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Phenylobacterium falsum sp. nov., an *Alphaproteobacterium* isolated from a nonsaline alkaline groundwater, and emended description of the genus *Phenylobacterium* ☆

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Abstract

A Gram-negative bacterium designated AC-49^T was isolated from an alkaline groundwater with a pH 11.4. This organism formed rod-shaped cells, was strictly aerobic, catalase and oxidase positive, with an optimum growth temperature of 35 °C and an optimum pH value of 8.0. Strain AC-49^T assimilated primarily amino acids and some Krebs cycle metabolites, did not use sugars for growth. The organism did not grow on L-phenylalanine or antipyrin. The G+C content of DNA was 66.9 mol%. The phylogenetic analyses based on the 16S rRNA sequencing showed that the closest relatives of strain AC-49^T were *Phenylobacterium lituiforme* and *Phenylobacterium immobile*, indicating that the organism is a member of the order *Caulobacterales* of the *Alphaproteobacteria*. Based on the phylogenetic analyses and distinct phenotypic characteristics, we are of the opinion that strain AC-49^T, represents a novel species of the genus *Phenylobacterium* for which we propose the name *Phenylobacterium falsum* sp. nov.

Keywords: *Alphaproteobacteria*; *Phenylobacterium falsum*; Alkaline groundwater

Introduction

Natural alkaline environments are uncommon geological features. Soda lakes and soda deserts represent the most stable naturally occurring alkaline environments on earth, with pH values generally higher than 10 and occasionally reaching pH 12 [12]. The alkalinity of these environments is generated by the accumulation of sodium carbonate. However, most of these sites contain high concentrations of NaCl and other salts giving rise to saline, and sometimes, hypersaline environments [12]. Nonsaline alkaline environments are much rarer. The genesis of these peculiar environments depends on complex geological formations that probably lead, in all cases to a single geochemical process known as serpentinization [27]. Basically this process can be depicted as the weathering, by CO_2-charged waters, of silicate minerals present in mafic or ultramafic rocks. This rare process can be found in some of environments such as: groundwater associated to kimberlite formation as in Lake Timiskaming and Kirkland Lake kimberlite

☆Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number(s): AJ717391 for strain AC-49^T.

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fields in Canada [22]; ground and surface water associated to some ophiolites in northern California [24], the Semail in Oman [4], Troodos ophiolictic complex in Cyprus [5], and the Maqarin site in Jordan [3]. Recently, serpentinization has been implicated in the formation of a new class of sea-floor hydrothermal system know as the Lost City Field, and considered as a potential environment for the emergence of life on the Earth's ocean floor [16]. Remarkably, water associated with ophiolites and serpentinization activity has also been considered as habitat analogs on Mars [24].

The alkalinity of these water environments is generated by high levels of $Ca(OH)_2$ and maintained, around pH 11, due to an equilibrium between the solid phase $Ca(OH)_2$ and the soluble Ca^{2+} and OH^-.

Recently, we investigated the bacterial diversity of a groundwater at Cabeço de Vide, in Southern Portugal. The ophiolite-like geological background of this aquifer and its chemical characteristics, strongly suggests serpentinization activity. This groundwater has a high alkalinity (pH 11.4) associated with an extremely low ionic concentration where Ca^{2+} and OH^- are major chemical constituents [27].

We isolated a large number of bacterial strains during a survey of the bacterial diversity present in the water samples collected from the Cabeço de Vide borehole which were mainly Gram-positive. Nevertheless, we also recovered a small number of Gram-negative isolates phylogenetically related to the caulobacters and particularly one organism closely related to the lineage containing the species *Phenylobacterium immobile* [18] and *Phenylobacterium lituiforme* [15].

In this study, we describe the morphological, physiological, chemotaxonomic and phylogenetic characteristics of this organism. On the basis of the data presented, strain AC-49^T should be placed in the genus *Phenylobacterium* as a new species for which the name *Phenylobacterium falsum* sp. nov. (AC-49^T = LMG 22693^T = CIP 108469^T) is proposed.

Materials and methods

Bacterial strains and culture conditions

Strain AC-49^T was isolated, as described previously, in 10 × diluted Alkaline-Buffered Medium 2 (ABM2), adjusted to pH 7.0, at 37 °C [27]. After isolation the strain was routinely cultured in an altered version of R3A medium (designated R3A-V) adjusted to pH 8.0, at 35 °C, and maintained at −70 °C in the same medium supplemented with 15% glycerol. The R3A-V contained the following components per liter of media: yeast extract (Difco), 1.0 g; proteose peptone (Difco no. 3), 1.0 g; casamino acids, 1.0 g; glucose, 1.0 g; K_2HPO_4, 0.6 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; Na pyruvate, 0.05 g; agar (Difco), 15.0 g; 50 ml of a macronutrients solution 10 × concentrated; 5 ml of a micronutrients solution 100 × concentrated; and 100 ml of a specific buffer solution (listed below) at a concentration of 1 M, autoclaved separately and mixed after cooling. The 10 × concentrated macronutrients solution contained per liter: nitrilotriacetic acid, 1.0 g; $CaSO_4 \cdot 2H_2O$, 0.6 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; NaCl, 0.8 g; KNO_3, 1.03 g; $NaNO_3$, 6.89 g; $NaHPO_4$, 1.11 g. The 100 × concentrated micronutrients solution contained per liter: $MnSO_4 \cdot H_2O$, 0.22 g; $ZnSO_4 \cdot 7H_2O$, 0.05 g; H_3BO_3, 0.05 g; $CuSO_4 \cdot 5H_2O$, 0.0025 g; $Na_2MoO_4 \cdot 2H_2O$, 0.0025 g; $CoCl_2 \cdot 6H_2O$, 0.0046 g.

The following buffer solutions, prepared according to Gomory [9], were used to adjust the medium to different pH values: citrate buffer was used to adjust the pH to 6.5, phosphate buffer was used to adjust the pH to 7.0 and 7.5, tris (hydroxymethyl) aminomethane–Tris buffer was used for pHs between 8.0 and 8.5, carbonate-bicarbonate buffer was used for pHs between 9.0 and 10.0 and carbonate-KOH was used to for pH values higher than 10.0. The pH value of each lot of solid medium was verified prior to using with a surface-testing Sentix® Sur pH electrode (WTW, Hoskin Scientific).

Morphological, physiological and biochemical tests

Cell morphology and motility were examined by phase-contrast microscopy. Gram reaction, the presence of cytochrome oxidase and catalase were determined as described by Smibert and Krieg [25]. All tests were performed after 24 h of incubation at 35 °C on R3A-V, pH 8.0.

The pH range for growth of strain AC-49^T was examined by determining the turbidity (610 nm) of cultures incubated in 300 ml Erlenmeyer flasks containing 100 ml of R3A-V, between pH 6.0 and 10.0, in a reciprocal water bath (170 rpm) at 35 °C. The growth temperature range of strain AC-49^T was examined in liquid medium at pH 7.0, 8.0 and 9.0 between 20 and 45 °C. Growth of AC-49^T, in the presence NaCl (w/v) ranging up to 9.0%, was examined in liquid medium at pH value 8.0 and 35 °C.

Single-carbon source assimilation was determined using API 50 CH test strips (Analytab Products Inc., Biomerieux, France), using 0.1 M tris (hydroxymethyl) aminomethane–Tris buffer pH 8.0 supplemented with 0.03 g agar (Difco); K_2HPO_4, 600 mg; $MgSO_4 \cdot 7H_2O$, 100 mg; NH_4Cl_2, 500 mg; vitamin B12, 0.03 mg; 50 ml of macronutrients solution and 5 ml of micronutrients solution (described above) per liter. Microorganisms were resuspended in sterilized water with a turbidity corresponding to the McFarland No. 5 and 6 standard [25]. The cell suspensions (3 ml) were added to 60 ml of