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Characterization of the Mineral Phosphate-Solubilizing Activity of *Pantoea aglomerans* MMB051 Isolated from an Iron-Rich Soil in Southeastern Venezuela (Bolívar State)

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Abstract The mineral phosphate-solubilizing (MPS) activity of a *Pantoea agglomerans* strain, namely MMB051, isolated from an iron-rich, acidic soil near Ciudad Piar (Bolívar State, Venezuela), was characterized on a chemically defined medium (NBRIP). Various insoluble inorganic phosphates, including tri-calcium phosphate [$Ca_3(PO_4)_2$], iron phosphate ($FePO_4$), aluminum phosphate ($AlPO_4$), and Rock Phosphate (RP) were tested as sole sources of P for bacterial growth. Solubilization of $Ca_3(PO_4)_2$ was very efficient and depended on acidification of the external milieu when MMB051 cells were grown in the presence of glucose. This was also the case when RP was used as the sole P source. On the other hand, the solubilization efficiency toward more insoluble mineral phosphates ($FePO_4$ and $AlPO_4$) was shown to be very low. Even though gluconic acid (GA) was detected on culture supernatants of strain MMB051, a consequence of the direct oxidation pathway of glucose, inorganic-P solubilization seemed also to be related to other processes dependent on active cell growth. Among these, proton release by ammonium (NH_4^+) fixation appeared to be of paramount importance to explain inorganic-P solubilization mediated by strain MMB051. On the contrary, the presence of nitrate (NO_3^-) salts as the sole N source affected negatively the ability of MMB051 cells to solubilize inorganic P.

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Introduction

Most tropical soils are acid, iron rich, and deficient in soluble forms of phosphorus (P), one of the essential elements in crop production [9]. In order to increase their fertility, chemical fertilizers containing soluble forms of P are applied in important amounts to the farmyard. Yet, a great proportion of soluble P is rapidly precipitated into forms of sparing solubility, particularly Fe-P and Al-P complexes, which can be regarded as unavailable to plants [11, 19]. As an alternative strategy, phosphatic-bearing minerals, particularly Rock Phosphate (RP) are also used [18]. RP, which usually contains some form of the mineral apatite, can be applied directly to the soil with varying agronomic efficiencies depending on the type of soil and crop. The use of such a natural resource constitutes an economic, environmentally friendly, and efficient way of fertilizing crops in many tropical and subtropical countries.

Many soil microorganisms, particularly those colonizing the rhizosphere of plants, are able to mobilize insoluble inorganic phosphates from their mineral matrix, including RP, to the soil solution, making them available to plant roots. In turn, the plants supply root-borne C compounds, mainly sugars and organic acids, which can be metabolized for bacterial growth [4, 6]. The mineral phosphate-solubilizing phenotype (MPS^+) exhibited by soil bacteria has been traditionally associated with the production of low-molecular-weight organic acids, mainly gluconic (GA) and ketogluconic acids (KGA) [4, 7, 21]. These acids are produced in the periplasm of many Gram-negative bacteria

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through a direct oxidation pathway of glucose (DOPG; nonphosphorylating oxidation) [1]. The enzymes of the DOPG pathway [i.e., 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 [1]. Consequently, the organic acids diffuse freely outside of the cells and might release high amounts of soluble P from mineral phosphates, by supplying both protons and metal-complexing organic acid anions [21].

The aim of this work was to characterize the P-solubilizing activity of a *Pantoea agglomerans* strain, previously isolated by us from an iron-rich, acidic soil near Ciudad Piar (Bolívar State, Venezuela), toward various insoluble inorganic phosphates, including RP. We also report on the mechanisms involved in P solubilization by *P. agglomerans* MMB051, as part of our efforts toward understanding the microbial mechanisms beneath P-cycling, particularly in iron-rich, acidic soils.

Materials and Methods

Bacterial Strains

One bacterial strain, namely MMB051, was isolated from an iron-rich, acidic soil in the southern region of Venezuela (Ciudad Piar, Bolívar State) and identified as *P. agglomerans* [17]. This strain exhibited a high MPS activity both on liquid and agarized NBRIP media.

Growth Media and Conditions

Strain MMB051 was routinely grown at 30°C on NBRIP medium [5 g/L $MgCl_2 \cdot 6H_2O$, 0.25 g/L $MgSO_4 \cdot H_2O$, 0.2 g/L KCl, 0.1 g/L $(NH_4)_2SO_4$], amended with glucose (10 g/L) [16]. Different inorganic phosphates were added to the NBRIP medium as sole sources of P (see below). The cultures were grown with continuous agitation on a reciprocal shaker at medium speed (80 cycles/min). In some experiments, 0.15 g/L KNO_3 was added instead of $(NH_4)_2SO_4$ as the sole N source.

Resting Cells Acidification Assay

One colony of strain MMB051 grown overnight in Luria Bertani (LB) medium was inoculated onto 50 mL of LB broth and grown overnight at 30°C with continuous agitation. Cells were collected by centrifugation, washed three times with a 0.85% NaCl sterile solution, and adjusted to an optical density (OD_{600}) of 0.6 ($\sim 5 \times 10^8$ cfu/ mL) in the same solution. Ten milliliters of these cell suspensions were poured inside a 50-mL plastic tube and magnetically stirred at room temperature. The pH of the suspensions were determined at a 5-min time interval after giving an initial pulse of glucose (100 µL of a concentrated solution in distilled water) for a final concentration of 10 mM (modified from [24]).

Mineral Phosphate-Solubilization Assays

The mineral phosphate-solubilization activities of *P. agglomerans* MMB051 was estimated from batch cultures in NBRIP medium supplemented with 1 g/L of either tricalcium phosphate [$Ca_3(PO_4)_2$], iron phosphate ($FePO_4$), or aluminum phosphate ($AlPO_4$). In some experiments, NBRIP medium was supplemented either with 50 mg/L KH_2PO_4 or 1 g/L RP from the Monte Fresco deposits (Táchira State, Venezuela) containing 27.7% P_2O_5, 38.6% CaO, 17.3% SiO_2, and 2.73% F. The experiments were carried out in 250-mL Erlenmeyer flasks, which were filled with 75 mL of NBRIP medium. Bacteria were inoculated by adding 0.15-mL aliquots of an overnight-grown culture in LB broth. The cells were washed twice in NBRIP salts (1X) before inoculation. A series of uninoculated flasks were used as controls. Flasks were incubated at 30°C on a reciprocal shaker at medium speed (80 cycles/min) and 3-mL aliquots were harvested at different times. The samples were serially diluted with NBRIP salts (1X) and streaked onto LB plates to estimate the bacterial density by titration (cfu/mL). The supernatant was collected after a centrifugation step at 12,000 *g* for 5 min and stored at −20°C until use. A subsample of this supernatant was used to determine the pH.

Glucose Dehydrogenase Assay

Whole cell suspensions were used as the source of enzyme in glucose dehydrogenase (GDH) assay carried out according to Matsushita and Ameyama [14]. For this, overnight-grown cells were harvested by centrifugation (3000 *g* for 20 min), washed twice with NBRIP salts, and stored at −20°C until use. GDH activity was measured spectrophotometrically at 610 nm in assay buffer [0.1 M Tris/HCl, pH 7.2, 1 mg/mL Würster's blue (*N*,*N*,*N*′,*N*′-tetramethyl-*ρ*-phenylenediamine; Sigma)] using 50 mM glucose as the substrate. Calculations of GDH activity were based on a molar extinction coefficient for Würster's blue of 12,400 M^{-1} cm^{-1} at 610 nm [5].

Analytical Procedures

Thin-layer-chromatography (TLC), performed according to the procedure described by Joveva et al. [12], was used for qualitative detection of organic acids. Organic acid detection was also performed by nuclear magnetic resonance

(NMR) spectroscopy. For this, cells were grown in NBRIP medium for 24 h as described earlier; the supernatant was collected by centrifugation at 4000 *g* for 10 min and filtered through 0.45-µm membrane filters (Millipore Corp.). The cell-free supernatant was freezed-dried and stored at − 80 °C until use. Freezed-dried supernatants were resuspended with D_2O and ^{1}H-NMR spectra were obtained using a BRUKER Advance DRX 400 MHz spectrometer. 3-Trimethyl silyl propane was used as an internal standard at 0 ppm. ^{13}C-NMR spectra were recorded on the same spectrometer at 100 MHz in D_2O. Water-soluble P was determined according to Murphy and Riley [15].

Results and Discussion

Growth, Glucose Consumption, and Supernatant Acidification of MMB051 Cultures

Pantoea agglomerans MMB051 cells inoculated in NBRIP medium containing $Ca_3(PO_4)_2$ as the sole P source started to grow exponentially after an initial lag phase of about 4 h and reached the stationary phase after 12 h of growth (Fig. 1A). As can be seen, following the first 3 h, the pH dropped from the initial values (~7.0) to less than 3.0 units (i.e., a shift of 4 pH units approximately). Supernatant acidification was also recorded irrespective of using $FePO_4$, $AlPO_4$, or KH_2PO_4 as sole P sources (Table 1). Surprisingly, the pH of the medium started to drop immediately after the cells were inoculated and continued to decrease for 4 more hours in the absence of any noticeable growth (Fig. 1A). When the cells were inoculated in NBRIP medium devoid of any source of P, a significant pH drop was also noticed even though there was no growth of the cultures (Fig. 1A). As can be seen, the pH decrease was concomitant to the consumption of glucose, at least during the first 5–6 h of incubation (Fig. 1B). In the uninoculated control experiments there was no significant variation of the pH during the period of study (not shown). In addition to this, we observed a rapid acidification of the extracellular medium by cell suspensions (nonproliferating) of *P. agglomerans* MMB051, from an initial value of 6.4 pH units to 3.7 in less than 45 min, when supplied with glucose (Fig. 2). This rapid acidification seemed to be related to glucose metabolism, as no variations in pH was observed when glucose was omitted.

Altogether, these results suggested that *P. agglomerans* MMB051 oxidized glucose through the DOPG, a metabolic pathway exhibited by some Gram-negative bacteria [6]. The products of this pathway can be GA, KGA, and/or 2,5 di-keto gluconic acid. Indeed, a spot with an R_f value of about 0.32 (identical to that of pure GA) was detected in supernatant samples subjected to TLC (not shown). Accordingly, when the same supernatants were subjected to ^{1}H- and ^{13}C-NMR, characteristic peaks corresponding to D-GA were recorded (Fig. 3). It was possible to reject the signals assigned to glucose after comparisons of ^{1}H- and ^{13}C-NMR spectra of pure D-glucose with the supernatant of MMB051.The absence of both KGA and 2,5 di-ketogluconic acid was also noticed. On the other hand, *P. agglomerans* MMB051 cells grown overnight in the presence of glucose and KH_2PO_4 as the sole source of P exhibited a GDH-specific activity of 0.36 µmol/min/mg protein. Membrane fractions of these cells exhibited a specific GDH activity of 2.35 µmol/min/mg protein. As expected, the specific activity exhibited by the soluble fraction of these cells was significantly lower (0.19 µmol/min/mg). These results confirm the expression of a membrane bound GDH by MMB051 cells.

Solubilization of Pure Inorganic Phosphates by Strain MMB051

The concentration of soluble P, as determined in the cell-free supernatants, showed no significant variations until the cultures reached the mid-log phase of growth (i.e., after 7 h of growth approximately) (Fig. 1B). From this point, there was a dramatic increase in soluble P reaching a maximum of 80 mg/L after 10 h of growth. The uninoculated control experiments showed no significant variation of the soluble-P concentration (not shown).

The GA-mediated acidification of the extracellular space has been frequently proposed as a common strategy to solubilize P from sparingly soluble-P-containing minerals in order to sustain both bacterial and plant growth [6, 21]. This was therefore our first assumption when looking at our results. However, a careful examination of our data led us to conclude that MMB051-mediated acidification by GA production did not seem to be the only mechanism beneath Ca_3PO_4 or RP solubilization, because (a) the soluble-P concentration remained very low and almost invariable during the first 7 h of culture, even though there was a pH fall of more than two units (= GA production and excretion) (Fig. 1A, B), (b) the solubilization of inorganic P occurred just after the glucose stopped being consumed and/or oxidized into GA, and (c) a positive correlation between P solubilization and culture growth (= biomass production) was observed, both in the presence of $Ca_3(PO_4)_2$ and RP (see below), suggesting a solubilization mechanism directly related to processes dependent on biomass production, not to GA production.

Therefore, it seemed plausible that a different physiological process was also responsible for P solubilization mediated by actively growing MMB051 cells. It is well

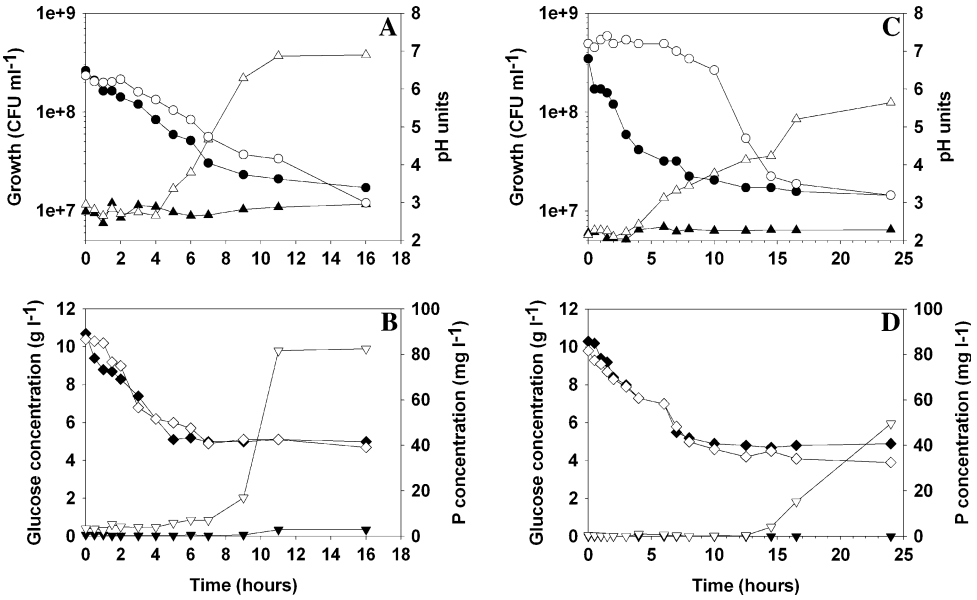

Fig. 1 Bacterial cell density (Δ,▲), pH changes (●,○), glucose concentration (◇,◆) and soluble-P concentration (▽,▼) during growth of *P. agglomerans* MMB051 in NBRIP medium supplemented with $Ca_3(PO_4)_2$ (**a**, **b**) or Rock Phosphate (**c**, **d**). Values are the means of three replicates. Solid symbols correspond to experiments carried without any source of P added to the cultures

Table 1 Final pH and soluble-P concentration of supernatants of *P. agglomerans* cultures in NBRIP medium supplemented with varying P and N sources

Condition	Final pH	Final P concentration (mg/L)
NBRIP + $Ca_3(PO_4)_2$ + NH_4^+	2.86 ± 0.21[a]	95.75 ± 2.94
NBRIP + $FePO_4$ + NH_4^+	2.94 ± 0.04	0.97 ± 0.06
NBRIP + $AlPO_4$ + NH_4^+	3.02 ± 0.01	3.11 ± 0.24
NBRIP + KH_2PO_4 + NH_4^+	2.75 ± 0.02	6.69 ± 0.45
NBRIP + $Ca_3(PO_4)_2$ + KNO_3	5.10 ± 0.15	58.15 ± 1.65

[a] Numbers given are the average of triplicate experiments ± SD

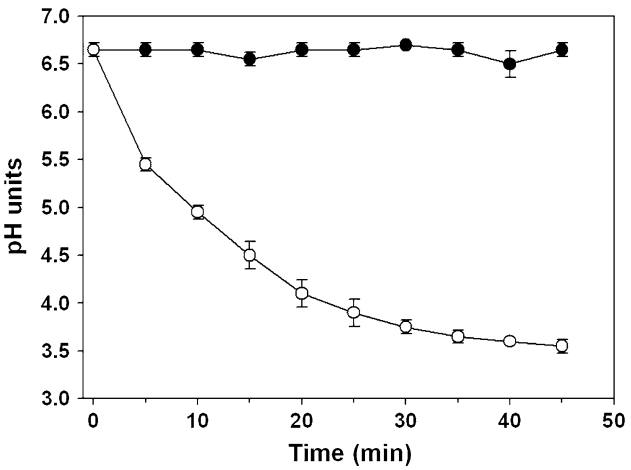

Fig. 2 pH curves of nonproliferating cell suspensions of *P. agglomerans* MMB051 supplemented with 10 mM glucose (○). Values for the control experiment (no glucose added) are represented by ●. Values are the average ± SD from triplicate experiments

known that some fungi excrete H^+ in exchange for NH_4^+ and, hence, solubilize P by drastically acidifying the medium (reviewed in [2]). Halder et al. [8] and, later, Illmer and Schinner [10] proposed that excretion of protons originating from NH_4^+ assimilation might also account for P solubilization mediated by some P-solubilizing bacteria (PSB). In agreement with such a proposal, we found that when growing the cells in the presence of KNO_3, as an alternative N source, instead of $(NH_4)_2SO_4$, (a) the final pH of the culture supernatant was almost two units above the one recorded when using $(NH_4)_2SO_4$ (5.1 ± 0.15 vs. 2.86 ± 0.21) and (b) the final concentration of soluble P was lower (58.15 vs. 95.75 ± 2.94 mg/L) (Table 1). Whereas NH_4^+ uptake by cells can lower the pH due to the release of protons, the opposite might happen when transporting NO_3^- ions into the cytoplasm, as they are negatively charged. In bacteria, although different NO_3^- transporter systems have been identified, some authors consider a nitrate/proton symporter as the main (and sometimes sole) transporter for NO_3^- [13, 22]. Hence, an increase in extracellular pH might arise as a consequence of NO_3^- uptake by bacterial cells, as happens during the initial stages of nitrate assimilation in plants [3]. This might explain the differences observed in the P-solubilizing abilities of MMB051 cells grown in the presence of NH_4^+ or NO_3^- ions.

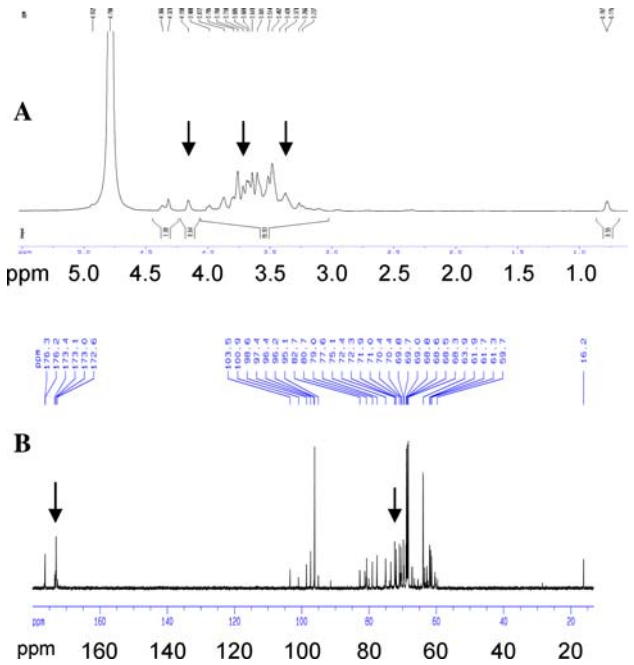

Fig. 3 NMR of *P. agglomerans* MMB051 culture supernatants: ^{1}H-NMR (**a**) and ^{13}C-NMR (**b**) spectra of concentrated *P. agglomerans* MMB051 supernatants. **a** The peaks between 3.37 and 4.81 ppm are for hydrogen atoms of D-gluconic acid (some peaks are not visible in **a** due to the overlapping peak, which corresponds to D_2O at 4.790 ppm). **b** The arrows indicate some characteristic gluconic acid peaks

The amount of P solubilized by *P. agglomerans* MMB051 varied according to the source of P present in the medium. When the cells were grown for 24 h in the presence of 1 g/L of $AlPO_4$ or $FePO_4$, the biomass yield at 24 h was much lower (not shown) and the soluble P concentration detected at the end of the growth period was equivalent to 3.21% and 1.01%, respectively, of the amount of P solubilized when using $Ca_3(PO_4)_2$ (Table 1). Strikingly, the final pH of the supernatants of these cultures showed no significant differences, even when cells were grown for 24 h in the presence of soluble P (KH_2PO_4). This is in agreement with the results reported in our previous work that suggested that strain MMB051 was not able to solubilize P compounds of a lesser solubility than $Ca_3(PO_4)_2$ [17] . Because $FePO_4$ and $AlPO_4$ are among the phosphate-bearing minerals most commonly found in iron-rich, acid soils [20], our results support the idea that *P. agglomerans* MMB051—and other strains previously isolated by us [17]—should be of minor importance from an ecological point of view (i.e., in terms of promoting plant growth by supplying roots with soluble P in iron-rich, acid soils). This also points to the need of developing more specific screening methods to identify MPS^+ microorganisms that are ecologically relevant in these types of soils.

Solubilization of Rock Phosphate

Pantoea agglomerans MMB051 was shown here to rapidly and efficiently solubilize P from RP, when supplied as the sole source of P. The kinetics of supernatant acidification exhibited an initial phase of about 7 h without any significant variation from the acidification kinetics discussed earlier (compare Fig. 1A, C). This might be the consequence of the buffering capacity of RP, possibly due to the presence of carbonate gangue minerals [23]. Moreover, the presence of other unknown, more soluble, P-bearing compounds in RP might have supported a rapid growth of MMB051 cultures. Soluble-P concentration started to increase in a steep way in the supernatant after 12 h of growth (i.e., when the culture pH dropped to less than 5 units) and reached 50 mg/L at 24 h (Fig. 1D). This is similar to what we have seen when using $Ca_3(PO_4)_2$. The glucose concentration in both experiments varied from 10 g/L to ~5 g/L during the first 7 h of growth. After reaching this value, the glucose concentration remained almost unvarying. Again, a mechanism involving both GA production and proton extrusion—as a consequence of NH_4^+ uptake—is, from our point of view, responsible for such a solubilization.

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