Treponema rectale sp. nov., a spirochete isolated from the bovine rectum.

Running Title: A novel spirochete isolated from the bovine rectum.

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Subject category: 'NEW TAXA: Other bacteria'.

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Genbank accession numbers: The Genbank accession numbers for the 16S rRNA gene sequence and the RecA gene sequence of *Treponema* strain CHPA^T are GU566699 and KX501214, respectively.

Abbreviations: GI, gastrointestinal; *RecA*, recombinase A; RS, rabbit serum; OTEB, oral treponeme enrichment broth; FAA, fastidious anaerobe agar.

Abstract.

A gram-negative, obligatory anaerobic spirochete, CHPA^T, was isolated from the rectal tissue of a Holstein-Friesian cow. On the basis of 16S rRNA gene comparisons, CHPA^T is most closely related to the human oral spirochete, *Treponema parvum,* with 88.8% sequence identity. Further characterisation on the basis of *recA* gene sequence analysis, cell morphology, pattern of growth and physiological profiling identified marked differences with respect to other recognised species of *Treponema*. Microscopically, the helical cells measured approximately 1-5 μm long and 0.15-0.25 μm wide, with 2-5 irregular spirals. Transmission electron microscopy identified 4 periplasmic flagella in a 2:4:2 arrangement. CHPA^T grew independently of serum, demonstrated no evidence of haemolytic activity and possessed an *in vitro* enzyme activity profile that is unique amongst recognised *Treponema* spp., exhibiting C4 esterase, α-galactosidase and β-galactosidase activity. Taken together, these data indicate that CHPA^T represents a novel species of the genus *Treponema*, for which the name *Treponema rectale* is proposed. The type strain of *Treponema rectale* is CHPA^T (=DSM 103679^T, =NCTC 13848^T).

Main text.

Treponema species are typically anaerobic, fastidious and highly motile microorganisms with a spiral morphology and are capable of occupying a diverse range of hosts and tissues, including the oral cavity and genital tract of humans, the gastrointestinal (GI) tract and feet of ruminants, and the digestive tract of insects [1, 2, 3, 4]. Whereas GI colonisation has been associated with commensalism, several taxa have been shown to play a pathogenic role in a number of diseases, including bovine digital dermatitis [5], periodontal disease [6] and syphilis [7]. To date, there are 28 valid *Treponema* species, with one of these, *Treponema socranskii*, having been delineated into 3 subspecies [8].

The mammalian GI tract harbours a complex symbiotic community of microorganisms, numerous in both abundance and diversity. Spirochetes are known to be a common inhabitant of the GI tract and occur to relatively high densities in healthy animals, including the rumen of cattle [9, 10]. Despite early confirmation of the presence of a large number of morphologically and physiologically diverse spirochete species in the bovine rumen [11], their fastidious nature has, for the most part, hindered their characterisation. Moreover, little is known about the spirochetes present in other regions of the bovine GI tract.

Treponema spp. in particular are thought to comprise a significant, yet poorly understood, proportion of the spirochetes that reside within the bovine GI tract. The bovine rumen harbours several *Treponema* phlyotypes, three taxa of which have been classified to date: *Treponema bryantii* [12], *Treponema saccharophilum* [13] and *Treponema ruminis* [14]. As part of an investigation into the microbial diversity of the bovine GI tract, Evans *et al.* [15] used 16S rRNA gene sequence comparisons to delineate bovine GI tract treponeme isolates into four novel phylotypes. Since all four novel phylotypes shared less than 97% sequence identity with established members of the *Treponema* genus, it is suggested that on the basis of current taxonomic criteria [16], they may each represent a novel species. In the present study,

these findings have been combined with new genotypic and phenotypic data to support the proposal that one of these phylotypes (phylotype 2; CHPA^T), represents a novel species of the genus *Treponema*.

Strain CHPA^T was recovered from a post mortem rectal tissue biopsy collected from a single Holstein-Friesian cow in Merseyside, United Kingdom, immediately after slaughter, as described previously [15]. CHPA^T was maintained in the laboratory by passage every 24 hours in Oral Treponeme Enrichment Broth (OTEB; Anaerobe Systems) supplemented with 10% (v/v) rabbit serum (RS; GE Healthcare Life Sciences, Buckinghamshire, UK), under anaerobic conditions ($N_2/H_2/CO_2$, 85:10:5, 36^{o}C). Phase contrast microscopy confirmed the presence of helically-coiled spirochete cells in the liquid media that displayed high levels of rotational and translational motility when observed in wet mounts. Cultured treponemes were stored at -80^{o}C in growth medium containing 10% (v/v) glycerol and were revived successfully. The bacterial morphology of this strain was examined by transmission electron microscopy and has been reported previously [17]. Cells, when grown in OTEB, were observed to be approximately 1 to 5 μm long and 0.15 to 0.25 μm wide. CHPA^T exhibited typical spirochaetal helical morphology, with 2 to 5 irregular spirals and 4 periplasmic flagella, originating at the poles and overlapping centrally, to yield a 2:4:2 arrangement.

Bacteria also grew when sub-cultured onto unsupplemented fastidious anaerobe agar (FAA) plates (LabM, Bury, UK), forming singular circular, convex, punctiform colonies of approximately 0.2 mm in diameter after 10 days' incubation. Inoculation onto FAA plates that did not contain serum failed to retard growth, and cells were thereafter successfully sub-cultured in OTEB without serum supplementation, indicating that these treponemes were serum-independent under the conditions of *in vitro* culture [15]. Colonies were observed to be translucent and lacked a metallic sheen and there was no evidence of local β-haemolysis. These colonies differed markedly in both size and appearance from those formed by other

treponemes of the GI tract, including the spherical, opaque colonies of *Treponema saccharophilum* ATCC 43261^T and *Treponema succinifaciens* ATCC 33096^T, with reported colony diameters of 3-4 mm [13] of 4-8 mm [18] respectively, and the irregular, greyish colonies of *Treponema berlinese* ATCC BAA-909^T, with a reported colony size of 1-2 mm in diameter [19]. The colonies of *Treponema ruminis* DSM 103462^T, although similar in appearance, varied somewhat in size (0.2-0.5 mm) [14], an observation not made for strain CHPA^T (0.2 mm). It is noted however that the extent to which variable culture conditions contribute to these differences remains undefined.

Genomic DNA preparation, 16S rRNA gene PCR amplification and sequencing were performed as described previously by Evans *et al.* [15]. Sequencing of this amplification product yielded 1309 base pairs (bp) of unambiguous sequence data (Genbank accession no. GU566699). A comparison of this sequence with the 16S rRNA sequences available in GenBank confirmed it to be most similar to the 16S rRNA gene sequences of the genus *Treponema*. A 1309bp 16S rRNA gene sequence alignment of CHPA^T and members of the *Treponema* genus was generated using CLUSTALW [20] and trimmed in the BioEdit sequence alignment editor [21]. The 16S rRNA gene of CHPA shared 84.4% and 88.0% sequence similarity with the two other previously identified bovine GI tract treponemes, *T. saccharophilum* ATCC 33096^T and *T. bryantii* ATCC 33254^T, respectively. In sharing 88.8% sequence identity, CHPA^T is most closely related to *T. parvum* ATCC 700770^T, a spirochete isolated from the human oral cavity that has been implicated in periodontal disease [22]. From a 16S rRNA gene sequence alignment of all valid treponemal species, phylogeny was inferred using the maximum likelihood method with nucleotide substitution rates calculated according to the Tamura-Nei model [23] in MEGA6 [24], selected as the best-fit evolutionary model using TOPALi 2.5 [25]. The robustness of the proposed tree branching was evaluated using bootstrap analysis (10,000 iterations).

In the proposed tree (fig. 1), the phylogenetic distance between CHPA^T and its nearest neighbour was at least that observed between several valid *Treponema* species. CHPA^T was observed to cluster with a number of commensal species of *Treponema* isolated from, or associated with, the GI tract of several mammalian hosts: *T. bryantii* ATCC 33254^T, isolated from the bovine rumen [12], T. *succinifaciens* ATCC 33096^T, isolated from the porcine colon [18] and *Treponema porcinum*, isolated from porcine faeces [19], sharing 88.0%, 85.4% and 88.7% 16S rRNA gene sequence identity, respectively. Phylogenetic reconstruction placed strain CHPA^T within a deep-rooted clade that is occupied by the aforementioned commensal treponemes as well as a number of oral *Treponema* species, including the closest known relative to CHPA^T, *T. parvum* ATCC 700770^T.

The phylogenetic position of CHPA within the *Treponema* genus was further explored using inferences derived from an alignment of recombinase A gene (*recA*) sequences. Degenerate primers suitable for the amplification of a *recA* fragment were used as described previously [14]. The PCR primers (*recA* forward 5'-GCAACYTTGTTCTTTACR-3' and *recA* reverse 5'-GAAATGTACGGTCCYGAA-3') and template DNA were added to a *Taq* polymerase master mix, prepared according to manufacturer's instructions (Qiagen, Manchester, UK). Temperature cycling consisted of an initial denaturation of 95ºC for 6 minutes, followed by 40 cycles of 95ºC for 15 seconds, 48.2ºC for 15 seconds and 72ºC for 1 minute, followed by a final extension of 72ºC for 7 minutes. Sequencing of the amplification product yielded 455 bp of unambiguous sequence data. Sequencing results were viewed and edited using ChromasPro 2.0.0. (Technelysium Pty Ltd, Helensville, Queensland, Australia), and submitted to Genbank™ (accession no. KX501214). This 455 bp fragment was then aligned against the *recA* genes of relevant characterised species of the genus *Treponema* using CLUSTALW [20] using sequences trimmed in the BioEdit sequence alignment editor [21]. TOPALi 2.5 [25] was utilised to identify the best-fit evolutionary model for phylogenetic

reconstruction. Phylogeny was subsequently inferred using the Kimura 2-parameter model [26] using MEGA6 [24]. The robustness of the proposed tree branching was evaluated using bootstrap analysis (10,000 iterations).

In contrast to the relatively high (>80 %) 16S rRNA sequence homology observed across a diverse range of *Treponema* species, *recA* gene sequence homology between CHPA and this selection of organisms was generally lower, ranging from 67.6-82.5%. Comparison of these data with *recA* sequences available from *Treponema* species revealed that CHPA^T shared highest *recA* sequence similarity with *T. succinfaciens* ATCC 33096^T (82.5%). Phylogenetic inference, performed on the available *Treponema recA* sequences as described above, resulted in CHPA being loosely clustered with the GI tract treponemes *T. saccharophilum* ATCC 43261^T, *T. succinifaciens* ATCC 33096^T and *T. ruminis* DSM 103462^T (Fig. 2).

The enzyme activity profile for CHPA was determined using the API® ZYM system (bioMérieux, Lyon, France). The results of this analysis (Table 1), whilst identifying the presence of saccharolytic activity in CHPA^T, confirmed a unique profile among the *Treponema species*. Moreover, these data reveal that this novel isolate is phenotypically distinct from its closest known relative, *T. parvum* ATCC 700770^T.

In summary, genotypic and phenotypic characterisation of the bovine GI tract spirochaetal isolate, CHPA^T, indicate that although undoubtedly a member of the *Treponema* genus, it cannot be satisfactorily accommodated into any of the currently valid *Treponema* species. On this basis, we present CHPA^T as *Treponema rectale*, a new member of the genus.

Description of *Treponema rectale* sp. nov. *Treponema rectale* (rec.ta'le. N.L. neut. adj. *rectale*, pertaining to the rectum, rectal, referring to the source of isolation). Cells are small gram-negative, obligatory anaerobic spirochetes of the genus *Treponema*, indigenous to the bovine GI tract. Under phase contrast microscopy, cells were identified as highly motile

spirochete cells with a helical coil. Cells measured approximately 1-5 μm long, 0.15-0.25 μm wide, with 2-5 irregular spirals. Transmission electron microscopy identified 4 periplasmic flagella, in a 2:4:2 arrangement. Cells require a 24-hour anaerobic incubation at 36^{o}C to reach stationary phase in OTEB. Cells do not require serum supplementation to grow. In culture, rotational and translational movement is evident; cells exhibit jerky flexing. When streaked onto FAA plates with or without 10% RS, colonies grow to approximately 0.2 mm in diameter after 10 days. There is no evidence of β-haemolysis after three weeks' incubation. API® ZYM analysis identified positive reactions for C4 esterase, α-galactosidase and β-galactosidase and negative reactions for alkaline phosphatase, C8 esterase lipase, C14 lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphtholphosphohydrolase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-Acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

The type strain, CHPA^{T} (=DSM 103679^{T}, =NCTC 13848^{T}) was isolated from the rectal tissue of a Holstein-Friesian cow from a farm in Cheshire, UK.

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Ethical statement.

All sampling undertaken was approved by the University of Liverpool Ethical Review Process under approved ethics application number VREC137.

Conflicts of interest.

There are no conflicts of interest to declare.

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Tables.

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

		Presence of enzyme activity¶																		
Treponema species	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Treponema rectale [#]	CHPA^T	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
Treponema ruminis [#]	DSM 103462^T	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	+	-	-	-
Treponema parvum [‡]	ATCC 700770^T	+	+	+	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-
Treponema berlinense [§]	ATCC BAA-909^T	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Treponema porcinum [§]	ATCC BAA-908^T	-	+	-	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-
Treponema pedis [+]	DSM 18691^T	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Treponema medium [+]	ATCC 700293^T	+	+	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Treponema brennaborense [|]	DSM 12168^T	+	+	+	-	-	-	-	-	-	+	+	-	+	-	+	-	+	-	-
Treponema pectinovorum [†]	ATCC 33768^T	-	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Treponema socranskii subsp. *socranskii* [†]	ATCC 35536^T	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Treponema socranskii subsp. *buccale* [†]	ATCC 35534^T	+	+	+	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-
Treponema socranskii subsp. *paredis* [†]	ATCC 35535^T	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Treponema maltophilum [†]	ATCC 51939^T	+	+	+	-	-	-	-	-	-	+	+	+	-	-	+	-	-	-	+
Treponema amylovorum [#]	ATCC 700288^T	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+
Treponema denticola [*]	ATCC 35405^T	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Treponema putidum [*]	ATCC 700334^T	+	+	+	-	+	-	-	+	+	+	+	+	+	-	+	+	-	-	-
Treponema lecithinolyticum [α]	ATCC 700332^T	+	+	+	-	-	-	-	-	-	+	+	-	+	+	-	-	+	-	+

¶ API® ZYM data previously reported by [#]Evans *et al.* [15], [‡]Wyss *et al.*,[22] , [§]Nordhoff *et al.*, [19], [+]Evans *et al*. [27], [|]Schrank *et al*. [28], [†]Wyss *et al*. [29], [#] Wyss *et al*. [30], *Wyss *et al*. [31], [α] Wyss *et al*. [32].

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.

Figure Legends.

Fig. 1. A molecular phylogenetic analysis of 16S rRNA sequences from all currently recognised species of *Treponema*, inferred using the maximum likelihood method based on the Tamura-Nei model, from gene sequence comparisons across 1309 aligned bases. Accession numbers are shown in parentheses. Bootstrap values, based on 10,000 iterations, are shown as percentages at the nodes. Bar, 0.05 nucleotide substitutions per site.

Fig. 2. A molecular phylogenetic analysis of available recombinase A (recA) sequences from recognised species of *Treponema*, inferred using the maximum likelihood method based on the Kimura 2-parameter model, from gene sequence comparisons across 293 aligned bases. Accession numbers are shown in parentheses. Bootstrap values, based on 10,000 iterations, are shown as percentages at the nodes. Bar, 0.05 nucleotide substitutions per site.

361

362 **Fig. 1.**

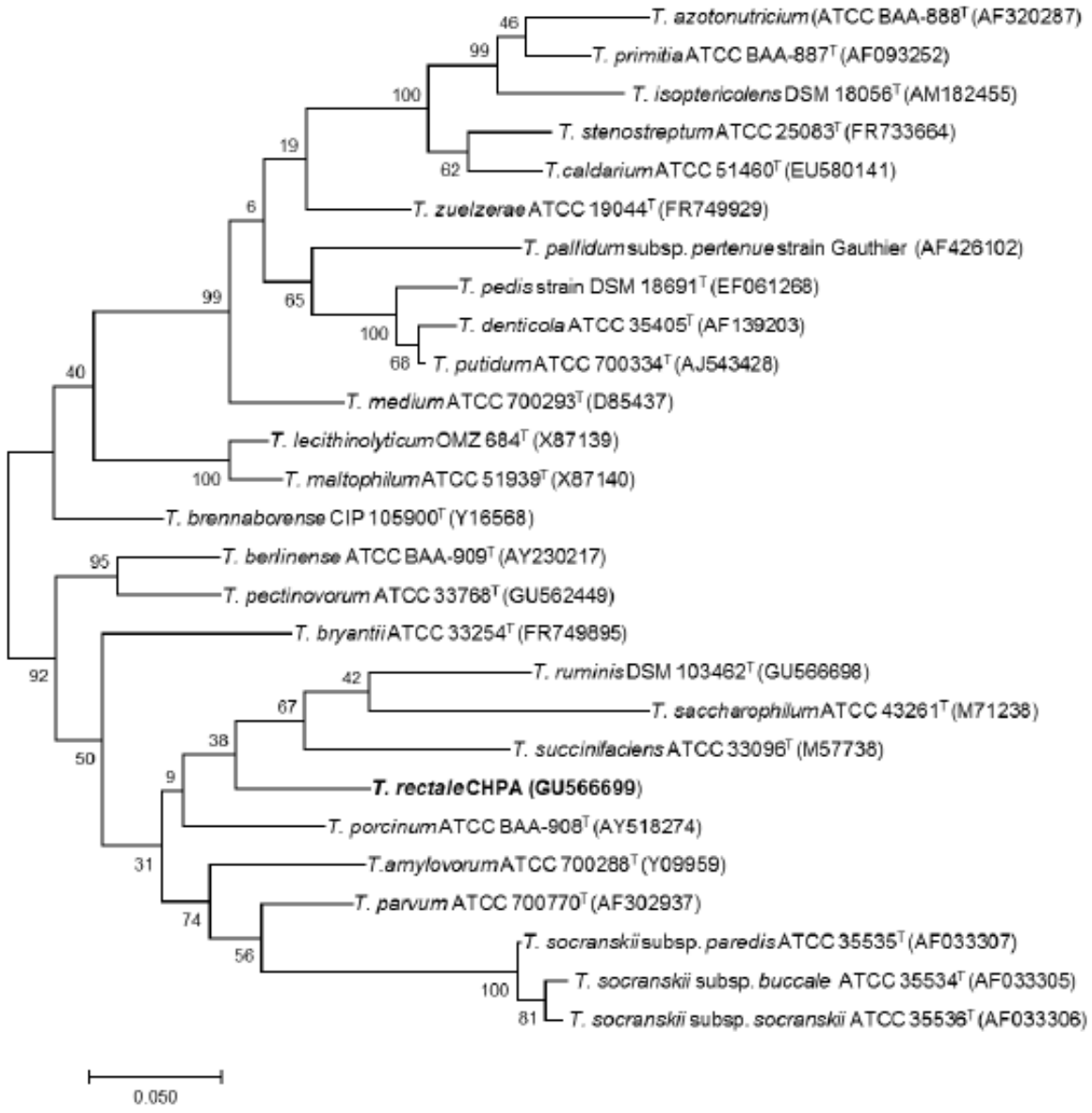

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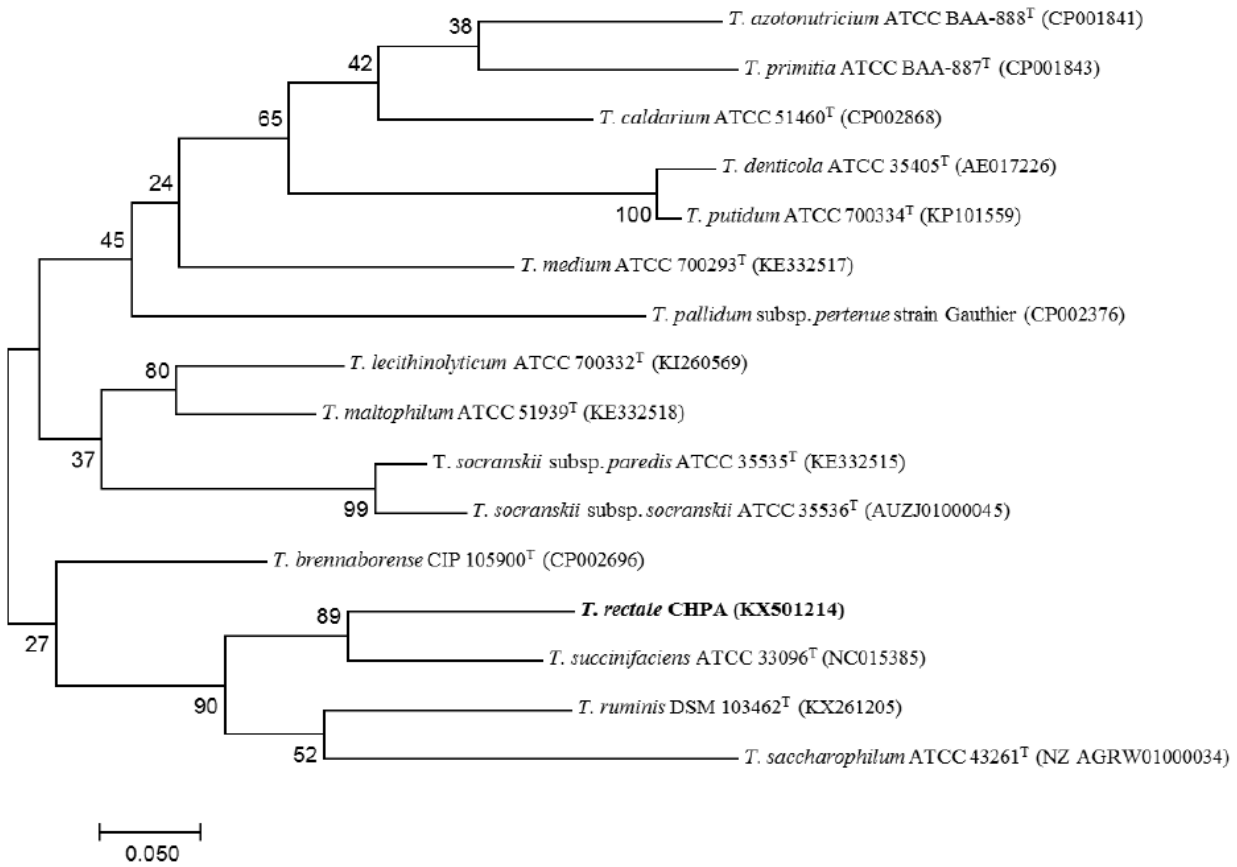
Fig. 2.


T. azotonutricium ATCC BAA-888T (CP001841)
T. primitia ATCC BAA-887T (CP001843)
T. caldarium ATCC 51460T (CP002868)
T. denticola ATCC 35405T (AE017226)
T. putidum ATCC 700334T (KP101559)
T. medium ATCC 700293T (KE332517)
T. pallidum subsp. pertenue strain Gauthier (CP002376)
T. lecithinolyticum ATCC 700332T (KI260569)
T. maltophilum ATCC 51939T (KE332518)
T. socranskii subsp. paredis ATCC 35535T (KE332515)
T. socranskii subsp. socranskii ATCC 35536T (AUZJ01000045)
T. brennaborense CIP 105900T (CP002696)
T. rectale CHPA (KX501214)
T. succinifaciens ATCC 33096T (NC015385)
T. ruminis DSM 103462T (KX261205)
T. saccharophilum ATCC 43261T (NZ AGRW01000034)
38
42
65
24
100
45
80
37
99
27
89
90
52
0.050