Putative β-barrel outer membrane proteins of the bovine digital dermatitis-associated treponemes: identification, functional characterisation and immunogenicity.

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Abbreviations: BDD, bovine digital dermatitis; OMP, outer membrane protein

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Abstract. Bovine Digital Dermatitis (BDD), an infectious disease of the bovine foot with a predominant treponemal aetiology, is a leading cause of lameness in dairy and beef herds worldwide. BDD is poorly responsive to antimicrobial therapy and exhibits a relapsing clinical course; an effective vaccine is therefore urgently sought. Using a ‘reverse vaccinology’ approach, the present study surveyed the genomes of the three BDD-associated *Treponema* phylogroups for putative β-barrel outer membrane proteins and considered their potential as vaccine candidates. Selection criteria included the presence of a signal peptidase I cleavage site, a predicted β-barrel fold and cross-phylogroup homology. Four candidate genes were overexpressed in *Escherichia coli* BL21 (DE3), refolded and purified. Consistent with their classification as β-barrel OMPs, circular dichroism spectroscopy revealed the adoption of a predominantly β-sheet secondary structure. These recombinant proteins, when screened for their ability to adhere to immobilised ECM components, exhibited a diverse range of ligand specificities. All four proteins specifically and dose-dependently adhered to bovine fibrinogen. One recombinant protein was identified as a candidate diagnostic antigen (disease specificity, 75%). Finally, when adjuvanted with aluminium hydroxide and administered to BDD-naïve calves using a prime-boost vaccination protocol, these proteins were immunogenic, eliciting specific IgG antibodies. In summary, we present the description of four putative treponemal β-barrel OMPs that exhibit the characteristics of multispecific adhesins. The observed interactions with fibrinogen may be critical to host colonisation and dissemination and it is hypothesised that vaccination-induced antibody blockade of these interactions will impede treponemal virulence and thus be of therapeutic value.
Introduction. Bovine digital dermatitis (BDD) is a painful, ulcerative disease of the bovine foot and a significant cause of lameness in dairy cattle. Clinically, BDD presents as a malodorous, focally-inflamed, circumscribed lesion of raised hyperkeratotic skin localised to the plantar/palmer aspect of the interdigital cleft, on or adjacent to the coronary band (1, 2). BDD is now considered to be the most common infectious cause of lameness in dairy cattle herds in the northern hemisphere and one of the most significant challenges to farm animal welfare. Moreover, the economic burden to the dairy industry resulting from production losses is considerable (3). In the UK, BDD is endemic, affecting an estimated 79% of UK dairy farms (4), and emergence of BDD in beef herds has also been recently described (5, 6). In addition, a new variant (contagious ovine digital dermatitis), noted for its particularly severe presentation in sheep, continues to spread through the UK’s national flock (7, 8). This disease therefore represents an additional and growing challenge to global food security. A substantial body of evidence supports the involvement of multiple *Treponema* spp. at various stages of BDD lesion development (9–12). Three treponeme taxa in particular have been consistently isolated from lesion biopsy material from cases in the US and the UK, namely the *Treponema medium* phylogroup, the *Treponema phagedenis* phylogroup and *Treponema pedis* (13, 14). The presence of these organisms deep within the lesion (15, 16), their clear association with necrotic changes in infected tissue (17), a failure to isolate these organisms from the feet of healthy animals (12, 18) and a disease-associated, specific IgG antibody response to these organisms (19–23) strongly implies an aetiopathogenic role in BDD.
Treponemes are gram-stain-negative bacteria exhibiting a spiral morphology and consist of an outer membrane (OM) that surrounds the axial filaments and the protoplasmic cylinder. The OM of these extracellular pathogens is a feature of considerable interest, given its surface exposure and the subsequent involvement of its components in host-pathogen interactions. Adhesins embedded in the OM play a critical role in bacterial cytoadherence to the host during colonisation. The host extracellular matrix (ECM) is an important adherence target for pathogenic microorganisms during the primary stages of infection. Previous studies investigating the ECM binding capacity of the two most relevant human pathogenic species, *T. pallidum* sub. *pallidum*, the causative agent of syphilis, and *Treponema denticola*, a key member of the polymicrobial consortium implicated in periodontal disease, reported specific treponemal cell adherence to a range of immobilised ECM components, including fibronectin, laminin, fibrinogen and collagen (25, 26). Subsequently, a growing number of adhesins are being identified and investigated to characterise the molecular basis of physical host-pathogen interactions (27–35). However, the fastidious nature of the BDD-associated treponemes has precluded any detailed characterisation of the OM components likely to promote such interactions. Employing recently available BDD-associated treponeme genome sequences, we sought to identify novel β-barrel OMP-encoding genes and characterise the function of the recombinantly expressed OMPs in ECM binding assays.

**METHODS.**

**Ethical approval.**

All experimental work involving animals was covered by a UK Home Office Project License PPL 70/8330.
In *silico* identification of Outer Membrane Proteins (OMPs).

Previously generated and annotated representative genomes of the three BDD treponemes, *T. medium* T19 (Accession CP027017), *T. phagedenis* T320A (Accession CP027018) and *T. pedis* T3552B\(^T\) (Accession CP045760) were subjected to *in silico* analysis to identify putative OMPs via prediction of encoded β-barrel structural motifs. Putative Coding Sequence (CDS) features for each genome were translated to their amino acid sequences using Artemis (36). All translated *T. medium* T19 CDS features were analysed for the presence of a signal peptidase I cleavage site using SignalP 4.1 (37). Sequences predicted to harbour a signal peptide were further scrutinised for signatures of β-barrel tertiary structure using three β-barrel prediction programs (BOMP (38), TMBETA-NET (39) and PRED-TMBB (40)). All *T. medium* T19 CDS features which were predicted to code a β-barrel tertiary structured protein by at least one of the β-barrel prediction programs were retained. Homologues of putative *T. medium* T19 OMPs were identified in *T. phagedenis* T320A and *T. pedis* T3552B\(^T\) genomes using a combination of a Markov cluster algorithm (41) and BLAST (42) and their OMP predictions verified independently. Putative OMP sequences which were conserved in all three genomes were examined for predicted adhesin functionality using SPAAN (43) and their tertiary structures modelled using I-TASSER (44).

**Cloning and expression of candidate antigens.**

*T. medium* T19, *T. phagedenis* T320A and the *T. pedis* T3552B\(^T\), isolated previously from BDD lesion biopsies (12, 14) and cryopreserved in 10% (v/v) glycerol at -80 °C, were cultured as described previously (12). Genomic DNA (gDNA) was extracted from the treponeme cultures at late exponential phase using Chelex® 100 resin (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) according to a previously described method (45).
Technologies, Paisley, UK) was utilised for gene cloning and expression. Putative OMP sequences, lacking their signal peptide sequences, were amplified from the gDNA using high-fidelity Phusion polymerase (Thermo Scientific, Hemel Hempstead, UK) in accordance with manufacturer instructions. Primers (Table 1) for amplification contained CACC overhangs to facilitate entry cloning. A well-characterised OMP (OmpL1) from *Leptospira interrogans* serovar *Copenhageni* strain M20 was selected (46–48) and produced as a recombinant protein control.

Amplified putative OMPs were inserted into the Gateway entry plasmid pENTR™/D-TOPO™ (Life Technologies, Paisley, UK) in accordance with the manufacturer’s instructions prior to chemical transformation into *Escherichia coli* Top10 cells. Positive transformants were selected on LB agar plates containing kanamycin (50 µg/ml) and plasmid DNA from successful transformants isolated using the Qiagen Plasmid MiniPrep Kit (Qiagen, Manchester, UK). Successful amplicon insertion was confirmed using EcoRV restriction digest analysis (ThermoFisher, Horsham, UK). Inserts were thereafter cloned into the Gateway expression vector, pDEST™17, using a site-directed integration reaction in accordance with the manufacturer’s instructions (Life Technologies, Paisley, UK) prior to chemical transformation into DH5α *E. coli*. Positive transformants were selected on LB agar plates containing ampicillin (100 µg/ml) and the plasmid DNA isolated as previously described. pDEST™17-gene constructs verified by EcoRI endonuclease restriction digest analysis and Sanger sequencing (Source Bioscience, Nottingham, UK).

**Protein expression, refolding and purification**

All protein expression was performed in *E. coli* BL21 DE3 (Life Technologies, Paisley, UK). *E. coli* BL21 DE3 cultures were grown at 37°C with shaking in LB medium (2 L) containing
ampicillin (100 µg/ml), until the OD$_{600}$ was 0.8-1. Protein expression was induced by the addition of 1 mM IPTG (Sigma Aldrich, Gillingham, UK). Cultures were grown for a further 4-5 hours and cells harvested by centrifugation (3500 x g, 4°C, 10 mins). *E. coli* BL21 DE3 cell pellets were re-suspended in 50 mM Tris-HCl pH 7.9 (20ml per 10 g wet cell weight) containing lysozyme (5 mg/g wet cell weight) (Sigma Aldrich, Gillingham, UK) and incubated on ice for 30 min prior to cellular disruption using a micro-sonicator tip (Soniprep-150, MSE, London, UK). Inclusion bodies (IB) containing recombinant proteins were subsequently harvested by centrifugation (10000 x g, 4°C, 30 mins). IB pellets were re-suspended in 150 ml of IB detergent buffer (4% (v/v) Tergitol™ 15-S-9 (Sigma-Aldrich, Dorset, UK), 50 mM Tris HCl, pH 7.9) with rapid stirring for a minimum of two hours, washed twice in 150 ml IB wash buffer (50 mM Tris HCl, pH 7.9) and re-suspended in solubilisation buffer (6M Guanidine hydrochloride, 50 mM Tris-HCl pH 7.9 and 1 mM EDTA; 40 ml per 500 mg of IB) for 1 hour with constant agitation. The suspension was centrifuged (10, 000 x g, 4°C, 30 mins) to remove insoluble material. Recombinant protein refolding was performed by rapid dilution (49) into refolding buffer (250 mM NaCl, 50 mM Tris-HCl pH 7.9, 5% N,N-Dimethyldodecylamine N-oxide solution (LDAO; Sigma-Aldrich, Dorset UK)) and subsequently dialysed against 10 volumes of dialysis buffer (250 mM NaCl, 50 mM Tris-HCl pH 7.9, 0.1% LDAO). Refolded, recombinant proteins were purified by standard immobilised metal affinity chromatography (50), sterilised through a 0.2 µM filter and stored at -80°C.

The purity of the recombinant proteins was assessed by SDS-PAGE.

**Evaluation of secondary structure.**

**Heat modifiability.** The sensitivity of the recombinant proteins to denaturation upon heating was determined by comparing the electrophoretic mobility of the refolded recombinant...
proteins prepared in SDS sample buffer without reducing agent (100mM Tris-HCl [pH 6.8] 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20 % (v/v) glycerol) and either incubated at ambient temperature for 10 minutes or heated to 100°C for 10 minutes prior to SDS-PAGE analysis, as described previously (51).

Circular dichroism (CD) spectroscopy. Far UV CD spectroscopy was performed using a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan), equipped with a Peltier unit for temperature control. Spectra were measured from 190 to 260 nm using 1-mm path length cell at intervals of 0.5-nm and presented as an average of three scans. Spectra were analysed by Beta Structure Selection (BestSel) software (http://bestsel.elte.hu/) (52) to calculate the percentage secondary structure content from the ellipticity experimental data.

Evaluation of immunogenicity during natural infection.

Cattle sera

An ELISA was performed to investigate systemic IgG seroreactivity to the putative OMPs in blood samples collected from cows naturally infected with BBD. Sera from sixteen adult Holstein-Friesian cows with a recent (<6 month) history of BBD, were collected from a dairy herd situated in Cheshire, UK. Similarly, sera from 5 healthy adult Holstein-Friesian cows were collected from a closed dairy herd situated in Monmouthshire, UK, and were included as a control group. In all cases, whole blood was collected from the coccygeal vein. Following clotting and centrifugation, the serum fraction was harvested and stored at -20°C for serological assessment.

Sero logical ELISA
Non-activated, 96-well microtitre plates (Microplate Immulon 2HB, Thermo Scientific, Hemel Hempstead) were coated with a single recombinant protein (5 μg/ml) in PBS (pH 7.2) and incubated for 1 hour (37°C) and overnight (4°C). Unbound antigen was removed by washing with PBST (PBS-Tween 20; 0.05%). All sera were diluted 1/100 in PBST and pipetted into ELISA plate wells in duplicate and incubated for 1 hour (37°C). Wells were washed as before and incubated for 1 hour (37°C) with 100 μl monoclonal mouse anti-bovine immunoglobulin class G subclass 1 (IgG1) antibody, clone IL-A60 (BioRad, Hemel Hempstead, UK) or monoclonal mouse anti-bovine immunoglobulin class G subclass 2 (IgG2) antibody, clone IL-A2 (BioRad, Hemel Hempstead, UK), diluted 1:1000. To ensure adherence of the antigen to the plate, 100 μl of mouse monoclonal anti-polyhistidine antibody, clone HIS-1 (Sigma-Aldrich, Dorset, UK), diluted 1:2000 in PBST, was added to recombinant control wells. Following washing, wells were incubated with 100 μl of horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (Sigma-Aldrich, Dorset, UK), diluted 1:10000 in PBST for 1 hour (37°C). Following washing, the presence of HRP-conjugated goat anti-mouse IgG antibodies was detected by the addition of 100 μl of the HRP substrate, 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma-Aldrich, Dorset, UK). The reaction was terminated after ~20 minutes by the addition of 100 μl 0.5 M hydrochloric acid. The optical density (OD) of each well was read at 450 nm using a microplate reader (Multiskan EX; Thermo Fisher Scientific, Loughborough, UK). All data was processed and analysed using GraphPad Prism 5 (GraphPad, San Diego, CA). In order to classify results as positive or negative, an ELISA OD value of less than or equal to the mean plus 3 standard deviations of the control sera was considered to be non-reactive (53).

Evaluation of adhesin function.
All ECM macromolecules were purchased from Sigma Aldrich (Dorset, UK), and prepared from the following sources: Collagen I from bovine skin; elastin from bovine neck filament; fibrinogen from bovine plasma; heparan sulphate from bovine kidney; chondroitin sulphate from bovine cartilage; laminin-1 from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma.

**ECM-binding ELISA.**

An ELISA was performed to screen the recombinant proteins for their ability to attach to individual ECM macromolecules using a previously described method (54). Briefly, Immulon 2HB plates (ThermoFisher, Horsham, UK) were coated with 5 μg/ml of the ECM component or the negative control protein (BSA) by incubation for 1 hour at 37°C and overnight at 4°C, washed with PBS containing 0.05% Tween 20 (PBST) and blocked with a 1% (w/v) BSA solution. Recombinant proteins, diluted in PBST, were added at 10 μg/ml to screen for ECM binding activity and a range of concentrations (from 0 to 6 μM) to determine the dose-dependency of these binding interactions. Following incubation, bound recombinant proteins were detected by addition of mouse monoclonal anti-polyhistidine IgG antibody (Sigma-Aldrich, Dorset, UK), diluted 1:2000, before proceeding as described before. KD values were estimated from curves fitted by non-linear regression analysis in GraphPad Prism v. 5, using the following equation: $K_D = (A_{max}[protein]) / A - [protein]$, where A is the absorbance at a given protein concentration, $A_{max}$ is the maximum plate reader absorbance (when the equilibrium is reached), [protein] is the protein concentration and $K_D$ is the dissociation equilibrium constant (55, 56).

**Far-western blotting.**
A far-western blotting technique was employed to further characterise the specific binding interactions between the recombinant proteins and bovine fibrinogen (57). To dissociate native bovine fibrinogen into its constituent polypeptide chains (Aα, Bβ and γ), 60 μl of bovine fibrinogen stock solution (1 mg/ml) was mixed with 350 μl of gel loading buffer (100 mM Tris-Cl [pH 6.8] 4% SDS, 0.2% bromophenol blue, 20 % glycerol, 200 mM dithiothreitol, heated at 95°C for 5 minutes and separated in Tris-glycine polyacrylamide gels by SDS-PAGE (4-20 % gradient gel) at a constant voltage of 180 V for 50 minutes. The fibrinogen chains were electroblotted onto a nitrocellulose membrane (100 V, 240 mA, 120 minutes) and the membrane was blocked with 5% (w/v) skimmed milk. Membranes were subsequently incubated with 30 μg/ml recombinant protein. Any bound protein was detected by incubation of the membrane with mouse anti-polyhistidine antibody (Sigma-Aldrich, Dorset, UK), diluted 1:2000 followed by goat anti-mouse antibody (Sigma-Aldrich, Dorset, UK) and development in 3,3′-diaminobenzidine membrane substrate (Sigma-Aldrich, Dorset, UK).

**Immunogenicity trial in calves.**

**Calves.**

The immunogenicity of the recombinant proteins was evaluated in two Holstein-Friesian calves. A control group of two additional calves was used to verify that any serological response did not result from environmental exposure to BDD-associated treponemes or to ubiquitous antigens.

The four calves were reared and maintained according to routine agricultural practice at the University’s Farm with housing conditions having increased biosecurity to reduce the risk of exposure to the BDD-associated *Treponema* spp.. Calves were bedded on straw and...
quarantined for 4 weeks before vaccine administration. Calves were bled immediately prior to vaccine administration to ascertain pre-immunisation antibody status.

**Formulation of the vaccine.**

The multivalent vaccine was formulated to deliver the four recombinant proteins simultaneously and comprised of 100 µg of each recombinant protein and 40 µl aluminium hydroxide adjuvant (Rehydragel®, Chemtrade Logistics, Toronto, Ontario), adjusted to a final volume of 2 ml using PBS. All vaccines were administered subcutaneously to the left flank. Calves received an initial 2 ml dose of the vaccine followed by a 2 ml booster dose two weeks later. Concurrently, Control animals (n=2) received a 2 ml dose of the vehicle only. Blood samples, obtained by jugular venepuncture, were collected at two-week intervals for four weeks beginning with a pre-immunisation draw at day zero. Serum was retained for serological studies.

**Detection of serum IgG antibodies by ELISA.**

Vaccinee IgG1 and IgG2 antibody seroreactivity to the recombinant proteins was determined as described previously in this manuscript. All data was processed and analysed using GraphPad Prism 5 (GraphPad, San Diego, CA). ELISA reactivity was confirmed by Western blot analysis using previously described methods (23).

**RESULTS.**

**In silico detection of putative treponemal OMPs.**

SignalP 4.1 analysis identified 182 *T. medium* T19 CDS features predicted to contain an N-terminal peptidase I cleavage site. These features were further analysed by three β-barrel prediction programs: BOMP, PRED-TMBB and TMBETA-NET. CDS features predicted to
encode β-barrel proteins by at least one these programs were selected for cross-phylogroup homology detection. In total, fifteen CDS features identified in the *T. medium* T19 genome matched the following selection criteria precisely: i) the presence of a signal peptidase I cleavage site, ii) a predicted β-barrel topology and iii) cross-phylogroup homology (Table 2). Four CDS features, two from the *T. medium* genome and two from the *T. pedis* genome (including one homologous pair: C5N99_10335 and DYQ05_13425; amino acid sequence identity 31.87%) were subsequently selected to evaluate their ability to bind to selected ECM components and to induce an IgG antibody response in calves.

**Prediction of 3-dimensional (3D) tertiary structure.**

To generate predicted 3D structural models, each protein sequence was submitted to the I-TASSER server. The highest-ranking model for each protein is shown in Fig. 1. The four putative OMPs were each predicted to contain a typical β-barrel domain, consistent with localisation to the outer membrane of gram-negative bacteria. Typical β-barrel structures were predicted for C5N99_10335, DYQ05_13425 and DYQ05_06810, whereas C5N99_02965 was predicted to adopt a β-solenoid fold.

**Heat-modifiability assay.**

Three of the proteins expressed a heat-modifiable electrophoretic mobility, consistent with the stability of a protein comprising predominantly of a β-structure (66). Fig. 2 shows the change in electrophoretic mobility observed under unheated versus heated conditions for C5N99_10335, C5N99_02965 and DYQ05_06810.

Heat modification of C5N99_02965 (Fig. 2A) led to a change in electrophoretic mobility and a change in the apparent MW, from ~30 to ~36 kDa. Similar changes in mobility were
observed for C5N99_10335 (Fig 2B; ~22 to ~24 kDa) and DYQ05_06810 (Fig 2C; ~21 to 23 kDa). Heat modifiability was not identified for DYQ05_13425 (data not shown).

**CD spectroscopic analysis of the treponemal recombinant OMP secondary structure.**

Far UV CD spectroscopy was employed to provide further evidence of the secondary structure fold of these proteins (Fig. 3). Consistent with a predicted β-barrel tertiary structure state, analysis of the four putative OMPs yielded spectral signatures typical of a predominantly β-sheet secondary structure, with spectral minima occurring at approximately 218 nm for C5N99_10335, C5N99_02965, DYQ05_06810 and DYQ05_13425 as well as the positive control protein, OmpL1.

**Serological response to putative OMPs during natural BDD infection.**

As demonstrated in Fig 4, IgG2 seroreactivity to DYQ05_06810 was detected in BDD-infected Holstein Friesian cows (n=12; 75%) relative to the healthy control animals, with no apparent IgG2 response observed against the remaining three putative OMPs. No IgG1 response was identified against any of the putative OMPs under investigation. A statistically significant decrease in the IgG1 ELISA ODs was observed in the sera of BDD-exposed animals, relative to controls, in the *T. medium*/T19 putative OMP analyses (P<0.05).

**Binding of the treponemal OMPs to ECM components.**

Statistically significant (P<0.05) adherence to fibrinogen, elastin and heparan sulphate was observed for both C5N99_10335 and C5N99_02965, with C5N99_10335 additionally adhering to fibronectin (Fig. 5).
The T. pedis homologue of C5N99_10335, namely DYQ05_13425, exhibited a similar binding profile to C5N99_10335, but was found to additionally bind to chondroitin. DYQ05_06810 bound to fibronectin, fibrinogen, elastin, chondroitin and heparan sulphate.

Next, given the ubiquitous fibrinogen binding amongst these putative treponemal OMPs (P <0.01), we sought to further characterise this interaction across a concentration range. The results of these analyses are shown in Fig. 6.

The binding interactions between fibrinogen and C5N99_10335, C5N99_02965, DYQ05_06810 and DYQ05_13425 were observed to be concentration-dependent. Binding saturation levels were achieved with recombinant protein concentrations of ~1 µM, ~1.5 µM, and ~2.0 µM for C5N99_02965, DYQ05_13425 and DYQ05_06810, respectively. Conversely, C5N99_10335, although showing a tendency towards reaching saturation, failed to do so up to a concentration of 2 µM. Further examination of this interaction was precluded by insufficient protein yield. K₀ values were estimated by non-linear regression analysis of the binding curves. Table 4 summarises the KD values calculated from these experiments. The positive control protein, OmpL1, was similarly observed to adhere to bovine fibrinogen in a dose-dependent and saturable manner as previously reported (47) (data not shown).

Far western blotting.

Far Western blotting identified interactions between the putative OMPs and individual chains of the fibrinogen molecule. The results of this analysis are shown in Fig. 7.

The results of the far western analysis revealed that DYQ05_13425 and DYQ05_06810 interacted with all three chains of the fibrinogen molecule, similarly to OmpL1. Conversely,
C5N99_10335 and C5N99_02965 were observed to interact with the β and γ chains of fibrinogen only.

**Immunogenicity.**

The immunogenic properties of these four fibrinogen-binding proteins were investigated following inoculation into two naïve bull calves as part of a multivalent antigen cocktail. ELISA analysis of pre-vaccination sera yielded broadly comparable ELISA ODs between calves.

This pilot immunogenicity trial demonstrated that this subcutaneous prime-boost vaccination protocol, involving an aluminium hydroxide-adjuvanted 100 µg dose of each treponemal recombinant OMP, is capable of eliciting IgG antibodies in BDD-naïve bull calves (Fig. 8). All animals vaccinated with the recombinant protein cocktail seroconverted rapidly, permitting treponemal OMP-specific IgG antibody detection by day 14. A second booster vaccination on day 14 enhanced the IgG response further and specific antibody titres peaked at day 28. No seroconversion was detected in the control animals up to day 56 (the last day of the trial; data not shown). The fold-change, calculated as the mean OD change on day 28 from pre-immunisation baseline (Table 5) was used to account for varying baseline ELISA ODs.

Whilst all recombinant proteins were shown to be immunogenic under the conditions of this trial, C5N99_10335 was found to be the weakest driver of both an IgG1 and IgG2 antibody response, with ELISA OD fold changes calculated to be 2.37 and 1.66 from baseline, respectively. DYQ05_13425, conversely, was found to the most potent immunogen, with ELISA OD fold-changes from baseline calculated to be 7.44 and 9.10, for IgG1 and IgG2, respectively. The specificity of the anti-sera was confirmed using Western Blotting and...
revealed the presence of treponemal putative OMP-specific IgG1 and IgG2 serum antibodies in immunised animals only (data not shown). Serological analysis on day 42 of the trial revealed a slight waning of the IgG antibody response in all cases. The trial vaccine was well tolerated in vaccinated calves.

DISCUSSION.

Using a bioinformatics-based approach, the present study sought to identify and characterise novel BDD-associated treponemal OMPs, bearing in mind the potential value of these molecules as BDD vaccine candidates. Post-purification analysis of the four refolded proteins by CD spectroscopy yielded spectra indicative of a predominantly β-strand secondary structure with three of the proteins (C5N99_10335, C5N99_02965 and DYQ05_06810) additionally demonstrated to be heat-modifiable. These findings are consistent with the characteristics of proteins with a β-barrel tertiary structure. Conversely, DYQ05_13425 was found to not be heat-modifiable. Owing to a highly resistant tertiary structure, some bacterial OMPs exhibit an unusual stability to heat in the presence of SDS and an extended heating period may be required to convert such proteins to their denatured form, as reported for Protein F from *P. aeruginosa* (67) and OmpL1 from *L. interrogans* (66).

Examination by ELISA of IgG seroreactivity in animals naturally infected with BDD revealed that only one protein (DYQ05_06810) was capable of discriminating clearly between the sera of animals with recent or active BDD infection and cattle presumed not to have been exposed to the BDD treponemes. In identifying a disease-specific IgG2 antibody response to DYQ05_06810, these findings are in concordance with the findings of a number of previous studies that demonstrated a strong anti-treponemal IgG2 bias in the antibody response of
animals naturally infected with BDD (68–70). These data suggest that whilst DYQ05_06810 is accessible to the immune system during treponemal infection (and thus capable of eliciting IgG2 antibodies), the remaining three putative treponemal OMPs may be expressed as subdominant antigens or their expression may be immunosubversive in nature. Unexpectedly, a reduction in OD was observed when comparing the IgG1 response to the two T. medium/T19 putative OMPs in cows exposed to BDD relative to healthy controls. The reasons for this are unclear, although treponemes exhibit a number of immunosuppressive functions capable of interfering with both innate (71) and adaptive immune activity (72), the utilisation of which is likely to contribute to infection chronicity. In this case, it is speculated that whereas previous colonisation with bacteria expressing orthologues of CSN99_10335 and CSN99_02965 leads to the production of cross-reactivity antibodies (which are detectable in control animals), subsequent infection with T. medium leads to a reduction in the ser titre of these antibodies via immunosuppressive mechanisms. Crucially, both proteins have been detected in the transcriptome of BDD lesions (73) and studies are required to investigate their capacity to modulate host immunity. Presently, no commercially available serological assay to detect BDD treponemes is available for field diagnostics and immunoassay-based serological assessment of BDD in research settings is currently dependent on the use of whole cell lysates. The T. phagedenis putative proline-rich repeat lipoprotein, PrrA, was previously identified as an immunogenic protein of T. phagedenis-like strain V1 (isolated from a BDD lesion) and as an antigenic target capable of discriminating between animals acutely infected with BDD (n=8) and BDD naïve animals (n=7) (74). An ELISA, capable of detecting bovine anti-PrrA antibodies in serum and milk, has been available to the research community for some time, although there are currently no reports detailing post-marketing experience. In addition, the PrrA gene and its product have
been absent from several *T. phagedenis*-like BDD lesion isolates (74, 75), potentially limiting the value of this ELISA as a means of determining herd infection status. It is therefore proposed that the IgG2 antibody response to DYQ05_06810 be further evaluated as a potential antigenic marker of seroconversion in BDD-infected animals. The diagnosis of BDD currently remains restricted to clinical examination of the lifted foot by means of individual restraint (76) and specificity and sensitivity are limited by subjectivity and observer-bias (77). A quantitative diagnostic ELISA is less labour-intensive, less time consuming, and is a more reproducible indicator of biological infection (78). Since these recombinant molecules can be readily synthesised in *E. coli* expression systems, the difficulties in cultivating BDD-associated *Treponema* spp. for whole-cell antigen preparations are bypassed.

An initial ECM binding screen demonstrated that the putative treponemal β-barrel OMPs identified in this study exhibited ECM-adhesive properties, supporting their role in bacterial cytoadherence to host tissues. Interestingly, these proteins exhibited multispecific ECM binding profiles. One of the most striking properties of the putative treponemal OMPs was an ability to adhere to immobilised bovine fibrinogen. Because of the highly significant nature of this interaction, these fibrinogen binding activities were investigated further and found to be concentration-dependent and saturable, suggesting the existence of fibrinogen-specific binding sites. The estimated dissociation constants (K<sub>D</sub>) (0.34 and 0.72 for C5N99_02965 and DYQ05_13425, respectively) broadly align with those calculated for other spirochaetal fibrinogen binding proteins, including the leptospiral proteins OmpL1 (0.223 µM) (47), OmpL37 (0.244 µM) (79) and Lsa33 (0.12 µM) (80), supporting the relevance of these interactions *in vivo*. DYQ05_06810 exhibited a higher estimated K<sub>D</sub> of 1.0 µM, and is of a similar magnitude to other biologically-relevant ECM binding interactions,
including those reported for OmpL1 interactions with laminin ($K_D = 2.10$ µM) and fibronectin ($K_D = 1.24$ µM) (46).

Bovine fibrinogen is a 340-kDa dimeric glycoprotein comprising of three pairs of non-identical $\alpha$ (~67 kDa), $\beta$ (~55 kDa) and $\gamma$ (~48 kDa) peptide chains. Fibrinogen is a major clotting factor and performs an essential role in preventing haemorrhage and facilitating vascular repair. At sites of tissue damage, fibrinogen is found embedded with the extracellular matrix (81). It has previously been demonstrated that successful and consistent experimental transmission of BDD requires abrasion of the skin (82). Abrasive trauma would be expected to lead to enhanced fibrinogen deposition and it is therefore hypothesized that ECM-associated fibrinogen represents an adherence target during the initial stage of host colonisation. In support of this hypothesis, it has been demonstrated previously that two $T. medium$-like treponemal strains, UB1467 and UB1090, isolated from a bovine and an ovine DD lesion, and a $T. pedis$-like strain, UB1466, isolated from an ovine DD lesion, were all capable of adhering to immobilised fibrinogen (83). Thus, adhesin-mediated treponemal adherence to the host via such interactions may be central to the treponemal infection, in which abrasive trauma may be necessary point of entry.

Soluble host proteins, including plasma fibrinogen, may act as a diffuse peptide nutrient source within inflamed tissues. However, fibrinogen additionally plays a direct role in antimicrobial host defence. Firstly, thrombin-catalysed conversion to insoluble fibrin clots leads to the formation of a structural protective barrier capable of containing bacteria and preventing dissemination (84). Secondly, this conversion to thrombin leads to the release of potent chemotactic elements, including fibrinopeptide B, which drives an influx of phagocytes (85). Amongst the treponemal OMPs, Chymotrypsin-Like Protease (CTLP) from...
T. denticola and Tp0751 (Pallysilin) from T. pallidum subs. pallidum, are both capable of adhering to fibrinogen whilst additionally exhibiting fibrinogenolytic protease activity, with important deleterious consequences for platelet homeostasis and clot formation (29, 86). BDD lesions tend to bleed readily upon palpation (87), and a disturbance in the coagulation pathway arising from treponemal fibrinogen targeting is suspected. The interactions observed between the recombinant proteins and specific chains of the fibrinogen molecule identified in the present study may support this hypothesis, since the four recombinant proteins exhibited the capacity to adhere to all three fibrinogen chains or the beta and gamma chains only. Crucially, platelet aggregation is dependent on the binding of platelet membrane glycoprotein IIa/IIIb to the fibrinogen Aα and γ chain (88) and previous studies have identified selective fibrinogen chain targeting by pathogenic OMPs. For instance, targeting of the γ chain of fibrinogen has been previously reported by S. aureus clumping factor A (89), leading to disturbances in platelet aggregation, fibrin clot formation and platelet-mediated clot retraction (90).

The mechanisms that underpin the observed interactions with fibrinogen are unknown and require further investigation. Further studies, using gene mutagenesis and critical binding domain mapping, are required to fully understand the molecular basis for these interactions. It is noteworthy that these proteins appear to adhere to a number of other ECM components, demonstrating the apparent multi-specificity of these putative treponemal adhesins. Although we have not yet explored these interactions further, this suggests that these molecules may be functionally similar to a number of other spirochaetal OMPs capable of adhering to multiple ligands, including Tp0751 (29) and OmpL1 (46). This presumably represents an evolutionary adaptation that minimises bacterial surface immunogenicity whilst preserving adhesive function.
Given the apparent importance of these putative OMPs to fibrinogen (and other ligands), and their potential roles in host colonisation and pathogenicity, we sought to assess their immunogenic properties. Previous studies have shown that anti-adhesin antibodies, elicited by vaccination, have the potential to protect the host (91, 92) and it is hypothesised that blockade of these putative OMPs would impede host colonisation and/or virulence. Previous attempts at designing a BDD vaccine have been limited to the use of treponemal whole-cell lysate ‘bacterin’ formulations of a single phylotype, and field trials have been disappointing (93). The subcutaneous vaccine formulation evaluated in the present study was designed specifically to induce an IgG response against putative treponemal fibrinogen-binding OMPs, which may be critical to host colonisation, yet exist as subdominant antigens with little or no immunogenic capacity in natural infection.

Since recombinant protein antigens tend to be relatively weak antigens (94), aluminium hydroxide was employed as an adjuvant. Both an IgG1 and an IgG2 response to the putative OMP antigens was generated for the four proteins tested. Moreover, fold-change in the ELISA ODs generated for IgG1, relative to IgG2, was generally greater. Aluminium compounds are considered to be principally promotores of Th2 polarisation, at least in humans and mice (95, 96), and may explain the IgG1 subclass bias observed in this study. A mixed IgG1/IgG2 or IgG1-polarised host response may prove to be an important correlate of protection against an infection that is usually considered to induce a non-protective, yet robust, IgG2 response (68–70). However, because a later study identified IgG1 as the predominant IgG subclass in cattle both naturally exposed to BDD and experimentally infected with BDD-associated treponemes (97), there exists considerable uncertainty of the nature of the bovine immune response in BDD and subsequently, its potential for manipulation.
The four proteins evaluated were found to be immunogenic in both calves in the treatment group; only C5N99_10335 was found to be non-immunogenic in one of the two calves immunised with this antigen, highlighting the heterogeneity of the immune response between vaccinees. Whether this apparent variation in immunogenicity has arisen from intrinsic differences in the molecular structures of these recombinant proteins, host-specific variations in immune response or potential contamination with endogenous endotoxin, has not been established. Moreover, whilst the immunogenic potential of these putative OMPs has been demonstrated here, neither the duration of the IgG response or its magnitude (in terms of absolute antibody titre) was established. Since the immunised calves were not assessed for post-vaccination susceptibility to BDD, it is unknown whether high titres of the IgG antibodies generated during this study correlate with protection against disease and future studies are warranted. However, these data indicate successful B cell priming after the initial vaccination with a boost effect following the second vaccination, both of which are important characteristics of a vaccine component.

Given the difficulties associated with the isolation, cultivation and purification of the BDD treponemes, the development of a vaccine against BDD has previously been substantially hindered. This \textit{in silico} approach to novel OMP identification overcomes the challenges of traditional vaccine design methods. To this end, we report on the identification and characterisation of four putative adhesins, selected from the sequenced genomes of \textit{T. medium} and \textit{T. pedis} phylogroups, two of the principle treponeme taxa associated with BDD. Further studies are justified to establish their value as BDD vaccine candidates.

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Figure legends.

Table 2. Each putative treponemal OMP was selected on the basis of predicted β-barrel topology by at least one of the β-barrel prediction programs. The results generated by BOMP, PRED-TMBB, TMBETA-NET and SPAAN were interpreted in accordance with default cut-off values. All four CDS features were predicted to share domain homology with known bacterial OMPs.

Fig. 1. The four putative β barrel-outer membrane proteins (a-d) were structurally modelled using I-TASSER. A lateral view of the top-ranking ribbon model, as determined by the C-score, is shown, with β-sheet depicted in blue and α-helix depicted in red.

Table 3. The amino acid sequence of each putative OMP was submitted to I-TASSER for 3D structural modelling and Protein Databank (PDB) structural analogue detection. ^C-Score: A confidence score for estimating the quality of predicted models.

Fig. 2. Gel A, C5N99_02965; Gel B, C5N99_10335; Gel C, DYQ05_06810. Gels A, B and C: 1, wide-range MW marker (kDa); 2, unheated sample; 3, heated sample. The heat-modified (unfolded) forms of the proteins are distinguished from the unmodified (folded) forms by the addition of an asterisk (*). Panels A and C: lane between marker and sample contained a wash fraction and has been removed for brevity.

Fig. 3. CD spectra of proteins encoded by a) C5N99_10335, b) C5N99_02965, c) DYQ05_06810, d) DYQ05_13425 and e) L. interrogans OmpL1 are shown. Far-UV CD spectra
are presented as an average of three scans recorded from 190 to 260 nm. Φ, molar ellipticity.

Fig. 4. Serological assessment of the IgG1 and IgG2 response to putative treponemal OMPs by ELISA. Error bars: standard error of the mean. Non-exp., Non-exposed; DD exp., digital dermatitis-exposed. Asterisks (*) indicates a significant difference in IgG seroreactivity relative to control sera as determined by Mann-Whitney U test (* P<0.05, ** P<0.005).

Fig. 5. Immobilised ECM component binding screens of four putative β-barrel OMPs and the positive control protein (OmpL1). Bars represent the mean OD of three independent experiments. Error bars: standard error of the mean. Asterisks (*) indicate a significant difference in binding compared to the negative control protein, BSA, as determined by one-way ANOVA and the Dunnett post-test (* P<0.05, **P<0.005, ***P<0.001).

Fig. 6. Binding affinity curves of the four putative β-barrel OMPs to bovine fibrinogen. Graph A, putative OMPs from T. medium; Graph B, putative OMPs from T. pedis. Data points represent the mean OD of replicate readings. Error bars: standard error of the mean.

Fig. 7. Specific binding interactions between the recombinant proteins and the α, β and γ chains of bovine fibrinogen, using monoclonal Anti-polyHistidine antibody as the probe.

Fig. 8. The serological IgG1 and IgG2 response to four treponemal putative OMPs was assessed in BDD-naïve calves (n=2). The mean OD of both immunised calves is shown. Error bars: standard error of the mean.
**Figures and Tables.**

Table 1. Primers used to amplify putative OMP genes for recombinant expression.

| Putative OMP locus tag | Treponema phylogroup | Primer sequence (5’-3’) | Predicted band size (kb) | Predicted mass (Kda) |
|------------------------|----------------------|-------------------------|--------------------------|----------------------|
| C5N99_10335            | T. medium            | Forward: CACCGAAGGATCGAGATTGTTGCG | 0.7                      | 27.1                 |
|                        |                      | Reverse: CTACAGCTAAAAGCGATCC |                          |                      |
| C5N99_02965            | T. medium            | Forward: CACCCAGGAAGAAGAAGGACAGG | 0.9                      | 35.0                 |
|                        |                      | Reverse: AGAGATACCCATTAGGTGG |                          |                      |
| DYQ05_13425            | T. pedis             | Forward: CACCTTAAGCGATATTTCAGGCGATG | 0.8                      | 29.8                 |
|                        |                      | Reverse: TTACAGCTTCATGAATACC |                          |                      |
| DYQ05_06810            | T. pedis             | Forward: CACCGAAAAGACTATCGGTCTTAATTG | 0.9                      | 22.1                 |
|                        |                      | Reverse: TTAAAAAAAAACTCTTAAAAACCG | 0.6                      |                      |
| OmpL1                  | L. interrogans       | Forward: CACAAAAATATGCAATGTAGGATTG | 0.8                      | 31.0                 |
|                        |                      | Reverse: TTAGATTCGTGTTTTATAACC |                          |                      |

Table 2. Bioinformatic analysis of four putative treponemal OMPs.

| Putative OMP locus tag | Treponema phylogroup | Signal peptide cleavage site | β-barrel and adhesin prediction | Homologous domain search | PDB Top Hit (% probability; E-value) | Function | Ref. |
|------------------------|----------------------|-----------------------------|--------------------------------|--------------------------|-----------------------------------|----------|------|
| C5N99_02965            | T. medium            | YES (20/21; LSA/QE)         | YES NO YES YES                | OmpU (57.16; 120)        | OM Porin                          | (58)     |
| C5N99_10335            | T. medium            | YES (21/22; VFS/DG)         | YES YES YES YES               | OmpA (97.71; 6.1e-5)     | OM Porin/adhesin                  | (59, 60) |
| DYQ05_13425            | T. pedis             | YES (21/22; AFN/LS)         | NO YES YES YES                | OmpA (98.07; 4.9e-6)     | OM Porin/adhesin                  | (59, 60) |
| DYQ05_06810            | T. pedis             | YES (221/22; LSA/QT)        | YES YES YES YES               | OprF (97.32; 0.0014)     | OM porin                          | (61)     |
**Figure 1. Prediction of the treponemal putative OMP 3-dimensional structure**

![Image](http://iai.asm.org/)

**Table 3. I-TASSER structural modelling of the treponemal OMPs: A summary of results.**

| Putative OMP  | C-score | Predicted topology | PDB Structural analog (bacterial species) | Structural analog function | Ref. |
|---------------|---------|--------------------|------------------------------------------|---------------------------|------|
| CSN99_10335   | 3.98    | 8-stranded β-barrel | OmpT (E. coli)                          | Protease                  | (62) |
| CSN99_02965   | 2.73    | β-solenoid barrel   | serine-rich repeat protein (Lactobacillus reuterin) | Cell adhesion             | (63) |
| DYQ05_13425   | 4.07    | 8-stranded β-barrel | OprG (Pseudomonas aeruginosa)            | Porin                     | (64) |
| DYQ05_06810   | 2.73    | 8-stranded β-barrel | OmpA (E. coli)                          | Porin                     | (65) |
Figure 2. Heat-modifiability of C5N99_10335, C5N99_02965 and DYQ05_06810.
Fig 3. Determination of protein fold state using far UV Circular Dichroism (CD).
Fig 4. Determination by ELISA of the serological response to four putative OMPs in BDD-positive adult Holstein-Friesian dairy cattle.
Fig 5. Analysis of the attachment of treponemal putative OMPs to ECM components.
Fig. 6. Binding affinity curves of the four putative β-barrel OMPs to bovine fibrinogen.

![Affinity Curves](image)

Table 4. Binding interactions between the putative recombinant OMPs and bovine fibrinogen.

| OMP           | Dissociation Constant ($K_D$) |
|---------------|-------------------------------|
| CSN99_02965   | 0.3370 ± 0.09753              |
| DYQ05_13425   | 0.7180 ± 0.08743              |
| DYQ05_16810   | 1.024 ± 0.2946                |
| OmpL1         | 0.3669 ± 0.04328 (0.223 ± 0.063 (47)) |

Fig. 7. Far western blotting analysis of the treponemal recombinant OMP-Bovine fibrinogen binding interactions.
Fig. 8. Serological IgG1 and IgG2 response to immunisation with four recombinant treponemal putative OMPs.

Table 5. Fold-change in calf seroreactivity to the inoculated treponemal recombinant putative OMPs.

| Antigen     | IgG1 Fold-change (day 28 post-immunisation) | IgG2 Fold-change (day 28 post immunisation) |
|-------------|--------------------------------------------|--------------------------------------------|
| C5N99 10335 | 2.37                                       | 1.66                                       |
| C5N99 02965 | 4.34                                       | 3.58                                       |
| DYQ05_13425 | 7.44                                       | 9.10                                       |
| DYQ05_06810 | 5.42                                       | 6.00                                       |