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

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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 758T = ATCC 700334T = CIP 108088T).
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 immunobubbling 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 CaCl2, 0·1 mM ZnSO4) of the fluorogenic substrate MU-NANA (methylumbelliferyl N-acetylmuraminic acid); after 3 h incubation at 37 °C, fluorescent product was determined at λex. 360 nm and λemiss. 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 CaCl2, pH 8·0) of the fluorogenic substrate SAAPFpNA (Suc-Ala-Ala-Pro-Phe-pNA) (λex. 360 nm and λemiss. 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 35405T, 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

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

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**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, 835, 844, 846, 847 and 848 lacked sialidase activity (MU-NANA), which distinguished them from all the 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 dendogram in Fig. 2; the strains are referred to as Treponema putidum sp. nov., with strain OMZ 758 as the type strain. The closest named relative of T. putidum is T. denticola, at a level of 98–99% 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 trepnomes ‘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.

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**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 phylogenetic 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 758T; 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, glycerogen, D-lactose, D-malose, D-mannitol, D-mannose, D-melibiose, starch, D-sucrose, D-trehalose, D-xylene, L-arabinose, L-fucose, L-rhamnose, L-sorbose and L-xylene. 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 I−1.

The type strain is strain JZC3T (= OMZ 758T = ATCC 700334T = CIP 108088T), isolated from subgingival plaque of a deep human periodontal lesion.

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