Treponema ruminis sp. nov., a spirochaete isolated from the bovine rumen.

Running title: A novel treponeme isolated from the bovine rumen.

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Abbreviations. GI, gastrointestinal; recA, recombinase A; RS, rabbit serum.
Abstract

A novel bacterium, Ru1T, was encountered during a survey of spirochaetes living in the gastrointestinal (GI) tract of ruminants. Comparative analysis of 16S rRNA gene sequence data indicated that Ru1T clustered within the Treponema genus but shared at most 86.1% sequence similarity with other recognised Treponema species. Further phylogenetic analysis based on partial recombinase A (recA) gene sequence comparisons, together with phenotypic characterisation, also demonstrated the divergence of Ru1T from other recognised Treponema species. Microscopically, Ru1T appeared as a very small, highly motile, helical spirochaete with four periplasmic flagella. It exhibited C8 esterase lipase, leucine arylamidase, β-galactosidase and β-glucosidase activity. A distinctive, serum-independent growth pattern was also observed, characterised by colonies with an absence of the local haemolysis that is typical of many pathogenic treponemes. On the basis of these data, Ru1T is considered to represent a new Treponema species for which the name Treponema ruminis sp. nov. is proposed. The type strain of Treponema ruminis is Ru1T (=DSM 103462=NCTC 13847T).

Main Text

Treponema species are fastidious, highly motile, helical, anaerobic microorganisms of the spirochaete phylum which have been identified within the gastrointestinal (GI) tract, oral cavity and genital areas of animals, humans and insects (Smirbert, 1984). Some treponemes are associated with infectious diseases including digital dermatitis, human periodontal disease, pinta, yaws and the venereal infection, syphilis (Choi et al., 1997; Dewhirst et al., 2000; Engelkens et al., 1991; Mitjà et al., 2013; Radolf et al., 2006) but others are commensal symbionts living in the GI tract of animals and insects.
Due to their fastidious nature, only a handful of GI treponemes have been characterised. *Treponema succinifaciens*, *Treponema porcinum* and *Treponema berlinense* were isolated from porcine GI contents (Cwyk & Canale-Parola, 1979; Nordhoff et al., 2005). Similarly, *Treponema isoptericolens*, *Treponema azotonutricium* and *Treponema primitia* have been isolated from the digestive tract of termites (Dröge et al., 2008; Graber et al., 2004). Metagenomic studies have identified a diverse variety of spirochaetes within the bovine rumen (Edwards et al., 2004; Paster & Canale-Parola, 1982; Tajima et al., 1999; Zinicola et al., 2015) and although several have been successfully isolated (Evans et al., 2011; Ziolecki, 1979; Ziolecki & Wojciechowicz, 1980), only two have been formally proposed as novel treponeme taxa, namely *Treponema bryantii* and *Treponema saccharophilum* (Paster & Canale-Parola, 1985; Stanton & Canale-Parola, 1980).

A recent study aimed to isolate and characterise spirochaetes from the GI tract of Holstein-Friesian cattle in the United Kingdom (UK) for comparison with bovine digital dermatitis treponemes (Evans et al., 2011). Seven 16S rRNA gene sequence variants were obtained that were found to cluster into four novel phylotypes within the *Treponema* genus. Each phylotype shared less than 97% 16S rRNA gene sequence identity to extant *Treponema* species, suggesting these isolates represent novel taxa. In the present study we further characterised one of these phylotypes, represented by strain Ru1T, and on the basis of this characterisation propose it as a novel *Treponema* species.

Strain Ru1T was isolated at slaughter from the rumen contents of a Holstein-Friesian bull from a UK dairy farm, as previously described (Evans et al., 2011). The isolate was initially grown anaerobically (85% nitrogen, 10% hydrogen, 5% carbon dioxide) at 36°C in oral treponeme enrichment broth (Anaerobe Systems, Morgan Hill, USA) supplemented with 10% (v/v) rabbit serum (RS; GE Healthcare Life Sciences, Buckinghamshire, UK). However, after isolation the spirochaete did not require RS for further growth and thereafter was successfully and routinely subcultured daily in the absence of serum. Under phase contrast microscopy, the cells exhibited morphological characteristics which are typical of many treponemes in culture including rotational and translational movement, jerky flexing and high motility. The isolate was successfully stored at -80°C in growth medium containing 10% (v/v) glycerol. Cells were additionally subcultured onto unsupplemented fastidious
anaerobe agar (LabM, Bury, UK) plates. Following anaerobic incubation for 10 days, colonies appeared which were circular, translucent, convex and pinprick-sized with a diameter of between 0.2-0.5mm. The colonies exhibited no metallic sheen or local haemolysis and were morphologically very different to those reported for several other taxonomically appraised GI treponemes, which are typically much larger in size. For example, T. succinifaciens ATCC 33096\textsuperscript{T} and T. saccharophilum ATCC 43261\textsuperscript{T} form spherical, opaque colonies with a diameter of 4-8mm and 3-4mm respectively, whereas colonies of T. porcinum ATCC BAA-908\textsuperscript{T} and T. berlinense ATCC BAA-909\textsuperscript{T} exhibit a 1-2mm diameter and are irregular and greyish in colouration (Cwyk & Canale-Parola, 1979; Nordhoff et al., 2005; Paster & Canale-Parola, 1985).

Genomic DNA was extracted from the cultured isolate for subsequent PCR amplification and sequencing of the 16S rRNA gene product, as previously described (Evans et al., 2008; Evans et al., 2011) and the near-complete 16S rRNA gene sequence (1309bp) of Ru1\textsuperscript{T} was aligned with the 16S rRNA gene sequences of extant Treponema species in the Bioedit Sequence Alignment Editor using CLUSTAL W (Hall, 2013; Thompson et al., 1994). Ru1\textsuperscript{T} shared highest sequence similarity (86.1\%) with T. porcinum ATCC BAA-908\textsuperscript{T}, a spirochaete isolated from porcine GI tract contents (Nordhoff et al., 2005). This level of sequence similarity is well below the proposed threshold for species delineation (Stackebrandt & Goebel, 1994). Phylogeny was inferred from this alignment, using ModelTest software in the TOPALi v2 program to predict the best-fit evolutionary model (Milne et al., 2009). The Tamura-Nei model was subsequently used to produce a bootstrapped maximum-likelihood tree based upon 10,000 reiterations, as implemented in MEGA 6.0 (Tamura et al., 2013; Tamura & Nei, 1993). Phylogenetic reconstruction revealed that Ru1\textsuperscript{T} formed a distinct phylotype within a wider, deep-branched region of porcine and bovine GI tract treponemes (Figure 1). Ru1\textsuperscript{T} clustered specifically with T. succinifaciens ATCC 33096\textsuperscript{T} and T. saccharophilum ATCC 43261\textsuperscript{T} (sharing 85.8\% and 84.1\% 16S rRNA gene sequence identity respectively) and then with T. porcinum ATCC BAA-908\textsuperscript{T}. Whilst clustering with these GI tract treponemes, Ru1\textsuperscript{T} had diverged markedly from them such that they were separated by phylogenetic distances akin to those observed among extant Treponema species.
A novel degenerate PCR assay was developed and optimised for amplification of the recombinase A (recA) gene from Ru1T, with this gene having recently been used in the phylogenetic typing of treponeme isolates (Clegg et al., 2016). The PCR assay incorporated the primer pair recA forward (5′-GCAACYTTGTTCTTTACR-3′) and recA reverse (5′-GAAATGTACGGTCCYGAA-3′), designed following the alignment of recA gene sequences from relevant Treponema of the bovine and porcine GI tract phylogenetic cluster (Evans et al., 2011) using CLUSTAL W within the Bioedit Sequence Alignment Editor. Genomic treponemal DNA (1µl) was incorporated into a 25µl PCR master mix containing 10µM of each degenerate primer, 20mM dNTP mix (5mM each of dATP, dCTP, dGTP, dTTP; Thermo Scientific™, Hemel Hempstead, UK) and Taq DNA polymerase according to the manufacturer’s instructions (Qiagen, Manchester, UK). Mixes were subjected to a thermal cycle of 95°C for 5 minutes; 40 cycles of 94°C for 1 minute, 49.1°C for 3 minutes, 72°C for 3 minutes; 72°C for 7 minutes. The presence of amplification products was verified by agarose gel electrophoresis, and, when present, these were purified and both strands were sequenced commercially (Source BioScience, Nottingham, UK). A partial (479bp) recA alignment of Ru1T and Treponema species was generated in the Bioedit Sequence Alignment Editor using CLUSTAL W. Treponemal recA gene sequences differed from one another markedly. The mean recA gene sequence similarity between extant Treponema species was 64.3% (range 56.3% to 93.8%) whilst the mean intra-species recA gene sequence similarities for two recognised Treponema spp. (with recA data available for a range of isolates) were calculated as 99.1% and 97.4% (for Treponema medium and Treponema pedis respectively) (Clegg et al., 2016). The recA gene sequence of Ru1T was most similar to that of T. succinifaciens ATCC 33096T (76.8%). It also shared 75.3% recA gene sequence similarity with T. brennaborense CIP 105900T (Schrank et al., 1999) and Treponema socranskii subsp. paredis ATCC 35535T (Smibert et al., 1984). A phylogeny was derived from the recA alignment using the Tamura-Nei model based upon 10,000 reiterations, as implemented in MEGA 6.0 (Tamura & Nei, 1993). In this phylogeny (Figure 2), Ru1T was again a distinct phylotype among bovine and porcine treponeme isolates.
The API® ZYM system (bioMérieux, Lyon, France) was used to generate an enzyme activity profile for Ru1T, as reported previously (Evans et al., 2011). Positive enzyme activity was detected for C8 esterase lipase, leucine arylamidase, β-galactosidase and β-glucosidase. However, no enzyme activity was detected for alkaline phosphatase, C4 esterase, C14 lipase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphtholphohydrolase, α-galactosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Among Treponema species, Ru1T was found to have a unique API® ZYM profile (Table 1).

The morphology of Ru1T was determined directly from liquid culture by transmission electron microscopy, as reported previously (Demirkan et al., 2006; Evans et al., 2011). Whilst sharing the common morphological characteristics of other Treponema species, Ru1T could be distinguished on the basis of being approximately 5-9µm in length, 0.4-0.5µm in width and having between 3-5 regular coils. Each cell possessed 4 periplasmic flagella.

Based upon the comparative data presented in this study, strain Ru1T is considered to represent a novel species within the Treponema genus, for which the name Treponema ruminis sp. nov. is proposed.

Description of Treponema ruminis sp. nov. Treponema ruminis (ru’mi.nis. L. neut. gen. n. ruminis, of the rumen). Anaerobic, gram-negative, helically coiled, motile and very small-sized treponemes. Cells are approximately 5-9µm long and 0.40-0.50µm wide, have between 3-5 even windings and each have 4 periplasmic flagella. Cells typically reach optimal growth following anaerobic incubation at 36°C for 1 day within oral treponeme enrichment broth without serum supplementation. Cells exhibit translational movement, rotation and jerky flexing in culture and typically sediment towards the bottom of the tube. Circular, translucent, convex, pinprick-sized colonies of 0.20-0.50mm diameter are observed after 10 days when streaked onto unsupplemented fastidious anaerobe agar plates. Colonies do not have a metallic sheen or exhibit local hemolysis. Cells can be stored at -80°C
in growth medium containing 10%(v/v) glycerol. The API® ZYM system identified enzyme activity for C8 esterase lipase, leucine arylamidase, β-galactosidase and β-glucosidase, while detecting no activity for alkaline phosphatase, C4 esterase, C14 lipase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphtholphohydrolase, α-galactosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

The type strain, Ru1T (=DSM 103462T=NCTC 13847T), was isolated from the rumen contents of a Holstein-Friesian bull from a Cheshire farm in the UK.

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References


Table 1. An enzyme activity profile comparison between the bovine GI tract isolate (Ru1) and other related bovine, porcine and human treponemes as determined by the API® ZYM system.

Enzymes tested: 1, alkaline phosphatase; 2, C4 esterase; 3, C8 esterase lipase; 4, C14 lipase; 5, leucine arylamidase; 6, valine arylamidase; 7, cystine arylamidase; 8, trypsin; 9, chymotrypsin; 10, acid phosphatase; 11, naphtholphohydrolase; 12, α-galactosidase; 13, β-galactosidase; 14, β-glucuronidase; 15, α-glucosidase; 16, β-glucosidase; 17, N-acetyl-β-glucosaminidase; 18, α-mannosidase; 19, α-fucosidase. +, positive; -, negative.

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<tr>
<th>Treponema strain</th>
<th>Presence of enzyme activity</th>
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<tr>
<td>Treponema ruminis Ru1†</td>
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<tr>
<td>Treponema porcinum ATCC BAA-908†</td>
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<td>Treponema berlinense ATCC BAA-909†</td>
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<td>Treponema pedis DSM 18691†</td>
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<td>Treponema brennaborense CIP 105900†</td>
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<td>Treponema pectinovorum ATCC 33768†</td>
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<td>Treponema lecithinolyticum OMZ 684†</td>
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API® ZYM profile reported by ‡Evans et al., 2011, †Nordhoff et al., 2005, ‡Evans et al., 2009, †Schrank et al., 1999, ‡Wyss et al., 1996, †Wyss et al., 1997, ‡Wyss et al., 2004, ‡Wyss et al., 2001, ‡Wyss et al., 1999.

Figure Legends

Fig. 1. A phylogenetic tree of maximum-likelihood illustrating 16S rRNA gene sequence comparisons over 1,312 aligned bases between the bovine GI tract isolate (Ru1) and all other recognised Treponema spp.. Bootstrap confidence intervals, based on 10,000 reiterations, are shown as percentages at the nodes; values below 40% were removed for clarity. Genbank accession numbers are given in parentheses next to each strain. Bar, 0.02 nucleotide substitutions per site.

Fig. 2. A phylogenetic tree of maximum-likelihood illustrating the gene sequence comparisons across 479 aligned bases encoding recombinase A (recA) between the bovine GI tract isolate (Ru1) and all available sequences from other recognised Treponema spp.. Bootstrap confidence intervals, based on 10,000 reiterations, are shown as percentages at the nodes; values below 40% were removed for clarity. Genbank accession numbers are given in parentheses next to each strain. Bar, 0.05 nucleotide substitutions per site.