Treponema putidum sp. nov., a medium-sized proteolytic spirochaete isolated from lesions of human periodontitis and acute necrotizing ulcerative gingivitis

Wyss, C; Moter, A; Choi, B-K; Dewhirst, F E; Xue, Yi; Schüpbach, P; Göbel, U B; Paster, B J; Guggenheim, B

Treponema putidum sp. nov., a medium-sized proteolytic spirochaete isolated from lesions of human periodontitis and acute necrotizing ulcerative gingivitis

Abstract

So far, little phenotypic heterogeneity has been detected in cultured oral treponemes with trypsin-like proteolytic activity, and all have been assigned to the species Treponema denticola. However, comparisons of protein patterns and antigen expression in our collection of proteolytic oral treponemes occasionally identified isolates with a unique phenotype; e.g. strain OMZ 830 (=ATCC 700768), which qualified as a 'pathogen-related oral spirochaete' due to the presence of a approximately 37 kDa protein reactive with the Treponema pallidum FlaA-specific mAb H9-2. In addition to such single isolates, a homogeneous group of seven independent strains is described that were highly motile, medium-sized, proteolytic but asaccharolytic spirochaetes and were cultured from human gingivitis, periodontitis and acute necrotizing ulcerative gingivitis in medium OMIZ-Pat supplemented with 1% human serum and antibiotics. Growth of these spirochaetes in OMIZ-Pat was not dependent on, but was stimulated by, human or bovine serum. Carbohydrates were neither required nor stimulatory for growth. The protein and antigen patterns of total cell extracts of these organisms separated by SDS-PAGE were distinct from those of all previously cultured spirochaetes, with highest similarity to T. denticola. The novel spirochaete has a 2 : 4 : 2 arrangement of the periplasmic flagella, similar to T. denticola. However, the flagellin pattern as detected by immunostaining or glycan staining of Western blots readily distinguished the novel group from T. denticola. Also, distinct from reference strains of T. denticola, none of the novel isolates displayed sialidase or dentilisin activities, both of which are expressed by most strains of T. denticola. Trypsin-like activity and other enzymes as detected by API ZYM test were similar to those of T. denticola. The status of a novel species is supported by the 16S rRNA gene sequence, with 98.5% similarity to its closest cultured relative, T. denticola. The name Treponema putidum sp. nov. is proposed (type strain OMZ 758T=ATCC 700334T=CIP 108088T).

Treponema putidum sp. nov., a medium-sized proteolytic spirochaete isolated from lesions of human periodontitis and acute necrotizing ulcerative gingivitis

C. Wyss,[1] A. Moter,[2] B.-K. Choi,[3] F. E. Dewhirst,[4] Yi Xue,[1]† P. Schüpbach,[1]‡ U. B. Göbel,[2] B. J. Paster[4] and B. Guggenheim[1]

Correspondence
C. Wyss
wyss.c@zzmk.unizh.ch

[1]Institut für Orale Mikrobiologie und Allgemeine Immunologie, Zentrum für Zahn-, Mund- und Kieferheilkunde der Universität Zürich, Plattenstrasse 11, CH-8028 Zürich, Switzerland

[2]Charité-Universitätsmedizin Berlin, Institut für Mikrobiologie und Hygiene, Dorotheenstrasse 96, D-10117 Berlin, Germany

[3]Department of Oromaxillofacial Infection and Immunity, College of Dentistry, Seoul National University, 110-749 Seoul, Korea

[4]Department of Molecular Genetics, The Forsyth Institute, 140 Fenway, Boston, MA 02115, USA

So far, little phenotypic heterogeneity has been detected in cultured oral treponemes with trypsin-like proteolytic activity, and all have been assigned to the species *Treponema denticola*. However, comparisons of protein patterns and antigen expression in our collection of proteolytic oral treponemes occasionally identified isolates with a unique phenotype; e.g. strain OMZ 830 (=ATCC 700768), which qualified as a 'pathogen-related oral spirochaete' due to the presence of a ~37 kDa protein reactive with the *Treponema pallidum* FlaA-specific mAb H9-2. In addition to such single isolates, a homogeneous group of seven independent strains is described that were highly motile, medium-sized, proteolytic but asaccharolytic spirochaetes and were cultured from human gingivitis, periodontitis and acute necrotizing ulcerative gingivitis in medium OMIZ-Pat supplemented with 1 % human serum and antibiotics. Growth of these spirochaetes in OMIZ-Pat was not dependent on, but was stimulated by, human or bovine serum. Carbohydrates were neither required nor stimulatory for growth. The protein and antigen patterns of total cell extracts of these organisms separated by SDS-PAGE were distinct from those of all previously cultured spirochaetes, with highest similarity to *T. denticola*. The novel spirochaete has a 2 : 4 : 2 arrangement of the periplasmic flagella, similar to *T. denticola*. However, the flagellin pattern as detected by immunostaining or glycan staining of Western blots readily distinguished the novel group from *T. denticola*. Also, distinct from reference strains of *T. denticola*, none of the novel isolates displayed sialidase or dentilisin activities, both of which are expressed by most strains of *T. denticola*. Trypsin-like activity and other enzymes as detected by API ZYM test were similar to those of *T. denticola*. The status of a novel species is supported by the 16S rRNA gene sequence, with 98·5 % similarity to its closest cultured relative, *T. denticola*. The name *Treponema putidum* sp. nov. is proposed (type strain OMZ 758^T=ATCC 700334^T=CIP 108088^T).

Published online ahead of print on 23 January 2004 as DOI 10.1099/ijs.0.02806-0.

†Present address: Bethune International Peace Hospital, Shijiazhuang, People's Republic of China.

‡Present address: Microphot, Horgen, Switzerland.

Abbreviations: ANUG, acute necrotizing ulcerative gingivitis; PROS, pathogen-related oral spirochaete.

Details of the clinical sources of the novel isolates and electron micrographs of cells of the novel species are available as supplementary material in IJSEM Online.

Spirochaetes are often the dominant morphotype in the microbiota of deep periodontal pockets and lesions of acute necrotizing ulcerative gingivitis (ANUG) and are therefore suspected to be aetiological agents; however, many deep periodontal pockets seem to be devoid of spirochaetes (Riviere *et al.*, 1995; C. Wyss, unpublished). Intensive studies relying on the isolation of bacteria in culture or, more recently, on their culture-independent detection by 16S rRNA gene sequencing have demonstrated the presence of hundreds of bacterial species in such oral

lesions (Paster *et al.*, 2001), an obvious impediment to the identification of aetiological agents. A large proportion of oral bacteria, notably most spirochaetes, are fastidious and refractory to *in vitro* cultivation and consequent investigation of their physiology and virulence potential (Choi *et al.*, 1994; Moter *et al.*, 1998a; Paster *et al.*, 1998; Willis *et al.*, 1999). A small number of the cultured oral organisms have acquired the status of suspected pathogens based both on epidemiological data and on the presence of potential virulence factors (Haffajee & Socransky, 1994; Page, 1995; Wyss *et al.*, 1999). In this list are the two spirochaetes *Treponema denticola*, with trypsin and dentilisin proteolytic activities, and *Treponema lecithinolyticum*, with dentilisin, neuraminidase and phospholipase A and C activities. Treponemes with a specific antigenic relationship to *Treponema pallidum* may also be associated with disease and have received the aetiologically suggestive name 'pathogen-related oral spirochaetes' (PROS; Riviere *et al.*, 1991).

Recent improvements in culture media and the application of limit-dilution techniques have allowed the routine isolation of oral spirochaetes (Wyss *et al.*, 1996, 1997, 1999, 2001). Several hundred treponemal isolates were initially assigned to a small number of apparently homogeneous groups containing no more than one of the presently accepted species, based on only a limited set of phenotypic characteristics. However, by using a more comprehensive analysis, we demonstrated significant heterogeneity within such groups. For example, based on further phenotypic and genetic analysis of small glucuronic or galacturonic acid-dependent treponemes, the *Treponema pectinovorum* group was clearly differentiated from the *Treponema parvum* group (Wyss *et al.*, 2001). Here, we report a comparable case concerning the group of medium-sized, asaccharolytic oral treponemes with trypsin-like activity.

Clinical isolates of spirochaetes from plaque of Swiss patients with periodontitis and from Chinese ANUG patients and gingivitis controls were cloned, cultured and phenotypically characterized in Zürich as described; phenotypic characterization comprised the determination of enzyme activities by API ZYM, nutritional studies, SDS-PAGE and immunolabelling or glycan staining of Western blots, electron microscopy, DNA isolation and 16S rRNA gene sequencing, performed as described previously (Wyss, 1992, 1998; Wyss *et al.*, 1996, 1997, 1999, 2001). To determine sialidase activity, 100 µl of the cell suspension used for the API ZYM tests was added to 100 µl of a 0·1 mM solution (in 200 mM Tris/HCl, pH 7·2, 158 mM NaCl, 10 mM $CaCl_2$, 0·1 mM $ZnSO_4$) of the fluorogenic substrate MU-NANA (methylumbelliferyl *N*-acetylneuraminic acid); after 3 h incubation at 37 °C, fluorescent product was determined at $\lambda_{excit.}$ 360 nm and $\lambda_{emiss.}$ 460 nm. Dentilisin activity was determined after aerobic incubation (6 h, 37 °C) of 100 µl of the cell suspension used for the API ZYM test mixed with 100 µl of a solution (1 mM in 100 mM NaCl, 50 mM Tris/HCl, 1 mM $CaCl_2$, pH 8·0) of the fluorogenic substrate SAAPFAMC (Suc-Ala-Ala-Pro-Phe-AMC) ($\lambda_{excit.}$ 360 nm and $\lambda_{emiss.}$ 460 nm). Additionally, dentilisin activity was assessed visually with 2 mM of the colour-yielding substrate SAAPFpNA (Suc-Ala-Ala-Pro-Phe-pNA) in place of SAAPFAMC.

Isolates with trypsin-like activity and with morphology comparable to that of the type strain of *T. denticola*, ATCC 35405^T, were routinely designated *T. denticola* according to the only named species contained in this group (cluster 11 in group 2 of oral treponemes according to the nomenclature used by Dewhirst *et al.*, 2000). However, upon closer examination, this group proved to be heterogeneous.

Table 1 lists the phenotypic characters of *T. denticola* reference strains and the related clinical isolates used in this study. Data on the patients harbouring these isolates as well as information on the spectrum of treponemes

Table 1. Enzyme activities displayed by proteolytic oral *Treponema* strains within group 2 of the oral treponemes, as defined by Dewhirst *et al.* (2000)

Enzyme activities: 1, sialidase; 2, dentilisin; 3, alkaline phosphatase; 4, esterase C8; 5, leucine arylamidase; 6, cystine arylamidase; 7, trypsin; 8, chymotrypsin; 9, acid phosphatase; 10, naphthol-phosphohydrolase; 11, α-galactosidase; 12, β-galactosidase; 13, α-glucosidase; 14, β-glucosidase. Sialidase activity was determined with the fluorogenic substrate MU-NANA and dentilisin activity was determined with the fluorogenic substrate SAAPFAMC; all other enzyme activities were assayed in the API ZYM test. The following API ZYM tests not listed gave negative results for all strains: lipase C14, valine arylamidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Esterase C4 (API ZYM) gave a positive result for all strains.

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14
ATCC 35405^T	+	+	−	−	−	−	+	+	−	−	−	−	−	−
ATCC 33521	+	−	+	+	+	−	+	+	+	+	+	+	−	−
ATCC 35404	+	+	+	+	+	−	+	+	+	+	+	−	−	−
ATCC 33520	+	+	+	+	+	−	+	+	+	+	+	−	−	−
ST10	+	−	+	+	+	+	+	+	+	+	+	+	−	+
OMZ 830	−	+	+	+	+	−	+	−	+	+	−	+	−	+
OMZ 834	−	−	+	+	+	+	+	+	+	+	−	+	−	+
OMZ 845	+	+	−	+	−	−	+	−	+	+	+	−	−	−
OMZ 849	+	+	+	+	+	−	+	−	+	+	−	−	−	−
OMZ 850	+	+	+	+	+	−	+	−	+	+	−	−	−	−
OMZ 852	+	+	+	+	+	+	+	+	+	+	+	+	−	+
OMZ 905	+	+	+	+	+	−	−	−	+	+	−	−	−	−
OMZ 730	−	−	−	+	+	−	+	−	+	+	+	+	−	+
OMZ 758^T	−	−	+	+	+	−	+	+	+	+	+	+	+	+
OMZ 835	−	−	+	+	+	−	+	+	+	+	+	+	−	+
OMZ 844	−	−	+	+	+	−	+	+	+	+	+	+	+	+
OMZ 846	−	−	+	+	+	−	+	+	+	+	+	+	−	+
OMZ 847	−	−	−	+	+	−	+	−	+	+	−	+	−	+
OMZ 848	−	−	−	+	+	−	+	−	+	+	−	+	−	+

cultured from each clinical sample are available in a Supplementary Table in IJSEM Online.

Some unique isolates were detected that had a different phenotype, such as OMZ 830 and OMZ 834 (Table 1; Fig. 1b), which displayed ~37 and ~75 kDa bands, respectively, reactive with mAb H9-2, which specifically labels the 37 kDa flagellar sheath protein FlaA in *T. pallidum* (Lukehart *et al.*, 1985; Wyss, 1998) and defines the PROS phenotype (Fig. 1b). In addition to this H9-2-antigenic difference, the protein pattern in strain OMZ 834 was different from that of *T. denticola* reference strains (Fig. 1a) and, in strain OMZ 830, the (glycosylated) flagellins had a higher apparent molecular mass than those of *T. denticola* reference strains (Fig. 1c). Isolates OMZ 830 and OMZ 834 have been deposited in the ATCC as ATCC 700768 and ATCC 700771.

A homogeneous group of seven strains was distinguished from *T. denticola* on SDS-PAGE blots stained for proteins (Fig. 1a) or for flagellins (Fig. 1c). The same grouping as obtained by immunostaining was seen when the blotted flagellins were revealed by a glycan stain (not shown).

Although the results presented in Fig. 1 show that, at the molecular level, there are differences between the flagellins (FlaB proteins) of the two subgroups, no distinguishing morphological features were recognized by electron microscopy. Cells of all strains listed in Table 1 [including the type strain of *T. denticola*, ATCC 35405^T, which has mistakenly been reported to have five flagella per pole (Chan *et al.*, 1993)] were of intermediate size and of a 2:4:2-type flagellation, as shown in a Supplementary Figure in IJSEM Online.

In terms of *in vitro* growth behaviour and nutrient requirements, no marked differences were observed between the strains listed in Table 1. For example, addition of carbohydrates did not influence growth and serum, while not required, was stimulatory. Human and fetal calf serum were equally effective, in contrast to *Treponema amylovorum*, *T. lecithinolyticum*, *Treponema maltophilum* and *Treponema socranskii*, which are completely inhibited by the addition of 1 % fetal calf serum to medium OMIZ-Pat (Wyss *et al.*, 1996, 1997, 1999).

Enzyme activities detected by API ZYM generally revealed

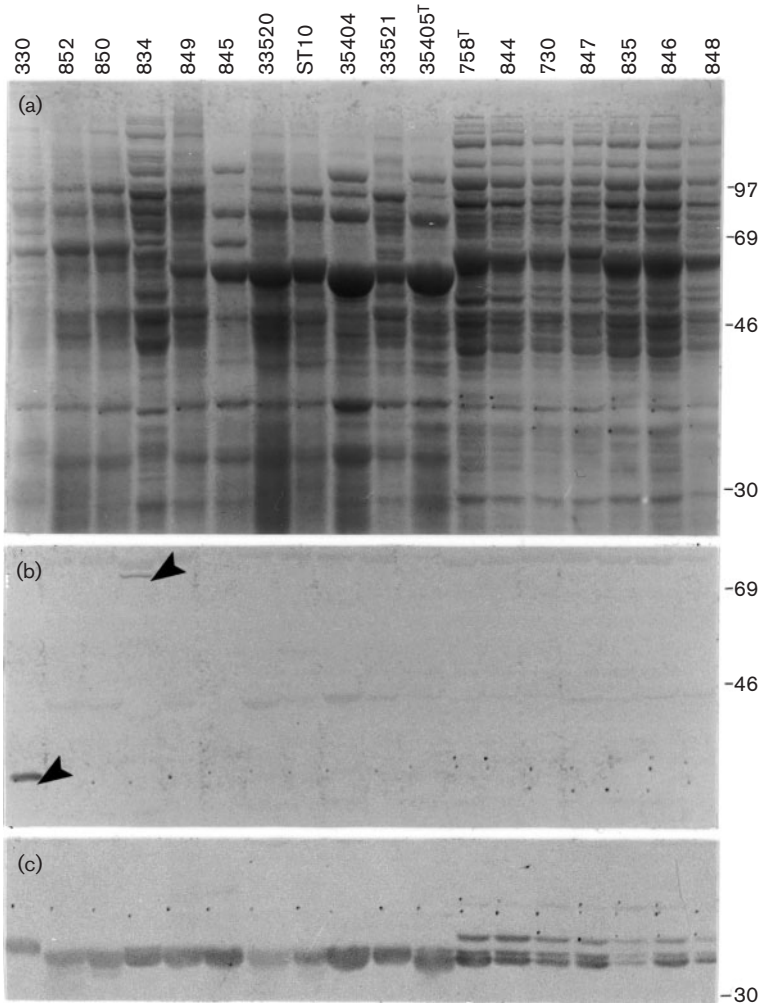

Fig. 1. SDS-PAGE of cellular extracts of reference strains of *T. denticola* (lanes 33520–35405^T) and of novel clinical isolates with proteolytic activity blotted on to nitrocellulose. (a) Gel stained for protein by the reversible copper phthalocyanine tetrasulphonic acid (CPTS) method (Bickar & Reid, 1992). (b) Same blot as (a), after removal of CPTS and immunolabelling with mAb H9-2 to detect PROS antigen (arrowheads at ~37 kDa in lane 830 and ~75 kDa in lane 834). (c) Parallel blot immunolabelled with an anti-flagellin antibody. Labels on the right indicate positions of molecular mass markers (kDa). The pencil spots visible in the 37 kDa region were added after CPTS staining to facilitate orientation on the immunolabelled blot after removal of CPTS.

only minor differences between the strains, with the notable exception of strain OMZ 905, which lacked trypsin-like activity (Table 1). However, with an expanded panel of artificial enzyme substrates, consistent differences in enzyme activity emerged; namely, the seven strains OMZ 730, 758^T, 835, 844, 846, 847 and 848 lacked sialidase activity (MU-NANA), which distinguished them from all other isolates with trypsin-like activity and from strain OMZ 905, except OMZ 830 and 834 (i.e. the two mAb H9-2-reactive strains mentioned above). The same seven strains also lacked dentilisin activity, which was found in all other strains except the three *T. denticola* strains ST10, ATCC 33521 and OMZ 834 (Table 1).

While such artificial enzyme substrates are useful for the characterization and differentiation of bacterial isolates, one should be cautious when extrapolating from such data to possible bacterial activity towards natural substrates *in vivo*. The expression of trypsin-like proteolytic activity is plausibly considered a potential virulence factor and has been reported as such in *Capnocytophaga gingivalis*, *Capnocytophaga sputigena*, *Tannerella forsythensis* (*Bacteroides forsythus*), *Porphyromonas gingivalis* and *T. denticola*. Whether or not the trypsin-like activities detected in these species and in the novel isolates show the same specificity towards natural substrates remains to be determined. Intuitively, the novel isolates would have to be considered of lower aggressive potential than *T. denticola*, which can combine the trypsin-like activity with that of dentilisin. However, definitive conclusions concerning their role in aetiology are not possible, as epidemiological evidence at present does not support the notion of a single specific pathogen responsible for either periodontal diseases or ANUG. A case in point has been previously reported with *Tannerella forsythensis*, which is highly proteolytic: it is unable to display its potential unless other bacteria in the oral cavity provide it with its essential nutrient *N*-acetylmuramic acid (Wyss, 1989).

The 16S rRNA gene sequences of the seven novel treponeme strains were essentially identical, differing in only 0–3 nucleotide positions. These strains all fell within group 2, one of the 10 groups of oral treponemes previously described (Dewhirst *et al.*, 2000), in the dendrogram in Fig. 2; the strains are referred to as *Treponema putidum* sp. nov., with strain OMZ 758^T as the type strain. The closest named relative of *T. putidum* is *T. denticola*, at a level of 98·5 % similarity. *T. denticola* represents cluster 11 within this group. Interestingly, *T. putidum* represents a novel cluster (labelled J in Fig. 2) not represented in the 16S rRNA gene sequence libraries assembled by culture-independent methods. Also indicated in Fig. 2 are the phylogenetic positions within group 2 of single strains with unique phenotypes. Thus, strain OMZ 905, isolated from a patient with periodontitis, represents the first cultivable member of cluster 2 : 10. Whether its exceptional combination of the absence of trypsin and chymotrypsin activities with a display of dentilisin activity (Table 1) is representative of this cluster cannot yet be decided. At an antigenic level, OMZ 830 represents the first cultured PROS that is more closely related to *T. denticola* than to any of the previously identified PROS, namely the two group 1 treponemes '*Treponema vincentii*' and *Treponema medium* (Wyss, 1998).

A possible role in pathogenesis for the novel proteolytic treponeme isolated from a few cases of ANUG and severe periodontitis cannot be deduced from the limited information available. The isolation of this novel phylotype permits its physiological characterization, and the availability of its 16S rRNA gene sequence provides an experimental basis for directed clinical studies applying techniques such as FISH (Moter *et al.*, 1998b) and DNA chequerboard hybridization (Becker *et al.*, 2002). Only such comprehensive epidemiological studies will be able to determine whether the novel proteolytic treponeme described here may form part of a pathogenic complex.

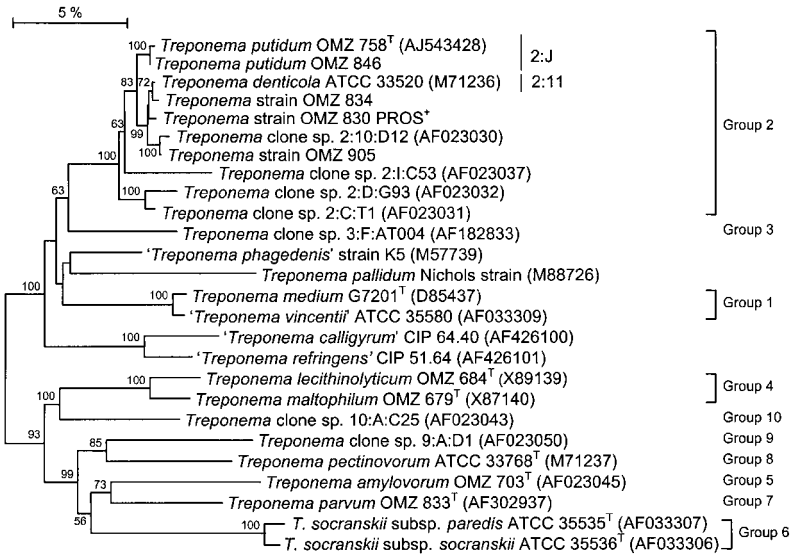

Fig. 2. Phylogenetic position of *T. putidum* sp. nov. based on 16S rRNA gene sequences. Strains of *T. putidum* form a novel, tight cluster (cluster 2 : J) within group 2 of the oral treponemes, as defined by Dewhirst *et al.* (2000). The other nine groups of oral treponemes are shown, together with representatives of the genus *Treponema*. Accession numbers are given. Bar, 5 % difference in nucleotide sequences. Two hundred bootstrap trees were generated, and bootstrap confidence levels are shown as percentages at nodes (only values over 40 % shown).

While cultivation techniques are unsuitable for obtaining quantitative results on treponeme prevalence, isolation and phenotypic characterization of treponemes significantly enhance the description of treponemal populations in the oral cavity. Complementing results from culture-independent studies, we were able to isolate and characterize phenotypically a wide phylotypic range of treponemes from most clinical samples (Supplementary Table; Wyss *et al.*, 2001). As regards treponemes with trypsin-like activity, we repeatedly isolated from a single sample, strains with distinct SDS-PAGE protein profiles (e.g. compare the *T. denticola* pairs 849/850 and 830/852, and the *T. denticola*/*T. putidum* pairs 834/835 and 845/847 in Fig. 1). An exception to this heterogeneity was seen in the two deep periodontal lesions of patient JZ (Supplementary Table): of more than 15 treponemal clones isolated from each site, all were nearly identical (c.f. strains OMZ 844 and OMZ 758^T; Fig. 1). In spite of obvious technological advances, cultural studies are unpredictable because they often fail to culture any treponeme even from plaque populations in which spirochaetes predominate microscopically (up to 63 %). Considering such failures and the fact that the majority of oral treponemal phylotypes recognized by their 16S rRNA gene trace (Dewhirst *et al.*, 2000) have still never been isolated *in vitro*, much more work is clearly required to understand the growth requirements of oral treponemes and their interactions with other members of the oral microbiota.

Description of *Treponema putidum* sp. nov.

Treponema putidum (pu′ti.dum. L. neut. adj. *putidum* stinking, fetid).

Obligately anaerobic, helically coiled, motile, asaccharolytic and proteolytic. The human oral cavity is so far its only known habitat. Cells are approximately 0·25 μm in diameter and approximately 10 μm long, with a wavelength of approximately 3 μm and amplitude of approximately 1·5 μm. They contain four periplasmic flagella, two originating at each cell end and overlapping in the central region of the cell (i.e. flagellation type 2 : 4 : 2). In liquid media of low viscosity, cells appear highly active with cellular rotation and jerky flexing but no directional motility. Translational movement, however, is seen in media of higher viscosity or when cells creep along a surface. Cells can be stored at temperatures below −70 °C in medium supplemented with 10–20 % glycerol. Within 5 days of anaerobic incubation at 37 °C when streaked onto OMIZ-Pat agar, dense, off-white subsurface colonies up to 3 mm in diameter are formed. Does not grow in the chemically defined OMIZ-W1 medium, but requires the addition of yeast extract and/or Neopeptone (or fractions thereof); addition of 1–10 % human or fetal bovine serum is highly stimulatory. Growth is neither dependent on nor stimulated by any of the following carbohydrates, each tested at 2 g l^{-1}: D-arabinose, D-cellobiose, D-fructose, D-fucose, D-galactose, D-galacturonic acid, D-glucose, D-glucuronic acid, glycogen, D-lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-ribose, starch, D-sucrose, D-trehalose, D-xylose, L-arabinose, L-fucose, L-rhamnose, L-sorbose and L-xylose. Neuraminidase and dentilisin activities are not detected. Using API ZYM strips, the following enzyme activities are always detected: esterase C4, esterase C8, leucyl arylamidase, trypsin, acid phosphatase, naphtholphosphohydrolase, β-galactosidase and β-glucosidase; none of the strains shows activity of lipase C14, valine arylamidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase. Other enzyme activities detectable by API ZYM are present only in some strains. Growth is resistant to 1 mg rifampicin, 100 mg fosfomycin, 30 mg nalidixic acid and 5 mg polymyxin l^{-1}.

The type strain is strain JZC3^T (=OMZ 758^T=ATCC 700334^T=CIP 108088^T), isolated from subgingival plaque of a deep human periodontal lesion.

Acknowledgements

We thank C. Hefenbrock, C. Weiss and V. Zängerle for expert technical assistance and Dr G. Riviere for strain ST10. This work was supported in part by a grant of the Deutsches Bundesministerium für Bildung und Forschung (01KI9138) to U. B. G. and grants DE-10374 and DE-11443 (B. J. P. and F. E. D.) from the National Institute of Dental and Craniofacial Research.

References

Becker, M. R., Paster, B. J., Leys, E. J., Moeschberger, M. L., Kenyon, S. G., Galvin, J. L., Boches, S. K., Dewhirst, F. E. & Griffen, A. L. (2002). Molecular analysis of bacterial species associated with childhood caries. *J Clin Microbiol* **40**, 1001–1009.

Bickar, D. & Reid, P. D. (1992). A high-affinity protein stain for Western blots, tissue prints, and electrophoretic gels. *Anal Biochem* **203**, 109–115.

Chan, E. C. S., Siboo, R., Keng, T., Psarra, N., Hurley, R., Cheng, S. L. & Iugovaz, I. (1993). *Treponema denticola* (ex Brumpt 1925) sp. nov., nom. rev., and identification of new spirochete isolates from periodontal pockets. *Int J Syst Bacteriol* **43**, 196–203.

Choi, B.-K., Paster, B. J., Dewhirst, F. E. & Göbel, U. B. (1994). Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis. *Infect Immun* **62**, 1889–1895.

Dewhirst, F. E., Tamer, M. A., Ericson, R. E., Lau, C. N., Levanos, V. A., Boches, S. K., Galvin, J. L. & Paster, B. J. (2000). The diversity of periodontal spirochetes by 16S rRNA analysis. *Oral Microbiol Immunol* **15**, 196–202.

Haffajee, A. D. & Socransky, S. S. (1994). Microbial etiological agents of destructive periodontal diseases. *Periodontol 2000* **5**, 78–111.

Lukehart, S. A., Tam, M. R., Hom, J., Baker-Zander, S. A., Holmes, K. K. & Nowinski, R. C. (1985). Characterization of monoclonal antibodies to *Treponema pallidum*. *J Immunol* **134**, 585–592.

Moter, A., Hoenig, C., Choi, B.-K., Riep, B. & Göbel, U. B. (1998a). Molecular epidemiology of oral treponemes associated with periodontal disease. *J Clin Microbiol* **36**, 1399–1403.

Moter, A., Leist, G., Rudolph, R., Schrank, K., Choi, B.-K., Wagner, M. & Göbel, U. B. (1998b). Fluorescence *in situ* hybridization shows spatial distribution of as yet uncultured treponemes in biopsies from digital dermatitis lesions. *Microbiology* **144**, 2459–2467.

Page, R. C. (1995). Critical issues in periodontal research. *J Dent Res* **74**, 1118–1128.

Paster, B. J., Dewhirst, F. E., Coleman, B. C., Lau, C. N. & Ericson, R. L. (1998). Phylogenetic analysis of cultivable oral treponemes from the Smibert collection. *Int J Syst Bacteriol* **48**, 713–722.

Paster, B. J., Boches, S. K., Galvin, J. L., Ericson, R. E., Lau, C. N., Levanos, V. A., Sahasrabudhe, A. & Dewhirst, F. E. (2001). Bacterial diversity in human subgingival plaque. *J Bacteriol* **183**, 3770–3783.

Riviere, G. R., Wagoner, M. A., Baker-Zander, S. A., Weisz, K. S., Adams, D. F., Simonson, L. & Lukehart, S. A. (1991). Identification of spirochetes related to *Treponema pallidum* in necrotizing ulcerative gingivitis and chronic periodontitis. *N Engl J Med* **325**, 539–543.

Riviere, G. R., Smith, K. S., Carranza, N., Jr, Tzagaoulaki, E., Kay, S. L. & Dock, M. (1995). Subgingival distribution of *Treponema denticola*, *Treponema socranskii*, and pathogen-related oral spirochetes: prevalence and relationship to periodontal status of sampled sites. *J Periodontol* **66**, 829–837.

Willis, S. G., Smith, K. S., Dunn, V. L., Gapter, L. A., Riviere, K. H. & Riviere, G. R. (1999). Identification of seven *Treponema* species in health- and disease-associated dental plaque by nested PCR. *J Clin Microbiol* **37**, 687–689.

Wyss, C. (1989). Dependence of proliferation of *Bacteroides forsythus* on exogenous N-acetylmuramic acid. *Infect Immun* **57**, 1757–1759.

Wyss, C. (1992). Growth of *Porphyromonas gingivalis*, *Treponema denticola*, *T. pectinovorum*, *T. socranskii*, and *T. vincentii* in a chemically defined medium. *J Clin Microbiol* **30**, 2225–2229.

Wyss, C. (1998). Flagellins, but not endoflagellar sheath proteins, of *Treponema pallidum* and of pathogen-related oral spirochetes are glycosylated. *Infect Immun* **66**, 5751–5754.

Wyss, C., Choi, B. K., Schüpbach, P., Guggenheim, B. & Göbel, U. B. (1996). *Treponema maltophilum* sp. nov., a small oral spirochete isolated from human periodontal lesions. *Int J Syst Bacteriol* **46**, 745–752.

Wyss, C., Choi, B. K., Schüpbach, P., Guggenheim, B. & Göbel, U. B. (1997). *Treponema amylovorum* sp. nov., a saccharolytic spirochete of medium size isolated from an advanced human periodontal lesion. *Int J Syst Bacteriol* **47**, 842–845.

Wyss, C., Choi, B.-K., Schüpbach, P., Moter, A., Guggenheim, B. & Göbel, U. B. (1999). *Treponema lecithinolyticum* sp. nov., a small saccharolytic spirochaete with phospholipase A and C activities associated with periodontal diseases. *Int J Syst Bacteriol* **49**, 1329–1339.

Wyss, C., Dewhirst, F. E., Gmür, R., Thurnheer, T., Xue, Y., Schüpbach, P., Guggenheim, B. & Paster, B. J. (2001). *Treponema parvum* sp. nov., a small, glucuronic or galacturonic acid-dependent oral spirochaete from lesions of human periodontitis and acute necrotizing ulcerative gingivitis. *Int J Syst Evol Microbiol* **51**, 955–962.