

Genetic analysis of *Treponema denticola* ATCC 35405 biofilm formation

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Treponema denticola is a major aetiological organism implicated in periodontal disease. The interaction of *T. denticola* with other oral bacteria, in particular *Porphyromonas gingivalis*, in biofilm formation is thought to be an important step in the onset of periodontal disease. The interaction between *T. denticola* and *P. gingivalis* has been examined using a panel of *T. denticola* mutants and their effects on mixed biofilm formation tested in a static biofilm model. *T. denticola* ATCC 35405 did not form detectable biofilms on various inert surfaces. However, the spirochaete was demonstrated to form a biofilm with preattached *P. gingivalis* 381. *T. denticola* *cfpA*, which lacks the cytoplasmic filament, was unable to produce a mixed biofilm with *P. gingivalis*. A *T. denticola* *flgE* mutant which lacks the flagella hook protein and is therefore non-motile displayed a reduced, but readily detectable, ability to form a mixed biofilm as did the *T. denticola* mutant which does not possess the major outer sheath protein (Msp). The *T. denticola* *IrrA* mutant was only moderately defective in forming mixed biofilms with *P. gingivalis*. However, the *T. denticola* methyl-accepting chemotaxis protein (DmcA) did not appear to play a major role in mixed biofilm formation. In contrast, *T. denticola* lacking the PrtP protein for prolyl-phenylalanine-specific protease, showed an increased ability to form mixed biofilms and a prolonged viability in the biofilm.

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INTRODUCTION

In vivo, most bacteria do not exist as discrete entities but as mixed populations, clearly indicated in the case of human periodontal diseases (Slots & Genco, 1984). These bacteria live in close association with inert surfaces as complex communities often referred to as biofilms. Biofilms are particularly important in the oral cavity where the primary colonizers, which attach to oral surfaces, can enhance colonization by other bacteria which are themselves poor colonizers (Kolenbrander *et al.*, 2002). Oral biofilms provide a sheltered environment for secondary colonizing bacteria that would otherwise be carried away with the saliva flow. A variety of molecular interactions enable interbacterial adhesion contributing to dental plaque formation (Kolenbrander *et al.*, 2002).

It has been shown by scanning electron microscopic investigations that oral treponemes and rod-like bacteria are the main morphological agents at the advancing front of subgingival plaque in sites of periodontitis (Saglie *et al.*, 1982). Oral spirochaetes have been implicated in the

aetiology of periodontal diseases (Loesche, 1988, 1993), and their numbers strongly correlate with the severity of periodontal inflammation. *Treponema denticola* is a small helically shaped obligate anaerobic spirochaete frequently isolated from the periodontal pocket and correlated with the severity of periodontal disease (Simonson *et al.*, 1988; Haffajee & Socransky, 1994; Asai *et al.*, 2002). Another major Gram-negative rod bacterium which has been associated with periodontitis is *Porphyromonas gingivalis* (Slots & Genco, 1984). It has been shown previously *in vitro* that there is a symbiotic relationship between these two periodontopathogens for nutrients (Grenier, 1992). The interaction between *T. denticola* and *P. gingivalis* in biofilm formation could play an important role in the initial stages of the onset of periodontal disease. Furthermore, no information is currently available regarding biofilm formation by any spirochaete.

In *T. denticola* the cytoplasmic filaments, a ribbon-like structure, span the cytoplasm at all stages of the cell division process. It has been suggested that the cytoplasmic filament protein, CfpA, may be involved in chromosome structure, segregation, or the cell division process in *T. denticola* 33520 (Izard *et al.*, 2001). Here, we report the construction of a *T. denticola* 35405 *cfpA* mutant and investigate its possible role in biofilm formation.

The structure and motility of treponemes such as *T. denticola*

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Abbreviations: r.f.u., relative fluorescence units; SAAPNA, Suc-Ala-Ala-Pro-Phe-AMC; SEM, scanning electron microscopy.

is an unusual feature which enables motility in a highly viscous environment (Kimsey & Spielman, 1990; Klitorinos *et al.*, 1993). Like other spirochaetes, the *T. denticola* flagellar filaments are located within the periplasmic space. A non-motile mutant, lacking flagellar hooks and flagella, was previously constructed by insertionally inactivating the *flgE* gene by allelic exchange (Li *et al.*, 1996). It was therefore of interest to utilize this mutant to assess the role of motility in biofilm formation by *T. denticola*. We also speculated that chemotaxis may be an important factor during the initial development of biofilm formation. A *T. denticola* mutant defective in the *T. denticola* methyl-accepting chemotaxis A (*dmcA*) gene has been shown to be defective in chemotaxis toward nutrients (Kataoka *et al.*, 1997). Therefore, this mutant was also examined to investigate the role of chemotaxis in mixed biofilm formation with *P. gingivalis*.

As earlier studies have found that the cell surface Msp may serve as a *T. denticola* adhesin (Fenno *et al.*, 1996), it was also of interest to examine the role of this putative outer sheath protein in mixed biofilm formation. Likewise, the prolyl-phenylalanine-specific serine protease (dentilisin; also called chymotrypsin-like protease) is a major extracellular protease produced by *T. denticola* (Fenno *et al.*, 1998). The gene encoding this protease, *prtP*, has been isolated and inactivated (Ishihara *et al.*, 1998) and the attenuated protease-deficient mutant was compared to the *T. denticola* type strain in its ability to form a mixed biofilm. It has already been suggested that dentilisin participates in adhesion to epithelial cells (Leung *et al.*, 1996) and also affects the organization of the Msp protein (Ishihara *et al.*, 1998).

The present study investigated the interaction between the two periodontopathogens *P. gingivalis* and *T. denticola* in an *in vitro* biofilm model. The aim of this study was first to devise a procedure to accurately quantitate *T. denticola* in a biofilm and second to identify the genes involved in mixed biofilms of *T. denticola* and *P. gingivalis* using a panel of *T. denticola* mutants. These results suggest that *T. denticola* requires several factors to form mixed biofilms with *P. gingivalis*.

METHODS

Culture media and conditions, strains and molecular methods. *T. denticola* (ATCC 35405) was grown in TYGVS medium (Arakawa & Kuramitsu, 1994) and *P. gingivalis* 381 was cultured in brain heart infusion (BHI) medium containing haemin ($10 \mu\text{g ml}^{-1}$) at 37°C in an anaerobic chamber (Forma Scientific) with an atmosphere of 85% nitrogen, 5% carbon dioxide and 10% hydrogen. *P. gingivalis* was grown for 1 day whereas *T. denticola* was cultured for 5 days. The *T. denticola* mutants were all constructed in the type strain ATCC 35405 by inactivating the particular gene using insertional inactivation. The genes inactivated were for the cytoplasmic filament, *cfpA* (this study), flagellar hook protein, *flgE* (Li *et al.*, 1996), methyl-accepting chemotaxis protein, *dmcA* (Kataoka *et al.*, 1997), major surface protein, *msp* (Fenno *et al.*, 1998), the leucine-rich repeat containing protein, *lrrA* (A. Ikegami & H. K. Kuramitsu, unpublished data) and polyphenylalanine-specific serine protease, *prtP* (Ishihara *et al.*, 1998).

Construction of the *cfpA* mutant in *T. denticola* 35405. A *T. denticola* 35405 *cfpA* mutant was constructed by electroporation using the DraDentErm 8 plasmid (unnamed in Izard *et al.*, 2001; supplied by J. Izard, New York State Department of Health, Albany, NY, USA) which contained a cloned *cfpA*-20H fragment from *T. denticola* 33520 disrupted at the *DraI* site by intergration of the ErmF/AM cassette (Izard *et al.*, 2001). Studies in our laboratory have shown that the Erm cassette does not produce polar mutations in *T. denticola* when cloned in the same direction as the inactivated gene. Briefly, $12 \mu\text{g}$ non-methylated plasmid DNA was used to electroporate *T. denticola* ATCC 35405 competent cells produced by growing 100 ml *T. denticola* to an OD_{600} of 0.3. The cells were washed three times with ice-cold 10% glycerol and resuspended in a final volume of 2 ml electroporation buffer. Electroporation was performed using $100 \mu\text{l}$ cells and $1 \mu\text{l}$ plasmid DNA in cuvettes with a 1 mm gap using a Bio-Rad Gene Pulser at 1.8 kV, 200Ω and $25 \mu\text{F}$ (Bio-Rad). The time constant was 4.3 ms. Immediately following electroporation 2 ml TYGVS was added followed by overnight anaerobic incubation at 37°C . Cells were then grown on TYGVS plates with SeaPlaque, 0.7% (w/v) (low-melting-point) agarose (Bio Whittaker) and $40 \mu\text{g}$ erythromycin ml^{-1} . After 7 days, colonies were visible and isolated. Disruption of the *cfpA* gene was confirmed using PCR with *cfpA* probes. Confirmation of mutant construction was carried out using PCR amplification outside the region of gene interruption using primers DENTCF4 (5'-AAATCGCTACCCTTCTTGATG-3') located in the *cfpA* sequence and DENTCF5 (5'-GCAGCCAAATCGTTAAAG-3') located outside the cloned sequence in the 3' end of the *cfpA* sequence. For PCR to confirm the relative position of the antibiotic resistance cassette we used DENTCF4 and ERMBGLF (Limberger *et al.*, 1999). An additional PCR to confirm the position of the antibiotic resistance cassette used ERMBGLR (Limberger *et al.*, 1999) and DENTCF5.

Quantification of *T. denticola* using SAAPNA assays. To accurately quantify the number of *T. denticola* attached to a surface or incorporated into a biofilm we needed to first establish a convenient and accurate procedure for quantification. In this regard the dentilisin activity of the oral spirochaete was utilized. The fluorogenic chymotrypsin substrate II, Suc-Ala-Ala-Pro-Phe-AMC (SAAPNA) (Calbiochem), was dissolved in 50 mM Tris/HCl, pH 8, to a concentration of 12.59 mM. For analysis, $12 \mu\text{l}$ SAAPNA substrate was added to $138 \mu\text{l}$ cell suspension in a 96-well microtitre plate and read in a Bio-Tek FLx 800 microplate fluorescence reader using excitation at 360 nm and emission at 460 nm. In each assay the SAAPNA activity of an aliquot of cells at an OD_{600} of 0.2 in PBS (10 mM potassium phosphate, 0.9% NaCl, pH 7.4) was measured to normalize the chymotrypsin activity of the mutants to *T. denticola* 35405. The results are presented as relative fluorescence units (r.f.u.).

Attachment of *T. denticola* to fibronectin. *T. denticola* was collected from a 5-day-old liquid culture by centrifugation ($10\,000 \text{g}$), washed once in PBS and adjusted to an OD_{600} of 0.2 (Haapasalo spectrophotometer) in PBS, corresponding to 5×10^8 cells ml^{-1} as determined with a Petroff-Hausser bacteria counter (Hausser & Son). Fibronectin (Sigma; $100 \mu\text{l}$) was used to coat polystyrene 96-well plates at a concentration of 1mg ml^{-1} for 12 h at 5°C . After washing three times with $200 \mu\text{l}$ PBS, $100 \mu\text{l}$ *T. denticola* ATCC 35405 (OD_{600} of 0.2 in PBS) was added, incubated and washed again. The number of attached cells was then determined using the SAAPNA assay outlined above.

Biofilm formation. An *in vitro* static biofilm model was used to quantify biofilm formation by *T. denticola* and *P. gingivalis*. A *P. gingivalis* biofilm was initially formed in 96-well polyvinylchloride (PVC) plates by inoculating 10 ml BHI+haemin with a $100 \mu\text{l}$ *P. gingivalis* 381 overnight starter culture and then aliquoting the culture at $100 \mu\text{l}$ per well. The plate was incubated at 37°C

anaerobically overnight. The following day planktonic *P. gingivalis* cells were removed by washing three times with 200 μ l PBS. The *P. gingivalis* biofilm was then incubated with 100 μ l *T. denticola* cells from an overnight culture resuspended in fresh TYGVS (OD₆₆₀ of 0.2).

Quantification of *T. denticola* using viability counts. The procedure outlined above for the production of an *in vitro* biofilm was followed with some modifications. Prior to the final washing step, a 25 μ l aliquot of planktonic cells was removed from the wells of the microtitre plate. Dilutions of the planktonic cells were made in PBS and spread onto TYGVS+low-melting-point SeaPlaque agarose (0.8% w/v) containing rifampicin (1 μ g ml⁻¹) selective medium. The wells were then washed three times with sterile PBS and resuspended with 100 μ l PBS. The biofilm was removed by mechanical disruption with a pipette tip and a homogeneous suspension was achieved by repeatedly pipetting PBS into the well containing the biofilm. Removal of the biofilm was evaluated by staining any residual biofilm with crystal violet dye. Dilutions of the biofilm were made in PBS and spread onto TYGVS+low-melting-point SeaPlaque agarose (0.8% w/v) containing rifampicin (1 μ g ml⁻¹) selective medium. The results were obtained in duplicate and the mean number of c.f.u. determined.

Scanning electron microscopy (SEM). Microscope glass cover-slips were incubated in the wells of 6-well polystyrene microtitre plates with a 1:10 dilution of a *P. gingivalis* 381 overnight culture in 2 ml TSB medium and incubated anaerobically at 37 °C for 8 h. The cover slips were then washed with 3 vols PBS and the preformed *P. gingivalis* biofilm was incubated with *T. denticola* 35045 resuspended in PBS to an OD₆₆₀ of 0.2 and incubated anaerobically at 37 °C for 1 h.

The mixed biofilm was prepared for SEM using the following protocol. The biofilm was washed three times with PBS and fixed using 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 30 min at room temperature. The biofilm was then washed three times for a total of 10 min with sodium cacodylate buffer, 0.2 M pH 7.4, followed by sequential dehydration in increasing concentrations of ethanol, 5 min in each (25, 50, 70, 90 and 100%). A final dehydration step in hexamethyl disilazane (HMDS) (Sigma) for 30 min was included, the biofilm was then coated with carbon and viewed using SEM (Hitachi S-4000 FESEM) operating at 15 kV.

RESULTS

Biofilm formation by *T. denticola*

Quantification of *T. denticola* using the SAAPNA protease assay was shown to be highly reproducible and representative of the number of *T. denticola* in culture (Fig. 1) and was therefore used as the method of choice for quantifying the number of bacteria attaching to solid surfaces. We did not demonstrate a difference in SAAPNA activity between biofilm cells and planktonic cells. The data for the *T. denticola* mutants was compared against ATCC 35405. Initially, attempts to demonstrate the formation of a *T. denticola* biofilm on a variety of inert surfaces (glass, polystyrene, PVC) were uniformly unsuccessful. Therefore, since earlier results (Dawson & Ellen, 1994) have demonstrated *T. denticola* attachment to fibronectin, this substrate was used in an attempt to form biofilms.

Attachment of *T. denticola* to fibronectin-coated plates and

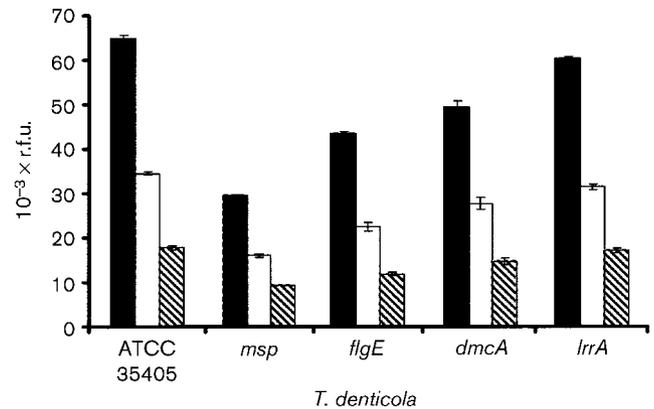


Fig. 1. Quantification of planktonic *T. denticola* at different cell densities using SAAPNA substrate. Black bars, OD₆₆₀ = 0.2; white bars, OD₆₆₀ = 0.1; hatched bars, OD₆₆₀ = 0.05.

to preformed *P. gingivalis* biofilms could be demonstrated by the oral spirochaete. *T. denticola* ATCC 35405 were first diluted to an OD₆₆₀ of 0.2, 0.1 and 0.05. Attachment to fibronectin at 37 °C for 3 h was quantified using the SAAPNA assay and gave r.f.u. values of 908 ± 125, 495 ± 7 and 231 ± 7.8, respectively. In a similar assay, attachment of *T. denticola* ATCC 35405 to a *P. gingivalis* monolayer was quantified. As before, *T. denticola* were first diluted to an OD₆₆₀ of 0.2, 0.1 and 0.05. Attachment after 4.5 h at 37 °C gave r.f.u. values of 17 807 ± 561, 9480 ± 694 and 4497 ± 426, respectively. When the number of *T. denticola* cells added was increased, the number of cells in the biofilms also increased proportionally. The main difference between the two surfaces was the magnitude of attachment. There was a 20-fold increase in attachment to *P. gingivalis* preformed biofilms compared to attachment to fibronectin. Control experiments demonstrated a very low background *P. gingivalis* SAAPNA activity which could be subtracted and did not affect the ability to quantify *T. denticola* using the enzyme assay (data not shown). *T. denticola* type strain ATCC 35405 was used throughout this study as it was found in preliminary experiments to have a significantly higher binding capacity to fibronectin and *P. gingivalis* than *T. denticola* ATCC 33520 (data not shown). Initial experiments looked at attachment during the early stages of the mixed biofilm which showed limited incorporation of *T. denticola* (data not shown).

Attachment of *T. denticola* to fibronectin was determined at three time points, 1, 2 and 3 h. When quantified using the SAAPNA substrate the biofilms gave r.f.u. values of 13 225 ± 2212, 10 611 ± 645 and 4288 ± 329, respectively. Analysis of the kinetics of biofilm formation indicated that *T. denticola* 35405 colonization of fibronectin-coated plates decreased over a 3 h period. This was undoubtedly the result of the degradation of fibronectin by dentilisin of strain 35405 (Ishihara *et al.*, 1996) as the opposite observation was made with the dentilisin mutant. In contrast, when

T. denticola was incubated with a preformed *P. gingivalis* biofilm the numbers of *T. denticola* recovered from the mixed biofilm after 5, 11.5 and 27 h gradually increased. When quantified using SAAPNA substrate, r.f.u. values of 1193 ± 188 , 4349 ± 435 and 8160 ± 633 , respectively, were obtained. Therefore, *T. denticola* biofilm formation on preformed *P. gingivalis* biofilms was examined in greater detail.

The number of *T. denticola* used in the biofilm studies was standardized to an OD₆₆₀ of 0.2. However, due to the highly sensitive SAAPNA substrate and detection system used there was a large variance in r.f.u. values on different days, largely due to variation in the fluorescence reader sensitivity setting. In contrast, quantification of *T. denticola* in biofilms run in triplicate and then quantified simultaneously in 96-well microtitre plates gave consistent results.

Biofilm formation by *T. denticola* mutants

To examine the genetic basis for initial *T. denticola* biofilm formation, the role of several potential colonization-dependent genes of the oral spirochaete in mixed biofilm formation was examined. We constructed a *T. denticola* 35405 *cfpA* mutant, PVUB7, which was confirmed by PCR to have a disrupted *cfpA* gene, resulting in reduced migration in TYGVS plates when compared to *T. denticola* 35405 (data not shown). These findings were consistent with the findings of Izard *et al.* (2001) and the corresponding *T. denticola* 33520 *cfpA* mutant. When the *cfpA* mutant was compared with parental strain 35405, it was observed that the mutant was attenuated in forming biofilms on fibronectin (70.1 % reduction) as well as *P. gingivalis*-coated surfaces (89.2 % reduction) when compared to ATCC 35405. Therefore, though localized cytoplasmically (Izard *et al.*, 2001), this filamentous protein affects biofilm formation. The *cfpA* mutant, and other *T. denticola* mutants tested, all displayed similar planktonic growth rates.

Chemotaxis, specifically involving the methyl-accepting chemotaxis protein DmcA, did not appear to play a significant role in the initial attachment of *T. denticola* into a mixed biofilm with *P. gingivalis* (Fig. 2). Recently, a novel leucine-rich repeat protein gene, *lrrA*, has been identified in our laboratory (A. Ikegami & H. K. Kuramitsu, unpublished data). This putative lipoprotein has been demonstrated to mediate interactions between strain 35405 and other oral bacteria, including *P. gingivalis*. A *T. denticola* *lrrA* mutant was constructed by insertional inactivation using a suicide plasmid with the cassette in the same orientation as the *lrrA* gene. However, only a slight reduction in the ability of the *lrrA* mutant to form a mixed biofilm was noted (Fig. 2). By contrast, the major surface protein of *T. denticola*, Msp, appears to play a major role in biofilm formation with *P. gingivalis* since the *msp* mutant was markedly attenuated in mixed biofilm formation (Fig. 2). Furthermore, the *flgE* mutant was also unable to initiate a biofilm (Fig. 2) with *P. gingivalis* and displayed reduced attachment to fibronectin (data not shown). These

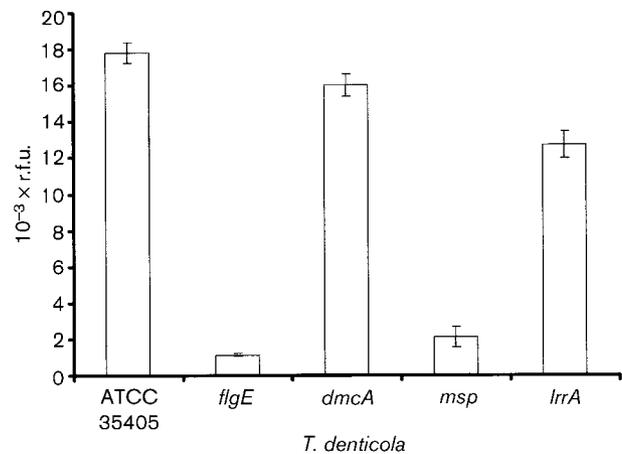


Fig. 2. Attachment of *T. denticola* 35405 to *P. gingivalis* biofilms compared to relevant mutants. Quantified using SAAPNA substrate after incubation for 4.5 h.

results suggest that in the *in vitro* mixed biofilm model the motility of *T. denticola* and its major outer sheath protein are both important factors in the formation of a mixed biofilm with *P. gingivalis*. Longer term biofilms with *T. denticola* mutants that are impaired in the initial stages of biofilm formation did not demonstrate an increased incorporation of *T. denticola* into the mature biofilm (data not shown).

Since it is not possible to quantify the *T. denticola* *prtP* mutant with SAAPNA assays, an alternative procedure was necessary to measure attachment of this mutant into biofilms. Viability counts were used to quantify the

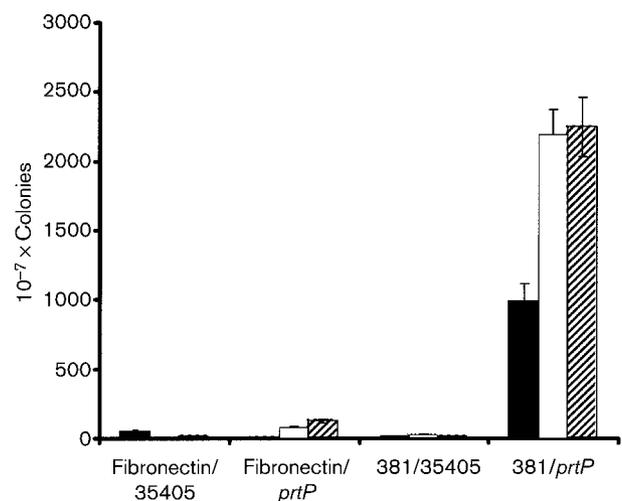


Fig. 3. Colonization of *T. denticola* to fibronectin and *P. gingivalis* 381 quantified using viability counts on TYGVS plates supplemented with rifampicin for *T. denticola* selection. Black bars, day 1; white bars, day 2; hatched bars, day 3.

prtP mutant in biofilm assays (Fig. 3). Interestingly, the *T. denticola prtP* mutant lacking the enzyme for prolyl-phenylalanine-specific protease activity showed increased numbers both on fibronectin-coated plates and in mixed biofilms with *P. gingivalis* when incubated over a prolonged period of 3 days (Fig. 3). The increased attachment of the *prtP* mutant K1 relative to strain 35405 to fibronectin is compatible with the degradation of the protein substrate.

Dawson & Ellen (1994) have examined the interaction of *T. denticola* with fibronectin-coated beads using SEM. Their data suggest that *T. denticola* may interact with fibronectin at its tip. To investigate if this is also the case with interactions with *P. gingivalis*, we examined a *T. denticola*-*P. gingivalis* mixed biofilm using SEM. The mixed biofilm was very dense, proving problematic when viewed with the electron microscope. Artifacts were apparent upon observation (data not shown) which most certainly occurred

during the dehydration of the biofilm causing the biofilm to crack and shrink. However, our data demonstrated some interesting observations in which individual *P. gingivalis* cells were observed, attached to the ends of each *T. denticola* cell (Fig. 4). This would also tend to suggest that *T. denticola* interacts not only with fibronectin, but also with *P. gingivalis*, in some instances, through the ends of the spirochaete.

DISCUSSION

The present results suggest that *T. denticola* biofilm formation *in vitro* can be conveniently monitored by assaying for the dentilisin activity of the organisms. Using this system it has been demonstrated that strain 35405 did not form detectable biofilms on inert surfaces (PVC, polystyrene, glass) commonly used to monitor this property in other micro-organisms (Pratt & Kolter, 1998). However,

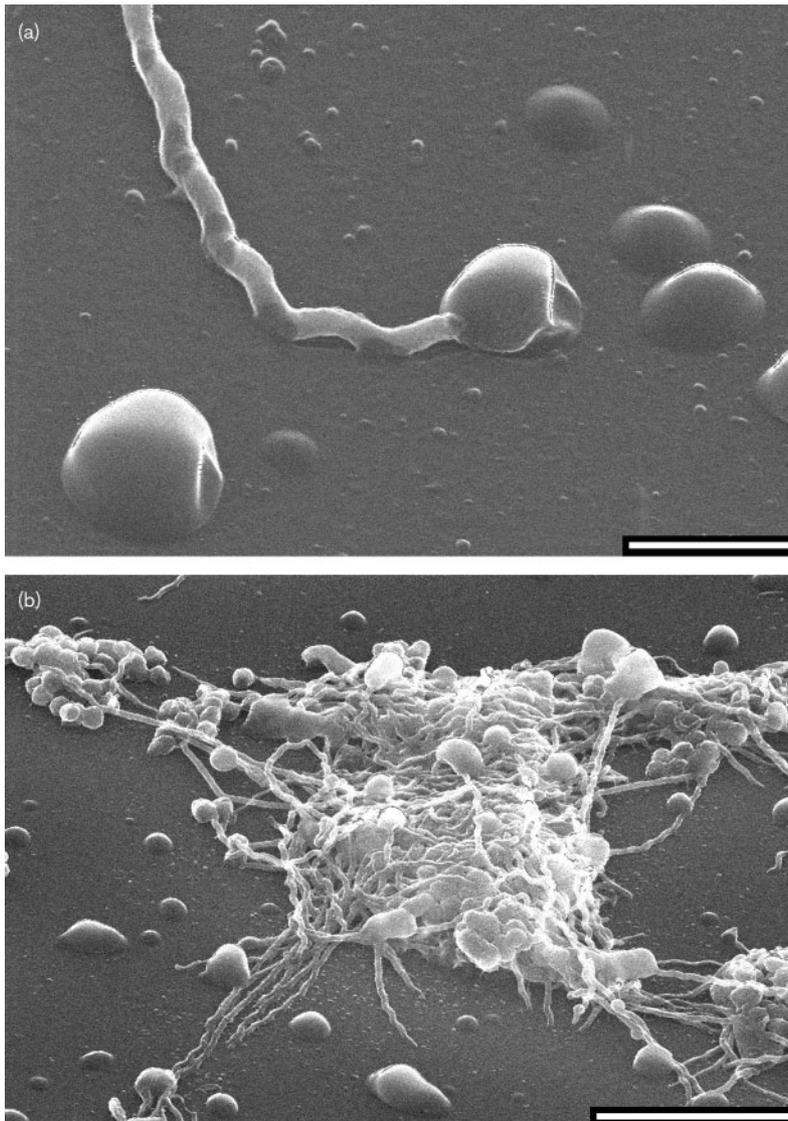


Fig. 4. SEM showing the interaction of *P. gingivalis* and *T. denticola* in a mixed biofilm. Bars, 1 (a) and 6 (b) μm .

strain 35405 was demonstrated to form detectable biofilms on preformed *P. gingivalis* and to a lesser extent on fibronectin-coated microtitre plates. Nevertheless, prolonged incubation of strain 35405 with the latter surface resulted in reduced colonization. Based upon a comparison of colonization by 35405 and its *prtP* mutant K1, it is likely that this reduction was the result of the degradation of the matrix protein by the strong dentilisin activity of the oral spirochaete. Interestingly, the K1 mutant also formed much more pronounced biofilms on *P. gingivalis* 381 compared to the wild-type organism. This may result from the surface alterations which were detected in the K1 mutant, leading to increased co-aggregation with other organisms (Ishihara *et al.*, 1998). Also, the protease has been demonstrated to be important in the organization of the protein components associated with the outer sheath of these organisms (Ishihara *et al.*, 1998). Since the interaction of *T. denticola* with *P. gingivalis* has not been defined at the molecular level, it is possible that multiple complex interactions are involved in the formation of the mixed biofilms. These interactions are currently under investigation in this laboratory.

The present results have also suggested the important role of several *T. denticola* genes in the formation of mixed biofilms with *P. gingivalis*. The cytoplasmic filament protein, CfpA, appears to play a key role in this process. Strain 35405 mutants which are defective in the *cfpA* gene are attenuated in colonizing preformed biofilms of *P. gingivalis* 381. The mutant appears to be also defective in spreading on agarose plates as observed for a similar mutant constructed in strain 33520 (Izard *et al.*, 2001). However, the motility of the *cfpA* mutant compared to parental strain 35405 did not appear to be altered when viewed under dark field microscopy. Whether or not the reduction in biofilm formation is related to the altered spreading property of the mutant in relatively viscous media still remains to be determined. Since spreading is considered to be an important factor in the development of biofilms (O'Toole *et al.*, 2000), this may be a reasonable explanation for this property of the mutant. Moreover, it is interesting that a cytoplasmic protein can affect the spreading properties of the organism in relatively viscous media. Whether or not this is a direct or indirect effect on this property is still uncertain.

The motility of *T. denticola* appears to play a role in mixed biofilm formation since the *flgE* mutant, which is non-motile (Li *et al.*, 1996), is markedly attenuated in colonizing preformed *P. gingivalis* biofilms. It may be that motility is required to overcome the repulsive forces present on the surface of *P. gingivalis* biofilms. However, we cannot rule out the possibility that some other defect in the *flgE* mutant is responsible for such alterations.

Although the methyl-accepting chemotaxis protein DmcA does not appear to play a role in forming mixed biofilms with *P. gingivalis*, the importance of chemotaxis cannot be ruled out as DmcA is not the only methyl-accepting

chemotaxis protein which exists in *T. denticola* (Kataoka *et al.*, 1997).

The Msp protein of strain 35405 also appears to play a major role in forming mixed biofilms with *P. gingivalis*. The *msp* mutant was markedly deficient in forming mixed biofilms in the static *in vitro* system. This is not surprising since this surface protein has been proposed to serve as a major adhesin of these organisms (Fenno *et al.*, 1996). Interestingly, since dentilisin was demonstrated to be involved in the organization of Msp into the outer sheath of *T. denticola* (Ishihara *et al.*, 1998), the *prtP* mutant was not attenuated in mixed biofilm formation as was the *msp* mutant. This suggests that the PrtP-mediated alteration of Msp maturation is not required for interaction with *P. gingivalis* in forming mixed biofilms.

Recently, a mutant defective in the LrrA leucine-rich repeat protein of *T. denticola* has been constructed and demonstrated to show increased co-aggregation with *P. gingivalis* 381 (A. Ikegami & H. K. Kuramitsu, unpublished data). Therefore, the present observation that the *lrrA* mutant was only moderately altered in forming mixed biofilms with *P. gingivalis* is compatible with this property.

The present results demonstrating that *T. denticola* can form mixed biofilms with preformed biofilms of *P. gingivalis in vitro* may be relevant to subgingival plaque formation by these periodontopathic organisms. An earlier *in situ* localization study of both organisms in subgingival plaque using monospecific antibodies has demonstrated that *T. denticola* is present in layers which appear to be exterior to *P. gingivalis* (Kigure *et al.*, 1995). This may suggest a sequential colonization of the subgingival region: attachment by *P. gingivalis* to other early colonizers followed by subsequent colonization by the oral spirochaete. However, since other organisms known to interact with both organisms are also present in subgingival plaque (i.e. *Fusobacterium nucleatum*) other interactions (Kolenbrander, 1988) may also play important roles in the incorporation of both organisms into subgingival plaque. Clearly, since dental plaque is composed of a variety of organisms, a multiplicity of interactions are likely to be involved in oral biofilm formation. Nevertheless, the present investigation suggests one of the interactions which could play an important role in the initial colonization of the gingival margin by *T. denticola*.

Future work in our laboratory is under way to develop an alternative method for studying the relationship between *P. gingivalis* and *T. denticola* using confocal scanning laser microscopy. Mixed biofilm formation in a continuous flow cell biofilm model will also be evaluated.

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