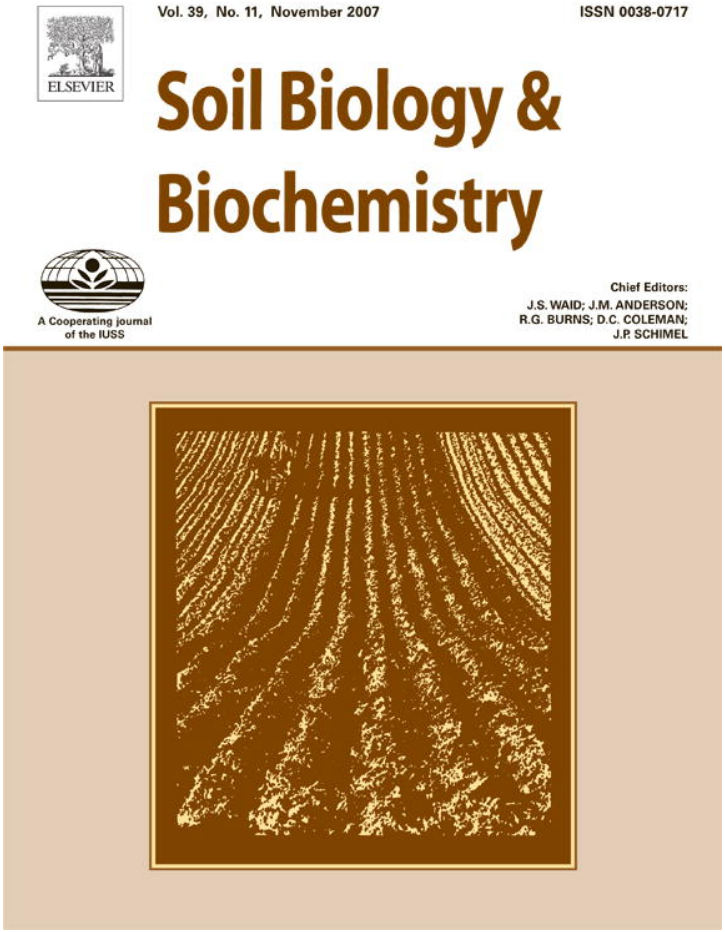

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Isolation and characterization of mineral phosphate-solubilizing bacteria naturally colonizing a limonitic crust in the south-eastern Venezuelan region

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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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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

of NBRIP plates supplemented with either $Ca_3(PO_4)_2$ or $FePO_4$. The plates were incubated at 30 °C for 48 h and assayed visually. The ratio between the diameter of the halo and the diameter of the colony was calculated and multiplied by 100 (Nguyen et al., 1992). Finally, the kinetics of $Ca_3(PO_4)_2$ solubilization mediated by each of the isolates was further monitored in liquid NBRIP medium.

2.4. PCR amplification of 16S rRNA and sequencing

The gene-encoding 16S rRNA was amplified from selected strains by the polymerase chain reaction (PCR) using bacterial universal primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′) (Weisburg et al., 1992). The PCR mix consisted of deoxynucleotides at 200 μM each, 0.25 μM of each primer, 2.5 mM $MgCl_2$, 1 × PCR buffer and 0.2 U of Taq DNA polymerase (New England Biolabs). A suspension of cells on MilliQ water, coming from a fresh colony grown on Nutrient Agar, was used as target DNA. The following cycle conditions were used: 95 °C for 3 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and a final extension step at 72 °C for 3 min (Lane, 1991).

The PCR products were purified from agarose gels with the PCR Clean-up Gel Extraction Kit (Macherey-Nagel, Germany) and sequenced. The nucleotide sequences were compared using the BlastN program (Altschul et al., 1997), and the closest match of known phylogenetic affiliation was used to assign the isolated strains to specific taxonomic groups. Species identification was improved by performing several classical microbiological and biochemical tests.

2.5. Detection of gdh and pqqE by PCR

Two primer sets were used to amplify by PCR both the *gdh* gene, encoding membrane GDH, and the *pqqE* gene, whose product is involved in the biosynthesis of the PQQ cofactor. Both genes are involved in the expression of the PS^+ phenotype, through the activation of the DOPG. The gene encoding the apoenzyme (*gdh*) was amplified using primers pqq2 and pqq4 and cycling conditions previously reported (Pujol and Kado, 1999).

The *pqqE* gene was chosen among the rest of the genes belonging to the *pqq* operon for two reasons: (i) it is present in all of the sequences available on the databases and (ii) it contains the most conserved regions comprising the entire *pqq* operon. The sequences available in public databases for *pqqE* (*Pantoea agglomerans*, *Klebsiella pneumoniae*, *Kluyvera intermedia*, *Rahnella aquatilis*, *Ralstonia eutropha* strain JMP134, *Pseudomonas aeruginosa* strain PAO1 and *P. fluorescens*) were aligned using the ClustalW program (Thompson et al., 1994) and degenerate primers were designed according to the sequences of the most conserved regions. Primers pqqF (5′-GARCTGACYTAYCGCTGYCC-3′) and pqqR2 (5′-TSAGSAKRARSGCCTGRCA-3′), were used to amplify an internal fragment of 900 bp from *pqqE* (R represents any purine, Y any pyrimidine, S represents G or C and K represents T or G). The reaction conditions were the following: 95 °C for 1 min, then 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. In preliminary experiments, fragments of the expected sizes were cloned in pBluescript SK^+ and sequenced. The results obtained validated the PCR protocols described above. Therefore, PCR products of the expected size were considered to correspond to the genes being amplified.

2.6. Thin-layer chromatography

Qualitative detection of organic acids was performed by thin-layer chromatography (TLC) with a modification of the procedure described by Joveva et al. (1991). The acids were separated from culture supernatants on Silica Gel plates (10 by 10 cm) impregnated with 5% metaphosphoric acid, using a two-step migration. The first migration was performed in solvent A (isopropanol–pyridine–water–acetic acid [8:8:1:4]) until the front of the solvent was 6 cm away from the starting point; then, the plate was air dried and the second migration took place in solvent B (isopropanol–pyridine–water–acetic acid [8:8:3:4]) until the front of the solvent was 8 cm away from the starting point. The plate was air dried before spraying freshly prepared reactive C (*ρ*-anisaldehyde 0.5% in methanol–sulfuric acid–acetic acid [9:5:5]). Finally, the plate was dried at 120 °C for 15 min and photographed. Standards consisting of glucose and several organic acids, prepared from pure chemicals (Sigma), were included in the chromatography.

2.7. Analytical procedures

The kinetics of phosphate solubilization during growth of the cultures in NBRIP medium were estimated both directly and indirectly by the following procedures: (a) soluble Pi concentration was determined colorimetrically by using the molybdenum blue method (Murphy and Riley, 1962); (b) to avoid an underestimation of solubilization rates by only measuring soluble Pi in the supernatant of actively grown cultures (Rodríguez and Fraga, 1999), the remaining insoluble $Ca_3(PO_4)_2$ at each time point was also measured. For this, samples were collected as mentioned above (see: Growth media and conditions), the cell-containing supernatant was completely removed after 15 min of settling, and the remaining $Ca_3(PO_4)_2$ was resuspended in an equal volume of MilliQ water and vortexed thoroughly. The O.D. of the particle' suspension was immediately monitored at 600 nm, against a blank containing medium salts. A standard curve was prepared by measuring the OD_{600} of homogenized suspensions of $Ca_3(PO_4)_2$ containing known amounts of the insoluble phosphate.

3. Results

3.1. Isolation of PSB from soil samples

The screening strategy employed during this research enabled the identification of PSB colonies only on NBRIP medium containing $Ca_3(PO_4)_2$ as a sole P source. No colonies exhibiting a clear halo were observed on agar plates supplemented with either $FePO_4$ or $AlPO_4$. Approximately 130 bacterial isolates showing clear halos of Ca_3PO_4 solubilization were selected among thousands of colonies. Some obvious differences in the size of the halos of different isolates were observed (not shown). This preliminary observation suggested the existence of bacterial isolates exhibiting different degrees of PS efficiencies in the soil samples collected. To confirm this observation, the 130 purified isolates were tested following the protocol of Mehta and Nautiyal (2001), a method previously shown to be a reliable and qualitative indicator of the PS activity of different bacterial isolates. Fig. 1 shows the OD_{600nm} shift of the culture supernatants of each of the 130 PSB isolates after a 3-day cultivation period in NBRIP-BPB medium. Again, clear differences in the acidification of the culture supernatants by different isolates were evident. Indeed, some isolates did not show any significant change in the absorbance of the supernatant (not shown), while others exhibited OD_{600} shifts of more than 1.5 units. Furthermore, we noticed that the most dramatic changes in the color of the supernatant correlated with a total solubilization of $Ca_3(PO_4)_2$ in the medium. Based on these results we selected ten isolates exhibiting the highest PS activities for further studies (see Fig. 1). The solubilization efficiencies of these isolates calculated according to Nguyen et al. (1992) are shown in Table 1. Three of them, namely MMB040, MMB047 and MMB051, showed the highest values.

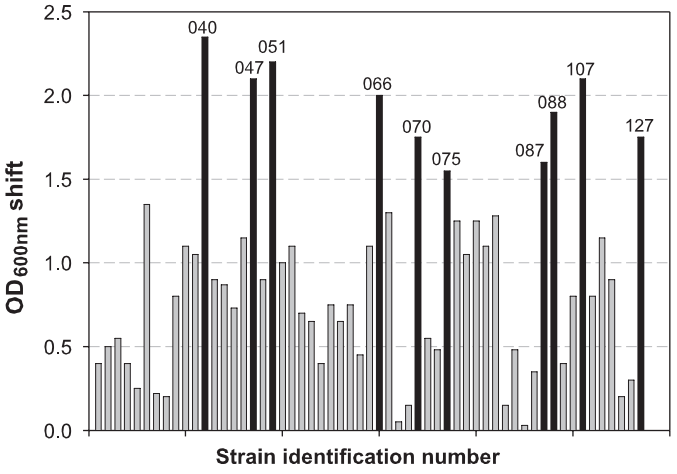

Fig. 1. Qualitative analysis of PS activity in NBRIP-BPB medium. The OD_{600} of the supernatant of the NBRIP-BPB medium at the end of the 3-day culture period was subtracted from the initial OD_{600} and the difference plotted for each strain. Isolates exhibiting an OD_{600} shift of >1.5 units were selected for further studies (black bars).

These results were further confirmed by determining the soluble Pi in the supernatant of liquid cultures (Table 1). When $Ca_3(PO_4)_2$ was added to the NBRIP medium as the sole source of P ($1\,g\,l^{-1}$), after 3 days a complete solubilization was observed in every case. In contrast, when $FePO_4$ was used, the amount of solubilized P by each of the strains was much lower, with isolates MMB066 and MMB070 showing the highest values of solubilized P (Table 1). Moreover, these two isolates were the only ones which exhibited a halo of solubilization when re-tested in agarized NBRIP medium containing $FePO_4$ for calculating solubilization efficiencies (see Materials and Methods; Table 1).

3.2. Identification of PSB isolates

All the PSB isolates selected in the previous steps were shown to be Gram-negative, motile rods. Some clear differences were noticed when studying colony phenotype (Table 1). Other biochemical and physiological characteristics were also studied and are shown on Tables 1 and 3. Nucleotide sequencing of PCR-amplified 16S rRNA genes and sequence comparison with available data in the GenBank using the BLAST algorithm (Altschul et al., 1997) allowed us to identify the majority of the PSB isolates (Table 2). Based on a sequence identity of 97% or greater (Van Waasbergen, 2004) they were all affiliated to the β- or γ- sub-divisions of the *Proteobacteria*: three isolates were similar to species of the *Burkholderia* genus, another three were similar to *Pantoea* species, two were closely related to *Serratia* species, and the last one is a member of the genus *Ralstonia*. Thus, preliminary phylogenetic placements based on the results of the BLAST analysis revealed that the majority of the isolates clustered together to members of well characterized and previously named bacterial species (not shown).

3.3. Characterization of PSB isolates

All the selected PSB isolates grew well at 30 °C in LB broth, reaching the stationary phase after 5–6 h (not shown). When grown in NBRIP medium (Nautiyal, 1999), most of the isolates reached the stationary phase after 12 h and exhibited similar growth rates and biomass yields (Fig. 2A). In all cases, bacterial growth was concomitant with a significant pH decrease (Fig. 2B). Indeed, after a 24 h period, the pH decreased from the initial values (6.8–7.0) to 3.2–4.0 pH units; that is, a variation of more than 3 pH units. Strikingly, one isolate acidified the culture supernatant to pH values below 3.0 after only 12–14 h (strain MMB066).

PSB-mediated $Ca_3(PO_4)_2$ solubilization in NBRIP medium followed two different kinetics (Fig. 2C): in one group of strains (MMB088, 087, 107, 070 and 066), the disappearance of the insoluble phosphate followed a steep curve (mean rate of P solubilization: ~0.05 mg P solubilized h^{-1}), and most of the $Ca_3(PO_4)_2$ was completely

Table 1
Phenotypic characteristics of the PSB isolates[a]

Isolate	Colony morphology[b]	Solubilization of phosphate		Gluconic acid production[e]	PCR detection	
		$Ca_3(PO_4)_2$ PSE[c]/ $mg\,l^{-1}$ sol. Pi[d]	$FePO_4$ PSE[c]/$mg\,l^{-1}$ sol. Pi[d]		*gdh*	*pqqE*
MMB040	C, E, R, Y, V	375/97.6±3.4	0/3.7±0.8	No	No	No
MMB047	C, E, Cr, Y,V	363/85.4±4.5	0/6.9±2.2	Yes	No	No
MMB051	C, E, Cr, Y,V	338/80.5±2.8	0/8.7±1.6	Yes	Yes	Yes
MMB066	C, I, R, W, Cy	188/78.6±5.6	175/42.3±3.4	No	No	Yes
MMB070	C, I, R, W, Cy	214/82.7±1.2	130/13.7±2.1	No	No	Yes
MMB075	C, E, R, OW, V	189/75.4±4.6	0/7.2±1.4	Yes	No	Yes
MMB087	C, E, R, OW, V	285/86.5±2.6	0/2.4±1.2	No	No	Yes
MMB088	C, E, R, OW, V	250/84.4±3.8	0/3.9±0.8	No	No	No
MMB107	C, I, R, W, Cy	313/92.3±2.2	0/2.7±0.4	Yes	Yes	Yes
MMB127	C, E, R, OW, V	216/78.9±4.6	0/2.1±0.6	Yes	Yes	Yes

[a]All PSB isolates were shown to be Gram-negative, motile straight rods.
[b]Colony morphology in LB medium: C: circular; E/I: entire/irregular edge; R/Cr: raised/crateriform; Y/W/OW: yellow/white/off-white; V/Cy: viscous/creamy.
[c]PSE: Phosphate-solubilizing efficiency calculated according to Nguyen et al. (1992) from colonies grown in NBRIP plates supplemented with either $Ca_3(PO_4)_2$ or $FePO_4$ (see Materials and Methods).
[d]Soluble Pi in the culture supernatants determined according to Murphy and Riley (1962) (results represent the mean of three replicates±SD).
[e]Determined from TLC plates developed according to Joveva et al. (1991).

Table 2
Identification of PSB isolates from iron-rich soil samples by 16S rDNA sequencing

Isolate	Length of 16S rRNA gene sequenced	GenBank accession no.	Most closely related organism		
			Species (strain)	Accession no.	% Gene identity
MMB040	979	DQ785817	*Burkholderia cepacia* (ATCC55792)	AY741359.1	99.6
MMB047	979	DQ785818	*Pantoea ananatis* (BD647)	DQ195525.1	98.3
MMB051	987	DQ785819	*Pantoea agglomerans* (SC-2)	AY924375.1	98.3
MMB066	391	DQ785820	*Serratia marcescens* (RZ01)	DQ832185.1	99.2
MMB070	549	DQ785821	*Serratia marcescens* (CECRIbio01)	DQ207558	99.8
MMB075	984	DQ785122	*Ralstonia picketii* (ATCC27511)	AY741342.1	98.9
MMB087	ND	ND	ND	ND	ND
MMB088	980	DQ785123	*Burkholderia cepacia* (ATCC39277)	AY741347.1	99.5
MMB107	986	DQ785124	*Pantoea agglomerans* (WAB1927)	AM184266.1	88.5
MMB127	982	DQ785125	*Burkholderia vietnamiensis* (LMG1029)	AF097534.1	99.5

Table 3
Biochemical characteristics of the PSB isolates[a]

Biochemical reactions	MMB040	MMB047	MMB051	MMB066	MMB070	MMB075	MMB087	MMB088	MMB107	MMB127
Citrate (Simmons)	−	−	−	+	+	+	+	+	−	+
Methyl-red	−	−	−	−	−	−	−	−	−	−
Voges–Proskauer	−	+	+	+	+	−	−	−	+	−
Glucose utilization	−	+	+	+	+	−	−	−	+	−
Lactose utilization	−	+	+	+	+	−	−	−	+	−
Gelatin hydrolysis	+	−	−	+	+	−	+	+	−	+
Oxidase	−	−	−	−	−	+	−	−	−	−
Nitrate test	+	+	+	+	+	−	+	+	+	+
Hugh–Leiffson	F−/O+	F+/O+	F+/O+	F+/O+	F+/O+	F−/O+	F−/O+	F−/O+	F+/O+	F−/O+
TSI	−	+	+	+	+	−	−	−	+	−

+, Tested positive/utilized as substrate; −, tested negative/non-utilized as substrate. F: fermentation; O: oxidation.
[a]All PSB isolates reacted positive for catalase and negative for H_2S production.

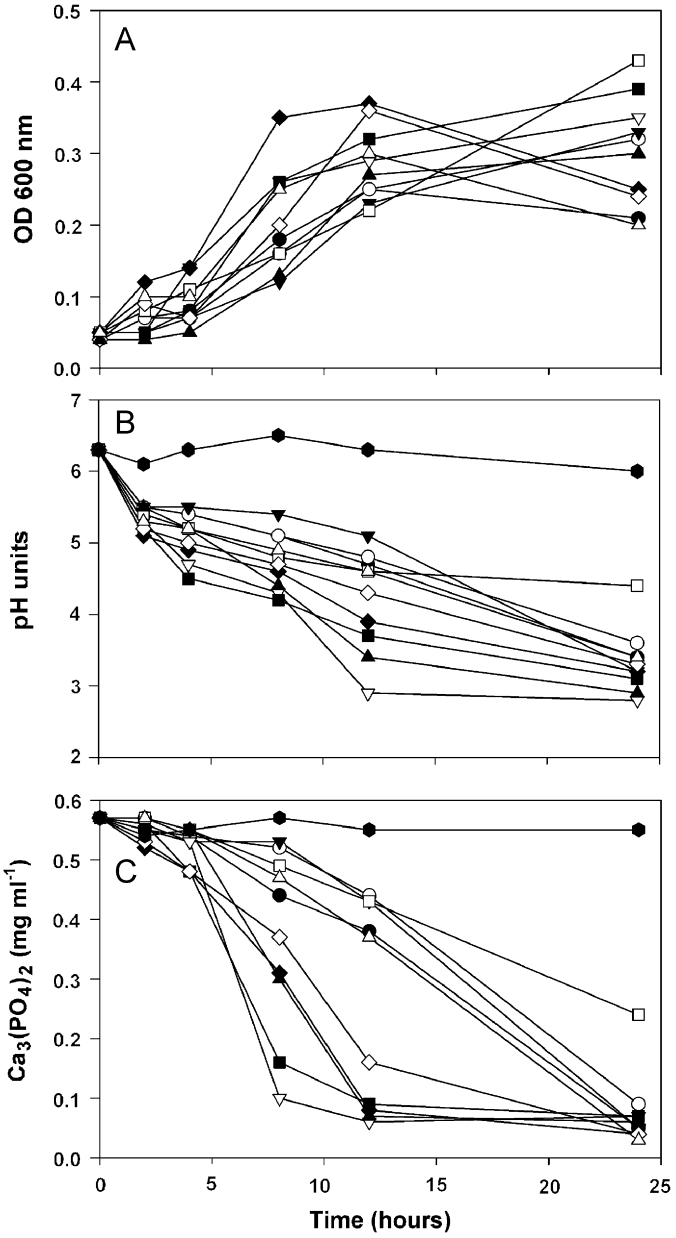

Fig. 2. PS activity of bacterial isolates in NBRIP medium. PSB isolates were inoculated into NBRIP medium and samples were harvested aseptically at different time intervals; bacterial density (A), pH (B) and remaining insoluble phosphate (C) were determined in each sample (by triplicate) and the mean values plotted against time. Symbols: ○, MMB040; ●, MMB047; ▼, MMB051; ▽, MMB066; ■, MMB070; □, MMB075; ♦, MMB087; ◇, MMB088; ▲, MMB107; △, MMB127; ●, uninoculated control.

solubilized after 12 h culture; the rest of the isolates (MMB040, 047, 051, 075 and 127) solubilized the mineral phosphate at a less pronounced rate (mean rate of P solubilization: ~0.02 mg P solubilized h^{-1}), showing complete solubilization after 24 h culture. In all cases, the solubilization of $Ca_3(PO_4)_2$ was concomitant with the acidification of the culture supernatants (see Fig. 2B and C). Indeed, TLC-analysis of culture supernatants revealed the presence of gluconic acid in five cases (see Table 1).

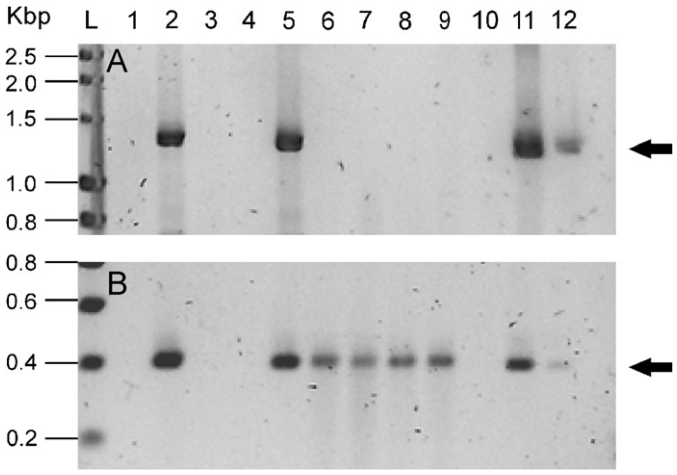

Fig. 3. PCR amplification of *gdh* and *pqqE* genes from PSB isolates. Agarose gel electrophoresis showing *gdh* (A) and *pqqE* (B) amplification products from PSB isolates. Lanes: L, SmartLadder (Eurogentec); 1, negative control; 2, positive control; 3, MMB040; 4, MMB047; 5, MMB051; 6, MMB066; 7, MMB070; 8, MMB075; 9, MMB087; 10, MMB088; 11, MMB107; 12, MMB127.

3.4. Screening of PS genes in bacterial isolates

In order to test for the presence of the genes encoding the enzymes responsible for gluconic acid production, we performed PCR amplification experiments using specific primers for *gdh* and *pqqE* genes. The results obtained show that seven PSB isolates exhibited a band corresponding to a fragment of the *pqqE* gene (Fig. 3B). Interestingly, the *gdh* gene was detected only in three of these isolates (namely MMB051, MMB107 and MMB127) (Fig. 3A).

4. Discussion

4.1. Screening and identification of PSB

The preliminary screening protocols used by many for the identification of PSB isolates rely, usually, on the use of $Ca_3(PO_4)_2$ or hydroxyapatite on indicator plates (Goldstein et al., 1999; Rodríguez and Fraga, 1999; Barroso and Nahas, 2005). However, in acid soils the available inorganic P fraction is found adsorbed to the surface of soil minerals or occurs as poorly soluble precipitates of $FePO_4$ and/or $AlPO_4$ (Richardson, 2001). Consequently, we decided to use three different synthetic compounds representative of phosphate minerals commonly found in soils as sole sources of P for the initial screening of PSB on agarized medium. Our results show, however, that even though several hundreds of colonies were able to grow on medium supplemented with each of the aforementioned phosphate compounds, solubilization halos surrounding bacterial colonies were observed only on plates containing $Ca_3(PO_4)_2$. This is in agreement with a previous report of Puente et al. (2004), which shows that bacteria isolated from the rhizoplane of desert plants, although very effective in dissolving Ca-phosphates on solid medium,

were not able to produce clear halos on agar plates amended with $AlPO_4$ or $FePO_4$. Therefore, for subsequent studies on phosphate solubilization we kept the best P solubilizers, selected from an initial group of 130 isolates showing clear halos in this type of plates. The isolates were shown to belong to bacterial genera previously demonstrated by different authors as to participate in the solubilization of several different mineral phosphates (Rodríguez and Fraga, 1999). Furthermore, the PS activities of these isolates in the presence of $Ca_3(PO_4)_2$, calculated according to Nguyen et al. (1992), did not exceed the values reported for closely related species (Rodríguez and Fraga, 1999).

Bacterial growth in the absence of any visible sign of phosphate solubilization in plates containing $FePO_4$ or $AlPO_4$ was also observed, and may have resulted from the solubilization of small amounts of P when placing these compounds in contact with the culture medium. This observation has been previously reported by Reyes et al. (1999) and Delvasto et al. (2006) when using $FePO_4$ and hydroxyapatite, respectively, in liquid cultures. Nevertheless, since it has been demonstrated that many isolates not producing any visible halo on agar plates are indeed able to solubilize different types of insoluble phosphates in liquid medium (Gupta et al., 1994), we decided to monitor the solubilization activities of the selected isolates when grown in liquid NBRIP containing $FePO_4$ as the sole source of P. Our results show that the mobilization of P from $FePO_4$ occurs at a much lesser extent when compared with $Ca_3(PO_4)_2$ (Table 2). This is in agreement with the available information on this matter which shows that, although very effective in mobilizing P from $Ca_3(PO_4)_2$, PSM initially identified by the standard approach exhibit a much lower efficiency when grown in the presence of sparingly soluble Fe–P or Al–P complexes (Banik and Dey, 1983; Jones et al., 1991; Illmer and Schinner, 1995; Whitelaw et al., 1999; Reyes et al., 1999; Chung et al., 2005; Barroso and Nahas, 2005; Son et al., 2006). Moreover, some authors have shown that even when testing PS^{+++} mutants with an increased PS activity, $FePO_4$ is hardly solubilized (Reyes et al., 1999). Interestingly, the two isolates exhibiting the highest levels of $FePO_4$ solubilization in liquid medium were also shown to produce small solubilization halos when re-tested in agar plates containing $FePO_4$ (Table 1). However, it is worth mentioning that due to the use of bacterial suspensions and not single cells as the inoculum in this assay, the size of the respective colonies were much larger than those observed in the initial screening tests conducted in the same type of medium.

Together, our results clearly show that care should be taken when monitoring the existence and prevalence of Fe–P and/or Al–P bacterial solubilizers in natural environments, by means of an experimental approach which is not suited for this specific purpose. Indeed, the screening of PSB in NBRIP medium containing $Ca_3(PO_4)_2$ as an indicator is biased towards the identification of strains which solubilize this particular P-salt mainly as a result of a drastic acidification of the colony surroundings. Furthermore, the use of this screening procedure reduces the possibility of identifying other PSB-strains which may exhibit different P-solubilization strategies towards less soluble forms of P.

4.2. Role of PSB in inorganic phosphate solubilization

Acidification seemed to be the main strategy followed by the PSB isolates identified in the present work for solubilizing P. Indeed, a clear relationship could be established between bacterial growth, supernatant acidification and P solubilization from $Ca_3(PO_4)_2$. It has been well established that, as a common strategy to scavenge P from insoluble mineral sources, microorganisms produce and exudate organic acids and/or chelating agents (Rodríguez and Fraga, 1999). In the particular case of Gram-negative bacteria, it has been shown that they may mobilize insoluble phosphates very efficiently as a consequence of the production of gluconic acid, which results from the extracellular oxidation of glucose via the quinoprotein glucose dehydrogenase (Goldstein, 1996). Interestingly, analysis of the culture supernatants allowed us to detect the presence of this particular organic acid in the supernatant of five cultures. Furthermore, the gene-encoding glucose dehydrogenase (*gdh*), the enzyme responsible for gluconic-acid production, was also detected by a PCR method in 3 of these isolates. The absence of a PCR product when trying to amplify *gdh* from the rest of the gluconic acid producing isolates does not necessarily mean that it is absent from their genomes; rather, this result may be attributed to an inefficient amplification reaction, possibly due to the presence of inhibitor compounds in the reaction mixture.

Nevertheless, neither the production of gluconic acid nor the presence of a *gdh* gene were detected when testing the rest of the isolates, even though they did also significantly acidify the culture supernatants after 24 h growth. We believe that, in these cases, the acidification might be due to the production and excretion of other organic acids, which may also act as chemical agents for mobilizing insoluble phosphates. For example, oxalic acid produced by a *Penicillium*-like fungus has been shown to partially solubilize phosphate from an iron ore (Parks et al., 1990). Moreover, citrate released by *Pseudomonas fluorescens* has been shown to effectively mobilize P from goethite, a common iron oxide/hydroxide (Hoberg et al., 2005). The carboxylate anions of these organic acids may compete with PO_4^{2-} for binding sites and chelate Fe and Al, liberating this way phosphates in solution (Cline et al., 1982; Fox et al., 1990; Gerke, 1992).This could explain the ability of the two strains of *Serratia* sp. (MMB066 and MMB070) to solubilize $FePO_4$, since it has been shown that members of this genera produce both citric and oxalic acids, among other organic acids (Solé et al., 2000). Furthermore, other mechanisms have also been proposed to explain bacterial-mediated phosphate solubilization, apart from the production and excretion of gluconic acid:

these include the production of chelating substances, the release of protons originated by NH_4^+ assimilation and the production of inorganic acids (Illmer and Schinner, 1995, Rodríguez and Fraga, 1999). We are currently attempting to identify the metabolites excreted by isolates MMB066 and MMB070 involved in P solubilization from $FePO_4$.

4.3. Kinetics of phosphate solubilization

During preliminary experiments to estimate phosphate solubilization rates in NBRIP medium, we noticed that the amount of soluble Pi in the cell-free culture supernatants did not match the values obtained by subtracting the remaining amount of insoluble $Ca_3(PO_4)_2$, at the time of sampling, from the initial values (results not shown). This result is perhaps not surprising, since a significant proportion of bacteria-solubilized Pi is incorporated into biomass during growth (Rodríguez and Fraga, 1999). Therefore, to avoid an underestimation of solubilization rates, we decided to quantify the remaining insoluble phosphate at each time point, instead of determining soluble Pi in the culture supernatants. The use of such an experimental approach allowed us to discriminate between two groups of bacterial isolates showing different PS kinetics: in one group, the complete solubilization of $Ca_3(PO_4)_2$ occurred in 12 h, approximately; the other group of isolates solubilized the same amount of P only after 24 h (and even after longer periods). In 1984, Babenko et al. differentiated PSB into four physiological groups based on studies of the kinetics and rates of P solubilization (Babenko et al., 1984). As they demonstrated, in some cases the increase of soluble P in the supernatant followed the growth of the culture in an almost linear relationship. On the contrary, another group of PSB mediated the appearance of oscillating levels of soluble Pi in the culture, a result that was attributed to co-precipitation of soluble Pi with some organic metabolites. In a recent paper, Delvasto et al. (2006) demonstrated that the oscillatory behavior of soluble-P kinetics in batch cultures of *Burkholderia caribiensis*, strain FeGL3 isolated from a Brazilian iron-ore, was related to the re-precipitation and re-dissolution of an intermediate Ca-phosphate phase, namely brushite. Our results adds to these observations, showing the existence of important differences between the kinetics of solubilization of $Ca_3(PO_4)_2$ mediated by PSB isolates, when monitored in batch cultures. Moreover, our data also suggest that P incorporation into biomass must be considered when estimating solubilization rates, since cultures may attain high cellular densities requiring thus significant amounts of P to sustain bacterial growth.

5. A final comment on P-mobilization from iron-rich, acidic soils

When considering microbial-mediated solubilization of insoluble P complexes commonly found in iron-rich, tropical and sub-tropical acid soils, two main phenomena should be taken into account: (i) microbial-mediated reductive dissolution of Fe(III)-oxides under anaerobic conditions (Hutchison and Hesterberg, 2004; Chacón et al., 2006); and, (ii) production and release of protons and chelating agents by microbes under aerobic conditions (Illmer and Schinner, 1995; Jones, 1998; He and Zhu, 1998; Rodríguez and Fraga, 1999). In the first case, seasonally flooding of soils may contribute to the release of P through the combined action of sulphate-reducing bacteria and Fe(III)-respiring microorganisms (Chacón et al., 2006). However, in well-drained, aerobic soils the second phenomenon should be of paramount importance in P mobilization from insoluble Al- and Fe-complexes. Nevertheless, the information available on this matter is scarce and even contradictory. Indeed, some studies show that PSM, including fungi and bacteria, have only a limited capacity to solubilize $FePO_4$ and $AlPO_4$ (Banik and Dey, 1983; Whitelaw et al., 1999). These studies suggest that where poorly soluble Fe–P and Al–P occur in high amounts, the microbial-mediated mobilization of P may be less effective. In turn, this may be one of the reasons explaining the low fertility of soils exhibiting those characteristics. On the other side, Illmer et al. (1995) demonstrated that a *Pseudomonas* strain was very efficient in solubilizing $AlPO_4$. A more recent study shows that PSB can effectively reduce the content of P adsorbed by goethite, a common ferric oxide (Hoberg et al., 2005), confirming previous observations of He et al. (2002), He and Zhu (1998) and Shang et al. (1996). These apparent contradictions require further studies to be clearly explained. Moreover, other factors affecting P availability in acid soils should also be taken into account when facing this issue: for example, the role of root-exudates in rhizosphere acidification, and the contribution of ecto- and endo-mycorrhiza in mobilizing P from sparingly soluble sources.

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