Title

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Hemagglutinin/Adhesin Domains of *Porphyromonas gingivalis* Play Key Roles in Coaggregation with *Treponema denticola*

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Running title: *P. gingivalis*-T. *denticola* coaggregation

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Abstract

Porphyromonas gingivalis and Treponema denticola are major pathogens of periodontal disease. Coaggregation between microorganisms plays a key role in the colonization of the gingival crevice and the organization of periodontopathic biofilms. We investigated the involvement of surface ligands of *P. gingivalis* in coaggregation. Two triple mutants of *P. gingivalis* lacking Arg-gingipain A (RgpA), Lys-gingipain (Kgp) and Hemagglutinin A (HagA) or RgpA, Arg-gingipain B (RgpB) and Kgp showed significantly decreased coaggregation with *T. denticola*, whereas coaggregation with a major fimbriae (FimA)-deficient mutant was the same as that with the *P. gingivalis* wild-type parent strain. *rgpA, kgp* and *hagA* code for proteins that contain 44 kDa Hgp44 adhesin domains. The coaggregation activity of a *rgpA kgp* mutant was significantly higher than that of the *rgpA kgp hagA* mutant. Furthermore, anti-Hgp44 IgG reduced coaggregation between *P. gingivalis* wild-type and *T. denticola*. *T. denticola* sonicates adhered to recombinant Rgp domains. Coaggregation following co-culture of the *rgpA kgp hagA* mutant expressing the RgpB protease with the *rgpA rgpB kgp* mutant expressing the unprocessed HagA protein was enhanced compared to that of each triple mutant with *T. denticola*. These results indicate that the processed *P. gingivalis* surface Hgp44 domains are key adhesion factors for coaggregation with *T. denticola*. 
Introduction

Chronic periodontitis is induced by anaerobic Gram-negative rods and spirochetes in dental plaque biofilms (Genco, et al., 1988, Ellen & Galimanas, 2005). Porphyromonas gingivalis and Treponema denticola have been shown to be major pathogens of chronic periodontitis (Lamont & Jenkinson, 1998, Ishihara & Okuda, 1999). The coaggregation of these periodontopathic bacteria to antecedent microorganisms on the tooth surface appears to play a pivotal role in the formation of periodontopathic biofilms (Kolenbrander, et al., 1993). A large number of microorganisms colonize periodontal biofilms by coaggregation (Kolenbrander, et al., 1993), with P. gingivalis being shown to coaggregate with T. denticola (Grenier, 1992, Onagawa, et al., 1994).

Interbacterial communication between the consortia of microorganisms is an essential factor in the organization of periodontopathic biofilms (Park, et al., 2006, Saito, et al., 2008). Socransky et al. (1998) designated the combination of P. gingivalis, T. denticola and Tannerella forsythia as the “red complex” and these three bacteria have been shown to increase proportionally in chronic periodontitis lesions. T. denticola and P. gingivalis are commonly detected together in chronic periodontitis lesions (Kigure, et al., 1995) and synergistic effects between these microorganisms have been demonstrated (Simonson, et al., 1992, Yamada, et al., 2005). In addition, Grenier (1992) suggested that coaggregation between P. gingivalis and T. denticola was bimodal. Several cell surface components of P. gingivalis contribute to its adhesive properties, including fimbriae, vesicles and hemagglutinins (Okuda & Takazoe, 1974, Okuda, et al., 1986, Lee, et al., 1993, Duchesne, et al., 1995). The proteases Arg-gingipain A (RgpA),
arg-gingipain B (RgpB) and lys-gingipain (Kgp) are also involved in hemagglutination (Shi, et al., 1999). RgpA and Kgp consist of propeptide, catalytic and C-terminal hemagglutinin/adhesion domains (Hgp44, Hgp15, Hgp17 and Hgp27), whereas RgpB contains a propeptide and a catalytic domain (Potempa, et al., 2003). The Hgp44 and Hgp15 domains of RgpA and Kgp mediate hemagglutination and hemoglobin-binding activity (Shi, et al., 1999). The Hgp44 domain is also encoded by the hemagglutinin gene (hagA) of P. gingivalis (Han, et al., 1996). Several motifs in the Hgp domains were also reported to adhere to intercellular matrix components such as collagen and fibronectin (O’Brien-Simpson, et al., 2005). Furthermore, P. gingivalis fimbriae mediate coaggregation with various plaque-forming bacteria such as Actinomyces viscosus, Streptococcus gordonii and Streptococcus oralis (Goulbourne & Ellen, 1991, Lamont, et al., 1993, Amano, et al., 1997). Hashimoto et al. (2003) showed that P. gingivalis fimbriae and T. denticola dentilisin were involved in coaggregation between these two bacteria. However, the molecular basis for the mechanism of this coaggregation still remains to be fully elucidated. The aim of this study was to further clarify periodontopathic biofilm formation by identifying the P. gingivalis ligands involved in coaggregation with T. denticola.

**Materials and Methods**

**Bacterial strains and culture conditions**

The bacterial strains used in this study are listed in Table 1. P. gingivalis ATCC 33277
and its mutants were grown in Tryptic soy broth (Becton Dickinson and Co., Sparks, MD) supplemented with hemin (5 μg/ml) and menadione (0.5 μg/ml) at 37 °C under anaerobic conditions (N₂: 80%, H₂: 10%, CO₂: 10%). T. denticola ATCC 35405 was grown in TYGVS medium (Ohta, et al., 1986) or modified TYGVS medium (E-TYGV) in which 1% EX-CYTE (Millipore, Billerica, MA) was used instead of 10% rabbit serum at 37 °C under anaerobic conditions. Growth rates in E-TYGV medium were found to be similar to those in TYGVS medium. Luria-Bertani (LB) broth and LB agar plates (Becton Dickinson and Co.) were used for growth of Escherichia coli strains. Antibiotics were used at the following concentrations: ampicillin (Am; 100 μg/ml), erythromycin (Em; 10 μg/ml), chloramphenicol (Cm, 20 μg/ml) and tetracycline (Tet; 0.7 μg/ml).

Coaggregation assay

Coaggregation between T. denticola and P. gingivalis was evaluated by the modified turbidimetric assay method of Nagata et al. (1990). Three-day cultures of T. denticola and P. gingivalis were harvested by centrifugation at 10,000 g for 20 min. Cells were washed twice with phosphate-buffered saline (PBS, pH 7.2) and resuspended in coaggregation buffer, which consisted of 1 mM phosphate buffer (pH 6.0) containing CaCl₂ (0.1 mM), MgCl₂ (0.1 mM) and 150 mM NaCl. Each cell suspension was adjusted to an optical density of 0.5 at 660 nm using a UV-visible spectrophotometer UV-2550 (Shimadzu, Kyoto, Japan). Fifteen hundred μl of P. gingivalis ATCC 33277 suspension and 1,000 μl of T. denticola were mixed in the cuvette of the spectrophotometer and the mixture then incubated at room temperature. Coaggregation was monitored by measuring the decrease of absorbance at OD660 in the
spectrophotometer for 60 min. When 20% or more autoaggregation of *P. gingivalis* or *T. denticola* was observed, the results were excluded. The optimal conditions for coaggregation (buffer pH, NaCl concentration and ratio of *P. gingivalis* to *T. denticola*) were determined in preliminary experiments. Coaggregation percentage was calculated according to the following equation: Coaggregation = [(pre-incubation value [OD660] – post-incubation value [OD660])/(pre-incubation value [OD660])] x 100.

The effects of each amino acid and inactivation of specific genes were expressed as a percentage of the coaggregation achieved with wild-type strains of *P. gingivalis* ATCC 33277 and *T. denticola* ATCC 35405. The effects of specific amino acids (L-arginine, L-lysine, L-cysteine, L-histidine, L-asparagine, L-proline, L-serine, L-glutamate and L-α-alanine) as well as the sugars (D-galactosamine, D-galactose, N-acetylgalactosamine, D-sorbitol and D-fructose) in addition to specific protease inhibitors (Na-p-tosyl-L-lysine chloromethyl ketone (TLCK) and leupeptin) were also evaluated. Coaggregation assays were performed in triplicate or more and repeated three times on separate days.

**Overlay assay and immunoblotting**

To determine the size of the *P. gingivalis* ligand involved in coaggregation, an overlay assay was performed. Three-day cultures of *T. denticola* were harvested by centrifugation at 10,000 g for 20 min. Cells were then washed twice with PBS and resuspended in the same buffer. Cell suspensions were disrupted by sonication on ice at 100 W for 5 min using a sonicator (SONIFIER 250, Branson, Danbury, CT). Unbroken cells were removed by centrifugation at 10,000 g for 20 min. Three-day cultures of *P. gingivalis* ATCC 33277 cells were washed twice with PBS, followed by
resuspension in SDS-solubilizing buffer containing 0.25 mM TLCK and boiling at 100 °C for 5 min, after which they were fractionated by SDS-polyacrylamide gel electrophoresis using 12.5% or 10-20% gradient gels. For immunoblotting, the proteins on the gels were electrophoretically transferred to a PVDF membrane (Immobiline, Millipore, Billerica, MA) by using a semi-dry blotting system (BIO-RAD, Hercules, CA) according to the manufacturer’s instructions. After blocking with Milk Diluent Blocking Solution Concentrate (KPL, Gaithersburg, MD) for 1 h, the membrane was washed with PBS containing 0.05% Tween 20 (PBST), incubated with sonic extracts of *T. denticola* ATCC 35405 (1 mg/ml) at room temperature for 1 h and washed with PBST. The membrane was immunostained using 1:1000 diluted rabbit antiserum against whole cells of *T. denticola* ATCC 35405 (Ishihara, *et al.*, 1998) for 1 h. The membrane was then washed thoroughly with PBST and reacted with peroxidase-conjugated anti-rabbit IgG (Bio-Rad). After washing with PBST, the membranes were developed with TMB Membrane Peroxidase Substrate (KPL). Immunoblotting was performed as described previously (Ishihara, *et al.*, 1998) using anti-Hgp44 serum (Yasaki-Inagaki, *et al.*, 2006). To perform immunoblot and overlay assays on the same membrane, the membrane was separately reacted with different solutions using Screenerblotter (SANPLATEC, Osaka, Japan).

**Construction of *P. gingivalis* mutant strains**

*P. gingivalis* genome sequence data were obtained from The Institute for Genomic Research website (http://www.tigr.org) to construct mutants. The *P. gingivalis* *rgpA* mutant (RK103) was constructed as follows: a DNA fragment corresponding to a 1.4-kb region within *rgpA* was generated by PCR using *P. gingivalis* ATCC 33277
chromosomal DNA as the template with a forward primer,

5'-GGGGGGGAATTCGGAGAGATCGCCACGCTTGATGATCCTTTT, containing an 
EcoRI site (underlined) and a reverse primer,

5'-GGGGGGGATCCTCAGACCTGTCAGATTGATTGTAGCTGTTC, containing a 
BamHI site (underlined). The resulting fragment was cloned into the pCR4 vector 
(Invitrogen, Carlsbad, CA) and the plasmid designated as pRK1. An ermF-ermAM 
DNA fragment (Fletcher, et al., 1995) was inserted into an EcoRV site of the rgpA 
fragment in pRK1, resulting in pRK2. pRK2 plasmid DNA was then linearized by 
NotI digestion and introduced into the P. gingivalis kgp mutant (KDP129) cells by 
electroporation (Kikuchi, et al., 2005).

The transformants were spread on Tryptic soy agar plates containing 10 µg/ml Em, 20 
µg/ml Cm, 5 µg/ml hemin and 0.5 µg/ml menadione, and incubated anaerobically for 7 
days. Insertion of the Em-resistant cassette following transformation (RK103) was 
verified by Southern and Western blot analyses.

**Inter-bacterial complementation.**

The RgpAB/Kgp-null mutant (P. gingivalis KDP 136) was co-cultured with the 
HagA/RgpA/Kgp-null mutant (P. gingivalis KDP 137) in Tryptic soy broth (Becton, 
Dickinson and Co.) supplemented with hemin (5 µg/ml) and menadione (0.5 µg/ml) 
under anaerobic conditions according to the method of Naito et al. (2006).

Coaggregation of cells co-cultured with T. denticola ATCC 35405 was analyzed as 
described above.

**Adherence of recombinant Rgp domains to T. denticola**
To determine which Rgp domains were involved in coaggregation with *T. denticola*, activity was measured using recombinant Rgp domains. Recombinant proteins of the catalytic domain, as well as the Hgp44 and Hgp15-27 domains, were purified as described previously (Inagaki, *et al.*, 2003). One hundred µl of each domain-containing coating buffer (10 µg/ml) was applied to 96-well EIA plates (Corning Incorporated, Corning, NY) at 37 °C for 2 h. Control wells were coated with 2% BSA using the same procedure. The wells were then washed with PBST and blocked with 2% BSA-PBS for 1 h. The wells were washed again with PBST and 100-µl sonicates of *T. denticola* (10 µg/ml) added and incubated for 1 h. After washing the plate with PBST, 1:500 diluted anti-*T. denticola* antibody (Ishihara, *et al.*, 1998) in 0.5% BSA-PBST was added and incubated for 1 h. Following washing of the plates, 1:2,000 diluted peroxidase-labeled goat anti-rabbit antibody in 0.5% BSA-PBST was added and incubated for 1 h. Finally, after washing the plate with PBST, color development was performed using the ABTS Peroxidase Substrate (KPL), and optical densities at 410 nm were measured with a microplate reader (BIO-RAD).

**Effects of anti-Hgp44 antibody on coaggregation**

Normal rabbit serum was obtained from Pel-Freez Biologicals (Rogers, AR, USA). Rabbit IgG of each serum was purified by protein G Sepharose Fast Flow chromatography (GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, UK) according to the supplier's instructions. *T. denticola* and *P. gingivalis* were then adjusted to an absorbance of 0.5 at 660 nm. *P. gingivalis* (750 µl) suspensions in the 24-well microtiter plates were incubated with 0.002-0.046 mg/ml protein G-purified normal rabbit IgG, anti-*P. gingivalis* ATCC 33277 whole cell IgG, or anti-Hgp44 IgG for
10 min, followed by addition of *T. denticola* ATCC 35405 (500 µl). The plates were then shaken gently at room temperature for 30 min. Coaggregation was evaluated visually based on the formation of large clumps of microorganisms.

**Statistical analysis**

Coaggregation activity among various *P. gingivalis* strains and attachment of *T. denticola* sonicates to *P. gingivalis* Rgp domains were subjected to a one-way analysis of variance and Tukey’s multiple comparison at a 5% level of significance. Reduction in coaggregation activity by treatment with amino acid, protease inhibitor and heat treatment were assessed with Student t-test.

**Results**

**Effects of amino acids and protease inhibitors on coaggregation reactions**

*P. gingivalis* ATCC 33277 strongly coaggregated with *T. denticola* ATCC 35405 between 15 min to 40 min, with no significant autoaggregation of either organism (data not shown). Heating of *P. gingivalis* cells at 75 °C for 30 min resulted in significant reduction in coaggregation activity (p < 0.01), whereas the same treatment for *T. denticola* cells showed no effects on coaggregation (Table 2). None of the tested sugars affected the coaggregation reactions at 100 or 200 mM (data not shown). However, L-arginine, L-lysine, L-histidine, leupeptin and TLCK strongly inhibited coaggregation in a dose-dependent manner (Table 2).

**Overlay assays**
The apparent sizes of the *P. gingivalis* ligands involved in coaggregation were determined by overlay assays. Sonicates of *T. denticola* adhered primarily to approximately 61-, 53-, 43-, 40 and 38.9-kDa *P. gingivalis* ATCC 33277 proteins separated by SDS-PAGE (Fig. 1A lane 1). As the 43 kDa protein was similar to the Hgp44 protein, overlay assays and immunoblotting were performed at the same time. The two bands obtained from the overlay assay were almost the same size as that detected by anti-Hgp44 antibody (Fig. 1B).

**Coaggregation activity of *P. gingivalis* mutants lacking cell surface components**

Inhibition of coaggregation by cysteine protease inhibitors, arginine or lysine suggested the involvement of gingipains in coaggregation between *P. gingivalis* and *T. denticola*. Therefore, to verify the involvement of gingipains in the coaggregation reaction, interactions between *P. gingivalis* mutants defective in these components and *T. denticola* ATCC 35405 were performed. Coaggregation with *T. denticola* was strongly reduced in *P. gingivalis* KDP137, which lacked intact *rgpA*, *kgp* and *hagA* genes, and in *P. gingivalis* KDP136, which was defective in the *rgpA*, *rgpB* and *kgp* genes (Fig. 2). The mutant defective in only *kgp* and the doubly-deficient mutant lacking *rgpA* and *rgpB* exhibited only weak attenuation of coaggregation activities. On the other hand, the coaggregation reaction was not reduced by mutation of the *fimA* gene.

**Adherence of recombinant Rgp domains to *T. denticola***

Adherence of *T. denticola* sonicates was significant for all three Rgp domains compared with the control (Fig. 3A). This suggested an interaction between Rgp domains and *T. denticola*. Among these, Hgp44 showed the most avid binding to *T. denticola* cellular
Inhibition of coaggregation by anti-Hgp44 IgG

To clarify the role of Hgp44 in coaggregation, we evaluated the effects of antibody against Hgp44 on these interactions. Anti-Hgp44 antibody at 0.012-0.046 mg/ml and anti-\textit{P. gingivalis} whole cell IgG at 0.006-0.046 mg/ml significantly inhibited coaggregation, whereas normal rabbit IgG at these concentrations did not (Fig. 3B). These results provide additional support for an important role for the Hgp44 domain in coaggregation.

Inter-bacterial complementation

The reduction in coaggregation with \textit{T. denticola} in the \textit{rgpA kgp hagA} mutant KDP137 suggested the involvement of mature Hgp domains. To confirm this, expression of the Hgp domains on the surface of the \textit{P. gingivalis} gingipain-null mutant KDP136 by co-culture of the HagA-deficient and RgpB-positive mutant KDP137 was evaluated. We hypothesized that, as the Rgp proteases are secreted by \textit{P. gingivalis} and involved in the processing of gingipains and HagA (Naito, \textit{et al.}, 2006), extracellular RgpB liberated by KDP137 might process the HagA proprotein on the surface of KDP136, leading to complementation of the weak coaggregation exhibited by the latter mutant. Indeed, coaggregation following co-culture of KDP136 and KDP137 was restored to 58.9\% of that of the wild-type parent strain, whereas coaggregation with KDP136 and KDP137 alone were 0\% and 20.1\% of that with the wild-type parent strain, respectively (Fig. 4) (P < 0.05).
Effect of Hgp44 on coaggregation reactions

Several serum components have been reported to adhere to *T. denticola* and *P. gingivalis*, suggesting their possible involvement in coaggregation between these microorganisms. To assess the effects of these components, coaggregation reactions were also evaluated using *T. denticola* grown in serum-free E-TYGV medium. Coaggregation between wild-type *P. gingivalis* and *T. denticola* was observed at almost the same level as in serum containing media (Fig. 5). We next examined the contribution of the Hgp44 domains to coaggregation. Coaggregation reactions decreased according to the copy number of the Hgp44 coding region. Coaggregation of the *rgpA kgp* mutant (RK103) was significantly stronger than that of the *rgpA kgp hagA* mutant KDP137, further suggesting the involvement of the Hgp44 domains in coaggregation (Fig. 5).

**Discussion**

The results of the present study indicate that gingipain Hgp domains such as Hgp44 also act as ligands in coaggregation between *P. gingivalis* and *T. denticola*. Biofilm formation is important in the colonization of the oral cavity by bacteria as this is a hostile environment in which saliva continuously flows across tissue surfaces and contains anti-microbial agents secreted from mucosal membranes (Marsh, 1989, Scannapieco, 1994). Therefore, oral bacteria colonizing the oral cavity must adhere tightly to the surfaces of oral tissue and teeth, as well as to microorganisms already present. Colonization of dental plaque by *P. gingivalis* may, therefore, be facilitated by its adhesion to other bacteria such as *Fusobacterium nucleatum* (Kolenbrander &
Andersen, 1989, Bradshaw, et al., 1998), T. denticola (Grenier, 1992, Onagawa, et al., 1994) and S. gordonii (Lamont, et al., 2002). Indeed, T. denticola is commonly isolated together with P. gingivalis (Kigure, et al., 1995, Socransky, et al., 1998). These two species were also reported to exhibit synergistic effects during biofilm formation in vitro (Yamada, et al., 2005).

P. gingivalis-T. denticola coaggregation activity was inhibited in a dose-dependent manner by cysteine protease inhibitors, as well as by arginine or lysine, which is consistent with previous reports (Grenier, 1992, Onagawa, et al., 1994). Loss of coaggregation activity in P. gingivalis with heating suggests that the adhesion molecule on the surface of this organism is heat labile. The inhibition profile also suggests the involvement of gingipains in the coaggregation reaction, since all gingipains are inhibited by TLCK and Rgps by leupeptin (Kadowaki, et al., 1994, Pike, et al., 1994). The low coaggregation activity exhibited by the gingipain-null mutant KDP136 observed in this study is consistent with earlier results (Grenier, 1992, Onagawa, et al., 1994). However, the interaction between hydrophobic tosyl groups of TLCK and cell surface components of T. denticola may act independently of gingipain inhibition. Such interactions may affect coaggregation reactions. Further study is required to clarify the mechanism of inhibition by protease inhibitors.

The binding ability of the major outer sheath protein (Msp) to fibrinogen and fibronectin and of a factor H-like protein 1 to complement has been reported in T. denticola (Edwards, et al., 2005, McDowell, et al., 2005). As T. denticola is usually grown in serum-containing medium, it was possible that serum components attach to T. denticola and are involved in its coaggregation activity. Therefore, in this study, the coaggregation reaction was evaluated with T. denticola grown in modified TYGVS
medium containing 1% EX-CYTE, which contains bovine lipoprotein and cholesterol in a liquid concentrate, or in serum-free TYGVS medium (data not shown). The results suggest that the coaggregation between P. gingivalis and T. denticola seen here was serum-independent.

Coaggregation was also significantly reduced in the rgpA kgp hagA triple mutant (adhesin-null mutant). The involvement of these genes in coaggregation between P. gingivalis and A. viscosus was also reported (Abe, et al., 2004). HagA has been shown to be a hemagglutinin of P. gingivalis (Han, et al., 1996). HagA contains domains which are highly homologous to some of the Hgp domains of RgpA and Kgp (Potempa, et al., 2003). These Hgp domains were reported to play a role in P. gingivalis adherence to type-V collagen, fibronectin and fibrinogen (O'Brien-Simpson, et al., 2005). The weak coaggregation displayed by the KDP137 triple mutant (rgpA kgp hagA) in this study suggests that the Hgp domains also play an important role in coaggregation between P. gingivalis and T. denticola. In the rgpA rgpB double mutant (KDP133), coaggregation showed no significant decrease in comparison with the parental strain. It is possible that Hgp domains from other molecules such as Kgp and HagA mediated the coaggregation reaction.

Overlay assays revealed that P. gingivalis 38.9- and 43- kDa proteins reacted with T. denticola. The molecular size of the proteins was very similar to that detected by anti-Hgp44 antibody. RgpA and Kgp have hemagglutinin/adhesion domains downstream from their catalytic domains, and these domains exhibit extensive homology with one another (Potempa, et al., 2003). Among these, the catalytic domains and Hgp44 of RgpA are approximately the same size. This latter domain also shares homology with the hemagglutinin (HagA) of P. gingivalis. These results
suggest that the Hgp44 domain is involved as a ligand in coaggregation with *T. denticola*.

Complementation assays indicated that coaggregation was restored 58.9% by inter-bacterial complementation with the adhesin-null but RgpB-positive mutant KDP137 and the HagA-positive but gingipain-null mutant KDP136. Naito *et al.* (2006) reported that complementation with these mutants resulted in maturation of Hgp44. Taken together, these data suggest that RgpB protease produced by KDP137 partially restores coaggregation activity of the gingipain-null mutant KDP136 by processing the proprotein of HagA.

Among the recombinant Rgp domains, recombinant Hgp44 showed the strongest adherence to *T. denticola* sonicates, and, furthermore, anti-Hgp44 antibody inhibited coaggregation. These results suggest that the Hgp44 domain is largely responsible for coaggregation with *T. denticola* and that the maturation of Hgp44 is important in this interaction. Maturation appears to be primarily dependent upon Rgp protease activity. We obtained evidence to support this following complementation of the *rgpA rgpB kgp* triple mutant with the *rgpA kgp hagA* triple mutant. The results suggest a role for Rgp proteases in such maturation and are compatible with the effects of protease inhibitors. A similar phenomenon was also observed for hemagglutination by *P. gingivalis* (Shah, *et al.*, 1992). In addition, coaggregation activity decreased according to the number of the Hgp44 coding genes. A comparison of the *rgpA kgp* double mutant (RK 103) with the *rgpA kgp* and *hagA* mutant (KDP137) also suggested the involvement of Hgp44 in coaggregation.

Several other surface components of *P. gingivalis* and *T. denticola* have been reported. FimA has been shown to be a structural component of the major fimbriae of
P. gingivalis and appeared to be involved in its coaggregation reactions (Hashimoto, et al., 2003). These reports indicated that FimA binds to the dentilisin protease of T. denticola according to overlay assays and noted that coaggregation was inhibited in the presence of a synthetic FimA peptide. Previously, we observed that a dentilisin-deficient mutant coaggregated normally with P. gingivalis (Ishihara, et al., 1998). The fimA mutant also coaggregated with T. denticola in the present study. Rosen et al. (2008) further reported that T. denticola mutants lacking the Msp, dentilisin, or a leucine-rich repeat protein adhered strongly to P. gingivalis cells. These results taken together suggest that FimA is not a major ligand for coaggregation. Grenier (1992) earlier suggested a bimodal coaggregation reaction between T. denticola and P. gingivalis. The adherence of Msp to P. gingivalis has also been reported (Rosen, et al., 2008). It is possible that several surface components are involved in this reaction. However, further analysis is required to fully clarify how these ligands interact during the coaggregation process.

Taken together, the present findings indicate that the Hgp44 domains of RgpA, Kgp and HagA are the adhesins involved in the coaggregation between P. gingivalis and T. denticola. Such interactions may play an important role in the incorporation of these periodontopathic bacteria into biofilms present in the gingival crevice, which may induce the formation of peridontitis lesions.

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Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Relevant characteristics</th>
<th>Source/Reference</th>
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<td>Wild type</td>
<td>American Type Culture Collection</td>
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<td>(Shoji, et al., 2004)</td>
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<td>kgp::Cm^r</td>
<td>(Shi, et al., 1999)</td>
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<td>(Shi, et al., 1999)</td>
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<td>(Shi, et al., 1999)</td>
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<td>(Shi, et al., 1999)</td>
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<tr>
<td><em>P. gingivalis</em> RK103</td>
<td>rgpA::Em^r, kgp::Cm^r</td>
<td>This study</td>
</tr>
<tr>
<td><em>T. denticola</em> ATCC 35405</td>
<td></td>
<td>American Type Culture Collection</td>
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<tr>
<td>Tested chemicals</td>
<td>Concentration (mM)</td>
<td>Inhibition (%)</td>
</tr>
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<tr>
<td><strong>Amino acids</strong></td>
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<td>L-arginine</td>
<td>100</td>
<td>98.1 ± 1.7*</td>
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<tr>
<td></td>
<td>50</td>
<td>94.0 ± 1.0*</td>
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<tr>
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<td>92.0 ± 1.0*</td>
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<tr>
<td>L-lysine</td>
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<td>98.5 ± 1.8*</td>
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<td>97.6 ± 3.6*</td>
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<td><strong>Inhibitor</strong></td>
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<tr>
<td>TLCK</td>
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<td>0.5</td>
<td>69.6 ± 7.2*</td>
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<td>43.0 ± 7.9*</td>
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<td>0.1</td>
<td>33.0 ± 8.6*</td>
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<td><strong>Heat treatment</strong></td>
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<td><em>P. gingivalis</em></td>
<td>-</td>
<td>90.8 ± 36.6*</td>
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<tr>
<td><em>T. denticola</em></td>
<td>-</td>
<td>-17.7 ± 1.38*</td>
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*P < 0.05
Figure legends

Figure 1.

A. Evaluation of *P. gingivalis* ligands involved in coaggregation by overlay assay and immunoblotting. *P. gingivalis* proteins were separated following electrophoresis and blotted membranes incubated with sonic extracts of *T. denticola* ATCC 35405. Membranes were then immunostained with rabbit anti-serum against whole cells of *T. denticola* ATCC 35405.

Lanes 1: *P. gingivalis* after reaction with *T. denticola* sonicate; 2: Control, *P. gingivalis* not incubated with *T. denticola* sonicate. Arrows indicate the 61-, 53-, 43-, 40 and 38.9-kDa bands.

B. Comparison of bands obtained with overlay assay and immunoblotting with anti-Hgp44 serum. Lane 1: Membrane transferred with *P. gingivalis* antigen was blocked with 3% BSA containing PBS, incubated with 3% BSA containing PBS, reacted with anti-Hgp44 rabbit antibody and the reacted antibody detected with peroxidase conjugated anti-rabbit antibody. Lane 2: Membrane transferred with *P. gingivalis* antigen was blocked with 3% BSA containing PBS, reacted with sonic extracts of *T. denticola* ATCC 35405 (1 mg/ml), reacted with anti-*T. denticola* rabbit antibody and the reacted antibody detected with peroxidase conjugated anti-rabbit antibody.

Figure 2.

Coaggregation of *T. denticola* with *P. gingivalis* gingipain- and adhesin-deficient mutants. Cells of each *P. gingivalis* strain were mixed with *T. denticola* ATCC 35405
and coaggregation activities evaluated after 60 min incubation. Autoaggregation (%) of *P. gingivalis* ATCC 33277, KDP150, KDP129, KDP133, KDP136 KDP137 and *T. denticola* ATCC35405 alone were 2.76 ± 1.52, 2.49 ± 1.19, 3.46 ± 0.67, 19.1 ± 1.93, 1.63 ± 0.81, 2.39 ± 0.46 and 14.98 ± 25.16, respectively.

*: P<0.05

Fig. 3

A. Adherence of recombinant Rgp domains to *T. denticola*. Each domain (10 µg/ml) of Rgp was coated onto 96-well EIA plates and sonicates of *T. denticola* (10 µg/ml) reacted for 1 h. Levels of adherent *T. denticola* proteins were evaluated using anti-*T. denticola* antibody.

Each experiment was performed 4 times using 5 samples. CAT: Catalytic subunit of RgpA, Hgp44: Hgp15-27, Non: Control  *

*: P<0.05

B. Inhibition of coaggregation between *T. denticola* and *P. gingivalis* by *P. gingivalis* Hgp44 antibody. *P. gingivalis* cell suspensions (OD660=0.5) in 24-well microtiter plates were incubated with anti-*P. gingivalis* ATCC 33277 whole cell IgG, anti-Hgp44 IgG or normal rabbit IgG (control) for 10 min, followed by addition of *T. denticola* ATCC 35405 (OD660=0.5). After 30 min, coaggregation was evaluated visually.

1. Anti-*P. gingivalis* ATCC 33277 whole cell IgG
2. Anti-Hgp44 IgG
3. Normal rabbit IgG (control)

PBS: *P. gingivalis* treated with PBS.
Figure 4

Coaggregation increased by coculture with *P. gingivalis* mutants KDP136 and KDP137. *P. gingivalis* KDP 136 was cocultured with *P. gingivalis* KDP 137 at 37°C for 1-2 days and coaggregation activity with *T. denticola* ATCC 35405 evaluated.

*: P<0.05

Figure 5

Coaggregation of *T. denticola* with *P. gingivalis* HGP-44 domain-deficient mutants.

Cells of each *P. gingivalis* HGP44 domain-deficient mutant strain were mixed with *T. denticola* ATCC 35405 grown in E-TYG medium and coaggregation activities evaluated after 60 min incubation. Autoaggregation (%) of *P. gingivalis* ATCC 33277, KDP129, RK103, KDP137 and *T. denticola* 35404 alone was 6.0 ± 3.37, 3.6 ± 1.8, 1.40 ± 0.62, 4.2 ± 1.6 and -5.6 ± 3.28, respectively.
Fig. 2

P. gingivalis strains
Fig. 3

A

B

IgG concentration (mg/ml)
Fig. 4

P. gingivalis strains
Fig. 5

* * *

Coaggregation activity (%)

33277  KDP 129  RK 103  KDP 137

* * *

* * *

P. gingivalis strains