Mineral phosphate solubilization defective mutants of *Pseudomonas* sp. express pleiotropic phenotypes

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***Pseudomonas* sp., Psd 201, which showed adequate mineral phosphate solubilization (MPS) activity on TCP and MSM assays was subjected to random mutagenesis through nitrosoguanidine (MNNG) to isolate mutants defective in MPS functioning. Simultaneously random Tn*5* insertions into Psd 201 genome were also caused using Tn*5* vector, pGS9. Four MPS$^-$ and two delayed solubilization (MPS^d) mutants were selected from MNNG-treated cultures. Tn*5* insertions yielded four Psd *201* : : Tn*5* derivatives with MPS$^-$ phenotypes. These mutants showed different phenotypic classes with respect to metabolic and cell surface properties. The nature of pleiotropies shown by these mutants indicated that these mutational lesions might have occurred in some of the regulatory *mps* loci as well, since the level of expression of zone and time of solubilization got affected in some mutants.**

PHOSPHORUS, a major nutrient for crop plants is involved in many essential processes including cell division, photosynthesis, breakdown of sugar, energy transfer and nutrient transfer within the plant[1]. At the same time the role of phosphorus in expression and maintenance of genetic material is also well established. Plants obtain phosphorus from the soil in the form of HPO_4^{2-} and $H_2PO_4^{1-}$. However a major portion of the applied phosphatic fertilizers gets fixed into unavailable forms in the soil, resulting in only 10% becoming available to the plants[2]. This sink of fixed phosphorus can be harnessed biologically, using the mineral phosphate solubilizing (MPS) bacteria that convert the fixed phosphorus into HPO_4^{2-} and $H_2PO_4^-$. Although inconsistent[3] inoculation of the MPS bacteria has resulted in increased Pi uptake and economic yield[4] in crop plants, further genetic improvement of strains in MPS bacteria to achieve stable, inheritable characteristics towards enhanced efficiency of the MPS activity is a desirable step.

Understanding the genetic regulation and mechanism of expression for MPS phenotype in specific bacteria thus can be useful to break the natural plateau of efficiency in MPS activity by uncoupling linkages with some undesirable traits. Reports on the genetic basis of phosphate solubilization is limited. Bacterial genes involved in mineral phosphate solubilization have been proposed as MPS functions in *Erwinia herbicola*[5]. Cloned gene necessary for gluconic acid production has been shown to express in *Escherichia coli* background through dissolution of hydroxyapatite[6]. The gene was later identified to be responsible for synthesis of PQQ – a cofactor for glucose dehydrogenase[7]. However, genetic characterization of MPS loci through isolation and analysis of MPS mutants is lacking. In *Pseudomonas* sp., which are well known for MPS activity, no genetic analysis has been attempted.

In the present study, efforts have been made to isolate and characterize MPS$^-$ mutants to get preliminary insight into the nature of gene(s) which might be involved in expressing and regulating the process of mineral phosphate solubilization. Since no direct information is available on either the genetic map or genes involved in the MPS functions in *Pseudomonas* sp., the approach of forward genetic analysis was employed. An MPS-efficient strain of *Pseudomonas* sp., Psd 201, was subjected to chemical mutagenesis using nitrosoguanidine (MNNG). Transposon mutagenesis was carried out in Psd 201 for random insertion mutations using pGS-9 (ref. 8), a suicidal vehicle containing Tn*5* which is useful in a number of gram-negative species[9]. The mutant banks were quickly screened on modified Sperbers medium[10] [MSM; glucose 10 g; $MgSO_4\,7H_2O$ (2.5%), 10 ml; $CaCl_2$ (1%), 10 ml; dH_2O, 1000 ml. To this sterile basal medium, sterile solutions of $CaCl_2$ (10%) and K_2HPO_4 (10%) were added @ 3 ml and 2 ml/100 ml for isolation of MPS$^-$ mutants]. Appearance of solubilization zone on MSM agar indicates ability of strains to solubilize insoluble mineral phosphates. The mutants were also screened for the release of Pi from tricalcium phosphate (TCP) essentially according to the procedure of Gaur[4].

Six independent mutants could be identified as MPS-defective upon screening 1761 MNNG surviving colonies from mutagenized culture. These mutants showed two distinct MPS phenotypes: (i) Non solubilizers (MPS$^-$), showing no solubilization zone on MSM (Figure 1) and releasing negligible Pi from TCP in the broth (Table 1). Two MPS$^-$ mutants were identified (Figure 1) and characterized further for pleiotropic phenotypes. (ii) Delayed solubilizers (MPS^d). Four mutants showed significantly delayed expression of MPS activity and produced smaller zone of solubilization on MSM agar (Table 2). In this screening, two other mutants showing hyper expression of MPS activity were also observed which produced larger zone of solubilization. On the other hand, screening of 1200 Kanamycin resistant (Psd *201* : : Tn*5*) colonies obtained through Tn*5* mutagenesis, could identify four MPS$^-$ mutants (Table 2).

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The reports available on MPS property in general, and particularly in *Pseudomonas* spp., do not mention isolation or analysis of MPS-defective mutants. These MPS^- and MPS^d mutants have distinct classes of phenotypes which could be useful in analysis of the process of mineral phosphate solubilization. External Pi is known to repress the MPS activity in *Erwinia herbicola*. Our analysis on *Pseudomonas* sp. strain Psd 201, indicates existence of an inducible repressible system that functions to solubilize mineral phosphates, since presence of 15 μM K_2HPO_4 in the medium completely inhibited the solubilization of mineral phosphate in MSM assays. MPS^d mutants may represent mutation in regulation of MPS genes or switch on an alternate pathway in the event of mutational block, resulting in delayed expression. MPS^- mutants perhaps represent lesions either in structural genes or their promoter elements. The transposon Tn*5*-tagged mutations will be useful in mapping these mutations and loci.

Phosphate-regulated genes have been shown to belong to a distinct regulon in *E. coli*, which may be involved in cellular metabolism controlling several other activities. Hence, these Psd 201-derived mutants defective in MPS phenotype, were also tested for other characteristics. The pleiotrophic phenotypes of these mutants are described in Table 3. The MPS^- mutants appeared to

Table 1. Release of Pi from TCP by Psd 201 and derived mutants

Strains	Available P (mg/50 ml medium)
Psd 201	13.35
Psd 205	2.05
Psd 207	3.25
Psd 208	18.85
Psd 209	17.25
Psd 210	2.85
Psd 212	2.65
Control (Uninoculated)	1.02
CV	15.07%
F test (0.01)	***
SEm ±	0.635

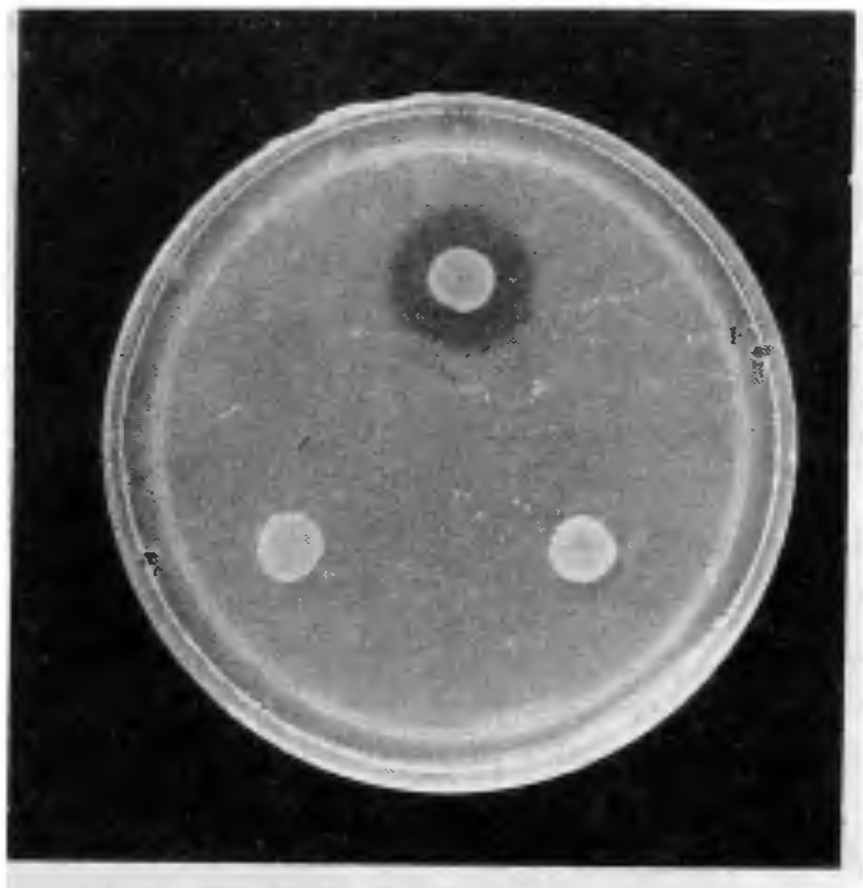

Figure 1. Mineral phosphate solubilization assay on TCP agar. The wild type strain Psd 201 shows a clearing solubilization zone (top) while the MPS^- mutants do not show any clearing around the bacterial growth spot (bottom two spots).

Table 2. Mineral phosphate solubilization by *Pseudomonas* sp. Psd 201 and mutant strains on modified Sperber's medium

	Solubilization zone (cm dia)			
Strains	24HAI	48HAI	72HAI	96HAI
Pseudomonas sp. Psd 201	1.00	1.5	2.0	7.0
NTG mutants				
Psd 202	–	1.0	1.0	6.0
Psd 203	–	0.5	1.5	5.0
Psd 204	–	0.5	1.5	6.5
Psd 205	–	–	–	–
Psd 206	–	0.5	0.5	5.0
Psd 207	–	–	–	–
Psd 208	1.5	2.0	2.5	8.5
Psd 209	1.5	2.0	2.5	8.5
Tn*5* mutants				
Psd 210	–	–	–	–
Psd 211	–	–	–	–
Psd 212	–	–	–	–
Psd 213	–	–	–	–

Hypers start showing signs of solubilization 14HAI.
WT takes > 18HAI
–: No solubilization

Table 3. Pleiotropic phenotypes of *Pseudomonas* sp. strain Psd 201, and derived mutants

Pseudomonas striata	Cell size	Colony morphology	Colony colour on		Fluorescence on M9 *medium* + calcafluor	*Siderophore* production
			MSM + BTB	MSM + aniline blue		
Psd 201 (WT)	++	Rod	Yellow	Blue	High	Sid^h
Mutants						
Psd 205	+	Cocci	White	White	Nil	Sid^r
Psd 207	+	Cocci	White	White	Reduced	Sid^h
Psd 208	+	Rod	Yellow	Blue	High	Sid$^+$
Psd 209	+	Rod	Yellow	Blue	High	Sid$^+$
Psd 210	++	Rod	White	White	Reduced	Sid$^+$
Psd 212	++	Rod	White	White	Reduced	Sid$^+$

^{h}High; ^{r}reduced; $^+$?.

be affected in synthesis of B (1–3) glucan as shown by their reduced binding to aniline blue. Aniline specifically binds to B (1–3) glucan in exopolysaccharide. Calcafluor is a dye that specifically binds to acidic polysaccharides and as a result fluoresce under UV light. MPS$^-$ mutants showed no fluorescence on M9-glucose calcafluor media, indicating thereby that the synthesis of acidic polysaccharide has been affected. The more mucoid nature of Psd 205 and Psd 212 could be due to excessive synthesis of slime. It is known that the regulation of a capsular physaccharide, alginate is multigenic and *alg* D is activated in P-deficient environments[11,12]. Variation was also seen in the ability to produce siderophore on CAS agar. Psd 205 showed reduced siderophore synthesis than the wild type.

A range of mutants affected in the MPS function could be isolated in this study with varying phenotypes, showing thereby independent mutational lesions. In addition, these mutants exhibited unique pleiotropic phenotypes, not reported so far. These pleiotropies and the two distinct classes namely, nonsolubilzers (MPS$^-$) and delayed expression types (MPS^d) clearly indicate the involvement of regulatory functions in the MPS process. Moreover independence of mutational lesions exhibited by distinct phenotypes indicates involvement of several structural genes which might be coordinatedly regulated in *Pseudomonas* sp., Psd 201. Thus the collection of MPS mutants obtained in this study would enable us to isolate both regulatory as well as structural genes involved in MPS activity, in addition to genetic mapping in *Pseudomonas*.

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