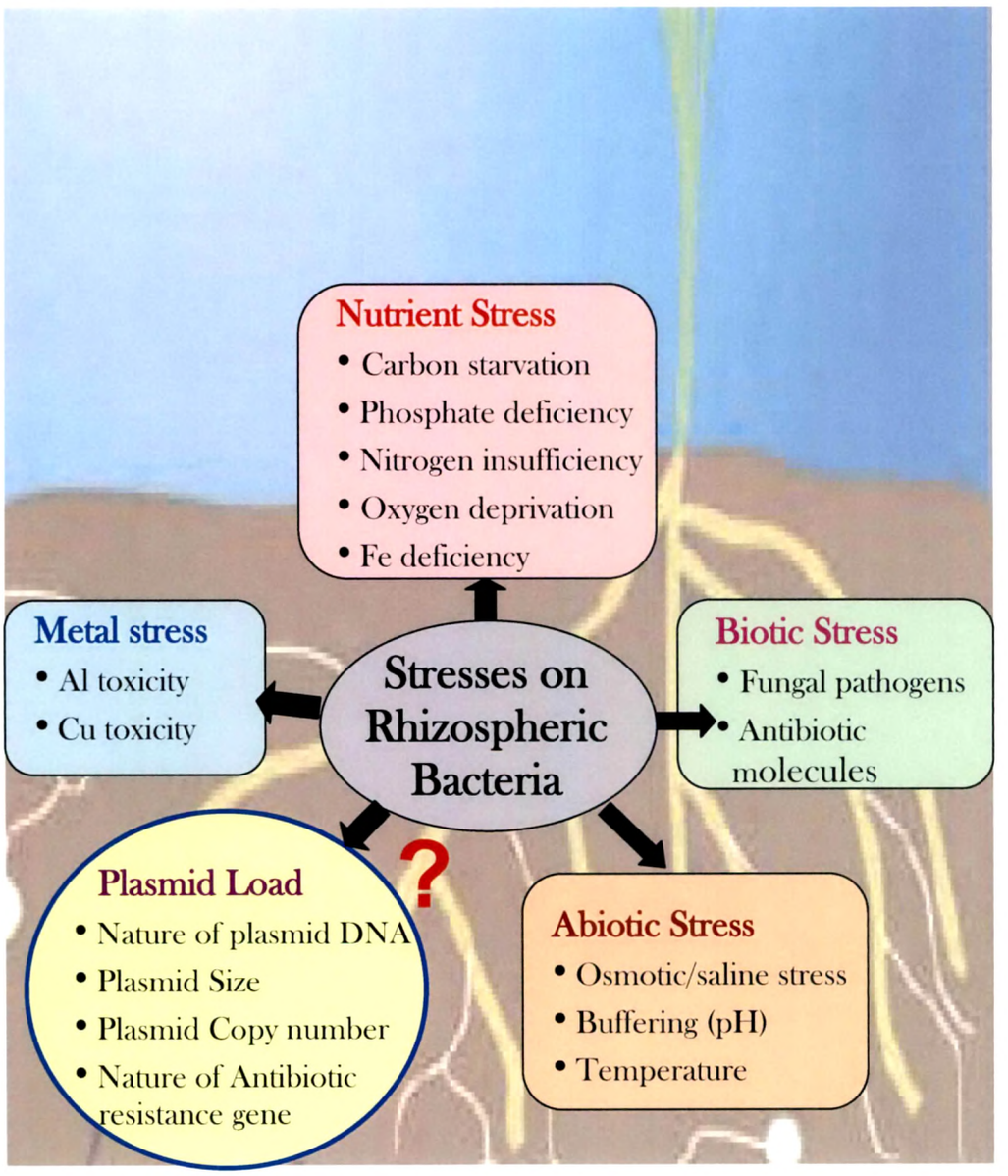
CHAPTER 3

Effect of the presence of plasmids on the P-solubilization by Enterobacter asburiae PSI3



3.1 INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) enhance plant growth by increasing nutrient availability, phytohormone production and/or by preventing deleterious effects of phytopathogenic microorganisms by the production of siderophores, hydrogen cyanide, antibiotics, etc. (Bloemberg and Lugtenberg, 2001; Compant et al., 2005). The potential of PGPRs as biofertilizers depends upon their ability to survive, grow and manifest PGPR traits in soils (Bashan, 1998; Vessey, 2003). The identification of genes involved in mechanisms of PGPR action has provided the potential to improve the performance of natural PGPR strains by genetic modification (Glick and Bashan, 1997). Strategies for genetic alterations of PGPR usually involve introduction of gene constructs under *tac* or *lac* promoters through broad host range plasmid cloning vectors or by integration of foreign genes into the chromosome using transposon or suicide vectors (Bloemberg and Lugtenberg, 2001).

Expression of foreign proteins and maintenance of vectors causes significant utilization of host resources (Glick, 1995). This phenomenon, termed as metabolic load or burden, adversely affects the growth of the microorganism and its magnitude depends upon the extent of foreign protein produced, copy number of plasmid, size of the cloning vector, metabolic state of the cell, composition and amount of dissolved oxygen etc. Metabolic load affects primary carbon and energy metabolism of the microorganism (Neubauer et al., 2003).

Mineral phosphate solubilization (MPS) is important for the maintenance of P sufficiency for plant growth (Rodriguez and Fraga, 1999; Gyaneshwar et al., 2002). Major mechanism of P solubilization is the secretion of low molecular weight organic acids such as succinate, gluconate, citrate, oxalate, etc (Khan et al., 2006). Gluconic acid-mediated P-solublization has been extensively studied in gram-negative bacteria (Goldstein, 1995).

Earlier efforts from this laboratory have shown that high buffering capacity of the growth medium or soils negatively affects the P-solubilization efficiency particularly for those phosphate solubilising microorganisms (PSMs) that secrete weaker acids or lower concentrations of the acids (Gyaneshwar et al., 1998a). Incorporation of Tris-Cl buffer of pH 8.0 in the medium facilitated the isolation of *E. asburiae* strains which

secrete high amounts of gluconic acid (Gyaneshwar et al., 1999). Mutant analysis the MPS phenotype of *E. asburiae* PSI3 demonstrated gluconic acid secretion by the action of phosphate-starvation-inducible glucose dehydrogenase (GDH) that has broad substrate specificity (Gyaneshwar et al., 1999).

Since the plant growth promoting abilities are influenced by the metabolic load including plasmid load, it is interesting to understand the effect of plasmid load on MPS phenotype. Present work demonstrates the effect of different plasmids on the MPS phenotype of *E. asburiae* PSI3.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and plasmids

Table 3.1: Bacterial strains and plasmids used in this study

Bacterial strains or plasmids	Genotype or relevant characteristics	References
***E. coli* strains**		
E. coli DH5α	*F' Phi80 Δ lacZ Δ M15 Δ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK-mK+) phoA supE44 lambda- thi-1*	Sambrook and Russell, 2001
E. c (pBluescript/SK)	*E. coli* DH5α strain containing Bluescript/SK plasmid, Ap^r	This study
E. c (pBR322)	*E. coli* DH5α strain containing pBR322 plasmid, Ap^r, Tc^r	This study
E. c (pGM160)	*E. coli* DH5α strain containing pGM160 plasmid, Gm^r	This study
E. c (pTOL)	*E. coli* DH5α strain containing TOL*gfp*mut3b plasmid, Km^r	This study
E. c (pACYC184)	*E. coli* DH5α strain containing pACYC184 plasmid, Tc^r, Cm^r	This study
E. c (pBSK-Gm)	*E. coli* DH5α strain containing pBSK-Gm plasmid, Ap^r, Gm^r	This study
E. c (pBR322-Gm)	*E. coli* DH5α strain containing pBR322-Gm plasmid, Ap^r; Gm^r, Tc^r	This study

Cont.....

Bacterial strains or plasmids	Genotype or relevant characteristics	References
***E. asburiae* strains**		
E. asburiae PSI3	rhizosphere isolate from *Cajanus cajan*	Gyaneshwar et al., 1999
E. a (pBSK-Gm)	*E. asburiae* PSI3 containing pBSK-Gm plasmid; Ap^r, Gm^r	This study
E. a (pBR322-Gm)	*E. asburiae* PSI3 containing pBSK-Gm plasmid; Ap^r, Gm^r, Tc^r	This study
E. a (pACYC184)	*E. asburiae* PSI3 containing pACYC184 plasmid; Cm^r, Tc^r	This study
E. a (pTOL)	*E. asburiae* PSI3 containing pTOL*gfp*mut3b plasmid; Km^r	This study
E. a (pGM160)	*E. asburiae* PSI3 containing pGM160 plasmid; Gm^r	This study
P. putida BBC443	*P. putida* SM1443 with TOL*gfp*mut3b plasmid; Km^r	Christensen et al., 1998
Plasmids used		
pBluescript/SK	Cloning vector for *E. coli*; Ap^r	Sambrook and Russell, 2001
pBSK-Gm	Cloning vector for *E. coli* containing gentamycin resistance marker gene; Ap^r, Gm^r	This study
pBR322	Sub cloning vector for *E. coli*; Ap^r, Tc^r	Sambrook and Russell, 2001
pBR322-Gm	Sub cloning vector for *E. coli* containing gentamycin resistance marker gene; Ap^r, Gm^r, Tc^r	This study
pGM160	Shuttle vector for streptomycetes and *E. coli*	Muth et al., 1989
pTOL*gfp*mut3b	pTOL derivative containing *gfp* under *lac* promoter and kanamycin resistance gene; Km^r	Christensen et al., 1998
pACYC184	Cloning vector for *E. coli*; Cm^r, Tc^r	Chang and Cohen, 1978

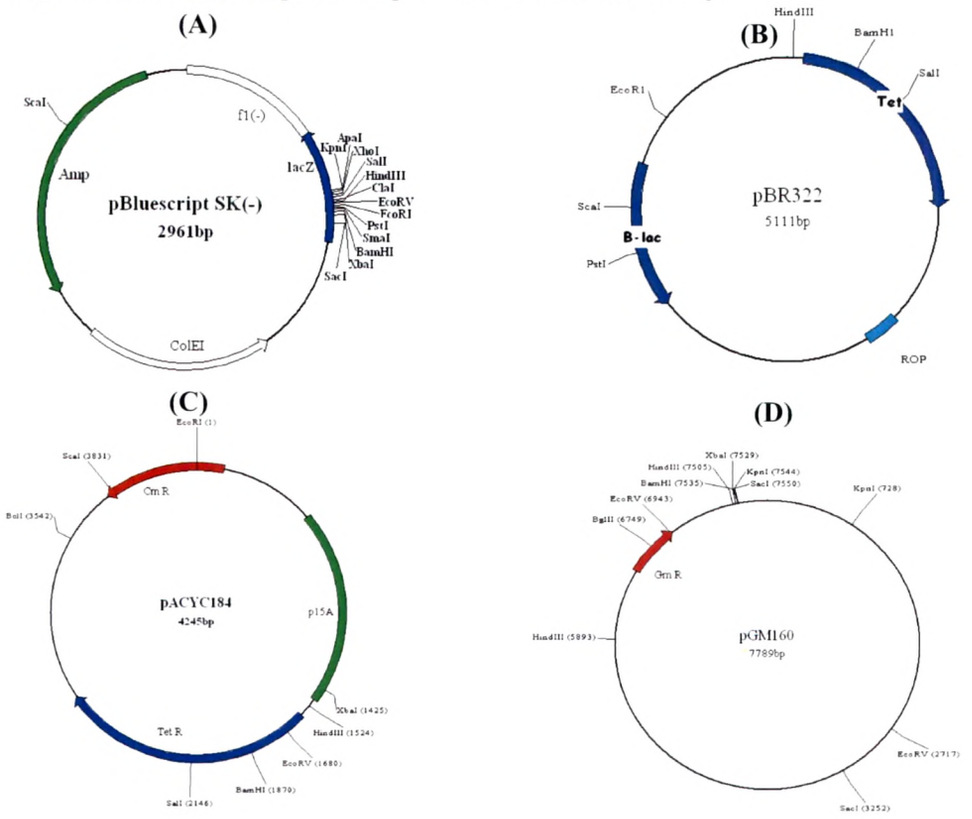
Ap= Ampicillin; Km= Kanamycin; Tc = Tetracycline; Gm= Gentamycin; Cm= Chloramphenicol; r= resistance

Pseudomonas putida SM1443 was a generous gift from Dr. **Soren molin**, Department of Microbiology, The Technical University of Denmark, Denmark

3.2.2 Description of plasmids used

pBluescript/SK is a high copy number cloning vector containing ColEI origin of replication. It has unique and broad multiple cloning site and direct selection of recombinant plasmids based on blue-white selection strategy (**Fig 3.1 A**). **pBR322** is a medium/ low copy number containing ColEI based ROP origin of replication. It has tetracycline and ampicillin resistance gene (**Fig 3.1 B**). **pACYC184** is low copy number cloning vector containing p15A origin of replication which allow it to coexist with ColEI based vectors (**Fig 3.1 C**). **pGM160** is a shuttle vector used for mutagenesis in streptomyces and is a source of gentamycin resistance gene for pBSK-Gm and pBR322-Gm (**Fig 3.1 D**). **pTOL*gfp*mut3b** is a 117Kb large self mobilisable plasmid carrying genes for degradation of xenobiotic compounds such as toluene, xylene, benzoates etc. having oriV origin of replication. It has been modified for kanamycin resistance gene and green fluorescence due to green fluorescence protein (GFP) cloned under modified *lac* promoter which is constitutive in nature (Christensen et al., 1998).

Fig 3.1 Restriction maps of the plasmids used in this study.



3.2.3 Media and Culture Conditions

E. coli and *E. asburiae* PSI3 were grown and maintained on luria broth and luria agar respectively. Both the cultures were grown at 37°C, for growth in liquid medium and shaking was provided at 200 rpm. For plasmid bearing transformants appropriate rich media were supplemented with kanamycin (25 μg ml^{-1}), tetracycline (30 μg ml^{-1}) chloramphenicol (20 μg ml^{-1}), ampicillin (50 μg ml^{-1}) and gentamycin (40μg ml^{-1}) (Sambrook and Russell, 2001) which was reduced to $1/4^{th}$ concentration when grown in minimal media. *Pseudomonas putida* SM1443 was maintained on pseudomonas agar and grown on luria broth at 30°C. Inoculum was prepared as described in Chapter 2 section 2.2.2.1.

3.2.4 Growth kinetics, acidification and solubilisation of RP

Rock phosphate (RP) solubilization experiments using Senegal RP (composition as given in Sharma et al., 2005) as the sole P-source were carried out in minimal medium as described earlier (Gyaneshwar et al., 1999) on agar plates as well as in liquid media containing 100mM Tris-Cl, pH 8.0 and 100mM glucose. 3μl of the inoculum was spotted on the Tris minimal solid medium. The P solubilization by acid production was determined by zone of pH reduction (change in colour from yellow to red) around the colony.

For experiments in liquid media, methyl red and agar were omitted. *E. asburiae* PSI3 strains containing different plasmids were grown on media containing RP alone and in the presence of 100μM of KH_2PO_4 as P source. In growth experiments, 10mM of KH_2PO_4 was used as P sufficient conditions. Aliquots taken at regular intervals were analyzed for $O.D._{600}$, and pH. Culture filtrate obtained after centrifugation at 9200 x *g* for 5min. was checked for P released as estimated by the ascorbate method (Ames, 1964).

3.2.5 Physiological parameters

The physiological parameters like specific growth rate, total glucose consumed and biomass yield (as described by Chao and Liao, 1993), organic acid yield and soluble P total P^{-1} (%) were calculated as described below.

3.2.5.1 Specific growth rate (h^{-1})

$$k = \frac{(Log_{10}Nt_2 - Log_{10}Nt_1) \times 2.303}{(t_2 - t_1)\ (h)} \quad \text{where,}$$

Nt_1 and Nt_2 are the number of cells at time t_1 and t_2 respectively and (t_2-t_1) is the corresponding time interval in h. The number of cells was calculated from $O.D._{600nm}$ using the correlation 1 $O.D._{600nm} = 8.3 \times 10^9$ cell ml^{-1} (Zheng et al., 2005) since the *E. asburiae* PSI3 belongs to enterobacteriaceae family.

3.2.5.2 Dry cell weight g L^{-1}

$$\textbf{dcw} = O.D._{600} \times 0.69$$

Where 0.69 is a factor derived from the subtracting wet weight from dry weight in particular media conditions.

3.2.5.3 Organic acid yield

$$\text{Organic acid yield} = \frac{\text{Amount of organic acid produced (g } L^{-1})}{\text{Total glucose consumed (g } L^{-1}) \times \text{dcw}} \quad \text{where,}$$

The amount of total glucose consumed was obtained by deducting the value of residual glucose concentration from the initial glucose concentration supplied in the medium.

The statistical analysis of all the parameters was done using Microsoft Excel (XP).

3.2.6 Molecular biology techniques

Plasmid extraction using the CTAB method, agarose gel electrophoresis, preparation of competent cells of *E. coli* and transformation were carried out as described by Sambrook and Russell (2001). Restriction enzymes, T4 DNA ligase from Bangalore Genei Private Ltd., India were used according to the manufacturer's instructions.

3.2.7 Construction of pBSK-Gm and pBR322-Gm

E. asburiae PSI3 is naturally resistant to ampicillin; hence, gentamycin resistance cassette was incorporated in to pBluescript/SK. To compare the effect of two antibiotic selection markers on maintenance of same plasmid, pBR322 was also modified so as to contain gentamycin resistance. pBSK and pBR322 were linearized with *Hin*dIII and ligated to 1,612 bp *aacCI* fragment containing gentamycin resistance marker from plasmid pGM160, released by HindIII digestion. Ligation with the gentamycin fragment gave rise to pBSK-Gm (4573 bp), pBR322-Gm (5973 bp) respectively.

3.2.8 Transformation of *E. asburiae* PSI3 by electroporation

Transformation of plasmids in *E. asburiae* PSI3 was carried out by electroporation with modifications (Sambrook and Russell, 2001). *E. asburiae* PSI3 was grown overnight on LB at 37°C with 200 rpm of shaking. Overnight grown cells were inoculated in 100ml of SOC medium containing (for 1L) tryptone 20g, Yeast extract 5g, NaCl 0.5g and KCl 2.5mM. pH of the media was adjusted to 7.0 with 5N NaOH and autoclaved at 15 psi for 20 min. The media was cooled to 42°C and 10mMof $MgCl_2$ and 20mM of glucose was added. The cells were allowed to grow at 37°C till $O.D._{600}$ comes to 0.6. The cells were pelleted at 9200 x g for 3 min. at 4°C, washed several times with decreasing volume of chilled sterile double distilled water. The cells were finally washed with chilled 300mM sucrose twice before suspending in 500µl of sucrose. 40µl of cells were taken in chilled electroporation quvette and mixed with approximately 300ng of DNA and subjected to electroporation. The electroporation parameters were same as *E. coli* (Sambrook and Russell, 2001).

3.2.9 Conjugative transfer of pTOL*gfp*mut3b to *E. asburiae* PSI3

TOL*gfp*mut3b plasmid was transferred from *Pseudomonas putida* SM1443 to *E. asburiae* PSI3 by biparental mating. *Pseudomonas putida* SM1443 and *E. asburiae* PSI3 were grown overnight at 30°C and 37°C respectively. Both donor and recipient cultures were mixed in 1:1 proportion and collected on 0.22 µm pore size nylon-66 membrane filter disc which was then placed on an LB agar plate, incubated at 30°C for 16 h. Cells were then scraped off the disc and suspended into normal saline. The transconjugants were obtained by plating on lactose agar containing kanamycin (25

μg ml^{-1}) as *P. putida* SM1443 does not utilize lactose as a sole carbon source. The transconjugants were further confirmed by growing them on Bushnell Haas (BH) minimal medium containing sodium benzoate as the sole carbon source under kanamycin (25 μg ml^{-1}) selection.

3.2.10 Determining the stability of the plasmid pACYC184 in *E. asburiae* PSI3

E. asburiae PSI3 containing pACYC184 was grown overnight in LB containing chloramphenicol (20μg ml^{-1}). Culture was washed twice with 0.85% saline solution and inoculated in minimal medium used for RP solubilization studies, aliquots were taken at different time intervals and colony forming units were counted on LB agar with and without the appropriate antibiotic after overnight incubation at 37°C.

3.3 RESULTS

Four plasmid vectors and a derivative of a natural plasmid coding for toluene degradation, pTOL*gfp*mut3b, were used in this study to monitor their effect on the MPS phenotype of *E. asburiae* PSI3. The plasmids differed in their copy number, size, and/or antibiotic resistance markers (**Table 3.2**).

Table 3.2: Plasmids used in this study and their relevant properties.

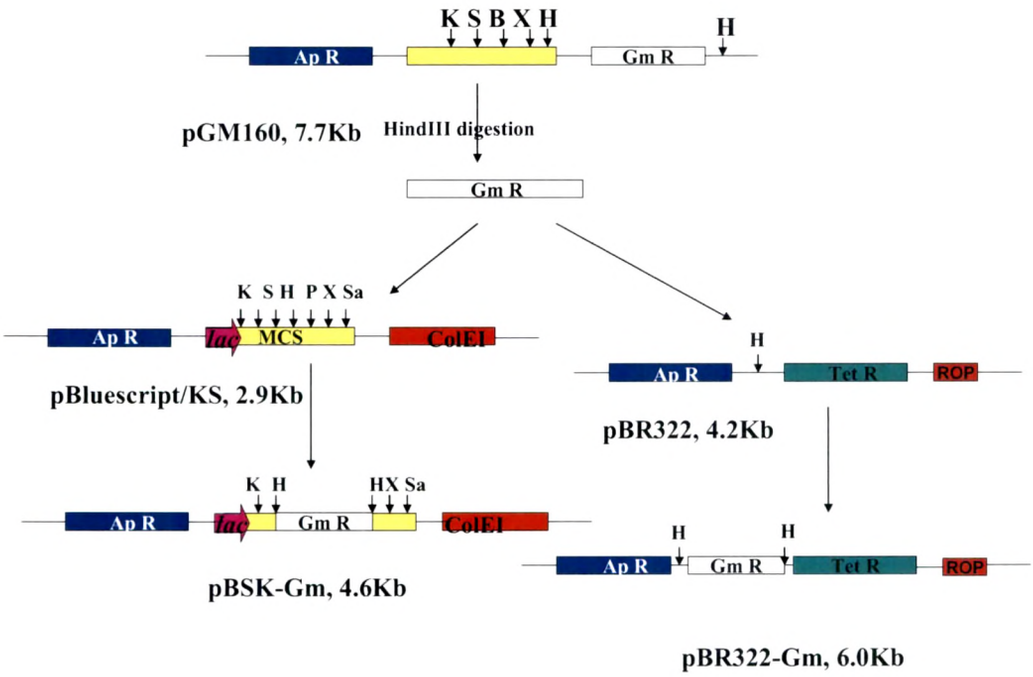
Plasmid	Antibiotic marker	Size (bp)	Copy No. in *E. coli*	Origin of replication	Reference
pBSK-Gm	Ap^r, Gm^r	4,570	500-700	Col E1	Sambrook and Russell, 2001 and this work
pBR322-Gm	Ap^r, Gm^r, Tc^r	5,973	15-20	Col E1	Sambrook and Russell, 2001 and this work
pGM160	Ap^r, Gm^r	7,789	Not known	Col E1	Muth et al., 1989
pACYC184	Cm^r, Tc^r	4,244	10-12	p15A	Chang and Cohen, 1978
pTOL*gfp*mut3b	Km^r	~117,000	1	oriV	Christensen et al., 1998

Ap= Ampicillin; Km= Kanamycin; Tc = Tetracycline; Gm= Gentamycin; Cm= Chloramphenicol; r=resistance

3.3.1 Construction of pBSK-Gm and pBR322-Gm

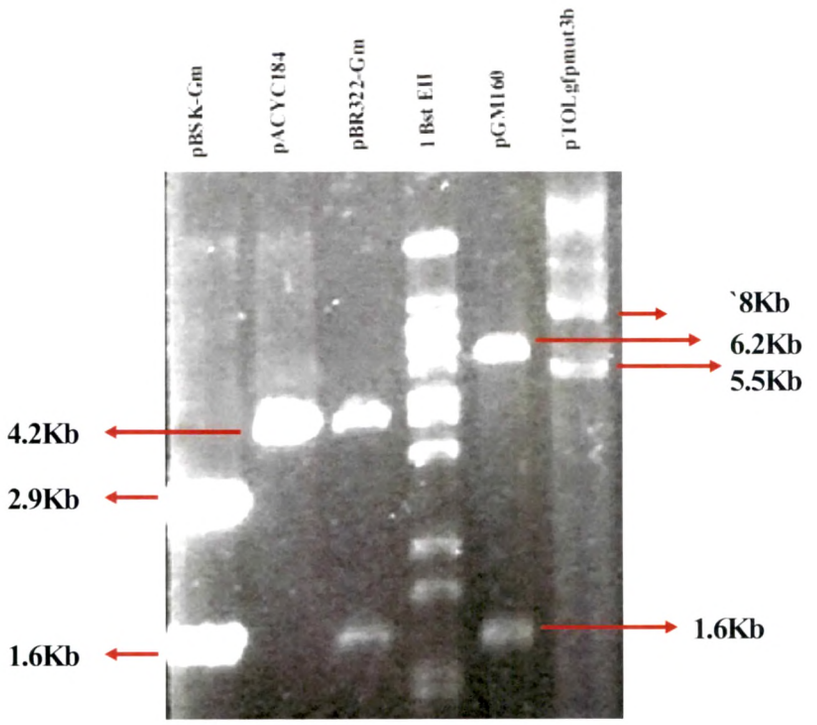
Plasmids pBSK-Gm and pBR322-Gm were constructed as mentioned in Materials and methods. The schematic representation of the cloning procedures is depicted in **Fig. 3.2, with Fig. 3.4 A and B** showing map of pBR322-Gm and pBSK-Gm. Plasmids were confirmed based on restriction digestion pattern along with digested pACYC184, pGM160 and pTOL*gfp*mut3b **(Fig. 3.3).** Restriction digestion of pBSK-Gm and pBR322-Gm with HindIII showed release of 1.6Kb *aaaCI* fragment containing gentamycin resistance marker. Similar release of 1.6Kb was also seen with pGM160 digested with HindIII.

Fig 3.2: Schematic representation of construction of plasmid pBSK-Gm and pBR322-Gm



Key: E=EcoRI; B=BamHI; K= KpnI; H=HindIII; S=SalI; P=PstI; X=XbaI; Sa=SacI P= promoter; RBS =Ribosomal binding site; Tet R= tetracycline resistance gene; Ap R= ampicillin resistance gene; GmR=gentamycin resistance gene; ROP= origin of Replication, CoLEI= origin of Replication; MCS= multiple cloning site.

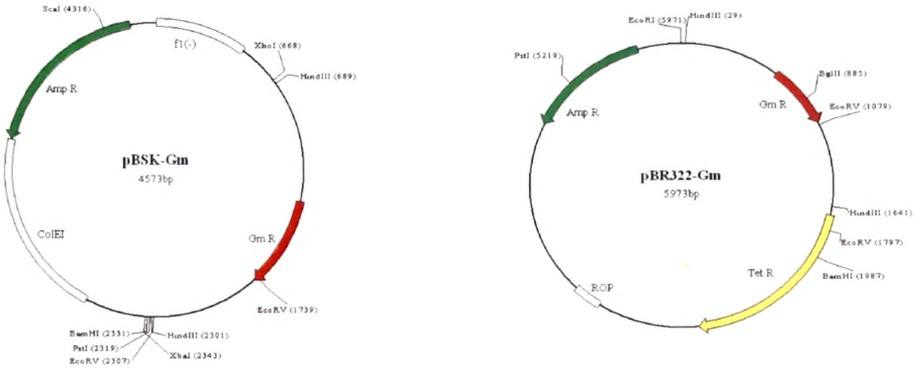
Fig 3.3: Restriction endonuclease analysis of pBSK-Gm and pBR322-Gm



Lane 1: pBSK-Gm digested with HindII; Lane 2: pACYC184 digested with HindIII; Lane 3: pBR322-Gm digested with HindIII; Lane 4: Molecular Weight Marker (MWM)-Lambda DNA cut with BstEII; Lane 6: pGM160 digested with HindIII; Lane 7: pTOL*gfp*mut3b digested with HindIII.

Fig 3.4: Restriction map of (A) pBSK-Gm and (B) pBR322-Gm

(A) **(B)**



3.3.2 Acid production of *E. asburiae* PSI3 containing different plasmids

E. asburiae PSI3 strain containing pACYC184 plasmid showed red zone of acidification on buffered minimal media containing 100mM glucose as sole carbon source when grown on RP as a sole P source (**Fig. 3.5 E**) whereas *E. asburiae* PSI3 containing other plasmids did not show red zone of acidification (**Fig. 3.5 A, C**). Acid production or red zone was observed with all the transformants when supplemented with 10mM KH_2PO_4 in place of RP as the P source (**Fig. 3.5 B, D, F**).

3.3.3 Growth Kinetics, acidification and mineral phosphate solubilization in liquid media by *E. asburiae* PSI3 strains with different plasmids

E. asburiae PSI3 transformants *E. a* (pBSKS-Gm), *E. a* (pBR322-Gm), *E. a* (pGM160) and *E. a* (pTOL*gfp*mut3b) did not show any growth as well as acidification upto 48 h when grown on 100mM glucose-RP minimal medium (**Fig 3.6 A and B**). However, *E. asburiae E. a* (pACYC184) showed growth and acidification comparable to untransformed strain (**Fig 3.6 A and B**). Culture supernatant of *E. asburiae E. a* (pACYC184) showed 860 ± 89 µM soluble P after 48 h which was similar to the soluble P released by *E. asburiae* PSI3 without any plasmid (786 ± 69 µM) (**Table 3.3**).

Table 3.3: P solubilization by *E. asburiae* PSI3 containing various plasmids.

Strains	Soluble P (mM)	
	0 h	48 h
E. asburiae PSI3	ND*	0.786 ± 0.069
E. a (pBSK-Gm)	ND	ND
E. a (pBR322-Gm)	ND	ND
E. a (pGM160)	ND	ND
E. a (pTOL)	ND	ND
E. a (pACYC184)	ND	0.860 ± 0.089

Results are expressed as Mean ± SD of nine independent experiments; *ND- Not Detectable

Fig 3.5: Acidification of methyl red plates by *E. asburiae* PSI3 containing different plasmids on Tris-Cl containing minimal media with RP (A,C,E) and 10mM KH_2PO_4 (B,D,F) as a sole P source (1) *E. a* (pBSK-Gm) (2) *E. a* (pBR322-Gm) (3) *E. a* (pGM160) (4) *E. a* (pTOL) (5) *E. a* (pACYC184).

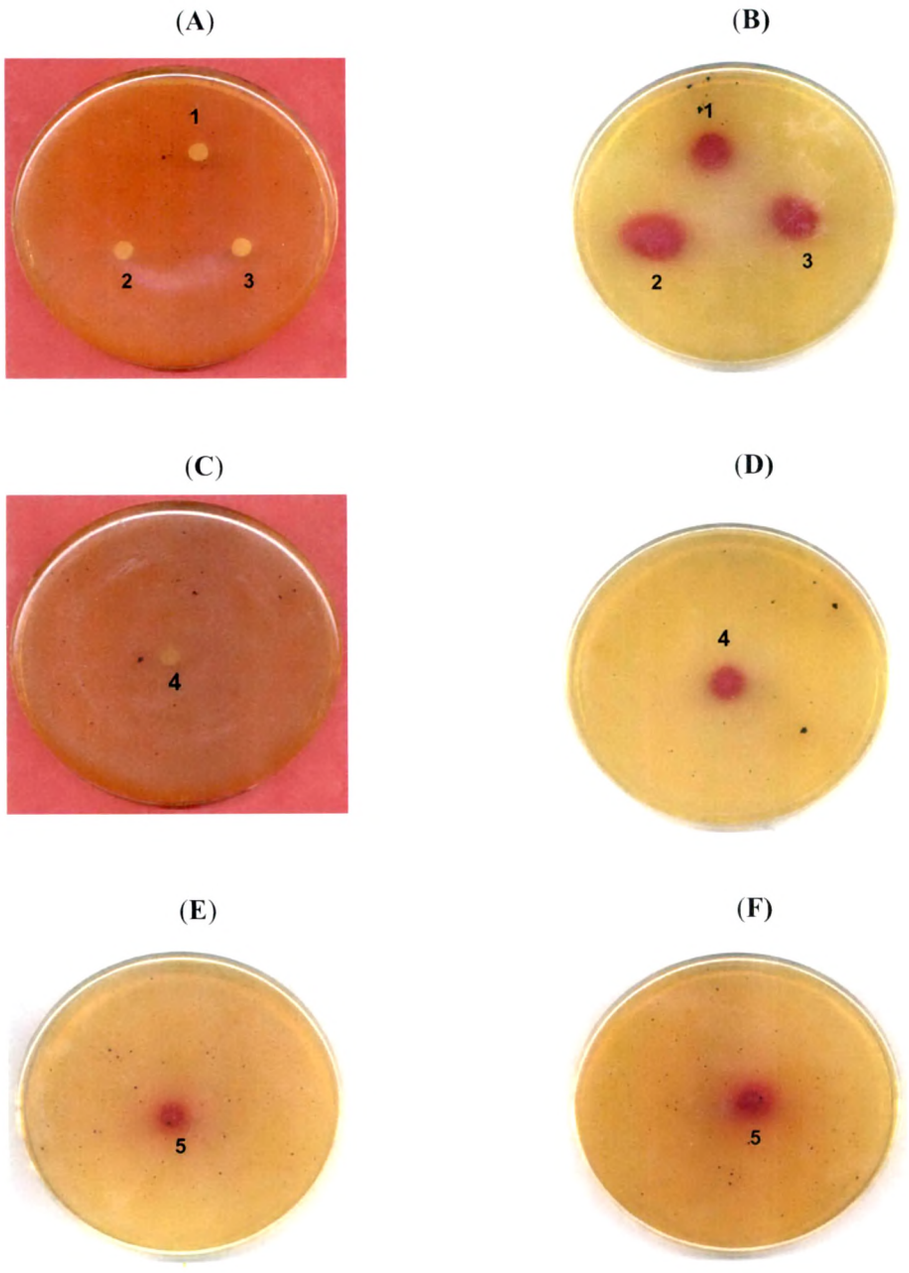
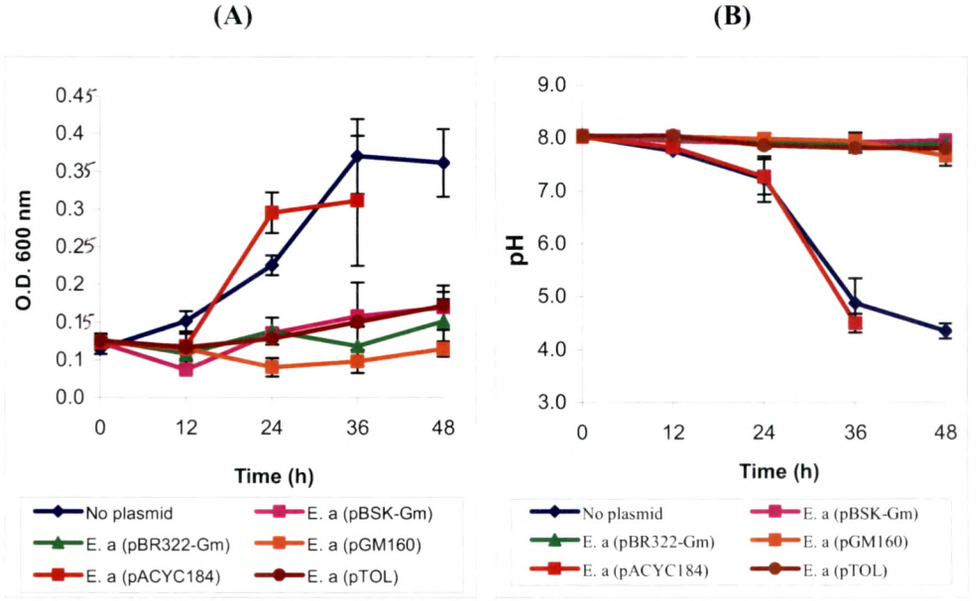


Fig. 3.6: Growth profile (A) and acidification pattern (B) of *E. asburiae* PSI3 containing different plasmids on Tris RP minimal media

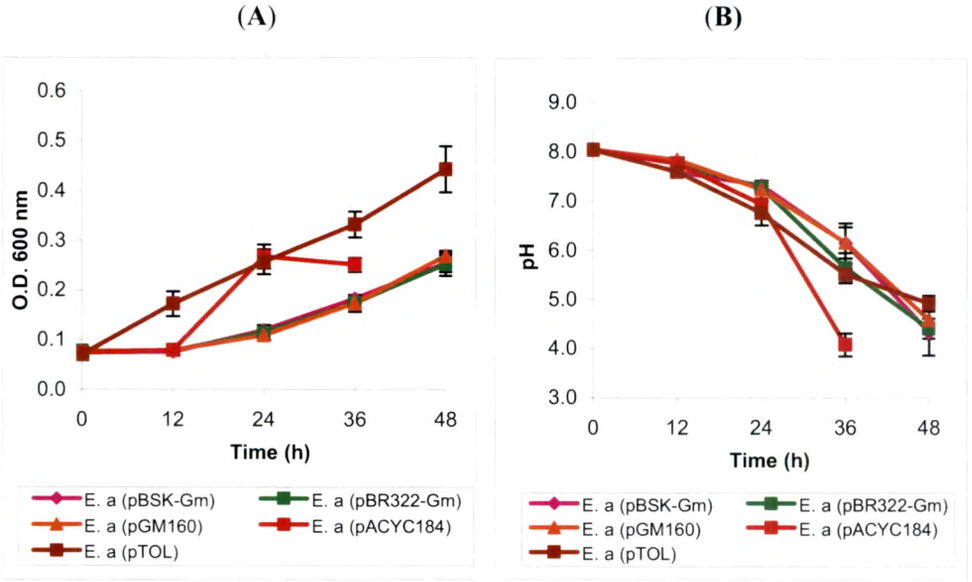


(A) Growth profile and (B) acidification pattern of *E. asburiae* PSI3 WT and containing plasmids pACYC184, pBSK-Gm, pBR322-Gm, pGM160 and pTOL*gfp*mut3b

3.3.4 Growth Kinetics and acidification pattern in liquid media by *E. asburiae* PSI3 strains with different plasmids on Tris minimal media containing soluble P.

In order to determine the role of phosphate availability on the acidification, *E. asburiae* PSI3 and its transformants containing different plasmids were grown in above-mentioned minimal medium with excess of P (10mM KH_2PO_4) in place of RP. Growth of *E. asburiae* PSI3 without any plasmid and *E. a* (pACYC184) was comparable (**Fig 3.7 A**). In contrast, in RP medium, *E. asburiae* PSI3 with all other plasmids could grow albeit slower than the native strain. Interestingly, all transformants could drop pH to below 5.0 by 48 h (**Fig 3.7 B**).

Fig. 3.7: Growth profile (A) and acidification pattern (B) of *E. asburiae* PSI3 containing different plasmids on Tris minimal media containing 10mM of KH_2PO_4



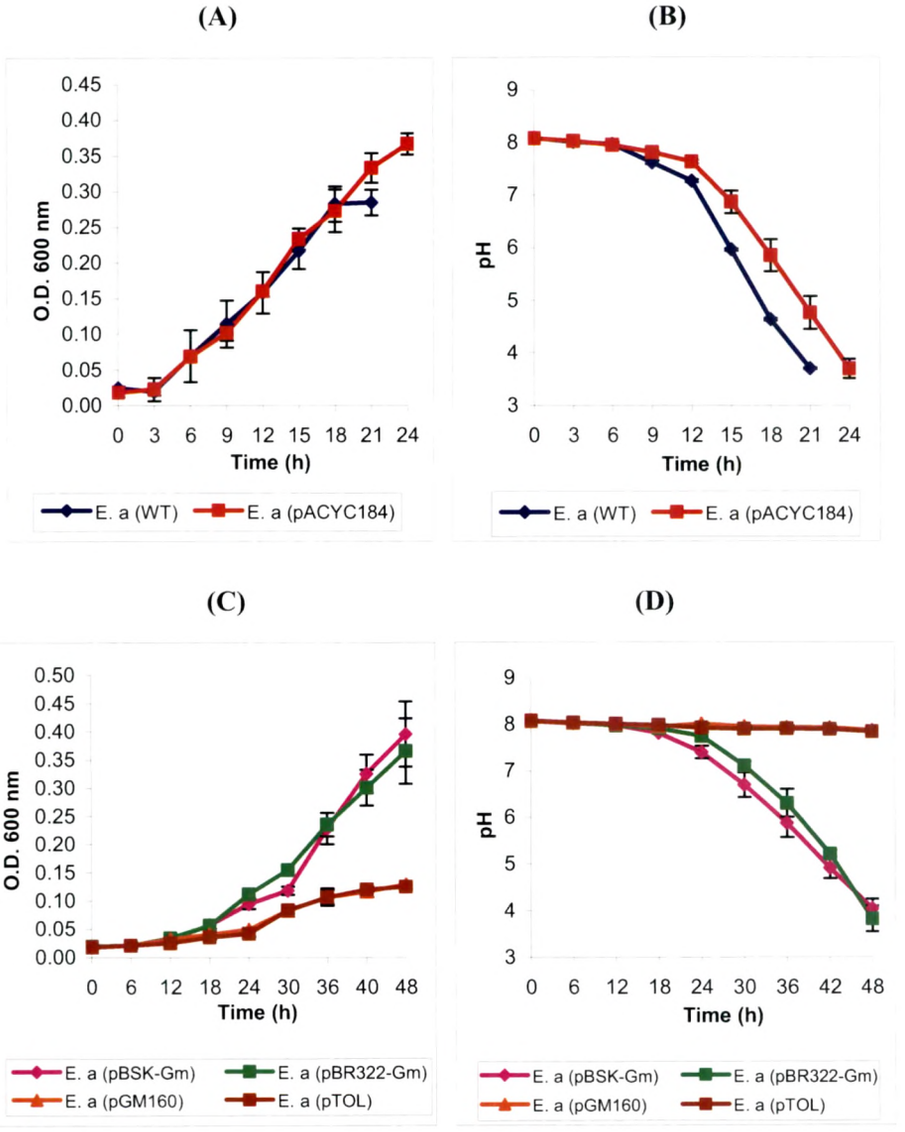
(A) Growth profile and (B) acidification pattern of *E. asburiae* containing plasmids pACYC184, pBSK-Gm, pBR322-Gm, pGM160 and pTOL*gfp*mut3b

3.3.5 Growth Kinetics, acidification pattern and P-solubilisation in liquid media by *E. asburiae* PSI3 strains with different plasmids on Tris RP media containing limited P.

In order to determine the role of phosphate availability on the acidification and P-solubilisation, *E. asburiae* PSI3 and its transformants containing different plasmids were grown in above mentioned minimal medium supplemented with 100: µM KH_2PO_4 of P along with RP. O.D. $_{600}$ of *E. asburiae* PSI3 WT and its transformants were less than 0.4 and were plotted with different time points (**Fig. 3.8 A and C).** Specific growth rates of *E. asburiae* PSI3 harboring plasmids pACYC184, pBSK-Gm and pBR322-Gm were found to be ~2 fold less than that of *E. a* (WT) (**Table 3.4**). Plasmids pGM160 and pTOL*gfpmut3b* further decreased the specific growth rate by ~5 fold compared to wild type (**Table 3.4**). *E. a* (pACYC184) could acidify the media below 5.0 after 24h, comparable to untransformed (**Fig. 3.8 B**). *E. a* (pBSK-Gm) and *E. a* (pBB322-Gm) could also acidify the medium to below pH 5.0 but only after 48h (**Fig. 3.8 D**). *E. asburiae* PSI3 harboring plasmids pGM160 and pTOLgfpmut3b

could not acidify the medium even after 48h supported by total glucose consumed (**Table 3.4**).

Fig. 3.8: Growth profile and acidification pattern of *E. asburiae* PSI3 containing different plasmids on Tris minimal media containing 100µM of KH_2PO_4



(A) and (B) Growth profile and acidification pattern of *E. asburiae* PSI3 WT and containing plasmid pACYC184; (C) and (D)) Growth profile and acidification pattern of *E. asburiae* PSI3 containing plasmids pBSK-Gm, pBR322-Gm, pGM160 and pTOL*gfp*mut3b

Table 3.4: Physiological variables of *E. asburiae* PSI3 and its various transformants grown on Tris RP medium with 100μM of KH_2PO_4 and 100mM glucose.

E. asburiae PSI3 Transformants	Sp. growth rate k $(h^{-1})^a$	Glucose consumed $(g\ L^{-1})^b$	Dry cell weight $(g\ L^{-1})^c$
E. a (WT)	0.15 ± 0.05	12.9 ± 0.19	0.20 ± 0.01
E. a (pACYC184)	0.07 ± 0.02	11.2 ± 0.88	0.25 ± 0.01
E. a (pBSK-Gm)	0.08 ± 0.01	9.8 ± 1.32	0.27 ± 0.04
E. a (pBR322-Gm)	0.06 ± 0.02	13.6 ± 1.04	0.25 ± 0.04
E. a (pGM160)	0.03 ± 0.01	3.20 ± 0.72	0.09 ± 0.00
E. a (pTOL)	0.04 ± 0.02	3.01 ± 0.87	0.09 ± 0.01

Values given are Mean ± SD of six independent experiments. ND: not detected; a: Specific growth rate (k) was determined from mid log phase of each experiment. b: Total glucose consumed was determined at the time of pH drop below 5.0; c: Dry cell mass calculated at the $O.D._{600}$ at the time of pH drop.

3.3.6 P-solubilisation by *E. asburiae* PSI3 containing different plasmids on Tris RP media containing limited P.

E. a (WT) released highest i.e 92.8 ± 8.2 mg L^{-1} P compared to other transformants supported by organic acid yield. *E. a* (pACYC184) and *E. a* (pBR322-Gm) released lesser P, 87.5 ± 2.1 mg L^{-1} and 85.5 ± 2.1 mg L^{-1}, respectively, compared to untransformed. However, *E. a* (pBSK-Gm) showed slightly higher organic acid yield compared to *E. a* (pACYC184) and *E. a* (pBR322-Gm) but yet could release ~1.3 fold less P, 66.2 ± 5.6 mg L^{-1} than the latter two. *E. a* (pGM160) and *E. a* (pTOL) did not release P (**Table 3.5**).

3.3.7 Stability of pACYC184 plasmid in *E. asburiae* PSI3 grown in the absence chloramphenicol

To find out the stability of pACYC184 in *E. asburiae* PSI3 in the absence of the selective pressure, *E. a* (pACYC184) was grown in the absence of chloramphenicol and cells were counted on LB agar with and without the antibiotic. As seen in **Table 3.6**, there was 99% loss of plasmid within 24 h.

Table 3.5: Organic acid yield and phosphate release of *E. asburiae* PSI3 and its various transformants grown on Tris RP medium with 100μM of KH_2PO_4 and 100mM glucose.

E. asburiae PSI3 transformants	Organic acid yield (g.g dcw^{-1})	Phosphate released (mg L^{-1})	Soluble P total P^{-1} (%)[a]
E. a (WT)	3.65 ± 0.06	92.6 ± 4.8	582.1 ± 30.0
E. a (pACYC184)	2.59 ± .059	87.0 ± 3.2	547.2 ± 20.1
E. a (pBSK-Gm)	2.94 ± 0.66	66.3 ± 4.1	416.7 ± 25.6
E. a (pBR322-Gm)	2.69 ± 0.17	85.1 ± 3.2	535.4 ± 20.4
E. a (pGM160)	ND	ND	ND
E. a (pTOL)	ND	ND	ND

Values given are Mean ± SD of six independent experiments. ND: not detected;
a: % of Soluble P (mg L^{-1}) released per g of total P in RP

Table 3.6: Stability of pACYC184 plasmid in *E. asburiae* PSI3 in absence of antibiotic selection

Time (h)	pH of the medium	Viable cells of *E. asburiae* PSI3 (Cfu ml^{-1})		Cells with plasmid (%)
		LB	LB + Cm	
0	8.02 ± 0.00	(4.10 ± 1.66) x 10^5	(4.57±1.35) x 10^5	111.5
24	5.73 ± 0.78	(3.08 ± 0.34) x 10^8	(3.71± 1.96) x 10^6	1.2
48	3.56 ± 0.09	(6.23 ± 1.25) x 10^8	(6.54± 1.94) x 10^5	0.1

Results are represented as Mean ± SD of nine independent experiments.

3.4 DISCUSSION

Cloning and characterization of genes conferring P-solubilizing ability (Goldstein and Liu, 1987; Liu et al., 1992; Babu-Khan et al., 1995; Gyaneshwar et al., 1998b; Krishnaraj and Goldstein, 2001; Kim et al., 2003) has provided the basis for imparting MPS phenotype to other PGPR strains (Rodriguez et al., 2000). Conversely, PGPR traits could also be incorporated into efficient PSMs. Since *E. asburiae* PSI3 is an efficient PSM, it is important to understand the consequences of maintenance of foreign DNA on P-solubilizing ability of the microbe.

Present study showed the presence of pBSK-Gm, pBR322-Gm, pGM160 and pTOL*gfp*mut3b plasmids resulted in diminished inherent MPS phenotype of *E. asburiae* PSI3 on RP under buffered conditions. Loss of MPS phenotype under buffered RP medium indicates the presence of plasmids could reduce the P-solubilization efficacy of *E. asburiae* PSI3 in soils. It is noteworthy that in spite of the simple uptake- independent pathway for gluconic acid production, it is yet subject to metabolic load by the maintenance of plasmids. The metabolic burden caused by the plasmids was more pronounced under conditions when growth was limited by P starvation. When limited (0.1mM) P was supplemented with RP, plasmid load was more pronounced with pGM160 and pTOL*gfp*mut3b plasmids which are supported from specific growth rate, gluconic acid yield and the time for acidification (**Table 3.4**). For instance, *Azotobacter vinelandii* transformants harboring broad host range plasmids could not grow well under conditions of iron stress (Glick et al., 1985). The presence of pRKLACC reduced the growth rate and IAA synthesis by *Azospirillum brasilense* in minimal medium but not in rich medium (Holguin and Glick, 2001).

Significant proportion of the metabolic burden imposed by plasmids that do not express foreign genes comes from the expression of plasmid-borne resistance genes and not necessarily the increased plasmid size (Rozkov et al., 2004). Protein expressed by antibiotic resistance genes could amount to 18 % of total cellular protein (for kanamycin resistance), thus imposing increased ATP requirements. A weaker expressed marker gene e.g. tetracycline resistance, exerts lower burden than a stronger counterpart (Lee and Edlin, 1985). The plasmid vectors pBSKS-Gm and pBR322-Gm and pGM160 were maintained during our experiments using a common selective marker i.e. gentamycin. The effect of metabolic load on *E. asburiae* PSI3 was seen with each of above three plasmid vectors but not with pACYC184 under RP conditions. The efficiency of P release with pACYC184 was less compared to untransformed but similar to other pBR322-Gm, however time taken for acidification was ~2 fold less compared to pBR322-Gm. Since the size and copy number of pACYC184 is comparable with pBR322-Gm, it is possible that the difference in the metabolic burden caused by the two is due to the presence of different antibiotic resistance genes. *E. asburiae* PSI3 harboring pBSK-Gm showed similar acidification time to pBR322-Gm but delayed compared pACYC184, showed less efficiency in P

release. pBSK-Gm however have similar antibiotic resistance marker as of pBR322-Gm but have higher copy number indicating apart from antibiotic marker copy number could also cause metabolic burden. *E. asburiae* PSI3 containing pGM160 and TOL*gfp*mut3b did not secrete gluconic acid and therefore release P even after 48h indicating, contrary to Rozkov et al., (2004), plasmid size also could play a role in imparting metabolic burden i.e. energy intensive maintenance of plasmid in the cell.

While naturally occurring plasmids are often stably maintained within the population in the absence of selection pressure, many cloning vectors are lost from the bacterial population when the appropriate selection is not exerted (Mcloughlin, 1994). If plasmid loss is found in absence of selection in laboratory media, it is very likely that it will be lost in rhizosphere environment as well (Van Der Bij et al., 1996). Plasmid pACYC184 is not stable in *E. asburiae* PSI3 in the absence of antibiotic selection which deters its use for gene expression experiments although it did not impart metabolic load on the bacterium.

The TOL*gfp*mut3b plasmid is a derivative of the TOL plasmid which is natural large catabolic plasmid conferring benzoate degradation (Christensen et al., 1998). TOL*gfp*mut3b additionally carries a constitutively expressed fluorescent reporter tag and a kanamycin resistance marker gene. Other large catabolic plasmids such as pJP4 have been previously shown to affect competitiveness of rhizobacteria (Eisenlohr and Baron, 2003). Loss of MPS phenotype of *E. asburiae* PSI3 due to maintenance of pTOL*gfp*mut3b raises an important aspect of the efficacy of PSMs in the field conditions. Natural plasmids similar to TOL are known to horizontally transfer across a variety of bacteria by conjugation in the soil (Ramos-Gonzalez et al., 1991). Lateral transfer of such plasmids into PSMs could interfere with their MPS phenotype. Although other PGPR traits including nitrogen fixation (Glick et al., 1985), indole acetic acid (IAA) production (Holguin and Glick, 2001), environmental fitness in soils (De Leij et al., 1998) and competitiveness in rhizosphere colonization (Eisenlohr and Baron, 2003) have been shown to be negatively affected by plasmid inflicted metabolic load, this is to our knowledge the first report on its effect on P-solubilizing ability.