***Propionibacterium microaerophilum* sp. nov., a microaerophilic bacterium isolated from olive mill wastewater**

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A new Gram-positive, facultative anaerobic, microaerophilic bacterium, designated strain M5^T, was isolated from a decantation reservoir of olive mill wastewater. The cells were rod-shaped, non-motile, non-spore-forming and catalase-negative. Growth occurred at pH ranging from 4.5 to 9.5, with optimum growth at 7.0. The optimum temperature for growth was around 30 °C. Although growth occurred under anaerobic and aerobic conditions, the optimum O₂ concentration for growth was determined as 5% in the gas phase of the culture. During anaerobic growth, glucose or lactate were mainly fermented to propionate, acetate and CO₂. In the presence of O₂ (more than 2%), glucose was oxidized completely to CO₂. The G+C content of the DNA was 67.7±0.6 mol% and 16S rRNA gene analysis revealed that the new isolate belonged to the cluster of 'dairy' propionibacteria, *Propionibacterium acidipropionici* being its closest phylogenetic relative (97.5% similarity). However, the level of DNA relatedness between strain M5^T and *P. acidipropionici* was 56.2%. Consequently, both the phenotypic (range of substrates used) and genotypic characteristics of strain M5^T allow it to be assigned as a new species of the genus *Propionibacterium*, *Propionibacterium microaerophilum* sp. nov. The type strain is strain M5^T (= CNCM I-2360^T = DSM 13435^T).

Keywords: microaerophilic, anaerobe, propionic fermentation, taxonomy, phylogeny

INTRODUCTION

Olive oil production is a significant industry in most Mediterranean countries, and 1800 million tons are produced per year. This activity generates large volumes of effluent (20–30 million m³ year⁻¹), rich in organic matter (420 000 tons BOD₅ year⁻¹, that is an important environmental pollutant (Balice *et al.*, 1992). In this respect, several bioremediation programs dealing with olive mill wastewater have been undertaken during recent decades. Studies on the chemical composition revealed the presence of phenolic compounds, which represent the main source of pollution (Hamdi *et al.*, 1992; Sayadi & Ellouz, 1993). Phenolics include monomers (e.g. hydroxyquinone, gallate, sal-

icylate, protocatechuate and *p*-hydroxybenzoate) and condensed aromatics (e.g. soluble tannins, complex tannins, flavonoids, lignins, catechol-melanines and flavolanes) (Harborne, 1980). Besides phenolic compounds, olive mill effluents also contain other organic substances such as sugars, nitrogen compounds and organic acids. These compounds provide a large range of substrates for micro-organisms present in olive mill wastewater.

Whereas most microbiological studies have paid attention to aerobic and anaerobic populations of micro-organisms from olive mill wastewater, no studies on microaerophiles have been reported so far. We report here the isolation of a new microaerophilic bacterium from an olive mill wastewater reservoir. This reservoir is a storage pond, approximately 2 m deep and 30 m³ in volume. The olive mill wastewater is stored in the reservoir for natural bioremediation before evacuation

The GenBank accession number for the 16S rDNA sequence of strain M5^T is AF234623.

into a river. Since the reservoir was open to the air, it constituted a good ecosystem from which to seek microaerophilic bacteria.

METHODS

Origin of strains. Strain M5^T was isolated from an olive mill wastewater reservoir in Raphèle-les-Arles (100 km north-west of Marseille) in the south of France. Samples were stored at 4 °C in serum bottles under anaerobic conditions under an atmosphere of O₂-free N₂. *Propionibacterium acidipropionici* DSM 20272, *P. acidipropionici* DSM 4900^T, *Propionibacterium jensenii* DSM 20535^T and *Propionibacterium thoenii* DSM 20276^T were obtained from the DSMZ, Braunschweig, Germany.

Culture methods and media. Unless indicated otherwise, all media were boiled, dispensed under a stream of O₂-free N₂ into 100 ml serum bottles (20 ml medium) or into Hungate tubes (5 ml medium) and stored with N₂ in the headspace. For the enrichment and purification procedures, a basal medium (BM) was used that contained (l⁻¹ reverse-osmosis water): 0.6 g KH₂PO₄, 0.2 g MgCl₂·6H₂O, 1 g NH₄Cl, 1 g NaCl and 10 ml trace-element solution (Balch *et al.*, 1979). The effects of vitamins on growth were tested in BM supplemented with 10 mM glucose and 0.8 ml Balch vitamin solution l⁻¹ (Balch *et al.*, 1979). For routine cultures and further characterization of strain M5^T, YEM medium (basal medium supplemented with 0.1 g yeast extract l⁻¹) was used. *P. acidipropionici*, *P. jensenii* and *P. thoenii* were cultivated in PM medium containing (l⁻¹ reverse-osmosis water): 2 g KH₂PO₄, 10 g bio-Tryptase (bioMérieux) and 10 g yeast extract. The pH of culture media was adjusted to 7.0 with a 10 M NaOH solution. Sterilization was performed by autoclaving at 110 °C for 45 min. Energy sources tested were injected into serum bottles and Hungate tubes from separate anaerobic stock solutions. Unless indicated otherwise, all experiments were conducted in duplicate at the optimum temperature for growth (30 °C) at pH 7.0 and under anaerobic conditions.

Enrichment and isolation procedure. Enrichment cultures were obtained by culture in deep agar (Weinberg *et al.*, 1937). Hungate tubes containing 10 ml BM supplemented with 4 g agar l⁻¹, 5 mM sodium benzoate and 10 mM glucose were boiled for 10 min under a stream of O₂-free N₂ to purge the O₂ dissolved in the medium. After cooling to approximately 40 °C, 0.5 ml samples of olive mill wastewater were dispensed with a syringe into the tubes containing semi-solid medium, homogenized and cooled to room temperature under a stream of sterile air. The tubes were then incubated at 30 °C. After approximately 1 week of incubation, significant growth was obtained at about 2.5 cm from the surface, suggesting the presence of microaerophilic micro-organisms. An aliquot (0.5 ml from this deep semi-solid culture) was taken by using a sterile syringe. After subculturing twice under the same conditions, a semi-solid aliquot of this enrichment culture was serially diluted in liquid BM. Thereafter, each dilution was spread on agar plates containing the same BM supplemented with 10 mM glucose, 5 mM sodium benzoate and 20 g agar l⁻¹. The inoculated agar plates were placed in jars under an atmosphere of N₂/O₂ (95:5%, v/v) at atmospheric pressure and incubated at 30 °C for 6 d. Single colonies were picked from the plates and subcultured at least twice on agar plates under the same conditions. In the last purification step, single colonies obtained from the plates were serially diluted in Hungate tubes containing 5 ml liquid BM and 10 mM

glucose as an energy source. The gas atmosphere was N₂/O₂ (95:5%, v/v). Incubation was performed at 30 °C without agitation for 1 week. The procedure was repeated until only one type of colony was observed.

Sporulation and heat-resistance tests. The presence or absence of spores was examined microscopically. In the absence of apparent spore formation, the presence of heat-resistant or heat-tolerant cells was tested. For this purpose, bacterial cultures were grown at 30 °C in YEM medium with 10 mM glucose. After 2, 7 and 14 d of incubation at 30 °C, the cells were observed with a phase-contrast microscope and the viability of cells was checked by subculturing. Duplicate cultures were then heated at 80 °C for 5 and 10 min, subcultured into fresh medium (20% inoculum) and incubated for 48 h at 30 °C.

Analytical techniques. Bacterial growth was monitored by measuring the increase in turbidity at 580 nm with a spectrophotometer (Shimadzu UV 160A). Glucose and fermentation end-products were determined by HPLC using a pump (Spectra Series P100; Spectra-Physics), an automatic sampler (Spectra Series AS100), an Aminex HPLC 87H (300 × 7.8 mm) column (Bio-Rad), a differential refractometer detector (Shimadzu RID 6A) and an integrator (Shimadzu C-R 6A Chromatopac). An aliquot of 20 µl cell-free supernatant was injected into the column, which was maintained at 35 °C. A 2.5 mM H₂SO₄ solution was used as solvent with a flow rate of 0.6 ml min⁻¹. Oxygen, H₂, N₂ and CO₂ levels were determined with a GC (Chrompack CP 9000) equipped with a thermal conductivity detector and a double column. The first column (1.5 m × 2 mm) was packed with silica gel 60–80 mesh. The second column (1.5 m × 2 mm) was packed with a molecular sieve 5 Å 60–80 mesh. The carrier gas was helium (15 ml min⁻¹). Temperatures were: injector, 50 °C; columns, 50 °C; detector, 150 °C. Gases were sampled and injected (0.5 ml) using 1 ml syringes.

DNA was isolated from exponentially growing cells and the G + C content was determined at the DSMZ by HPLC, as described previously (Cashion *et al.*, 1977; Mesbah *et al.*, 1989). Temperature, pH and NaCl profiles for growth were determined under anaerobic conditions using YEM medium containing glucose as an energy source. The temperature range was determined by using thermostatically controlled water baths. Determination of the pH profile was performed in a 2 l fermenter (Labo 2000; Interscience) at 30 °C with stirring at 200 r.p.m. The pH was controlled by the addition of 0.5 M NaOH with an automatic pH regulator (Interscience). Catalase activity was tested by resuspending cells, harvested by centrifugation of 1.5 ml culture, in 3% H₂O₂. The culture was incubated for 5 d in the presence of 5% O₂ in the gas phase. Nitrate reduction was determined by using Griess' reagent (Sigma). H₂S was measured photometrically as colloidal CuS after reacting with a mixture containing 50 mM HCl and 5 mM CuSO₄ (Cord-Ruwisch, 1985). The fermentation patterns were determined in triplicate by using the API 50 CH kit (bioMérieux) with YEM medium supplemented with 2 g KH₂PO₄ l⁻¹ for strain M5^T and PM medium for *P. acidipropionici*, *P. jensenii* and *P. thoenii*; both media were supplemented with 0.018% phenol red, used as a pH indicator. Anaerobic conditions were ensured by filling wells with paraffin oil.

Effects of O₂ on growth and product fermentation patterns. Experiments were performed in 100 ml serum bottles containing 20 ml YEM medium supplemented with 20 mM of glucose, with N₂ in the headspace. Bottles were inoculated (5% inoculum) with exponentially grown cultures of strain M5^T and various volumes of sterile air were added to the gas

Fig. 1. (a) Phase-contrast photomicrograph of strain M5^T from a culture in stationary phase. Bar, 10 µm. (b) Electron microscope photomicrograph of negatively stained cells of strain M5^T. Bar, 2 µm. (c) Electron microscope photomicrograph of an ultrathin section of strain M5^T, showing the cell wall structure. cm, Cytoplasmic membrane; cw, cell wall. Bar, 0.2 µm.

phase. The cultures were incubated at 30 °C with 200 r.p.m. agitation. The gas phase was replaced every 12 h throughout the course of the experiment. Growth and metabolic end-products were monitored at 24 h intervals by turbidity measurements (580 nm) and HPLC analysis, respectively. All the experiments were conducted in duplicate and repeated at least once.

Morphological characteristics. Morphological properties were determined by phase-contrast microscopy, using slides coated with a thin layer (0.5 mm) of purified agar (Difco). For electron microscopy, preparations were negatively stained with 1% sodium phosphotungstate (pH 7.2). For preparation of thin sections, exponentially grown cells were centrifuged and fixed for 0.5 h in 4% (w/v) glutaraldehyde prepared in 0.025 M HCl/cacodylate buffer (pH 6.0). The cells were then washed in the same buffer containing 0.3 M sucrose and post-fixed in osmium tetroxide prepared in the same buffer with 0.25 M sucrose. After fixation, the cells were washed with water, embedded in 2% agarose and stained with 4% uranyl acetate. The agar was cut into small cubes, dehydrated in acetone and embedded in Araldite. Thin sections were stained with 5% uranyl acetate for 20 min and with 2% lead citrate for 10 min. Micrographs were taken with a Hitachi model H600 electron microscope at an accelerating voltage of 75 kV.

DNA–DNA hybridization. DNA was isolated by the method of Cashion *et al.* (1977). DNA–DNA hybridization was performed at the DSMZ as described by De Ley *et al.* (1970), with the modifications of Escara & Hutton (1980) and Huß *et al.* (1983), using a Gilford System model 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program of Jahnke & Bahnweg (1986) and Jahnke (1992).

DNA extraction and amplification of 16S rRNA gene. DNA was extracted from the isolate as described previously (Andrews & Patel, 1996; Redburn & Patel, 1993). The universal primers Fd1 and Rd1 were used to obtain a PCR product of approximately 1.5 kb, corresponding to positions 8–1542 (*Escherichia coli* numbering) of the 16S rDNA (Winker & Woese, 1991). A 50 µl reaction contained 1–20 ng genomic DNA, 1 µM of each primer, 5 µl 10× buffer, 200 µM dNTPs, 3.5 mM MgCl₂ and 2.5 U *Taq* polymerase (Promega). PCR was performed by an initial denaturation at 94 °C for 4 min, followed by 30 cycles of annealing at 55 °C for 2 min, extension at 72 °C for 4 min and denaturation at 94 °C for 1 min and finally an extension cycle of 55 °C for 2 min and 72 °C for 20 min.

Direct sequencing of PCR products. PCR products were purified using a QIAquick kit (Qiagen). The DNA concentration of the purified PCR product was estimated by comparison with a Low-Mass ladder (Gibco-BRL) on an agarose gel containing ethidium bromide. QIAquick-purified PCR products were sequenced using the ABI PRISM Dye Terminator cycle sequencing kit, containing AmpliTaq FS DNA polymerase, and an ABI 373A sequencer. A 10 µl reaction contained 35 ng PCR product, 4 µl cycle-sequencing reaction mix, 3.2 pmol primer and 2.5 µg BSA. Thermal cycling was performed using a RapidCycler (Idaho Technology) at a temperature transition slope of 2, an initial denaturation of 94 °C for 15 s and then 25 cycles of denaturation at 94 °C for 0 s, annealing at 50 °C for 10 s and extension at 60 °C for 3 min.

Sequence alignments and phylogenetic inferences. The new sequence was aligned to an almost full length consensus 16S rRNA gene sequence, assembled and checked for accuracy manually using the alignment editor ae2 (Maidak *et al.*, 1996). These were compared with other sequences in the

GenBank database (Benson *et al.*, 1993) using BLAST (Altschul *et al.*, 1997) and in the Ribosomal Database Project, version 7.0, using SIMILARITY_RANK and SUGGEST_TREE (Maidak *et al.*, 1996). Pairwise evolutionary distances based on 1253 unambiguous nucleotides were computed by using the DNADIST (Jukes & Cantor, 1969) and NEIGHBOR programs that form part of the PHYLIP suite of programs (Felsenstein, 1993).

RESULTS

Enrichment and isolation

In the initial enrichment cultures in deep agar, a ring or disc of growth was formed at about 2.5 cm under the surface within 1 week of incubation at 30 °C. The disc was white and 1–2 mm thick. After two subcultures under the same conditions, an aliquot of the disc was spread on agar plates and incubated under N₂/O₂ (95:5%, v/v). After 1 week, single colonies were obtained and subcultured once again under the same conditions. One isolate, designated strain M5^T, was used for further characterization.

Colony and cell morphology

Colonies obtained after 1 week of incubation on solid YEM medium under microaerophilic conditions (95:5% N₂/O₂, v/v) were white, lens-shaped with

smooth edges and 2–3 mm in diameter. Cells of strain M5^T were single, or in short chains in aged cultures, pleomorphic rods (0.5–0.7 µm wide and 2–3.5 µm long) with rounded ends (Fig. 1a,b). They exhibited no motility under phase-contrast microscope examination. Ultrathin electron microscope sections revealed a thick, not very dense, Gram-positive cell envelope (Fig. 1c). Spores were not observed at any stage of growth. Cultures incubated at 80 °C for 5 or 10 min could not be subcultured, indicating the absence of spores or heat-resistant forms.

Growth and metabolic properties

Strain M5^T grew on BM containing glucose or lactate as the sole energy source in the absence of growth factors including vitamins, amino acids and peptides. However, growth was stimulated in the presence of yeast extract. Addition of vitamins had no significant effect on growth or cellular yield. During fermentation of glucose, strain M5^T produced mainly propionate, acetate and CO₂ as end-products, but traces of succinate and formate were also detected. No H₂ was detected. Otherwise, with more than 2% O₂ in the gas phase, glucose was oxidized completely to CO₂. However, under anaerobic conditions, strain M5^T did not grow with propionate, acetate, succinate or formate as the energy source. The fermentation balance on

Table 1. Comparison of sugar fermentation by strain M5^T and phylogenetically related *Propionibacterium* species

Fermentation patterns for the four strains were determined in this study. Sugar utilization was assessed out by using API 50 CH strips with phenol red as pH indicator. A drop of oil was added to each cupule after inoculation to create anoxic conditions. Strips were incubated at 30 °C for 4 d, after which results were recorded. The other sugars of the API 50 CH strip gave the same fermentation profiles for all four strains.

Carbon source	Strain M5 ^T	<i>P. acidipropionici</i> DSM 20272	<i>P. jensenii</i> DSM 20535 ^T	<i>P. thoenii</i> DSM 20276 ^T
D-Arabinose	—	+	—	—
L-Arabinose	+	+	—	—
D-Xylose	—	+	—	—
L-Sorbose	+	—	—	—
Rhamnose	+	+	—	—
Inositol	+	+	+	—
Mannitol	+	+	+	—
Sorbitol	+	+	—	+
Methyl α-D-mannoside	—	—	—	+
Methyl α-D-glucoside	+	+	—	+
N-Acetylglucosamine	+	+	—	+
Amygdalin	—	+	+	—
Lactose	—	+	—	+
Melibiose	—	+	+	—
Melezitose	+	+	+	—
D-Raffinose	—	+	+	—
Starch	+	+	—	+
Glycogen	+	+	—	—
D-Fucose	—	+	—	—
Gluconate	+	+	+	—
Aesculin hydrolysis	—	+	+	+

Fig. 2. Effect of oxygen concentration (%) on the specific growth rate (■) and growth yield on glucose (△) of *Propionibacterium microaerophilum* M5^T.

glucose, accounting for a level of carbon recovery of 87.3 %, was 3.0 glucose → 4.2 propionate + 1.8 acetate + 1.8 CO₂. On lactate, accounting for a level of carbon recovery of 98.67 %, the fermentation balance

was 3.0 lactate → 2.1 propionate + 0.9 acetate + 0.9 CO₂. The optimum temperature for growth of strain M5^T was 30 °C, with growth occurring at temperatures ranging from 20 to 45 °C. The organism grew at pH values ranging from 4.5 to 9.5; the optimum pH was 7.0. The optimum NaCl concentration for growth was 5 g l⁻¹; growth occurred at concentrations ranging from 0 to 20 g l⁻¹.

Sulfate, thiosulfate, sulfite, elemental sulfur and fumarate were not used as terminal electron acceptors. Nitrate was reduced into N₂. The range of carbohydrates catabolized was determined with the API 50 CH kit (bioMérieux). Among the pH indicators tested for growth (bromthymol blue, bromcresol purple, phenol red) of *Propionibacterium* strains, only phenol red did not inhibit growth of any of the strains studied. It was therefore chosen for further growth studies. The range of carbohydrates used by each strain is reported in Table 1.

Effect of O₂ on growth and catabolism products of glucose

Growth rate ($\mu_{\max}$) and cellular yield ($Y_{x/s}$) values (Fig. 2) showed that strain M5^T grew at O₂ concentrations ranging from 0 (anaerobic conditions) to 21 %, the optimum O₂ concentration for growth being 5 %.

Fig. 3. The position of *Propionibacterium microaerophilum* M5^T in relation to its phylogenetic relatives in the family *Propionibacteriaceae*. Strain numbers from culture collections and their corresponding 16S rRNA gene sequence accession numbers (in parentheses) extracted from GenBank/EMBL are shown. Phylogenetic analysis was performed on 1196 unambiguous nucleotides using the DNADIST and NEIGHBOR programs that form part of the PHYLIP suite of software. Bar, 2 nucleotide changes per 100 nucleotides. Abbreviations: T, type strain; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; ACM, Australian Collection of Microorganisms; IAM, Institute of Applied Microbiology; and NCTC, National Collection of Type Cultures.

Below 2%, propionate and acetate were detected. For O_2 concentrations higher than 2%, glucose was oxidized completely to CO_2 . However, strain M5^T did not possess detectable catalase activity.

16S rDNA sequence analysis and DNA relatedness

The mean of triplicate determinations of G + C content of strain M5^T DNA was 67.7 ± 0.6 mol % (HPLC). Using eight primers, a nearly complete sequence was determined consisting of 1524 bases of the 16S rRNA gene of isolate M5^T. The sequence of positions 8–1524 (*E. coli* numbering; Winker & Woese, 1991) was aligned with sequences representative of the family *Propionibacteriaceae*. Phylogenetic analysis showed that strain M5^T was related to species of the genus *Propionibacterium*, *P. acidipropionici* being its closest relative (similarity of 97.5%) (Fig. 3). The level of DNA relatedness between strain M5^T and *P. acidipropionici* DSM 4900^T, its closest relative, was 56%.

DISCUSSION

Strain M5^T, isolated from olive mill wastewater, was a mesophilic, catalase-negative, facultative anaerobe and microaerophilic bacterium. The results of 16S rRNA gene sequence studies indicated that strain M5^T is phylogenetically related to the *Propionibacterium* species group and that it is closely related to *P. acidipropionici* DSM 20272, with 97.5% sequence similarity. Using the multiplex-PCR method based on 16S–23S ribosomal spacer DNA sequences, Meile *et al.* (1999) showed that the phylogeny of *Propionibacterium* species was similar to that established with only 16S rRNA, even if the distances were quite different. DNA–DNA hybridization experiments between strain M5^T and its closest relative, *P. acidipropionici*, showed reassociation of only 56%. Phenotypically, cells of strain M5^T were single or in short chains, pleomorphic rods, non-motile, non-spore-forming, with a Gram-positive cell envelope profile. The isolate had a DNA G + C content of 67.7 ± 0.6 mol %. The strain required no complex nitrogen nutrients such as yeast extract, amino acids or peptides for growth. It could grow with only ammonium salts as the nitrogen source. Yeast extract improved the growth of strain M5^T, whereas the presence of other growth factors such as pantothenate, biotin or thiamin had no effect on growth. Under anaerobic conditions, the strain produced large amounts of propionate, acetate and CO_2 during fermentation of glucose or lactate. Under these conditions, the stoichiometric balances of glucose and lactate fermentations were consistent with the glucose and lactate fermentation pathways described for members of the genus *Propionibacterium* (Wood, 1981). Furthermore, the ranges of temperature, pH and NaCl concentration for growth and the morphological and physiological characteristics of strain M5^T were also consistent with the general description of the genus *Propionibacterium* (Cummins & Johnson, 1986). The phenotypic characteristics of

strain M5^T supported the phylogenetic results and confirmed that it belongs to the genus *Propionibacterium*.

Several phenotypic differences were observed between strain M5^T and other species of the genus *Propionibacterium*. It has been reported that pantothenate is required for growth of all *Propionibacterium* species. The presence of thiamin, nicotinamide, oleate and biotin improved the growth of propionibacteria in general, *p*-aminobenzoic acid being required by some strains (Delwiche, 1949; Ferguson & Cummins, 1978; Holland *et al.*, 1979; Cummins & Johnson, 1986). In contrast to the auxotrophic behaviour of *Propionibacterium* species, strain M5^T did not require growth factors and was therefore prototrophic. Strain M5^T was catalase-negative, whereas the propionibacteria are generally catalase-positive, with the exception of *Propionibacterium propionicus* (Schaal, 1986; Charfreitag *et al.*, 1988) and *Propionibacterium cyclohexanicum* (Kusano *et al.*, 1997). Moreover, strain M5^T reduced nitrate to N_2 , whereas other *Propionibacterium* species reduce nitrate only to nitrite (Table 2). While strain M5^T was microaerophilic, growing better in an atmosphere containing 5% oxygen, *Propionibacterium* species are generally considered as anaerobic to aerotolerant, preferring anaerobic conditions for growth (Cummins & Johnson, 1986). *Propionibacterium avidum* and *Propionibacterium granulosum* are the only two current species of *Propionibacterium* described as microaerophilic, and *P. propionicus* was described as facultatively anaerobic (Cummins & Johnson, 1986; Schaal, 1986; Charfreitag *et al.*, 1988). In the presence of O_2 , during the growth of strain M5^T, the energy source (glucose or lactate) was oxidized completely to CO_2 . Moreover, the cellular yield of strain M5^T in the presence of 5% O_2 was higher than under anaerobic conditions, suggesting that strain M5^T used O_2 as an electron acceptor, generating energy through oxidation of the energy source. Although it has been confirmed that several *Propionibacterium* species possess the components of a typical aerobic electron transport chain, including membrane-bound dehydrogenase and cytochromes (Sone, 1972; De Vries *et al.*, 1977; Pritchard *et al.*, 1977; Ye *et al.*, 1999), the metabolic pathways of propionibacteria growing under aerobic conditions are still not clear. Understanding of the metabolic pathways of strain M5^T that generate ATP in the presence of O_2 will require further specific studies.

More accurate definitions are needed to qualify the ' O_2 –bacteria' relationship. Up to now, five terms have commonly been used to qualify the phenotype of bacteria according to their requirements for and tolerance and sensitivity to oxygen: 'aerobe', 'facultative anaerobe', 'aerotolerant anaerobe', 'microaerophile' and 'strict anaerobe' (Stanier *et al.*, 1976; Marty *et al.*, 1988). Microaerophiles are described as requiring O_2 as the terminal electron acceptor, but growing exclusively or best at O_2 levels below atmospheric concentrations (21% O_2). However, some

Table 2. Characteristics that differentiate strain M5^T and *Propionibacterium* species

Taxa are identified as: 1, strain M5^T; 2, *P. acidipropionici*; 3, *P. jensenii*; 4, *P. thoenii*; 5, *P. acnes*; 6, *P. avidum*; 7, *P. granulosum*; 8, *Propionibacterium lymphophilum*; 9, *P. propionicus*; 10, *P. cyclohexanicum*; 11, *P. freudenreichii*. Data were taken from this study (strain M5^T), Cummins & Johnson (1986) (*P. acidipropionici*, *P. jensenii*, *P. thoenii*, *P. acnes*, *P. avidum*, *P. granulosum* and *P. lymphophilum*), Charfreitag *et al.* (1988) and Schaal (1986) (*P. propionicus*) or Kusano *et al.* (1997) (*P. freudenreichii*) unless otherwise indicated. Characters are scored as: +, positive; d+, 40–90% of strains are positive; d–, 10–40% of strains are positive; d, 11–89% of strains are positive; –, negative. ND, No data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Habitat	Olive mill wastewater	Dairy products	Dairy products	Cheese and other dairy products	Human skin, intestinal contents, blood, pus	Infected sinuses, submaxillary abscesses	Human skin, blood, intestinal contents	Urinary tract infection	Lachrymal canaliculitis	Spoiled orange juice	Raw milk, Swiss cheese and other dairy products
Catalase	–	d+	d+	+	d+	+	+	d+	–	–	+
Aesculin hydrolysis	–	+	+	+	–	+	–	–	–	+	+
L-Sorbose fermentation	+	–*	–*	–*	–	–	–	–	–	–	–
Oxygen tolerance	Microaerophilic facultative anaerobe	Anaerobic to aerotolerant	Anaerobic to aerotolerant	Anaerobic	Anaerobic to aerotolerant	Microaerophilic facultative anaerobe	Anaerobic to aerotolerant	Anaerobic	Facultative anaerobe	Aerotolerant	Anaerobic to aerotolerant
Nitrate reduction to nitrite	Reduction to N ₂	+	–	–	d+	–	–	d	+	–	d
Toxicity of bromocresol purple†	–	+	+	+	ND	ND	ND	ND	ND	ND	ND

* Data from this study.

† Bromocresol purple was tested at 0.018%.

species previously described as anaerobes have been shown to use O_2 under microaerobic conditions and have been redefined as microaerophiles. For example, *Wolinella succinogenes*, using fumarate as terminal electron acceptor in anaerobiosis, was shown to use O_2 at low concentrations (approximately 2% O_2) and was redefined as microaerophilic (Wolin *et al.*, 1961; Jacobs & Wolin, 1963a,b). Similarly, *Wolinella recta*, *Wolinella curva* and *Bacteroides ureolyticus*, bacteria described previously as anaerobic, using fumarate or nitrate as terminal electron acceptors, exhibited microaerophilic growth under 4–14% O_2 in liquid medium and 2–8% in solid medium (Han *et al.*, 1991). Thus, the authors asserted that both species must be considered as anaerobic and microaerophilic rather than simply anaerobic. Strain M5^T, which grew both under anaerobic and aerobic conditions, could be described as a facultatively anaerobic bacterium. However, the term 'facultative anaerobe' gives no information on the optimal O_2 concentration for growth. Facultative anaerobes were defined as bacteria growing under aerobiosis with O_2 as an electron acceptor and under anaerobiosis using another endogenous or exogenous electron acceptor. In the case of *E. coli*, the term 'facultative anaerobe' is appropriate because the species grows both under anaerobic and aerobic conditions and the cellular yield increases with O_2 concentration, with no inhibition at atmospheric O_2 concentrations. In contrast, the growth of strain M5^T was reduced under O_2 concentrations higher than 5%. Considering (i) the weak definition of the term 'facultative anaerobe' and (ii) the recent redefinition of some anaerobes as microaerophiles, we propose to combine the terms 'facultative anaerobe' and 'microaerophile' to designate the respiratory type of strain M5^T and to indicate clearly the preference of the isolate for low O_2 concentrations.

Strain M5^T differed from *P. acidipropionici*, *P. jensenii* and *P. thoenii* in the range of substrates used (Table 1). L-Sorbose was fermented by strain M5^T only and not by *P. acidipropionici*, *P. jensenii* or *P. thoenii*. In contrast, aesculin was hydrolysed by *P. acidipropionici*, *P. jensenii* and *P. thoenii*, but not by strain M5^T. Bromocresol purple inhibited specifically the growth of *P. acidipropionici*, *P. jensenii* and *P. thoenii*, but not the growth of strain M5^T (Table 2). The propionibacteria belong to the class *Actinobacteria* (Stackebrandt *et al.*, 1997) and are divided into two main groups (Cummins & Johnson, 1986, 1992), the 'cutaneous' propionibacteria, such as *Propionibacterium acnes*, *P. granulosum* and *P. avidum*, and the 'classical' or 'dairy' propionibacteria, such as *P. acidipropionici*, *P. jensenii*, *P. thoenii* and *Propionibacterium freudenreichii*. The cutaneous propionibacteria were isolated from human skin, whereas dairy propionibacteria were isolated mainly from dairy products. Although strain M5^T was not isolated from a dairy product, it is closely related to *P. acidipropionici* and clearly belongs to the phylogenetic cluster of 'dairy' propionibacteria. The presence of strain M5^T in olive mill wastewater is not

surprising since it contains (i) a wide range of carbohydrates released from the olive pulp during the oil-extraction process and (ii) organic acids, and in particular lactic acid, resulting from the fermentation of sugars by the lactic acid bacteria. It is well known that lactic and propionic acid bacteria may develop simultaneously in environments rich in sugars such as dairy by-products (Rehberger & Glatz, 1998). Moreover, besides dairy products, classical propionibacteria have been isolated from soils (Van Niel, 1957; Prevot & Fredette, 1966) and anaerobic digestors (Riedel & Britz, 1993) and also from fermenting olives (Cancho *et al.*, 1970, 1980; Vaughn, 1981). Our results, together with those of Cancho *et al.* (1970, 1980), suggest strongly that classical propionibacteria are also involved in the decomposition of organic compounds of olives. In addition, the presence of strain M5^T, as a microaerophilic bacterium, validates the prior hypothesis that the design of the open-air reservoir permits the growth of microaerophilic bacteria.

On the basis of a polyphasic approach including both genotypic and phenotypic characteristics, we propose to designate strain M5^T as a new species of the genus *Propionibacterium*, *Propionibacterium microaerophilum* sp. nov.

Description of *Propionibacterium microaerophilum* sp. nov.

Propionibacterium microaerophilum (mi.cro.ae.ro'phi.lum. Gr. adj. *mikros* small, Gr. n. *aer* air; Gr. adj. *philos* loving; N.L. neut. adj. *microaerophilum* slightly air-loving).

Colonies on agar plates (basal medium) incubated under an atmosphere of N_2/O_2 (95:5%, v/v) are white and lens-shaped with smooth edges and 2–3 mm in diameter. Cells are Gram-positive, pleomorphic rods with rounded ends, non-motile, non-spore-forming, facultatively anaerobic, microaerophilic and catalase-negative. Although growth occurs under anaerobic and aerobic conditions, the optimum O_2 concentration for growth is 5% in the gas phase of the culture. Nitrate is reduced to N_2 . The strain requires no complex nitrogen nutrients such as yeast extract, amino acids or peptides for growth and can grow with ammonium salts as the only nitrogen source. However, yeast extract stimulates growth. Under anaerobic conditions, large amounts of propionate, acetate and CO_2 are produced from glucose or lactate fermentation. Lactate, formate and succinate can also be detected from glucose fermentation, depending on the pH or the medium composition. Neutrophilic and mesophilic. The pH for growth ranges from 4.5 to 9.5, with optimum growth at 7.0. The temperature for growth ranges from 20 to 45 °C, with optimum growth at 30 °C. The DNA G + C content is 67.7 ± 0.6 mol %. Results of 16S rRNA gene sequence analysis indicate that the isolate is related to members of genus *Propionibacterium* and is closely related to *P. acidipropionici*.

propionici (97.5% similarity). DNA–DNA hybridization with this species showed 56% reassociation. Isolated from the decantation reservoir of olive mill wastewater of an olive oil factory in Raphèle-les-Arles (south of France). The type strain is M5^T (= CNCM I-2360^T = DSM 13435^T).

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