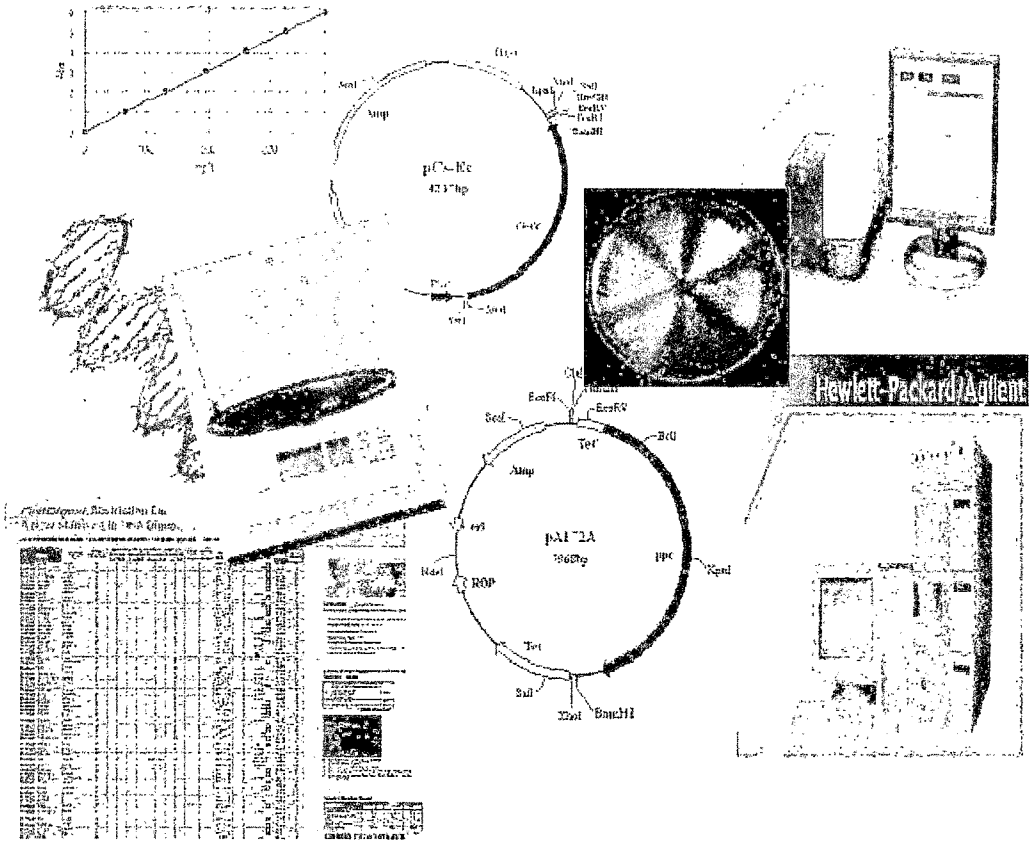


CHAPTER 2

Materials and Methods

The true method of knowledge is experiment."
-William Blake

2.1: Bacterial strains / Plasmids

All the wild type and genetically modified *E. coli* and *Pseudomonas* strains are listed in **Table 2.1 and 2.2**. The plasmids used in the present study and their restriction maps are given in **Table 2.3** and **Fig. 2.1**. *E. coli* JM101 was used for all the standard molecular biology experiments wherever required. The *ppc* mutant strain, *E. coli* JWK3928, was a generous gift from NARA Institute of Science and Technology (Japan) due to kind recommendation of Prof. H. Mori. The *glt*A (citrate synthase gene) mutant of *E. coli* was obtained from *E. coli* Genetic Stock Center (CGSC), Yale University, U.S.A.

Bacterial Strains	Genotype	Reference
E. coli JM101	F′ *traD36 proA*$^{+}$*B*$^{+}$ *lacI*^{q} Δ*(lacZ) M15/* Δ*(lac-proAB) glnV thi*	Sambrook and Russell, 2001
E. coli JWK3928	*lacI*^{q} *rrnBT14 DlacZWJ16 hsdR514 DaraBADAH33,DrhaBADLD78* ***ppc****::Km*; Km^{r}	Peng et al., 2004
E. coli W620	CGSC 4278 - *glnV44* ***gltA6*** *galK30 LAM-pyrD36 relA1 rpsL129 thi*$^{-1}$; Str^{r}	*E. coli* Genetic Stock Center
E. coli S17.1	*thi pro hsdR recA RP4-2* (Tet::Mu) (Km::Tn7); Tmp^{r}	Simon et al., 1983
JM101 (pAB2)	*E. coli* JM101 with pAB2 plasmid; Ap^{r}, Tc^{r}	Ch.3
JM101 (pAB3)	*E. coli* JM101 with pAB3 plasmid; Ap^{r}, Tc^{r}	Ch.3
JM101 (pAB4)	*E. coli* JM101 with pAB4 plasmid; Ap^{r}, Tc^{r}	Ch.3
JM101 (pAB7)	*E. coli* JM101 with pAB7 plasmid; Ap^{r}, Km^{r}	Ch.5
JM101 (pAB8)	*E. coli* JM101 with pAB8 plasmid; Ap^{r}, Km^{r}	Ch.5
JM101 (pAB9)	*E. coli* JM101 with pAB9 plasmid; Km^{r}	Ch.5
JM101 (pBBR1MCS-2)	*E. coli* JM101 with pBBR1MCS-2 plasmid; Km^{r}	Ch.5
JWK3928 (pAB2)	*E. coli* JWK3928 with pAB2 plasmid; Km^{r}, Ap^{r}, Tc^{r}	Ch.3
JWK3928 (pAB3)	*E. coli* JWK3928 with pAB3 plasmid; Km^{r}, Ap^{r}, Tc^{r}	Ch.3
JWK3928 (pAB4)	*E. coli* JWK3928 with pAB4 plasmid; Km^{r}, Ap^{r}, Tc^{r}	Ch.3
W620 (pAB7)	*E. coli* W620 with pAB7 plasmid; Str^{r}, Ap^{r}, Km^{r}	Ch.5
W620 (pAB8)	*E. coli* W620 with pAB8 plasmid; Str^{r}, Ap^{r}, Km^{r}	Ch.5
W620 (pAB9)	*E. coli* W620 with pAB9 plasmid; Str^{r}, Km^{r}	Ch.5
W620 (pBBR1MCS-2)	*E. coli* W620 with pBBR1MCS-2 plasmid; Str^{r}, Km^{r}	Ch.5
S17.1 (pAB3)	*E. coli* S17.1 with pAB3 plasmid; Tmp^{r}, Ap^{r}, Tc^{r}	Ch.7
S17.1 (pAB4)	*E. coli* S17.1 with pAB4 plasmid; Tmp^{r}, Ap^{r}, Tc^{r}	Ch.7
Enterobacter asburiae PSI3	Pigeon pea rhizosphere isolate *	Gyaneshwar et al., 1999

Table 2.1: List of *E. coli* strains used in the present study. Ap=Ampicillin; Km=Kanamycin; Tc=Kanamycin; Str^{r} =Streptomycin; Tmp =Trimethoprim; ^{r} = resistant. * *E. asburiae* PSI3 belonged to *Enterobactericiae* family and hence has been included in this table.

Bacterial Strains	Genotype/Relevant characteristics	Reference
P. fluorescens ATCC 13525	Wild Type	ATCC
P. putida KT2440	*hsdRl hsdM*+, Plasmid free derivative of *P. putida* mt-2	Franklin et al., 1981; Yuste et al ; 2006
P. putida BBC443	*P. putida* SM1443 with TOL*gfp*mzut3b plasmid; Km^r	Christensen et al., 1998
P. fluorescens-TOL	*P. fluorescens* 13525 with TOL*gfp*mut3b plasmid; Km^r	Ch.3
Pf (pAB3)	*P. fluorescens* 13525 with pAB3 plasmid; Tc^r	Ch.3, Ch.4
Pf (pAB4)	*P. fluorescens* 13525 with pAB4 plasmid; Tc^r	Ch.3, Ch.4
Pf (pAB7)	*P. fluorescens* 13525 with pAB7 plasmid; Km^r	Ch.5
Pf (pAB8)	*P. fluorescens* 13525 with pAB8 plasmid; Km^r	Ch.5
Pf (pAB9)	*P. fluorescens* 13525 with pAB9 plasmid; Km^r	Ch.5
Pf (pBBR1MCS-2)	*P. fluorescens* 13525 with pBBR1MCS-2 plasmid; Km^r	Ch.5
Pf (pAB37)	*P. fluorescens* 13525 with pAB3 and pAB7 plasmids; Tc^r, Km^r	Ch.6
Pf (pAB48)	*P. fluorescens* 13525 with pAB4 and pAB8 plasmids; Tc^r, Km^r	Ch.6
Pf (pAB39)	*P. fluorescens* 13525 with pAB3 and pAB9 plasmids; Tc^r, Km^r	Ch.6
Pf (pAB4 BBR1MCS-2)	*P. fluorescens* 13525 with pAB4 and pBBR1MCS-2; Tc^r, Km^r	Ch.6
Fp585 (pAB4)	Fp585 with pAB4 plasmid; Tc^r	Ch.7
Fp585 (pAB3)	Fp585 with pAB3 plasmid; Tc^r	Ch.7
Fp315 (pAB4)	Fp315 with pAB4 plasmid; Tc^r	Ch.7
Fp315 (pAB3)	Fp315 with pAB3 plasmid; Tc^r	Ch.7
P109 (pAB4)	P109 with pAB4 plasmid; Tc^r	Ch.7
P109 (pAB3)	P109 with pAB3 plasmid; Tc^r	Ch.7
Pseudomonas P4	Native P-solubilizing isolate of fluorescent pseudomonads	Ch.8

Native isolates	P-solubilizing ability	ACC Deaminase Activity	Reference
A48	$P+^{+++}$	ACC^{+++}	Ch.7
P109	$P+^{+++}$	ACC^{-}	Ch.7
Fp315	P+	ACC^{-}	Ch.7
Fp366	P+	ACC^{-}	Ch.7
Fp636	P+	ACC^{+}	Ch.7
Fp585	P+	ACC^{-}	Ch.7
Fp587	$P+^{+}$	ACC^{-}	Ch.7
Fp441	P++	ACC^{-}	Ch.7
Fp600	$P+^{++}$	ACC^{+++}	Ch.7
Fp561	P+	ACC^{-}	Ch.7

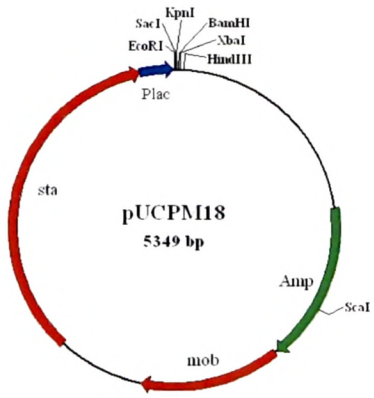
Table 2.2: List of *Pseudomonas* strains used in the present study. *P. fluorescens* ATCC 13525 and selected native isolates were used to incorporate genetic modifications and for further physiological studies.

Fluorescent pseudomonads isolated from wheat rhizosphere were a generous gift from **Prof. B. N. Johri, Pantnagar University**. These 10 isolates were already characterized with respect to P-solubilization ability on Pikovaskya's (PVK) Agar and ACC Deaminase activity as a marker for plant growth promoting ability as shown in **Table 2.2**.

Plasmids	Features	Reference
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r	Hester et al., 2000
pME6010	pVS1-p15A shuttle vector; Tc^r	Heeb et al., 2000
pA172A	pBR322 with ~3.5kb *Synechococcus elongatus* PCC 6301 genomic DNA fragment harboring *ppc gene*	Kodaki et al., 1985
pBBR1MCS-2	Broad-Host-Range vector; Km^r	Kovach et al., 1995
pCS-Ec	pBluescript with *cs* gene of *E. coli*; Ap^r	Delhaize, E
pYanni3	Cloning vector for *E. coli*; contains *npt*II gene conferring kanamycin resistance; Ap^r, Km^r	Wackernagel, W.
pBluescript/SK	Cloning vector for *E. coli*; Ap^r	Sambrook and Russell, 2001
pAB1	pA172A with tc^r gene; Ap^r, Tc^r	Ch.3
pAB2	pUCPM18 with promoterless *S. elongatus* PCC 6301 *ppc* gene; Ap^r/Tc^r	Ch.3
pAB3	pUCPM18 with *S. elongatus* PCC 6301 *ppc* gene under P_{lac} and tc^r gene; Ap^r/Tc^r	Ch.3
pAB4	pUCPM18 with tc^r gene; Ap^r, Tc^r	Ch.3
pAB5	pUCPM18 with tc^r gene; Ap^r Tc^r	Ch.3
pAB6	pUCPM18 with *cs* gene of *E. coli* under P_{lac}; Ap^r	Ch.5
pAB7	pUCPM18 with *E. coli cs* gene under P_{lac} and km^r gene; Ap^r, Km^r	Ch.5
pAB8	pUCPM18 with km^r gene; Ap^r, Km^r	Ch.5
pAB9	pBBR1MCS-2 with *cs* gene of *E. coli* under P_{lac}; Km^r	Ch.5
pX2, pX6, pX15, pX20, pX25	Plasmids derived from self ligation of XbaI digestion derived *Pseudomonas* P4 genomic DNA; Ap^r	Ch.8
pA2, pA18, pA51, pA55, pA56	Plasmids derived from self ligation of ApaI digestion derived *Pseudomonas* P4 genomic DNA; Ap^r	Ch.8
pBSK(1.1)	~1.1kb genomic DNA fragment from pA55 cloned in pBluescript/SK; Ap^r	Ch.8
pA172Aself(5.4)	Self ligated pA55 plasmid backbone; Ap^r	Ch.8

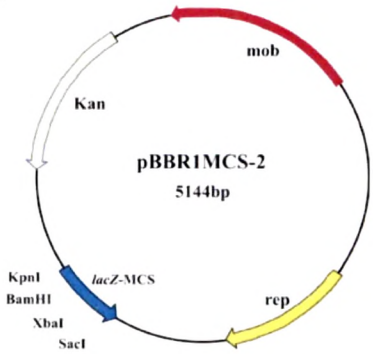
Table 2.3: List of plasmids used in the present study. All the plasmids were stored as stocks at -20°C in the form of ethanol precipitates.

(a)



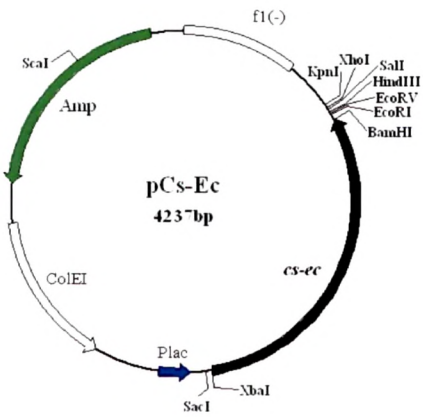
Generous gift from **Dr. J. R. Sokatch**, University of Oklahoma Health Sciences

(b)



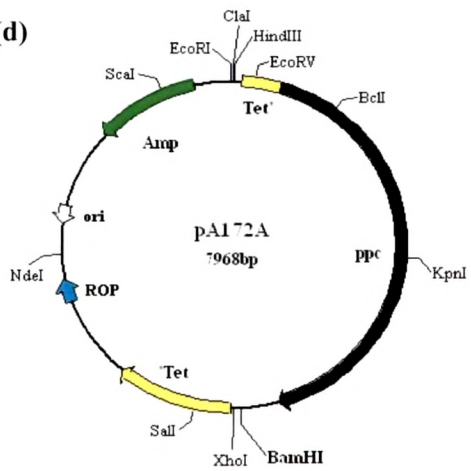
Generous gift from **Dr. Kovach M. E.,** Louisiana State University Medical Center, U.S.A

(c)



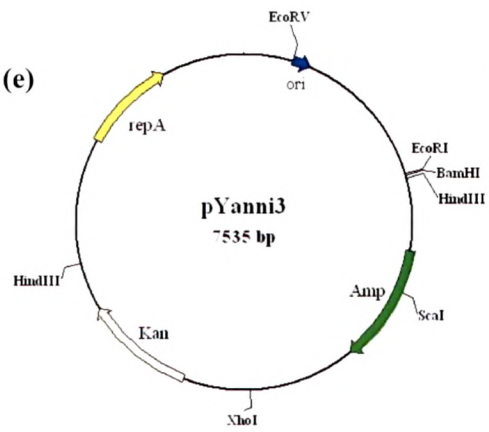
Generous gift from **Dr. Delhaize, E.**, CSIRO Plant Industry, Australia

(d)



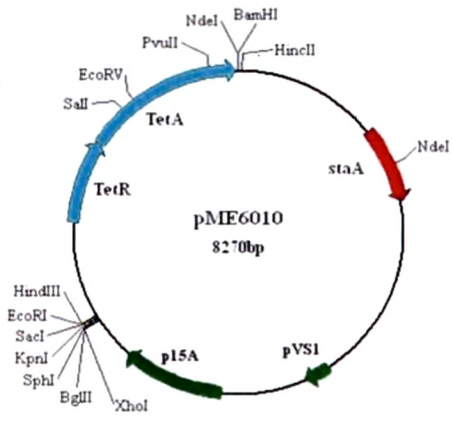
Generous gift from **Dr. S. K. Apte**, BARC, Mumbai

(e)



Generous gift from **Dr. D. W. Wackernagel**, University of Oldenberg, Germany

(f)



Generous gift from **Dr. D. Haas**, Université de Lausanne, Switzerland

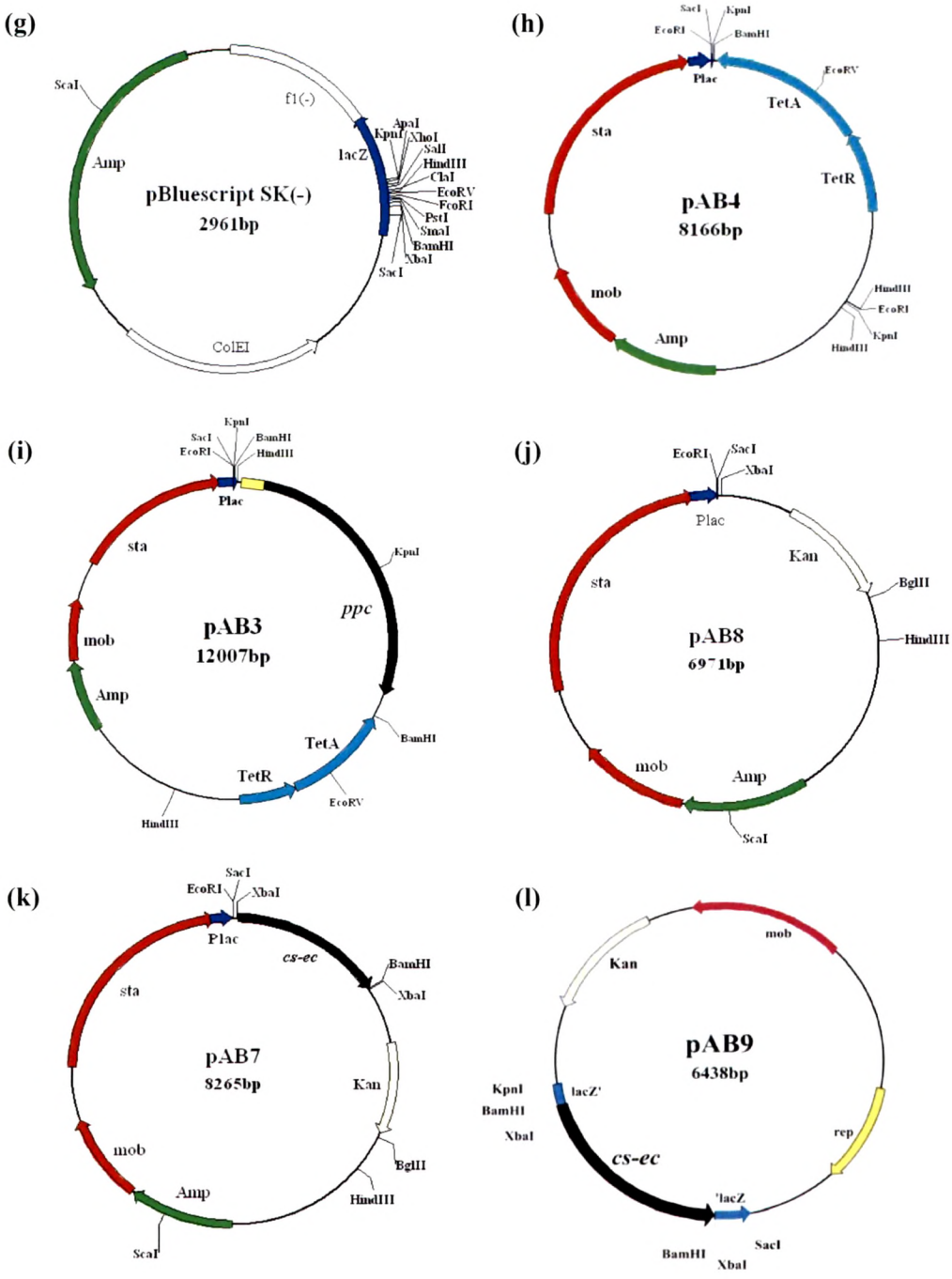


Fig. 2.1: Restriction maps of the plasmids used in this study. (a) and (b) are the broad-host-range vectors used for expression of heterologous genes under *lac* promoter in *P. fluorescens* 13525. (c), (d), (e) and (f) are the plasmids used to obtain *cs*, *ppc*, Km^r and Tc^r genes. (g) is the standard pBluescript plasmid used for conventional cloning in *E. coli*. (h), (i), (j), (k) and (l) are the final constructs of the plasmids used for physiological studies in *P. fluorescens* 13525.

Broad host range, multicopy plasmid vectors, pUCPM18 and pBBR1MCS-2, were selected for expression in pseudomonads on account of their small size and versatile multiple cloning sites. **pUCPM18** plasmid is derived from a pUC-derivative, pUCP18 plasmid which is stably maintained in both *E. coli* and *Pseudomonas* species (Schweizer, 1991). pUCPM18 was developed from pUCP18 (GenBank accession number: U07164) by incorporating a 750bp mob fragment from pLAFRI (broad host range vector used for genetic analysis of gram-negative bacteria; Vanbleu et al., 2004), in order to enable convenient mobilization within *Pseudomonas* species (Hester et al, 2000). It replicates in *E. coli* using ColE1 origin of replication (*ori*) while in *Pseudomonas* it replicates owing to a pRO1614 derived DNA fragment encoding a putative *ori* and a replication-controlling protein (West et al., 1994).

pBBR1MCS-2 (GenBank accession number: U23751) plasmid conferring kanamycin resistance is originally derived from pBBR1 plasmid of *Bordetella bronchiseptica* (Kovach et al., 1995). This plasmid facilitates cloning due to availability of unique restriction sites and direct selection of recombinant plasmids based on blue-white selection strategy. pBBR1MCS based vectors are mobilizable when the RK2 transfer functions are provided in *trans*, are compatible with IncP, IncQ and IncW group plasmids, as well as with ColE1- and P15a-based replicons, can stably replicate in a variety of hosts including *E. coli*, *P. fluorescens*, *P. putida*, *Rhizobium meliloti* and *R. leguminosarum bv. Viciae* and are stably retained *in vivo* for more than 4 weeks in absence of antibiotic selection (Kovach et al., 1995).

pYanni3 (GenBank accession number: AJ247371) is a broad host range cloning vector (Graupner and Wackernagel, unpublished data). **pME6010** (GenBank accession number: AF118810) is a shuttle vector for use in plant associated gram-negative bacteria. It can replicate in *E. coli* owing to p15A (pACYC177) moiety while uses pVS1 replicon to replicate in *Pseudomonas* species (Heeb et al., 2000). It contains a multiple cloning site downstream of a constitutive kanamycin resistance promoter (P_k) and a repressible tetracycline resistance gene (tc^r) and has been shown to be 100% stable in biocontrol strain *P. fluorescens* CHAO for 100 generations without antibiotic selection. **pA172A** plasmid contains *ppc* gene on ~3.5kb genomic DNA fragment of *S. elongatus* PCC 6301 inserted under the promoter of tetracycline resistance gene of pBR322 plasmid. This plasmid could functionally complement the *E. coli ppc* mutant (Kodaki et al., 1985).

2.2: Media and Culture conditions

The *E. coli* strains and pseudomonads were cultured and maintained on Luria Agar (LA) and Pseudomonas Agar respectively (Hi-Media Laboratories, India). *E. coli* cultures were grown at 37°C while all *Pseudomonas* cultures were grown at 30°C. For growth in liquid medium, shaking was provided at the speed of 200rpm. The plasmid transformants of both *E. coli* and pseudomonads were maintained using respective antibiotics at the final concentrations as mentioned in **Table 2.4** as and when applicable. Both *E. coli* and *Pseudomonas* wild type strains and plasmid transformants grown in 3ml Luria broth (LB) containing appropriate antibiotics were used to prepare glycerol stocks which were stored at -20°C.

Antibiotic	Rich medium	Minimal medium
Tetracycline	30µg/ml	7.5µg/ml
Kanamycin	50µg/ml	12.5µg/ml
Streptomycin	10µg/ml	2.5µg/ml
Trimethoprim	60µg/ml	15µg/ml
Ampicillin*	50µg/ml	12.5µg/ml
Erythromycin	100µg/ml	-
Chloramphenicol*	20µg/ml	-
Spectinomycin	50µg/ml	-
Gentamycin*	20µg/ml	-

Table 2.4: Recommended dozes of antibiotics used in this study (Sambrook and Russell, 2001). The antibiotic dozes were maintained same for both *E. coli* and pseudomonads. All the antibiotics were prepared in sterile distilled water or recommended solvent at the stock concentrations of 1000x or 2000X (for antibiotics marked with *) and were used accordingly to have the desired final concentrations.

The compositions of different minimal media used in this study are as described below. Antibiotic concentrations in all the following minimal media were reduced to $1/4^{th}$ of that used in the above mentioned rich media (**Table 2.4**).

2.2.1: Bushnell-Haas (BH) medium

The ingredients of BH agar include Ammonium nitrate, 1.0g/L; calcium chloride, 0.02g/L; dipotassium phosphate, 1.0g/L; ferric chloride, 0.05g/L; magnesium sulphate, 0.2g/L; monopotassium phosphate, 1.0g/L and agar, 20.0g/L. The readymade media was obtained from Hi-Media Laboratories, India, and was used according to manufacturer's instructions. Sterile sodium benzoate at the final concentration of 1mM was supplemented as the sole carbon source.

2.2.2: Koser's Citrate medium

Composition of the Koser's Citrate medium included magnesium sulphate, 0.2g/L; monopotassium phosphate, 1.0g/L; sodium ammonium phosphate, 1.5g/L; sodium citrate, 3.0g/L and agar, 15g/L. The readymade media was obtained from Hi-Media Laboratories, India, and was used according to manufacturer's instructions.

2.2.3: M9 minimal medium

Composition of M9 minimal broth was according to Sambrook and Russell (2001) including Na_2HPO_4 $7H_2O$, 34g/L; KH_2PO_4, 15g/L; NH_4Cl, 5g/L; NaCl, 2.5g/L; 2mM $MgSO_4$; 0.1mM $CaCl_2$ and micronutrient cocktail. The micronutrient cocktail was constituted of $FeSO_4.7H_2O$, 3.5 mg/L; $ZnSO_4.7H_2O$, 0.16 mg/L; $CuSO_4.5H_2O$, 0.08 mg/L; H_3BO_3, 0.5 mg/L; $CaCl_2.2H_2O$, 0.03 mg/L and $MnSO_4.4H_2O$, 0.4 mg/L. Carbon sources used were glucose, xylose, fructose and lactose as and when required. For solid media, 15g/L agar was added in addition to above constituents.

5X M9 salts, micronutrients (prepared at 1000X stock concentration) and carbon source (2M stock) were autoclaved separately. Fixed volumes of these were added aseptically into pre-autoclaved flasks containing distilled water to constitute the complete media with the desired final concentrations. Volume of water to be autoclaved per flask was calculated by subtracting the required volumes of each ingredient from the total volume of the media to be used.

2.2.4: Tris buffered medium

The media composition included Tris-Cl (pH=8.0), 100mM; NH_4Cl, 10mM; KCl, 10mM; $MgSO_4$, 2mM; $CaCl_2$, 0.1mM; micronutrient cocktail; Glucose, 100mM and phosphate (P) sources (Sharma et al., 2005). 1mg/ml Senegal Rock phosphate (RP) or KH_2PO_4 were used as insoluble and soluble P sources respectively. Each ingredient was separately autoclaved at a particular stock concentration and a fixed volume of each was added to pre-autoclaved flasks containing sterile distilled water (prepared as in Section 2.2.3) to constitute complete media.

2.2.5: Pikovskaya's (PVK) Agar

The media composition included Ammonium sulphate, 0.5g/L; Calcium phosphate, 5.0g/L; Dextrose, 10.0g/L; Ferrrous sulphate, 0.0001g/L; Magnesium

sulphate, 0.1g/L; Manganese sulphate, 0.0001g/L; Potassium chloride, 0.2g/L; Yeast extract, 0.5g/L and agar, 15.0g/L. Dextrose was substituted by same amount of xylose and fructose as and when mentioned. The readymade media was obtained from Hi-Media Laboratories, India, and was used according to manufacturer's instructions.

2.2.6: Murashige-Skoog's Medium

Murashige-Skoog's (MS) medium was composed of macro elements and micro elements. The macro elements included $CaCl_2.2H_20$, 0.440g/L; KH_2PO_4, 0.17g/L; KNO_3, 1.9g/L; $MgSO_4.7H_2O$, 0.37g/L; NH_4NO_3, 1.65g/L and 10g/L agar. The micro elements included the essential trace elements. Micronutrients and desired carbon source (autoclaved separately), were added aseptically to reconstitute the complete media. The readymade micronutrient was obtained from Hi-Media Laboratories, India, and was used according to manufacturer's instructions while the macronutrients were reconstituted as and when required.

2.3: Morphological characterization and antibiotic sensitivity profile

Primary fluorescence of the native isolates and the *P. fluorescens* 13525 transformants was checked on Pseudomonas agar (for fluorescein production) plates. The antibiotic sensitivity profile was obtained for all the above 10 native isolates by checking the growth of these cultures by streaking on LA plates containing antibiotics. The same was also confirmed by inoculating single colonies of all the strains independently in 3ml LB containing recommended dose of antibiotics (Sambrook and Russell, 2001) and checking the growth after overnight shaking at 30°C. The antibiotics used were erythromycin, ampicillin, kanamycin, spectinomycin, chloramphenicol, gentamycin, trimethoprim and tetracycline at the final concentrations as described in **Table 2.4.**

2.4: Molecular biology tools and techniques

2.4.1: Isolation of plasmid and genomic DNA

2.4.1.1: Plasmid DNA isolation from *E. coli* and *P. fluorescens* 13525

The plasmid DNA from *E. coli* was isolated by the boiling lysis method using CTAB while that from *P. fluorescens* 13525 was isolated using standard alkali lysis method (Sambrook and Russell, 2001).

2.4.1.2: Isolation of large size plasmid DNA from native pseudomonads

The native plasmids in the soil isolates of fluorescent pseudomonads, which are generally known to be of large size, were isolated by the protocol described for the isolation of ~117kb TOL plasmid (Ramos-Gonzalez et al., 1991). Single colonies of the pseudomonads were inoculated in 3ml of LB and were allowed to grow at 30°C under shaking conditions (200rpm). 0.5ml of freshly grown cultures was centrifuged at 9, 200x *g* for 2 minutes and the pellet was re-suspended homogenously in 200µl of sterile STE [20% sucrose in 25mM Tris-Cl containing 25mM EDTA (pH=8.0) and 1mg/ml lysozyme]. Complete lysis of the cells was achieved by adding 100µl of alkaline SDS [0.3N NaOH containing 2% SDS]. After gentle mixing, the resulting viscous solution was incubated at 55°C for 5 minutes to minimize the chromosomal DNA contamination. Following this the solution was treated with equal volume of phenol-chloroform and after gentle mixing was subjected to centrifugation at 9, 200x *g* for 10 minutes. The aqueous layer recovered was subjected to chloroform extraction, centrifugation at 9, 200x *g* for 10 minutes, recovery of the aqueous phase and finally DNA precipitation using 0.6 volumes of isopropanol. The DNA was allowed to precipitate at room temperature for ~30-40 minutes, washed with 70% ethanol and finally dissolved in 30µl Tris-EDTA (TE) buffer (Sambrook and Russel, 2001). Of this, 15µl DNA solution was subjected to agarose gel electrophoresis using 1% agarose (Section 2.4.4).

2.4.1.3: Genomic DNA isolation from *Pseudomonas*

Fresh *Pseudomonas* culture obtained by growing a single colony inoculated in 3ml LB under shake conditions at 30°C was dispensed in 1.5ml sterile centrifuge tubes, pelleted at 9, 200x *g* and washed twice with sterile normal saline. Following this, the cells were used for genomic DNA isolation performed using genomic DNA extraction kit (Cat.# FC46, Bangalore Genei, India) according to the manufacturer's instructions. The DNA was finally re-suspended in 40µl of sterile double distilled water.

2.4.2: Transformation of plasmid DNA

2.4.2.1: Transformation of plasmid DNA in *E. coli*

The transformation of plasmids in *E. coli* using $MgCl_2$-$CaCl_2$ method and blue-white selection of the transformants using IPTG and X-Gal (as and when applicable) was carried out according to Sambrook and Russell (2001).

2.4.2.2: Transformation of plasmid DNA in *P. fluorescens* 13525

Plasmid transformation in *P. fluorescens* 13525 was done using the $NaCl$-$CaCl_2$ method (Cohen et al., 1972) with slight modifications which are as follows. *P. fluorescens* 13525 was grown at 30°C in LB broth to an O.D. $_{600}$ of 0.6-0.8. At this point, the cells were chilled for about 10minutes, centrifuged at 5000rpm for 5 minutes and washed once in 0.5 volume 10mM NaCl (chilled). After centrifugation (5000rpm for 5 minutes), bacteria were re-suspended in half the original volume of chilled 0.1M $CaCl_2$, incubated on ice-bath for 1hr, centrifuged (5000rpm for 5 minutes) and then re-suspended in $1/10^{th}$ of the original culture volume of chilled 0.1M $CaCl_2$. 0.2ml of competent cells treated with $CaCl_2$ was used per vial (microcentrifuge tube) to add DNA samples (minimum 0.8-1.0µg is required) and were further incubated on ice-bath for 1hr. Competent cells were then subjected to a heat pulse at 42°C for 2 min to enable DNA uptake, immediately chilled for 5 minutes and then were supplemented with 1.8ml of sterile LB broth followed by incubation for 1hour at 30°C under shake conditions. These cells were then centrifuged and plated on Pseudomonas Agar plates containing appropriate antibiotics. The colonies obtained after overnight incubation of the plates at 30°C were then subjected to fluorescence check and plasmid DNA isolation.

2.4.3: Transfer of plasmid DNA by conjugation

The plasmids were transformed in *E. coli* S17.1, for mediating the conjugal transfer of the plasmids used (Section 2.4.2.1) and the resultant transformant strain was used as the donor strain. *E. coli* S17.1 harboring the plasmid and the recipient pseudomonad strain were separately grown in 3ml LB broth with respective antibiotics at 30°C under shake conditions for approximately 16h. The freshly grown cultures of recipient and the donor strains were aseptically mixed in 1:1 ratio (v/v) in a sterile centrifuge tube and the cells were centrifuged at 5000rpm for 5 minutes. The media supernatant was discarded to remove the antibiotics and the pellet was re-suspended in 0.2ml of fresh sterile LB and the bacteria were allowed to mate at 30°C. After 16h, the bacterial culture mix was centrifuged at 5000rpm for 5 minutes and the resultant pellet was re-suspended in 0.2ml of sterile normal saline. About 30µl of this cell suspension were plated on Pseudomonas agar containing the appropriate antibiotics for selection (antibiotic dose was as described in **Table 2.4**) to obtain the transconjugants. Conjugation mediated plasmid transfer was employed only for the native isolates of fluorescent pseudomonads.

2.4.4: Agarose gel electrophoresis

The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue, and 40% sucrose in water) and subjected to electrophoresis through 0.8% agarose (containing 1μg/ml ethidium bromide) gel in Tris-acetate-EDTA (TAE) buffer at 5v/cm for 0.5-2h. The DNA bands were visualized by fluorescence under the UV-light using UV transilluminator.

2.4.5: Restriction enzyme digestion analysis

0.5-1.0μg DNA sample was used for each restriction enzyme digestion. 1-3U of the restriction endonuclease (RE) was used with the appropriate 10X buffers supplied by the manufacturer in a final reaction volume of 10μl. The reaction mixture was incubated overnight at 37°C. The DNA fragments were visualized by ethidium bromide staining after electrophoresis on 0.8% agarose gels and were subsequently photographed. In case of double digestion, a compatible buffer for the two REs was essentially checked. If not available, digestion with one enzyme is performed followed by purification and subsequent digestion with the other enzyme, using respective buffers.

2.4.6: Gel elution and purification

The DNA fragments of desired sizes were recovered from the gel by cutting the agarose gel slab around the DNA band. The agarose piece was weighed in a sterile microcentrifuge tube and was solubilized in 2.5 volumes of 6M sodium iodide (NaI, freshly made) [e.g. for 200mg of agarose piece, 500μl of NaI was added]. Once completely dissolved, 15μl of silicon dioxide suspension (50% w/v, stored at 4°C) was added and was incubated at room temperature for 15-20 minutes. The DNA bound to silica was recovered by centrifuging at 9, 200x *g* for 2 minutes; the pellet was washed twice with 70% ethanol, dried and finally re-suspended in 20-30μl sterile double distilled water. The microcentrifuge tube was incubated at 55°C for 10 minutes to allow complete dissociation of DNA from silica beads into the solution and then was subjected to centrifugation at 9, 200x *g* for 2 minutes. The resultant supernatant was gently recovered using sterile micropipette tip and was transferred to fresh sterile tube. The purification efficiency was checked by subjecting 2μl DNA solution to gel electrophoresis and visualizing the sharp DNA band of desired size. The purified DNA was used for ligation experiments only if >50ng/μl DNA was recovered after purification.

2.4.7: Ligation

The ligation reaction was usually done in 10µl volume containing the following constituents: Purified vector and insert DNA (volume varied depending on the respective concentrations); 10X T4 DNA Ligase buffer, 1µl; T4 DNA ligase (MBI Fermentas), 0.5-1.0U and sterile double distilled water to make up the volume. The cohesive end ligation reaction was carried out at 16°C for 12-16h. The vector to insert molar ratio (molar concentrations calculated by the under mentioned formula) of 1:4 was maintained, with a total of 50-100ng of DNA in each ligation system.

$$\text{pmoles of DNA} = \frac{\text{Amount of DNA (µg) x 1, 515}}{\text{Size of the DNA fragment (no. of base pairs)}}$$

2.4.8: SDS-PAGE

(A) Monomer solution (30%) (Store at 4° C in dark)		**(B) Resolving gel buffer-1.5M Tris (pH 8.8)** Adjust pH with HCl		**(C) Stacking gel buffer 1.0M Tris (pH 6.8)** Adjust pH with HCl	
Acrylamide	14.6 gm	Tris base	9.1 gm	Tris base	3.02 gm
Bisacrylamide	0.4 gm	SDS	0.2 gm	SDS	0.20 gm
D. H_20	Till 50 ml	D. H_20	Till 50 ml	D. H_20	Till 50ml
(D) Tank Buffer (pH 8.3)		**(E) Sample Loading buffer (2X)**		**(F) Other reagents**	
Tris base	6.0 gm	SDS	4%	APS (fresh)	10% (w/v)
Glycine	28.8 gm	Glycerol	20%	TEMED	2-3 µl
SDS	2.0 gm	Tris-Cl (pH6.8)	0.125M	Water saturated n-butanol	
D. H_20	Till 2 L	Bromophenolblue	0.05%w/v	Sigma protein molecular weight marker SDS6H2-(30,000-200,000).	
Adjust the pH with HCl		β-mercaptoethanol	10mM		
		D. H_20	Till 10ml		
(G) Separating Gel (8%, 10ml)		**(H) Stacking Gel (3.9%, 5ml)**		**(I) Staining Solution**	
30% Monomer	2.7 ml	30% Monomer	0.65 ml	0.025% Commassie Blue R-250 in 40% Methanol and 7% Acetic acid	
Separating gel buffer (pH 8.8)	2.5 ml	Stacking gel buffer (pH 6.8)	1.25 ml		
D. H_20	2.3 ml	D. H_20	3.05 ml	**(J) De-staining solution**	
10% APS	50 µl	10% APS	25 µl	(10% methanol and 10% Acetic acid)	
TEMED	2 µl	TEMED	3 µl		

Table 2.5: Composition of SDS-PAGE reagents (Sambrook and Russell, 2001)

SDS-PAGE slab gel electrophoresis was carried out using 8% acrylamide gel by following the procedures described by Sambrook and Russell (2001). After electrophoresis the gel was stained using the staining solution (**Table 2.5**) for about 1h and then de-stained with de-staining solution (**Table 2.5**) by incubating overnight under mild shaking conditions. Result was recorded by direct scanning of gel.

2.4.9: Polymerase Chain Reaction (PCR)

The PCR reaction set up was based on the guidelines given in Roche Laboratory Manual. The assay system and the temperature profile used are described in **Table 2.6.**

Assay system used		Temperature Profile	
Sterile DDW	38 µl	Initial denaturation	94°C- 5 min
dNTP (10mM with 2.5mM each)	3 µl	Denaturation	94°C- 30 sec
Reverse Primer 20pmoles	1 µl	Annealing	Varies from 55-62°C for 30sec.*
Forward Primer 20pmoles	1 µl		
Template DNA (100ng/µl)	1 µl	Elongation	72°C for 45sec-2.5min*
Taq PCR buffer (10X)	5 µl	Final Elongation	72°C- 10 min
Taq DNA Polymerase (1 unit/µl)#	1.0 µl	For plasmid	(30 cycles)
Total System	**50 µl**	For genomic DNA	(40 cycles)

Table 2.6: PCR conditions used in the present study. PCR amplifications were performed in Techne TC-312 thermal cycler. *Exact primer annealing temperature and primer extension time varied with primers (designed with respect to different templates) and has been specified in the text as and when applicable. # Processivity of Taq polymerase is ~1000 bases per min. Taq DNA polymerase and its buffer, dNTPs and primers were obtained respectively from Bangalore Genei Pvt. Ltd., India, Sigma Chemicals Pvt. Ltd. and MWG Biotech. Pvt. Ltd, India, respectively and were used according to manufacturer's instructions.

The theoretical validation of the primers with respect to absence of intermolecular and intramolecular complementarities to avoid primer-primer annealing and hairpin structures and the appropriate %G-C was carried out with the help of online primer designing software Primer 3. The sequence, length and %G-C content of primers are subject to variation depending on the purpose of PCR and will be given as and when applicable in the following chapters. The PCR products were analyzed on 1.0% agarose gel along with appropriate molecular weight markers (Section 2.4.4)

2.4.10: DNA sequencing

The DNA sequencing service was obtained from MWG Biotech. Pvt. Ltd. (India) for ~1.1kb *Pseudomonas* genomic DNA and ~750bp of 16SrDNA (details in Chapter 8.)

2.4.11: BLAST Search

The online computational tool "BLAST" for the DNA sequence alignments and homology search was used for microbial classification of a *Pseudomonas* isolate P4 and for characterization of a genomic integration event in the same (details in Chapter 8).

These molecular biology tools were collectively applied, as and when applicable, to construct recombinant plasmids with *ppc/cs* genes under *lac* promoter, confirmation of the recombinant clones and expression of respective proteins and ultimately development of pseudomonads harboring the appropriate recombinant plasmids; for further investigations.

2.5: Aromatic hydrocarbon utilization

Aromatic hydrocarbon utilizing ability of several *Pseudomonas* strains was checked by monitoring the growth at 30°C on BH Agar (Section 2.2.1) containing 1mM of Na-benzoate as sole carbon source. Kanamycin was used at the final concentration of 7.5μg/ml. Accompanying phenotype of green fluorescence (attributed to expression of GFP) was monitored under UV light.

2.6: Lactose and Citrate utilization

Ability of the pseudomonads to utilize citrate as carbon source and inability to assimilate lactose (Lessie and Phibbs, 1984) was exploited as a selection tool for pseudomonads in several experiments. Growth at 30°C on Koser's Citrate medium (Kiyohara et al., 1982) and M9 minimal medium containing 100mM lactose agar plates was monitored to indicate citrate and lactose utilizing abilities respectively (Section 2.2.2; 2.2.3). Tetracycline and kanamycin were used at final concentrations of 7.5μg/ml and 12.5μg/ml respectively, as and when applicable.

2.7: P-solubilization phenotype

P-solubilizing ability of the native as well as the transformant pseudomonads was tested on **(i)** Pikovskaya's (PVK) agar (Section 2.2.5; Pikovskaya, 1948) to monitor the

ability to solubilize di-calcium phosphate and **(ii)** Tris buffered RP-Methyl red (TRP) agar plates which represents a much more stringent condition of screening for PSMs (Gyaneshwar et al, 1998). Liquid medium for RP solubilization is described in Section 2.2.4 with an addition of methyl Red as pH indicator dye and 1.5% agar for all the plate experiments. *Pseudomonas* cell suspension for these experiments was prepared as described in Section 2.9.1 and 3µl of it was aseptically spotted on the above mentioned agar plates and was allowed to dry completely followed by incubation at 30°C for 5-7 days. P solubilization was determined by monitoring the zone of clearance on PVK agar and red zone on the TRP agar plates. Media acidification from pH=8.0 to pH<5 on TRP broth was used as an indicator for P-solubilization and organic acid secretion.

2.8: Mutant Complementation Phenotype

E. coli ppc (JWK3928) and *cs* (W620) mutants were used to confirm the functionality of the cloned *ppc* and *cs* genes, respectively. The recombinant plasmids containing *ppc* and *cs* genes independently under *lac* promoter were transformed into respective mutants. The *ppc* transformants were selected on kanamycin and tetracycline while *cs* transformants were selected on streptomycin and kanamycin. These transformants were grown on M9 minimal medium with 0.2% (11.11mM) glucose as carbon source in presence and absence of 340µg/ml sodium glutamate, under shaking conditions at 30°C for *E. coli* JWK3928 and at 37°C for *E. coli* W620. Kanamycin, tetracycline and streptomycin were used at the final concentrations of 12.5µg/ml, 7.5µg/ml and 2.5µg/ml ($1/4^{th}$ of the concentration used for growth in LB broth). Both *ppc* and *cs* mutants exhibited glutamate auxotrophy (Izui et al., 1986; Underwood et al., 2002) as they could not grow on glucose as the sole carbon source in the absence of glutamate supplementation.

2.9: Physiological experiments

The physiological experiments were carried out using various WT and transformant pseudomonads which included growth, pH profile and enzyme assays.

2.9.1: Inoculum preparation

The inoculum for M9 and Tris minimal media containing free Pi was prepared by growing the *Pseudomonas* cultures overnight at 30°C in 3ml LB broth. Inoculum for the

buffered RP broth (TRP) was prepared by growing the *Pseudomonas* cultures overnight at 30°C in 10ml of M9 minimal medium. Cells were harvested aseptically, washed twice by normal saline and finally re-suspended in 1ml normal saline under sterile conditions. Freshly prepared inoculum was used for all the experiments.

2.9.2: Growth characteristics and pH profile

Growth parameters and pH profile of the native as well as transformant pseudomonads were determined using three different media conditions including **(i)** TRP medium with RP as P source (Gyaneshwar et al, 1998). The media composition in this case was same as mentioned in Section 2.2.4; **(ii)** Tris buffered medium with KH_2PO_4 as P source: The media composition was same as mentioned in section 2.2.4 except for RP being substituted by free Pi in the form of KH_2PO_4 at the concentrations including 0.1mM, 1.0mM, 10.0mM and 20mM as and when required; and **(iii)** M9 minimal medium (Section 2.2.3). 100mM glucose was used as the carbon source for all the experiments unless and until stated categorically. In some experiments, xylose (100mM) and fructose (100mM) were also used as carbon source.

In 150ml conical flasks, 30ml of relevant minimal broth containing free Pi was inoculated with cell suspensions to have 0.01-0.03 $O.D_{600nm}$ initially (0 hour O.D.). Similarly, for the TRP broth, the initial $O.D._{600nm}$ was about 0.09-0.15. The batch culture studies were performed under aerobic conditions in Orbitek rotary shaker maintained at 30°C with agitation speed kept constant at 200rpm. 1ml samples were aseptically harvested at regular intervals (varying with every set of batch culture depending on media conditions) and were subjected to various analytical techniques.

2.9.3: Analytical techniques

The cell density determinations were done at 600nm as monitored spectrophotometrically. Change in absorbance was considered as the measure of growth and drop in pH of the media was taken as the measure of acid production. In all the cases, the observations were continued till the media pH reduced to less than 5. 1ml aliquots withdrawn aseptically at regular time intervals were immediately frozen at -20°C until further used for biochemical estimations. The stored samples were centrifuged at 9, 200x *g* for 1 min at 4°C and the culture supernatants derived were used to estimate residual glucose and organic acid analysis using HPLC. For HPLC analysis, the culture

supernatant was passed through 0.2μm nylon membranes (MDI advanced microdevices, India) and the secreted metabolites were quantified using RP-18 column. The column was operated at room temperature using mobile phase of 0.01M H_2SO_4 at a flow rate of 1.0 ml min^{-1} and the column effluents were monitored using a UV detector at 210 nm. For citric acid estimation the same column was operated at room temperature using mobile phase of 20mM Na_2HPO_4 with 2.5% acetonitrile at a flow rate of 1.0 ml min^{-1}. Standards of organic acids were prepared in double distilled water, filtered using 0.2μm membranes and were subjected to chromatography for determining the individual retention time. Measurements of area under peak with an external standard were used for quantification. The glucose concentration in the medium was estimated using enzymatic kit (Reckon Diagnostics, India).

The physiological parameters like specific growth rate, specific total glucose utilization rate and biomass yield (as described by Chao and Liao, 1993) and the organic acid yield were calculated as described below.

(i) Specific growth rate (h^{-1}):

$$k = \frac{(Log_{10}Nt_1 - Log_{10}Nt_2) \times 3.3}{(t_1 - t_2)\ (h)} \quad \text{where,}$$

N_1 and N_2 are the number of cells at time t_1 and t_2 respectively and (t_1-t_2) is the corresponding time interval in hours. 3.3 is the factor derived from the formula- number of generations $(n) = (Log_{10}N - Log_{10}N_0)/Log_{10}2$. The number of cells was calculated from $O.D._{600nm}$ using the correlation 1 $O.D._{600nm} = 1.5 \times 10^9$ cell/ml (Koch et al., 2001)

(ii) Specific total glucose utilization rate (Q_{Glc}):

$$Q_{Glc} = \frac{\Delta Glucose\ (t_1 - t_2)\ (g/L)}{\Delta dcw\ (t_1 - t_2)\ (g/L) \times Time\ interval\ (t_1 - t_2)\ (h)} \quad \text{where,}$$

$\Delta Glucose\ (t_1-t_2)$ is the amount of glucose consumed over the time interval t_1-t_2; $\Delta dcw\ (t_1-t_2)$ is the difference in the dry cell weight (dcw) of the cells over the time interval t_1-t_2. Q_{Glc} is expressed as g glucose utilized/g dcw/h). Dry cell weight was calculated using the correlation 1 $O.D._{600nm} = 0.382$mg/ml (Bugg et al., 2000).

(iii) Biomass yield

$$Y_{dcw/Glc} = \frac{\Delta dcw\ (t_1\text{-}t_2)\ (g/L)}{\Delta Glucose\ (t_1\text{-}t_2)\ (g/L) \times Time\ interval\ (t_1\text{-}t_2)\ (h)} \quad \text{where,}$$

All the parameters were as described for Specific glucose utilization rate. $Y_{dcw/Glc}$ is expressed as g of dry cell weight produced/ g glucose utilized/h.

(iv) Organic acid yield

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

The amount of total glucose utilized was obtained by deducting the value of residual glucose concentration from the initial glucose concentration supplied in the medium. The difference between the total glucose utilized and gluconic acid produced was considered as glucose consumed. Hence, the total glucose utilized and not glucose consumed was taken into account for calculating specific glucose utilization rate. The statistical analysis of all the parameters was done using Graph Pad Prism (version 3.0) software.

2.10: Enzyme assays

2.10.1: Preparation of cells and cell free extracts

Glucose grown cells under above mentioned minimal media conditions were harvested in appropriate growth phase from 30ml of cell culture by centrifugation 9,200x *g* for 2 minutes at 4°C. Unlike citrate synthase (CS), isocitrate lyase (ICL) and isocitrate dehydrogenase (ICDH) which were assayed in the stationary phase, all the enzyme were assayed from mid-late log phase cell cultures. The preparation of cell free extracts for PPC, CS, PYC, G-6-PDH, ICDH and ICL assays was carried out according to Kodaki et al (1985) with an addition of 5mM $MgCl_2$ and 1mM EDTA for PYC assay. The cell pellet was washed once with 80mM phosphate buffer (pH=7.5) followed by re-suspension in same buffer containing 20% glycerol and 1mM DTT. The cells were then subjected to lysis by sonicating for maximum 1-1.5 minute in an ice bath, followed by centrifugation at 9,200x *g* at 4°C for 30 minutes to remove the cell debris. The

supernatant was then used as cell-free extract for the enzyme assays. The whole cell preparation for GDH assay was done by washing the harvested cells (mid-late log phase cultures) thrice with normal saline to remove the residual glucose of the medium and re-suspending in 0.01M phosphate buffer (pH 6.0) with 5mM $MgCl_2$.

2.10.2: Enzyme Assay Protocols

2.10.2.1: PPC assay

PPC (EC 4.1.1.31) activity was estimated spectrophotometrically by monitoring the oxidation of NADH in a coupled assay with malate dehydrogenase (MDH) as described by Kodaki et al (1985) with modifications as follows. The coupled assay was divided in two steps. The assay mixture for first step of the reaction contained following ingredients in total volume of 0.9ml: Tris-H_2SO_4 (pH 8.0), 100mM; potassium PEP, 2mM; $MgSO_4$, 10mM; $KHCO_3$, 10mM and the enzyme solution (cell lysate).

After 20 minutes incubation at 30°C, the reaction was terminated using 25% TCA followed by 15 minutes incubation on ice bath. The supernatant recovered after centrifuging the resultant reaction mix at 9,200x *g* for 30 minutes at 4°C, was brought to pH 8.0 by using 7µl of 10N NaOH which was then used as the source of OAA in second step of the reaction for which the assay system contained 950µl of reaction mix from first step of reaction, 5units of MDH and 0.12mM NADH. The assay system volume was adjusted to 1ml using distilled water. The rate of oxidation of NADH recorded at 340nm was used to indicate concentration of OAA formed. Molar absorbance of NADH was taken as $6.22mM^{-1}cm^{-1}$ at pH 8.0.

The absence of glycerol in the sonication buffer led to complete loss of PPC activity (Kodaki et al., 1985).

2.10.2.2: PYC assay

PYC (EC 6.4.1.1) activity was estimated by monitoring NADH oxidation in a coupled assay with MDH (Taylor et al., 1972) with modifications as follows. The assay mixture for first step of the reaction contained following ingredients in total volume of 0.9ml: Tris-HCl (pH 8.0), 100mM; Na-pyruvate, 5mM; ATP, 5mM; $MgCl_2$, 5mM; $NaHCO_3$, 50mM and the enzyme solution (cell lysate). Remaining steps and specific activity determination were same as in case of PPC activity.

2.10.2.3: G-6-PDH assay

G-6-PDH (EC 1.1.1.49) activity was determined spectrophotometrically at room temperature by following the reduction of NADP at 340nm as a function of time (Eisenberg and Dobrogosz, 1967). Molar absorbance of NADP was taken as 6.22mM^{-1}cm^{-1} at pH 8.0. The reaction mixture of 1ml included: Tris-Cl (pH 8.2), 200mM; glucose-6-phosphate, 3.3mM; $MgCl_2$, 10mM; NADP, 0.1mM and cell extract.

2.10.2.4: GDH assay

GDH (D-glucose phenazine methosulphate oxidoreductase, (EC 1.1.5.2) was determined spectrophotometrically by following the coupled reduction of 2,6-dichlorophenolindophenol (DCIP) at 600nm (Quay et al., 1972). Molar absorbance of DCIP was taken as 15.1 mM^{-1} cm^{-1} at pH 8.75. The reaction mixture included: Tris-Cl buffer (pH 8.75), 16.66mM; D-glucose, 66mM; DCIP, sodium salt, 0.05mM; phenazine methosulfate, 0.66mM; sodium azide, 4mM; whole cells, and distilled water to 3.0ml.

2.10.2.5: ICL assay

ICL (4.1.3.1) activity was measured by a modified method of Dixon and Kornberg (1959). ICL catalyzes the hydrolysis of isocitrate into glyoxylate and succinate. The glyoxylate formed in the presence of phenylhydrazine was measured as glyoxylic acid phenylhydrazone at 324nm. The reaction mixture of 1ml consisted of following ingredients: potassium phosphate buffer (pH=7.0), 100mM; $MgCl_2$, 6mM; cysteine HCl, 12mM; phenylhydrazine HCl, 4mM; isoctrate; 8mM and cell extract. Molar absorbance coefficient of phenylhydrazine was taken as 17.4 mM^{-1}cm^{-1}. The rate of increase in absorbance in the linear range was used to calculate ICL activity.

2.10.2.6: ICDH assay

ICDH (1.1.1.42) activity was measured by following NADPH formation at 340nm (Garnak and Reeves, 1979). The reaction mixture contained the following in 1ml: Tris-HCl (pH 7.5), 150mM; $MnCl_2$, 0.25mM; NADP, 0.65mM, isocitrate, 2.5mM and cell extract. Molar absorbance coefficient of NADP was taken as 6.22 mM^{-1}cm^{-1}.

2.10.2.7: CS assay

CS (4.1.3.7) activity was estimated by following the absorbance of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) at 412nm which would change due to its reaction

with sulfhydryl group of CoA (Serre, 1969). The assay mixture per cuvette contained following ingredients in 1.0ml: Tris-HCl (pH=8.0), 93mM; acetyl CoA, 0.16mM; OAA, 0.2mM; DTNB, 0.1mM and cell lysate. The reaction was started by addition of OAA. Molar absorbance coefficient was taken as 13.6 $mM^{-1}cm^{-1}$ at 412nm. The rate of increase in absorbance was used to calculate CS activity.

All the enzyme activities were determined at 30°C and were expressed per mg total protein. Total protein concentration of the crude extract as well as whole cell suspensions was measured by modified Lowry's method (Peterson, 1979) using bovine serum albumin as standard. Corrections were made for Tris buffer. Enzyme activities were calculated using following formula

$$\text{Specific enzyme activity (U)} = \frac{\Delta A_{y\,nm}/\text{min}}{\epsilon \times \text{enzyme (sample) aliquot (ml)} \times \text{Total protein (mg/ml)}} \quad \text{where,}$$

$\Delta A_{y\,nm}$ is the difference in the absorbance at any given wavelengths ($_{y\,nm}$) and ϵ is the millimolar extinction coefficient at *y* nm.

One unit of enzyme activity was defined as the amount of protein required to convert 1 nmole of substrate per minute unless stated in the figure legend.

2.11: Enzymatic estimation of organic acids

Organic acids levels determined were confirmed by using enzymatic analysis. Culture supernatants were filtered through 0.2µm nitrocellulose membrane and different aliquots were used to assay for pyruvic and citric acids.

2.11.1: Pyruvic acid

Culture supernatant was used as source of pyruvate in reaction with lactate dehydrogenase (LDH) following the rate of NADH utilization (Cocaign-Bousquet et al., 1996) with several modifications. The rate of reduction in absorbance of NADH at 340nm was proportional to the amount of pyruvic acid present. The assay mixture per cuvette contained following ingredients in 1ml: Tris-HCl (pH=7.5), 200mM; NADH, 0.12mM; LDH, 5 U; pyruvate (variable). The assay volume was adjusted using distilled water. The standard curve was prepared using known concentrations of Na-pyruvate

(4mM stock solution) while 5 and 10µl aliquots of the filtered culture supernatant were used to determine the extracellular pyruvic acid. Sample aliquots were selected so as to work within the linear range of standard curve.

2.11.2: Extracellular citric acid

Citric acid in the culture supernatants was analyzed using the method involving citrate lyase mediated cleavage of citrate to OAA which is subsequently utilized in MDH catalyzed reaction requiring NADH. Change in NADH absorbance would be proportional to citrate concentration (Petrarulo et al., 1995; Boehringer Mannheim/R-Biopharm Enzymatic BioAnalysis/Food Analysis Manual of Citric acid determination kit, Cat. # 10 139 076 035). The assay system per cuvette contained- 50mM phosphate buffer mix, 1.0ml; citrate lyase, 0.27 units (0.02ml of 13.3 units/ml stock); citric acid standard or test sample; 5U MDH; 0.1M NADH. Citric acid standards of 5µM, 10µM, 15µM and 20µM concentrations were used to generate standard curve. Difference in O.D. at 340nm after 4 minutes (time required for complete OAA utilization) of addition of citrate lyase was used for the calculations.

2.11.3: Intracellular citric acid

Pseudomonas transformant cultures grown on appropriate media were harvested in the late stationary phase and were pelleted by centrifuging at 9, 200x *g* for 2 minutes at 4°C. The pellet was washed with 1ml of 80mM phosphate buffer (pH=7.5) and the washed cells were suspended in the same buffer containing 20% glycerol. This homogenous cell suspension was subjected to sonication for maximum 1-1.5 minute in an ice bath to have complete cell lysis, followed by centrifugation at 9, 200x *g* and 4°C for 30 minutes to remove cell debris. The recovered supernatant was filtered through 0.2µm nitrocellulose membrane and frozen immediately till further analysis (Petrarulo et al., 195; Aoshima et al., 2003). This filtrate was used to estimate the amount of intracellular citric acid using the method described by Petrarulo et al (1995) with minor modifications.

According to this method citrate lyase converts citrate to OAA and acetate and the resultant OAA can rapidly react with phenylhydrazine at slightly acidic pH to form the corresponding phenylhydrazone which can absorb ultraviolet (UV) light. Increase in the absorbance at 330nm would be an expression of the citrate concentration. The assay

system per cuvette contained- 50mM phosphate buffer mix, 1.0ml; 246mM phenylhydrazine, 0.02ml; citrate lyase, 0.27U (0.02ml of 13.3 units/ml stock); citric acid standard or test sample. Buffer mix contained 50mM phosphate buffer (pH=6.5), 0.1mM $ZnSO_4.7H_2O$ and 0.2g/L sodium azide. Citric acid standards of 5μM, 7.5μM, 10μM, 15μM and 20μM concentrations were used to generate standard curve. Difference in $O.D._{330nm}$ after 3 minutes of addition of citrate lyase was used for the calculations. To calculate the intracellular citrate concentration (in mM), cellular volume was assumed to be 1.63μl mg dcw^{-1} (Emmerling et al., 1999).

$$\text{Intracellular concentration (mM)} = \frac{\text{X nmoles/ml}}{\text{Dry cell mass (mg)} \times C_f \times 1.63} \quad \text{where,}$$

X nmoles/ml is calculated as follows- $\dfrac{\Delta O.D._{330nm}/3\text{min}_{(test)} \times \text{Std (nmoles)}}{\text{Test aliquot (ml)} \times \Delta O.D._{330nm}/3\text{min}_{(Std.)}}$

C_f= concentration factor of the 25 ml cell culture

Dry cell mass (dcw) = $O.D_{600nm}$ x 0.382, where 0.382 is the factor correlating $O.D_{600}$ was with dry cell weight (Bugg et al., 2000)

2.12: Inoculation of mung beans (*Vigna radiata*)

Mung bean seeds were surface sterilized by treating with 0.1% (w/v) mercuric chloride for ~3-5 minutes followed by 5 rounds of thorough washing with 100ml freshly sterilized distilled water (Ramakrishna et al., 1991). These seeds were placed in autoclaved petri-dishes containing moist filter paper and were allowed to germinate in dark for 24h at room temperature. *Pseudomonas* P4 was inoculated in 10ml sterile LB broth and was allowed to grow at 30°C with overnight shaking. Fresh culture was harvested by centrifuging at 9, 200x *g* for 2 minutes; the pellet was aseptically washed twice with sterile normal saline and finally was re-suspended in 4 ml of normal saline (Ramos et al., 2000). Healthy germinated seeds were incubated with this cell suspension in a sterile petri-dish under aseptic conditions for 10-15 minutes. Root tips of hence obtained *Pseudomonas* P4 coated mung bean seeds were implanted with the help of sterile forceps in autoclaved sugar tubes containing 50 ml of solidified Murashige-Skoog's (MS) Agar with the desired phosphate and carbon sources. Plants were allowed to grow

at room temperature with sufficient light conditions for 5-7 days after which effect of *Pseudomonas* P4 inoculation was monitored in terms of overall plant growth.

MS medium was amended with 1% sucrose and 100mM glucose used as carbon sources while 1mg/ml RP was used as insoluble P source to replace KH_2PO_4 in the media, as and when required. RP containing media after autoclaving was cooled by continuous shaking (swirling) till the commencement of complete solidification so that the RP particles are homogenously distributed through the media. Effect of Al was checked in the presence of 400μM Al by supplemented in the form of $AlCl_3$.

2.13: Estimation of plasmid copy number

Single colonies of all the plasmid bearing *P. fluorescens* 13525 strains were inoculated independently in 3ml sterile LB broth with respective antibiotics in appropriate doses and cultures were allowed to grow under overnight shaking conditions at 30°C. Freshly obtained 3ml cultures were subjected to plasmid isolation by alkali lysis method (Sambrook and Russell, 2001) with elimination of phenol-chloroform purification step. The supernatant recovered after lysis and potassium acetate treatment was directly subjected to DNA precipitation by isopropanol, followed by 70% ethanol wash and final re-suspension in 40μl of TE (10mM Tris-HCl, pH=8.0 + 1.0mM EDTA, pH=8.0). Of this, 10μl DNA solution of each plasmid was linearized by treating with BamHI (as described in Section 2.4.4) in total system of 20μl. RNA interference was avoided by treating the DNA with 0.1mg/ml RNAase (Sambrook and Russell, 2001). Out of this, 10μl of each sample was used for agarose gel electrophoresis (Section 2.4.4) along with 500ng of lambda DNA digested with BstEII used as molecular weight marker. Resultant ethidium bromide-stained agarose gels were used for determination of plasmid copy numbers by fluorimetric densitometry (Lupski et al., 1986). Following formula was used to calculate the plasmid copy number in *P. fluorescens* 13525.

$$\text{Plasmid copy number/cell} = \frac{\text{Total Amount of DNA in 40μl (A) (ng)}}{\text{Total number of cells used (N) x Mass of the single plasmid (ng)}}$$

The band intensities of the linearized plasmids were determined by online free software Image J1.38x and were compared with those of known amount and size of

DNA molecular weight marker to calculate the total amount of DNA (**A** in the formula) present in total 40µl (. The $O.D._{600nm}$ of each culture was converted to cell number by the relation 1 O.D=$1.5x10^9$ cells/ml (Koch et al., 2001) to determine the total number of cells in 3ml (**N** in the formula) for extracting the plasmid. Mass of a single plasmid was determined based on its size using the correlation 1 nucleotide bp=610Da and the resultant mass in Daltons was converted to ng.

Note:

(i)All the centrifugations were carried out using Heraeus Biofuge *Stratos*. The spectrophotometric measurements of $O.D._{600nm}$ were carried out using Helios γ spectrophotometer, Thermospectronics (path length of 1cm) while the enzymatic determinations were done using JASCO V-530 spectrophotometer. The cell-lysis by sonication was achieved using Branson Sonifier Model 450. HPLC equipment from Merck, India was used for organic acid analysis.

(ii) The fine chemicals like NADH, NADP, acetyl-CoA, PEP potassium salt, DCIP, pure enzymes like MDH, LDH and citrate lyase were obtained from Sigma Chemicals Pvt. Ltd. while other routine chemicals and solvents were procured from Hi-media Laboratories, Qualigens Fine Chemicals, Sisco Research Laboratories and Merck, India.

(iii) All molecular biology grade reagents and kits were procured from Hi-Media Laboratories, India; Sisco Research Lab (SRL), India; Bangalore Genei Pvt. Ltd., India, or Sigma Chemical Pvt. Ltd. until and unless specified.