation buffer (1 mM Tris buffer [pH 8.0], 0.1 mM CaCl_2 , 0.1 mM MgCl_2 , 150 mM NaCl) at room temperature. Then, cells were resuspended in coaggregation buffer and normalized to $\text{OD}_{600}=1.2$. Equal volumes (0.1 mL) of each cell suspension were mixed in a micro-centrifuge tube and mixed by vortexing briefly. The tubes were kept at room temperature until aggregates formed, usually between 15 – 120 min. Tubes containing single species cell suspension alone (0.2 mL) were included as controls.

Microscopic analysis of *V. atypica* adherence to oral buccal cells

V. atypica OK5 cells were grown in BHIL overnight. Fresh human buccal cells were obtained by swabbing the buccal mucosa with a sterile cotton swab and suspended in 1 mL PBS. For the adherence assay, 0.5 ml of *V. atypica* cell culture was added to the buccal cells and mixed by gentle vortexing. The mixture was allowed to stand at room temperature for 10 min to allow buccal cells to settle at the bottom. The supernatant was then removed, and the pellet resuspended in 0.5 mL PBS. Adherence of *V. atypica* cells to the buccal cells was examined microscopically.

RESULTS

Coaggregation of *V. atypica* OK5 with oral bacteria

Coaggregation between *Veillonella* sp PK1910 and oral streptococci has been well studied (Hughes, *et al.*, 1992), however, the coaggregation spectrum of the transformable strain *V. atypica* OK5 was unknown. Using the well-established in vitro coaggregation assay and a panel of laboratory strains, we found that strain OK5 coaggregated with all 3 strains of *S. gordonii*, all 3 strains of *S. parasanguinis*, each of an ATCC strain of *S. oralis* and *S. cristatus*, and *P. gingivalis* 33277. Surprisingly, none of the >30 strains of *S. mutans*, *S. sanguinis*, *S. sobrinus*, and *S. salivarius* in our collection coaggregated with *V. atypica* OK5 in vitro, although all supported OK5 growth in co-cultures without lactate supplementation (data not shown).

Identification of putative hemagglutinins in *V. atypica* OK5

To determine if coaggregation of OK5 with its partners is mediated by surface proteins (adhesin) or other molecules, OK5 cells were treated with proteinase K before mixing with *S. gordonii* in an in vitro coaggregation assay. This treatment completely abolished coaggregation (Fig. 1A), suggesting a proteinaceous substance is involved. As a putative hemagglutinins (radD) in the other bridging species, *F. nucleatum*, was shown to be an adhesin binding to the early colonizers (Kaplan, *et al.*, 2009), we started our search for the adhesin from putative hemagglutinins in *V. atypica* OK5. To do this, we sequenced the genome of strain OK5 (unpublished data). Using BLASTN and BLASTP, we then mapped the contigs of OK5 against the draft genomes of three *V. atypica* strains (Col7a, Sch6, KON) in the HOMD database (<http://www.homd.org/>). This allowed us to identify 8 putative hemagglutinin genes in OK5 (Table 2). Interestingly, the same number of hemagglutinins were also found in *F. nucleatum* (Kaplan, *et al.*, 2009). Each of these genes was then inactivated using single-crossover insertional inactivation, as we have not been able to transform *V. atypica* using double-crossover recombination. After confirming the correct insertion by PCR, each mutant was tested using in vitro coaggregation assays against the known coaggregation partners described above. One of the mutants (*hag1*) completely abolished coaggregation with *S. gordonii*, *S. oralis*, *S. cristatus*, and *P. gingivalis* (Fig. 1B), while the remaining mutants had no effect. None of the mutants affected coaggregation with *S. parasanguinis* (data not shown), suggesting a different gene is involved. Fortuitously, in a separate study searching for surface proteins involved in adherence to oral epithelial cells (buccal cells), we found the *hag1* mutation to be the only one that abolished OK5 adherence to buccal cells (Fig. 1C).

Characterization of Hag1 mediated OK5 coaggregation

The finding that the same hemagglutinin (Hag1) is involved in coaggregation with Gram-positive streptococci, Gram-negative *P. gingivalis*, and human buccal cells was intriguing. We hypothesized that different mechanisms could be involved in binding to these vastly different cell types. Hughes *et al.* used inhibition assays with lactose, protease, and heat to characterize the types of coaggregation between *Veillonella* species and streptococci (Hughes, *et al.*, 1988). They categorized these coaggregations into lactose-sensitive and lactose-insensitive groups. Coaggregation properties of fusobacteria have also been characterized using sugar and amino acid inhibitors such as lysine and arginine (Merritt, *et al.*, 2009). To determine whether the same mechanism is used in OK5 coaggregation with bacteria as well as human buccal cells, coaggregation assays were performed using lactose, galactose, arginine, lysine, proteinase K, heat, glucosamine, fetuin, and neuraminidase as inhibitors. The last 3 treatments were used to test whether glycosylated or sialylated proteins are involved in binding. As shown in Table 3, no sugar or aminosugar inhibited coaggregation with any partner. Coaggregation with *P. gingivalis* was completely inhibited by arginine, lysine, heat and proteinase K treatment. In the presence of fetuin, aggregates formed after 2 hr of incubation instead of 15 min seen in the untreated control, and the amount of aggregates was smaller compared with the control. This was given a semi-quantitative value of one plus in Table 3. In contrast, none of the treatments inhibited OK5 binding to buccal cells. Interestingly, the inhibition profiles for the three streptococcal species are different. While coaggregation with *S. gordonii* was fully inhibited by arginine, heat, and proteinase K treatment, coaggregation with *S. oralis* was fully inhibited only by heat. However, complete inhibition by proteinase K was observed when 5 x proteinase K (5 µg/ml) was used. In contrast, coaggregation with *S. cristatus* was inhibited only by proteinase K.

Sequencing of the *hag1* gene locus

To further understand the mechanism of coaggregation involving Hag1, we sought to obtain the complete sequence of the *hag1* gene. The *hag1* gene was distributed among 3 contigs in our original draft sequence of strain OK5. Using the draft sequence of Va-Sch6 (contig00063) in the HOMD database as reference, we closed the gaps of the 3 contigs. It turned out that the *hag1* ORF in OK5 is 1542 bp larger than its counterpart in strain Sch6. Sequence analysis showed that *hag1* is located in a single gene operon (Fig. 2A), with an ORF of 21,561 bp encoding a protein of 7187 amino acids (aa) (Fig. 2B). BLASTP analysis and domain annotation using the online software da-TAA (Szczesny & Lupas, 2008) (<http://toolkit.tuebingen.mpg.de/dataa>) revealed that Hag1 is a predicted trimeric autotransporter with 3 major “head” domains and 8 minor head domains (Fig. 2B). This domain organization is consistent with its involvement in OK5 coaggregation with a diverse array of bacterial and eukaryotic cells.

Surface structure of OK5 wild-type and *hag1* mutant

Since Hag1 is the largest bacterial surface protein described so far, we were curious to see if the *hag1* mutation changed surface structure of the mutant cells. Transmission electron microscopy (TEM) was performed with mid-log cells of the wild-type and *hag1* mutant

(Fig. 3). Contrary to our expectation, the *hag1* mutant **appears** to have a much denser, though thinner outer layer compared with the wild-type. This suggested that the Hag 1 protein may also play an organizational role in the outer membrane structure.

DISCUSSION

Two characteristics of the *Veillonella* species make them one of the most intriguing bacteria in the oral biofilm. One is their unique metabolic property, *i.e.* utilizing lactate as a preferred carbon and energy source. This property facilitates their specific association with lactic acid producing bacteria in the dental plaque, thus implicating an important role in caries development (Gross, *et al.*, 2012). Another is their prolific coaggregation ability with many initial, early, middle, and late colonizers, thus implicating a role in development of periodontal diseases. Indeed, in Socransky's color scheme of the microbiology of periodontal diseases (Socransky, *et al.*, 1998), *Veillonella* belongs to the "purple complex", which acts as "bridging species leading to the orange complex", in which *Fusobacterium* is a member, and "ultimately, at some site, to the red complex", which includes *P. gingivalis*, *Treponema denticola*, and *Tannerella forsythia*. It is now well established from in vitro and in vivo studies that what allowed *Veillonella* to function as bridging species is their attachment to the initial colonizing streptococci, and their ability to recruit later colonizers like *P. gingivalis* to the biofilm community through cell-cell coaggregation. However, due to the previous lack of a genetic transformation system in this genus, the adhesins mediating these coaggregations were completely unknown.

In this study, we used a reverse genetics approach to identify a putative hemagglutinin (Hag1) from the transformable strain *V. atypica* OK5 that is responsible for coaggregation with *S. gordonii*, *S. oralis*, *S. cristatus*, *P. gingivalis*, and human buccal cells. This approach was used because the low transformation efficiency of OK5 made it unfeasible to construct a random-mutant library, and because we have not found a transposon mutagenesis system that worked in *Veillonella* to create a random mutant library. We chose the eight putative hemagglutinin genes as our initial targets based on the findings of surface adhesins in fusobacteria (Kaplan, *et al.*, 2009). In subsequent studies attempting to determine the adhesion for *S. parasanguinis*, we mutagenized three more surface proteins annotated as *fliS*, *fibB*, and *sspE*, none of which affected coaggregation with any of the coaggregation partners of OK5 (Liu *et al.*, unpublished).

Hag1 consists of 7187 aa, making it the largest bacterial surface protein reported thus far. A BLASTP search of the Hag1 protein found a YadA-like N-terminal Extended Signal Peptide sequence of Type V secretion system (Henderson, *et al.*, 2004); members of this system include autotransporters (Desvaux, *et al.*, 2004), the TpsA exoproteins of the two-partner system (Jacob-Dubuisson, *et al.*, 2004), and trimeric autotransporters (Cotter, *et al.*, 2005). The N-terminal signal peptide allows the protein to be transported across the inner membrane. Downstream from the signal peptide are five trimer interface domains, suggesting that Hag1 could be a trimeric autotransporter. The C-terminus contains a typical translocator domain (Fig. 2B), which translocates then inserts the protein into the outer membrane. This domain organization suggests that the Hag 1 protein is likely located in the outer membrane. The *hag1* mutation, which should produce a Hag 1 protein with a C-

terminal 294-aa truncation, completely abolished OK5 coaggregation with its partners *S. gordonii*, *S. oralis*, *S. cristatus*, *P. gingivalis*, and human buccal cells, indicating that the truncated protein failed to translocate to the outer membrane. Two more pieces of evidence further support the surface localization of Hag1. First, proteinase K treatment of OK5 completely abolished its coaggregation with *S. gordonii* (Fig. 1A) as well as with other partners (data not shown). Second, a translational fusion of Gfp in between the N- and C-terminal domains of Hag1 was able to target Gfp to the cell surface of the transformed *E. coli* cells (Fig. S1).

Hag 1 is annotated as an autotransporter. A typical domain structure of an autotransporter consists of a “head”, which is usually the target binding domain; a “stalk”, which protrudes the “head” out from the outer membrane; and a “neck”, which is a flexible region connecting the head to the stalk (Fig. 2C) (Lyskowski, *et al.*, 2011). Domain annotation of Hag1 using the da-TAA software identified 3 large and 5 small head domains at the N-terminal non-repeat region (1-2659 aa, Fig. 2B). These head domains are all followed by the “neck” sequence, consistent with the general structure of autotransporters. Interestingly, the da-TAA software could not assign domain structures to the majority of the repeat region (2660 – 6489 aa, Fig. 2B), although it suggested that it could form a coiled-coil, a typical structure for the stalk. Furthermore, there are three intermediate sized head domains interspersed with the large putative stalk region, followed by two small neck regions next to the C-terminal membrane anchor domain (Fig. 2B). Although further genetic studies need to be done to determine which domain is responsible for binding to which partner, this domain organization already provided us with some clues on the protein configuration.

The extremely large size of *hag1* made it technically unfeasible to complement the *hag1* mutation. Nonetheless, *hag1* is located on a single gene operon, suggesting that the insertion is unlikely to pose any polar effect on the downstream gene. In fact, sequence analysis of the intergenic region between *hag1* and the downstream gene *metE* identified a strong Rho-independent transcription terminator following the translation stop codon of *hag1* (Fig. S2). Downstream of this terminator is a typical sigma70 promoter with an extended -10 sequence for the *metE* gene (Fig. S2). Furthermore, the mutant strain did not show any defect in cell growth, although the mutant cells appeared to be more sensitive to oxidative stress (data not shown).

Perhaps the most intriguing results are the different coaggregation properties mediated by the same Hag1 protein (Table3). The experiments were designed not only to distinguish coaggregation with different partners, but also to provide us with clues for the type of target molecules on the partner cell's surface. For example, we observed that treating OK5 with proteinase K for 2 h completely abolished its coaggregation with *S. gordonii* (Fig. 1A), and it turned out that Hag1 is the protein responsible for coaggregation. Similarly, treating *S. gordonii* with the same condition also abolished its coaggregation with OK5 cells (Table 3). We have recently identified the *S. gordonii* adhesin as the sialic acid binding protein Hsa (data will be published elsewhere). Interestingly, although Hsa has been shown to be a sialic acid binding adhesin (Bensing, *et al.*, 2004, Takamatsu, *et al.*, 2005, Urano-Tashiro, *et al.*, 2008, Yajima, *et al.*, 2008, Petersen, *et al.*, 2010), *S. gordonii* coaggregation with OK5 is not inhibited by fetuin, a highly sialylated glycoprotein containing terminal sialic acid linked in

both $\alpha(2-3)$ and $\alpha(2-6)$ linkages (Cointe, *et al.*, 1998), nor is affected by neuraminidase treatments of either *S. gordonii* or OK5 cells (Table 3). These results indicate that coaggregation between *S. gordonii* and *V. atypica* is unlikely to involve sialic acid binding, although Hsa is known to bind to sialic acid on the surface of mammalian cells.

In contrast, the coaggregation with *P. gingivalis* is abolished by proteinase K and heat treatment on *P. gingivalis* cells, as well as by adding lysine, arginine, and fetuin to the coaggregation buffer (Table 3), although fetuin only exerted weak inhibition. This suggested that coaggregation with *P. gingivalis* may involve sialic acid, at least partially. However, neuraminidase treatment on *P. gingivalis* or OK5 cells did not affect coaggregation (Table 3). The nature of the target molecule on the *P. gingivalis* cell surface remains unknown at present.

More interestingly, although *S. oralis* and *S. cristatus* are closely related to *S. gordonii*, their coaggregation properties with *V. atypica* OK5 are different. *S. oralis* coaggregation was not inhibited by arginine as for *S. gordonii*, and 5 times proteinase K was needed to completely inhibit coaggregation, although heat treatment has the same effect as for *S. gordonii*. This result suggested that either the protein target on the *S. oralis* surface is somehow resistant to proteinase K or only partially accessible to proteinase K. The result with *S. cristatus* is the most interesting. Here, coaggregation with OK5 is inhibited only by proteinase K treatment. This suggests that the target on the *S. cristatus* cell surface could be a sugar moiety of a glycoprotein, or the protein could be heat resistant. The most perplexing result is the Hag1 mediated OK5 binding to the buccal cells, which is not inhibited by any of the treatments (Table 3), especially the neuraminidase treatment on the buccal cells, although the same treatment abolished *S. gordonii* binding to the buccal cells (data not shown). *S. gordonii* binding to mammalian cells has been known to be mediated by sialic acid binding proteins especially Hsa (Takahashi, *et al.*, 2002, Bensing, *et al.*, 2004, Jakubovics, *et al.*, 2005, Yajima, *et al.*, 2005, Kerrigan, *et al.*, 2007, Yajima, *et al.*, 2008, Jakubovics, *et al.*, 2009), thus, it is likely that binding to the buccal cell by *V. atypica* does not involve sialic acid either.

In summary, we have identified a largest known bacterial hemagglutinin Hag1 in *V. atypica* OK5. Hag1 likely mediates coaggregation with the initial colonizers *S. gordonii*, *S. oralis*, *S. cristatus*, and the periodontal pathogen *P. gingivalis*, as well as adhesion to human buccal cells. To the best of our knowledge, this is the first surface protein definitively identified in the *Veillonella* genus. This study will provide new avenues for future studies on the mechanisms of oral biofilm development, especially the roles played by bridging species like veillonellae.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A: Effect of proteinase K treatment of *V. atypica* OK5 (Va) on coaggregation with *S. gordonii* (Sg). OK5 cells were treated with proteinase K at 1 µg/ml, 50 °C/2 hr, and

coaggregation was performed as described in Materials and Methods. Without proteinase K treatment, cells form aggregates and precipitate to the bottom; with proteinase K treatment, cells stay in solution. B. Coaggregation of *V. atypica* OK5 wild-type (wt) and *hag1* mutant (*hag1*) with streptococci and *P. gingivalis*. As in (A), coaggregation positive pairs form aggregates that precipitate to the bottom of the tube (arrowhead), while cells of the single species cultures or mixed cultures with the *hag1* mutant remain in solution. Sg: *S. gordonii*; So: *S. oralis*; Sc: *S. cristatus*; Pg: *P. gingivalis*; Va: *V. atypica* OK5; hag1: OK5-hag1 mutant. C. Adhesion of OK5 and the hag1 mutant to human buccal cells. Wild-type OK5 cells attached to the buccal cell as shown by accumulated bacterial cells on the cell surface (arrowhead in the enlarged portion above the middle panel), while the hag1 mutant cells could not attach to surface as shown by the smooth buccal cell surface (enlarged portion above the right panel).

Fig. 2. Gene organization and predicted protein domain organization of Hag1

A. The *hag1* gene is located on a single-gene operon with putative promoter and transcription terminator sequences indicated. B. The total length of the protein is 7187 aa with a calculated molecular weight of 714,781 Da. NR1: N-terminal non-repeat region of 2659 aa; included in the NR1 region is a signal peptide for the Type V secretion system (blue bar), 3 major (purple line), 5 minor head domains (purple bar), and 7 neck domains (green bar). NR2: C-terminal, non-repeat region of 696 aa; included in this region is a membrane anchor domain (gray bar) and a neck domain (green bar). RR: repeat region, which include the putative stalk domains (orange lines) and 3 minor head domains (purple bar). The domain annotation was performed using da-TAA (<http://toolkit.tuebingen.mpg.de/dataa>). C. General structure of a typical autotransporter.

Fig. 3. Transmission electron microscopy (TEM) of *V. atypica* OK5 wild-type (wt) and *hag1* mutant

TEM was performed with lead staining and a slide thickness of 100 nm. Note the difference in density of the outer layers.

Table 1

Bacterial strains and plasmids used in this study

Strains and plasmids	Characteristics	Reference
Strains		
<i>E. coli</i> DH5a	Cloning strain	
<i>V. atypica</i> OK5	Wild type	(Liu, <i>et al.</i> , 2012)
hag1	OK5 <i>hag1</i> ; Tc ^r	This work
hag2	OK5 <i>hag2</i> ; Tc ^r	This work
hag3	OK5 <i>hag3</i> ; Tc ^r	This work
hag4	OK5 <i>hag4</i> ; Tc ^r	This work
hag5	OK5 <i>hag5</i> ; Tc ^r	This work
hag6	OK5 <i>hag6</i> ; Tc ^r	This work
hag7	OK5 <i>hag7</i> ; Tc ^r	This work
hag8	OK5 <i>hag8</i> ; Tc ^r	This work
<i>S. gordonii</i> DL1	Wild type	(Pakula, <i>et al.</i> , 1963)
Plasmids		
pBluescript II KS(+)	Cloning vector; Ap ^r	Stratagene
pBST	Suicide vector of <i>V. atypica</i> , the beta-lactamase gene in pBluescript II KS(+) was replaced by tetM cassette; Tc ^r	This work
pBST-hag1	pBST+ <i>hag1</i> ; Tc ^r	This work
pBST-hag2	pBST+ <i>hag2</i> ; Tc ^r	This work
pBST-hag3	pBST+ <i>hag3</i> ; Tc ^r	This work
pBST-hag4	pBST+ <i>hag4</i> ; Tc ^r	This work
pBST-hag5	pBST+ <i>hag5</i> ; Tc ^r	This work
pBST-hag6	pBST+ <i>hag6</i> ; Tc ^r	This work
pBST-hag7	pBST+ <i>hag7</i> ; Tc ^r	This work
pBST-hag8	pBST+ <i>hag8</i> ; Tc ^r	This work

Erm^r: erythromycin resistanceTc^r: tetracycline resistance

Table 2Putative hemagglutinins in *V. atypica* OK5

Name	Hag1	Hag2	Hag3	Hag4	Hag5	Hag6	Hag7	Hag8
Size (aa)	7187	3838	3349	2424	2367	2079	743	528
MW (kD)	/714.8	/395.4	/338.2	/262.2	/244	/215.3	/76.7	/53.1

Table 3

Coaggregation of OK5 with different partners in the presence of various inhibitors

Partner ¹	Inhibitor ²								
	Lac	Gal	GluAN	Fetuin	Arg	Lys	NMDase	Heat	Proteinase
S. g	++++	++++	++++	++++	–	++++	++++	–	–
S. o	++++	++++	++++	++++	++++	++++	++++	–	++++*
S. c	++++	++++	++++	++++	++++	++++	++++	++++	–
P. g	++++	++++	++++	+	–	–	++++	–	–
BC	++++	++++	++++	++++	++++	++++	++++	ND	ND

¹ S. g: *S. gordonii*; S. o: *S. oralis*; S. c: *S. cristatus*; P. g: *P. gingivalis*; BC: buccal cell

² Lac: lactose (60 mM), Gal: galactose (60 mM); GluAN: glucosamine (60 mM); Fetuin: fetuin from fetal bovine serum (1mg/ml); Arg: arginine (60 mM); Lys: lysine (60 mM); NMDase: neuraminidase (1.25 U, 37 °C/16 h); Heat: 85 °C/1 hr; Proteinase: proteinase K (1 µg/ml, 50 °C/2 hr). Proteinase K and heat treatment was only performed on the OK5 coaggregation partners, not on OK5 cells.

++++: 100% coaggregation as seen in the untreated control;

+: weak coaggregation

–: no coaggregation as seen in the single species culture control

* complete inhibition was observed when 5 µg/ml proteinase K was used.