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Dentilisin involvement in coaggregation between *Treponema denticola* and *Tannerella forsythia*

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Abstract

Periodontitis arises from a biofilm consisting of gram-negative anaerobic rods and spirochetes. *Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia,* termed the Red complex, have been co-isolated with high frequency from chronic periodontitis lesions, and these microorganisms are thought to be major pathogens of the disease. Coaggregation is an important strategy in the colonization of dental plaque biofilm by these bacteria. In the present study, we investigated the coaggregation of *T. denticola* strains with *T. forsythia* ATCC 43037 by use of visual grading or spectrophotometry. *T. denticola* ATCC 35405 coaggregated with *T. forsythia*, reaching a plateau at approximately 60 min. This coaggregation was inhibited by heat treatment of *T. denticola* ATCC 35405, but not of *T. forsythia*. Disaccharides such as sucrose, maltose, and lactose inhibited coaggregation by approximately 50%. The coaggregation reaction varied among *T. denticola* strains. There was somewhat less coaggregation between *T. denticola* ATCC 33520 and *T. forsythia* than between *T. denticola* ATCC 35405 and *T. forsythia*, although this difference was not statistically significant; *T. denticola* ATCC33521 showed a trace level of coaggregation with *T. forsythia*. The magnitude of coaggregation among the three *T. denticola* strains was proportional to their dentilisin activities. Inactivation of dentilisin abolished coaggregation activity, but inactivation of the major outer sheath protein did not. Phenylmethylsulfonyl fluoride (PMSF) also did not affect coaggregation. These results indicate that dentilisin is involved indirectly in the coaggregation between *T. denticola* and *T. forsythia*, because its proteolytic activity is not required, possibly via ligand maturation.
**Key word:** biofilm; coaggregation; periodontal disease; adherence; colonization; protease
1. Introduction

Periodontal disease is characterized by polymicrobial infection associated with progressive destruction of the tissue surrounding and supporting the teeth [1, 2]. More than 700 taxa of bacteria have been isolated from subgingival plaque biofilm [3], among which *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* are the major pathogens of chronic periodontitis [4]. These three species are frequently isolated together from periodontitis lesions [5]. The combination of these three microorganisms is termed the *Red complex*, and it is believed to be involved in the progression of chronic periodontitis [5]. *T. denticola* possesses several potential pathogenic properties including immunosuppressive activity, a major outer sheath protein (Msp), and proteases [6]. Its surface protease, dentilisin, is a major pathogenic factor [7-10]. *T. forsythia* is also an important and consistent member of the microbial biofilm in periodontal disease and possessed several potential pathogenic factors such as Bacteroides surface protein A (BspA) [11]; however, there is limited information regarding its colonization strategy.

Coaggregation is an essential step in adherence and colonization on the surface of established biofilm [12]. Most periodontopathic bacteria are late colonizers that exhibit coaggregation activity [12]. *T. denticola* is reported to coaggregate with several oral microorganisms such as *Fusobacterium nucleatum*, *P. gingivalis*, and *T. forsythia* [13-16]. Co-isolation of *T. denticola*, *T. forsythia*, and *P. gingivalis* from chronic periodontitis samples suggests that there is an interaction such as coaggregation among the three species. Although coaggregation of *T. denticola* with *P. gingivalis* has been characterized [15, 17-19], the coaggregation reaction between *T. denticola* and *T.
*denticola* has yet to be fully clarified. The purpose of this study was to investigate the coaggregation reaction between *T. denticola* and *T. forsythia* and to clarify its mechanism.

2. Materials and methods

2.1. Bacterial strains and plasmids

The bacterial strains used are listed in Table 1. *T. denticola* was propagated in TYGVS medium [20], and *T. denticola* K1, KpSano7, and DMSP3 were propagated in the same medium containing 40 μg/mL erythromycin. *Tannerella forsythia* ATCC 43037 was grown in TF broth [21]. Both were incubated at 37 °C under anaerobic conditions as described previously [22]. *Escherichia coli* TOP10 and SCS110 were grown in Luria–Bertani (LB) broth or on LB agar plates.

2.2. Coaggregation assays

Coaggregation between *T. denticola* and *T. forsythia* was first determined visually by using the turbidimetric method of Cisar et al. [23] and subsequently by modifying the method of Nagata et al. [24]. Briefly, cells were washed with phosphate-buffered saline (PBS, pH7.2) and suspended in coaggregation buffer (1 mM sodium phosphate buffer [pH 8.0] containing 0.1 mM CaCl$_2$, 0.1 mM MgCl$_2$ and 150 mM NaCl) [23]. Aliquots (500 μL) of each bacterial and coaggregation partner suspension were vortexed for 10 sec, and OD$_{660}$ was evaluated with a UV-2550 spectrophotometer (Shimazu, Tokyo, Japan). Coaggregation was monitored at room temperature by measuring the decrease in absorbance at 660 nm for 60 min. Coaggregation was calculated by using the following
equation: coaggregation (%) = \{(OD_{660 \text{ at } 0 \text{ min}} - OD_{660 \text{ at } 60 \text{ min}})/ \text{OD}_{660 \text{ at } 0 \text{ min}}\} \times 100\%.

When autoaggregation of *T. denticola* and *T. forsythia* reached 20% or more, the result was excluded.

2.3. Effect of putative inhibitors or heat treatment on coaggregation

We also evaluated the effects of sugars (glucose, fucose, lactose, mannitol, sorbitol, rhamnose, and mannose) on coaggregation. Concentrations were adjusted to 100 mM and coaggregation was determined visually [23] as described above. To investigate the effect of heat treatment on coaggregation, cell suspensions of *T. denticola* and *T. forsythia* were heated at 75 °C for 30 min and then evaluated in the coaggregation assay.

2.4. Measurement of dentilisin activity

Dentilisin activity was measured with the synthetic substrate *N*-succinyl-L-alanyl-Lalanyl-L-prolyl-L-phenylalanine p-nitroanilide (SAAPNA; Sigma Chemical Company, St. Louis, Mo). *T. denticola* cells were washed and suspended in PBS. The protein concentration of the suspension was determined by using the DC protein assay (Bio-Rad Laboratories, Hercules, Calif.). A 5-µL suspension was mixed with 150 µL of 100 mM Tris-HCl buffer (pH 8.0) containing 1.0 mM SAAPNA. The mixture was then incubated at 37 °C for 15 min. The reaction was stopped by the addition of 50 µL of 20% acetic acid. Release of p-nitroaniline was determined by measuring \text{OD}_{405}. One unit of enzyme was defined as the amount required to release 1.0 \text{µmol p-nitroaniline in 1 min at 37 °C under these conditions.}

2.5. Construction of deletion mutants of *T. denticola* ATCC 33520 and ATCC 35405

Dentilisin- and Msp-deficient mutants were constructed as described previously [25]. Briefly, the sequences of the 5’ end of prcA and the 3’ end of prtP were amplified by PCR from *T. denticola* ATCC 33520 by using the synthetic oligonucleotide primers
listed in Table 2. The two pairs of primers, 1st ORFD and 520 prcAU, and 520 prtPD and 520 prtPU, have EcoRI, SmaI, Sall, and SphI restriction sites, respectively, at their 5’ ends. Amplified fragments obtained by using these two primer pairs were cloned into pMCL191. An ermF–ermAM cassette from plasmid pVA2198 was then cloned between the two previously cloned fragments. The resulting plasmid was designated as pSano3. The plasmid was amplified in E. coli SCS110. Plasmid DNA was linearized after EcoRI digestion [25] and transformed into T. denticola ATCC 33520 by electroporation, and transformants were isolated on TYGVS agar containing 40 µg/mL erythromycin.

The Msp-deficient mutant was constructed in the same way by using primers MspK1–4 (Table 2). These primers contained EcoRI, KpnI, BamHI, and SphI restriction sites, respectively, at their 5’ ends. The fragments amplified from T. denticola ATCC 35405 by these primers were located from 57 bp upstream of the start codon to the 412th base of the msp open reading frame and from the 437th to the 945th base of the msp open reading frame. The amplified fragments and ermF–ermAM cassette were cloned into pMCL191, and transformation of the fragment to T. denticola ATCC35405 and isolation of the transformants were performed as described above. Inactivation of prtP and msp was confirmed by Southern blotting and immunoblotting (Supplementary figures 1 and 2) with an anti-dentilisin antibody or an anti-Msp antibody as described previously [26]. After confirmation of the mutants, the prtP mutant from T. denticola ATCC 33520 and the msp mutant from T. denticola ATCC 35405 were designated as KpSano7 and DMSP3, respectively.

2.6. Phenylmethylsulfonyl fluoride (PMSF) treatment of bacteria

T. denticola ATCC 35405 were treated with the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) at 1 or 10 µM for 10 min. Coaggregation of
PMSF-treated *T. denticola* ATCC35405 and ATCC33520 with *T. forsythia* ATCC 4303 was evaluated as described above.

2.7. Statistical analysis

To investigate differences in coaggregation between various *T. denticola* strains and *T. forsythia*, a one-way ANOVA followed by a Turkey’s multiple comparison were performed for comparisons among the three strains, and a Student's t-test was performed for the comparison between strains. The level of significance for all statistical tests was set at $P < 0.05$.

3. Results

3.1. Effect of chemicals and heat treatment on coaggregation

The coaggregation between *T. forsythia* ATCC 43037 and *T. denticola* ATCC35405 increased visually from 10 min and reached a plateau at approximately 60 min (Fig. 1), achieving a visual coaggregation score of 4 (data not shown). Coaggregation was dramatically reduced for heat-treated *T. denticola* ATCC 35405; however, heat treatment of *T. forsythia* ATCC 43037 did not affect coaggregation (Table 3).

The effect of sugar on coaggregation, evaluated according to OD$_{660}$, is shown in Table 4. The monosaccharides tested failed to inhibit coaggregation, whereas the disaccharides including maltose, sucrose, and lactose reduced coaggregation by approximately 50%.

3.2. Comparison of coaggregation reactions between *T. denticola* strains and *T. forsythia*
Msp antigenicity and dentilisin activity vary among strains [27-29]. We therefore investigated the effect of this variation on coaggregation. The amount of coaggregation between *T. forsythia* ATCC 43037 and *T. denticola* ATCC 33520 was somewhat lower than that between *T. forsythia* ATCC 43037 and *T. denticola* ATCC 35405, although this difference was not statistically significant (Fig. 2). *T. denticola* ATCC33521 showed a trace level of coaggregation with *T. forsythia* ATCC43037.

As stated above, dentilisin activity is reported to differ among *T. denticola* strains [28]. Therefore, to investigate the relationship between dentilisin activity and coaggregation, we evaluated the proteolytic activity of dentilisin by using SAAPNA. *T. denticola* ATCC 33520 possessed dentilisin activity; however the level was about one-tenth that of *T. denticola* ATCC 35405 (Table.5). *T. denticola* ATCC 33521 showed significant less activity than *T. denticola* ATCC 33520 and ATCC 35405.

3.3. Coaggregation of dentilisin- or Msp-deficient mutants of *T. denticola* with *T. forsythia*

To determine the role of dentilisin and Msp in coaggregation, isogenic mutants defective in *prtP* or *msp* were constructed by means of allelic exchange mutagenesis. As shown in Table 3, the *prtP* mutants KpSano7 and K1 did not possess dentilisin activity, whereas the *msp* mutant had similar dentilisin activity to that of the wild-type strain *T. denticola* ATCC35405. The dentilisin-deficient mutants K1 and KpSano7 did not coaggregate with *T. forsythia*, whereas both wild-type strains coaggregated with *T. forsythia* (Fig. 3A and B). The Msp-deficient mutant DMSP3 coaggregated with *T. forsythia* and the magnitude of the coaggregation reaction was almost the same as that for the wild-type *T. denticola* ATCC35405 (Fig. 3A). To determine whether the proteolytic activity of dentilisin was directly involved in the coaggregation reaction,
coaggregation between PMSF-treated *T. denticola* ATCC 35405 and *T. forsythia* ATCC 4303 was investigated. Treatment of *T. denticola* ATCC 35405 with 1–10 mM PMSF did not affected the coaggregation (data not shown).

**4. Discussion**

Periodontopathic bacteria such as *P. gingivalis, T. forsythia,* and *T. denticola* colonize dental plaque biofilm that has been established by early colonizers such as streptococci [30]. For periodontopathic bacteria, coaggregation is a major colonization strategy for establishing dental plaque biofilm [31]. In the present study, *T. denticola* coaggregated with *T. forsythia*. Heat treatment of *T. denticola* ATCC 35405, but not of *T. forsythia*, dramatically reduced coaggregation, suggesting that the coaggregation ligand on the surface of *T. denticola* is a proteinaceous material. Coaggregation was also inhibited by maltose, lactose, and sucrose. These results indicate that lectin-like activity is involved in the coaggregation of *T. denticola* with *T. forsythia*. Lactose-sensitive coaggregation has been reported for several oral microorganisms, the receptor for which was a cell surface polysaccharide containing galactose [12]. In the current study, the coaggregation was inhibited by a glucose-containing disaccharide, which suggests that the receptor involved here is different from that involved in “lactose-sensitive coaggregation.”

*P. gingivalis, T. denticola,* and *T. forsythia* are associated with chronic periodontitis [5]. Among these species, coaggregation and synergistic effects between *T. denticola* and *P. gingivalis* have been reported [14, 15, 17, 19, 32-34]; however, to date, only LrrA
involvement in the coaggregation of *T. denticola* with *T. forsythia* has been reported [16]. In the present study, we showed that *T. denticola*, which possess dentilisin activity, coaggregate with *T. forsythia* ATCC43037. The reduction in coaggregation by inactivation of IrrA was approximately 10% [16], whereas the inactivation of dentilisin resulted in the complete loss of coaggregation, indicating that dentilisin is also involved in coaggregation. Given that these two microorganisms are frequently co-isolated from chronic periodontitis lesions [5], dentilisin-related coaggregation of *T. denticola* with *T. forsythia* may contribute to the co-colonization of these species in the periodontal pocket.

The level of coaggregation between *T. denticola* and *T. forsythia* differed depending on the strain of *T. denticola*. Among the surface molecules of *T. denticola*, variations in Msp antigenicity and in dentilisin activity across strains has been reported [27, 35]. In this study, dentilisin activity differed among the strains tested. The degree of coaggregation for each strain was proportional to it level of dentilisin activity. In addition, dentilisin-knockout mutants of *T. denticola* ATCC 33520 and ATCC 35405 showed a loss of coaggregation. These results indicate that dentilisin is involved in the coaggregation of *T. denticola* with *T. forsythia*. Msp is reported to be involved in the coaggregation of *T. denticola* with *Fusobacterium nucleatum* or *Porphyromonas gingivalis* [18]. However, coaggregation of Msp-deficient *T. denticola* DMSP3 with *T. forsythia* occurs at the same level as that seen with wild-type *T. denticola* and *T. forsythia*, indicating that Msp is not a coaggregation ligand for these microorganisms.

There are three potential ways in which dentilisin may be involved in coaggregation: 1) by degrading the surface protein of *T. forsythia*, thereby exposing a cryptic receptor for coaggregation; 2) by directly binding to the surface of *T. forsythia*;
or 3) by participating in the maturation of the coaggregation ligand on the surface of *T. denticola* via its proteolytic activity. Kontani et al. [36] reported that *P. gingivalis* adheres to matrix proteins via a fimbrial cryptic receptor exposed by its own arginine-specific protease. In the present study, treatment of *T. denticola* with PMSF did not affect coaggregation. Similar results were reported with respect to adherence during biofilm formation by *T. denticola* [37, 38]. Dentilisin-deficient mutants of *T. denticola* reduce binding to fibrinogen, whereas adherence of *T. denticola* to fibrinogen is not affected by PMSF treatment [37]. Dentilisin is involved in adherence to *P. gingivalis, Prevotella intermedia,* and *F. nucleatum*; however, PMSF treatment does not affect this adherence [38]. The data from the present study and, as well as those from previous reports, indicate that degradation of the surface protein of *T. forsythia* by the dentilisin protease is not involved in coaggregation. Dentilisin forms a complex with PrcA and PrcB [10, 39], its inactivation of the dentilisin affects oligomeric protein formation including that of Msp [26]. The interaction between dentilisin and fimbriae (FimA) of *P. gingivalis* is involved in the coaggregation of *T. denticola* with *P. gingivalis* [17]. It is possible that dentilisin is involved in the maturation of the ligand protein on the surface of *T. denticola* or it may be that some domain of dentilisin other than its protease domain has the ability to adhere to *T. forsythia*; further analysis is clearly required.

In the present study we investigated the coaggregation reaction between *T. denticola* and *T. forsythia* in vitro. A large number of microorganisms colonized in dental plaque by adhering to antecedent microorganisms [30]. It is possible that the adherence between *T. denticola* and *T. forsythia* involves colonization of these microorganisms. In mouse model, most rats were infected with *P. gingivalis, T. denticola,* and *T. forsythia* as a consortium after mix infection of the three
microorganisms [40]. *P. gingivalis, T. forsythia* and *T. denticola* were frequently co-isolated from lesion of periodontitis [5]. *T. denticola* and *T. forsythia* have fastidious growth requirements for culture [6, 11]. Several bacterial pairs were reported to require cell to cell contact for enhancement of biofilm formation [41, 42]. Taken together with these reports, the coaggregation reaction between *T. denticola* and *T. forsythia* may work for adhesion and colonization to dental plaque and involve the increase of *T. denticola* or *T. forsythia* in dental plaque.

5. Conclusion

*T. denticola* coaggregates with *T. forsythia*. The coaggregation ligand on the surface of *T. denticola* appears to be a proteinaceous material, and a lectin-like mechanism is involved in the reaction. Dentilisin is involved indirectly in the coaggregation between *T. denticola* and *T. forsythia*, because its proteolytic activity is not required, possibly via ligand maturation.

**Acknowledgments:** This work was supported in part by grant 24592778 from the Ministry of Education, Science, Sport, Culture, and Technology of Japan. We thank Dr. Naoya Matsumoto for his assistance with the experiments.
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*Treponema denticola* prtP gene encoding a prolyl-phenylalanine-specific protease (dentilisin).


Figure legends

Fig. 1. Coaggregation reaction between *T. denticola* ATCC 35405 and *T. forsythia* ATCC 43037.

Coaggregation (%) = \[\frac{(OD_{660 \text{ at } 0 \text{ min}}) - (OD_{660 \text{ at } 60 \text{ min}})}{(OD_{660 \text{ at } 0 \text{ min}})} \times 100\%\].

Data are shown as means ± standard error.

Fig. 2. Coaggregation reactions between wild-type *T. denticola* strains and *T. forsythia* ATCC43037 after 60 min.

Coaggregation (%) = \[\frac{(OD_{660 \text{ at } 0 \text{ min}}) - (OD_{660 \text{ at } 60 \text{ min}})}{(OD_{660 \text{ at } 0 \text{ min}})} \times 100\%\],

Data are shown as means ± standard deviation. 35405 + Tf = *T. denticola* ATCC 35405 and *T. forsythia* ATCC 43037; 33520 + Tf = *T. denticola* ATCC 33520 and *T. forsythia* ATCC 43037; and 33521 + Tf = *T. denticola* ATCC 33521 and *T. forsythia* ATCC 43037. *p < 0.05, against *T. denticola* ATCC35405; §p < 0.05 against *T. denticola* ATCC33520 by one-way ANOVA, followed by Tukey’s multiple-comparison test.

Fig. 3. Coaggregation between dentilisin- or msp-deficient mutants of *T. denticola* and *T. forsythia* ATCC43037 after 60 min.

Data are shown as means ± standard deviation. Coaggregation (%) = \[\frac{(OD_{660 \text{ at } 0 \text{ min}}) - (OD_{660 \text{ at } 60 \text{ min}})}{(OD_{660 \text{ at } 0 \text{ min}})} \times 100\%\].

A: Comparison among *T. denticola* ATCC 35405 and dentilisin- or msp-deficient mutants derived from *T. denticola* ATCC 35405. 35405 + Tf = *T. denticola* ATCC 35405 and *T. forsythia* ATCC 43037; DMSP3 + Tf = *T. denticola* DMSP3 and *T. forsythia*
ATCC 43037; and K1 + Tf = T. denticola K1 and T. forsythia ATCC 43037. One-way ANOVA for repeated measurements was used for inter-group comparisons. Tukey’s multiple comparison test was used for multiple comparisons. *p < 0.05, against T. denticola ATCC35405 and §p < 0.05, against T. denticola DMSP3.

B: Comparison among T. denticola ATCC 33520 and dentilisin-deficient mutants derived from T. denticola ATCC 33520. 33520 + Tf = T. denticola ATCC 33520 and T. forsythia ATCC 43037; and KpSano7 + Tf = T. denticola KpSano7 and T. forsythia ATCC 43037 The Student's t-test was used for comparisons. *P < 0.05, against T. denticola ATCC 33520.

Supplementary figure 1. Southern blot analysis of T. denticola strains
A. Chromosomal DNA from T. denticola ATCC33520 (lanes 1 and 3) and KpSano (lanes 2 and 4) was digested with BamHI (lanes 1 and 2) or HindIII (lanes 3 and 4). DNA fragments between primers 520 prtPD and 520prtPU were labeled with digoxigenin by PCR and used as probes.
B. Chromosomal DNA from T. denticola ATCC35405 (lane 1) and DMSP3 (lane 2) was digested with HindIII The open reading frame of Msp was labeled with digoxigenin by PCR and used as a probe.

Supplementary figure 2. Immunoblot analysis of wild-type T. denticola and its mutants
A. Dentilisin-deficient mutants. Lane 1 = ATCC33520; lane 2 = KpSano; lane 3 = ATCC35405; and lane 4 = K1. Primary antibody = anti-dentilisin rabbit serum
B. Msp-deficient mutants. Lane 1 = ATCC33405; and lane 2 = DMSP3. Primary antibody = anti-Msp rabbit serum.
Coaggregation (%) vs. min.

Fig. 1
Fig. 2.
Supplementary material 1.
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△ dentilisin

△ Msp