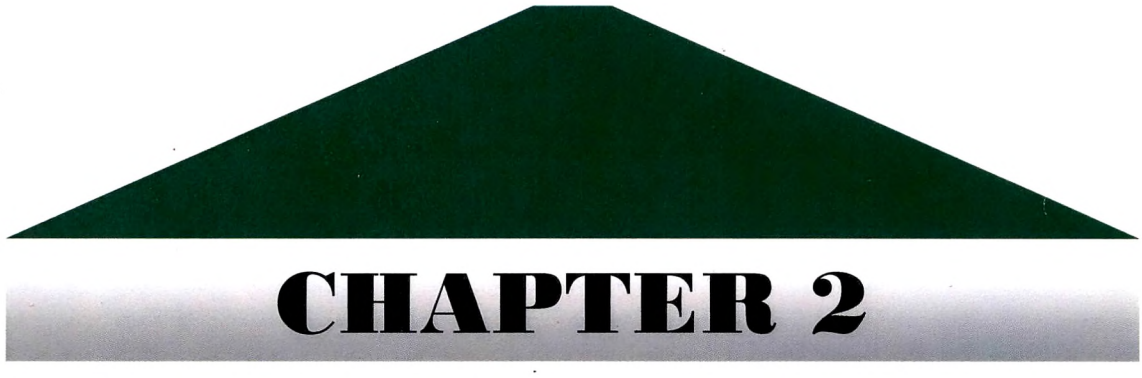
MATERIALS and METHODS



CHAPTER 2

2 Materials and Methods

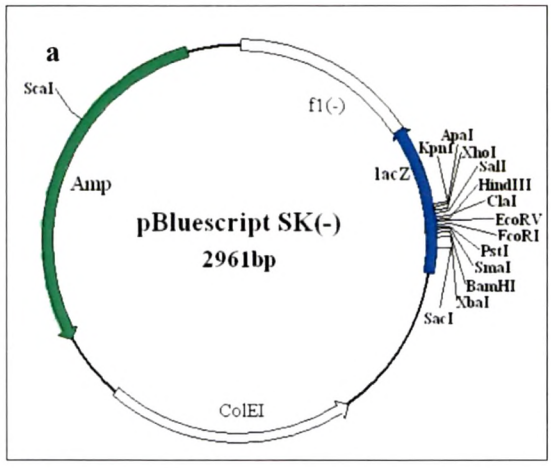
2.1 Bacterial strains / Plasmids

All the plasmids use in the present study and their restriction maps are given in **Table 2.1** and **Fig. 2.1**.The wild type and genetically modified *E. coli* and *Pseudomonas* strains are listed in **Table 2.2 and 2.3**. *E. coli* DH5α was used for all the standard molecular biology experiments wherever required. The NADH insensitive *cs* mutant plasmid was a generous gift from Prof Harry Duckworth, University of Manitoba, Canada. The *glt*A (citrate synthase gene) mutant of *E. coli* was obtained from *E. coli* Genetic Stock Center. The mini Tn7 delivery and helper plasmid for genomic integration was a generous gift from Prof. Soren Molin, Technical University of Denmerk.

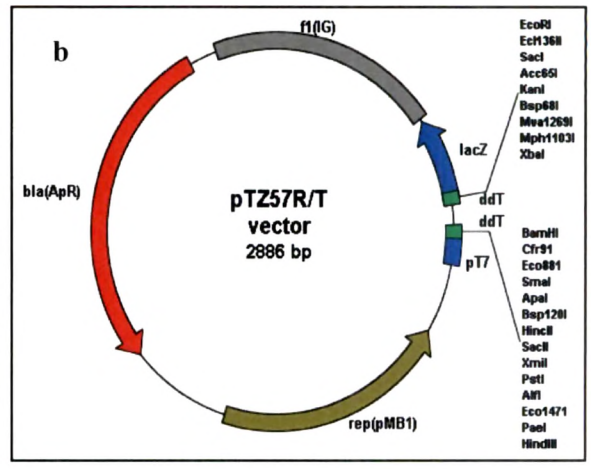
Table 2.1: List of plasmids used in the present study.

Plasmids	Features	Reference
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r	Hester et al., 2000
pUCPM18 Gm	pUC18 derived Broad-Host-Range vector; Ap^r Gm^r	Ch 3
pAB8	pUCPM18 with *kan*^{r} gene; Amp^r, Km^r	Buch et al., 2009
pBluescript/KS	Cloning vector for *E. coli*; Ap^r	Sambrook et al, 2000
pTZ57R/T	T vector	MBI Fermentas
pTZ57R/T *R163L*	T vector with *E. coli* NADH insensitive gene R163L	Ch 3
pTZ57R/T *K167A*	T vector with *E. coli* NADH insensitive gene R163L	Ch 3
pTZ57R/T *Y145F*	T vector with *E. coli* NADH insensitive gene R163L	Ch 3
pTZ57R/T *CitM*	T vector with *E. coli* NADH insensitive gene R163L	Ch 4

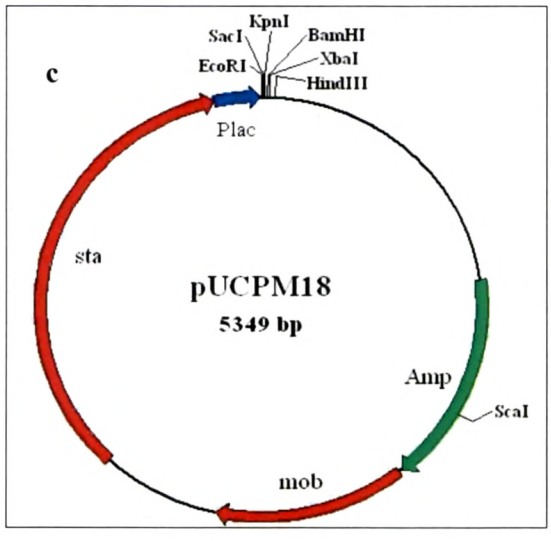
pCS-Ec	pBluescript with *cs* gene of *E. coli*; Ap^r	Delhaize, E
pAB7	pUCPM18 with *E. coli cs* gene under P_{lac} and *kan*^{r} gene; Ap^r, Km^r	Buch et al., 2009
R163L	NADH insensitive cs gene	Duckworth, H., 2003
K167A	NADH insensitive cs gene	Duckworth, H., 2003
Y145F	NADH insensitive cs gene	Duckworth, H., 2003
pR163L	pUCPM18 with NADH insensitive cs gene, Amp^r, Km^r	Ch3
pK167A	pUCPM18 with NADH insensitive cs gene, Amp^r, Km^r	Ch3
pY145F	pUCPM18 with NADH insensitive cs gene, Amp^r, Km^r	Ch3
pCitC	pUCPM18 with *CitC* gene, Amp^r, Gm^r	Ch4
pCitM	pUCPM18 with *CitM* gene, Amp^r, Gm^r	Ch4
pYC	pUCPM18 with Y145F and CitC gene under p*lac*, Amp^r, Gm^r	Ch4
AKN68	pUXBF-13 , Helper plasmid providing the Tn7 transposase proteins	Lambertsen et al., 2004
AKN69	Mini Tn7(Gm)$P_{A1/04/03\text{-}eyfp}$, plasmid Amp^r,Gm^r, Cm^r	Lambertsen et al., 2004
pYC Int	p*lac yc* operon cloned into AKN69 plasmid, Amp^r,Gm^r, Cm^r	Ch5



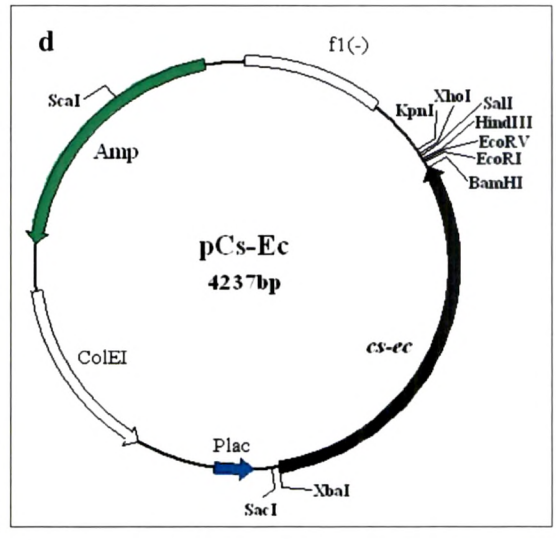
pBluescript KS: cloning vector Amp^r



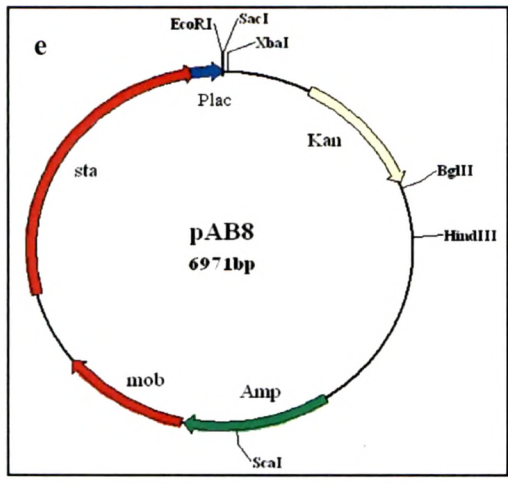
pTRZ57/R: TA cloning vector Amp^r



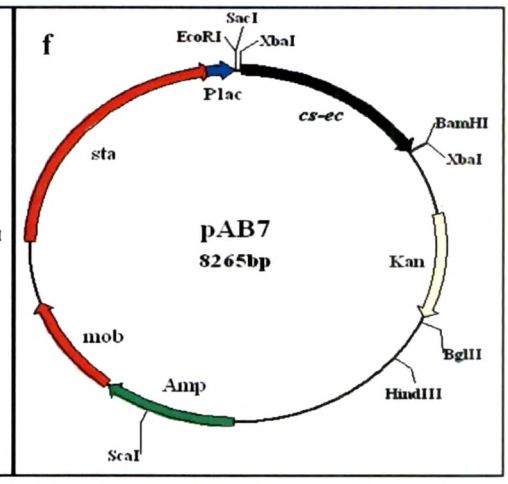
Generous gift from Dr. J. R. Sokatch,
University of Oklahoma Health Sciences



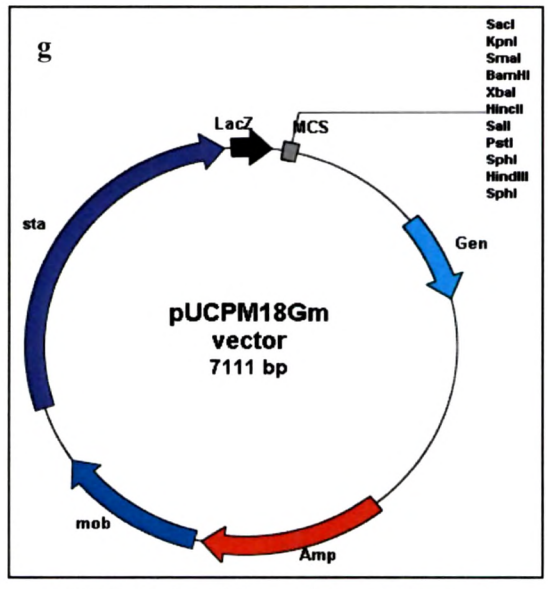
Generous gift from Dr. Delhaize, E., CSIRO
Plant Industry, Australia



pUCPM18 plasmid containing kanamycin resistance gene (Buch et al., 2008)



pUCPM18 plasmid containing wild type *E. coli* citrate synthase gene, Km^r (Buch et al., 2008)



pUCPM18 plasmid containing gentamycin resistance gene (Buch et al., 2008)

Fig. 2.1: Restriction maps of the plasmids used in this study. (a) and (b) are standard pBluescript and TA plasmid used for conventional cloning in *E. coli*.,(c),(e) and (g), are the broad-host-range vectors used for expression of heterologous genes under *lac* promoter in *P. fluorescens*., (d) and (f) are plasmids used to obtain *cs*, genes.

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-controling protein (West et al., 1994).

Table 2.2: List of *E. coli* strains used in the present study

Bacterial Strains	Genotype	Reference
E. coli DH5α	*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
DH5α (pAB8)	*E. coli* DH5α with pAB8 plasmid; Ap^r, Km^r	Buch et al
DH5α (pAB7)	*E. coli* DH5α with pAB7 plasmid; Ap^r, Km^r	Buch et al
DH5α (pR163L)	*E. coli* DH5α with pR163L plasmid; Ap^r, Km^r	Ch 3
DH5α (pK167A)	*E. coli* DH5α with pK167A plasmid; Ap^r, Km^r	Ch 3
DH5α (pY145F)	*E. coli* DH5α with pY145F plasmid; Ap^r, Km^r	Ch 3

W620 (pAB8)	*E. coli* W620 with pAB7 plasmid; Str^r, Ap^r, Km^r	Ch 3
W620 (pAB8)	*E. coli* W620 with pAB8 plasmid; Str^r, Ap^r, Km^r	Ch 3
W620 (pR163L)	*E. coli* W620 with (pR163L) plasmid; Str^r, Km^r	Ch 3
W620 (pK167A)	*E. coli* W620 with (pK167A)plasmid; Str^r, Km^r	Ch 3
W620 (pY145F)	*E. coli* W620 with (pY145F) plasmid; Str^r, Km^r	Ch 3
S17.1 (pAB7)	*E. coli* S17.1 with pAB7 plasmid; Tmp^r, Amp^r, km^r	Ch 3
S17.1 (pAB8)	*E. coli* S17.1 with pAB8 plasmid; Km^r, Amp^r,	Ch 3
S17.1 (p R163L)	*E. coli* S17.1 with pAB8 plasmid; Km^r, Amp^r,	Ch 3
S17.1 (p K167A)	*E. coli* S17.1 with pAB8 plasmid; Km^r, Amp^r,	Ch 3
S17.1 (p Y145F)	*E. coli* S17.1 with pAB8 plasmid; Km^r, Amp^r,	Ch 3
E.coli JM105 AKN69	*E. coli* JM105 with AKN69 plasmid Gm^r, Cm^r	Klausen et al., 2003
E.coli SM10λpir AKN68	*E. coli* SM10λpir with AKN68 plasmid	Bao et al., 1991

Amp=Ampicillin; Km=Kanamycin; Gm=Gentamycin; Str^r =Streptomycin; Cm= Chloramphenicol;

^{r} = resistant.

Table 2.3: List of *Pseudomonas* strains used in the present study

P. fluorescens **Strains**	**Genotype/Relevant characteristics**	**Reference**
ATCC 13525	Wild Type	Ch5, Ch6
PfO-1	Wild Type	Ch3-Ch6
Pf-5	Wild Type	Ch5,Ch6
CHAO-1	Wild Type	Ch5,Ch6
P109*	Wild Type	Ch5,Ch6
Fp315*	Wild Type	Ch5,Ch6
PfO-1 (AB7)	*P. fluorescens* PfO-1with pAB7 plasmid; Tc^r	Ch3
PfO-1 (pAB8)	*P. fluorescens* PfO-1with pAB8 plasmid; Tc^r	Ch3
PfO-1 (pR163L)	*P. fluorescens* PfO-1with pR163L plasmid; Km^r	Ch3
PfO-1 (pK167A)	*P. fluorescens* PfO-1with pK167A plasmid; Km^r	Ch3
PfO-1 (pY145F)	*P. fluorescens* PfO-1with pY145F plasmid; Km^r	Ch3
PfO-1 (pYFpCitC)	*P. fluorescens* PfO-1with pY145F and pCitC plasmid; Amp^r,Km^r Gm^r	Ch4
PfO-1 (pYFpCitM)	*P. fluorescens* PfO-1with pY145F and pCitM plasmid; Amp^r,Km^r Gm^r	Ch4
PfO-1 (pYC)	*P. fluorescens* PfO-1with pYC plasmid; Gm^r	Ch4
PfO-1 (Int)	*P. fluorescens* PfO-1 genomic integrant Amp^r, Gm^r	Ch5
ATCC 13525 (pYC)	*P. fluorescens* ATCC 13525 with *pYC* plasmids;Amp^r, Gm^r	Ch6
ATCC 13525 (Int)	*P. fluorescens* ATCC 13525 genomic integrant Amp^r, Gm^r	Ch5,Ch6

Pf-5(pYC)	*P. fluorescens* Pf-5 with *pYC* plasmids; Amp^r, Gm^r	Ch6
Pf-5 (Int)	*P. fluorescens* Pf-5 genomic Integrant Amp^r, Gm^r	Ch5, Ch6
CHAO-1(pYC)	*P. fluorescens* CHAO-1 with *pYC* plasmids; Amp^r,Gm^r	Ch6
CHAO (Int)	*P. fluorescens* CHAO-1 genomic Integrant Amp^r, Gm^r	Ch5, Ch6
P109 (pYC)	*P. fluorescens* P109 with *pYC* Amp^r, Gm^r	Ch6
P109 (Int)	*P. fluorescens* P109 genomic Integrant Amp^r, Gm^r	Ch5, Ch6
Fp315(pYF)	*P. fluorescens* Fp315with *pYF* Amp^r, Km^r	Ch5
Fp315(pYC)	*P. fluorescens* Fp315with *pYC* Amp^r, Gm^r	Ch6
. Fp315(Int)	*P. fluorescens* Fp315 genomic Integrant Amp^r, Gm^r	Ch5, Ch6

Km^r = Kanamycin; Gm^r = Gentamycin; resistant. *P. fluorescens* strains including 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 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

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.

Table 2.4: Recommended dozes of antibiotics used in this study (Sambrook et al 2000).

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	-

The antibiotic dozes were maintained same for both *E. coli* and pseudomonads. All the antibiotics were prepared in sterile distilled water 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 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.

2.2.2 M9 minimal medium

Composition of M9 minimal broth was according to Sambrook et al (2000) 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.

2.2.3 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.

2.2.4 Pikovskaya's (PVK) Agar

The media composition included Ammonium sulphate, 0.5g/L; Calcium phosphate, 5.0g/L; Dextrose, 10.0g/L; Ferrous 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.

2.3 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 et al., 2000) 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

The routine molecular biology experiments were carried out following the standard protocol of Sambrook et al.(2004) with modifications as when necessary

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 et al., 2000).

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 et al., 2000). 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 et al (2000).

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 (micro-centrifuge 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 enzymes (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

Table 2.5: Composition of SDS-PAGE reagents (Sambrook et al, 2000)

(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	3.02 gm
Bisacrylamide	0.4 gm	SDS	0.2 gm	SDS	0.20 gm
D. H_2O	Till 50 ml	D. H_2O	Till 50 ml	D. H_2O	Till 50ml

(D) Tank Buffer (pH 8.3)		**(E) Sample Loading buffer (2X)**		**(F) Other reagents**	
Tris base	6.0 gm	SDS	4%	APS	10% (fresh)
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	Bromophenol blue	0.05%w/v	Sigma protein molecular weight marker SDS6H2-(30,000-200,000).Used 14µg total protein/well	
Adjust the pH with HCl		β-mercapto ethanol	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		

SDS-PAGE slab gel electrophoresis was carried out using 8% acrylamide gel by following the procedures described by Sambrook et al (2000). After electrophoresis the gel was stained using the staining solution (**Table 2.5**) for approximately 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 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.

2.6 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.

2.7 Physiological experiments

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

2.7.1 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.

2.7.2 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.

2.7.3 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 0.02% orthophosphoric acid was used as mobile phase. at a flow rate of 0.8 ml min^{-1} and the column effluents were monitored using a UV detector at 210 nm. 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. The organic acids were quantified by reference to the peak areas obtained for the authentic standards for Gluconic acid, 2 keto gluconic acid, Lactic acid, Oxalic acid, Maleic acid, Succinic acid, Formic acid, Citric acid, Malonic acid, Acetic acid, Propionic acid, Pyruvic acid and Tartaric acid (Merck, Germany and Hi-Media Laboratories and Sigma Pvt. Ltd.). The values were presented as the mean of three replicates. 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 (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

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

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

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

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.

2.8 Enzyme assays

2.8.1 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 $MgCl_2$.

2.8.2 Enzyme Assay Protocols

2.8.2.1 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., 2005).

2.8.2.2 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.

2.8.2.3 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.

2.8.2.4 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: DCIP, sodium salt, 0.05mM; phenazine methosulfate, 0.66mM; sodium azide, 4mM; Tris-Cl buffer (pH 8.75), 16mM; D-glucose, 66mM; whole cells, and distilled water to 3.0ml.

2.8.2.5 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.

2.8.2.6 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 $mM^{-1}cm^{-1}$.

2.8.2.7 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

$$\text{Units/mg} = \frac{\Delta A_{y\ nm}/\text{min}}{\epsilon \times \text{enzyme (sample) aliquot (ml)} \times \text{Total protein (mg/ml)}}$$

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.

2.9 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.

2.9.1 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.

2.9.2 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.

2.9.3 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., 1995; 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 cdw^{-1} (Emmerling et al., 1999).

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

where,

X nmoles/ml is calculated as follows-

$$\frac{\Delta O.D._{330nm}/3\text{min}_{(test)} \times \text{Std (nmoles)}}{\text{Test aliquot (ml)} \times \Delta O.D._{330nm}/3\text{min}_{(Std.)}}$$

$\mathbf{C_f}$= 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)

2.10 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 $AlCl_3$.

2.11 Pot experiments- Interaction with mung beans (*Vigna radiata* GM4)

2.11.1 Preparation of inoculums:

A loopful of each of the seven bacterial strains was inoculated into a separate 50 ml aliquot of PB and incubated at 28±2°C for 4 days in an incubator shaker.

2.11.2 Growth Analysis: Above ground parts

Plant height:

Measurements of plant height were taken at the crop maturity in three replicates of five plants each. Plant height was measured from the soil line to shoot tip. Plant height was measured by placing the plant on a centimeter scale. Plant population was uniform at the time of maturity of crop.

Leaf area:

The area of the leaf was taken by pressing the leaf on millimeter graph paper and tracing the exact outline. The area was measured by weighing the graph cuttings of the leaf (Shine et al., 2011). The calibration curve was prepared by weighing a 0–100 cm^2 area of graph paper.

Dry matter production

At maturity fifteen plants (five plants from each replicate) were randomly selected and the data on component part dry weights (leaf, root and shoot) were recorded. Leaf, stem and root were dried at 60 °C for 72 hours. Pods were separated and the total numbers of branches were recorded. Dry weights of seed components were recorded after drying at 35–40 °C for 10 days.

2.11.3 Growth Analysis: Below ground parts

Root length:

The measurements of root length were done in plants of all the eight varieties at 40 and 50 DAE. Roots were taken out carefully, washed and measured against a cm scale.

Root fresh and dry weight

Plant roots with nodules at each sampling were washed and dried on filter paper and weighed for the fresh weight. For dry weight roots were dried at 60°C for 72 hours and weighed.

Number of root nodules/plant

Nodules on each root were counted carefully and recorded per plant.

Nodule fresh weight

Nodules were taken out at each sampling, washed and dried on filter paper. Weight was recorded in gm/plant for all treatments.

2.11.4 Antioxidant Enzymes / ROS scavenging enzyme activity:

(1) Superoxide Dismutase (SOD)

Two hundred mg of leaves were homogenized in a pre-chilled mortar and pestle under ice cold condition using 3.0 ml of extraction buffer, containing 50 mM sodium phosphate buffer (pH 7.4), 1 mM EDTA and 1% (W/V) polyvinylpyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatants used for the assay (Costa *et al.*2002).

Total SOD (EC 1.15.1.1) activity was measured spectrophotometrically based on inhibition in the photochemical reduction of nitroblue tetrazoilum (NBT). The 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8),13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA and 0.1 ml enzyme extract, riboflavin was added last (Van Rossun *et al.*1997).After addition of all these components and mixing, test tubes were placed on stand 30 cm below a light source consisting of four 15-w fluorescent lamps. The photochemical inhibition was allowed to happen for 10 minutes and stopped by switching off the light source. The photoreduction in NBT was measured as increase in absorbance at 560 nm. Blanks and controls were run the same way but without illumination and enzyme,

respectively. One unit of SOD was defined as the quantity of enzyme required to inhibit the reduction of NBT by 50% in a reaction mixture. Enzyme unit of SOD was calculated according to formula given by Constantine and Stanley (1977).

$$\text{SOD unit} = \frac{\text{O.D. control (without enzyme)}}{\text{O.D. sample}} - 1 \text{ x } \frac{1}{\text{enzyme conc. (g)}}$$

$$\text{SOD U/g protein} = \frac{\text{SOD unit}}{\text{mg/g protein}}$$

(2) Catalase

Two hundred mg of acetone powder homogenized with a pre-chilled mortar and pestle under ice cold condition in 2.0 ml of extraction buffer, containing 0.1 M sodium phosphate buffer (pH 7.2) with the addition of 1 mM EDTA and 1% (w/v) polyvinylpyrolidone (PVP) and a pinch of activated charcoal. The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatants were used for the assay (Mahatma *et al.* 2011).

Total catalase (EC 1.11.1.6) activity was determined in the supernatants by measuring the decrease in absorption at 240 nm as H_2O_2 ($\varepsilon = 39.4\ mM^{-1}\ cm^{-1}$) got consumed according to the method of Aebi (1984) and enzyme activity expressed as mmol H_2O_2 oxidized $min^{-1}\ g^{-1}$ protein. The 3 ml assay mixture contained 50 mM sodium phosphate buffer (pH 7.0), 30mM H_2O_2 and 50 µl enzyme extract. Enzyme unit of CAT was defined as:

$$\text{Mmol/min/g protein} = \frac{\Delta \text{ O.D.}}{\text{Enzyme conc.(g) x mg/g protein x } \varepsilon}$$

Where, ε = Extinction coefficient

Enzyme conc. (g) = Amount of enzyme in 3 ml reaction mixture

(3) Guaiacol Peroxidase (POX)

Two hundred mg of leaves were homogenized in a pre-chilled mortar and pestle under ice cold condition in 2.0 ml of extraction buffer, containing 0.1M sodium phosphate buffer (pH 7.2) with the addition of 1 mM β-mercaptoethanol and 1% (w/v) polyvinylpyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatants used for the assay.

POX (EC 1.11.1.7) activity was determined in the supernatants of centrifuged homogenates by measuring the increase in absorption at 470 nm due to the formation of tetraguaiacol (ϵ=26.6$mM^{-1}cm^{-1}$) in a reaction mixture containing 50 mM sodium phosphate buffer pH 7.0, 0.1 mM EDTA, 0.05 ml enzyme extract, and 10 mM H_2O_2 (Costa *et al.*2002).

(4) Ascorbate peroxidase (APX)

Maize leaf samples were crushed with chilled acetone in pre-chilled mortar-pestle. To obtain fine acetone powder the homogenates were filtered and stored immediately at -20° C. Hundred mg of acetone powder was then homogenized in a pre-chilled mortar and pestle under ice cold condition using 2.0 ml of extraction buffer, containing 0.1 M sodium phosphate buffer (pH 7.2), and 1% (w/v) polyvinylpyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatants used for the assay (Mahatma et al., 2011).

APX (EC 1.11.1.11) activity was measured immediately in fresh crude extracts and assayed by procedure described by Nakano and Asada (1981). Three ml of the reaction mixture contained 50 mM sodium phosphate buffer pH 7.0, 0.1 mM H_2O_2, 0.5 mM ascorbic acid, 0.1 mM EDTA and 0.1 ml enzyme extract. The hydrogen peroxide dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (ε = 2.8 mM^{-1} cm^{-1}). The enzyme unit of APX was defined similar to that of CAT activity as described.

2.11.5 Acid phosphatase

1 g of leaves were homogenized in 10ml ice cold 50mM citrate buffer (pH5.3) in a prechilled pestle and mortar.The homogenate is filtered through four layer of cheese clothe and the filtrate is centrifuged at 10,000g for 10 min. The supernatant is ready to use as enzyme source.

Acid phosphatase (3.1.3.2) hydrolyzes a number of phosphomonoesters and phosphoproteins.The enzyme phosphatase hydrolyzes p-nitrophenol phosphate. The released p-nitrophenol is yellow in colour in alkaline medium and is measured at 405nm. The optimum pH for acid and alkaline phosphatase are 5.3 and 10.5 respectively.3ml of substrate solution containing 1.49g EDTA,0.84 g citric acid and 0.03 g p-nitrophenyl phosphate in 10 ml water(pH 5.3)incubated at 37 for 5 min.0.5 ml of enzyme extract added and mixed well.0.05 ml sample immediately removed from the mixture and mixed with 9.5 ml of sodium hydroxide solution(0.085N).This correspond to zero time assay(blank). The remaining solution is incubated for 15 min at 37. 0.5 ml sample were drawn from the mixture and mix wit 9.5 ml of sodium hydroxide solution.Absorbance of the balk and incubated tubes were measured at 405 nm.Take 0.2-1.0 ml (4-20mM) of the standard ie. 100mM p-nitrophenol dilute to 10 ml with NaOH solution.Read the colour and draw the standard curve. Specific activity is expressed as m moles of p-nitrophenol released per min per mg protein.

2.11.6 Nitrate Assimilating Enzyme

Nitrate Reductase Activity. (Bhatnagar *et al.* 2007)

Two hundred mg leaf tissues were cut into small slices, and then suspended in reaction mixture (1 ml 5 % isopropanol, 1 ml 1 M potassium nitrate and 3 ml phosphate buffer, 0.1 M, pH 7.5). Incubation was carried out at 30^0C for two hrs in dark. After incubation 1.0 ml aliquots were withdrawn, to which 0.2 ml sulfanilamide, 0.2 ml of 0.2% NEDH solution was added. After 20 minutes, 4.0 ml of millipore water was added to each test tube. The intensity of the colour developed was recorded at 570 nm in a spectrophotometer. A standard curve was prepared using sodium nitrite in the concentration

range of 1-5 µg. Enzyme units are expressed in terms of µg nitrite released per minute per g tissue and calculated as:

$$\frac{\text{Conc. of µg nitrite derived from graph for unit O.D. value X O.D. value of sample}}{0.04 \times 120}$$

(1.0 ml reaction mixture is equivalent to 40 mg tissue)

2.11.7 Estimation of Water Soluble Protein Content

Protein concentration of each enzyme extract was estimated by method of Lowry *et al.* (1951).

(a) Reagents for Lowery's Method

(i) Solution A: 2% Na_2CO_3 in 0.1 N NaOH

(ii) Solution B: (a) 1% $CuSO_4.5H_2O$ solution

(b) 2% sodium potassium tartarate solution

Working solution of B : Prepared fresh before use by mixing equal volume of solution B (a) and B (b).

(iii) Solution C: Prepared fresh before use by mixing 50 ml of solution A and 1 ml of working solution of B.

(iv) Solution D: Folin & Ciocalteu reagent (1N).

Procedure (Folin Lowery's Method)

Enzyme extracts (25 µl) were taken in test tube and volume was made up to 1 ml with millipore water. A tube with 1 ml of water served as blank. Five ml of solution C was mixed by vortexing and kept for 10 min. Then 0.5 ml of solution D (Folin & Ciocalteu reagent) was added and vortexed. The tubes were allowed to stand at room temperature for 30 min. Absorbance was read at 660 nm. A standard curve was prepared using bovine serum albumin (BSA) in the concentration range of 10-80 µg.