Characterization of a novel family of fibronectin-binding proteins with M23 peptidase domains from *Treponema denticola*

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SUMMARY

Interactions with fibronectin are important in the virulence strategies of a range of disease-related bacteria. The periodontitis-associated oral spirochaete *Treponema denticola* expresses at least two fibronectin-binding proteins, designated Msp (major surface protein) and OppA (oligopeptide-binding protein homologue). To identify other *T. denticola* outer membrane fibronectin-binding proteins, the amino acid sequence of the *Treponema pallidum* fibronectin-binding protein Tp0155 was used to survey the *T. denticola* genome. Seven *T. denticola* genes encoding orthologous proteins were identified. All but two were expressed in *Escherichia coli* and purified recombinant proteins bound fibronectin. Using antibodies to the N-terminal region of Tp0155, it was demonstrated that *T. denticola* TDE2318, with highest homology to Tp0155, was cell surface localized. Like Tp0155, the seven *T. denticola* proteins contained an M23 peptidase domain and four (TDE2318, TDE2753, TDE1738, TDE1297) contained one or two LysM domains. M23 peptidases can degrade peptidoglycan whereas LysM domains recognize carbohydrate polymers. In addition, TDE1738 may act as a bacteriocin based on homology with other bacterial lysins and the presence of an adjacent gene encoding a putative immunity factor. Collectively, these results suggest that *T. denticola* expresses fibronectin-binding proteins associated with the cell surface that may also have cell wall modifying or lytic functions.

Keywords

fibronectin; LysM; M23 peptidase; periodontal disease; *Treponema*

INTRODUCTION

Periodontal disease is prevalent within the human population and results from complex communities of microorganisms, combined with the host inflammatory response, causing damage to gingival and supporting tissues. Periodontal pockets, which are indicative of periodontitis, enable the persistence of anaerobic conditions, which facilitate growth of organisms including *Fusobacterium*, *Porphyromonas*, *Prevotella* and *Treponema* species. Recent advances in understanding the growth requirements of oral *Treponema* have allowed the isolation and characterization of a number of different species associated with periodontal disease (Choi *et al*., 1994). However, there is evidence that many not yet

cultivable treponemes are also present at diseased sites (Choi *et al.*, 1994; Dewhirst *et al.*, 2000). Possibly the best characterized of the disease-associated oral spirochaetes is *Treponema denticola*. There is strong evidence linking this pathogenic treponeme with disease initiation and progression. Gingivitis was more likely to develop from healthy sites where *T. denticola* was detected, than when it was absent (Riviere *et al.*, 1998). *Treponema denticola* is most regularly detected in deep periodontal pockets and evidence suggests that it persists predominantly towards the leading edge of subgingival plaque providing direct contact with periodontal tissues (Kigure *et al.*, 1995).

Damage and inflammation within infected periodontal pockets leads to host macromolecules, such as extracellular matrix and plasma components such as fibronectin, becoming accessible to colonizing bacteria. Fibronectin is a vital matrix component, existing either as an insoluble glycoprotein, which polymerizes to form part of the extracellular matrix thereby adding structural support to host tissues (matrix fibronectin), or as a soluble disulphide-linked plasma protein with a role in wound healing (Couchman *et al.*, 1990). Both matrix and plasma fibronectin are found in abundance during blood clotting, tissue repair, cell migration and embryogenesis, as occurs within periodontal pockets.

Adhesion to, or degradation of, fibronectin is important in the virulence strategies of a number of pathogenic organisms. Adhesion promotes colonization (Pasula *et al.*, 2002; Pracht *et al.*, 2005), whereas proteolysis provides a source of peptides (Fenno *et al.*, 2000; Samen *et al.*, 2004) or may hamper or obstruct wound-healing processes (Heilmann *et al.*, 2004). The capacity to avoid host immune defence mechanisms by penetrating host tissues is also enhanced by interactions with fibronectin (Dziewanowska *et al.*, 1999; Sinha *et al.*, 1999). Adherence to either matrix or plasma fibronectin could therefore be an essential resource in the pathogenesis of *T. denticola*.

Fibronectin-binding by *T. denticola* was first described by Dawson & Ellen (1990) and was later quantified (Haapasalo *et al.*, 1991). Fluid-phase, but not immobilized, forms of fibronectin were bound by an OppA (oligopeptide-binding protein) homologue, involved in internalization of oligopeptides for nutrition (Fenno *et al.*, 2000). Immunoblot overlays (Umemoto *et al.*, 1993) suggested that *T. denticola* expressed three outer membrane fibronectin-binding proteins. The same technique was used by Haapasalo *et al.* (1992) in characterization of the 53-kDa major surface protein (Msp) of *T. denticola* ATCC 35405. Further analysis of Msp functions (Fenno *et al.*, 1996; Edwards *et al.*, 2005) suggested involvement of this surface-associated protein in fibronectin interactions.

The causative agent of syphilis, *Treponema pallidum*, interacts with fibronectin (Peterson *et al.*, 1983; Thomas *et al.*, 1986) but the molecular mechanisms involved are not well understood. Two fibronectin-binding proteins were identified in *T. pallidum* using a bioinformatics approach. Ten genes encoding potential outer membrane proteins were cloned and expressed and the recombinant proteins were purified (Cameron, 2003) and characterized for their abilities to bind plasma or matrix forms of fibronectin (Cameron *et al.*, 2004). Two proteins, designated Tp0155 and Tp0483, bound fibronectin although Tp0155 bound preferentially to matrix fibronectin whereas Tp0483 showed greater attachment to the plasma form.

Comparisons between the *T. pallidum* Nichols and *T. denticola* ATCC 35405 genomes have become possible because they have both been fully sequenced and annotated. Analysis of open reading frames suggests that *T. pallidum* and *T. denticola* are derived ancestrally. The *T. denticola* genome contains 2786 open reading frames and *T. pallidum* carries 1039 open reading frames. This suggests that gene elimination may have occurred in *T. pallidum*, whereas duplication and expansion occurred in the *T. denticola* genome (Seshadri *et al.*,

2004). It is also interesting to note that the *T. denticola* genome is predicted to encode 156 lipoproteins but *T. pallidum* encodes only 16. This may reflect the fact that *T. denticola* has adapted to grow and survive in an oral environment where there is strong competition with other microorganisms.

It has previously been shown that Msp has a role in mediating *T. denticola* binding to fibronectin (Haapasalo *et al*., 1992; Fenno *et al*., 1996; Edwards *et al*., 2005). However, an isogenic Msp-deficient strain (Fenno *et al*., 1998) was only reduced by ~ 30% in adherence to fibronectin (C.V. Bamford, unpublished data), suggesting that other factors were present that bound fibronectin. As the OppA homologue is reported to bind only fluid-phase fibronectin, additional fibronectin-binding proteins may be expressed on the surface of *T. denticola*. In this article we describe a family of *T. denticola* proteins with homology to a *T. pallidum* fibronectin-binding protein, Tp0155, (Cameron *et al*., 2004) was identified. These proteins are able to bind both matrix and plasma fibronectin, and protein sequence characteristics suggest a possible multifunctional role in *T. denticola* pathogenesis.

METHODS

Bacterial strains and growth conditions

Treponema denticola ATCC 35405 was maintained in pre-reduced New Oral Spirochete medium (Bamford *et al*., 2007) at 37°C in an anaerobic atmosphere (80% N_2: 10% H_2: 10% CO_2). Cultures were grown for 3–4 days to late exponential phase, corresponding to an optical density (OD_{600}) of 0.4–0.6, and were harvested by centrifugation (10,000 *g*, 10 min). *Escherichia coli* XL-1 Blue was used as host to prepare plasmid DNA, and *E. coli* M15 was used for production of recombinant polypeptides from pQE30 plasmids (Table 1). The *E. coli* was cultured on Luria Bertani (LB) agar (Sambrook *et al*., 1989) or in LB broth, containing appropriate antibiotics (ampicillin, 100μg ml^{-1}; kanamycin 25μg ml^{-1}).

Bioinformatics analyses

Treponema denticola ATCC 35405 and *T. pallidum* Nichols peptide sequences were acquired from the TIGR Comprehensive Microbial Resource (http://cmr.tigr.org.tigr-scripts/cmr/CmrHomePage.cgi) and analysed using a number of *in silico* investigations. BLAST analyses to identify homologous peptide sequences were achieved using appropriate tools at http://cmr.tigr.org.tigr-scripts/cmr/CmrHomePage.cgi. Properties of individual peptide sequences were studied to assess peptide mass (www.us.expasy.org/cgi-bin/peptide-mass.html) and presence of signal sequence (http://bioinformatcs.leeds.ac.uk/prot_analysis/signal.html) and to examine other structural or functional domains (www.ebi.ac.uk/InterProScan/) within the peptide sequence.

Expression and purification of recombinant Tp0155 orthologues

Coding sequences of *T. denticola* DNA that corresponded to potential orthologues of *T. pallidum* Tp0155 were identified from predicted open reading frames of the *T. denticola* genome (Seshadri *et al*., 2004) using BLAST similarity analyses (Altschul *et al*., 1990). Chromosomal DNA of *T. denticola* was extracted using a method adapted from Nelson & Selander (1994) and a polymerase chain reaction (PCR) was carried out with a Platinum Pfx PCR system (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The PCR amplimers were generated using specific forward and reverse primers for each gene (see Supporting information, Table S1) and, where relevant, without inclusion of sequences encoding leader peptides that may result in products toxic to *E. coli* (Fenno *et al*., 1996; Edwards *et al*., 2005). Included within the forward primer sequences was an *Sph*I restriction site, and within the reverse primer a *Sal*I site to ensure in-frame insertion of coding sequences within the His$_6$-tag expression vector pQE-30 (Qiagen). The PCR products were

purified using a PCR cleanup kit (Qiagen), then digested and ligated into *Sph*I/*Sal*I-digested and dephosphorylated pQE-30 vector. *Escherchia coli* XL-1 Blue was transformed with each ligation mix and colonies were screened first by PCR, then potential recombinant plasmids were purified (Qiagen miniprep) and sequenced to confirm in-frame fusion and sequence identity (Seqlon, Goettingen, Germany). The expression host *E. coli* M15 was then transformed with appropriate constructs and recombinant His$_6$-tagged proteins were expressed after addition of 1 mM isopropyl-β-D-thiogalactopyranoside. Purification was achieved under denaturing conditions (8 M urea) using nickel-nitrilotriacetic acid resin (Qiagen) as described previously (Edwards *et al*., 2005). Recombinant proteins were dialysed against 4 M and 2 M urea and finally against distilled H$_2$O, and then freeze-dried for storage.

Extraction of *T. denticola* outer sheath proteins

Proteins of *T. denticola* ATCC 35405 outer membranes were isolated as previously described (Bamford *et al*., 2007). Briefly, exponential phase *T. denticola* cells were harvested and washed twice in TE buffer (10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid, pH 8) in the presence of 0.05 mM phenylmethylsulphonyl fluoride (PMSF). Approximately 3.6×10^9 cells ml^{-1} were incubated for 16 h at 4°C with TE buffer containing 1% Triton-X 114 and 0.05 mM PMSF. Outer membrane proteins were separated from non-periplasmic material by centrifugation at 21,000 g for 1 h at 4°C and membrane proteins in supernatants were analysed by sodium dodecyl sulphate–polyacrylamide gel eletrophoresis (SDS–PAGE).

SDS–PAGE and Western blot analysis

SDS–PAGE was carried out as described by Laemmli (1970). Rehydrated purified recombinant His$_6$-tagged *T. denticola* polypeptides or *T. denticola* outer sheath proteins were mixed with sample buffer containing 2% SDS and 5 mMβ-mercaptoethanol and incubated at 20°C or 100°C before electrophoresis within a gel containing 10% acrylamide. Proteins were visualized by silver nitrate staining or electroblotted onto nitrocellulose membrane (Hybond ECL, Amersham, Amersham, UK). The membrane was incubated for 16 h at 4°C in Tris-buffered saline (TBS) containing 5% [weight/volume (w/v)] Fraction V bovine serum albumin (BSA) or 10% (w/v) skimmed milk powder and washed with TBS-0.1% Tween 20 (TBST). Membranes were then incubated with either anti-tetra His antibodies (Qiagen) diluted 1: 2000, or antibodies raised in chickens to the N terminus (amino acids 46–187) of Tp0155 diluted 1: 50 in TBST-0.1% BSA (TBSTB) or 1% skimmed milk powder (TBSTM) for 2 h at 20°C. The membranes were washed twice with TBSTB or TBSTM as appropriate and incubated with either horseradish peroxidase (HRP) - conjugated secondary anti-mouse antibodies (1: 1000) or HRP-Protein A for 2 h at 20°C. Visualization of proteins was achieved using 4-chloro-1-naphthol solution or enhanced chemiluminescence (Amersham).

Fibronectin adherence assay

Human plasma (Roche, Basel, Switzerland) or fibroblast (Calbiochem, San Diego, CA) fibronectin (5μg) in carbonate coating buffer (0.02 M NaHCO$_3$, 0.02 M Na$_2$CO$_3$, pH 9.3) were adsorbed on to the surface of 96-well plastic plates (Greiner, Frickenhausen, Germany) for 16 h at 4°C (confirmed using anti-fibronectin antibodies, data not shown). Wells were blocked for 16 h at 4°C with 5% (w/v) BSA in phosphate-buffered saline (PBS). After removal of the blocking agent, 50μl His$_6$-tagged purified recombinant *T. denticola* peptides (0–200μg/ml) were added to the wells, and incubated for 2 h at 20°C. Wells were washed twice with PBS containing 0.1% Tween 20 and 0.1% (w/v) BSA (PBSTB), and once with PBS for 10 min at 20°C. Recombinant peptides were detected using anti-tetra His antibodies diluted 1: 1000 in PBSTB for 2 h at 20°C. Wells were washed twice with PBSTB and once

with PBS for 10 min each and HRP-conjugated secondary antibodies diluted 1: 1000 in PBSTB were added. After 2 h incubation at 20°C the wells were washed twice with PBSTB and once with PBS for 10 min. Antibody binding to recombinant protein was detected by incubating with *o*-phenylenediamine colour reagent and developing with 0.65 M H_2SO_4 (Edwards *et al*., 2005). Experiments were carried out in triplicate, and results are expressed as means with standard deviations from the mean.

Immunofluorescence microscopy

Stationary-phase *T. denticola* cells were pelleted by centrifugation (10,000 ***g*** for 10 min) and washed with PBS. Sterile glass coverslips (13 mm) were coated with filter-sterilized 0.1 mg ml^{-1} poly-lysine (Sigma, St Louis, MO) in borate buffer (0.1 M, pH 8.5) for 30 min at 20°C, washed thoroughly in sterile distilled H_2O and dried. Approximately 4.8×10^7 cells were added to the poly-lysine coated coverslips in a 24-well plate and this was centrifuged at 3000 ***g*** for 5 min. Coverslips were gently washed twice with PBS, blocked with 5% BSA in PBS (1 h at 20°C) and washed with 0.1% Tween 20 in PBS. Antibodies to Tp0155 and to Tp0249, a *T. pallidum* flagellar sheath protein (Cameron *et al*., 2008) were pre-absorbed with *Treponema phagedenis* (Kazan). Antibodies and corresponding preimmune sera controls in PBSTB were added at suitable dilutions and incubated for 1 h at 20°C. Excess antibodies were removed by washing twice with PBSTB and once with PBS and then fluorescein isothiocyanate-conjugated immunoglobulin G was added for 1 h at 20°C. Coverslips were washed as above, placed onto slides with a drop of PermaFluor (Thermo Electron), and imaged using phase and fluorescence microscopy.

Statistics

To establish statistical significance, Student's *t*-test was used on paired samples.

RESULTS

Identification of *T. denticola* proteins with homology to Tp0155 of *T. pallidum*

The amino acid (aa) sequence of *T. pallidum* Tp0155 fibronectin-binding protein (371 aa residues) was compared with the *T. denticola* genome (Seshadri *et al*., 2004) using BLAST similarity analyses. Significant similarities between Tp0155 and seven *T. denticola* predicted proteins were identified (Table 2), with TDE2318 showing the highest overall identity (43% identity) and TDE1136 being the least similar (14% identity). The aa sequences of the *T. pallidum* and *T. denticola* proteins were analysed to identify potential structural or functional regions. A number of distinct domains were evident in Tp0155 and in the seven *T. denticola* proteins (Fig. 1A). Tp0155 was composed of a leader peptide (38 aa residues), two N-terminal LysM domains, labelled for clarity LysM(A) (44 aa residues) and LysM(B) (42 aa residues), and an M23 peptidase sequence (95 aa residues) at the C-terminal end. The C-terminal M23 peptidase domain was present in all of the *T. denticola* polypeptide sequences and showed the highest levels of homology (Table 2) with each other and with the M23 sequence of Tp0155. Three of the seven *T. denticola* proteins contained two predicted N-terminal LysM domains (TDE2318, TDE2753, TDE1297), whereas TDE1738 polypeptide had only one LysM domain (Fig. 1A). TDE0110 and TDE2189 contained no potential LysM domains but TDE1136 appeared to consist solely of an extended M23 peptidase sequence of 144 aa residues (Fig. 1A). Alignment of the seven proteins with Tp0155 and the positions of the various domains within the sequences are shown in supplementary material (see Supporting information, Fig. S1 and Fig. S2, respectively).

Other *T. pallidum* fibronectin-binding proteins have been described including Tp0483 (Cameron *et al*., 2004) and Tp0136 (Brinkman *et al*., 2008). An orthologous protein to

Tp0483 was identified in *T. denticola* (TDE1984). However, the low sequence identity (21%) between these proteins, by comparison to the Tp0155 orthologues, led to no further functional investigations at this time. No orthologues to Tp0136 were found.

Generation of recombinant *T. denticola* proteins

To determine the fibronectin-interacting activities of *T. denticola* proteins, genes were cloned and proteins were expressed in *E. coli*. The appropriate gene sequences were amplified by PCR with proof-reading enzymes (see Supporting information, Fig. S3) and primers based on the sequence of the *T. denticola* ATCC 35405 genome on the Comprehensive Microbial Resource (http://cmr.tigr.org.tigr-scripts/cmr/CmrHomePage.cgi). Forward primers were constructed such that start codons, and where appropriate signal sequences, were absent from the expressed proteins to counteract possible expression problems in *E. coli*. Previous work suggests that some leader sequences of *T. denticola* polypeptides may be toxic to *E. coli* (Fenno *et al*., 1996; Edwards *et al*., 2005). Termination of translation was effected by including the stop codon of each gene within the reverse PCR primer (see Supporting information, Table S1). *In silico* restriction analyses were carried out (www.restrictionmapper.org) to identify enzymes that did not cut any of the *T. denticola* sequences, so allowing a common strategy to be used for cloning and expression of the genes.

Recombinant His_6-tagged *T. denticola* proteins were expressed and purified from *E. coli* for five proteins, as cloning was unsuccessful for TDE0110 and TDE1297 (Fig. 2A). All polypeptides migrated closely to their predicted molecular mass and were detected using HRP-conjugated anti-tetra His antibodies and appropriate HRP-conjugated secondary antibodies (Fig. 2B). A peptide doublet was observed for rTDE2318, which may suggest degradation at the C terminus because the 6×His Tag was at the N terminus.

Adherence to fibronectin

Differential adherence of *T. pallidum* protein Tp0155 to matrix or plasma fibronectin has already been characterized (Cameron *et al*., 2004), with matrix fibronectin being preferentially bound. Quantitative assessment of binding to fibronectin immobilized to wells of a plastic plate by the recombinant *T. denticola* proteins indicated that all, except rTDE2753, were better able to adhere to matrix fibronectin (Fig. 3). Significant differences ($P < 0.05$) in binding matrix versus plasma fibronectin were observed for rTDE1738, rTDE2189 and rTDE1136 (Fig. 3). This figure was based on dose-dependent (Fig. S4) adherence to matrix or plasma fibronectin ($p < 0.05$) that was shown for all proteins tested, compared with binding to blocked wells (controls). The recombinant *T. denticola* proteins were also immobilized by Western blot on nitrocellulose and assessed for their ability to bind fluid-phase fibronectin detected using anti-fibronectin antibodies. None of the recombinant proteins bound fibronectin under these conditions (data not shown), suggesting that affinity towards fluid-phase fibronectin was much lower than to immobilized fibronectin. These results identify five novel fibronectin-binding proteins from *T. denticola* with greater ability to bind immobilized forms of fibronectin.

Surface localization of Tp0155 orthologues in *T. denticola*

Sequence analyses of the *T. denticola* proteins indicated that TDE2318, TDE1738, TDE2189 and TDE1297 had leader peptides (Fig. 1) suggesting cell surface localization or secretion. To investigate surface exposure of TDE2318, which has the highest homology to *T. pallidum* fibronectin-binding protein Tp0155 on the basis of aa sequence (Table 1), antibodies specific to Tp0155 were used to probe *T. denticola* outer membrane proteins in Western blot analyses and immunofluorescence microscopy investigations of *T. denticola* cells. These antibodies were shown to detect both TDE2318 and TDE2753 in Western blot

analyses (Fig. 2C) but lack of predicted leader sequence in TDE2753 indicates that this protein might not be secreted. Polyclonal antibodies to Tp0155 were first pre-adsorbed to a closely related organism *T. phagedenis* (Kazan) to reduce non-specific binding. Preimmune sera were similarly preadsorbed against *T. phagedenis*.

A single peptide of ~ 33 kDa was visualized in *T. denticola* outer sheath proteins, although only in a sample heated to 100°C before separation. This peptide showed similar molecular mass to that predicted for TDE2318. This suggests that this peptide is excreted to the *T. denticola* outer membrane but may be a multimeric complex in its native form.

Immunofluorescence analyses demonstrated control (preimmune) antibodies did not react with *T. denticola* cells in analyses (Fig. 4A,B). However, antibodies to Tp0155 reacted with intact *T. denticola* cells (Fig. 4C,D). Based on fluorescence distribution, it appeared that the orthologous proteins may not be expressed by all cells of the population. In addition, proteins may not be expressed uniformly across the cell surface (Fig. 4D). When cells were permeabilized with Triton X-114, all cells present reacted with Tp0155 antibody (Fig. 4E,F). To demonstrate that *T. denticola* cells were all intact in Fig. 4(C,D), the *T. denticola* cells were reacted with anti-Tp0249 (FlaA) antibodies generated to the flagella sheath proteins (Cameron *et al.*, 2008) that are beneath the outer membrane, and so not exposed in intact cells. Neither preimmune antibody (Fig. 4G,H) nor anti-FlaA antibody (Fig. 4I,J) reacted with *T. denticola* cells, but cells permeabilized with Triton X-114 were all reactive (Fig. 4K,L). These were as reactive as whole cell antibodies to intact cells (Fig. 4M,N). The results show that TDE2318 is surface exposed in *T. denticola*.

DISCUSSION

Normal epithelial shedding or tissue damage, caused by trauma or infection of mucosal surfaces as witnessed deep within the periodontal pocket, will expose plasma and extracellular matrix proteins including fibronectin. It has been known for some time that *T. denticola* can bind both matrix fibronectin (Ellen *et al.*, 1994) and plasma fibronectin (Haapasalo *et al.*, 1991; Fenno *et al.*, 1996; Edwards *et al.*, 2005). The major surface protein Msp has been shown to bind fibronectin (Haapasalo *et al.*, 1992; Edwards *et al.*, 2005) but an Msp mutant of *T. denticola* (Fenno *et al.*, 1998) still retains the ability to adhere to fibronectin (C.V. Bamford, unpublished data) suggesting that other factors are involved. To identify these molecules we used comparative sequence analyses, based on the *T. pallidum* fibronectin-binding protein Tp0155 we identified seven potential fibronectin-binding proteins from the *T. denticola* genome. Five of these were produced as recombinant polypeptides and shown to adhere to matrix or plasma fibronectin. Protein TDE2318, the closest homologue to Tp0155, was shown to be cell surface exposed and others with signal sequences, but not detected here, may be similarly localized to the cell membrane. A role for these proteins in colonization of damaged host tissues by *T. denticola* is suggested. The ability of these proteins to also bind plasma fibronectin may interfere with tissue regeneration and disrupt healing processes.

Distinct domains are present in all of the seven *T. denticola* proteins described in this study. However, it is not clear at this stage which of these domains might be involved in adherence to fibronectin. None of the protein sequences contain the consensus bacterial fibronectin-binding protein domain motif (Schwarz-Linek *et al.*, 2004) that is often a component of amino acid sequence repeats. Four of the polypeptides contain a LysM domain, and all of them contain the M23 peptidase domain (Fig. 1).

LysM domains consist of about 40 aa residues, containing a lysin motif sequence YxxxxGxx(Hyd) (Turner *et al.*, 2004). The LysM domains of Tp0155 and the *T. denticola*

proteins all showed high homology and carried the lysin motif (Fig. 1B). LysM domains are commonly found in proteins with modular functions including autolysis (MurA, *Listeria monocytogenes*, Carroll *et al*., 2003). A surface protein (Sep) of *Lactobacillus fermentum* BR11 (Turner *et al*., 2004) and the autolysin, Aae, of *Staphylococcus epidermidis* (Heilmann *et al*., 2003) use the peptidoglycan-binding LysM domains as cell wall anchors. LysM domains of proteins with accompanying lytic domains may act to target activity to a specific substrate (Steen *et al*., 2003).

The LysM domains of autolysins Aae (*Staphylococcus epidermidis*) and Aaa (*Staphylococcus aureus*), were shown to bind vitronectin, the Aα and Bβ chains of fibrinogen and the 29-kDa fragment of fibronectin (Heilmann *et al*., 2003, 2005). This may indicate a similar role for the LysM domains within the *T. denticola* polypeptide. However, ability to bind either matrix or plasma fibronectin did not correlate with the numbers of LysM domains present. For example, TDE2318 and TDE2753 both have two LysM domains, but show a much lower adherence level to fibronectin compared with TDE1136. The latter consists almost entirely of an M23 peptidase domain with no LysM sequence and is able to bind fibronectin to a higher level than TDE2318 or TDE2753. A development of this work will be to produce truncated recombinant polypeptides of TDE1738 and TDE1136 to try to delineate more precisely the fibronectin-binding domains.

Interactions of LysM sequences with peptidoglycan, or peptidoglycan precursors, appear to be one property, so potentially targeting LysM-containing proteins to the cell surface. In *T. denticola* it has been shown that binding to immobilized fibronectin occurs through interactions with the bacterial cell tip (Dawson & Ellen, 1990). Binding levels were reduced ~50% at 4°C compared with 37°C, suggesting that metabolic activity was important (Dawson & Ellen, 1990). Recent work shows that the cell tips of *T. denticola* and *T. pallidum* have complex morphologies with *T. pallidum* having higher structural complexity (Izard *et al*., 2008, 2009). Peptidoglycan forms a thin layer between the cytoplasmic membrane and outer membrane, and is thicker at the cell ends (Izard *et al*., 2009). It is possible therefore that the LysM domain proteins described in this article may be involved in peptidoglycan modelling as well as fibronectin adherence.

All the *T. denticola* polypeptides contained M23 peptidase domains which are zinc metalloproteases for which the active sites are HxH or HxxxD. Analysis of Tp0155 and the seven *T. denticola* polypeptides with M23 peptidase domains indicated that all except TDE1297 had conserved HxH (HLH or HVH) and HxGxD sequences (Fig. S2). Characterization of other M23 peptidases has suggested activity against glycine–glycine bonds (Schindler & Schuhardt, 1965). Many proteins containing the M23 domain have specific hydrolytic activity on peptidoglycan, resulting in bacteriolytic capabilities (Schindler & Schuhardt, 1964; Thumm & Gotz, 1997). Perhaps the best characterized of these lytic M23 peptidase-containing proteins is lysostaphin produced by *Staphylococcus simulans* biovar *staphylolyticus* (Schindler & Schuhardt, 1964; Naverre & Schneewind, 1999). It specifically cleaves between the third and fourth Gly residueS of the pentaglycine cross bridges which are unique to the peptidoglycan of staphylococcal species (Thumm & Gotz, 1997; Recsei *et al*., 1987). Other known lytic proteins with similar peptidoglycanolytic activities are Zoocin A (*Streptococcus equi* subsp. *zooepidemicus* 4881, Simmonds *et al*., 1996, 1997) and ALE-1 (*Staphylococcus capitis* EPK1, Sugai *et al*., 1997a). These proteins are all conserved for the M23 protease motifs (Fig. 5A). It is possible therefore that the *T. denticola* proteins have lytic activity against other closely related or closely co-habiting bacteria. We have tested the recombinant polypeptides for their ability to bind to, or degrade, cells walls from *S. aureus* or *T. denticola*, but have been unable to demonstrate any clumping or lytic activities (data not shown).

Many bacteriolysins are targeted to bind peptidoglycan via a substrate-binding domain (e.g. LysM) at the C-terminal end of the protein with the M23 peptidase domain at the N terminus (Baba & Schneewind, 1996; Simmonds *et al*., 1996; Nilsen *et al*., 2003). In four of the *T. denticola* proteins there is opposite orientation, with LysM at the N-terminal and M23 peptidase domain at the C-terminal ends. We speculate that the LysM domain is responsible for anchoring the protein to the cell surface environment of *T. denticola* through binding peptidoglycan-like material associated with the outer membranes. Such a function for LysM sequences has been shown for surface protein Sep of *Lactobacillus fermentum* BR11 (Turner *et al*., 2004) and the Aae autolysin of *Staphylococcus epidermidis* (Heilmann *et al*., 2003).

Bacteria that produce M23 peptidase domain bacteriolytic proteins all appear to produce immunity factors. These factors protect the producer organism from self-peptidoglycanolysis. Some immunity factors (Sugai *et al*., 1997b; Beatson *et al*., 1998) are enzymic and replace peptidoglycan glycines with serines (DeHart *et al*., 1995; Heath Farris *et al*., 2003), which prevents successful targeting of the substrate-binding domain and inhibiting hydrolysis (Lai *et al*., 2002). The genes encoding for immunity factors are commonly adjacent to the bacteriocin peptidase gene (Fig. 5B) although they may not always be in the same orientation (Recsei *et al*., 1987; Heath *et al*., 1989, 2004; Sugai *et al*., 1997b). Analysis of the *T. denticola* genome shows that immediately downstream of TDE1738, the gene TDE1739 encodes a protein with similarity to lysostaphin immunity factor (Fig. 5B). The TDE1739 gene is annotated within the genome as encoding a FemAB homologue. FemAB imparts resistance to methicillin by altering the glycine ratio of peptidoglycan in a similar manner to other immunity factors (Maidhof *et al*., 1991; Heath *et al*., 2005). This strongly suggests that TDE1738 could be a bacteriocin, with the gene product of TDE1739 necessary to protect *T. denticola* from autolysis. The periodontal pocket is a highly competitive environment for bacteria and *T. denticola* bacteriocins could lyse organisms to release otherwise unavailable nutrients. It has been shown that *Prevotella intermedia*, *Eubacterium nodatum*, *Veillonella parvula* and *Fusobacterium nucleatum* all stimulate growth of *T. denticola* (ter Steeg & van der Hoeven, 1990). The possibility that *T. denticola* has the ability to lyse anaerobic bacteria is currently being investigated along with attempted genetic knockouts of the relevant genes to further develop functional analyses.

The comparative analyses carried out in this article have identified seven *T. denticola* proteins that show various degrees of homology to Tp0155. Further investigation of the *T. pallidum* genome reveals that six of these *T. denticola* proteins have corresponding homologous proteins in *T. pallidum* (Table S2). Close phylogenetic relationships between individual *T. pallidum* and *T. denticola* proteins is retained, with each containing similar structural arrangements of LysM and M23 peptidase domains (Table S2). These observations suggest that this gene set is functionally conserved within *Treponema*. The *T. pallidum* genome also contains a gene, Tp0806, that encodes a protein with homology to lysostaphin immunity factor. The ZoocinA immunity factor (Zif) acts as a penicillin-binding protein (Heath *et al*., 2004) capable of peptidoglycan reorganization, and downstream and in the same orientation as the *T. pallidum* M23 peptidase gene Tp0706 (homologous to TDE1738) is a gene, Tp0705 (Fig. 5C), annotated as a penicillin-binding protein, suggesting a role in protection from autolysis.

In summary, this paper describes a family of seven conserved *T. denticola* genes. The products of five of these genes bind fibronectin and TDE2318, the orthologue of *T. pallidum* protein Tp0155, is cell-surface exposed. Six of the *T. denticola* genes have orthologues in *T. pallidum*. Based on sequence similarities, and genetic organization of adjacent genes, one of these polypeptides (TDE1738), and the orthologue *T. pallidum* (Tp0706), may be a bacteriocin. The other proteins may have autolytic or peptidoglycan remodelling activities in

addition to recognizing fibronectin. It will be important to determine that these molecules are expressed *in vivo* and if their expression correlates with virulence or disease severity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990; 215:403–10. [PubMed: 2231712]

2. Baba T, Schneewind O. Target cell specificity of a bacteriocin molecule: a C-terminal signal directs lysostaphin to the cell wall of *Staphylococcus aureus*. EMBO J. 1996; 15:4789–97. [PubMed: 8890152]

3. Bamford CV, Fenno JC, Jenkinson HF, Dymock D. The chymotrypsin-like protease complex of *Treponema denticola* ATCC 35405 mediates fibrinogen adherence and degradation. Infect Immun. 2007; 75:4364–72. [PubMed: 17591786]

4. Beatson SA, Sloan GL, Simmonds RS. Zoocin A immunity factor: a *femA*-like gene found in group C streptococcus. FEMS Microbiol Lett. 1998; 163:73–7. [PubMed: 9631548]

5. Brinkman MB, McGill MA, Pettersson J, et al. A novel *Treponema pallidum* antigen, Tp0136, is an outer membrane protein that binds human fibronectin. Infect Immun. 2008; 76:1848–57. [PubMed: 18332212]

6. Cameron CE. Identification of a *Treponema pallidum* laminin binding protein. Infect Immun. 2003; 71:2525–33. [PubMed: 12704124]

7. Cameron CE, Brown EL, Kuroiwa JM, Schnapp LM, Brouwer NL. *Treponema pallidum* fibronectin-binding proteins. J Bacteriol. 2004; 186:7019–22. [PubMed: 15466055]

8. Cameron CE, Kuroiwa JMY, Yamada M, Francescutti T, Chi B, Kuramitsu HK. Heterologous expression of the *Treponema pallidum* laminin-binding adhesin Tp0751 in the culturable spirochete *Treponema phagedenis*. J Bacteriol. 2008; 190:2565–71. [PubMed: 18263731]

9. Carroll SA, Hain T, Technow U, et al. Identification and characterisation of a peptidoglycan hydrolase, MurA, of *Listeria monocytogenes*, a muraminidase needed for cell separation. J Bacteriol. 2003; 185:6801–8. [PubMed: 14617644]

10. Chan ECS, Siboo R, Keng T, et al. *Treponema denticola* (ex Brumpt 1925) sp. nov., nom. rev., and identification of new spirochete isolates from periodontal pockets. Int J Sys Bacteriol. 1993; 43:196–203.

11. Choi BK, Paster BJ, Dewhirst FE, Gobel UB. Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis. Infect Immun. 1994; 62:1889–95. [PubMed: 8168954]

12. Couchman JR, Austria MR, Woods A. Fibronectin-cell interactions. J Invest Dermatol. 1990; 94:7S–14S. [PubMed: 2191056]

13. Dawson JR, Ellen RP. Tip-oriented adherence of *Treponema denticola* to fibronectin. Infect Immun. 1990; 58:3924–8. [PubMed: 2254019]

14. DeHart HP, Heath HE, Heath LS, LeBlanc PA, Sloan GL. The lysostaphin endopeptidase resistance gene (epr) specifies modification of peptidoglycan cross bridges in *Staphylococcus simulans* and *Staphylococcus aureus*. Appl Env Microbiol. 1995; 61:1475–9. [PubMed: 7747966]

15. Dewhirst FE, Tamer MA, Ericson RE, et al. The diversity of periodontal spirochetes by 16S rRNA analysis. Oral Microbiol Immunol. 2000; 15:196–202. [PubMed: 11154403]

16. Dziewanowska K, Patti JM, Deobald CF, Bayles KW, Trumble WR, Bohach GA. Fibronectin binding protein and host cell tyrosine kinase are required for internalisation of *Staphylococcus aureus* by epithelial cells. Infect Immun. 1999; 67:4673–8. [PubMed: 10456915]

17. Edwards AM, Jenkinson HF, Woodward MJ, Dymock D. Binding properties and adhesion-mediating regions of the major sheath protein of *Treponema denticola* ATCC 35405. Infect Immun. 2005; 73:2891–8. [PubMed: 15845495]

18. Ellen RP, Song M, McCulloch CA. Degradation of endogenous plasma membrane fibronectin concomitant with *Treponema denticola* 35405 adhesion to gingival fibroblasts. Infect Immun. 1994; 62:3033–3037. [PubMed: 8005694]

19. Fenno JC, Muller KH, McBride BC. Sequence analysis, expression, and binding activity of recombinant major outer sheath protein (Msp) of *Treponema denticola*. J Bacteriol. 1996; 178:2489–97. [PubMed: 8626313]

20. Fenno JC, Tamura M, Hannam PM, Wong GW, Chan RA, McBride BC. Identification of a *Treponema denticola* OppA homologue that binds host proteins present in the subgingival environment. Infect Immun. 2000; 68:1884–92. [PubMed: 10722578]

21. Fenno JC, Wong GW, Hannam PM, McBride BC. Mutagenesis of outer membrane virulence determinants of the oral spirochete *Treponema denticola*. FEMS Microbiol Lett. 1998; 163:209–15. [PubMed: 9673024]

22. Haapasalo M, Muller KH, Uitto VJ, Leung WK, McBride BC. Characterization, cloning, and binding properties of the major 53-kilodalton *Treponema denticola* surface antigen. Infect Immun. 1992; 60:2058–5. [PubMed: 1563796]

23. Haapasalo M, Singh U, McBride BC, Uitto VJ. Sulfhydryl-dependent attachment of *Treponema denticola* to laminin and other proteins. Infect Immun. 1991; 59:423–7.

24. Heath Farris M, Heath LS, Heath HE, LeBlanc PA, Simmonds RS, Sloan GL. Expression of the genes for lysostaphin and lysostaphin resistance in streptococci. FEMS Microbiol Lett. 2003; 288:115–19. [PubMed: 14612246]

25. Heath HE, Heath LS, Nitterauer JD, Rose KE, Sloan GL. Plasmid-encoded lysostaphin endopeptidase resistance of *Staphylococcus simulans* biovar *staphylolyticus*. Biochem Biophys Res Comm. 1989; 15:1106–9. [PubMed: 2730641]

26. Heath LS, Gargis SR, Smithberg SR, et al. Plasmid-specified FemBX-like immunity factor in *Staphylococcus sciuri* DD4747. FEMS Microbiol Lett. 2005; 249:227–31. [PubMed: 16006076]

27. Heath LS, Heath HE, LeBlanc PA, et al. The streptococcolytic enzyme ZoocinA is a penicillin-binding protein. FEMS Microbiol Lett. 2004; 236:205–11. [PubMed: 15251198]

28. Heilmann C, Hartleib J, Hussain MS, Peters G. The multifunctional *Staphylococcus aureus* autolysin, Aaa, mediates adherence to immobilised fibrinogen and fibronectin. Infect Immun. 2005; 73:4793–802. [PubMed: 16040992]

29. Heilmann C, Niemann S, Sinha B, Herrmann M, Kehrel BE, Peters G. *Staphylococcus aureus* fibronectin-binding protein (FnBP)-mediated adherence to platelets, and aggregation of platelets induced by FnBPA but not by FnBPB. J Infect Dis. 2004; 190:321–9. [PubMed: 15216468]

30. Heilmann C, Thumm G, Chhatwal GS, Hartleib J, Uekotter A, Peters G. Identification and characterisation of a novel autolysin (Aae) with adhesive properties from *Staphylococcus epidermidis*. Microbiology. 2003; 149:2769–78. [PubMed: 14523110]

31. Izard J, Hsieh CE, Limberger RJ, Mannella CA, Marko M. Native cellular architecture of *Treponema denticola* revealed by cryo-electron tomography. J Struct Biol. 2008; 163:10–17. [PubMed: 18468917]

32. Izard J, Renken C, Hsieh CE, et al. Cryo-electron tomography elucidates the molecular architecture of *Treponema pallidum*, the syphilis spirochete. J Bacteriol. 2009; 191:7566–80. [PubMed: 19820083]

33. Kigure T, Saito A, Seida K, Yamada S, Ishihara K, Okuda K. Distribution of *Porphyromonas gingivalis* and *Treponema denticola* in human subgingival plaque at different periodontal pocket depths examined by immunohistochemical methods. J Periodontal Res. 1995; 30:332–41. [PubMed: 7494175]

34. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227:680–5. [PubMed: 5432063]

35. Lai AC, Tran S, Simmonds RS. Functional characterisation of domains found within a lytic enzyme produced by *Streptococcus equi* subsp. *zooepidemicus*. FEMS Microbiol Lett. 2002; 215:133–8. [PubMed: 12393213]

36. Maidhof H, Reinicke B, Blumel P, Berger-Bachi B, Labischinski H. *femA*, which encodes a factor essential for expression of methicillin resistance, affects glycine content of peptidoglycan in methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains. J Bacteriol. 1991; 173:3507–13. [PubMed: 2045371]

37. Naverre WW, Schneewind O. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol Rev. 1999; 63:174–229.

38. Nelson K, Selander RK. Analysis of genetic variation by polymerase chain reaction-based nucleotide sequencing. Methods Enzymol. 1994; 235:174–83. [PubMed: 8057894]

39. Pasula R, Wisniowski P, Martin WJ 2nd. Fibronectin facilitates *Mycobacterium tuberculosis* attachment to murine alveolar macrophages. Infect Immun. 2002; 70:1287–92. [PubMed: 11854212]

40. Peterson KM, Baseman JB, Alderete JF. *Treponema pallidum* receptor binding proteins interact with fibronectin. J Exp Medicine. 1983; 157:1958–70.

41. Pracht D, Elm C, Gerber J, et al. PavA of *Streptococcus pneumoniae* modulates adherence invasion and meningeal inflammation. Infect Immun. 2005; 73:2680–9. [PubMed: 15845469]

42. Recsei PA, Gruss AD, Novick RP. Cloning, sequence and expression of the lysostaphin gene from *Staphylococcus simulans*. Proc Natl Acad Sci USA. 1987; 84:1127–31. [PubMed: 3547405]

43. Riviere GR, DeRouen TA. Association of oral spirochetes from periodontally healthy sites with development of gingivitis. J Periodontol. 1998; 69:496–501. [PubMed: 9609381]

44. Sambrook, J.; Fritsch, EF.; Maniatis, T. Molecular Cloning: a Laboratory Manual. 2. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1989.

45. Samen U, Gottschalk B, Eikmanns BJ, Reinscheid DJ. Relevance of peptide uptake systems to the physiology and virulence of *Streptococcus agalactiae*. J Bacteriol. 2004; 186:1398–408. [PubMed: 14973032]

46. Schindler CA, Schuhardt VT. Lysostaphin: a new bacteriolytic agent for the staphylococcus. Proc Natl Acad Sci USA. 1964; 51:414–21. [PubMed: 14171453]

47. Schindler CA, Schuhardt VT. Purification and properties of lysostaphin – a lytic agent of *Staphylococcus aureus*. Biochem Biophys Acta. 1965; 97:242–50. [PubMed: 14292833]

48. Schwarz-Linek U, Hook M, Potts JR. The molecular basis of fibronectin-mediated bacterial adherence to host cells. Mol Microbiol. 2004; 52:631–41. [PubMed: 15101971]

49. Seshadri R, Myers GS, Tettelin H, et al. Comparison of the genome of the oral pathogen *Treponema denticola* with other spirochete genomes. Proc Natl Acad Sci USA. 2004; 101:5646–51. [PubMed: 15064399]

50. Simmonds RS, Simpson WJ, Tagg JR. Cloning and sequence analysis of *zooA*, a *Streptococcus zooepidemicus* gene encoding a bacteriocin-like inhibitory substance having a domain structure similar to that of lysostaphin. Gene. 1997; 189:255–61. [PubMed: 9168135]

51. Simmonds RS, Pearson L, Kennedy RC, Tagg JR. Mode of action of a lysostaphin-like bacteriolytic agent produced by *Streptococcus zooepidemicus* 4881. Appl Env Microbiol. 1996; 62:4536–41. [PubMed: 8953725]

52. Sinha B, Francois PP, Nusse O, et al. Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin α5β1. Cell Microbiol. 1999; 1:101–17. [PubMed: 11207545]

53. Steen A, Buist G, Leenhouts KJ, et al. Cell wall attachment of a widely distributed peptidoglycan binding domain is hindered by cell wall constituents. J Biol Chem. 2003; 278:23874–81. [PubMed: 12684515]

54. Sugai M, Fujiwara T, Akiyama T, et al. Purification and molecular characterisation of glycineglycine endopeptidase produced by *Staphylococcus capitis* EPK1. J Bacteriol. 1997a; 179:1193–202. [PubMed: 9023202]

55. Sugai M, Fujiwara T, Ohta K, Komatsuzawa H, Ohara M, Suginaka H. *epr*, which encodes glycylglycine endopeptidase resistance, is homologous to *femAB* and affects serine content of

peptidoglycan cross bridges in *Staphylococcus capitis* and *Staphylococcus aureus*. J Bacteriol. 1997b; 179:4311–18. [PubMed: 9209049]

56. ter Steeg PF, van der Hoeven JS. Growth stimulation of *Treponema denticola* by periodontal microorganisms. Antonie Van Leeuwenhoek. 1990; 57:63–70. [PubMed: 2321929]

57. Thomas DD, Baseman JB, Alderete JF. Enhanced levels of attachment of fibronectin-primed *Treponema pallidum* to extracellular matrix. Infect Immun. 1986; 52:736–41. [PubMed: 3519458]

58. Thumm G, Gotz F. Studies on prolysostaphin processing and characterization of the lysostaphin immunity factor (Lif) of *Staphylococcus simulans* biovar *staphylolyticus*. Mol Microbiol. 1997; 23:1251–65. [PubMed: 9106216]

59. Turner MS, Hafner LM, Walsh T, Giffard PM. Identification and characterisation of the Novel LysM domain-containing surface protein Sep from *Lactobacillus fermentum* BR11 and its use as a peptide fusion partner in *Lactobacillus* and *Lactococcus*. Appl Env Microbiol. 2004; 70:3673–80. [PubMed: 15184172]

60. Umemoto T, Nakatani Y, Nakamura Y, Namikawa I. Fibronectin-binding proteins of a human oral spirochete *Treponema denticola*. Microbiol Immunol. 1993; 37:75–8. [PubMed: 8474361]
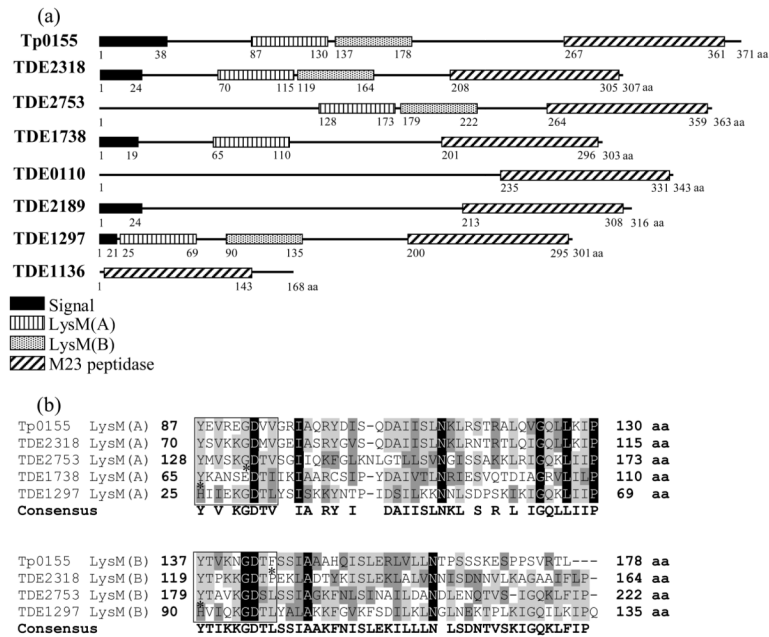
Figure 1.
(A) Predicted identifiable peptide motifs (www.ebi.ac.uk/InterProScan/) of *Treponema pallidum* fibronectin-binding protein Tp0155 and potential orthologues identified from the *Treponema denticola* genome using BLAST similarity searches. LysM domains are shown with vertical bars (A) or dots (B). *Treponema pallidum* and *T. denticola* M23 peptidase domains (diagonal bars) retain 37% identity. Potential signal sequences (solid black) were identified using http://bioinformatics.leeds.ac.uk/prot_analysis/signal.html. (B) Alignments of LysM(A) and LysM(B) domain sequences from *T. pallidum* Tp0155 and potential *T. denticola* orthologues showing high degree of consensus across the different peptide sequences. Degree of identity is shown from highest (e.g. ▮) to lowest (e.g. H). The LysM motif **Y**xxxx**G**xx(**Hyd**) (Turner *et al.*, 2004) is boxed. Asterisk indicates residues that do not fit the specified motif.
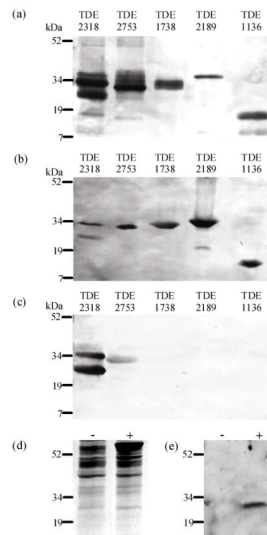
Figure 2.
Recombinant *Treponema denticola* His-tagged proteins (5μg), which show homology to fibronectin-binding protein Tp0155 of *Treponema pallidum*, purified using nickel affinity chromatography, analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) stained with silver nitrate (A), and by Western blot, using anti-tetra His antibodies to detect each protein (B) or antibodies to the *T. pallidum* protein Tp0155, which had first been pre-adsorbed with *T. phagedenis* (Kazan) (C). *Treponema denticola* ATCC35405 outer membrane proteins extracted with 1% Triton X-114 incubated at 20°C or 100°C before separation by SDS–PAGE analyses (d) exposed to antibodies to *T. pallidum* Tp0155 (E). Molecular mass standards (kDa) are indicated to the side of the figure.
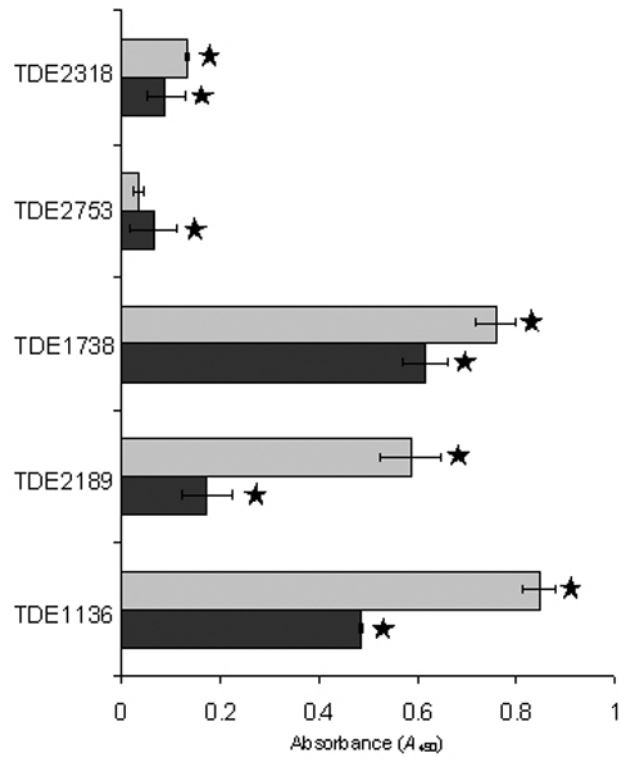
Figure 3.
Adhesion of recombinant *Treponema denticola* proteins to matrix (grey bars) or plasma fibronectin (black bars). Recombinant protein (5 μg) was added to each well, except for TDE2753 and TDE1738, which appeared to bind non-specifically to the substrates when more than 1.25μg was added. Stars denote significant difference ($P < 0.05$) between adhesion to either matrix or plasma fibronectin and bovine serum albumin-blocked wells. Error bars indicate ± SD of the mean from triplicates of a representative experiment.
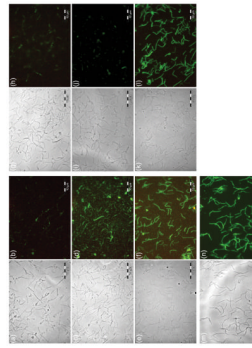
Figure 4.
Detection of proteins on the surface of *Treponema denticola* ATCC 35405 using antibodies to the *Treponema pallidum* protein Tp0155 which had first been pre-adsorbed against *Treponema phagedenis* (Kazan). (A, C, E, G, I, K and M) Phase-contrast images; (B, D, F, H, J, L and N) corresponding immunofluorescence. (A, B) Preimmune serum for Tp0155-specific antibodies; (C, D), Tp0155-specific antibodies, non-detergent-treated *T. denticola*; (E, F), Tp0155-specific antibodies, Triton X-114-treated *T. denticola*; (G, H), preimmune serum for Tp0249-specific antibodies; (I, J), Tp0249-specific antibodies, non-detergent-treated *T. denticola* (negative control); (K, L), Tp0249-specific antibodies, Triton X-114-treated *T. denticola*; (M, N), anti-*T. denticola* antibodies (control) (Edwards *et al.*, 2005).

```
TDE1738M23 (202) HPGIDIAAPTGSPVMACAAGRVKEISYNK-------VYGNYIILTHTDG
Lysostaphin (279) HYGVDFFMNIGTPVKAISSGKIVEAGWSNYG------GGNQIGLIENDG
   Zoocin A (45) HVGVDYAVPVGTPVRAVANGTVKFAGNGANHPWMLWMAGNCVLIQHADG
        ALE1 (150) HYGVDFFMNVGTPVRAISDGKIVEAGWTNYG------GGNEIGLVENDG
   Consensus      HYGVDFFMPVGTPVRAIAAGKIVEAGWSNYG      MGGN IGLIHNDG


TDE1738M23 (243) RA-SLYGHLSKVYASLNETIKSGTIIGAVGSTGMSTGPHLHFEIHEQGI
Lysostaphin (323) VHRQWYMHLSKYNVKVGDYVKAGQIIGWSGSTGYSTAPHLHFQ------
   Zoocin A (95) MH-TGYAHLSKISVSTDSTVKQGQIIGYTGATGQVTGPHLHFEMLPANP
        ALE1 (194) VHRQWYMHLSKFNVKVGDRVKAGQIIGWSGSTGYSTAPHLHFQRMTNSF
   Consensus      VHRQWYMHLSKVNSVGDTVKAGQIIGWSGSTGYSTAPHLHFQIL N


TDE1738M23 (293) PKNPA
Lysostaphin  (-) -----
   Zoocin A  (-) -----
        ALE1 (243) SNNTA
   Consensus        N A
```
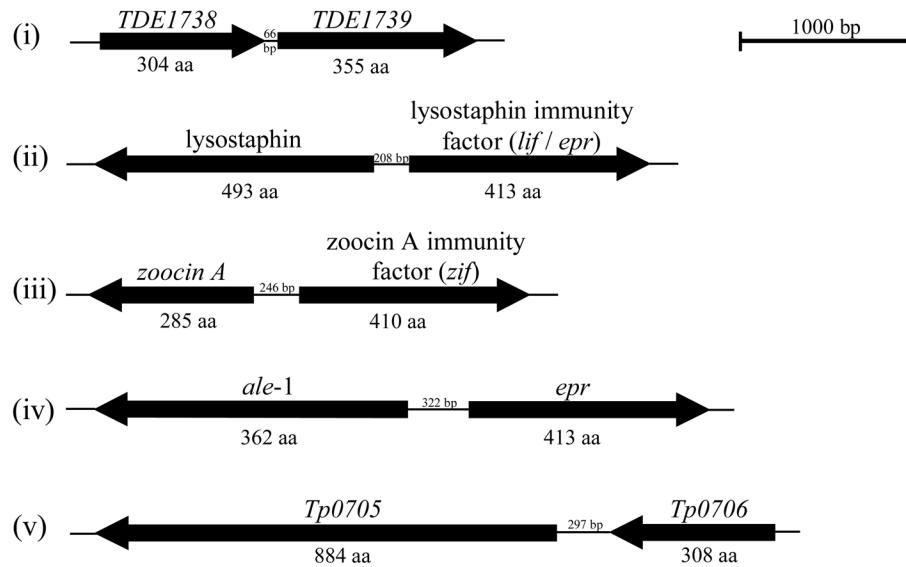
(b)

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        Lif (323) EVYYLSSGSNPKYNAYMGAYRLQWEMIQFAKNKGINRYNFYGITG-DFS
    TDE1739 (252) KLYYPYGASSNSHRELMPNNLIMWKAIQYGKKLNCSVFDLWGCLGPNPD
   Consensus      LYY  AS  H  M    I W  IQFAK      F  WG  G


        Lif (372) EDAEDFGVQKFKEGFNAHVEEYVGDFIKPIKPLFYKIHQLLNR
    TDE1739 (302) TENSWYGFHKFKAGYNPQLVEYIGTFDFVYKPFMYKLFNLADK
   Consensus      D   FG  KFK GFN  L EYIG F   KP YKI NL  K
```

(c)

(i) TDE1738 66 bp TDE1739
304 aa   355 aa   1000 bp

(ii) lysostaphin   lysostaphin immunity factor (*lif* / *epr*)
493 aa   208 bp   413 aa

(iii) *zoocin A*   zoocin A immunity factor (*zif*)
285 aa   246 bp   410 aa

(iv) *ale*-1   *epr*
362 aa   322 bp   413 aa

(v) *Tp0705*   *Tp0706*
884 aa   297 bp   308 aa

Figure 5.
(A) Alignments of M23 peptidase domains from TDE1738 of *Treponema denticola* and homologous regions of peptidoglycan-degrading enzymes lysostaphin from *Staphylococcus simulans* biovar *staphylolyticus*, zoocin A from *Streptococcus equi* subsp *zooepidemicus* and ALE-1 from *Staphylococcus capitis*. The M23 peptidase domain of TDE1738 retains 43% identity to lysostaphin, 43% identity to zoocin A and 40% identity to ALE-1 with conservation across the HxGxD and HLH active sites. (B) Alignments of a 92 amino acid (aa) residue portion regions of TDE1739 of *T. denticola* and lysostaphin immunity factor from *S. simulans* biovar *staphylolyticus*, demonstrating homology (29% identity) between these peptides. Sequence consensus is shown as is degree of identity from highest (e.g. ◼) to lowest (e.g. A). (C) Diagram depicting genes encoding potential and known peptidoglycan

degradation enzymes and adjacent immunity factors which provide protection from autolysis by the peptidoglycanolytic enzyme. (i) The chromosomal gene TDE1738 from *T. denticola* encodes for a potential peptidoglycan-degrading enzyme, and TDE1739 for a potential immunity factor. (ii) Lysostaphin and adjacent lysostaphin immunity factor (Lif/Epr) are encoded on the plasmid pACK1 of *S. simulans* biovar *staphylolyticus*. (iii) The gene encoding *zoocin* A and corresponding zoocin A immunity factor (Zif) is on the chromosome of *Streptococcus equi* subsp *zooepidemicus*, while (iv) *ale*-1 and adjacent endopeptidase resistance (*epr*) genes are located on a plasmid in *Staphylococcus capitis*. (v) Tp0706 from the *Treponema pallidum* chromosome, with homology to TDE1738 may also be an enzyme with peptidoglycan-degrading activity and the adjacent gene Tp0705 is annotated as a pencillin-binding protein in the same manner as zoocin A immunity factor (Zif).

Table 1

Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source
Treponema denticola		
ATCC 35405	Type strain	(Chan *et al.*, 1993)
Escherichia coli		
XL-1 Blue	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac* [*F 'proAB lacI^{q}ZΔM15 Tn10* (Tet^R)]	Stratagene
M15	(pREP-4 Kn^R) *nal^s str^s rif^s thi$^-$ lac$^-$ ara$^+$ gal$^+$ mtl$^-$F$^-$ recA$^+$ uvr$^+$ lon$^+$*	Qiagen
Plasmids		
pQE30	*bla* ColE1*ori* (Ap^R)	Qiagen
pQE30-TDE2318	*bla* ColE1*ori*	This study
pQE30-TDE2753	*bla* ColE1*ori*	This study
pQE30-TDE1738	*bla* ColE1*ori*	This study
pQE30-TDE0110	*bla* ColE1*ori*	This study
pQE30-TDE2189	*bla* ColE1*ori*	This study
pQE30-TDE1136	*bla* ColE1*ori*	This study
pQE30-TDE1297	*bla* ColE1*ori*	This study

Table 2

Comparative bioinformatic analyses of *Treponema pallidum* (Tp) fibronectin-binding protein Tp0155 and *Treponema denticola* (TDE) homologues

| ORF | aa residues | Predicted molecular mass (Da) | % identity to Tp0155 | | | |
			Complete	LysM(A)[1]	LysM(B)[2]	M23[3]
Tp0155	**371**	**40,664**	**100**	**100**	**100**	**100**
TDE 2318	307	33,607	42.8	73.3	31.4	61.1
TDE 2753	363	39,731	29.7	34.8	26.9	52.6
TDE 1738	303	33,766	26.7	33.3	_[4]	43.4
TDE 0110	343	38,172	23.6	–	–	36
TDE 2189	316	36,052	20.8	–	–	34.3
TDE 1297	301	32,820	16.8	28.9	18	23.6
TDE 1136	168	18,642	13.9	–	–	20.4

[1]The LysM domain located closest to the N terminus (Fig. 1) has been designated LysM(A).

[2]Where a second LysM domain sequence was identified (Fig. 1), it was assigned the title LysM(B). % identity of *T. denticola* LysM sequences were calculated through comparison with the most similar domains in Tp0155.

[3]The eight polypeptides all carry an M23 peptidase domain, see Fig. 1A.

[4]Indicates no LysM domain present