Macellibacteroides fermentans gen. nov., sp. nov., a member of the family *Porphyromonadaceae* isolated from an upflow anaerobic filter treating abattoir wastewaters

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A novel obligately anaerobic, non-spore-forming, rod-shaped mesophilic bacterium, which stained Gram-positive but showed the typical cell wall structure of Gram-negative bacteria, was isolated from an upflow anaerobic filter treating abattoir wastewaters in Tunisia. The strain, designated LIND7H^T, grew at 20–45 °C (optimum 35–40 °C) and at pH 5.0–8.5 (optimum pH 6.5–7.5). It did not require NaCl for growth, but was able to grow in the presence of up to 2 % NaCl. Sulfate, thiosulfate, elemental sulfur, sulfite, nitrate and nitrite were not used as terminal electron acceptors. Strain LIND7H^T used cellobiose, glucose, lactose, mannose, maltose, peptone, rhamnose, raffinose, sucrose and xylose as electron donors. The main fermentation products from glucose metabolism were lactate, acetate, butyrate and isobutyrate. The predominant cellular fatty acids were anteiso-$C_{15:0}$, $C_{15:0}$, $C_{17:0}$ 2-OH and a summed feature consisting of $C_{18:2}\omega6,9c$ and/or anteiso-$C_{18:0}$, and the major menaquinones were MK-9, MK-9(H_2) and MK-10. The G+C content of the genomic DNA was 41.4 mol%. Although the closest phylogenetic relatives of strain LIND7H^T were *Parabacteroides merdae*, *Parabacteroides goldsteinii* and *Parabacteroides gordonii*, analysis of the *hsp60* gene sequence showed that strain LIND7H^T was not a member of the genus *Parabacteroides*. On the basis of phylogenetic inference and phenotypic properties, strain LIND7H^T (=CCUG 60892^T=DSM 23697^T=JCM 16313^T) is proposed as the type strain of a novel species in a new genus within the family *Porphyromonadaceae*, *Macellibacteroides fermentans* gen. nov., sp. nov.

The phylum *Bacteroidetes* [formerly the *Cytophaga–Flavobacterium–Bacteroides* group (Gherna & Woese, 1992)] constitutes the dominant bacterial group of the gastrointestinal microbiota (23 % of the human intestinal microbiota) followed by members of the phylum *Firmicutes* (Eckburg *et al.*, 2005; Gill *et al.*, 2006). The family *Porphyromonadaceae* has been proposed to encompass the genera *Porphyromonas* (type genus), *Barnesiella*, *Dysgonomonas*, *Paludibacter*, *Petrimonas*, *Proteiniphilum*, *Tannerella* and *Parabacteroides* (Shah & Collins, 1988; Sakamoto *et al.*, 2002). Members of the family *Porphyromonadaceae* are obligately anaerobic, heterotrophic, non-spore-forming, non-motile, Gram-staining-negative rods that ferment sugars (Krieg, 2011). In this paper, we propose a novel species in a new genus in the family *Porphyromonadaceae* to accommodate a mesophilic, anaerobic strain, designated LIND7H^T.

Strain LIND7H^T was isolated from an upflow anaerobic filter treating abattoir wastewaters in Tunisia (Gannoun

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LIND7H^T is HQ020488.

A supplementary table is available with the online version of this paper.

et al., 2009). The digester was operated under both mesophilic (37 °C) and thermophilic (55 °C) conditions. A sample was taken during the mesophilic phase of the process in order to isolate fermentative micro-organisms.

For enrichment and isolation, the basal medium contained (per litre of distilled water) 0.3 g KH_2PO_4, 0.3 g K_2HPO_4, 1 g NH_4Cl, 1 g NaCl, 0.1 g KCl, 0.1 g $CaCl_2.2H_2O$, 1 g $MgCl_2.6H_2O$, 0.5 g L-cysteine.HCl, 2 g yeast extract (Difco), 10 ml trace mineral element solution (Balch *et al.*, 1979) and 1 ml of 0.1 % resazurin (Sigma). The pH was adjusted to 7.2 with 10 M KOH solution. The medium was boiled under a stream of O_2-free nitrogen and cooled to room temperature. Aliquots of 5 ml were dispensed into Hungate tubes, degassed under N_2/CO_2 (80 : 20, v/v) and subsequently sterilized by autoclaving at 120 °C for 20 min. Prior to inoculation, 0.1 ml of 10 % (w/v) $NaHCO_3$, 0.1 ml of 2 % (w/v) $Na_2S.9H_2O$ and 20 mM glucose from sterile stock solutions were injected into the tubes. The Hungate technique (Hungate, 1969) was used throughout the study.

A 0.5 ml aliquot of the sample was inoculated into the Hungate tubes that were subsequently incubated at 37 °C. To obtain pure cultures, the enrichment was subcultured several times under the same growth conditions prior to isolation. For isolation, the culture was serially diluted tenfold in roll tubes (Miller & Wolin, 1974) containing the same culture medium supplemented with 2 % (w/v) agar. Several colonies developed after incubation at 37 °C and were picked separately. The process of serial dilution was repeated several times until the isolates were deemed to be axenic. Several strains were isolated; their morphology and metabolic profile were similar and the same phylogenetic inference was obtained for all of them. One strain, designated LIND7H^T, was selected and used for further metabolic and physiological characterization.

Methods for purification of the DNA, PCR amplification and sequencing of the 16S rRNA gene were described previously (Thabet *et al.*, 2004). The partial sequences generated were assembled using BioEdit v. 5.0.9. (Hall, 1999) and the consensus sequence of 1502 nt was corrected manually for errors. The sequence was compared with available sequences in GenBank (version 178) using a BLAST search (Altschul et *al.*, 1990). The consensus sequence was then manually adjusted to conform to the 16S rRNA secondary structure model (Winker & Woese, 1991). Nucleotide ambiguities were omitted and evolutionary distances were calculated using the Jukes and Cantor option (Jukes & Cantor, 1969). Phylogenetic trees were constructed with the TREECON program using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony and maximum-likelihood algorithms. Tree topology was evaluated by a bootstrap analysis using 1000 resamplings of the sequences (Felsenstein, 1985). In addition, sequence analysis of the *hsp60* gene, an alternative phylogenetic marker for the classification of Gram-negative anaerobic rods, was performed as described previously (Sakamoto & Ohkuma, 2010).

On the basis of 16S rRNA gene sequence analysis, strain LIND7H^T (HQ020488) formed a separate branch within the family *Porphyromonadaceae* (Fig. 1). Its closest phylogenetic relatives were micro-organisms of human origin (faeces, intestine and blood), i.e. *Parabacteroides merdae* (Johnson et *al.*, 1986), *Parabacteroides goldsteinii* (Song *et al.*, 2005; Sakamoto & Benno, 2006) and *Parabacteroides gordonii* (Sakamoto *et al.*, 2009), with sequence similarities of 91.4, 91.3 and 91.2 % between the type strains of these three species and the novel isolate, respectively. In addition, *hsp60* gene sequence similarities between strain LIND7H^T and *P. gordonii*, *P. goldsteinii*, *Parabacteroides johnsonii*, *P. merdae* and *Parabacteroides distasonis* were 82.3, 81.4, 78.7, 78.5 and 78.1 %, respectively, while sequence similarities with members of other *Parabacteroides* species were 83.3–97.1 % (Table S1 available in IJSEM Online), confirming that the novel isolate did not belong to any of these species.

The Gram reaction was performed with heat-fixed liquid cultures stained with Difco kit reagents. Cellular morphology and purity of the strain were assessed under an Optiphot (Nikon) phase-contrast microscope. For transmission electron microscopy studies, cells were negatively stained with sodium phosphotungstate, as described previously (Fardeau *et al.*, 1997). The presence of spores was assessed: (i) by microscopic observation of cultures; and (ii) by pasteurization tests performed at 80, 90 and 100 °C for 10 and 20 min.

Growth experiments were performed in duplicate using Hungate tubes containing the above-mentioned basal medium. The pH of the medium was adjusted to pH 5.0–9.0 (at 0.5 pH unit intervals) by injecting aliquots of anaerobic stock solutions of 1 M HCl, 10 % $NaHCO_3$ or Na_2CO_3 into the Hungate tubes. The pH of the medium was verified after autoclaving. Water baths were used for incubating bacterial cultures at 20–55 °C (at 5 °C intervals). To assess the requirement of and tolerance to NaCl, NaCl (0–10 %, at 0.5 % intervals) was weighed directly into the tubes before the medium was dispensed. The strain was subcultured at least twice under the same experimental conditions before growth rates were determined. Arabinose, cellobiose, glucose, glycerol, lactose, maltose, mannitol, mannose, raffinose, rhamnose, sucrose, xylose, peptone, Casamino acids, acetate, lactate and pyruvate, were tested as electron donors. Each substrate was added to the basal medium at a final concentration of 20 mM. H_2/CO_2 (80 : 20) alone or in the presence of acetate (2 mM) as a carbon source was also tested as an electron donor at 2 bars (200 kPa). The effects of yeast extract (Panreac), biotrypcase (Panreac) and Balch's vitamins (Balch *et al.*, 1979) on growth were tested with and without substrate added. Other biochemical tests were performed using the API 20A anaerobe test kit as recommended by the manufacturer (bioMérieux). Resistance to ampicillin, vancomycin and kanamycin was tested between 10 and 500 μg ml^{-1} in Hungate tubes incubated at 37 °C for 4 days. Elemental sulfur (1 % w/v), sodium sulfate

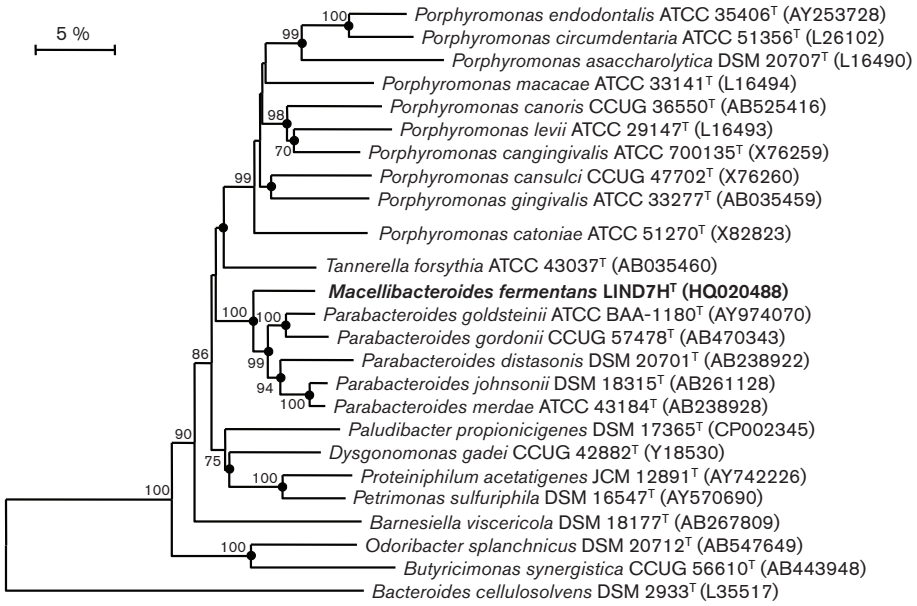

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain LIND7H^T and related representatives of the family *Porphyromonadaceae*. Bootstrap values of 70 % or higher (based on 1000 repetitions) are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony and maximum-likelihood algorithms. *Bacteroides cellulosolvens* DSM 2933^T was used as an outgroup. Bar, 5 substitutions per 100 nt.

(20 mM), sodium thiosulfate (20 mM), sodium sulfite (2 mM), sodium nitrate (10 mM) and sodium nitrite (2 mM) were tested as terminal electron acceptors. H_2S production was determined photometrically as colloidal CuS by using the method of Cord-Ruwisch (1985). Bacterial growth was monitored by measuring the increase in turbidity at 580 nm by insertion of Hungate tubes into the cuvette holder of a spectrophotometer (Cary 50; Varian). End products of metabolism were measured by HPLC and GC of the gases released after 2 weeks of incubation at 37 °C (Fardeau *et al.*, 2000).

Cells of strain LIND7H^T were non-motile, non-spore-forming rods. Although staining Gram-positive, they had a multilayered cell-wall and an outer membrane that was typical of Gram-negative bacteria (Fig. 2). The isolate required yeast extract for growth; this could not be replaced by biotrypcase or Balch's vitamins. Other phenotypic characteristics of strain LIND7H^T are listed in the genus and species descriptions.

Analysis of the respiratory quinones and polar lipids was carried out by Dr Brian Tindall at the Identification Service of the DSMZ (Braunschweig. Germany). Polar lipids was separated by two-dimensional TLC and detected using molybdatophosphoric acid and heating at 200 °C for 10 min. For fatty acid analysis, the biomasses of strain LIND7H^T and *P. distasonis* CCUG 4941^T were standardized for their physiological age at the point of harvest according to Technical Note 101 of MIDI (http://www.microbialid.com/PDF/TechNote_101.pdf). Fatty acids were extracted using the method of Miller (1982) with the modifications of Kuykendall *et al.* (1988) and analysed by GC (model 6890N; Agilent Technologies) using the Microbial Identification System (MIDI, Sherlock version 6.1; database, TSBA40; Sasser, 1990). DNA was isolated and purified by chromatography on hydroxyapatite using the procedure of Cashion *et al.* (1977) and the G+C content was determined at the DSMZ by using HPLC as described by Mesbah *et al.* (1989).

The complete fatty acid profile of strain LIND7H^T and *P. distasonis* CCUG 4941^T is given in Table 1. The major fatty acids of strain LIND7H^T were anteiso-$C_{15:0}$ (30.1 %), $C_{15:0}$ (10.4 %), $C_{17:0}$ 2-OH (8.0 %) and a summed feature consisting of $C_{18:2}\omega6,9c$ and/or anteiso-$C_{18:0}$ (8.3 %). The proportions of several fatty acids differed significantly between the two strains; moreover, the novel isolate contained 3.2 % of an unidentified fatty acid, the chain length of which could not be determined. There were also significant differences in the respective proportions of the different menaquinones [especially MK-9(H_2) and MK-10] between strain LIND7H^T and the six *Parabacteroides* species (Table 2). In addition, the DNA G+C content of strain LIND7H^T was significantly lower than those reported for *Parabacteroides* species (Table 2). The polar lipid profile of strain LIND7H^T consisted of phosphatidylethanolamine, phosphatidylglycerol, one unidentified glycolipid and several unidentified phospholipids (Fig. 3).

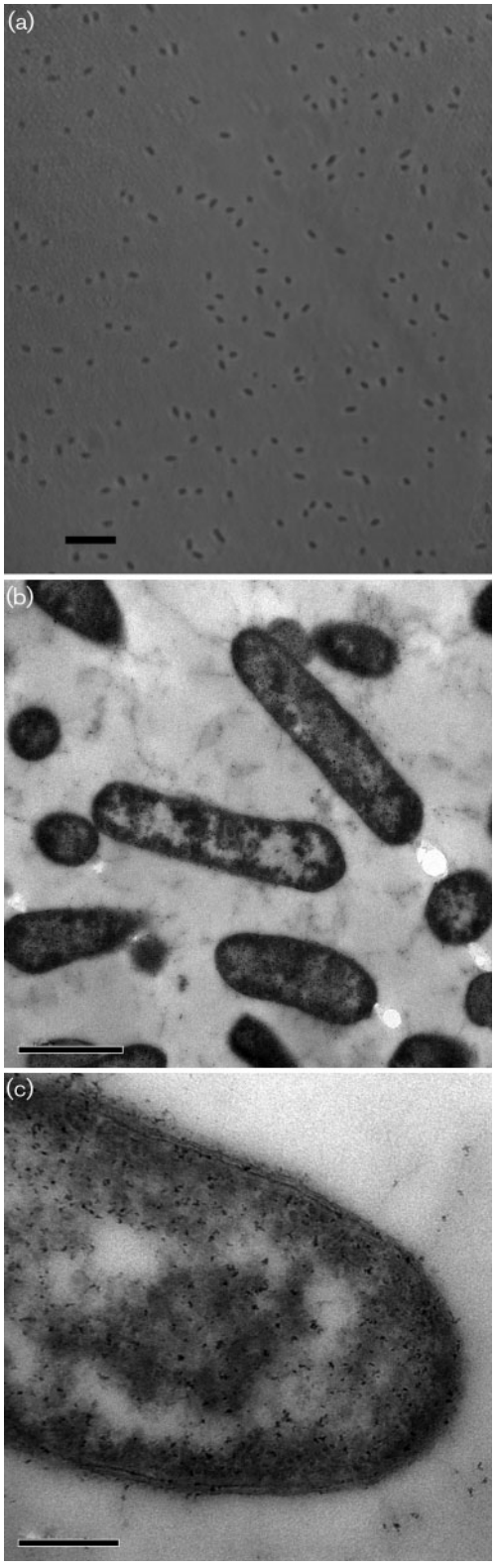

Fig. 2. Cell morphology of strain LIND7H^T. Phase-contrast micrograph (a) and thin-section electron micrographs (b, c) showing the Gram-negative cell wall type. Bars: a, 10 μm; b, 1 μm; c, 0.2 μm.

Table 1. Cellular fatty acid composition (%) of strain LIND7H^T and *P. distasonis* CCUG 4941^T

Strains 1, LIND7H^T; 2, *P. distasonis* CCUG 4941^T. Fatty acids amounting to less than 1% in the two strains are not shown. Tr, Trace (<1%); −, not detected. All data are from this study. ECL, Equivalent chain-length; unidentified, length of fatty acid could not be determined.

Fatty acid	1	2
iso-$C_{13:0}$	1.1	Tr
anteiso-$C_{13:0}$	2.7	Tr
ECL 13.566	2.3	−
iso-$C_{15:0}$	5.4	8.5
anteiso-$C_{15:0}$	30.1	43.5
$C_{15:0}$	10.4	6.8
$C_{16:0}$	1.8	4.2
$C_{15:0}$ 3-OH	3.4	Tr
$C_{16:0}$ 3-OH	1.1	5.6
Summed feature*	8.3	4.8
iso-$C_{17:0}$ 3-OH	6.3	13.6
$C_{17:0}$ 2-OH	8.0	1.3
Unidentified	3.2	−

*The summed feature comprises $C_{18:2}\omega6,9c$ and/or anteiso $C_{18:0}$.

Therefore, on the basis of phylogenetic inference and differential characteristics (Tables 1 and 2), we propose that strain LIND7H^T represents a novel species in a new genus within the family *Porphyromonadaceae*, *Macellibacteroides fermentans* gen. nov., sp. nov.

Description of *Macellibacteroides* gen. nov.

Macellibacteroides (Ma.cel.li.bac.te.ro′i.des. L. n. *macellum* a butcher's stall, meat-market, slaughterhouse; N.L. masc. n. *Bacteroides* a bacterial genus name; N.L. masc. n. *Macellibacteroides* a relative of the genus *Bacteroides* isolated from a slaughterhouse).

Cells stain Gram-positive but have a Gram-negative type of cell wall. Non-motile, non-spore-forming, mesophilic rods with a fermentative and obligately anaerobic type of metabolism. The major respiratory quinones are MK-9 and MK-9(H_2) and the major fatty acids are anteiso-$C_{15:0}$ and $C_{15:0}$. The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol and several unidentified phospholipids. The type species is *Macellibacteroides fermentans*. A member of the family *Porphyromonadaceae*, phylum *Bacteroidetes*, according to 16S rRNA gene sequence analysis.

Description of *Macellibacteroides fermentans* sp. nov.

Macellibacteroides fermentans (fer.men′tans. L. part. adj. *fermentans* fermenting).

Table 2. Comparison of the menaquinone composition and DNA G+C content of strain LIND7H^T and *Parabacteroides* species

Taxa: 1, strain LIND7H^T (this study); 2, genus *Parabacteroides* [data for the type strains of *P. merdae*, *P. goldsteinii*, *P. gordonii*, *P. johnsonii*, *P. distasonis* (values for *P. distasonis* JCM 5825^T are shown in parentheses) taken from: Sakamoto & Benno, 2006; Sakamoto *et al.*, 2007, 2009].

Characteristic	1	2
Menaquinone (%)		
MK-8	0	1–5 (4)
MK-9	52	10–54 (24)
MK-9(H_2)	33	0
MK-10	15	37–72 (67)
MK-11	0	tr*–13 (4)
DNA G+C content (mol%)	41.4	Approx. 44–48 (47.1)

*tr, Trace amount.

Displays the following characteristics in addition to those listed in the genus description. Cells are approximately 2.0–3.0 μm in length and 0.5–1.0 μm in diameter, occurring singly or in pairs. Colonies are yellowish and circular with entire edges, 1.0–2.0 mm in diameter after 3–5 days of incubation at 37 °C. Growth occurs at 20–45 °C (optimum 35–40 °C) and at pH 5.0–8.5 (optimum pH 6.5–7.5). Does not require NaCl for growth, but tolerates up to 2 % NaCl. Yeast extract is required for growth. Catalase-negative. Cellobiose, glucose, lactose, mannose, maltose, peptone, rhamnose, raffinose, sucrose and xylose are used as electron donors, but not arabinose, glycerol, mannitol, Casamino acids, acetate, lactate, pyruvate, H_2/CO_2 or H_2/CO_2 in the presence of acetate. Sodium sulfate, sodium thiosulfate, elemental sulfur, sodium sulfite, sodium nitrate and sodium nitrite are not used as terminal electron acceptors. The main fermentation products from glucose metabolism are lactate, acetate, butyrate and isobutyrate. In the API 20A system, the following reactions are positive: hydrolysis of gelatin and aesculin and production of acid from glycerol, glucose, mannitol, lactose, sucrose, maltose, xylose, arabinose, cellobiose, mannose, melezitose, raffinose, sorbitol, rhamnose and trehalose. The following reactions in the API 20A system are negative: urease activity and production of acid from salicin. In addition to the major fatty acids and respiratory quinones listed in the genus description, significant amounts (>5 %) of iso-$C_{17:0}$ 3-OH, iso-$C_{15:0}$, a summed feature consisting of $C_{18:2}\omega6,9c$ and/or anteiso $C_{18:0}$ and MK-10 are also present. In addition to the major polar lipids listed in the genus description, one unidentified glycolipid and several unidentified phospholipids are present. Susceptible to ampicillin (10 μg ml^{-1}) and vancomycin (10 μg ml^{-1}) but resistant to kanamycin (400 μg ml^{-1}).

The type strain, LIND7H^T (=CCUG 60892^T=DSM 23697^T=JCM 16313^T), was isolated from an upflow anaerobic filter treating abattoir wastewaters in Tunisia. The DNA G+C content of the type strain is 41.4 mol%.

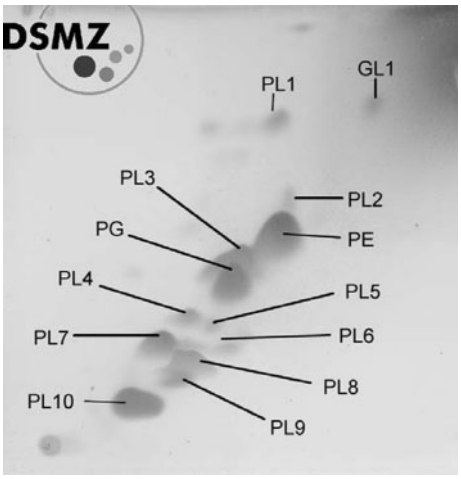

Fig. 3. Total polar lipids of strain LIND7H^T after two-dimensional TLC and detection with molybdatophosphoric acid and heating at 200 °C for 10 min. PG, Phosphatidylglycerol; PE, phosphatidylethanolamine; PL1–10, unidentified phospholipids; GL1, unidentified glycolipid.

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