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# Phosphate-solubilizing microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon

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**Abstract** The phosphate-solubilizing potential of the rhizosphere microbial community in mangroves was demonstrated when culture media supplemented with insoluble, tribasic calcium phosphate, and incubated with roots of black (*Avicennia germinans* L.) and white [*Laguncularia racemosa* (L.) Gaertn.] mangrove became transparent after a few days of incubation. Thirteen phosphate-solubilizing bacterial strains were isolated from the rhizosphere of both species of mangroves: *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus atrophaeus*, *Paenibacillus macerans*, *Vibrio proteolyticus*, *Xanthobacter agilis*, *Enterobacter aerogenes*, *Enterobacter taylorae*, *Enterobacter asburiae*, *Kluyvera cryocrescens*, *Pseudomonas stutzeri*, and *Chryseomonas luteola*. One bacterial isolate could not be identified. The rhizosphere of black mangroves also yielded the fungus *Aspergillus niger*. The phosphate-solubilizing activity of the isolates was first qualitatively evaluated by the formation of halos (clear zones) around the colonies growing on solid medium containing tribasic calcium phosphate as a sole phosphorus source. Spectrophotometric quantification of phosphate solubilization showed that all bacterial species and *A. niger* solubilized insoluble phosphate well in a liquid medium, and that *V. proteolyticus* was the most active solubilizing species among the bacteria. Gas chromatographic analyses of cell-free spent culture medium from the various bacteria demonstrated the presence of 11 identified, and several unidentified, volatile and nonvolatile organic acids. Those most commonly produced by different species were lactic, succinic, isovaleric, isobutyric, and acetic acids. Most of the bacterial species produced more than one organic acid whereas *A. niger* produced only succinic acid. We propose the production of organic acids by these mangrove rhizosphere microorganisms as a possible mechanism involved in the solubilization of insoluble calcium phosphate.

**Key words** Mangrove · *Bacillus* spp. · *Enterobacter* spp. · Inorganic phosphate-solubilizing microorganisms · Rhizosphere

Dedicated to Prof. K. Vlassak on the occasion of his 65th birthday

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## Introduction

Dissolved inorganic phosphate exists in the sea mainly as ionic forms of orthophosphoric acid. Because of the negative charge of phosphate ions, they are quickly absorbed after weathering of clays or detritus particles, forming insoluble forms of aluminum, calcium, or iron phosphates, all unavailable to mangroves. Fungi and bacteria have the ability to solubilize these compounds (Illmer 1995). Although several mechanisms may be involved, the main one is through the production of organic acids (Nahas 1996). It is assumed that these organic acids solubilize insoluble forms of phosphate to a useable form, such as orthophosphate, thus increasing the potential availability of phosphate for plants (Kucey et al. 1989). Proton-excretion accompanying ammonium ion assimilation is thought to be the most probable explanation for microbial solubilization without acid production (Illmer 1995).

Inorganic-phosphate-solubilizing bacteria (IPSB) have been isolated from the rhizosphere of many terrestrial plants (Sundara-Rao and Sinha 1963). These bacteria may benefit crop plants like legumes, maize, and lettuce by increasing their phosphate content when inoculated alone (Chabot et al. 1996), or in combination with mycorrhizal fungi (Singh and Kappor 1998).

Phosphate-solubilizing fungi from both cultivated and virgin soils are well known (Kucey 1983).

In the marine environment, IPSB have been found in the water column (Promod and Dhevendaran 1987), in sediments (Devendran et al. 1974), and associated with the seagrass *Zostera Marina*, whose root exudates support these bacteria (Craven and Hayasaka 1982). To our knowledge, there are no reports of marine phosphate-solubilizing fungi.

Mangrove swamps are among the most productive marine ecosystems (Flores-Verdugo et al. 1990). They are feeding, spawning, and reproduction areas for numerous economically and ecologically important marine species (Robertson and Blaber 1992). Mangroves also export considerable amounts of organic matter and nutrients to adjacent coastal waters (Alongi et al. 1993). Some mangrove ecosystems are nutrient deficient, especially in nitrogen and phosphorus (Sengupta and Chaudhuri 1991). Nitrogen fixation in mangrove sediments (Zuberer and Silver 1978), in the rhizosphere (Holguin et al. 1992), and associated with aerial roots (Toledo et al. 1995) may provide the nitrogen necessary for the sustenance of these semiarid ecosystems. Large phosphate-rock deposits exist in Baja California Sur, Mexico (Galli 1993), including at our study site. However, these phosphates are in forms unavailable for plants. There are no scientific reports on phosphate-solubilizing activity of microorganisms in the mangrove rhizosphere. Such activity might explain the apparent contradiction of mangrove forests proliferating in phosphorus-deficient waters.

Our aims were to demonstrate the presence of insoluble-phosphate-solubilizing microorganisms in the rhizosphere of the semiarid mangrove species *Avicennia germinans* L. (black mangrove) and *Laguncularia racemosa* (L.) Gaertn.(white mangrove), to isolate and identify the microbial species, to measure their phosphate-solubilizing potential in vitro, and to identify the organic acids produced by these microorganisms.

## Materials and methods

### Study site

The semiarid mangrove ecosystem (approximately 180 mm rainfall annually) is at Laguna de Balandra, about 25 km north of La Paz, Baja California Sur, Mexico. The ecosystem has been described geographically (Holguin et al. 1992), geologically (Pedrin-Avilés et al. 1992), and edaphically (Giani et al. 1996).

### Soil and water sampling and analysis

Eight 30-cm-deep soil cores were obtained at low tide with a stainless steel core sampler (inner diameter 39 mm) at a distance $<20$ cm from young black mangrove trees ($<50$ cm tall), and seven samples were taken from the sediment at a distance $>50$ m from the mangrove forest. The samples were transported to the laboratory in an ice box within 1 h. The samples were dried at 90 °C for 48 h in a forced-draft oven, ground in a porcelain mortar and pestle, and sieved. The fraction containing particles $<250$ μm were analyzed. Total phosphorus and inorganic and organic phosphorus in the sediment were measured by using the method of Saunders and Williams (1955) as modified by Walker and Adams (1958) with the colorimetric detection described by Chapman and Pratt (1984). The quantity of organic phosphorus was calculated by subtracting the quantity of inorganic phosphorus from total phosphorus. pH was measured with a pH meter (Beckman pH meter $\Phi$ 44, Irvine, Calif.) (Chapman and Pratt 1984), and salinity with a salinity refractometer (Aquafauna; Biomarine, Hawthorne, Calif.). To determine soluble orthophosphates in the overlying seawater, sterile, white polyethylene bottles (500 ml) were used to sample water (about 1 l/sample, four samples). Water samples were frozen at –20 °C until analyzed. To analyze soluble phosphate in the pore water, five wet sediment samples (4 kg each) were taken during low tide with a garden shovel to a depth of 20 cm, 10 cm from the small trees. Each sample was placed in a plastic bag containing numerous 2-mm holes. Each of these was placed in a second, larger plastic bag to collect the water dripping from the wet sediment. Additionally a manual press was used on the samples to collect 200 ml of pore water from each sample. This water was further filtered through Whatman glass microfibre filters (GF/F). Fifty-milliliter samples were used for analyses according to Strickland and Parsons (1972).

### Isolation of microorganisms from mangrove roots

Young mangrove plants of the two species, *A. germinans* and *L. racemosa*, were harvested from the study site with a spade. Their root-sediment ball was kept intact, and they were transferred immediately to the laboratory in sterile plastic bags. There, the adhering sediment was carefully removed from the roots, without touching the roots, and the roots were then rinsed with two consecutive baths (approximately 10 l each) of seawater. Seawater was obtained from the experimental aquaculture facility of CIB (seawater was pumped from the sea into a large dirt cistern and later pumped into a large sedimentation tank to eliminate denser particles). The seawater was passed through a 5-mm Aqua-Pure AP 110/HC filter cartridge (CUNO, Meridan, Conn.), and flowed slowly through a UV irradiator (SL-1 sterilizer; Aquafine, Valencia, Calif.). Finally, the roots were rinsed once with sterile (standard autoclaving) artificial seawater ASN-III (Rippka et al. 1979). After clipping the seedling foliage with sterile scissors, the entire root system of the seedlings was placed in separate Erlenmeyer flasks [125 ml containing 50 ml of a modified standard SRSM1 enrichment medium of Sundara Rao and Sinha (1963) specific for the isolation of phosphate-solubilizing microorganisms]. The modified medium consisted of: glucose, 10 g/l; tribasic calcium phosphate, 5 g/l; ammonium sulfate, 0.5 g/l; potassium chloride, 0.2 g/l; magnesium sulfate heptahydrate, 0.3 g/l; manganese sulfate, 0.004 g/l; ferrous sulfate, 0.002 g/l; sodium chloride, 20 g/l; yeast extract (Difco), 0.5 g/l; bromocresol purple, 0.1 g/l. The tribasic calcium phosphate was autoclaved first. Then, the other sterile ingredients were aseptically mixed after autoclaving. The final pH was adjusted, after autoclaving, to 7.2 with sterile 1 N sodium hydroxide. The flasks containing the roots were incubated in a rotary shaker for 5 days at $30 \pm 2$ °C (incubator shaker series 25; New Brunswick, Edison, N.J.) at 150 rpm. For the isolation of fungi, similar flasks with intact plants were incubated at $25 \pm 2$ °C (pH 7.2) at a light intensity of 50 μmol/m$^2$ per s, without shaking, for 21 days. Phosphate-solubilizing activity was considered positive when the medium appeared transparent to the eye. These transparent flasks were sampled and plated. Colonies of morphotypes showing clear and large solubilization halos were purified on successive Petri dishes containing SRSM1 solid medium (1.6% purified agar, Sigma St. Louis, Mo.).

Because it has been reported that after successive transfers, isolates of IPSB lose their phosphate-solubilizing capacity (Craven and Hayasaka 1982), the isolates were stored at $-40 \pm 3$ °C in SRSM2 liquid medium (a SRSM1 medium, where 1.1 g/l of dipotassium phosphate-trihydrate replaced the tribasic calcium phosphate), supplemented with 30% glycerol.

### Characterization and identification of the phosphate-solubilizing microorganisms

The colonial bacterial morphotypes were selected by using a stereoscope STEMI SR (Zeiss, Germany). The cellular morphology of the pure isolates was determined in wet mounts with light microscopy (Zeiss) from colonies grown in SRSM1 solid medium. Bacterial identification was done by gas chromatography of methyl esters of cell fatty acids (FAME analysis). These FAME analyses were done as a commercial service by the Department of Plant Pathology, Auburn University, Alabama.

Fungus identification was done with a light-phase-contrast microscope (Nikon, Japan), and confirmed by Dr. Joaquin Cifuentes of the Herbarium of the Faculty of Science, National Autonomous University of Mexico (UNAM), Mexico City. Identification criteria were the presence of conidiophores emerging from foot cells. Each conidiophore had typical bottle-shaped sterigma supporting black spherical conidiospores.

### In vitro determination of phosphate-solubilizing capacity of microorganisms

Each of the bacterial species was grown as a preinoculum for 16 h in 50 ml SRSM2 liquid medium (containing soluble phosphate) in 250-ml Erlenmeyer flasks under rotary agitation of 120 rpm at $30 \pm 2$ °C. The cultures were washed 3 times at $27 \pm 2$ °C by centrifugation at 20,000 *g* for 10 s with 2% sodium chloride solution. The optical density at 540 nm of each bacterial culture was adjusted to $1.0 \pm 0.1$ in the saline solution by using a spectrophotometer (Spectro Master model 415, Fisher Scientific, Chicago, Ill.). An aliquot of 250 μl of this bacterial suspension was added to the 125-ml flasks containing 25 ml of SRSM1 liquid medium containing insoluble calcium phosphate. The control consisted of uninoculated flasks. All cultures were incubated for 24 h as described above.

Two samples of 500 μl each were taken from each culture and centrifuged (20,000 *g*, $27 \pm 2$ °C, 10 s). The pellet was discarded. Twenty to 50 ml (depending on a predetermined level of phosphate solubilization by each species) was sampled and diluted with 50 ml distilled water. The total concentration of soluble phosphate was determined according to the standard method of Strickland and Parsons (1972).

Viable counts in triplicate of the same cultures were made on SRSM2 solid medium. Because it was highly aggregated, enumeration of *Xanthobacter agilis* was impossible.

*Bacillus* spores were stained by the Schaeffer-Fulton method (Bradshaw 1979) and were counted in ten different microscopic fields (at ×1000 magnification) every 24 h by light microscopy.

The fungus was grown in liquid SRSM1 medium at $25 \pm 2$ °C and at 140 rpm in a rotary shaker for 3 days. Twenty microliters of culture samples was taken to analyze phosphate solubilization (after filtration of the hyphae). For dry biomass determination, aliquots of 30 ml were filtered through Whatman filter paper no. 1. The biomass accumulated on the filter was dried at $70 \pm 3$ °C for 48 h. Aliquots (5 ml) were used for measuring pH at 24 h intervals.

### Extraction of organic acids from microbial cultures

Small modifications of the extraction technique of Smibert and Krieg (1981) were used. Uninoculated medium was used as a control in both bacterial and fungal experiments.

#### *Volatile organic acids*

The samples (1 ml) were acidified with 0.2 ml of 50% sulfuric acid. Sodium chloride (0.4 g) was added and the mixture was thoroughly mixed in a Vortex-Genie (X-2; Scientific Industries, Bohemia, N.Y.). One milliliter of diethylether was then added. The contents of the tube were mixed by inverting the tube gently about 20 times to extract the free organic acids into the ether phase. The emulsion was allowed to settle for 20 min at $8 \pm 2$ °C. Once the two phases formed, the upper, ether phase was removed with a Pasteur pipette and transferred to an Eppendorf tube containing calcium chloride (approximately one fourth of the volume of the ether phase). The ether phase (containing the organic acids) was transferred to another Eppendorf tube and sealed with Parafilm. Samples (1 μl) of this extract were injected into the gas chromatograph (GC).

#### *Nonvolatile organic acids*

These acids were first converted to methyl esters before gas chromatography. Methanol (2 ml) and 0.4 ml of 50% sulfuric acid were added to 1-ml samples in test tubes. Each mixture was heated for 30 min at $60 \pm 2$ °C. Then, 1 ml of distilled water was added, the solution was cooled to ambient temperature, and 0.5 ml chloroform was added. The tubes were tightly closed and inverted gently approximately 20 times. The lower chloroform phase containing the nonvolatile organic acid methylester was extracted with a Pasteur pipette and transferred to an Eppendorf tube. Samples of one microliter were injected into the GC for organic acid detection and identification. Organic acid standards (Sigma, St. Louis, Mo.) were used for identification. The concentration of mixed standards for volatile organic acids was 0.5 mEq/ml, and a sample of 0.5 μl was injected into the GC. The concentration of mixed standards for nonvolatile organic acids varied. For oxalic, malonic, methylmalonic, and fumaric acids it was 1 mEq/ml and an 0.5-μl aliquot was injected. For determination of succinic acid, it was 0.5 mEq/ml and for lactic acid 0.25 mEq/ml. Both these latter acid standards were injected into the GC at a volume of 0.25 μl.

### Detection of organic acids by gas chromatography

Samples (1 μl) were injected into a VARIAN 6000 GC (Varian Instrument Group, Sunnyvale, Calif.) equipped with a hydrogen flame ionization detector (FID). The GC operating conditions were as follows: a capillary column (Nukol fused silica; Supelco wax 10, 15 m; 0.53-mm inner diameter; and 0.53-mm film thickness); $N_2$ at a flow rate of 2 ml/min; $H_2$ and air flow rate of 30 ml/min and 300 ml/min, respectively, and injector and detector temperatures of 240 °C. The initial temperature of 70 °C was programmed to remain constant for 5 min, then to increase at a rate of 8 °C/min to reach 140 °C, remain at that temperature for 5 min, and then revert to 70 °C. Graph speed was 5 mm/min and the detector sensitivity was set at $8 \times 10^{-11}$. To improve separation of very close peaks, initial temperatures of 50 °C and 62 °C and a retention time of 10 min were also used. The organic acids were identified by comparing the retention time of standards of volatile organic acids (acetic, propionic, butyric, isobutyric, valeric, isovaleric, isocaproic, caproic, and heptanoic; Sigma) and of nonvolatile organic acids (pyruvic, lactic, oxalacetic, oxalic, malonic, methylmalonic, fumaric, and succinic; Sigma) to that of the samples. This method does not allow the quantification of organic acids.

### Experimental design, statistical analysis, and data presentation

Four replicates were used for the enrichment of IPSB and fungi from the roots of each mangrove species. Viable bacterial counts and determination of the phosphate-solubilization potential of IPSB were made for each species, in triplicate. A replicate consisted of one flask. From each replicate, two samples were taken for the quantification of soluble phosphate. All experiments, regardless of the microorganism or organic acid determination, were repeated twice. Results presented are the means of all replicates from both repetitions and the bars represent the SEs. Organic acid chromatograms are from one GC run for each bacterial species.

## Results

### Sediment and water analyses

The pH of mangrove seawater (8.2) was higher than that of the sediments of both black (7.2) and white mangroves (7.8). The salinity of the mangrove lagoon (37‰) was higher than that of the coastal seawater (33‰). The concentration of orthophosphates in mangrove-lagoon seawater was low (18.6 ± 2 μg/l) compared to 73 μg/l commonly found in the coastal seawater (Martin 1970). The level of total phosphorus in the sediment was 15 times higher than that in seawater, and most of it was inorganic phosphorus. The level of soluble phosphate in the pore water was higher than its common value in seawater (122 vs 73 μg/l), and over 6 times higher than its level in the overlying seawater in the swamp. No organic phosphorus was found in sediment far from the mangrove forest, and the level of total inorganic phosphorus was slightly higher than in samples taken close to the mangrove forest (Table 1).

### Isolation of phosphate-solubilizing bacteria from mangrove roots

After 24 h of incubation, only two flasks containing the root system of white mangrove and one flask containing the root system of black mangrove showed solubilization of calcium phosphate. After 5 days, three of four flasks containing black mangrove roots and three flasks containing white mangrove seedling roots showed visible phosphate solubilization (Fig. 1).

The following bacterial species were isolated from roots of black mangroves: *Bacillus atrophaeus*, *Bacillus amyloliquefaciens*, *Vibrio proteolyticus*, *Paenibacillus macerans*, and *Xanthobacter agilis*. Roots of white mangroves yielded only *Bacillus licheniformis*.

These strains were used to determine organic acid production and phosphate solubilization. Additionally, six more species of IPSB previously isolated were identified, and their capacity to solubilize phosphate was qualitatively determined. From black mangroves, we obtained *Enterobacter aerogenes*, *Enterobacter taylorae*, *Enterobacter asburiae*, *Kluyvera cryocrescens*, and an unidentified bacterium, and from white mangroves, *Pseudomonas stutzeri*, *Chryseomonas luteola*, and an unidentified fungus. The assessment of the quantitative capacity of the last group of bacterial species to solubilize phosphate was not completed since all were lost in a laboratory accident in 1996.

### Isolation and identification of phosphate-solubilizing fungus from mangrove roots

Only those flasks incubated with *A. germinans* seedlings showed positive phosphate-solubilizing activity. After the enrichment, light-microscopy observations of thin sections of mangrove roots showed fungal hyphae associated with the roots. After inoculating pieces of these roots on SRSM1 solid medium, a dense fungal growth was seen, and a halo around the fungal colony, indicating phosphate-solubilizing activity. Only one fungal isolate was obtained and was identified as *Aspergillus niger* Van Tiegh, designated as strain B 9411-1.

### Bacterial growth curves in medium containing insoluble phosphate

All of the five bacterial species growing in medium containing insoluble calcium phosphate as the sole P source reached the stationary phase within 24 h of growth (Fig. 2A–E). *B. amyloliquefaciens* and *B. licheniformis* reached the stationary phase after 36 h (data not presented).

In long incubations, spore formation was observed in both *B. amyloliquefaciens* and *B. licheniformis*. In *B. amyloliquefaciens*, endospore formation started after 24 h. At 48 h, 45% of the observed bacterial population was composed of vegetative cells and 55% of cells contained endospores. After 96 h of incubation, the cultures contained free spores almost exclusively. In *B. licheniformis*, endospore formation (55%) was also visible, but only at 72 h. After 96 h of incubation, the culture contained 98% of free spores and 2% of cells with endospores.

**Table 1** Phosphate content in the mangrove ecosystem at Laguna de Balandra, Baja California Sur, Mexico. *dw* Dry weight, *ND* not determined

| Phosphorus type | Near black mangrove (young trees) | Far from black mangrove trees |
|---|---|---|
| Total phosphorus in sediment | 1092 ± 95 (μg/g dw sediment)[a] | 1317 ± 70 (μg/g dw sediment)[d] |
| Inorganic phosphorus in sediment | 1047 ± 31 (μg/g dw sediment)[a] | 1317 ± 70 (μg/g dw sediment)[d] |
| Organic phosphorus in sediment | 112 ± 40 (μg/g dw sediment)[b] | 0 |
| Soluble phosphate in pore water | 112 ± 48 (μg/l)[c] | ND |
| Soluble phosphate in overlying water | ND | 18.59 ± 2 (μg/l)[e] |

[a] Eight samples, three replicates per sample
[b] Ten samples, three replicates per sample
[c] Five samples, three replicates per sample
[d] Seven samples, three replicates per sample
[e] Four water samples

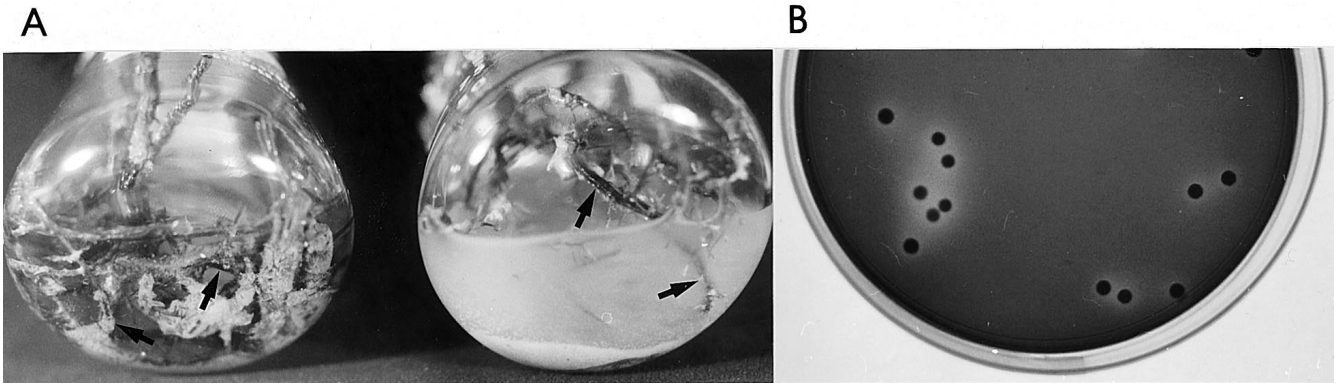


**Fig. 1A, B** Phosphate solubilization by a rhizosphere microbial community of black mangroves. **A**. Solubilization of tricalcium phosphate of the opaque medium *on the right*. *Arrows* indicate mangrove roots. **B** V*ibrio proteolyticus* growing on medium rendered opaque by tricalcium phosphate. *Halos* indicate phosphate solubilization

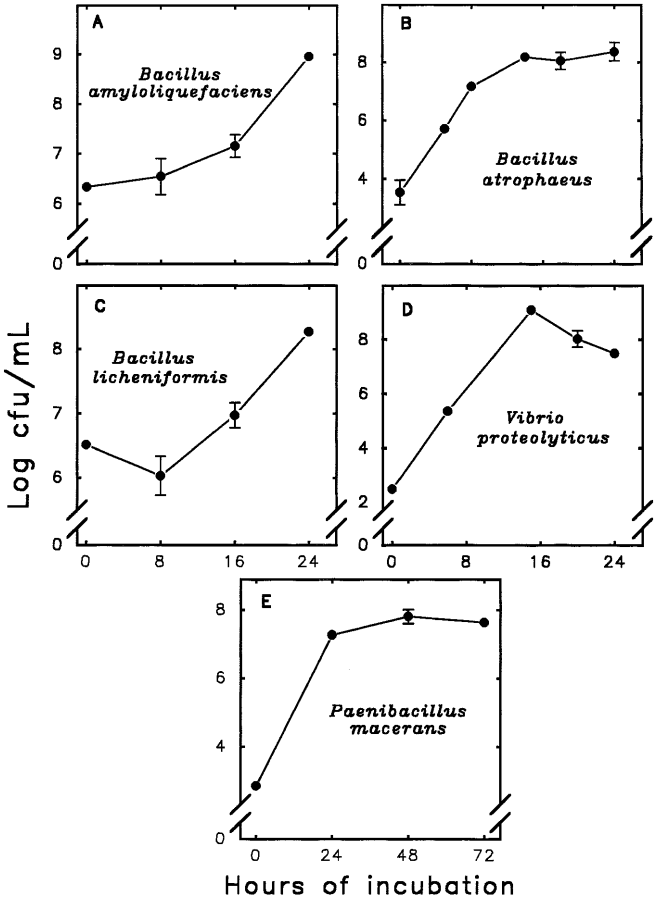


**Fig. 2** Growth of five phosphate-solubilizing bacteria in culture medium containing insoluble calcium phosphate. *Bars* represent SEs. When the SE bar is absent, the SE is smaller than the point. *cfu* Colony forming unit

## Phosphate-solubilization capacity of six species of phosphate-solubilizing bacteria

All six bacterial species were capable of dissolving insoluble phosphate, albeit to a different extent (Fig. 3). The species with highest phosphate-solubilizing capacity were *V. proteolyticus* and *X. agilis* (Fig. 3D, F), whereas the four *Bacillus* strains dissolved less (Fig. 3A–C, E). The greatest solubilization was after 18–24 h of incubation, corresponding roughly to the logarithmic phase of growth of the bacteria (compare Figs. 2 and 3). Longer incubation periods usually yielded no additional phosphate solubilization (data not shown), whereas in two species, *B. amyloliquefaciens* and *P. macerans*, phosphate solubilization was reduced after 18 h (Fig. 3A, E). We can provide no explanation for this decrease. When the capacity of single cells to solubilize phosphate was calculated, the best bacterial species was *V. proteolyticus* and the worst was *B. amyloliquefaciens* (Fig. 4).

## Phosphate solubilization by *A. niger*

Qualitative determination of the phosphate-solubilizing capacity of *A. niger* on solid medium showed 5-mm halos around the periphery of each fungal colony after 24 h. The entire dish turned clear within 48 h of incubation. Quantitative analysis showed that the concentration of soluble phosphate in liquid culture increased abruptly with time (Fig. 5A), and the pH in the culture showed a drastic drop (from 6.5 to 3). The solubilization of phosphate was correlated to the fungal dry biomass (Fig. 5B).

## Identification of organic acids in the microbial cultures

All bacterial species produced volatile and nonvolatile organic acids. Most of the species produced several organic acids (Fig. 6). The most common ones were lactic, succinic, isobutyric, isovaleric, and acetic acids.

Although 11 different organic acids were produced among the six bacterial species (butyric and caproic

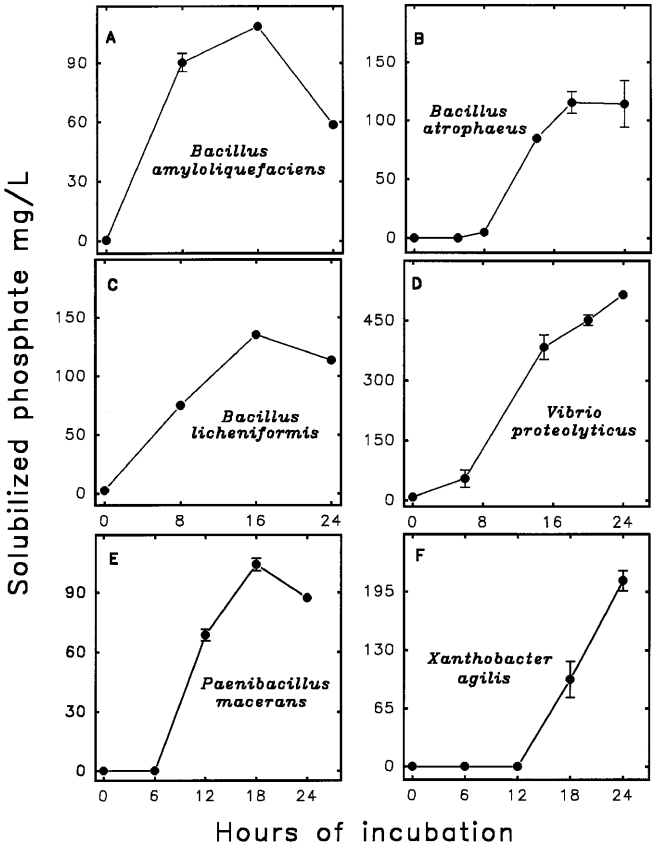


**Fig. 3** Phosphate solubilization of six phosphate-solubilizing bacterial species in culture medium containing insoluble calcium phosphate. *Bars* represent SEs. When the SE bar is absent, the SE is smaller than the point

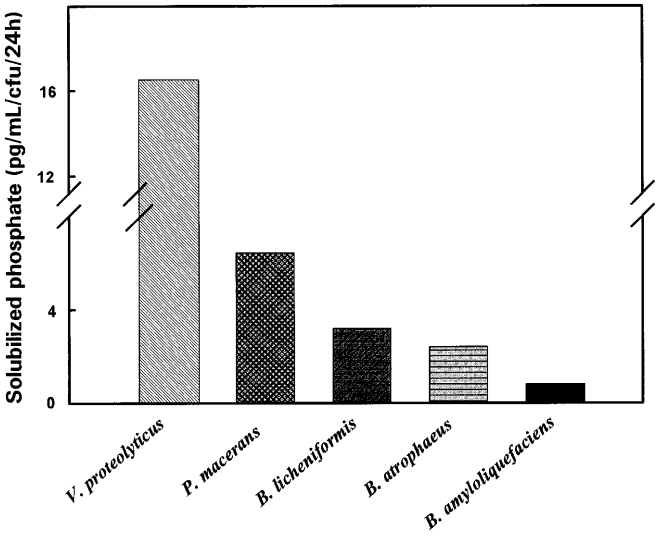


**Fig. 4** Phosphate-solubilization capacity per cell of five species of phosphate-solubilizing bacteria

acids are not shown in the chromatograms), it should be noted that several, as yet unidentified, organic acids were also in the culture filtrates (peaks marked "0" in Fig. 6A–F).

GC analysis detected only succinic acid in the fungal culture (Fig. 5C). The production of succinic acid increased with time from $5\times10^{-2}$ mEq/l after 48 h to $26\times10^{-2}$ mEq/l after 72 h.

## Discussion

The average concentration of dissolved orthophosphates in seawater is 73 μg/l (Martin 1970). Values below 31 μg/l are considered low (Boto 1982), therefore the concentrations found at the study site (18 μg/l) were very low. The study site is in an area in Mexico

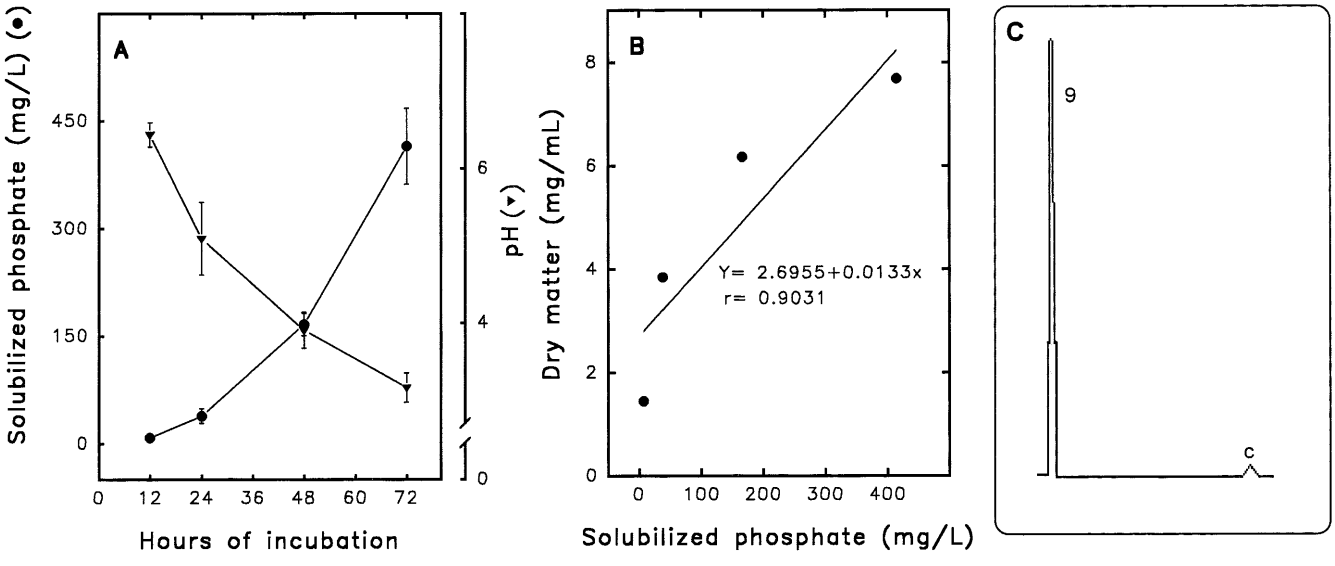


**Fig. 5** **A** Phosphate solubilization and pH changes by *Apergillus niger*. *Bars* represent SEs. **B** Linear correlation between fungal dry weight and phosphate solubilization. *r* is significant at $P \leq 0.05$. **C** Gas chromatographic analysis of organic acids in culture filtrate of *A. niger*. Peak labeled *C* is an impurity in solvents used in the analyses. *9* succinic acid

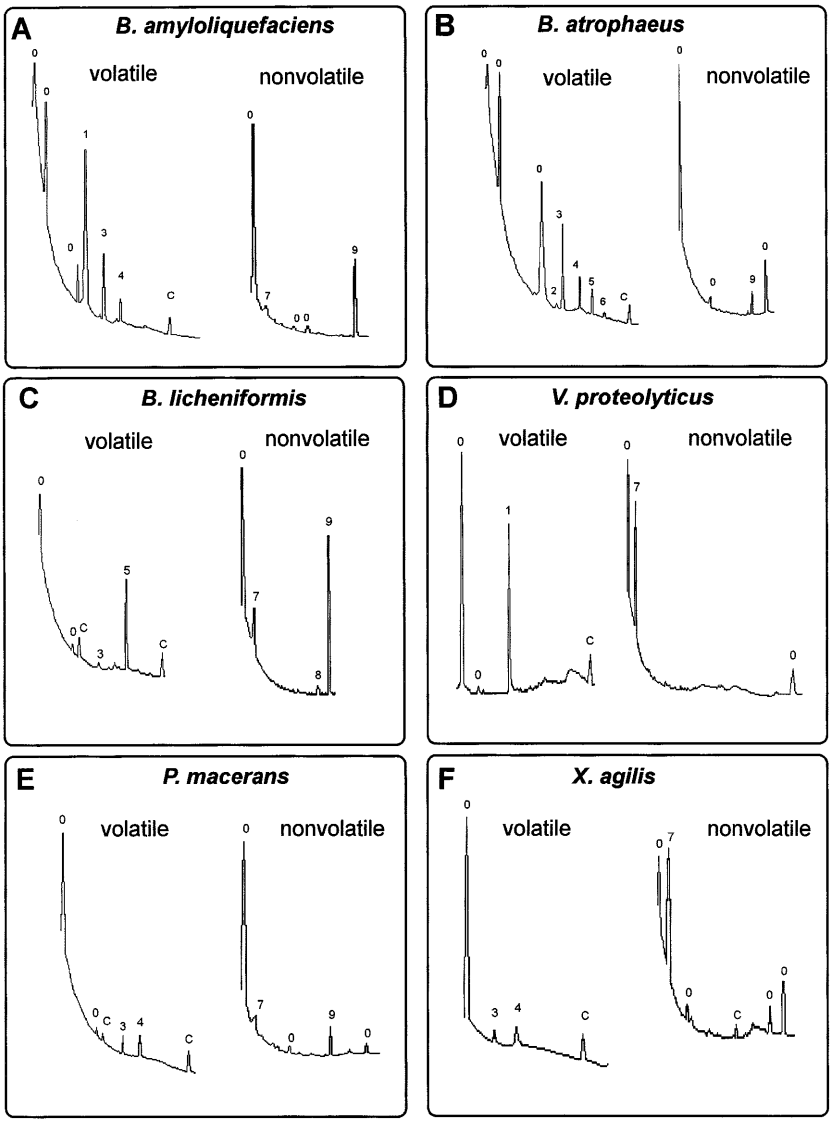


**Fig. 6** Gas chromatographic analyses of organic acids produced by six species of phosphate-solubilizing bacteria. Peaks labeled *C* are impurities in solvents used in the analyses. All analysis were repeated twice. Results presented are from one set of analyses. *0* Unidentified organic acids, *1* acetic acid, *2* propionic acid, *3* isobutyric acid, *4* isovaleric acid, *5* valeric acid, *6* isocaproic acid, *7* lactic acid, *8* fumaric acid, *9* succinic acid

characterized by abundant phosphate-rock deposits (Galli 1993). The total phosphorus in the sediment of our mangrove ecosystem was much higher than phosphorus in seawater, and was composed mainly of insoluble phosphorus and to a lesser extent organic phosphorus. The latter was present only at sampling sites close to the mangrove forest.

Muddy mangrove soils have a strong capacity to absorb nitrates and phosphates carried in by the tides (Hesse 1962). Most of the inorganic phosphate present in the sediments is bound to calcium, iron, and aluminum ions as insoluble phosphates (Alongi et al. 1992). We suggest that the fungi and IPSB present in the mangrove rhizosphere participate in releasing soluble phosphate into the pore water. A higher level of soluble phosphate in the pore water than in common seawater was found in this study. Some of this plant-available phosphate may reach the mangrove plants. Because the level of soluble phosphate in the pore water of the sediment is over 6 times higher than in the overlying seawater (112 compared to 18 μg P/l) at the same site, we propose, albeit not proved, that at least part of this soluble phosphate was available to the mangrove roots completely filling the sediment.

The flow of nutrients, including phosphorus, between sediments and water is a complex phenomenon. It includes biological, physical, and chemical processes and depends on pH, temperature, and redox potential, which are themselves influenced by bacterial activity (Carlton and Wetzel 1988). Because bacteria can represent up to 91% of the total microbial mass in mangrove sediments (Alongi 1988), the participation of bacteria in the flow of nutrients in mangrove ecosystems is crucial. In the Fly river in Australia, Alongi (1994) found a significant correlation between the growth rate of bacteria and dissolved phosphate in the interstitial (pore) waters. Mangrove trees, through root exudates, not only fuel the microbial activity in the rhizosphere but

also may support high rates of microbial activity in sediments (Alongi et al. 1993).

The ability of *Bacillus* spp. and *Vibrio* spp. to solubilize phosphate is known for seawater and marine sediments (Promod and Dhevendaran 1987). Terrestrial IPSB like *B. amyloliquefaciens* have been used together with ectomycorrhizal fungi for inoculation of Douglas fir seedlings to promote their growth (Duponnois and Garbaye 1991), and the weathering of calcium phosphate rocks has been attributed to ectomycorrhizal fungi and bacteria (Chang and Li 1998). However, there are no previous reports on the phosphate-solubilizing ability of any of the species isolated in this study except for *B. licheniformis* isolated from Brazilian soils (Nahas 1996).

IPSB *Bacillus* strains from wheat rhizosphere solubilized 112–157 mg/l of phosphate after 14 days (Sundara-Rao and Sinha 1963). Marine sediment IPSB *Vibrio* sp. and *Pseudomonas* sp. solubilized 0.5–0.55 mg/l (Promod and Dhevendaran 1987), where maximum growth coincided with the maximum amount of solubilized phosphate. The highest reported phosphate solubilization was by an unidentified marine bacterium, 300 mg/l, isolated from the rhizosphere of the seagrass *Zostera marina* (Craven and Hayasaka 1982). Our results of phosphate-solubilizing activity, but with identified bacterial species, are comparable to this study.

*A. niger* is a well-known phosphate-solubilizing fungal species from terrestrial environments (Nahas et al. 1990; Vassilev 1997). The average phosphate solubilization of our marine isolate of *A. niger* was higher than that of any of the bacterial species, but was similar to the solubilization capacity of other terrestrial *A. niger* strains isolated from soil (Nahas 1996). It is unknown whether all these in vitro phosphate solubilizations also occur in situ, and whether mangrove plants may benefit from this microbial activity.

As far as we know, there are no previous reports on organic acid production by the bacterial species to which our IPSB isolates belong. This is probably because their phosphate-solubilizing capacity has not been reported earlier. However, *Bacillus* spp. strains produced oxalic, 2-ketogluconic, and succinic acids capable of solubilizing phosphate (Banik and Dey 1983).

The phosphate-solubilization potential of acetic, lactic, isovaleric, and succinic acids found in our study was demonstrated decades ago in a study showing that solubilization depends not only on the pH but also the structure and type of the organic molecule, but did not depend on the acid concentration (Johnston 1952). The simultaneous production of different organic acids by the IPSB isolated in this study may enhance their potential for solubilizing insoluble phosphate.

In conclusion, we demonstrated microbial phosphate-solubilizing activity associated with mangrove roots. This is the first report on IPSB and a fungus associated with mangrove roots, and the phosphate-solubilizing potential of these species. Our microbial isolates solubilized phosphate probably through the production of organic acids. This solubilized phosphate increased in the pore water of the sediment, where mangrove roots are dense, and was possibly available to the plants.

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