Treponema isoptericolens sp. nov., a novel spirochaete from the hindgut of the termite *Incisitermes tabogae*

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A novel spirochaete, *Treponema* sp. strain SPIT5^T, was isolated from hindgut contents of the drywood termite *Incisitermes tabogae* (Snyder). The cells of strain SPIT5^T were motile, helical in shape, 0.4–0.5 µm in diameter and generally 12–20 µm long. The strain is obligately anaerobic and ferments different mono-, di- and oligosaccharides by forming ethanol as the main liquid fermentation end product. Furthermore, strain SPIT5^T was able to grow anaerobically with yeast extract as sole carbon and energy source. Fastest growth was obtained at 30 °C, the temperature at which the termites were also grown. The optimum pH for growth was 7.2, with a range of pH 6.5–8.0. The cells possessed various enzyme activities that are involved in the degradation of lignocellulose in the termite hindgut, such as β-D-glucosidase, α-L-arabinosidase and β-D-xylosidase. The G+C content of the DNA was 47.7 mol%. Based on 16S rRNA gene sequence analysis, strain SPIT5^T was shown to belong to the so-called 'termite cluster I' of the genus *Treponema*. The closest relative of strain SPIT5^T was *Treponema primitia* ZAS-2^T, with 92.3 % sequence similarity. On the basis of its phenotypic and genotypic properties, strain SPIT5^T can be distinguished from other described species of the genus *Treponema*. Therefore, strain SPIT5^T represents a novel species of *Treponema*, for which the name *Treponema isoptericolens* sp. nov. is proposed. The type strain is strain SPIT5^T (=DSM 18056^T =JCM 13955^T).

Introduction

The genus *Treponema* is composed of host-associated, strictly anaerobic or microaerophilic, helical-shaped bacteria which live as symbionts, commensals or parasites in animals and humans (Smibert, 1984). One of the habitats of treponemes is the digestive tract of termites and wood-eating cockroaches. The diverse microbial gut community of the termites, consisting of bacteria, archaea, yeasts and unique flagellates, is known to support the decomposition of complex organic compounds and thus enables the termites to feed on wood or soil (Eutick *et al.*, 1978; Breznak & Brune, 1994; Varma *et al.*, 1994; König *et al.*, 2002; König & Varma, 2005). Morphologically diverse spirochaetes (0.2–1.0 µm wide and 3–100 µm long) are present consistently in the hindgut of all termites (Breznak,

1984), and they represent one of the most abundant bacterial groups in the gut (Paster *et al.*, 1996). In the hindgut of the so-called lower termites, spirochaetes have also been found as ectosymbionts attached to the surface of cellulose-digesting protists, which inhabit the digestive tract of these species in great numbers (Cleveland & Grimstone, 1964; Wenzel *et al.*, 2003).

The evolutionary distance between spirochaetes from termite guts and other members of this group has been clearly demonstrated by several studies based on culture-independent investigations of 16S rRNA gene sequences from different termite guts (Berchtold *et al.*, 1994; Berchtold & König, 1996; Paster *et al.*, 1996; Ohkuma *et al.*, 1999). Phylogenetic analyses have shown that most symbiotic spirochaetes obtained so far form a distinct branch within the *Treponema* group of the spirochaetes.

The lack of spirochaetal isolates from termite hindguts has limited our understanding of their physiological properties and thus of their specific role within the symbiotic

This article is dedicated to the memory of Dr Terry J. Beveridge, who died on 10 September 2007.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SPIT5^T is AM182455.

community for many decades. Only a few years ago, the first pure cultures of hindgut spirochaetes were obtained, from the lower termite *Zootermopsis angusticollis* (Leadbetter *et al.*, 1999; Lilburn *et al.*, 2001; Graber & Breznak, 2004; Graber *et al.*, 2004). Interestingly, these isolates display physiological pathways that were previously unknown within the spirochaetal group, including acetogenesis from H_2 plus CO_2 and nitrogen fixation. Both processes are beneficial for termites, because acetate is their major carbon and energy source (Odelson & Breznak, 1983) and nitrogen fixation by symbiotic hindgut bacteria can supply up to 60 % of the nitrogen in termite biomass (Lilburn *et al.*, 2001). More recently, a novel spirochaete of the genus *Spirochaeta* was isolated from gut contents of the termite *Neotermes castaneus* (Dröge *et al.*, 2006). This strain showed different glycolytic activities that are involved in the further breakdown of oligosaccharides derived from cellulose and hemicellulose in the termite gut. These findings imply an important role of symbiotic spirochaetes in the nutrition of the termites. In this study, we describe a novel member of the genus *Treponema* isolated from the hindgut of the termite *Incisitermes tabogae*.

Methods

Strain SPIT5^T was isolated from hindgut contents of the lower drywood termite *Incisitermes tabogae* (Snyder) (Isoptera: Kalotermitidae). The termites were obtained from the Federal Institute for Material Research and Testing (BAM, Berlin, Germany). The animals were fed with poplar wood and cultured in metallic vessels containing humid vermiculite at 30 °C. Techniques for the preparation of termite hindguts have been described previously (Dröge *et al.*, 2006). To obtain enrichment cultures, hindgut contents of 10 individuals were transferred into screw-capped tubes containing a modified medium described previously by Leadbetter *et al.* (1999). The medium contained (l^{-1}) 1 g NaCl, 0.5 g KCl, 0.4 g MgCl₂·6H₂O, 0.1 g CaCl₂·2H₂O, 0.3 NH₄Cl, 0.2 g KH₂PO₄, 0.15 g Na₂SO₄, 2.0 g peptone, 2.0 g yeast extract, 5.0 g D-glucose and 1 mg resazurin. Aliquots of the basal medium were dispensed in screw-capped tubes and autoclaved. A mixture of nitrogen and CO₂ (80:20 v/v) was used for the gas phase. Prior to use, the medium was supplemented with the following sterile solutions (per 5 ml): 0.3 ml NaHCO₃ (10 % w/v), 0.05 ml trace element solution (tenfold-diluted; Tschsch & Pfennig, 1984), 0.05 ml seven-vitamin solution (tenfold diluted), 0.1 ml cofactor solution (Leadbetter *et al.*, 1999) and 0.05 ml DTT (0.5 M). The tubes were incubated for 2–3 weeks at 30 °C. Microscopic examination of enrichment cultures showed mainly larger and loosely coiled spirochaetes, whereas smaller and/or tightly coiled morphotypes were rare or absent. The isolated strain SPIT5^T was obtained by repeated deep-agar dilution series after serial dilution of the enrichment cultures in fresh liquid medium. Visible colonies in deep agar (0.9–1 % w/v) developed after 6–7 weeks of incubation at 30 °C. No growth was observed in media containing agar concentrations higher than 1 % (w/v).

The morphology, size range and ultrastructure of the cells were determined by phase-contrast microscopy and transmission electron microscopy (TEM). For TEM, cells were harvested by centrifugation and fixed in a solution of 4 % paraformaldehyde and 2.5 % glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2). After 60 min incubation, the cells were washed three times in buffer and post-fixed

in 2 % OsO₄ for 1.5 h. The cells were dehydrated in a series of ethanol and embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate prior to examination with a Philips CEM 120 Bio-Twin transmission electron microscope.

Utilization of different growth substrates was tested in screw-capped tubes with the medium described above, which was modified according to the carbon source (without glucose, 0.5 g yeast extract l^{-1} , 1 g peptone l^{-1}). The utilization of carbohydrates and production of fermentation end products were analysed by HPLC (Shimadzu LC 10AD VP) with refractive index detection (RI detector ERC-7515B; Erma CR). Analysis of substrates and fermentation products was performed by using an Aminex HPX 87 H column (300 × 7.8 mm; Bio-Rad). The injection volume was 5 µl, the column temperature was 65 °C and the flow rate was 0.6 ml min⁻¹. Concentrations of ethanol determined with HPLC were checked by enzyme analysis. Gases were measured with a gas chromatograph (GC 14A; Shimadzu) with a thermal conductive detector and a Sulpeco Carbosieve column (100/120 Carbosieve S-II). Amounts of fermentation products were calculated from the means of three parallel determinations. Growth rates, as well as temperature and pH optima, were determined by measuring the increase of optical density at 578 nm by using a spectrophotometer (1101M; Eppendorf). The activities of enzymes that are involved in the degradation of oligosaccharides were tested qualitatively by using nitrophenol-labelled carbohydrates (Sigma-Aldrich). Actively growing cells were incubated with the *p*-nitrophenol derivatives (final concentration 0.3 mg ml⁻¹) over 24 h. Enzyme activity led to the development of a yellow colour as a result of free nitrophenol in the medium. Negative controls were performed with cell-free medium and with inactivated cells (10 min at 80 °C).

Genomic DNA was isolated by ethanol precipitation after cell lysis and subsequent phenol/chloroform extraction following a modified Marmur protocol (Johnson, 1991). The bacterial SSU rRNA gene was amplified by PCR using the primers Eubak5 and Eubak3 (Berchtold *et al.*, 1994). The amplified 16S rRNA gene was sequenced commercially (GENTenterprise). For phylogenetic analysis, the novel 16S rRNA gene sequence was added to the ARB database and aligned against existing similar sequences using the fast aligner of the ARB software package (Ludwig *et al.*, 2004). The alignment was checked manually and corrected where necessary. Highly variable regions of the 16S rRNA gene were excluded from the phylogenetic analysis by using only those nucleotide positions that were identical in at least 50 % of all sequences of the alignment. Pairwise distances between taxa were calculated using Kimura's two-parameter model (Kimura, 1980). To determine the evolutionary relationship of the newly isolated organism, the neighbour-joining method (Saitou & Nei, 1987), included in the PHYLIP 3.5 program package (Felsenstein, 1993), was used. The reliability of the reconstructed tree was evaluated by bootstrap analysis (Felsenstein, 1985).

The G+C content of the DNA was determined as described by Mesbah *et al.* (1989) after degradation of the DNA to nucleosides by P1 nuclease and alkaline phosphatase and subsequent separation of the nucleosides by HPLC.

Results and Discussion

In deep agar, strain SPIT5^T formed spheroid white colonies with diffuse edges and a higher density toward the centre (cotton ball-like). Cells of strain SPIT5^T were highly motile, with wavy or rotating movement. The morphological features of strain SPIT5^T are shown in Fig. 1 and are indicated in the species description. The novel isolate grew only under strictly anaerobic conditions and exhibited no

Fig. 1. Morphology of strain SPIT5^T (*Treponema isoptericolens* sp. nov.). (a) Phase-contrast micrograph of a single cell with the wavelength indicated above the cell. Bar, 10 µm. (b) Scanning electron micrograph of a cell with three flagella released from the periplasmic space. Bar, 1 µm. (c) Transmission electron micrograph of an ultrathin cross-section shows six flagella in the periplasmic space. Bar, 0.5 µm.

Table 1. Morphological and physiological properties of strain SPIT5^T in comparison with other *Treponema* species

Strains: 1, SPIT5^T (*Treponema isoptericolens* sp. nov.); 2, *Treponema primitia* ZAS-2^T (data from Graber *et al.*, 2004); 3, *Treponema azotonutricium* ZAS-9^T (Graber *et al.*, 2004); 4, *Treponema bryantii* DSM 1788^T (Stanton & Canale-Parola, 1980); 5, *Treponema saccharophilum* DSM 2985^T (Paster & Canale-Parola, 1985). +, Positive; –, negative; ND, not determined; NR, not reported. All strains produce acetate as an end product of fermentation.

Characteristic	1	2	3	4	5
Cell size (µm)	0.4–0.5 × 12–20	0.2 × 3–7	0.2–0.3 × 10–12	0.3 × 3–8	0.6–0.7 × 12–20
Number of axial filaments	6	2	2	2	32
Optimum pH (range)	7.2 (6.5–8)	7.2 (6.5–7.8)	NR	NR	NR
Optimum temperature (°C)	30	30	30	37	37–39
Use as sole carbon source for growth					
Arabinose	+	–	–	+	+
Ribose	–	–	+	–	–
Xylose	+	+	+	+	–
Fructose	+	NR	+	–	+
Galactose	–	NR	NR	+	+
Glucose	–	+	+	+	+
Mannose	–	NR	NR	+	+
Cellobiose	+	–	+	+	+
Lactose	ND	NR	NR	+	+
Maltose	+	+	+	–	+
Sucrose	–	NR	–	+	+
Trehalose	+	NR	–	NR	NR
H ₂ plus CO ₂ *	–	+	–	NR	NR
Fermentation products					
Formate	–	–	–	+	+
Ethanol	+	–	+	–	+
Succinate	–	–	–	+	–
H ₂	–	–	+	–	–
CO ₂	+	–	+	–	–
DNA G + C content (mol%)	47.7	50.9	50	36	54

*Homoacetogenic growth.

catalase activity. Strain SPIT5^T was a chemoheterotroph and showed a fermentative metabolism. Under optimal conditions (with maltose at 30 °C), the doubling time of the organism was 45 h. Strain SPIT5^T required yeast extract (at a minimum concentration of 0.5 g l⁻¹) and a cofactor solution (Leadbetter *et al.*, 1999) for growth. At higher concentrations, yeast extract could serve as a sole carbon and energy source. This was probably a result of the relatively high trehalose content of the yeast extract which was used for cultivation and growth studies. Analysis of yeast extract solutions with trehalase (Sigma) and HPLC revealed a trehalose content of about 85 mg per gram dry powder, resulting in a final concentration of about 0.5 mM trehalose in the medium used for the isolation of strain SPIT5^T (which contained 2 g yeast extract l⁻¹). Growth of strain SPIT5^T was improved by the addition of peptone but, in contrast to yeast extract, it could not be used as a sole growth substrate. Further physiological properties are summarized in Table 1.

The di- and oligosaccharide-degrading capacity of strain SPIT5^T was tested with different nitrophenol-labelled carbohydrates. Strain SPIT5^T possessed enzyme activities of α -L-arabinosidase, β -D-cellobiosidase, β -D-fucosidase, α -D-galactosidase, α -D-glucosidase, β -D-glucosidase and β -D-xylosidase. No activities of β -D-galactosidase, β -D-glucuronidase or α -D-mannosidase were detected. The tested enzyme activities seemed to be cell-bound, because no glycolytic activity was found in the supernatant of the growth medium.

To determine the phylogenetic relationship between the novel isolate and other members of the order *Spirochaetales*, an almost-complete sequence of the 16S rRNA gene was amplified by PCR and sequenced. A comparison with sequences available in GenBank showed that the sequence of strain SPIT5^T was similar to sequences from the spirochaetal genus *Treponema*. The highest similarities were observed with molecular clones obtained from hindgut contents of the termite *Neotermes koshunensis* (about 95 % sequence identity with NkS53, NkS97 and NkS-Oxy3; Noda *et al.*, 2003). The closest relative of strain SPIT5^T with a validly published name was *Treponema primitia* ZAS-2^T, isolated from hindgut contents of the termite *Zootermopsis angusticollis*, with 92.3 % sequence similarity. Earlier investigations with culture-independent molecular approaches had shown that the majority of termite spirochaetes belonged to one of the so-called termite clusters I or II of the treponemes (Fig. 2), whereas few could be assigned to the genus *Spirochaeta* (Dröge *et al.*, 2006). Based on the phylogenetic analysis, strain SPIT5^T belonged to termite cluster I of the treponemes.

Strain SPIT5^T was identified as a member of the order *Spirochaetales* by its phenotypic characteristics. Based on phylogenetic analysis, it could be verified clearly that strain SPIT5^T belonged to the genus *Treponema* within this bacterial group. The main differences between strain SPIT5^T and related *Treponema* species are summarized in

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain SPIT5^T and other spirochaetes. The tree was constructed by using the neighbour-joining method. Bootstrap values above 50 (from 100 resamplings) are shown for each node. Accession numbers (GenBank) are given in parentheses. Termite clusters I and II of the treponemes are indicated. A sequence of *Leptonema illini* 3055^T (GenBank accession no. Z21632) was used as an outgroup (not shown). Bar, 5 % sequence divergence.

Table 1. Strain SPIT5^T could be distinguished from its closest relatives in both morphological and physiological characteristics. Strain SPIT5^T differs from *T. primitia* ZAS-2^T and *Treponema azotonutricium* ZAS-9^T in cell size and number of axial filaments. Significant differences were also found in important metabolic characteristics, such as the substrate spectra and main fermentation end products. Finally, based on its 16S rRNA gene sequence, strain SPIT5^T was only distantly related to known species of the genus *Treponema* (>5 % sequence divergence). These results indicated that strain SPIT5^T represents a novel species of the genus *Treponema*, for which we propose the name *Treponema isoptericolens* sp. nov.

Description of *Treponema isoptericolens* sp. nov.

Treponema isoptericolens [i.so.ptē'ri.co.lens. N.L. pl. neut. n. *Isoptera* scientific name of an order which encompasses the termites; L. part. adj. *colens* (from L. v. *colere* to inhabit) inhabiting; N.L. n. (participial adjective used as a substantive) *isoptericolens* inhabitant of Isoptera, termites].

Cells are helical-shaped with a wavelength of 6–7 µm, an amplitude of 1.5–1.8 µm, a diameter of 0.4–0.5 µm and generally 12–20 µm in length. Motile by six periplasmic flagella, inserted near opposite ends of the protoplasmic cylinder. Gram-negative. Spherical bodies are formed at the end of the exponential growth phase. Strictly anaerobic and catalase-negative. Yeast extract is required for growth and can serve as a sole energy and carbon source. Optimal temperature for growth is 30 °C. The pH range for growth

is pH 6.5–8.0 with optimum growth at pH 7.2–7.4. Chemoheterotroph with fermentative metabolism. Grows on arabinose, xylose, fructose, maltose, cellobiose, trehalose, maltotriose and yeast extract. Maltose is fermented to ethanol and CO₂ as main fermentation products. Glucose, galactose, lactate, pyruvate, fatty acids, amino acids and polysaccharides are not utilized. Activities of α -L-arabinosidase, β -D-cellobiosidase, β -D-fucosidase, α -D-galactosidase, α -D-glucosidase, β -D-glucosidase and β -D-xylosidase are exhibited. The G+C content of the DNA of the type strain is 47.7 mol%.

The type strain, SPIT5^T (=DSM 18056^T =JCM 13955^T), was isolated from hindgut contents of the lower drywood termite *Incisitermes tabogae* (Snyder) (Isoptera: Kalotermitidae).

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