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Microbacterium endophyticum sp. nov. and *Microbacterium halimionae* sp. nov., endophytes isolated from the salt-marsh plant *Halimione portulacoides* and emended description of the genus *Microbacterium*

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ABSTRACT

Fifteen actinobacterial isolates retrieved from the endophytic community of the salt-marsh plant *Halimione portulacoides* were characterised in this study. ERIC-PCR fingerprinting analysis divided these isolates into two groups represented by strains PA15^T and PA36^T, respectively.

16S rRNA gene sequence analyses showed the isolates belonged to the genus *Microbacterium*, with *Microbacterium saccharophilum* NCIMB 14782^T as the closest phylogenetic relative to which they have pairwise sequence similarities of 98.7–98.8%. Strains PA15^T and PA36^T are closely related having a pairwise sequence similarity of 99.8%. However, DNA–DNA hybridization result between both was well under 70% confirming them as distinct genomic species. Both strains have a B2β peptidoglycan type with ornithine as diaminoacid, MK-10 and MK-11 as major menaquinones, anteiso-C15:0, anteiso-C17:0 and iso-C16:0 as major fatty acids. These features as well as DNA–DNA hybridization results clearly separate them from *M. saccharophilum*.

On the basis of physiological, chemotaxonomic and genetic characteristics we propose that the isolates studied represent two novel species of the genus *Microbacterium*, *Microbacterium endophyticum* sp. nov. (type strain PA15^T = DSM 27099^T = CECT 8354^T) and *Microbacterium halimionae* sp. nov. (type strain PA36^T = DSM 27576^T = CECT 8593^T).

The genus *Microbacterium* includes Gram-stain positive, rod-shaped and non-spore-forming bacteria [22,28] and currently consists of more than 80 species that have been described from a wide diversity of terrestrial and aquatic environments, including the phyllosphere and rhizosphere of plants [4,5,18,23,36]. Several *Microbacterium* strains have also been isolated from wild legume nitrogen fixing nodules collected in different geographical sites in the infra-arid zone of Tunisia [35]. Although these strains did not induce nodule formation in the legumes tested, *Macroptilium atropurpureum* and *Retama raetam*, the presence of *nifH*-like gene sequences was reported for several of these isolates [35]. Thus, although the occurrence of *Microbacterium* strains as endophytes has not been widely studied, present data suggest that this microorganism may be common in plant tissues.

In a study on the diversity of the bacterial endophytic communities from the salt-marsh halophyte plant *Halimione portulacoides*, a set of actinobacterial isolates represented by strains PA5, PA8, PA15^T, PA17, PA22, PA24, PA29, PA32, PA36^T, RZ26, RZ29, RZ42, RZ45, RZ46 and RZ49 was isolated from the plant samples collected from Ria de Aveiro estuary in Portugal. On the basis of phenotypic and genotypic characteristics, we propose that these strains represent two novel species of the genus *Microbacterium*, and the names *Microbacterium endophyticum* sp. nov. (PA5, PA8, PA15^T, PA17, PA22, PA24, PA29, PA32, RZ26, RZ29, RZ42, RZ45, RZ46 and RZ49) and *Microbacterium halimionae* sp. nov. (PA36^T) are proposed.

Samples of *H. portulacoides* (sea purslane) were collected in the estuary Ria de Aveiro in the area of Largo do Laranjo, Murtosa (40°43′48.0″N 8°36′45.5″W). Plant tissues (roots and aerial parts) were surface sterilized by washing with ethanol and sodium hypochlorite [2] and macerated on phosphate buffered saline (PBS). The extracts were serially diluted and plated on Tryptic Soy Agar

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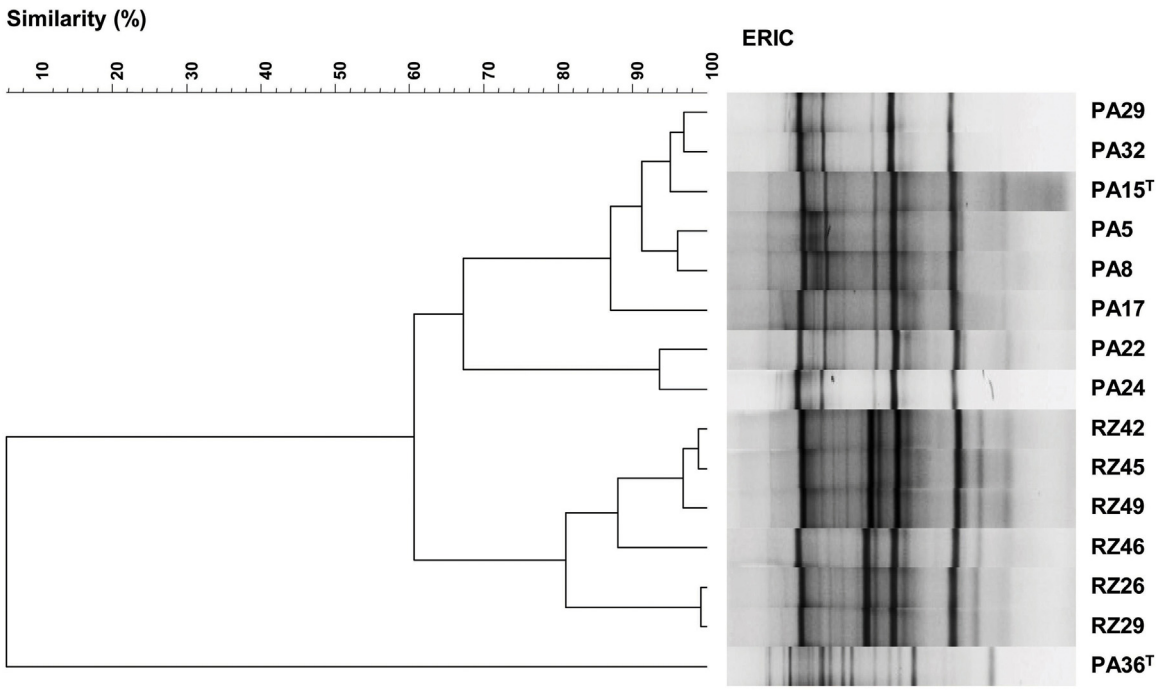

Fig. 1. UPGMA cluster analysis based on the Pearson coefficient of ERIC-PCR fingerprints of *Microbacterium* spp. strains isolated from *H. portulacoides*. Similarity is indicated as a percentage.

(TSA, Merck). After incubation at 28 °C during 72 h colonies were transferred and purified on TSA.

For morphological and physiological characterisation strains were cultured on TSA medium at 28 °C for 72 h. The ability to grow at different temperatures (10, 15, 20, 25, 30, 37, 44 °C), salinities (0.5, 1, 2, 4, 6, 8%, w/v NaCl) and pH values (4.5, 5, 5.5, 6, 6.5, 8, 8.5, 9, 10, 11, 12) was tested on TSA agar as the basal medium. In the case of pH tests the medium was adjusted using appropriate buffer solutions [3,19]. Oxidase and catalase activities were carried out as described previously [33]. Gram reaction was determined using a Gram Staining Kit (Merck) according to the manufacturer's instructions. Physiological characteristics of the strains were determined using API 20NE, API ZYM and API 50CH test strips (Biomérieux) following the manufacturer's recommendations.

Biomass for chemotaxonomic studies was prepared by cultivating the strains in Tryptic Soy Broth at 28 °C for 72 h on a rotary shaker until reaching stationary phase. Cells were harvested by centrifugation and washed with 0.9% (w/v) NaCl. The analyses of peptidoglycan structure were carried as described by Schumann [26]. Menaquinones were extracted from freeze-dried cells using the two stage method described by Tindall [31,32], separated by TLC on silica gel and further analysed by HPLC.

For fatty acid methyl ester analysis, all strains were grown on TSA agar at 28 °C for 3 days or until good growth was obtained on the third quadrant [25]. Fatty acids were extracted, methylated and analysed according to the standard protocol of the Sherlock microbial identification system (MIDI) and peaks were named using the database SACTIN6. The type strains *Microbacterium pumilum* DSM 21018^T [13], *Microbacterium flavum* DSM 18909^T [14] and *M. saccharophilum* NCIMB 14782^T [21] were also analysed for comparative studies.

The DNA base composition of strains PA15^T and PA36^T was determined by HPLC [20]. For DNA–DNA hybridization studies, DNA was isolated by the modified procedure of Gevers et al. [10] and hybridizations in the presence of 50% formamide at 53 °C were carried out using the modified microplate method [7,8,11]. Hybridization pairs were as follows: strain PA15^T and *M. saccharophilum* NCIMB 14782^T; PA15^T and PA36^T; *M. saccharophilum* and PA36^T.

Genomic DNA for ERIC-PCR and 16S rRNA gene sequencing was extracted using the Genomic DNA Purification Kit (Thermo Scientific) according to the manufacturer's instructions. Genetic relatedness of the 15 isolates was evaluated by ERIC-PCR fingerprinting using primers ERIC1 and ERIC2 [1]. Cluster analysis of ERIC fingerprints was carried out with the software GelCompar II (Applied Maths). The 16S rRNA gene was amplified using the universal primers 27F and 1492R [17]. Purified PCR products were sequenced at GATC Biotech. The identification of phylogenetic neighbours was carried out by searching against the database containing type strains with validly published prokaryotic names and representatives of uncultured phylotypes (http://www.ezbiocloud.net/eztaxon) [15]. 16S rRNA gene pairwise sequence similarities were determined using the programme PHYDIT v3.1 [6].

Nearly full-length 16S rRNA gene sequences were aligned with sequences from type strains of all *Microbacterium* species retrieved from GenBank database using ClustalX v2.1 [30]. Phylogenetic analyses were performed with MEGA v 6.0 [29] using the neighbour-joining (NJ) [24] and maximum likelihood (ML) [9] algorithms. Evolutionary distances were calculated with the Kimura 2-parameter model [16] and *Clavibacter michiganensis* DSM 46364^T and *Plantibacter flavus* DSM 14012^T were used as the outgroup taxa. A bootstrap analysis [12] based on 1000 replicates was done to evaluate the robustness of the trees topologies.

ERIC-PCR analysis showed that the majority of the strains (14) exhibited very similar ERIC-PCR fingerprints but they were not clonal, while PA36^T had a quite distinctive fingerprint (Fig. 1). Nearly full-length 16S rRNA gene sequences (1438 bp) were obtained for the new strains and pairwise sequence comparisons showed that strains PA5, PA8, PA15^T, PA17, PA22, PA24, PA29, PA32, RZ26, RZ29, RZ42, RZ45, RZ46 and RZ49 shared identical sequences.