Treponema berlinense sp. nov. and *Treponema porcinum* sp. nov., novel spirochaetes isolated from porcine faeces

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Limit-dilution procedures were used to isolate seven, helically coiled bacterial strains from faeces of swine that constituted two unidentified taxa. Comparative 16S rRNA gene sequence analysis showed highest similarity values with species of the genus *Treponema* indicating that the isolates are members of this genus. Strain 7CPL208^T, as well as five further isolates, and 14V28^T displayed the highest 16S rRNA gene sequence similarities with *Treponema pectinovorum* ATCC 33768^T (92.3%) and *Treponema parvum* OMZ 833^T (89.9%), respectively. Polar lipid profiles distinguished 7CPL208^T and 14V28^T from each other as well as from related species. Based on their phenotypic and genotypic distinctiveness, strains 7CPL208^T and 14V28^T are suggested to represent two novel species of the genus *Treponema*, for which the names *Treponema berlinense* sp. nov. and *Treponema porcinum* sp. nov. are proposed. The type strain for *Treponema berlinense* is 7CPL208^T (= ATCC BAA-909^T = CIP 108244^T = JCM 12341^T) and for *Treponema porcinum* 14V28^T (= ATCC BAA-908^T = CIP 108245^T = JCM 12342^T).

Due to difficulties in the culture of *Treponema* species, only a small number have been cultivated (Sakamoto *et al.*, 1999; Siqueira & Rocas, 2003). As a result, most oral *Treponema* species have been identified only by their 16S rRNA gene sequences (Dewhirst *et al.*, 2000). In the gastrointestinal tract of termites, comparative 16S rRNA gene sequence analyses have revealed more than 67 different treponemal phylotypes (Lilburn *et al.*, 1999) of which only two distinct species have been isolated and characterized (Lilburn *et al.*, 2001; Graber *et al.*, 2004). Recent improvements in cultivation techniques have resulted in the identification and characterization of several novel *Treponema* species (Smibert & Burmeister, 1983; Umemoto *et al.*, 1997; Schrank *et al.*, 1999; Wyss *et al.*, 1996, 1997, 1999, 2001, 2004). In the gastrointestinal tract of animals, *Treponema bryantii* and *Treponema saccharophilum* have been cultivated from the rumen of cows (Stanton & Canale-Parola, 1980; Paster & Canale-Parola, 1985). Although the presence

of *Treponema*-related spirochaetes in swine intestine and faeces has been reported (Smibert & Claterbaugh, 1972; Livermore & Johnson, 1975), only one species, *Treponema succinifaciens*, has been isolated and described in swine (Cwyk & Canale-Parola, 1979).

As part of a study on the microbial diversity of the gastrointestinal tract of swine, strains 7CPL208^T, 11IV56, 25AKT66, 27VII56, 33AKL503, 13VII56 and 14V28^T were recovered from faeces. Faeces were suspended in OMIZ-Pat medium supplemented with 10% (v/v) brain heart infusion (BHI), 10% (v/v) trypticase soy yeast extract (TSYE), rifampicin (1 µg ml⁻¹) and phosphomycin (100 µg ml⁻¹) using limit-dilution procedures and were incubated anaerobically for 3–4 days at 37 °C (Wyss, 1992; Wyss *et al.*, 1996). When spirochaete bacteria were visible by dark-field microscopy, subcultures were performed on supplemented OMIZ-Pat agar plates (1.3% w/v). To obtain pure cultures, single colonies were purified from agar plates and transferred to liquid medium for further analyses. Agar plates were supplemented with 5% (v/v) sheep blood for strain 7CPL208^T and with 5% (v/v) egg yolk for strain 14V28^T.

Cell morphology was examined by transmission electron microscopy as described previously (Cwyk & Canale-Parola, 1979). Cells showed typical spirochaete morphology,

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Treponema berlinense* strain 7CPL208^T and *Treponema porcinum* strain 14V28^T are AY230217 and AY518274, respectively.

Transmission electron micrographs, SDS-PAGE analyses, genomic fingerprinting gels and polar lipid profiles are available as supplementary material in IJSEM Online.

exhibiting two to three windings and two subterminally inserted flagella (see Supplementary Fig. S1 available in IJSEM Online).

Genomic DNA was purified as described by Schrank *et al.* (1999). Amplified PCR products were sequenced commercially (MWG Biotech). Phylogenetic analyses were performed using the software package DNASTAR for multiple alignment of sequences and MEGA (Kimura *et al.*, 1993). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining method were determined using bootstrap values based on 1000 reiterations.

Continuous 16S rRNA gene sequences of 1450 and 1500 bp were obtained for strains 7CPL208^T and 14V28^T, respectively, and partial 16S rRNA gene sequences of about 650 bp (data not shown) were obtained from 11IV56, 25AKT66, 27VII56, 33AKL503 and 13VII56. Partial 16S rRNA gene sequences of 11IV56, 25AKT66, 27VII56, 33AKL503 and 13VII56 were identical to the corresponding 16S rRNA gene sequence of 7CPL208^T, indicating a possible relationship at the species level. Sequence similarities indicated that the closest relatives of strains 7CPL208^T and 14V28^T were *Treponema pectinovorum* ATCC 33768^T (92.3 %) and *Treponema parvum* OMZ 833^T (89.9 %), respectively. Strains 7CPL208^T and 14V28^T shared sequence similarities of 89.2 %.

Phylogenetic calculations after neighbour-joining analyses confirmed these relationships (Fig. 1). Strain 7CPL208^T was placed in group 8 of the oral treponemes, of which *T. pectinovorum* ATCC 33768^T is so far the only representative (Dewhirst *et al.*, 2000). Strain 14V28^T could not be assigned to any of these groups.

Culture characteristics were examined using either

glucuronic or galacturonic acid supplementation of carbohydrate-free OMIZ-Pat medium. In both cases, growth was observed for strain 7CPL208^T and related isolates. In carbohydrate-supplemented OMIZ-Pat medium without the addition of glucuronic and galacturonic acid, growth was observed for strain 14V28^T. In contrast, *T. parvum* required supplementation with at least one of these substances (Wyss *et al.*, 2001).

For strain 7CPL208^T and related isolates, no visible growth was observed in glucuronic and galacturonic acid-free medium, with one exception, strain 25AKT66, which showed only a decreased growth rate. These results indicated that addition of either glucuronic or galacturonic acid promoted growth of 7CPL208^T and related isolates, as previously described for *T. pectinovorum* (Smibert & Burmeister, 1983). In contrast to *T. pectinovorum*, no growth was observed for either strain with pectin as the sole carbon source.

The ability to use a single carbohydrate source was investigated by the addition of one of the following compounds to carbohydrate-free OMIZ-Pat medium containing galacturonic and glucuronic acid: D-glucose, D-fructose, D-maltose, D-mannitol, D-mannose, D-arabinose, L-fucose, D-trehalose, D-sucrose or L-rhamnose. For isolate 14V28^T, growth was only observed in presence of D-maltose, whereas the growth rate of 7CPL208^T was enhanced by the addition of any one of these carbohydrates to OMIZ-Pat medium.

Enzyme profiles were determined using the API ZYM and Rapid ID 32A systems (bioMérieux) according to the manufacturer's instructions. Using the Rapid ID 32A system, isolate 14V28^T showed a positive reaction only for α -glucosidase, which was also consistent with the results from the API ZYM system. Strain 7CPL208^T was negative

Fig. 1. Phylogenetic analysis based on 16S rRNA gene sequences constructed after multiple alignment of data by CLUSTAL W (accession numbers in parentheses). Distances were calculated (distance options according to the Kimura-2 model) and clustering with the neighbour-joining method was performed using the software package MEGA version 2.1.

for all enzyme reactions tested. Identical profiles were obtained for 7CPL208^T, 11IV56, 25AKT66, 27VII56, 33AKL503 and 13VII56 using the API ZYM system and these profiles clearly distinguished them from 14V28^T and other closely related *Treponema* species previously examined by this method (Table 1). The detailed enzyme profiles are indicated in the species descriptions.

In order to examine relationships at the species level, 7CPL208^T, 11IV56, 25AKT66, 27VII56, 33AKL503 and 13VII56 were compared with each other and with 14V28^T by SDS-PAGE of whole-cell protein patterns and genomic fingerprinting analyses using RAPD- and ERIC-PCR. SDS-PAGE of whole-cell proteins using standard protocols (Sambrook *et al.*, 1989) showed essentially identical protein profiles for these related isolates (see Supplementary Fig. S2 in IJSEM Online). Genomic fingerprinting was performed by both RAPD- and ERIC-PCR. The RAPD-PCR utilized 1.5 mM MgCl₂, 0.2 mM dNTPs, 12.5 pmol primer (random primer 5'-GCGGCTGGAG-3'), 100 ng genomic DNA and reaction buffer supplied by the manufacturer (Rapidozym) in a total volume of 50 µl. Samples were

preheated at 94 °C for 5 min followed by amplification with 94 °C for 30 s, 40 °C for 30 s and 72 °C for 45 s. A total of 45 cycles was carried out with a final elongation step at 72 °C for 10 min. ERIC-PCR using primers ERIC-1R (5'-ATGTAAGCTCCTGGGGATTAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') was conducted in 50 µl containing 2 mM MgCl₂, 0.2 mM dNTPs, 12.5 pmol primer and 50 ng genomic DNA. Samples were preheated at 94 °C for 5 min followed by amplification with 94 °C for 30 s, 46 °C for 5 min and 72 °C for 5 min. A total of 45 cycles was carried out with a final elongation step at 72 °C for 10 min. The RAPD fingerprints of 7CPL208^T, 11IV56, 25AKT66, 27VII56, 33AKL503 and 13VII56 shared at least two major bands at approximately 550 and 1050 bp. Several shared bands were present in the ERIC fingerprints, with one major band at approximately 1200 bp as determined by gel electrophoresis. These fingerprints also distinguished this group of isolates from 14V28^T and other related *Treponema* species (Supplementary Fig. S3 in IJSEM Online). Strain 25AKT66 was unique among this set of isolates as indicated by slightly different growth characteristics and slightly varying protein and genomic fingerprint patterns.

Table 1. Biochemical characteristics of type strains of *Treponema* species as described using the API ZYM system

Species: 1, '*Treponema vincentii*' ATCC 35580 (data from Schrank *et al.*, 1999); 2, *T. denticola* 51B2 (Schrank *et al.*, 1999); 3, *T. brennaborensis* DSM 12168^T (Schrank *et al.*, 1999); 4, *T. maltophilum* ATCC 51939^T (Wyss *et al.*, 1996); 5, *T. amylovorum* ATCC 700288^T (Wyss *et al.*, 1996); 6, *T. pectinovorum* ATCC 33768^T (Schrank *et al.*, 1999); 7, *T. socranskii* sub sp. *socranski* ATCC 35536^T (Schrank *et al.*, 1999); 8, *T. parvum* ATCC 700770^T (Wyss *et al.*, 2001); 9, *T. lecithinolyticum* ATCC 700332^T (Wyss *et al.*, 1999); 10, *T. berlinense* sp. nov. (six isolates); 11, *T. porcinum* sp. nov. 14V28^T. All strains are positive for acid phosphatase and naphthol-AS-BI-phosphohydrolase and negative for lipase (C14), valine arylamidase and α-mannosidase. +, Positive; -, negative; (+), weakly positive.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Alkaline phosphatase	-	+	+	+	+	-	+	(+)	+	-	-
Esterase (C4)	-	+	+	+	+	+	+	(+)	+	-	+
Esterase lipase (C8)	-	+	+	+	-	+	-	(+)	+	-	-
Leucine arylamidase	+	+	-	-	-	-	-	-	-	-	-
Cystine arylamidase	-	+	-	-	-	-	-	-	-	-	-
Trypsin	-	+	-	-	-	-	-	-	-	-	-
α-Chymotrypsin	-	+	-	-	-	-	-	-	-	-	-
α-Galactosidase	-	+	-	+	-	-	-	-	-	-	-
β-Galactosidase	+	+	+	-	-	-	-	+	+	-	-
β-Glucuronidase	-	-	-	-	-	-	-	-	+	-	-
α-Glucosidase	-	-	+	+	-	-	-	-	-	-	+
β-Glucosidase	-	+	-	-	-	-	-	-	-	-	-
N-Acetyl-β-glucosaminidase	+	-	+	-	-	-	-	-	+	-	-
α-Fucosidase	-	-	-	+	+	-	-	-	+	-	-

Although several reports have been published on the presence and variability of polar lipids in *Treponema* species (Livermore & Johnson, 1974; Matthews *et al.*, 1979), polar lipid profiles have not been introduced into their classification. These early studies prompted us to apply this approach to the classification of strain 7CPL208^T and 14V28^T. For analysis of the polar lipid profiles, strains 7CPL208^T, 14V28^T, *Treponema socranskii* ATCC 3553^T and *T. pectinovorum* ATCC 33768^T were grown in supplemented OMIZ-Pat medium at 37 °C under anaerobic conditions. Analysis of their polar lipids (Tindall, 1990; Altenburger *et al.*, 1997) revealed a unique, complex profile for each strain (Supplementary Fig. S4 in IJSEM Online), further demonstrating that strains 7CPL208^T and 14V28^T represented novel species of the genus *Treponema*. Strain 7CPL208^T displayed a polar lipid profile consisting of the major compounds diphosphatidylglycerol, phosphatidylethanolamine, an unknown aminophospholipid and an unknown, highly hydrophobic compound. Moderate or minor amounts of phosphatidylglycerol, several unknown aminophospholipids, phospholipids, aminolipids, polar lipids and a glycolipid were also present. The closest phylogenetic relative of 7CPL208^T, *T. pectinovorum* ATCC 33768^T, shared the majority of the major compounds except for the predominant aminophospholipid APL4 and several of the other lipids present in moderate to minor amounts.

In contrast to these two strains, no phosphatidylethanolamine was detected in extracts of 14V28^T or *T. socranskii* ATCC 33768^T. Strain 14V28^T displayed a polar lipid profile consisting mainly of phospholipids, including diphosphatidylglycerol and phosphatidylglycerol, glycolipid GL1 and some unknown polar lipids, but completely lacking aminolipids (Supplementary Fig. S4c in IJSEM Online). The

major lipids present were three unknown phospholipids (PL1, PL14 and PL15) and an unknown, highly hydrophobic lipid, L1. *T. socranskii* ATCC 33768^T and strain 14V28^T both contained ten phospholipids, glycolipid GL1 and unknown lipid L1. An unknown aminophospholipid, APL1, and two unknown aminolipids, AL1 and AL2, were also found in the profile of *T. socranskii* ATCC 33768^T (Supplementary Fig. S4d in IJSEM Online). Major compounds were diphosphatidylglycerol, phosphatidylglycerol, unknown phospholipid PL5 and unknown lipid L1.

A comparison of the polar lipid profiles of the four strains analysed indicated a higher degree of similarity between the strains of the close phylogenetic relative pairs 7CPL208^T/*T. pectinovorum* ATCC 33768^T and 14V28^T/*T. socranskii* ATCC 33768^T than between strains of different pairs. Our data indicate that analysis of polar lipids is a useful approach for the characterization and differentiation of *Treponema* strains. This conclusion is supported by the analysis of the polar lipids of two additional species, *Treponema denticola* ATCC 35521 and *Treponema brennaborensense* DSM 12168^T, each of which displayed a unique profile (data not shown). These results may also indicate that polar lipid profiles display, to some degree, the phylogenetic relationships within species of the genus *Treponema*. In the future, such profiles may serve as important markers for the dissection of this genus that might be necessary due to the deep phylogenetic branching of species found within the genus. Identical or near-identical phenotypic and genotypic characteristics indicated that strains 7CPL208^T, 11IV56, 25AKT66, 27VII56, 33AKL503 and 13VII56 are members of a single species.

Strains 7CPL208^T and 14V28^T were distinguished from related *Treponema* species by their 16S rRNA gene sequences, which exhibited the highest sequence similarities with *Treponema pectinovorum* ATCC 33768^T (92.3%) and *Treponema parvum* OMZ 833^T (89.9%), respectively, whereas strains 7CPL208^T and 14V28^T shared 89.2% sequence similarity. Using different genomic fingerprinting methods (RAPD- and ERIC-PCR) these strains could be clearly differentiated from related *Treponema* species by their unique fingerprint patterns. In addition, strains 7CPL208^T and 14V28^T differed from other *Treponema* species in their phenotypic characteristics. In contrast to *T. pectinovorum* ATCC 33768^T, strain 7CPL208^T showed positive enzyme reactions only for acid phosphatase and naphthol-AS-BI-phosphohydrolase and no activity for esterase C4 or esterase lipase C8. No growth occurred with pectin as the sole carbohydrate source. Unlike *Treponema parvum* OMZ 833^T, strain 14V28^T exhibited a positive reaction for α -glucosidase but lacked activity for β -galactosidase, alkaline phosphatase and esterase lipase C8. In addition, while neither glucuronic nor galacturonic acids were essential for growth, D-maltose was required.

These results suggest that strains 7CPL208^T and 14V28^T constitute two distinct genomic species that are clearly differentiated from other *Treponema* species. We therefore

propose the names *Treponema berlinense* sp. nov. and *Treponema porcinum* sp. nov. for strains 7CPL208^T and 14V28^T, respectively.

Description of *Treponema berlinense* sp. nov.

Treponema berlinense (ber.li.nen'se. N.L. neut. adj. *berlinense* pertaining to Berlin, Germany, where the type strain was isolated).

Cells show typical spirochaete morphology exhibiting two to three windings with two periplasmic, subterminally inserted flagella. Cells are approximately 6 μ m in length and 0.3 μ m in width. Strictly anaerobic. Good growth is observed in liquid OMIZ-Pat medium at 37 °C supplemented with 10% (v/v) BHI and 10% (v/v) TSYE. On OMIZ-Pat agar plates (1.3% w/v) supplemented with 5% (v/v) sheep blood, 10% (v/v) BHI and 10% (v/v) TSYE, species forms small, irregular, greyish swarms up to 1–2 mm in diameter, visible after 3–4 days. Addition of galacturonic or glucuronic acid promotes growth, which is enhanced further by addition of any of the following carbohydrates: D-glucose, D-fructose, D-maltose, D-mannitol, D-mannose, D-arabinose, L-fucose, D-trehalose, D-sucrose and L-rhamnose. No visible growth is observed with pectin as the sole carbon source. Using the API ZYM and Rapid ID 32A systems, positive enzyme reactions are obtained only for acid phosphatase and naphthol-AS-BI-phosphohydrolase. Negative in tests for alkaline phosphatase, esterase C4, esterase lipase C8, leucine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -fucosidase, urease, arginine dihydrolase, α -arabinosidase, mannose and raffinose fermentation, glutamic acid decarboxylase, α -fucosidase, arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase and serine arylamidase. Reduction of nitrates and indole production are not detected. The polar lipid profile contains diphosphatidylglycerol, phosphatidylethanolamine, an unknown aminophospholipid and an unknown highly hydrophobic compound as major components. Moderate or minor amounts of phosphatidylglycerol, several unknown aminophospholipids, phospholipids, amino lipids, polar lipids and a glycolipid are also present.

The type strain, 7CPL208^T (ATCC BAA-909^T=CIP 108244^T=JCM 12341^T), was isolated from swine faeces in Berlin, Germany.

Description of *Treponema porcinum* sp. nov.

Treponema porcinum (por.ci'num. L. neut. adj. *porcinum* pertaining to swine, from which the type strain was isolated).

Cells exhibit typical spirochaete morphology and are approximately 6–8 µm in length and 0.3 µm in width with two to three windings. Transmission electron microscopy reveals two periplasmic, subterminally inserted flagella. Strictly anaerobic. Best growth is obtained in liquid OMIZ-Pat medium at 37 °C supplemented with 10 % (v/v) BHI and 10 % (v/v) TSYE. Growth is independent of glucuronic or galacturonic acid. D-Maltose is essential for growth, whereas no growth is observed in the presence of any of the following carbohydrates as the sole carbohydrate source: D-glucose, D-fructose, D-mannitol, D-mannose, D-arabinose, L-fucose, D-trehalose, D-sucrose and L-rhamnose. Does not grow with pectin as a sole carbon source. On supplemented OMIZ-Pat (1.3 % w/v) agar supplemented with 5 % egg yolk, 10 % BHI and 10 % TSYE, the species forms small, greyish, irregular swarms up to 2 mm in diameter, visible after 3–4 days. Positive enzyme reactions using the API ZYM and Rapid ID 32A system are obtained for acid phosphatase, esterase C4, naphthol-AS-BI-phosphohydrolase and α -glucosidase, whereas reactions for alkaline phosphatase, esterase lipase C8, leucine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -fucosidase, urease, arginine dihydrolase, α -arabinosidase, mannose and raffinose fermentation, glutamic acid decarboxylase, α -fucosidase, arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase and serine arylamidase are negative. Reduction of nitrates and indole production are not detected. In the polar lipid profile, three unknown phospholipids and a highly hydrophobic compound predominate. Diphosphatidylglycerol, phosphatidylglycerol as well as phospholipids are present in moderate amounts. Additionally, a glycolipid and several phospholipids are present in minor amounts.

The type strain, 14V28^T (deposited as ATCC BAA-908^T=CIP 108245^T=JCM 12342^T), was isolated from swine faeces in Berlin, Germany.

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