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Isolation and characterization of a diazotrophic, oxalate-oxidizing bacterium from sour grass (*Oxalis pes-caprae* L.)

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Abstract

A new type of nitrogen-fixing, oxalate-oxidizing *Azospirillum* sp. was isolated from the roots of *Oxalis pes-caprae*. Polyphasic taxonomy was performed, including auxanography using API galleries, physiological tests and 16S rRNA sequence comparison. Optimum growth occurred at 30 °C, pH 7.5. Growth was observed at 37 and 42 °C with oxalate and in the presence of 3–4% NaCl and 2% potassium oxalate. In liquid culture, the doubling time (t_d) with oxalate was 9 h. Its closest phylogenetic neighbors, as deduced by 16S rDNA-based analysis, were *Azospirillum brasilense*, *Azospirillum doebereinerae* and *Azospirillum lipoferum*, with 99.5, 98.4 and 96.7% sequence similarity, respectively. The strain differed from *A. brasilense* by its ability to use *N*-acetylglucosamine, D-glucose and D-mannitol. It may be a variant strain of *A. brasilense*. Oxalotrophic, N_2-fixing species of the genus *Azospirillum* may be important contributors to soil formation, soil fertility, and retention and/or cycling of elements necessary for plant growth.

Keywords: *Azospirillum*; Oxalate-bacteria; Oxalotrophic; Oxalate; Taxonomy

1. Introduction

Azospirillum species are important rhizosphere bacteria and represent the best characterized genus of plant growth-promoting rhizobacteria. Since the first detailed description of the genus *Azospirillum* with two species, *Azospirillum lipoferum* and *Azospirillum brasilense* [39], members of *Azospirillum* have been isolated from the roots of numerous wild and cultivated grasses, cereals, food crops and soils in tropical, subtropical and temperate regions all over the world [3,35,41]. In addition, various genera of diazotrophic bacteria, such as *Beijerinckia*, *Azotobacter* and *Derxia* spp., have been isolated from the rhizosphere of several gramineous plants [4].

At present, seven species have been described: *Azospirillum lipoferum*, *A. brasilense* [39], *A. amazonense* [25], *A. halopraeferens* [29], *A. irakense* [19], *A. largimobile* [8] and *A. doebereinerae* [9]. They display versatile C and N metabolism, which makes them well adapted to the competitive environment of the rhizosphere [13].

Several plant genera accumulate substantial amounts of oxalate crystals. Oxalic acid is first prepared from the expressed juice of sorrel. The common species in Europe and North America is *Oxalis acetosella*, the wood sorrel. *Oxalis pes-caprae* L. (syn. *O. cornua*) is a widespread herb which has caused a large number of deaths among sheep in Australian pastures. In the Mediterranean it is sometimes used for cattle feed [24]. Typically, roots contain many organic acids varying in chain length with lactate, acetate, oxalate, succinate, fumarate, malate, citrate, isocitrate and aconitate being the primary anion components [17]. *Azospirilla* exhibit positive chemotaxis towards organic acids, sugars, amino acids and aromatic compounds as well as towards root exudates [14].

Several species of aerobic bacteria are known to be able to grow with oxalate. Most of them are facultative methylotrophs and/or facultative hydrogen-oxidizing chemolitho-

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autotrophs [16,31–33,38]. Thus far, a limited number of diazotrophic strains have been reported, mainly from soil litter close to oxalate-producing plants, which are able to utilize oxalate as sole carbon and energy source; they belong to the genera *Xanthobacter*, *Mesorhizobium*, *Burkholderia*, *Beijerinckia* and *Azospirillum*, and the taxonomic position of most strains described is unclear [33]. Little attention has been paid to the occurrence of oxalotrophic bacteria in the rhizoplanes or rhizospheres of plants.

With the purpose of providing additional new pure cultures of oxalotrophic bacteria for further work in the field of oxalate metabolism and taxonomy, 14 novel strains have been reported [32]. Among them, a non-pigmented strain deriving from independent enrichment procedures was isolated on N-free calcium-oxalate agar plates. The present report is focused on its physiological properties and phylogenetic position.

2. Materials and methods

2.1. Pure culture isolation

Oxalis pes-caprae were collected from the Izmir province in April–May intact with root zone sediments and transported to the laboratory. Roots and rhizomes were shaken and washed briefly with distilled water to remove loosely associated sediments; then 1 g of fresh material was macerated and inoculated into Erlenmeyer flasks containing N-free Schlegel's basal mineral medium [2]. In addition, 4 g/l potassium oxalate was added to the liquid cultures which were then incubated with agitation (105 t/min) at 28 °C for 5 days. All enrichment cultures were transferred three times to the same medium at 5-day intervals. Pure cultures were obtained by streak plating of suspensions (approx. 0.1 ml) of the enrichment culture on N-free solidified Schlegel's basal mineral medium containing calcium oxalate. Consumption of oxalate was indicated by the formation of a clear zone around the colonies due to the dissolution of calcium oxalate and morphologically distinct colonies that developed clear zones were selected. For morphological and physiological characterization, the purified cultures were maintained on Schlegel's basal mineral medium agar slants containing 4 g/l potassium oxalate as carbon source and stored at 4 °C or as glycerol suspensions (20%, v/v) of cells at −25 °C.

2.2. Strain characterization

Preliminary characterization of strains was by colony morphology, cell morphology and motility, Gram and PHB staining using standard methods [22]. Carbon source assimilation tests were performed as described earlier [38]. Stock solutions of different carbon sources were filter-sterilized and added to Schlegel's basal mineral medium to a final concentration of 1–2 g/l. The pH of the media was adjusted to 7 with HCl or NaOH for routine analysis or to 5, 6, 7 or 8 for pH profile analysis. Cultures were grown in 50 ml of medium in 250 ml flasks incubated with agitation (105 t/min) in the dark. The growth was monitored turbidimetrically, using a Shimadzu 1601 VIS scanning spectrophotometer at 600 nm. All experiments were performed in triplicate.

Auxonagraphic features were determined by using the API 20E and API 20NE gallery methods (BioMerieux). The bacterial inoculum was diluted in minimal medium according to the manufacturer's instructions, incubated aerobically at 30 °C and examined visually for growth daily over a period of 5 d. Additionally, the API ZYM gallery method was performed for determination of extracellular enzymatic activities. Hydrogenase activity was determined by using the 1% TTC (triphenyltetrazolium salt) impregnated membrane filter method [2].

2.3. Cellular lipid analysis

Cells were grown at 27 °C on nutrient agar plates until good growth were observed. The cellular lipids were extracted overnight at 37 °C from cells with 250 µl chloroform/methanol 2:1 (v/v). To avoid possible losses of polar glycolipids, no attempt was made to remove non-lipid contaminants from the extracts. Lipid solutions were stored in the dark at −20 °C until analyzed. Lipids were separated by TLC and two-dimensional thin-layer chromatography (2D-TLC) with Silica Gel $60F_{254}$ TLC plates (Merck). The solvent for the first dimension (solvent I) was chloroform-methanol-water (65:25:4, by vol.); that for the second dimension (solvent II) was chloroform–methanol–acetic acid–water (100:20:12:5, by vol.) [30]. A 50% sulfuric acid solution followed by heating at 110 °C was used to detect spots of all types of lipids.

2.4. Resistance to antibiotics

Resistance to antibiotics was determined on Mueller–Hinton agar (Difco 0252) using standard antibiotic disks (Oxoid). The inhibition zone was noted after 48 h incubation. Strains were considered susceptible when the inhibition zone was 12 mm or more in diameter [32]. Tests were performed in triplicate.

2.5. Effect of heavy metals on growth of isolates

To determine heavy metal sensitivity (Zn^{2+}, Ni^{2+}, Pb^{2+}, CrO_4^{2-}, Co^{2+} and Cu^{2+}), one ml of a culture of the strain, which was grown to exponential phase in mineral medium, was transferred to 20-ml vials containing 10 ml Schlegel's mineral medium supplemented with 1 mM heavy metals. In the experiment to determine heavy metal sensivity the cells were incubated at 30 °C for 7 days and harvested by centrifugation.