CHAPTER 5

CLONING OF *MPS* GENES FROM *SYNECHOCYSTIS* 6803 IN *E. COLI*

5.1 : INTRODUCTION

The ability of PSMs to solubilize mineral phosphates under laboratory conditions may not reflect upon their potential under field conditions as inoculation of plants with PSMs have not resulted in consistent enhancement of P uptake and plant growth. In the previous chapters we have shown that the inability to solubilize P under soil condition is mainly due to the buffering action of the alkaline soils which results in the neutralization of the acids produced by the PSMs. The concentration of acid required to solubilize P from soil is much higher than that required for *in vitro* P solubilization. We have also shown that PSMs secreting high amounts of acid(s) can be isolated form soil using buffered media condition during screening. The PSMs isolated using this modified procedure are able to reduce the pH of alkaline soil and thus could be very effective phosphate biofertilizers in such soils.

The limitation of the above screening procedure is that it selects only the existing population of microorganisms. A PSM isolated at one particular location may not be effective at other locations. Also organisms isolated from bulk soil may not be able to establish themselves in the rhizosphere. Additionally, the results presented earlier in this study have shown that not many "effective" PSMs are present in the rhizosphere of various plants. Therefore, it is of immense importance to develop a strategy to incorporate organic acid secreting ability (which would result in *mps* phenotype) into any rhizospheric microorganism. The modified isolate will be able to establish itself

successfully in the rhizosphere and the released P would be in the close vicinity of plant roots. The *mps* genes are considered to revolutionaries the agriculture (Netzer, 1987).

There have been attempts to genetically engineer phosphate solubilizing microorganisms. A *mps* gene has been cloned from *Erwinia herbicola* in to *E. coli* (Goldstein & Liu, 1987). The cloned gene coded for the enzyme PQQ synthase thereby allowing *E. coil* to produce PQQ, a cofactor for glucose dehydrogenase. This resulted in the transformed *E.coli* producing gluconic acid and thus solubilizing HAP (Liu *et al.*, 1992). Another gene was cloned from *Pseudomonas cepacia* which could code for either protein involved in membrane transport, probably of PQQ or a regulatory protein involved in the regulation of PQQ biosynthesis (Babukhan *et al.*, 1995). Attempts have also been made to deregulate the phosphorus starvation induced (psi) genes of plants. An efficient screening procedure has been developed to isolate such mutants by using a toxic compound X-P which can be cleaved by plant phosphatases thus making it nontoxic. Mutants are selected on the basis of their growth in the presence of free Pi (Goldstein *et al.*, 1989). This procedure has limited application, since it only deregulate the existing system but cannot enhance the P solubilizing potential of plants.

The genetic basis of secretion of other organic acids by PSMs remains obscure. Therefore, it is essential to clone the gene(s) from microorganisms that can result in the overproduction of the desired organic acid. Metabolic engineering of microorganisms has immense potential in industrial applications and would also help to understand the mechanisms of metabolic control exerted by the organisms. The genetic engineering has made it possible to change the levels of a particular enzyme thereby giving a better picture about the role of the enzyme in a particular pathway. It

could be possible to change the fluxes going through a particular pathway by altering the levels of key enzymes involved. In order to achieve a desired modification, it is essential to understand how the fluxes through a particular pathway are altered and how the organism responds to the metabolic changes that are force upon it by genetic manipulations. The use of R-DNA technology has contributed enormously towards understanding metabolic regulation (Nimmo & Cohen, 1987).

Can Overexpression of Single or Set of Enzyme(s) Result in Overproduction of a Desired Metabolite ?

Although many studies have involved qualitative concept of 'rate limiting step', it has been suggested that a single enzyme is rarely, if not never, truly rate limiting in a particular pathway (Kascer & Burns, 1973; Heinrich & Rapopart, 1974; Goren *et al.*, 1982). The metabolic control analysis predicts that increasing the level of a enzyme should have little effect on flux, but lowering of an enzyme concentration may have a major impact (Blakeley & Dennis, 1994). This view is further supported by the observations that in *S. cereviciae*, overexpression of various enzymes involved in glycolysis, either separately or in combination, were not able to either increase the fluxes through the pathway or increase the concentration of key metabolites. Overexpression of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) in logarithmic phase also did not result in increased alcohol production (Schaaff *et al.*, 1989). However, a mutant of *S. cervaciae* overexpressing PDC and ADH did produce high amount of alcohol, in stationary phase and anaerobic conditions (Sharma & Tauro 1986).

In recent years, many examples of overexpression of one or more than one enzyme resulted in overproduction of desired metabolite have been reported. In *E. coli,* which do not show alcohol fermentation, expression of ADH and PDC from *Zymomonas*

mobilis resulted in efficient conversion of any sugar to ethanol by the modified strain (Alterthum & Ingram, 1989; Ingram & Conway, 1988; Ingram *et al.*, 1987, 1989 Otha *et al.*, 1993). *Zymomonas* genes have also been transferred to other organisms like *Erwinia chrysanthemi* (Tolan & Finn, 1987) and *Klebsiella planticola* (Tolan & Finn 1987; Feldmann *et al.*, 1989). The yield of ethanol was always higher in *E. coli* recombinants expressing both PDC and ADH than those of *K. planticola* expressing only PDC (Ohta *et al.*, 1991). A strain of *Klebsiella oxytoca* overexpressing both PDC and ADH of *Z. mobilis* could produce almost the theoretical maximum of ethanol (Ohta *et al.*, 1991). Enhancement of acetone forming enzymes by using a synthetic acetone operon could produce 95%, 37% and 90% higher concentration of acetone, butanol and ethanol than the wild type. The recombinant also showed 50% higher yield of solvents on glucose and lower amounts of residual carboxylic acids (Mermelstein *et al.*, 1993). Overexpression of PEP carboxylase in *E. coli* led to increased production of succinic acid under anaerobic conditions (Millard *et al.*, 1996).

A tryptophan producing *Cornybacterium glutamicum* strain was metabolically engineered to produce tyrosine or phenylalanine by overexpressing feedback control insensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, the first enzyme in the common pathway of biosynthesis of aromatic amino acids and the branch point enzymes, chorismate synthase, leading to the production of prephenate and prephenate dehydratase, which leads ultimately to the production of phenylalanine (Ikeda & Katsumata, 1992). The recombinant transformed with plasmid overexpressing DS and CS resulted in the redirecting the flow to the production of tyrosine whereas the recombinant overexpressing all the three genes produced high amounts of phenylalanine. This showed that the metabolic flow through any pathway can be altered by changing the levels of enzymes involved in the branch points.

Transgenic tobacco plants expressing Mannitol-1-phosphate dehydrogenase from *E. coli* were able to accumulate mannitol thereby becoming more salt tolerant (Tarczynski *et al.*, 1993). Similarly transgenic tomato expressing cDNA for sucrose phosphate synthase accumulated high levels of sucrose and decreased levels of starch as compared to controls (Worrell *et al.*, 1991). Potato tubers expressing bacterial ADP-glucose pyrophosphorylase, which has different regulatory properties, high levels of starch as the normal regulatory circuit was bypassed (Stark *et al.*, 1992). This result also demonstrated the importance of using heterologus enzyme in overcoming the regulatory steps in metabolism.

Since nothing much is known about the genetic basis of acid secretion of PSMs, it is difficult to predict the desired modification for organic acid secretion. Hence, it will be interesting to explore whether enhanced organic acid production could be achieved by overexpression of gene of an organism. Consequently, we have taken a genomic DNA library of *Synechocystis* present in plasmid bluescript to screen for organic acid producing genes in *E. coli*. *Synechocystis* was chosen as it is a photoautotroph and the properties of the enzymes of the central carbohydrate metabolism is different from that of a heterotrophic bacteria like *E. coli*. Since the plasmid bluescript is a high copy plasmid, the genes located in this plasmid will be present in a few hundred copies, such as high copy number may also had to overexpression of gene. Thus, the transformants were screened for enhanced acid production by determining the zone of P solublization on DCP plates. Since there are differences in the metabolism of different C sources like mannitol and glyerol, transformants were selected on both these C sources.

5.2 : MATERIALS AND METHODS

Strains and Plasmids :

E. coli DH5α was used for transformation by the genomic DNA library of *Synechocystis* 6803 which was kindly provided by Dr. Parag Chitnis, Dept. of Biology, Kanas State University, Kanas, U.S.A.. The library is constructed in multicopy plasmid bluescript and the average insert size is 3 Kb (Zhao *et al.*, 1994).

Growth of *E. coli* on DCP :

E.coli DH5α was grown on minimal media containing 50mM Mannitol, 5mM NH_4Cl and 0.1mM K_2HPO_4 and the micronutrient solution as described in the previous chapter till late stationary phase. The cells were then inoculated on plates of same composition except that the free P was substitued with DCP. DCP was precipitated *in situ* as described earlier (Goldstein & Liu, 1987). The media was buffered with different concentrations of Tris-HCl pH 8.0 as indicated. P solubilization was monitored by measuring the zone of clearance.

Selection of *mps* Clones :

The transformed *E. coli* were plated on minimal media containing 50 mM of either Mannitol or glycerol, 5mM ammonium chloride and DCP. The media was buffered with 40mM Tris-HCl pH 8.0 when mannitol was provided as C source and with 10mM Tris-HCl pH 8.0 in plates containing glycerol. The colonies showing zone of DCP solubilization were picked up for further analysis.

Restriction Analysis of *mps* Clones :

The plasmids was isolated from the colonies showing acid production and were analysed by restriction digestion with various restriction enzymes. Plasmid

preparation was done by alkaline SDS method (Sambrook *et al.*, 1989). Restriction digestions and transformations were performed by standard methods (Sambrook *et al.*, 1989).

Mineral P Solubilization by *mps* Clones :

E. coli containing the *mps* clones selected on one particular C source were tested for mineral P solubilization acid production in minimal media with the other C sources in both liquid and solid media. In studies using liquid media, DH5α containing various clones was grown in media containing 50mM of either glycerol or mannitol, 5 mM ammonium chloride. RP was provided as sole source of P. The media was buffered with Tris-HCl buffer pH 8.0 at 10mM for glycerol and 50mM for mannitol and glucose, respectively. The bacteria were allowed to grow and the O.D. and pH were monitored after 24 and 48 hours.

5.3 : RESULTS

Growth of *E. coli* on DCP :

E. coli DH5α could solubilize DCP to various levels depending upon the carbon source used in the medium. With mannitol as C source, it showed zone of DCP solubilization till media buffered with 30mM Tris-HCl pH 8.0 but not in media buffered upto 40 mM of the same buffer. However when glycerol was the carbon source, it could show zone of DCP solubilization only uder unbuffered conditions but failed to do so in the presence of 10mM Tris-HCl pH 8.0 (Table 5.1). Therefore, 40mM and 10mM Tris-HCl pH 8.0 were chosen for selecting *mps* clones form mannitol and glycerol, respectively.

Selection of *mps* Clones :

E. coli DH5α was transformed with the genomic DNA library of synchocystis and 10^4 transformants were screened for mps phenotype on plates containing glycerol and mannitol as C source. 3 mps clones (clones 2,3 and 5) were selected on mannitol plates and 2 (clone 1 and 4) on glycerol. The clonies showing zone of clearance were picked up and plasmid was extracted. The plasmid was retransformed into *E. coli* DH5α and phenotype was confirmed (Fig. 5.1).

Characterization of *mps* Clones :

The *mps* clones were grown in both solid and liquid media with RP as sole P source and their ability to show zone of pH reduction on methyl red indicator plates and drop the pH of the media and release P in liquid media was monitored. The *mps* clones which were isolated based on their ability to solubilize DCP also could solubilize rock phosphate in both liquid and solid media (Table 5.2 and Fig. 5.2).

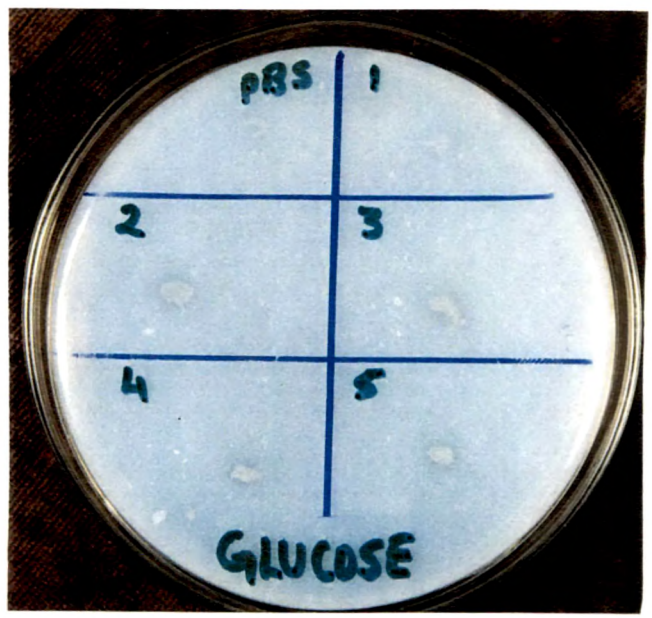
Table 5.1

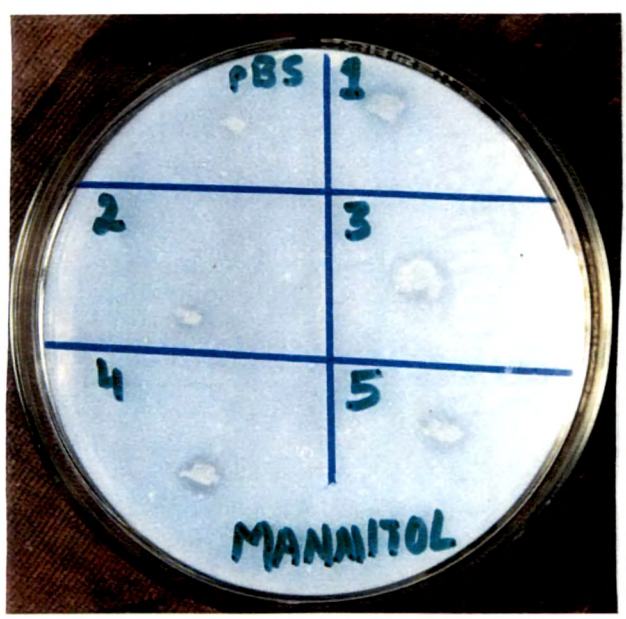
Solubilization of DCP by *E. coli* DH5α

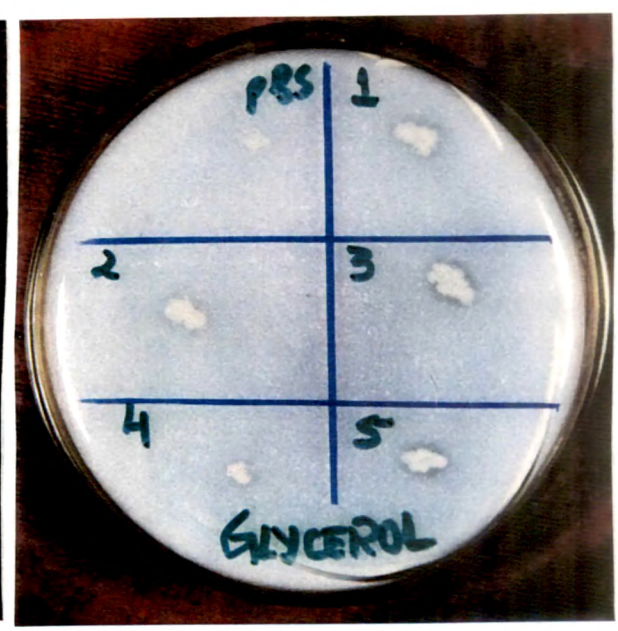
C Source	Zone of Clearance (cm) Buffer Concentration				
	0	10	20	30	40
Mannitol	1.5	1.0	0.7	0.3	UD
Glycerol	0.5	UD	UD	UD	UD
Glucose	2.0	1.5	1.0	0.4	UD

The results are mean of three independent measurements.

Fig. 5.1 : DCP solubilization by mps clones on Mannitol, Glucose and Glycerol as C source.

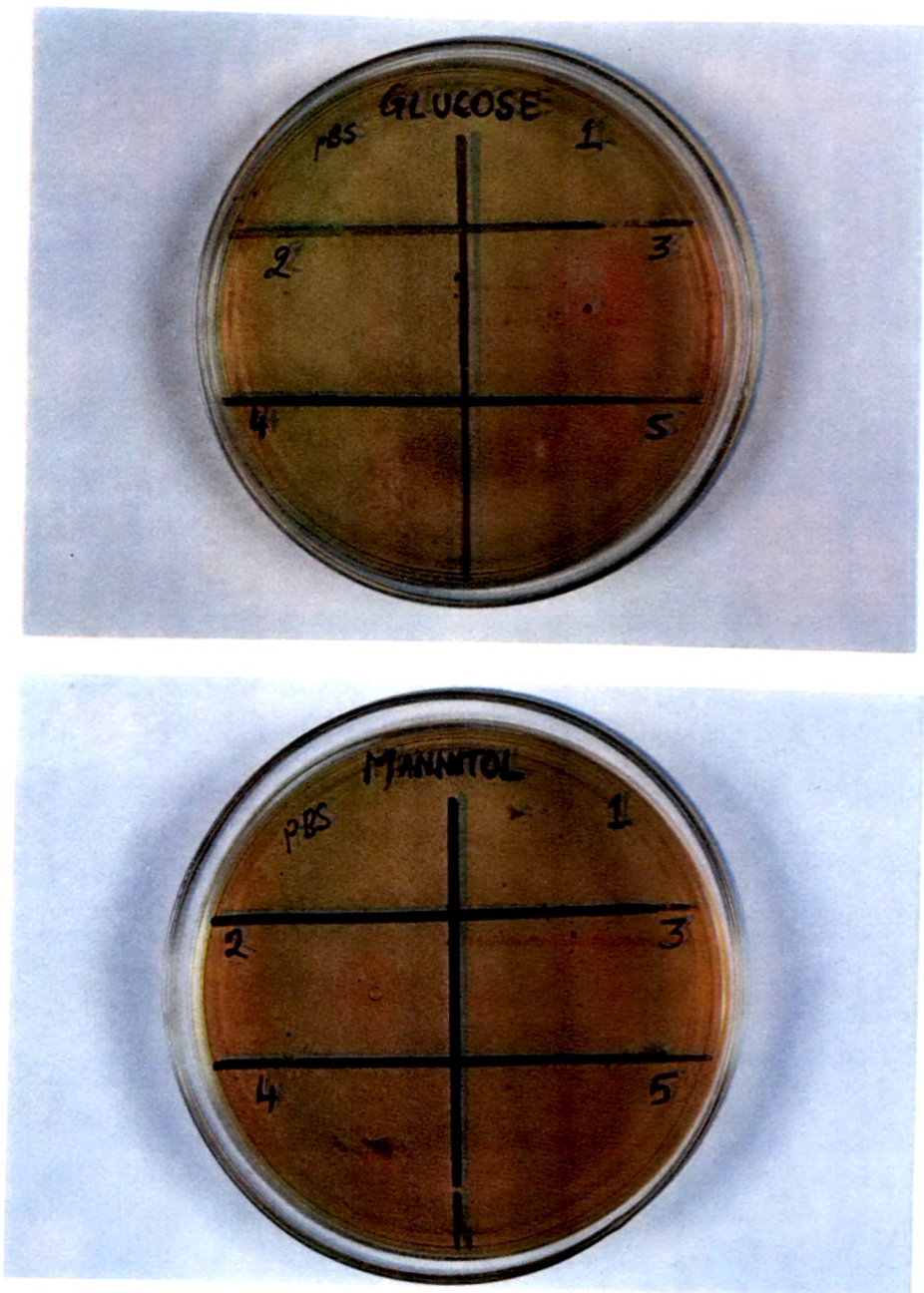






pBS DH5α containing plasmid bluescript.
1-5 DH5α containing various *mps* clones.

Fig. 5.2: RP solubilization by *mps* clones.



pBS DH5α containing plasmid bluescript.
1-5 DH5α containing various *mps* clones.

Table 5.2

Solubilization of RP by *mps* clones

Clone No.	Time					
	12 h		24 h		48 h	
	pH	P	pH	P	pH	P
Glucose						
pBS	7.0	0.1	6.0	0.15	5.5	0.25
1	6.0	0.2	5.0	0.6	4.8	0.7
2	6.5	0.1	5.5	0.5	4.5	0.8
Mannitol						
pBS	7.0	0.1	7.0	0.1	6.5	0.2
1	6.5	0.2	5.0	0.6	5.0	0.6
2	6.0	0.25	4.8	0.7	4.8	0.65
Glycerol						
pBS	8.0	UD	7.5	0.05	7.5	0.05
1	7.0	0.1	6.0	0.25	5.0	0.65
2	6.5	0.25	5.0	0.7	4.8	0.6

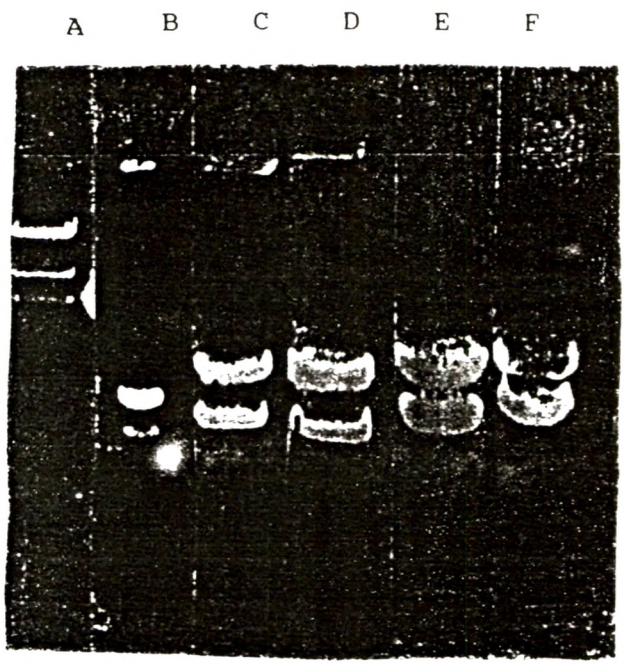
pBS : DH5α containing plasmid bluescript.

1 & 2 : DH5α containing '*mps*' clones.

Restriction Analysis of the Clones :

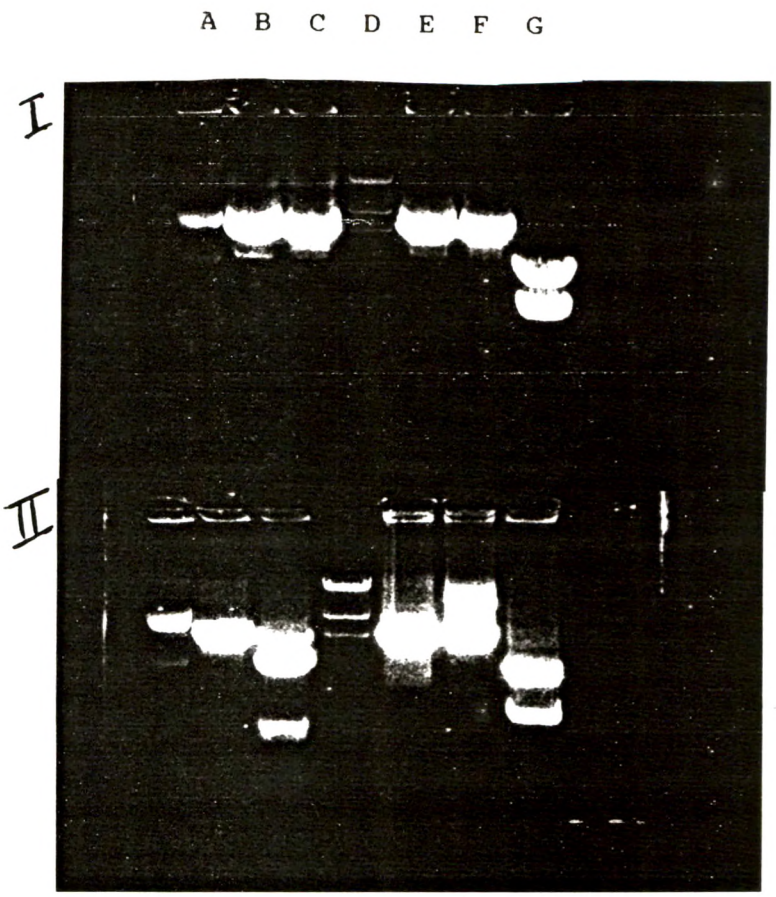
All the clones showed insert size of 3-4 kb as determined by digestion with Pvu II which has got a site on either end of MCS of plasmid bluescript. The average insert size of the genomic DNA library is 3 Kb (Zhao *et al.*, 1994) (Fig. 5.3). The clones did not have an internal site However, there was an internal site of Hind III in clone number 2 (Fig. 5.4).

Fig. 5.3 : Average insert size of *mps* clones.



A : λDNA/HIII Marker
B-F : *mps* clones cut with Pvu II

Fig.5.4 : Restriction analysis of *mps* clones.



I : CLONE No. 1 ; II: CLONE No. 2

A : Plasmid DNA; B : Cut with EcoR I; C: Cut with Hind III;

D : λ Hind III Marker DNA; E : Cut with BamH I;

F : Cut with Pst I; G : Cut with Pvu II

5.4 : DISCUSSION

The results presented in Chapter 2 have shown that there are not many effective PSMs in the rhizosphere of various plants and therefore there is a need to understand the genetic basis of organic aicd secretion and in turn P solubilzation by microorganisms. This could help in engineering any rhizospheric predominant bacteria into PS bacteria.

In an effort to clone the genes when present in multiple copies give rise to enhanced acid production, we transformed *E. coli* with the genomic DNA library of a cyanobacteria which is a photosynthetic autotroph as compared to chemoheterotropic *E. coli.* It is known that enzymes form heterologus sources can overcome the regulatory mechanisms of the host.

We were able to isolate various clones which could give *mps*+ phenotype on DCP plates buffered with different concentrations of Tris-HCl pH 8.0 to neutralize the basal level of acid production by *E. coli.* These clones could drop the media pH and solubilize RP in both solid and liquid media indicating that these clones were producing more acid as compared to *E. coli* containing just the vector. Though *mps* genes have been cloned earlier form PS bacteria (Liu *et al.*, 1989; Babukhan, *et al.*, 1996) this is the first time that this novel approach has been employed to select *mps* genes form a non-PS bacteria.

It would of interest to further characterize these clones for the encoded products and effect of further overexpression on acid production and mineral phosphate solubilization. This approach could be used in identifying the gene(s) necessary for increasing the acid production directly in PGPR like *Pseudomonas* and *Rhizobium.* cDNA library present under the control of promoters that are expressed at moderately

high levels in these bacteria like *lac* (Labes *et al.*, 1990) could be used for this purpose. The advantage of this type of phenotypic selection over conventional mutagenesis is the direct cloning of the DNA fragment responsible for the *mps*+ phenotype.

In conclusion the results presented in this chapter show that it is possible to isolate the gene(s) which could give *mps*+ phenotype to the organism. This method of phenotypic selection would be of immense importance in understanding the genetic basis of organic acid production by PS microorganisms.