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Propionibacterium acidifaciens sp. nov., isolated from the human mouth

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Three strains of anaerobic, pleomorphic, Gram-positive-staining bacilli, which were isolated from human carious dentine, were subjected to a comprehensive range of phenotypic and genotypic tests and were found to comprise a homogeneous group. The strains were saccharolytic and produced acetic and propionic acids in large amounts, and succinic acid in moderate amounts, as the end products of fermentation. 16S rRNA gene and RpoB protein sequence analyses revealed that the strains constituted a novel group within the genus *Propionibacterium*, most closely related to *Propionibacterium australiense* but sharing only 8 % DNA–DNA relatedness with the type strain of that species. Therefore, a novel species, *Propionibacterium acidifaciens* sp. nov., is proposed to accommodate these strains. The DNA G+C content of the type strain is 70 mol%. The type strain is C3M_31^T (=DSM 21887^T =CCUG 57100^T).

Dental caries is one of the commonest bacterial diseases of man and results from the dissolution of tooth enamel by acids produced by bacterial members of the dental plaque biofilm (Bowden & Edwardsson, 1994). *Streptococcus mutans* is the organism principally implicated in the pathogenesis of the disease, although many other bacterial species are capable of playing a similar pathogenic role in producing acid from fermentable carbohydrates and being tolerant of acidic conditions (Beighton, 2005). If carious lesions are not treated, the destruction of a tooth can continue into the dentine. Obligate anaerobes predominate in carious dentine and proteolytic activity may be important in the progression of the lesion as it approaches the pulp, where the bacteria can utilize proteins leaking into the dentine (Chhour *et al.*, 2005). Members of the genus *Propionibacterium* are commonly detected in carious dentine (Aas *et al.*, 2008; Chhour *et al.*, 2005; Edwardsson, 1974; Hoshino, 1985), with *Propionibacterium acnes* being the species most commonly reported.

In a combined cultural and molecular analysis of the microbiota in dentinal caries, we found that an as-yet unnamed taxon was the dominant *Propionibacterium* taxon present and was one of only three taxa at species level to be found in every lesion (Munson *et al.*, 2004). The aim of the present study was to perform a genetic and phenotypic characterization of three strains that are representative of this unnamed *Propionibacterium* taxon.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains C3M_31^T, C2A_34 and C5A_34 are EU979537, FJ746023 and FJ746024, respectively, and those for the *rpoB* gene sequences of strains C3M_31^T and C5A_34 are FJ409978 and FJ746025, respectively.

Strains C3M_31^T, C2A_34 and C5A_34 were isolated from the dentine of carious lesions in three subjects, as described previously (Munson *et al.*, 2004). Strains were grown at 37 °C on fastidious anaerobe agar (FAA; LabM) supplemented with 5 % horse blood in an anaerobic workstation (Don Whitley Scientific) under anaerobic conditions (80 % N_2/10 % H_2/10 % CO_2). Colonial morphologies of 6-day-old FAA plate cultures were viewed by means of a dissecting microscope. The morphology of Gram-stained cells harvested from 2-day-old FAA plate cultures was determined by light microscopy at ×1000 magnification. Cell motility in hanging-drop preparations of 18 h cultures from peptone-yeast extract-glucose (PYG) broth was determined by examination using phase-contrast microscopy at ×400 magnification.

Biochemical and physiological tests were performed using standard methods (Holdeman *et al.*, 1977; Jousimies-Somer *et al.*, 2002). Fermentation tests were performed using prereduced, anaerobically sterilized sugars prepared in an anaerobic workstation (Holdeman *et al.*, 1977). Bile sensitivity was determined by growth on tryptone soy agar (TSA; Oxoid) with and without 2 % oxgall (Sigma) (equivalent to 20 % bile). Susceptibility to special-potency antibiotic discs containing vancomycin (5 μg), kanamycin (1 mg) and colistin (10 μg) was determined on FAA (Jousimies-Somer *et al.*, 2002). Presence and amounts of volatile and non-volatile fatty acids produced as metabolic end products in 7-day-old PYG broth cultures were determined by gas chromatography (Holdeman *et al.*, 1977). Enzyme profiles were generated with the Rapid ID 32A anaerobe identification kit (bioMérieux), according to the manufacturer's instructions, using bacteria harvested from 2-day-old Columbia agar plates (LabM) supplemented with 5 % horse blood.

Table 1. Actinobacterial genome sequences used for the design of *rpoB* gene-specific PCR primers

Strain	Accession number
Propionibacterium acnes KPA171202	AE017283
Streptomyces avermitilis MA-4680^T	BA000030
Kineococcus radiotolerans SRS30216^T	CP000750
Saccharopolyspora erythraea NRRL 2338^T	AM420293
Nocardioides sp. JS614	CP000509
Mycobacterium vanbaalenii PYR-1^T	CP000511
Rhodococcus jostii RHA1	CP000431
Kocuria rhizophila DC2201	AP009152
Nocardia farcinica IFM 10152	AP006618
Frankia sp. EAN1pec	CP000820
Corynebacterium jeikeium K411	CR931997
Arthrobacter sp. FB24	CP000454
Salinispora arenicola CNS-205	CP000850
Bifidobacterium adolescentis ATCC 15703^T	AP009256

The G+C content of the DNA of strain C3M_31^T was determined by an HPLC method as described previously (Wade *et al.*, 1999). The 16S rRNA genes of the strains were sequenced as described previously (Downes *et al.*, 2005). Sequences were assembled with the BioEdit program (Hall, 2004) and their closest relatives were determined by BLAST interrogation of the GenBank database (Altschul *et al.*, 1990). Sequences were aligned using CLUSTAL W within BioEdit and phylogenetic trees were constructed in MEGA version 4 (Tamura *et al.*, 2007) using the neighbour-joining method from distance matrices prepared with the Jukes–Cantor correction. Pairwise estimations of DNA–DNA relatedness were performed using a thermal denaturation method (Huß *et al.*, 1983). Primers that were specific for part of the *rpoB* gene were designed from an alignment of *rpoB* sequences from actinobacterial genome sequences, as detailed in Table 1. The primers were Prop_rpoB_551F, 5′-GCACCTTCATYATCAACGGCAC-3′, and Prop_rpoB_1489R, 5′-CACATVCGRCCGTAGTG-3′, and were predicted to give an amplicon of around 950 bases. The amplified genes were sequenced and translated and a dendrogram based on the amino acid sequences was constructed in MEGA with the neighbour-joining method using the Poisson correction.

The results of the phenotypic tests are given in the species description. The three strains were anaerobic, non-motile, non-spore-forming, Gram-positive-staining bacilli that were 0.7–0.8 μm wide by 1.2–4 μm long. Cells were pleomorphic with straight, slightly curved or club-shaped forms, arranged singly, in pairs or in short chains with some branched diphtheroidal arrangements. After anaerobic incubation for 6 days on FAA plates, colonies were 0.7–1.1 mm in diameter, circular, entire, high-convex to dome-shaped, smooth, shiny, white to pale cream and opaque with a solid non-translucent internal appearance when viewed under a plate microscope. There was no detectable growth on FAA plates incubated in air with 5 % CO_2. The strains grew on TSA with 20 % bile and the amount of growth was similar to that on TSA without bile, although the size of the colonies was reduced in the presence of bile. Strains were sensitive to vancomycin and kanamycin but resistant to colistin. There was good growth of all strains in peptone-yeast extract broth (3+ on a scale of 0–4+). Growth was enhanced markedly (4+) by the addition of 1 % fermentable carbohydrates.

Strains were saccharolytic and produced acid from fructose, glucose, lactose, maltose, mannitol, mannose and sucrose, resulting in a final pH of 4.37–4.79. Fermentation of melibiose, raffinose, rhamnose and ribose produced a final pH of 5.07–5.60. L-Arabinose, cellobiose, melezitose and salicin were not fermented (pH 6.06–6.72).

Table 2. Phenotypic characteristics that differentiate *Propionibacterium acidifaciens* sp. nov. from other *Propionibacterium* species

Strains: 1, *Propionibacterium acidifaciens* sp. nov. (data from this study for three strains including C3M_31^T); 2, *P. acidipropionici* (four strains including ATCC 25562^T); 3, *P. acnes* (several hundred strains including ATCC 6919^T); 4, *P. australiense* (Bernard *et al.*, 2002; six strains including CCUG 46075^T); 5, *P. avidum* (43 strains including ATCC 25577^T); 6, *P. cyclohexanicum* ATCC 700429^T (Kusano *et al.*, 1997); 7, *P. freudenreichii* (several strains including ATCC 6207^T); 8, *P. granulosum* (68 strains including ATCC 25564^T); 9, *P. jensenii* (13 strains including ATCC 4868^T); 10, *P. microaerophilum* DSM 13435^T (Koussemon *et al.*, 2001); 11, *P. propionicum* (several strains including ATCC 14157^T); 12, *P. thoenii* (three strains including ATCC 4874^T). +, Positive; −, negative; V, variable; ND, no data available. Unless indicated, data were obtained from Cummins & Johnson (1986).

Character	1	2	3	4	5	6	7	8	9	10	11	12
Hydrolysis of:												
Aesculin	−	+	−	+	+	+	+	−	+	−	−	+
Gelatin	−	−	+	−	+	−	−	−	−	ND	V	−
Production of:												
Catalase	−	V	V	−	+	−	+	+	V	−	−	+
Indole	−	−	+	−	−	−	−	−	−	ND	−	−
Reduction of nitrate	−	+	+	+	−	−	V	−	−	+	+	−
Fermentation of sucrose	+	+	−	−	+	+	−	+	+	ND	+	+

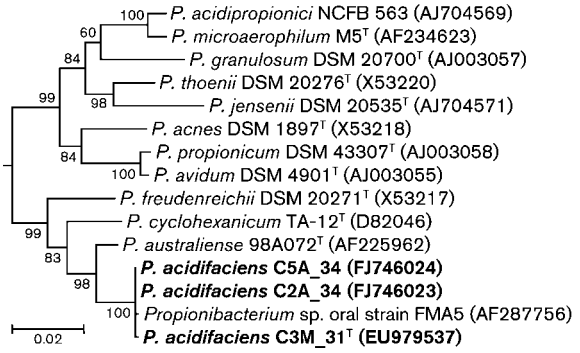

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequence comparisons of 1321 aligned bases and constructed using the neighbour-joining method, showing the relationship between strains of *Propionibacterium acidifaciens* sp. nov. and related *Propionibacterium* species. Percentages at nodes are levels of bootstrap support based on 500 resamplings. Bar, 2 substitutions per 100 nucleotide positions.

The DNA G+C content of strain C3M_31^T was 70 mol%. The phenotypic characteristics that distinguish these strains from the type strains of related *Propionibacterium* species are shown in Table 2.

All three strains gave positive reactions in the Rapid ID 32A panel for α-galactosidase, β-galactosidase, α-glucosidase, arginine arylamidase, proline arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase and fermentation of mannose and raffinose, resulting in a profile of 4506 0331 00.

The almost-complete sequences of the 16S rRNA genes of all three strains were obtained. Over 1442 unambiguously aligned bases, the sequences of strain C2A_34 and C5A_34 differed from that of C3M_31^T by one and two bases, respectively. Phylogenetic analysis of the 16S rRNA gene sequence of strain C3M_31^T revealed that this organism belonged to the genus *Propionibacterium* (Fig. 1), with >99.5 % sequence identity to subgingival plaque strain *Propionibacterium* sp. oral strain FMA5. The most closely related type strain was *Propionibacterium australiense* 98A072^T, with which strain C3M_31^T shared 97.7 % sequence identity. However, the DNA–DNA relatedness value between strain C3M_31^T and *P. australiense* CCUG 46075^T was 8±4 %, indicating that the group of strains under study constitutes a novel species. The phylogenetic distinctiveness of C3M_31^T was confirmed by RpoB protein sequence analysis with species from within the phylum *Actinobacteria*, which showed strain C3M_31^T to be most closely related to *Propionibacterium acnes* KPA171202 (Fig. 2). Partial *rpoB* gene sequences for strains C3M_31^T and C5A_34 were identical over 789 bases.

The strains constitute a homogeneous group and are clearly distinct from all species of the genus *Propionibacterium* with validly published names. We therefore propose the name *Propionibacterium acidifaciens* sp. nov.

Description of *Propionibacterium acidifaciens* sp. nov.

Propionibacterium acidifaciens (a.ci.di.fa′ci.ens. N.L. n. *acidum* acid; L. v. *facio* to produce; N.L. part. adj. *acidifaciens* acid-producing).

The description is based on three strains isolated from the human oral cavity. Cells are anaerobic, non-spore-forming, non-motile, pleomorphic, Gram-positive-staining bacilli (0.7–0.8 × 1.2–4 μm). After incubation for 6 days on FAA plates, colonies are 0.7–1.1 mm in diameter, circular, entire, high-convex to dome-shaped, white to pale cream and opaque with a solid non-translucent internal appearance. Growth in broth media produces a moderate turbidity that is markedly enhanced by the addition of fermentable carbohydrates. Cells are saccharolytic and ferment fructose, glucose, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, ribose and sucrose; L-arabinose, cellobiose, melezitose and salicin are not fermented. Large amounts of acetic and propionic acids and moderate amounts of succinic acid are produced as the end products of metabolism in peptone-yeast extract-glucose broth. Arginine, aesculin, gelatin and urea are not hydrolysed. Indole and catalase are not produced. Nitrate is not reduced. There is less growth with 20 % bile. The Rapid ID 32A profile is 4506 0331 00. The DNA G+C content of the type strain is 70 mol%.

The type strain, C3M_31^T (=DSM 21887^T =CCUG 57100^T), was isolated from carious lesions in the human mouth.

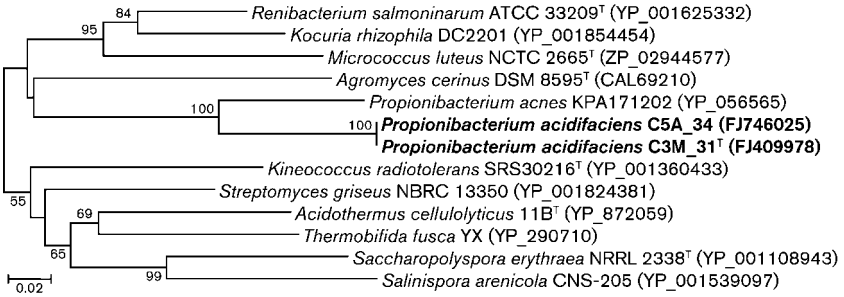

Fig. 2. Phylogenetic tree, based on RpoB amino acid sequence comparisons over 202 aligned amino acids and constructed using the neighbour-joining method, showing the relationship between strains of *P. acidifaciens* sp. nov. and related species. Percentages at nodes represent bootstrap values for each branch based on data for 500 trees. Bar, 2 substitutions per 100 amino acid positions.

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