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Bulleidia extructa gen. nov., sp. nov., isolated from the oral cavity

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Five strains of anaerobic non-sporing Gram-positive bacilli isolated from advanced periodontitis (four strains) and a dentoalveolar abscess (one strain) that did not correspond to existing species were subjected to phenotypic and genetic characterization. Following 16S rDNA sequence analysis, they were found to constitute a novel branch of the low G+C Gram-positive division of the phylogenetic tree related to *Erysipelothrix rhusiopathiae* and *Holdemania filiformis*. A new genus *Bulleidia*, and the species *Bulleidia extructa*, are proposed. Growth of *B. extructa* in broth media was poor but was enhanced by the addition of fructose, glucose or maltose together with Tween 80. Glucose and maltose were fermented and arginine was hydrolysed. Acetate, lactate and trace amounts of succinate were the end products of glucose fermentation. The G+C content of the DNA of the type strain is 38 mol%. The type strain of *Bulleidia extructa* is DSM 13220^T.

Keywords: *Bulleidia*, *Eubacterium*, taxonomy, phylogeny

INTRODUCTION

Anaerobic non-sporing Gram-positive bacilli currently assigned to the genus *Eubacterium* are an extremely diverse group of organisms (Moore & Holdeman Moore, 1986). A number of new species have recently been proposed (Cheeseman *et al*., 1996; Poco *et al*., 1996a, b; Uematsu *et al*., 1993) and it is estimated that there are at least 25 un-named taxa (Wade, 1997). Phylogenetic analysis of 16S rRNA gene sequence data has placed most of these new species in either the Actinobacteria or the low G+C Gram-positive division of the phylogenetic tree (Cheeseman *et al*., 1996; Wade *et al*., 1999). In this study, a group of isolates from oral infections, which could not be identified as belonging to an existing species, were studied.

METHODS

Bacterial strains. The strains included in the study had been provisionally identified as *Eubacterium* spp. (Wade *et al*., 1990; Olsvik, 1995). Strains GF10, MK1, UM3 and W1219^T were isolated from periodontal pockets and W2274 was isolated from a dentoalveolar abscess. *Erysipelothrix rhusiopathiae* NCTC 8163^T (= ATCC 19414^T) was obtained from the NCTC and *Holdemania filiformis* ATCC 51649^T from the ATCC.

Morphology. Strains were grown at 37 °C on fastidious anaerobe agar (FAA, LabM) supplemented with 5% horse blood under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2). Colonial morphologies were determined using a plate microscope after incubation for 7 d. Cellular morphology was recorded after Gram-staining of 3 d plate cultures. Hanging-drop preparations of 18 h cultures of peptone/yeast extract/glucose (PYG) broth supplemented with 0·5% Tween 80 were examined by phase-contrast microscopy for cellular motility.

Ultrastructure. Transmission electron microscopy was used to examine the cell-wall ultrastructure. Cells were fixed in 2·5% glutaraldehyde in 0·1 M Sorensen's buffer, then centrifuged and washed in the same buffer. The cells were post-fixed in 1% osmium tetroxide, dehydrated by a graded series of ethanol, treated with propylene oxide and embedded in Taab epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate for transmission electron microscopy.

Biochemical and physiological tests. Fermentation tests were performed using pre-reduced, anaerobically sterilized

†Sadly, Bente Olsvik passed away in December 1998.

Abbreviation: PRAS, pre-reduced aerobically sterilized.

The GenBank accession number for the 16S rDNA sequence of *Bulleidia extructa* is AF220064.

(PRAS) sugars according to the methods of Holdeman *et al.* (1977) except that the PRAS media were prepared in an anaerobic workstation using pre-reduced distilled water. Other biochemical tests were performed as described by Holdeman *et al.* (1977) and Summanen *et al.* (1993). Sensitivity to bile was determined by comparing growth of strains on FAA with and without 2% oxgall after 5 d incubation. Spore formation was assessed by both direct microscopic examination and culture following killing of vegetative cells by either heating to 80 °C for 10 min or treatment with 95% ethanol for 30 min. Temperature optima were determined by incubation of PYG + 0·5% Tween 80 broth cultures at 30, 37 and 42 °C.

Metabolic-end-product analysis. Bacterial strains were grown in PYG broth supplemented with 0·5% Tween 80 and short-chain volatile and non-volatile fatty acids extracted by standard methods (Holdeman *et al.*, 1977). Analysis was performed by GC with a capillary column coated with CP-Wax 58 (free fatty acid) solid phase.

Protein profiles. Protein 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) except that the cells were pre-treated with lysozyme (50 μg ml^{-1}) for 3 h at 37 °C and vortexed for 6 min with 1/10 vol. 100 μm diameter glass beads before boiling.

Enzyme profiles. Enzyme profiles were generated with the Rapid ID32A anaerobe identification kit (bioMérieux) according to the manufacturer's instructions. Bacteria were harvested from blood agar plates [Blood Agar Base No. 2 (LabM) supplemented with 5% horse blood] incubated anaerobically at 37 °C for 72 h.

16S rRNA gene PCR and sequencing. DNA was isolated from the bacteria by standard methods. The 16S rRNA gene was amplified by PCR using primers 27F and 1525R (Lane, 1991). PCR amplification was performed with an Uno II Thermocycler (Biometra) using PCR buffer (Bioline) containing 1·5 mM $MgCl_2$, 200 μM dNTPs, 1 mM of each oligonucleotide primer, 1 U *Taq* DNA polymerase (Bioline) and template DNA (100 ng) in a total volume of 100 μl. Thirty amplification cycles were performed with denaturing at 94 °C for 1 min, annealing at 54 °C for 1 min and elongation at 72 °C for 2 min. PCR products were sequenced directly using a dye terminator cycle sequencing kit with Ampli*Taq* FS (Perkin Elmer) and 60 ng template DNA, according to the manufacturer's instructions. Sequencing was performed using an automated sequencer (ABI 377; Perkin Elmer) with primers 27F, 342R, 357F, 519R, 907R, 926F, 1100R, 1114F, 1392R and 1525R (Lane, 1991).

Sequence analysis. Sequences were connected using DNASIS (Hitachi) and then submitted to the Ribosomal Database Project via the World Wide Web for provisional identification using the Similarity_Rank program. From the phylogenetic position indicated by Similarity_Rank, related sequences were selected and aligned by means of CLUSTAL W (Thompson *et al.*, 1994). Further analysis was performed using the PHYLIP suite of programs (Felsenstein, 1993). Specifically, DNADIST was used to compare sequences by the Jukes–Cantor algorithm and NEIGHBOR was used for neighbour-joining cluster analysis.

Estimation of mol % G + C content of DNA. This was done by an HPLC method as described previously (Wade *et al.*, 1999).

RESULTS AND DISCUSSION

Five strains of Gram-positive bacilli were studied. They were found to be obligately anaerobic, non-motile, non-spore-forming short bacilli (0·5 × 0·8–2 μm). Colonies on FAA were 0·8 mm in diameter, circular, entire, low convex and opaque after 7 d incubation. Growth in peptone/yeast extract broth was poor but was enhanced by the addition of 0·5% Tween 80 together with 1% glucose, maltose or fructose. Glucose and maltose were fermented, with acetate, lactate and trace amounts of succinate produced as the end products of metabolism. Cells grew in PYG + 0·5% Tween 80 at 30 and 37 °C but not 42 °C.

Transmission electron microscopic examination revealed a typical Gram-positive cell wall composed of a single layer of peptidoglycan approximately 55 nm thick surrounding the cytoplasmic membrane (Fig. 1).

All strains hydrolysed arginine. Catalase, indole and H_2S were not produced and nitrate was not reduced. Aesculin and urea were not hydrolysed and gelatin was not liquefied. All strains were positive for arginine dihydrolase, arginine arylamidase and leucine arylamidase in the Rapid ID 32A panel. The G + C content of the DNA of strain W1219^T was 38 mol%.

16S rRNA gene PCR products obtained from strain W1219^T with primers 27F and 1525R were fully sequenced. Approximately 550 bases of 16S rDNA were sequenced for strains GF10, MK1 and UM3 between bases 357 and 907 (*Escherichia coli* numbering). This stretch of sequence includes the variable regions V3–5 (Neefs *et al.*, 1993). Over 545 aligned bases, each strain had >99% sequence similarity with each other strain. Phylogenetic analysis of the sequence data assigned W1219^T to the clostridial subphylum within the low G + C Gram-positive bac-

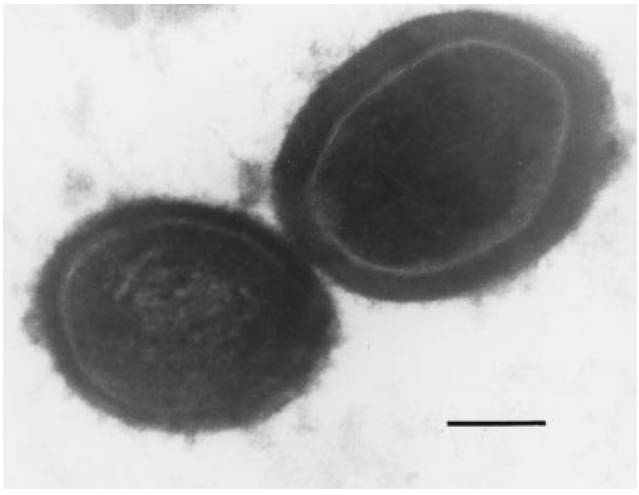

Fig. 1. Transmission electron micrograph of *Bulleidia extructa* DSM 13220^T. Ultrathin section showing the Gram-positive cell wall and the cytoplasmic membrane. Bar, 0·2 μm

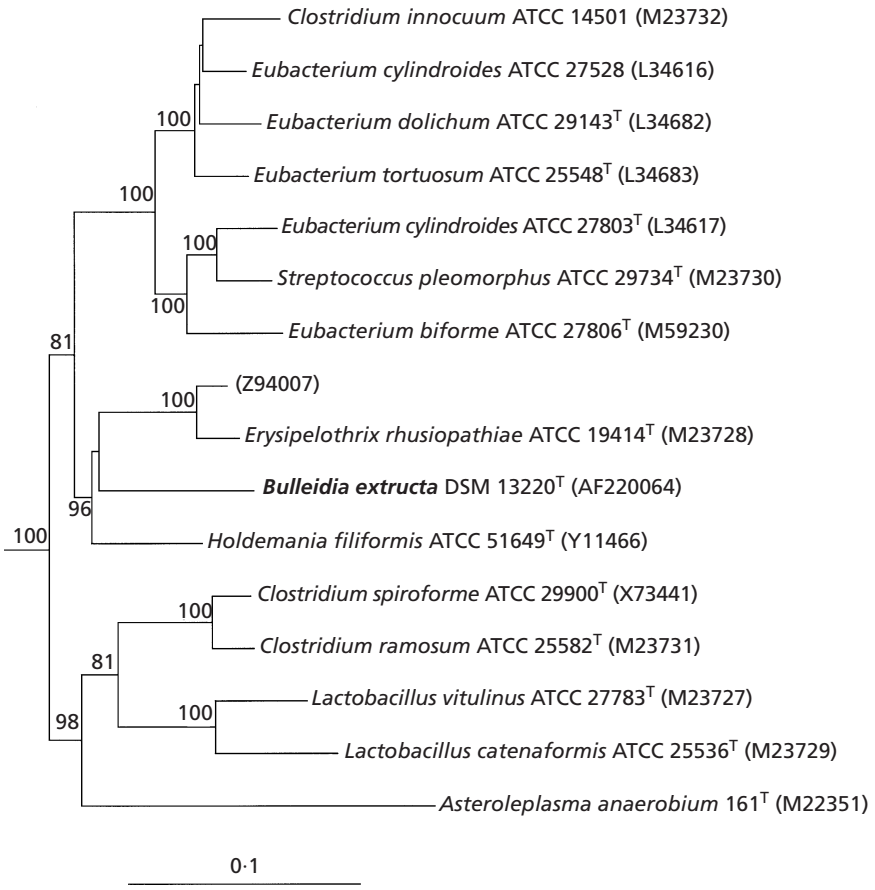

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence comparisons over 1344 aligned bases showing the relationship between *B. extructa* and related species. The tree was constructed using the neighbour-joining method following distance analysis of aligned sequences and was rooted with *Anaeroplasma bactoclasticum*. The numbers at branch points represent bootstrap values for each branch based on data for 1000 trees. Accession numbers for 16S rRNA sequences are given in parentheses for each strain. The scale bar shows the number of nucleotide substitutions per site. Z94007 is a cloned sequence from activated sludge (Snaidr *et al.*, 1997).

Table 1. Biochemical characteristics of *Bulleidia extructa* and related species

w, weak.

	Bulleidia extructa	***Holdemania filiformis***	***Erysipelothrix rhusiopathiae***
Rapid ID32A profile	2000 0120 00	0500 0041 20	2103 417$^3/_7$ 05
Growth in air + CO_2	−	−	+
H_2S production	−	w	+
Aesculin hydrolysis	−	+	−
Arginine hydrolysis	+	−	+
Growth in 20% bile	−	w	+
Vancomycin susceptibility (5 μg disc)	S	R	R
DNA G+C content (mol%)	38	38	36–40
Habitat	Oral, human	Faecal, human	Animal faecal, environmental, human infection

teria division (Fig. 2). Nearest relatives were *Erysipelothrix rhusiopathiae* and the recently described *Holdemania filiformis* (Willems *et al.*, 1997). 16S rDNA sequence similarities between the strains sequenced in this study and both *E. rhusiopathiae* and *H. filiformis* were below 90%, and therefore the new strains were sufficiently distinct to warrant proposal of a new genus and species.

Members of the proposed taxon, for which the name *Bulleidia extructa* is proposed below, were obligate anaerobes and sensitive to metronidazole, unlike *E. rhusiopathiae*. Strains were also sensitive to 20% bile, unlike both *E. rhusiopathiae* and *H. filiformis*. Biochemical characteristics which enable the new species to be distinguished from *H. filiformis* and *E. rhusiopathiae* are given in Table 1. In addition, *B. extructa*

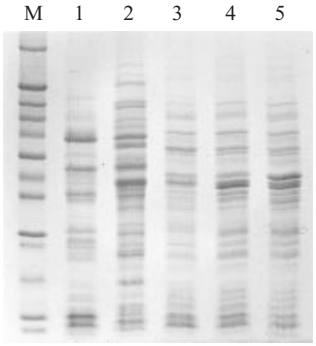

Fig. 3. Protein profiles of *B. extructa*, *E. rhusiopathiae* and *H. filiformis*. Lanes: 1, *E. rhusiopathiae* NCTC 8163^T; 2, *H. filiformis* ATCC 51649^T; 3, *B. extructa* DSM 13220^T (= W1219^T); 4, *B. extructa* UM3; 5, *B. extructa* GF10; M, molecular mass markers (6·5, 14·2, 20·1, 24, 29, 36, 45, 55, 66, 84, 97·4, 116 and 205 kDa).

can be distinguished from *E. rhusiopathiae* and *H. filiformis* by comparison of their protein profiles (Fig. 3).

Description of *Bulleidia* gen. nov.

Bulleidia (Bull.eid′ia. M.L. n. *Bulledia* named to honour Arthur Bulleid, distinguished British oral microbiologist).

Cells are obligately anaerobic, non-spore-forming, non-motile, Gram-positive short bacilli. Principal end products of glucose fermentation are acetate and lactate, with trace amounts of succinate produced. Growth in broth media is poor but is stimulated by 0·5% Tween 80 in the presence of fermentable carbohydrates. Arginine is hydrolysed and there is no growth in 20% bile. Catalase and indole are not produced and nitrate is not reduced. The type species is *Bulleidia extructa*.

Description of *Bulleidia extructa* sp. nov.

Bulleidia extructa (ex.truc′ta. L. adj. *extructus* slow, referring to the slow growth of the organism).

Description is based on four strains isolated from the oral cavity. Cells are obligately anaerobic, non-spore-forming, non-motile, Gram-positive short bacilli (0·5 × 0·8–2 μm) occurring singly and in pairs, aligned side by side. After 7 d incubation on FAA plates, colonies are 0·8 mm in diameter, circular, entire, low convex, grey to off-white, opaque and glossy. Growth in PRAS media + 0·5% Tween 80 is poor with arabinose, cellobiose, lactose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose or xylose. Growth is stimulated by fructose, glucose and maltose in the presence of 0·5% Tween 80. Glucose and maltose are fermented; fructose is not fermented. Moderate amounts of acetate and lactate and trace amounts of succinate are produced as the end products of glucose metabolism in PYG. Arginine is hydrolysed. Catalase and indole are not produced; nitrate is not reduced. Aesculin and urea are not hydrolysed. There is no growth in 20% bile. Gelatin is not liquefied and H_2S is not produced. The G+C content of the DNA of the type strain is 38 mol%. The type strain is DSM 13220^T (W1219^T). Isolated from human periodontal pockets and dentoalveolar infections.

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