1 **Putative β-barrel outer membrane proteins of the bovine digital**

2 **dermatitis-associated treponemes: identification, functional**

3 **characterisation and immunogenicity.**

4

5 Staton, G.J.[1], Carter, S.D. [1], Ainsworth, S.[1][¶], Mullin, J.[1], Smith R. F.[2] and Evans, N.J.[1]

6   1. Department of Infection Biology, Institute of Infection & Global Health, University of

7   Liverpool, Leahurst Campus, Chester High Road, Neston, Cheshire, UK, CH64 7TE

8

9   2. Department of Livestock Health and Welfare, Institute of Veterinary Science,

10   University of Liverpool, Leahurst Campus, Chester High Road, Neston, Cheshire, UK,

11   CH46 7TE

12

13 ¶Current affiliation: Centre for Snakebite Research and Interventions, Liverpool School of

14 Tropical Medicine, Pembroke Place, Liverpool, UK, L3 5QA.

15

16 **Corresponding author:** Evans N.J., Department of Infection Biology, Institute of Infection &

17 Global Health, University of Liverpool, Leahurst Campus, Chester High Road, Neston,

18 Cheshire, UK, CH64 7TE. Email: evansnj@liverpool.ac.uk

19

20 **Running Title:** β-barrel proteins of digital dermatitis-associated treponemes

21

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

23

24 **Word count:** 5846

25

26

27

28   **Abstract.**

29   Bovine Digital Dermatitis (BDD), an infectious disease of the bovine foot with a predominant
30   treponemal aetiology, is a leading cause of lameness in dairy and beef herds worldwide. BDD
31   is poorly responsive to antimicrobial therapy and exhibits a relapsing clinical course; an
32   effective vaccine is therefore urgently sought. Using a 'reverse vaccinology' approach, the
33   present study surveyed the genomes of the three BDD-associated *Treponema* phylogroups
34   for putative β-barrel outer membrane proteins and considered their potential as vaccine
35   candidates. Selection criteria included the presence of a signal peptidase I cleavage site, a
36   predicted β-barrel fold and cross-phylogroup homology. Four candidate genes were
37   overexpressed in *Escherichia coli* BL21 (DE3), refolded and purified. Consistent with their
38   classification as β-barrel OMPs, circular dichroism spectroscopy revealed the adoption of a
39   predominantly β-sheet secondary structure. These recombinant proteins, when screened for
40   their ability to adhere to immobilised ECM components, exhibited a diverse range of ligand
41   specificities. All four proteins specifically and dose-dependently adhered to bovine fibrinogen.
42   One recombinant protein was identified as a candidate diagnostic antigen (disease specificity,
43   75%). Finally, when adjuvanted with aluminium hydroxide and administered to BDD-naïve
44   calves using a prime-boost vaccination protocol, these proteins were immunogenic, eliciting
45   specific IgG antibodies. In summary, we present the description of four putative treponemal
46   β-barrel OMPs that exhibit the characteristics of multispecific adhesins. The observed
47   interactions with fibrinogen may be critical to host colonisation and dissemination and it is
48   hypothesised that vaccination-induced antibody blockade of these interactions will impede
49   treponemal virulence and thus be of therapeutic value.

50
51
52
53
54

55

56

57

58

59

60

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.

83   Treponemes are gram-stain-negative bacteria exhibiting a spiral morphology and consist of

84   an outer membrane (OM) that surrounds the axial filaments and the protoplasmic cylinder

85   (24). The OM of these extracellular pathogens is a feature of considerable interest, given its

86   surface exposure and the subsequent involvement of its components in host-pathogen

87   interactions. Adhesins embedded in the OM play a critical role in bacterial cytoadherence to

88   the host during colonisation. The host extracellular matrix (ECM) is an important adherence

89   target for pathogenic microorganisms during the primary stages of infection. Previous studies

90   investigating the ECM binding capacity of the two most relevant human pathogenic species,

91   *T. pallidum* sub. *pallidum*, the causative agent of syphilis, and *Treponema denticola*, a key

92   member of the polymicrobial consortium implicated in periodontal disease, reported specific

93   treponemal cell adherence to a range of immobilised ECM components, including fibronectin,

94   laminin, fibrinogen and collagen (25, 26). Subsequently, a growing number of adhesins are

95   being identified and investigated to characterise the molecular basis of physical host-

96   pathogen interactions (27–35). However, the fastidious nature of the BDD-associated

97   treponemes has precluded any detailed characterisation of the OM components likely to

98   promote such interactions. Employing recently available BDD-associated treponeme genome

99   sequences, we sought to identify novel β-barrel OMP-encoding genes and characterise the

100   function of the recombinantly expressed OMPs in ECM binding assays.

101   **METHODS.**

102   **Ethical approval.**

103   All experimental work involving animals was covered by a UK Home Office Project License PPL

104   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). The Gateway System (Life

128   Technologies, Paisley, UK) was utilised for gene cloning and expression. Putative OMP

129   sequences, lacking their signal peptide sequences, were amplified from the gDNA using high-

130   fidelity Phusion polymerase (Thermo Scientific, Hemel Hempstead, UK) in accordance with

131   manufacturer instructions. Primers (Table 1) for amplification contained CACC overhangs to

132   facilitate entry cloning. A well-characterised OMP (OmpL1) from *Leptospira interrogans*

133   serovar *Copenhageni* strain M20 was selected (46–48) and produced as a recombinant

134   protein control.

135   Amplified putative OMPs were inserted into the Gateway entry plasmid pENTR™/D-TOPO™

136   (Life Technologies, Paisley, UK) in accordance with the manufacturer's instructions prior to

137   chemical transformation into *Escherichia coli* Top10 cells. Positive transformants were

138   selected on LB agar plates containing kanamycin (50 µg/ml) and plasmid DNA from successful

139   transformants isolated using the Qiagen Plasmid MiniPrep Kit (Qiagen, Manchester, UK).

140   Successful amplicon insertion was confirmed using EcoRV restriction digest analysis

141   (ThermoFisher, Horsham, UK). Inserts were thereafter cloned into the Gateway expression

142   vector, pDEST™17, using a site-directed integration reaction in accordance with the

143   manufacturer's instructions (Life Technologies, Paisley, UK) prior to chemical transformation

144   into DH5α *E. coli*. Positive transformants were selected on LB agar plates containing ampicillin

145   (100 µg/ml) and the plasmid DNA isolated as previously described. pDEST™17-gene

146   constructs verified by EcoRI endonuclease restriction digest analysis and Sanger sequencing

147   (Source Bioscience, Nottingham, UK).

148   **Protein expression, refolding and purification**

149   All protein expression was performed in *E.coli* BL21 DE3 (Life Technologies, Paisley, UK). *E.coli*

150   BL21 DE3 cultures were grown at 37°C with shaking in LB medium (2 L) containing ampicillin

151   (100 µg/ml), until the OD_{600} was 0.8-1. Protein expression was induced by the addition of 1

152   mM IPTG (Sigma Aldrich, Gillingham, UK). Cultures were grown for a further 4-5 hours and

153   cells harvested by centrifugation (3500 x g, 4°C, 10 mins). *E. coli* BL21 DE3 cell pellets were

154   re-suspended in 50 mM Tris-HCl pH 7.9 (20ml per 10 g wet cell weight) containing lysozyme

155   (5 mg/g wet cell weight) (Sigma Aldrich, Gillingham, UK) and incubated on ice for 30 min prior

156   to cellular disruption using a micro-sonicator tip (Soniprep-150, MSE, London, UK). Inclusion

157   bodies (IB) containing recombinant proteins were subsequently harvested by centrifugation

158   (10000 x g, 4°C, 30 mins). IB pellets were re-suspended in 150 ml of IB detergent buffer (4%

159   (v/v) Tergitol™ 15-S-9 (Sigma-Aldrich, Dorset, UK), 50 mM Tris HCl, pH 7.9) with rapid stirring

160   for a minimum of two hours, washed twice in 150 ml IB wash buffer (50 mM Tris HCl, pH 7.9)

161   and re-suspended in solubilisation buffer (6M Guanidine hydrochloride, 50 mM Tris-HCl pH

162   7.9 and 1 mM EDTA; 40 ml per 500 mg of IB) for 1 hour with constant agitation. The

163   suspension was centrifuged (10, 000 x g, 4°C, 30 mins) to remove insoluble material.

164   Recombinant protein refolding was performed by rapid dilution (49) into refolding buffer (250

165   mM NaCl, 50 mM Tris-HCl pH 7.9, 5% N,N-Dimethyldodecylamine N-oxide solution (LDAO;

166   Sigma-Aldrich, Dorset UK)) and subsequently dialysed against 10 volumes of dialysis buffer

167   (250 mM NaCl, 50 mM Tris-HCl pH 7.9, 0.1% LDAO). Refolded, recombinant proteins were

168   purified by standard immobilised metal affinity chromatography (50), sterilised through a 0.2

169   µM filter and stored at -80°C. The purity of the recombinant proteins was assessed by SDS-

170   PAGE.

171   **Evaluation of secondary structure.**

172   *Heat modifiability*. The sensitivity of the recombinant proteins to denaturation upon heating

173   was determined by comparing the electrophoretic mobility of the refolded recombinant

174 proteins prepared in SDS sample buffer without reducing agent (100mM Tris-HCl [pH 6.8] 4%

175 (w/v) SDS, 0.2% (w/v) bromophenol blue, 20 % (v/v) glycerol) and either incubated at ambient

176 temperature for 10 minutes or heated to 100°C for 10 minutes prior to SDS-PAGE analysis, as

177 described previously (51).

178 *Circular dichroism (CD) spectroscopy*. Far UV CD spectroscopy was performed using a Jasco J-

179 810 spectropolarimeter (Japan Spectrocopic, Tokyo, Japan), equipped with a Peltier unit for

180 temperature control. Spectra were measured from 190 to 260 nm using 1-mm path length

181 cell at intervals of 0.5-nm and presented as an average of three scans. Spectra were analysed

182 by Beta Structure Selection (BestSel) software (http://bestsel.elte.hu/) (52) to calculate the

183 percentage secondary structure content from the ellipticity experimental data.

184 **Evaluation of immunogenicity during natural infection.**

185 *Cattle sera*

186 An ELISA was performed to investigate systemic IgG seroreactivity to the putative OMPs in

187 blood samples collected from cows naturally infected with BDD. Sera from sixteen adult

188 Holstein-Friesian cows with a recent (<6 month) history of BBD, were collected from a dairy

189 herd situated in Cheshire, UK. Similarly, sera from 5 healthy adult Holstein-Friesian cows were

190 collected from a closed dairy herd situated in Monmouthshire, UK, and were included as a

191 control group. In all cases, whole blood was collected from the coccygeal vein. Following

192 clotting and centrifugation, the serum fraction was harvested and stored at -20°C for

193 serological assessment.

194 *Serological ELISA*

195   Non-activated, 96-well microtitre plates (Microplate Immulon 2HB, Thermo Scientific, Hemel

196   Hempstead) were coated with a single recombinant protein (5 µg/ml) in PBS (pH 7.2) and

197   incubated for 1 hour (37°C) and overnight (4°C). Unbound antigen was removed by washing

198   with PBST (PBS-Tween 20; 0.05%). All sera were diluted 1/100 in PBST and pipetted into ELISA

199   plate wells in duplicate and incubated for 1 hour (37°C). Wells were washed as before and

200   incubated for 1 hour (37°C) with 100 µl monoclonal mouse anti-bovine immunoglobulin class

201   G subclass 1 (IgG1) antibody, clone IL-A60 (BioRad, Hemel Hempstead, UK) or monoclonal

202   mouse anti-bovine immunoglobulin class G subclass 2 (IgG2) antibody, clone IL-A2 (BioRad,

203   Hemel Hempstead, UK), diluted 1:1000. To ensure adherence of the antigen to the plate, 100

204   µl of mouse monoclonal anti-polyhistidine antibody, clone HIS-1 (Sigma-Aldrich, Dorset, UK),

205   diluted 1:2000 in PBST, was added to recombinant control wells. Following washing, wells

206   were incubated with 100 µl of horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG

207   antibodies (Sigma-Aldrich, Dorset, UK), diluted 1:10000 in PBST for 1 hour (37°C). Following

208   washing, the presence of HRP-conjugated goat anti-mouse IgG antibodies was detected by

209   the addition of 100 µl of the HRP substrate, 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma-

210   Aldrich, Dorset, UK). The reaction was terminated after ~20 minutes by the addition of 100 µl

211   0.5 M hydrochloric acid. The optical density (OD) of each well was read at 450 nm using a

212   microplate reader (Multiskan EX; Thermo Fisher Scientific, Loughborough, UK). All data was

213   processed and analysed using GraphPad Prism 5 (GraphPad, San Diego, CA). In order to

214   classify results as positive or negative, an ELISA OD value of less than or equal to the mean

215   plus 3 standard deviations of the control sera was considered to be non-reactive (53).

216   **Evaluation of adhesin function.**

217   *ECM macromolecules.*

9

218   All ECM macromolecules were purchased from Sigma Aldrich (Dorset, UK), and prepared from

219   the following sources: Collagen I from bovine skin; elastin from bovine neck filament;

220   fibrinogen from bovine plasma; heparan sulphate from bovine kidney; chondroitin sulphate

221   from bovine cartilage; laminin-1 from the basement membrane of Engelbreth-Holm-Swarm

222   mouse sarcoma.

223   *ECM-binding ELISA.*

224   An ELISA was performed to screen the recombinant proteins for their ability to attach to

225   individual ECM macromolecules using a previously described method (54). Briefly, Immulon

226   2HB plates (ThermoFisher, Horsham, UK) were coated with 5 µg/ml of the ECM component

227   or the negative control protein (BSA) by incubation for 1 hour at 37°C and overnight at 4°C,

228   washed with PBS containing 0.05% Tween 20 (PBST) and blocked with a 1% (w/v) BSA

229   solution. Recombinant proteins, diluted in PBST, were added at 10 µg/ml to screen for ECM

230   binding activity and a range of concentrations (from 0 to 6 µM) to determine the dose-

231   dependency of these binding interactions. Following incubation, bound recombinant proteins

232   were detected by addition of mouse monoclonal anti-polyhistidine IgG antibody (Sigma-

233   Aldrich, Dorset, UK), diluted 1:2000, before proceeding as described before. K_D values were

234   estimated from curves fitted by non-linear regression analysis in GraphPad Prism v. 5, using

235   the following equation: K_D = (Amax [protein])/A) – [protein], where A is the absorbance at a

236   given protein concentration, Amax is the maximum plate reader absorbance (when the

237   equilibrium is reached), [protein] is the protein concentration and K_D is the dissociation

238   equilibrium constant (55, 56).

239   *Far-western blotting.*

240   A far-western blotting technique was employed to further characterise the specific binding

241   interactions between the recombinant proteins and bovine fibrinogen (57). To dissociate

242   native bovine fibrinogen into its constituent polypeptide chains (Aα, Bβ and γ), 60 µl of bovine

243   fibrinogen stock solution (1 mg/ml) was mixed with 350 µl of gel loading buffer (100 mM Tris-

244   Cl [pH 6.8] 4% SDS, 0.2% bromophenol blue, 20 % glycerol, 200 mM dithiothreitol, heated at

245   95°C for 5 minutes and separated in Tris-glycine polyacrylamide gels by SDS-PAGE (4-20 %

246   gradient gel) at a constant voltage of 180 V for 50 minutes. The fibrinogen chains were

247   electroblotted onto a nitrocellulose membrane (100 V, 240 mA, 120 minutes) and the

248   membrane was blocked with 5% (w/v) skimmed milk. Membranes were subsequently

249   incubated with 30 µg/ml recombinant protein. Any bound protein was detected by incubation

250   of the membrane with mouse anti-polyhistidine antibody (Sigma-Aldrich, Dorset, UK), diluted

251   1:2000 followed by goat anti-mouse antibody (Sigma-Aldrich, Dorset, UK) and development

252   in 3,3'-diaminobenzidine membrane substrate (Sigma-Aldrich, Dorset, UK).

253   **Immunogenicity trial in calves.**

254   *Calves.*

255   The immunogenicity of the recombinant proteins was evaluated in two Holstein-Friesian

256   calves. A control group of two additional calves was used to verify that any serological

257   response did not result from environmental exposure to BDD-associated treponemes or to

258   ubiquitous antigens.

259   The four calves were reared and maintained according to routine agricultural practice at the

260   University's Farm with housing conditions having increased biosecurity to reduce the risk of

261   exposure to the BDD-associated *Treponema* spp.. Calves were bedded on straw and

262 quarantined for 4 weeks before vaccine administration. Calves were bled immediately prior

263 to vaccine administration to ascertain pre-immunisation antibody status.

264 *Formulation of the vaccine.*

265 The multivalent vaccine was formulated to deliver the four recombinant proteins

266 simultaneously and comprised of 100 µg of each recombinant protein and 40 µl aluminium

267 hydroxide adjuvant (Rehydragel®, Chemtrade Logistics, Toronto, Ontario), adjusted to a final

268 volume of 2 ml using PBS. All vaccines were administered subcutaneously to the left flank.

269 Calves received an initial 2 ml dose of the vaccine followed by a 2 ml booster dose two weeks

270 later. Concurrently, Control animals (n=2) received a 2 ml dose of the vehicle only. Blood

271 samples, obtained by jugular venepuncture, were collected at two-week intervals for four

272 weeks beginning with a pre-immunisation draw at day zero. Serum was retained for

273 serological studies.

274 *Detection of serum IgG antibodies by ELISA.*

275 Vaccinee IgG1 and IgG2 antibody seroreactivity to the recombinant proteins was determined

276 as described previously in this manuscript. All data was processed and analysed using

277 GraphPad Prism 5 (GraphPad, San Diego, CA). ELISA reactivity was confirmed by Western blot

278 analysis using previously described methods (23).

279 **RESULTS.**

280 *In silico detection of putative treponemal OMPs.*

281 SignalP 4.1 analysis identified 182 *T. medium* T19 CDS features predicted to contain an N-

282 terminal peptidase I cleavage site. These features were further analysed by three β-barrel

283 prediction programs: BOMP, PRED-TMBB and TMBETA-NET. CDS features predicted to

284 encode β-barrel proteins by at least one these programs were selected for cross-phylogroup

285 homology detection. In total, fifteen CDS features identified in the *T. medium* T19 genome

286 matched the following selection criteria precisely: i) the presence of a signal peptidase I

287 cleavage site, ii) a predicted β-barrel topology and iii) cross-phylogroup homology (Table 2).

288 Four CDS features, two from the *T. medium* genome and two from the *T. pedis* genome

289 (including one homologous pair: C5N99_10335 and DYQ05_13425; amino acid sequence

290 identity 31.87%) were subsequently selected to evaluate their ability to bind to selected ECM

291 components and to induce an IgG antibody response in calves.

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

293 To generate predicted 3D structural models, each protein sequence was submitted to the I-

294 TASSER server. The highest-ranking model for each protein is shown in Fig. 1. The four

295 putative OMPs were each predicted to contain a typical β-barrel domain, consistent with

296 localisation to the outer membrane of gram-negative bacteria.

297 Typical β-barrel structures were predicted for C5N99_10335, DYQ05_13425 and

298 DYQ05_06810, whereas C5N99_02965 was predicted to adopt a β-solenoid fold.

299 *Heat-modifiability assay.*

300 Three of the proteins expressed a heat-modifiable electrophoretic mobility, consistent with

301 the stability of a protein comprising predominantly of a β-structure (66). Fig. 2 shows the

302 change in electrophoretic mobility observed under unheated versus heated conditions for

303 C5N99_10335, C5N99_02965 and DYQ05_06810.

304 Heat modification of C5N99_02965 (Fig. 2A) led to a change in electrophoretic mobility and a

305 change in the apparent MW, from ~30 to ~ 36 kDa. Similar changes in mobility were observed

306   for C5N99_10335 (Fig 2B; ~22 to ~24 kDa) and DYQ05_06810 (Fig 2C; ~21 to 23 kDa). Heat

307   modifiability was not identified for DYQ05_13425 (data not shown).

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

309   Far UV CD spectroscopy was employed to provide further evidence of the secondary structure

310   fold of these proteins (Fig. 3). Consistent with a predicted β-barrel tertiary structure state,

311   analysis of the four putative OMPs yielded spectral signatures typical of a predominantly β-

312   sheet secondary structure, with spectral minima occurring at approximately 218 nm for

313   C5N99_10335, C5N99_02965, DYQ05_06810 and DYQ05_13425 as well as the positive

314   control protein, OmpL1.

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

316

317   As demonstrated in Fig 4, IgG2 seroreactivity to DYQ05_06810 was detected in BDD-infected

318   Holstein Friesian cows (n=12; 75%) relative to the healthy control animals, with no apparent

319   IgG2 response observed against the remaining three putative OMPs. No IgG1 response was

320   identified against any of the putative OMPs under investigation. A statistically significant

321   decrease in the IgG1 ELISA ODs was observed in the sera of BDD-exposed animals, relative to

322   controls, in the T. *medium*/T19 putative OMP analyses (P<0.05).

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

324   Statistically significant (P<0.05) adherence to fibrinogen, elastin and heparan sulphate was

325   observed for both C5N99_10335 and C5N99_02965, with C5N99_10335 additionally adhering

326   to fibronectin (Fig. 5).

327   The *T. pedis* homologue of C5N99_10335, namely DYQ05_13425, exhibited a similar binding

328   profile to C5N99_10335, but was found to additionally bind to chondroitin. DYQ05_06810

329   bound to fibronectin, fibrinogen, elastin, chondroitin and heparan sulphate.

330   Next, given the ubiquitous fibrinogen binding amongst these putative treponemal OMPs (P

331   <0.01), we sought to further characterise this interaction across a concentration range. The

332   results of these analyses are shown in Fig. 6.

333   The binding interactions between fibrinogen and C5N99_10335, C5N99_02965,

334   DYQ05_06810 and DYQ05_13425 were observed to be concentration-dependent. Binding

335   saturation levels were achieved with recombinant protein concentrations of ~1 µM, ~1.5 µM,

336   and ~2.0 µM for C5N99_02965, DYQ05_13425 and DYQ05_06810, respectively. Conversely,

337   C5N99_10335, although showing a tendency towards reaching saturation, failed to do so up

338   to a concentration of 2 µM. Further examination of this interaction was precluded by

339   insufficient protein yield. K_D values were estimated by non-linear regression analysis of the

340   binding curves. Table 4 summarises the KD values calculated from these experiments. The

341   positive control protein, OmpL1, was similarly observed to adhere to bovine fibrinogen in a

342   dose-dependent and saturable manner as previously reported (47) (data not shown).

343   *Far western blotting.*

344   Far Western blotting identified interactions between the putative OMPs and individual chains

345   of the fibrinogen molecule. The results of this analysis are shown in Fig. 7.

346   The results of the far western analysis revealed that DYQ05_13425 and DYQ05_06810

347   interacted with all three chains of the fibrinogen molecule, similarly to OmpL1. Conversely,

348 C5N99_10335 and C5N99_02965 were observed to interact with the β and γ chains of

349 fibrinogen only.

350 *Immunogenicity.*

351 The immunogenic properties of these four fibrinogen-binding proteins were investigated

352 following inoculation into two naïve bull calves as part of a multivalent antigen cocktail. ELISA

353 analysis of pre-vaccination sera yielded broadly comparable ELISA ODs between calves.

354 This pilot immunogenicity trial demonstrated that this subcutaneous prime-boost vaccination

355 protocol, involving an aluminium hydroxide-adjuvanted 100 μg dose of each treponemal

356 recombinant OMP, is capable of eliciting IgG antibodies in BDD-naïve bull calves (Fig. 8). All

357 animals vaccinated with the recombinant protein cocktail seroconverted rapidly, permitting

358 treponemal OMP-specific IgG antibody detection by day 14. A second booster vaccination on

359 day 14 enhanced the IgG response further and specific antibody titres peaked at day 28. No

360 seroconversion was detected in the control animals up to day 56 (the last day of the trial; data

361 not shown). The fold-change, calculated as the mean OD change on day 28 from pre-

362 immunisation baseline (Table 5) was used to account for varying baseline ELISA ODs.

363 Whilst all recombinant proteins were shown to be immunogenic under the conditions of this

364 trial, C5N99_10335 was found to be the weakest driver of both an IgG1 and IgG2 antibody

365 response, with ELISA OD fold changes calculated to be 2.37 and 1.66 from baseline,

366 respectively. DYQ05_13425, conversely, was found to the most potent immunogen, with

367 ELISA OD fold-changes from baseline calculated to be 7.44 and 9.10, for IgG1 and IgG2,

368 respectively. The specificity of the anti-sera was confirmed using Western Blotting and

369 revealed the presence of treponemal putative OMP-specific IgG1 and IgG2 serum antibodies

370 in immunised animals only (data not shown). Serological analysis on day 42 of the trial

371   revealed a slight waning of the IgG antibody response in all cases. The trial vaccine was well

372   tolerated in vaccinated calves.

373   **DISCUSSION.**

374   Using a bioinformatics-based approach, the present study sought to identify and characterise

375   novel BDD-associated treponemal OMPs, bearing in mind the potential value of these

376   molecules as BDD vaccine candidates. Post-purification analysis of the four refolded proteins

377   by CD spectroscopy yielded spectra indicative of a predominantly β-strand secondary

378   structure with three of the proteins (C5N99_10335, C5N99_02965 and DYQ05_06810)

379   additionally demonstrated to be heat-modifiable. These findings are consistent with the

380   characteristics of proteins with a β-barrel tertiary structure. Conversely, DYQ05_13425 was

381   found to not be heat-modifiable. Owing to a highly resistant tertiary-structure, some bacterial

382   OMPs exhibit an unusual stability to heat in the presence of SDS and an extended heating

383   period may be required to convert such proteins to their denatured form, as reported for

384   Protein F from *P. aeruginosa* (67) and OmpL1 from *L. interrogans* (66).

385   Examination by ELISA of IgG seroreactivity in animals naturally infected with BDD revealed

386   that only one protein (DYQ05_06810) was capable of discriminating clearly between the sera

387   of animals with recent or active BDD infection and cattle presumed not to have been exposed

388   to the BDD treponemes. In identifying a disease-specific IgG2 antibody response to

389   DYQ05_06810, these findings are in concordance with the findings of a number of previous

390   studies that demonstrated a strong anti-treponemal IgG2 bias in the antibody response of

391   animals naturally infected with BDD (68–70). These data suggest that whilst DYQ05_06810 is

392   accessible to the immune system during treponemal infection (and thus capable of eliciting

393   IgG2 antibodies), the remaining three putative treponemal OMPs may be expressed as

394 subdominant antigens or their expression may be immunosubversive in nature.

395 Unexpectedly, a reduction in OD was observed when comparing the IgG1 response to the two

396 *T. medium*/T19 putative OMPs in cows exposed to BDD relative to healthy controls. The

397 reasons for this are unclear, although treponemes exhibit a number of immunosuppressive

398 functions capable of interfering with both innate (71) and adaptive immune activity (72), the

399 utilisation of which is likely to contribute to infection chronicity. In this case, it is speculated

400 that whereas previous colonisation with bacteria expressing orthologues of C5N99_10335

401 and C5N99_02965 leads to the production of cross-reactivity antibodies (which are

402 detectable in control animals), subsequent infection with *T. medium* leads to a reduction in

403 the serotitre of these antibodies via immunosuppressive mechanisms. Crucially, both proteins

404 have been detected in the transcriptome of BDD lesions (73) and studies are required to

405 investigate their capacity to modulate host immunity.

406 Presently, no commercially available serological assay to detect BDD treponemes is available

407 for field diagnostics and immunoassay-based serological assessment of BDD in research

408 settings is currently dependent on the use of whole cell lysates. The *T. phagedenis* putative

409 proline-rich repeat lipoprotein, PrrA, was previously identified as an immunogenic protein of

410 *T. phagedenis*-like strain V1 (isolated from a BDD lesion) and as an antigenic target capable of

411 discriminating between animals acutely infected with BDD (n=8) and BDD naïve animals (n=7)

412 (74). An ELISA, capable of detecting bovine anti-PrrA antibodies in serum and milk, has been

413 available to the research community for some time, although there are currently no reports

414 detailing post-marketing experience. In addition, the PrrA gene and its product have been

415 absent from several *T. phagedenis*-like BDD lesion isolates (74, 75), potentially limiting the

416 value of this ELISA as a means of determining herd infection status. It is therefore proposed

417 that the IgG2 antibody response to DYQ05_06810 be further evaluated as a potential

418 antigenic marker of seroconversion in BDD-infected animals. The diagnosis of BDD currently

419 remains restricted to clinical examination of the lifted foot by means of individual restraint

420 (76) and specificity and sensitivity are limited by subjectivity and observer-bias (77). A

421 quantitative diagnostic ELISA is less labour-intensive, less time consuming, and is a more

422 reproducible indicator of biological infection (78). Since these recombinant molecules can be

423 readily synthesised in *E. coli* expression systems, the difficulties in cultivating BDD-associated

424 *Treponema* spp. for whole-cell antigen preparations are bypassed.

425 An initial ECM binding screen demonstrated that the putative treponemal β-barrel OMPs

426 identified in this study exhibited ECM-adhesive properties, supporting their role in bacterial

427 cytoadherence to host tissues. Interestingly, these proteins exhibited multispecific ECM

428 binding profiles. One of the most striking properties of the putative treponemal OMPs was an

429 ability to adhere to immobilised bovine fibrinogen. Because of the highly significant nature of

430 this interaction, these fibrinogen binding activities were investigated further and found to be

431 concentration-dependent and saturable, suggesting the existence of fibrinogen-specific

432 binding sites. The estimated dissociation constants (K_D) (0.34 and 0.72 for C5N99_02965 and

433 DYQ05_13425, respectively) broadly align with those calculated for other spirochaetal

434 fibrinogen binding proteins, including the leptospiral proteins OmpL1 (0.223 μM) (47),

435 OmpL37 (0.244 μM) (79) and Lsa33 (0.12 μM) (80), supporting the relevance of these

436 interactions *in vivo*. DYQ05_06810 exhibited a higher estimated K_D of 1.0 μM, and is of a

437 similar magnitude to other biologically-relevant ECM binding interactions, including those

438 reported for OmpL1 interactions with laminin ($K_D = 2.10$ μM) and fibronectin ($K_D = 1.24$ μM)

439 (46).

440   Bovine fibrinogen is a 340-kda dimeric glycoprotein comprising of three pairs of non-identical

441   Aα (~67 kDa), Bβ (~55 kDa) and γ (~48 kDa) peptide chains. Fibrinogen is a major clotting

442   factor and performs an essential role in preventing haemorrhage and facilitating vascular

443   repair. At sites of tissue damage, fibrinogen is found embedded with the extracellular matrix

444   (81). It has previously been demonstrated that successful and consistent experimental

445   transmission of BDD requires abrasion of the skin (82). Abrasive trauma would be expected

446   to lead to enhanced fibrinogen deposition and it is therefore hypothesized that ECM-

447   associated fibrinogen represents an adherence target during the initial stage of host

448   colonisation. In support of this hypothesis, it has been demonstrated previously that two *T.*

449   *medium*-like treponemal strains, UB1467 and UB1090, isolated from a bovine and an ovine

450   DD lesion, and a *T. pedis*-like strain, UB1466, isolated from an ovine DD lesion, were all

451   capable of adhering to immobilised fibrinogen (83). Thus, adhesin-mediated treponemal

452   adherence to the host via such interactions may be central to the treponemal infection, in

453   which abrasive trauma may be necessary point of entry.

454   Soluble host proteins, including plasma fibrinogen, may act as a diffuse peptide nutrient

455   source within inflamed tissues. However, fibrinogen additionally plays a direct role in

456   antimicrobial host defence. Firstly, thrombin-catalysed conversion to insoluble fibrin clots

457   leads to the formation of a structural protective barrier capable of containing bacteria and

458   preventing dissemination (84). Secondly, this conversion to thrombin leads to the release of

459   potent chemotactic elements, including fibrinopeptide B, which drives an influx of phagocytes

460   (85). Amongst the treponemal OMPs, Chymotrypsin-Like Protease (CTLP) from *T. denticola*

461   and Tp0751 (Pallysilin) from *T. pallidum* subs. *pallidum*, are both capable of adhering to

462   fibrinogen whilst additionally exhibiting fibrinogenolytic protease activity, with important

463   deleterious consequences for platelet homeostasis and clot formation (29, 86). BDD lesions

464 tend to bleed readily upon palpation (87), and a disturbance in the coagulation pathway

465 arising from treponemal fibrinogen targeting is suspected. The interactions observed

466 between the recombinant proteins and specific chains of the fibrinogen molecule identified

467 in the present study may support this hypothesis, since the four recombinant proteins

468 exhibited the capacity to adhere to all three fibrinogen chains or the beta and gamma chains

469 only. Crucially, platelet aggregation is dependent on the binding of platelet membrane

470 glycoprotein IIa/IIIb to the fibrinogen Aα and γ chain (88) and previous studies have identified

471 selective fibrinogen chain targeting by pathogenic OMPs. For instance, targeting of the γ chain

472 of fibrinogen has been previously reported by *S. aureus* clumping factor A (89), leading to

473 disturbances in platelet aggregation, fibrin clot formation and platelet-mediated clot

474 retraction (90).

475 The mechanisms that underpin the observed interactions with fibrinogen are unknown and

476 require further investigation. Further studies, using gene mutagenesis and critical binding

477 domain mapping, are required to fully understand the molecular basis for these interactions.

478 It is noteworthy that these proteins appear to adhere to a number of other ECM components,

479 demonstrating the apparent multi-specificity of these putative treponemal adhesins.

480 Although we have not yet explored these interactions further, this suggests that these

481 molecules may be functionally similar to a number of other spirochaetal OMPs capable of

482 adhering to multiple ligands, including Tp0751 (29) and OmpL1 (46). This presumably

483 represents an evolutionary adaptation that minimises bacterial surface immunogenicity

484 whilst preserving adhesive function.

485 Given the apparent importance of these putative OMPs to fibrinogen (and other ligands), and

486 their potential roles in host colonisation and pathogenicity, we sought to assess their

487   immunogenic properties. Previous studies have shown that anti-adhesin antibodies, elicited

488   by vaccination, have the potential to protect the host (91, 92) and it is hypothesised that

489   blockade of these putative OMPs would impede host colonisation and/or virulence. Previous

490   attempts at designing a BDD vaccine have been limited to the use of treponemal whole-cell

491   lysate 'bacterin' formulations of a single phylotype, and field trials have been disappointing

492   (93). The subcutaneous vaccine formulation evaluated in the present study was designed

493   specifically to induce an IgG response against putative treponemal fibrinogen-binding OMPs,

494   which may be critical to host colonisation, yet exist as subdominant antigens with little or no

495   immunogenic capacity in natural infection.

496   Since recombinant protein antigens tend to be relatively weak antigens (94), aluminium

497   hydroxide was employed as an adjuvant. Both an IgG1 and an IgG2 response to the putative

498   OMP antigens was generated for the four proteins tested. Moreover, fold-change in the ELISA

499   ODs generated for IgG1, relative to IgG2, was generally greater. Aluminium compounds are

500   considered to be principally promotors of Th2 polarisation, at least in humans and mice (95,

501   96), and may explain the IgG1 subclass bias observed in this study. A mixed IgG1/IgG2 or IgG1-

502   polarised host response may prove to be an important correlate of protection against an

503   infection that is usually considered to induce a non-protective, yet robust, IgG2 response (68–

504   70). However, because a later study identified IgG1 as the predominant IgG subclass in cattle

505   both naturally exposed to BDD and experimentally infected with BDD-associated treponemes

506   (97), there exists considerable uncertainty of the nature of the bovine immune response in

507   BDD and subsequently, its potential for manipulation.

508   The four proteins evaluated were found to be immunogenic in both calves in the treatment

509   group; only C5N99_10335 was found to be non-immunogenic in one of the two calves

510 immunised with this antigen, highlighting the heterogeneity of the immune response

511 between vaccinees. Whether this apparent variation in immunogenicity has arisen from

512 intrinsic differences in the molecular structures of these recombinant proteins, host-specific

513 variations in immune response or potential contamination with endogenous endotoxin, has

514 not been established. Moreover, whilst the immunogenic potential of these putative OMPs

515 has been demonstrated here, neither the duration of the IgG response or its magnitude (in

516 terms of absolute antibody titre) was established. Since the immunised calves were not

517 assessed for post-vaccination susceptibility to BDD, it is unknown whether high titres of the

518 IgG antibodies generated during this study correlate with protection against disease and

519 future studies are warranted. However, these data indicate successful B cell priming after the

520 initial vaccination with a boost effect following the second vaccination, both of which are

521 important characteristics of a vaccine component.

522 Given the difficulties associated with the isolation, cultivation and purification of the BDD

523 treponemes, the development of a vaccine against BDD has previously been substantially

524 hindered. This *in silico* approach to novel OMP identification overcomes the challenges of

525 traditional vaccine design methods. To this end, we report on the identification and

526 characterisation of four putative adhesins, selected from the sequenced genomes of *T.*

527 *medium* and *T. pedis* phylogroups, two of the principle treponeme taxa associated with BDD.

528 Further studies are justified to establish their value as BDD vaccine candidates.

529 **Acknowledgements**

References

534 1.   Blowey RW, Sharp MW. 1988. Digital dermatitis in dairy cattle. Vet Rec 122:505–8.

535 2.   Read DH, Walker RL. 1998. Papillomatous digital dermatitis (footwarts) in California

536   dairy cattle: clinical and gross pathologic findings. J Vet Diagnostic Investig 10:67–76.

537 3.   Zinicola M, Lima F, Lima S, Machado V, Gomez M, Döpfer D, Guard C, Bicalho R. 2015.

538   Altered Microbiomes in Bovine Digital Dermatitis Lesions, and the Gut as a Pathogen

539   Reservoir. PLoS One 10:e0120504.

540 4.   Barker ZE, Leach KA, Whay HR, Bell NJ, Main DCJ. 2010. Assessment of lameness

541   prevalence and associated risk factors in dairy herds in England and Wales. J Dairy Sci

542   93:932–941.

543 5.   Sullivan LE, Carter SD, Blowey R, Duncan JS, Grove-White D, Evans NJ. 2013. Digital

544   dermatitis in beef cattle. Vet Rec 173:582.1-582.

545 6.   Sullivan LE, Evans NJ, Blowey RW, Grove-White DH, Clegg SR, Duncan JS, Carter SD.

546   2015. A molecular epidemiology of treponemes in beef cattle digital dermatitis

547   lesions and comparative analyses with sheep contagious ovine digital dermatitis and

548   dairy cattle digital dermatitis lesions. Vet Microbiol 178:77–87.

549 7.   Davies IH, Naylor RD, Martin PK. 1999. Severe ovine foot disease. Vet Rec 145:646.

550 8.   Harwood DG, Cattell JH, Lewis CJ, Naylor R. 1997. Virulent foot rot in sheep. Vet Rec

551   140:687.

552 9.   Walker RL, Read DH, Loretz KJ, Nordhausen RW. 1995. Spirochetes isolated from dairy

553   cattle with papillomatous digital dermatitis and interdigital dermatitis. Vet Microbiol

554 47:343–55.

555 10. Krull AC, Shearer JK, Gorden PJ, Cooper VL, Phillips GJ, Plummer PJ. 2014. Deep

556 Sequencing Analysis Reveals Temporal Microbiota Changes Associated with

557 Development of Bovine Digital Dermatitis. Infect Immun 82:3359–3373.

558 11. Nielsen MW, Strube ML, Isbrand A, Al-Medrasi WD, Boye M, Jensen TK, Klitgaard K.

559 2016. Potential bacterial core species associated with digital dermatitis in cattle herds

560 identified by molecular profiling of interdigital skin samples. Vet Microbiol 186:139–

561 149.

562 12. Evans NJ, Brown JM, Demirkan I, Singh P, Getty B, Timofte D, Vink WD, Murray RD,

563 Blowey RW, Birtles RJ, Hart CA, Carter SD. 2009. Association of Unique, Isolated

564 Treponemes with Bovine Digital Dermatitis Lesions. J Clin Microbiol 47:689–696.

565 13. Evans NJ, Brown JM, Demirkan I, Murray RD, Birtles RJ, Hart CA, Carter SD. 2009.

566 Treponema pedis sp. nov., a spirochaete isolated from bovine digital dermatitis

567 lesions. Int J Syst Evol Microbiol 59:987–991.

568 14. Evans NJ, Brown JM, Demirkan I, Murray RD, Vink WD, Blowey RW, Hart CA, Carter

569 SD. 2008. Three unique groups of spirochetes isolated from digital dermatitis lesions

570 in UK cattle. Vet Microbiol 130:141–150.

571 15. Rasmussen M, Capion N, Klitgaard K, Rogdo T, Fjeldaas T, Boye M, Jensen TK. 2012.

572 Bovine digital dermatitis: Possible pathogenic consortium consisting of Dichelobacter

573 nodosus and multiple Treponema species. Vet Microbiol 160:151–161.

574 16. Moter A, Leist G, Rudolph R, Schrank K, Choi B-K, Wagner M, Gobel UB. 1998.

575 Fluorescence in situ hybridization shows spatial distribution of as yet uncultured

576   treponemes in biopsies from digital dermatitis lesions. Microbiology 144:2459–2467.

577   17.   Choi B-K, Natterman H, Grund S, Haider W, Gobel UB. 1997. Spirochetes from Digital

578   Dermatitis Lesions in Cattle Are Closely Related to Treponemes Associated with

579   Human Periodontitis. Int J Syst Bacteriol 47:175–181.

580   18.   Evans NJ, Timofte D, Isherwood DR, Brown JM, Williams JM, Sherlock K, Lehane MJ,

581   Murray RD, Birtles RJ, Anthony Hart C, Carter SD. 2012. Host and environmental

582   reservoirs of infection for bovine digital dermatitis treponemes. Vet Microbiol

583   156:102–109.

584   19.   Gomez A, Anklam KS, Cook NB, Rieman J, Dunbar KA, Cooley KE, Socha MT, Döpfer D.

585   2014. Immune response against Treponema spp. and ELISA detection of digital

586   dermatitis. J Dairy Sci 97:4864–4875.

587   20.   Elliott MK, Alt DP. 2009. Bovine immune response to papillomatous digital dermatitis

588   (PDD)-associated spirochetes is skewed in isolate reactivity and subclass elicitation.

589   Vet Immunol Immunopathol 130:256–261.

590   21.   Trott DJ, Moeller MR, Zuerner RL, Goff JP, Waters WR, Alt DP, Walker RL,

591   Wannemuehler MJ. 2003. Characterization of Treponema phagedenis-like spirochetes

592   isolated from papillomatous digital dermatitis lesions in dairy cattle. J Clin Microbiol

593   41:2522–9.

594   22.   Moe KK, Yano T, Misumi K, Kubota C, Yamazaki W, Muguruma M, Misawa N. 2010.

595   Analysis of the IgG immune response to Treponema phagedenis-like spirochetes in

596   individual dairy cattle with papillomatous digital dermatitis. Clin Vaccine Immunol

597   17:376–383.

598   23.   Demirkan I, Walker RL, Murray RD, Blowey RW, Carter SD. 1999. Serological evidence

599   of spirochaetal infections associated with digital dermatitis in dairy cattle. Vet J 69–

600   77.

601   24.   Smirbert RM. 1984. Genus III Treponema, p. 49–57. *In* Krieg, NR, Holt, JG (eds.),

602   Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore, MD.

603   25.   Dawson JR, Ellen RP. 1990. Tip-Oriented Adherence of Treponema denticola to

604   Fibronectin. Infect Immun 58:3924–3928.

605   26.   Fitzgerald TJ, Repesh LA, Blanco DR, Miller JN. 1984. Attachment of Treponema

606   pallidum to fibronectin, laminin, collagen IV, and collagen I, and blockage of

607   attachment by immune rabbit IgG. Br J Vener Dis 60:357–63.

608   27.   Brinkman MB, McGill MA, Pettersson J, Rogers A, Matejková P, Smajs D, Weinstock

609   GM, Norris SJ, Palzkill T. 2008. A novel Treponema pallidum antigen, TP0136, is an

610   outer membrane protein that binds human fibronectin. Infect Immun 76:1848–57.

611   28.   Parker ML, Houston S, Pětroš ová H, Lithgow K V, Hof R, Wetherell C, Kao W-C, Lin Y-

612   P, Moriarty TJ, Ebady R, Cameron CE, Boulanger MJ. 2016. The Structure of

613   Treponema pallidum Tp0751 (Pallilysin) Reveals a Non-canonical Lipocalin Fold That

614   Mediates Adhesion to Extracellular Matrix Components and Interactions with Host

615   Cells. PLOS Pathog 12:e1005919.

616   29.   Houston S, Hof R, Francescutti T, Hawkes A, Boulanger MJ, Cameron CE. 2011.

617   Bifunctional role of the Treponema pallidum extracellular matrix binding adhesin

618   Tp0751. Infect Immun 79:1386–1398.

619   30.   Cameron CE, Brouwer NL, Tisch LM, Kuroiwa JMY. 2005. Defining the Interaction of

620   the Treponema pallidum Adhesin Tp0751 with Laminin. Infect Immun 73:7485–7494.

621 31. Cameron CE. 2003. Identification of a Treponema pallidum laminin-binding protein.

622   Infect Immun 71:2525–33.

623 32. Haapasalo M, Müller KH, Uitto VJ, Leung WK, McBride BC. 1992. Characterization,

624   cloning, and binding properties of the major 53-kilodalton Treponema denticola

625   surface antigen. Infect Immun 60:2058–65.

626 33. Fenno JC, Müller KH, McBride BC. 1996. Sequence analysis, expression, and binding

627   activity of recombinant major outer sheath protein (Msp) of Treponema denticola. J

628   Bacteriol 178:2489–97.

629 34. Anand A, Luthra A, Edmond ME, Ledoyt M, Caimano MJ, Radolf JD. 2013. The major

630   outer sheath protein (Msp) of Treponema denticola has a bipartite domain

631   architecture and exists as periplasmic and outer membrane-spanning conformers. J

632   Bacteriol 195:2060–71.

633 35. Gaibani P, Pellegrino MT, Rossini G, Alvisi G, Miragliotta L, Prati C, Sambri V. 2010.

634   The central region of the msp gene of Treponema denticola has sequence

635   heterogeneity among clinical samples, obtained from patients with periodontitis.

636   BMC Infect Dis 10:345.

637 36. Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, Barrell B. 2000.

638   Artemis: sequence visualization and annotation. Bioinformatics 16:944–5.

639 37. Petersen TN, Brunak S, von Heijne G, Nielsen H. 2011. SignalP 4.0: discriminating

640   signal peptides from transmembrane regions. Nat Methods 8:785–786.

641 38. Berven FS, Flikka K, Jensen HB, Eidhammer I. 2004. BOMP: a program to predict

642   integral -barrel outer membrane proteins encoded within genomes of Gram-negative

643   bacteria. Nucleic Acids Res 32:W394–W399.

644 39.   Gromiha MM, Ahmad S, Suwa M. 2005. TMBETA-NET: discrimination and prediction

645   of membrane spanning -strands in outer membrane proteins. Nucleic Acids Res

646   33:W164–W167.

647 40.   Bagos PG, Liakopoulos TD, Spyropoulos IC, Hamodrakas SJ. 2004. PRED-TMBB: a web

648   server for predicting the topology of -barrel outer membrane proteins. Nucleic Acids

649   Res 32:W400–W404.

650 41.   Enright AJ, Van Dongen S, Ouzounis CA. 2002. An efficient algorithm for large-scale

651   detection of protein families. Nucleic Acids Res 30:1575–84.

652 42.   Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment

653   search tool. J Mol Biol 215:403–410.

654 43.   Sachdeva G, Kumar K, Jain P, Ramachandran S. 2005. SPAAN: a software program for

655   prediction of adhesins and adhesin-like proteins using neural networks.

656   Bioinformatics 21:483–491.

657 44.   Zhang Y. 2008. I-TASSER server for protein 3D structure prediction. BMC

658   Bioinformatics 9:40.

659 45.   Chua PKB, Corkill JE, Hooi PS, Cheng SC, Winstanley C, Hart CA. 2005. Isolation of

660   Waddlia malaysiensis, a novel intracellular bacterium, from fruit bat (Eonycteris

661   spelaea). Emerg Infect Dis 11:271–7.

662 46.   Fernandes LG V., Vieira ML, Kirchgatter K, Alves IJ, Morais ZM de, Vasconcellos SA,

663   Romero EC, Nascimento ALTO. 2012. OmpL1 Is an Extracellular Matrix- and

664   Plasminogen-Interacting Protein of Leptospira spp. Infect Immun 80:3679.

665   47.   Robbins GT, Hahn BL, Evangelista K V., Padmore L, Aranda PS, Coburn J. 2015.

666   Evaluation of Cell Binding Activities of Leptospira ECM Adhesins. PLoS Negl Trop Dis

667   9:e0003712.

668   48.   Haake DA, Champion CI, Martinich C, Shang ES, Blanco DR, Miller JN, Lovett MA.

669   1993. Molecular cloning and sequence analysis of the gene encoding OmpL1, a

670   transmembrane outer membrane protein of pathogenic Leptospira spp. J Bacteriol

671   175:4225–34.

672   49.   Prince SM, Feron C, Janssens D, Lobet Y, Achtman M, Kusecek B, Bullough PA, Derrick

673   JP. 2001. Expression, refolding and crystallization of the OpcA invasin from Neisseria

674   meningitidis. Acta Crystallogr D Biol Crystallogr 57:1164–6.

675   50.   Porath J, Carlsson J, Olsson I, Belfrage G. 1975. Metal chelate affinity

676   chromatography, a new approach to protein fractionation. Nature 258:598–9.

677   51.   Schweizer M, Hindennach I, Garten W, Henning U. 1978. Major proteins of the

678   Escherichia coli outer cell envelope membrane. Interaction of protein II with

679   lipopolysaccharide. Eur J Biochem 82:211–7.

680   52.   Micsonai A, Wien F, Kernya L, Lee Y-H, Goto Y, Réfrégiers M, Kardos J. 2015. Accurate

681   secondary structure prediction and fold recognition for circular dichroism

682   spectroscopy. Proc Natl Acad Sci 112:E3095–E3103.

683   53.   Ji B, Collins MT. 1994. Seroepidemiologic survey of Borrelia burgdorferi exposure of

684   dairy cattle in Wisconsin. Am J Vet Res 55:1228–31.

685   54.   Cameron CE. 2003. Identification of a Treponema pallidum laminin-binding protein.

686   Infect Immun 71:2525–33.

687 55. Pathirana RD, O'Brien-Simpson NM, Veith PD, Riley PF, Reynolds EC. 2006.

688   Characterization of proteinase-adhesin complexes of Porphyromonas gingivalis.

689   Microbiology 152:2381–2394.

690 56. Lin Y-P, Lee D-W, McDonough SP, Nicholson LK, Sharma Y, Chang Y-F. 2009. Repeated

691   Domains of *Leptospira* Immunoglobulin-like Proteins Interact with Elastin and

692   Tropoelastin. J Biol Chem 284:19380–19391.

693 57. Wu Y, Li Q, Chen X-Z. 2007. Detecting protein–protein interactions by far western

694   blotting. Nat Protoc 2:3278–3284.

695 58. Li H, Zhang W, Dong C. 2018. Crystal structure of the outer membrane protein OmpU

696   from Vibrio cholerae at 2.2 Å resolution. Acta Crystallogr Sect D, Struct Biol 74:21–29.

697 59. Zhao H, Sequeira RD, Galeva NA, Tang L. 2011. The host outer membrane proteins

698   OmpA and OmpC are associated with the Shigella phage Sf6 virion. Virology 409:319–

699   27.

700 60. Torres AG, Kaper JB. 2003. Multiple Elements Controlling Adherence of

701   Enterohemorrhagic Escherichia coli O157:H7 to HeLa Cells. Infect Immun 71:4985–

702   4995.

703 61. Zahn M, D'Agostino T, Eren E, Baslé A, Ceccarelli M, van den Berg B. 2015. Small-

704   Molecule Transport by CarO, an Abundant Eight-Stranded β-Barrel Outer Membrane

705   Protein from Acinetobacter baumannii. J Mol Biol 427:2329–39.

706 62. Vandeputte-Rutten L, Kramer RA, Kroon J, Dekker N, Egmond MR, Gros P. 2001.

707   Crystal structure of the outer membrane protease OmpT from Escherichia coli

708   suggests a novel catalytic site. EMBO J 20:5033–9.

709   63. Sequeira S, Kavanaugh D, MacKenzie DA, Šuligoj T, Walpole S, Leclaire C, Gunning AP,

710   Latousakis D, Willats WGT, Angulo J, Dong C, Juge N. 2018. Structural basis for the

711   role of serine-rich repeat proteins from Lactobacillus reuteri in gut microbe-host

712   interactions. Proc Natl Acad Sci U S A 115:E2706–E2715.

713   64. Touw DS, Patel DR, van den Berg B. 2010. The crystal structure of OprG from

714   Pseudomonas aeruginosa, a potential channel for transport of hydrophobic molecules

715   across the outer membrane. PLoS One 5:e15016.

716   65. Bushell SR, Mainprize IL, Wear MA, Lou H, Whitfield C, Naismith JH. 2013. Wzi Is an

717   Outer Membrane Lectin that Underpins Group 1 Capsule Assembly in Escherichia coli.

718   Structure 21:844–853.

719   66. Shang ES, Exner MM, Summers TA, Martinich C, Champion CI, Hancock RE, Haake DA.

720   1995. The rare outer membrane protein, OmpL1, of pathogenic Leptospira species is

721   a heat-modifiable porin. Infect Immun 63:3174–81.

722   67. Mizuno T, Kageyama M. 1978. Separation and Characterization of the Outer

723   Membrane of Pseudomonas aeruginosa. J Biochem 84:179–191.

724   68. Demirkan I, Walker R. L, Murray R. D, Blowey R. W, Carter S. D. 1999. Serological

725   evidence of spirochaetal infections associated with digital dermatitis in dairy cattle.

726   Vet J 157:69–77.

727   69. Murray R. D, Downham D. Y, Demirkan I, Carter S. D. 2002. Some relationships

728   between spirochaete infections and digital dermatitis in four UK dairy herds. Res Vet

729   Sci 73:223–230.

730   70.   Dhawi A, Hart CA, Demirkan I. 2005. Bovine digital dermatitis and severe virulent

731   ovine foot rot : A common spirochaetal pathogenesis. Vet J 169:232–241.

732   71.   Hashimoto M, Asai Y, Ogawa T. 2003. Treponemal Phospholipids Inhibit Innate

733   Immune Responses Induced by Pathogen-associated Molecular Patterns. J Biol Chem

734   278:44205–44213.

735   72.   Shenker BJ, Listgarten MA, Taichman NS. 1984. Suppression of human lymphocyte

736   responses by oral spirochetes: a monocyte-dependent phenomenon. J Immunol

737   132:2039–2045.

738   73.   Marcatili P, Nielsen MW, Sicheritz-Pontén T, Jensen TK, Schafer-Nielsen C, Boye M,

739   Nielsen M, Klitgaard K. 2016. A novel approach to probe host-pathogen interactions

740   of bovine digital dermatitis, a model of a complex polymicrobial infection. BMC

741   Genomics 17:987.

742   74.   Rosander A, Guss B, Frykberg L, Bjö Rkman C, Nä Slund K, Rit Pringle M, Björkman C,

743   Näslund K, Pringle M. 2011. Identification of immunogenic proteins in Treponema

744   phagedenis-like strain V1 from digital dermatitis lesions by phage display. Vet

745   Microbiol 153:315–322.

746   75.   Pringle M, Bergsten C, Fernström L-L, Höök H, Johansson K-E. 2008. Isolation and

747   characterization of Treponema phagedenis-like spirochetes from digital dermatitis

748   lesions in Swedish dairy cattle. Acta Vet Scand 50:40.

749   76.   Stokes JE, Leach KA, Main DCJ, Whay HR. 2012. The reliability of detecting digital

750   dermatitis in the milking parlour. Vet J 193:679–684.

751   77.   Rodriguez-Lainz A, Melendez-Retamal P, Hird DW, Read DH. 1998. Papillomatous

752   digital dermatitis in Chilean dairies and evaluation of a screening method. Prev Vet

753   Med 37:197–207.

754   78.   Vink WD, Jones G, Johnson WO, Brown J, Demirkan I, Carter SD, French NP. 2009.

755   Diagnostic assessment without cut-offs : Application of serology for the modelling of

756   bovine digital dermatitis infection. Prev Vet Med 92:235–248.

757   79.   Pinne M, Choy HA, Haake DA. 2010. The OmpL37 surface-exposed protein is

758   expressed by pathogenic Leptospira during infection and binds skin and vascular

759   elastin. PLoS Negl Trop Dis 4:e815.

760   80.   Oliveira R, Domingos RF, Siqueira GH, Fernandes LG, Souza NM, Vieira ML, de Morais

761   ZM, Vasconcellos SA, Nascimento ALTO. 2013. Adhesins of Leptospira interrogans

762   Mediate the Interaction to Fibrinogen and Inhibit Fibrin Clot Formation In Vitro. PLoS

763   Negl Trop Dis 7:e2396.

764   81.   Rybarczyk BJ, Lawrence SO, Simpson-Haidaris PJ. 2003. Matrix-fibrinogen enhances

765   wound closure by increasing both cell proliferation and migration. Blood 102:4035–

766   4043.

767   82.   Krull AC, Cooper VL, Coatney JW, Shearer JK, Gorden PJ, Plummer PJ. 2016. A Highly

768   Effective Protocol for the Rapid and Consistent Induction of Digital Dermatitis in

769   Holstein Calves. PLoS One 11:e0154481.

770   83.   Edwards AM, Dymock D, Woodward MJ, Jenkinson HF. 2003. Genetic relatedness and

771   phenotypic characteristics of Treponema associated with human periodontal tissues

772   and ruminant foot disease. Microbiology 149:1083–1093.

773   84.   Levi M, van der Poll T, Büller HR. 2004. Bidirectional Relation Between Inflammation

774   and Coagulation. Circulation 109:2698–2704.

775   85.   Ponnuraj K, Bowden MG, Davis S, Gurusiddappa S, Moore D, Choe D, Xu Y, Hook M,

776   Narayana SVL. 2003. A "dock, lock, and latch" Structural Model for a Staphylococcal

777   Adhesin Binding to Fibrinogen. Cell 115:217–228.

778   86.   Bamford C V, Fenno JC, Jenkinson HF, Dymock D. 2007. The chymotrypsin-like

779   protease complex of Treponema denticola ATCC 35405 mediates fibrinogen

780   adherence and degradation. Infect Immun 75:4364–72.

781   87.   Berry SL, Read DH, Famula TR, Mongini A, Döpfer D. 2012. Long-term observations on

782   the dynamics of bovine digital dermatitis lesions on a California dairy after topical

783   treatment with lincomycin HCl. Vet J 193:654–658.

784   88.   Farrell DH, Thiagarajan P, Chung DW, Davie EW. 1992. Role of fibrinogen alpha and

785   gamma chain sites in platelet aggregation. Proc Natl Acad Sci U S A 89:10729–32.

786   89.   Hawiger J, Timmons S, Strong DD, Cottrell BA, Riley M, Doolittle RF. 1982.

787   Identification of a region of human fibrinogen interacting with staphylococcal

788   clumping factor. Biochemistry 21:1407–13.

789   90.   Liu C-Z, Shih M-H, Tsai P-J. 2005. ClfA221–550, a fibrinogen-binding segment of

790   Staphylococcus aureus clumping factor A, disrupts fibrinogen function. Thromb

791   Haemost 94:286–94.

792   91.   Ghosh S, Chakraborty K, Nagaraja T, Basak S, Koley H, Dutta S, Mitra U, Das S. 2011.

793   An adhesion protein of Salmonella enterica serovar Typhi is required for pathogenesis

794   and potential target for vaccine development. Proc Natl Acad Sci U S A 108:3348–53.

795   92.   Lithgow K V, Hof R, Wetherell C, Phillips D, Houston S, Cameron CE. 2017. A defined

796   syphilis vaccine candidate inhibits dissemination of Treponema pallidum subspecies

797   pallidum. Nat Commun 8:14273.

798   93. Berry SL, Ertze RA, Read DH HD. 2003. Field evaluation of prophylactic and

799   therapeutic effects of a vaccine against (papillomatous) digital dermatitis of dairy

800   cattle in two California dairiesProceedings of the 13th International Symposium on

801   Ruminant Lameness. Maribor, Slovenia.

802   94. O'Hagan DT, MacKichan ML, Singh M. 2001. Recent developments in adjuvants for

803   vaccines against infectious diseases. Biomol Eng 18:69–85.

804   95. Grun JL, Maurer PH. 1989. Different T helper cell subsets elicited in mice utilizing two

805   different adjuvant vehicles: The role of endogenous interleukin 1 in proliferative

806   responses. Cell Immunol 121:134–145.

807   96. Brewer JM, Conacher M, Hunter CA, Mohrs M, Brombacher F, Alexander J. 1999.

808   Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the

809   absence of IL-4- or IL-13-mediated signaling. J Immunol 163:6448–54.

810   97. Elliott MK, Alt DP. 2009. Bovine immune response to papillomatous digital dermatitis

811   (PDD)-associated spirochetes is skewed in isolate reactivity and subclass elicitation.

812   Vet Immunol Immunopathol 130:256–261.

813

814

815

816

817   **Figure legends.**

818   *Table 2. Each putative treponemal OMP was selected on the basis of predicted β-barrel*

819   *topology by at least one of the β-barrel prediction programs. The results generated by BOMP,*

820   *PRED-TMBB, TMBETA-NET and SPAAN were interpreted in accordance with default cut-off*

821   *values. All four CDS features were predicted to share domain homology with known bacterial*

822   *OMPs.*

823   *Fig. 1. The four putative β barrel-outer membrane proteins (a-d) were structurally modelled*

824   *using I-TASSER. A lateral view of the top-ranking ribbon model, as determined by the C-score,*

825   *is shown, with β-sheet depicted in blue and α-helix depicted in red.*

826   *Table 3. The amino acid sequence of each putative OMP was submitted to I-TASSER for 3D*

827   *structural modelling and Protein Databank (PDB) structural analogue detection. [1]C-Score: A*

828   *confidence score for estimating the quality of predicted models.*

829   *Fig. 2. Gel A,* C5N99_02965; *Gel B,* C5N99_10335; *Gel C,* DYQ05_06810. *Gels A, B and C: 1,*

830   *Wide-range MW marker (kDa); 2, unheated sample; 3, heated sample. The heat-modified*

831   *(unfolded) forms of the proteins are distinguished from the unmodified (folded) forms by the*

832   *addition of an asterisk (*). Panel C: lane between marker and sample contained a wash*

833   *fraction and has been removed for brevity.*

834   *Fig. 3. CD spectra of proteins encoded by a)* C5N99_10335, *b)* C5N99_02965, *c)* DYQ05_06810,

835   *d)* DYQ05_13425 and e) *L. interrogans* OmpL1 *are shown. Far-UV CD spectra are presented as*

836   *an average of three scans recorded from 190 to 260 nm. φ, molar ellipticity.*

837

838 *Fig. 4. Serological assessment of the IgG1 and IgG2 response to putative treponemal OMPs by*

839 *ELISA. Error bars: standard error of the mean. Non-exp., Non-exposed; DD exp., digital*

840 *dermatitis-exposed. Asterisks (*) indicates a significant difference in IgG seroreactivity relative*

841 *to control sera as determined by Mann-Whitney U test (* P<0.05, ** P<0.005).*

842 *Fig. 5. Immobilised ECM component binding screens of four putative β-barrel OMPs and the*

843 *positive control protein (OmpL1). Bars represent the mean OD of three independent*

844 *experiments. Error bars: standard error of the mean. Asterisks (*) indicate a significant*

845 *difference in binding compared to the negative control protein, BSA, as determined by one-*

846 *way ANOVA and the Dunnett post-test (* P<0.05, **P<0.005, ***P<0.001).*

847 *Fig. 6. Binding affinity curves of the four putative β-barrel OMPs to bovine fibrinogen. Graph*

848 *A, putative OMPs from T. medium; Graph B, putative OMPs from T. pedis. Data points*

849 *represent the mean OD of replicate readings. Error bars: standard error of the mean.*

850 *Fig. 7. Specific binding interactions between the recombinant proteins and the α, β and γ*

851 *chains of bovine fibrinogen, using monoclonal Anti-polyHistidine antibody as the probe.*

852 *Fig 8. The serological IgG1 and IgG2 response to four treponemal putative OMPs was assessed*

853 *in BDD-naïve calves (n=2). The mean OD of both immunised calves is shown. Error bars:*

854 *standard error of the mean.*

855

856

857

858

859
860
861

862 **Figures and Tables.**

863 Table 1. Primers used to amply 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: CACCGATGGGGTCGATTTTTCG	0.7	27.1
		Reverse: CTACAGCTTAAAAGCGATCC		
C5N99_02965	*T. medium*	Forward: CACCCAGGAAGAAGGAGCAGAGG	0.9	35.0
		Reverse: AGAGATACCCATTAGTTGTTG		
DYQ05_13425	*T. pedis*	Forward: CACCTTAAGCGATATTTCAGGCGATG	0.8	29.8
		Reverse: TTACAGCTTCCATGCAATACC		
DYQ05_06810	*T. pedis*	Forward: CACCGCAAAGACTATCGGTCTTAATTG	0.9	22.1
		Reverse: TTAAAAATAAACTCTTAAACCCGC	0.6	
OmpL1	*L. interrogans*	Forward: CACCAAAACATATGCAATTGTAGGATTTG	0.8	31.0
		Reverse: TTAGAGTTCGTGTTTATAACCG		

864

865 Table 2. Bioinformatic analysis of four putative treponemal OMPs.

Putative OMP	*Treponema* phylogroup	*Signal peptide cleavage site*	*β-barrel and adhesin prediction*				*Homologous domain search*		
			BOMP	PRED-TMBB	TMBETA-NET	SPAAN	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)

866

867

868

869

870

871

872

873　*Figure 1. Prediction of the treponemal putative OMP 3-dimensional structure*

a) C5N99_10335   b) C5N99_02965

c) DYQ05_06810   d) DY105_13425
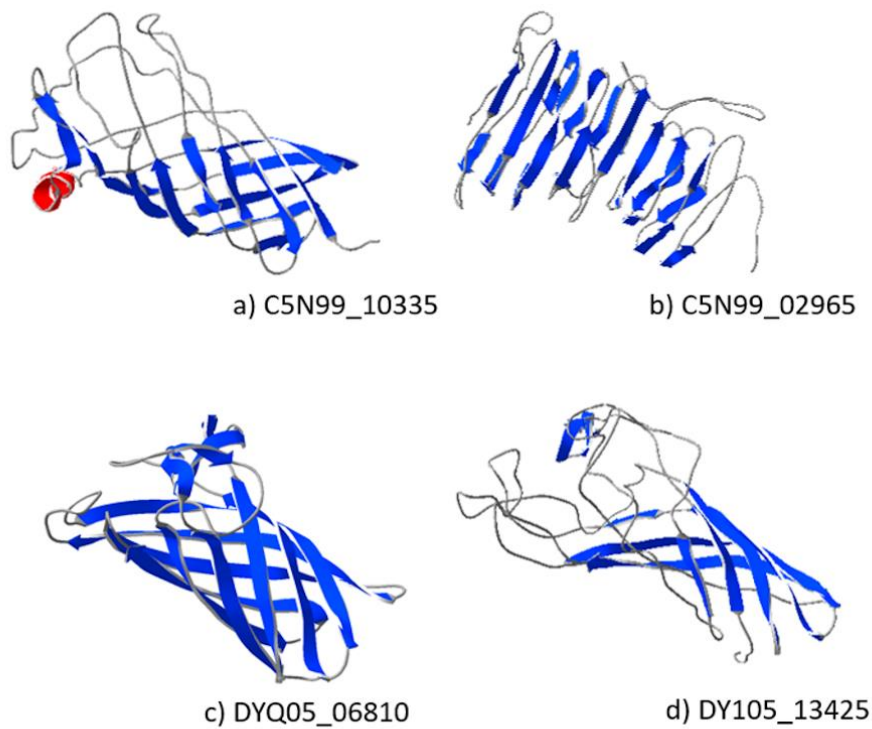
874

875

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

Putative OMP	*C*-score[1]	Predicted topology	PDB Structural analog (bacterial species)	Structural analog function	Ref.
C5N99_10335	3.98	8-stranded β-barrel	OmpT (*E. coli*)	Protease	(62)
C5N99_02965	2.73	β-solenoid barrel	serine-rich repeat protein (*Lactobacillus reuteriin*)	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)

877

878

879

880

881

882   Figure 2. Heat-modifiability of C5N99_10335, C5N99_02965 and DYQ05_06810.
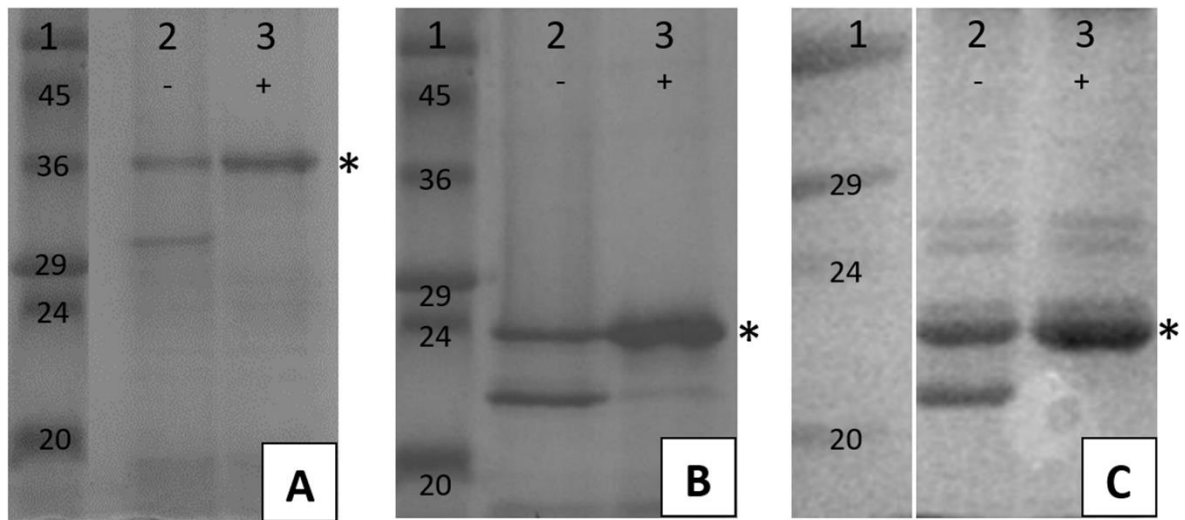
883

884

885

886

887

888

889

890

891

892

893

894

895

896

897

898

899

900

901

902

904 Fig 3. *Determination of protein fold state using far UV Circular Dichroism (CD).*
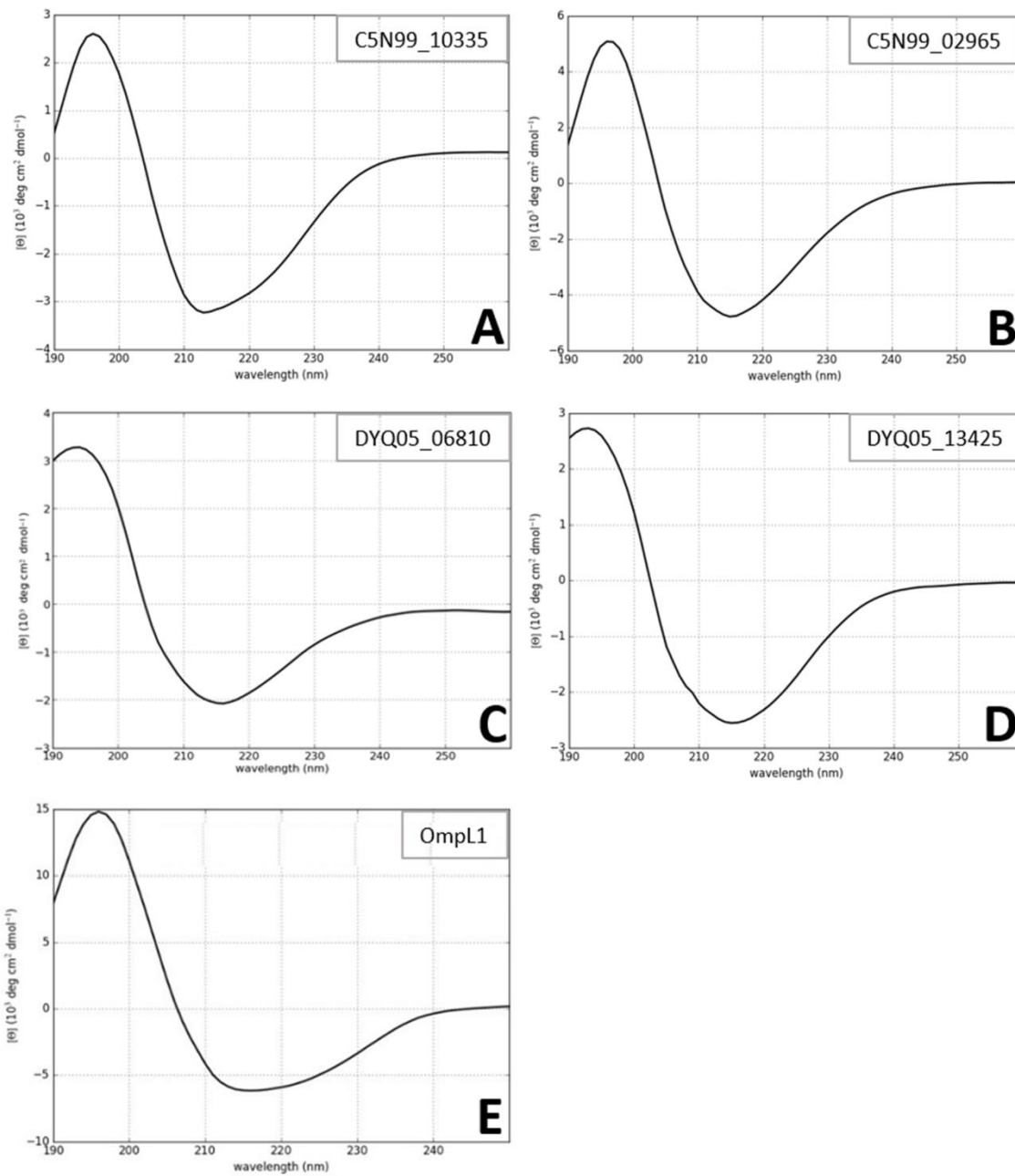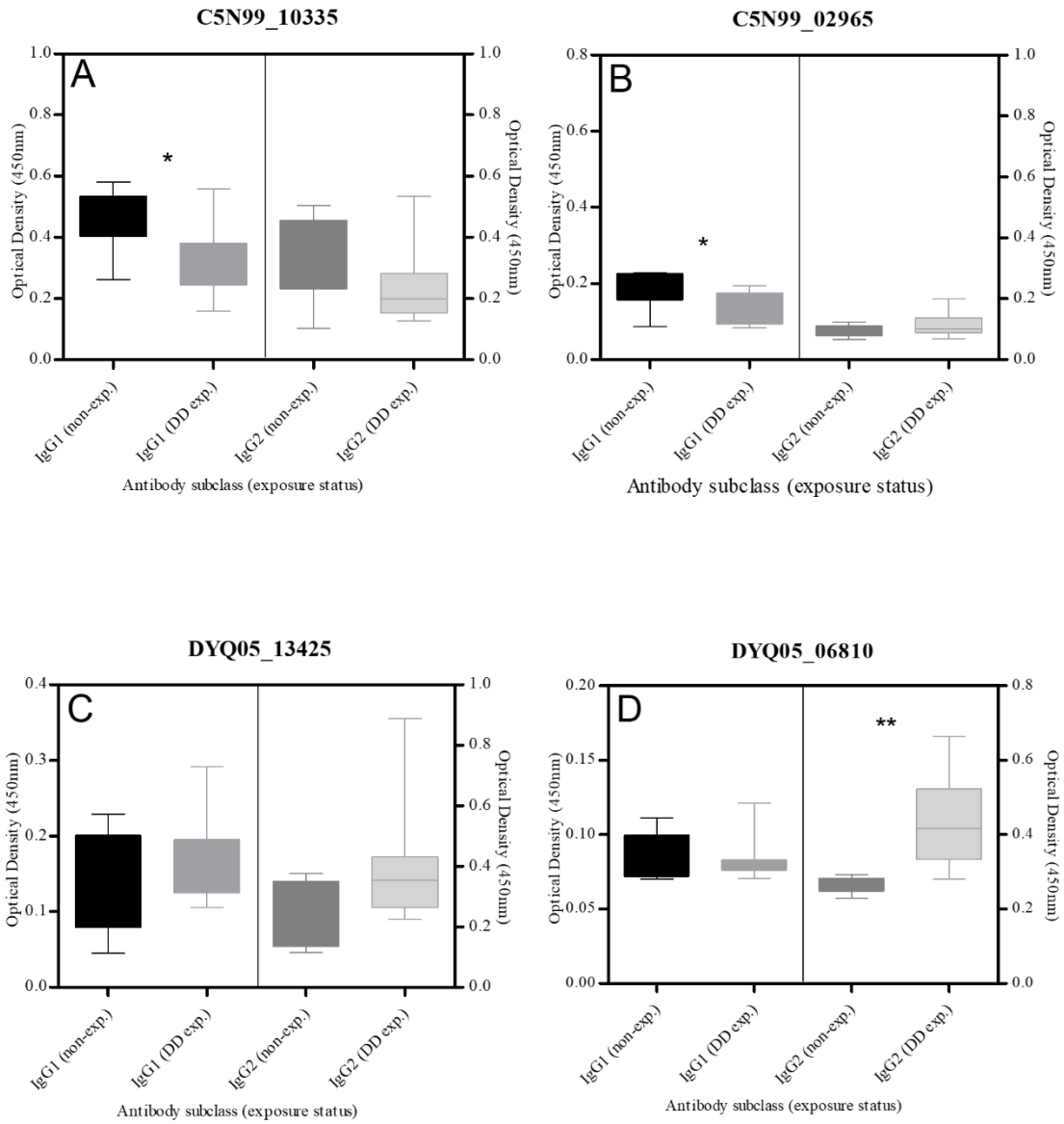

905
906
907
908
909

910

911

912

42

913   Fig 4. *Determination by ELISA of the serological response to four putative OMPs in BDD-*
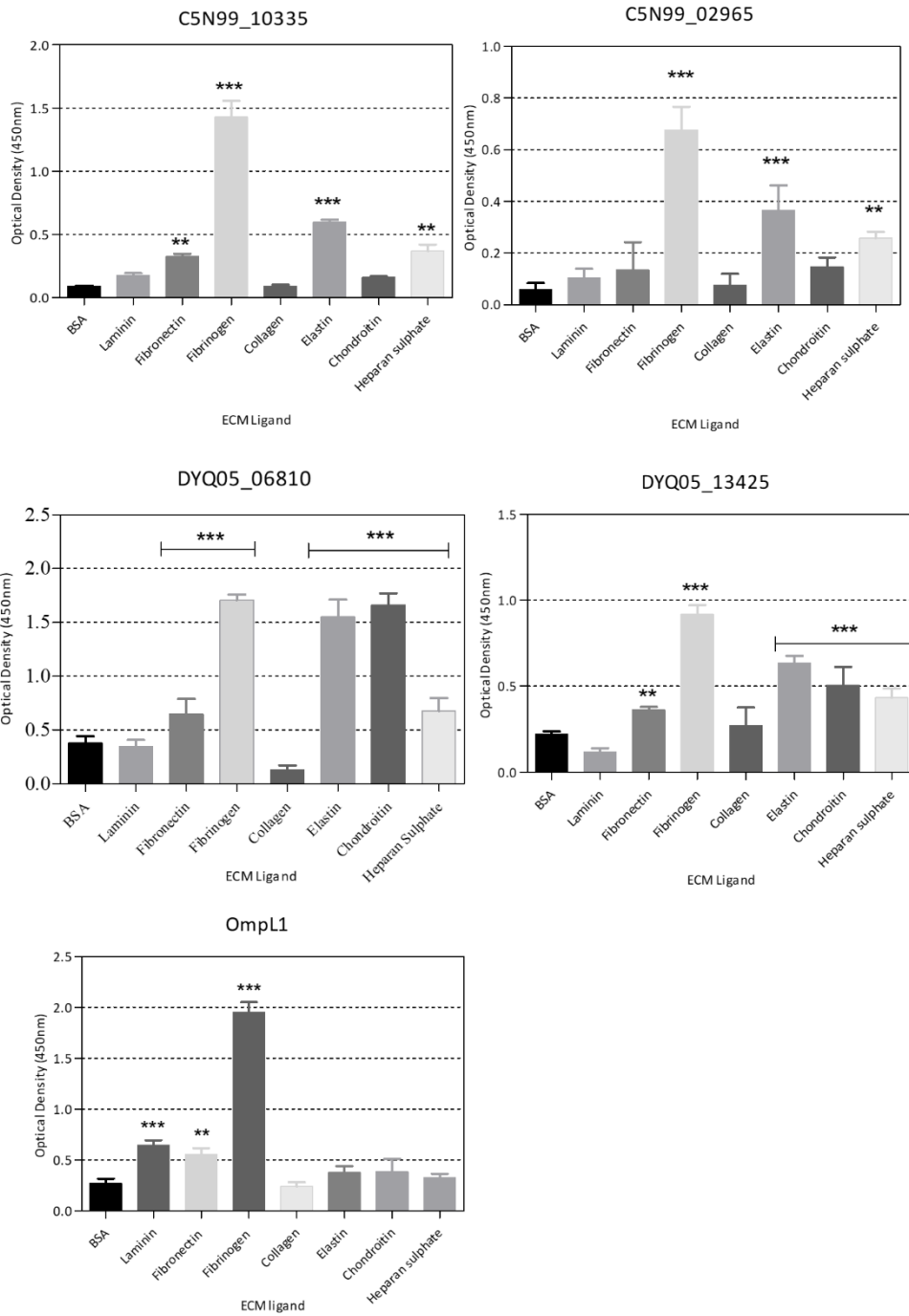914   *positive adult Holstein-Friesian dairy cattle.*

915

916

917

918

919

920

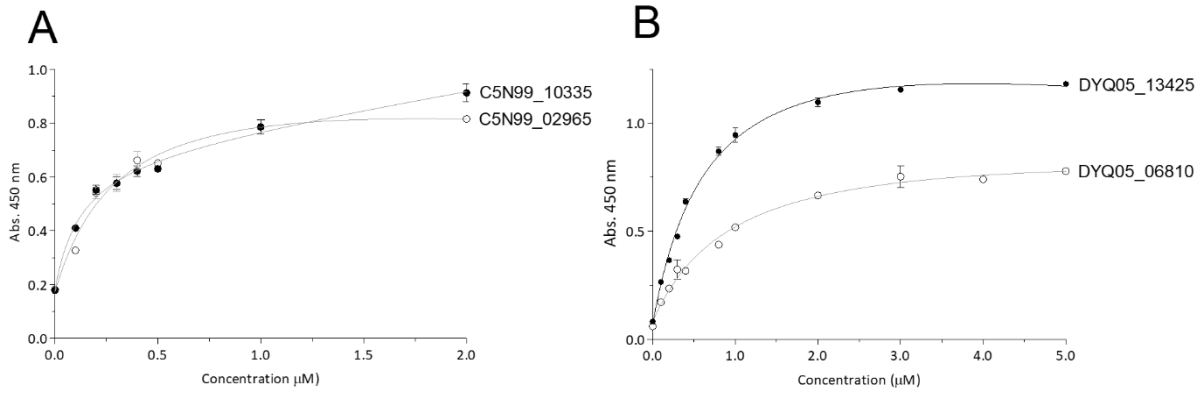*Fig 5. Analysis of the attachment of treponemal putative OMPs to ECM components.*

922

923

924

925

926   *Fig. 6. Binding affinity curves of the four putative β-barrel OMPs to bovine fibrinogen.*

927

928

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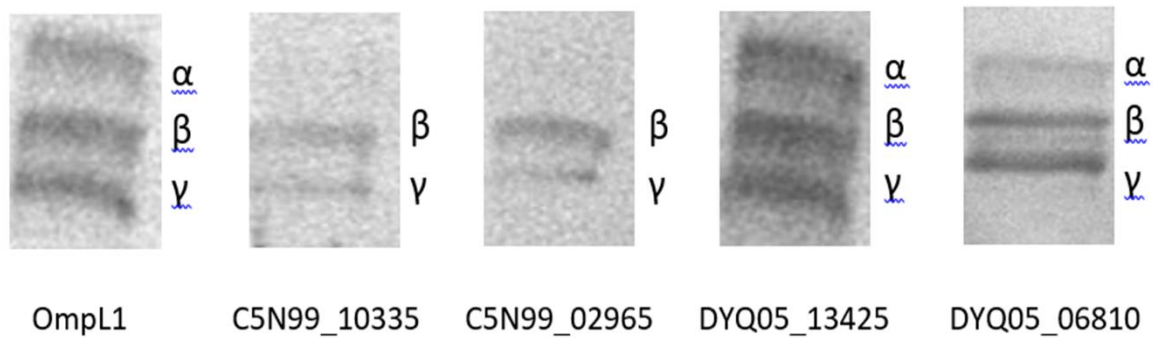930   fibrinogen.

931

OMP	*Dissociation Constant (K_D)*
C5N99_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))

932

933   Fig. 7. Far western blotting analysis of the treponemal recombinant OMP-Bovine fibrinogen
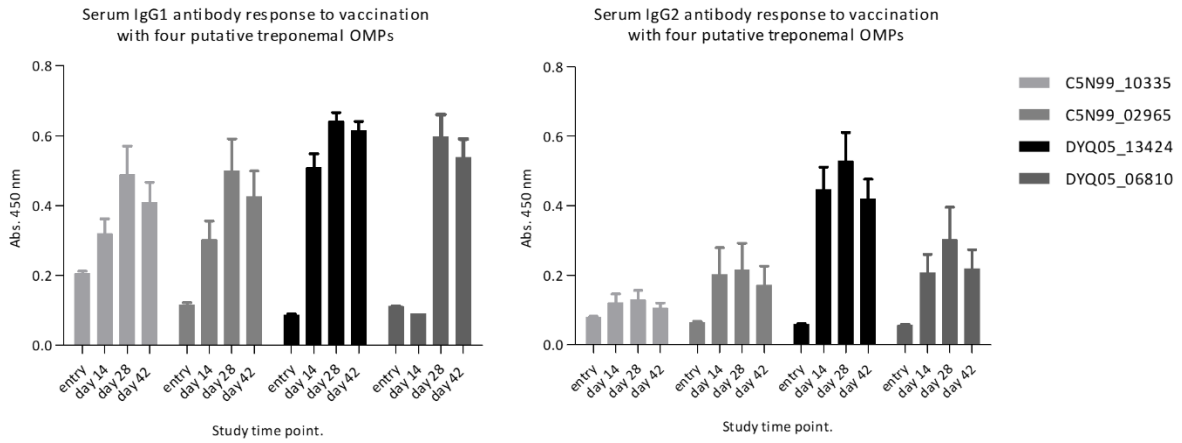
934   binding interactions.

935

936

937   Fig. 8. Serological IgG1 and IgG2 response to immunisation with four recombinant treponemal

938   putative OMPs.

939

940

941

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

944

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

945

946

947

948

949

950

951

952

953