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Treponema denticola chymotrypsin-like proteinase (CTLP) integrates spirochaetes within oral microbial communities

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Treponema denticola is found ubiquitously in the human oral cavity and is mainly associated with bacterial communities implicated in the establishment and development of periodontal disease. The ability to become integrated within biofilm communities is crucial to the growth and survival of oral bacteria, and involves inter-bacterial coaggregation, metabolic cooperation, and synergy against host defences. In this article we show that the chymotrypsin-like proteinase (CTLP), found within a high-molecular-mass complex on the cell surface, mediates adherence of *T. denticola* to other potential periodontal pathogens, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Prevotella intermedia* and *Parvimonas micra*. Proteolytic activity per se did not appear to be required for the interactions, and expression of the major outer-sheath protein (Msp) was not necessary, except for binding *Parv. micra*. Biofilms of densely packed cells and matrix, up to 40 μm in depth, were formed between *T. denticola* and *P. gingivalis* on salivary pellicle, with *T. denticola* cells enriched in the upper layers. Expression of CTLP, but not Msp, was critical for dual-species biofilm formation with *P. gingivalis*. *T. denticola* did not form dual-species biofilms with any of the other three periodontal bacterial species under various conditions. Synergy between *T. denticola* and *P. gingivalis* was also shown by increased inhibition of blood clotting, which was CTLP-dependent. The results demonstrate the critical role of CTLP in interactions of *T. denticola* with other oral micro-organisms, leading to synergy in microbial community development and host tissue pathogenesis.

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INTRODUCTION

The human oral cavity harbours a diverse and complex microbiota (Paster *et al.*, 2001), with over 700 taxa now identified (Dewhirst *et al.*, 2010). Investigations of bacterial species detected in subgingival plaque from subjects affected by periodontitis have consistently revealed the presence of bacteria such as *Treponema denticola*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Fusobacterium nucleatum*, *Prevotella intermedia* and *Parvimonas micra* (Ali *et al.*, 1994; Socransky *et al.*, 1998; Mayanagi *et al.*, 2004). More recent studies have indicated that bacteria such as *Filifactor alocis* (Aruni *et al.*, 2011; Moffatt *et al.*, 2011) may also be associated with the development of periodontal disease. *In vitro* models of infection suggest that synergistic interactions between these periodontal bacteria can be related to severity of disease (Kesavalu *et al.*, 2007; Bakthavatchalu *et al.*, 2011; Orth *et al.*, 2011). Therefore the abilities of micro-organisms to interact with one another are probably crucial to progression of periodontal disease. However, the molecular basis of such interactions is only just beginning to be understood.

Spirochaetes are detected in most samples of subgingival plaque microbiota taken from subjects with a range of periodontal conditions (Paster *et al.*, 2001; Colombo *et al.*, 2009; Visser & Ellen, 2011). *T. denticola* has been the most frequently isolated and best-characterized oral spirochaete (Moore *et al.*, 1982; Riviere *et al.*, 1992), but it is evident that multiple phylotypes are found in individual subjects (Dewhirst *et al.*, 2000). A number of virulence factors have been described for *T. denticola* (Dashper *et al.*, 2011). One of these is a surface-localized prolyl-phenylalanine-specific chymotrypsin-like protease (CTLP) with a broad repertoire

Abbreviations: CTLP, chymotrypsin-like protease; HRP, horseradish peroxidase; SAAPFNA, *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide; TCT, thrombin clotting time.

Three supplementary figures are available with the online version of this paper.

of cytopathic activities (Fenno *et al.*, 1998b; Ellen *et al.*, 2000). The CTLP, also designated dentilisin (Ishihara *et al.*, 1996), has been reported to degrade a range of host proteins (Bamford *et al.*, 2007; McDowell *et al.*, 2009), hydrolyse bioactive peptides (Mäkinen *et al.*, 1995; Miyamoto *et al.*, 2006) and help spirochaetes penetrate epithelial cell monolayers (Fenno *et al.*, 1998b; Ellen *et al.*, 2000; Chi *et al.*, 2003). Formation of the CTLP functional complex on the cell surface requires expression of three genes, *prcB*, *prcA* and *prtP* (Godovikova *et al.*, 2010). PrcB is a lipoprotein that interacts with PrtP pre-proteinase and is required for enzymic activity. The CTLP complex on the surface of *T. denticola* comprises PrtP, PrcA1 and PrcA2 (processed forms of PrcA), PrcB and Msp (major outer-sheath protein) (Godovikova *et al.*, 2011).

T. denticola is almost always found in association with other oral micro-organisms, including recognized periodontal pathogens (Faveri *et al.*, 2009; Söder *et al.*, 1993). *T. denticola* has been shown to coaggregate with a number of these micro-organisms (Grenier, 1992; Onagawa *et al.*, 1994; Yao *et al.*, 1996; Kolenbrander *et al.*, 1995) and, in particular, there seems to be a strong link between *P. gingivalis* and *T. denticola* in subgingival plaque samples (Pederson *et al.*, 1994; Simonson *et al.*, 1992). It has been suggested that the occurrence of *T. denticola* at a site of periodontitis might in fact depend upon the presence of *P. gingivalis* (Simonson *et al.*, 1992). Furthermore, immunohistochemical techniques have shown that *P. gingivalis* is localized beneath *T. denticola* in human subgingival plaque, while in deeper subgingival plaque their coexistence has been observed (Kigure *et al.*, 1995). These various observations have been reproduced in dual-species biofilm experiments, which show that *P. gingivalis* acts as an initial colonizer of the substratum, with subsequent incorporation of *T. denticola* (Yamada *et al.*, 2005). Interactions between these bacteria have been suggested to be mediated by *P. gingivalis* fimbriae recognizing dentilisin (Hashimoto *et al.*, 2003) or by gingipains (Ito *et al.*, 2010), and to involve the major surface protein (Msp) of *T. denticola* (Vesey & Kuramitsu, 2004; Rosen *et al.*, 2008). However, there are still many discrepancies between findings, and the critical processes of adherence and microbial community development are not fully understood. Since *P. gingivalis* produces several proteinases, e.g. RgpA (arginine-specific gingipain) and Kgp (lysine-specific gingipain), which degrade extracellular matrix (ECM) proteins and dysregulate host immune functions (O'Brien-Simpson *et al.*, 2003; McAlister *et al.*, 2009), it is envisaged that the concerted activities of *P. gingivalis* and *T. denticola* proteinases are highly disruptive to host tissues.

The aims of this study were to investigate the ability of *T. denticola* to interact with several species of periodontal bacteria that have been found associated with treponemes in clinical infection (Siqueira & Rôças, 2009; Joshi & Vandana, 2007), and to determine the role of CTLP in the interactive processes. The results show that CTLP expression is critical for interactions of *T. denticola* with a range of oral micro-organisms, and is essential for biofilm formation with *P. gingivalis*. The identification of a pivotal factor, such as CTLP, that is important for a range of pathogenic properties, including microbial community development, establishes this as a potential target to control destructive periodontal disease.

METHODS

Bacterial strains and growth conditions. *Treponema* strains used in this study were *T. denticola* ATCC 35405 wild-type, isogenic mutant strains CKE Δ*prcAprtP* (CTLP-negative) (Fenno *et al.*, 1998a) and MHE Δ*msp* (Msp-negative) (Fenno *et al.*, 1998a), and *Treponema vincentii* ATCC 35580. Treponemes were grown and maintained in TYGVS medium (Ohta *et al.*, 1986) or in OBGM medium (Orth *et al.*, 2010) at 37 °C under an atmosphere of $N_2/CO_2/H_2$ (8 : 1 : 1) in a Don Whitley Mk3 anaerobic workstation. *P. gingivalis* ATCC 33277, *F. nucleatum* subsp. *vincentii* ATCC 49256, *Prev. intermedia* ATCC 25261 and *Parv. micra* ATCC 33270 were grown and maintained in Fastidious Anaerobe Broth (FAB; Oxoid) anaerobically at 37 °C, as above.

Chymotrypsin-like activity. Enzymic activities of *Treponema* strains were tested by hydrolysis assay of the chymotrypsin substrate *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (SAAPFNA) (Ohta *et al.*, 1986). Exponential phase *Treponema* cells were collected by centrifugation (10 000 ***g***, 10 min), washed three times by suspension in PBS followed by centrifugation, and suspended in PBS at OD_{600} 0.5 (1.2×10^9 cells ml^{-1}). Cell suspensions were incubated with substrate solution (1.0 mM SAAPFNA in 0.5 M Tris/HCl, pH 7.9, containing 2 mM DTT) for 1 h at 37 °C (Bamford *et al.*, 2007). Bacteria were sedimented by centrifugation and the A_{405} of the supernatant was measured.

Outer-membrane protein extraction and zymography. Gelatin zymography was used to identify proteolytic activities associated with outer-membrane proteins of *Treponema*. Outer-membrane proteins were extracted from *Treponema* cells as described previously (Edwards *et al.*, 2003). Briefly, exponential phase bacterial cells were harvested by centrifugation, washed twice with TE buffer (50 mM Tris/HCl, pH 7.5, containing 1 mM EDTA), suspended at OD_{600} 2.0 in TE buffer containing 1 % Triton X-114, and incubated for 16 h at 4 °C with end-over-end mixing. Samples were centrifuged (21 000 ***g***, 1 h, 4 °C) to sediment debris, and portions of supernatants were subjected to SDS-PAGE through gels containing 0.25 mg gelatin ml^{-1} for zymography (Bamford *et al.*, 2007). Following electrophoresis, the gel was washed extensively in 50 mM Tris/HCl, pH 7.4, to remove SDS and then incubated in 50 mM Tris/HCl, pH 7.4, containing 10 mM $CaCl_2$ at 37 °C for 16 h with gentle agitation to allow proteolysis. The gel was then stained with Coomassie brilliant blue and destained, such that transparent protease active bands appeared within a blue background.

Adherence of *Treponema* to oral bacteria. Exponential phase *Treponema* cells in PBS at OD_{600} 0.2 were biotinylated using Ez-Link Sulfo-NHS-LC-Biotin (5 μg ml^{-1}, Pierce), as described previously (Edwards *et al.*, 2003). Exponential phase cultures of *P. gingivalis*, *F. nucleatum* subsp. *vincentii*, *Prev. intermedia* and *Parv. micra* were centrifuged, cells were washed three times with PBS/centrifugation, and suspended at OD_{600} 0.5. Serial twofold dilutions of bacterial cells were applied to flat-bottomed wells of a 96-well microtitre plate (50 μl per well), the plates were centrifuged (1500 r.p.m., 5 min, 4 °C) to sediment the cells, and the cells were fixed with 0.25 % glutaraldehyde (30 min, 22 °C). The fluid was aspirated from the wells and remaining plastic binding sites were blocked with 5 % BSA

in PBS (16 h, 4 °C). Wells were washed once with PBST (PBS containing 0.1 % Tween 20), and then *Treponema* biotinylated cell suspension (50 µl) was added to wells in triplicate and incubated for 2 h at 22 °C. In some experiments biotinylated cells were incubated with the protease inhibitor PMSF (0.1 mM, 37 °C, 30 min) before the adherence assay. After incubation, bound *Treponema* cells were detected by adding horseradish peroxidase (HRP)–streptavidin (BD Bioscience Pharmingen) diluted 1 : 1000 in PBSTB (PBST containing 0.1 % BSA) as described previously (Edwards *et al.*, 2003). A_{490} values were proportional to cell numbers, for each batch of biotinylated cells prepared.

Coaggregation assay. Mid-exponential phase *Treponema* cells in PBS were suspended in FITC solution (7.3 mg FITC dissolved in 12.5 ml 0.05 M Na_2CO_3 and 0.1 M NaCl) and incubated for 30 min with gentle rocking to fluorescently label the bacteria. Cells were collected by centrifugation, washed three times in PBS, and suspended at OD_{600} 0.5. The coaggregating partner bacteria were harvested from mid-exponential phase cultures and washed twice with PBS, and the OD_{600} was adjusted to 0.5. Equal volumes of labelled *Treponema* and unlabelled bacterial cell suspensions were then end-over-end mixed in a sealed glass tube for 30 min in the dark. Coaggregation was scored on the basis of visual assessment, where (++) indicated formation of clumps that settled rapidly, (+) indicated granular appearance with slow settling, and (−) indicated no obvious coaggregation. Portions of the cell suspensions were air-dried onto a glass microscope slide, fixed with ethanol/acetone (50 : 50), and covered with 0.5 mM propidium iodide for 30 min in the dark. After two rinses in water, the bacteria were examined by fluorescence microscopy using a Leica DMLB microscope.

Biofilm analysis. Monospecies biofilms of *P. gingivalis*, *F. nucleatum* subsp. *vincentii*, *Prev. intermedia* and *Parv. micra* were grown in FAB medium. Dual-species biofilms of *T. denticola* and *P. gingivalis* were grown in OBGM medium. All biofilms were prepared as follows: mid-exponential phase cells at OD_{600} 1.0 in appropriate medium (0.1 ml) were added to 0.8 or 0.9 ml fresh medium in wells of a 12-well plate containing 19 mm diameter sterile glass coverslips that had been previously coated with 10 % human saliva in water (16 h at 4 °C). Human saliva was collected from up to eight subjects (who provided informed consent) in accordance with approval from the Faculty Ethics Committee. Biofilms were grown for up to 4 days under anaerobic conditions. The planktonic cell suspensions were removed at 24 h intervals and replaced with fresh medium. Coverslips were recovered at intervals for microscopic and biomass analyses by aspirating the planktonic bacteria from the well, removing the coverslips and washing them carefully with PBS. Biofilms were fixed with 25 % formaldehyde (30 min), washed with PBS and stained with FITC as described above. Coverslips were inverted onto Vectashield on a glass microscope slide and visualized with a fluorescence microscope (Leica DMLB). Images were captured using CellD imaging software. Biofilms were also observed using a ×63 oil immersion objective with a Leica TCS SP2 confocal imaging system attached to a Leica DMIRBE inverted microscope with a 488 nm excitation wavelength to excite FITC. *Z*-slices were obtained every 0.5–1.0 µm. Data were analysed using Volocity image analysis software (Improvision). To estimate biomass, biofilms on coverslips were stained with 0.5 % crystal violet solution for 15 min, excess stain was removed by washing with distilled H_2O, and then the crystal violet was released with 10 % acetic acid (0.4 ml). A_{595} is proportional to biofilm biomass (Maddocks *et al.*, 2011).

Thrombin clotting time (TCT). Human blood from a donor was mixed with 3.2 % sodium citrate (9 : 1 ratio) and centrifuged (150 ***g***, 10 min, 20 °C). The supernatant or platelet-rich plasma (PRP) was diluted 1 : 1 in 0.9 % saline (100 µl), mixed with 0.1 ml Owren's (sodium barbital) buffered saline, pH 7.35, and warmed (37 °C, 5 min). Human thrombin (5 U ml^{-1}) (Sigma) in Owren's buffered saline (0.1 ml) with 10 mg BSA ml^{-1} was added, and the time taken to produce a clot was recorded (Bamford *et al.*, 2007). To investigate the effects of mixtures of bacteria on TCT, exponential phase cells of each species were suspended in PBS at OD_{600} 0.5. Bacterial cell suspensions, either individually or in combination (0.1 ml), were mixed with 50 µl Owren's buffered saline and incubated with diluted fresh frozen plasma (0.1 ml) for 5 min at 37 °C. Thrombin was added immediately, and the time taken to form a first visible clot was recorded. Assays were carried out in triplicate and mean TCTs were calculated ± SD (n=3).

Statistical analyses. All data are reported as mean ± SD, unless indicated otherwise. Significance between samples was determined using the paired two-tailed Student's t test, with a value of $P<0.01$ accepted as indicating significance. Data were analysed with GraphPad Prism v5 software.

RESULTS

CTLP activities of *Treponema* strains

The proteolytic activity of the CTLP complex of *T. denticola* can be conveniently measured using the substrate SAAPFNA (Uitto *et al.*, 1988). Accordingly, chymotrypsin-like activities associated with *T. denticola* ATCC 35405, *T. denticola* MHE, *T. denticola* CKE and *T. vincentii* ATCC 35580 were assessed. High levels of chymotryspin-like activity were detected in *T. denticola* ATCC 35405 and *T. denticola* MHE (Fig. 1a), whereas no chymotryspin-like activity was detected in *T. denticola* CKE and *T. vincentii* ATCC 35580. Confirmatory results were obtained when the abilities of these strains to degrade gelatin were investigated by gelatin zymography. Zymographic analyses revealed that the approximately 95 kDa protein band in *T. denticola* ATCC 35405 extracts, corresponding to the CTLP complex (Bamford *et al.*, 2007), had strong gelatinolytic activity (Fig. 1b). This activity was present in extracts of mutant MHE (Msp^-) but absent in extracts from strain CKE ($CTLP^-$), and from *T. vincentii* ATCC 35580 (Fig. 1b).

Coaggregation of *Treponema* with periodontopathogens

Since associations between bacteria are believed to be crucial to the establishment of functional communities (Kolenbrander *et al.*, 2006), the ability of *T. denticola* to interact with *P. gingivalis*, *F. nucleatum* subsp. *vincentii*, *Prev. intermedia* and *Parv. micra* was determined. To visualize coaggregates by microscopy, *Treponema* bacteria were fluorescently stained with FITC (green) and the partner bacteria were stained with propidium iodide (red) (Supplementary Fig. S1). *T. denticola* ATCC 35405 was found to coaggregate strongly with *P. gingivalis* ATCC 33277 and *F. nucleatum* subsp. *vincentii* ATCC 49256, whereas it failed to coaggregate with *Prev. intermedia* ATCC 25261 (Table 1). In addition *Parv. micra* ATCC 33270, a Gram-positive anaerobic coccus frequently detected in primary root canal infection (Sundqvist, 1992), coaggregated with *T. denticola* (Table 1). *T. denticola* CKE and MHE mutants

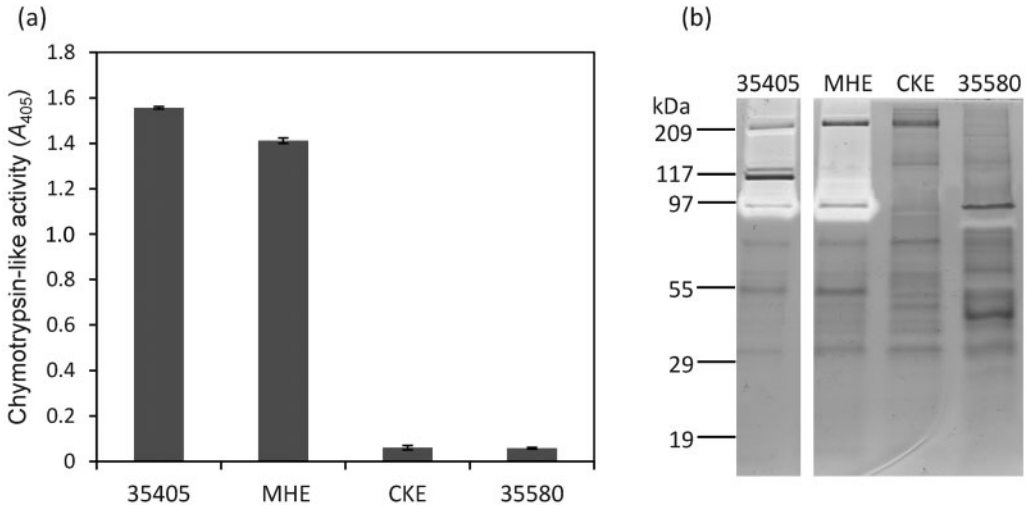

Fig. 1. Activity and electrophoretic profiles of CTLP. (a) SAAPFNA hydrolysis by *T. denticola* and *T. vincentii* cell suspensions was detected spectrophotometrically at 405 nm, as described in Methods. Error bars, SD of the mean calculated from triplicate experiments repeated three times. (b) SDS-PAGE of unheated outer-membrane proteins, extracted with Triton X-114 from *T. denticola* and *T. vincentii*, to detect gelatin degradation (zone of clearing) by zymography. Note that proteolysis occurs during the electrophoresis process to cause trailing of gelatinolytic activity. The positions of molecular mass standards (kDa) are indicated.

showed no significant differences in abilities to coaggregate compared with parental *T. denticola* wild-type (Table 1). This suggested that CTLP and Msp were not necessary for fluid-phase (planktonic) coaggregation. *T. vincentii* showed a different coaggregation profile from that of *T. denticola* ATCC 35405. Cells of *T. vincentii* did not coaggregate with those *Prev. intermedia*, *Parv. micra* or *P. gingivalis* (Table 1), but were able to coaggregate with *F. nucleatum* (Table 1), as has been shown elsewhere (Kolenbrander *et al.*, 1995).

Coadhesion of *T. denticola*

To quantify their abilities to adhere to selected oral bacteria, *Treponema* cells were biotinylated and then assayed for coadherence to immobilized bacteria, with binding detected by HRP–streptavidin (Edwards *et al.*, 2003). These assays were all saturable (Supplementary Fig. S2), and *T. denticola* cells adhered to each bacterial species tested. Knockout of *msp* resulted in reduced attachment levels to *Prev. intermedia* and *Parv. micra* (Fig. 2). However, the Msp mutant was not affected in binding to *F. nucleatum*. Adhesion levels of *T. denticola* CKE to *P. gingivalis*, *F. nucleatum*, *Parv. micra* and *Prev. intermedia* were all greatly reduced (by 50 % or more) compared with wild-type, implying that CTLP plays an important role in the adherence process (Fig. 2). However, adherence was not ablated, suggesting other contributory factors. *T. vincentii*, which contains an incomplete CTLP gene locus and does not

Table 1. Coaggregation of oral *Treponema* species with other periodontopathogenic bacteria

Treponema* strain	**Periodontopathogen coaggregation score†**			
	***P. gingivalis* ATCC 33277**	***F. nucleatum* ATCC 49256**	***Prev. intermedia* ATCC 25261**	***Parv. micra* ATCC 33270**
T. denticola ATCC 35405	++	++	−	+
T. denticola CKE	++	++	−	+
T. denticola MHE	++	++	−	+
T. vincentii ATCC 35580	−	+	−	−

**Treponema* strains were grown in TYGVS medium and the other bacteria in FAB medium.

†Coaggregation was assessed by microscopic visualization after 30 min incubation. ++, Strong coaggregation with large clumps that settled quickly; +, weak coaggregation with granular appearance; −, no obvious coaggregation. See Supplementary Fig. S1 for supporting visual data.

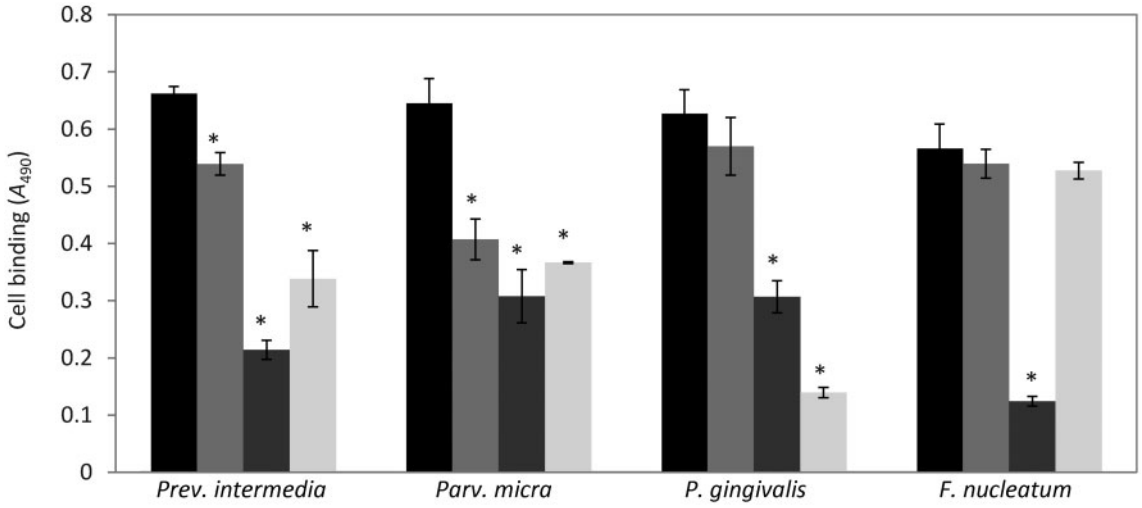

Fig. 2. Adherence levels of *Treponema* to other periodontopathogens. The input cell number was 1.2×10^7 cells per well of *T. denticola* ATCC 35405 (black bars), *T. denticola* MHE (mid-grey bars), *T. denticola* CKE (dark-grey bars) and *T. vincentii* ATCC 35580 (light-grey bars). Asterisks denote significant differences ($P<0.01$) in adhesion levels between *T. denticola* CKE and *T. denticola* wild-type. Error bars, SD of the mean calculated from triplicate experiments repeated three times.

express chymotrypsin-like activity, adhered strongly to *F. nucleatum*, weakly to *Prev. intermedia* and *Parv. micra*, and failed to adhere to *P. gingivalis* (Fig. 2).

Adherence does not depend upon CTLP activity

Since CTLP expression was found to be necessary for coadhesion, we investigated whether enzymic activity per se was required for the adherence process. Accordingly, biotinylated *T. denticola* cells were pre-incubated with PMSF before measuring adherence to immobilized *P. gingivalis*. PMSF treatment led to >95% inactivation of proteolytic activity, but had no effect on adherence of *T. denticola* to *P. gingivalis*, suggesting that the catalytic activity of the protease was not required in the adherence process (Fig. 3). Similarly, PMSF treatment of *T. denticola* cells did not affect their adherence levels to *Prev. intermedia*, *Parv. micra* and *F. nucleatum* (data not shown).

Biofilm formation

It was then of relevance to the establishment of subgingival communities to investigate the ability of *T. denticola* to form biofilms in the presence or absence of coadhering bacteria. *P. gingivalis*, *Prev. intermedia* and *F. nucleatum*, but not *Parv. micra*, grown in FAB medium each formed a dense 72 h biofilm on saliva-coated surfaces (Supplementary Fig. S3). *T. denticola* grows poorly in FAB and was unable to form a biofilm. Although these bacterial species could all be cultivated in OBGM medium, only *P. gingivalis*

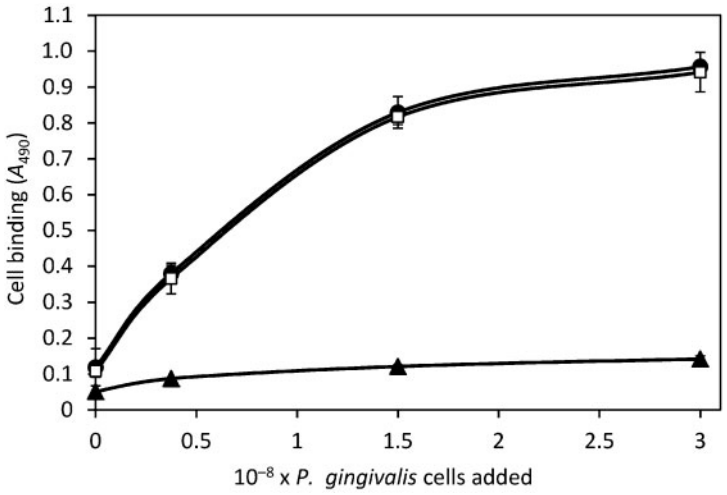

Fig. 3. Effect of the protease inhibitor PMSF on adhesion. Biotinylated *T. denticola* cells were incubated with 0.1 mM PMSF for 30 min at 37 °C (□), or left untreated (●), and then assayed for adhesion to glutaraldehyde-fixed *P. gingivalis* ATCC 33277 cells. Bound cells were detected with HRP–streptavidin. Control, no cells (▲). Error bars, SD of the mean calculated from triplicates of a representative experiment repeated three times.

showed a propensity to form a biofilm (data not shown). We were therefore unable to establish medium conditions under which all organisms grew individually and formed biofilms.

Investigation of dual-species biofilm formation in OBGM medium led to the finding that only *Treponema* spp. and *P. gingivalis* were able to form a mixed-species biofilm on saliva-coated coverslips. Similar biofilms were formed on coverslips coated with fibrinogen (data not shown), which is a major substrate for CTLP (Bamford *et al.*, 2007). Significantly greater biofilm biomass was formed by the dual-species biofilm than by the monoculture biofilm of *P. gingivalis* in OBGM medium (Fig. 4a, b), confirming a synergistic effect in biofilm formation between these two organisms. On the other hand, *T. denticola* CKE ($CTLP^-$) was abrogated in the ability to form dual-species biofilms with *P. gingivalis* (Fig. 4a, b). To rule out the possibility that this was related to aberrant expression or oligomerization of Msp, the ability of *T. denticola* MHE (*msp*) mutant to form biofilms was also tested. However, *T. denticola* MHE was apparently unaffected in ability to form dual-species biofilms with *P. gingivalis* (Fig. 4a, b). Thus CTLP, but not Msp, appeared to be essential for *T. denticola* biofilm formation with *P. gingivalis*.

Imaging of *Treponema*/*P. gingivalis* biofilms

Since fluorescence microscopy analyses did not provide data on biofilm architecture, mature biofilms (96 h) were grown upon saliva-coated coverslips and examined in more detail by CLSM. Wild-type *T. denticola* ATCC 35405 formed a dense biofilm when co-cultured with *P. gingivalis* ATCC 33277 (Fig. 5a, b), while strain CKE did not (Fig. 5c, d). The dual-species biofilms of wild-type *T. denticola* and *P. gingivalis* ATCC 33277 showed almost uninterrupted coverage of the salivary pellicle substratum, with an overall thickness in the range 30–35 μm (Fig. 5b). The biofilms were of uneven height, and what appeared to be pillars of dense microbial growth existed within these biofilms. These CLSM images suggested that *P. gingivalis* cells grew mainly within the deeper layers adjacent to the substratum (Fig. 6a, b), with spirochaetes predominant in the outer layers (Fig. 6c). The pillars comprised mainly *P. gingivalis* cells, on top of which multiple layers of *Treponema* cells attached.

TCT

It has been hypothesized that CTLP plays an important role in interference with the blood coagulation cascade (Bamford *et al.*, 2007). Since *T. denticola* and *P. gingivalis* appeared to have synergy in biofilm formation, we examined the dual effects of these bacteria on TCT. Addition of *T. denticola* ATCC 35405 cells led to an increased TCT, whereas mutant CKE or *T. vincentii* ATCC 35580 cells did not affect the time taken to induce clot formation (Fig. 7). When treponemes were incubated with fresh human plasma, in combination with equal numbers of *P. gingivalis* ATCC 33277 cells, TCTs were found to be significantly ($P<0.01$) greater than those observed with each individual species (Fig. 7), and were greatest only when CTLP was expressed. To rule out that the effects resulted simply from greater numbers of bacteria being present in the dual-species experiments, controls were included with twice the numbers of *T. denticola* or *P. gingivalis* ATCC 33277 cells, but the TCTs for the individual species were not increased. This implied that the increased dual-species effect on TCT was synergistic as opposed to related to bacterial load.

DISCUSSION

T. denticola is thought to be an important periodontal pathogen, based upon observations that it is frequently detected in active periodontal lesions (Mineoka *et al.*, 2008), and has been defined as a component of the so-called red complex that includes *P. gingivalis* and *T. forsythia*. In PCR-based methods of bacterial detection, *T. denticola* and other *Treponema* phylotypes are almost always isolated from infected root canals (Montagner *et al.*, 2010) and periodontal pockets (Byrne *et al.*, 2009), and within a single pocket there may be multiple phylotypes (Choi *et al.*, 1994; Dewhirst *et al.*, 2000). An important question that is addressed in this paper concerns the factors that enable *T. denticola* to be competitive and to survive in the mixed periodontal communities of micro-organisms. Interactions between plaque micro-organisms are associated with the development of efficient and robust communities (Jenkinson, 2011). A wide spectrum of coaggregation and coadhesion reactions has been characterized (Kolenbrander *et al.*, 2002, 2010), and some of these are now clearly identified as being metabolically driven (Periasamy *et al.*, 2009). Synergistic interactions between oral micro-organisms have been suggested to contribute to disease progression in models of periodontal disease (Kesavalu *et al.*, 2007; Orth *et al.*, 2011; Bakthavatchalu *et al.*, 2011), and synergism in proteolysis by *T. denticola* and *P. gingivalis* to promote bleeding is implicated by data presented in this article.

Major surface proteins on *T. denticola* include CTLP and Msp, which are known virulence factors (Dashper *et al.*, 2011), and were potential candidates for mediating interactions with other bacteria. The CTLP knockout mutant showed >50 % reduction in adherence levels to all of the bacteria tested, suggesting some common functional features of the CTLP complex in mediating adherence. Although expression of Msp is affected in this mutant (Bamford *et al.*, 2007), the Msp knockout, which still expresses CTLP, was unaffected in adherence under the conditions employed. This is in contrast to earlier work suggesting that Msp (not CTLP) is necessary for *T. denticola* adherence to *P. gingivalis* 381 and incorporation into a biofilm (Vesey & Kuramitsu, 2004). These divergent findings could be related to the different strains of *P.*

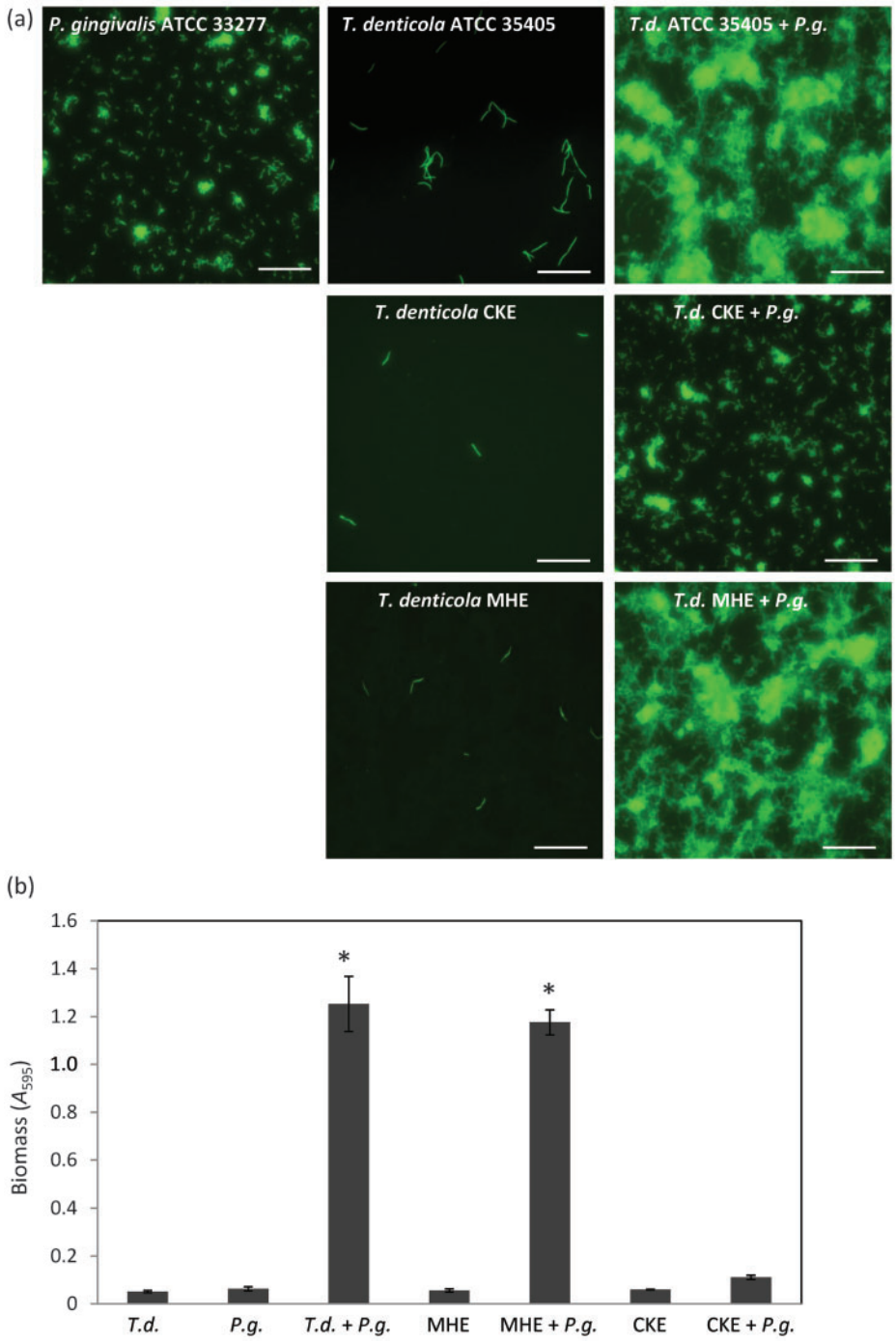

Fig. 4. Fluorescence microscopy of monospecies or dual-species biofilms of *T. denticola* ATCC 35405, *T. denticola* CKE or *T. denticola* MHE, and *P. gingivalis* ATCC 33277, and corresponding biomass data. (a) Biofilms formed upon saliva-coated coverslips after 72 h in OBGM medium were FITC-stained and visualized by fluorescence microscopy as described in Methods. Images are representative of experiments performed in triplicate. Bars, 25 μm. (b) Bacterial strains were grown in OBGM medium, and biofilms were formed upon saliva-coated coverslips for 72 h as described in Methods. Biofilm biomass was quantified by crystal violet staining assay at A_{595}. Asterisks denote significant differences ($P<0.01$) for dual-species biofilms compared with single species. Error bars, SD of the mean calculated from triplicate experiments repeated three times. *T.d.*, *T. denticola*; *P.g.*, *P. gingivalis*.

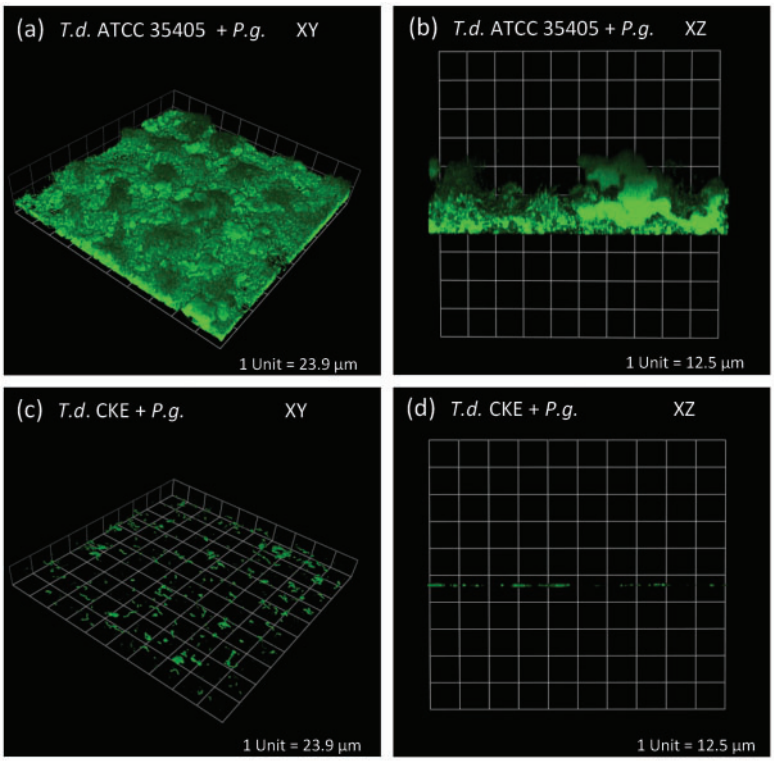

Fig. 5. Role of the CTLP complex in the formation of *T. denticola*/*P. gingivalis* dual-species biofilms. Representative CLSM images of dual-species biofilms of (a, b) *T. denticola* ATCC 35405 and (c, d) *T. denticola* CKE, with *P. gingivalis* ATCC 33277. Biofilms were formed upon saliva-coated coverslips for 96 h, with an OBGM medium change every 24 h, as described in Methods. Biofilms were fixed with 25 % formaldehyde before being stained with FITC and visualized by CLSM. The data obtained were analysed using Volocity image analysis software (Improvision). *T.d.*, *T. denticola*; *P.g.*, *P. gingivalis*.

gingivalis utilized or to the medium conditions employed. In earlier work the cytoplasmic filament was also identified as being necessary for adherence to *P. gingivalis*. The Msp was shown in other studies to act as an adhesin in coaggregation of *T. denticola* ATCC 35404 and *F. nucleatum* (Rosen *et al.*, 2008). However, in our studies of *T. denticola* ATCC 35405, the Msp knockout mutant was unaffected in binding *F. nucleatum*.

The results presented strongly suggest therefore that CTLP is necessary for adherence of *T. denticola* to at least four other species of oral bacteria that are found subgingivally. This would be important for incorporation of *T. denticola* into periodontal bacterial communities. Furthermore, the adherence properties of *T. denticola* to *P. gingivalis* were unaffected by the serine protease inhibitor PMSF, which is an effective enzymic function inhibitor for intact cells

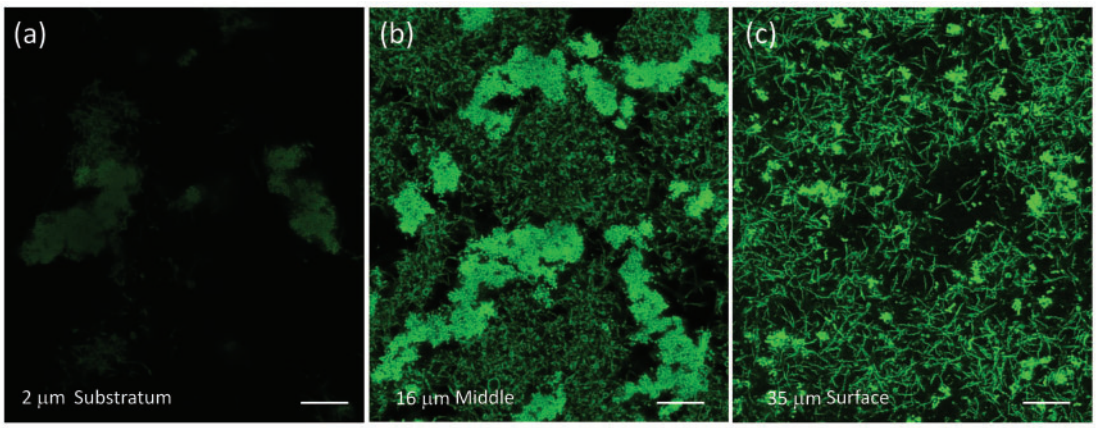

Fig. 6. Representative CLSM images of *T. denticola* and *P. gingivalis* dual-species biofilms formed on saliva-coated coverslips (72 h). The images show that *P. gingivalis* cells are mainly localized attached to the substratum [(a); depth, 2 µm], whereas *T. denticola* cells are predominant in the outer layers of the biofilm [(c); depth, 35 µm]. Within the middle region [(b); depth, 16 µm], the two bacterial species coexist. Bars, 25 µm (a, b) and 10 µm (c).

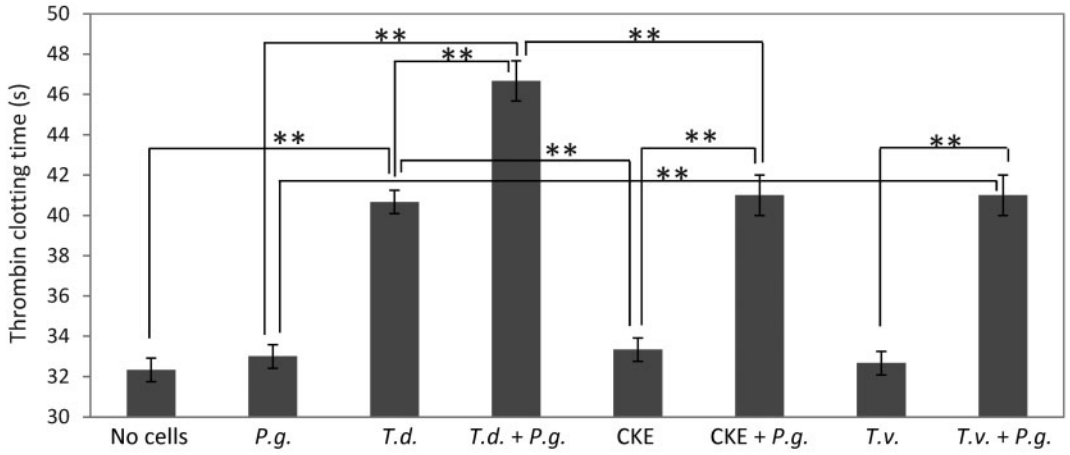

Fig. 7. Effect of *Treponema* bacteria, individually or in combination with *P. gingivalis* ATCC 33277, on TCT. Equal numbers of *Treponema* or *P. gingivalis* cells, either individually or combined, were incubated with human fresh frozen plasma for 5 min at 37 °C before addition of 5 U thrombin ml^{-1}. TCTs were determined as described in Methods. Double asterisks denote significant differences ($P<0.01$) in TCTs between single species and the control (no bacteria), and between dual species and single species alone. Error bars, SD of the mean calculated from triplicate experiments repeated three times. *T.d.*, *T. denticola*; *P.g.*, *P. gingivalis*; *T.v.*, *T. vincentii*.

(Bamford *et al.*, 2007), implying that adherence functions may not be linked to proteolysis. PMSF inhibited bacterial growth, so it was not possible to directly test the effects of protease inhibition on biofilm formation. In *P. gingivalis*, the Arg-specific and Lys-specific proteases (gingipains) are multi-domain proteins with catalytic and adhesion regions that function independently (Lamont & Jenkinson, 1998; Guo *et al.*, 2010). The CTLP complex may fit the same kind of paradigm, whereby a hydrolytic enzyme carries separate substrate-binding and catalytic sites. In a similar way to CTLP, the gingipains have been implicated in *P. gingivalis* interactions with other bacteria (Ito *et al.*, 2010).

T. vincentii carries an incomplete CTLP gene locus. The *prtP* gene is truncated, with the product predicted to lack ~160 C-terminal amino acid residues compared with *T. denticola* PrtP. Utilizing a range of protease substrates, Correia *et al.* (2003) were also unable to detect proteolytic activity in *T. vincentii*. Therefore the biological activity and specificity of the analogous *T. vincentii* CTLP locus remain unclear. The strain of *T. vincentii* used in this work was not able to adhere to *P. gingivalis*, in keeping with the notion that CTLP is important in this interaction. However, *T. vincentii* showed intermediate adherence levels to *Prev. intermedia* and *Parv. micra*, and adherence levels to *F. nucleatum* similar to those of *T. denticola*. These results provide an *in vitro* basis for the findings that *T. denticola* is almost always found associated with *P. gingivalis in vivo*, while *T. vincentii* is isolated from subgingival microbial communities (Siqueira & Rôças, 2004; Visser & Ellen, 2011) but not necessarily associated with *P. gingivalis* or with periodontal disease. Overall, *T. vincentii* appeared to have a less adherent phenotype than *T. denticola*. However, *T. vincentii* shows greater motility than *T. denticola* in migration experiments through semisolid medium (Edwards *et al.*, 2003). Thus it might be speculated that while *T. denticola* shows firmer adherence, the greater motility of *T. vincentii* facilitates movement into tissues. *T. vincentii*-related strains have been suggested to be more invasive than *T. denticola* in murine models (Riviere *et al.*, 1991).

In dual-species biofilms, *P. gingivalis* was shown to form a bottom layer close to the saliva-coated substratum, while *T. denticola* migrated towards the surface layers. Because these biofilms were relatively fragile, we were unable to develop a method of dual fluorescence for CLSM that would distinguish between the two organisms, without modifying the biofilm structure. However, the cell morphologies were sufficiently distinct to satisfactorily distinguish the species when stained with FITC. These biofilms showed areas of dense *P. gingivalis* growth surrounded by *T. denticola*, suggesting the possible development of a metabolic synergy through the initial abilities of these organisms to coaggregate. This synergy in biofilm formation was absolutely reliant upon expression of CTLP by *T. denticola*. The notion that these two important periodontal bacteria function cooperatively in colonization is extended also to pathogenesis, with our observation that their combined proteolytic activities were more effective in interfering with blood clotting (promoting bleeding) than individually.

The results presented in this article suggest that *T. denticola* has a broader capacity to adhere to other periodontal micro-organisms than had been previously realised. Our findings extend the information known about CTLP in its capacity to promote colonization and virulence in *T. denticola*, and synergy in pathogenesis. CTLP provides a mechanism for *T. denticola* to disrupt tissues and to invade

deeper layers, but it also appears to promote the integration of *T. denticola* into biofilm communities. Future studies will focus on attempting to reconstruct more complex communities by incorporating the coadhering partners *F. nucleatum*, *Prev. intermedia* and *Parv. micra* into *P. gingivalis*/*T. denticola* biofilms. The ability to mediate adherence to a range of periodontal pathogens suggests that CTLP is an important factor in colonization. In this regard, CTLP would be a crucial component for growth and survival of *T. denticola*, and thus presents a therapeutic target that would render *T. denticola* highly vulnerable to exclusion from periodontal communities.

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