

Dialister invisus sp. nov., isolated from the human oral cavity

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Six strains of anaerobic, Gram-negative coccobacilli isolated from the root canals of patients with endodontic infections (five strains) and from a deep periodontal pocket (one strain) were subjected to a comprehensive range of phenotypic and genetic tests and were found to comprise a homogeneous group. Following 16S rRNA gene sequence analysis, they were found to be most closely related to *Dialister pneumosintes*, with 93% sequence similarity between the two taxa. A novel species, *Dialister invisus* sp. nov., is proposed. Biochemically, the species is largely unreactive and is asaccharolytic, with only traces of acetate and propionate detected as metabolic end-products. The G + C content of the DNA of *D. invisus* strains is 45–46 mol%. The type strain is E7.25^T (= CCUG 47026^T = DSM 15470^T).

Despite the taxonomic advances of recent years, a substantial number of taxa isolated from the human oral cavity remain unnamed. For example, it has long been recognized that there is a group of anaerobic, Gram-negative, small coccobacilli that are unreactive in commonly used biochemical tests. In a study of the microflora associated with periocoronitis, four isolates of this group were isolated from three patients (Wade *et al.*, 1991). Subsequent 16S rRNA gene sequence analysis of these strains revealed their identity to be *Dialister pneumosintes* (Milsom, 1997; Milsom *et al.*, 1996). This species was originally named as '*Bacterium pneumosintes*' (Olitsky & Gates, 1921), before being transferred to the genus *Dialister* (Bergey *et al.*, 1923). The species was subsequently moved to the genus *Bacteroides* (Holdeman & Moore, 1970), but the name *Dialister* was revived by Moore & Moore (1994). The species was formerly included in the family *Bacteroidaceae*, by virtue of being a Gram-negative, anaerobic bacillus, but a phylogenetic analysis of 16S rRNA gene sequences by Willems & Collins (1995) showed that the species belonged to the *Sporomusa* sub-branch of the Gram-positive bacterial phylum and was closely related to the genus *Veillonella*.

D. pneumosintes is a frequent isolate from the oral cavity and has been implicated in periodontitis (Ghayoumi *et al.*, 2002). It is also found at non-oral sites as part of mixed flora associated with purulent infections, including brain

abscesses and bite-wound infections (Goldstein *et al.*, 1984; Rousee *et al.*, 2002).

The introduction of culture-independent analyses of the oral microflora and the concomitant use of 16S rRNA gene sequence analysis for identification have led to increased precision in the identification of isolates from oral infections (Dymock *et al.*, 1996; Kroes *et al.*, 1999; Paster *et al.*, 2001). Such analyses have revealed that there are a number of taxa related to *D. pneumosintes* that are commonly isolated from oral infections. One of these, designated *Dialister* E1, was found to be the only organism detected in each of five samples obtained from chronic endodontic lesions (Munson *et al.*, 2002).

The aim of this study was to characterize six isolates of taxon *Dialister* E1 by a variety of phenotypic and genetic tests. *Dialister* E1 strains E2.20, E3.07, E7.25^T, E9.48 and E10.39 were isolated from the root canals of patients with endodontic infections. In each case, the strains were recovered together with a variety of other, predominantly anaerobic, taxa (Munson *et al.*, 2002). Strain P2.65 was isolated from a periodontal pocket (8 mm deep) in an adult with periodontitis. *D. pneumosintes* ATCC 33048^T was obtained from the ATCC (Manassas, VA, USA). *D. pneumosintes* strains E9.19 and E10.18 were isolated from endodontic infections. Strains were grown at 37 °C on fastidious anaerobe agar (FAA; LabM) supplemented with 5% horse blood under anaerobic conditions (80:10:10, N₂/H₂/CO₂). Colonial morphologies were determined using a plate microscope after 7 days incubation. Cellular morphology was recorded after Gram-staining of 3-day plate cultures. Transmission electron microscopy was used to examine the cell-wall ultrastructure as described previously (Downes *et al.*, 2002). Fermentation tests were performed

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Abbreviation: PRAS, pre-reduced anaerobically sterilized.

The GenBank accession number for the 16S rRNA gene sequence of *Dialister invisus* E7.25^T is AY162469.

using pre-reduced, anaerobically sterilized (PRAS) sugars (Holdeman *et al.*, 1977) and other biochemical tests, including metabolic end-product analysis by GC, were performed as described by Holdeman *et al.* (1977) and Summanen *et al.* (1993). In addition, Rosco diagnostic tablets (Rosco Diagnostica) were used, according to the manufacturer's instructions, to test for the following: aesculin and arginine hydrolysis, indole and urease production and fermentation of glucose, lactose, mannitol, melibiose, sorbitol and trehalose. Susceptibility to special-potency antibiotic discs (vancomycin, 5 µg; kanamycin, 1 mg; colistin, 10 µg) was determined on FAA (Summanen *et al.*, 1993). Temperature optima for growth were determined after 5 days incubation on FAA at 25, 30, 37, 42 and 45 °C. Profiles of whole-cell proteins were generated by SDS-PAGE using 10–15% gradient gels and the PhastSystem (Pharmacia) as described previously (Slayne *et al.*, 1990). Enzyme profiles were generated with the Rapid ID 32A anaerobe identification kit (bioMérieux) according to the manufacturer's instructions. The G + C content of the DNA was estimated by using an HPLC method as described previously (Wade *et al.*, 1999). A thermal denaturation method (Huß *et al.*, 1983) was used to determine the DNA–DNA relatedness between strains.

16S rRNA gene sequences were determined as described previously (Downes *et al.*, 2002). Sequences were submitted to Ribosomal Database Project II (Maidak *et al.*, 2001) for provisional identification using the program SEQUENCE_MATCH. From the phylogenetic position indicated by SEQUENCE_MATCH, related sequences were selected from sequence databases and aligned by means of CLUSTAL X (Thompson *et al.*, 1997). Further analysis was performed using the PHYLIP suite of programs (Felsenstein, 1993). Specifically, DNADIST was used to compare sequences, using the Jukes–Cantor algorithm, and NEIGHBOR was used to construct phylogenetic trees, which were viewed using TreeView (Page, 1996).

The 16S rRNA gene sequences of *Dialister* E1 strains E2.20, E3.07 and P2.65 had >99.5% sequence similarity to that of strain E7.25^T over 1504 bp. The two closest matches (both >99.5% sequence similarity) for the 16S rRNA gene sequence of strain E7.25^T were *Dialister* isolate GBA27 and *Dialister* clone BS095 from subgingival plaque (Paster *et al.*, 2001). Phylogenetic analysis revealed that the most closely related named species (93% sequence similarity) was *D. pneumosintes* within the family 'Acidaminococcaceae' (Fig. 1). The relatedness between DNA preparations of E7.25^T and *D. pneumosintes* ATCC 33048^T was found to be 42%.

The *Dialister* E1 strains were Gram-negative, obligately anaerobic, non-motile coccobacilli (0.3–0.4 × 0.3–0.6 µm), occurring singly, in pairs, in short chains and in small clumps. Their cellular morphology therefore resembled that of *D. pneumosintes*. After 7 days incubation on FAA plates, *Dialister* E1 colonies were 0.5–0.7 mm in diameter, circular and entire. Colony morphology varied from translucent, umbonate colonies to transparent, low-convex colonies with

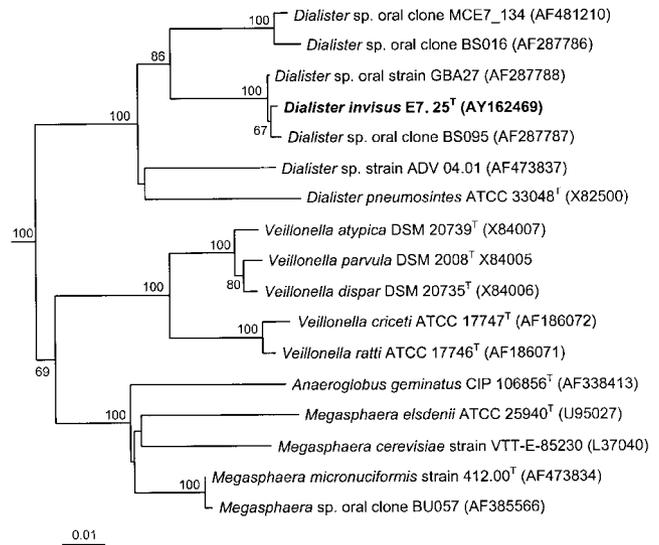


Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequence comparisons over 1299 aligned bases, showing the relationship between *D. invisus* sp. nov. and related species. The tree was constructed using the neighbour-joining method after distance analysis of aligned sequences. Numbers represent bootstrap values for each branch, based on data for 100 trees. Accession numbers for 16S rRNA sequences are given for each strain. The scale bar shows the number of nucleotide substitutions per site.

a narrow, marginal, translucent fringe. *D. pneumosintes* strains exhibited larger colonies, 1.0–1.2 mm in diameter. Transmission electron microscopic examination of ultrathin sections through *Dialister* E1 cells showed the presence of a typical Gram-negative cell wall composed of a thin peptidoglycan layer surrounded by an outer membrane (Fig. 2).

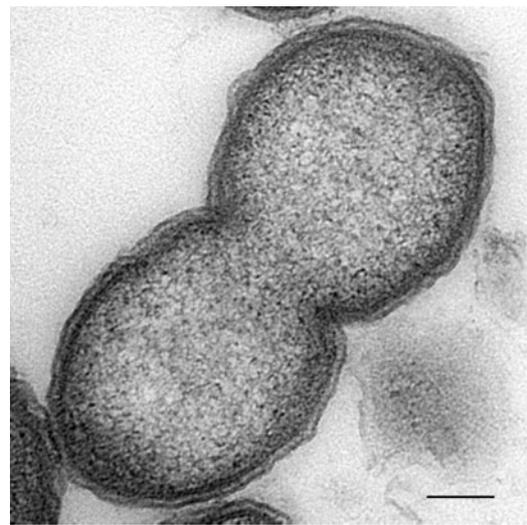


Fig. 2. Transmission electron micrograph of *D. invisus* E7.25^T: ultrathin section showing the Gram-negative cell wall and the cytoplasmic membrane. Bar, 100 nm.

The growth of both *Dialister* E1 and *D. pneumosintes* strains in peptone/yeast extract broth produced only a slight turbidity that was not visibly improved by the addition of 1% (w/v) carbohydrates. *Dialister* E1 strains were asaccharolytic and did not ferment arabinose, cellobiose, fructose, glucose, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose or xylose in the PRAS system. The use of PRAS media relies on growth of the organism in the system to determine reactions, and growth giving rise to at least 2+ turbidity (on a scale of 0 to 4+) is recommended for results to be reliable. In the case of the strains studied here, however, growth in PRAS media resulted in only slight turbidity. This lack of visible growth may not necessarily mean that the organism is not growing in the broth media, and may be due to the small size of the bacterial cells and their consequent poor light-refractive qualities. A growth-independent system (Rosco diagnostic tablets) that utilizes preformed enzymes was used to overcome this problem. However, the results obtained from the Rosco system confirmed those from the PRAS media, in that acid was not produced from glucose, lactose, mannitol, melibiose, sorbitol or trehalose. Trace amounts of acetate and propionate were detected as end-products of metabolism in peptone/yeast extract/glucose medium. Aesculin, arginine and urea were not hydrolysed and indole and catalase were not produced. There was no growth in 20% bile. Equivalent growth occurred at 30 and 37 °C and growth was only slightly reduced for some strains at 42 °C, whereas no significant growth was detected at 25 or 45 °C. The *Dialister* E1 strains were unreactive for all tests in the Rapid ID 32A identification system, resulting in a Rapid ID 32A profile of 0000 0000 00. *D. pneumosintes* strains ATCC 33048^T, E9.19 and E10.18 all gave a profile of 0000 0100 00; the single positive reaction was for arginine arylamidase. All *Dialister* E1 strains were resistant to vancomycin and sensitive to kanamycin and colistin, whereas the type strain and two clinical isolates of *D. pneumosintes* were resistant to colistin, confirming the findings of Doan *et al.* (2000), who reported that 9/9 clinical isolates of *D. pneumosintes* were resistant to colistin. The G+C contents of the DNAs of strains E7.25^T and E2.20 were 45 and 46 mol%, respectively. The G+C content of the DNA of *D. pneumosintes* ATCC 33048^T was 35 mol%. Protein profiles of strains E2.20, E7.25^T, E9.48 and *D. pneumosintes* ATCC 33048^T are shown in Fig. 3. The profiles for E2.20, E7.25^T and E9.48 were virtually identical, but showed significant differences with respect to that of *D. pneumosintes*.

The relatively large differences in DNA G+C content and 16S rRNA gene sequence similarity between *Dialister* E1 and *D. pneumosintes* could be interpreted as indicating that these two taxa should be placed in different genera. However, because of the lack of phenotypic differences between the taxa, we propose that the novel taxon should be named as a novel species within the genus *Dialister*. We therefore propose the name *Dialister invisus* sp. nov. for strains formerly described as *Dialister* E1. There appear to be additional novel taxa in this branch of the phylogenetic tree.

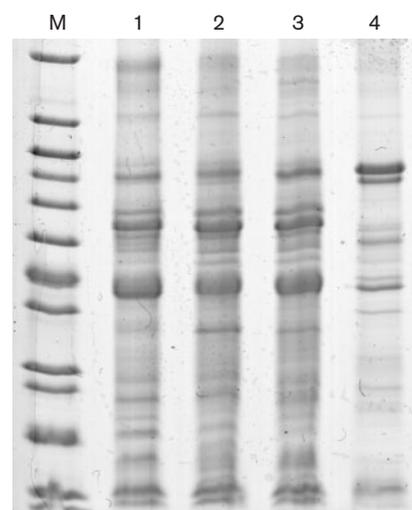


Fig. 3. Protein profiles of *D. invisus* and *D. pneumosintes* strains. Lanes: 1, *D. invisus* E2.20; 2, *D. invisus* E7.25^T; 3, *D. invisus* E9.48; 4, *D. pneumosintes* ATCC 33048^T. M, Molecular mass markers (6.5, 14.2, 20.1, 24, 29, 36, 45, 55, 66, 84, 97.4, 116 and 205 kDa).

Fig. 1 shows two cloned sequences, MCE7_134 and BS016, respectively recovered from an endodontic infection and subgingival plaque. The 16S rRNA gene sequences of these clones were very similar (>99% relatedness): BS016 exhibited 91% relatedness to *D. pneumosintes* and 94% to *D. invisus*. They therefore appear to represent another novel *Dialister* taxon, but since no cultivable representatives of this group have yet been identified, no species proposal is possible at present.

Emended description of *Dialister* (ex Bergey *et al.* 1923) Moore and Moore 1994

Dialister (Di.a.lis'ter. Etymology unknown).

Cells are 0.2–0.4 × 0.3–0.6 μm, Gram-negative, obligately anaerobic, non-motile, non-sporing and non-fermentative. Growth in broth media is only slightly turbid at best. Aesculin and urea are not hydrolysed; indole and catalase are not produced. There is no growth in 20% bile. The G+C content of the DNAs ranges from 35 to 46 mol%. The major cellular constituents of the type species *D. pneumosintes* include fatty acids C_{18:1}cis-9, C_{16:0}, C_{18:0} and C_{16:1}cis-9. The cellular constituents of the other species in the genus, *D. invisus*, have not been determined. The type species is *Dialister pneumosintes* (Olitsky and Gates 1921) Moore and Moore 1994.

Description of *Dialister invisus* sp. nov.

Dialister invisus (in.vis'us. L. masc. adj. *invisus* unseen, referring to the lack of turbidity of broth cultures of this organism).

The etymology, and therefore the gender, of the genus name is unknown. We therefore propose that the gender of the genus name be assigned as male, as allowed under Rule 65(3) of the *Bacteriological Code*, and hence the male form of the adjective selected as the specific epithet is given.

The description is based on six strains isolated from the human oral cavity. Cells are obligately anaerobic, non-motile, Gram-negative, small cocci or ovoid cocci (0.3–0.4 × 0.3–0.6 µm) that occur singly, in pairs, in short chains and in small clumps. After 7 days incubation on FAA plates, colonies are 0.5–0.7 mm in diameter, circular, entire and either translucent and umbonate or transparent and low-convex with a narrow marginal, translucent fringe. Growth in broth media produces only a slight turbidity and is not visibly stimulated by the addition of 1% carbohydrates. Cells are asaccharolytic and only trace amounts of acetate and propionate are detected as end products of metabolism in peptone/yeast extract/glucose medium. Aesculin, arginine and urea are not hydrolysed. Indole and catalase are not produced. No growth in 20% bile. The G + C content of the DNA of the type strain is 45 mol%.

The type strain is E7.25^T (= CCUG 47026^T = DSM 15470^T). Isolated from the human oral cavity in patients with endodontic and periodontal infections.

Emended description of *Dialister pneumosintes* (Olitsky and Gates 1921) Moore and Moore 1994

The description is as described by Moore & Moore (1994), with the following additions: aesculin and urea are not hydrolysed; indole and catalase are not produced. There is no growth in 20% bile. The Rapid ID 32A profile is 0000 0100 00, which corresponds to a positive reaction for arginine arylamidase. The G + C content of the DNA is 35 mol%.

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