Treponema lecithinolyticum sp. nov., a small saccharolytic spirochaete with phospholipase A and C activities associated with periodontal diseases

Wyss, C; Choi, B K; Schüpbach, P; Moter, A; Guggenheim, B; Göbel, U B

Treponema lecithinolyticum sp. nov., a small saccharolytic spirochaete with phospholipase A and C activities associated with periodontal diseases

Abstract

Strong phospholipase A (PLA) and phospholipase C (PLC) activities as potential virulence factors are the outstanding characteristics of eight strains of small oral spirochaetes isolated from deep periodontal lesions. By qualitative dot-blot DNA-DNA hybridization and 16S rDNA sequence comparison, these spirochaetes form a distinct phylogenetic group, with Treponema maltophilum as its closest cultivable relative. Growth of these treponemes, cells of which contain two endoflagella, one at each pole, was autoinhibited by the PLA-mediated production of lysolecithin unless medium OMIZ-Pat was prepared without lecithin. N-Acetylglucosamine was essential and D-ribose was stimulatory for growth. All isolates were growth-inhibited when 1% foetal calf serum was added to the medium. Growth on agar plates supplemented with human erythrocytes produced haemolysis. In addition to PLA and PLC, the new isolates displayed strong activities of alkaline and acid phosphatases, beta-galactosidase, beta-glucuronidase, N-acetyl-beta-glucosaminidase and sialidase, intermediate activities of C4- and C8-esterases, naphthol phosphohydrolase and alpha-fucosidase and a distinctive 30 kDa antigen detectable on Western blots. This phenotypically and genotypically homogeneous group is proposed as a novel species, Treponema lecithinolyticum sp. nov., with isolate OMZ 684T designated as the type strain. A molecular epidemiological analysis using a T. lecithinolyticum-specific probe showed this organism to be associated with affected sites when compared with unaffected sites of periodontitis patients. This association was more pronounced in patients with rapidly progressive periodontitis than in those with adult periodontitis.

International Journal of Systematic Bacteriology (1999), **49**, 1329–1339 Printed in Great Britain

Treponema lecithinolyticum sp. nov., a small saccharolytic spirochaete with phospholipase A and C activities associated with periodontal diseases

C. Wyss,[1] B.-K. Choi,[2,3] P. Schüpbach,[1] A. Moter,[2] B. Guggenheim[1] and U. B. Göbel[2]

Author for correspondence: C. Wyss. Tel: +411 634 3322. Fax: +411 634 4310.
e-mail: 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] Universitätsklinikum Charité, Institut für Mikrobiologie und Hygiene, Dorotheenstrasse 96, D-10117 Berlin, Germany

[3] Department of Oral Biology, Yonsei University College of Dentistry, Seoul, Republic of Korea

Strong phospholipase A (PLA) and phospholipase C (PLC) activities as potential virulence factors are the outstanding characteristics of eight strains of small oral spirochaetes isolated from deep periodontal lesions. By qualitative dot-blot DNA–DNA hybridization and 16S rDNA sequence comparison, these spirochaetes form a distinct phylogenetic group, with *Treponema maltophilum* as its closest cultivable relative. Growth of these treponemes, cells of which contain two endoflagella, one at each pole, was autoinhibited by the PLA-mediated production of lysolecithin unless medium OMIZ-Pat was prepared without lecithin. *N*-Acetylglucosamine was essential and D-ribose was stimulatory for growth. All isolates were growth-inhibited when 1% foetal calf serum was added to the medium. Growth on agar plates supplemented with human erythrocytes produced haemolysis. In addition to PLA and PLC, the new isolates displayed strong activities of alkaline and acid phosphatases, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase and sialidase, intermediate activities of C4- and C8-esterases, naphthol phosphohydrolase and α-fucosidase and a distinctive 30 kDa antigen detectable on Western blots. This phenotypically and genotypically homogeneous group is proposed as a novel species, *Treponema lecithinolyticum* sp. nov., with isolate OMZ 684^T designated as the type strain. A molecular epidemiological analysis using a *T. lecithinolyticum*-specific probe showed this organism to be associated with affected sites when compared with unaffected sites of periodontitis patients. This association was more pronounced in patients with rapidly progressive periodontitis than in those with adult periodontitis.

Keywords: Phospholipase, periodontal disease, phylogeny, cultivation, virulence

INTRODUCTION

Dental plaque is implicated in the development of periodontal diseases and is thought to induce tissue damage both by direct bacterial effectors and by misguided host response mechanisms (Holt & Bramanti, 1991; Socransky & Haffajee, 1991). The search for aetiological agents of periodontitis has resulted in the identification of some 300 bacterial species in dental plaque by cultural methods (Moore *et al.*, 1991). However, it has not been possible so far to identify a specific pathogen responsible for any form of periodontitis. Therefore, more complex models of pathogenesis, invoking mixed and/or sequential infections, have been plausibly proposed (Caldwell *et al.*, 1997; Page, 1995). Nevertheless, the specific pathogen model cannot be dismissed, since numerous bacterial species present in dental plaque have yet to be evaluated in epidemiological studies and because

Abbreviations: AP, adult periodontitis; Bis-BODIPY, 1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoyl)-*sn*-glycero-3-phosphocholine; MU-NANA, 4-methylumbelliferyl *N*-acetylneuraminic acid; MU-PC, 4-methylumbelliferyl phosphocholine; PLA, phospholipase A; PLC, phospholipase C; pNP-PC, *p*-nitrophenylphosphorylcholine; RPP, rapidly progressive periodontitis.

The EMBL/GenBank accession numbers for the 16S rRNA sequences of strains OMZ 684^T and OMZ 702 are X89139 and AJ007740.

within any species clones of widely differing virulence may exist (Choi *et al*., 1994; Haffajee & Socransky, 1994; Riviere *et al*., 1991).

Spirochaetes are prominent in subgingival plaque of many periodontitis patients. At least 25 species-level phylotypes of oral treponemes have been recognized (Choi *et al*., 1994; Paster *et al*., 1998) and their clinical prevalence has been studied using specific 16S rDNA oligonucleotide probes (Moter *et al*., 1998). However, most of their phenotypic characteristics are unknown, since to date only seven species have been studied extensively in culture (Paster *et al*., 1998; Umemoto *et al*., 1997; Wyss *et al*., 1996, 1997). Among these, the proteolytic activity of *Treponema denticola* is the physiological activity most widely accepted as having pathogenetic potential (Holt & Bramanti, 1991).

Using novel techniques for the cultivation of fastidious anaerobes from subgingival plaque, we have isolated large numbers of oral spirochaetes, including members of the recently described species *Treponema maltophilum* and *Treponema amylovorum* (Paster *et al*., 1998; Wyss *et al*., 1996, 1997). We now describe eight small spirochaetal isolates possessing prominent phospholipase A (PLA) and phospholipase C (PLC) activities that form a phenotypically distinct homogeneous group and represent a novel genotype, as defined by qualitative dot-blot DNA–DNA hybridization and 16S rRNA sequencing. Association of this group with disease was more pronounced in patients with rapidly progressive periodontitis than in those with adult periodontitis. We propose the designation of a new species, *Treponema lecithinolyticum* sp. nov., with strains OMZ 684^T and OMZ 685 as type and reference strains, respectively.

METHODS

Bacteria. Reference strains were obtained and maintained as described previously (Wyss *et al*., 1996). Novel isolates were named as follows: the first two letters designate patient origin, the third position designates plaque sample (e.g. FO1DAA and FOC6C1 were isolated from two different plaque samples of the same patient; BL2A and BL2B are isolates from the same plaque sample; see Table 1). Two of the new isolates, maintained in medium OMIZ-Pat-w/oPC, were designated type and reference strains and deposited in the ATCC: the proposed type strain is ATCC 700332^T (= PFB4G^T = OMZ 684^T) and the proposed reference strain is ATCC 700333 (= MH1DD = OMZ 685).

Culture media. For the cultivation of lecithinolytic treponemes, medium OMIZ-Pat (Wyss *et al*., 1996) was prepared without dipalmitoyl phosphatidylcholine (OMIZ-Pat-w/o PC). For all other strains, OMIZ-Pat and OMIZ-Pat-w/oPC could be used interchangeably.

Enzyme activities

For the determination of enzyme activities, cells from actively growing cultures were centrifuged and resuspended as recommended for Api Zym tests. Aliquots of these suspensions were added to substrates as indicated and incubated aerobically at 35 °C.

Api Zym tests. These tests were performed as described previously (Wyss *et al*., 1996), except that cells were grown in OMIZ-Pat-w/oPC.

Fluorogenic assay for sialidase. The fluorogenic sialidase substrate 4-methylumbelliferyl *N*-acetylneuraminic acid (MU-NANA; Sigma), dissolved at 100 μg ml^{-1} in buffer B of Kurioka & Matsuda (1976), was mixed with an equal volume of cell suspension and product formation was determined after 4 h with a Cytofluor 2350 fluorimeter (Millipore) set at excitation and emission wavelengths of 360 and 460 nm, respectively.

Assays for PLC. The turbidimetric assay of Jolivet-Reynaud *et al*. (1988), with egg lecithin (Merck) or synthetic dipalmitoyl phosphatidylcholine (Sigma) as substrate, was modified by substituting desoxycholate for cholate in order to disperse the lecithin. PLC from *Clostridium perfringens* (Sigma) was used as a positive control. A spectrophotometric assay using a water-soluble substrate analogue, *p*-nitrophenyl phosphorylcholine (pNP-PC; Sigma), was performed as described by Kurioka & Matsuda (1976). A fluorometric assay was developed using a 1:1 mixture of cell suspension and a solution of 10 μg ml^{-1} of the water-soluble substrate analogue 4-methylumbelliferyl phosphocholine (MU-PC; Molecular Probes) in buffer B of Kurioka & Matsuda (1976); product formation was determined after 4 h with a Cytofluor 2350 fluorimeter (Millipore) set at excitation and emission wavelengths of 360 and 460 nm, respectively (Freeman *et al*., 1985).

Assays for PLA. Phospholipase activity was identified by TLC product analysis (Tigyi & Miledi, 1992) after incubation for 24 h of 50 μl cell suspension (or reference phospholipase) with 2 ml of 5 mg ml^{-1} synthetic dipalmitoyl lecithin in buffer B of Kurioka & Matsuda (1976) supplemented with 5 mg desoxycholate ml^{-1}. Reference lipids, including palmitoyl lysolecithin and dipalmitin, were obtained from Sigma. Phospholipase A2 from *Crotalus adamantus* (Sigma) was used as a positive control.

A more convenient screening of PLA activity was accomplished using a fluorochrome assay with the synthetic substrate analogue 1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoyl)-*sn*-glycero-3-phosphocholine (Hendrickson, 1994) (Bis-BODIPY; Molecular Probes) dissolved (2 μg ml^{-1}) in buffer B of Kurioka & Matsuda (1976) supplemented with 10 μg cholesterol ml^{-1}. A 1:1 mixture of cell suspension and substrate solution was incubated for 4 h. BODIPY-acyl release through cleavage by PLA leads to increased fluorescence, which was measured using a Cytofluor 2350 fluorimeter set at excitation and emission wavelengths of 485 and 530 nm, respectively.

Qualitative whole DNA–DNA dot-blot hybridization. Digoxigenin (DIG) labelling and detection of strain OMZ 684^T DNA was performed using a commercial kit (Boehringer) according to the manufacturer's instructions. Dot hybridization at 68 °C to cellular DNA denatured with NaOH/EDTA on grid filters (Millipore HAWG 047 S3) with a stringent wash in 0·2× SSC containing 0·1% SDS was performed according to the method of Burnens & Nicolet (1992).

Assay for haemolytic activity. Haemolysis was determined either by the addition of 2% washed human erythrocytes plus 1·5% agarose to OMIZ-Pat-w/oPC or in a 96-well plate assay according to Jolivet-Reynaud *et al*. (1988) using human erythrocytes.

Sugar stimulation of growth. These semi-quantitative tests were performed as described previously (Wyss, 1992; Wyss

et al., 1996), but in media lacking dipalmitoyl phosphatidylcholine.

SDS-PAGE gels and Western blots: These were done as described previously (Wyss *et al*., 1996) using extracts prepared from cells grown in OMIZ-Pat-w/oPC. Flagellins were detected as described previously (Wyss, 1998).

Limit dilution culture of plaque. This was performed as described previously (Wyss *et al*., 1996) with medium OMIZ-Pat-w/oPC supplemented with rifampicin (1 μg ml^{-1}) and fosfomycin (100 μg ml^{-1}).

Electron microscopy. Specimen preparation was done as described previously (Wyss *et al*., 1996) using cells grown in OMIZ-Pat-w/oPC.

16S rDNA sequencing and phylogenetic analysis. Treponemes from 10 ml cultures in OMIZ-Pat-w/oPC were pelleted by centrifugation and washed once in PBS. The pellet was resuspended in 400 μl lysis buffer (500 mM Tris/HCl, pH 9·0, 20 mM EDTA, 10 mM NaCl, 1% SDS) containing proteinase K (final concentration 200 μg ml^{-1}; Boehringer Mannheim) and incubated at 37 °C for 2 h. Phenol/chloroform extraction and all further steps were done as described previously (Choi *et al*., 1994). 16S rDNA was amplified using eubacterial universal primers, TPU1 (5′ AGAGTTTGATCMTGGCTCAG 3′) and RTU8 (5′ AAGGAGGTGATCCAKCCRCA 3′). The same primers were used for sequencing the PCR products by a modified Sanger dideoxynucleotide chain-termination method using Sequenase (USB) and a model 4000 LI-COR automated sequencer (MWG-Biotech) according to the manufacturers' instructions.

Phylogenetic analysis of 16S rRNA. The nearly complete 16S rRNA sequences were compared with those of all sequences currently accessible from public databases (EMBL, GenBank) and private databases (Choi *et al*., 1994; Paster *et al*., 1991). A phylogenetic tree was constructed by using the TREECON software package (Jukes & Cantor, 1969) and including all nucleotide positions for distance calculation. The neighbour-joining method of Saitou & Nei (1987) was used and multiple base changes at single positions were corrected by the method of Jukes and Cantor (Van de Peer & De Wachter, 1993).

Epidemiological study

Clinical samples. A total of 244 subgingival plaque specimens (200 specimens from deep periodontal pockets and 44 specimens from healthy control sites) from 53 patients suffering from rapidly progressive periodontitis (RPP) were collected as described previously (Moter *et al*., 1998). A further 325 specimens from previously untreated patients with adult periodontitis (AP) were investigated (268 samples from periodontal pockets plus 67 samples from unaffected sites from the same patients). Plaque samples were collected as described previously (Moter *et al*., 1998). Diagnoses of RPP and AP were made according to established criteria (Page *et al*., 1983).

DNA extraction and amplification. Aliquots of plaque suspensions were pelleted and bacteria were resuspended in 100 μl lysis buffer. Eubacterial amplification of 16S rDNA was performed using universal primers TPU1 and RTU3 (5′ GWATTACCGCGGCKGCTG 3′) as described previously (Moter *et al*., 1998).

Specific dot-blot hybridizations. After denaturation, PCR-amplified plaque material was spotted onto nylon membranes. Oligonucleotide probe TLECI (5′ CACTCTCAGAAAGGAGCAAGCTCC 3′) was designed according to the phylogenetic tree retrieved from an earlier comparative 16S rRNA analysis (Choi *et al*., 1994) and all 16S rDNA entries currently available at the EMBL and GenBank databases. The probe was labelled with DIG-ddUTP (Boehringer Mannheim). Bound probe was detected by chemiluminescence according to the manufacturer's recommendations. To adjust the hybridization specificity of probe TLECI, PCR-amplified 16S rDNA from 34 related and unrelated bacteria, including cultivable and uncultured treponemes as well as other oral micro-organisms, served as controls (Moter *et al*., 1998). Stringency was adjusted by varying the washing temperature and SSC concentration (0·1–5·0× SSC) in the washing buffer (1× SSC is 0·15 M NaCl, 0·015 M sodium citrate, 0·2% SDS). The detection limit with cultured cells of strain OMZ 684^T was in the range of 100 organisms, but this may differ with subgingival plaque material.

RESULTS

Isolation from subgingival plaque

During the screening of limit dilution cultures derived from subgingival plaque samples using medium OMIZ-Pat supplemented with rifampicin and fosfomycin (Wyss *et al*., 1996), we encountered a clone of small spirochaete, OMZ 684^T, that was difficult to subculture and that produced a notable precipitate. Analysis of the nutritional requirements of this unusual isolate revealed that its growth was inhibited by

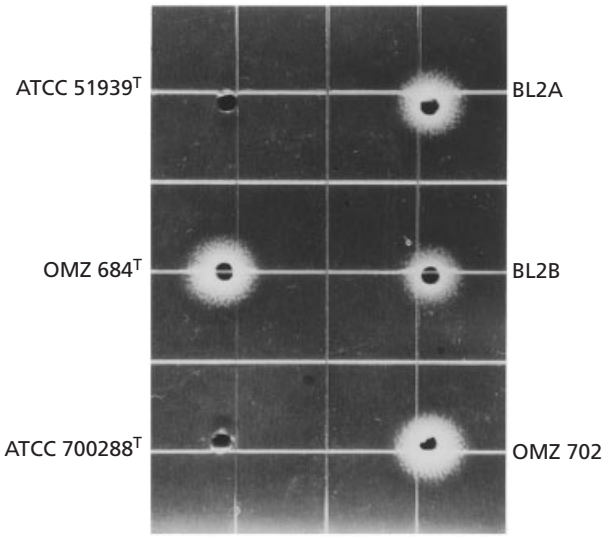

Fig. 1. PLC-dependent production of turbidity in lecithin-agarose after 4 h aerobic incubation at 37 °C. Wells in lecithin-agarose contained cell suspensions prepared as for the Api Zym tests of the strains indicated (15 ml agar per Integrid dish, grid size 13 mm). Positive reactions, such as those seen for OMZ 684^T, BL2A, BL2B and OMZ 702, were also observed with the other six novel isolates (EL3M1A, FO1DAA, FOC6C1, HA2B, OMZ 685 and MH3C). Negative reactions, as shown here for *T. maltophilum* ATCC 51939^T and *T. amylovorum* ATCC 700288^T, were seen with all other tested treponemes, '*T. vincentii*' ATCC 35580, *T. denticola* ATCC 35405^T, *T. socranskii* subsp. *socranskii* ATCC 35536^T, *T. socranskii* subsp. *paredis* ATCC 35535^T, *T. socranskii* subsp. *buccale* ATCC 35534^T and *Treponema pectinovorum* ATCC 33768^T.

Table 1. Origins of 10 oral *Treponema* isolates with lecithinolytic activity isolated from seven patients with refractory periodontitis

Strain	Patient age/gender	Previous treatment	Spirochaetes (%)*	Isolation dilution (log_{10})	Other *Treponema* species isolated from the same sample
BL2A	60/f	Mechanical	73	−7	BL2B, *T. denticola*
BL2B	60/f	Mechanical	73	−6	*T. maltophilum* (BL2A), *T. denticola*
EL3M1A	20/f	Mechanical	1	−3	*T. socranskii* subsp. *socranskii*, *T. denticola*
FO1DAA	43/f	Mechanical	17	−3	*T. socranskii* subsp. *socranskii*
FOC6C1	43/f	Mechanical	48	−6	*T. socranskii* subsp. *buccale*, *T. denticola*, *T. medium*
HA2B	54/m	Mechanical	62	−4	*T. amylovorum*, *T. denticola*, *T. maltophilum*
OMZ 685	35/f	Metronidazole + ciprofloxacin, 3 month control	16	−4	*T. socranskii* subsp. *socranskii*, *T. medium*
MH3C	35/f	Metronidazole + ciprofloxacin, 3 month control	45	−4	'*T. socranskii* subsp. 04', *T. maltophilum*
OMZ 684^T	33/m	Mechanical	56	−4	*T. pectinovorum*
OMZ 702	40/f	Metronidazole + ciprofloxacin, 4 month control	8	−4	*T. denticola*

* The percentage of spirochaetes as a proportion of bacteria observed was determined by dark-field microscopy.

Table 2. Phospholipase activities and classification of oral spirochaetes

The 16S rRNA clusters to which each isolate belongs, as defined by Choi *et al.* (1994), are shown. ND, Not done.

Isolate	16S rRNA cluster	Haemolysis	Relative enzyme activity (substrate)				
			PLC (lecithin)	PLC (pNP-PC)	PLC (MU-PC)	PLA (Bis-BODIPY)	Sialidase (MU-NANA)
'*T. vincentii*' OMZ 800	1	−	0	4	1	0	0
'*T. vincentii*' ATCC 35580	1	−	0	8	9	0	0
T. medium OMZ 805	2	ND	0	6	4	0	9
T. medium OMZ 806	2	ND	0	3	1	0	9
T. denticola ATCC 35405^T	11	+	0	5	4	0	9
T. denticola BL2C	11	ND	0	3	2	0	9
T. denticola HA2R12	11	ND	0	7	8	0	9
T. maltophilum ATCC 51939^T	17	−	0	4	0	0	0
T. maltophilum ATCC 51941	17	−	0	6	2	0	0
T. maltophilum BL2A	17	ND	9	9	1	0	0
T. maltophilum OMZ 702	17	+	9	9	2	0	0
T. lecithinolyticum OMZ 685	17	+	9	2	1	9	9
T. lecithinolyticum OMZ 684^T	17	+	9	3	2	9	9
T. lecithinolyticum BL2B	17	ND	9	1	0	9	9
T. amylovorum ATCC 700288^T	19	−	0	0	0	0	3

the lecithin contained in medium OMIZ-Pat and that precipitate formation depended on the presence of lecithin. OMZ 684^T cells displayed a strong PLC activity in the turbidimetric test, which relies on the insolubility of the diacylglycerol product (Fig. 1). However, neither diacylglycerol nor phosphoryl-choline, the products of PLC action on lecithin, could reproduce the growth-inhibitory effect of lecithin. Growth inhibition was seen with palmitoyl lyso-lecithin, one of the products of PLA action on dipalmitoyl lecithin, but not with palmitic acid. TLC analysis of dipalmitoyl lecithin (R_F 0·5) incubated with

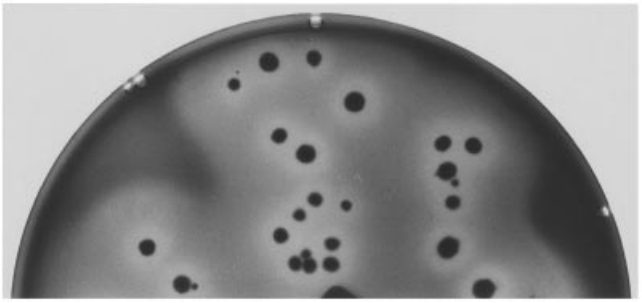

Fig. 2. Haemolytic zones around colonies of strain OMZ 684^T grown for 12 d on OMIZ-Pat-w/oPC agar with human erythrocytes in a 9 cm Petri dish.

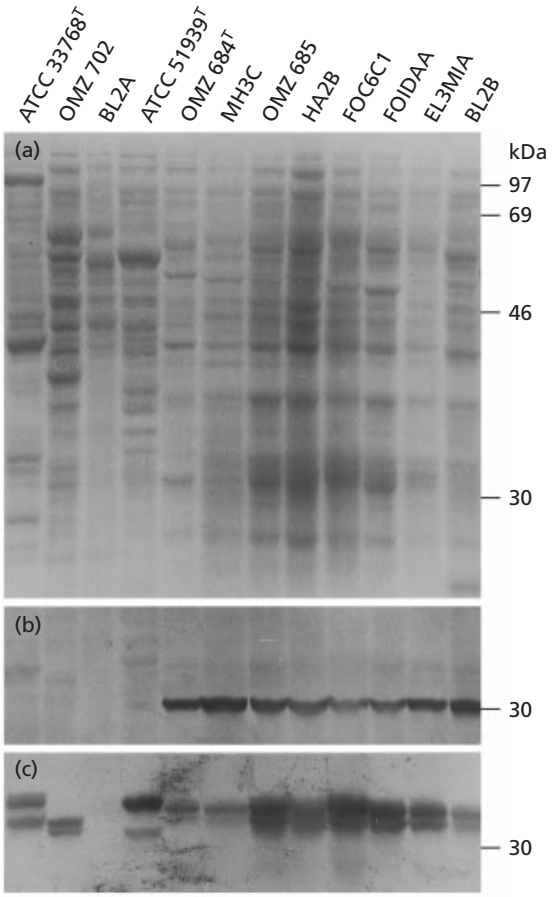

Fig. 3. Western blots of the 10 novel isolates and the type strains of *T. maltophilum*, ATCC 51939^T, and *T. pectinovorum*, ATCC 33768^T, after SDS-PAGE (10% gel) stained for protein (a), for a ~30 kDa OMZ 684 group-specific antigen (b) and for glycosylated flagellin (c) [same blot as (a), labelled after removal of the protein stain]. Numbers on the right indicate positions of molecular mass markers (in kDa).

intact cells of strain OMZ 684^T revealed the production of dipalmitin (R_F 0·95) and palmitoyl lysolecithin (R_F 0·25), demonstrating activities of both PLC and PLA (data not shown).

On the basis of this information, we omitted lecithin from the culture medium (OMIZ-Pat-w/oPC) and have now isolated and characterized a total of 10 strains with PLC activity from nine subgingival plaque samples from seven patients with refractory periodontitis that did not respond to scaling and root planing (or, in two patients, even to antibiotic treatment). From each of these samples, we could also isolate at least one other species of oral treponeme, and there appeared to be no obvious correlation between the percentage of spirochaetes in microscopically observed bacteria and the nominal plaque dilution at which treponemes were isolated (Table 1).

Growth characteristics

Growth of all 10 lecithinolytic strains was dependent on *N*-acetylglucosamine and was stimulated by D-ribose; growth of eight of these strains, including OMZ 684^T, was also promoted by L-fucose and by D-arabinose, whereas growth of two strains (OMZ 702 and BL2A) was inhibited by L-fucose. Growth of some strains was also enhanced by D-fructose (excluding OMZ 684^T) and/or D-xylose (including OMZ 684^T). Proliferation of all strains was independent of asialofetuin but was completely inhibited by the addition of 1% foetal bovine serum, whereas 1% human serum had growth-promoting activity.

Enzyme activities

All strains showed a strong PLC activity towards egg or synthetic lecithin (Fig. 1), while their activities towards PLC substrate analogues pNP-PC and MU-PC differed considerably (Table 2). The results of the determination of further enzymic activities divided the 10 strains into two homogeneous groups: eight strains (OMZ 684 group; OMZ 684^T plus BL2A, EL3M1A, FO1DAA, FOC6C1, HA2B, OMZ 685 and MH3C) had not only PLC but also PLA activity, as indicated by the Bis-BODIPY fluorochrome assay (confirmation of this activity as PLA by TLC, by the production of lysolecithin from synthetic dipalmitoyl lecithin, was made for strains OMZ 684^T and OMZ 685 only). These strains also showed strong activities for alkaline phosphatase, acid phosphatase, β-galactosidase, β-glucuronidase and *N*-acetyl-β-glucosaminidase and moderate activities for C4-esterase, C8-esterase, naphthol phosphohydrolase and α-fucosidase. The three strains tested (OMZ 684^T, OMZ 685 and BL2B) showed strong sialidase activity (Table 2).

The remaining two strains (OMZ 702 and BL2A) differed from the OMZ 684 group by the absence of PLA, β-glucuronidase and *N*-acetyl-β-glucosaminidase activities and by only weak to intermediate activities of alkaline phosphatase and β-glucuronidase. A weak α-glucosidase activity was found in BL2A, only; no sialidase activity was detected (Table 2).

As shown in Table 2, representative strains of other cultivated oral treponemes, including type or other

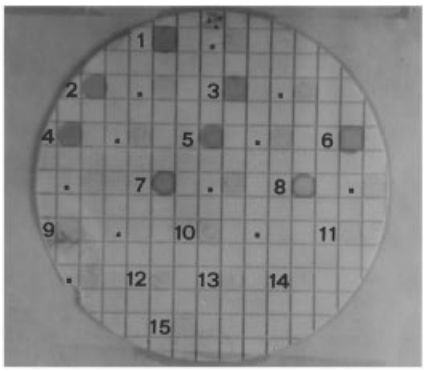

Fig. 4. Hybridization between DIG-labelled OMZ 684^T DNA and lysates of cultured oral treponemes immobilized on nitrocellulose. NaOH cell lysates were spotted to the right of the numbered squares; 1:10 dilutions of lysates 1–11 were spotted to the right of squares marked with a dot. 1, OMZ 684^T; 2, BL2B; 3, EL3M1A; 4, FO1DAA; 5, FOC6C1; 6, HA2B; 7, OMZ 685; 8, MH3C; 9, BL2A; 10, OMZ 702; 11, *T. maltophilum* ATCC 51939^T; 12, '*T. vincentii*' ATCC 35580; 13, BL3G2 (a proteolytic treponeme clinical isolate); 14, *T. denticola* ATCC 35405^T, 15, *T. amylovorum* ATCC 700288^T.

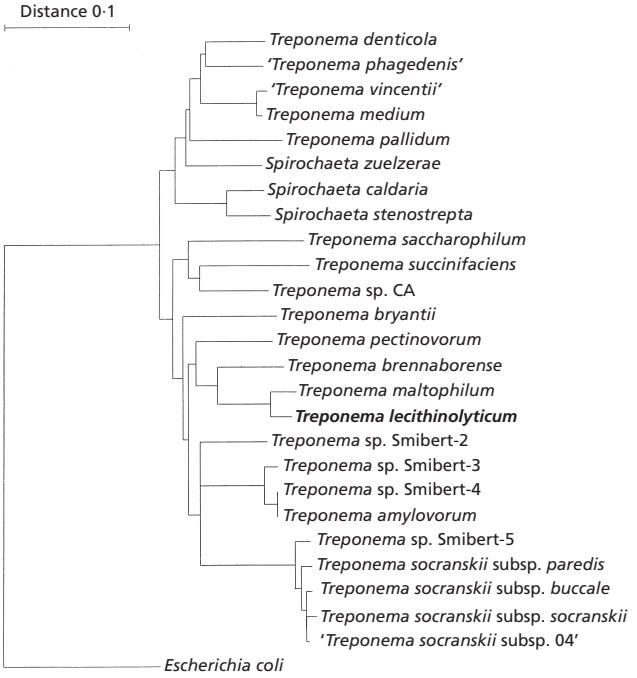

Fig. 5. Dendrogram based on comparative 16S rRNA sequence analysis showing the phylogenetic position of group IV treponeme *T. lecithinolyticum* relative to other spirochaetes, with *Escherichia coli* as the outgroup. Bar indicates 10% difference in nucleotide sequence as determined by measuring the lengths of the horizontal lines connecting two sequences. The accession numbers of the sequences used for tree construction were: *T. denticola* M71236, '*Treponema phagedenis*' M57739, '*T. vincentii*' AF033309, *Treponema medium* D85437, *Treponema pallidum* M34266, *Spirochaeta zuelzerae* M34265, *Spirochaeta caldaria* M71240, *Spirochaeta stenostrepta* M34264, *Treponema saccharophilum* M71238, *Treponema* sp. CA M59294, *Treponema succinifaciens* M57738, *Treponema bryantii* M57737, *T. pectinovorum* M71237, *T. maltophilum* X87140, *T. lecithinolyticum* OMZ 684^T X89139, *Treponema brennaborense* Y16568 (Schrank *et al.*, 1999), *Treponema* sp. Smibert-2 AF033304, *Treponema* sp. Smibert-3 AF023044, *Treponema* sp. Smibert-4 AF023045, *T. amylovorum* Y09959, *Treponema* sp. Smibert-5 AF033303, *T. socranskii* subsp. *paredis* AF033307, *T. socranskii* subsp. *buccale* AF033305, *T. socranskii* subsp. *socranskii* AF033306, '*Treponema socranskii* subsp. 04' AF033308, *E. coli* J01859.

ATCC strains of *T. amylovorum*, *T. denticola*, *T. maltophilum*, *Treponema socranskii* subsp. *socranskii* and '*Treponema vincentii*', showed differing activities towards the artificial hydrophilic PLC substrates pNP-PC and MU-PC, but none in the turbidimetric assay for PLC; PLA activity detectable by the fluorochrome assay with Bis-BODIPY was found only in the OMZ 684 group.

Haemolysis

All five strains examined (FO1DAA, OMZ 684^T, OMZ 685, OMZ 702 and BL2A) showed haemolytic activity when grown on solid OMIZ-Pat-w/oPC supplemented with 1% heat-inactivated human serum and 2% washed human erythrocytes (Fig. 2).

Protein and antigen patterns

For further phenotypic characterization and for comparison with other treponemes, protein and antigen profiles of SDS-PAGE-separated cell extracts were determined (Fig. 3). The results support the division of the ten lecithinolytic strains into two groups, with a dominant ~ 30 kDa antigen specific for the OMZ 684 group (Fig. 3b).

Phylogenetic classification

To estimate the genetic relationship among the novel isolates and towards known species of oral treponemes, we prepared DIG-labelled DNA of strain OMZ 684^T by random-primed Klenow fragment synthesis. As shown in Fig. 4, the eight OMZ 684 group isolates were readily distinguished from the two other strains with PLC activity (OMZ 702 and BL2A) and representatives of other oral *Treponema* species. In a complementary experiment, DIG-labelled DNA of strain OMZ 702 hybridized to dots of the novel isolates OMZ 702 and BL2A as well as *T. maltophilum* ATCC 51939^T, but not to OMZ 684^T, OMZ 685 or representative strains of *T. amylovorum*, *T. denticola* or '*T. vincentii*' (data not shown).

The nearly complete 16S rRNA sequences of strains OMZ 684^T and OMZ 702 were determined. The sequence of OMZ 684^T showed 96·5% identity to its closest cultured relative, *T. maltophilum* ATCC 51939^T, but also clustered in phylogenetic group IV according to the classification scheme of Choi *et al.* (1994) (Fig. 5). In contrast, the sequence of OMZ 702 showed 99·5% identity to that of *T. maltophilum*. On

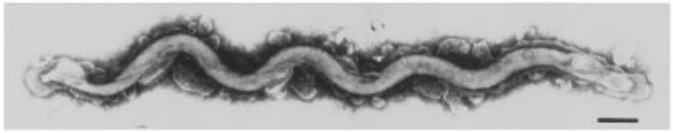

Fig. 6. Electron micrograph of a negative-contrasted cell of strain OMZ 684^T. Bar, 0·2 μm.

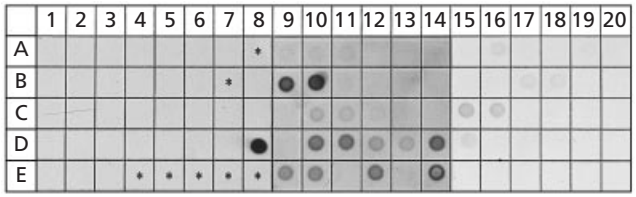

Fig. 7. Dot-blot hybridizations with OMZ 684^T-specific probe TLECI. In columns 1–8, PCR products of the following strains, kindly provided by R. Mutters[1] (Marburg) and B. Wilske[2] (München) where indicated, were applied: *Actinobacillus actinomycetemcomitans*[1] MCCM 02638 (A1), *Capnocytophaga gingivalis*[1] MCCM 00858 (A2), *Capnocytophaga ochracea*[1] MCCM 00238 (A3), *Eubacterium lentum*[1] ATCC 25559^T (A4), *Fusobacterium nucleatum*[1] ATCC 25586^T (A5), *Porphyromonas gingivalis*[1] ATCC 33277 (A6), *Prevotella intermedia*[1] MCCM 00407 (A7); '*T. vincentii*' ATCC 35580 (B1), *T. denticola* ATCC 35405^T (B2), *T. socranskii* subsp. *socranskii* ATCC 35536^T (B3), *T. socranskii* subsp. *buccale* ATCC 35534^T (B4), *T. maltophilum* ATCC 51939^T (B5), '*T. phagedenis* subsp. *reiterii*'[2] (B6), clinical isolate, highest homology to clone NZM 3142 (B8), *T. pectinovorum* ATCC 33768^T (E1), *T. lecithinolyticum* OMZ 684 (D8); recombinant clones: group I: NZM3D292 (C1), NZM3D464 (C5), NZM3112 (C6), NZM3142 (D2), NZM3147 (D4), NZM 3166 (D7); group II: NZM3106 (C7), NZM3158 (D6); group III: NZM3143 (D3), NZM3D298 (C3), NZM3D527 (C4); group IV: NZM3122 (C8), NZM3D505 (C2); group V: NZM3124 (D1), NZM3155 (D5); group VI: NZM3104 (E2); group VII: NZM3D384 (E3). In columns 9–14 and 15–20, PCR products from subgingival plaque samples of RPP and AP patients, respectively, were applied. TLECI does not detect treponemes of the OMZ 702 group since the sequences differ at three positions. *, No sample applied.

the basis of partial sequences (628 bp), the sequence of non-motile isolate BL2A showed 99·5% identity to that of OMZ 702; these two strains are therefore considered to belong to *T. maltophilum* (Table 2).

Morphology

As shown in Fig. 6 for strain OMZ 684^T, nine of the lecithinolytic isolates are small spirochaetes with two endoflagella, one per pole, which sometimes overlap in the middle of the cell. The tenth isolate, BL2A, was observed in the initial well to form a floating, compact colony of non-motile cells. When streaked onto solid medium for recloning, all nine motile isolates formed fluffy subsurface colonies, whereas strain BL2A formed raised colonies that did not penetrate into the agar. The non-motile cells of strain BL2A are helically coiled like the other novel isolates, but neither flagella nor Fla B proteins could be detected (Fig. 3c).

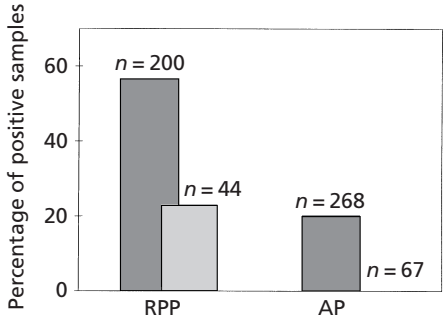

Fig. 8. Presence of *T. lecithinolyticum* OMZ 684^T-complementary sequences in subgingival plaque specimens from periodontal pockets (dark bars) or control sites (light bars) of periodontitis patients, analysed by dot-blot hybridization of PCR-amplified samples using oligonucleotide probe TLECI.

Epidemiology

To assess the prevalence of the novel OMZ 684 group strains in periodontitis patients, dot-blot hybridizations were performed with an OMZ 684-specific probe (TLECI). OMZ 684 group treponemes were detected in both patient groups (Fig. 7). However, the novel species was detected more frequently in deep periodontal pockets from RPP patients (56·5%) than from AP patients (19·8%). Only 22·7% in RPP patients and 0% in AP patients of the respective control sites were TLECI-positive (Fig. 8).

DISCUSSION

Numerous previously undetected bacterial species have been revealed in all natural environments that have been investigated using refined molecular biological techniques. However, these techniques provide no information regarding the expected phenotypes and therefore the possible biological roles of these unknown species. Here, we report the isolation and phenotypical characterization of a group of oral treponemes, the presence of which had hitherto only been inferred from the detection of 16S rRNA in dental plaque of a periodontitis patient (Choi *et al.*, 1994). Very recently, Paster *et al.* (1998) identified strains of *T. maltophilum* (Smibert-1), *T. amylovorum* (Smibert-4) and three new species (Smibert-2, -3 and -5) in the Smibert culture collection of oral treponemes by phylogenetic analysis; however, they postponed definitive species description because of insufficient phenotypic data. As shown in Fig. 5, these are all phylogenetically distant from OMZ 684^T. This raises the number of cultured species of oral treponemes to 11 but still leaves the majority of known phylotypes to be isolated *in vitro*.

Phospholipase activity

To our knowledge, the eight novel isolates are the first bacteria reported to show sufficient PLA activity to be detected with intact cells. Low levels detected in concentrated extracts of other bacteria are assumed to function in the turnover of cellular lipids. Previously reported microbial PLAs include a membrane-associated PLA of *Campylobacter coli* that may be involved in haemolysis (Grant *et al*., 1997), an outer-membrane PLA of *Enterobacteriaceae* required for secretion of bacteriocins (Merck *et al*., 1997) and a PLA activity of *Helicobacter pylori* that may help this organism to reach the gastric epithelium (Slomiany *et al*., 1992).

In contrast, strong PLC activity is widely distributed amongst bacteria and is recognized as an important virulence factor (Titball, 1993). Our novel isolates are the first oral treponemes to show activity in the turbidimetric PLC assay, as used for the α-toxin of *Clostridium perfringens* (Jolivet-Reynaud *et al*., 1988), although all strains except *T. amylovorum* hydrolysed the hydrophilic PLC substrate analogue pNP-PC (Table 2; Siboo *et al*., 1989). This latter activity has been purified from *T. denticola* on a lecithin-affinity matrix (Chan *et al*., 1991), suggesting that it is not simply due to a general phosphodiesterase; its specificity may be towards a phospholipid different from lecithin or its activity may be sensitive to the conditions of the turbidimetric assay.

Growth inhibition

Caution is essential with regard to results concerning environmental conditions required for growth of treponemes. Growth inhibition of the novel isolates by addition of 1 % foetal calf serum to OMIZ-Pat medium is in line with previous results for *T. socranskii* (Wyss, 1992), *T. maltophilum* (Wyss *et al*., 1996) and *T. amylovorum* (Wyss *et al*., 1997) but contradicts results obtained in other culture systems (Smibert *et al*., 1984) and is contrary to physiological expectations. Similarly, the toxic effects of lysolecithin seen here and in other *in vitro* systems may be neutralized *in vivo*. This is suggested by the observation that this phospholipid is the only lipid consumed by *Borrelia hermsii* (Pickett & Kelly, 1974) and several *Treponema* species (Trevathan *et al*., 1982).

Role of phospholipase activity *in vivo*

The physiological target of the strong activities of both PLC and PLA expressed by the novel isolates is most likely a component of the vertebrate host, since no such activity seems to be required for lipid metabolism by other treponemes. Direct action on host cell membranes may increase the local availability of nutrients and lead to enhanced tissue invasion by treponemes. The resulting production of potent regulators of eukaryote cell metabolism and inflammation such as ceramide, diglycerides, lysolecithin, arachidonic acid and platelet-activating factor (Divecha & Irvine, 1995; Prescott *et al*., 1990; Serhan *et al*., 1996; Songer, 1997; Titball, 1993) as well as the release of processed antigens from accessory cells is likely to affect host physiology severely (Falo *et al*., 1987).

Furthermore, in the subgingival niche there is ample opportunity for ecologically important interactions between host and the complex microbiota. These include the enhancement of cell damage through phospholipase action by strongly proteolytic organisms like *T. denticola*, *Bacteroides forsythus*, *Porphyromonas gingivalis* and *Capnocytophaga* species, which all rank high in the list of suspected periodonto-pathogens (Haffajee & Socransky, 1994), or the cohaemolytic activity of phospholipase with Christie–Atkins–Munch–Petersen (CAMP)-active bacteria (Frey *et al*., 1989).

Taxonomic position

The strong PLC activity of the 10 clinical isolates reported in this study clearly separates them from all previously studied oral treponemes. On the basis of phylogenetic and additional phenotypic data, however, they represent two distinct groups, both located within group IV according to Choi *et al*. (1994) (Fig. 5). The larger group represented by OMZ 684^T comprises eight strains and is phenotypically and genotypically homogeneous. Therefore, we propose group OMZ 684 organisms as a novel species, *Treponema lecithinolyticum* sp. nov.

The remaining two isolates, one of which lacked endoflagella since its primary isolation, are phylogenetically closely related to *T. maltophilum*, although previously described strains of that species are not known to display PLC activity (Paster *et al*., 1998; Wyss *et al*., 1996).

Isolation of novel organisms from complex microbial consortia not only allows their phenotypic and phylogenetic characterization but also serves in the development and validation of cultivation-independent methods for epidemiological studies on the aetiology of periodontal diseases.

Epidemiology

The primary aim of our cultural studies was to gain a view of the physiological diversity of oral treponemes. The data summarized in Table 1 confirm previous experience, that cultural analysis is unlikely to give an adequate quantitative picture of the spirochaetal population of plaque samples. Nevertheless, it may be noted that we also recovered proteolytic *T. denticola* from six of the nine plaque samples yielding PLC-active isolates.

Only patients of strictly characterized status were included in the clinical studies on AP and RPP using culture-independent methods. The results of this and a previous report (Moter *et al*., 1998) provide evidence

for an association of group IV treponemes with AP and even more clearly with RPP. The association of *T. denticola* with specific forms of periodontitis appeared less pronounced than in previous studies (Riviere *et al.*, 1992). The same 244 RPP plaque samples have been analysed in this and a previous study (Moter *et al.*, 1998); of the 217 samples containing TREIV-complementary sequences, both *T. maltophilum* and *T. lecithinolyticum* were detected in 69 plaque samples, whereas neither of these two phylotypes were detected in 64 of the plaque samples. These latter samples may contain other as yet uncultured members of phylotype group IV.

In conclusion, our physiological and molecular epidemiological data suggest that small PLA- and PLC-active oral treponemes, comprising the proposed novel species *T. lecithinolyticum*, may contribute to the development and/or progression of RPP and, to a lesser degree, of AP.

Description of *Treponema lecithinolyticum* sp. nov.

Treponema lecithinolyticum (le.ci.thi.no.ly′ti.cum. Gr. n. *lekithos* egg yolk; Gr. v. *lytikos* able to loose, dissolve; M.L. adj. *lecithinolyticum* effecting the breakdown of egg yolk).

An obligately anaerobic, helically coiled, motile treponeme. Cells are approximately $5 \times 0{\cdot}15$ μm, with a wavelength of 0·7 μm and an amplitude of 0·3 μm. They contain two periplasmic flagella, one originating at each cell end and overlapping in the central region of the cell. In liquid media, flexing and rotation of cells does not appear to result in directional motility. However, in media of higher viscosity, or when cells creep along a surface, translational movement is readily detectable. Cells can be stored frozen (liquid nitrogen or mechanical freezer) in OMIZ-Pat-w/oPC medium supplemented with 10–20 % glycerol.

When streaked onto OMIZ-Pat-w/oPC agarose, *T. lecithinolyticum* forms off-white, diffuse subsurface colonies up to 3 mm in diameter within 7 d incubation at 37 °C. *T. lecithinolyticum* does not grow in the chemically defined medium OMIZ-W1, but requires addition of yeast extract and/or Neopeptone (or fractions thereof). Growth is inhibited by lecithin, which must be omitted from the media formulation (OMIZ-Pat-w/oPC). Growth is strictly dependent on *N*-acetylglucosamine and is strongly enhanced by further addition of D-arabinose, L-fucose or D-ribose; some strains also respond to D-fructose (excluding OMZ 684^T) and/or to D-xylose (including OMZ 684^T). Growth is not influenced by L-arabinose, D-cellobiose, D-fucose, D-galactose, D-galacturonic acid, D-glucose, D-glucuronic acid, D-lactose, D-maltose, D-mannitol, D-melibiose, L-rhamnose, L-sorbose, D-sucrose, D-trehalose or L-xylose. Heat-inactivated human serum (1 % v/v) is tolerated or stimulatory, whereas 1 % foetal calf serum is completely inhibitory. All strains are resistant to rifampicin (1 μg ml^{-1}) and fosfomycin (100 μg ml^{-1}).

In all eight isolates, activities of alkaline phosphatase, acid phosphatase, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, phospholipase A and phospholipase C are prominent, whereas only intermediate activities of C4-esterase, C8-esterase, naphthol phosphohydrolase and α-fucosidase are expressed. Catalase is not produced. OMZ 684^T, OMZ 685 and BL2B have strong sialidase activity (the other five strains were not tested).

T. lecithinolyticum is phylogenetically distinct from other cultivable treponemes on the basis of its 16S rRNA sequence. Protein and antigen patterns (SDS-PAGE) are also readily distinguished from those of other cultivable treponemes, though more conventional criteria may suffice to distinguish it from the seven previously characterized oral spirochaetes. Simultaneous expression of strong activities of phospholipase C, phospholipase A, alkaline phosphatase, acid phosphatase, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase and sialidase and intermediate activities of C4-esterase, C8-esterase, naphthol phosphohydrolase and α-fucosidase distinguish *T. lecithinolyticum* from all other oral spirochaetes. Size, flagellation and growth characteristics additionally distinguish it from *T. amylovorum*, *T. denticola*, *T. medium* and '*T. vincentii*'. Finally, *T. lecithinolyticum* is phenotypically distinguished from the two other lecithinolytic isolates described in this report, OMZ 702 and BL2A (which are phylogenetically classified as *T. maltophilum*), by its protein profile after SDS-PAGE, by a prominent antigen of ~ 30 kDa and by activities of phospholipase A, sialidase, β-glucuronidase and *N*-acetyl-β-glucosaminidase.

T. lecithinolyticum has thus far been detected only in human subgingival plaque, with a strong association suggested for diseased versus control sites in patients with adult periodontitis and rapidly progressive periodontitis. Strains OMZ 684^T and OMZ 685, isolated from subgingival plaque of human deep periodontal lesions, have been designated as type and reference strains and deposited under accession numbers ATCC 700332^T and ATCC 700333, respectively.

ACKNOWLEDGEMENTS

We thank C. Weiss, V. Zängerle and M. Kachler for expert technical assistance and S. Shapiro for critical review of the manuscript. We also thank two anonymous reviewers for constructive comments. This work was supported in part by a grant to U. B. G. from the Deutsches Bundesministerium für Bildung und Forschung.

REFERENCES

Burnens, A. P. & Nicolet, J. (1992). Detection of *Campylobacter upsaliensis* in diarrheic dogs and cats, using a selective medium with cefoperazone. *Am J Vet Res* **53**, 48–51.

Caldwell, D. E., Atuku, E., Wilkie, D. C., Wivcharuk, K. P., Karthikeyan, S., Korber, D. R., Schmid, D. F. & Wolfaardt, G. M.

(1997). Germ theory vs. community theory in understanding and controlling the proliferation of biofilms. *Adv Dent Res* **11**, 4–13.

Chan, E. C. S., Siboo, I. R. & Siboo, R. (1991). Purification of phospholipase C by hydrophobic interaction affinity chromatography. *J Chromatogr* **568**, 85–92.

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.

Divecha, N. & Irvine, R. F. (1995). Phospholipid signaling. *Cell* **80**, 269–278.

Falo, L. D., Jr, Haber, S. I., Herrmann, S., Benacerraf, B. & Rock, K. L. (1987). Characterization of antigen association with accessory cells: specific removal of processed antigens from the cell surface by phospholipases. *Proc Natl Acad Sci USA* **84**, 522–526.

Freeman, S. J., Shankaran, P., Wolfe, L. S. & Callahan, J. W. (1985). Phosphatidylcholine and 4-methylumbelliferyl phosphorylcholine hydrolysis by purified placental sphingomyelinase. *Can J Biochem Cell Biol* **63**, 272–277.

Frey, J., Perrin, J. & Nicolet, J. (1989). Cloning and expression of a cohemolysin, the CAMP factor of *Actinobacillus pleuropneumoniae*. *Infect Immun* **57**, 2050–2056.

Grant, K. A., Belandia, I. U., Dekker, N., Richardson, P. T. & Park, S. F. (1997). Molecular characterization of *pldA*, the structural gene for a phospholipase A from *Campylobacter coli*, and its contribution to cell-associated hemolysis. *Infect Immun* **65**, 1172–1180.

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

Hendrickson, H. S. (1994). Fluorescence-based assays of lipases, phospholipases, and other lipolytic enzymes. *Anal Biochem* **219**, 1–8.

Holt, S. C. & Bramanti, T. E. (1991). Factors in virulence expression and their role in periodontal disease pathogenesis. *Crit Rev Oral Biol Med* **2**, 177–281.

Jolivet-Reynaud, C., Moreau, H. & Alouf, J. E. (1988). Assay methods for alpha toxin from *Clostridium perfringens*: phospholipase C. *Methods Enzymol* **165**, 293–297.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Kurioka, S. & Matsuda, M. (1976). Phospholipase C assay using *p*-nitrophenylphosphoryl-choline together with sorbitol and its application to studying the metal and detergent requirement of the enzyme. *Anal Biochem* **75**, 281–289.

Merck, K. B., de Cock, H., Verheij, H. M. & Tommassen, J. (1997). Topology of the outer membrane phospholipase A of *Salmonella typhimurium*. *J Bacteriol* **179**, 3443–3450.

Moore, W. E. C., Moore, L. H., Ranney, R. R., Smibert, R. M., Burmeister, J. A. & Schenkein, H. A. (1991). The microflora of periodontal sites showing active destructive progression. *J Clin Periodontol* **18**, 729–739.

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

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

Page, R. C., Altman, L. C., Ebersole, J. L., Vandesteen, G. E., Dahlberg, W. H., Williams, B. L. & Osterberg, S. K. (1983). Rapidly progressive periodontitis. A distinct clinical condition. *J Periodontol* **54**, 197–209.

Paster, B. J., Dewhirst, F. E., Weisburg, W. G. & 7 other authors (1991). Phylogenetic analysis of the spirochetes. *J Bacteriol* **173**, 6101–6109.

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.

Pickett, J. & Kelly, R. (1974). Lipid catabolism of relapsing fever borreliae. *Infect Immun* **9**, 279–285.

Prescott, S. M., Zimmerman, G. A. & McIntyre, T. M. (1990). Platelet-activating factor. *J Biol Chem* **265**, 17381–17384.

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., Elliot, K. S., Adams, D. F., Simonson, L. G., Forgas, L. B., Nilius, A. M. & Lukehart, S. A. (1992). Relative proportions of pathogen-related oral spirochetes (PROS) and *Treponema denticola* in supragingival and subgingival plaque from patients with periodontitis. *J Periodontol* **63**, 131–136.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Schrank, K., Choi, B.-K., Grund, S., Moter, A., Heuner, K., Nattermann, H. & Göbel, U. B. (1999). *Treponema brennaborense* sp. nov., a novel spirochaete isolated from a dairy cow suffering from digital dermatitis. *Int J Syst Bacteriol* **49**, 43–50.

Serhan, C. N., Haeggström, J. Z. & Leslie, C. C. (1996). Lipid mediator networks in cell signaling: update and impact of cytokines. *FASEB J* **10**, 1147–1158.

Siboo, R., al-Joburi, W., Gornitsky, M. & Chan, E. C. S. (1989). Synthesis and secretion of phospholipase C by oral spirochetes. *J Clin Microbiol* **27**, 568–570.

Slomiany, B. L., Piotrowski, J. & Slomiany, A. (1992). Effect of sucralfate on the degradation of human gastric mucus by *Helicobacter pylori* protease and lipases. *Am J Gastroenterol* **87**, 595–599.

Smibert, R. M., Johnson, J. L. & Ranney, R. R. (1984). *Treponema socranskii* sp. nov., *Treponema socranskii* subsp. *socranskii* subsp. nov., *Treponema socranskii* subsp. *buccale* subsp. nov., and *Treponema socranskii* subsp. *paredis* subsp. nov. isolated from the human periodontia. *Int J Syst Bacteriol* **34**, 457–462.

Socransky, S. S. & Haffajee, A. D. (1991). Microbial mechanisms in the pathogenesis of destructive periodontal diseases: a critical assessment. *J Periodontal Res* **26**, 195–212.

Songer, J. G. (1997). Bacterial phospholipases and their role in virulence. *Trends Microbiol* **5**, 156–161.

Tigyi, G. & Miledi, R. (1992). Lysophosphatidates bound to serum albumin activate membrane currents in *Xenopus* oocytes and neurite retraction in PC12 pheochromocytoma cells. *J Biol Chem* **267**, 21360–21367.

Titball, R. W. (1993). Bacterial phospholipases C. *Microbiol Rev* **57**, 347–366.

Trevathan, C. A., Smibert, R. M. & George, H. A. (1982). Lipid catabolism of cultivated treponemes. *Can J Microbiol* **28**, 672–678.

Umemoto, T., Nakazawa, F., Hoshino, E., Okada, K., Fukunaga, M. & Namikawa, I. (1997). *Treponema medium* sp. nov., isolated

from human subgingival dental plaque. *Int J Syst Bacteriol* **47**, 67–72.

Van de Peer, Y. & De Wachter, R. (1993). TREECON: a software package for the construction and drawing of evolutionary trees. *Comput Appl Biosci* **9**, 177–182.

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.