Murdochiella asaccharolytica gen. nov., sp. nov., a Gram-stain-positive, anaerobic coccus isolated from human wound specimens

Nurver Ulger-Toprak,[1,2] Chengxu Liu,[1] Paula H. Summanen[1] and Sydney M. Finegold[3,4]

Correspondence
Paula H. Summanen
carlsonph@aol.com

[1]Research Service, VA Medical Center West Los Angeles, Los Angeles, CA 90073, USA

[2]Marmara University, Faculty of Medicine, Department of Microbiology, Istanbul, Turkey

[3]Infectious Diseases Section, VA Medical Center West Los Angeles, Los Angeles, CA 90073, USA

[4]Departments of Medicine and of Microbiology, Immunology, and Molecular Genetics, UCLA School of Medicine, Los Angeles, CA, USA

Two strains of previously unknown Gram-stain-positive, anaerobic, coccus-shaped bacteria from human wound specimens were characterized using phenotypic and molecular taxonomic methods. Comparative 16S rRNA gene sequencing studies and distinguishable biochemical characteristics demonstrated that these two unknown strains, WAL 1855C^T and WAL 2038E, are genotypically homogeneous and constitute a novel lineage within *Clostridium* cluster XIII. There was 13–14 % 16S rRNA gene sequence divergence between the novel strains and the most closely related species, *Parvimonas micra*, *Finegoldia magna* and species of *Helcococcus*. Based on the phenotypic and phylogenetic findings, a novel genus and species, *Murdochiella asaccharolytica* gen. nov., sp. nov., are proposed. Strain WAL 1855C^T (=ATCC BAA-1631^T =CCUG 55976^T) is the type strain of *Murdochiella asaccharolytica*.

Gram-positive anaerobic cocci (GPAC) are part of the commensal flora of humans and animals and are also commonly associated with a variety of human infections. They account for about 25–30 % of all anaerobic isolates recovered from clinical samples (Finegold, 1977). Extensive taxonomic changes have occurred recently among this group of bacteria with the addition of novel species and renaming of existing species (Finegold *et al.*, 2002; Murdoch, 1998). *Peptostreptococcus productus* was transferred to the genus *Ruminococcus* and the genus *Peptostreptococcus* was divided into new groups. Currently, five genera of GPAC that contain former members of the genus *Peptostreptococcus* that may be isolated from humans have been designated: *Parvimonas*, *Finegoldia*, *Peptoniphilus*, *Anaerococcus* and *Gallicola* (Ezaki *et al.*, 2001). The only species remaining in the genus *Peptostreptococcus* are *Peptostreptococcus anaerobius* and a novel species, *Peptostreptococcus stomatis*, isolated recently from the human oral cavity (Downes & Wade, 2006). Three of the new genera, *Parvimonas*, *Finegoldia* and *Gallicola*, have only one species each, *Parvimonas micra*, *Finegoldia magna* and *Gallicola barnesae*, respectively. The type species of the two other genera are *Peptoniphilus asaccharolyticus* and *Anaerococcus prevotii*.

We have isolated two GPAC strains, WAL 2038E and WAL 1855C^T, from a human abdominal wall abscess and a sacral pilonidal cyst aspirate. Comparative 16S rRNA gene sequencing studies demonstrated that the unknown strains were genotypically homogeneous but did not appear to correspond to any of the recognized species of GPAC genera. In this article, we report the phenotypic characteristics of the strains and the results of a phylogenetic analysis and, based on the results presented, a new genus and species are described.

Strains WAL 2038E and WAL 1855C^T and the reference strains *Parvimonas micra* ATCC 33270^T, *Finegoldia magna* CCUG 17636^T, *Helcococcus sueciensis* CCUG 47334^T and *Helcococcus kunzii* CCUG 32213^T were included in the present study. Clinical specimens were obtained at Olive View–UCLA Hospital. The specimens were collected, transported and processed as outlined by Jousimies-Somer *et al.* (2002). Anaerobic conditions consisted of a gas mixture of 5 % CO_2, 5 % H_2 and 90 % N_2. Microaerophilic conditions were obtained using an automated evacuation-replacement system [Anoxomat (Mart BV Laboratorium); microaerophilic cycle]. All testing was done on 24–72 h pure cultures on plates of Brucella blood agar (Anaerobe Systems), CDC blood agar (Becton

Abbreviations: GPAC, Gram-positive anaerobic cocci; SPS, sodium polyanethol sulfonate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain WAL 1855C^T is EU483153.

Dickinson Microbiology) or trypticase soy blood agar (TSBA; Becton Dickinson Microbiology). Single colonies were selected, described and subcultured to assure purity and to determine whether the organism was aerobic, anaerobic or microaerophilic, and were confirmed as anaerobes if there was no growth in aerobic or microaerophilic environments.

The strains were characterized biochemically by using a combination of conventional tests and commercially available biochemical kits. Conventional identification of bacteria was performed based on Gram stain, colony morphology, susceptibility to special-potency antibiotic discs [colistin, vancomycin, kanamycin and sodium polyanethol sulfonate (SPS)] and other biochemical reactions according to Jousimies-Somer *et al.* (2002). The commercial biochemical kits Rapid ID 32A and API ZYM (bioMérieux) were used according to the manufacturer's instructions and the results were graded using a colour chart supplied by the manufacturer. All biochemical tests were performed in duplicate. Carbohydrate fermentation tests were performed using pre-reduced, anaerobically sterilized peptone-yeast (PY) broth tubes (Anaerobe Systems) with the addition of different carbohydrates. The strains were grown in PY and peptone-yeast-glucose (PYG) broth (Anaerobe Systems) for analysis of metabolic end products (short-chain volatile and non-volatile fatty acids) by GLC.

Genomic DNA was extracted and purified from bacterial cells in the mid-exponential growth phase by using a QIAamp DNA Mini kit (Qiagen). 16S rRNA gene fragments were amplified by standard methods (Song *et al.*, 2003). Two subregions of the 16S rRNA gene were amplified by using two pairs of primers. The first part of the 16S rRNA gene was defined as an approximately 800 bp region between primers 8UA (5′-AGAGTTTGATCCTGGCTCAG-3′) and 907B (5′-CCGTCAATTCMTTTAGTTT-3′). The second part, defined as an approximately 700 bp sequence between primers 774A (5′-GTAGTCCACGCTGTAAACGATG-3′) and 1485B (5′-TACGGTTACCTTGTTACGAC-3′), was sequenced to obtain the complete 16S rRNA gene sequence. PCR was performed for 35 cycles of 30 s at 95 °C, 30 s at 45 °C and 1 min at 72 °C, with a final extension at 72 °C for 5 min. The PCR products were excised from a 1 % agarose gel after electrophoresis and purified using a QIAquick Gel Extraction kit (Qiagen). Purified PCR products were sequenced directly with BigDye Terminator cycle sequencing kits (Applied Biosystems) on an ABI 3100 *Avant* Genetic System (Applied Biosystems). The sequences were analysed by comparison of the consensus sequences with GenBank sequences by using Ribosomal Database Project (RDP-II) and BLAST (Benson *et al.*, 1997) software, and the percentage similarity to other sequences was determined. Closely related sequences were retrieved from GenBank and were aligned with the newly determined sequences by using the program CLUSTAL W (Thompson *et al.*, 1994). Phylogenetic and molecular evolutionary analyses were constructed by using the neighbour-joining algorithm within MEGA version 4 (Tamura *et al.*, 2007).

Strain WAL 1855C^T was isolated from a sacral pilonidal cyst aspirate from an immunocompetent patient and strain WAL 2038E was obtained from an abdominal wall abscess of a morbidly obese diabetic patient at an insulin injection site; no other clinical information was given. Strain WAL 1855C^T was isolated together with strains of *Solobacterium moorei*, *Streptococcus anginosus/Streptococcus constellatus*, *Parvimonas micra* and *Bilophila wadsworthia* and strains showing 98 % 16S rRNA gene sequence similarity to an uncultured member of the *Clostridiales*, and WAL 2038E was isolated with strains of *Corynebacterium simulans*, *Actinomyces europaeus*, *Mobiluncus curtisii*, *Peptoniphilus asaccharolyticus*, *Actinobaculum schaalii* and *Anaerococcus vaginalis*. The majority of these isolates were present in counts of $\geqslant 10^5$ c.f.u. ml^{-1} (WAL 1855C^T, 2×10^5 c.f.u. ml^{-1}; WAL 2038E, 1×10^5 c.f.u. ml^{-1}). Comparative 16S rRNA gene sequencing studies on the isolates demonstrated that the novel strains did not correspond to any of the recognized species in the GenBank database. The novel species were found to be Gram-stain-positive, coccoid organisms. Cells were 0.5–0.6 μm in diameter and occurred in pairs and short chains. Colonies on Brucella blood agar plates at 5 days were grey, flat or low-convex, circular, entire, white and opaque with a diameter of 2–3 mm. They grew well anaerobically, but no growth occurred following subculture in air or in atmospheres of 2 or 6 % O_2. The novel strains were sensitive to special-potency identification discs containing kanamycin (1000 μg) and vancomycin (5 μg) and resistant to discs containing colistin sulfate (10 μg) and SPS (1000 μg).

The novel strains were negative for catalase, urease and reduction of nitrate and produced indole. The unknown strains and *Parvimonas micra* ATCC 33270^T and *F. magna* CCUG 17636^T were asaccharolytic; they did not produce acid from glucose or other carbohydrates. The two tested *Helcococcus* strains, *H. kunzii* CCUG 32213^T and *H. sueciensis* CCUG 47334^T, produced acid from lactose and trehalose. Based on results from the literature (Collins *et al.*, 1999), *Helcococcus ovis* produced acid from glucose but not from lactose or trehalose. In PYG broth, major amounts of lactic and oxaloacetic acids and moderate amounts of acetic, butyric and succinic acids were produced by the novel isolates. *Parvimonas micra* ATCC 33270^T and *F. magna* CCUG 17636^T produced major amounts of acetic acid and small amounts of lactic and succinic acids.

Using the Rapid ID 32A kit, the two novel isolates produced identical profiles. Positive reactions were obtained for arginine dihydrolase, arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase (weak), leucine arylamidase, tyrosine arylamidase (weak), alanine arylamidase, glycine arylamidase, histidine arylamidase and serine arylamidase. All the other tests were negative. Neither strain hydrolysed

aesculin or gelatin. According to the API ZYM system, the unknown strains were positive for leucine arylamidase and esterase (C4) only. Distinguishing results from the Rapid ID 32A kit and other biochemical tests between the novel strains and members of the phylogenetically most closely related genera are given in Table 1.

To assess the genotypic affinity between the unknown bacteria and their relationship with other taxa, their 16S rRNA gene sequences were determined. The sequences of the novel strains were identical (100 % sequence similarity) and were most closely related to that of bacterium N14-24 (approx. 99 % sequence similarity) in GenBank. Bacterium N14-24 was isolated by Hooper *et al.* (2006) from oral squamous cell carcinoma tissue, but the properties of the strain have not been described. Investigation of the most closely related described species revealed that the novel strains were members of the phylum *Firmicutes* and represent a previously unknown lineage within *Clostridium* cluster XIII, exhibiting 87 % sequence similarity with *Parvimonas micra* ATCC 33270^T and 86 % sequence similarity with strains of *F. magna*, *H. sueciensis*, *H. kunzii* and *H. ovis*. The novel strains could be distinguished from *Parvimonas micra* and *F. magna* by the presence of indole production and the absence of pyroglutamic acid arylamidase activity. Further, the obligately anaerobic novel isolates could be differentiated from facultatively anaerobic *Helcococcus* species according to atmospheric growth requirements (Collins *et al.*, 1993, 1999, 2004); *Helcococcus* species produce heavy growth of pinpoint colonies on 5 % sheep blood agar and chocolate agar plates under a CO_2-enriched (5 %) atmosphere, whereas the novel strains only grow under anaerobic conditions. A tree depicting the phylogenetic affinity of strains WAL 1855C^T and WAL 2038E is shown in Fig. 1 and confirmed the placement of the unknown bacterium in *Clostridium* cluster XIII. On the basis of low level of 16S rRNA gene sequence similarity (<87 %), it is evident from the branching pattern in the tree that the strains constitute a novel lineage within *Clostridium* cluster XIII.

Based on the previously mentioned characteristics, the isolates showed some resemblance to members of a number of genera that encompass species formerly assigned to the genus *Peptostreptococcus* (e.g. *Parvimonas*, *Finegoldia*, *Peptoniphilus*, *Gallicola* and *Anaerococcus*) within *Clostridium* cluster XIII (Collins *et al.*, 1994; Ezaki *et al.*, 2001). However, the genotypic and phenotypic test results clearly demonstrated that the unknown cocci were distinct from the phylogenetically closest members of GPAC genera (Fig. 1; Table 1). Therefore, we propose that the unidentified coccus-shaped bacterium be assigned to a novel genus and species, *Murdochiella asaccharolytica* gen. nov., sp. nov.

Table 1. Phenotypic characteristics of strain WAL 1855C^T and the type strains of the phylogenetically most closely related species

Strains: 1, *Murdochiella asaccharolytica* gen. nov., sp. nov. WAL 1855C^T; 2, *Parvimonas micra* CCUG 46357^T; 3, *F. magna* CCUG 17636^T; 4, *H. kunzii* CCUG 32213^T; 5, *H. sueciensis* CCUG 47334^T; 6, *H. ovis* CCUG 37441^T. Data for reference strains were obtained from Collins *et al.* (1999). +, Positive; −, negative; V, variable; ND, no data available.

Characteristic	1	2	3	4	5	6
Acid production from:						
Lactose	−	−	−	+	+	−
Trehalose	−	−	−	+	+	−
Glucose	−	−	−	+	+	+
Production of:						
Alkaline phosphatase	−	+	V	−	+	+
β-Glucosidase	−	−	−	+	−	−
Indole	+	−	−	−	−	ND
Pyroglutamic acid arylamidase	−	+	+	+	−	−
N-Acetyl-β-glucosaminidase	−	−	−	+	+	−
Metabolic end products*	L, a, b, s	A, l, s	A, l, s	a, s	a	ND

*A/a, Acetic acid; b, butyric acid; L/l, lactic acid; s, succinic acid. Capitals indicate major amounts produced; lower-case letters indicate small amounts produced.

Description of *Murdochiella* gen. nov.

Murdochiella (Mur.do.chi.el′la. N.L. fem. dim. n. *Murdochiella* named to honour Dr David A. Murdoch, British microbiologist, who has contributed so much to our knowledge of anaerobic bacteriology).

Cells are cocci, Gram-stain-positive and non-motile. Obligately anaerobic. Indole-positive. Catalase- and urease-negative. Nitrate is not reduced. Carbohydrates are not fermented. In PY and PYG broth, major amounts of lactic acid and moderate amounts of acetic, butyric and succinic acids are produced. Bile-sensitive. Do not hydrolyse aesculin or gelatin. The type species is *Murdochiella asaccharolytica*.

Description of *Murdochiella asaccharolytica* sp. nov.

Murdochiella asaccharolytica (a.sac.cha.ro.ly′ti.ca. Gr. pref. *a-* not; Gr. n. *saccharon* sugar; N.L. fem. adj. *lytica* from Gr. adj. *lutikos* able to lyse, able to loose; N.L. fem. adj. *asaccharolytica* not digesting sugar).

Displays the following properties in addition to those described for the genus. Cells are 0.5–0.6 μm in diameter and occur in pairs and short chains. Colonies on Brucella blood agar plates at 5 days are grey, flat or low-convex, circular, entire, white and opaque with a diameter of 2–3 mm. Asaccharolytic; acid is not produced from glucose or other sugars. Using the Rapid ID 32A kit, positive reactions are obtained for arginine dihydrolase, arginine arylamidase, phenylalanine arylamidase (weak), proline arylamidase, leucine arylamidase, leucyl glycine arylamidase, alanine arylamidase, tyrosine arylamidase (weak), histidine arylamidase and serine arylamidase. According to

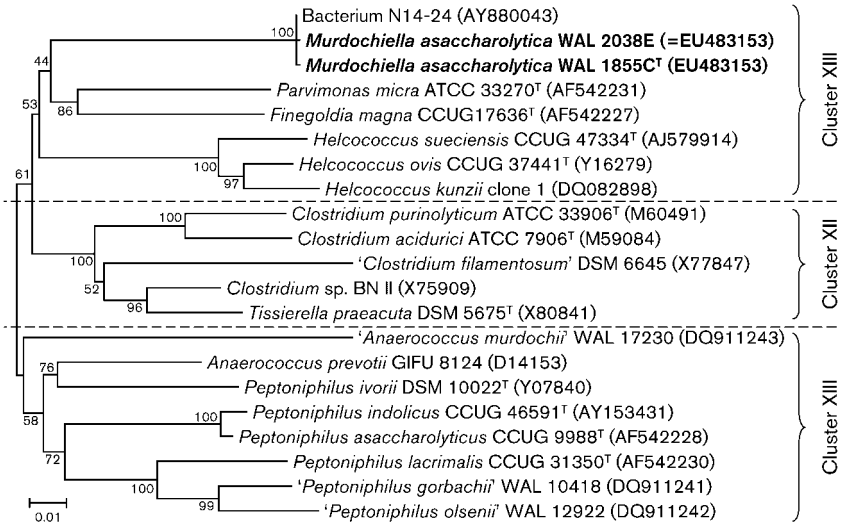

Fig. 1. Phylogenetic position of strains WAL 1855C^T and WAL 2038E within the *Clostridiales*. The alignment was performed with 16S rRNA gene sequences of the most closely related species of *Clostridium* clusters XII and XIII. Accession numbers are indicated in parentheses. Unrooted tree constructed using the neighbour-joining algorithm established by MEGA version 4 (Tamura *et al.*, 2007). Bootstrap values are displayed as percentages on the relevant branches. Bar, 0.01 inferred substitutions per nucleotide position.

the API ZYM system, leucine arylamidase and esterase (C4) are positive. All the other tests are negative; aesculin and gelatin are not hydrolysed. Sensitive to vancomycin (5 μg) and kanamycin (1000 μg), resistant to colistin sulfate (10 μg) and SPS (1000 μg) identification discs. Strains have been isolated from human clinical specimens.

The type strain is WAL 1855C^T (=ATCC BAA-1631^T =CCUG 55976^T). Strain WAL 2038E is a second strain of the species.

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