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SHORT COMMUNICATION

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Gluconic acid production and phosphate solubilization by the plant growth-promoting bacterium *Azospirillum* spp.

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Abstract In vitro gluconic acid formation and phosphate solubilization from sparingly soluble phosphorus sources by two strains of the plant growth-promoting bacteria *A. brasilense* (Cd and 8-I) and one strain of *A. lipoferum* JA4 were studied. Strains of *A. brasilense* were capable of producing gluconic acid when grown in sparingly soluble calcium phosphate medium when their usual fructose carbon source is amended with glucose. At the same time, there is a reduction in pH of the medium and release of soluble phosphate. To a greater extent, gluconic acid production and pH reduction were observed for *A. lipoferum* JA4. For the three strains, clearing halos were detected on solid medium plates with calcium phosphate. This is the first report of in vitro gluconic acid production and direct phosphate solubilization by *A. brasilense* and the first report of P solubilization by *A. lipoferum*. This adds to the very broad spectrum of plant growth-promoting abilities of this genus.

Introduction

The ability to produce organic acids or to induce proton and organic acid extrusion from plant roots are important, but relatively less well explored, traits of several plant growth-promoting bacteria (PGPB) of the genus *Azospirillum* and other PGPBs (Amooaghaie et al. 2002; Bashan 1990; Carrillo et al. 2002; Chabot et al. 1996; Chang and Li 1998; Deubel et al. 2000; Puente et al. 2004; Raven et al. 1990). The main mechanism for mineral phosphate (P) and other mineral solubilization useful for plant growth is the production of organic acids (Rodriguez and Fraga 1999).

Under in vitro conditions, most strains of *A. brasilense* and *A. lipoferum* use organic acids, such as malic, succinic, α-ketoglutaric, gluconic, or lactic (Hartmann and Zimmer 1994; Rodelas et al. 1994; Westby et al. 1983) as their preferred carbon source, rather than producing them. Additionally, gluconic acid is one of the favored carbon sources of *A. lipoferum* for producing siderophores (Shah et al. 1993).

Goebel and Krieg (1984) showed that gluconic acid was not formed during growth of either *A. brasilense* or *A. lipoferum* on fructose (a common carbon source for both), and was detected only during growth of *A. lipoferum* on glucose, a carbon source that *A. brasilense* cannot use. Deubel et al. (2000) reported on gluconic acid production by *Azospirillum* sp. CC 322, which was probably not *A. brasilense,* as it grew on glucose. As far as we know, there are no other reports or confirmation of these observations on gluconic acid production by *A. brasilense*. On the other hand, in vitro mineral P solubilization in this genus was documented by Seshadri et al. (2000) for *A. halopraeferans*, indirectly for *A. brasilense* Cd (Janzen and McGill 1995), and by Chang and Li (1998) and Deubel et al. (2000) for *Azospirillum* sp.

This study explored (1) the possibility that *A. brasilense,* the most common *Azospirillum* species used as an agricultural inoculant (Bashan et al. 2004), can produce gluconic acid in vitro when grown on fructose and amended with glucose as an inducer for gluconic acid production with a common strain of *A. lipoferum* as a positive control, and (2) whether these strains have in vitro phosphate-solubilizing capability.

Materials and methods

Bacterial strains

Three PGPB strains were studied: (1) *A. brasilense* Cd (DSM 1843, Germany), (2) *A. brasilense* 8-I, isolated from the rhizosphere of sugarcane at the National Institute of Sugarcane Research, Havana,

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Cuba [identified by R. Fernandez, personal communication, Universidad Benemerita de Puebla, Mexico, according to Bergey's Manual of Systematic Bacteriology (1989)], and (3) *A. lipoferum* JA4 (donated by V.L.D Baldani, CNPBS, Rio de Janeiro, Brazil). The strains were conserved and maintained according to the standard methods for this genus (Bashan et al. 1993). Medium used with sparingly soluble phosphate for growth was composed of (g l^{-1}): glucose (10); fructose (10); NH_4NO_3 (0.373); $MgSO_4$ (0.41); KCl (0.295); NaCl (0.2); $FeCl_3$ (0.003); $Ca_3(PO_4)_2$ (0.7) (Diagnostic Biochems, Oakville, Ontario, Canada). Agar (20 g l^{-1}) was added for solid plate cultures. Additionally, the same medium without glucose was used.

Cultivation and analyses

Cultures were grown in 250-ml Erlenmeyer flasks containing 70 ml culture medium on a rotary shaker at 200 rpm at 37±1°C for 48 h. Inoculation rate was 1:8 (v/v) from an overnight pre-inoculum culture grown in 150-ml Erlenmeyer flasks containing 15 ml medium. Every 24 h, 10-ml samples were processed. Growth was determined by optical density at 540 nm after sample dilution 1:1 (v/v), using 1 N HCl to dissolve the residual sparingly soluble phosphate, (Rodriguez et al. 2000). The pH was measured with an MV 870 digital pH-meter (Pracitronic, Dresden, Germany). Soluble phosphate was analyzed from the supernatant of the culture after removing cells by centrifugation for 10 min at 10,000 *g*, using the method of Chen et al. (1956). Organic acid production was evaluated by HPLC (Knauer, Berlin, Germany) equipped with a diode array detector (DAD) and a Hyperfil RP-18 column at a flow rate of 0.6 ml/min. Standards of organic acids were purchased from Sigma. The following organic acids were analyzed: D-gluconic, L-malic, L-lactic, acetic, citric, succinic, propionic, glycolic, and T-aconitic.

Experimental design and statistical analysis

The experiment was performed in a completely randomized fashion with three replicates. A replicate consisted of one Erlenmeyer flask. Each analysis was done on three samples from each replicate. The experiment was repeated four times. Results are from a representative experiment and were analyzed by ANOVA, using Statistica software (StatSoft, Tulsa, Okla.).

Results

After growing for 72 h at 37°C, the three *Azospirillum* strains showed P-solubilizing capacity on plates containing minimal medium supplemented with both glucose and fructose with a sparingly soluble P source. After 5 days of incubation, clearing halos reached 11 mm diameters for the two *A. brasilense* strains and 20 mm diameters for *A. lipoferum* JA4 (data not shown).

The three *Azospirillum* strains were able to grow on minimal liquid medium supplemented with sparingly soluble P as the sole P source. Population densities were far greater for *A. brasilense* strains Cd (ANOVA; $F_{2,6}$=578.8; P=0.0001) and 8-I (ANOVA; $F_{2,6}$=31.45; P=0.0006) than *A. lipoferum* JA4 (ANOVA; $F_{2,6}$=92.45; P=0.0001) (Fig. 1A, C, E). A significant decrease in pH occurred in cultures of *A. lipoferum* JA4 (Δ pH 1.9) (ANOVA; $F_{2,6}$=19.96; P=0.002), smaller for *A. brasilense* strain 8-I (Δ pH 0.7) (ANOVA; $F_{2,6}$=122.59; P=0.0001), and minimal for strain Cd (Δ pH 0.1) (ANOVA; $F_{2,6}$=5.58; P=0.042) (Fig. 1A, C, E). In the two strains of *A. brasilense*, soluble phosphate in the medium significantly increased after incubation for 24 h in the medium and later declined (ANOVA; $F_{2,6}$=21.72; P=0.002, for strain 8-1) (ANOVA; $F_{2,6}$=55.9; P=0.0001 for strain Cd) (Fig. 1B, D). *A. lipoferum* JA4 cultures grew less, but soluble phosphate accumulated (ANOVA; $F_{2,6}$=22.03; P=0.002) (Fig. 1F) to higher values than in *A. brasilense* cultures. Significant amounts of gluconic acid were detected in cultures of *A. lipoferum* JA4 after incubation for 24 and 48 h (ANOVA; $F_{2,6}$=7.53; P=0.023) (Fig. 1F), and lesser quantities in cultures of the two strains of *A. brasilense*, but only after incubation for 48 h (ANOVA; $F_{2,6}$=20.85; P=0.001, for strain 8-1) (ANOVA; $F_{2,6}$= 270.7; P=0.0001 for strain Cd) (Fig. 1B, D). No other organic acids were detected during cultivation in any strain. Longer incubation periods did not yield additional production of gluconic acid or additional P solubilization (data not shown).

Discussion

The release of soluble phosphate from calcium phosphate usually involves production of organic acids and a decrease in pH of the medium (Carrillo et al. 2002; Illmer and Schinner 1995; Illmer et al. 1995; Puente et al. 2004). Although this study does not provide direct evidence that production of gluconic acid is responsible for the dissolution of sparingly soluble calcium phosphate, it nevertheless was the sole organic acid detected. Furthermore, no halos were formed on plates by *A. brasilense* strains Cd and 8-I when glucose was not in the medium. Glucose is the precursor for synthesis of gluconic acid. This suggests that P solubilization in these strains is mediated by gluconic acid metabolism.

As solubilization of phosphate preceded detection of gluconic acid in the medium, perhaps even low levels of the acid (below the detection level of our HPLC method) started to dissolve the sparingly soluble phosphate. Alternatively, consumption of gluconic acid by growing cells could also take place.

In *A. brasilense,* reduction in the quantity of soluble phosphate after incubation for 48 h can be explained as auto-consumption of soluble phosphate by the growing bacterial population (Rodriguez et al. 2000). In *A. lipoferum*, P solubilization is combined with a decrease in pH. The latter may result from production of gluconic acid and NH_4^+ uptake, which may release protons to the medium. In the faster growing *A. brasilense* strains, perhaps the cells used more NO_3^- at the end of the incubation time, thereby releasing OH^-, which may account for the higher pH after 48 h. The metabolic mechanism by which gluconic acid was produced was not explored.

In summary, this study demonstrated that two strains of *A. brasilense* were capable of producing gluconic acid when growing on sparingly soluble calcium phosphate, provided that the usual fructose carbon source is amended with glucose. This confirms an old report (Goebel and Krieg 1984) that *A. lipoferum* is capable of producing this organic acid, as well. At the same time, there is a lowering of the pH of the medium and release of soluble

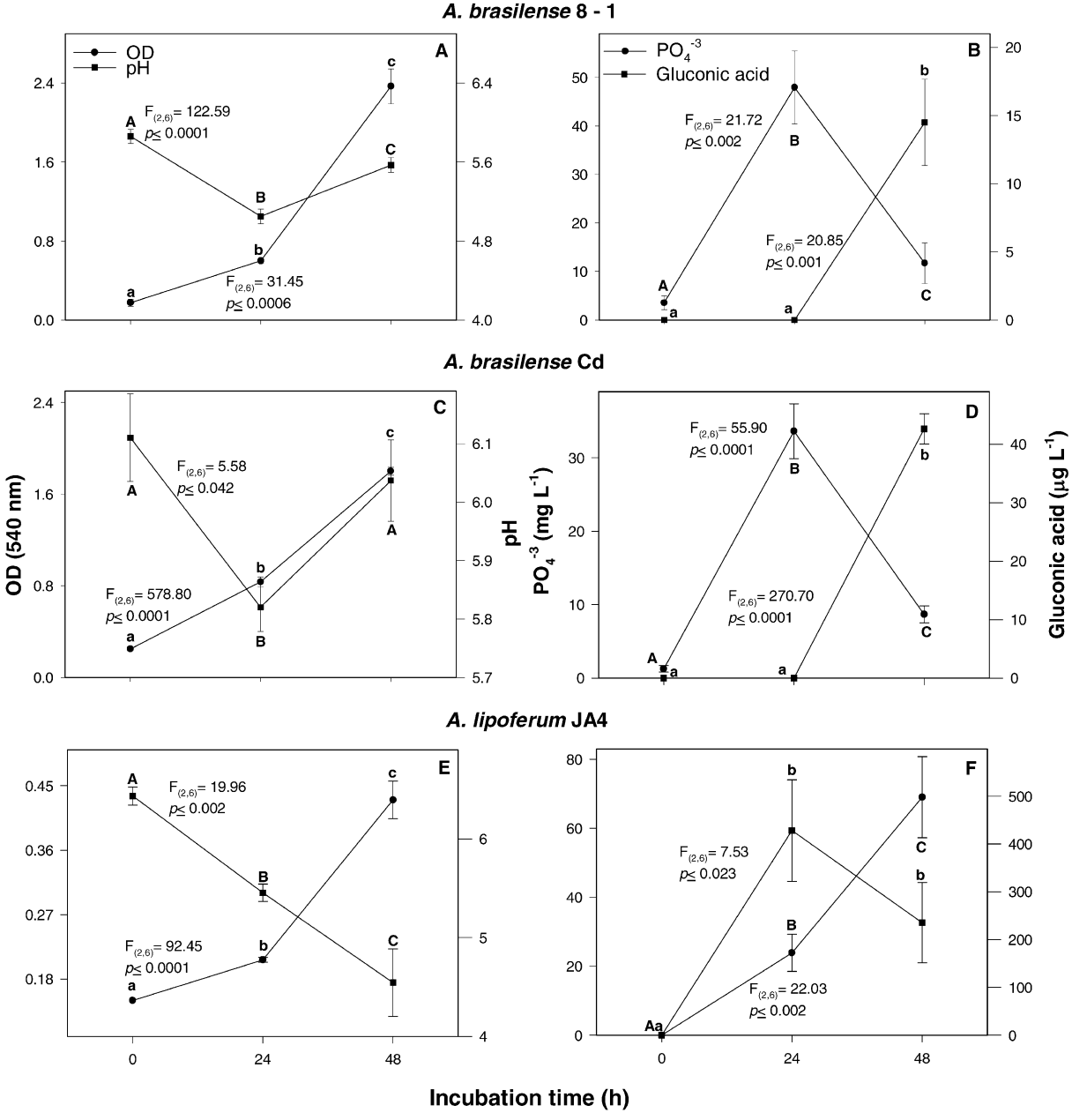

Fig. 1 Changes in bacterial growth, pH of the medium (**A**, **C**, **E**), soluble phosphate and gluconic acid production (**B**, **D**, **F**) by strains *A. brasilense* Cd and 8-I and *A. lipoferum* JA4, when the bacteria were grown in culture having fructose as the main carbon source, glucose as inducer of gluconic acid production, and sparingly soluble calcium phosphate as the sole P source. *Different letters accompanying each line*, in each subfigure separately, differ significantly by one-way ANOVA. *Bars* represent standard error

phosphate. These facts, together with halo formation on the plates, indicate P-solubilizing activity by *A. brasilense* and *A. lipoferum* strains. These capacities add to the already broad spectrum of plant growth-promoting abilities (hormone and siderophore production, N_2-fixation, general mineral uptake, mitigation of stressors, and proton extrusion) of this genus.

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