Microcella alkaliphila sp. nov., a novel member of the family *Microbacteriaceae* isolated from a non-saline alkaline groundwater, and emended description of the genus *Microcella*

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A high-G + C-content Gram-positive bacterium, designated as strain AC4r^T, was isolated from a highly alkaline, non-saline groundwater environment (pH 11.4). This organism formed small rod-shaped cells, was aerobic, heterotrophic, catalase-positive and oxidase-negative and had an optimum growth temperature of 35 °C and an optimum pH of 9.5. The strain possessed a B2β-type cell-wall peptidoglycan, with D-Orn as the diagnostic diamino acid. The major respiratory quinones were unsaturated menaquinones with 13 and 14 isoprene units. The predominant fatty acids were anteiso-15 : 0, iso-16 : 0, iso-14 : 0 and iso-15 : 0. The G + C content of the DNA was 67.1 mol%. In a 16S rRNA gene sequence analysis, strain AC4r^T showed the highest level of similarity (99.2%) to the type strain of *Microcella putealis*; however, the DNA–DNA reassociation value between these two organisms was low (38.3%). On the basis of phylogenetic analysis, the DNA–DNA reassociation value and distinct phenotypic characteristics, strain AC4r^T represents a novel species within the genus *Microcella*, for which the name *Microcella alkaliphila* sp. nov. is proposed. The type strain is AC4r^T (=LMG 22690^T = CIP 108473^T).

The ophiolite-like geological context of the aquifer at Cabeço de Vide in southern Portugal, together with its chemical characteristics, strongly suggests serpentinization activity. The groundwater vents at a temperature of 20.5 °C and has high alkalinity (pH 11.4) associated with an extremely low ionic concentration, with Ca²⁺ and OH[−] as major chemical constituents (Tiago *et al.*, 2004). The majority of the bacterial isolates recovered from Cabeço de Vide groundwater belonged to the recently described species *Microcella putealis* (Tiago *et al.*, 2005). In this study, we report the taxonomic characterization of another Cabeço de Vide isolate that represents a novel species of the genus *Microcella*.

Strain AC4r^T was isolated from borehole water at Cabeço de Vide on alkaline buffered medium 2 (ABM2), adjusted to pH 9.5, at 30 °C, as described previously (Tiago *et al.*, 2004). The isolate was routinely cultured under the same conditions and maintained at −70 °C in the same

medium supplemented with 15 % glycerol. Unless otherwise stated, all morphological, biochemical and tolerance tests were performed on that medium at 35 °C, with incubation for up to 6 days as described previously (Tiago *et al.*, 2005).

The growth-temperature range of strain AC4r^T was examined in ABM2 liquid medium buffered at pH 9.5; the pH range for growth was determined at 35 °C in the same medium, and growth in the presence of NaCl was examined in liquid medium at pH 9.5 and 35 °C as described previously (Tiago *et al.*, 2005).

Assimilation of single carbon sources was determined using API 50 CH test strips (bioMérieux), with 0.1 M carbonate/bicarbonate buffer (pH 9.5) supplemented with 0.3 % (w/v) agar (Difco), 0.05 % NH₄Cl (Merck) and macronutrient and micronutrient solutions as described previously (Tiago *et al.*, 2005). Anaerobic growth was assessed at 35 °C in anaerobic chambers with a H₂/CO₂ atmosphere (bioMérieux).

Strain AC4r^T formed yellow-pigmented colonies and Gram-positive, small, rod-shaped, non-motile cells (0.3 µm in width and 3–4 µm in length). The isolate had an optimum growth temperature of 35 °C and did not grow at 20 or 45 °C.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AC4r^T is AJ717385.

The fatty acid compositions of strain AC4r^T and *Microcella putealis* CV2^T and a phylogenetic dendrogram are available as supplementary material in IJSEM Online.

The optimum pH for growth was 9.5; no growth was observed at pH 7.5 or pH 10.5. Optimal growth was observed in the absence of NaCl, but growth occurred in ABM2 containing up to 8.0% NaCl. Despite having the same optimal temperature as the type strain of *M. putealis*, strain AC4r^T had a narrower temperature range for growth, a higher optimal pH for growth and did not grow below pH 8.0 (Tiago *et al.*, 2005).

Strain AC4r^T and the type strain of *M. putealis* utilized several sugars and proteinaceous substrates, but several differences between the two strains were detected that can be used to distinguish the strains (Table 1).

Purified cell-wall preparations were obtained by using the method of Schleifer & Kandler (1972). The amino acids and peptides in cell-wall hydrolysates were analysed as described previously (Schleifer & Kandler, 1972; Schleifer, 1985). Lipoquinones were extracted (from 300 mg freeze-dried cells) and detected as described previously (Tindall, 1989). Cultures used for fatty acid analyses were grown on ABM2 medium, adjusted to pH 9.5, in sealed plastic bags submerged in a water bath at 35 °C for 24 h. Fatty acid methyl esters were obtained from fresh wet biomass by saponification, methylation and extraction as described

previously and were separated, identified and quantified with standard MIS Library Generation Software as described by the manufacturer (Microbial ID).

The peptidoglycan of strain AC4r^T was of the B2β type (according to the nomenclature of Schleifer & Kandler, 1972), with L-homoserine in position 3 of the tetrapeptide and an interpeptide bridge containing glycine and D-ornithine. Although the peptidoglycan structure determined for the novel strain and for *M. putealis* CV2^T is of type B, the diagnostic diamino acid of *M. putealis* strain CV2^T is Lys, although the complete peptidoglycan structure of *M. putealis* remains unknown (Tiago *et al.*, 2005). The respiratory quinones found in strain AC4r^T were menaquinones: MK-13 (47%), MK-14 (35%) and MK-12 (18%). The menaquinone composition and the relative amounts of the menaquinones constitute additional distinctive features of the novel isolate in comparison with the species *M. putealis* (Table 1). The fatty acid composition of strain AC4r^T was characterized by the predominance of branched fatty acids, namely anteiso-15:0, iso-16:0, iso-14:0 and iso-15:0, which constituted 32.6, 30.8, 12.5 and 9.8%, respectively, of the total fatty acids. The fatty acid profiles of AC4r^T and of the type strain of *M. putealis* are similar (see Supplementary Table S1 available in IJSEM Online).

Table 1. Phenotypic characteristics that differentiate strain AC4r^T from *M. putealis*

Data for *M. putealis* are based on three strains and were taken from Tiago *et al.* (2005). Characteristics are scored as follows: +, positive; –, negative; w, weak reaction; v, variable among strains (reaction of type strain in parentheses).

Characteristic	Strain AC4r ^T	<i>M. putealis</i>
Cell size (µm)	0.3 × 3.0–4.0	0.4 × 0.8–1.6
Growth temperature range (°C)	25–40	15–40
Optimum pH	9.5	8.5–9.0
pH range	8.0–10.0	7.5–10.0
Urease	+	w
β-Galactosidase	+	–
Degradation/hydrolysis of:		
Casein	–	+
Starch	w	+
Utilization of sole carbon sources		
D-Arabinose	+	–
D-Mannose	–	+
L-Rhamnose	+	–
Inulin	–	v (+)
Starch	–	+
Glycogen	–	+
Xylitol	–	+
L-Fucose	+	–
Gluconate	–	+
2-Ketogluconate	+	–
5-Ketogluconate	–	+
Major menaquinones	MK-13, MK-14, MK-12	MK-12, MK-13, MK-14, MK-11
Cell-wall diamino acid	D-Orn	Lys

DNA for determination of the G + C content was isolated as described by Nielsen *et al.* (1995). The G + C content of the DNA was determined by HPLC as described by Mesbah *et al.* (1989). The DNA relatedness between the type strain of *M. putealis* and novel strain AC4r^T was determined by DNA–DNA hybridization. For this purpose, DNA was isolated and purified as described by Cashion *et al.* (1977) and reassociation reactions were carried out as described by De Ley *et al.* (1970) with the modifications described by Huß *et al.* (1983).

The 16S rRNA gene was amplified and sequenced as described by Tiago *et al.* (2004) and phylogenetic analysis was performed using the software package MEGA3 (Kumar *et al.*, 2004) after multiple alignments of the sequence data had been obtained using CLUSTAL_X (Thompson *et al.*, 1997). Evolutionary distances were calculated by using the method of Jukes & Cantor (1969), phylogenetic dendrograms were constructed using the neighbour-joining method (Saitou & Nei, 1987) and tree topologies were evaluated by performing a bootstrap analysis (Felsenstein, 1985) of 1000 datasets.

The DNA G + C content determined for strain AC4r^T was 67.1 mol%. Comparative analyses of 1505 nucleotide positions of the 16S rRNA gene sequence of strain AC4r^T with respect to representatives of the main lines of descent within the domain *Bacteria* indicated that this strain is a member of the family *Microbacteriaceae* (see Supplementary Fig. S1 available in IJSEM Online). The most closely related organism was *M. putealis* CV2^T (99.2 % similarity). Despite the close phylogenetic relationship, the two strains exhibited a DNA–DNA reassociation value of only 38.3 %. This fact, together with the distinctive phenotypic characteristics of strain AC4r^T, clearly indicates that it represents a novel species of the genus *Microcella*, for which we propose the name *Microcella alkaliphila* sp. nov.

Description of *Microcella alkaliphila* sp. nov.

Microcella alkaliphila (al.ka.li'phi.la. N.L. n. *alkali* alkali; Gr. adj. *philos* loving; N.L. fem. adj. *alkaliphila* loving alkaline environments).

Cells are small, rod-shaped, 0.3 × 3.0–4.0 µm, non-motile and Gram-positive. Non-endospore-forming. Aerobic and heterotrophic. Colonies are small, smooth, convex and yellow. Oxidase-negative and catalase-positive. The optimum temperature for growth is about 35 °C; no growth occurs at 10 or 45 °C. The optimum pH is 9.5; no growth occurs at pH 7.5 or 10.5. Grows in medium containing up to 8.0 % NaCl, but the growth rate is higher without added NaCl. Peptidoglycan is type B2β with D-Orn as the diagnostic diamino acid. The major respiratory quinones are MK-13 and MK-14. The predominant fatty acids (i.e. constituting approx. 10 % or more) are anteiso-15:0, iso-16:0, iso-14:0 and iso-15:0. The type strain does not hydrolyse elastin, aesculin, gelatin, arbutin or casein. Hydrolyses starch and arginine. Urease, β-galactosidase and DNase are present; xylanase is not detected. Nitrate is not reduced. The type

strain assimilates glycerol, arabinose, ribose, D-xylose, galactose, glucose, fructose, L-rhamnose, mannitol, cellobiose, maltose, sucrose, D-turanose and 2-ketogluconate. The DNA G + C content of the type strain is 67.1 mol%.

The type strain, AC4r^T (= CIP 108473^T = LMG 22690^T), was isolated from water from the borehole at Cabeço de Vide in southern Portugal.

Emended description of the genus *Microcella* Tiago *et al.* 2005

The genus description is as given by Tiago *et al.* (2005), with the following modifications and additions. The cell-wall peptidoglycan is of the B-type, with Lys or Orn as the diamino acid. The major isoprenologues are unsaturated menaquinones with 12–14 isoprene units.

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