***Treponema brennaborens* sp. nov., a novel spirochaete isolated from a dairy cow suffering from digital dermatitis**

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A novel *Treponema* species was isolated from an ulcerative lesion of a cow suffering from digital dermatitis (DD), a disease which causes painful ulcerations along the coronary band. Among other anaerobic bacteria, high numbers of spirochaetes have been regularly found in DD lesions. Here data are presented of a spirochaete isolated from a DD ulcer. By chemotaxonomy, protein analysis and comparative 16S rDNA sequence analysis this isolate was classified as a treponeme that differed from all *Treponema* species described previously. The only isolate, DD5/3^T, for which the name *Treponema brennaborens* is proposed, is designated the type strain of the novel species. The strain is a small, highly motile spirochaete that has two periplasmic flagella, one flagellum being attached at each cell pole. Strain DD5/3^T exhibits α -glucosidase and *N*-acetyl- β -glucosaminidase activity and growth is inhibited by rabbit serum. *T. brennaborens* was phylogenetically most closely related (89.5% 16S rRNA similarity) to *Treponema maltophilum*, an oral spirochaete isolated from a periodontitis patient.

Keywords: treponemes, digital dermatitis, cattle, 16S rRNA

INTRODUCTION

Digital dermatitis (DD) is an acute or chronic inflammatory disease of the bovine foot and was first described by Cheli & Mortellaro (1974). The infection occurs with high incidence and leads to a significant decrease in body weight and milk production, causing significant economic losses worldwide (Blowey & Sharp, 1988). The painful DD lesions, typically found along the coronary band, are red, ulcerative and have a characteristic foul odour (Blowey & Sharp, 1988). Due to a loss of keratin, the superficial layers of the epidermis are eroded, leading to epithelial hyperplasia and sometimes to a granulomatous surface similar to a strawberry.

Gram-negative rods and significant numbers of spirochaetes were found by microscopic analyses in touch-preparations of specimens taken from DD lesions

Abbreviations: DD, digital dermatitis; FISH, fluorescence *in situ* hybridization.

The GenBank/EMBL accession number for the 16S rDNA sequence of the novel isolate *Treponema brennaborens* is Y16568.

(Choi *et al.*, 1997; Done *et al.*, 1993; Döpfer *et al.*, 1997; Read *et al.*, 1992). *Porphyromonas levii*, *Fusobacterium necrophorum*, *Fusobacterium nucleatum*, *Prevotella oralis*, *Prevotella denticola*, *Prevotella bivia* (Nattermann *et al.*, 1996) and as yet unclassified spirochaetes (Walker *et al.*, 1995) were isolated from DD specimens. Recently, we identified by electron microscopy different spirochaetal morphotypes in DD lesions (Grund *et al.*, 1995). The presence of high numbers of spirochaetes suggested a causative role of these bacteria in the aetiopathogenesis of this infection (Collighan & Woodward, 1997; Döpfer *et al.*, 1997; Read *et al.*, 1992; Walker *et al.*, 1997). However, the aetiology of this mixed bacterial infection is still under discussion.

Previous molecular analyses of DD material revealed five spirochaetal phylotypes all clustering within the genus *Treponema* (Choi *et al.*, 1997), some of which were closely related to treponemes associated with human periodontitis (Choi *et al.*, 1997; Collighan & Woodward, 1997; Rijpkema *et al.*, 1997).

Here we describe a novel strain isolated from DD lesions in cattle. Phenotypic and genotypic charac-

terization indicates that this strain represents a novel treponeme species. In this paper we analysed its protein pattern and morphological and biochemical characteristics.

METHODS

Reference strains. The following type and reference strains were maintained as described previously (Wyss *et al.*, 1996): *Treponema denticola* CD-1, *Treponema pectinovorum* ATCC 33768^T (T = type strain), *Treponema socranskii* subsp. *buccale* ATCC 35534^T, *Treponema socranskii* subsp. *paredis* ATCC 35535^T, *Treponema socranskii* subsp. *socranskii* ATCC 35536^T, *Treponema vincentii* LA-1 (= ATCC 355-80), *Treponema maltophilum* ATCC 51939^T and *Treponema phagedenis* biotype Reiter, kindly provided by B. Wilske, Ludwig-Maximilians-Universität München, Germany.

Material, media and culture conditions. A biopsy (1 × 2 × 0.5 cm) from an infected dairy cow (ear-clip: 1219, Dretzener Landprodukte, Land Brandenburg) was taken from the plantar site of a bulbous that showed the typical clinical appearance of DD and was transported to the laboratory immediately. It was cut into pieces of 0.5 × 0.3 × 0.3 cm and incubated in 5 ml OMIZ (Oral Microbiologie and Immunology Zürich)-Pat medium for 1 h at 37 °C. This chemically defined liquid culture medium, developed by Wyss (1992), supports growth of a variety of *Treponema* strains (Wyss *et al.*, 1996).

After incubation, aliquots (100 µl) were mixed with 200 µl fresh medium supplemented with antibiotics (rifampin and phosphomycin, 1 and 100 mg l⁻¹, respectively), transferred into 96-well microtitre plates and subjected to a limit dilution procedure (Leschine & Canale-Parola, 1980; Wyss, 1992). After 14 d incubation at 37 °C in GasPak anaerobic jars (AnaeroGen), the samples were examined by dark-field microscopy (Olympus BH2-RFCA; Carl-Zeiss) to determine growth and cell morphology.

Aliquots from wells containing spirochaetes were plated onto OMIZ-Pat agar (1.5%, w/v) plates. Within 5–10 d diffuse colonies were observed within the agarose. Single colonies were streaked out onto fresh agar plates. To obtain a pure culture, the procedure was repeated three times. Afterwards the strain was removed, transferred to liquid OMIZ-Pat medium and incubated anaerobically until the late-exponential growth phase (6.5 × 10⁸ bacteria ml⁻¹) was reached. Bacteria were stored at -80 °C in liquid growth medium supplemented with 15% (v/v) glycerol.

Electron microscopy. For scanning electron microscopy, colony material (1 × 1 mm) was fixed according to the OTOTO method (Kelley *et al.*, 1973), dried with Peldri II (Ted Pella) and sputter-coated with gold-palladium (5 nm) (SC 501; Emscope).

For transmission electron microscopy, bacteria grown in liquid culture or material of one colony were directly applied to collodion-coated copper grids. After sedimentation of the bacteria and removal of remaining fluid, the samples were directly negatively stained by the method of Nermut (1973), or after fixation in a moist chamber with glutaraldehyde and 2% (v/v) phosphotungstic acid for 1–5 min. The grids were washed with PBS and double-distilled water several times and then air dried. The samples were examined with a Phillips EM 400 electron microscope.

Enzyme activities. For enzyme activity testing, 20 ml bacteria grown in OMIZ-Pat was centrifuged (1305 g, 10 min). To determine catalase activity, cell pellets were covered with 3% (v/v) H₂O₂ and observed for 15 min for bubble evolution. For API ZYM systems and Rapid ID 32A systems (bioMérieux) the cells were resuspended in double-distilled water and examined according to the manufacturer's recommendations.

SDS-gel electrophoresis. SDS-PAGE was performed by the method of Laemmli (1970). For protein pattern analysis all strains were grown in OMIZ-Pat at 37 °C. Bacteria were harvested by centrifugation (1305 g, 10 min). The pellet was resuspended in 100 µl lysis buffer (500 mM Tris/HCl, pH 9.0; 20 mM EDTA; 10 mM NaCl; 1%, w/v, SDS). The protein concentration of the lysate was determined by the method of Lowry (DC Protein Assay; Bio-Rad). An aliquot containing 10 µg whole-cell protein was taken into two volumes of loading buffer, incubated for 5 min at 95 °C and loaded onto an SDS-polyacrylamide (10%, v/v) gel. After electrophoresis the gel was stained with Coomassie brilliant blue R-250.

DNA isolation. For isolation of genomic DNA, 8 ml treponeme culture grown to exponential phase in OMIZ-Pat was pelleted by centrifugation (1305 g, 10 min, 4 °C). The pellet was suspended in 100 µl lysis buffer containing proteinase K (final concentration of 200 µg ml⁻¹; Boehringer Mannheim) and incubated for 1 h at 52 °C. Phenol/chloroform extraction and all further steps have been described previously (Choi *et al.*, 1997). Genomic DNA was resuspended in 50 µl TE buffer (10 mM Tris; 1 mM EDTA) and stored at -20 °C.

16S rDNA sequencing and phylogenetic analysis. 16S rDNA was amplified by PCR using universal eubacterial primer TPU1 [5' AGAGTTTGATC(A/C)TGGCTCAG 3'; corresponding to positions 8–27 in *Escherichia coli* 16S rRNA] and biotinylated eubacterial primer RTU8 [5' AAGGAGGTGATCCA(T/G)CC(G/A)CA 3'; corresponding to positions 1541–1522 in *E. coli* 16S rRNA] as described previously (Choi *et al.*, 1994). For sequence determination of the reverse strand, primer RTU8 and biotinylated primer TPU1 were used to amplify the appropriate almost full-length 16S rDNA fragment. Biotinylated strands were separated by using streptavidin-coated paramagnetic beads (Dynabeads; Dynal Hamburg). Infrared labelled universal eubacterial primers were used as sequencing primers (Lane *et al.*, 1985; Weisburg *et al.*, 1991). A Thermo Sequenase fluorescence-labelled primer cycle sequencing kit (Amersham) and a model 4000 LI-COR automated sequencer (MWG-Biotech) were used according to the manufacturers' instructions.

The 16S rDNA sequence of the new isolate was compared with those of all currently available sequences from the public databases (EMBL, GenBank) using the sequence analysis program Husar 4.0 (Deutsches Krebsforschungszentrum, Heidelberg, Germany). For phylogenetic analysis the TREECON version 3.1 software package was used (van de Peer & de Wachter, 1993). A phylogenetic tree was constructed using the neighbour-joining method of Saitou & Nei (1987). Multiple base changes at single positions were corrected by the method of Jukes & Cantor (1969). All positions were included in the distance calculation.

Phylotype-specific rDNA-based oligonucleotide probe. Specific oligonucleotide probe DDK5/3 (5' CCTCACAGCTCTCTAACCTC 3'; corresponding to positions 184–204 in *E. coli* 16S rRNA) was designed. The specificity of probe DDK5/3 was assessed by dot-blot hybridization using PCR-amplified 16S rDNA from 34 related and unrelated bacteria.

Fig. 1. (a) Scanning electron micrograph of strain DD5/3^T cells grown in liquid medium. Different morphotypes are visible: cells with loose irregular windings and helical and circular forms. Bar, 2 µm. (b) Transmission electron micrograph of a cell of strain DD5/3^T with the outer cell membrane visible. The two periplasmic flagella are subterminally attached at each pole. The preparation was negatively stained. Bar, 0.5 µm.

The controls included sequences from uncultured oral treponemes derived from a 16S rDNA library, culturable *Treponema* species and oral pathogens as described by Moter *et al.* (1998). This probe was used to identify the appropriate organisms by fluorescence *in situ* hybridization (FISH).

FISH. For FISH, bacteria were grown in OMIZ-Pat, pelleted by centrifugation (4000 g, 5 min, 4 °C; Heraeus Labofuge 400) and washed in cold PBS. Fixation and preparation of bacteria were performed as described previously (Gersdorf *et al.*, 1993), except the final methanol and formaldehyde fixation step was omitted. The oligonucleotide DDK5/3 was enzymically labelled at the 3' end with Cy3 (indocarbocyanin)-dUTP (Amersham) by using terminal transferase according to the digoxigenin-labelling protocols (Boehringer Mannheim) and hybridization was performed as described previously (Choi *et al.*, 1997). '*T. vincentii*', *T. maltophilum*, '*T. phagedenis*', *T. denticola* CD-1 and *E. coli* served as negative control strains to assess specificity of probe DDK5/3. Control strains were included in every *in situ* hybridization experiment.

The FISH preparations were examined under oil immersion with a ×100 Neofluar objective on an Axioskop epifluorescence microscope (Carl-Zeiss) equipped with a high-pressure mercury bulb (HBO 50, Osram) and narrow-band filter sets HQ-F41-007 and HQ-F41-001 (AHF). Photo-

micrographs were taken on a Kodak Ektachrome HC 400 film.

Nucleotide sequence accession numbers. The EMBL accession numbers for the reference spirochaetal 16S rRNA sequences used in this study are as follows: *Treponema medium*, Y09959; DDKL-13, Y08896; '*T. phagedenis*', M57739; DDKL-4, Y08894; *T. denticola*, M71236; DDKL-3, Y08893; *Treponema pallidum*, M88726; *Spirochaeta* sp., M71240; *T. maltophilum*, X87140; *T. pectinovorum*, M71237; *Treponema bryantii*, M57737; *Treponema saccharophilum*, M71238; *Treponema* sp., M59294; *Treponema succinifaciens*, M57738; *Spirochaeta aurantia*, M57740; *Spirochaeta isovalerica*, M88720; *Borrelia burgdorferi*, L36-160; *Borrelia anserina*, M72397; *Borrelia hermsii*, M60968; *Brachyspira aalborgi*, Z22781; *Brachyspira* (formerly *Serpulina*) *hyodysenteriae*, M57742; *Brachyspira* (formerly *Serpulina*) *innocens*, M57744; *Leptonema illini*, Z21632; *Leptonema biflexa*, Z12821; *Leptonema interrogans*, X17547; isolate 1-9185MED, L78125; isolate 2-1498, L78126; *E. coli*, J01859.

RESULTS

Cultural and morphological characteristics

Using a chemically defined culture medium, OMIZ-Pat, we isolated by limit dilution a novel small spirochaete. The strain was isolated from a biopsy of a

dairy cow with clinical appearance typical for DD. The temperature for growth of the new isolate was 37 °C in GasPak anaerobic jars. After 5 d incubation the strain formed small white diffuse colonies with a diameter up to 3 mm that penetrated completely into the agar on OMIZ-Pat agar plates.

The cells were 5–8 µm long and 0.25–0.55 µm wide, as shown in Fig. 1. They had a cytoplasmic cylinder surrounded by two periplasmic flagella that originated subterminally at each pole.

In liquid medium the cells exhibited high motility. The cells varied in length depending upon the age of the culture. No growth was seen under aerobic conditions. At a growth temperature of 37 °C the culture reached maximum cell density (8×10^8 bacteria ml⁻¹; pH 5.5) after 21 h incubation, whereas the cell density at the beginning was 8.5×10^6 bacteria ml⁻¹; pH 6.9. In the stationary phase of liquid culture the cells developed circular forms (Fig. 2b). The addition of 2–10% (v/v) rabbit serum or a growth temperature of 30 °C resulted in a decreased growth rate and the serum has therefore been omitted.

Protein profile

The protein profile of the new isolate exhibited major bands in the region between 31 and 66 kDa (Fig. 3).

Fig. 3. Coomassie-stained polypeptide patterns of whole cells of novel strain DD5/3^T and of representative strains of eight cultivable treponemes on an SDS-10% polyacrylamide gel. Lanes: 1, strain DD5/3^T; 2, *T. maltophilum* ATCC 51939^T; 3, *T. socranskii* subsp. *socranskii* ATCC 35536^T; 4, *T. socranskii* subsp. *paredis* ATCC 35535^T; 5, *T. socranskii* subsp. *buccale* ATCC 35534^T; 6, *T. pectinovorum* ATCC 33768^T; 7, *T. denticola* CD-1; 8, '*T. vincentii*' LA-1; 9, '*T. phagedenis*' biotype Reiter; 10, molecular mass standards (in kDa).

These were clearly different from those of the reference strains *T. denticola*, *T. pectinovorum*, *T. socranskii* subsp. *buccale*, *T. socranskii* subsp. *paredis*, *T. socranskii* subsp. *socranskii*, '*T. vincentii*', *T. maltophilum*,

Fig. 2. FISH analysis of strain DD5/3^T cultures grown to exponential (a) and stationary (b) phase using the specific oligonucleotide probe DDK5/3. Hybridization clearly showed helical forms (a) and circular forms (b) of the novel strain. Bar, 5 µm.

Table 1. Enzyme activities in treponemes as determined by the API ZYM system

Enzymes: 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, naphtholphosphohydrolase; 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.

Taxon	Strain	Source	Enzyme activity																		
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Novel isolate	DD5/3 ^T		+	+	+	—	—	—	—	—	—	+	+	—	+	—	+	—	+	—	—
Veterinary treponemes	Isolates*	†	+	+	+	—	—	—	—	—	—	+	+	—	+	+	—	—	+	—	—
	1-9185MED	†	+	+	+	—	—	—	—	+	+	+	+	—	—	—	—	—	—	—	—
<i>T. maltophilum</i>	BR ^T	‡	+	+	+	—	—	—	—	—	—	+	+	+	—	—	+	—	—	—	+
	HO2A	‡	+	+	+	—	—	—	—	—	—	+	+	+	+	+	+	+	—	—	—
	PNA1	‡	+	+	+	—	—	—	—	—	—	+	+	+	+	+	+	+	—	—	—
<i>T. denticola</i>	CD-1	‡	+	+	+	—	+	—	—	+	+	+	+	+	—	—	—	—	—	—	—
	51B2	‡	+	+	+	—	+	—	+	+	+	+	+	+	—	—	+	—	—	—	—
<i>T. pectinovorum</i>	ATCC 33768 ^T	‡	—	+	+	—	—	—	—	—	—	+	+	—	—	—	—	—	—	—	—
<i>T. socranskii</i> subsp. <i>buccale</i>	ATCC 35534 ^T	‡	+	+	+	—	—	—	—	—	—	+	+	—	—	+	—	—	—	—	—
<i>T. socranskii</i> subsp. <i>paredis</i>	ATCC 35535 ^T	‡	+	+	+	—	—	—	—	—	—	+	+	—	—	—	—	—	—	—	—
<i>T. socranskii</i> subsp. <i>socranskii</i>	ATCC 35536 ^T	‡	+	+	—	—	—	—	—	—	—	+	+	—	—	—	—	—	—	—	—
' <i>T. vincentii</i> '	LA-1	‡	—	—	—	—	+	—	—	—	—	+	+	—	+	—	—	—	+	—	—
	Ritz A	‡	—	+	+	—	+	—	—	—	—	+	+	—	+	—	—	—	+	—	—
<i>T. amylovorum</i>		§	+	+	—	—	—	—	—	—	—	+	+	—	—	—	—	—	—	—	+

* All published isolates except 1-9185MED.

† Walker *et al.* (1995).

‡ Wyss *et al.* (1996).

§ Wyss *et al.* (1997).

'*T. phagedenis*' biotype Reiter (Fig. 3). These results demonstrate that the new isolate, designated *Treponema brennaborensis*, could be clearly distinguished from the reference species by its protein pattern.

Enzyme activities

In Table 1 phenotypic characteristics of strain DD5/3^T as detected by the API ZYM system are shown in comparison with the enzyme activities of cultivated oral treponemes, described by Wyss *et al.* (1996, 1997), and DD treponemes, as published by Walker *et al.* (1995). The following enzyme activities were found: alkaline phosphatase, C₄ esterase, C₈ esterase lipase, acid phosphatase, naphtholphosphohydrolase, β -galactosidase, α -glucosidase and *N*-acetyl- β -glucosaminidase. Using the Rapid ID 32A system, the new isolate exhibited the enzyme activities alkaline phosphatase, β -galactosidase, α -glucosidase, *N*-acetyl- β -glucosaminidase and arginine arylamidase and it fermented raffinose and mannose. The catalase test was negative.

Phylogenetic classification

The almost complete 16S rRNA sequence (1400 bases) was determined for phylogenetic classification. Comparative 16S rDNA sequence analysis showed highest homology to (cluster 17) group IV oral treponemes according to the phylogenetic tree derived from a periodontitis patient (Choi *et al.*, 1994). The new strain showed sequence similarity of 89.5% to *T. maltophilum*, the as yet only cultured species of group IV (Fig. 4).

The overall levels of sequence similarity between *T. brennaborensis* 16S rRNA and those of other cultivable oral treponemes ranged from 83.7 to 89.5% (Table 2).

FISH

Among all cultivable treponeme species tested, oligonucleotide probe DDK5/3 specifically detected the novel isolate. FISH analysis showed helical (Fig. 2a) and circular (Fig. 2b) forms of strain DD5/3^T.

Fig. 4. Phylogenetic tree showing the relationship of novel strain DD5/3^T to other spirochaetes based on a comparison of 16S rRNA sequences (positions 67–1467 in *E. coli* 16S rRNA). *E. coli* was used as the outgroup. Scale bar, 10% difference in nucleotide sequences as determined by measuring the lengths of the horizontal lines connecting two sequences. DDKL sequences were obtained from cloned PCR products of uncultured bacteria.

DISCUSSION

The aetiology of DD, a chronic ulcerative dermatitis of the feet of cattle, is still under discussion. A variety of bacterial species including anaerobic Gram-negative rods have been isolated from DD lesions. However, spirochaetes appear to be the predominant organisms in DD lesions and they have been found to invade the stratum spinosum and dermal papillae (Kimura *et al.*, 1993; Read *et al.*, 1992). Microscopic analysis of touch preparations of infected tissue showed different spirochaetal morphotypes (Choi *et al.*, 1997; Done *et al.*, 1993; Döpfer *et al.*, 1997; Read *et al.*, 1992). A report of a significant humoral response in cattle suffering from DD, as compared to healthy animals without detectable lesions, further suggested a possible role of spirochaetes in the aetiopathogenesis of DD (Walker *et al.*, 1997).

Because of the fastidious nature of spirochaetes only few strains associated with DD in cattle have been

cultured so far (Walker *et al.*, 1995). Based upon their enzyme activities and morphological or antigenic characteristics, which were clearly different from those of known spirochaetes, they were classified as members of the genus *Treponema* (Walker *et al.*, 1995). Recently, we used comparative 16S rDNA analysis to phylogenetically classify DD spirochaetes. All of the spirochaetal sequences of a DD-specific 16S rDNA library clustered within the genus *Treponema* and were closely related to oral treponemes (Choi *et al.*, 1997).

Here we present data of a novel isolate, derived from a DD lesion of an infected cattle, including biochemical, enzymic, morphological and phylogenetic comparisons to known spirochaetes.

By using culture media developed primarily for the isolation of oral spirochaetes, we successfully cultivated treponemes from DD material. After cloning by limit dilution we obtained an isolate with typical spirochaetal morphology and motility. Electron microscopic analysis showed the presence of a cytoplasmic cylinder and two periplasmic endoflagella, inserting subterminally at each pole. Strain DD5/3^T could be clearly distinguished by its flagellation type from all but one (strain 1-9185MED) of the isolates described by Walker *et al.* (1995). In addition, all isolates differed by their enzyme activities from our new strain (Table 1). This was also true for strain 1-9185MED, which exhibited trypsin and chymotrypsin activity, but lacked β -galactosidase, α -glucosidase and *N*-acetyl- β -glucosaminidase activity. None of the oral treponeme species showed an identical enzyme pattern in the API ZYM system (Table 1). Furthermore, the novel isolate showed a different protein pattern compared to all treponemes investigated (Fig. 3).

Comparative 16S rDNA sequence analysis allowed the unambiguous phylogenetic classification of the new isolate as a member of the genus *Treponema*, different from all strains published by Walker *et al.* (1995). The data of the similarity matrix constructed from the sequence of strain DD5/3^T and those of 17 other spirochaetes clearly support the species status of the new isolate. As seen in Table 2 our strain exhibited highest homology (89.5%) to *T. maltophilum*, recently isolated from human subgingival plaque (Wyss *et al.*, 1996).

Multiple sequence alignment allowed us to design an oligonucleotide probe specific for the new strain. The specificity of the probe was tested using FISH on different treponeme cultures. Using FISH and electron microscopic analysis on a *T. brennaborensis* culture, circular morphotypes were detected (Fig. 2b). These forms were primarily found in older cultures of DD isolates (Walker *et al.*, 1995) and other treponeme species (Walker *et al.*, 1995; Wecke *et al.*, 1995; Wolf *et al.*, 1993; Wolf & Wecke, 1994). However, the mechanisms leading to circular forms and their possible biological role are still unknown.

Table 2. 16S rRNA sequence similarity matrix for novel treponeme strain DD5/3^T and reference organisms

The similarity values were determined by sequence pair comparison.

Taxon	Phylogenetic group	16S rRNA similarity (%)																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 <i>T. vincentii</i> [*]	I																		
2 DDKL-13*	I	98.7																	
3 <i>T. medium</i>	I	98.8	99.6																
4 <i>T. phagedenis</i> [*]		91.0	91.3	91.4															
5 DDKL-4*		90.6	91.2	91.4	99.4														
6 <i>T. denticola</i>	II	90.4	91.4	91.6	92.3	93.0													
7 DDKL-3*	II	89.3	90.2	90.4	92.0	92.1	95.3												
8 <i>T. pallidum</i>		87.6	87.7	87.7	90.4	90.5	89.4	89.1											
9 <i>T. maltophilum</i>	IV	86.6	86.3	86.9	86.4	86.3	87.2	87.1	84.5										
10 Strain DD5/3 ^T	IV	87.5	87.5	88.0	85.9	86.3	87.3	86.6	84.2	89.5									
11 <i>T. bryantii</i>		85.0	83.9	84.4	84.8	84.4	85.1	85.7	84.2	86.4	87.0								
12 <i>T. pectinovorum</i>		84.5	85.1	85.5	84.6	85.1	85.4	85.4	84.1	87.9	89.2	87.0							
13 <i>T. amylovorum</i>		82.8	83.2	83.8	84.2	83.7	84.8	84.3	83.0	85.6	86.9	86.8	88.5						
14 <i>T. succinifaciens</i>		79.5	79.8	80.5	80.8	81.5	81.0	80.9	82.6	81.3	83.7	83.5	83.6	85.5					
15 <i>Treponema</i> sp.		82.2	83.4	83.7	83.9	83.5	83.8	83.6	84.7	84.6	85.2	86.0	86.6	86.2	88.2				
16 <i>T. saccharophilum</i>		82.4	83.6	84.1	82.9	83.2	83.7	83.9	82.8	82.7	83.7	83.6	86.2	83.5	82.7	87.9			
17 <i>T. socranskii</i>		82.8	82.1	82.8	83.8	83.8	84.2	82.8	82.9	83.9	83.8	84.1	84.9	86.6	83.9	85.1	81.3		
18 <i>Spirochaeta</i> sp.		87.6	87.6	88.3	86.1	87.5	87.0	87.9	87.0	85.2	87.0	85.3	83.9	84.9	82.4	84.9	83.0	83.2	

* 16S rDNA clone sequences (Choi *et al.*, 1997).

At present, we have no information about the epidemiology of this species. Interestingly, the new isolate was most closely related to the phylogenetic group IV of oral treponemes but was not found in our DD-specific 16S rDNA library published earlier (Choi *et al.*, 1997), suggesting a higher diversity of DD treponemes as compared to oral treponemes in human periodontitis. Molecular epidemiological analyses using the specific oligonucleotide probe are needed to provide information about the incidence and distribution of *T. brennaborens* in DD-infected animals. Phenotypic characteristics and 16S rDNA gene sequence data indicate that DD5/3^T represents a new species for which we propose the name *Treponema brennaborens*.

Description of *Treponema brennaborens* sp. nov.

Treponema brennaborens (bren.na.bo.ren'se. Brennabor, medieval name of Brandenburg, Germany; M. L. neut. adj. *brennaborens* referring to Brennabor, where the cow was raised from which the organism was first isolated).

Anaerobic, Gram-negative, helically coiled, motile treponeme isolated from a DD biopsy of a dairy cow. Bacterial cells are 5–8 µm long and 2.5–5.5 µm wide. They have a cytoplasmic cylinder and two periplasmic flagella that originate subterminally at each cell pole. In stationary-phase liquid cultures the bacteria develop circular forms. In liquid culture the bacteria exhibit rotational movement. Growth of strain DD5/3^T is accompanied by acid production. The optimum growth temperature is 37 °C and maximum cell density of approximately 8 × 10⁸ bacteria ml⁻¹ is reached after 21 h incubation. Cells can be stored frozen (–80 °C)

in OMIZ-Pat medium supplemented with 15% (v/v) glycerol. Using semi-solid agarose plates *T. brennaborens* forms diffuse, submersed white colonies up to 3 mm in diameter within 5 d incubation. Strain DD5/3^T ferments raffinose and mannose and exhibits the enzyme activities alkaline phosphatase, C₄ esterase, C₈ esterase lipase, acid phosphatase, naphtholphosphohydrolase, β-galactosidase, α-glucosidase, N-acetyl-β-glucosaminidase and arginine arylamidase, as determined by the API ZYM and Rapid ID 32A systems. Catalase is not produced. The addition of 2–10% (v/v) rabbit serum leads to decreased growth rate. The strain is resistant to rifampin (1 mg l⁻¹) and phosphomycin (100 mg l⁻¹). Strain DD5/3^T is designated the type strain and has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) as DSM 12168^T.

Distinguishing characteristics. All previously described treponemes are genetically distinct from *T. brennaborens* as determined by comparative 16S rDNA sequencing. *T. brennaborens* could be clearly distinguished by its morphology, protein pattern and enzyme activities from the other cultivable *Treponema* species. Its flagellation type was different from most of the other cultivable *Treponema* species. Only *T. denticola*, *T. pectinovorum*, veterinary isolate 1-9185MED and *T. maltophilum* exhibited the same flagellation type, but they exhibited different enzyme activities. Furthermore *T. brennaborens* is distinguishable from *T. maltophilum* by its lack of α-galactosidase activity and its N-acetyl-β-glucosaminidase activity. *T. brennaborens* is clearly distinguishable from veterinary isolate 1-9185MED by its lack of trypsin and chymotrypsin activities.

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