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Phenotypic and proteomic characterization of treponemes associated with bovine digital dermatitis.

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Abstract

Bovine digital dermatitis (BDD) is a multifactorial polymicrobial infectious disease associated with multiple species and phylotypes of treponemes. However, despite the abundance of molecular signatures for treponemes that are identified in bovine lesions, relatively few isolates are cultured, and even fewer have been characterized at the level of protein expression. Here we report the successful isolation and characterization of novel strains of T. brennaborense and T. phagedenis from cases of BDD in Iowa dairy cows, and compare them to a well characterized strain of T. phagedenis, and the type strain of the more recently recognized T. pedis. Propagation of T. brennaborense was only possible at room temperature in Cooked Meat Medium, and not in oral treponeme enrichment medium at 37°C as used for T. phagedenis and T. pedis. A prominent and rapid motility is observed by T. brennaborense under dark-field microscopy. The highly motile T. brennaborense strain 11-3 has an identical enzymatic profile to that of the only other isolate of T. brennaborense to be cultured from a lesion of BDD. Outer membrane protein profiles of each strain were compared by 2-D gel electrophoresis, and the five most abundant proteins in each strain were identified by mass spectrometry. All identified proteins are predicted to have signal peptides. Results identified outer membrane proteins specific to each strain including predicted membrane lipoproteins, ABC transporters and, as yet, uncharacterized proteins. Collectively, our results provide for the identification and characterization of outer membrane components of multiple phylotypes of treponemes associated with BDD which can facilitate development of vaccines and diagnostics in our efforts to eradicate the disease.

Keywords

Bovine digital dermatitis; Treponema; outer membrane proteins

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Introduction

Digital dermatitis (DD) is a multifactorial polymicrobial infectious disease originally described in dairy cattle, but is increasingly recognized in beef cattle, sheep, and more recently, elk and goats (Wilson-Welder et al., 2015). Bovine digital dermatitis (BDD) is a global disease that results in significant economic losses every year due to lameness associated with decreased milk yield (Losinger, 2006). BDD is readily observed as a cause of acute lameness; lesions range from the early M1 stage of a small circumscribed granulomatous area, to the M2 classical ulceration close to the coronary band, and the M3 healing lesion covered by a scab (Döpfer et al., 1997). Additional modifications to this scoring system comprise the presence of chronic lesions (M4), including those that contain small active foci (M4.1), as well as a system that correlates morphological stage of lesion development with unique microbiota (Berry et al., 2012; Krull et al., 2014a).

No single bacterial aetiology of BDD has been identified; it is a polybacterial disease as evidenced by the detection of multiple bacterial agents associated with clinical lesions. Most commonly, bacteria associated with BDD comprise multiple species and phylotypes from the genus Treponema. The diversity and dynamics of treponemes associated with BDD lesions has been confirmed by several molecular approaches, including in situ hybridization and high throughput DNA sequencing technologies, and suggest that up to 45 species of Treponema are associated with BDD lesion progression (Choi et al., 1997; Klitgaard et al., 2008; Klitgaard et al., 2014; Knappe-Poindecker et al., 2013; Krull et al., 2014b; Rasmussen et al., 2012).

In contrast to the large number of species and phylotypes of Treponema associated with BDD that are detected by molecular methods, to date, only a few have been cultured. This likely reflects the fastidious nature of treponemes, many of which are anaerobic, as well as the degree of contamination expected with a typical bovine foot lesion. Nevertheless, isolates of treponemes associated with BDD are essential for continued research to define effective intervention strategies. Improved methods to isolate Treponema associated with BDD continue to be pursued by pioneers in the field. To date, cultured isolates include representatives of several clusters such as 1) T. medium/T. vincentii-like, 2) T. phagedenis-like, and 3) T. pedis which are grouped according to 16S rDNA and flaB2 gene homology (Evans et al., 2008; Pringle et al., 2008; Trott et al., 2003). Cultures may also comprise mixed species of treponemes (Sayers et al., 2009), and need to be plated out to select individual colonies for expansion of clonal isolates. Only a single isolate of T. brennaborense has ever been cultured; the type strain from this novel species was cultured from an infected dairy cow in Germany (Schrank et al., 1999). Though T. brennaborense was detected in some BDD lesions by in situ hybridization (Klitgaard et al., 2008), it was not detected in others, leading to the suggestion that this species may not be a significant cause of BDD disease pathogenesis (Nordhoff et al., 2008).

In this study, we have successfully cultured a second strain of T. brennaborense, strain 11-3, from a lesion of BDD, as well as another strain of T. phagedenis, strain 1452. The phenotype and outer membrane proteome of both strains was compared to other bovine isolates of Treponema associated with BDD; the well characterized T. phagedenis strain 4A, and the

more recent and newly classified T. pedis strain T3552B (Evans et al., 2009). Mass spectrometry was performed to identify proteins in the OM fraction of each strain, highlighting similarities and differences between strains, and to provide for their further evaluation in understanding pathogenic mechanisms of BDD, and the development of diagnostics and vaccines.

Methods

Bacteria

Treponema phagedenis strain 1452 was isolated from a dairy cow at the National Centers for Animal Health, Ames, IA, U.S.A. Lesion material was obtained by punch biopsy, placed in a glass screw capped tube containing 10 ml Oral Treponeme Isolation (OTI) medium (Smibert, 1991) with enrofloxacin (5 μg/ml), phosphomycin (100 μg/ml), and rifampicin (25 μg/ml) and transported to the laboratory for processing. The biopsy material was placed in an anaerobic chamber and macerated in a petri dish in the OTI medium. OTI agar plates containing antibiotics were inoculated with 100 μl of the macerated lesion material and incubated at 37°C. Individual colonies were picked and observed by dark-field microscopy. A colony, demonstrating typical T. phagedenis morphology and motility (Trott et al., 2003; Wilson-Welder et al., 2013), was clonally purified three times on OTI plates without antibiotics. Treponema phagedenis strain 4A was isolated from a dairy cow with bovine digital dermatitis (BDD) as previously described (Trott et al., 2003). Treponema brennaborense strain 11-3 was isolated from a punch biopsy of a lesion from an Iowa dairy cow with BDD. The lesion biopsy was placed in a glass screw capped tube containing 10 ml OTI medium with enrofloxacin (5 μg/ml), phosphomycin (100 μg/ml), and rifampicin (25 μg/ml), transported on ice and placed at 4°C for 2 days before processing as described above. One of the colonies picked from OTI plates demonstrated a very rapid motility under dark-field examination, but attempts to expand this colony in OTI liquid at 37°C failed. However, the colony did grow well at room temperature in commercial Cooked Meat Medium (Hardy Diagnostics, Santa Maria, CA); these conditions were used to expand Treponema brennaborense strain 11-3. Treponema pedis strain T3552B was sourced from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Culture (Evans et al., 2009).

T. phagedenis strains 1452 and 4A, and T. pedis strain T3552B, were propagated in commercial Oral Treponeme Enrichment Broth (OTEB) (Anaerobe Systems, Morgan Hill, CA) at 37°C. T. brennaborense strain 11-3 was propagated in Cooked Meat Medium at room temperature. All cells were harvested at mid to late logarithmic phase of growth, at approximately 72 hours after media inoculation, by centrifugation at 12,000g for 30 mins at 4°C. Treponeme morphology and motility at this time point was documented at 400X using dark-field microscopy on a Leica DMi8 microscope with a Leica MC170 HD microscope camera and Leica Application Suite V 4.8 software. Treponemes were enumerated by dark-field microscopy as previously described (Miller, 1971). The species of each strain was confirmed by sequencing of 16S rDNA, Supplementary Figure 1, as previously described (Han et al., 2019). The gene sequence of 16S rDNA from T. phagedenis strain 4A is available from NCBI; assembly GCA_000513775.1.

Enzymatic Profiles

Enzyme profiles were determined using API®ZYM test strips (bioMérieux Inc.) according to manufacturer's instructions.

Transmission Electron Microscopy

A 10μl aliquot of culture was taken directly from each tube of media, at mid to late logarithmic phase of growth, and placed directly on a copper 200 mesh formvar carbon film grid (Electron Microscopy Sciences, Hatfield, PA) for a negative stain. After 60 seconds, the sample was overlaid with 0.5% Phosphotungstic acid (PTA) pH 7.0, for 3 (T. phagedenis) or 10 (T. brennaborense and T. pedis) seconds. Images were viewed with a ThermoFisher FEI Tecnai G^2 BioTWIN electron microscope (FEI Co., Hillsboro, OR) and images taken with a side mount ORCA-HR camera (Advanced Microscopy Techniques Woburn, MA). Measurements provided are values obtained from 6 different images for each strain.

Enrichment for outer membrane proteins

Fractions of treponemes were enriched for outer membrane proteins using Triton X-114 similar to that previously described (Nally et al., 2001; Radolf et al., 1988). In brief, from 5×10^9 to 1×10^{10} treponemes were resuspended in 2 ml 2% Tx-114 (Sigma) in 10mM Tris, 1mM EDTA (TE) buffer, pH 7.4 and rotated overnight at 4°C. Insoluble materials, including protoplasmic cylinders, were removed by centrifugation at 20,000 × g, for 30 min at 4°C. The supernatant was phase separated by warming the solution to 37°C for 10 min followed by centrifugation at 13,000 × g at room temperature for 10 min. The separated detergent phase was washed three times with ice-cold TE buffer, while the aqueous phase was washed three times by adding ice-cold 10% Triton X-114 to bring the final concentration of Triton X-114 to 2%, followed by warming to 37°C and centrifugation as described above. Chloroform-methanol precipitation was used to remove detergent contaminants (Wessel and Flügge, 1984).

1 and 2-D gel electrophoresis and immunoblotting

Protein samples were processed for one-dimensional (1-D) SDS-PAGE on 12% acrylamide gels (BioRad) as per manufacturer's guidelines. Protein samples (20μg) were processed for two-dimensional (2-D) gel electrophoresis as previously described using pH 4-7 7cm strips (Nally et al., 2005; Nally et al., 2007). Protein concentrations were determined using the RC/DC protein assay kit (Bio-Rad). Proteins were visualized by staining with Sypro Ruby (Invitrogen, CA) as per manufacturer's guidelines. For immunoblotting, samples were transferred to Immobilon-P transfer membrane (Millipore, 220 Bedford, MA) and blocked overnight at 4°C with 5% non-fat dried milk in PBS containing 0.1% Tween 20 (PBS-T). Membranes were individually incubated with anti-Treponema FlaA at 1:2000 in PBS-T for 1 hour at room temperature, followed by incubation with horseradish-peroxidase anti-rabbit immunoglobulin G conjugate (1:5,000 in PBS-T) (Sigma, MO). Bound conjugates were detected using Clarity Western ECL substrate (BioRad, CA) and images acquired using a Bio-Rad ChemiDoc MP imaging system.

Protein identification by mass spectrometry

Selected protein spots were excised from 2-D gels using a 2.5 mm single use sterile biopsy punch. Gel spots were processed using the In-gel Tryptic Digestion kit (Thermo Scientific) as per manufacturer's instructions. Eluted peptides were further cleaned-up using Pierce C18 Spin Columns (Thermo Scientific) and samples dried in a vacuum evaporator. Samples were reconstituted in 100 μL of a buffer solution (2% acetonitrile, 0.5% acetic acid) and desalted with C18 stage tips as previously described (Capri and Whitelegge, 2017). Eluted samples were lyophilized and solubilized with 10 μL of 2% acetonitrile, 0.1% formic acid and then were placed into injection vials for MS analysis. Samples were analyzed with an Eksigent 2D nanoLC system attached to a Q-Exactive Plus (Thermo Fisher Scientific). Peptides were injected into a laser-pulled nanobore 20 cm × 1.8 pm C18 column (AcuTech Scientific) in buffer A (2% acetonitrile, 0.15% formic acid) and were eluted using a 3-h linear gradient from 3 to 80% buffer B (98% acetonitrile, 0.15% formic acid). The Q-Exactive Plus was operated in Full MS/dd-MS2 mode with a resolution of 70,000 and an auto gain control target of 3e6 for the parent scan. The top 20 ions above +1 charge were subjected to higher collision dissociation (HCD) set to a value of 30 with a resolution of 17,500 and an auto gain control target of 1e5 as well as a dynamic exclusion of 15 s. Tandem mass spectrometry data were matched to a protein fasta file (UniProt-Treponema, comprising 138,141 entries on February 2019) using Mascot software (version 2.4; Matrix Sciences). Identified proteins, with a Mascot score greater than 100 and ordered by exponentially modified protein abundance index (emPAI), are provided (Supplementary Table 1). emPAI offers approximate, label-free, relative quantitation of the proteins in the mixture based on protein coverage by the peptide matches in the database search result. Signal peptides in identified proteins were predicted using SignalP (Armenteros et al., 2019); http://www.cbs.dtu.dk/services/SignalP/. Protein alignments were generated with Clustal Omega (Sievers et al., 2011); https://www.ebi.ac.uk/Tools/msa/clustalo/.

Results

Typing of bovine Treponema isolates

The species identification, and clonal nature of, T. brennaborense strain 11-3 and Treponema phagedenis strain 1452 that were cultured from lesions of cows with digital dermatitis during this work was confirmed by sequencing of the gene encoding 16S rDNA, Supplementary Figure 1. The enzymatic profile of T. phagedenis strain 1452 is very similar to that of T. phagedenis strain 4A, Table 1; both strains are positive for alkaline phosphatase, C_4 esterase, C_8 esterase lipase, acid phosphatase, Naphtholphosphohydrolase, β-galactosidase and N-acetyl-β-glucosamidase. Some variability is observed for the detection of β-glucuronidase, α-glucosidase, N-acetyl-β-glucosamidase and α-fucosidase, and as similarly described for other strains of T. phagedenis isolated from lesions of bovine digital dermatitis (Pringle et al., 2008; Wilson-Welder et al., 2013). The enzymatic profile of T. brennaborense strain 11-3 is identical to that previously described for the type strain of T. brennaborense DD5/3^T (Schrank et al., 1999). The enzymatic profile of T. pedis strain T3552B is identical to that previously described (Evans et al., 2009). In contrast to T. phagedenis and T. pedis, expansion of T. brennaborense strain 11-3 was not possible using

Oral Treponeme Enrichment Broth at 37°C; growth was only maintained when cultured at room temperature in commercial Cooked Meat Medium.

Motility of bovine Treponema isolates

T. brennaborense strain 11-3 was selected for expansion as it displayed an unusually rapid motility compared to that typically observed for other strains of T. phagedenis. Representative movie clips demonstrating the motility of each species, as observed by dark-field microscopy, are provided as supplementary information.

Transmission electron microscopy of bovine Treponema isolates

T. phagedenis strain 1452 ranged in length from 9.3 to 11.2 μm (average = 10.6μm) and 0.5μm in thickness and was similar to T. phagedenis strain 4A which ranged in length from 7.7 to 12.3 μm (average = 10.0μm) and 0.4μm in thickness, and as previously described (Wilson-Welder et al., 2013). Multiple flagella were observed in each of the T. phagedenis strains and ranged from 6 to 12, Figure 1. T. brennaborense strain 11-3 ranged in length from 8.8 to 13.7 μm (average = 11.3 μm) and 0.2μm in thickness. In contrast to T. phagedenis, no more than two flagella were observed for the highly motile T. brennaborense. Finally, T. pedis strain T3552B ranged in length from 8.0 to 12.8 μm (average = 10.4μm) and 0.2μm in thickness; up to 6 flagella were observed.

Outer membrane proteins of bovine Treponema isolates

Whole treponemes were fractionated using Triton X-114 to provide for a fraction enriched for outer membrane (OM) proteins. Fractionation of proteins from whole treponemes results in three fractions; a detergent-rich phase (D) enriched for outer membrane proteins containing hydrophobic domains, a detergent-poor aqueous phase (A) containing water soluble proteins associated with the outer membrane and periplasmic space, and the remaining insoluble proteins associated with the protoplasmic cylinder (P), Figure 2, A, B, C & D. Comparison of protein profiles in the detergent phase clearly demonstrate enrichment of specific proteins when compared to whole treponemes and other fractions. The periplasmic space of Treponema contains the flagellar apparatus which is anchored to the inner membrane of the protoplasmic cylinder; as expected for FlaA, minimal amounts were detected in the OM fraction when compared to the protoplasmic cylinder and whole treponemes by immunoblot, Figures 2 F, G & H. Significant differences in protein composition of the OM are evident not only between each species, but also between each of the two strains of T. phagedenis, Figure 2E.

To further evaluate the protein composition of the outer membrane from each strain, 2-D gel electrophoresis was performed to provide increased separation and resolution of protein content, Figure 3. Five of the most abundant protein spots from each of the 4 strains were selected for additional analysis and identification by mass spectrometry. The identification of the most significant protein identification for each protein spot is provided, Table 2. All significant protein identifications are provided in Supplementary Table 1.

T. phagedenis strain 4A and strain 1452 have similar 2-D OM protein profiles, Figure 3 A & B respectively. Protein spots with similar molecular mass and pi in both strains include spot

numbers 1&6, 2&7 and 4&9, which were identified as a basic membrane protein, a glucose/galactose binding lipoprotein and an uncharacterized protein, respectively. There also exists apparent differential protein expression between each of these T. phagedenis strains as evidenced by protein spot numbers 3 and 5 which are abundantly expressed by strain 4A but not 1452, circled in Figure 3 A & B. The identification of these proteins are VpsB and an uncharacterized protein respectively. In contrast, protein spot number 8 is apparently expressed in greater amounts by strain 1452 compared to strain 4A, the identification of which is also an uncharacterized protein. Protein spot number 10, which appears common to both strains, had three significant hits including an ABC transporter solute-binding protein (Table 1) and two uncharacterized proteins (Supplementary Table 1). In all cases, protein spots identified in strains of T. phagedenis are predicted by SignalP to have lipoprotein signal peptides, Table 2.

The 2-D profile of the OM protein enriched fraction from T. brennaborense had a profile that was very different to that of T. phagedenis, Figure 3C. Protein spots 11 and 12 were identified as a Flavocytochrome and uncharacterized protein respectively. Protein spots 13 and 14 were identified as an extracellular ligand-binding protein and a 5'-nucleotidase respectively. All are predicted to have lipoprotein signal peptides. Protein spot 15 was identified as an uncharacterized protein which is predicted to have a non-lipoprotein signal peptide.

The OM protein profile of T. pedis was more similar to that of T. phagedenis than that of T. brennaborense. Protein spot 16 has a similar molecular mass and pI to that of protein spots 2 & 7 from T. phagedenis. Similarly, it was also identified as a glucose/galactose binding lipoprotein. An alignment of these three protein sequences showing significant homology is provided, Supplementary Figure 2. Additional protein identifications in T. pedis include protein spots 17, 18, 19 and 20 that were identified as an Oligopeptide ABC transporter substrate-binding protein, a membrane lipoprotein, an uncharacterized protein and an Endo-1,4-beta-xylanase, respectively. All are predicted to have a lipoprotein signal peptide except the Endo-1,4-beta-xylanase which has a non-lipoprotein signal peptide. The uncharacterized protein in spot 19 has a similar mass and pI to that of the uncharacterized protein identified in spots 4 and 9 from T. phagedenis, though they only have a 54.5% homology (data not shown).

Discussion

Here we report a second successful isolation of T. brennaborense from a lesion of BDD. Expansion of this isolate required the use of a commercial Cooked Meat Medium maintained at room temperature, in contrast to the more commonly used Oral Treponeme Enrichment medium used at 37°C for growth of T. phagedenis and T. pedis. An identical enzymatic profile was identified in T. brennaborense strain 11-3 as the reference strain, Treponema brennaborense strain DD5/3^{T}, despite being isolated 20 years later, and on a different continent (Schrank et al., 1999). Molecular signatures for this new species have been identified in some lesions of BDD but not others and coupled with the lack of additional clinical isolates, this has led to suggestions that this species of treponeme may not be important in BDD disease progression (Nordhoff et al., 2008). However, our results

report that T. brennaborense is associated with BDD and that there is a need to reassess its importance in the pathogenesis of BDD. T. brennaborense strain 11-3 was selected for expansion due to the unusual rapid motility observed by dark-field microscopy compared to other treponemes (see supplementary movies). When examined by transmission electron microscopy, it was similar in length to other treponemes but only two flagella were observed compared to the much larger number (up to 12) observed in T. phagedenis and T. pedis. Nevertheless, visual observation of lesion material by dark-field microscopy may be useful to determine its presence amongst other treponemes.

Despite the universal observation that treponemes are not only observed at the interface of healthy and diseased tissue, but as highly invasive within the stratum corneum, and as recently reviewed (Wilson-Welder et al., 2015), few studies have looked to identify those components of the outer membrane that facilitate such host-pathogen interactions. To address this, the outer membrane protein components of treponemes cultured from lesions of BDD were separated from the protoplasmic cylinder, and enriched using Triton-X-114. Control immunoblots confirm that there is minimal protein carry-over of the periplasmic flagella into the aqueous and detergent phase validating the OM protein enrichment strategy, Figure 2. This was also confirmed by mass spectrometry; the 5 most abundant proteins from the OM enriched fraction in each strain were identified and all are predicted to have signal peptides, Table 2 & Supplementary Table 1.

The OM protein profile for both T. phagedenis strain 4A and 1452 were very similar, Figure 3 A & B, and in both strains the most abundantly expressed proteins included a basic membrane protein, a glucose/galactose binding lipoprotein and an uncharacterized protein. All three are predicted periplasmic ABC transporter proteins. The basic membrane protein (BMP) belongs to a BMP family ABC transporter substrate-binding protein conserved amongst several species of treponemes and has a conserved periplasmic binding domain homologous to the basic membrane lipoprotein PnrA in T. pallidum which transports purine nucleosides (Deka et al., 2006). The glucose/galactose binding lipoprotein belongs to a Type 1 periplasmic binding fold superfamily that are the primary receptors for chemotaxis and transport of many sugar based solutes. The uncharacterized protein is also predicted to be an ABC transporter substrate-binding protein, with a conserved substrate-binding domain of nickel/dipeptide/oligopeptide transport systems, which function in the import of nickel and peptides, and other closely related proteins. It is also apparent that there were significant differences in OM protein profiles between both strains of T. phagedenis. Large amounts of VpsB (protein spot number 3) were readily detected in strain 4A but not strain 1452. The gene that encodes VpsB was identified in the genome of T. phagedenis since it had a similar putative promoter sequence as the proline rich repeat protein PrrA (Mushtaq et al., 2016). Interestingly, VpsB was found only in bovine isolates of T. phagedenis and not in a human isolate. VpsB is an immunogenic protein that reacts with sera from BDD infected animals. VpsB is hypothesized to facilitate antigenic variation since its expression is controlled by a promoter with variable numbers of TA repeats. Protein spot number 5 also appeared to be expressed in large amounts by strain 4A compared to strain 1452; this protein was identified as an uncharacterized protein which contains a haloacid dehalogenase (HAD)-like hydrolase domain. In contrast, protein spot number 8 was abundantly expressed in strain 1452 compared to strain 4A; this is an uncharacterized protein with no conserved domains

identified at present. The observed differential protein expression patterns amongst different strains of T. phagedenis isolated from bovine samples highlight the importance of examining the genetic content of multiple bovine isolates (Mushtaq et al., 2016); this can provide for the identification of unique factors that differentiate them from human commensal isolates of T. phagedenis, and provide insights into pathogenic mechanisms of BDD.

All identified proteins are predicted to have a signal peptide and thus to be integral to the outer membrane. However, many are also predicted to be periplasmic. Additional assays will be required to determine whether the C-terminus of OM proteins are surface exposed or not. Nevertheless, our results have identified specific proteins unique to each strain of Treponema examined. It will be interesting to determine whether these novel identifications are reactive with sera from convalescent animals: in our hands, analysis of whole treponemes with sera from convalescent cattle and sheep is limited due to significant cross reactivity with secondary antibodies (unpublished data). The identification of specific OM proteins provides for their use as potential diagnostic assays (Mushtaq et al., 2016; Rosander et al., 2011), in vaccine development, as well as investigations to further elucidate pathogenic mechanisms of BDD.

Conclusion

T. brennaborense is associated with some cases of BDD, and requires alternative growth conditions when compared with T. phagedenis and T. pedis. Dark-field microscopy of T. brennaborense indicates it is highly motile relative to other treponemes. Enrichment for outer membrane proteins from each strain of treponeme confirmed the presence of a range of predicted lipoproteins, ABS transporter proteins and uncharacterized proteins. Though very similar, the OM profile of both strains of T. phagedenis indicates variable antigen expression as exemplified by large amounts of VpsB being expressed by strain 4A but not strain 1452. OM proteins specific for T. pedis and T. brennaborense were identified. Further evaluation of these outer membrane components can aid in understanding pathogenic mechanisms of BDD and in turn facilitate the development of vaccines and diagnostics to prevent and eradicate this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Armenteros JJA, Tsirigos KD, Sønderby CK, Petersen TN, Winther O, Brunak S, von Heijne G, Nielsen H, 2019 SignalP 5.0 improves signal peptide predictions using deep neural networks. Nature biotechnology, 1.

Berry SL, Read DH, Famula TR, Mongini A, Dopfer D, 2012 Long-term observations on the dynamics of bovine digital dermatitis lesions on a California dairy after topical treatment with lincomycin HCl. Veterinary journal (London, England : 1997) 193, 654–658.

Capri J, Whitelegge JP 2017 Full membrane protein coverage digestion and quantitative bottom-up mass spectrometry proteomics, In: Proteomics. Springer, 61–67.

Choi B-K, Nattermann H, Grund S, Haider W, Gobel U, 1997 Spirochetes from digital dermatitis lesions in cattle are closely related to treponemes associated with human periodontitis. International Journal of Systematic and Evolutionary Microbiology 47, 175–181.

Deka RK, Brautigam CA, Yang XF, Blevins JS, Machius M, Tomchick DR, Norgard MV, 2006 The PnrA (Tp0319; TmpC) lipoprotein represents a new family of bacterial purine nucleoside receptor encoded within an ATP-binding cassette (ABC)-like operon in Treponema pallidum. Journal of Biological Chemistry 281, 8072–8081. [PubMed: 16418175]

Döpfer D, Koopmans A, Meijer F, Szakall I, Schukken Y, Klee W, Bosma R, Cornelisse J, Van Asten A, Ter Huurne A, 1997 Histological and bacteriological evaluation of digital dermatitis in cattle, with special reference to spirochaetes and Campylobacter faecalis. Veterinary Record 140, 620–623. [PubMed: 9228692]

Evans NJ, Brown JM, Demirkan I, Murray RD, Birtles RJ, Hart CA, Carter SD, 2009 Treponema pedis sp. nov., a spirochaete isolated from bovine digital dermatitis lesions. International journal of systematic and evolutionary microbiology 59, 987–991. [PubMed: 19406779]

Evans NJ, Brown JM, Demirkan I, Murray RD, Vink WD, Blowey RW, Hart CA, Carter SD, 2008 Three unique groups of spirochetes isolated from digital dermatitis lesions in UK cattle. Veterinary microbiology 130, 141–150. [PubMed: 18243592]

Han S, Mansfield KG, Bradway DS, Besser TE, Read DH, Haldorson GJ, Alt DP, Wilson-Welder JH, 2019 Treponeme-Associated Hoof Disease of Free-Ranging Elk (Cervus elaphus) in Southwestern Washington State, USA. Veterinary pathology 56, 118–132. [PubMed: 30244661]

Klitgaard K, Boye M, Capion N, Jensen TK, 2008 Evidence of multiple Treponema phylotypes involved in bovine digital dermatitis as shown by 16S rRNA gene analysis and fluorescence in situ hybridization. Journal of Clinical Microbiology 46, 3012–3020. [PubMed: 18562583]

Klitgaard K, Nielsen MW, Ingerslev H-C, Boye M, Jensen TK, 2014 Discovery of bovine digital dermatitis-associated Treponema spp. in the dairy herd environment by a targeted deep-sequencing approach. Appl. Environ. Microbiol. 80, 4427–4432. [PubMed: 24814794]

Knappe-Poindecker M, Gilhuus M, Jensen TK, Klitgaard K, Larssen R, Fjeldaas T, 2013 Interdigital dermatitis, heel horn erosion, and digital dermatitis in 14 Norwegian dairy herds. Journal of dairy science 96, 7617–7629. [PubMed: 24140335]

Krull AC, Shearer JK, Gorden PJ, Cooper VL, Phillips GJ, Plummer PJ, 2014a Deep sequencing analysis reveals temporal microbiota changes associated with development of bovine digital dermatitis. Infect Immun 82, 3359–3373. [PubMed: 24866801]

Krull AC, Shearer JK, Gorden PJ, Cooper VL, Phillips GJ, Plummer PJ, 2014b Deep sequencing analysis reveals temporal microbiota changes associated with development of bovine digital dermatitis. Infection and immunity 82, 3359–3373. [PubMed: 24866801]

Losinger WC, 2006 Economic impacts of reduced milk production associated with papillomatous digital dermatitis in dairy cows in the USA. Journal of dairy research 73, 244–256. [PubMed: 16569275]

Miller JN, 1971 Spirochetes in Body Fluids and Tissues. Charles C. THomas, Springfield, Illinois, U.S.A.

Mushtaq M, Bongcam-Rudloff E, Loftsdottir H, Pringle M, Segerman B, Zuerner R, Rosander A, 2016 Genetic analysis of a Treponema phagedenis locus encoding antigenic lipoproteins with potential for antigenic variation. Veterinary microbiology 189, 91–98. [PubMed: 27259832]

Nally JE, Timoney JF, Stevenson B, 2001 Temperature-regulated protein synthesis by Leptospira interrogans. Infection and immunity 69, 400–404. [PubMed: 11119530]

Nally JE, Whitelegge JP, Aguilera R, Pereira MM, Blanco DR, Lovett MA, 2005 Purification and proteomic analysis of outer membrane vesicles from a clinical isolate of Leptospira interrogans serovar Copenhageni. Proteomics 5, 144–152. [PubMed: 15672460]

Nally JE, Whitelegge JP, Bassilian S, Blanco DR, Lovett MA, 2007 Characterization of the outer membrane proteome of Leptospira interrogans expressed during acute lethal infection. Infection and immunity 75, 766–773. [PubMed: 17101664]

Nordhoff M, Moter A, Schrank K, Wieler LH, 2008 High prevalence of treponemes in bovine digital dermatitis-A molecular epidemiology. Veterinary microbiology 131, 293–300. [PubMed: 18524502]

Pringle M, Bergsten C, Fernström L-L, Höök H, Johansson K-E, 2008 Isolation and characterization of Treponema phagedenis-like spirochetes from digital dermatitis lesions in Swedish dairy cattle. Acta Veterinaria Scandinavica 50, 40. [PubMed: 18937826]

Radolf JD, Chamberlain N, Clausell A, Norgard M, 1988 Identification and localization of integral membrane proteins of virulent Treponema pallidum subsp. pallidum by phase partitioning with the nonionic detergent triton X-114. Infection and immunity 56, 490–498. [PubMed: 3276627]

Rasmussen M, Capion N, Klitgaard K, Rogdo T, Fjeldaas T, Boye M, Jensen TK, 2012 Bovine digital dermatitis: possible pathogenic consortium consisting of Dichelobacter nodosus and multiple Treponema species. Veterinary microbiology 160, 151–161. [PubMed: 22698300]

Rosander A, Guss B, Frykberg L, Björkman C, Näslund K, Pringle M, 2011 Identification of immunogenic proteins in Treponema phagedenis-like strain V1 from digital dermatitis lesions by phage display. Veterinary microbiology 153, 315–322. [PubMed: 21763087]

Sayers G, Marques P, Evans N, O'grady L, Doherty M, Carter S, Nally J, 2009 Identification of spirochetes associated with contagious ovine digital dermatitis. Journal of Clinical Microbiology 47, 1199–1201. [PubMed: 19204100]

Schrank K, Choi B-K, Grund S, Moter A, Heuner K, Nattermann H, Göbel UB, 1999 Treponema brennaborense sp. nov., a novel spirochaete isolated from a dairy cow suffering from digital dermatitis. International Journal of Systematic and Evolutionary Microbiology 49, 43–50.

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, 2011 Fast, scalable generation of high- quality protein multiple sequence alignments using Clustal Omega. Molecular systems biology 7, 539. [PubMed: 21988835]

Smibert R, 1991 Anaerobic spirochetes, p 572–578 Manual of clinical microbiology. American Society for Microbiology, Washington, DC.

Trott DJ, Moeller MR, Zuerner RL, Goff JP, Waters WR, Alt DP, Walker RL, Wannemuehler MJ, 2003 Characterization of Treponema phagedenis-like spirochetes isolated from papillomatous digital dermatitis lesions in dairy cattle. Journal of clinical microbiology 41, 2522–2529. [PubMed: 12791876]

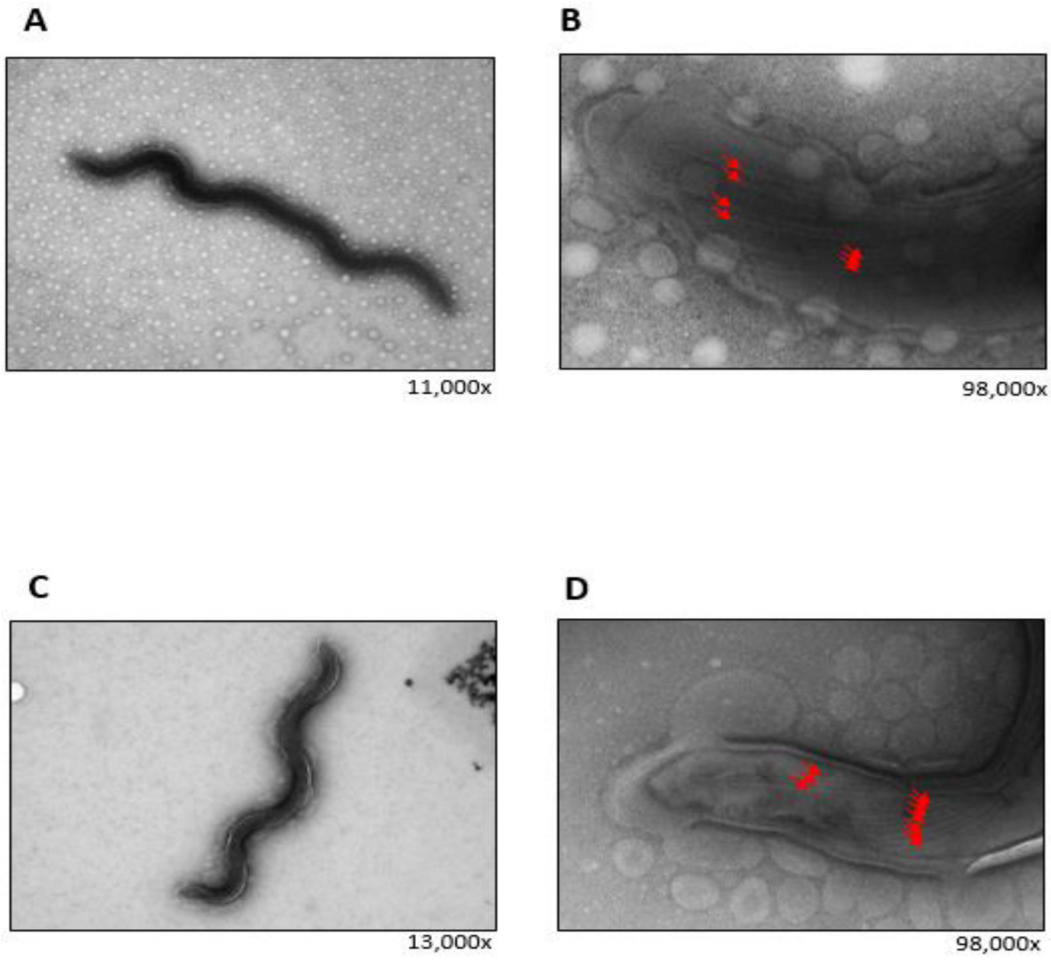
Wessel D.m., Flügge U, 1984 A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Analytical biochemistry 138, 141–143. [PubMed: 6731838]

Wilson-Welder JH, Alt DP, Nally JE, 2015 The etiology of digital dermatitis in ruminants: recent perspectives. Veterinary Medicine: Research and Reports 6, 155.

Wilson-Welder JH, Elliott MK, Zuerner RL, Bayles DO, Alt DP, Stanton TB, 2013 Biochemical and molecular characterization of Treponema phagedenis-like spirochetes isolated from a bovine digital dermatitis lesion. BMC microbiology 13, 280. [PubMed: 24304812]

Highlights

- A novel strain of *T. brennaborense* was isolated from a lesion of bovine digital dermatitis (BDD).
- *T. brennaborense* is highly motile as observed by dark-field microscopy.
- *T. brennaborense* requires different growth factors than *T. phagedenis* and *T. pedis*.
- The outer membrane (OM) of treponemes associated with BDD contains predicted lipoproteins, ABS transporter proteins and uncharacterized proteins.
- Variable protein and antigen expression is observed in the OM for different strains of *T. phagedenis*.



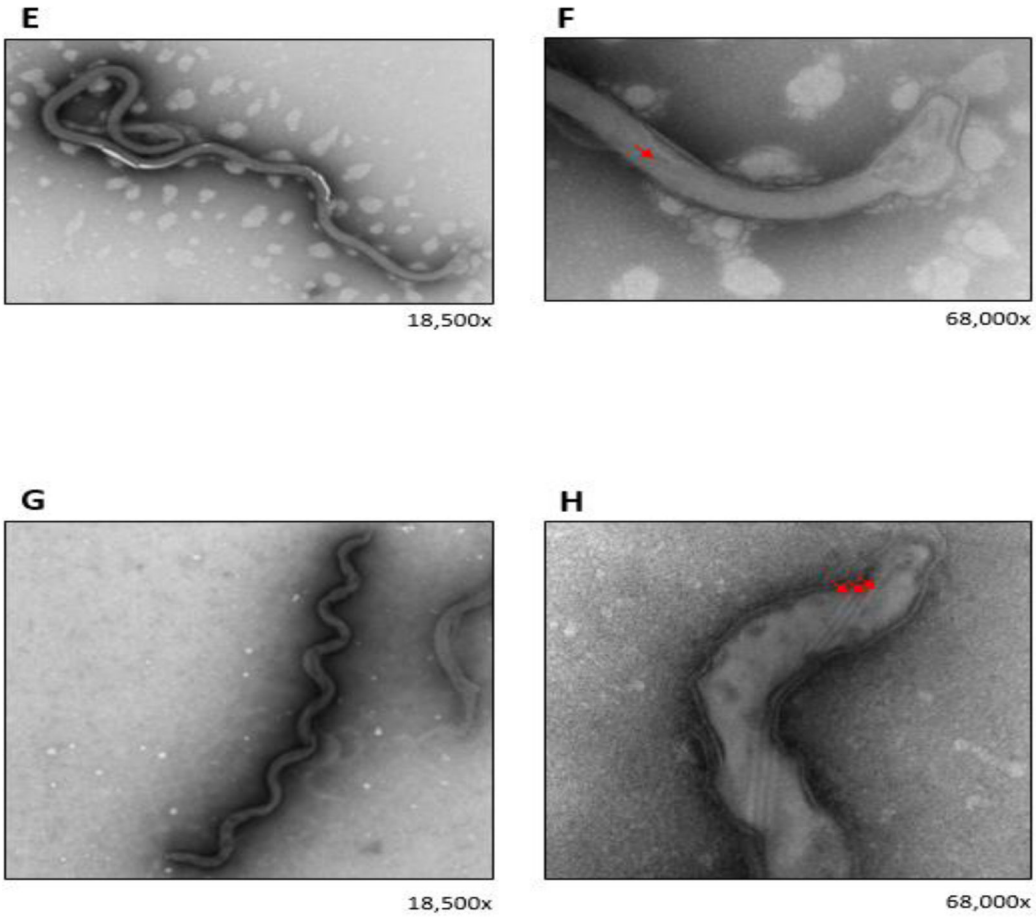


Figure 1.

Transmission electron micrographs of T. phagedenis strain 1452 (A&B), T. phagedenis strain 4A (C&D), T. brennaborense (E&F) and T. pedis (G&H). Red arrows indicate periplasmic flagellae. Direct magnification is indicated.

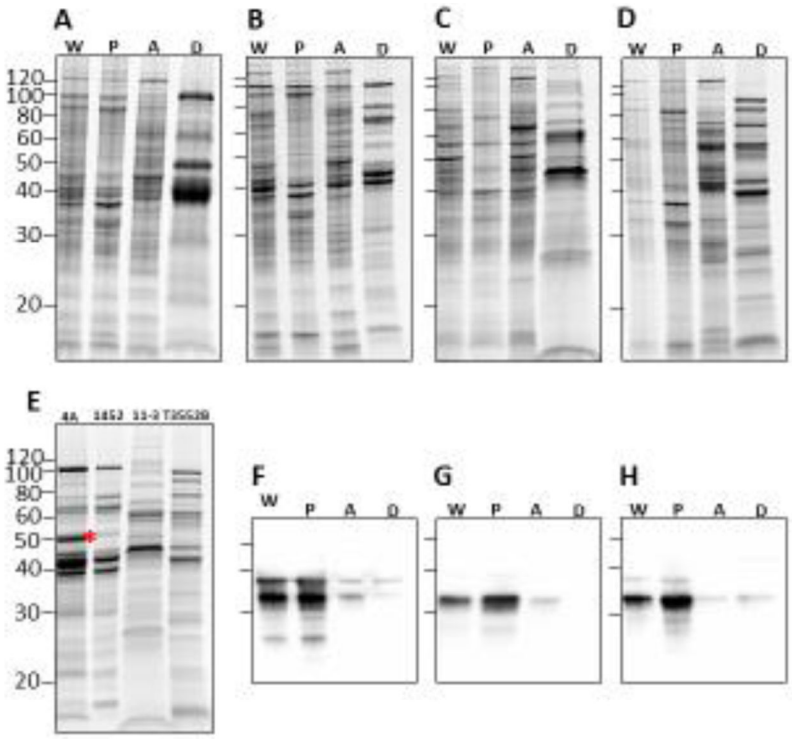


Figure 2.

Analysis of the protein content by 1-D gel electrophoresis of T. phagedenis strain 4A (A), T. phagedenis strain 1452 (B), T. brennaborense strain 11-3 (C) and T. pedis strain T3552B (D). Sample lanes include whole treponemes (W) compared to the protoplasmic cylinder (P), aqueous phase (A) and detergent phase (D). Detergent phase from each strain is compared directly in E; *indicates a protein readily detected in strain 4A compared to strain 1452. Representative immunoblots of T. phagedenis (F), T. brennaborense (G) and T. pedis (H) probed with anti-FlaA are provided to indicate the presence of flagella is minimal in detergent and aqueous phase fractions compared to the protoplasmic fraction and whole cells. Molecular mass markers are indicated (kDa).

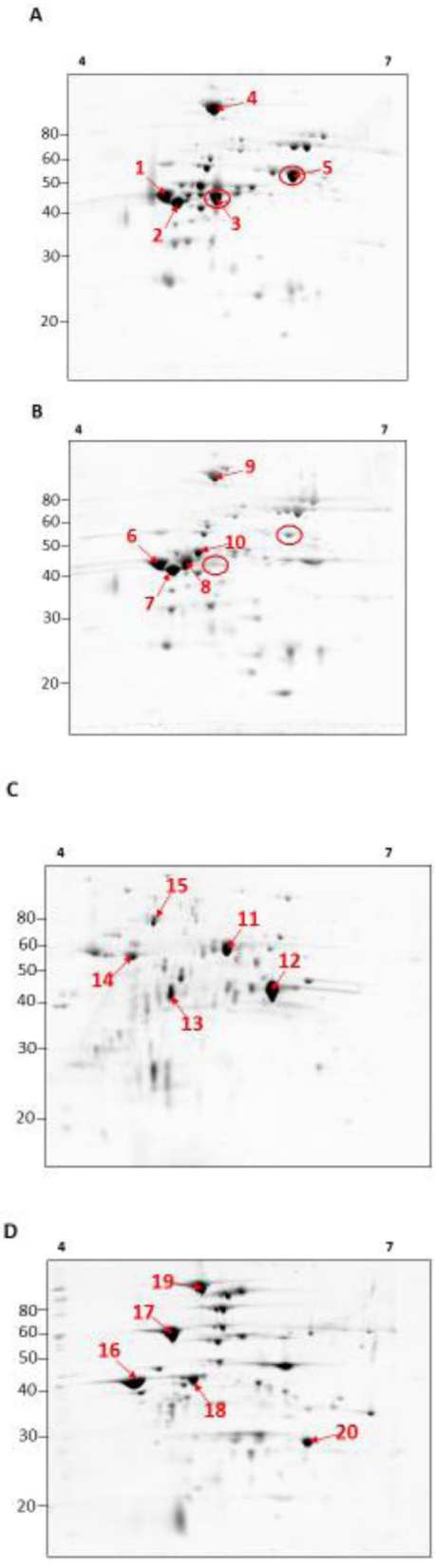


Figure 3.

Analysis of the protein content by 2-D gel electrophoresis of OM enriched fractions from T. phagedenis strain 4A (A), T. phagedenis strain 1452 (B), T. brennaborense strain 11-3 (C) and T. pedis strain T3552B (D). Molecular mass markers are indicated (kDa). Protein spot numbers correspond to protein identifications listed in Table 2. Circles indicate proteins readily detected in strain 4A compared to strain 1452.

Table 1:

Enzymatic profile of each strain of treponeme associated with bovine digital dermatitis as determined using the API®ZYM system.

Enzyme	T. phagedenis strain 4A	T. phagedenis strain 1452	T. brennaborense strain 11-3	T. pedis strain T3552B
Negative Control	-	-	-	-
Alkaline phosphatase	S	S	W	-
C_4 esterase	W	W	W	S
C_8 esterase lipase	W	W	W	W
C_{14} lipase	-	-	-	-
Leucine arylamidase	-	-	-	-
Valine arylamidase	-	-	-	-
Cystine arylamidase	-	-	-	-
Trypsin	-	-	-	W
α-chymotrypsin	-	-	-	W
Acid phosphatase	S	S	W	-
Naphtholphosphohydrolase	W	W	S	-
α-galactosidase	-	-	-	-
β-galactosidase	S	S	W	-
β-glucuronidase	W	S	-	-
α-glucosidase	-	W	S	-
β-glucosidase	-	-	-	-
N-acetyl-β-glucosamidase	S	W	S	-
α-mannosidase	-	-	-	-
α-fucosidase	-	W	-	-

S = strong reaction, W = weak reaction, − = negative.

Table 2:

Identifications of protein spots by mass spectrometry. Spot numbers correspond to those shown in Figure 3.

Treponema strain (& corresponding 2-D gel)	Spot ID	Accession number[1]	Mass[1]	Protein name[1]	Mascot score[2]	Number peptides matched[2]	Treponeme species of identified protein[3]	SignalP[4]
T. phagedenis 4A (Figure 3A)	1	E7NTC8	41149	Basic membrane protein	1600	61	TREPH	Sec/SPII
	2	A0A0B7GVQ8	42588	Glucose/galactose-binding lipoprotein (mglB)	4016	130	TREPH	Sec/SPII
	3	A0A191VM28	41355	VpsB	6523	206	TREPH	Sec/SPII
	4	A0A0B7GZT4	94372	Uncharacterized protein	9054	294	TREPH	Sec/SPII
	5	A0A0B7GUH7	47961	Uncharacterized protein	1136	104	TREPH	Sec/SPII
T. phagedenis 1452 (Figure 3B)	6	E7NTC8	41149	Basic membrane protein	941	55	TREPH	Sec/SPII
	7	E7NRL5	42679	Putative Glucose/ galactose-binding lipoprotein	5975	200	TREPH	Sec/SPII
	8	A0A0B7GVB5	35444	Uncharacterized protein	3420	116	TREPH	Sec/SPII
	9	A0A0B7GZT4	94372	Uncharacterized protein	6677	217	TREPH	Sec/SPII
	10	A0A0B7H298	45911	ABC transporter, solute-binding protein	3692	153	TREPH	Sec/SPII
T. brennaborense 11-3 (Figure 3C)	11	F4LP33	58466	Flavocytochrome c	6684	153	TREBD	Sec/SPII
	12	F4LMZ8	44535	Uncharacterized protein	3975	156	TREBD	Sec/SPII
	13	F4LLN2	40825	Extracellular ligand-binding receptor	2876	96	TREBD	Sec/SPII
	14	F4LMV1	55415	5'-nucleotidase	1622	86	TREBD	Sec/SPII
	15	F4LQA3	74672	Uncharacterized protein	740	59	TREBD	Sec/SPI
T. pedis T3552B (Figure 3D)	16	S5ZXP6	42621	Galactose/glucose-binding lipoprotein	17044	507	TPE	Sec/SPII
	17	S6A4N0	61740	Oligopeptide ABC transporter substrate-bindingprotein	4456	203	TPE	Sec/SPII
	18	S6A3W3	38900	Membrane lipoprotein TmpC	2749	125	TPE	Sec/SPII
	19	S3KLN3	95894	Uncharacterized protein	345	27	TREDN	Sec/SPII
	20	S5ZKA9	25431	Endo-1,4-beta-xylanase	1710	59	TPE	Sec/SPI

[1] As assigned by Uniprot.

[2] As assigned by Mascot.

[3] As assigned by Uniprot; TREPPH=T. phagedenis, TREBD=T. brennaborense, TPE=T. pedis, TREDN=T. denticola.

[4] As predicted by SignalP (Armenteros et al., 2019); http://www.cbs.dtu.dk/services/SignalP/