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.

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 phyotypes 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...
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 (lysin-specific gingipain), which degrade extracellular matrix (ECM) proteins and dysregulate host immune functions (O’Brien-Simpson et al., 2003; McAllister 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 53405 wild-type, isogenic mutant strains CKE ΔprcA ΔprtP (CTLP-negative) (Fenno et al., 1998a) and MHE Δmsp (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 OBG medium (Orth et al., 2010) at 37 °C under an atmosphere of N₂/CO₂/H₂ (8:1:1) in a Don Whitley Mk3 anaerobic workstation. P. gingivalis ATCC 33277, F. nucleatum subs. vincentii ATCC 49256, Prev. intermedia ATCC 25261 and Parv. microa 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 (10000 g, 10 min), washed three times with PBS by centrifugation, and suspended in PBS at OD₆₀₀ 0.5 (1.2 × 10⁹ 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 A₄₅₅ 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 OD₆₀₀ 0.5 in TE buffer containing 1 % Triton X-114, and incubated for 16 h at 4 °C with end-over-end mixing. Samples were centrifuged (21000 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 CaCl₂ 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 OD₆₀₀ 0.2 were biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (5 mg ml⁻¹, Pierce), as described previously (Edwards et al., 2003). Exponential phase cultures of P. gingivalis, F. nucleatum subs. vincentii, Prev. intermedia and Parv. microa were centrifuged, cells were washed three times with PBS/centrifugation, and suspended at OD₆₀₀ 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). A490 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 Na2CO3 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 OD600 0.5. The coaggregating partner bacteria were harvested from mid-exponential phase cultures and washed twice with PBS, and the OD600 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/acetic (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. microa were grown in FAB medium. Dual-species biofilms of T. denticola and P. gingivalis were grown in OGBM medium. All biofilms were prepared as follows: mid-exponential phase cells at OD600 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 CellID 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 H2O, and then the crystal violet was released with 0.5% acetic acid (0.4 ml). A490 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 at 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 OD600 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 chymotrypsin-like activity were detected in T. denticola ATCC 35405 and T. denticola MHE (Fig. 1a), whereas no chymotrypsin-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. microa 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. microa 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
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

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

<table>
<thead>
<tr>
<th><em>Treponema</em> strain*</th>
<th><em>P. gingivalis</em> ATCC 33277</th>
<th><em>F. nucleatum</em> ATCC 49256</th>
<th><em>Prev. intermedia</em> ATCC 25261</th>
<th><em>Parv. micra</em> ATCC 33270</th>
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<tr>
<td><em>T. denticola</em> ATCC 35405</td>
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<td>++</td>
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<tr>
<td><em>T. denticola</em> CKE</td>
<td>++</td>
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<tr>
<td><em>T. denticola</em> MHE</td>
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<td><em>T. vincentii</em> ATCC 35580</td>
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*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.
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*...
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.*
In earlier work the cytoplasmic filament was also identified as being necessary for adherence to \textit{P. gingivalis}. The Msp was shown in other studies to act as an adhesin in coaggregation of \textit{T. denticola} ATCC 35404 and \textit{F. nucleatum} (Rosen \textit{et al.}, 2008). However, in our studies of \textit{T. denticola} ATCC 35405, the Msp knockout mutant was unaffected in binding \textit{F. nucleatum}.

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