

Phylogenetic analysis of cultivable oral treponemes from the Smibert collection

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Dr Robert Smibert from the Virginia Polytechnic Institute, USA, isolated and collected over 200 strains of oral treponemes over a 20-year period. Dr Smibert, Dr W. E. C. Moore and Dr L. V. Moore separated these isolates and reference strains into different groups on the basis of cellular fatty acid analysis. In this study, the 16S rRNA genes were sequenced for 47 strains that were representative of these groups. Five distinct species were identified on the basis of 16S rRNA sequence comparisons; two of these species are newly named and three have not yet been characterized. The first species, designated *Treponema* Smibert-1, was represented by the single strain D4B-1 and was later identified as the newly described *Treponema maltophilum*. However, strain D4B-1 possessed a different flagellar arrangement to that of *T. maltophilum*. The second species, *Treponema* Smibert-2, was represented by nine isolates that possessed identical 16S rRNA gene sequences. The closest relatives of this species were *Treponema* Smibert-3 and *Treponema* Smibert-4 at approximately 90% sequence similarity. Within *Treponema* Smibert-2, there was no correlation between phylogenetic analysis and cellular fatty acid analysis since six different cellular fatty acid groups represented the nine strains. *Treponema* Smibert-3 (strain D36ER-1) and *Treponema* Smibert-4 (D62CR-12) were each represented by only a single strain and were closely related to each other at 98% sequence similarity. Strain D36ER-1 of *Treponema* Smibert-3 was identified as belonging to the not-yet-cultivated phylotype 20 [Choi, B. K., Paster, B. J., Dewhirst, F. E. & Göbel, U. B. (1994). *Infect Immun* 62, 1889–1895]. Strain D62CR-12 of *Treponema* Smibert-4 was nearly identical in sequence to the newly described *Treponema amylovorum*. The fifth species, *Treponema* Smibert-5, was represented by a single strain, D120CR-1, and was closely related at about 98% sequence similarity to the three subspecies of *Treponema socranskii*. The phylogenetic analyses of strains of *Treponema vincentii* and of subspecies of *T. socranskii* are also reported. The closest oral relatives of *T. vincentii* were *Treponema medium* at 98.7% sequence similarity and *Treponema denticola* at 91.5% sequence similarity. *T. socranskii* subspp. *socranskii*, *buccale* and *paredis* formed three separate phylogenetic branches with sequence similarities of about 98% to each other. The closest relative of the subspecies of *T. socranskii* and of Smibert-5 was Smibert-2 at about 86% sequence similarity. Historic reference strains Fuji, '*Treponema ambigua*', Fm, Ichelson-2, N-39, TD2, TRRD, MRB, IPP, Jethro and T32A, as well as an unknown strain designated only as *Treponema oralis*, were identified as strains of *T. denticola*. Reference strains Fuji, Jethro, T32A and IPP plus three isolates of the Smibert collection were also contaminated with a mycoplasma as determined by 16S rRNA comparative analysis. Consequently, spirochaetal cultures should be screened for mycoplasmas. There are presently at least ten species of cultivable oral species of *Treponema* with the cut-off for separate species

The GenBank accession numbers for the 16S rRNA sequences reported in this paper are given in Table 1.

designation at about 98% sequence similarity. However, DNA–DNA reassociation experiments are necessary to differentiate species when 16S rDNA sequence similarities are at about this level.

Keywords: phylogeny, 16S rDNA, oral spirochaete, *Treponema*

INTRODUCTION

It has been reported from microscopic examinations that oral treponemes can represent up to 50% of the detectable flora in subgingival plaque from diseased sites (1, 23). Spirochaetes are present in elevated numbers in subgingival plaque of subjects with a variety of oral diseases that include gingivitis (19, 25), acute necrotizing ulcerative gingivitis (ANUG) (8, 14, 18), juvenile periodontitis (3, 28), chronic periodontitis (20, 21), rapidly progressing periodontitis (27, 29, 38), periocoronitis (6) and endodontic abscesses (43). HIV-positive subjects with gingivitis and adult periodontitis also have elevated numbers of spirochaetes (26, 35). In sites of ANUG, juvenile periodontitis and advanced periodontal disease, spirochaetes have been observed invading the connective tissue or intact epithelium (3, 11, 18, 33, 34, 36). In some instances, nearly confluent monolayers of spirochaetes have been observed adhering to the epithelial lining of inflamed periodontal pockets (16, 18, 19).

By comparison of 16S rRNA or 16S rDNA sequences, spirochaetes are divided into two major phylogenetic groupings (7, 13, 31). The first grouping, which corresponds to the family *Spirochaetaceae*, contains species of the genera *Treponema*, *Spirochaeta*, *Borrelia*, *Serpulina* (and *Brachyspira*) and *Brevinema*. However, a few species of *Spirochaeta* are more closely related to the treponemes than to other members of the genus *Spirochaeta* (31). The second grouping, which corresponds to the family *Leptospiraceae*, contains species of the genera *Leptospira* and *Leptonema*. Thus far, spirochaetes isolated from the oral cavity all fall within the genus *Treponema*.

Although spirochaetes have been associated with oral diseases for many years, only seven species have been cultured and validly named: *Treponema denticola* (39), *Treponema pectinovorum* (40), *Treponema socranskii* (including three subspecies) (41), *Treponema vincentii* (39), and, more recently, *Treponema maltophilum* (48), *Treponema medium* (45) and *Treponema amylovorum* (49). Despite recent development of improved culture conditions and artificial media (17, 32, 44, 48), many of the spirochaetal types often observed microscopically, i.e. the 'medium to large' spirochaetes (9, 21, 47), have still resisted efforts for long-term *in vitro* cultivation. However, over 20 species of not-yet-cultivated oral treponemes have been identified by sequencing cloned spirochaetal 16S rRNA genes that were amplified from plaque DNA from a patient with severe periodontitis (5). In addition, species of not-yet-cultivated trepo-

nemes from digital dermatitis lesions in cattle have been shown to be related to some oral treponemes (4).

Robert Smibert (Virginia Polytechnic Institute, Blacksburg, VA, USA) isolated and collected oral spirochaetes over a 20-year period. The Smibert collection contains approximately 250 spirochaetal isolates including some reference strains. W. E. C. Moore and L. V. Moore (also of the Virginia Polytechnic Institute) classified the Smibert isolates into lettered groups (A through V) based upon their biochemical characteristics and cellular fatty acid profiles (25, 26, 27, 28, 40). In this study, 16S rRNA gene sequence comparisons were used to determine the phylogenetic identity of representatives of the cellular fatty acid groups and of other oral cultivable *Treponema* species not previously analysed, such as *T. vincentii* and subspecies of *T. socranskii*. Another goal was to determine the correlation between phylogenetic analysis and cellular fatty acid analysis of oral treponemes.

A preliminary report of these results was recently published (12).

METHODS

Bacterial strains. Dr W. E. C. Moore and Dr L. V. Moore of Virginia Polytechnic Institute provided strains to us and to ATCC as frozen cell pellets or suspensions. Strains analysed in this study are listed in Table 1. Cells were initially grown in NOS media supplemented with pectin (40). Cultures were incubated in an anaerobic environment at 37 °C for 3–5 d. In this study, cells from the frozen samples were used directly for analysis. Strains are available from ATCC, although specific accession numbers have not yet been assigned.

DNA isolation. Approximately $1-5 \times 10^7$ cells from frozen cell samples were suspended in lysis buffer (50 mM Tris/HCl pH 7.6, 1 mM EDTA, 0.5% Tween 20, 200 µg proteinase K ml⁻¹) and incubated at 55 °C for 2 h. The proteinase K was subsequently inactivated by heating at 95 °C for 10 min. Alternatively, 5 µl of a suspension containing 10^8 cells ml⁻¹ was treated with 15 µl GeneReleaser (Bioventures) and microwaved on high for 5 min. Aliquots of the supernatant were used for the DNA template.

Amplification of 16S rRNA cistrons. Universally conserved primers [primers C70 and B37 (10)] were used to amplify 16S rRNA genes. A spirochaetal selective reverse primer, C90 (5' GTT ACG ACT TCA CCC TCC T 3'), was used with a universal forward primer, C75 (5' GAG AGT TTG ATY CTG GCT CAG 3'), if the universal primers did not produce PCR product. PCR reactions were performed in thin-walled tubes using a Perkin-Elmer 480 thermal cycler. Three microlitres of the crude DNA and 1 µM primers were added to the reaction mixture (as suggested by the manufacturer),

Table 1. Sources and accession numbers of the strains sequenced

Bacterial species	Strain	GenBank accession no.
Reference species		
<i>Spirochaeta caldaria</i>	H1 ^T	M71240
<i>Spirochaeta stenostrepta</i>	ATCC 25083 ^T	M34264
<i>Spirochaeta zuelzeriae</i>	ATCC 19044 ^T	M34265
<i>Treponema amylovorum</i>	HA2P ^T	Y09959
<i>Treponema bryantii</i>	ATCC 33254 ^T	M57737
<i>Treponema denticola</i>	ATCC 33520	M71236
<i>Treponema maltophilum</i>	BR ^T	X87140
<i>Treponema medium</i>	G7201 ^T	D85437
<i>Treponema pectinovorum</i>	ATCC 33768 ^T	M71237
<i>Treponema pallidum</i>	Nichols	M34266
<i>Treponema phagedenis</i>	K5	M57739
<i>Treponema saccharophilum</i>	ATCC 43261 ^T	M71238
<i>Treponema</i> sp.	CA	M59294
<i>Treponema succinifaciens</i>	ATCC 33096 ^T	M57738
Species isolated in this study		
<i>Treponema socranskii</i> subsp. <i>socranskii</i>	ATCC 35536 ^T	AF033306
<i>Treponema socranskii</i> subsp. <i>buccale</i>	ATCC 35534 ^T	AF033305
<i>Treponema socranskii</i> subsp. <i>paredis</i>	ATCC 35535 ^T	AF033307
<i>Treponema socranskii</i> subsp. 04	D11B-2	AF033308
<i>Treponema</i> sp. Smibert-1	D4B-1	AF023039
<i>Treponema</i> sp. Smibert-2	D96NR-3	AF033304
<i>Treponema</i> sp. Smibert-3	D36ER-1	AF023044
<i>Treponema</i> sp. Smibert-4	D62CR-12	AF023045
<i>Treponema</i> sp. Smibert-5	D120CR-1	AF033303
<i>Treponema vincentii</i>	N-9 (ATCC 700013)	AF033310
<i>Treponema vincentii</i>	ATCC 35580	AF033309

which had a final volume of 82 µl. Ampliwax PCR Gem 100s (PE Applied Biosystems) were used in a hot-start protocol. The following conditions were used for amplification using primers C70 and B37: denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s and elongation at 72 °C for 90 s with an additional 5 s added for each cycle. A total of 30 cycles were performed followed by a final elongation step at 72 °C for 15 min. Conditions for amplification using primers C90 and C75 were identical except that the annealing temperature was 60 °C. After removal of Ampliwax, 0.6 vol. 20% PEG 8000 (Sigma) in 2.5 M NaCl was added and the mixture incubated at 37 °C for 10 min to precipitate the DNA. The sample was centrifuged for 15 min at 15000 g and the pellet was washed with 80% ethanol. The pellet was dissolved in 35 µl sterile filtered water. For automated sequencing, amplified DNA was purified by washing on a QiaQuick PCR Purification Kit (Qiagen), following the manufacturer's protocol.

Sequencing methods. The DNA sample from PCR was directly sequenced using a TAQuence cycle-sequencing kit (USB) or a *fmol* DNA Sequencing System kit (Promega). Primers (10) were end-labelled with [γ -³³P]ATP (NEN/Dupont) using the manufacturer's protocol. Purified DNA (25–80 ng) from the PCR was used for each sequencing reaction. Reaction products were run electrophoretically

onto 8% polyacrylamide/urea gels and subsequently detected by exposure of dried gels to X-ray film for 24–48 h. More recently, purified DNA from PCR was sequenced using an ABI prism cycle-sequencing kit (dRhodamine Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase FS, Perkin-Elmer). The manufacturer's protocol was followed. Sequencing was performed using an ABI 377 DNA sequencer.

16S rRNA sequence analysis. Programs for data entry, editing, sequence alignment, secondary structure comparison, similarity matrix generation and phylogenetic tree construction were written in Microsoft QuickBASIC for use on IBM PC-AT and compatible computers (30). Over 5000 sequences from our database, the Ribosomal Database Project (22) and GenBank were available for sequence comparisons. Sequences of all known cultivable and not-yet-cultivated species of *Treponema* have been deposited in GenBank. Similarity matrices were constructed from the aligned sequences by using only those sequence positions for which 90% of strains had data. The similarity matrices were corrected for multiple base changes by the method of Jukes & Cantor (15). Phylogenetic trees were constructed using the neighbour-joining method of Saitou & Nei (37).

Electron microscopy. Cells were suspended in 10 mM Tris

Table 2. Phylogenetic and cellular fatty acid identity of sequenced strains from the Smibert collection

Phylogenetic identity	VPI or reference strain no.*	Cellular fatty acid identity†	Clinical data‡	Sequence§
<i>Treponema</i> Smibert-1 (<i>T. maltophilum</i>)	D4B-1	F or C	Not available	Full
<i>Treponema</i> Smibert-2	D52CR-1	S	Supragingival, severe	Full
	D79TR-1	F	Subgingival, moderate, 4 mm	Partial
	D79WRI1	U	Subgingival, moderate	Partial
	D80BRII-5	<i>T. socranskii</i> subsp. <i>socranskii</i>	Subgingival, health	Full
	D82MRI-1	E	Subgingival, moderate, 5 mm	Partial
	D83PR1	T	Subgingival, moderate, 6 mm	Partial
	D83RR-I1	F	Subgingival, moderate, 3 mm	Partial
	D84AR-1	S	Subgingival, moderate, 8 mm	Partial
<i>Treponema</i> Smibert-3	D36ER-1	F	Supragingival, severe	Full
<i>Treponema</i> Smibert-4 (<i>T. amylovorum</i>)	D62CR-I2	N	Supragingival, JP, 9 mm	Full
<i>Treponema</i> Smibert-5	D120CR-1	<i>T. socranskii</i> subsp. <i>buccale</i>	Subgingival, health, 3 mm	Full
<i>T. denticola</i>	D3A-1	<i>T. denticola</i>		Partial
	D46DR-I1	N		Partial
	D64BPPI-1	<i>T. denticola</i>		Full
	D79ZR-1	F		Partial
	Fm	<i>T. denticola</i>		Full
	Fuji¶	<i>T. denticola</i>		Partial
	Ichelson 2	<i>T. denticola</i>		Full
	IPP¶	<i>T. denticola</i>		Partial
	Jethro-monkey¶	<i>T. denticola</i>		Partial
	MRB	<i>T. denticola</i>		Partial
	N-39	<i>T. denticola</i>		Partial
	<i>T. ambigua</i>	<i>T. denticola</i>		Partial
	<i>T. oralis</i>	<i>T. denticola</i>		Full
	T32A¶	<i>T. denticola</i>		Partial
	TD2	<i>T. denticola</i>		Full
	TRRD	<i>T. denticola</i>		Partial
<i>T. pectinovorum</i>	D48BR-1	<i>T. pectinovorum</i>		Partial
	D73CR-4	<i>T. pectinovorum</i>		Full
<i>T. socranskii</i> subsp. <i>buccale</i>	ATCC 35534 ^T	<i>T. socranskii</i> subsp. <i>buccale</i>		Full
	D64AR-5	F		Partial

(continued on facing page)

buffer (pH 7.4) at a concentration of approximately 10^8 cells ml^{-1} . Samples were negatively stained with 1% (w/v) phosphotungstic acid (pH 6.6) for 20–30 s. Specimens were examined with a JEOL model JEM-1200EX transmission electron microscope operating at 100 kV.

RESULTS AND DISCUSSION

Dr W. E. C. Moore and Dr L. V. Moore divided the spirochaete strains in the Smibert collection into lettered groups (A–V) based upon their biochemical

Table 2. (cont.)

Phylogenetic identity	VPI or reference strain no.*	Cellular fatty acid identity†	Clinical data‡	Sequence§
<i>T. socranskii</i> subsp. <i>paredis</i>	ATCC 35535 ^T	<i>T. socranskii</i> subsp. <i>paredis</i>		Full
	D45APE-1	C		Partial
	D46CPE-1 ^{T¶}	<i>T. socranskii</i> subsp. <i>paredis</i>		Partial
	D45DPEI1	L		Full
	D46CPE-5	L		Partial
	D83BR1	L		Full
	D130AR-1	V		Full
	D151CRII-1	<i>T. socranskii</i> subsp. <i>paredis</i>		Full
	E1H-3	C		Full
	E1F-1	E		Partial
<i>T. socranskii</i> subsp. <i>socranskii</i>	ATCC 35536 ^T	<i>T. socranskii</i> subsp. <i>socranskii</i>		Full
	D46DR-I3	N		Full
	D50AR-IIa	<i>T. socranskii</i> subsp. 04		Partial
	D84BR-1	G		Partial
<i>T. socranskii</i> subsp. 04	D97RR-4	Not available		Partial
	D09B-1	<i>T. socranskii</i> subsp. 04		Partial
	D11B2	<i>T. socranskii</i> subsp. 04		Full
	D36FPE-1	<i>T. socranskii</i> subsp. 04		Partial
	D83DR-5	<i>T. socranskii</i> subsp. 04		Partial
<i>T. socranskii</i> unknown subspecies	D108AR-4	<i>T. socranskii</i> subsp. 04		Partial
	D08B-1	C		Partial
	D75BR-I3	G		Full
	D75BR-III7	F		Full
	D75BR-III8	G		Partial
	D77DR-5	E		Partial
	D79VR-1	F		Partial
	D116CR-1	V		Partial
D135AR-I1 [¶]	DB		Partial	
<i>T. vincentii</i>	E2D-II2	E		Partial
	N-9	<i>T. vincentii</i>		Full

* VPI strains begin with letters D or E followed by a patient number. The letter after the number represents the visit number; for example C is the third visit by that patient. The following suffixes after the patient ID are as follows: PE, isolated from pectin agar medium; R, isolated from pectin agar medium supplemented with rifampicin and polymyxin; PP, isolated from peptone/yeast extract/glucose agar medium; I, colony type; II, a single isolate from that colony type; IIa, a re-isolation of that particular strain.

† Data from R. M. Smibert, Virginia Polytechnic Institute, VA, USA. Cellular fatty acid groups are designated as letters or known species.

‡ Data from R. M. Smibert, Virginia Polytechnic Institute, VA, USA. Clinical data, where known, are reported only for new species described in this study. Subgingival, subgingival plaque; supragingival, supragingival plaque; moderate, moderate adult periodontitis; severe, severe adult periodontitis; JP, juvenile periodontitis; health, healthy sites. Pocket depths, where known, are indicated by numbers.

§ Full indicates that a nearly complete 16S rRNA sequence (approximately 1450 bases) of the strain was determined. Partial indicates that approximately 350–500 bases were determined.

¶ Strain contaminated with a mycoplasma as determined by sequence analysis. Only mycoplasma sequences were obtained from strains D113DR-1 and D156DR-4.

Table 3. Similarity matrix for the spirochaetal strains constructed from 16S rRNA sequences

Numbers above the diagonal represent percentages of similarity and those below the diagonal are percentages of difference corrected for multiple base changes.

Bacterial species	Tde	Tph	Tpa	Tvi	Tme	Szu	Ste	Sca	Sm-1	Tma	Tbr	Tpe	Tsa	Tsu	CA	Sm-2	Sm-3	Sm-4	Tam	Sm-5	Tsb	Tss	Tsp	T04
<i>Treponema denticola</i>	–	93.1	88.7	91.5	91.5	91.1	86.7	87.6	87.5	87.4	86.0	85.9	84.2	81.3	84.0	86.6	84.1	84.8	84.7	83.9	84.0	83.9	83.4	84.4
<i>Treponema phagedenis</i>	7.3	–	90.4	92.0	91.8	91.4	87.6	87.0	86.7	86.9	85.2	86.0	84.0	81.8	83.6	86.3	84.7	85.2	85.0	84.3	84.1	83.7	83.6	84.3
<i>Treponema pallidum</i>	12.2	10.2	–	88.4	88.2	88.0	86.0	86.2	84.5	84.5	83.4	83.6	82.3	81.8	83.6	84.4	83.6	83.2	83.1	82.7	82.3	82.3	81.9	82.9
<i>Treponema vincentii</i>	9.0	8.4	12.6	–	98.7	90.9	87.2	87.8	87.3	87.3	85.4	85.9	83.6	80.7	83.5	86.2	84.2	84.3	84.2	83.5	83.2	83.2	82.7	83.3
<i>Treponema medium</i>	9.0	8.7	12.8	1.3	–	91.0	87.2	88.2	87.5	87.5	85.2	85.4	83.7	80.6	83.5	85.8	83.8	84.0	84.1	83.6	83.2	83.0	82.7	83.2
<i>Spirochaeta zuelzeriae</i>	9.5	9.1	13.1	9.7	9.6	–	88.7	88.9	87.9	87.9	86.5	86.3	84.7	82.4	84.4	86.7	84.7	84.9	84.9	83.6	83.2	83.2	82.9	83.5
<i>Spirochaeta stenostrepta</i>	14.6	13.6	15.5	14.0	14.0	12.3	–	94.2	84.7	84.7	85.4	84.4	82.9	82.1	85.5	84.4	84.6	84.1	84.1	83.8	83.5	83.4	83.3	84.0
<i>Spirochaeta caldaria</i>	13.5	14.2	15.2	13.3	12.8	12.0	6.1	–	86.0	85.8	86.4	85.4	83.8	82.5	85.2	84.6	84.4	84.3	84.4	84.1	84.0	84.1	84.0	84.0
<i>Treponema Smibert-1</i>	13.7	14.7	17.4	13.9	13.7	13.2	17.1	15.5	–	99.8	86.9	87.8	82.8	81.7	84.2	87.6	84.7	85.6	85.5	84.2	84.3	84.1	84.1	84.0
<i>Treponema maltophilum</i>	13.8	14.4	17.3	13.9	13.7	13.3	17.1	15.7	0.2	–	86.9	88.0	83.0	81.4	84.2	87.7	84.8	85.7	85.6	84.2	84.4	84.2	84.2	84.2
<i>Treponema bryantii</i>	15.5	16.4	18.8	16.3	16.5	14.9	16.2	14.9	14.4	14.4	–	88.1	83.8	84.7	86.4	88.3	86.8	87.3	87.3	84.6	84.6	84.4	84.9	84.9
<i>Treponema pectinovorum</i>	15.7	15.5	18.5	15.6	16.2	15.1	17.5	16.3	13.3	13.1	13.0	–	86.3	83.8	86.9	88.9	88.4	88.3	88.5	84.8	84.7	85.0	85.1	84.7
<i>Treponema saccharophilum</i>	17.8	18.0	20.2	18.5	18.4	17.2	19.4	18.3	19.5	19.3	18.3	15.2	–	82.9	88.0	83.5	83.7	83.6	83.6	81.2	81.3	81.2	81.2	81.3
<i>Treponema succinifaciens</i>	21.5	20.8	20.9	22.3	22.4	20.0	20.4	19.9	21.0	21.4	17.1	18.3	19.4	–	87.7	84.6	85.2	85.6	85.5	84.1	84.0	83.8	84.4	84.2
<i>Treponema</i> sp. CA	18.0	18.5	18.5	18.6	18.6	17.5	16.1	16.5	17.7	17.7	15.0	14.4	13.0	–	86.2	85.5	85.9	85.8	85.5	85.5	85.5	85.5	85.6	85.6
<i>Treponema Smibert-2</i>	14.8	15.1	17.4	15.3	15.7	14.6	17.5	17.2	13.5	13.4	12.7	12.0	18.6	17.2	15.2	–	89.3	90.2	90.3	86.3	86.4	86.5	87.0	86.8
<i>Treponema Smibert-3</i>	17.8	17.1	18.5	17.7	18.2	17.1	17.3	17.6	17.2	17.0	14.5	12.6	18.4	16.5	16.1	11.6	–	98.0	98.0	86.6	86.4	86.6	86.7	86.1
<i>Treponema Smibert-4</i>	17.0	16.5	19.1	17.7	18.0	16.8	17.9	17.7	16.0	15.9	13.9	12.8	18.5	15.9	15.7	10.4	2.0	–	99.9	86.2	86.1	86.1	86.1	85.8
<i>Treponema amylovorum</i>	17.1	16.7	19.2	17.8	17.8	16.9	17.9	17.7	16.1	16.0	13.9	12.5	18.5	16.1	15.7	10.3	2.0	0.1	–	86.2	86.0	86.0	86.1	85.7
<i>Treponema Smibert-5</i>	18.1	17.6	19.7	18.6	18.5	18.5	18.2	17.4	17.8	17.7	17.2	17.0	21.6	17.8	16.1	15.1	14.7	15.2	15.3	–	97.8	98.0	97.4	98.2
<i>Treponema socranskii</i> subsp. <i>buccale</i>	18.0	17.9	20.2	19.0	19.0	19.0	18.6	17.8	17.7	17.5	17.2	17.2	21.5	18.0	16.1	15.0	15.0	15.4	15.5	2.2	–	98.6	98.3	99.3
<i>Treponema socranskii</i> subsp. <i>socranskii</i>	18.1	18.4	20.2	19.0	19.3	19.0	18.7	18.0	17.9	17.7	17.4	16.8	21.7	18.2	16.1	14.9	14.8	15.4	15.5	2.0	1.4	–	98.4	98.9
<i>Treponema socranskii</i> subsp. <i>paredis</i>	18.7	18.6	20.7	19.6	19.7	19.4	18.9	17.8	17.9	17.7	16.9	16.6	21.6	17.5	16.0	14.3	14.6	15.3	15.4	2.7	1.7	1.6	–	98.2
<i>Treponema socranskii</i> subsp. 04	17.5	17.6	19.4	18.8	19.0	18.6	18.0	18.0	18.0	17.8	16.8	17.1	21.5	17.8	15.9	14.5	15.3	15.8	15.9	1.9	0.7	1.1	1.9	–

characteristics and cellular fatty acid profiles (25, 26, 27, 28, 40). In this study, the 16S rRNA genes were sequenced for 47 strains that were representative of these groups. If strains were identified definitively from analysis of partial sequences (i.e. approximately 350–500 bases), then full sequences of these strains were not determined. Nearly complete sequences of the 16S rRNA genes were determined for 26 strains (Table 2). At least one full sequence for each group has been submitted to GenBank (Table 1). The sequences of all other strains are available from the corresponding author. Many strains from the collection could not be identified because a PCR product of the DNA was not obtained using the primers described above. Preliminary studies using a newly designed, spirochaetal-selective forward primer (primer F29; 5' CGG YGC GTC TTA AVC ATG CAA 3', *E. coli* position 40–60) indicated that the sequences of one or more of the original primers used for amplification were not complementary to target sequences of some of the isolates. DNA from these samples is presently being amplified for sequence analysis.

Table 3 shows a similarity matrix that was constructed on the basis of 1445 base position comparisons for 16S rRNA sequences of the spirochaetal strains under examination. A phylogenetic tree (Fig. 1) was constructed from the corrected distance matrix using the neighbour-joining method. Based on these analyses, five distinct species were identified from the Smibert collection with the cut-off for separate species designation at about 98% sequence similarity. These new

species are designated as *Treponema* Smibert-1 through *Treponema* Smibert-5 (Fig. 1). As described in detail below, two of these species are newly named and three have not yet been characterized. Other strains were identified as previously described species, such as *T. socranskii*, *T. pectinovorum*, *T. denticola* and *T. vincentii*.

Strain D4B-1, the sole isolate of *Treponema* Smibert-1, was nearly identical in 500 base comparisons to spirochaetal clone NZM3125 (phylogroup 17), previously described from clonal analysis of plaque DNA from a patient with severe periodontitis (5). The closest relative of D4B-1 was *T. pectinovorum* at about 88% sequence similarity. While this work was in progress, this strain was shown to be nearly identical in sequence to the recently described species *T. maltophilum* (48). However, cells of D4B-1 had two (and occasionally three) periplasmic flagella inserted at each end of the cell (Fig. 2), whereas strains of *T. maltophilum* were reported to have only one flagellum inserted at each end (48). Strain D4B-1 was defined as belonging to cellular fatty acid group F or C. No clinical data were available for this strain.

Treponema Smibert-2 contained nine strains that possessed identical partial sequences. The closest relatives of *Treponema* Smibert-2 were *Treponema* Smibert-3 and *Treponema* Smibert-4 at about 90% sequence similarity. Cells measured about 0.15–0.2 µm by 3–5 µm and had a single periplasmic flagellum inserted at each end of the cell. Smibert-2 did not

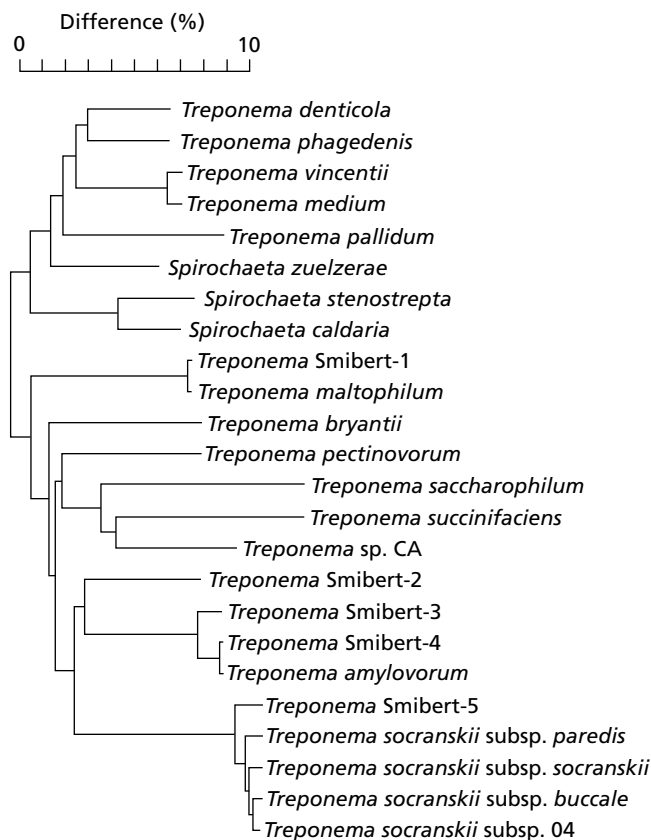


Fig. 1. Phylogenetic tree of the spirochaetes illustrating the phylogenetic positions of oral cultivable species of *Treponema*. The tree was constructed from comparisons of sequences of the specific strains listed in Table 1. *T. vincentii* strain N9 (not ATCC 35580) was used for the analysis. The scale bar represents a 10% difference in nucleotide sequence as determined by taking the sum of all of the horizontal lines connecting two species.

match with any of the phylotypes previously described from clonal analysis of plaque DNA (5). These isolates were previously described as belonging to six separate cellular fatty acid groups – *T. socranskii* subsp. *socranskii*, E, F, S, T and U. Strains of this new species were isolated from supragingival and subgingival plaque from healthy and diseased sites (Table 2).

Treponema Smibert-3 was represented by a single strain, D36ER-1, and was identical in 500 base comparisons to spirochaetal clone NZM3109 (cluster 20) previously described from clonal analysis of plaque DNA (5). *Treponema* Smibert-3 was identified previously as belonging to cellular fatty acid group F. As described below, other strains belonging to cellular fatty acid group F were identified as *Treponema* Smibert-2 (Table 2). Cells of *Treponema* Smibert-3 measured about 0.2 µm by 3–5 µm and had four to five periplasmic flagella inserted at each end of the cell. *Treponema* Smibert-3 was isolated from supragingival plaque from a site characterized as juvenile periodontitis. As shown in Table 3 and Fig. 1, the closest cultivable relative of *Treponema* Smibert-3 was

Treponema Smibert-4 at about 98% sequence similarity.

Treponema Smibert-4 was represented by a single strain, D62CR-I2, and differed by two bases in 500 base sequence comparisons to spirochaetal clone NZM3124 (cluster 19) previously described from clonal analysis of plaque DNA (5). While this work was in progress, this strain was shown to be identical in sequence to the recently described species *T. amylovorum* (49). Consequently, strain D62CR-I2 represents the second isolate of *T. amylovorum*. As with the type strain of *T. amylovorum*, strain D62CR-I2 was also isolated from plaque from a site characterized as severe periodontitis. Smibert-4 was identified previously as belonging to cellular fatty acid group N (Table 2). Other strains of cellular fatty acid group N were not related to *Treponema* Smibert-2 (Table 2).

Treponema Smibert-5 was represented by a single strain, D120CR-1, which was identified from cellular fatty acid analysis as *T. socranskii* subsp. *buccale*. However, *Treponema* Smibert-5 probably represents a new species that is closely related to the three subspecies of *T. socranskii* each at about 98% sequence similarity (Fig. 1). DNA–DNA reassociation experiments will be necessary to determine if separate species (or subspecies) designation of *Treponema* Smibert-5 is warranted. Cells of Smibert-5 measured about 0.2 µm by 3–5 µm and had one periplasmic flagellum inserted at each end of the cell. This ultrastructure is similar to that of cells of subspecies of *T. socranskii*. *Treponema* Smibert-5 was isolated from subgingival plaque from a healthy site.

Strains belonging to cellular fatty acid groups C, DB, E, some F, G, L, some N and V were found to be nearly identical to subspecies of *T. socranskii* and did not represent new species (Table 2). The type strains of subspecies of *T. socranskii* – subsp. *socranskii*, *buccale* and *paredis* – formed three separate phylogenetic branches with intragenic sequence similarities of over 98% (Table 2, Fig. 1). Preliminary studies have shown that at least five members of a fourth subspecies of *T. socranskii* (referred to as subsp. 04) cluster together to form a separate fourth branch (Table 2). However, to determine if each subspecies is a phylogenetically coherent group, comparisons of complete 16S rRNA gene sequences for multiple strains of each subspecies will be necessary.

The two strains of *T. vincentii* that were sequenced had nearly identical sequences, differing by four bases. The closest relative of *T. vincentii* was *T. medium* at 98.7% sequence similarity. However, the levels of DNA–DNA homology between these species was reported to be 28% (46), thus warranting separate species designation. Both *T. vincentii* and *T. medium* had about 92% sequence similarity to *T. denticola* and *Treponema phagedenis* (Table 3, Fig. 1).

We previously reported that 16S rRNA sequences of some spirochaetes (including the treponemes) possess

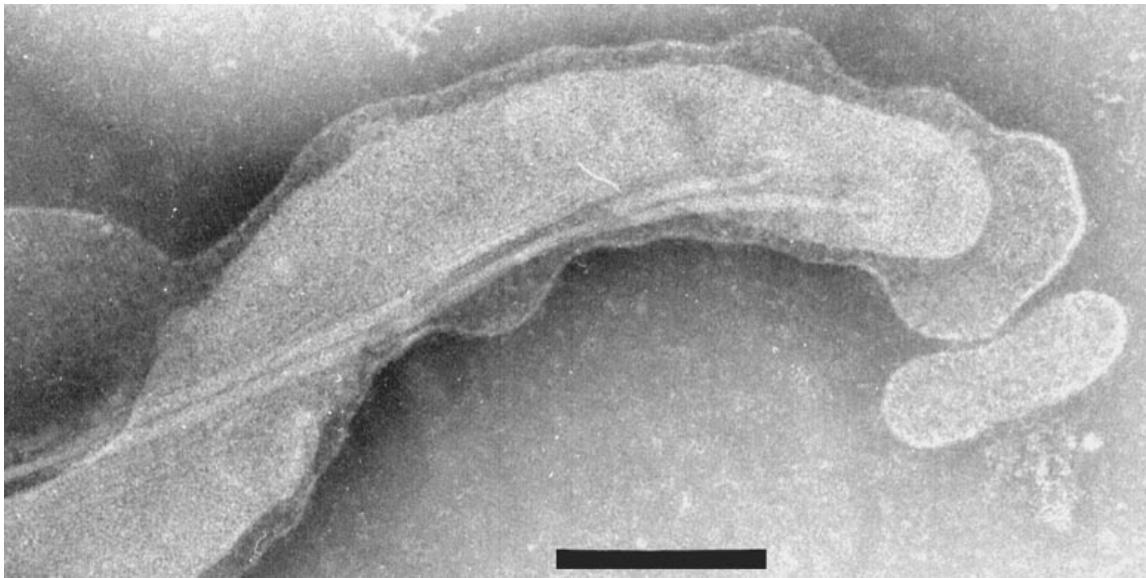


Fig. 2. Transmission electron micrograph of a negatively stained cell of strain D4B-1 (*Treponema* sp. Smibert-1), which is phylogenetically identical to *T. maltophilum*. Two periplasmic flagella are inserted at the end of the cell. Cells of *T. maltophilum* were reported to have only one flagellum at each end (48). Bar, 0.2 μ m.

a highly variable 5' extension of 20–40 bases (31). These extensions, which are unique among prokaryotes and eukaryotes, were determined by directly sequencing 16S rRNA using reverse transcriptase (31). The rRNA sequences of *T. vincentii* ATCC 35580 and *T. socranskii* subsp. *socranskii* ATCC 35536 were determined using this methodology and they did possess unique 5' extensions. It is not known if the other strains examined possess a 5' extension. The present PCR methodology utilizes a forward primer that begins at position 8, and thus the 5' extension would not be detected.

Historic reference strains *Treponema oralis* (no strain designation), TRRD, MRB, Fuji, IPP, Jethro and T32A were identified as strains of *T. denticola*. There were four or fewer base differences in the sequences of any historic strain as compared to the sequence of *T. denticola* ATCC 33520. Strains of *T. oralis* were previously reported to have a flagellar arrangement of 1-2-1 (42); however the strain available from the Smibert collection had a 2-4-2 arrangement.

Four of the historic strains (Fuji, IPP, Jethro and T32A) and three of the Smibert isolates [D156DR-4, D113DR-1 and D46CPE-1 (type species of *T. socranskii* subsp. *paredis*)] were contaminated with *Mycoplasma orale* as deduced from sequence analysis. The presence of mycoplasmas is not surprising since treponemes were cultured on medium supplemented with filter-sterilized bovine serum. Mycoplasmas are able to pass through a 0.22 μ m filter. Mixed cultures of organisms are immediately evident by 16S rRNA sequencing as double reads on the sequencing gels. If the sequence for one of the species is known in a binary

mixture, then the sequence of the second can be determined and the organism can be placed phylogenetically. It is unlikely that our laboratory is the source of the contamination since DNA was extracted directly from the frozen samples.

Cellular fatty acid analysis can be used to reliably differentiate many bacterial species and is considered a useful taxonomic tool (24). However, the grouping of human oral treponemes by cellular fatty acid analysis did not always correlate with 16S rRNA gene sequence comparative analysis (Table 2). As described above, six cellular fatty acid groups are represented in the phylogenetically coherent *Treponema* Smibert-2. Cellular fatty acid groups C, E, G, L, V, F, DB and one strain of N were closely related to subspecies of *T. socranskii* (Table 2). One strain (D62CR-I2) of group N formed the new species *Treponema* Smibert-4, whereas another strain (D46DR-I1) of cellular fatty acid group N was identified as *T. denticola*. Strain D36ER-1 and strain D79VR-1 of cellular fatty acid group F were identified as *Treponema* Smibert-3 and *T. socranskii* subsp. *socranskii*, respectively, whereas most strains of cellular fatty acid group F belonged to the new species *Treponema* Smibert-2. One possibility for these discrepancies is that cellular fatty acid compositions of micro-organisms vary with growth media, age of the culture and the temperature used for incubation (24). Cells were initially grown in media containing serum, which contains fatty acids that cannot be washed easily from cells. Efforts were made to remove these fatty acids by transferring cells into medium without fatty acids for 24 h and then transferring cells to standardized media for 2 h. Since

Treponema pallidum, *Borrelia burgdorferi* and some oral treponemes are capable of only limited synthesis of cellular fatty acids, they incorporate fatty acids into their lipids relatively nonselectively from their environment (2, 46). If this requirement for fatty acids was true for most oral treponemes, then cellular fatty acid analysis would reflect the fatty acids present in the culture medium rather than from synthesis of fatty acids by the organism.

Whilst many species of oral treponemes have not yet been cultivated, Dr Smibert had isolated at least five novel species. 16S rRNA sequence analysis has allowed the unambiguous identification and phylogenetic placement of each of these species. Three of these new species (*Treponema* Smibert-1, *Treponema* Smibert-3 and *Treponema* Smibert-4) corresponded to species postulated to exist based upon 16S rRNA analysis of clones obtained from PCR amplification of DNA from plaque of a patient with severe periodontal disease (5). Consequently, there are now at least ten cultivable oral species of *Treponema*.

To formally name the three novel species reported in this study, a detailed description of phenotypic and other characteristics of strains is necessary. Furthermore, two of the species (Smibert-3 and Smibert 5) are each represented by only a single isolate. Formal naming of these latter two species should be avoided until additional isolates are obtained and characterized. Furthermore, a cut-off value of about 98% sequence similarity was used to differentiate species of oral treponemes. At this cut-off level, it is suggested that DNA-DNA reassociation experiments still be used to confirm separate species designation.

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