POSTERS & PUBLICATIONS

- **Poster presentation** at the Indian Science Academy Sponsored seminar on **'Modern Biology: Facets and Prospects',** Jointly Organized by Department of Biochemistry, The M.S. University of Baroda and Department of Biological science,Tata Institute of Fundamental Research, Mumbai, at Baroda, India during October10-11, 2005 on **Cloning And Characterization Of Mineral Phosphate Solubilizing [mps] Genes From *Arabidopsis thaliana* cDNA Library.**

  ***This poster received 2nd prize at the symposium**

- **Poster presentation** at **the 6th International PGPR Workshop** held at Kozhikode (Calicut), Kerala, India during October 5-10,2003 on **Nature Of Organic Acids Secreted On Various Carbon Sources In Relation To Rock Phosphate Solubilization By Rhizobacteria.**

- **Oral presentation** at **National symposium on Mineral Phosphate Solubilisation-2002** held at university of agricultural sciences, Dharwad during November 14-16, 2002 on **Repression Of Mineral Phosphate Solubilising (Mps) Phenotype In The Presence Of Organic Acids In *P. fluorescens* P4.**

- **Divya Patel**, Archana G, Naresh Kumar G (2008) **Variation In The Nature Of Organic Acid Secretion And Mineral Phosphate Solubilization By *Citrobacter* sp. DHRSS In The Presence Of Different Sugars.** Curr Microbiol. 56: 168–174

- **Patel DK**, Gattupalli A, Gattupalli NK (2006) ***Citrobacter* sp. DHRSS 16S ribosomal RNA gene, partial sequence.** NCBI Nucleotide Databank Accession No **DQ486057**

- **Divya Patel**, Shweta Chaudhary , Priti Chaudhari M, Surobhi Lahiri, Dhruvdev Vyas, Naresh Kumar G, Archana G (2003) **Nature Of Organic Acids Secreted On Various Carbon Sources In Relation To Rock Phosphate Solubilization By Rhizobacteria. I**n: Proceedings of 6th International PGPR Workshop, Kozhikode (Calicut), Kerala, India pp 127-132

- **Patel DK**, Parmar S, Darpalli KD, Joshi FR, Naresh Kumar G, Archana G (2002) **Repression Of Mineral Phosphate Solubilising (Mps) Phenotype In The Presence Of Organic Acids In *Pseudomonas fluorescens* P4.** In: Proceedings of the National symposium on Mineral Phosphate Solubilisation-2002, Dharwad, Karnataka, India pp 186-190

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Variation in the Nature of Organic Acid Secretion and Mineral Phosphate Solubilization by *Citrobacter* sp. DHRSS in the Presence of Different Sugars

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Abstract A novel phosphate solubilizing bacterium (PSB) was isolated from the rhizosphere of sugarcane and is capable of utilizing sucrose and rock phosphate as the sole carbon and phosphate source, respectively. This PSB exhibited mineral phosphate solubilizing (MPS) phenotype on sugars such as sucrose and fructose, which are not substrates for enzyme glucose dehydrogenase (GDH), along with GDH substrates, viz., glucose, xylose, and maltose, as carbon sources. PCR amplification of the rRNA gene and sequence analysis identified this bacterium as *Citrobacter* sp. DHRSS. On sucrose and fructose *Citrobacter* sp. DHRSS liberated 170 and 100 μM free phosphate from rock phosphate and secreted 49 mM (2.94 g/L) and 35 mM (2.1 g/L) acetic acid, respectively. Growth of *Citrobacter* sp. DHRSS on sucrose is mediated by an intracellular inducible neutral invertase. Interestingly, in the presence of GDH substrates like glucose and maltose, *Citrobacter* sp. DHRSS produced approximately 20 mM (4.36 g/L) gluconic acid and phosphate released was 520 and 570 μM, respectively. *Citrobacter* sp. DHRSS GDH activity was found when grown on GDH and non-GDH substrates, indicating that it is constitutive and could act on a wide range of aldose sugars. This study demonstrates the role of different organic acids in mineral phosphate solubilization by rhizobacteria depending on the nature of the available carbon source.

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Introduction

Phosphorus (P) is the second major macronutrient required for plant growth [29, 33]. P is present in soil in abundance in both organic and inorganic forms, but the majority of it is immobilized and rendered unavailable for plant uptake as it complexes with cations of Ca, Fe, and Al, depending on the type of soils. Thus, only the phosphate in a soluble ionic form (P_i) is effective as a mineral nutrient [1, 15]. Several attempts to overcome the P deficiency problem including the application of chemical P fertilizers have not been very effective due to the high refixing ability of phosphate in the soil [6].

Rhizospheric bacteria are known to play a very significant role in plant growth promotion by different mechanisms, one of them being the ability to solubilize mineral phosphate in the rhizosphere, thus making it available for plant uptake [10, 15]. A variety of bacteria, belonging to diverse genera, and several groups of fungi have been reported to show mineral phosphate solubilizing (MPS) ability [5, 28, 31, 35]. The MPS property is due to a drop in pH, which has been associated with their ability to secrete low molecular weight organic acids such as gluconic, 2-ketogluconic, oxalic, citric, acetic, malic, and succinic, etc., [3, 7, 28]. *Aspergillus niger* and some *Penicillium* species have been shown to solubilize mineral phosphates by the secretion of organic acids such as gluconic, citric, and oxalic acid [4, 5, 24, 32].

Solubilization of mineral phosphates mediated by gram-negative rhizobacteria has been extensively studied using glucose as the carbon source [7, 9]. Gluconic acid is one of the prominent organic acids responsible for P solubilization and is produced by direct oxidation of glucose via membrane-bound quinoprotein GDH enzyme. The GDH enzyme is known to exhibit broad substrate specificity in

some organisms [28] in that it can also convert other aldose sugars such as xylose, galactose, and maltose, in addition to glucose, to their corresponding aldonic acids, which can also bring about efficient P solubilization.

Root exudates are known to serve as a substantial source of reduced carbon compounds which are released in the rhizosphere [13, 17]. Microbes in the rhizosphere utilize root exudates as their major nutrient source and this forms the basis for rhizosphere colonization. Apart from glucose, many different carbon sources are found in root exudates and sucrose is one of the common sugars [11]. Sucrose has been detected in large amounts in the soil near the root tip and large numbers of bacteria occur near the root area, with the highest sucrose and tryptophan exudation. Cowpea root exudates also contain arabinose, ribose, glucose, and sucrose as the main constituents [21]. Glucose and fructose were the major components in all growth stages of stone-wool-grown tomato [12]. Thus rhizobacteria demonstrating MPS ability using sucrose and fructose as carbon sources for P solubilization could be very effective in field conditions. For such sugars which are not glucose dehydrogenase (GDH) substrates, the organic acid secreted is of interest since organic acids other than aldonic acids are expected. Here we report the isolation and characterization of bacteria from the sugarcane rhizosphere with MPS ability on aldo- and keto-sugars.

Materials and Methods

Screening of Phosphate Solubilizing Bacterium (PSB) Using Buffered Media

Rhizospheric soil samples were collected by gently uprooting the sugarcane. The loosely adhering soil was removed by gentle shaking and the soil that remained attached to the roots was suspended in sterile saline. Serially diluted soil suspension was used for screening on buffered minimal medium with 100 mM sucrose as the sole carbon (C) source for isolation of potential PSB [8]. Commercial-grade Senegal rock phosphate (RP; 15.94% P; composition given in Ref. 28) at a concentration of 1.0 g/L was used as source of insoluble mineral phosphate and 1% methyl red as indicator dye for visualization of color change on organic acid production.

Identification of PSB by 16S rRNA Gene Sequence Analysis

Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was done using genomic DNA of the isolate as template in an Eppendorf Personal Mastercycler. The forward primer was 5′-AGAGTTTGATCCTGGCTCA-3′, corresponding to bases 8–26 of 16S rRNA of *Escherichia coli*, and the reverse primer was 5′-CTCGTTGCGGG ACTTAACC-3′, corresponding to 1107–1088 bases of 16S rRNA of *E. coli* [23]. The PCR kit and primers were obtained from Bangalore Genie Pvt. Ltd, India. The amplified product was subcloned in pTZ57R vector (MBI Fermentas) according to the manufacturer's instructions. The nucleotide sequence was determined by the automated DNA sequencing service provided by Bangalore Genie Pvt. Ltd., using the M13 reverse primer. The partial 16S rRNA gene sequence has been deposited in GenBank (accession number DQ486057). Molecular biology techniques were performed according to standard methods [27].

Characterization of MPS Ability of the Isolate

The MPS ability of the isolate was determined on minimal medium with Senegal RP as the sole P source and varying concentrations of Tris-Cl, pH 8.0 (0, 25, 50, 75, and 100 mM). Detailed studies were done using the highest buffer concentration which allowed a drop in the pH of the growth medium. Thus, 25 mM Tris-Cl, pH 8.0, buffer was used in the case of sucrose and fructose, whereas 50 mM Tris-Cl, pH 8.0, was selected for maltose and glucose. All the C sources were used at a 100 mM concentration. Aliquots were withdrawn at 24-h intervals. Absorbance at 600 nm and pH drop were used as parameters for growth pattern and acidification, respectively. Culture supernatant when the pH dropped below 5.0 was used for identification of organic acids and for estimating soluble P_i. Results are means of three independent experiments performed in duplicate.

Measurement of GDH Activity

Bacterial isolate was grown on RP-containing minimal medium with 100 mM either glucose or sucrose as the C source and 50 or 25 mM Tris-Cl, pH 8.0, respectively. After the pH dropped below 5.0, cells were harvested (5000g for 10 min), washed with sterile saline, and resuspended in 50 mM Tris-HCl, pH 8.75, and the whole-cell suspension was used as the source of enzyme in GDH assays done according to Matsushita and Ameyama [18]. To determine the substrate specificity of the GDH enzyme, glucose was replaced with a 100 mM concentration of other sugars such as maltose, xylose, arabinose, galactose, and mannose in the assay mixture. Units of activity of GDH are defined as micromoles of 2,6-dichlorophenol-indophenol (DPIP) reduced per minute. Specific activity is defined as units per milligram of protein.

Invertase Assay

The bacterial isolate was grown overnight in M9 minimal medium with sucrose or glucose as the carbon source. Cells were harvested by centrifugation at 5000*g* for 10 min, then washed with and resuspended in sterile saline. An aliquot of the cells was used for toluenization to permeabilize the cells. Whole cells as well as toluenized cells were used for enzyme assay. The assay system (2 ml) consisted of 0.01 M sucrose, 0.1 M buffer (acetate buffer, pH 5.0, phosphate buffer, pH 7.0, or Tris-Cl buffer, pH 8.0), with an appropriate amount of cells as the source of enzyme. The reaction system was incubated at 37°C for 30 min and the reducing sugar produced was measured by the method of Miller [19]. One unit of activity is defined as the amount of enzyme that produced reducing sugar equivalent to 1 μmol of glucose per hour. Specific activity is defined as units per milligram of protein.

Analytical Methods

Organic acids were analyzed by LaChrom Merck Hitachi high-performance liquid chromatography (HPLC). The culture supernatant was filtered through a 0.22-μm nylon filter. The organic acids were separated using an RP-18 column. The mobile phase consisted of 0.1% phosphoric acid at a flow rate of 1 ml/min. Acids were detected by absorbance at 210 nm. The organic acids were identified and quantitated by comparing the retention times and peak areas with those of standard acids.

Phosphate estimations were done according to the Ames method using KH_2PO_4 as standard [2]. Total whole-cell protein was estimated using a modified Lowry's method [22]. Cell growth was estimated by optical density measurements at 600 nm. Dry cell mass was calculated considering that 1 unit of OD at 600 nm corresponds to a dry cell weight of 0.50 ± 0.01 g/L [36]. C source consumed was determined by measuring the amount of residual sugars in the culture supernatant as reducing sugar by the method of Miller [19]. Sucrose was estimated by the same method after acid hydrolysis with 2 N HCl.

Results

Isolation and Identification of PSB Using Sucrose-Containing Buffered Minimal Media

Sucrose was used as the preferred C source for the isolation of PSMs from the rhizosphere. Many isolates were obtained from the rhizospheres of different plants by screening in the presence of RP as the sole P source under buffered conditions. The isolates were characterized on the basis of their ability for growth and acidification on methyl red-containing minimal medium plates.

About 13 PSMs were obtained from the rhizospheres of tuver and chili plants, an almost-equal number was obtained from the rhizospheres of cotton, and a few from sugarcane and jowar. These PSMs were further characterized based on the time taken to grow and acidify the plates and the extent and intensity of the zone of clearance. An isolate, namely, DHRSS, obtained from the rhizospheric soil of sugarcane, was selected for further characterization on the basis of its higher efficiency to show a red zone of acid secretion and, thereby, P solubilization (Fig. 1a). Approximately 1.1 kb of the hypervariable region of the 16S rRNA gene was amplified from the genomic DNA of DHRSS by PCR using primers corresponding to the highly conserved region of the *E. coli* 16S rRNA gene sequence. About 400 bp of the rDNA was sequenced using the M13 reverse primers from the plasmid backbone. The sequence information obtained was subjected to sequence alignment in the Ribosomal Database Project (RDP). The highest homology was detected with *Citrobacter freundii* (0.988%), indicating that the isolate belonged to the genus *Citrobacter* of family *Enterobacteriaceae*. Preliminary biochemical characterization of DHRSS was performed according to Bergey's manual of determinative bacteriology and it was shown to be Gram-negative, motile short rods belonging to the *Enterobacteriaceae* family.

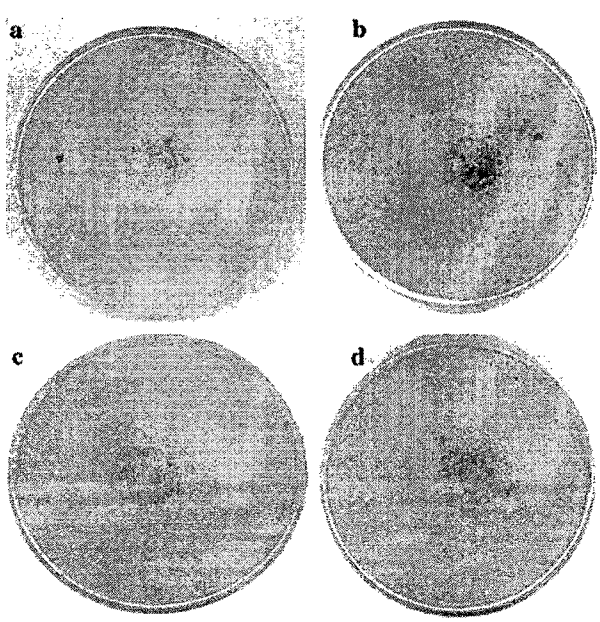

Fig. 1 Acidification on RP minimal medium containing methyl red by *Citrobacter* sp. DHRSS with (**a**) sucrose and 25 mM Tris-Cl (pH 8.0), (**b**) fructose and 25 mM Tris-Cl (pH 8.0), (**c**) glucose and 50 mM Tris-Cl (pH 8.0), and (**d**) maltose and 50 mM Tris-Cl (pH 8.0)

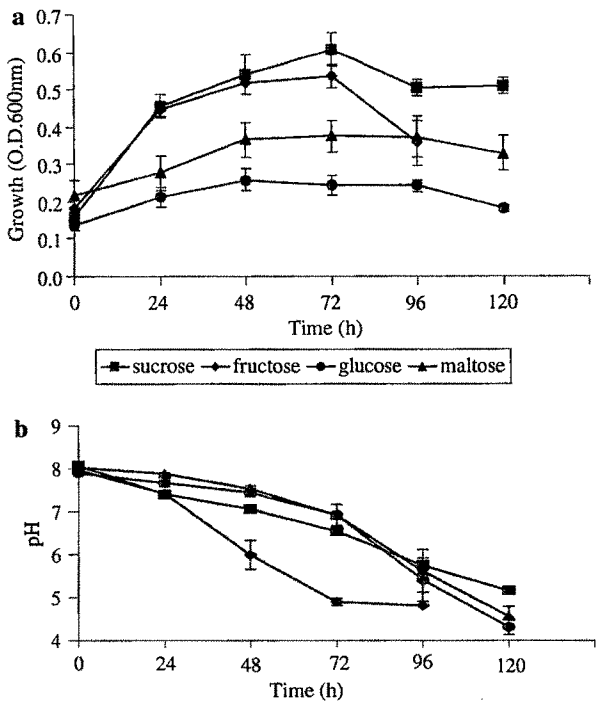

Fig. 2 (**a**) Growth and (**b**) acidification pattern of *Citrobacter* sp. DHRSS on buffered RP medium with different C sources: 25 mM Tris-Cl (pH 8.0) medium was used for fructose and sucrose, and 50 mM Tris-Cl (pH 8.0) medium was used for glucose and maltose. Values given are the mean ± SD of three independent experiments performed in duplicate

Characterization of the MPS Ability of *Citrobacter* sp. DHRSS

In addition to sucrose, *Citrobacter* sp. DHRSS showed a red zone of acidification on buffered RP minimal medium plates containing methyl red and fructose, glucose, or maltose as C source (Figs. 1b–d). The level of buffering that allowed a drop in the medium pH was found to vary with the C source used. On sucrose and fructose, *Citrobacter* sp. DHRSS was unable to decrease the medium pH beyond 25 mM Tris-Cl (pH 8.0) buffering, whereas with glucose and maltose as C sources, the concentration to which *Citrobacter* sp. DHRSS was able to grow and acidify the medium was 50 mM. Medium was acidified to pH <5.0 by 72 h on fructose and 120 h on sucrose, glucose, and maltose (Figs. 2a and b). A maximum of 570 μM (54.7 mg/L) P was released in the presence of maltose, followed by glucose 520 μM (50 mg/L), amounting to about 30%–35% of the total P. The specific RP solubilizing ability was also found to be highest with these C sources (Table 1). Thus, the amount of RP solubilized was found to depend on the nature of the sugar in the order of maltose > glucose > sucrose > fructose.

Determination of the Nature of Organic Acids

HPLC analysis of the medium supernatant was done to determine the nature and amount of organic acids secreted by *Citrobacter* sp. DHRSS. Approximately 49 mM (2.94 g/L) and 35 mM (2.1 g/L) acetic acid was secreted when sucrose and fructose were used as C source, respectively. A low level (~4 mM; 0.44 g/L) of pyruvic acid was also detected on fructose. On the contrary, approximately 20 mM (4.36 g/L) gluconic acid alone was secreted on glucose and maltose (Table 1). However, no gluconic acid secretion was detected in the presence of sucrose or fructose. Acid production was not found to be growth related. The acid accumulation was detected only in the late log phase when the pH of the medium fell below 5.0. The maximum yields of organic acids per gram of C source utilized were obtained on fructose and glucose, whereas the productivity of the organic acids (expressed per unit biomass) was found to be maximum in the presence of glucose and maltose.

In order to account for the nature of organic acids produced in the presence of different C sources, invertase and GDH activity of *Citrobacter* sp. DHRSS was monitored. *Citrobacter* sp. DHRSS was grown on sucrose minimal medium and invertase assay was done using toluenized cells as well as whole cells. No activity was observed in the case of whole cells. In the case of toluenized cells invertase activity was 94 ± 13 and 62 ± 6 units at pH 7.0 and 8.0, respectively, but no activity was found at pH 5.0. Invertase activity was also not found on glucose-grown cells, indicating that the intracellular invertase is induced in the presence of sucrose.

GDH activity was measured after growing the culture on buffered RP minimal medium with either glucose or sucrose as C source. The isolate showed low levels of GDH activity but exhibited a broad substrate range (Fig. 3). The enzyme was able to utilize xylose, arabinose, and galactose in addition to glucose and maltose as substrates.

Discussion

Citrobacter sp. DHRSS, a facultative anaerobe belonging to the *Enterobacteriaceae* family, was isolated from the rhizosphere of sugarcane using stringent buffered conditions with sucrose as the C source and RP as the P source, with the intention of obtaining a PSB that could exploit the sugar sucrose, which is present in abundance in the rhizosphere of a variety of plants [11], and show P solubilization. This isolate also showed P-solubilizing ability when provided with fructose as the C source. In both cases the isolate *Citrobacter* sp. DHRSS showed similar growth, acidification, and P solubilization properties.

Table 1 Rock phosphate (RP) solubilization and organic acid production by *Citrobacter* sp. DHRSS on different C sources

	Sucrose	Fructose	Glucose	Maltose
C source consumed mM (g/L)	52.1 ± 1.4 (17.8 ± 0.5)	34.4 ± 1.1 (6.2 ± 0.2)	73.9 ± 2.9 (28.8 ± 0.2)	80 ± 0.6 (13.3 ± 0.5)
Dry cell mass, g/L[a]	0.255 ± 0.01	0.178 ± 0.03	0.1 ± 0.004	0.165 ± 0.02
Gluconic acid (2.36 min)[b] mM (g/L)	ND	ND	19 ± 2.5 (4.1 ± 0.5)	20 ± 5.7 (4.4 ± 1.2)
Pyruvic acid (3.21 min)[b] mM (g/L)	ND	4.28 ± 0.8 (0.4 ± 0.1)	ND	ND
Acetic acid (4.11 min)[b] mM (g/L)	49 ± 6.4 (2.9 ± 0.4)	35 ± 4.5 (2.1 ± 0.3)	ND	ND
Organic acid yield (g/g)[c]	0.16 ± 0.03	0.41 ± 0.02	0.31 ± 0.02	0.15 ± 0.01
Organic acid productivity (g/g)[d]	11.5	14.2	41.4	26.4
P released (μM)	170 ± 2.8	100 ± 4.2	520 ± 31.1	570 ± 28.2
Phosphate released (mg/L)	16.3 ± 0.3	9.6 ± 0.3	50 ± 0.5	54.7 ± 0.6
Sp. RP solubilization (mg/g)[e]	64 ± 4	54 ± 3	500 ± 3.1	331 ± 7.5
Soluble P/total P (%)	10.24	6.02	31.36	34.33

Note. Values given are mean ± SD of three independent experiments performed in duplicate. ND, not detected

[a] Calculated considering1.0 OD = 0.5 ± 0.01 g/L dry cell mass

[b] In parentheses, retention time in minutes for standard organic acids as determined by HPLC analysis

[c] Calculated as grams of organic acid produced per gram of C source consumed

[d] Calculated as grams of organic acid produced per gram of dry cell mass

[e] Calculated as milligrams of phosphate released per gram of dry cell mass

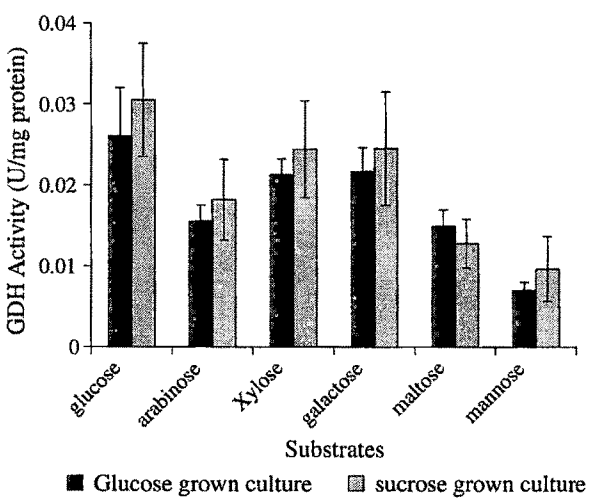

Fig. 3 Substrate specificity of glucose dehydrogenase activity of *Citrobacter* sp. DHRSS using different sugars as substrates. Units of activity of GDH are defined as micromoles of DPIP reduced per minute. Specific activity is defined as units per milligram of protein. Values given are the mean ± SD of three independent experiments performed in duplicate

However, the time taken to achieve P solubilization was faster in the presence of fructose than on sucrose. In addition to sucrose and fructose, *Citrobacter* sp. DHRSS also showed acid production and P-releasing ability on glucose and maltose in buffered medium. Several fungi have been reported to solubilize P in the presence of sugars such as sucrose, fructose, maltose, and glucose [4, 20, 24, 30]. Among bacteria, only *Azotobacter chroococcum* isolate from the wheat rhizosphere showed P solubilization on sucrose as the C source with tricalcium phosphate (TCP) and Mussoorie RP (MRP) as insoluble inorganic P sources [16].

P solubilization by *Citrobacter* sp. DHRSS was found to be much higher in the presence of maltose and glucose compared to sucrose and fructose. Narsian et al. [20] have also reported the use of sucrose, fructose, maltose, and glucose for P solubilizing activity of *Aspergillus aculeatus* and found it to be best on glucose. Although growth and acidification were similar on all the C sources, a clear difference in the nature and amount of organic acid profile was observed in the case of different sugars, which was also reflected in the amount of RP solubilized by *Citrobacter* sp. DHRSS. The P solubilization ability of *Citrobacter* sp. DHRSS, on sucrose and fructose was brought about predominantly by the secretion of acetic acid in the medium, whereas GDH-mediated gluconic acid production was the main mechanism of P solubilization in the presence of glucose and maltose. It has been shown using pure organic acids that the P solubilizing ability is related to the nature of the organic acid and that the kind of mineral P also influences the type and amount of organic acid requirement [3, 14]. However, gluconic acid was not included in these studies. In another report using alkaline vertisol as the source of mineral P, the efficacy of organic acids in P solubilization was in the order citric = oxalic > gluconic > succinate > acetate [8]. In gram-negative

bacteria, gluconic and 2-ketogluconic acids are the main acids implicated in P solubilization [7]. In agreement with these findings, it was observed that although *Citrobacter* sp. DHRSS produced higher molar concentrations of organic acids on sucrose and fructose, the P released was higher on glucose and maltose, sugars which supported gluconic acid production. In contrast, the P-solubilizing fungus *P. rugulosum* has been shown to secrete gluconic and citric acids on sucrose [25]. In terms of the amount of P released as a fraction of the total P and specific RP solubilizing abilities, fungal species are better than *Citrobacter* sp. DHRSS, possibly due to secretion of strong acids such as citric and oxalic in addition to gluconic acid [4, 5, 31, 32, 34].

Citrobacter sp. DHRSS is distinct from *Citrobacter koseri* in its ability to produce gluconic acid on glucose; the former instead produces 0.1 mM oxalic, 0.8 mM succinic, and 1.2 mM citric acid on glucose [8]. Gluconic acid secretion being an important mechanism of P solubilzation [7], *Citrobacter* sp. DHRSS is an efficient PSM compared to *C. koseri*, which did not show P solubilization under buffered conditions [8]. *Enterobacter asburiae* PSI3 has been shown to secrete a higher amount of gluconic acid (55 mM; 11.99 g/L) on glucose and can liberate a higher amount of soluble P (800 μM) than *Citrobacter* sp. DHRSS [9]. However, on sucrose and fructose, *E. asburiae* PSI3 did not show RP solubilization [28], whereas *Citrobacter* sp. DHRSS was proficient at using these two C sources for P solubilization, indicating it to be more versatile than *E. asburiae* PSI3. In addition, *Citrobacter* sp. DHRSS showed significant GDH activity using a variety of aldose sugars as substrates, converting them to their corresponding aldonic acid extracellularly, which may contribute to the P-solubilizing ability on GDH substrates [28]. This is the first report of a bacterial isolate to show P solubilizing ability on both GDH substrates, such as glucose and maltose, and non-GDH substrates, such as sucrose and fructose. On both types of C sources the mechanism of P solubilization with respect to the organic acids involved is different. Another unique property of *Citrobacter* sp. DHRSS is its ability to solubilize P on these sugars under buffered medium conditions, which mimic alkaline vertisol soil conditions [8].

Citrobacter sp. DHRSS could be an effective P solubilizer in the rhizosphere, as variation exists in the nature and amount of sugars in the root exudates of different plants [21]. Glucose and fructose have been shown to be the most frequent and abundant sugars detected in plant root exudates [26]. The major exudates in tomato root were glucose, fructose and maltose, along with small amounts of sucrose [17]. Thus, the ability of *Citrobacter* sp. DHRSS to solubilize RP in the presence of a variety of sugars could be of importance regarding the rhizosphere colonization, and further work is needed to test its efficacy under field conditions, especially alkaline vertisols.

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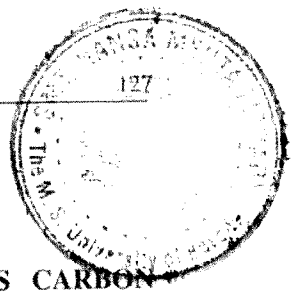

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NATURE OF ORGANIC ACIDS SECRETED ON VARIOUS CARBON SOURCES IN RELATION TO ROCK PHOSPHATE SOLUBILIZATION BY RHIZOBACTERIA

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ABSTRACT

Phosphate solubilizing microorganisms (PSMs) are known to solubilize mineral phosphates chiefly by production of low molecular weight organic acids. Among phosphate solubilizing bacteria (PSB), gluconic acid secretion is the most well-studied mechanism of P-solubilization. Gluconic acid is produced by direct oxidation of glucose via membrane-bound glucose dehydrogenase (Gcd) enzyme. This enzyme is known to act not only on glucose but also on several other aldo sugars such as xylose, arabinose maltose etc. and oxidizes them to their corresponding acids. We report here the isolation of two novel PSB capable of utilizing non-Gcd substrates like sucrose and fructose along with glucose, xylose and maltose as C-sources for exhibiting mineral phosphate solubilizing phenotype. DHRSS and ST10 strains were isolated from the rhizosphere of sugarcane and pigeon pea, respectively, by screening on buffered minimal medium with Senegal Rock Phosphate (RP) as the sole P-source and sucrose as C-source. Biochemical characterization and PCR amplification of rDNA was done to characterize the isolates. Both are Gram negative, motile, short rods. DHRSS is non-fluorescent while ST10 shows fluorescence. rDNA of both strains showed differences in the restriction digestion pattern. P-solubilizing and organic acid producing ability of the isolate DHRSS was checked on unbuffered as well as buffered (25mM and 50mM Tris pH 8.0) medium with RP as the P-source and different C-sources. On sucrose and fructose the P solubilized was170 µM and 100 µM, respectively. HPLC analysis of the medium supernatant showed the presence of 47 mM acetic acid along with a minor peak of an unidentified acid in media with sucrose as C-source. In case of fructose approximately 45 mM acetic, 3 mM lactic and additional major unidentified acid was produced. When RP was replaced with KH_2PO_4, a comparable profile was obtained along with some other unidentified peaks. On Gcd substrates like glucose and maltose approximately 20mM of gluconic acid was predominantly produced and P released was 520 µM and 570 µM, respectively. This study demonstrates the role of different organic acids in P-solubilization by rhizobacteria depending upon the nature of the C-source available.

INTRODUCTION

Rhizospheric bacteria are known to play a very significant role in plant growth promotion by different mechanisms, one of them being the ability to solubilize phosphorous (P) in the soil thus making it available for the plant uptake (Kucey et al., 1989; Gyaneshwar et al., 2002). The

mineral phosphate solubilizing (Mps) property is associated primarily with the production of low molecular weight organic acids which form complexes with the metal ions such as Fe, Al, Ca of the phosphate ore. The metabolic and genetic basis for the high efficiency solubilization of P by majority of the gram-negative PSB, studied so far, has been attributed to the production of gluconic acid by direct oxidation of glucose *via* membrane bound quinoprotein glucose dehydrogenase (Gcd) enzyme. In some species gluconic acid may further undergo one or two additional oxidations resulting in the production of 2-ketogluconic acid or 2,5-diketogluconic acid (Goldstein, 1995). The Gcd enzyme exhibits broad substrate specificity, converting other aldose sugars such as xylose, galactose, maltose etc. to their corresponding acids which can also bring about efficient P solubilization. Thus PSB producing gluconic acid can show Mps phenotype using several C-sources that are Gcd substrates (Unpublished data, this lab.). Here we report the isolation and characterization of novel phosphate solubilizing bacteria (PSB) capable of utilizing non-Gcd substrates such as sucrose and fructose as C source for P solubilization.

MATERIALS AND METHODS

Isolation of PSB using buffered media

Rhizospheric soils were collected by gently uprooting the sugarcane and pigeon pea plant. The soil attached to the roots was suspended in sterile saline and used for screening on buffered minimal medium (Gyaneshwar et al., 1998) with 100mM sucrose as C source.

Characterization of PSB

Biochemical characterization of the isolates was performed according to standard methods(Bergey, 1994).

PCR amplification of the 16S rDNA was done using genomic DNA of the isolates as template in 'Personal Mastercycler' from Eppendorf. The forward primer was 5'-AGAGTTTGATCCTGGCTCA-3' corresponding to bases 8-26 of 16S rRNA of *Escherichia coli* and the reverse primer was 5'-CTCGTTGCGGGACTTAACC-3' corresponding to 1107-1088 bases of 16S rRNA of *E. coli*. The PCR kit and primers were obtained from Bangalore Genie Pvt. Ltd, India. The amplified product was subcloned in T Vector (MBI Fermentas) according to manufacturers instructions. Further characterization by restriction digestion pattern analysis was by standard methods (Sambrook and Russel, 2000).

Mps ability of DHRSS

P solubilizing ability of the isolate was determined using buffered minimal medium with Senegal RP as the P source and 100mM sucrose, fructose, maltose or glucose as C source. Growth and acidification were monitored at an interval of every 24h. Results are mean of three independent experiments performed in duplicates.

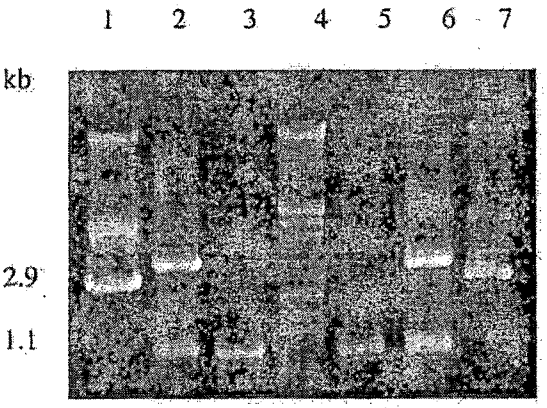
Analytical methods

Organic acids were analysed by LaChrom Merck Hitachi HPLC. The culture supernatant was filtered through 0.22µ nylon filter. The organic acids were separated using RP-18 column. The mobile phase consisted of 0.1% phosphoric acid with a flow rate of 1ml/min. Acids were detected by absorbance at 210nm. The organic acids were identified and quantitated by comparing the retention times and peak areas with those of multiple standards.

Phosphate estimations were done according to Ames method (Ames, 1964) using KH_2PO_4 as standard.

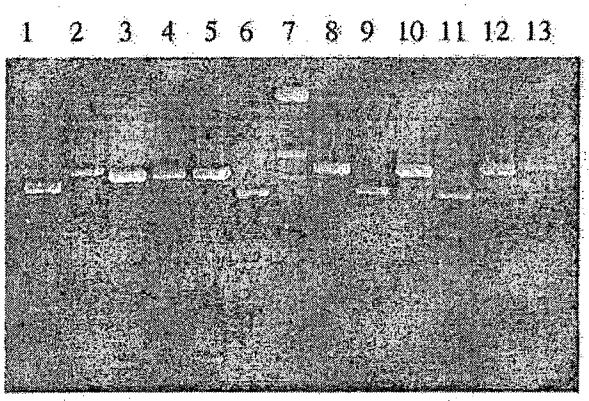
RESULTS AND DISCUSSION

Two isolates namely DHRSS and ST10, were obtained from the rhizospheric soil of sugarcane and pigeon pea, respectively, by screening on buffered minimal medium with sucrose as the C source on the basis of their P solubilizing ability. Both the isolates are Gram-negative, motile short rods and showed difference in their antibiotic sensitivities and biochemical characteristics. ST10 shows fluorescence while DHRSS does not. PCR amplification of rDNA using universal eubacterial primers and their RFLP analysis was done to further differentiate the two isolates. Approximately 1.1kb amplicons were obtained from both the isolates. These were cloned into plasmid vector for characterization (Fig. 1). The restriction digestion pattern of the rDNA was determined by



Lane1: Undigested rDNA of DHRSS in T vector
Lane2: *Hind*III/*Xba*I digested rDNA of DHRSS in T vector
Lane3: PCR amplified rDNA of DHRSS
Lane4: Lambda *Eco*RI/*Hind*III double digest
Lane5: PCR amplified rDNA of ST10
Lane6: *Eco*RI/*Bam*HI digested rDNA of ST10 in T vector
Lane7: Undigested rDNA of ST10 in T vector

Fig. 1. PCR amplification and cloning of rDNA of DHRSS and ST10



Lanes 1/2: *Eco*RI digests of DHRSS/ST10
Lanes 3/4: *Hinc*II digests of DHRSS/ST10
Lanes 5/6: *Sal*I digests of DHRSS/ST10
Lane 7: Lambda *Eco*RI/*Hind*III double digest
Lanes 8/9: *Xba*I digests of DHRSS/ST10
Lanes 10/11: *Hind*III digests of DHRSS/ST10
Lanes 12/13: *Bam*HI digests of DHRSS/ST10

Fig. 2. Restriction digestion pattern of rDNA clones of DHRSS and ST10

checking the presence or absence of sites for six different restriction enzymes (Fig. 2). The rDNA of DHRSS has restriction sites for *Eco*RI, *Hinc*II and *Sal*I but not for *Xba*I, *Hind*III and *Bam*HI whereas ST10 rDNA shows presence of sites for *Xba*I, *Hind*III and *Sal*I but not for *Bam*HI, *Eco*RI and *Hinc*II clearly indicating the difference between the two isolates.

Table 1. Rock phosphate (RP) solubilisation and organic acid production by isolate DHRSS on different C and P sources

C source (P source)	Tris-Cl pH 8.0 (mM)	Organic acids (mM)							P-Released (µM)
		Gluconic (2.36)[c]	UI[a]-1 (2.84)	UI[a]-2 (3.21)	Lactic (3.87)	Acetic (4.11)	UI[a]-3 (4.87)	UI[a]-4 (6.95)	
Sucrose (RP)	25	-	-	-	-	47	-	-	170
Sucrose (KH_2PO_4)	50	-	-	[	-	21	[	[	N.A.
Fructose (RP)	25	-	-	[	-	35	-	[	100
Fructose (KH_2PO_4)	50	-	-	[	3	30	[	[	N.A.
Maltose (RP)	50	20	[	-	-	-	-	-	570
Glucose (RP)	50	19	[	-	-	-	-	-	520

a :Unidentified organic acid - Minor peak b :Unidentified organic acid - Major peak
c :Numbers in brackets denote retention time in minute
[: Presence of organic acid -: Absence of organic acid N.A.: Not Applicable

Acid secretion and P solubilizing ability was monitored for the isolate DHRSS. DHRSS was able to grow and acidify the medium (Fig. 3) using both non-Gcd substrates like sucrose, fructose and Gcd substrates such as glucose and maltose. The medium pH reached below 5.0 within 120h in presence of 50mM of buffer with glucose and maltose whereas with sucrose and fructose it took a longer time to achieve the same drop in the pH. When the buffer concentration was lowered to 25mM acidification was achieved within 120h in case of the latter two sugars. The amount of P solubilized was found to be in the order of maltose> glucose>fructose>sucrose (Table 1). Thus it was observed that the ability to release P differed with the C source although growth and acidification was similar.

Kpomblekou and Tabatabai (1994) have shown that the P solubilizing ability is related to the nature of the organic acid and that the kind of the phosphate ore also influences the type and amount of organic acid requirement. P released depends on the strength of organic acids in the order of tricarboxylic acid> dicarboxylic acid> monocarboxylic acid (Bolan et al., 1994). HPLC analysis was done to determine the nature of organic acid produced and quantitate them under different substrate conditions (Table 1). A clear difference in the nature of the organic acid produced was observed in the HPLC profile of non- Gcd and Gcd substrates. Acetic acid along with an unidentified acid eluting at 3.21min were the major acids secreted in the presence of sucrose and fructose while gluconic acid predominated in the presence of glucose and maltose. The results correlated with the difference in the P solubilizing ability of the isolate in the presence of different C sources. Similar results were

obtained in the presence of KH_2PO_4 indicating that the acid production was not influenced by the P status in the medium.

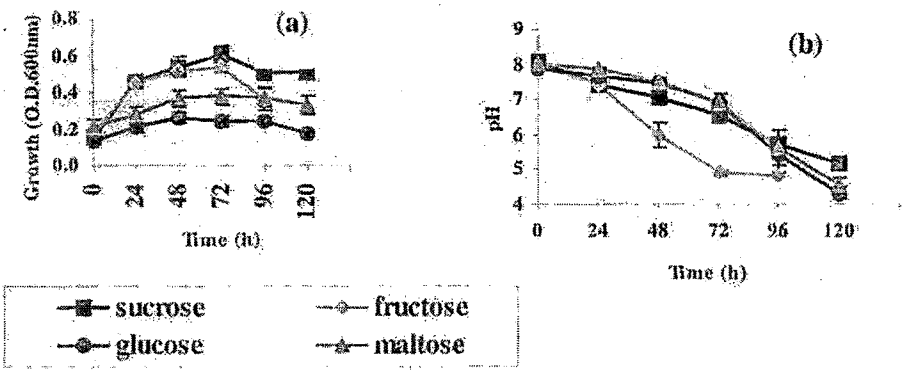


Fig. 3. (a) Growth and (b) acidification pattern of DHRSS isolate on buffered RP medium with different C sources.

Previous studies have shown that the nature of C source influences the P solubilizing ability of the organism (Tsay and To, 1987; Cerezine et al., 1988). The present study shows that the basis of this difference lies in the nature of organic acid produced under various C source conditions. It can thus be concluded that a relationship exists between the metabolic activity of the rhizobacteria and its P solubilizing capacity which would determine the efficacy of the rhizobacteria in response to carbon substrates released by root exudates.

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ANTAGONISM OF ENDOPHYTIC BACTERIA AGAINST *FUSARIUM OXYSPORUM* F.SP. *CUBENSE* CAUSING PANAMA DISEASE OF BANANA

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ABSTRACT

Forty strains of endophytic bacteria were isolated from the corm of banana plants were tested for the presence of plant growth promotion in rice. Among the isolates EB22 and EB5 performed well and showed more growth promotion than the other isolates. The promising isolates were used for testing the antagonistic activity against *Fusarium oxysporum* f.sp. *cubense*. Among the isolates EB 22 showed maximum inhibition of the mycelial growth of the wilt pathogen. These endophytes were tested for their ability to control the wilt disease under greenhouse condition. Among the different treatments EB22 + Pf1 showed maximum disease reduction when compared to all the other treatments. Thus it is clear that strain mixtures were found to be more promising in controlling the wilt pathogen than with individual strain.

INTRODUCTION

Banana (*Musa* spp.) is the most important fruit crop in the world. Panama wilt caused by *Fusarium oxysporum* f. sp. *cubense* is a major constraint to the production of bananas in many tropical and subtropical parts of the world (Persley and Delanghe, 1987). The vascular wilt pathogen penetrates the plant root system and eventually occludes the vascular vessels. The use of chemicals for the management of the disease is not only cost worthy but it is also not environmentally safe. The use of bio control agents is an alternative method in the management of the disease.

Repression of Mineral Phosphate Solubilizing Phenotype in the Presence of Organic Acids in *Pseudomonas fluorescens* P4.

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Abstract

In *Pseudomonas* species, organic acids such as acetate or a tricarboxylic cycle intermediate are the preferred carbon sources over carbohydrates and a "reverse catabolite repression control (CRC)" is exhibited by the repression of the genes involved in carbohydrate utilization in the presence of acids. We report here that the effect of CRC in *Pseudomonas* species can also be manifested as repression of mineral phosphate solubilising (mps) phenotype in the presence of organic acids. *P. fluorescens* P4 is a phosphate solubilising derivative obtained by the transformation of wild-type strain with the gene encoding phosphoenol pyruvate carboxylase from *Synchococcus* 7942 (formerly known as *Anacystis nidulans).* Addition of 20 mM of succinate, citrate, malate or acetate in Pikovskaya's medium was found to eliminate the zone of P-solubilisation normally displayed by *P. fluorescens* P4 on these plates. P-solubilisation by strain P4 has been attributed to production of organic acids when grown on sugars such as glucose, xylose and galactose. Addition of exogenous organic acids (as salts) decreased the secretion of acids on all of the above sugars in the order of citrate = malate > succinate > acetate. When grown on a mixture of glucose and either succinate or citrate, the P-solubilsed from rock phosphate was significantly reduced. HPLC analysis of the organic acid profile under these conditions showed both qualitative as well as quantitative changes.

The results signify that in rhizospheric soils, which are normally expected to contain a mixture of carbohydrates as well as organic acids both originating from root exudates, catabolite repression may adversely affect the P-solubilising efficacy and a *crc* mutant may show better field performance.

Introduction

Many Phosphate Solubilizing Bacteria (PSB) when grown on simple carbohydrates produce organic acids such as acetic, lactic, gluconic, succinic, ketogluconic etc. in the growth medium resulting in the dissolution of poorly soluble mineral phosphates (Kucey *et al.*,1989; Goldstein, 1986). The secreted organic acids are intermediate products of carbohydrate metabolism and efficient P-solubilising strains release significant amount outside by mechanisms not fully understood. Details about the genetics and biochemical basis of acid secretion are available only for gluconic acid secreting PSBs (Goldstein, 1995) such as *Erwinia herbicola* (Goldstein and Liu,1987), *Pseudomonas cepacia* (Babu-Khan *et al.*, 1995) and *Enterobacter asburiae* (Gyaneshwar *et al.*, 1999). In these bacteria, the cell-envelope bound glucose dehydrogenase (Gcd) along with the requisite cofactor pyrroloquinoline quinone (PQQ) has been shown to be responsible for the conversion of glucose to gluconic acid. Mutants defective in the apoenzyme or cofactor biosynthesis fail to show P-solubilisation (Gyaneshwar *et al.*, 1999). Cloning and expression of genes encoding enzymes for PQQ synthesis confers mineral phosphate solubilisation phenotype to *Escherichia coli* which harbours the Gcd apoenzyme but is unable to synthesize the cofactor (Goldstein and Liu,1987).

The use of any single strain as a universal phosphate solubilising bioinoculant is limited by the fact that the isolated organism has adapted over the years to survive, grow and function in its local conditions and hence may not be suitable for other environments. From this point of view, it could be advantageous to

incorporate mineral phosphate solubilizing (MPS) ability into native bacteria by genetic manipulations because they are well-adapted to colonize the root environment of specific crops and soils. Since fluorescent pseudomonads are efficient root colonizers and many plant growth-promoting strains are known (Lugtenberg *et al.*, 2001), we undertook genetic modification of *Pseudomonas fluorescens* so as to render this organism efficient at mineral phosphate solubilisation. Work in this direction has lead to the development of an organic acid producing, P-solubilizing strain of *P. fluorescens* by the incorporation of the gene for phosphoenol pyruvate carboxylase (PEPC) of *Synchococcus* 7942 (formerly known as *Anacystis nidulans*) The genetically modified strain *P. fluorescens* P4 releases P from rock phosphate (RP) by bringing about a drop in pH using carbon sources such as glucose and xylose, but not lactose, fructose and sucrose. (unpublished results).

Unlike the enterics, in *Pseudomonas* species the preferred carbon/energy source is an organic acid such as acetate or a tricarboxylic acid (TCA) cycle intermediate and a strong repression of the enzymes of carbohydrate catabolism is observed in their presence (MacGregor *et al.*, 1992). In *P. aeruginosa*, CRC has been shown to act upon the *hex* regulon and the independently regulated glucose transporter, the gluconate regulon, mannitol utilization regulon and the fructose regulon. In addition, enzymes of other catabolic pathways such as amidase, allantoinase, histidase are also subjected to CRC. Several evidences indicate that the 'reverse CRC' that operates in *Pseudomonas* species is cyclic-AMP independent (Collier *et al.*, 1996).

The vicinity of the plant root where the *Pseudomonas* spp. colonize is rich in organic compounds exuded by the plant roots and contains carbohydrates, amino acids and organic acids in various proportions (Lugtenberg *et al.*, 1999). It is thus expected that in their natural milieu, *Pseudomonas* spp. face CRC allowing the preferential utilization of one carbon source prior to others. In addition, the organic acid secreted by P-solubilising strains of *Pseudomonas* might itself cause CRC. In view of the above possibilities, it was of interest to study the effect of CRC on P-solubilisation phenotype of *P. fluorescens* P4. We report here that the MPS phenotype shown by *P. fluorescens* P4 is responsive to the effect of CRC.

Materials and methods

Bacterial Strain and culture conditions : *P. fluorescens* P4 was used through out this study. Strain P4 was routinely maintained on Luria-Bertrani agar plates (Hi-Media, India).

P-solubilisation studies : Acid production and RP solubilization was studied using a minimal medium containing NaCl, 0.5 g/l and KNO_3 0.5 g/l, $MgSO_4$, 25 μM, $CaCl_2$ 25 μM, Glucose, 100 mM. The medium was buffered with Tris-HCl pH 8.0 at 50 mM concentration for broth experiments and 100 mM for plate experiments. Phosphate source was either 1 mM KH_2PO_4 or 1 mg/ml Senegal rock phosphate. Glucose was replaced with xylose or galactose at 100 mM each wherever mentioned. Neutralised solutions of organic acids were added at concentrations indicated. In solidified media, methyl red indicator was added to check for acid production. Pikovskaya's medium (Hi Media, India) was used to study calcium phosphate solubilisation. Wherever mentioned, organic acids were added to Pikovskaya's medium as above. The supernatant obtained after centrifugation of the cell suspension was used to estimate P released.

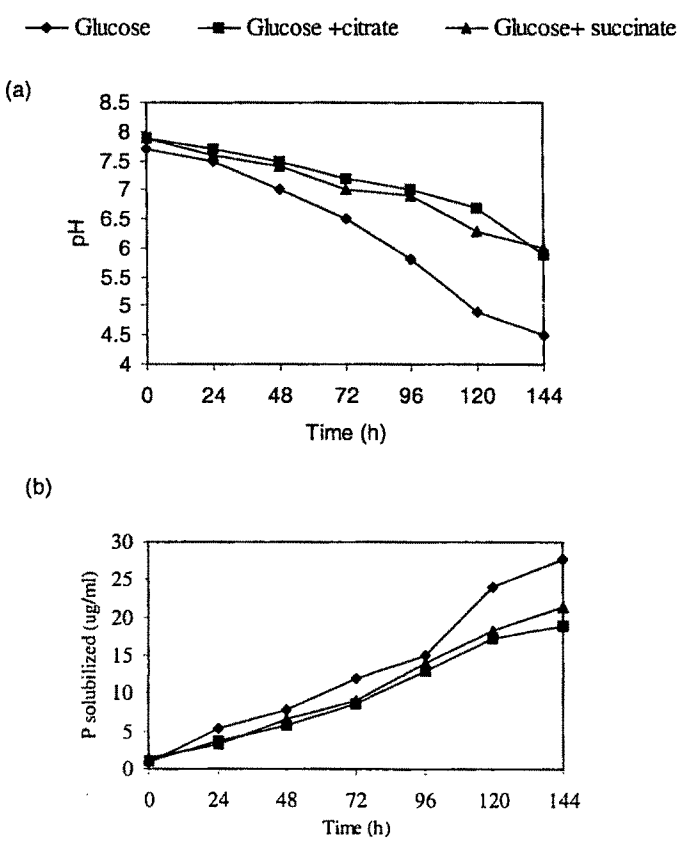
HPLC analysis : HPLC analysis was done using Shimadzu LC 10 Vp equipped with Winchrom software. Column used was YMC "J" sphere C-18. Mobile phase was 0.1% phosphoric acid at a flow rate of 1ml/min. Detection was in the UV range at 210 nm.

Results and discussion

In order to study the effect of exogenously added organic acids on the P-solubilsation by *P.fluorescens* P4, 20 mM each of succinate, malate, citrate and acetate were added separately to Pikovskaya's medium. A zone of clearance was produced by strain P4 on unamended medium but was absent when the organic acids were added. Growth was unaffected in the presence of succinate and citrate but was impaired upon the addition of malate and acetate. Since P-solubilisation in strain P4 has been attributed to secretion of organic acid, it was of interest to check the effect of exogenously added organic acids on acid production by P4. Acidification of glucose-containing medium could be visualised as zone of red coloration on methyl red containing RP-minimal medium. The red coloration was reduced on medium containing acetate and was faint or absent on media containing succinate, citrate and malate. Similar results were seen in the medium containing xylose or galactose as the carbon source.

The results on addition of succinate and citrate to liquid RP-minimal medium indicated that the drop in pH was slower upon the exogenous addition of the organic acids (Fig 1a) and was reflected in the reduced ability of the organism to solubilize rock phosphate in their presence (Fig 1b). The characterisation of the organic acids produced in the culture supernatant obtained after 144h was carried out by HPLC analysis. The strain P4 when grown on glucose as the carbon source produced a single major peak with retention time of 2.6 min and minor peaks corresponding to retention times 3.58 and 6.04 min. Comparison with standard

Fig. 1 : Effect of citrate and succinate on (a) acid production and (b) RP solubilisation in minimal glucose medium by *P. fluorescens* P4.



acids run under the same conditions allowed the identification of the major peak as gluconic acid. When citrate or succinate were exogenously added to the glucose-containing medium, the gluconic acid content was reduced to 55.3 and 49 per cent respectively as compared to the sample without addition of citrate or succinate. In addition, the profile of the minor peaks was different. The added organic acids apparently were utilised by the organism by the time the sample was assayed. The additional major peak in the sample with citrate with a retention time of 7.56 min could not be identified.

Pseudomonas aeruginosa exhibits diauxic growth in media containing both glucose and any one of the variety of additional carbon sources including acetate, citrate, a-ketoglutarate, succinate and fumarate or malate (MacGregor *et al.*, 1992). In case of glucose and succinate, and other glucose mixtures, activities of the *hex* regulon enzymes (the central pathway of hexose catabolism) such as glucose-6-phosphate dehydrogenase (G-6-PDH) and 2-keto-3-deoxy-6-phosphogluconate (KDPG) are repressed until the succinate is depleted (Collier *et al.*, 1996).

The work presented here shows that the catabolism of glucose is under CRC control in the phosphate solubilizing strain *P. fluorescens* P4 and this results in the repression of its mineral phosphate solubilising (MPS) phenotype. 20 mM of the organic acids tested could repress MPS phenotype shown with approximately 50 mM of glucose. Citrate, malate and succinate seem to be strongly repressing acids while acetate brings about a weak repression. The major organic acid produced by strain P4 was found to be gluconic acid. It may also be 2-ketogluconate, or a mixture of both, as these two acids could not be effectively resolved under the conditions of HPLC used here. The decrease in gluconate (and/or 2-ketogluconate) levels in the presence of citrate and succinate suggests that the enzyme Gcd (and/or gluconate dehydrogenase) of this organism is under CRC control. Repression of GCD by succinate has been reported in *P. aeruginosa* (MacGregor *et al.*, 1992). Our results show similar repression in *P. fluorescens.*

In *E. asburiae* PSI3, another PSB studied in our laboratory, the enzyme GCD has been found to be induced under phosphate starvation conditions and gluconic acid production is reduced under P-sufficiency (Gyaneshwar *et al.*, 1999). However unlike *P. fluorescens* P4, its MPS phenotype is not repressed by organic acids (unpublished results). Thus, although *P. fluorescens* P4 and *E. asburiae* PSI3 solubilize mineral phosphates by the same mechanism i.e. gluconic acid secretion, their MPS phenotype is differently affected by the presence of organic acids. This reflects the inherent differences in the regulation of the carbohydrate utilisation pathways in both the organisms and could manifest as variation in their field performance.

Although very little is known about the molecular aspects of CRC in *Pseudomonas, P. aeruginosa* mutants defective in CRC (crc^-) have been isolated (Wolff *et al.*, 1991). It would be interesting to isolate crc^- mutant of *P fluorescens* P4 and study its P-solubilization characteristics.

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