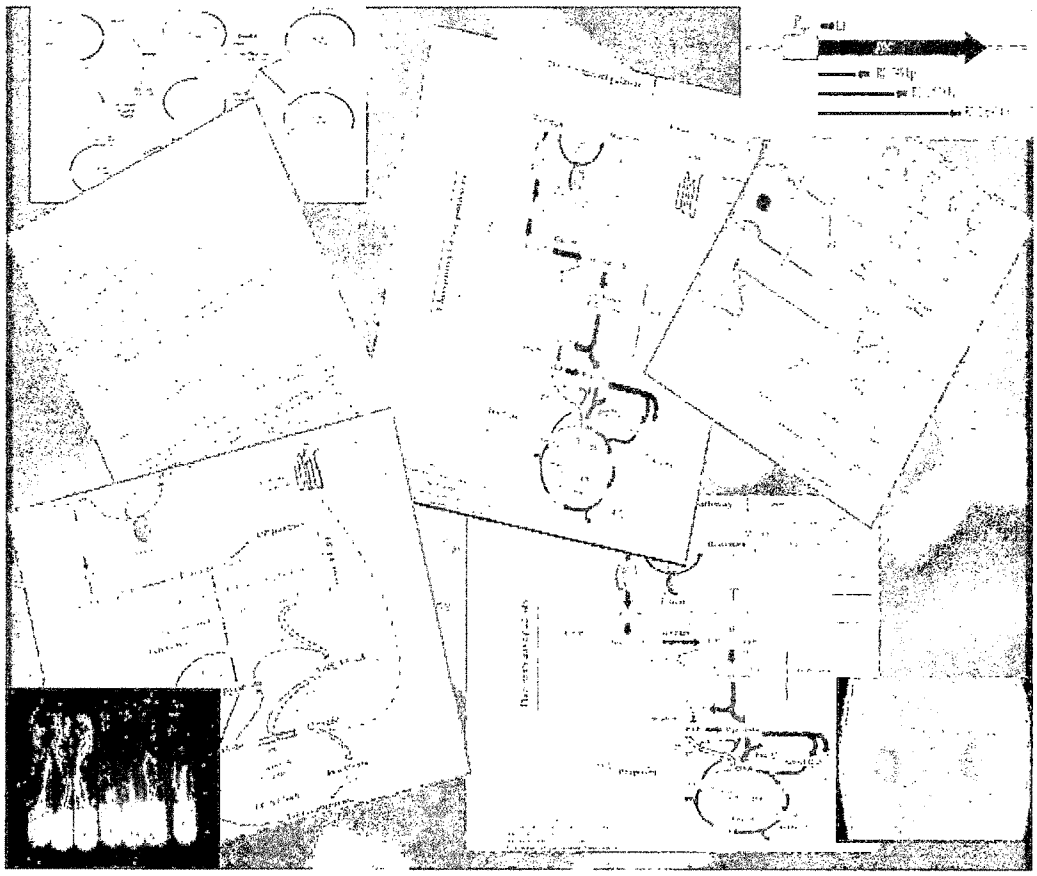

SUMMARY

"A conclusion is the place where you got tired of thinking."
-Arthur Bloch

Pseudomonads possess high ecological advantage due to their bioremediation ability and agricultural importance attributed to their effectiveness as biocontrol agents and biofertilizer. Hence, understanding its central carbon metabolism and regulatory mechanisms is necessary to enhance their potential. Foremost challenge in choosing *Pseudomonas* as a model system for engineering the central carbon metabolism was lack of integrated information on genetic, metabolic and regulatory networks, particularly when they were known to vary significantly amongst species. Furthermore, carbon metabolism in *Pseudomonas* is distinct from the very well explored *E. coli*. However, genome sequencing of *Pseudomonas aeruginosa* PAO1, *P. aeruginosa* UCBPP-PA14, *P. putida* KT2440, *P. putida* F1, *P. putida* GB-1, *P. fluorescens* Pf-5 and *P. fluorescens* PfO-1 and other experimental details facilitated the understanding of carbon metabolism.

Unlike *E. coli*, glucose is not the preferred carbon source in pseudomonads. Pseudomonads lack the phosphoenolpyruvate dependant phosphotransferase system (PEP-PTS) for glucose uptake and phosphofructokinase (PFK) of the traditional EMP mediated glucose catabolism. Instead, glucose catabolism in pseudomonads principally occurs by Entner-Doudoroff (ED) pathway in which 6-phosphogluconate (6-PG) is the key metabolite. Conversion of glucose to 6-PG can occur by periplasmic direct oxidation pathway and intracellular phosphorylative oxidation pathway. The crucial metabolic link between the glycolytic/gluconeogenic pathways and TCA cycle is the **PEP-Pyruvate-Oxaloacetate (OAA)** node (anaplerotic node) which acts as a key switch governing the balance between energy generation and anabolism.

The present study dealt with fluorescent pseudomonads as the model organism for investigating the flexibility/rigidity of the central glucose metabolism at the anaplerotic node of *Pseudomonas* and at the junction of citric acid formation from OAA and acetyl-CoA, by the overexpression of phosphoenolpyruvate carboxylase (*ppc*) and citrate synthase (*cs*) genes, respectively. Since, the central carbon metabolism is an energy intensive process fuelled by high energy co-substrates like ATP/PEP/Acetyl-P the genetic modifications in presence of low Pi might result into much more profound effects on the overall cellular physiology and metabolism, especially *ppc* overexperession as PPC reaction per se liberates free Pi. Pseudomonads have the potential to solubilize mineral phosphates by organic acid secretion; hence understanding the effect of above mentioned genetic modifications on the P-solubilzing ability of pseudomonads becomes

necessary. Alternative strategy of investigating the control of central carbon metabolism involved determining the biochemical basis of organic acid secretion in P-solubilizing *Pseudomonas* strains.

The recombinant pAB3 plasmid was developed that contained *ppc* gene of *Synechococcus elongatus* PCC 6301 under *lac* promoter of a pseudomonad stable vector pUCPM18 with tetracycline resistance gene. The pAB3 plasmid complemented the phenotype of *E. coli ppc* mutant suggesting expression of a functional PPC protein. Expression of *ppc* gene from constitutive *lac* promoter resulted into ~12 fold increase in PPC activity in *P. fluorescens* 13525 harboring pAB3 [*Pf* (pAB3)] when grown on M9 minimal medium with excess glucose. Increased PPC activity did not alter the specific growth rate, specific total glucose utilization rate and total amount of glucose utilized as compared to the control but reduced glucose consumption. However improved biomass yield in response to increased PPC activity in spite of high inherent PYC activity suggested that PPC has an anaplerotic role in *P. fluorescens* 13525. Reduction in acetic and pyruvic acid secretion indicated a decrease in metabolic overflow mechanism suggesting an improved metabolic status. These results were very similar to those reported for *E. coli*. Increased GDH activity and gluconic acid yield in *Pf* (pAB3) indicated increased contribution of direct oxidation pathway in response to increase in PPC activity.

On the other hand, specific growth rate of *Pf* (pAB4) control increased with Pi levels. At 0.1mM Pi no media acidification was seen while from 1mM onwards organic acid secretion was observed and the time required to acidify the medium (to pH<5) decreased with increase in Pi. Accordingly, 1mM Pi (TrP1 medium with 1mM KH_2PO_4) was used as low Pi condition as compared to M9 medium (~50mM Pi; high Pi). On low Pi, the specific growth and total glucose utilization rates of *Pf* (pAB4) were drastically reduced with a 24% increase in total glucose utilization as compared to that on high Pi. Direct oxidation pathway catabolized almost half of the total glucose used by *Pf* (pAB4) under low Pi unlike on high Pi where contribution of direct oxidation pathway was only ~11% and most of the glucose was used by phosphorylative oxidation. These results are in agreement with the higher GDH and lower PYC activities on TrP1 medium. G-6-PDH activity on TRP1 and M9 media was similar while reduced ICDH activity on TrP1

medium indicated low TCA cycle operation explaining low growth rates. These observations suggested that Pi limitation led *Pf* (pAB4) to minimize energy intensive processes and favoured ATP generating reactions.

Under these low Pi conditions, ~14 fold increase in PPC activity in *Pf* (pAB3) enhanced the biomass yield without affecting the aerobic growth and total glucose utilization rates. Glucose consumption increased by about 22% with concomitantly reduced GDH and increased G-6-PDH activities indicating increased operation of intracellular phosphorylative oxidation pathway for glucose catabolism as compared to the direct oxidation pathway. Additionally, decreased gluconic acid and increased pyruvic acid yields demonstrated that on low Pi, *ppc* overexpression favored phosphorylative glucose oxidation, probably because of Pi liberation in PPC reaction. Thus, *ppc* overexpression in *P. fluorescens* apart from enhancing the anaplerotic pathways, interestingly and typically redistributed the glucose flux between phosphorylative and direct oxidation pathways of glucose catabolism in a Pi dependant manner. This effect was quite distinct from the effects of *ppc* overexpression in *E. coli* and *C. glutamicum*. Moreover, our results demonstrated significant influence of Pi levels on the glucose distribution between the two catabolic pathways, which earlier has been shown to be affected by glucose and oxygen availability as well as temperature.

Citrate synthase (CS) is a non-redundant enzyme governing the carbon flux into TCA cycle and is responsible for citric acid production. The *E. coli cs* gene was incorporated under *lac* promoter of pUCPM18 with kanamycin resistance gene to generate pAB7 and the functionality of the CS protein was confirmed by complementation *of E. coli* W620 (*cs* mutant). CS activity increased upto about 2 fold in *Pf* (pAB7) grown on M9 minimal medium containing 100mM glucose. *cs gene* overexpression did not alter the specific growth rate, glucose utilization rate and biomass yield but reduced the glucose consumption. Increased GDH and PYC activities in response to elevated CS activity, respectively suggested increased contribution of direct oxidation pathway towards total glucose catabolism and increased requirement of OAA which is one of the substrates for CS. G-6-PDH, ICL and ICDH activities remained unaltered. Increased gluconic and acetic acid yields with concomitantly reduced pyruvic acid yields were in agreement with the observed metabolic alterations. Interestingly, *Pf* (pAB7) accumulated upto ~2 fold higher intracellular citric acid and secreted ~1.3mM

citric acid extracellularly which was ~20 fold higher than its control *Pf* (pAB8). Similar results were obtained with another recombinant pAB9 plasmid which contained *cs* gene under *lac* promoter of pBBR1MCS-2 and differed from pAB7 in terms of copy number. However, CS activity in *Pf* (pAB9) increased only by ~1.6 fold and citric acid was not detected in the culture supernatants.

P. fluorescens 13525 simultaneously overexpressing *ppc* and *cs* genes from pAB3-pAB7 [*Pf* (pAB37)] and pAB3-pAB9 [*Pf* (pAB39)] were developed, both of which showed comparatively lesser increase in PPC and CS activities as compared to the transformants independently expressing these genes. The specific growth and glucose utilization rates, glucose consumption and biomass yield as well as the GDH, PYC, G-6-PDH, ICL and ICDH activities remained unaffected which was in accordance with the unaltered yields of gluconic, pyruvic and acetic acids. Probably the effects exerted by the overexpressed genes were masked by much more profound effects exerted by the nature and the copy number of the plasmids.

In this context, presence of pUCPM18 based plasmids (pAB4 and pAB8) extensively altered the *P. fluorescens* 13525 metabolism unlike the pBBR1MCS-2 based plasmids, despite their comparatively lower copy number. Presence of pAB4 and pAB8 independently increased the direct oxidation pathway in *P. fluorescens* 13525 as compared to the wild type, which further increased when pAB4 and pAB8 were simultaneously present. Conversely, presence of pBBR1MCS-2 alone or in combination with pAB4 did not alter any of the physiological and biochemical parameters as compared to the wild-type. Although the difference in the nature of antibiotic resistance did not significantly influence the copy number of pAB4 and pAB8, it had differentially effected glucose utilization, G-6-PDH and CS activities as well as citric acid accumulation and secretion. Collectively, the physiology and metabolism of *P. fluorescens* is extensively modified by cumulative effects exerted by plasmid copy number, nature of origin of replication as well as the antibiotic resistance gene.

In order to understand effect of overexpression of *ppc* and *cs* genes on mineral phosphate solubilization ability, three isolates *viz.* P109, Fp585 and Fp315 were transformed with pAB3 expressing *ppc* gene. On Pikovaskya's (PVK) agar, *ppc* transformants of non-P-solubilizer and weak P-solubilizer strains like *P. fluorescens*

13525, P109 and Fp585 showed enhancement in P-solubilization upto varying extents while P-solubilizing ability of the inherently efficient P-solubilzer Fp315 was not significantly altered. *P. fluorescens* 13525 and P109 *ppc* transformants showed a very poor growth on RP suggesting no significant P-solubilization. Presence of plasmids in Fp315 reduced the growth rate as compared to the wild-type, however; the *ppc* transformant required lesser time for medium acidification as compared to its control. The growth and RP solubilization was almost unaffected in case of Fp585. Overexpression of *cs* gene independently enhanced the P-solubilizing ability of *P. fluorescens* 13525 on PVK agar while it did not affect when expressed in combination with *ppc* gene.

Biochemical basis of P-solubilization by pseudomonads (and most other MPS bacteria) is periplasmic PQQ-GDH catalyzed gluconic acid production. *Pseudomonas* P4 was an isolate with pA172A plasmid (containing *ppc* gene of *S. elongatus* PCC 6301) integrated into the genome and was confirmed to be *Pseudomonas aeruginosa*. Since this strain showed very good P-solubilizing ability, the characteristics of the integration of pA172A and the biochemical basis of P-solubilizing ability were determined.

Pseudomonas P4 genomic DNA was used to retrieve the integrated pA172A plasmid by following a carefully designed strategy and the resultant clones had sizes of about 5-6kb (less than the original pA172A). Inability of these clones to complement *E. coli ppc* mutant phenotype as well as PCR amplifications using primers corresponding to different regions of the *ppc* gene, confirmed that the *ppc* gene present in the *Pseudomonas* P4 genome was truncated. The sequencing of host genomic DNA fragment adjacent to the truncated *ppc* gene in the genome-derived plasmids showed that pA172A plasmid was not integrated into any gene directly associated with glucose metabolism or organic acid secretion.

However, significant physiological and biochemical differences were found between non-P-solubilizing *P. fluorescens* 13525 and P-solubilizing *Pseudomonas* P4 (*P. aeruginosa* spp.) under Pi-sufficient and -deficient conditions. GDH, G-6-PDH, PYC and PPC activities in both these *Pseudomonas* strains in correlation with organic acid profiles under Pi-sufficient and -deficient conditions demonstrated that under Pi limitation direct oxidation pathway was predominant while the contribution of

intracellular phsophorylative pathway increased under Pi sufficiency. Higher G-6-PDH activity in *Pseudomonas* P4 accounted for its higher growth rate as compared to *P. fluorescens* 13525 under both Pi-deficient and sufficient conditions. Significant GDH activity under Pi-sufficient conditions in *Pseudomonas* P4 indicated that GDH is constitutive. The metabolic perturbations under Pi deficiency *i.e.* the shift to direct oxidation pathway appears to be much higher in *Pseudomonas* P4 enabling high gluconic acid secretion and P-solubilization.

In conclusion, *P. fluorescens* 13525 appears to adapt to the metabolic stresses imposed in the form of incorporation of extrachromosomal plasmid DNA or Pi and glucose limitation by adjusting the glucose distribution between the periplasmic direct oxidation pathway and intracellular phosphorylative pathway. Metabolic perturbations at the anaplerotic node overall suggest that *P. fluorescens* metabolism is not optimized for efficient glucose utilization. Unlike *E. coli*, CS appears to be rate-limiting step on glucose in *P. fluorescens* as evident from even 2 fold increase leading to increased citric acid secretion. This presented a rare illustration of bacterial citric acid production, that too by targeted genetic manipulation, which otherwise has been a domain of fungi and yeasts. Several naturally occurring metabolic perturbations also appear to underlie the extensively characterized phenomenon of gluconic acid mediated mineral phosphate solubilziation by pseudomonads. The present study also draws attention towards influence of the nature and copy number of the plasmids on the central metabolism. A systematic detailed flux analysis of *P. fluorescens* 13525 engineered at crucial metabolic nodes would give a clearer insight into its metabolic rigidity/flexibility which might further open novel targets for metabolic engineering.