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Soil Biology & Biochemistry 39 (2007) 2905–2914

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Isolation and characterization of mineral phosphate-solubilizing bacteria naturally colonizing a limonitic crust in the south-eastern Venezuelan region

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Received 18 January 2007; received in revised form 7 June 2007; accepted 19 June 2007
Available online 20 July 2007

Abstract

With the aim to explore the possible role of mineral phosphate-solubilizing bacteria (PSB) in phosphorus (P) cycling in iron-rich, acidic soils, we conducted a survey of PSB naturally colonizing a limonitic crust in the south-east region of Venezuela (Bolívar State). A total of 130 heterotrophic bacterial isolates showing different degrees of mineral tri-calcium phosphate ($Ca_3(PO_4)_2$)-solubilizing activities were isolated from NBRIP plates. In contrast, no isolates showing iron phosphate ($FePO_4$)- or aluminum phosphate ($AlPO_4$)-solubilizing activities were detected by this experimental approach. The 10 best $Ca_3(PO_4)_2$-solubilizers were selected for further characterization. These isolates were shown to belong to the genera *Burkholderia*, *Serratia*, *Ralstonia* and *Pantoea* by partial sequencing analysis of their respective 16S rRNA genes. All the PSB isolates were able to mediate almost complete solubilization of $Ca_3(PO_4)_2$ in liquid cultures; in contrast, the PSB isolates were less effective when solubilizing $FePO_4$. Two groups of PSB isolates were clearly differentiated on the basis of their $Ca_3(PO_4)_2$ solubilization kinetics. Acidification of culture supernatants seemed to be the main mechanism for P solubilization. Indeed, gluconic acid was shown to be present in the supernatant of five isolates. Furthermore, detection of genes involved in the production of this organic acid was possible in three isolates by means of a PCR protocol.

Keywords: Phosphate solubilization; Phosphate-solubilizing bacteria; Iron-rich acidic soils; GDH

1. Introduction

Natural solubilization of mineral phosphates is a phenotype exhibited by many soil-borne microorganisms (known as PSM, for phosphate-solubilizing microorganisms). In natural environments, i.e. the rhizosphere of different plant species, phosphate-solubilizing bacteria (PSB) are considered to play an important ecophysiological role: indeed, PSB mobilize insoluble inorganic phosphates from their mineral matrix to the bulk soil where they can be absorbed by plant roots. In turn, the plants supply root-borne C compounds, mainly sugars, which can be metabolized for bacterial growth (Goldstein, 1995; Deubel et al., 2000). The discovery of this mutual relationship between plants and PSB encouraged the development of new technologies, such as the use of PSM for biofertilization to improve crop yield (Richardson, 2001; Niranjan Raj et al., 2006; Saghir Khan et al., 2007). Moreover, the development of commercial bioinoculants and the large-scale bioprocessing of rock phosphate ores—through the action of PSB—has resulted in the highly efficient, low-cost and successful commercial technologies now used by the agroindustry worldwide (Goldstein et al., 1993; Goldstein, 2000; Matsushita et al., 2002).

The phenotype exhibited by PSB has been traditionally associated with the production of low-molecular-weight organic acids, mainly gluconic and keto-gluconic acids (Rodríguez and Fraga, 1999; Goldstein, 2000; Deubel et al., 2000). These acids are produced in the periplasm of many Gram-negative bacteria through a direct oxidation pathway of glucose (DOPG, non-phosphorylating oxidation) whose physiological role remains uncertain (Anthony, 2001, 2004;

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0038-0717/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2007.06.017

Matsushita et al., 2002). The enzymes of the DOPG, the quinoproteins glucose dehydrogenase (GDH) and gluconate dehydrogenase (GADH), are oriented to the outer face of the cytoplasmic membrane so that they oxidize their substrates in the periplasmic space (Anthony, 2004). Consequently, the organic acids diffuse freely outside the cells and may release high amounts of soluble phosphorus (P) from mineral phosphates, by supplying both protons and metal complexing organic acid anions (Gadd, 1999).

Studies dealing with the mobilization of insoluble phosphates by PSM in harsh environments have been conducted by only a few research groups (Goldstein et al., 1999; Johri et al., 1999; Vázquez et al., 2000; Puente et al., 2004; Son et al., 2006). However, to our knowledge no studies concerning this topic have been conducted in ferric-iron-rich, acidic and P-deficient soils, despite the fact that almost 80% of tropical soils in Latin America share these characteristics (López-Hernández, 1977). In the case of Venezuela, these types of soils may account for nearly 32% of the national territory (Casanova et al., 1992). The low fertility of acidic soils is mainly due to the transformation of soluble forms of P into forms of poor solubility, particularly Fe–P and Al–P complexes, which can be regarded as unavailable to plants (Rengel and Marschner, 2005; Johnson and Loeppert, 2006). The presence of these complexes reduces the nutrient capacity of these soils for sustaining plant and microbial growth (Tiessen et al., 1996; Richardson, 2001). Therefore, bacteria colonizing P-deficient environments should exhibit high P-mobilizing abilities in order to sustain their own growth; this is the basis of the so-called "stress physiology paradigm" proposed by Goldstein et al. (1999). According to these authors, the rhizosphere of plants able to grow under these conditions should include one or more unique populations of PSM, which may contribute to P nutrition of plants.

The long-term goal of our work is to explore the possible role of PSB in P cycling in iron-rich, acidic soils. With this objective in mind, in the present work we conducted a survey of PSB naturally colonizing a limonitic crust, containing very low amounts of inorganic P, at the surface of an iron deposit in the south east region of Venezuela (Bolívar State). Bacteria showing the highest degrees of phosphate-solubilizing (PS) activities were selected according to standard procedures, further identified by molecular methods and characterized at the physiological and biochemical levels. Moreover, the PS activities of some selected strains were tested against two insoluble P salts: tri-calcium phosphate ($Ca_3(PO_4)_2$) and iron phosphate ($FePO_4$). This is the first step of a series aimed towards a better understanding of microbial-mediated P-mobilization in ferric-iron-rich soils.

2. Materials and methods

2.1. Soil samples and isolation of PSB

Soil samples were collected at the surface of an iron deposit located near Ciudad Piar (Bolívar State, Venezuela) (7°27′0″ North, 63°19′0″ West). In this area, the soils are classified as acid, Ultisols, according to the Instituto Geográfico de Venezuela Simón Bolívar (http://www.igvsb.gov.ve/site2006/imagenes/mapas/suelos.jpg). The samples were mainly composed of limonite and contained 58–60% Fe oxides/hydroxides and 0.82% Al_2O_3. The total inorganic P (Pi) content was shown to be very low and tightly associated with metals (Fe and Al) in the form of insoluble complexes (M. Benavides, personal communication).

The samples were stored at 4 °C in sterile containers. For each soil sample, several sub-samples were taken, homogenized in sterile MilliQ water containing 0.85% NaCl (wt/vol) and serially diluted. Aliquots of each dilution were spread on NBRIP medium (Nautiyal, 1999), and incubated at 30 °C for 24–48 h. Additionally, modified NBRIP media, containing either $FePO_4$ or aluminum phosphate ($AlPO_4$) as a sole source of P, were also used for the initial screening step. Colonies were selected from the plates on the basis of the appearance of a clear halo; the clones were further purified on minimal medium based on AT salts (Katznelson et al., 1962). Once purified, each isolate was stored as a glycerol stock at −80 °C.

2.2. Growth media and conditions

NBRIP liquid medium ($MgCl_2 \cdot 6H_2O$ (5 g l^{-1}), $MgSO_4 \cdot H_2O$ (0.25 g l^{-1}), KCl (0.2 g l^{-1}), $(NH_4)_2SO_4$ (0.1 g l^{-1}), $Ca_3(PO_4)_2$ (5 g l^{-1}) amended with glucose (10 g l^{-1}; Nautiyal, 1999) was inoculated with a 1% (v/v) inoculum coming from pre-cultures grown in the same medium. In some experiments, $FePO_4$ or $AlPO_4$ were used instead of $Ca_3(PO_4)_2$ as sole sources of P. Flasks containing 200 ml of inoculated media were incubated at 30 °C on a Kottermann 4020 shaker at medium speed (80 cycles min^{-1}). Samples (2 ml) were taken aseptically at different times and used to determine pH and growth (O.D. at 600 nm). Due to the presence of suspended particles of insoluble $Ca_3(PO_4)_2$ in the supernatant, the samples were first allowed to sediment for 15 min at RT and then were centrifuged at very low speed (350*g*) for 1 min 30 s. The supernatant was removed and diluted 1:1 with 1 N HCl in order to dissolve the residual insoluble phosphate. The sediment containing the remaining insoluble $Ca_3(PO_4)_2$ at each time point was used to determine the kinetics of solubilization (see below).

2.3. Mineral phosphate solubilization assays

The PS activity of each of the isolates was determined by following the protocol of Mehta and Nautiyal (2001). In brief, the isolates were grown in NBRIP medium containing a pH indicator (Bromophenol Blue) for 3 days at 30 °C with continuous agitation. At the end of the incubation period the final OD_{600} values were subtracted from the initial values. The solubilization efficiencies were determined by spotting 10 µl of overnight-grown cultures on top