

Genetic relatedness and phenotypic characteristics of *Treponema* associated with human periodontal tissues and ruminant foot disease

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Treponema have been implicated recently in the pathogenesis of digital dermatitis (DD) and contagious ovine digital dermatitis (CODD) that are infectious diseases of bovine and ovine foot tissues, respectively. Previous analyses of treponemal 16S rDNA sequences, PCR-amplified directly from DD or CODD lesions, have suggested relatedness of animal *Treponema* to some human oral *Treponema* species isolated from periodontal tissues. In this study a range of adhesion and virulence-related properties of three animal *Treponema* isolates have been compared with representative human oral strains of *Treponema denticola* and *Treponema vincentii*. In adhesion assays using biotinylated treponemal cells, *T. denticola* cells bound in consistently higher numbers to fibronectin, laminin, collagen type I, gelatin, keratin and lactoferrin than did *T. vincentii* or animal *Treponema* isolates. However, animal DD strains adhered to fibrinogen at equivalent or greater levels than *T. denticola*. All *Treponema* strains bound to the amino-terminal heparin I/fibrin I domain of fibronectin. 16S rDNA sequence analyses placed ovine strain UB1090 and bovine strain UB1467 within a cluster that was phylogenetically related to *T. vincentii*, while ovine strain UB1466 appeared more closely related to *T. denticola*. These observations correlated with phenotypic properties. Thus, *T. denticola* ATCC 35405, GM-1, and *Treponema* UB1466 had similar outer-membrane protein profiles, produced chymotrypsin-like protease (CTLP), trypsin-like protease and high levels of proline iminopeptidase, and co-aggregated with human oral bacteria *Porphyromonas gingivalis* and *Streptococcus crista*. Conversely, *T. vincentii* ATCC 35580, D2A-2, and animal strains UB1090 and UB1467 did not express CTLP or trypsin-like protease and did not co-aggregate with *P. gingivalis* or *S. crista*. Taken collectively, these results suggest that human oral-related *Treponema* have broad host specificity and that similar control or preventive strategies might be developed for human and animal *Treponema*-associated infections.

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INTRODUCTION

Spirochaetes of the genus *Treponema* are anaerobic, motile Gram-negative rods with spiral morphology. They colonize

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Abbreviations: CODD, contagious ovine digital dermatitis; CTLP, chymotrypsin-like protease; DD, digital dermatitis.

The GenBank accession numbers for the 16S rDNA sequences reported in this paper are AY119692 (*Treponema denticola* GM-1), AY119690 (*Treponema vincentii* D2A-2), AF363634 (*Treponema* sp. UB1466, previously designated G179) and AY119691 (*Treponema* sp. UB1467).

a range of animal hosts and vary considerably in their pathogenic properties. For example, while *Treponema pallidum* subsp. *pallidum* infections may give rise to syphilis, *Treponema phagedenis* is a normal commensal of the human urogenital tract. Many *Treponema* species have been difficult to cultivate and this has seriously hampered taxonomic and phenotypic analyses. Increasing numbers of not yet cultivated bacterial species, particularly from the human oral cavity, have been assigned to the genus *Treponema* based on PCR-derived 16S rDNA sequences (Dewhirst *et al.*, 2000; Paster *et al.*, 2001).

Treponema denticola is strongly associated with progression of adult human periodontitis (Sela, 2001), a chronic

inflammatory disease of the gums and gingival tissues that can lead to bone resorption and tooth loss (Page *et al.*, 1978). Disease severity is correlated with the presence, within periodontal lesions, of *T. denticola* in association with obligately anaerobic Gram-negative bacteria such as *Porphyromonas gingivalis* and *Bacteroides forsythus* (*Tannerella forsythensis*) (Socransky *et al.*, 1998). The interactions of these organisms with periodontal tissues involves adhesion to epithelial cells and extracellular matrix components, proteolysis and modulation of host immune functions (Loesche, 1993; Lamont & Jenkinson, 1998). A number of studies have demonstrated that *T. denticola* adheres avidly to epithelial cells (Ellen *et al.*, 1994), causes cytopathic effects mediated in part via the combined activities of proteases (Grenier *et al.*, 1990; Fenno *et al.*, 1998) and an outer-membrane protein designated Msp (Fenno *et al.*, 1996), and actively penetrates tissue layers by chemotaxis (Lux *et al.*, 2001). However, on the basis of 16S rDNA analysis data from periodontal pocket samples, it appears that *T. denticola* may be only one of more than 20 different species of *Treponema* found within a single active disease site (Choi *et al.*, 1994). The contributions of these other species, including the more well-characterized species *Treponema pectinovorum* and *Treponema vincentii*, to periodontal disease pathogenesis is currently not understood.

Digital dermatitis (DD), which is a bovine foot disease, and contagious ovine digital dermatitis (CODD, formerly known as severe virulent ovine footrot) are both ulcerative conditions that lead to lameness in affected animals (Blowey & Sharp, 1988; Naylor *et al.*, 1998). Lesions originate in the interdigital cleft and if left unchecked, spread to encompass the entire foot (Blowey *et al.*, 1994). Outbreaks of these diseases within herds or flocks are common, particularly in winter, and result in profound welfare and economic problems (Read & Walker, 1998). Spirochaetes have been identified within these lesions and based upon 16S rDNA sequence data, have been shown to be related to human oral isolates of *T. denticola* and *T. vincentii* (Choi *et al.*, 1997; Collighan *et al.*, 2000). Although aetiological roles for *Treponema* in bovine or ovine foot diseases have yet to be proven, treponemes tend to be found only in active lesions (Dopfer *et al.*, 1997). Furthermore, serum samples from diseased animals contain elevated antibody levels to *Treponema* antigens (Demirkan *et al.*, 1999b). There are thus many parallels with the pathology, immunology and inferred bacterial aetiology of these human and animal diseases of collagenous tissues (Edwards *et al.*, 2003).

In this article we describe the genotypic and phenotypic properties of three *Treponema* isolates that we have successfully cultivated from animal foot disease lesions. The properties of these isolates have been compared with *T. denticola* and *T. vincentii* strains originating from the human oral cavity. The results show that the animal isolates are taxonomically distinct from the major periodontal pathogen *T. denticola*. However, two of the animal strains are very closely related to *T. vincentii*, while the other is more similar to *T. denticola*. These organisms have a number of

common adhesive and putative virulence properties that may be related to broad host specificity of colonization.

METHODS

Bacterial strains and culture conditions. *T. denticola* strains ATCC 35405 and GM-1 were kindly provided by R. Lamont (University of Florida, Gainesville, FL, USA) and *T. vincentii* strains ATCC 35580 and D2A-2 were kindly provided by P. E. Greenberg (University of Iowa, USA). Spirochaete isolate UB1090 was obtained from a foot biopsy of a sheep with CODD (Naylor *et al.*, 1998). Spirochaete isolates UB1466 (see Demirkan *et al.*, 2001) and UB1467 were isolated from CODD and bovine DD lesions, respectively, using immunomagnetic enrichment (Demirkan *et al.*, 1999a). Treponemes were grown and maintained in new oral spirochaete (NOS) medium at 37 °C (Haapasalo *et al.*, 1991) in an anaerobic atmosphere of N₂/CO₂/H₂ (8:1:1). Exponential-growth-phase cultures of *Treponema* spp. were preserved in NOS medium containing 15% glycerol at -70 °C. Late-exponential-phase cultures of *Treponema*, corresponding to an OD₆₀₀ of between 0.4 and 0.6, were obtained following a 3- to 4-day incubation at 37 °C. *Porphyromonas gingivalis* ATCC 33277 was grown in Trypticase Soy Broth (TSB, Difco) supplemented with 1 g yeast extract l⁻¹, 5 mg haemin l⁻¹ and 1 mg menadione l⁻¹. *Streptococcus crista* (formerly *cristatus*) CC5A and fusobacteria were grown anaerobically as described above in TSB containing 5 g yeast extract l⁻¹ and 0.5% (w/v) glucose. *Escherichia coli* XL-1 Blue cells were grown in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) or on LB agar, containing ampicillin (100 µg ml⁻¹) when appropriate.

DNA purification. *Treponema* chromosomal DNA was extracted essentially as described by Nelson & Selander (1994). Bacteria from late-exponential-phase cultures (10 ml) were harvested by centrifugation at 10000 g for 5 min, suspended in 0.5 ml TE buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA) containing 0.25% (w/v) SDS, and the suspension was incubated at 65 °C for 1 h. RNase A (10 µl, 5 mg ml⁻¹) was added and the mixture incubated at 37 °C for 30 min, followed by addition of Proteinase K (5 µl, 10 mg ml⁻¹). To the lysate was then added 5 M NaCl (0.1 ml) and 0.08 ml 10% (w/v) CTAB (cetyltrimethylammonium bromide) solution in 0.7 M NaCl and the mixture was incubated at 65 °C for 10 min to facilitate precipitation of bacterial lipopolysaccharides. The suspension was cooled at 4 °C, emulsified with an equal volume of TE-saturated chloroform and centrifuged at 12000 g for 10 min. The aqueous phase containing DNA was recovered, subjected to phenol/chloroform (1:1) extraction and DNA within the aqueous phase was precipitated by adding an equal volume of propan-2-ol. The recovered DNA was suspended in TE buffer, precipitated with 95% (v/v) ethanol in the presence of 3.25 M ammonium acetate, washed with 70% (v/v) ethanol and suspended in TE buffer.

PCR and rDNA analysis. Universal eubacterial 16S rDNA primers 27F (5'-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3'), 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1392R (5'-CACGGATCCACGGCCGGTGTGTRC-3') were utilized in PCR amplifications as described by Harper-Owen *et al.* (1999) to isolate *Treponema* 16S rDNA fragments. The amplimers generated were cloned into pGEM-T (Promega) in *Escherichia coli* XL-1 Blue. Plasmids containing 16S rDNA inserts were purified with the Concert rapid extraction kit (Life Technologies) and inserts were sequenced using an automated ABI sequencer. An edited CLUSTAL W alignment of 1196 nt of each sequence was phylogenetically analysed using the PHYLIP suite of programs (Felsenstein, 1993) available at the UK Human Genome Mapping Project Resource Centre (<http://menu.hgmp.mrc.ac.uk>). More specifically, DNADIST was used to compare sequences by the

Jukes–Cantor algorithm, NEIGHBOR was used for neighbour-joining cluster analysis (Saitou & Nei, 1987), and SEQBOOT and CONSENSE were used for bootstrap analysis.

Protein extraction and electrophoresis. Treponemal surface proteins were extracted by the method of Cunningham *et al.* (1988) as modified by Fenno *et al.* (1998). Briefly, bacteria from late-exponential-phase cultures were harvested by centrifugation and washed twice with TE buffer. Bacteria were resuspended at a concentration of approximately 2.5×10^9 cells ml⁻¹ in Triton X-114 solution (1%, v/v, in TE buffer) and incubated with shaking on a rotary mixer at 4 °C for 16 h. The suspension was then centrifuged at 21 000 g at 4 °C for 1 h to sediment non-periplasmic material (cytoplasmic cylinders) and to obtain an outer-membrane-protein-enriched supernatant. A portion of supernatant was mixed with an equal volume of sample buffer (10 mM Tris/HCl, pH 6.8, containing 2% SDS and 2 mM 2-mercaptoethanol) and incubated for 5 min at either 100 or 20 °C. Proteins were separated by SDS-PAGE in 10% (w/v) acrylamide and stained with Coomassie blue. Molecular masses of proteins were estimated by reference to the mobilities of pre-stained molecular mass marker proteins (Bio-Rad).

Biotinylation of cells. Late-exponential-phase cells were harvested by centrifugation, washed three times in ice-cold PBS and resuspended at an OD₆₀₀ of 0.1 (approx. 2×10^9 cells ml⁻¹) in ice-cold PBS. Sulfo-NHS-LC-biotin (0.5 mg; Pierce Biochemicals) was added and the suspension was incubated at 4 °C for 30 min. Non-conjugated biotin was removed by three rounds of alternate centrifugation and washing of cells with ice-cold PBS. Cells were stored on ice and assayed for adhesion properties (see below) as soon as possible and within 4 h of labelling. A standard reference plot was generated for each *Treponema* strain relating cell numbers of immobilized biotin-labelled cells to HRP-conjugated streptavidin reactivities (A_{490}).

Adhesion assays. Human plasma fibronectin and human laminin were obtained from Roche Molecular Biochemicals, and human collagen type I was purchased from BD Biosciences. Bovine fibronectin, BSA, bovine fetuin, human fibrinogen, porcine gelatin, human keratin, human lactoferrin, porcine heparin and human hyaluronan (hyaluronic acid) were obtained from Sigma. Substrates to be immobilized were dissolved in coating buffer (50 µl; 0.02 M NaHCO₃, 0.02 M Na₂CO₃, pH 9.3), added to the wells of Immulon 2HB (Dyner Technologies) 96-well plastic plates (0–0.5 µg per well) and incubated at 4 °C for 16 h. Non-specific binding sites were blocked with 1% (w/v) BSA in PBS at 4 °C for 16 h. Wells were washed once with PBS and then *Treponema* cell suspensions in PBS (50 µl) containing between 1×10^7 and 2×10^8 biotinylated cells were applied in triplicate wells and incubated at 20 °C for 2 h. Unbound cell suspensions were aspirated and wells were washed twice with PBS (0.2 ml). Horseradish-peroxidase-conjugated streptavidin (Dako) diluted 1:4000 in PBSTB (PBS containing 0.1%, v/v, Tween 20 and 0.1%, w/v, BSA) was added to each well and incubated at 37 °C for 45 min. Liquid was aspirated from the wells, which were then washed once in PBSTB and twice with PBS. Colour reagent (*o*-phenylenediamine) was then added to the wells (50 µl), incubated in the dark for 10 min, developed by the addition of 0.65 M H₂SO₄ and the A_{490} measured with a Bio-Rad Benchmark plate reader. Numbers of cells bound were calculated from standard curves relating A_{490} to cell numbers (microscopic count) for each *Treponema* isolate.

Blot overlay assay. To generate tryptic fragments of fibronectin, human plasma fibronectin (30 µg) was suspended in 0.1 M Tris/HCl, pH 8.1, containing 10 mM L-cysteine hydrochloride, 10 mM CaCl₂ and 11 U trypsin (Sigma), and incubated at 37 °C for 1 h. The reaction was stopped by addition of PMSF (final concentration 0.25 mM) and then stored at –20 °C. Portions of trypsin digest were mixed with sample buffer, heated at 100 °C for 5 min and

subjected to SDS-PAGE as described above. Identification of fibronectin fragments was based on the banding patterns described by Hayashi & Yamada (1983). Proteins were electroblotted onto nitrocellulose membrane (Amersham) at 8 V cm⁻¹ for 1.5 h. To assay bacterial cell binding to protein bands, the nitrocellulose was blocked with 1% (w/v) BSA in PBS containing 0.1% (v/v) Tween 20 at 4 °C for 16 h. The blot was washed twice (5 min each) with PBS, cut into 5 mm wide strips and strips were incubated with biotinylated *Treponema* cells (or no cells, controls) at 20 °C for 2 h with shaking. The blots were then washed three times (5 min each) with PBS, incubated with HRP-conjugated streptavidin (diluted 1:2000 in PBSTB) at 20 °C for 1.5 h, washed once with PBSTB (5 min) and twice with PBS (5 min each). Biotinylated *Treponema* cells that were bound to the blots were detected following development with 3.5 mM 4-chloro-1-naphthol solution containing 0.03% (v/v) H₂O₂. Protein bands on control strips (no cells added) did not bind streptavidin-HRP.

Enzyme assays. Washed cell suspensions (0.1 ml, 5×10^8 cells) were mixed with 0.4 ml of a solution of chromogenic substrate and incubated at 37 °C for 1 h. Activities were assayed as follows: chymotrypsin-like activity with 1 mM *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide in 0.5 M Tris/HCl, pH 7.2, containing 2 mM dithiothreitol (Grenier *et al.*, 1990); trypsin-like activity with 1 mM *N*-benzoyl-DL-arginine *p*-nitroanilide hydrochloride in 0.1 M Tris/HCl, pH 8.1, containing 10 mM cysteine and 10 mM CaCl₂ (Aduse-Opuku *et al.*, 1998); proline iminopeptidase activity with 1 mM proline *p*-nitroanilide in 0.05 M Tris/HCl, pH 7.8, containing 150 mM NaCl (Makinen *et al.*, 1996); neutral phosphatase activity with 1 mM *p*-nitrophenyl phosphate in 0.025 M cacodylate buffer, pH 6.4 (Ishihara & Kuramitsu, 1995). Following incubation with substrate, *Treponema* cells were collected by centrifugation and the A_{405} of the supernatant was measured against a blank (no cells) taken through the assay. Enzyme activities were expressed in Units (U) where 1 U is defined as the amount of enzyme that produced 1 µmol *p*-NP min⁻¹ at 37 °C, based on a value of ϵ_{405} for *p*-NP of 3.8×10^3 M⁻¹ cm⁻¹. All assays were performed in triplicate on at least three individual bacterial cultures.

Coaggregation assay. Late-exponential-phase suspensions of bacteria were prepared at densities of approximately 10^{10} cells ml⁻¹ in coaggregation buffer (1 mM Tris/HCl, pH 8.0, containing 0.1 mM CaCl₂, 0.1 mM MgCl₂, 0.02%, w/v, Na₂S₂O₃ and 0.15 M NaCl) (Cisar *et al.*, 1979). Equal volumes of each cell type (0.2 ml) were mixed in clear plastic cuvettes and incubated at room temperature for 15 min. Coaggregation was scored visually as described by Kolenbrander *et al.* (1990) on a scale of 0–4 defined as follows: 0, no coaggregation; 1, finely dispersed aggregates; 2, definite aggregates but not settled; 3, larger clumps settling but suspension remaining turbid; 4, flocculant coaggregates settling to give a clear supernatant.

RESULTS

Genetic relatedness of human and animal-derived *Treponema* isolates

To establish the relatedness of two new animal-derived strains of *Treponema*, 16S rRNA gene sequences were determined following PCR amplifications with universal 16S rDNA primers. Sequences were already available for human oral-derived *T. denticola* ATCC 35405 and *T. vincentii* ATCC 35580, and for *Treponema* ovine strain UB1090. PCR amplification using eubacterial primers 27F and 1392R generated 1259 bp 16S rDNA products from *T. denticola* GM-1, *T. vincentii* D2A-2 and ovine strain

UB1466. 16S rDNA amplicons from bovine strain UB1467 were obtained following PCR amplification with eubacterial primers 63F and 1392R. The various 16S rDNA fragments were cloned into pGEM-T (Promega) and each was sequenced to obtain three times full sequence coverage for the entire fragment. To compare these sequences with 16S rDNA gene sequences in the GenBank database they were aligned and trimmed to the length of the smallest sequence used in the analysis (1223 bp) corresponding to the 155–1382 region within the *E. coli* 16S rRNA gene (accession no. NC 000913). The aligned sequences were then subjected to a cluster analysis to generate a phylogenetic tree (Fig. 1). As expected, the new sequences for *T. denticola* GM-1 and *T. vincentii* D2A-2 were most closely related to sequences from *T. denticola* ATCC 35405 and *T. vincentii* ATCC 35580, respectively (Fig. 1). The ovine isolate

Treponema UB1090 was more closely related to *T. vincentii*, as previously observed (Collighan *et al.*, 2000), and clustered with the new bovine isolate, *Treponema* UB1467, and with *Treponema medium* (Fig. 1). Conversely, the new ovine isolate *Treponema* spp. UB1466 (also designated G179) appeared more closely related to a bovine DD isolate 1-9185MED (Walker *et al.*, 1995) and fell within a cluster that is phylogenetically more closely related to *T. denticola* (Fig. 1). These data support the evidence that bacteria closely related to *T. denticola* and *T. vincentii* are widely dispersed across animal species.

Outer-membrane protein profiles

Outer-membrane proteins from *T. denticola*, *T. vincentii* and *Treponema* animal isolates were extracted by partitioning

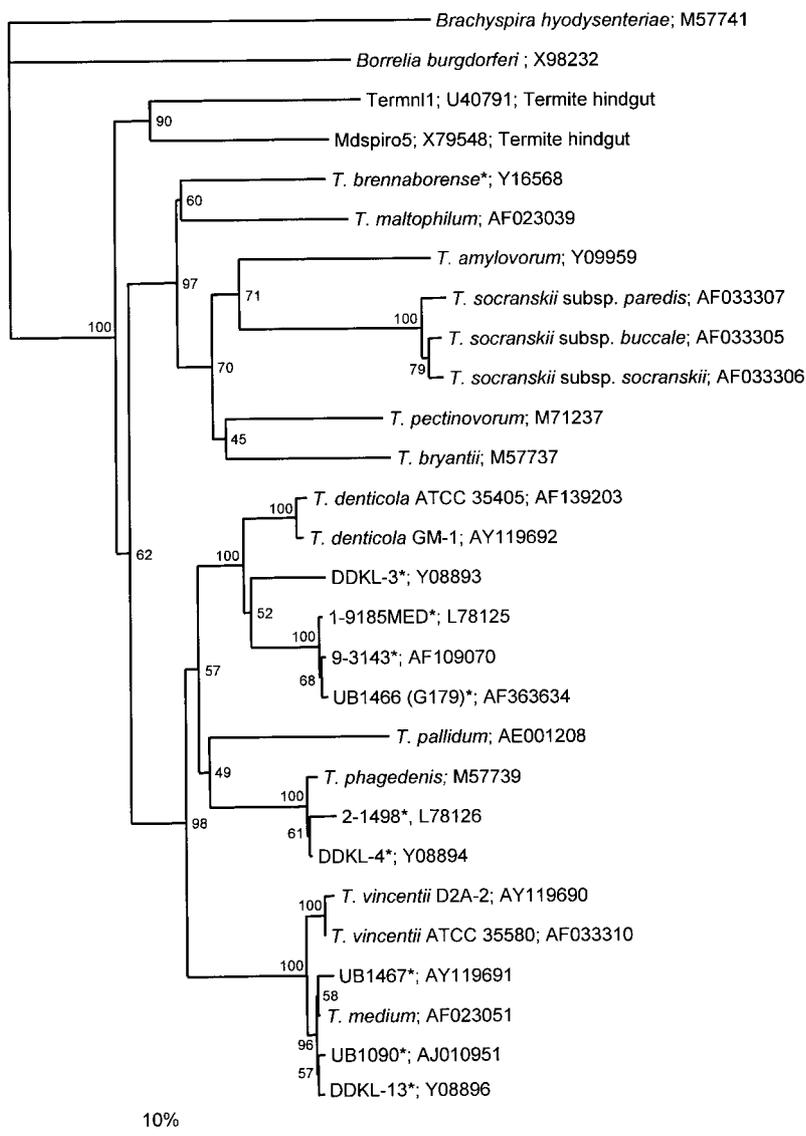


Fig. 1. Phylogenetic tree of *Treponema* showing close relatedness of animal *Treponema* isolates to human oral species on the basis of 16S rDNA sequences. CLUSTAL W alignments of each sequence were analysed by the PHYLIP suite of programs (Felsenstein, 1993) (see Methods). The dendrogram was drawn in TREEVIEW for Windows (v1.0a). Bootstrap values at each branch point are means of 100 replicates. *Brachyspira hyodysenteriae* is included as the outgroup. GenBank accession numbers are shown. Sequences derived from animal *Treponema* isolates or derived from direct PCR amplification of biopsy material from DD lesions are designated with an asterisk. The bar represents a 10% difference in nucleotide sequence.

into Triton X-114 and subjected to SDS-PAGE analysis. The outer-membrane protein profiles of *T. denticola* ATCC 35405 and GM-1 each contained, in unheated samples, a major band with an approximate molecular mass of 200 kDa (Fig. 2, lanes 1 and 2). This band was modified in samples that had been heated at 100 °C before loading and was replaced by a major band with an approximate molecular mass of 60 kDa and several other minor bands (Fig. 2, lanes 1 and 2). These results are consistent with the description by Rosen *et al.* (1999) of a *T. denticola* surface protein complex comprising the major surface protein (Msp, ~60 kDa) and a chymotrypsin-like protease (CTLP), the latter being composed of 72, 43 and 38 kDa polypeptides (Ishihara *et al.*, 1996). In the heated sample profiles of *T. denticola* ATCC 35405 and GM-1 the putative Msps may be identified as 56 and 62 kDa bands, respectively (Fig. 2, lanes 1 and 2). The 72 kDa bands in these profiles are likely to represent the largest CTLP component. In *T. vincentii* extracts, which generally contained a much larger number of bands, an approximately 130 kDa heat-modifiable protein was identified (Fig. 2, lanes 3 and 4). The outer-membrane profiles from ovine strain UB1090 and bovine strain UB1467 were very similar to those of *T. vincentii* (Fig. 2, lanes 3, 4, 5 and 7). However, the ovine *Treponema* strain UB1466 had a distinct outer-membrane protein profile (Fig. 2, lane 6) that

was more similar to *T. denticola* than to *T. vincentii*, or to the other animal isolates.

Adhesion of *Treponema* strains to fibronectin

Binding of *T. denticola* to extracellular matrix components is believed to play an essential role in treponemal invasion of oral tissues (Ellen *et al.*, 1994). In particular, fibronectin binding provides a common means by which treponemes adhere to epithelial or endothelial cells (Dawson & Ellen, 1994). Accordingly, we determined the abilities of human- or animal-derived *Treponema* strains to adhere to immobilized human and bovine fibronectin. All strains tested showed saturable binding to immobilized fibronectins. For *T. denticola* ATCC 35405, which showed the highest affinity for binding fibronectin, a maximum of approximately 2.9×10^6 cells adhered to 0.1 µg immobilized human fibronectin (Fig. 3a). The adhesion levels of *T. denticola* GM-1 were lower than for strain ATCC 35405, while *T. vincentii* strains and bovine or ovine *Treponema* strains all bound fibronectin at levels <20% of strain ATCC 35405 (Fig. 3a). Rather similar patterns of *Treponema* binding to immobilized bovine fibronectin were observed, but notably the adhesion levels of *T. denticola* were significantly lower (about 30%) than to human fibronectin (Fig. 3b). In addition, *Treponema* UB1466 bound significantly better to human than to bovine fibronectin, while the converse was true for *Treponema* UB1090 (Fig. 3). These data reveal subtle differences in fibronectin recognition with the *T. denticola*-related strains showing a preference for human fibronectin over bovine fibronectin.

Region of fibronectin bound by *Treponema*

To determine the region(s) of fibronectin recognized by these *Treponema* strains, tryptic fragments of human fibronectin were Western-blotted onto nitrocellulose to generate a defined banding pattern corresponding to various fibronectin domains (Fig. 4). Binding of biotinylated *Treponema* cells to these bands was then visualized. All strains of *Treponema* tested were found to bind exclusively to a single 30 kDa band (Fig. 4) that contains the N-terminal heparin I/fibrin I binding region of fibronectin (Hayashi & Yamada, 1983). To corroborate this finding, specificities of binding to fibronectin by *T. denticola* ATCC 35405 (highest affinity, see Fig. 3) or *Treponema* UB1090 (lowest affinity) were assessed. Both strains adhered to immobilized 30 kDa fragment as well as, if not better than, to the intact fibronectin molecule (Table 1). Adhesion of cells to fibronectin was significantly reduced following pre-incubation of fibronectin with heparin (Table 1). Pre-treatment of fibronectin with collagen type I did not affect adhesion levels of strain UB1090 cells, but enhanced adhesion of *T. denticola* ATCC 35405 cells presumably by providing additional sites for attachment (Table 1). In further experiments it was observed that adhesion of strain ATCC 35405 cells to immobilized fibronectin was unaffected by pre-incubation of cells with fluid-phase fibronectin or the 30 kDa fragment, demonstrating higher affinity or specificity for immobilized

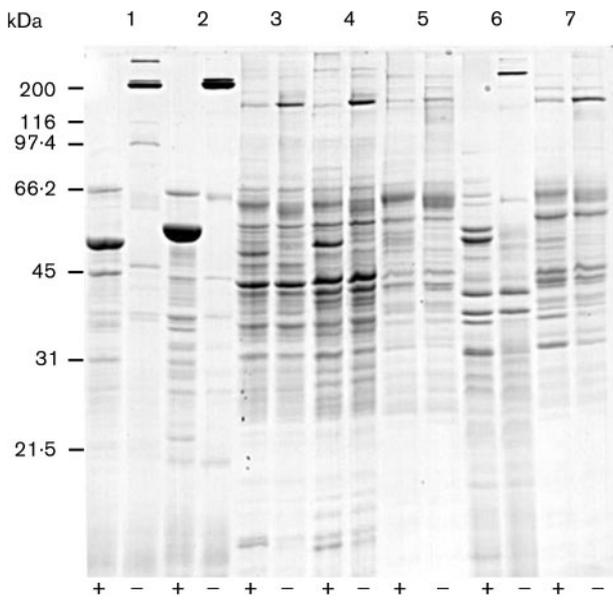


Fig. 2. SDS-PAGE profiles of outer-membrane proteins of human or animal *Treponema*. Triton X-114-solubilized outer-membrane protein extracts were mixed with SDS sample buffer and incubated for 5 min at either 100 (+) or 20 °C (–) before subjecting them to SDS-PAGE. Lanes: 1, *T. denticola* ATCC 35405; 2, *T. denticola* GM-1; 3, *T. vincentii* ATCC 35580; 4, *T. vincentii* D2A-2; 5, *Treponema* UB1090 (ovine); 6, *Treponema* UB1466 (ovine); 7, *Treponema* UB1467 (bovine). Each lane contained 20 µg protein. The positions of molecular mass markers are indicated.

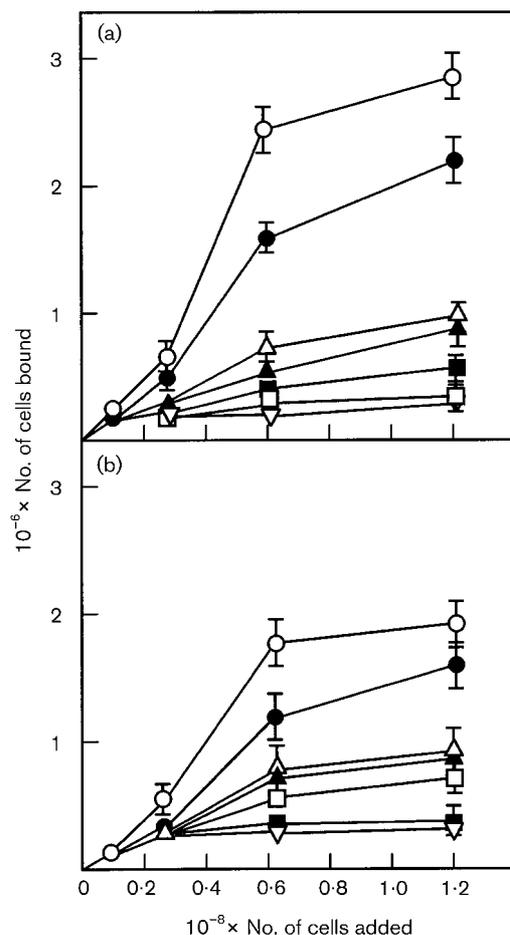


Fig. 3. Binding curves for adhesion of biotinylated *Treponema* cells to 0.1 μ g immobilized human plasma fibronectin (a) or bovine plasma fibronectin (b). Open circles, *T. denticola* ATCC 35405; filled circles, *T. denticola* GM-1; open triangles, *T. vincentii* ATCC 35580; filled triangles, *T. vincentii* D2A-2; open squares, ovine *Treponema* UB1090; filled squares, ovine *Treponema* UB1466; inverted triangles, bovine *Treponema* UB1467. Error bars indicate SD of triplicates from two independent experiments.

fibronectin. Interestingly, adhesion levels of UB1090 cells to fibronectin were reduced in the presence of the fluid-phase 30 kDa fragment (Table 1), possibly reflecting the lower binding affinity of these cells for immobilized fibronectin. In addition, pre-incubation of *Treponema* cells with Arg-Gly-Asp-Ser (RGDS) peptide did not affect adhesion levels (Table 1). Therefore, these results are entirely consistent with the evidence that *Treponema* recognize the N-terminal heparin I/fibrin I domain of fibronectin.

Adhesion of treponemes to tissue-associated proteins

To compare the adhesion properties of human- or animal-derived *Treponema* isolates in more detail their binding

levels to a range of host-associated proteins were determined. The *T. denticola* strains adhered significantly better to all substrates tested, except to fibrinogen (Fig. 5). *T. denticola* cells bound with higher affinity to laminin than to fibronectin (Fig. 5) and showed significant levels of adhesion to fibrinogen, collagen type I, gelatin, keratin and lactoferrin. By contrast, *T. vincentii* and the animal-derived strains bound only weakly, or not significantly, to collagen, gelatin and keratin (Fig. 5). Low levels of binding of all *Treponema* strains tested were observed for heparin, hyaluronic acid and fetuin (Fig. 5). All strains, except UB1467, showed binding to lactoferrin. An important observation was that the animal-derived *Treponema* isolates all bound fibrinogen equally well, or better than, *T. denticola* (Fig. 5), and that *T. vincentii* was least adherent. These data suggest that different adhesion mechanisms are employed by *Treponema* in recognizing fibrinogen and fibronectin.

Enzymic characterization

The virulence properties of *T. denticola* have been linked to adhesion ability, motility and production of proteinases (Sela, 2001). Therefore, the activities of CTLP, trypsin-like peptidase and proline iminopeptidase were measured, together with neutral phosphatase as control. Both strains of *T. denticola* produced significant levels of CTLP and trypsin-like peptidase (OpdB) (Fenno *et al.*, 2001) activities, whereas *T. vincentii* strains did not (Table 2). *T. denticola* expressed about 10-fold higher proline iminopeptidase activity than *T. vincentii*. Ovine strain UB1090 and bovine strain UB1467 showed enzymic levels similar to *T. vincentii* (Table 2). However, ovine strain UB1466 produced high levels of trypsin-like peptidase and proline iminopeptidase activities, and lower but easily detectable levels of CTLP (Table 2). All *Treponema* strains tested produced similar amounts of neutral phosphatase. These findings demonstrate further the close relatedness of *Treponema* ovine strain UB1466 to *T. denticola*.

Coaggregation properties

In their natural habitats, *Treponema* exist in close associations with other bacteria and are co-isolated with specific organisms from diseased sites. Co-aggregation between bacteria may be important in these associations (Kolenbrander, 2000) and so the abilities of *Treponema* strains to co-aggregate with other relevant bacteria were determined. In periodontal disease, *T. denticola* is frequently isolated with *P. gingivalis*, while in CODD *Fusobacterium necrophorum* is present along with *Treponema* (Naylor *et al.*, 1998). It is known that *T. denticola* specifically co-aggregates with *P. gingivalis*, *S. crista* and *Fusobacterium nucleatum* (Grenier, 1992; Yao *et al.*, 1996) and this was confirmed for both *T. denticola* strains in this study (Table 3). *T. vincentii* did not co-aggregate with *P. gingivalis* or *S. crista*, and nor did animal strains UB1090 and UB1467. However, ovine *Treponema* strain UB1466 co-aggregated strongly with *P. gingivalis* and *S. crista* (Table 3), which is consistent with results demonstrating the closer relatedness of this

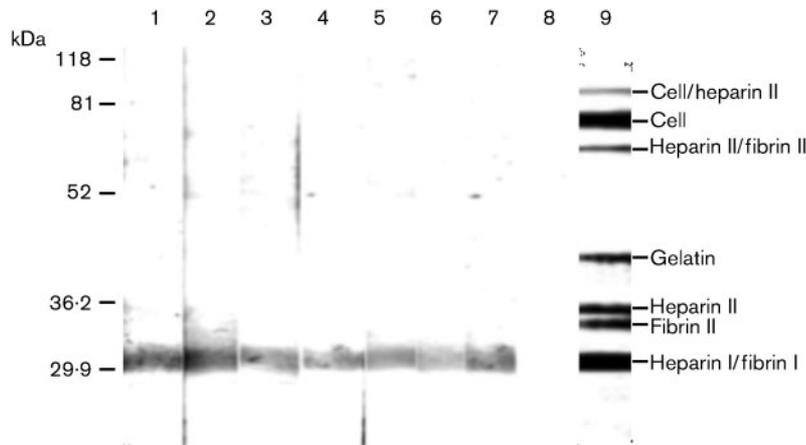


Fig. 4. *Treponema* cell adhesion to fragments of human fibronectin. Tryptic digest fragments encompassing various binding domains of fibronectin as indicated were separated by SDS-PAGE and blotted onto nitrocellulose. Blots were overlaid with biotinylated treponemal cells and binding was detected with streptavidin-linked HRP and colour reagent. Lanes: 1, *T. denticola* ATCC 35405; 2, *T. denticola* GM-1; 3, *T. vincentii* ATCC 35580; 4, *T. vincentii* D2A-2; 5, *Treponema* UB1090; 6, *Treponema* UB1466; 7, *Treponema* UB1467; 8, control (no biotinylated *Treponema* cells); 9, corresponding SDS-PAGE pattern of tryptic digest fragments and associated binding domains.

strain to *T. denticola*. All *Treponema* strains co-aggregated strongly with *F. nucleatum*, and also with *F. necrophorum* but to more varied extents.

DISCUSSION

Periodontal disease of humans, and DD or CODD in animals, are conditions that involve inflammatory processes and destruction of collagenous and connective tissues. These diseases are associated with mixed bacterial infections and *Treponema* species are strongly implicated in disease progression. There is growing evidence from *in situ* PCR analyses that multiple types or species of *Treponema* may be present within these lesions (Choi *et al.*, 1994; Dewhirst *et al.*, 2000; Stamm *et al.*, 2002), but the activities of the different species in contributing to pathogenesis are not yet understood. *In vitro* studies suggest that *T. denticola* has a high capability to penetrate tissue layers of human gingival

keratinocytes, whereas *T. phagedenis* does not (Lux *et al.*, 2001). *T. denticola* also produces high levels of CTLP and trypsin-like protease activities, compared with other oral *Treponema* species, that provide potential for tissue penetration and destruction (Fenno *et al.*, 1998; Ellen *et al.*, 2000). These and other virulence-related properties of *T. denticola* tend to support the notion that this species may be a major periodontal pathogen. To understand more about the potential pathogenic and virulence properties of related *Treponema* species we have characterized in detail three strains of *Treponema*, independently isolated from foot lesion biopsies, and compared them with human oral isolates of *T. denticola* and *T. vincentii*.

Phylogenetic data, based on 16S rDNA sequence analyses, suggest that *Treponema* present in DD or CODD lesions are closely related to *T. denticola*, *T. vincentii* and *T. medium* that are found at human periodontal sites. Interestingly, the

Table 1. Specificity of *Treponema* binding to immobilized fibronectin

Values are the means \pm SD of quadruplicate determinations from a representative experiment repeated three times.

Binding substrate*	$10^{-6} \times$ No. of cells bound	
	<i>T. denticola</i> ATCC 35405	<i>Treponema</i> ovine UB1090
Fibronectin	2.93 \pm 0.28	0.26 \pm 0.03
30 kDa heparin I/fibrin I fragment	3.32 \pm 0.27	0.39 \pm 0.05
Fibronectin + fluid-phase fibronectin†	2.75 \pm 0.25	0.21 \pm 0.03
Fibronectin + fluid-phase 30 kDa fragment†	2.74 \pm 0.30	0.19 \pm 0.05§
Fibronectin + RGDS peptide†	2.90 \pm 0.30	0.26 \pm 0.04
Fibronectin + fibronectin + heparin‡	1.76 \pm 0.22§	0.15 \pm 0.02§
Fibronectin + acid-soluble collagen type I‡	3.95 \pm 0.29§	0.23 \pm 0.02

*Protein (0.1 μ g) immobilized onto plastic wells.

†Bacterial cells (input of 1×10^8 cells) preincubated with 5 μ g protein or peptide.

‡Immobilized substrate pretreated with 5 μ g protein.

§Significantly different from top value within column; $P < 0.05$.

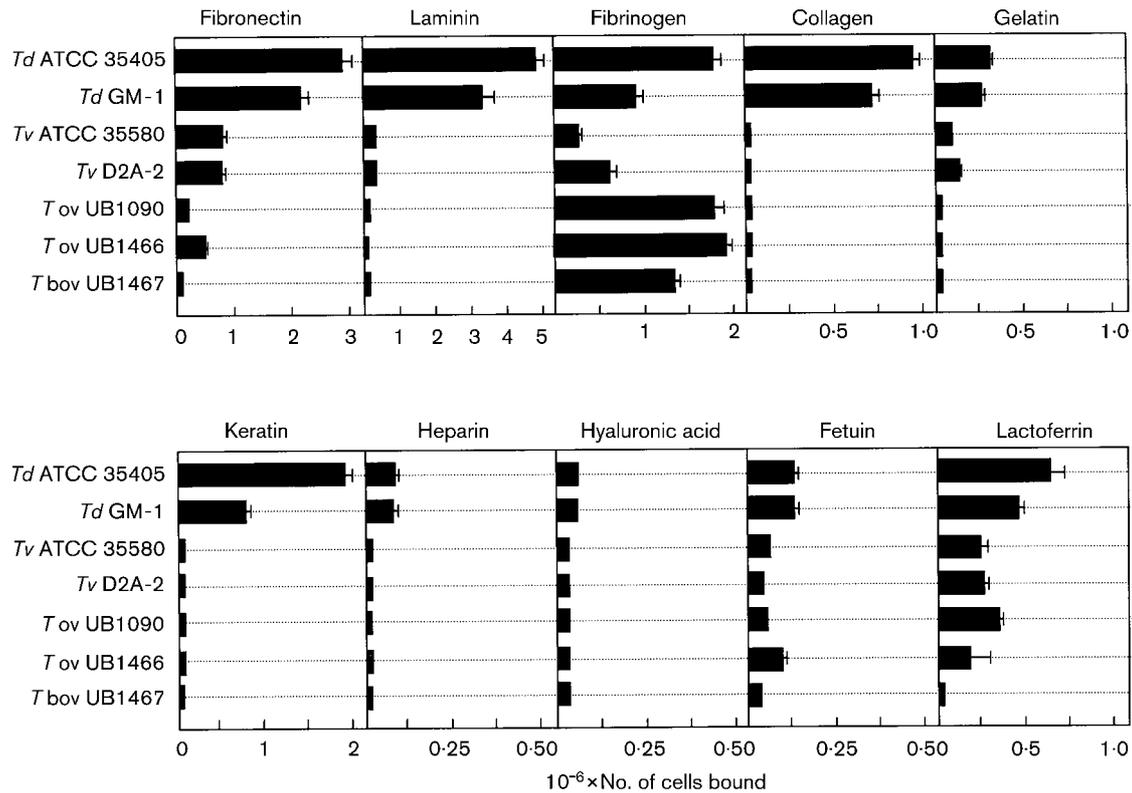


Fig. 5. *Treponema* adhesion levels to proteins and host tissue components immobilized onto plastic wells. Biotinylated treponemal cells (6×10^7) were added to wells coated with $0.1 \mu\text{g}$ substrate and the number of bound cells was determined as described in Methods. Error bars indicate SD of triplicates from three independent experiments. *Td*, *T. denticola*; *Tv*, *T. vincentii*; *T ov*, *Treponema ovine*; *T bov*, *Treponema bovine*.

isolates obtained from sheep and cattle did not themselves form a distinct phylogenetic group. However, more sensitive methods than 16S rDNA sequence analysis, such as 16S–23S rDNA intergenic spacer region analysis (Stamm *et al.*, 2002), could be used to determine if there were distinct lines of descent for strains isolated from the different hosts. Thus, ovine strain UB1466 was more closely related to *T. denticola*, while ovine strain UB1090 and bovine strain UB1467 appeared very closely related to *T. medium*, and clustered

with *T. vincentii* (Fig. 1). The various genetic relationships were corroborated by phenotypic data on outer-membrane protein profiles and proteolytic enzymic activities. Unfortunately, *T. medium* was not available to us at the time, and a more detailed comparison of strains UB1090 and UB1467 with *T. medium* would be most valuable. These results indicate that mixed *Treponema* species ecologies occur at diseased sites in both humans and animals, and that isolates from DD or CODD lesions share common ancestors

Table 2. Proteolytic enzymic activities associated with cells of human or animal *Treponema* strains

Values are the means \pm SD of triplicate determinations from a representative experiment repeated three times.

<i>Treponema</i> strain	Specific enzyme activity (mmol min^{-1} per 10^9 cells)			
	CTLP	Trypsin-like protease	Proline iminopeptidase	Neutral phosphatase
<i>T. denticola</i> ATCC 35405	3.17 ± 0.22	2.07 ± 0.13	4.58 ± 0.30	0.50 ± 0.03
<i>T. denticola</i> GM-1	4.27 ± 0.15	3.02 ± 0.12	3.07 ± 0.28	0.55 ± 0.03
<i>T. vincentii</i> ATCC 35580	<0.01	<0.01	0.40 ± 0.28	0.50 ± 0.03
<i>T. vincentii</i> D2A-2	<0.01	<0.01	0.37 ± 0.27	0.48 ± 0.02
<i>Treponema ovine</i> UB1090	<0.01	<0.01	0.38 ± 0.27	0.57 ± 0.02
<i>Treponema ovine</i> UB1466	0.28 ± 0.07	4.48 ± 0.47	5.25 ± 0.13	0.52 ± 0.03
<i>Treponema bovine</i> UB1467	<0.01	<0.01	0.37 ± 0.26	0.50 ± 0.02

Table 3. Coaggregation of human or animal *Treponema* strains with human oral bacteria

Coaggregation was scored visually across the range from 4 (bacterial clumps that freely sedimented) to 0 (no aggregates) as described in Methods.

<i>Treponema</i> strain	Coaggregation score with:			
	<i>P. gingivalis</i> ATCC 33277	<i>S. crista</i> CC5A	<i>F. nucleatum</i> ATCC 25586	<i>F. necrophorum</i> NCTC 10575
<i>T. denticola</i> ATCC 35405	3	3	3	3
<i>T. denticola</i> GM-1	4	4	4	3
<i>T. vincentii</i> ATCC 35580	0	0	3	1
<i>T. vincentii</i> D2A-2	1	1	4	2
<i>Treponema</i> ovine UB1090	0	0	3	2
<i>Treponema</i> ovine UB1466	4	4	4	1
<i>Treponema</i> bovine UB1467	1	1	4	1

with human oral strains. They also raise the possibility that *Treponema* strains may be able to move between sheep and cattle, which has implications for foot disease progression in mixed husbandry situations.

The precise mechanisms by which *T. denticola* and other oral *Treponema* adhere to epithelial cells in the process of colonizing oral tissue sites are largely unknown. Different species vary widely in their attachment properties (Carranza *et al.*, 1997) and in their abilities to induce changes in host-cell morphology and cytopathic effects (Ellen *et al.*, 1994). *T. denticola* has been shown to bind avidly to fibronectin via the cell tips (Dawson & Ellen, 1994) and this interaction may be important in facilitating adhesion to epithelial tissues via bridging of integrins on the epithelial cell membrane. In addition, the abilities of *Treponema* cells to bind laminin and collagen (Haapasalo *et al.*, 1991), as well as fibronectin, may promote treponemal invasion of basement membrane connective tissue. In this article we have confirmed the abilities of two strains of *T. denticola* to bind immobilized human fibronectin with high affinity and to bovine fibronectin with slightly lower affinity. Much lower (5- to 10-fold) binding levels to fibronectin were observed for *T. vincentii* and animal *Treponema* strains. All *Treponema* were shown to bind the fibrin I/heparin I binding domain of fibronectin and, consistent with these findings, adhesion of *Treponema* cells to fibronectin was inhibitable by heparin, but not by collagen. By contrast with another study (Dawson & Ellen, 1990), we obtained no evidence for recognition by *Treponema* of the cell-binding domain of fibronectin, as evidenced by the inability to achieve inhibition of adhesion to fibronectin in the presence of RGDS peptide, and inability to demonstrate treponemal cell adhesion to this domain fragment in blot overlays. The high affinity of *T. denticola* binding to fibronectin may be related to the expression of the CTLP/Msp cell-surface complex by this species in contrast to the *T. vincentii*-like strains that did not express CTLP. Since Msp has been shown to also bind laminin (Fenno *et al.*, 1996) and collagen (Umemoto & Namikawa, 1994), this could also account for the higher binding levels of *T. denticola* to these substrates.

For all substrates tested, with the exception of fibrinogen, highest levels of adhesion were obtained for *T. denticola*. However, binding of *T. denticola* to heparin, hyaluronic acid and fetuin was especially weak. Fetuin was included in these experiments because it has been reported that *Treponema* may recognize sialic acid residues on the host-cell surface (Keulers *et al.*, 1993). In our analyses fetuin, which is highly sialylated, did not support significant levels of *Treponema* cell adhesion. In general, our data suggest that *Treponema* do not have a high affinity for these negatively charged substrates (Haapasalo *et al.*, 1996). On the other hand, six of the seven strains tested in this article demonstrated relatively high levels of adhesion to lactoferrin. This has been reported for *T. denticola* (Staggs *et al.*, 1994) and may be relevant to oral cavity colonization either in inactivating innate defence properties of lactoferrin or in acquisition of iron. We have also shown a unique ability of *T. denticola* cells to bind keratin. This was surprising since we anticipated that this property might be exhibited by *Treponema* isolated from DD and CODD lesions, where there is an abundance of keratin associated with the hoof. Clearly, these results add further support to the notion that the animal isolates have not acquired a specificity in adhesion properties that sets them apart from human periodontal isolates. Interestingly though, all strains tested showed relatively high binding abilities to fibrinogen, and the animal isolates all bound to fibrinogen at higher levels than did *T. vincentii*. The molecules that mediate *Treponema* adhesion to fibrinogen are unknown and are currently under investigation. However, the evidence suggests that the mechanism is broadly distinct from the mechanisms mediating adhesion of *T. denticola* to fibronectin, laminin, collagen and keratin. Adhesion to fibrinogen may be of major importance in *Treponema* pathogenesis, since modulation of fibrinogen functions at diseased sites could promote bleeding, avoidance of immune cell recognition and further tissue destruction.

In summary, we have described in detail a range of adhesion properties for *Treponema* strains isolated from human periodontal tissues and from animal foot tissues. Our data indicate that there are no fundamental differences between

these animal isolates and human isolates, and that the animal isolates show no obvious predisposition for interaction with animal-derived proteins or animal-derived bacteria such as *F. necrophorum*. Indeed, one of the animal isolates designated strain UB1466, from a CODD lesion, more closely resembled *T. denticola* in a number of properties. This strain, unlike *T. vincentii* and the other animal isolates, produced CTLP, which is a major virulence factor for *T. denticola* (Fenno *et al.*, 1998). It also produced high levels of trypsin-like protease which, in *T. denticola*, has been identified as a prolyl oligopeptidase (OpdB) that may function as a convertase to modulate host inflammatory response proteins (Fenno *et al.*, 2001). Furthermore, strain UB1466 co-aggregated specifically with *P. gingivalis* and *S. crista*. Interactions of *T. denticola* with these bacteria may be significant in the establishment of oral bacterial communities associated with the initiation and progression of periodontal disease (Yao *et al.*, 1996). Taken collectively, these results suggest that *Treponema* related to the designated species *denticola*, *vincentii* and *medium* have broad host specificity. Indeed, it seems reasonable to suggest that these *Treponema* represent a cluster, within this heterologous genus, the members of which may be transmissible from humans to animals (and vice versa). This raises the intriguing possibility that anti-treponemal strategies currently being developed to control or prevent human oral periodontal disease may be directly applicable, or adaptable, to the control or prevention of ruminant foot diseases.

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