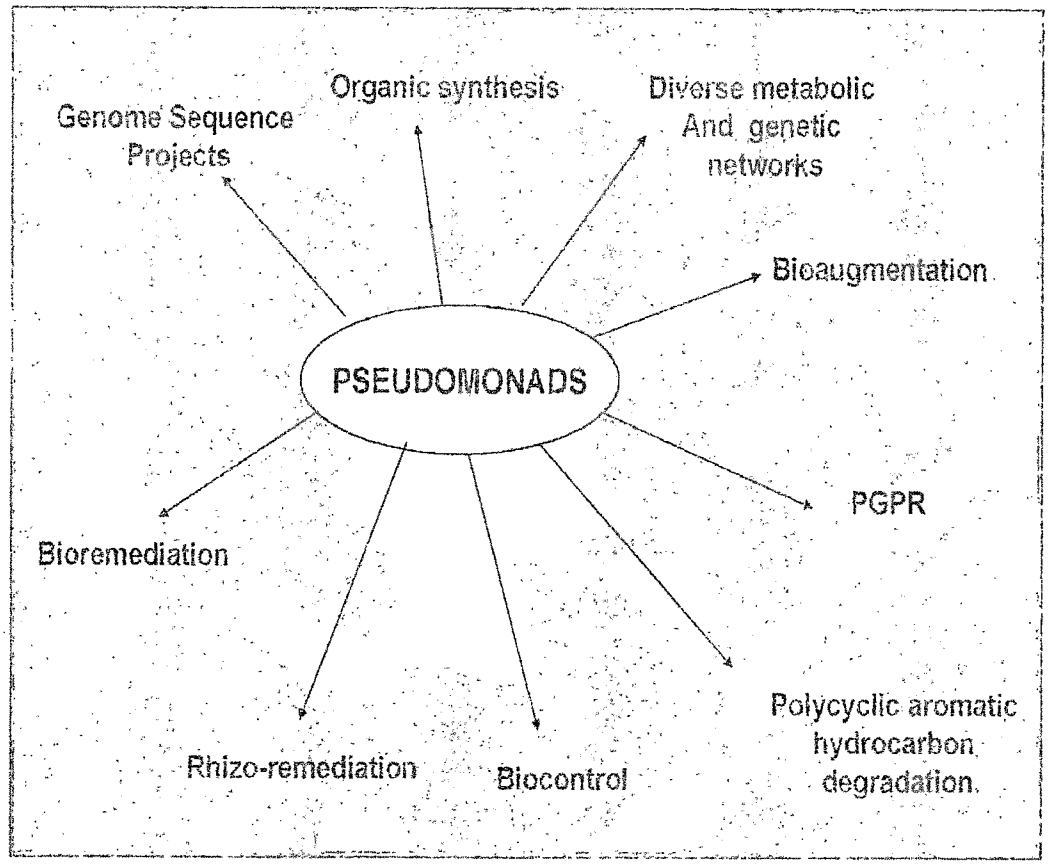

CHAPTER 1

Review of Literature and Introduction

"Learning is the discovery that something is possible."
– Fritz Perls

1.1: METABOLIC ENGINEERING

Metabolic engineering is referred to as the "directed improvement of cellular properties through the modification of specific biochemical reaction(s) or the introduction of new ones, with the use of recombinant DNA technology" (Stephanopoulos, Aristodou and Nielsen, 1998; Stephanopoulos, 1999). It can be explained as the targeted and purposeful alteration of metabolic pathways found in an organism in order to understand and utilize cellular pathways for chemical transformation, energy transduction, and supramolecular assembly (Lessard, 1996). This multidisciplinary field involves implementation of principles from chemical engineering, biochemistry, mathematical and computational sciences, and owes its existence to fast developing molecular biology techniques.

Earlier strategies for genetic modifications were based on random chemical mutagenesis and selection of strains exhibiting desired phenotypes but its success relied heavily on mutagens and creative selection techniques (Koffas et al., 1999). Studies by Bailey (1991) and Stephanopoulos and Vallino (1991) pioneered the major transition of this classical approach to a more systematic and rational approach called *Genetic Engineering* involving the use of recombinant DNA technology. This approach allowed precise intentional manipulations in the targeted metabolic reactions to redirect the cellular metabolism for enhancing desirable features and establishing completely novel metabolic configurations with beneficial characteristics. The technical manifestation of genetic engineering involving manipulation of enzymatic, transport and regulatory functions of the cell by using recombinant DNA technology was better referred to as "**Metabolic Engineering**" (Stephanopoulos and Vallino, 1991; Koffas et al., 1999). The novel contribution of metabolic engineering lies in its directionality and emphasis on complete metabolic networks rather than individual reactions. That is why it soon emerged with the need of more rational approach towards identifying promising targets of metabolic manipulation, replacing the previous, mostly extemporized phenotype selection process.

1.1.1: Modern Metabolic Engineering

Like all traditional fields of engineering, modern metabolic engineering too encompasses the two defining steps of analysis and synthesis applied to metabolic

pathways to attain a particular goal. The process conventionally begins with preliminary analysis of concerned metabolic pathways and identification of the "rate-limiting step" followed by introducing a specific genetic modification using a variety of molecular and recombinant biology techniques, to overcome these rate limiting steps (Vemuri and Aristidou, 2005) (**Fig. 1.1**). Subsequently, the effects or alterations in the cellular metabolism are analyzed considering the reactions in their entirety rather than in isolation. Similarly through several rounds of genetic engineering an ideal pathway is designed to enhance metabolic flux through the pathway of interest and minimize metabolic flow to undesired biosynthetically related products.

However, high complexity and elasticity of metabolic network sometimes may reflect as unrelated response to engineering attempts thereby posing difficulties in predicting the outcome of any specific modification. For example overexpression of enzymes producing phosphoenolpyuvate *in E. coli* inhibited heat shock response and nitrogen regulation (Liao et al., 1996). Metabolic engineering was therefore redefined as the approach that alters not only the metabolism itself but also the regulatory and gene-expression networks of the target organism. It should be directed at optimizing both the production flux and the functioning of the organism itself, such that most of the unfavorable homeostatic responses and malfunctioning of the metabolism are prevented.

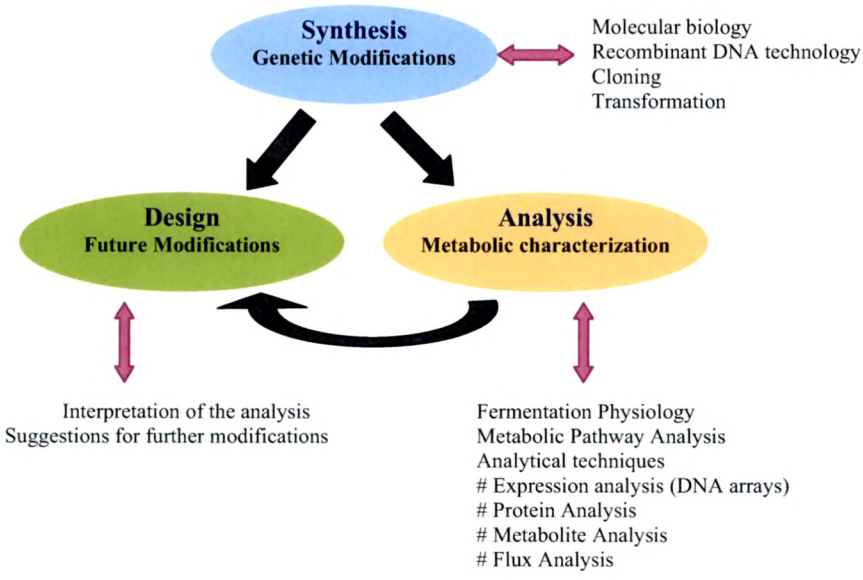

Fig. 1.1: The cycle of metabolic engineering (Nielsen, 2001)

1.1.2: Strategies of Metabolic Engineering

Emergence, development and success of metabolic engineering has been extensively elaborated upon by Stephanopoulos, Aristodou and Nielsen (1998) and Nielsen (2001)

(i) Constructive metabolic engineering

Based on the knowledge of the metabolic framework and the desired output, engineering rational and defined genetic modification(s) hypothesized to be beneficial to the system in some way has been referred to as ***constructive metabolic engineering*** by Bailey et al (1996). Different successful strategies for achieving the desired metabolic alterations have been reviewed by Nielsen (2001) (**Table 1.1**) which includes some of the landmark targets attained using metabolic engineering. Apart from these many other examples have been analyzed by Yang et al (1998).

Strategies	Aim of the work
Heterologous protein production	Engineer the protein synthesis pathway and strain to obtain improved productivity. *eg.* Production of hormones, antibodies, vaccines and novel enzymes
Extension of substrate range	Engineer organisms for broader substrate utilization to bring about efficient utilization of raw materials
Pathway leading to new products	To make a particular host to produce several different products **technique**-by using heterologous genes or gene shuffling to generate new pathways
Pathways for xeno-biotics degradation	To make few organisms to degrade several different xenobiotic compounds including TNT **techniques**-by inserting pathways from other organisms
Engineering of cellular physiology for process improvement	To make cells tolerant to low oxygen and sensitive to high glucose, increasing flocculation **techniques**-by expression of heterologous genes, disruption of genes, over expression of homologous genes
Elimination/reduction of by-product formation	To prevent carbon loss, to avoid toxicity, enhancing better purification **technique**-gene disruption strategies.
Improvement of yield or productivity	To improve the production of low-value added products, **techniques**-by inserting additional gene copies or engineering the central metabolic pathway *eg.* ethanol production

Table 1.1: Strategies of metabolic engineering and its typical targets (Nielsen, 2001)

Methods like single-gene overeexpresssion or overexpression of all the pathway genes lead to pathway optimization only in special cases (Jung and Stephanopoulos, 2004). In contrast, there are many cases where the consequences of the incorporated genetic change are very diverse rather than being in the desired direction. These effects have been referred to as ***secondary responses*** of metabolic engineering (Bailey et al, 1996). Such failures are mostly due to incomplete knowledge of the metabolic circuits, regulation in terms of rate-limiting steps, reaction kinetics, intermediate metabolite concentrations or the complicated interactions between different metabolic pathways.

Hence, considering metabolic network at more of a global scale becomes essential. Furthermore, the constructive metabolic engineering is also complicated by other factors like enzymes catalyzing reactions using substrate analogs, improper sub-cellular localization, mis-folding or selective degradation of the heterologous protein and unavailability of essential co-factors (often taken for granted in the constructive metabolic engineering approach). All these collectively imply that the introduced genetic change need not necessarily result in the anticipated phenotype/activity. This led to emergence of an alternate approach termed as ***inverse metabolic engineering***.

(ii) Inverse metabolic engineering (*IME*)

The *IME* begins with identifying the desired phenotype in any heterologous host or a related model organism followed by defining/hypothesizing the genetic or environmental basis responsible for conferring the phenotype and finally applying this information in the organism of interest to achieve the desired goal (**Fig. 1.2**) (Bailey et al, 1996; Gill et al., 2003).

One of the earliest successes was expression of *Vitreoscilla* hemoglobin (VHb) in *E. coli* to achieve higher cell densities in micro-aerobic conditions (Khosla and Bailey, 1988). According to a report, synthesis of higher levels of heme (for haemoglobin) by aerobe *Vitreoscilla,* suggested that VHb synthesis was the genetic alteration adopted by *Vitreoscilla* to improve its metabolism and growth under oxygen limitation. With this information, it was hypothesized that cloning of VHb gene and its subsequent expression in variety of industrial aerobic microorganisms could increase the cell biomass under oxygen limiting conditions. Numerous other examples have been discussed in detail by Bailey et al (1996).

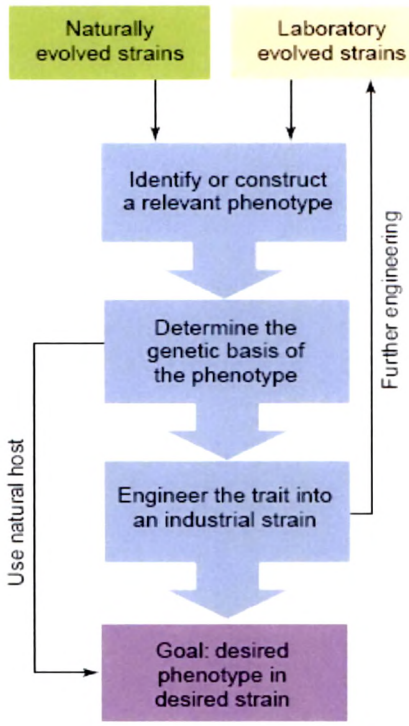

Fig. 1.2: The inverse metabolic engineering approach (Gill et al, 2003). First, evolutionary mechanisms operating in nature or in the laboratory result in the generation of the phenotype of interest. Genetic studies are performed to elucidate the basis of the phenotype, which provides guidance for further metabolic engineering. The genes are either engineered into a strain more suited to the intended application or the natural host is used for industrial production. Alternatively, further laboratory evolution may be pursued and the IME approach executed recursively until the desired phenotype is obtained.

1.1.3: Metabolic Engineering in the Post-Genomic Era

The importance and accurate implementation of the basic molecular biology, analytical methods, mathematical and computational tools in a sequential manner has been reviewed in detail (Cameron and Chaplen, 1997; Keasling, 1999). The major challenge for metabolic engineering in the post-genomic era is to broaden its design methodologies to incorporate genome-scale biological data. Research in the fields of genomics, proteomics and metabolomics has made enormous new promising tools and models available (Rohlin et al., 2001; Sanford, 2002; Gill et al., 2003; Herrgard et al., 2004; Patil et al., 2004; Vemuri and Aristidou, 2005).

The molecular biology tools in addition to choosing/designing suitable cloning vectors and promoter systems, now encompasses newer techniques like DNA microarrays, 2-D gels with MALDI-TOF mass spectrometry, probe hybridization based techniques, protein evolution using error-prone PCR or gene shuffling, immuno-precipitation of mRNA complexes, etc, reflecting the indispensable contribution of genomics and proteomics in refining the metabolic engineering approach. These advancements established that metabolic fluxes are a function of gene expression,

translation, post translational protein modifications and that the complicated interactions between genes-proteins-metabolites prevailing in the system are the ultimate representation of the cellular phenotype expressed under a given condition (Nielsen, 2003). The experimental techniques employed to estimate intracellular metabolite pools are mostly based on nuclear magnetic resonance (NMR) or gas chromatography-mass spectrometry (GC-MS) (Kelleher, 2001; Rohlin et al., 2001; Antoniewicz et al., 2007) while the use of marker gene/phenotype e.g. bioluminescence, allows real time analysis. Genome sequences find major applications in selecting the metabolic engineering targets. Goodacre et al (2004) have excellently reviewed the importance, current status and predicted potentials of metabolome profiling in various aspects of fundamental and applied biology. However this field is still in its infancy. Advancement in biostatistics and bioinformatics has led to increasing applications of database resources, metabolic control analysis (MCA), metabolic flux analysis (MFA) and network simulation programs in proposing stoichiometric models representing biological networks (Varner et al., 2000).

Metabolomics along with two major –omics, genomics and proteomics, will have a major contribution to the development of "Systems Biology" to understand the systems as a whole (Sanford et al., 2002; Goodacre et al., 2004; Stephanopoulos et al., 2004; Carlson, 2007). All these technical and theoretical advancements have still not translated into successful metabolic engineering applications, mainly due to the limitations in our understanding the regulatory phenomena from a global perspective (Vemuri and Aristidou, 2005; Carlson, 2007).

To conclude the whole discussion it is apt to say that "To understand the whole, one must study the whole"- **Henrik Kacser**, one of the architects of metabolic control analysis. But at the same time, Stephen Oliver also calls metabolic engineering, a frustrating occupation (2002).

1.1.4: Model System for Metabolic Engineering

Of the usually employed microorganisms for genetic manipulation, *E. coli* is the researcher's pet due to several reasons. The easy-to-handle *E. coli* has an advantage of fast replication, comparatively less complex metabolic network and most importantly vast knowledge available at biochemical, genetic and molecular level. In addition, the

molecular processes prevailing in the cell in combination with the normal physiological metabolic processes including the stress response mechanisms etc. are very well studied in *E. coli*. Thus, the possible outcome of the targeted genetic modifications could be reasonably well-predicted.

Most of the metabolic alterations have been standardized and optimized using *E. coli* as the model organism over so many years due to which *E. coli* is an important organism not only for experimental study, but also for constructing a mathematical model of the entire metabolic and regulatory mechanisms. Glucose (most preferred carbon source) metabolism in *E. coli* has been extenstively investigated, from uptake level to the transcriptional level, to understand the regulations employed for maintaining optimal intracellular metabolite pools and the energy status.

1.2: GLUCOSE METABOLISM IN MODEL ORGANSIM *Escherichia coli*

Escherichia coli, a Gram-negative facultative aerobe can utilize a variety of substrates and adopts three different metabolic modes to support growth under aerobic and anaerobic conditions depending on the availability of electron acceptors. Of all the carbon sources it can utilize, glucose is the preferred carbon source.

1.2.1: Glucose uptake in *E. coli*

Initially glucose diffuses through porins in the outer membrane which is followed by an active transport mechanism internalizing glucose into the cytoplasm. This process proceeds via phosphoenolpyruvate:sugar phosphotransferase system (PTS) which simultaneously transports and phopshorylates sugars in phosphoenolpyruvate (PEP) dependent manner (Gosset, 2005). The transport system is composed of cytoplasmic and non sugar-specific protein components Enzyme I (EI) and the phosphohistidine carrier protein (HPr) (encoded by genes *ptsHI*) which relay a phosphoryl group from PEP to the sugar-specific enzyme components IIA and IIB **(Fig. 1.3).** The integral membrane protein permease, IIC recognizes and transports the sugar molecules, which are phosphorylated by component IIB. Glucose can also be actively transported into the cytoplasm by systems that are normally involved in galactose internalization. These PTS dependent uptake systems are under the catabolite repression control.

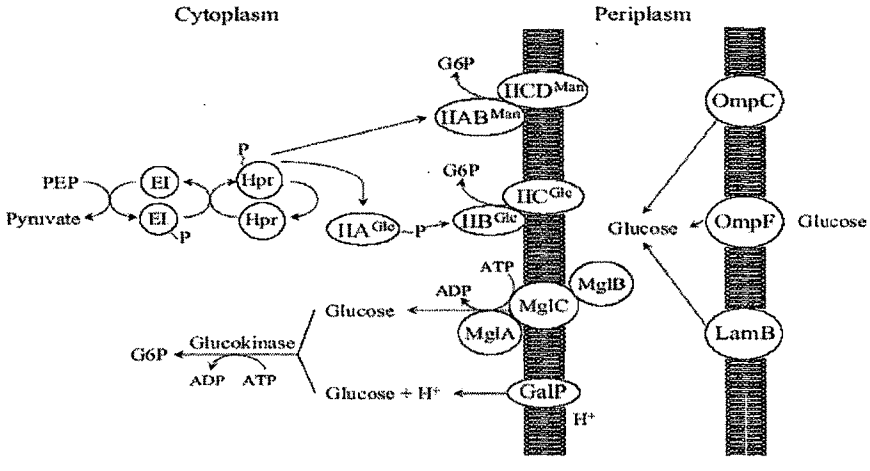

Fig. 1.3: The PEP:sugar phosphotransferase system (PTS) and other uptake mechanisms for sugar transport in *Escherichia coli* (Gosset, 2005)

1.2.2: Glucose catabolic pathways

Glucose-6-phosphate (G-6-P), formed from the PEP dependent uptake of glucose, is chiefly catabolized through **Embden-Meyerhof-Parnas** (EMP) pathway (Fuhrer et al., 2005; **Fig. 1.4**). Phosphofructokinase (PFK) and pyruvate kinase (PYK) catalyzing irreversible reactions are the key control enzymes of the glycolytic pathway (Emmerling et al., 1999; Sauer et al., 1999). The flux of glycolytic end-product, pyruvate is distributed to TCA cycle for energy generation and to anabolic pathways, depends on the redox status of the cell. Detailed flux analysis revealed that of the total flux going to TCA cycle; relatively less carbon is subjected to complete oxidation while major portion (10 to 30% of carbon flux from glucose) is commonly excreted as incompletely oxidized product acetate (Farmer and Liao, 1997; Holms, 2001; Fuhrer et al., 2005). Under aerobic conditions, this acetate is mainly produced from acetyl-CoA (formed from pyruvate in pyruvate dehydrogense catalyzed reaction) by phosphotransacetylase (Pta) and acetate kinase (AckA) and from pyruvate by pyruvate oxidase (PoxB) in the stationary phase (El-Mansi and Holms, 1989; Kleman and Strohl, 1994; Abdel-Hamid et al., 2001). Common explanation for acetate accumulation is the high carbon flux through glycolysis which exceeds the TCA cycle capacity, especially when glucose is in excess. Under anaerobic conditions, >95% of pyruvate is channeled for NADH oxidation by mixed acid fermentation while <5% goes for biosynthesis (Causey et al., 2004). Under anaerobic conditions, pyruvate formate lyase (PFL) majorly contributes to acetyl-CoA formation (Abdel-Hamid et al., 2001; Gosset et al., 2005; Wolfe, 2005).

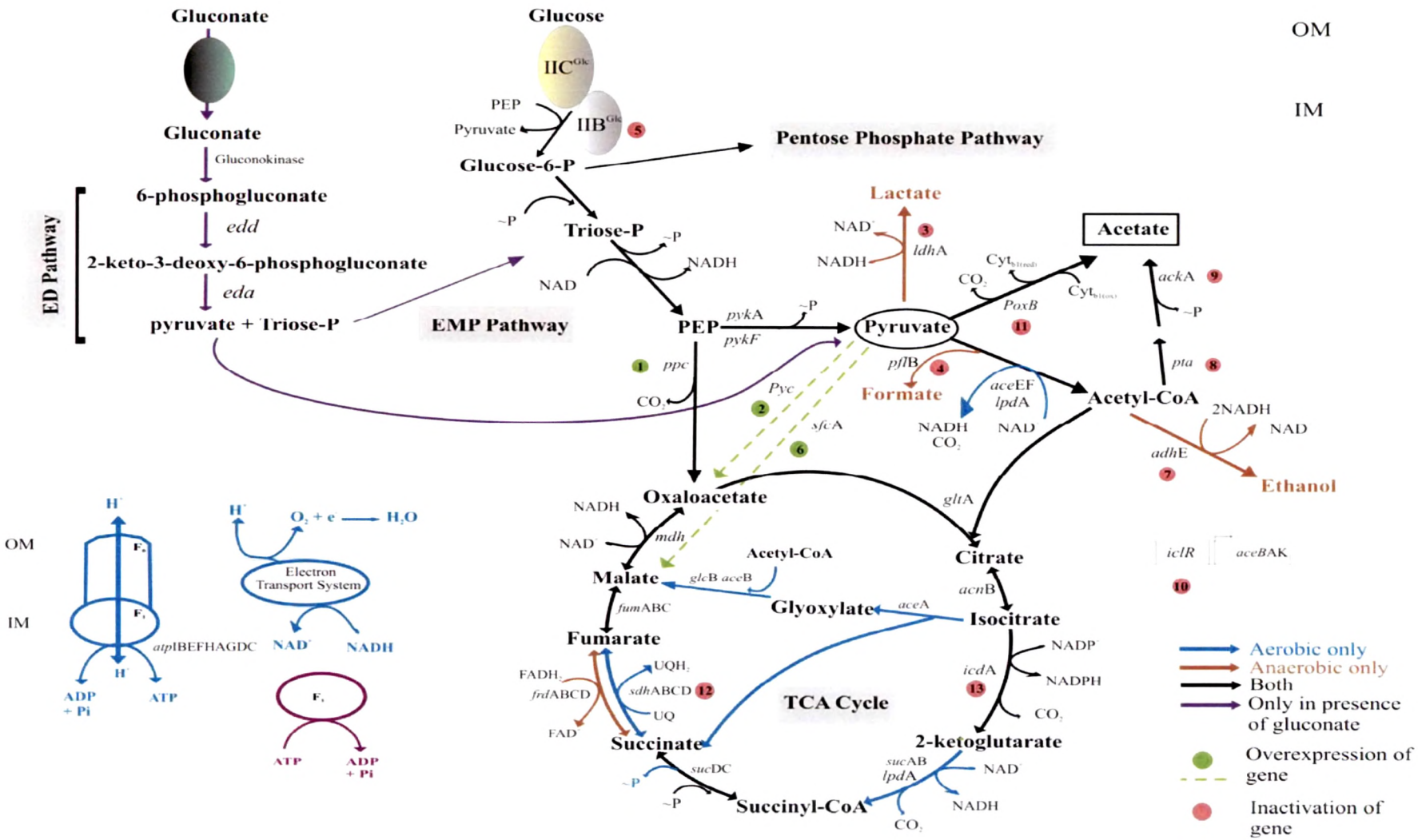

Fig. 1.4: Glucose metabolism in *E. coli*

Carbon flux analysis in *E. coli* also revealed that along with EMP pathway, the carbon flux through pentose phosphate pathway (PPP) is high and substantially contributes to the glucose catabolism (Fuhrer et al., 2005). Another major catabolic pathway known in *E. coli* is the Entner-Doudoroff's (ED) pathway which operates in a linear fashion only in the presence of gluconate as the carbon source (Fuhrer et al., 2005). Glucose cannot be converted to gluconate in *E. coli* as it lacks the functional periplasmic pyrroloquinoline quinone dependent glucose dehydrogenase (PQQ-GDH) required for the conversion. *E. coli* has apo-GDH but lacks the PQQ biosynthetic genes thereby having a functionally inactive GDH enzyme (van Schie et al., 1985).

One of the most challenging tasks is engineering of the central carbon metabolic pathway to overproduce any of its key intermediates as the metabolites of the central pathways are critically regulated and their imbalance may affect the survival.

1.3: METABOLIC ENGINEERING OF CENTRAL CARBON METABOLISM IN *E. coli* FOR ENHANCED SUCCINATE PRODUCTION

Metabolic engineering of *E. coli* secreting succinic acid at near theoretical maximum is one of the most successful cases. In absence of any alternative electron acceptors, *E. coli* undergoes mixed-acid-fermentation producing succinate as one of the minor products. Under anaerobic conditions, pyruvate also gets converted into lactate, acetate, formate and ethanol in addition to succinate. The biosynthesis of oxaloacetate (OAA), which is the precursor of succinate, involves the key reaction mediated by PEP-carboxylase (PPC) catalyzing the conversion of PEP to OAA. Hence the genetic manipulations were targeted to overcome the tight regulatory controls maintaining the optimal PEP-Pyruvate levels in order to increase succinate production and simultaneously decrease other fermentation products.

In order to enhance succinate production, the approach used was to minimize pyruvate formation as well as increase the synthesis of OAA. Pyruvate formation was blocked by deletion of *pts*G (encoding enzyme II of the glucose PTS), *pyk*A (pyruvate kinase A) and *pyk*F (pyruvate kinase F) which increased the succinate yield to ~0.23g/g glucose (17.4mM from 50mM glucose) being ~7 higher than the wild type while reduced

the formation of other fermentation products; though had a slower growth compared to the wild type under anaerobic conditions (Lee et al., 2005a). Simultaneously OAA supply was increased by heterologous overexpression of *Sorghum vulgare ppc* gene; resistant to feedback inhibiton by malate, and *Lactococcus lactis pyc* gene in *E. coli* which increased the succinate production to 0.11g/g glucose which was ~4.3fold higher than the wild type with a concomitant decrease in the lactate formation (Wendisch et al., 2006). Further deletion of lactate dehydrogenase (*ldh*A), acetate kinase (*ack*A) and phosphotransacetylase (*pta*) genes increased the succinate yield to 0.3g/g glucose.

On the other hand, homologous overexpression of *ppc* gene (modification 1 in **Fig. 1.4**) in *E. coli* resulted in ~3.75 fold increase in the succinate yield (~0.2g/g glucose) under anaerobic conditions along with reduction in all other fermentation products but it also reduced the glucose consumption rate as PEP is required for PEP-PTS mediated glucose uptake (Millard et al., 1996). Hence, *Rhizobium etli* pyruvate carboxylase (*pyc*) gene was overexpressed (modification 2 in **Fig. 1.4**) which increased succinate yield (~0.21g/g glucose) by 2.6 fold as compared to the wild type without affecting the glucose uptake but could not prevent the significant accumulation of other fermentation products, specially lactate although its levels were reduced (Gokarn et al., 1998; 2000; 2001) (**Table 1.2**). However, overexpression of *R. etli pyc* gene in *E. coli ldh* mutant, although eliminated lactate formation, increased succinate yield only by 1.7fold (modification 2 and 3 in **Fig. 1.4; Table 1.2**) which was hypothesized to be due to accumulation of pyruvate and NADH which affect activity of pyruvate formate-lyase (*pfl*) enzyme. In addition, formate levels reduced slightly while the acetate and ethanol levels remained unaffected. Vemuri et al (2002) demonstrated that the *E. coli* ***ldh⁻ pfl⁻*** double mutant had higher accumulation of succinate and pyruvate as compared to the wild type (**Table 1.3**). Increase in *pyc* activity in the double mutant drastically reduced pyruvate accumulation and increased the succinate yield to 0.81g/g (modification 2, 3 and 4 in **Fig. 1.4**).

Subsequently, the *E. coli* ***ldh⁻ pfl⁻ ptsG⁻*** triple mutant (additionally defective in an enzyme of glucose phophotransferase system) was constructed to overcome the reduced growth in *ldh⁻ pfl⁻* double mutant; which produced 0.88g/g glucose succinate with increase in acetate levels (**Table 1.3**). Overexpression of *pyc* gene in this triple mutant with H_2 gas in the headspace (modification 2, 3, 4 and 5 in **Fig. 1.4**) increased succinate yields to as high as 0.91g/g but still accumulated significant amount of acetate.

Moreover, co-expression of *S. vulgare ppc* and *L. lactis pyc* genes in *E. coli ldh⁻ pfl⁻* double mutant accumulating significant amount of pyruvate, increased succinate production by depleting the pyruvate accumulation (Lin et al., 2005b).

Strains	Yield (g per g of glucose)					
	Pyruvate	Succinate	Lactate	Formate	Acetate	Ethanol
WT	0.0	0.08	0.21	0.21	0.17	0.12
WT + *pyc* gene	0.0	0.21	0.08	0.15	0.17	0.12
***ldh⁻* (Mutant)**	0.03	0.09	0.00	0.32	0.21	0.16
***ldh⁻* + *pyc* gene**	0.02	0.15	0.00	0.27	0.21	0.13

Table 1.2: Fermentation products of *E. coli* overexpressing *pyc* gene of *Rhizobium etli* (Gokarn et al., 2001)

E. coli Strains	Yield of by-products on glucose (g/g of glucose)				
	Succinate	Pyruvate	Acetate	Ethanol	Fumarate
ldh⁻ pfl⁻	0.53	0.76	0.06	0.06	0.00
ldh⁻ pfl⁻ pyc⁺	0.81	0.19	0.11	0.05	0.00
ldh⁻ pfl⁻ ptsG⁻	0.88	0.00	0.22	0.07	0.00
***ldh⁻ pfl⁻ ptsG⁻ pyc⁺* (CO_2)**	**0.35**	0.00	**0.09**	0.06	0.47
***ldh⁻ pfl⁻ ptsG⁻ pyc⁺* (H_2)**	0.91	0.00	0.11	0.07	0.00

Table 1.3: Alterations in organic acid production upon *pyc* overexpression in *E. coli pfl, ldh* and *ptsG* triple mutant (Vemuri et al., 2002)

In an alternate approach, succinic acid production upto an yield of 0.48g/g glucose was achieved in *E. coli **ldh⁻ pfl⁻*** double mutant strain by amplifying the malic enzyme (ME) activity (modifications 3, 4 and 6 in **Fig. 1.4**); however, it also produced a considerable amount of malic acid (Hong and Lee, 2001). Simulations and metabolic control analysis predicted that supplying additional reducing power could enhance succinic acid production. Use of sorbitol instead of glucose as a carbon source produced highest succinic acid concentration and productivity with maximun *in silico* succinic acid yield reaching 1g/g sorbitol (Hong and Lee, 2002; Lee et al., 2002; Lin et al., 2005a). This succinic acid yield was 85% of the maximum theoretical yield which is 1.31g

succinic acid/g glucose (Lee et al., 2002). Use of xylose instead of glucose conserves the intracellular PEP pool, since its transport does not require the PTS system, leading to comparatively higher succinate production (Lin et al., 2005a). On the other hand, *E. coli ldh, pta* and alcohol dehydrogenase (*adh*) mutant overexpressing *M. succiniciproducens* MBEL55E phsophoenolpyruvate carboxykinase (*pck*) gene achived succinate yield of 0.72g/g on glucose while attained highest succinic acid yield glycerol (1.3 g/g glycerol) but with low succinic acid concentration and productivity.

One mole of succinate synthesis requires one mole of phosphoenolpyruvate (PEP), one mole of CO_2, and two moles of NADH. *E. coli adh*E-*ldh*A double mutant diverted NADH for succinate synthesis. Overexpression of *L. lactis pyc* gene in this double mutant *E. coli* yielded 0.85g succinate/g glucose (Sanchez et al., 2005a). Furthermore, to balance the available NADH and maximize the carbon conversion to succinate, the strategy of using a combination of the two pathways for succinate synthesis, *viz.* the traditional fermentative pathway and the glyoxylate pathway (which has lower NADH requirement), was also employed. According to this strategy, *E. coli adhE*, *ldh*A and *ack-pta* mutant with constitutive glyoxylate pathway through the inactivation of *icl*R (modifications 3, 7, 8, 9 and 10 in **Fig. 1.4**), which encodes a transcriptional repressor protein of the glyoxylate bypass, was developed (Sanchez et al., 2005b). This *E. coli* strain produced succinate at the yield of 1.05 g/glucose and could ferment high levels of glucose within 24h. This *E. coli* mutant has also been analyzed for the optimal flux distribution at different branch points in the metabolism, specifically the OAA node at the glyoxylate cycle and fermentation (Sanchez et al., 2006). Collectively all these approaches demonstrate the optimal metabolic design for efficient succinate production under anaerobic conditions even though when it is one of the minor products of mixed acid fermentation.

Considering the industrial scale, the drawback of this strategy was low biomass yield, limited NADH availability and slow microbial growth (Lin et al., 2005c). To overcome this, another strategy was designed based on pathway modeling and simulations performed on glycolytic pathway, TCA cycle and glyoxylate bypass, to allow *E. coli* to efficiently produce and accumulate succinate under aerobic conditions. *E. coli* when grown aerobically on glucose, mainly acetate and not succinate is secreted. Hence, various blocks were introduced in the metabolic pathways that would channel

more flux through TCA and reduce acetate formation e.g. mutations in *pox*B, *ack*A-*pta*, block the succinate utilization (inactivation of *sdh*AB) and increase the diversion towards succinate biosynthesis by activating glyoxylate bypass (mutations in *icd*A and *icl*R; **Fig. 1.4**). This aerobic *E. coli* system (modifications 8-9, 10, 11, 12 and 13 in **Fig. 1.4**) is advantageous, as it produced 14.28mM succinate with yield of (~0.22 g/g glucose) using 55mM glucose through pathways which do not require NADH (**Fig. 1.5**).

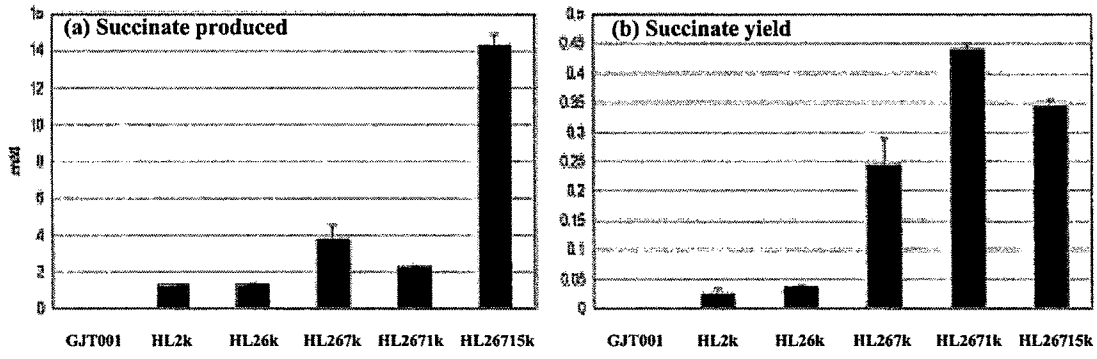

Fig. 1.5: Improvement in aerobic succinate production with subsequently incorporated genetic mutations in *E. coli* (Lin et al., 2005c). GJT001-Spontaneous *cad*R mutant of MC4100(ATC35695); HL2k GJT001-(ΔsdhAB::Km^R); HL26k GJT001-(ΔsdhAB, ΔpoxB::Km^R); HL267k GJT001-(ΔsdhAB, ΔpoxB, Δ(ackA-pta)::Km^R); HL2671k GJT001-(ΔsdhAB, ΔpoxB, Δ(ackA-pta), Δicd::Km^R); HL26715k GJT001- (ΔsdhAB, ΔpoxB, Δ (ackA-pta), Δicd, ΔiclR::Km^R).

In aerobic batch reactor studies, succinate production rate was faster, reaching 0.5mole/mole (~0.33g/g glucose) in 24h with a concentration of 22.12mM which on further cultivation increased to 43mM with a yield of 0.7mole/mole (~0.46g/g glucose). This metabolic system had 5 mutations leading to highly activated glyoxylate bypass and no flux through TCA as result of which although succinate yield increased, it was not up to the maximum theoretical levels due to accumulation of pyruvate and TCA cycle C_6 intermediates (citrate and isocitrate).

Hence, to further improvise the succinate yield, *E. coli* with 4 of the above 5 mutations was selected (Δ*sdh*AB, Δ*pox*B, Δ(*ack*A-*pta*), Δ*icl*R) (modifications 8-9, 10, 11 and 12 in **Fig. 1.4**) which could achieve aerobic succinate production either by

glyoxylate pathway or by oxidative TCA cycle (Lin et al., 2005c; 2005d). Succinate yield was higher than pentamutant system without any accumulation of TCA cycle intermediates (**Table 1.4**). Inactiavtion of *pts*G and simultaneous overexpression of *S. vulgare ppc* gene (mutant insensitive to malate feedback inhibition) further enhanced the succinate production to maximum theoretical yield (1.0mole succinic acid/mole of glucose consumed or 0.66g succinic acid/g glucose, **Table 1.4**). Studies using fed-batch and chemostat cultures demonstrated that this *E. coli* mutant constituted the most efficient large-scale aerobic succinate overproduction system with highest succinate productivity, no pyruvate accumulation and least acetate secretion as compared to other mutant systems that also showed succinate yield of ~1.0 mole/mole glucose (Lin et al., 2005e; 2005f) (modifications 8-9, 10, 11, 12, 1 and 5 in **Fig. 1.4**).

Genetic modifications in *E. coli*	$Y_{S/G}$ (mol/mol)	Q_p (g/lh)	q_p (mg/gh)
Δ*icl*R, Δ*icd*, Δ*sdh*AB, Δ(*ack*A-*pta*), Δ*pox*B	0.65	0.057	24.04
Δ*icl*R, Δ*icd*, Δ*sdh*AB, Δ(*ack*A-*pta*), Δ*pox*B, Δ*pts*G	0.87	0.086	35.47
Δ*icl*R, Δ*icd*, Δ*sdh*AB, Δ(*ack*A-*pta*), Δ*pox*B + *ppc*	**1.09**	0.140	44.26
Δ*icl*R, Δ*icd*, Δ*sdh*AB, Δ(*ack*A-*pta*), Δ*pox*B, Δ*pts*G +*ppc*	**0.96**	0.094	45.23
Δ*sdh*AB, Δ (*ack*A-*pta*), Δ*pox*B, Δ*icl*R	0.67	0.094	26.84
Δ*sdh*AB, Δ (*ack*A-*pta*), Δ*pox*B, Δ*icl*R, Δ*pts*G	0.78	0.130	32.82
Δ*sdh*AB, Δ (*ack*A-*pta*), Δ*pox*B, Δ*icl*R, Δ*pts*G +*ppc*	**0.95**	0.270	73.66

Table 1.4: Aerobic succinate yield and productivity as a result of various subsequently introduced genetic modifications (Lin et al., 2005c). YS/G is molar succinate yield at the end of fermentation; Qp and q_p are the average volumetric succinate productivity {mass concentration of succinate (g/l) over time (h)} and average specific succinate productivity {mass of succinate (mg) per mass of biomass (g) over time (h)} at the end of fermentation.

In addition to succinic acid, *E. coli* has been engineered for large scale production of other metabolic by-products like ethanol, acetate and pyruvate (Underwood et al., 2002; Causey et al., 2004; Wendisch et al., 2006; Zhou et al., 2008).

Apart from *E. coli*, major focus has also been on the improvement of industrially important bioprocesses occurring in organisms like *Corynebacterium glutamicum*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Zymomonas mobilis*. *S. cerevisiae* has

been an organism of choice for industrial ethanol production from glucose. This organism has been engineered to produce ethanol from xylose and glucose (Ostergaard et al., 2000; Bro et al., 2006). Similarly *Z. mobilis* has been genetically engineered to have much more efficient ethanol production from pentoses by introducing the entire xylose utilization pathway (Zhang et al., 1995; Rogers et al., 2007). Disruption of lactate dehydrogenase (*ldh*) gene by chromosomal insertion of *Z. mobilis* pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adh*B) genes under native *ldh* promoter along with acetolactate synthase (*als*S) mutation, enhanced ethanol production in *B. subtilis* (Romero et al., 2007). *C. glutamicum* has been used for improving the pathways for overproduction of industrially important L-lysine and L-glutamate (Sahm et al., 2000; de Graaf et al., 2001). These examples indicate that the choice of model system, apart from being conventional, is also influenced by the bioprocess being targeted and its relevance.

1.4: PSEUDOMONADS AS MODEL SYSTEM FOR METABOLIC ENGINEERING

The genus *Pseudomonas* comprises of a large group of highly diverse Gram-negative bacteria that are found abundantly as free-living organisms in soils, fresh water and marine environments, and in many other natural habitats. According to the microbial classification based on rRNA similarities, the largest group comprises of fluorescent species including *P. aeruginosa*, *P. fluorescens* (several biovars), *P. putida*, *P. chlororaphis*, *P. syringae* (many pathovars), *P. cichorii*, *P. stutzeri*, *P. mendocina*, *P. alcaligenes*, *P. pseudoalcaligenes*, *P. agarici*, etc., (predicted according to Palleroni et al. 1973).

The great catabolic versatility of pseudomonads has conferred an important ecological advantage which has allowed them (i) to colonize new habitats, including those toxic for most micro-organisms, (ii) to acquire and develop the specific mechanisms responsible for their natural resistance to harmful compounds and adaptations against metal stresses (Schleissner et al., 1997; Hamel et al, 1999), (iii) to promote plant growth and (iv) control plant pathogens by secretion of several antibiotics and antifungal molecules (Preston, 2004; **Fig. 1.6**).

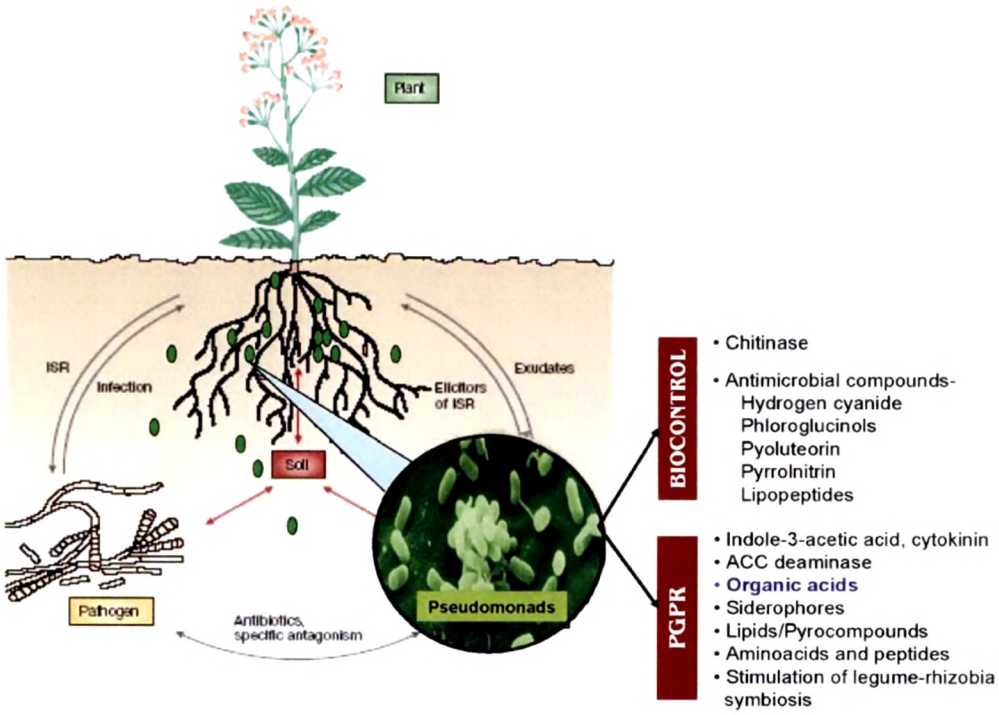

Fig. 1.6: Production of a variety of metabolites and other rhizospheric interactions of fluorescent *Pseudomonads* spp. These elements interact with one another through biotic and abiotic signals, many of which are still unknown. ISR, induced systemic resistance.

Variations in the catabolic pathways occurring within the members of this genus are attributed to evolutionary pressures and adaptations to certain adverse environmental and nutritional conditions (Ramos and Timmis, 1987). Presence of a single specific enzyme or an entire catabolic pathway could account for the extensive environmental adaptability and corresponding physiological differences observed within species of same genus and even strains of same species of pseudomonads (Schleissner et al., 1997; Ballerstedt et al., 2007). Moreover, comparing the genome sequences for opportunistic pathogen *Pseudomonas aeruginosa* PAO1, plant commensal *P. fluorescens* Pf-5 and *Pseudomonas fluorescens* PfO-1, all known for occurring in different environments, it is clear that not only physiological differences exist but they are also genetically diverse (Stover et al., 2000; Paulsen et al., 2005; Copeland et al., unpublished-submitted 2005). On account of the diverse survival and catabolic mechanisms, pseudomonds have been preferred targets of genetic engineering for the following applications:

(i) To enhance their use for the fermentative production of various industrially and commercially important biomolecules and secondary metabolites

For example, improved yield of medium-chain polyhydroxyalkanoates (PHAs, biodegradable polyesters) from triacylglycerols has been achieved by homologous overexpression of lipase gene in *P. corrugata, P. putida* and *P. oleovorans* (Solaiman et al., 2001; 2002). *P. putida* KT2442 mutant of *fad*A and *fad*B genes encoding 3-ketoacyl-CoA thiolase and 3-hydroxyacyl-CoA dehydrogenase, respectively, increased the production of PHAs with high 3-hydroxydodecanoate monomer content which improved crystallinity and tensile strength (Ouyang et al., 2007). On the other hand, heterologous expression of biosynthetic operon consisting of three genes, methylmalonyl-CoA (mm-CoA) epimerase, mm-CoA mutase (MCM) and *mea*B of MCM pathway in *P. putida* enabled it to synthesize novel complex polyketides like myxothiazol, requiring mm-CoA as the extender unit (Gross et al., 2006).

(ii) To use as effective biocontrol and plant growth promoting agents

Exploitation of beneficial plant-microbe interactions in the rhizosphere can result in the promotion of plant health and biocontrol. *Pseudomonas fluorescens* CHA0-Rif (pME3424) that is genetically modified to overproduce antimicrobial polyketides 2, 4-diacetylphloroglucinol (Phl) and pyoluteorin (Plt), displayed improved biocontrol efficacy (Girlanda et al., 2001). The gene cluster responsible for Phl biosynthesis in *P. fluorescens* F113 when integrated into the genome of a non-Phl producing *P. fluorescens* strain SBW25 EeZY6KX enabled the strain to exhibit dual biocontrol mechanisms by producing Phl and antifungal agents (Bainton et al., 2004). The advancements in '-Omics' technology has increasingly helped to understand the microbe-plant interactions and thereby overcome the existing limitations in designing improved strategies for the development of novel *Pseudomonas* biocontrol inoculant consortia (Mark et al., 2006).

(iii) **To use in bioremediation treatment strategies**

Metabolic engineering of bacteria for environmental bioremediation is still a challenging task of increasing environmental concern. One of the several examples is the introduction of plasmid pWW0 into *P. putida* F39/D (a derivative of *P. putida* F1, which was unable to transform cis-glycol intermediates to corresponding catechols) allowed simultaneous mineralization of BTX mixture *via* engineered *tol* pathway of aromatic hydrocarbon degradation (Lee et al., 2004).

In another study addressing the biodegradation of 2-chlorotoluene, a gene cassette containing two catabolic segments, one encoding the toluene dioxygenase of the TOD system of *P. putida* F1 (*tod*C1C2BA), and the other encoding the entire upper TOL pathway from pWW0 plasmid of *P. putida* mt-2 was inserted in the chromosome of the 2-chlorobenzoate degraders *P. aeruginosa* PA142 and *P. aeruginosa* JB2. These recombinant strains could mineralise 2-chlorotoluene, however, they failed to grow on 2-chlorotoluene as the only carbon source (Haro and de Lorenzo, 2001). *P. putida* KT2442 has been engineered to use the organophosphate pesticide parathion, a compound similar to other organophosphate pesticides and chemical warfare agents, as a source of carbon and energy (Walker and Keasling, 2002).

Considering the versatility and the high potentials of pseudomonads as discussed above, understanding its central carbon metabolism and regulatory mechanisms can help further explore the potential of this organism. Hence, fluorescent ***Pseudomonas* spp.** was chosen for metabolic engineering of the central carbon metabolism to investigate its flexibitly/rigidity. However, detailed genetic and metabolic network and regulations of pseudomonads were not integrated and were known to vary significantly from species to species. Furthermore, *Pseudomonas* carbon metabolism has been shown to be distinct from the well-studied *E. coli* carbon metabolism.

1.5: CARBOHYDRATE METABOLISM IN PSEUDOMONADS

The metabolic details and relative flux distribution discussed above for the model organism *E. coli* are very much different from the obligate aerobe *Pseudomonas*. Compared to facultative anaerobe *E. coli*, the glucose metabolism of *Pseudomonas* differs chiefly in the following aspects-(i) Absence of PTS mediated glucose uptake; (ii) ED pathway being almost exclusive catabolic route; (iii) PP pathway exhibiting completely biosynthetic functions; (iv) The respiratory mechanisms being highly efficient with very low overflow metabolism; (v) Significantly functional pyurvate bypass instead of the malate dehydrogenase (MDH) in TCA cycle; (vi) Glucose is extracellualrly converted to gluconate and 2-ketogluconate and simultaneously is also internalized by an active mechanism; and (vii) Absence of cAMP dependent glucose mediated catabolite repression (MacGregor et al., 1992; del Castillo and Ramos, 2007).

1.5.1: Glucose catabolic pathways in pseudomonads

In pseudomonads although organic acids are the preferred carbon sources presence of glucose as the sole carbon source does induce the glucose metabolizing pathways. Pseudomonads do not catabolize glucose to triose phosphate via the traditional EMP pathway as they lack the key glycolytic enzyme PFK (Lessie and Phibbs, 1984). Unlike *E. coli*, pseudomonads generally lack PEP-PTS system for glucose uptake (Romano et al., 1970). Instead, pseudomonads catabolize glucose by two different routes: the direct oxidative pathway which acts on glucose extracellularly and the simultaneously operating intracellular phosphorylative pathway. Early glucose dissimilation studies showed that in most of the pseudomonads glucose oxidation occurs in two successive reactions forming D-gluconate and 2-keto-D-gluconate (2-KG) catalyzed by a membrane-bound PQQ-GDH and gluconate dehydrogenase (GADH) respectively, in the periplasm (Lessie and Phibbs, 1984). Since then direct oxidative pathway has been demonstrated to occur in *Pseudomonas fluorescens* 52-1C (Fuhrer et al., 2005), *P. putida* U (Schleissner et al., 1997), *P. putida* KT2442 (Basu and Phale, 2006) and *P. aeruginosa* 2F32 (Midgley and Dawes, 1973). Although the direct oxidation pathway was not found in *P. putida* KT2440 (Lessie and Phibbs, 1984; Fuhrer et al., 2005), its genome sequence showed the presence of the PQQ-GDH encoding *gcd* gene (Nelson et al., 2002).

Furthermore, gluconate and 2-KG after being transported into the cell are acted upon by ATP dependent gluconokinase and 2-ketogluconokinase, respectively and 2-keto-6-phosphogluconate reductase finally producing 6-phosphogluconate (6-PG) to enter the central metabolism **(Fig. 1.7;** Lessie and Phibbs, 1984; Swanson et al., 2000; Fuhrer et al., 2005). This complex mechanism for glucose uptake has been demonstrated earlier for *P. aeruginosa* PAO (Lessie and Phibbs, 1984), *P. aeruginosa* M60 (Williams et al., 1996) and *P. fluorescens* A3.12 (Narrod and Wood, 1956). Generally, the genes responsible for direct oxidation of glucose and subsequent intracellular metabolism of gluconate and 2-KG occur variably as evident from **Table 1.5** (Stover et al., 2000; Nelson et al., 2002; Buell et al., 2003; Joardar et al., 2005; Paulsen et al., 2005; Lee et al., 2006; Vodovar et al., 2006; Yan et al., submitted 2006; Copeland et al., submitted 2005; 2007a; 2007b; 2008). Alternately, pseudomonads can also accumulate glucose *via* an active transport mechanism (Midgley and Dawes, 1973; Eisenberg et al., 1974; Guymon and Eagon, 1974) which is induced by glucose and transports glucose in the

form of free sugar; requiring a periplasmic glucose binding protein in *P. aeruginosa* (Lessie and Phibbs, 1984; Cuskey, 1985). Thus, direct oxidation is not the obligatory step for glucose metabolism. Intracellular glucose is rapidly phosphorylated by glucokinase (*glk*) followed by oxidation to 6-PG by glucose-6-phosphate dehydrogenase (*zwf*). These reactions comprise of the intracellular phosphorylative pathway.

***Pseudomonas* strain**	**GDH**	**GAD**	**GLK**	**6-PGDH**	**GNK**	**KGK**	**KGR**
P. aeruginosa PAO1	+	+	+	-	+	-	-
P. aeruginosa UCBPP-PA14	+	+	+	-	+	-	+
P. putida KT2440	+ *	putative	+	+	+	-	+
P. putida F1	+	+	+	+	-	-	-
P. putida GB-1	-	-	+	+	-	-	-
P. fluorescens Pf5	putative	putative	+	-	putative	-	putative
P. fluorescens PfO-1	-	-	+	+	-	-	-
P. syringae pv. phaseolicola 1448A	+	-	+	putative	putative	-	-
P. stutzeri A1501	+	-	+	-	-	-	-
P. mendocina ymp	+	-	+	+	-	-	-
P. syringae pv. tomato DC3000	putative	-	+	+	+	-	-
P. entomophila L48	+	-	+	putative	+	-	-

Table 1.5: Distribution of the essential glucose catabolism genes across the partially and completely sequenced genomes of *Pseudomonas* spp. GDH, glucose dehydrogenase; GAD, gluconate dehydrogenase; GLK, Glucokinase; GNK, gluconokinase; KGK, 2-ketogluconokinase; KGR, 2-ketogluconate-6-phosphate reductase; 6-PGDH, 6-phosphogluconate dehydrogenase. *GDH in *P. putida* KT2440 remains controversial as genome sequence reveals presence of *gcd* gene while Fuhrer et al (2005) demonstrated absence of the GDH mediated direct oxidation pathway in the same strain.

Contribution of periplasmic and intracellular pathways in the glucose catabolism varies in pseudomonads. *P. putida* C5V86 (Basu and Phale, 2006) and *P. citronellolis* (O'Brien, 1975; Lessie and Phibbs, 1984; Fuhrer et al., 2005) exclusively catalyze glucose by intracellular phosphorylative oxidation as they lack the GDH and GADH activities whereas *P. acidovorans* lacks GLK as well as GDH thereby failing to assimilate glucose (Lessie and Phibbs, 1984). In most pseudomonads both the glucose oxidation pathways are operative and their contribution alters depending on the physiological conditions. Glucose flux distribution between the direct and phosphorylative oxidation pathways in *P. aeruginosa* has been shown to be largely

dependent on oxygen concentration (Mitchell and Dawes, 1982) and glucose availability (Whiting et al., 1976) while on the temperature in *P. fluorescens* (Lynch et al., 1975).

P. fluorescens can utilize gluconate and 2-ketogluconate only when glucose in the medium becomes limiting (Fuhrer et al., 2005). In contrast, *P. putida* has been shown to metabolize glucose by three simultaneously operating pathways (i) ABC uptake system mediated internalization and phosphorylative oxidation initiated by GLK, (ii) direct oxidation to gluconate which can be transported into the cytoplasm and subsequently phosphorylated by GNK to 6-PG (iii) gluconate oxidation to 2-ketogluconate followed by transport to the cytoplasm and subsequent phosphorylation and reduction to 6-PG (del Castillo et al, 2007). Microarray and enzymatic analysis revealed that in presence of glucose although all three pathways were induced simultaneously, GLK mediated pathway being the main catabolic route.

The central metabolite of glucose catabolism in pseudomonads is 6-PG, a point of convergence of several catabolic pathways like direct and phosphorylative glucose oxidation as well as pentose phosphate pathway (Lee and Lessie, 1974). 6-PG is ultimately catabolized by ED pathway (Quay et al., 1972) that serves as the principal route of glucose catabolism. The ED pathway was first elucidated in *P. saccharophila* (Entner and Doudoroff, 1952) and subsequently established as a major glucose dissimilation mechanism in pseudomonads. Gluconate, the product of direct oxidation pathway serves as the inducer of the ED pathway in *P. fluorescens*; however, 6-PG has been suggested to be the possible physiological inducer of ED pathway in *P. aeruginosa* and *P. cepacia* (Ornston, 1971; Vicente and Canovas, 1973; Eisenberg et al., 1974, Lessie and Phibbs, 1984). 6-PG dehydratase (encoded by *edd*) and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (encoded by *eda*) are the two enzymes comprising the ED pathway **(Fig. 1.7).**

Pseudomonads exhibit, due to the lack of PFK, a cyclic operation of ED pathway at the level of fructose-1,6-bisphosphate (F-1,6-BP) formation during which glyceraldehyde-3-phosphate (G-3-P) derived form cleavage of KDPG is recycled to 6-PG *via* F-1,6-BP, fructose-6-phosphate (F-6-P) and G-6-P instead of being directly converted to pyruvate *via* PEP (Lessie and Phibbs, 1984; Fuhrer et al., 2005). Cyclic operation of ED pathway is also known to occur in *Acetobacter*, *Agrobacterium*,

Azotobacter, *Rhizobium*, *Paracoccus*, and *Xanthomonas* spp., which preferentially utilize organic acids rather than sugars and secrete exopolysaccharides (Portais and Delort, 2002). The cyclic pathway operation may facilitate the formation of the polysaccharide precursor F-6-P in cases where the EMP pathway is absent (Fuhrer et al., 2005).

1.5.2: Contribution of other metabolic pathways in glucose dissimilation

G-3-P formed from ED pathway is converted to PEP and pyruvate by the lower glycolytic (EMP) pathway (**Fig. 1.7**). Along with ED pathway, TCA cycle constitutes the central glucose catabolism pathways (Fuhrer et al., 2005). The enzymes involved at the junction of the glucose catabolism and the TCA cycle responsible for maintaining the optimal levels of key metabolites like PEP, pyruvate and OAA, again vary from strain to strain but unlike *E. coli*, pseudomonads have another enzyme pyruvate carboxylase (PYC) to catalyze the conversion of pyruvate to OAA). Unusually, *P. fluorescens* employs a so-called pyruvate shunt for malate-to-pyruvate conversion bypassing the regular malate dehydrogenase reaction (**Fig. 1.7**; Fuhrer et al., 2005; del Castillo et al., 2007). The flux of 6-PG between the ED pathway and hexosemonophosphate (HMP) shunt (pentose phosphate pathway; PPP) is regulated by intracellular NADPH levels as demonstrated in *Pseudomonas multivorans* (Lee and Lessie, 1974). Apparently, PPP plays a biosynthetic role and does not contribute significantly towards energy generation in *P. saccharophila, P. fluorescens,* and *P. putida* but significantly contributes to glucose metabolism in *P. cepacia* and *P. marginata* (Lessie and Phibbs, 1984; Vicente and Canovas, 1973; Eisenberg et al., 1974, Fuhrer et al., 2005). PPP plays no role in glucose dissimilation in *P. aeruginosa* as it is naturally deficient in 6-PG dehydrogenase (Lessie and Phibbs, 1984; Stover et al., 2000; Lee et al., 2006; **Table 1.5**).

1.5.3: Dissimilation of fructose and other carbohydrates

Fructose is the only carbohydrate which is uptaken in pseudomonads via PEP:fructophosphotransferase system, except in *P. cepacia* and certain strains of *P. saccharophila* which accumulate fructose by active transport (Lessie and Phibbs, 1984). PFK-1 is induced and the hence formed F-1,6-BP either forms triose phosphate via EMP reaction forming the minor branch while the major catabolism occurs through conversion to F-6-P and subsequent metabolism through ED pathway. The metabolic pathways of other carbohydrates like mannitol, glycerol, galactose, lactose etc. and the nodes of their merger to the central catabolic pathway are as depicted in **Fig. 1.7.**

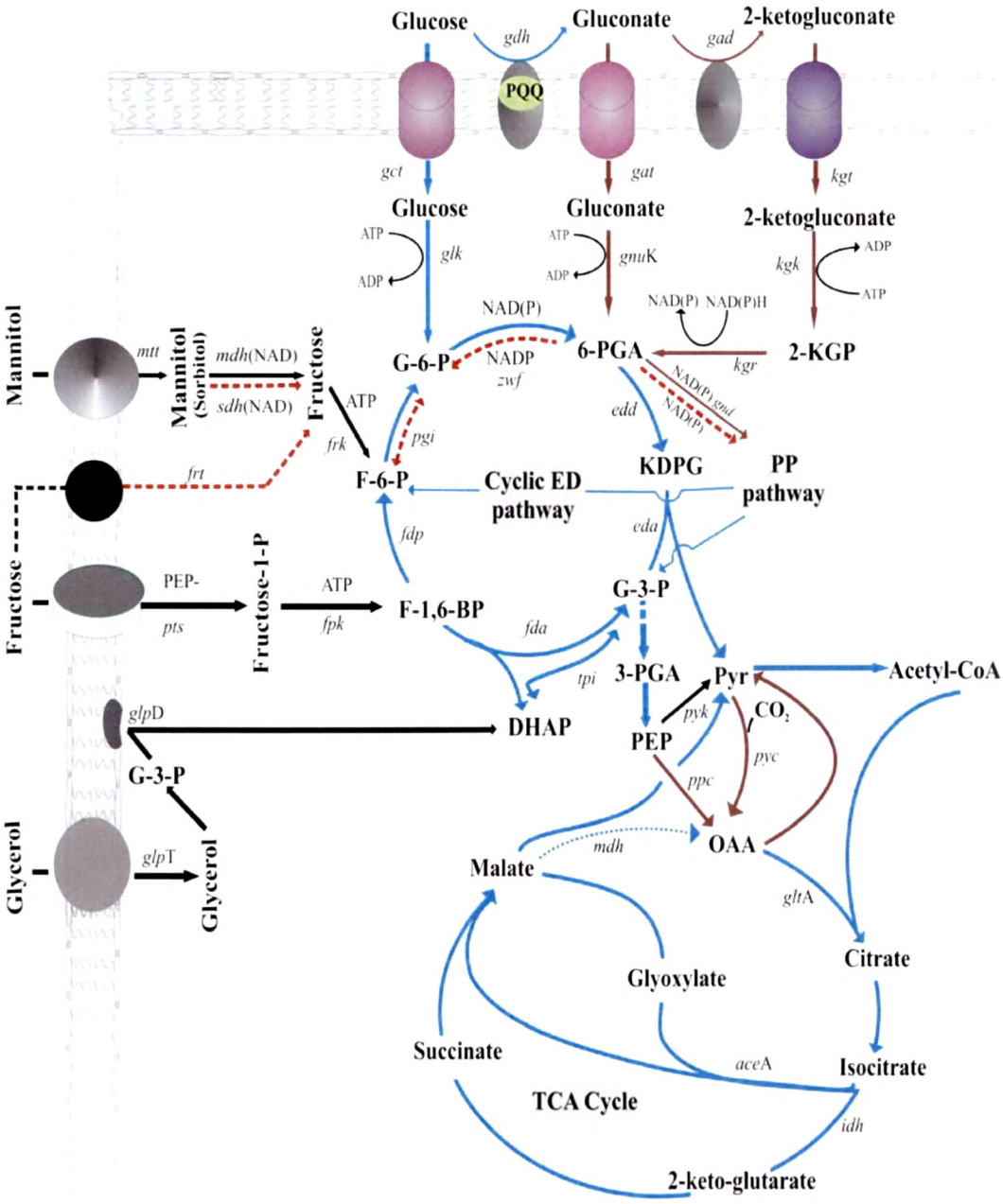

Fig. 1.7: Carbohydrate metabolism in pseudomonads

Key to the pathway: Blue lines/arrows=Pathway operating in presence of glucose; **Bold Black arrows**=Reactions occurring when carbon source is other than glucose; Blue dashed arrow=Flux through that reaction is very low; Brown arrows=Occurrence of those genes is highly variable from strain to strain; Red dashed arrows=Reactions specific to *Pseudomonas cepacia*.

Under any given condition, the metabolic network and its regulatory circuits synchronize in order to balance the catabolic and anabolic reactions to meet the requirements of energy and biomass. For smooth functioning of the metabolism, there are several check-points existing in the system at the junction of any two metabolic pathways. These branch points are sufficiently flexible so as to maintain a tight control over the carbon flux through a particular path under a particular condition. The following few sections discuss the importance of these check-points in the metabolism of several organisms and their role as potential new targets for metabolic engineering.

1.6: IMPORTANCE OF PEP-PYRUVATE-OAA BRANCH POINT IN THE CELLULAR METABOLISM

In most aerobic and facultatively anaerobic bacteria the chief metabolic pathways are the EMP pathway (glycolysis), ED pathway and the TCA cycle. The former two are involved in breakdown of carbohydrate to PEP and pyruvate which serve as precursors for biosynthesis of several cellular components. The pyruvate dehydrogenase (PDH) complex links the glycolytic/ED pathway and TCA cycle by further breakdown of pyruvate to acetyl-CoA which enters directly into TCA cycle. TCA cycle also performs dual functions of complete catabolism of acetyl-CoA for respiratory ATP formation as well as supplying the precursors for anabolism. On the other hand, when grown on TCA cycle intermediates or substrates that enter the central metabolism via acetyl-CoA, the cell diverts the metabolism towards gluconeogenic pathways for synthesis of the PEP and pyruvate to synthesize the essential sugar phosphates. Hence, the balance in the cellular physiology is highly dependent on the interactions between the catabolic and anabolic pathways.

The crucial metabolic link between the glycolytic / gluconeogenic / ED pathway and TCA cycle is the **PEP-Pyruvate-OAA** node often referred to as the anaplerotic node (Sauer and Eikmanns, 2005). The set of reactions operating at this node decide the carbon flux in a particular direction depending on the growth condition, thus acting as a key switch governing the overall cellular metabolism. Under glycolytic conditions, PEP and pyruvate enter the TCA cycle by two routes, one by oxidative decarboxylation forming acetyl-CoA and second by C3 carboxylation to form OAA which together

energize the first reaction of TCA cycle. The formation of OAA by carboxylation of PEP or pyruvate is defined as **anaplerosis**, a process that replenishes the TCA intermediates utilized for anabolic purposes.

In spite of being so critical, this node is quite flexible with respect to the metabolite pool and the prevailing biomolecular regulatory circuits. In certain organisms like *E. coli*, the regulation at this node is very simply mediated by catabolite repression that does not allow gluconeogenic enzymes to express in presence of sugars. But in certain organisms like *Bacillus, Corynebacterium* and specific strains of *Pseudomonas*, more than one enzyme is responsible for C3 carboxylation and C4 decarboxylation, resulting into much more complex regulation at the anaplerotic node (Sauer and Eikmanns, 2005). The collective information regarding the metabolic activities at the PEP-Pyruvate-OAA node based on biochemical, genetic and regulatory studies carried out in different bacterial species is as summarized in **Fig. 1.8**.

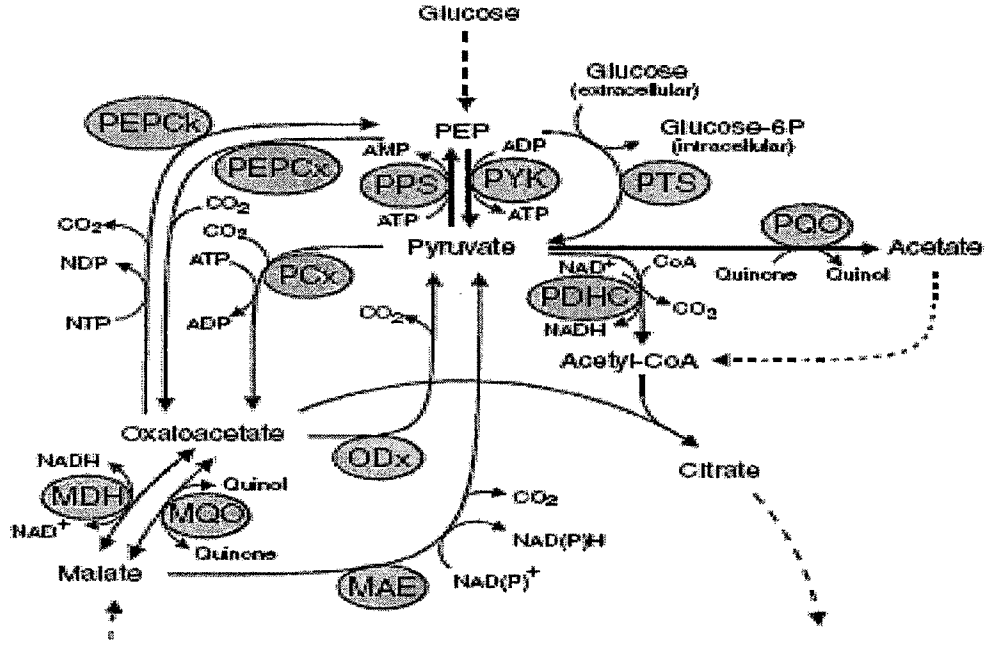

Fig. 1.8: Enzymes and pathways implicated in regulation at the PEP-Pyruvate-OAA node of different aerobic bacteria (Sauer and Eikmanns, 2005). Abbreviations denote the gene products that catalyze a given reaction: MAE, malic enzyme; MDH, malate dehydrogenase; MQO, malate: quinone oxidoreductase; ODx, oxaloacetate decarboxylase; PCx, pyruvate carboxylase; PDHC, pyruvate dehydrogenase complex; PEPCk, PEP carboxykinase; PEPCx, PEP carboxylase; PPS, PEP synthetase; PQO, pyruvate: quinone oxidoreductase; PTS, phospho-transferase system; PYK, pyruvate kinase.

However, a particular species contains only a subset of these reactions. Apart from this there are certain typical features observed in different organism which are reviewed in detail by Sauer and Eikmanns (2005). Commonly, enzymes involved in C3 carboxylation are PEP carboxylase (PPC) and pyruvate carboxylase (PYC). C4 decarboxylation is irreversibly catalyzed by OAA decarboxylase (ODx) while PEP carboxykinase (PEPCk), PEP carboxytransphosphorylase and malic enzyme perform the same function in a reversible manner. The differential occurrence of these enzymes in some of the commonly known bacterial sp. is as listed in **Table 1.6.**

Organism	PEPCK	PPC	PYC	ODx	MAE	PPS	PDHC
E. coli	1(ATP)	1	0	0	1(NAD),1(NADP) 1(NADP)	1	1
C. glutamicum	1 (GTP)	1	1	1	1 (NADP)	0	1
B. subtilis	1 (ATP)	0	1	0	2(NAD) 1(NADP)	0	1
Rhizobium etli	1 (ATP)	1	1		1(NAD) 1(NADP)	1	1
Sinorhizobium meliloti	1 (ATP)	0	1		1(NAD) 1NADP	1	1
Rhododpseudomonas palustris	1 (ATP)	1			1(NAD)	1	1
Pseudomonas citronellolis	0	1	1	1		1	1
Pseudomonas fluorescens	1	1	1		1(NADP)	1	1
Zymomonas mobilis		1			1		1

Table 1.6: Distribution of enzymes acting at PEP-pyruvate-OAA node in different bacteria (Sauer and Eikmanns, 2005). The numbers indicate the number of isozymes present in a given organism. Zero means that the organism has been tested for the enzyme or the respective gene however no activity is found so far. Empty space means that there is lack of evidence for the enzyme or the functional gene.

1.6.1: PEP-Pyruvate-OAA node in *E. coli*

The carbon flux at this node in *E. coli* is much more stringently regulated because PEP is involved in three major metabolic processes like PTS mediated sugar transport, in the PPC mediated anaplerotic reaction and as a precursor in the biosynthesis of amino acids (Clark, 1989; Gokarn et al., 2001). The enzymes participating in the PEP-Pyruvate-OAA interconversion in *E. coli* are as configured in **Fig. 1.9**. PPC is the exclusive C3 carboxylating enzyme while ATP-dependent PEPCk is primarily involved in C4

decarboxylation and gluconeogenesis (Yang et al., 2003). Other options for C4 decarboxylation are NADP dependent malic enzyme (ME) *mae*A and *mae*B whereas *sfc*A encodes NAD dependent ME which convert malate to pyruvate under physiological conditions but upon pyruvate accumulation can also act in reverse but thermodynamically favorable direction (Stols and Donnelly, 1997). However, malic enzymes are dispensable because PEP formation can be mediated by malate dehydrogenase and PEPCk. Interconversion of PEP and pyruvate is mediated by PYK and PEP synthetase (*pps*A, especially during growth on C3 acids like lactate and pyruvate). When grown on acetate, glyoxylatye shunt also contributes to anaplerosis replenishing the essential C4 intermediates of TCA cycle.

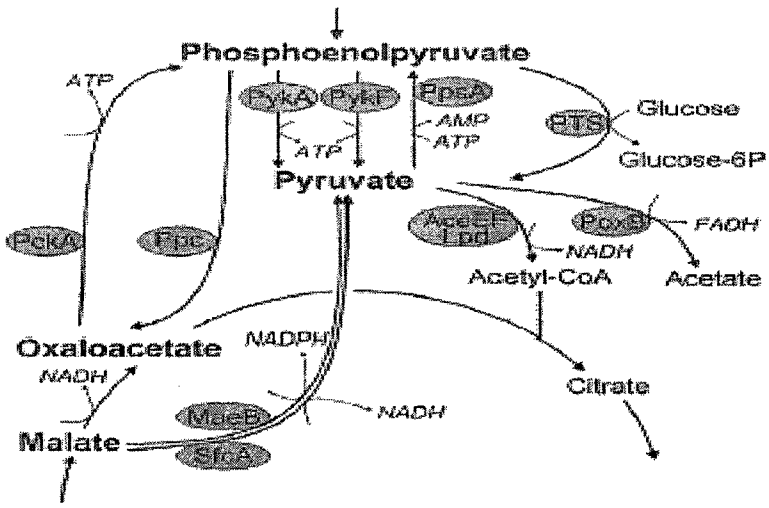

Fig. 1.9: The PEP-Pyruvate-OAA node in aerobic *E. coli*. Abbreviations denote the gene products that catalyze a given reaction: PykA(F), pyruvate kinase(s); PpsA, PEP synthetase; PTS, phosphotransferase system (for glucose uptake); AceEF and Lpd, pyruvate dehydrogenase; PoxB, pyruvate oxidase; Ppc, PEP carboxylase; PckA, PEP carboxykinase; MaeB and SfcA, malic enzyme(s).

Pyruvate apart from being converted to acetyl-CoA by PDH complex is acted upon by pyruvate oxidase to form acetate in the stationary phase (Dittrich et al., 2005). Hence, more than one metabolic reactions or enzymes are competing for the same metabolite to regulate this node. Additionally, the enzymes are individually regulated at the allosteric and transcriptional level. The regulation and expression of these enzymes also varies with aerobic and anaerobic conditions depending on the carbon source. Nevertheless the expression level of several genes of the PEP-pyruvate-OAA node is

apparently insufficient for optimal unrestricted growth on glucose or on gluconeogenic substrates like pyruvate and succinate (Sauer et al., 1999).

Simultaneous functioning of opposite reactions, owing to multiple involvements of the metablolites might result into significant futile fluxes. For example, PPC/PEPCk catalyzed ATP dissipating futile cycle in *E. coli* adversely effects under glucose limitation but not under glucose sufficient conditions (Chao and Liao, 1994; Sauer et al., 1999). In glucose-limited cultures of *E. coli* acetyl-CoA is completely oxidized to CO_2 *via* the PEPCk-glyoxylate cycle, which is normally done by TCA cycle (Fischer and Sauer, 2003). Such a bi-functional catabolic and anabolic role is contradictory to their classical function of gluconeogenesis and anaplerosis. However, requirement of this novel circuit is unclear as it is functionally redundant with the PPC and the TCA cycle (Sauer and Eikmanns, 2005).

1.6.2: PEP-Pyruvate-OAA node in *Bacillus subtilis and Corynebacterium glutamicum*

Bacillus is different from *E. coli* in being aerobic Gram-positive organism. In spite of its biotechnological and industrial relevance, not much is known about C3-carboxylating and C4-decarboxylating enzymes. The variations in the enzymes catalyzing the metabolic activity at this node in *B. subtilis* are as evident in **Fig. 1.10a**. The major difference is that instead of PPC, PYC acts as the sole anaplerotic enzyme to synthesize OAA. Due to absence of glyoxylate shunt, the organism fails to utilize carbon source that are metabolized via acetyl-CoA. PEPCk serves dual functions, primarily being involved in gluconeogenesis while performs a minor catabolic role in PYK and certain other mutants, by acting in reverse direction, despite being thermodynamically unfavorable (Sauer and Eikmanns, 2005). *B. subtilis* contains four paralogues *mle*A, *yts*J, *mal*S and *mae*A encoding putative ME, of which *yts*J encodes the major NADP-ME which is expressed constitutively on either glucose or malate (**Fig. 1.10a**). PYK and ME(s) constitute *pyruvate shunt* that substantially contributes to glucose uptake rate on carbon-limited conditions. Under these conditions, PEPCk flux is high which along with PYC and PYK constitutes an ATP dissipating futile cycle. Under gluconeogenic conditions PEPCk and MEs play a major role in redirecting the flux through PEP-Pyruvate-OAA node. Major regulation at this point is brought about by allosteric mechanisms and not by transcriptional control, unlike *E. coli*.

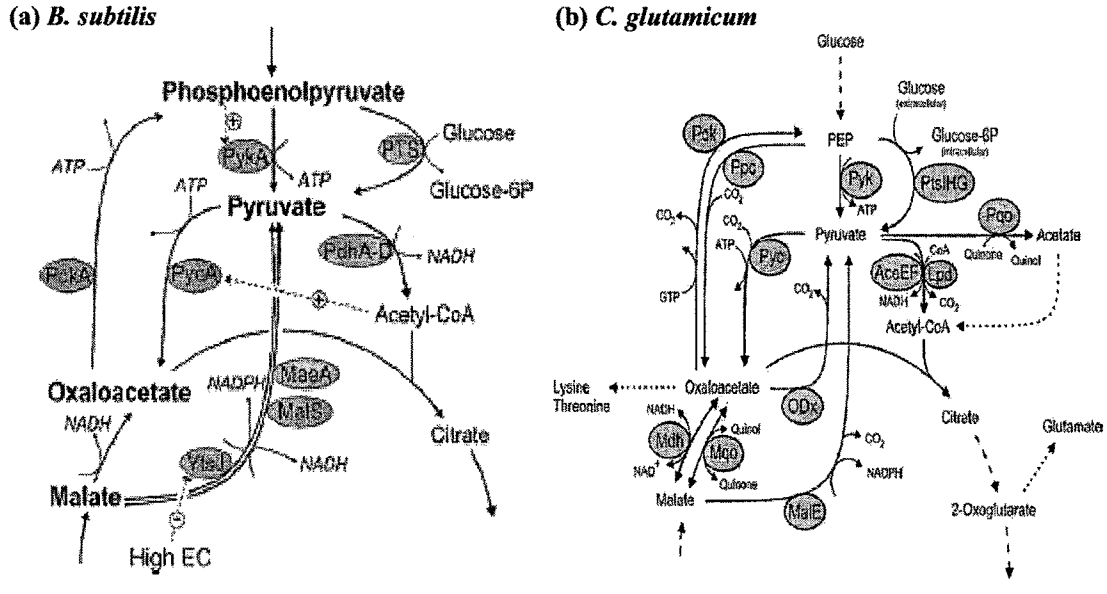

Fig. 1.10: The PEP-Pyruvate-OAA node in *Bacillus subtilis* and *Corynebacterium glutamicum*. Abbreviations denote gene products that catalyze a given reaction: AceEF, E1 and E2 subunits of the PDH complex; EC, energy charge; Lpd, subunit E3 of the PDH complex; MalE, MaeA, MalS and YtsJ, malic enzyme(s); Mdh, malate dehydrogenase; Mqo, malate: quinone oxido-reductase; ODx, oxaloacetate decarboxylase (gene not annotated); Pck, PEP carboxykinase; PckA, PEP carboxy-kinase; PdhABCD, pyruvate dehydrogenase complex; Pqo, pyruvate: quinone oxidoreductase; PTS and PtsIHG, phospho-transferase system; Pyc and PycA, pyruvate carboxylase; Pyk and PykA, pyruvate kinase

C. glutamicum exhibits some of the major differences in the type of enzyme occurring at this node as compared to *E. coli* and *B. subtilis* (**Fig. 1.10b**). It possesses both PPC and PYC as anaplerotic enzymes for C3 carboxylation, which are regulated by different allosteric effectors. PPC and PYC enzymes may have different affinities to HCO_3^- and thus the use of either of the two might be conditional (Koffas et al., 2002). Additionally, PEPCk, ME or ODx function for C4 decarboxylation converting OAA or malate to PEP or pyruvate (**Fig. 1.10b; Table 1.6**) of which PEPCk (GTP dependent) is the main enzyme with no anaplerotic functions. Unlike *E. coli* and *B. subtilis*, there is only one NADP dependent ME which acts for malate decarboxylation rather than for the reverse pyruvate carboxylation. ODx activity has been detected in several *C. glutamicum* strains but its function and role in growth and amino acid production is unclear. These enzymes constitute an unusual metabolic circuit with 5 enzymes directly participating in interconversion of the metabolites at the PEP-Pyruvate-OAA node.

In glucose grown cultures of *C. glutamicum*, the major anaplerotic role is played by PPC while PYC is the bottle neck enzyme for glutamate and lysine production (Shirai et al., 2007). Under glycolytic conditions, PYC, PEPCk and PYK are responsible for an energy (ATP/GTP) consuming (futile) cycle (**Fig. 1.10b**) but its physiological significance is unclear. The PDH complex is exclusively involved in oxidative decarboxylation of pyruvate to acetyl-CoA and is surprisingly not subjected to any allosteric regulation for unclear reasons. The reaction of the PDH complex may be bypassed by the combined activities of pyruvate:quinone oxidoreductase (catalyzing the oxidative decarboxylation of pyruvate with a naphtoquinone as electron acceptor), acetate kinase and phosphotransacetylase (both constitutively expressed and function to form acetyl-CoA from acetate), but the bypass will be thermodynamically unfavorable due to ATP requirement of acetate kinase. On acetate, the glyoxylate cycle was found to be the essential anaplerotic pathway (Reinscheid et al., 1994; Gerstmeir, R., 2003).

1.6.3: Genetic manipulations at the anaplerotic node in *E. coli, B. subtilis* and *C. glutamicum*

Anaplerotic node has been manipulated not only for understanding the regulatory network but also for altering molecular fluxes for improving the bioprocesses. One of the most successful examples is the case of fermentative and aerobic succinate overproduction in *E. coli* (detailed in Section 1.3). Pyruvate overproduction was achieved in various *E. coli* mutants having block in conversion of PEP to OAA and pyruvate to acetyl-CoA, PEP, acetate, lactate and ethanol by deletion of the genes coding for the PDH complex (*ace*EF), pyruvate formate lyase (*pfl*B), PEP synthetase (*pps*), pyruvate: quinone oxidoreductase (*pox*B), acetate kinase, lactate dehydrogenase (*ldh*A), PPC and alcohol dehydrogenase (Wendisch et al., 2006). Anaplerotic node in *E. coli* has been successfully engineered for optimizing the amino acid production as PEP forms a key precursor molecule (Bongaerts et al., 2001; Kramer et al., 2003). These strategies include avoiding the drain of PEP to pyruvate by mutation in gene encoding PYK; a non-PTS sugar uptake and increasing the gluconeogenic fluxes to PEP (e.g. overexpression of PEP synthetase), coupled with overexpression of transketolase which increases erythrose-4P level (Patnaik and Liao, 1994; Flores et al., 1996).

Several mutations in the enzymes acting at this node affect the physiological state of other relevant enzymes and *in vivo* pathway fluxes. Deletion of PYK gene developed a

local catabolic loop involving PPC and ME which jointly function for both anapleriosis and catabolism thereby highlighting a newer function of the two enzymes (Emmerling et al., 2002) in *E. coli* but not in *B. subtilis*. *E. coli ppc* mutant exhibits auxotrophy for TCA cycle intermediates like succinate when grown on glucose because glyoxylate bypass that can theoretically substitute the PPC reaction is inactive due to catabolite repression as well the competition of isocitrate dehydogenase (ICDH) and isocitrtae lyase (ICL) for the common substrate isocitrate. The anaplerotic function in *ppc* mutant could be fully restored by overexpressing pyruvate carboxylase (*pyc*) which is otherwise absent in *E. coli* (Gokarn et al., 2000; 2001). Similarly *ppc* gene overexpression in *E. coli* under aerobic conditions reduced acetate formation and a significantly increased biosynthetic efficiency (Farmer and Liao, 1997). Collectively, these results suggest that the anaplerotic reaction in *E. coli* is not optimized for unhampered growth on glucose and that some of the enzymes apart from their classically recognized functions in catabolism, anaplerosis and gluconeogenesis play novel roles in the metabolism of some bacteria.

Although extensive information on the regulatory mechanisms operating at the anaplerotic node is available for *B. subtilis* (Sauer and Eikmanns, 2005), no genetic manipulations are reported at this node. *C. glutamicum* is an aerobe industrially important for production of L-Lysine and L-glutamate (Kiefer et al., 2004). The fact that PEP-Pyruvate-OAA node is crucial for the supply of precursors for amino acid biosynthesis, a lot of focus has been there on the enzymes and their regulations involved at this node. Increase in PYC activity and abolition of PEPCk activity in *C. glutamicum* independently resulted in increased production of TCA cycle-derived amino acids like glutamate and lysine (Sauer and Eikmanns, 2005). The levels of ME activity affected the growth pattern of *C. glutamicum* on lactate but not on glucose or acetate (Gourdon et al., 2000). *C. glutamicum* overexpressing ME accumulated high levels of pyruvate in the medium. *C. glutamicum ppc* mutant grown under biotin limitation accumulated pyruvate due to which ME functioned in the reverse direction by utilizing pyruvate to replenish the TCA cycle intermediates (Gourdon et al., 2000).

All these bacteria, discussed above, metabolize glucose via the traditional EMP pathway yet exhibit such a diversified anaplerotic node. Pseudomonads, as discussed in earlier sections, are metabolically distinct with respect to non-PTS glucose uptake, two glucose catabolic routes ultimately following the ED pathway and exhibiting strain

specific variations in the occurrence of enzymes of central carbon metabolism and hence are likely to display a completely different interplay between the enzymes at the anaplerotic node. On account of the agricultural importance of pseudomonads and metabolic versatility, detailed analysis of prevailing regulations at the PEP-Pyruvate-OAA node by various genetic perturbations would not only add to fundamental knowledge but also might discover novel targets for metabolic engineering.

1.6.4: PEP-Pyruvate-OAA node in *Pseudomonas citronellolis*

The complex regulatory network at the PEP-Pyruvate-OAA node in *Pseudomonas citronellolis* with 5 different metabolically related enzymes had been elucidated by O'Brien and his co-workers (1977) (**Fig. 1.11**). Activities of C_3 carboxylating enzymes PPC and PYC, C_3-C_3 interconverting enzymes PEP synthase and PYK and C_4 decarboxylating ODx were detected when grown on glucose or lactate whereas PEPCk was not detected at all (**Table 1.7**). Four of these enzymes were found to be constitutive and hence obviously the circuit would be under tight control mediated by various metabolites.

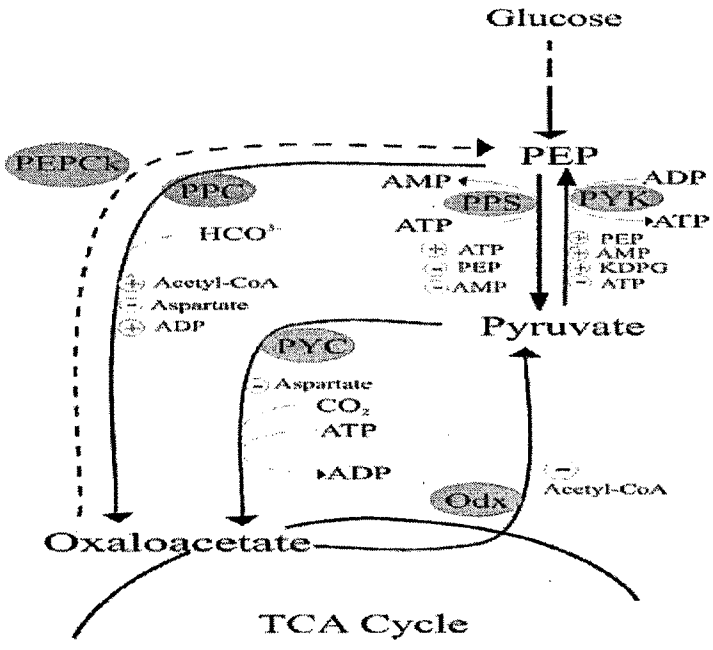

Fig. 1.11: PEP-Pyruvate-OAA node in *Pseudomonas citronellolis* (O'Brien et al., 1975). Abbreviations denote gene products that catalyze a given reaction: PYK, pyruvate kinase; PYC, pyruvate carboxylase; PEPCk, PEP carboxy-kinase; ODx, OAA decarboxylase; KDPG, 2-keto-3-deoxy-phosphogluconate. The minus sign indicates inhibitory while the plus sign indicates the activation effect of the adjacent metabolite, respectively.

Enzyme	Carbon source					
	Glucose	Lactate	Acetate	Citrate	Glutamate	Aspartate
	µmol/min/mg protein					
Oxalaocetate decarboxylase	1.15	1.10	1.25	1.20	1.70	1.25
Phosphoenolpyruvate carboxylase	0.053	0.034		0.031	0.043	0.039
Pyruvate kinase	0.22	0.12		0.055	0.070	0.078
Phosphoenolpyruvate synthase	0.023	0.026	0.022	0.033	0.030	0.035
Pyruvate carboxylase	0.044	0.031	0.015	0.012	0.009	0.002

Table 1.7: Activities of enzymes involved in interconversion of PEP-pyruvate-OAA in *P. citronellolis* cells grown on different carbon sources (O'Brien et al., 1977).

Most of the regulatory metabolites, shown in **Fig. 1.11,** exerted reciprocal but reinforcing effects under *in vitro* conditions but their physiological relevance is not known. For example, an increase in acetyl-CoA could lead to an increase in the concentration of OAA by two ways, either by stimulating the formation of OAA by PPC or by inhibiting OAA decarboxylation by ODx. As another example, the PEP levels might be lowered by an increase in the concentration of 5'-AMP which activates PYK and inhibits PEP synthase. Conversely, increase in ATP concentration increases the physiological levels of PEP in the cell. Unusual metabolic circuit at anaplerotic node in pseudomonads necessitates further understanding of the ongoing regulatory mechanism in different *Pseudomonas* strains.

1.6.5: Influence of PEP-pyruvate-OAA node on other branch points in the cellular metabolism

Apart from the PEP-Pyruvate-OAA branch point several other check points are also functional in the cellular metabolism, which might be influenced by altered flux distribution at the anaplerotic node. The direct involvement of the anaplerotic node in the TCA cycle is evident because the interplay of enzymatic reactions utilizing PEP and pyruvate at this node supplies the substrates OAA and acetyl-CoA for citrate synthase (CS) which is non-redundant for catalyzing the first step of TCA cycle to form citrate. CS catalyzes a crucial step at the branch-point of oxidative, lipogenic, and anaplerotic pathways (Walsh and Koshland, 1985a; 1985b). CS activity is regulated in *E. coli* at the transcriptional and allosteric level depending on the nature of the available carbon source (Park et al., 1994). Because of its key position as the first enzyme of the TCA cycle, CS

had been assumed to be an important control point for determining the metabolic rate of the cell and the carbon flux at the anaplerotic node (PEP levels) which could directly determine the flux through TCA cycle (Peng et al., 2004).

Another branch point in the central metabolism occurs between TCA cycle and the glyoxylate shunt which is mainly governed by the two enzymes NADP dependent ICDH and ICL competing for the common metabolite isocitrate. ICL in *E. coli* is mainly regulated at the level of expression depending on the growth conditions (acetate or glucose as carbon source) while ICDH is regulated by phosphorylation/ dephosphorylation (Walsh and Koshland, 1985b). Low PEP levels increase flux via glyoxylate cycle (Yang et al., 2003; Peng et al., 2004) and PEP *in vitro* inhibits ICDH as well as ICL; however its physiological significance is yet unclear (Ogawa et al., 2007). As compared to *E. coli*, the overall TCA flux is higher in pseudomonads along with very low acetate overflow metabolism (Fuhrer et al., 2005). The ICL and ICDH activities at TCA-glyoxylate branch-point in *P. fluorescens* as well the activities of malate synthase and CS alter significantly in response to aluminum stress (Hamel and Appanna, 2001).

1.6.6: Fate of engineering the central carbon metabolism in pseudomonads?

Metabolic and genetic diversity indicates that genetic perturbations in pseudomonads similar to that in *E. coli,* e.g. at the anaplerotic node, may lead to different responses. Discontinuous and unintegrated information regarding the metabolic networks in pseudomonads would complicate the predictions of the outcomes of targeted genetic alterations. However, exploring the metabolic flexibility at central carbon metabolism of pseudomonads would not only augment the fundamanetal understanding but could also help in enhancing their agricultural and ecological worth. Unavailability of soluble P is one of the major problems of agriculture and hence developing efficient biofertlizers with P-solubilizng ability is one of the major challenges to genetic engineering.

1.7: P-SOLUBILIZATION- A GLOBAL CHALLENGE

Phosphorus (P) is the second major nutrient required by the plants comprising of 0.2% of the plant's total dry weight and is an important constituent of essential molecules like nucleic acids, phospholipids, ATP etc. thus, is absolutely essential for optimal growth of plants (Theodorou and Plaxton, 1993).

1.7.1: Soil P and reasons for its deficiency

The soil P is in a dynamic equilibrium involving the geochemical and biological P transformations. Predominantly P is uptaken by plants in the form of $H_2PO_4^-$ (Vance et al., 2003). But a greater part of total soil P, almost 85-90%, is fixed in the form of insoluble phosphates mainly mineralized as Ca-P, Fe-P and Al-P or as organic bound forms like phytates, RNA etc. and hence cannot be utilized by plants (Holford, 1997; Khan et al., 2006). The degree of preicipitation and solubilization of Ca-P (predominant in vertisols), Fe-P and Al-P (predominant in alfisols) depends on the buffering capacity of a particular soil (Gyaneshwar et al., 2002). The soil P solution is in equilibrium with relatively labile form of P (present as very small portion) which is adsorbed to clay, calcium carbonates, organic matter etc. (Holford, 1997; Whitelaw, 2000; Arcand and Schneider, 2006). Soil P fixed as organic pool could supply P to the plants depending on the prevailing plant-microbial interactions (Arcand and Schneider, 2006).

P deficiency is one of the main biophysical constraints to crop production globally which engraved due to soil weathering and intensive nutrient-extracting agricultural practices (Akinrinde, 2006; Arcand and Schneider, 2006; Khan et al., 2006). Efficiency of the chemical fertilizers is low as the supplemented free Pi gets fixed into the soil due to pH dependent precipitation with cations and adsorption to soil particles (Rodríguez and Fraga, 1999). The use of conventional P fertilizers is highly limited in developing regions due to involvement of high expenditure and is prohibited for use by organic farmers as it harms the soil quality in long run (Khan et al., 2006; Harris et al., 2006). Additionally, soluble P in soil moves to plant roots mainly by diffusion which rate of which (10^{-12} to 10^{-15} m^2 s^{-1}) may be too low to meet the high plant P-uptake rates thereby creating a zone of P-depletion around the roots (Arcand and Schneider, 2006).

1.8: P-SOLUBILIZING MICROORGANISMS -AN ECOFRIENDLY TOOL

Phosphate solubilizing microorganisms (PSMs) are present naturally in plain soils and have been found to comprise 0.1 to 0.5% of the total soil microbial population (Kucey et al., 1983). The ability of microorganisms to act as PSMs was first demonstrated by Gerretsen in 1948. Since then, extensive reports are available which discuss the isolation, characterization and role of PSMs in enhancing the P availability in

the rhizosphere of plants (Kucey et al., 1989). Under diverse soil and agro-climatic conditions, the organisms with phosphate-solubilizing abilities have proved to be an economically sound alternative to the more expensive superphosphates and possess a greater agronomic utility. Rhizospheric microorganisms including bacteria like *Pseudomonas* sp., *Serratia*, *Bacillus* sp, *Rhizobium* sp., *Azotobacter*, *Azospirullum* and fungi like *Aspergillus*, *Penicillium*, etc (Rodriguez and Fraga, 1999; Whitelaw, 2000; Compant et al., 2005; Haas and Defago, 2005; Pradhan and Sukla, 2005; Khan et al., 2006) are now known to act as powerful PSMs. Although many PSMs have been isolated and characterized from soils, plant inoculation experiments resulted in variable effects on P supply, plant growth and crop yields (Gyaneshwar et al., 2002). These varied effects are attributed to the nature of the soil and survival inoculated microbes in the rhizosphere and their colonizing abitliy. Insertion of P-solubilizing genes in agriculturally important microorganisms lacking P-solubilizing ability has been an attractive approach to develop beneficial microbes with improved utility as soil inoculants (Rodriguez et al., 2006).

The principal mechanism for mineral phosphate solubilization by PSMs is by secretion of organic acids which can either directly dissolve the mineral phosphate as a result of anion exchange of PO_4^{2-} by acid anion or can chelate both Fe and Al ions associated with phosphate (Omar, 1998). These PSMs reportedly produce organic acids such as acetate, lactate, oxalate, tartarate, succinate, citrate, gluconate, ketogluconate, glycolate, etc (Rodriguez and Fraga, 1999; Khan et al., 2006). Some PSMs have proved even more advantageous as they not only increase the availability of soluble phosphate but also possess additional plant growth promoting abiltilies (Vessey, 2003; Haas and Defago, 2005; Ponmurugan and Gopi, 2006; Vassilev et al., 2006). With the increasing need of efficient PSMs, apart from isolation, identification of the organic acids responsible for P-solubilization and elucidation of mechanisms underlying production of these organic acids has been of prime interest.

Fluorescent pseudomonads are efficient biocontrol PGPR which also find application in bioremediation. Additional mineral phosphate solubilizing ability would help enhancing the efficacy of these pseudomonads. In order to enable pseudomonads to secrete organic acids which could solubilize mineral phosphate, such as gluconic and citric, it is necessary to clearly understand the primary and secondary metabolic circuits operating in the organism along with their regulatory controls.

1.9: RATIONALE

As discussed earlier, in most gram negative microorganisms including *Pseudomonas* and *E. coli,* phosphoenolpyruvate (PEP), pyruvate and OAA are the key metabolites at the anaplerotic junction of catabolism and biosynthesis. Diverse set of enzymes regulate the PEP-Pyruvate-OAA node in response to the physiological requirement of these metabolites in different organisms. The key junction has been well-studied in *E. coli*, *B. subtilis* and *C. glutamicum* which catabolized glucose primarily via EMP pathway. Pseudomonads which catabolize glucose chiefly *via* ED pathway could demonstrate a different regulatory pattern at the PEP-Pyruvate-OAA node. Genome sequences of several *Pseudomonas* strains and other metabolic data reveals that there is a lot of inter-species diversity in occurrence of the enzymes functional at the PEP-Pyruvate-OAA node. Some information pertaining to the existence and regulation of enzymes at the anaplerotic node in *P. citronellolis* is available as discussed in Section 1.6.4. But this organism is unique amongst pseudomonads as it lacks the periplasmic direct oxidative pathway, which therefore catabolizes glucose exclusively via intracellular phosphorylative pathway.

P. fluorescens, in addition to the intracellular phosphorylative pathway, is known to possess periplasmic glucose dehydrogenase (GDH) to catalyze direct oxidation of glucose to gluconic acid which is not internalized unless glucose becomes limiting (Fuhrer et al., 2005). Hence the glucose flux gets distributed between these two pathways. Also, the GDH mediated pathway can support the electron transport chain (ETC) for energy generation, which otherwise is a function of TCA cycle. The carbon flux through TCA cycle in turn is directly influenced by the metabolite pools and enzyme regulations at the anaplerotic PEP-Pyruvate-OAA node. Hence, in presence of direct oxidation pathway of glucose catabolism, the PEP-Pyruvate-OAA junction could have a different physiological significance.

The present work examines outcomes of altering the key enzymatic activities at PEP-pyruvate-OAA branch point and the TCA cycle in fluorescent pseudomonads with the help of targeted genetic manipulations in the form of overexpression of *Synechococcus elongatus* PCC 6301 phosphoenolpyruvate carboxylase (*ppc*) and *E. coli* citrate synthase (*cs*) genes as depicted in **Fig. 1.12.**

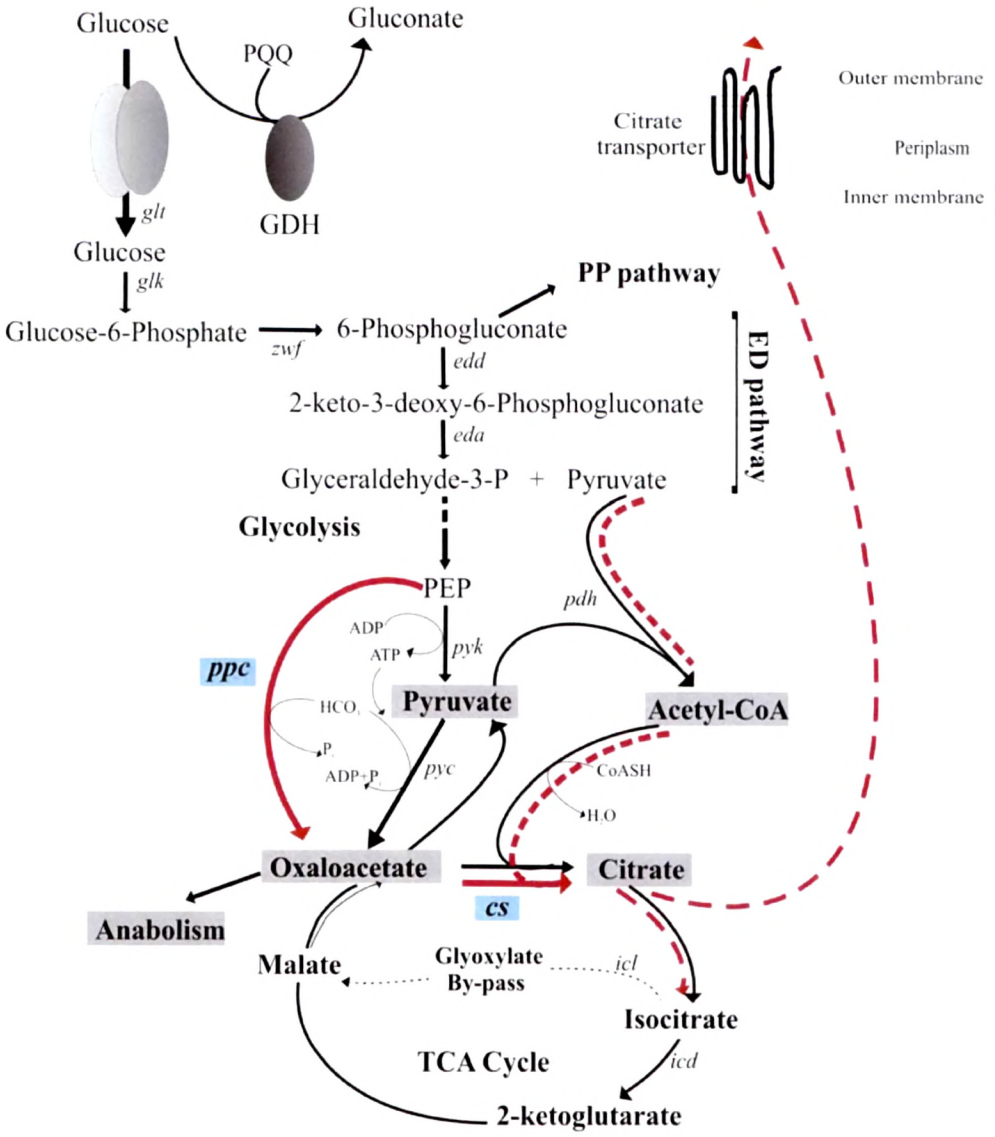

Fig. 1.12: Metabolic basis for designing the genetic modifications in fluorescent pseudomonads- Rationale. The metabolic network has been considered mainly as given by Fuhrer et al (2005) for *P. fluorescens* and partly as described by Lessie and Phibbs (1984). Blue boxes indicate the genes heterologously (over)expressed. Red lines (bold) indicate metabolic alterations imposed by the engineered genetic manipulations. Red lines (dashed) indicate the reactions through which flux might be altered as a consequence of engineered genetic manipulations. Grey boxes indicate the metabolites whose levels were likely to be affected. Abbreviations: *ppc*, phosphoenolpyruvate carboxylase; *cs*, citrate synthase; *pyc*, pyruvate carboxylase; *pyk*, pyruvate kinase; *icl*, isocitrate lyase; *icd*, isocitrate dehydrogenase; *pdh*, pyruvate dehydrogenase complex, *glt*, glucose transporter; *glk*, glucokinase; *zwf*, glucose-6-phosphate dehydrogenase; *edd*, 6-phosphogluconate dehydratase and *eda*, 2-keto-6-phosphogluconate aldolase.

1.10: OBJECTIVES OF THE PRESENT STUDY

The objectives of the present study were:

1. Effect of constitutive heterologous overexpression of phosphoenolpyruvate carboxylase (*ppc*) gene of *Synechococcus elongatus* PCC 6301 on physiology and glucose metabolism of *P. fluorescens* ATCC 13525.
2. Effect of Pi levels on glucose metabolism and organic acid secretion by *P. fluorescens* ATCC 13525 in the presence and absence of *ppc* gene overexpression.
3. Effect of constitutive heterologous overexpression of *E. coli* citrate synthase (*cs*) gene on glucose metabolism of *P. fluorescens* ATCC 13525.
4. Effect of simultaneous overexpression of *ppc-cs* genes on glucose metabolism of *P. fluorescens* ATCC 13525.
5. Effect of *ppc* and *cs* overexpression on the mineral phosphate solubilizing (MPS) ability of fluorescent pseudomonads
6. Genetic, phenotypic and biochemical characterization of *ppc* genomic integrant *Pseudomonas* P4.