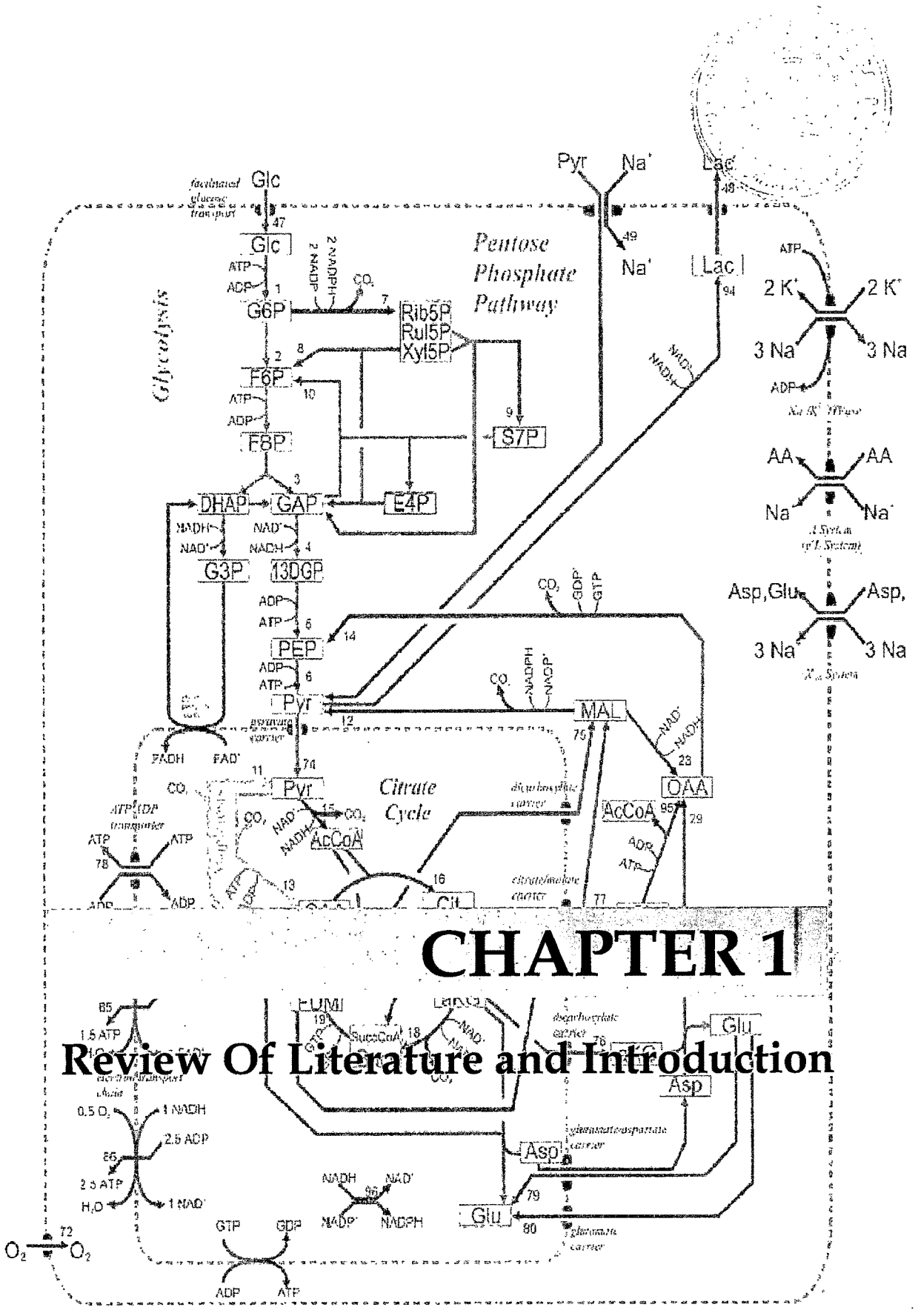

CHAPTER 1

Review Of Literature and Introduction

1.1: METABOLLIC ENGINEERING

Metabolic engineering is a coherent modification of the genetic architecture of the organisms to achieve a desired phenotype using the tools for genetic engineering (Bailey, 1991; Stephanopaulous et al., 1998; Nielsen, 1998; 2001, Shimizu, 2002). This process consists of developing a desired recombinant strain, analyzing this recombinant strain based on its performance compared to the original strain background, further designing the next target of possible modification thus giving rise to a cyclic process of strain improvement through several rounds of modifications (**Fig.1.1**) (Nielsen, 2001). This cyclic process is an assimilation of various disciplines like mathematics, chemical engineering, biochemistry, microbiology, bioinformatics and the most emerging field of recombinant DNA technology.

Classical approach of genetic modification involved use of chemical mutagens leading to random mutagenesis and screening for strains with superior properties related to product formation or growth. This method was well exploited for the production of antibiotics, solvents and vitamin production (Cameron and Tong, 1993; Koffas et al., 1999; Nielson, 2001). In 1973, Cohen and coworkers reported the first successful genetic modification of *E. coli*. Development in the field of genetic engineering has given a new dimension to the field of metabolic engineering to confer organisms with novel configuration resulting in beneficial properties. Today genetic engineering allows directed modification in the enzymatic reaction of the metabolic network leading to better product formation or redirection of cellular metabolism thus leading to enhanced product formation. The technical manifestation of metabolic engineering involves manipulations of enzymatic, transport and regulatory functions of cell using r-DNA technology (Bailey, 1991; Stephanopaulos and Vallino, 1991; Koffas et al., 1999). Hence, metabolic engineering has two main stages (i) carefully analyzing cellular networking to suggest possible targets, and (ii) introducing directed genetic modification using r-DNA technology (Ostergaard et al., 2000). "The directed improvement of product formation or cellular properties through the modification of specific modification of specific biochemical reaction(s) or introduction of new gene(s) with the use of recombinant DNA technology" was coined as **Metabolic engineering** (Stephanopaulous et al., 1998).

1.1.1: Concepts Of Metabolic Engineering

Metabolic engineering has become a fast emerging field and its major contributions lie in the directionality and emphasis on the complete metabolic network rather than individual reactions. The basic steps involving metabolic engineering are: identifying the rate limiting steps in the biochemical network based on the carbon-flux distributions and using this information for further applications or construction of recombinant strain for efficient product formation or lesser by-product outflow and based on the results targeting the second modification. Gathering the information from both the strategies further modification employed are overexpression(s) of the rate limiting enzyme or down regulation of the inefficient pathway contributing to high by-product formation (Stephanopaulous and Sinskey, 1993; Nielson, 2001; Vemuri and Aristodou, 2005). Thus metabolic engineering involves analysis, synthesis and designing of a strain with the help of molecular biology tools and information acquired of the cellular network.

Concept of metabolic rigidity includes identifying and removing the rigid nodes in the metabolic network (Stephanopoulos and Vallino, 1991). Rigidity of the metabolic network or its resistance to manipulations has been attributed to the control architecture of the cellular systems that ensure proper growth. This has often led to difficulties in bringing about the desired changes to improve cellular systems. Hence, it is important to understand the host system to determine the kind of genetic modifications needed and its probable effects on the physiological properties of the cell like growth and unrelated systems. Overexpression of phosphoenolpyruvate forming enzymes led to inhibit heat shock response and nitrogen regulation (Liao et al., 1996). Metabolic engineering thus involves a holistic approach of understanding cellular networking and cellular physiology.

Applications of metabolic engineering involve developing efficient strains producing products that are new to the host, or increase the production of the chemicals already produced by them or efficient product formation using lesser feedstock.

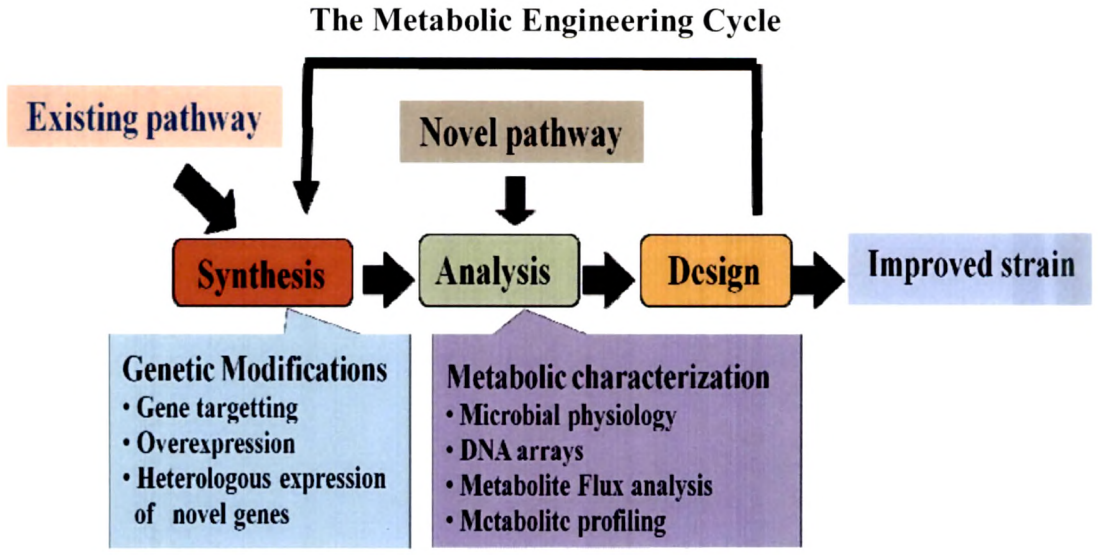

Fig. 1.1: The cycle of metabolic engineering (Thykaer and Nielsen, 2003).

1.1.2: Strategies of Metabolic Engineering

Various reviews have described the transition of metabolic engineering to a more defined and rational approach, the application of the field and the advances in the field and future of the field (Bailey, 1991; Stephanolpoulos and Vallino, 1993; Cameron and Tong, 1993; Ostergaard et al., 2000). Successful application of metabolic engineering involves designing good strategies based on the host metabolism and nature of the product (**Table 1.1**).

(i) Constructive metabolic engineering

Engineering the host based on the information gathered regarding the metabolic framework and desired product and hypothesizing a series of directed genetic modification(s) is defined as **constructive metabolic engineering.** This approach has been divided into seven categories (Cameron and Tong, 1993; Nielsen, 2001) (**Table: 1.1**). Although this approach was successful in many cases, it fails to address the engineering problems leading to multi component systems of the cells that strive to maintain homeostasis making it difficult to engineer these systems for maximum product yield.

(ii) Inverse metabolism (IME)

The concept of inverse metabolism (IME) starts with identification and construction of a desired phenotype, followed by determining the genetic or the particular environmental factors conferring that phenotype and incorporating the phenotype on another strain or organism by directed genetic or environmental manipulations (**Fig.1.2**).

One of the well studied examples is the expression of *Vitreoscella* hemoglobin (Vhb) in *E. coli* to improve growth under micro aerobic conditions (Bailey, 1996; Wang et al., 1998). Other examples are rendering phenotypes of improved pentose metabolism or incorporating unnatural amino acids, pH, acetate resistance or improving cellular energy state (Patnaik et al., 2002; Steiner and Sauer, 2003; Sauer and Schlattner, 2004). Advantage of inverse metabolic engineering is its access to the less understood cell subsystems that are necessary to bring out the desire change in phenotype.

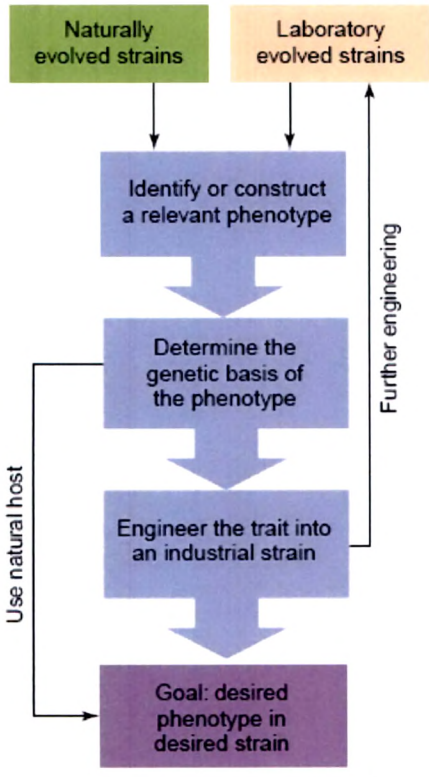

Fig. 1.2: The inverse metabolic engineering approach (Gill et al, 2003).

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.

Strategies of metabolic engineering	Aim of the work	References
Heterologous protein production	Engineer the protein synthesis pathway and strain to obtain improved productivity. *eg.* production of hormones, antibodies, vaccines and novel enzymes	Cameron and Tong, 1993; Shio, 1986; Aiba, et al., 1980; Weiss, 1992
Extension of substrate range	Engineer organisms for broader substrate utilization to bring about efficient utilization of raw materials	Preito et al., 1996; Sprenger, 1996
Pathway leading to new products	To make a particular host to produce several products; **Technique**-by using heterologous genes or gene shuffling to generate new pathways	Ingram 1998; Misawa and Shimada 1998; Berry et al., 2002.
Pathways for degradation of xenobiotics	To make few organisms to degrade several different xenobiotic compounds including TNT; **Techniques**-by inserting pathways from other organisms	Keasling et al.,1998; Chen and Wilson, 1996
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	Stephanopoulos and Sinskey, 1993, Farmer and Liao, 1996
Elimination or reduction of by-product formation	To prevent carbon loss, to avoid toxicity, enhancing better purification; **Technique**-gene disruption strategies.	Aristidou et al., 1994; Cameron et al.,1998, Chaplen at al., 1996
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	Pines et al., 1997; Shimada et al., 1998; Lu and Liao; 1997; Lin et al., 2005
Deregulation of existing enzymes	To overcome the existing control mechanisms of a rigid node; **Techniques**-by antisense gene expression under inducible promoters	Desai and Paupostakis 1999 ; Szafranski et al., 1997; Tummala et al., 2003

Table 1.1: Strategies of metabolic engineering and typical aim of the work (Neilson 2001; Wang et al., 1998).

1.1.3: Metabolic engineering in the -Omics era

Genome sequencing has paved a new direction to metabolic engineering. This generated the need to assign function to various orphan genes in order to generate potential targets for drug therapy and to develop biomarkers of diseases. Flux control nodes begin with transcription (induction-repression mechanism mRNA degradation) followed by translation (protein activation and proteolysis) and enzyme activity (allostery) or sometimes involves signaling cascade (Vemuri and Aristodou, 2005). Hence, understanding the regulation of these control nodes can develop efficient strategies for metabolic engineering. Recent developments in genome sequencing, transcription, protein analysis and metabolite profiling have been successful in improving metabolic engineering. Integrating the information from these three *omes* (genome, proteome, metabolome) can probably develop better rationale for engineering the metabolism of any organism. On the other hand, the emerging field of the three *omics* led to the development of various high throughput analytical techniques and a large amount of data that needed to be integrated in useful information.

1.1.4: Tools of Metabolic engineering

Rapid expansion in the field of metabolic engineering can be attributed to the development and availability of high throughput techniques for successful genetic modifications and various programs developed for the analysis of cellular functions.

1.1.4.1: Metabolic flux analysis

Metabolic flux analysis (MFA) summarizes series of metabolic events involving conversion of a feedstock to biomass and by-product which requires precise values of feedstock input, generation of biomass and excretion of by-product (Holms, 1996). The concept of **Metabolite balancing** for quantification of metabolic fluxes has been demonstrated for various microbial systems (Vallino et al., 1993; Sauer et al., 1996; Pramanik and Keasling, 1997; Pedersen et al., 1999). The flux depends on the co-factor balances e.g. NADH, NADPH or ATP hence it becomes important that all the reactions involving these cofactors are included. Since it is unlikely that all co-factors of the reactions are identified, the metabolic fluxes may be under-estimated. Hence, an

alternative approach was the use of ^{13}C labeled glucose which measured intracellular metabolites by the monitoring the labeling pattern deduced using NMR or GC-MS (Zupke and Stephanopoulos, 1994; Kelleher, 2001; Rohlin et al., 2001; Antoniewicz et al., 2007). This technique enabled to apply balance of individual carbon atoms in addition to the metabolite balances and does not involve balances of co-factors.

Quantification of the metabolic fluxes through the different branches in the network does not suggest the flux control at all these nodes. Hence, it is necessary to quantify the flux distribution across the network for designing suitable strategies. Flux control depends on the kinetic, thermodynamic, stoichiometry, flux capacity, and regulatory restraints under which reactions operate in the metabolic network (Varma and Palsson, 1994; Bonarius et al., 1997; Edwards et al., 2002; Price et al., 2003). Hence, material balance concept used to calculate fluxes does not provide information on the flux control nodes. Flux control can be deciphered from the regulation of enzymes of the branch points in the metabolic network. Further, the metabolite levels along with the affinities of the enzymes can provide valuable information on the *in vivo* regulation.

Metabolite levels could be obtained by two steps: (i) rapid quenching of the metabolite since the rapid turnover of the cellular metabolite, and (ii) efficient determination of the metabolite (Maharjan and Ferenci, 2003). Sample collection is a crucial event. Rapid sampling in cold methanol or boiling ethanol inactivates cellular metabolism. Metabolites are quantitated using enzyme assays, chromatographic techniques like GC-MS, liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS). **Fig. 1.3** summarizes the various methodologies employed for metabolome analysis.

Metabolic flux control concept is used for Metabolic Flux analysis. This involves the flux control coefficient (FCCs) which quantifies the relative increase in the network based on the enzyme activities. FCCs have been derived for the glycolytic pathway in yeast (Galazzo and Bailey, 1990; Rizzi et al., 1997) from the enzyme activities of certain pathways and the metabolite concentrations.

A shift towards system biology has generated the need for many genome based models which can evaluate and study intrinsic biological properties that operate in the cell. Constraint based models are the first step to such a study. Many reviews have put forward various models like METAFoR analysis, Mixed integer linear program (MILP), Minimization of metabolic adjustment (MOMA) for flux control calculations (Reed and Palsson, 2003).

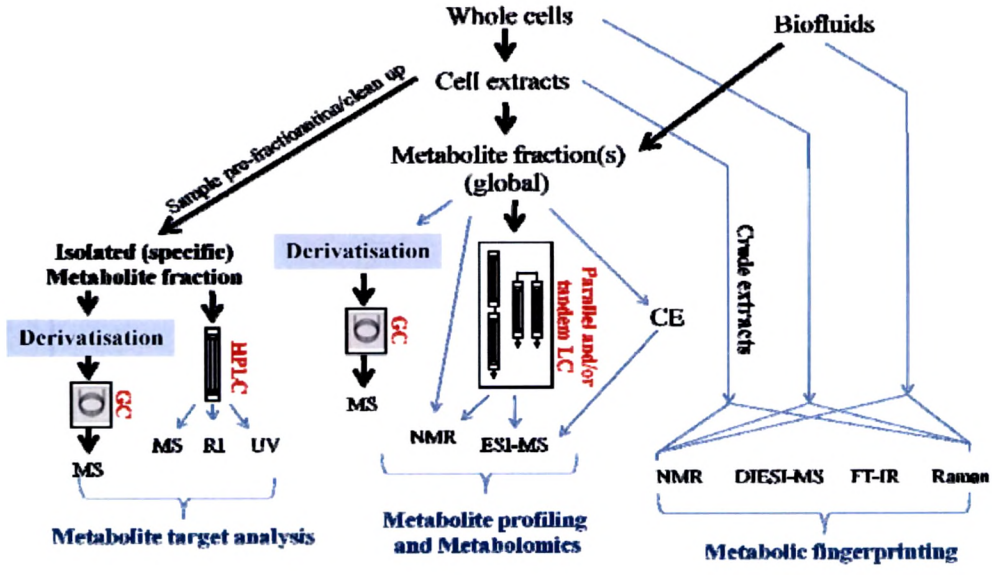

Fig. 1.3: General strategies of metabolome analysis (Goodacre et al., 2004**)** CE-capillary electrophoresis, DIESI-direct infusion ESI, FT-ICR –MS -Fourier transform ion cyclotron resonance mass spectrometry, NMR- nuclear magnetic resonance; RI- refractive index detection; UV-ultraviolet detection.

1.1.4.2: Genetic engineering tools

1.1.4.2.1: Vectors and Promoters for high level gene expression

An ideal gene expression tool should have some important characteristics: (i) the promoter must show tight and consistent control in all cells of the culture. Inducible promoters should respond linearly to the amount of the inducer; (ii) the genetic control system must be capable of regulating the expression of multiple genes simultaneously but at different levels; (iii) the vector carrying the genes must be maintained in the host indefinitely in absence of extreme selective pressures.

Promoters of varying strengths play an important tool for metabolic engineering. Makrides (1996) reviewed various strategies for achieving high level gene expression. Examples of the inducible promoters are P_{lac} (Isopropyl thiogalactoside (IPTG)), P_{BAD} (arabinose), P_m (benzoate), P_{sal} (salicylate). Variation in the -10 or -35 region of a promoter altered the strength which helped in developing constitutive promoters for *E. coli* and *Lacctococcus lactis* (**Table 1.2**) (Jensen and Hammer, 1998a;1998b). Some of the glycolytic promoters are usually strong, constitutive and have been used for overexpression of cloned genes *eg.* promoter of glyceraldehyde-3-phosphate dehydrogenase gene (Neilson, 2003). Inducible promoters using IPTG, as an inducer, is expensive for the industrial scale production. To resolve this problem phosphate inducible promoters (gene expressed when phosphate is depleted) were employed (Scharfstein et al., (1996).

Apart from the promoter, copy number of the plasmid defines efficient gene expression. Heterologous genes are cloned usually in high copy number plasmid (100 per cell). These plasmids although small in size (2-3kb) are unable to replicate very large sequences of DNA thus cause metabolic load on the cell (Keasling et al., 1991). Medium copy number plasmids (5-20 per cell) like broad host range plasmids RK2 have shown to be stable to 200 generations in *Pseudomonas* in the absence of antibiotic selection but are less stable in other bacteria. On the contrary, low copy number plasmids (1-5 per cell) have proved to be excellent alternative to high copy number plasmids. They are highly stable and impose low metabolic burden on the cell (Jones and Keasling, 1998).

Although plasmids are demonstrated as efficient tools for metabolic engineering transposons have emerged as effective expression systems (de Lorenzo et al., 1993). They are extremely stable and are integrated as single or multiple copies into the chromosome of the host. The limitation of the tool is that the integrating into the chromosome may affect the expression of the neighboring gene and more over the level of the expression of the gene in eukaryotes may depend on the location of the transposon on chromosome. Recently inducible expressing systems have been introduced into the transposons to allow regulated expression of foreign genes (de Lorenzo, 1993; Herrero et al., 1993; Perez-Martin and de Lorenzo, 1996).

Promoter (source)	Regulation	Induction
lac (*E. coli*)	*lacI*, *lacI*q	IPTG
	lacI (Ts)[a], *lacI*q (Ts)[a]	Thermal
	lacI (Ts)[b]	Thermal
trp (*E. coli*)		Trp starvation, indole acrylic acid
lpp (*E. coli*)		IPTG, lactose[c]
phoA (*E. coli*)	*phoB* (positive), *phoR* (negative)	Phosphate starvation
recA (*E. coli*)	*lex*A	Nalidixic acid
araABD (*E. coli*)	*ara*C	L-arabinose
proU (*E. coli*)		Osmolarity
cst-1 (*E. coli*)		Glucose starvation
*tet*A (*E. coli*)		Tetracycline
*cad*A (*E. coli*)	*cad*R	pH
nar (*E. coli*)	*Fnr* (FNR, NARL)	Anaerobic conditions, nitrate ion
tac, hybrid (*E. coli*)	*lac*I, *lac*I^q,	IPTG
	*lac*I^d	Thermal
Trc, hybrid (*E. coli*)	*lac*I, *lac*I^q	IPTG
	*lac*I(Ts)[a], *lac*I^q (Ts)[a]	Thermal
lpp-lac, hybrid (*E. coli*)	*lac*I	IPTG
P_{syn}, synthetic (*E. coli*)	*lac*I, *lac*I^q	IPTG
Starvation promoters (*E. coli*)		
p_L (λ)	λcIts857	Thermal
p_L-9G-50,mutant (λ)		Reduced temperature (<20^{0}C)
*csp*A (*E. coli*)		Reduced temperature (<20^{0}C)
p_R,p_L, tandem (λ)	λcIts857	Themal
T7 (T7)	λcIts857	Thermal
T7-*lac* operator (T7)	*lac*I^q	IPTG
λp_L, p_{T7}, tandem (λ, T7)	λcIts857, *lac*I^q	Thermal, IPTG
T3-*lac* operator (T3)	*lac*I^q	IPTG
T5-*lac* operator (T5)	*lac*I^q, *lac*I	IPTG
T4 gene32 (T4)		T4 infection
*npr*M-*lac* operator (Bacillus spp.)	*lac*I^q	IPTG
VHb (*Vitreoscilla* spp.)		Oxygen, cAMP-CAP[e]
Protein A (*Staphylococcus aureus*)		

[a] *lac*I gene with single mutation, (Gly-187→Ser . [b] *lac*I gene with three mutations. Ala-241→Thr, Gly-265 →Asp, and Ser -300→Asn . [c] The constitutive lpp promoter (P_{lpp}) was converted into an inducible promoter by insertion of *lac* UV5 promoter-operator region downstream of P_{lpp}, Thus, expression occurs only in the presence of a lac inducer [d] Wild-type *lac*I gene, [e] cAMP-CAP, cyclic AMP- catabolic activator protein.

Table 1.2 : Promoters used for high level Protein Expression (Makrides et al., 1996).

1.1.4.2.2: Gene disruption and mutation strategy

Genetic transfer system have been developed for efficient overexpression (heterologous and homologous) of key target genes in the pathway blocking or down-regulation of a particular gene or pathway for efficient product accumulation or minimize by-product formation resulting in overall high product formation (Mermelstein et al., 1992; Boynton et al., 1996). Mutations using chemical mutagens, gene disruption or gene replacement strategies have been developed. Guildener et al. (1996) demonstrated the use of *lox*P-*kan* MV-*lox*P gene cassette for gene disruption in *S. cerevisiae*. Bacteriophage P2 induction or imprecise excision by Tn10 methods were used for generation of random mutants (Bochner et al., 1980; Kleckner et al., 1977; Sunshine and Kelly, 1971). Several methods include F' homogenotization , the formation and resolution of ColEl plasmid cointegrates in a polAl genetic background, transformation of a *recBC sbcB* , or a *recD* strain with linear DNA, integration and induction of precisely engineered XcI857 vectors, and transformation and mating via an Hfr cross (Kupor and Fraenkel, 1971; Gutterson and Khosland, 1983; Saarilathi and Palva, 1985; Parker and Marinus, 1988). Balakrishnan and Backman (1988) have developed a derivative of XcI857 for use as an excision vector, primarily to make the deletion phenotype of a strain inducible *in vivo*.

Another technique depends on the intrinsic instability of multicopy plasmids that carry large insertions of chromosomal DNA to introduce mutations by homologous recombination (Kiel et al., 1987). Hamilton et al. (1989) demonstrated a technique based on homologous recombination where homologous regions of the chromosome were cloned on to a plasmid and then transformed into host and selected at 44^0C subsequent growth of these cointegrants at 30^0C leads to second recombination resulting in deletion of gene. Datsenko and Wanner (2001) developed a simple and highly efficient technique for gene disruption where PCR primers provided homology for target genes. This method has been successfully employed for gene deletions in *E. coli* for both single and multiple deletion (Mori et al. NARA Institute a collection centre for *E. coli* mutants http://www.ttck.keio.ac.jp/IAB/english/research/index.htm; Lee et al., 2005).

1.1.4.2.3: Antisense RNA in metabolic engineering

Antisense RNA technique has been successfully demonstrated in filamentous fungi and plants (Christensen, 1994; Borque, 1995; Zhen et al., 1998) but was difficult in *S. cerevisiae* (Atkins et al., 1994; Olsson et al., 1997). The possible mechanisms for *as*RNAs function are shown in **Fig. 1.4** - (i) inhibition of translation as duplex RNA structure prevents the identification of ribosome binding site. (ii) rapid degradation of duplex RNA by specific duplex RNA RNAases. (iii) inhibition of transcription of mRNA due to premature termination (Simons, 1988; Simons and Kleckner, 1988; Wagner and Simons, 1994 ; Delhihas, 1994).

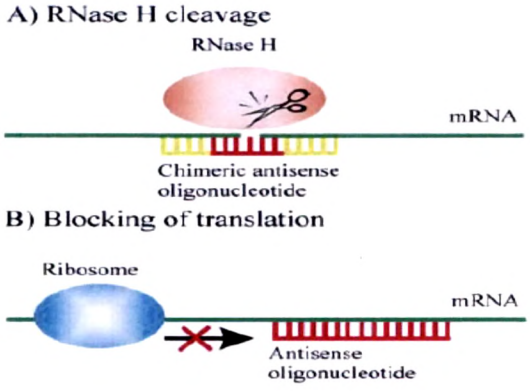

Fig 1.4 : Mechanism of Antisense RNA

An antisense RNA strategy holds various advantages as it can overcome lethal mutation, as complete inhibition of protein production is not likely. Moreover, *as*RNA can be used to inducibly repress protein synthesis for *e.g.* use of *lac* promoter to decrease lipoprotein production 2-16 fold in the presence of IPTG (Coleman et al., 1984).

The mechanism of action has not been thoroughly understood but their effective role in down regulation of gene has been studied in prokaryotes (Coleman et al., 1984; Pestka et al., 1984, Ellison et al., 1985; Engdahl et al., 1997; Kernodle et al., 1997). Studies were mostly focused on determining the effectiveness of *as*RNA strategies and their role in down regulation. Van den Berg et al. (1991) examined the role of hydrogenases in the metabolic of lactate by *Desulfovibrio vulgaris* and the results proved the strategy capable of affecting primary metabolism of prokaryotes.

Antisense RNA strategies have been exploited for metabolic engineering in *Clostridium* to increase solvent production (butanol, acetone and ethanol) and solvent resistance (Desai and Papoustakis, 1999; Nakayama, 2008; Sillers, 2009). The effectiveness of this strategy lies on the efficient association rate between *as*RNA and

target mRNA a phenomena well investigated by Tummala et al. (2000). Antisense strategies have been successfully demonstrated in down regulation of σ^{32}, a transient metabolic controller resulting in high specific orhophosphorus hydrolase activity as compared to non-antisense producing cultures (Srivastava et al., 2000). Kim and Cha (2003) showed the regulation of antisense for acetate kinase (ACK) and phosphotransacetylase (PTA) by intrinsic *ack*A promoter could lower the acetate secretion and thus improve protein expression in *E. coli* BL21.

1.1.4.2.4: Selection for model organisms

Selection of the model organisms for metabolic engineering depends on the product produced by the organisms and its industrial application. Moreover, the strain should be easy to handle with respect to carrying out genetic modifications and enough information should be available regarding the strain so that bioinformatics tools can be employed to design efficient strategies. Some of the commonly used organisms of industrial importance are *Corynebacterium*, *Aspergillus*, *Bacillus subtilus* and *Saccharomyces*.

Among the industrially important strains, gram-negative *E. coli* has gained more importance besides yeast. It is easy to handle, has fast replication, lesser complex metabolic network and above all exhaustive information is available on the central carbon metabolism and physiology which forms the basis for metabolic engineering to synthesize various reduced and oxidized metabolites. In addition to the information available on the molecular microbial physiology of the *E. coli*, responses to various stresses are known. Hence, this treasure of information can help in developing efficient strategies and analyzing the outcome of a particular alteration. Genetic engineering tools are well standardized for *E. coli* making it a preferred organism for metabolic engineering and developing computational programs or algorithms for the metabolic and regulatory networks. Carbon (glucose) metabolism has been well explored in *E. coli* with respect to its uptake, transcription and the global regulatory factors involved in maintaining an optimal intracellular metabolite level and energy status. These properties made it a desired model organism in carrying out the present study.

1.2: CENTRAL CABON METABOLISM WITH GLUCOSE AS A CARBON SOURCE IN *E. coli*

E. coli being a facultative anaerobe, can metabolize a wide variety of substrates operating under aerobic, anaerobic and fermentation based on the availability of electron acceptors. Of all glucose is the most preferred carbon source but cannot utilize sucrose and citrate.

1.2.1: Glucose transport mechanisms in *E. coli*

E. coli has more than 10^5 channels (located in outer membrane) with specialized porins of which OmpF and OmpC facilitate glucose entry into the peripalsm when high glucose (0.2 mM) levels are present (Death et al., 1993). *E. coli* can grow at low glucose concentrations by induction of a glucose-binding porin (LamB), and a high affinity periplasmic binding protein (Mgl) to actively transport glucose with an apparent K_Mt ~0.2 μM (Ferenci, 1996). Glucose diffuses into the outer membrane through porins as a passive transport and into periplasm with help of active transport systems available in the cytoplasm.

Glucose enters the cytoplasm through phosphoenolpyruvate:sugar phosphotransferase transport system (PTS) (**Fig. 1.5**, Gosset 2005). The bacterial PTS includes a collection of proteins that accomplish phosphoryl transfer from phosphoenolpyruvate (PEP) to a sugar in the course of transport. The PTS consists of Enzyme I (EI) and phosphohistidine carrier protein (HPr), as well as a collection of sugar-specific Enzyme II complexes, each of which comprises a transport system or permease. Each sugar-specific PTS permease contains at least three proteins or protein domains, termed the Enzymes IIA, IIB and IIC. Enzyme I, I-B, IIA and IIB are all phosphoryl transfer proteins of the PTS, whereas IIC is the actual membrane-embedded sugar permease that catalyzes transport of the sugar across the membrane as well as phosphoryl transfer from IIB-P to sugar. Enzyme I and the HPr (encoded by genes *ptsHI*) co-ordinate the transfer of a phosphoryl group from PEP to the sugar-specific enzyme components IIA and IIB. 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. Although an efficient uptake mechanism, PTS creates physiological constraints hindering the production processes.

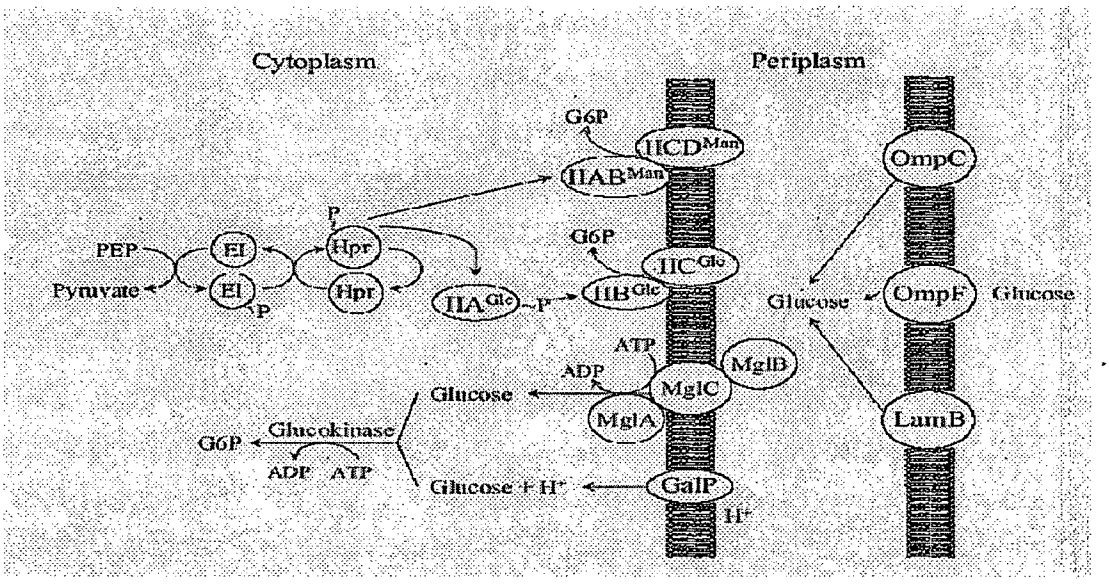

Fig. 1.5: The PTS and other glucose transport systems in *E. coli* (Gosset 2005).

1.2.2: Characteristics of PTS that affect productivity

PTS mediated uptake of glucose demonstrates a strong association between phosphorylation and glucose metabolism. PEP, on one hand plays a donor for phosphorylation, and on the other hand acts as a precursor for various metabolites. PEP is also involved in various energy generating processes, like substrate level phosphorylation of ADP and indirectly acts as a precursor for Acetyl CoA generation. *E. coli* grown on minimal medium, 50% of PEP is consumed by PTS and the remaining is utilized by PEP carboxylase, pyruvate kinases and other reaction where PEP acts as a substrate (Holms 1986; Flores et al., 2002).

Levels of PEP regulate the production of various aromatic amino acids like tyrosine, phenylalanine (produced by the Shikimate pathway) which are of commercial importance. *ptsG-crr* mutant produces high (theoretical) levels of aromatic compounds based on the stochiometric models. In the absence of the PTS uptake mechanism, the organism shifts to alternate mechanism like galactose permease (GalP) and glucokinse

(GlK) where ATP acts as a phosphate donor but results in poor growth and low productivity (Flores et al, 1996). These strains when engineered to direct the carbon flow towards Shikimate pathway resulted in better growth and high yield of phenylalanine (57%) (Baez et al., 2001; Baez et al., 2004).

PtsG mutations (Chou et al., 1994) / *Mlc* overexpression (represses *PtsHI* and *PtsG* genes)/ ptsHI operon deletion (Wong et al., 2008) have also lowered acetate level, thus improved the growth and recombinant protein expression. Similar strategies have also improved succinate and lactate yields. These results demonstrate that PTS plays an important role in the cellular metabolism of *E. coli* and it also poses an important target for strain improvement.

1.2.3: Glucose catabolism in *E. coli*

Glucose-6-phosphate (G-6-P), formed from the PEP dependent uptake of glucose, is primarily catabolized through **Embden-Meyerhof-Parnas** (EMP) pathway (Fuhrer et al., 2005; **Fig. 1.6**). The key check point enzymes of the glycolytic pathway catalyzing irreversible reactions are phosphofructokinase (PFK) and pyruvate kinase (PYK) (Emmerling et al., 1999; Sauer et al., 1999). Distribution of pyruvate, the end product of glycolysis, to TCA for energy generation and to anabolic processes depends on the redox status of the cell. Flux analysis revealed that of the total flux going to TCA cycle relatively less carbon oxidised while major portion (10 to 30% of carbon flux from glucose) is commonly excreted as incompletely oxidized acetate (Farmer and Liao, 1997; Holms, 2001; Fuhrer et al., 2005). Under aerobic conditions, acetate is 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). This outflow of acetate is usually attributed to 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) also significantly contributes to acetyl-CoA formation (Abdel-Hamid et al., 2001; Gosset et al., 2005; Wolfe, 2005).

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).

Methyl Glyoxal (MG) pathway operates under unfavourable conditions like, uptake of glucose-6-phosphate and other carbon substrates are down regulated or higher cAMP levels (Tempest and Neijssel, 1992; Inoue and Kimura, 1995; Ferguson et al., 1998; Kalapos, 1999). High concentrations of phosphate inhibit MG synthesis which is formed from Dihydroxyacetone phosphate (DHAP). MG synthesis is unfavorable energy generating bypass from the lower EMP pathway (Cooper and Anderson, 1970; Hopper and Cooper, 1971; Kadner et al., 1992; Ferguson et al., 1998; Totemeyer et al., 1998) and MG is also toxic to the cells as it can bind to protein, RNA, DNA (Lo et al., 1994, Papoulis et al., 1995) and affect cell growth. Thus, MG synthesis is very tightly regulated which is evident from the fact that by even upon 900 fold overexpression of MG synthase the accumulation MG level was low (Totemeyer et al., 1998).

1.2.4: *E. coli* metabolism on other carbon sources

Glucose is the preferred carbon source of *E. coli* amongst various other carbon substrates transported by PTS dependent or independent uptake mechanisms (**Fig 1.7**). Glycerol and xylose have been used in industrial application for the production of biofuels or ethanol. Hence, detailed metabolism on these carbon sources has become significant.

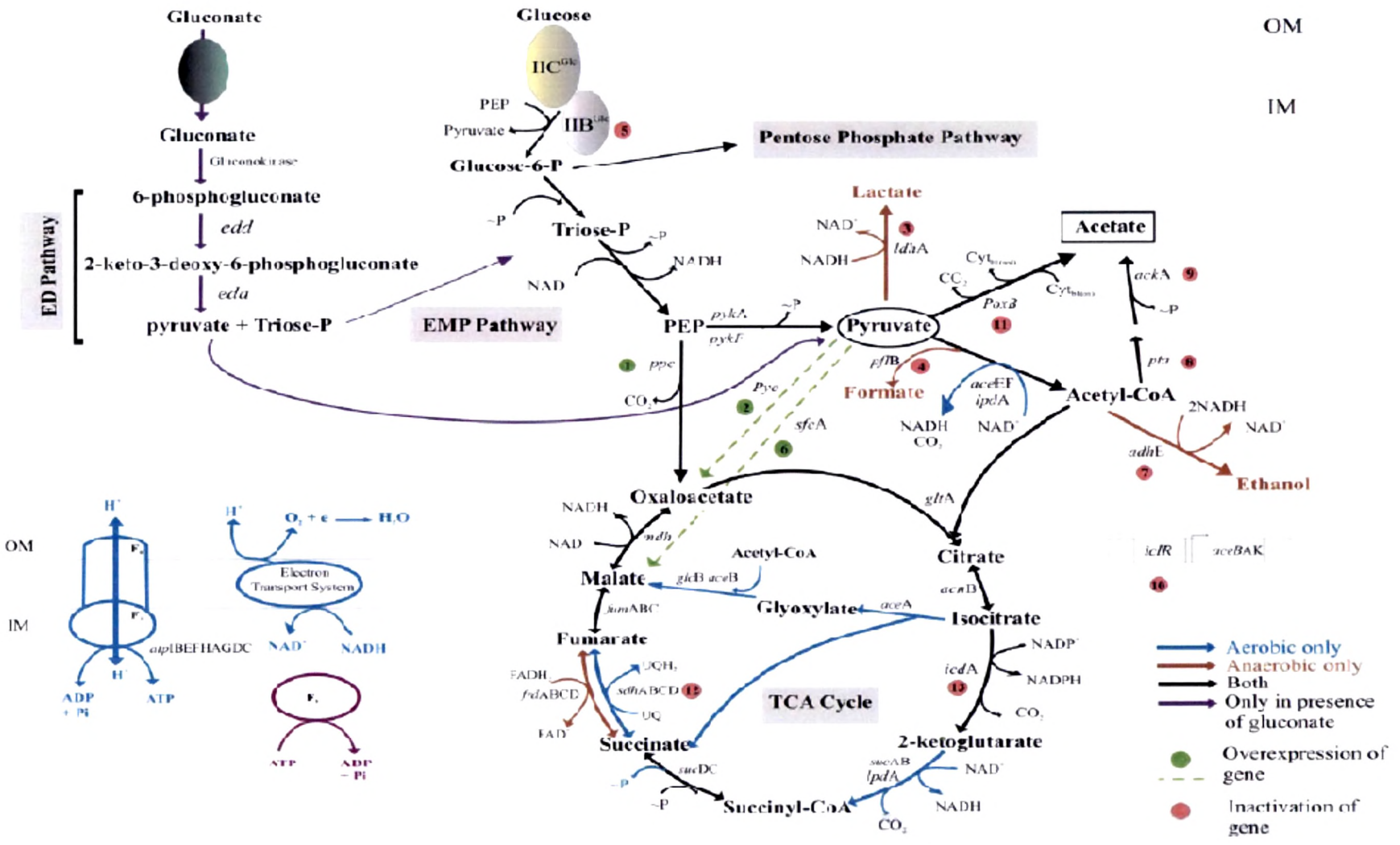

Fig. 1.6: Glucose catabolism in *E. coli*

1.2.4.1: Fructose catabolism

Fructose uptake is mediated by PTS uptake mechanism. Fructose enters as Fructose-1-phosphate into the cytoplasm where phosphofructokinase catalyses the conversion to fructose-1,6-bisphosphate and enters the EMP.

In comparison to glucose, fructose uptake is poor, growth rate is less, flux to pyruvate kinase is less and TCA cycle activity doubled resulting in low acetate outflow (Holms 2001).

1.2.4.2: Glycerol catabolism

Uptake of glycerol is mediated by facilitated diffusion hence it is independent of the PTS uptake. Glycerokinase converts glycerol to glycerolphosphate and enters the central metabolic pathway, this enzyme helps in maintaining a gradient across the cell. Glycerol enters the central metabolic pathway in the middle of the EMP after the phosphofrutokinase (PFK). Fructose-1,6-bisphosphate acts as a non-competitive allosteric inhibitor of the glycerokinase, this tight regulation governs the flux with respect to the need of the central carbon metabolism, resulting in slow growth rate . The slow growth rate may be beneficial as uncontrolled glycerol flux can produce methyl glyoxal which is toxic. Comparing the metabolism to fructose the growth rate is 97%, uptake 92% and Krebs cycle (MDH) 81% and no acetate outflow (Holms 2001; Lin 1976).

1.2.4.3: Xylose metabolism

Xylose is second largest sugar present after glucose hence; utilizing xylose for industrial application becomes more important. *E. coli* has been modified for xylose uptake (Kawaguchi et al., 2006). Xylose uptake is mediated by two transport mechanisms existing in *E. coli* first is a high affinity (Km ~ 0.2-0.4 μM) ATP binding cassette (ABC) class of transporters XylFGH -XylF is a periplasmic receptor protein, XylG is the ATP receptor binding protein and XylH is the membrane receptor of the ABC transport and secondly, a low affinity (Km ~63-169 μM) proton mediated symporter XylE (**Fig. 1.8**) (Sophia et al., 1994; Sumiya et al., 1995). Xylose is converted to xylulose-5-P which is an ATP requiring step and from here it enters the pentose phosphate (PPP) pathway (aerobic).

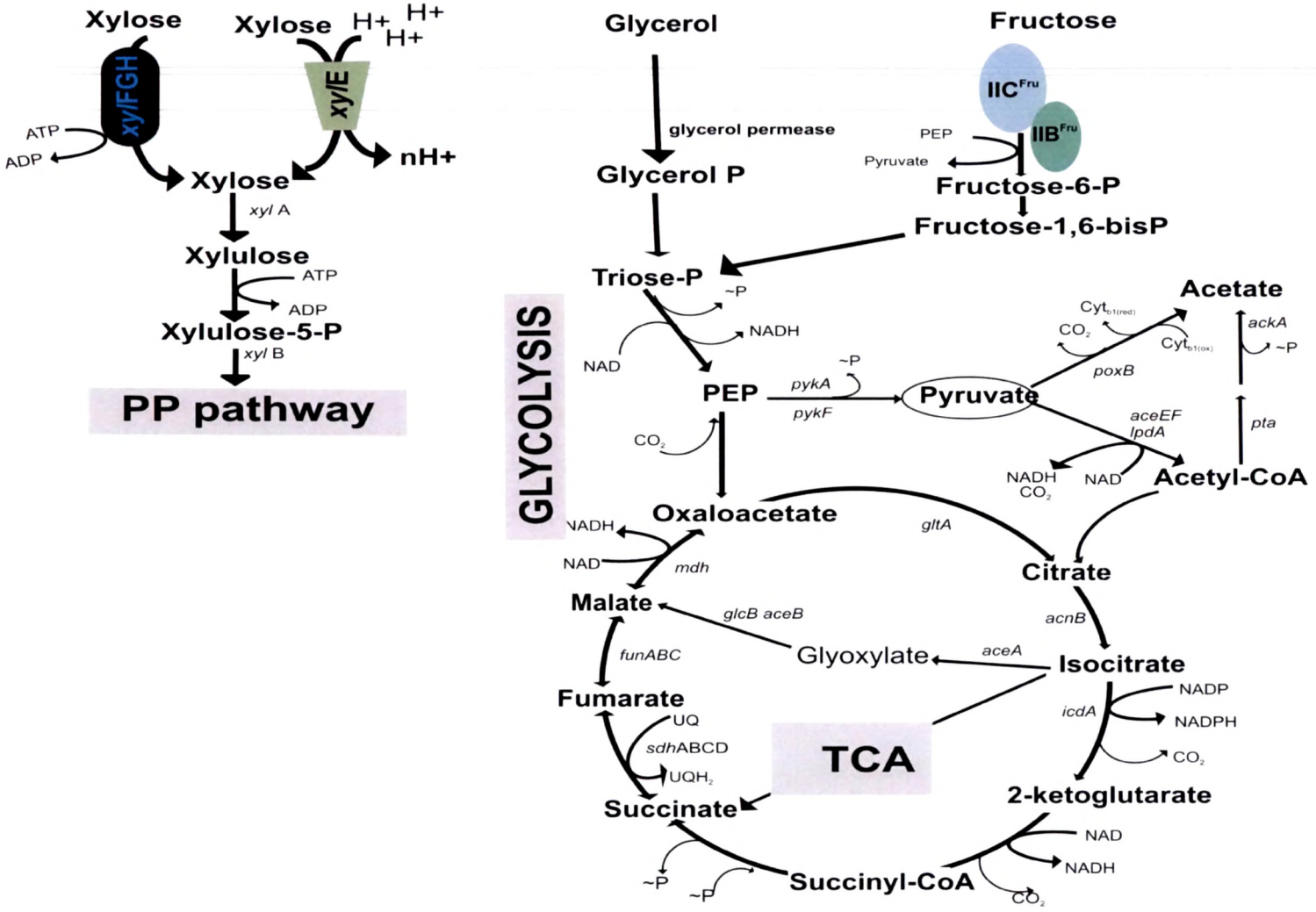

Fig. 1.7: Carbon metabolism of *E. coli* on other carbon sources

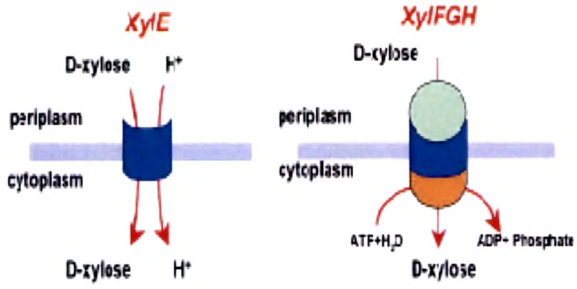

Fig. 1.8: Xylose uptake mechanisms in *E. coli* XylE- proton mediated symporter, XylFGH- requires one ATP for transport.

Various intermediates of PPP enter EMP to produce pyruvate (anaerobic fermentation). Xylose uptake is an energy utilizing pathway and the amount of ATP is only 33% of that generated on glucose. Thus on xylose, poor growth rate was observed as compared compared to glucose. However, xylose is the most preferred carbon source for ethanol production (Underwood et al., 2002).

1.2.4.4 : Acetate metabolism

Acetate is phosphorylated by acetate kinase and added to Coenzyme A by a phosphothiotransacetylase (Ack-Pta) forming acetyl CoA which enters the central metabolic pathway. Acetyl CoA pools, regulates carbon biosynthesis and oxidation pathways. Acetyl CoA is oxidized via the Krebs cycle; Acetyl CoA condenses with oxaloacetate forming isocitrate via citrate through citrate synthase. Isocitrate is further metabolized by isocitrate lyase (ICL) and malate synthase (MS) forming the anaplerotic glyoxalate bypass giving glyoxalate, succinate and malate which then enters Kreb cycle (Cozzone, 1998; Holms, 2001; Guest et al., 2004). Acetate can freely permeate in to the cell; assimilation does not require a transport mechanism (Kihara and Macnab, 1981; Repaske and Adler, 1981). Some studies have shown that in presence of saturated acetate levels a probable transport system could exist (Kakuda et al., 1994). This was further supported by the presence of acetate permease (ActP, formerly called YjcG) (Gimenez et al., 2003). Transport system appears to play a critical role in presence of millimolar concentration of acetate.

1.2.4.5: Why *E. coli* excrete acetate ?

Acetate excretion (acetogenesis) can be attributed to the regeneration of NAD^+ utilized in glycolysis and for recycling coenzyme A (CoASH), required converting pyruvate to acetyl CoA. Complete oxidation of acetyl CoA to carbon dioxide is mediated

by TCA, acetate excretion occurs when TCA does not operate or when the influx of the carbon exceeds the capacity of TCA (Salmond et al., 1984; el-Mansi and Holms, 1989; Rossman et al., 1991; Kessler and Knappe, 1996; Lee, 1996; Farmer and Liao, 1997; Xu et al., 1999; Chang et al., 1999). Under aerobic growth acetogenesis is due to high assimilation of glucose (high glucose in medium) that inhibits respiration (Holms, 1986; Holms, 2001) a behavior called **Crabtree** (Crabtree, 1929; Doelle et al., 1982; Lull and Strohl, 1990; Rinas et al., 1989). As a response to crabtree effect more than 15% of glucose is thrown out as acetate (Holms, 2001). Acetate is also excreted when *E. coli* is grown under anaerobic conditions by mixed acid fermentation (Bock and Sawers, 1996). Acetate excretion was since long referred to as carbon overflow of metabolism (Holms, 1986; 2001). This phenomenon may help in obtaining high cell densities by providing α-ketoglutarate dehydrogenase (αKGDH) with CoASH (el-Mansi, 2004). Acetate activation pathways are given in **Fig. 1.9**.

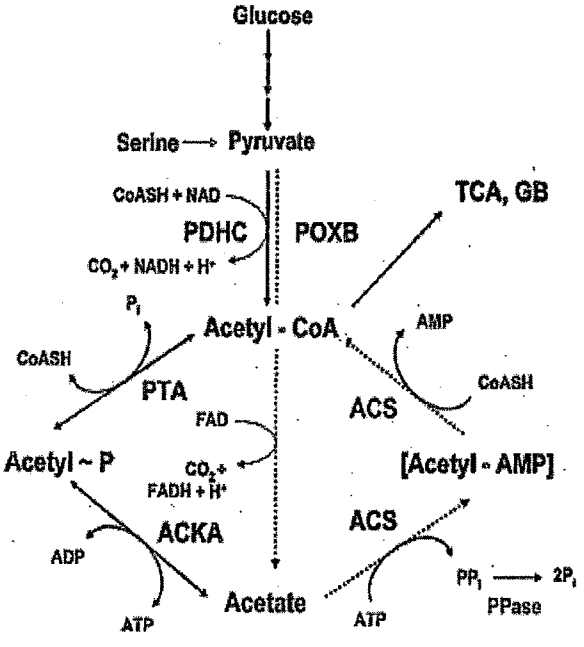

Fig. 1.9 : Acetate activation pathways (Wolfe, 2005). PDHC, pyruvate dehydrogenase complex; POXB, pyruvate oxidase; PTA, phosphotransacetylse; ACKA, acetate kinase; ACS, AMP-forming acetyl- CoA synthetase; PPase, pyrophosphatase; TCA, tricarboxylic acid cycle; GB, glyoxylate bypass. The dotted arrows denote the proposed PDHC bypass formed by POXB and AMP-ACS.

Acetate excretion requires decarboxylation of pyruvate to acetyl CoA which can occur oxidatively under aerobic conditions and non-oxidatively under anaerobic conditions. Oxidative decarboxylation is catalysed by pyruvate dehydrogenase (PDHC) generates NADH which in excess can inhibit PDHC. Hence in anaerobic conditions, were regeneration of NAD is less PDHC has reduced activity (Quail et al., 1994). Under

these conditions pyruvate is decarboxylated to acetyl CoA and formate mediated by pyruvate formate lyase; formate thus formed is either excreted as formate or decomposed to carbon dioxide or both carbon dioxide and dihydrogen by formate hydrogenase (FDO) or formate-hydrogen lyase (FHL) respectively depending on the environmental pH and oxygen availability (Rossman et al., 1991; Alexeeva et al., 2000). Acetyl CoA thus formed is excreted either as acetate (PTA-ACKA energy generating process does not consume reducing equivalents) or ethanol (alcohol dehydrogenase (ADH) energy consuming process and consumes reducing equivalents) which in turn regulates NAD^+ regeneration depending on the need of energy. Acetate can also be excreted by converting pyruvate directly to acetate through pyruvate oxidase (POXB).Although POXB has been regarded as a wasteful and nonessential, it has been reported to provide energy and acetyl CoA under microaerophilic condition. POXB is primarily expressed under aerobic conditions and plays a significant role in improving the growth efficiency (Abdel-Hamid et al., 2001). Null mutant of POXB have reported to have poor growth while constitutive or overexpression of POXB has shown to replace PDHC which usually function less efficiently especially under excess glucose conditions (Grabau and Cronan, 1984). Hence, acetate secretion is regulated by two routes: PTA-ACKA (expressed under log phase and repressed by acidic conditions) and POXB expressed under stationary phase and activated by acidic conditions (Dittrich, et al., 2005).

Acetate thus excreted can permeate through the membrane and once across the membrane it is converted into a proton and an anion (Kihara and Macnab, 1981; Booth, 1985). The proton acidifies the cytoplasm while the anion interferes with the internal osmotic pressure thus interfering with methionine synthesis (Roe et al., 1998; 2002). However acetate can only be partially oxidized hence can be utilized as carbon source and energy. Overview of acetate metabolism is depicted in **Fig. 1.10**. Hence few organisms including *E. coli* have demonstrated a phenomenon called acetate switch to overcome the toxicity of acetate by consuming it. **Acetate switch** is defined as a condition when the rate of dissimilation (when extracellular glucose concentration decreases) equals rate of assimilation (excreted acetate is taken up as carbon source) and this phenomenon has been reviewed well for *E. coli* and other organism like *Bacillus subtillus* and *Corynebacterium spp.* both which are industrially important (Wolfe, 2005). This phenomenon is usually observed during growth on acetogenic substrates like

glucose. The acetate switch is triggered on during the late stationary phase when the primary carbon source has been fully consumed, the oxygen and carbon dioxide evolution cease and the intracellular levels of Acetyl CoA diminish significantly (El-Mansi et al., 2006).

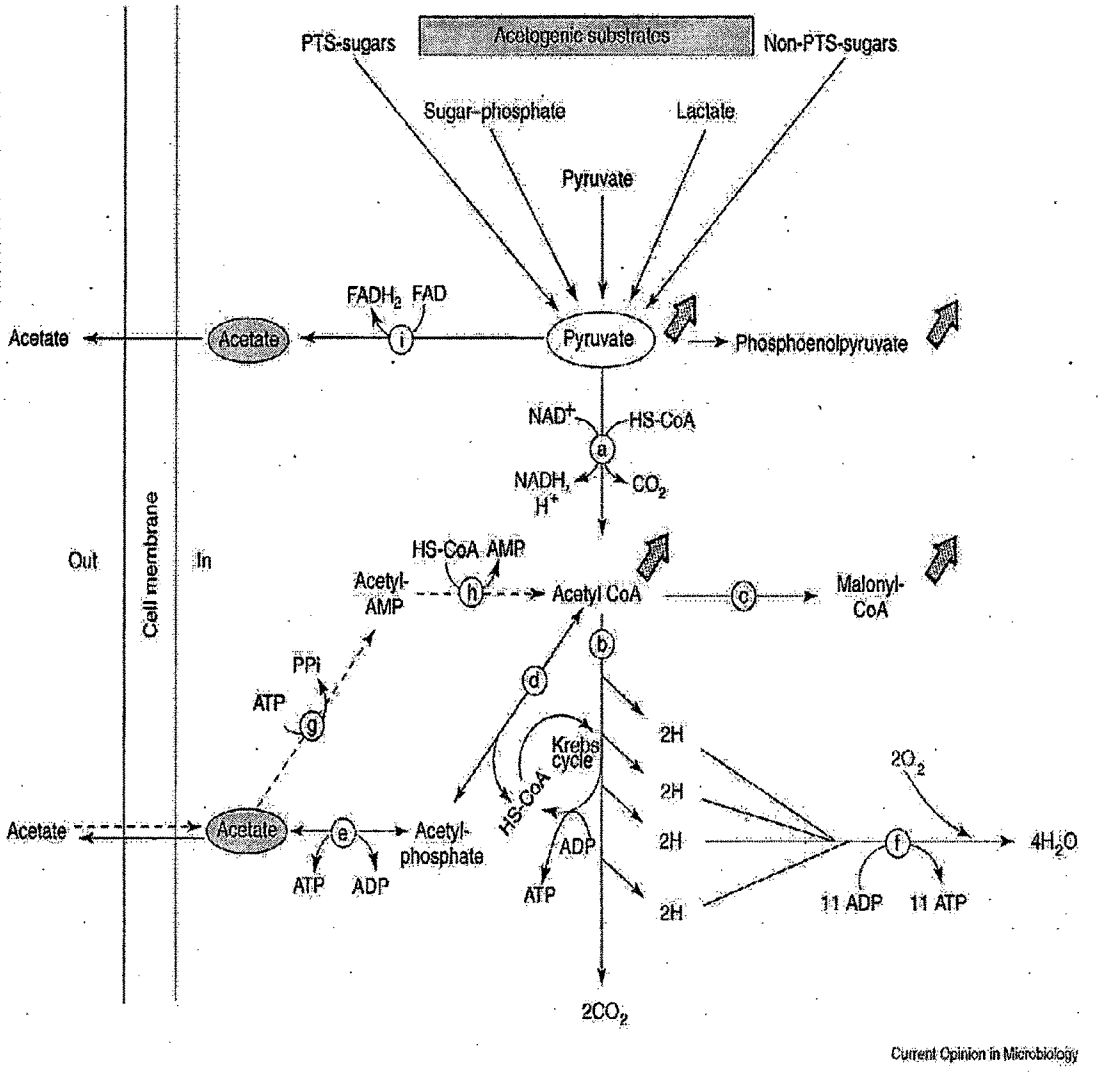

Fig. 1.10: An overview of the central metabolic pathways in *E. coli* on acetate (El-Mansi et al., 2006). Dashed lines indicate the routes for acetate utilization, whereas solid lines indicate the routes for acetate excretion. Large grey arrows indicate flux to biosyntheses (intermediary metabolism). Enzymes are as follows: a, pyruvate dehydrogenase complex; b, citrate synthase; c, acetyl CoA carboxylase; d, PTA; e, AK; f, the electron transport chain; g and h, ACS (AMP-forming); i, PO.

1.3: TRANSCRIPTIONAL REGULATION IN *E. coli*

The ever changing environment with respect to nutrient availability has always helped many organisms to evolve mechanisms that enable them to survive under wide range of environments. It puts up a strong selective pressure regulating and coordinating gene expression thus adjusting the rate of synthesizing important cellular components that are necessary for a particular condition. This regulation mainly circulates around the enzyme concentration and enzyme activities which are in turn regulated by allosteric regulation or kinetics. Regulation of enzyme activities can be mediated by different levels of transcription, translation and post translational modifications. This culminates to the fact that transcription regulation is the key regulation in most of the bacteria and it is only a part of an even more complex regulatory system.

Transcription regulation is mainly mediated by the transcription factors, synthesis of which depends on the size of the genome and lifestyle of the bacteria. Encountering a constantly changing environment a large of regulatory proteins are required to enable the free living organism to switch on to the changes with respect to the physiochemical changes and amount or quantity of the nutrients available. Referring to *E. coli* as a commensal organism comprises of 4288 open reading frames (Blattner, 1997) and roughly 300 transcription factors (Perez-Rueda and Collado-Vides, 2000) which are more in number compared to other free living microorganisms with the same genome size. This commensal organism can grow on amino acids provided in media and can synthesize amino acid when they are absent. It has various utilization pathways operating for a wide range of carbon substrates. It can also grow aerobically and anaerobically. It can also survive slight DNA damage or nutrient limitation condition. This shown that it has a very controlled cellular network as it will end up **wasting** energy resources it all the genes are expressed at the same time when they are not required.

The transcriptional network regulation involves various levels (**Fig. 1.11**) (i) an **operon** – it includes a set of genes regulating a particular pathway arranged closely in a transcription unit, with a single promoter. Therefore, operons help in regulating genes with related functions. The operon model has its own limitations, some of the cellular process involve too many genes to be accommodated in a single operon and secondly virtually every complex bacterial processes involves a number of genes that must be

subject to both independent regulation and coordinated control. Hence, a further level of organization, called **regulon**, is required. **Regulons** help in coordinating control of operons having their own unique controls, but it varies in their level of induction (extent and time) from operon and operon in a given **regulon**. Looking at the complexity of the network there exists, a regulatory mechanism over the regulon called **global regulators**. These global regulators, regulates a group of independent operons under the control of different **regulon**. Thus, the global regulators regulate operons distributed across the genome, with diverse functions.

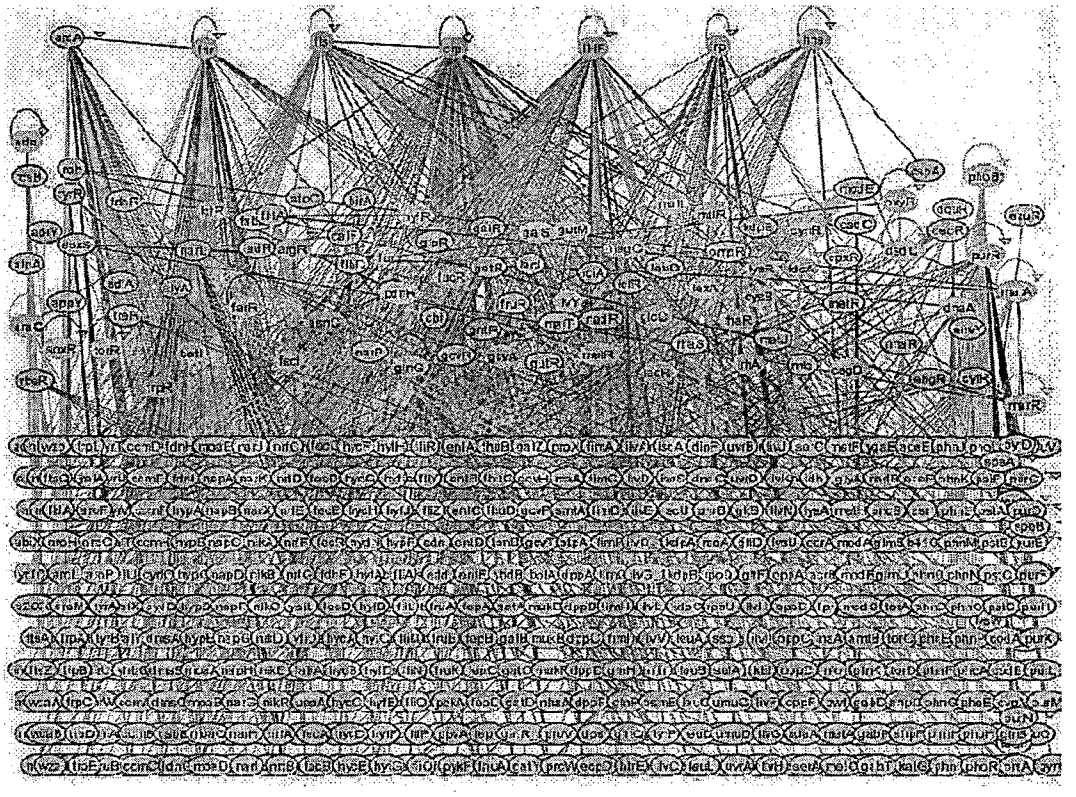

Fig. 1.11: Overview of the transcriptional network in *E. coli* (Martinez-Antonio and Collado-Vides, 2003)**.** In the figure the six top ovals represent global transcriptional regulators, the middle ovals are transcriptional regulators and all the lower ovals represent the regulated genes.

Regulatory proteins act as positive (activators) and negative (repressors) or both. Moreover, the promoter might have binding sites for both an activator and a repressor. On the whole transcriptional regulation involves a complex network of global and

specific regulators. Hence it becomes very important to understand the transcriptional regulatory networks.

E. coli has seven global regulators (Crp, Fnr, Ihf, Hns, ArcA, NarL, Lrp) that modulate the functions of nearly half of the genes (Martinez-Antonio and Collado-Vibes, 2003). Of these few regulators, one fifth of them regulate probably only one or two gene and a few might influence a larger set of genes (Thieffry et al. 1998; Oosawa and Savagaeu, 2002). Multiple regulations by transcriptional factors were observed for a larger population of genes one such example is Fis which regulates more than 67% of its genes. In contrast, ArcA and NarL have all their genes coregulated by global regulators such as Fnr and Crp (Martinez-Antonio and Collado-Vibes, 2003). Such kinds of co-regulation of transcriptional regulators help the organism to tune in to different environmental conditions.

Transcriptional regulators need to have essential properties like binding to DNA or metabolite, thus helping the organism to sense the changes in the environmental conditions and regulate its growth and gene expression. A set of histidine kinase senses the changes in the environmental conditions and pass on the message to the response regulators via a series of autophosphorylation, phosphorylation and de-phosphorylation, thus coordinating sensing and appropriate regulation (Hoch, 1995).

"**Global**" is addressed to those transcriptional regulators that regulate genes at more than 3 functional classes and most of the seven global regulators are shown to regulate at 15 functional classes. Sigma (σ) factors, components of RNA polymerase are also included in the list of global regulators as changes in the synthesis and competitiveness to bind to RNA polymerase lead to induction and repression of various gene expressions (Record et al., 1996).

1.3.1: Genetic regulation sugar metabolism in *E. coli*

E. coli has a large number of global regulatory factors functioning towards controlling genes expressed for carbon utilization and energetics. Growing *E. coli* on glucose reduces the transcription of genes and operons involved in the utilization of other

carbon sources. This phenomenon is called **carbon catabolite repression** (CCR) or **catabolite activation (Fig. 1.12)**. CCR in *E. coli* is mediated by cAMP dependent and cAMP independent mechanisms. In presence of glucose, *E. coli* demonstrated catabolite repression or activation mediated by cAMP and its counterpart catabolite repression Protein (CRP). cAMP levels are low on glucose, this is regulated by the glucose transport component (IIA^{glc}), which down regulates adenylate cyclase on glucose (Saier and Feucht, 1975). Low levels of glucose phosphorylates glucose specific component of the PTS system which allosterically activates adenylate cyclase, the enzyme synthesizing cAMP which binds to CRP (cAMP-CRP) protein forming a complex. This complex interacts with RNA polymerase and activates the transcription of genes from more than 100 promoters under its control (Saier, 1996; Gosset et al., 2004). This mechanism is predominant in *E. coli* under glucose limiting conditions (Nanchen et al., 2008). In the presence of high levels of glucose, IIA^{glc} becomes dephosphorylated (free form) leading to inactivation of adenylate cyclase and inhibiting certain permeases (lactose and maltose) and catabolic enzymes (glycerol kinase and arabinose isomerase) (Postma et al., 1993; Saier, 1993) giving rise to a phenomenon called **inducer exclusion**.

cAMP-independent system called **Catabolite Repressor Activator** (CRA) protein was initially referred as fructose repressor (FruR). Mutants defective in the *cra* gene showed a phenotype that was unable to grow with gluconeogenic substrates as a sole carbon source (Chin et al., 1987). Moreover mutation in *cra* resulted in increased levels of enzyme required for uptake and catabolism of sugars like glucose and fructose. Various studies reported that *cra* gene controlled the transcriptional expression of numerous genes involved in carbon and energy metabolism (Chin et al., 1987; Geerse et al., 1989). Cra is known to repress enzymes of the Embden-Meyerhoff-Parnas pathway (EMP pathway or glycolytic pathway) and Entner-Doudoroff pathway (ED pathway) while it induces gene encoding enzymes required for growth in organic acids and amino acids, that is, enzymes of the citric acid cycle, glyoxylate cycle and gluconeogenic pathway (Saier and Ramsaier, 1996).

Cra, recongnises a palindromic sequences (TGAAWCSNTHHW - R, A or G; S, C or G; W, A or T; H, A or C or T; N, any nucleotide) on the DNA, to which it can bind asymmetrically. It regulates the activation and repression of genes depending upon

where it binds to the DNA with respect to the RNA polymerase binding site. If it sits upstream then it acts as an activator and if it sits downstream then it behaves as transcription inhibitor. This model is supported by the sequence analysis studies of Cra binding site which places sites downstream for negatively controlled promoters and placed upstream for positively controlled promoters (Ramsaier, 1996). Certain catabolites derived from glucose or fructose, *i.e.* fructose-1-phosphate (micromolar concentration) and fructose-1,6-bisphosphate (millimolar concentration), bind to Cra and remove it from DNA therefore diminishing its effect thus repressing the genes activated by Cra and activating the genes repressed by Cra (Saier and Ramsaier, 1996).

ArcAB (two component system) and Fnr proteins regulate the gene expression based on the oxygen availability in *E. coli*. More than one-third of the genes expressed on aerobic condition are altered under anaerobic condition (Salmon et al., 2003). Fnr, plays an activator of a number of genes whose products are involved in anaerobic respiration, whereas ArcAB modulates the transcription under microaerobic and anaerobic conditions. These global regulators play a crucial role in optimizing energy generation by aerobic, anaerobic or fermentation of simple sugars (Gunsalus and Park, 1994; Lynch and Lin, 1996).

Sigma factors, components of RNA polymerase regulate transcription of genes by altering the binding specificity of the RNA polymerase to the promoters. Most the sigma factors are expressed under stress condition. *rpoS* (σ^S), a sigma factor expressed during stationary conditions, low pH or high osmolarity, plays a dual role as a emergence coordinator and a master regulator helping in adapting to various physiological conditions (Hengge-Aronis, 1999). Hns, histone like protein, is also a global regulator. It is small chromatin associated that is able to condense DNA like histones in eukaryotes. Mutation in *hns* has shown to affect genes involved in transcription or translation or those coding for proteins responsible for adaptation under changing physiological condition (Hommais et al., 2001). RpoS and Hns are global regulators as they have broad range, less specific action as compared to CRA, CAMP-CRP, ArcAB and Fnr which directly affect a large number of genes regulating carbon metabolism. Hence they are referred to as **global metabolic regulators**.

1.3.2: Regulation of central carbon metabolism

The central carbon metabolism in *E. coli* has a complex regulatory network involving various transcriptional regulators (global regulators); most of them being inactive under all growth conditions (**Fig. 1.13**). The two component system ArcAB regulated gene expression in response to redox status of the cell (Lynch and Lin, 1996).

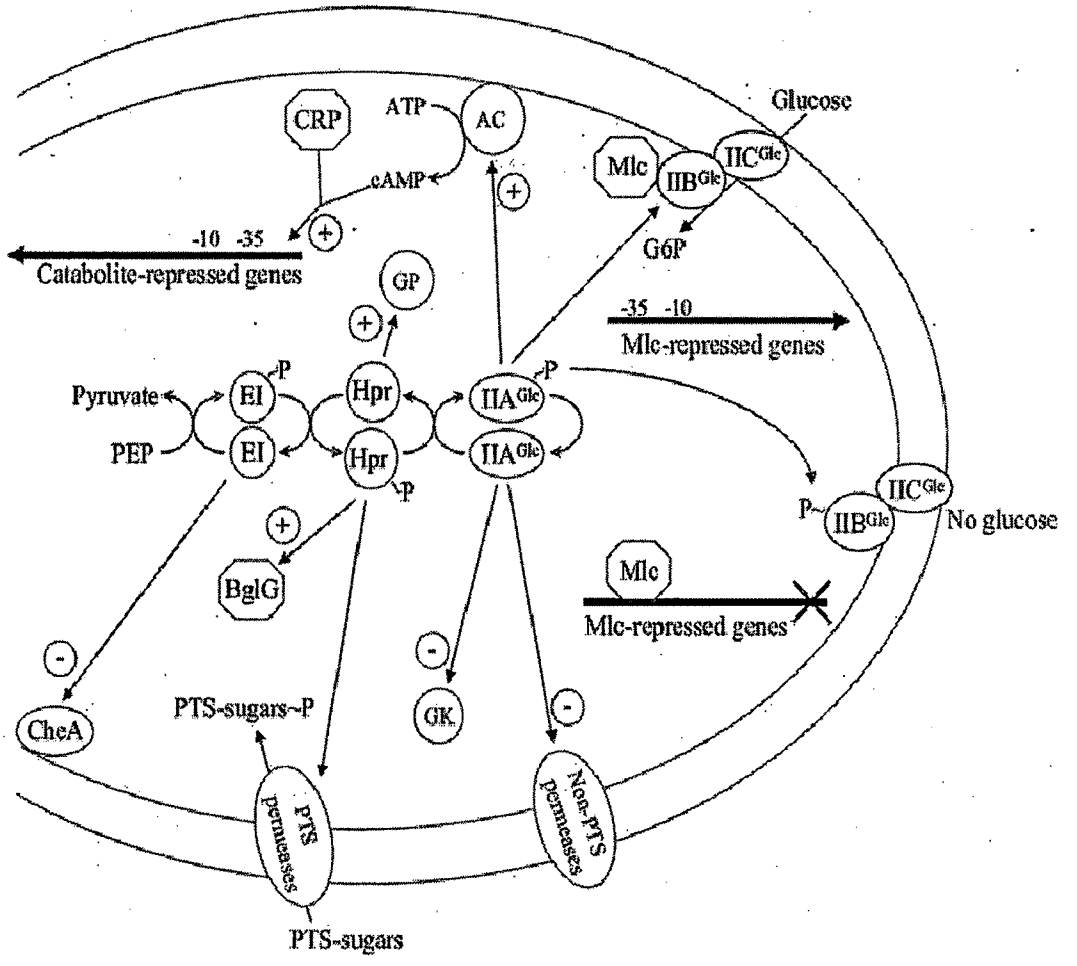

Fig. 1.12: Carbon catabolite repression in *E. coli* (Gosset, 2005).

The ArcB was found to be up regulated under microaerobic and anaerobic conditions (Georgellis et al., 2001). On the other hand Fnr, regulates the central carbon metabolism under anaerobic condition as presence of oxygen inactivates it (Khoroshilova et al., 1997). cAMP-CRP and CRA are inactive under high glucose. Under these conditions cAMP levels are low (Notley-Macrabb et al., 1997), thus

affecting the cAMP-CRP complex formation. While Cra binding to the DNA, depends on the fructose-1-phosphate and fructose-1,6-bisphosphate levels, which are usually high on carbon sources like glucose and fructose (Saier and Ramsaier, 1996).

CreBC, a two component system, regulates catabolite repression on minimal medium (Avison et al., 2001). Mlc, one of the global regulators is not directly responsive to glucose per se but regulates PTS levels. On glucose and other PTS sugars, Mlc get detached from the binding sites and sequesters the repressor to membranes by binding to dephosphorylated PtsG (Plumbridge, 2001). Mlc acts as a repressor for several operons encoding proteins of the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS), including *ptsHI*, *ptsG* and *manXYZ* as well as genes involved in the metabolism of certain non-PTS sugars, such as maltose. Hns showed less effect on the growth of the organism. Sigma factor, RpoS increases in the stationary phase (Hengge-Aronis, 1999).

Apart from these global regulators (transcriptional regulating), CsrA a translational regulator, has a key regulatory role in central carbon metabolism, both as an activator of glycolysis and as a potent repressor of glycogen biosynthesis and gluconeogenesis (Sabnis, 1995; Romeo, 1998). Allosteric regulation of the enzymes also plays a significant role in regulating the central carbon metabolism. This regulation is mediated by intermediates produced in the central carbon metabolism, to name a few, fructose-1,6-bisphosphate (pyruvate kinase (*pyk*F), phosphenolpyruvate carboxylase (*ppc*)) (Martinez and Collado-Vides, 2003; Waygood and Sanwal, 1974), phosphenolpyruvate (phosphofructokinase (*pfk*), phosphenolpyruvate carboxykinase (*pck*), phosphenolpyruvate synthase (*pps*)) (Blangy et al., 1968), acetyl CoA (*ppc*) (Morikawa et al., 1980) or sugars of the pentose phosphate pathway (*pykA*) (Waygood et al., 1975). Transcription or/translation for other enzymes show growth rate-dependent profile (*zwf, gnd*) (Wolfe, et al., 1979).

Regulation of the central carbon metabolism majorly depends on the combination of transcription and transcriptional regulation (enzyme levels) and the allosteric control (enzyme activity). It can also be suggested the regulation may be a condition dependent event. Although a transcriptional regulator, if active, will change the expression levels of

a transcript or a protein level it need not co-relate to the *in-vivo* flux which is the outcome of various regulation and metabolic events going on in the cell.

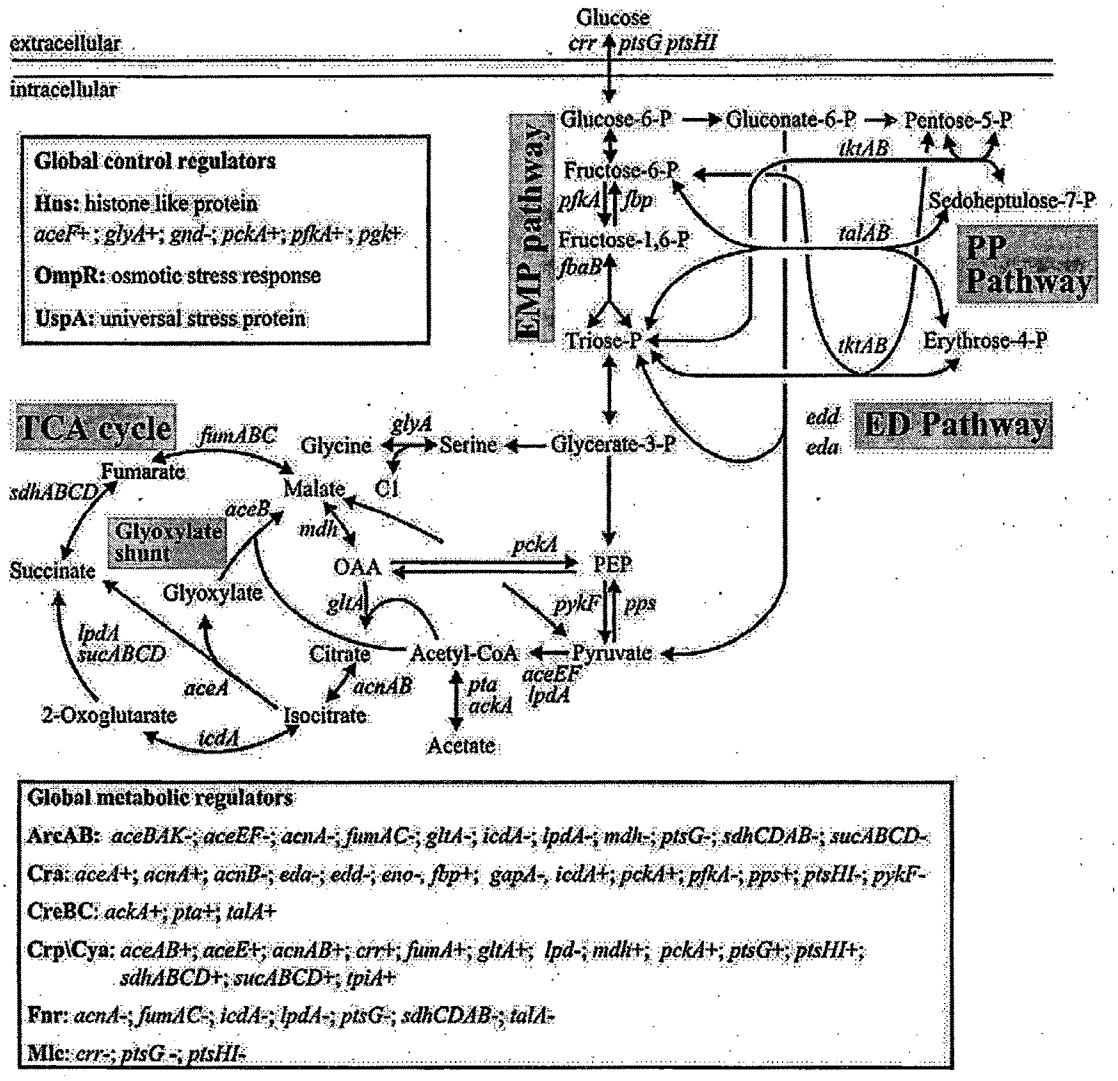

Fig. 1.13: Biochemical reaction network of central carbon metabolism in *E. coli.* (Nanchen, et al., 2008) Arrowheads indicate the assumed reaction reversibility. The inset affords an overview on central metabolic genes that are regulated by the global regulators investigated here. Plus and minus signs indicate positive and negative transcriptional regulation, respectively. Only regulated genes in the central metabolism are shown for clarity. PP, pentose phosphate; CoA, coenzyme A; OAA, oxaloacetate; P, phosphate.

Comparision of metabolic flux and gene expression on various substrates have suggested that in some cases the flux through central carbon metabolism correlate

qualitatively to the expression level. On the other hand several studies using molecular genetics have come to the conclusion that ATP levels controls the glycolytic flux in *E. coli* which in turn depends on the growth condition (Koebmann et al., 2002a; 2002b; Causey et al., 2003).

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

Engineering *E. coli* for succinate production at near theoretical maximum is one of most successful examples of metabolic engineering. Succinate production has been engineered for both aerobic and anaerobic conditions. Succinate is produced under anaerobic conditions (mixed acid fermentation) along with lactate, acetate, formate and ethanol. Oxaloacetate (OAA), a precursor of succinate is synthesized from PEP catalysed by PEPcarboxylase (PPC) hence the genetic manipulations were targeted for optimizing the PEP-pyruvate pools thus increasing succinate production and reducing other fermentation products apart from these various simulations were designed to optimize succinate production in *E. coli* which included genetic/or environmental operational constraints (Cox et al., 2006). Succinate production in *E. coli* (with various mutation) and other organisms are summarized in (**Table 1.7**).

1.4.1: Aerobic Succinate Production

Under anaerobic growth conditions *E. coli* secretes acetate and not succinate. Hence, primary targets involved to reduce acetate formation by generating mutants deficient in 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. 1.6**). Under aerobic condition *E. coli* (modifications 8-9, 10, 11, 12 and 13 in **Fig. 1.6**) is produced 14.28mM succinate with yield of (~0.22 g/g glucose) using 55mM glucose through pathways which do not require NADH (**Fig. 1.14**).

Aerobic batch reactor studies demonstrated faster succinate production rate, reaching 0.5mole/mole (~ 0.33g/g glucose) in 24h with a concentration of 22.12 mM

which on further cultivation increased upto 43mM with a yield of 0.7mole/mole (~0.46g/g glucose). To achieve this *E. coli* with five mutations was developed which had negligible flux through TCA and a highly activated glyoxylate bypass which resulted in increased succinate yield although not up to the maximum theoretical levels due to accumulation of pyruvate and TCA cycle C6 intermediates (citrate and isocitrate).

To further improve 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, 11and 12 in **Fig. 1.6**) which could achieve aerobic succinate production either by glyoxylate pathway or by oxidative TCA cycle (Lin et al., 2005c; 2005d). Succinate yield was higher in *E. coli* with five mutations which showed no accumulation of TCA cycle intermediates (**Table 1.3**).

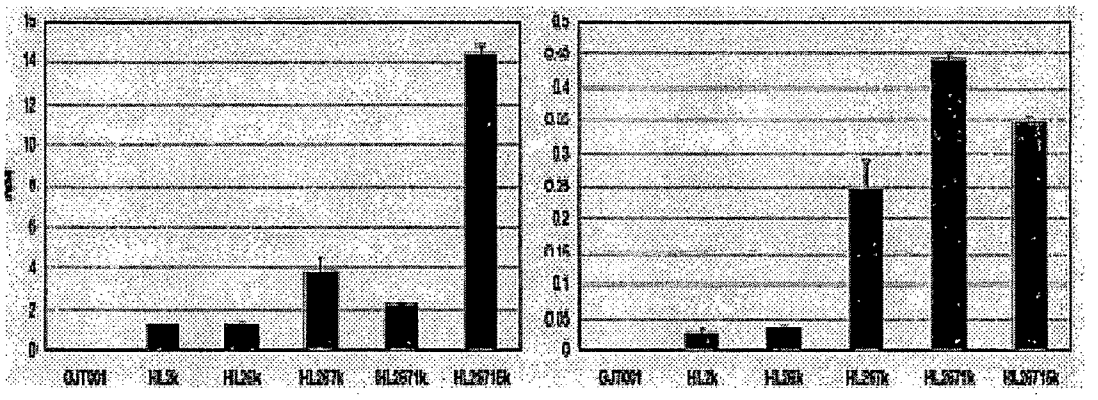

Fig. 1.14: Improvement in aerobic succinate production with subsequently incorporated genetic mutations in *E. coli* (Lin et al., 2005c). GJT001-Spontaneous *cad*R mutant of MC4100(ATC35695); HL2k GJT001-(Δ*sdh*AB::KmR); HL26k GJT001-(Δ*sdh*AB, Δ*pox*B::KmR); HL267k GJT001-(Δ*sdh*AB, Δ*pox*B, Δ(*ack*A-*pta*)::KmR); HL2671k GJT001-(Δ*sdh*AB, Δ*pox*B, Δ(*ack*A-*pta*), Δ*icd*::KmR); HL26715k GJT001- (Δ*sdh*AB, Δ*pox*B, Δ (*ack*A-*pta*), Δ*icd*, Δ*icl*R::KmR).

Inactiavtion of *pts*G and coupled with 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 1.3**). Fed-batch and chemostat cultures conditions demonstrated that this *E. coli* mutant was most efficient in large-scale

aerobic succinate overproduction with high succinate production, no pyruvate accumulation and least acetate secretion as compared to other mutants that 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. 1.6**).

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 1.3: Aerobic succinate yield and productivity as a result of various subsequently introduced genetic modifications (Lin et al., 2005c). $Y_{S/G}$ is molar succinate yield at the end of fermentation; Q_p 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.

1.4.2: Anaerobic Succinate Production

To enhance succinate production under anaerobic condition, the approach involved minimizing pyruvate formation and increasing OAA synthesis. Pyruvate formation was blocked by a series of mutation involving *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 upto ~0.23g/g glucose (17.4mM from 50mM glucose) which was ~7 times higher than the wild type and reduced the formation of other fermentation products but had a slower growth compared to the wild type (Lee et al., 2005a). Heterologous overexpression of *Sorghum vulgare ppc* (resistant to feedback inhibiton by malate) gene and *Lactococcus lactis pyc* gene in *E. coli* enhanced the OAA supply and also 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.

Homologous overexpression of *ppc* gene (modification 1 in **Fig. 1.6**) in *E. coli* resulted in ~3.75 fold increased succinate yield (~0.2g/g glucose) with reduction in all other fermentation products. This modification also led to reduced glucose consumption rate as PEP was also required for glucose uptake mediated by PEP-PTS (Millard et al., 1996). Hence, pyruvate was channeled to OAA synthesis by overexpressing *Rhizobium etli* pyruvate carboxylase (*pyc*) gene (modification 2 in **Fig. 1.6**) this strategy also resulted in increased succinate yield (~0.21g/g glucose) by 2.6 fold compared to the wild type without affecting glucose uptake but it also led to significant accumulation of other fermentation products, specially lactate although its levels were reduced (**Table 1.4**) (Gokarn et al., 1998; 2000; 2001). This strategy was further modified by overexpressing of *R. etli pyc* gene in *E. coli ldh* mutant. Although the strategy successfully eliminated lactate formation it could increase succinate yield by only 1.7fold (modification 2 and 3 in **Fig. 1.6; Table 1.4**). This was supported by the hypothesis that accumulation of pyruvate and NADH affected the activity of pyruvate formate-lyase (*pfl*) enzyme which was supported further by the reduced formate levels although not significant but had no affect on the acetate and ethanol levels. *E. coli* ***ldh- pfl-*** double mutant demonstrated high accumulation of succinate and pyruvate as compared to the wild type (**Table 1.5**). Overexpression of *pyc* in the double mutant drastically reduced pyruvate accumulation and increased the succinate yield to 0.81g/g (modification 2, 3 and 4 in **Fig. 1.6**) but showed a poor growth (Vemuri et al 2002).

To overcome the poor growth in *ldh- pfl-* double mutant *E. coli* ***ldh- pfl- ptsG-*** triple mutant (additionally defective in an enzyme of glucose phophotransferase system) was constructed. The three mutations resulted in 0.88g/g glucose of succinate with increased acetate levels (**Table 1.5**). Overexpression of *pyc* gene in this triple mutant with H_2 gas in the headspace (modification 2, 3, 4 and 5 in **Fig. 1.6**) increased succinate yields to as high as 0.91g/g but still accumulated acetate. On the other hand 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). Similarly, amplifying the malic enzyme (ME) activity in *E. coli* ***ldh- pfl-*** double mutant strain by (modifications 3, 4 and 6 in **Fig. 1.6**) resulted in succinic acid production upto an yield of 0.48g/g glucose but it also showed malic acid production (Hong and Lee, 2001).

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

Apart from developing mutant efficient in succinate production various simulations and metabolic control analysis were also predicted that emphasized on supplying additional reducing power to enhance succinic acid production. Change in carbon source was experimented to achieve high succinate yields. One such example was use of sorbitol instead of glucose as a carbon source which demonstrated high succinic acid concentration and productivity correlated with maximun *in silico* yield of succinic acid reaching 1g/g sorbitol (Hong and Lee, 2002; Lee et al., 2002; Lin et al., 2005a).

In this was one could achieve succinate yield that was 85% of the maximum theoretical yield that is 1.31g. succinic acid/g glucose (Lee et al., 2002). Other sorbitol, xylose (non PTS sugar) was also used. On xylose the intracellular PEP pool were conserved as PEP was required for sugar transport through PTS also led to high succinate production (Lin et al., 2005a).

Overexpression of phsophoenolpyruvate carboxykinase (*pck*) from *M. succiniciproducens* MBEL55E in *E. coli ldh, pta* and alcohol dehydrogenase (*adh*) mutant could achieve succinate yield of 0.72g/g on glucose; on glycerol high succinic

acid yield (1.3 g/g glycerol) was observed but also accumulated of other acid thus resulting in 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 could divert NADH for succinate synthesis hence; overexpression of *L. lactis pyc* gene was carried out in this double mutant resulting in 0.85g succinate/g glucose (Sanchez et al., 2005a).

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

Another strategy targeting to maximize the carbon conversion to succinate by balancing the NADH available involved a combination of two pathway, *viz.* the traditional fermentative pathway and the glyoxylate pathway (which has lower NADH requirement), was 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. 1.6**), which encodes a transcriptional repressor protein of the glyoxylate bypass, was developed (Sanchez et al., 2005b). This *E. coli* strain demonstrated succinate 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).

As most of the above studies employed plasmid a recent report on developing *E. coli* C which carried a series of mutation was subjected to metabolic evolution (2000 generation) and the best strain producing high succinate (1.2 -1.6 moles of succinate/ mole of glucose metabolized was selected (Jantama et al., 2008).

Objective	Products	Refernces
New products produced by *E. coli*	Biodiesel	Lu et al., 2008
	Hyaluronic acid	Yu and Stephanopoulos, 2008
	Dihydrogen	Do et al., 2009
	1-butanol and 1-propanol	Atsumi et al., 2007; Shen and Liao, 2008,
	Curcuminoids	Katsuyama et al., 2008
	Plant flavanoids	Leonard et al., 2008
	Hydrogen from glucose	Maeda et al., 2008
	Carotenoids	Das et al., 2007
	Polyketides	Boghigian and Pfeifer, 2008
	Hydroxystyrene	Qi et al., 2007
	Glucosamine and N-acetylglucoseamine	Deng et al., 2005
	Spider silk	Arcdiacono et al., 1998
	polyhydorxyalkanoates	Lee, 1996; Lee and Cho, 1999
Strain improvement of *E. coli* for various products	Methionine	Krömer et al., 2006
	L-Threonine	Lee et al., 2009
	Pyruvate	Zhu et al., 2008
	Ethanol	Alterthum and Ingram, 1989, Martinez et al., 2007; Yazdani and Gonzalez, 2008; Yomano et al., 2008; Beristain et al., 2008; Zhou et al., 2008
	succinate	Lin et al., 2005; Sanchez et al., 2006; Anderson et al., 2007;

		Jantama et al., 2008
	xylitol	Cirino et al., 2006; Khankal et al., 2008; Akinterinwa and Cirino, 2009; Chin et al., 2009
	L-tyrosine	Eversloh and Stephanopoulos, 2008
	L-lysine	Ishikawa et al., 2008
	L-alanine	Zhang et al., 2007
	Phenylalanine	Yakandawala et al., 2008
	5-aminolevulinic acid	Shin et al., 2007

Table 1.6: Examples of metabolic engineering of *E. coli* producing novel products or improving product yield through strain improvement.

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). Other than *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.

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

Chief metabolic pathways are the EMP pathway (glycolysis), ED pathway and the TCA cycle operating in most of the aerobic and facultative anaerobic bacteria. EMP and ED are involved in breakdown of carbohydrate to PEP and pyruvate which serve as precursors for biosynthesis of several cellular components. The pyruvate dehydrogenase (PDHC) 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 performs dual functions of complete catabolism of acetyl-CoA for respiratory ATP formation (aerobic) as well as supplying the precursors for anabolism (anaerobic).

Organisms when grown on TCA cycle intermediates or substrates that enter the central metabolism via acetyl-CoA, the cell diverted the metabolism towards gluconeogenisis for synthesis of the PEP and pyruvate which further synthesize 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 certain organisms like *E. coli*, the regulation at this node is mediated by catabolite repression that does not allow gluconeogenic enzymes to express in presence of sugars. But in certain organisms like *Bacillus, Corynebacterium*

and *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. 1.15.**

On the other hand certain species contains only a subset of these reactions and the characteristic features of the OAA-PEP node in different organisms is well reviewed in detail by Sauer and Eikmanns (2005).

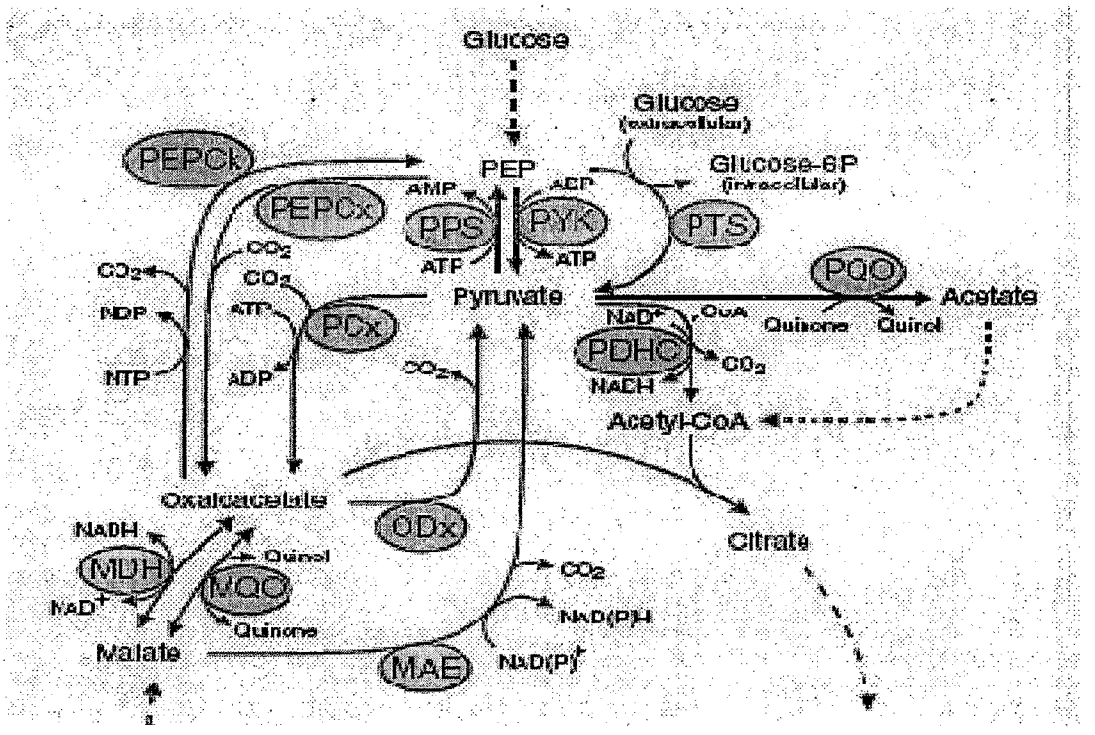

Fig. 1.15: Enzymes and pathways implicated in regulation at the PEP-Pyruvate-OAA node of different aerobic bacteria (Sauer and Eikmanns, 2005). Abbreviations denote the gene products that catalyze a given reaction: MAE, malic enzyme; MDH, malate dehydrogenase; MQO, malate: quinone oxidoreductase; ODx, oxaloacetate decarboxylase; PCx, pyruvate carboxylase; PDHC, pyruvate dehydrogenase complex; PEPCk, PEP carboxykinase; PEPCx, PEP carboxylase; PPS, PEP synthetase; PQO, pyruvate: quinone oxidoreductase; PTS, phosphotransferase system; PYK, pyruvate kinase.

Organism	Medium/condition	Succinate titer (mM)[b]	Succinate yield (mol/mol)
E. coli KJ060 (*ldhA, adhE, ackA, focA, pflB*)	Glucose AM1 (100 g/L) with 10 g/L $NaHCO_3$, simple batch fermentation, 120 h incubation, pH maintained with 1:1 mixture of 6 M KOHþ3 M $K2CO_3$	733 [0.90]	1.41
E. coli KJ073 (*ldhA, adhE, ackA, focA, pflB, mgsA, poxB*)	Glucose AM1 (100 g/L) with 10 g/L $NaHCO_3$, simple batch fermentation, 96 h incubation, pH maintained with 1:1 mixture of 6 M KOHþ3 M $K2CO_3$	668 [0.82]	1.20
E. coli KJ060 (*ldhA, adhE, ackA, focA, pflB*) high inoculum (200 mg CDW L^{-1})	Glucose AM1 (100 g/L) with 10 g/L $NaHCO_3$, simple batch fermentation, 120 h incubation,pH maintained with 1:1 mixture of 6 M KOHþ3 M $K2CO_3$	622 [0.61]	1.61
Actinobacillus succinogenes FZ53	Glucose (130 g/L) supplemented with 15 g/L CSL and 5 g/L YE, 80 g/L $MgCO_3$, anaerobic batch fermentation, 78 h incubation	898 [1.36]	1.25
E. coli AFP111 (*pflAB, ldhA, ptsG*) Rhizobium etli *pyc* overexpressed	Glucose (40 g/L; 90 g total glucose) in medium supplemented with 20 g/L tryptone, 10 g/L YE and 40 g/L $MgCO_3$, dual phase-fed batch fermentation, 76 h incubation	841 [1.31]	1.68
Anaerobiospirillum succiniciproducens ATCC 53488	Glucose (120 g/L) in peptone/YE-based medium, integrated membrane-bioreactor-electrodialysis with CO_2 sparging, 150 h incubation	703 [0.55]	1.35
A. succinogenes 130Z	Glucose (100 g/L) supplemented with 15 g/L CSL and YE, 80 g/L $MgCO_3$, anaerobic batch fermentation, CO_2 sparging, 39 h incubation	678 [2.05]	1.37
E. coli HL27659k/pKK313 (*iclR, sdhAB,ackA-pta, poxB, pstG*) *S. vulgare ppc* overexpressed	Glucose (106 g/L) in medium supplemented with 20 g/L tryptone, 32 g/L YE and 2 g/L $NaHCO_3$, fed batch fermentation under complete aerobic condition, 59 h incubation	499 [1.00	0.89
A. succiniciproducens ATCC 53488	Glucose (50 g/L) and 10 g/L CSL, CO_2 sparging and 300 mM $Na2CO_3$, batch fermentation, 24 h incubation	426 [2.09]	1.37

Mannheimia succiniciproducens (*ldhA, pflB, pta-ackA*)	Glucose (63 g/L) in MMH3 (yeast extract based medium), fed batch fermentation, 0.25 vol/vol/min CO_2 sparging, 30 h incubation	444 [1.75]	1.16
Bacterial isolate 130Z ATCC 55618	Glucose (50 g/L) supplemented with 1% CSL, 0.6% YE, and 2 g/L $MgCO_3$ neutralized with 10 N NaOH, 0.3 atm of CO_2, 29.5 h incubation	388 [1.55]	1.40
E. coli SBS550MG (*ldhA, adhE, iclR, ackA-pta*), *L. lactis pyc*, *Bacillus subtilis citZ*	Glucose (20 g/L; 100 g total glucose) LB supplemented with 1 g/L $NaHCO_3$, 200 mg/L ampicillin, and 1 mM IPTG. 100% CO_2 at 1 L/min STP headspace, repeated fed-batch fermentation, 95 h incubation	339 [0.42]	1.61[c]
E. coli AFP184 (*pflB, ldhA pts*)	Glucose (102 g/L) supplemented with 15 g/L CSL, dual phase aerobic growth and anaerobic production, sparging with air followed by CO_2, 32 h incubation	339 [1.27]	0.72[c]
A. succinogenes ATCC 55618	Glucose (70 g/L) with flour hydrolysate and 5 g/L YE, anaerobic batch fermentation with 4% inoculum, 65 h incubation	302 [0.55]	1.18
A. succiniciproducens ATCC 53488	Glucose (50 g/L), 2% CSL, and 25 ppm tryptophan, neutralized with 5.5 M $NaCO_3$, saturated medium of 0.3 atm partial pressure of CO_2, 29.5 h incubation	289 [1.16]	1.04
Succinivibrio dextrinosolvens ATCC 19716	CSL (15 g/L) and YE (15 g/L), 100 g/L glucose, and 80 g/L $MgCO_3$, batch fermentation, 36 h	226 [0.74]	NR
Corynebacterium glutanicum R	Glucose (40 g/L; 121 g total glucose) in defined mineral salt medium with 400 mM $NaHCO_3$, fed batch fermentation, 6 h incubation	195 [3.83]	0.29
Prevotella ruminocola ATCC 19188	CSL (15 g/L) and YE (15 g/L), 100 g/L glucose, and 80 g/L $MgCO_3$, batch fermentation, 36 h incubation	160 [0.52]	NR
E. coli SBS550MG (*ldhA, adhE, iclR, ackA-pta*), *L. lactis pyc*, *B. subtilis citZ*	Glucose LB (20 g/L) supplemented with 1 g/L $NaHCO_3$, 200 mg/L ampicillin, and 1 mM IPTG. 100% CO_2 at 1 L/min STP headspace, batch fermentation, 24 h incubation	162.6 [0.80]	1.61[c]
M. succiniciproducens MBEL55E KCTC 0769BP	Glucose (18 g/L) in MH_4 (YE-based medium) supplemented with 119 mM $NaHCO_3$, a continuous-cell-recycle membrane reactor with the	144 [2.83]	1.44

	CO2 partial pressure of 101.3 kPa gas (100% CO2), 6 h incubation		
E. coli SBS110MG (*ldhA, adhE*), *Lactococcus lactis pyc*	Glucose LB (20 g/L) supplemented with1.5 g/L $NaHCO_3$ and 0.5 g $MgCO_3$, 200 mg/L ampicillin, and 1 mM IPTG. Dual phase with 100% CO_2 at 1 L/min STP headspace, 168 h incubation	130 [0.09]	1.24[c]
E. coli NZN111 (W1485 *pflB, ladhA*), *E. coli mdh* overexpressed	Glucose LB (20 g/L) supplemented with 0.5 g $MgCO_3$, 1.5 g/L NaOAc, 0.1 g/L ampicillin, and 10 mM IPTG, 44 h incubation, sealed serum tube	108 [0.22]	0.98[c]
E. coli JCL1208, *E. coli ppc* overexpressed	Glucose LB (11 g/L) supplemented with 0.15 g $MgCO_3$, 0.1 g/L carbenicillin, and 0.1 mM IPTG, 44 h incubation, anoxic CO_2 charging at 1 atm headspace, 18 h incubation	91 [0.60]	0.44[c]
E. coli GJT–Sorghum *ppc*	Glucose LB (40 g/L) supplemented with 27.78 g/L $MgCO_3$, simple batch fermentation in sealed airtight flask	80[no data]	0.42[c]
E. coli HL51276k (*iclR, icd, sdhAB, ackA-pta, poxB, pstG*), Sorghum sp. *ppc* S8D mutation	Glucose LB (10.8 g/L) supplemented with 2 g/L $NaHCO_3$, 50 mg/L kanamycin, 1 mM IPTG, aerobic batch reactor, 50 h incubation	68 [0.16]	1.09[c]
E. coli SBS880MG (*ldhA, adhE, DfdhF*), *L. lactis pyc*	Glucose LB (20 g/L) supplemented with1.5 g/L $NaHCO_3$ and 0.5 g $MgCO_3$, 200 mg/L ampicillin, and 1 mM IPTG. Dual phase with 100% CO_2 headspace, 168 h incubation	60 [0.04]	0.94[c]

Table 1.7: Comparison of succinate production by microbial biocatalysts (Jantama et al., 2008). [a]Abbreviations: CSL, corn steep liquor; YE, yeast extract; NR, not reported.[b]Average volumetric productivity is shown in brackets [g L_1 h_1]. [c]The molar yield was calculated based on the production of succinate from metabolized sugar during both aerobic and anaerobic conditions. Biomass was generated predominantly during aerobic growth. Succinate was produced primarily during anaerobic incubation with CO2, H_2, or a mixture of both

Enzymes involved in C3 carboxylation are PEP carboxylase (PPC) and pyruvate carboxylase (PYC) and of C4 decarboxylation are 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 1.8.**

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

Bacillus is an aerobic Gram-positive organism. In spite of its biotechnological and industrial relevance, not much is known about C^3- 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.1.16.** The major difference is that instead of PPC, PYC acts as the sole anaplerotic enzyme to synthesize OAA. Absence of glyoxylate shunt restricts the organism 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. 1.16**). 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 PEPPyruvate-OAA node. Major regulation at this point is brought about by allosteric mechanisms and not by transcriptional control, unlike *E. coli.*

C. glutamicum possesses both PPC and PYC as anaplerotic enzymes for C^3 carboxylation, regulated by different allosteric effectors. PPC and PYC enzymes may have different affinities to HCO_3 - and thus their use remains conditional (Koffas et al., 2002). Additionally, PEPCk, ME or ODx function for C^4 decarboxylation converting

OAA or malate to PEP or pyruvate (**Fig. 1.16, Table 1.8**) 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.

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
Rhodopseudomonas 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 1.8 : 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.

Under glucose grown conditions *C. glutamicum* operates through 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. 1.16**) 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).

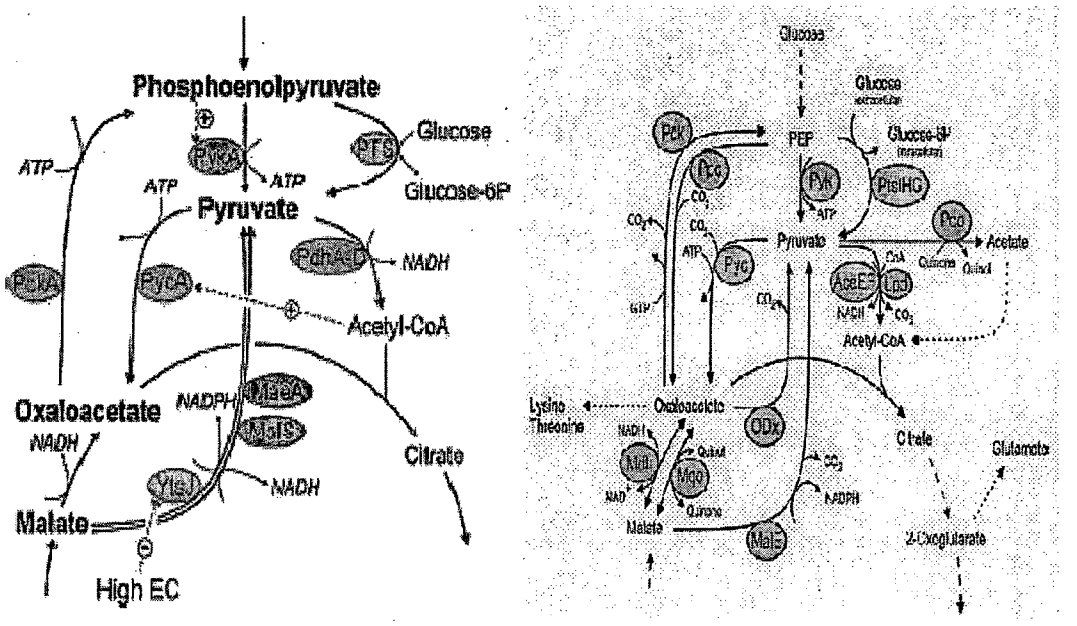

Fig. 1.16: The PEP-Pyruvate-OAA node in *Bacillus subtilis* and *Corynebacterium glutamicum*. Abbreviations denote gene products that catalyze a given reaction: *Ace*EF, E1 and E2 subunits of the PDH complex; EC, energy charge; *Lpd*, subunit E3 of the PDH complex; *Mal*E, *Mae*A, *Mal*S and *YtsJ*, malic enzyme(s); *Mdh*, malate dehydrogenase; *Mqo*, malate: quinone oxidoreductase; ODx, oxaloacetate decarboxylase (gene not annotated); *Pck*, PEP carboxykinase; *Pck*A, PEP carboxy-kinase; *Pdh*ABCD, pyruvate dehydrogenase complex; *Pqo*, pyruvate: quinine oxidoreductase; PTS and *Pts*IHG, phospho-transferase system; *Pyc* and *Pyc*A, pyruvate carboxylase; *Pyk* and *Pyk*A, pyruvate kinase.

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

In *E. coli*, 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 thus playing a critical role in directing the carbon flux (Clark, 1989; Gokarn et al., 2001). The enzymes participating in the PEP-Pyruvate-OAA

interconversion in *E. coli* are as configured in **Fig. 1.17**. PPC is the exclusive C^3 carboxylating enzyme while ATP-dependent PEPCk is involved in C4 decarboxylation and gluconeogenesis (Yang et al., 2003). Other PEPCK C4 decarboxylation enzymes 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 act reversibly in a thermodynamically favorable direction (Stols and Donnelly, 1997). Malic enzymes are considered to be 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. 1.17**).

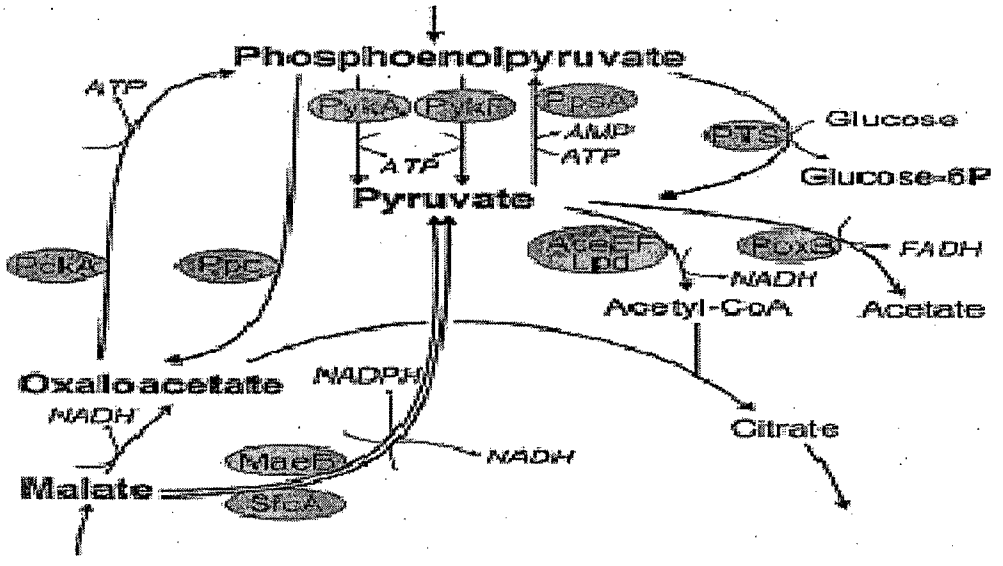

Fig. 1.17: The PEP-Pyruvate-OAA node in aerobic *E. coli*. Abbreviations denote the gene products that catalyze a given reaction: *pyk*A(F), pyruvate kinase(s); *pps*A, PEP synthetase; PTS, phosphotransferase system (for glucose uptake); *ace*EF and *lpd*, pyruvate dehydrogenase; *pox*B, pyruvate oxidase; *ppc*, PEP carboxylase; *pck*A, PEP carboxykinase; *mae*B and *sfc*A, 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).

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) this could be due to possible futile cycling. Under glucose-limited cultures *E. coli* completely oxidizes acetyl-CoA to CO_2 *via* the PEPCk-glyoxylate cycle, which is normally carried out 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).

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

Succinate overproduction is one of the most successful examples of engineering the flux at the anaplerotic node under both fermentative and aerobic conditions in *E. coli* (detailed in Section 1.3). Few reports of successful pyruvate overproduction 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 have been achieved (Wendisch et al., 2006). Anaplerotic node in *E. coli* has been 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).

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 failed to grow on glucose and required supplementation with TCA cycle intermediates like succinate and glutamate. In the absence of PPC, glyoxylate bypass can theoretically substitute the PPC reaction and meet the OAA requirement but it is inactive on glucose due to catabolite repression moreover there exists a competition of isocitrate dehydogenase (ICDH) and isocitrtae lyase (ICL) for the common substrate isocitrate. Overexpression of pyruvate carboxylase (*pyc*) in *ppc* mutant could functionally complement the anaplerotic function which is otherwise absent in *E. coli* (Gokarn et al., 2000; 2001). Under aerobic conditions *ppc* gene overexpression in *E. coli* reduced acetate formation and increased biosynthetic efficiency (Farmer and Liao, 1997). 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 is available on the regulatory mechanisms operating at the anaplerotic node for *B. subtilis* (Sauer and Eikmanns, 2005), no genetic manipulations are reported at this node. *C. glutamicum* is an aerobe holding a commercial importance with respect to L-Lysine and L-glutamate production (Kiefer et al., 2004). As 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. Increasing in PYC activity and knocking out PEPCk activity in *C. glutamicum* independently resulted in increased production of glutamate and lysine (Sauer and Eikmanns, 2005). The activity levels of ME affected 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.

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

There seems to be a direct involvement of the anaplerotic node in the TCA cycle which is evident from the interplay of enzymatic reactions utilizing PEP and pyruvate at this node that plays a critical role in suppling 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 in *E. coli* is regulated both transcriptional and allosterically 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 sets up a 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. In *E. coli* ICL 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). These reports suggest that PEP-pyruvate-OAA node plays a critical role in regulating the cellular metabolism especially with respect to the central carbon metabolism.

1.6 Rationale

E. coli has been demonstrated as one of the most preferred microorganism for employing various strategies of metabolic engineering depending on the kind of product desired. As referred in the above sections a vast range of information and tools have been

developed for *E. coli* and with the long list of successful metabolic engineering strategies in *E. coli* especially with respect to succinate production the present study selects *E. coli* as a model of study. Moreover PEP-pyruvate-OAA node has demonstrated to play a critical role in central carbon metabolism involving PPC, CS and ICDH and various reports have been demonstrated in exploiting this node for succinate production; the present study explores the role of PEP-pyruvate-OAA node in citrate accumulation. To achieve this, the strategy involves overexpression of *Synechococcus elongatus* PCC 6301 phosphoenolpyruvate carboxylase (*ppc*) and *E. coli* citrate synthase (*cs*) genes along with downregulation of ICDH mediated by antisense for isocitrate dehydrogenase (*icd*) gene under both, a conditionally regulated promoter or an inducible promoter. As *E. coli* lacks a citrate efflux mechanism the present study explores the strategies independently in absence and presence of citrate transporter in both *E. coli* K and *E. coli* B strains (**Fig. 1.18**).

1.6.1 Objectives for the present study.

The objectives for the present study were:

1. Effect of conditional expression of antisense isocitrate dehydorgenase (P_{fruB}-*asicd*) gene on organic acid secretion in *E. coli* DH5α.
2. Effect of conditional expression of antisense isocitrate dehydorgenase (P_{fruB}-*asicd*) gene on organic acid secretion in *E. coli* BL21 (λDE3).
3. Effect of P_{tac}-a*sicd* on the growth and acidification in *E. coli* BL21 on glucose.
4. Effect of overexpression of phosphoenol pyruvate carboxylase (*ppc*), citrate transporter genes in the *E. coli* MA1935 (*icd*) mutant on the growth and organic acid secretion.
5. Effect of overexpression of citrate synthase (*cs*), citrate transporter genes in the *E. coli* MA1935 (*icd*) mutant on the growth and organic acid secretion.
6. Effect of overexpression of citrate synthase (*cs*), phosphoenol pyruvate carboxylase (*ppc*), citrate transporter genes in the *E. coli* MA1935 (*icd*) mutant on the growth and organic acid secretion.

7. Effect of overexpression of citrate synthase (*cs*), antisense isocitrate dehydorgenase (P_{tac}-as*icd*), citrate transporter genes in the *E. coli* BL21 on the growth and organic acid secretion.
8. Effect of overexpression of citrate synthase (*cs*), antisense isocitrate dehydorgenase (P_{fruB}-*asicd*), citrate transporter genes in the *E. coli*DH5α on the growth and organic acid secretion.

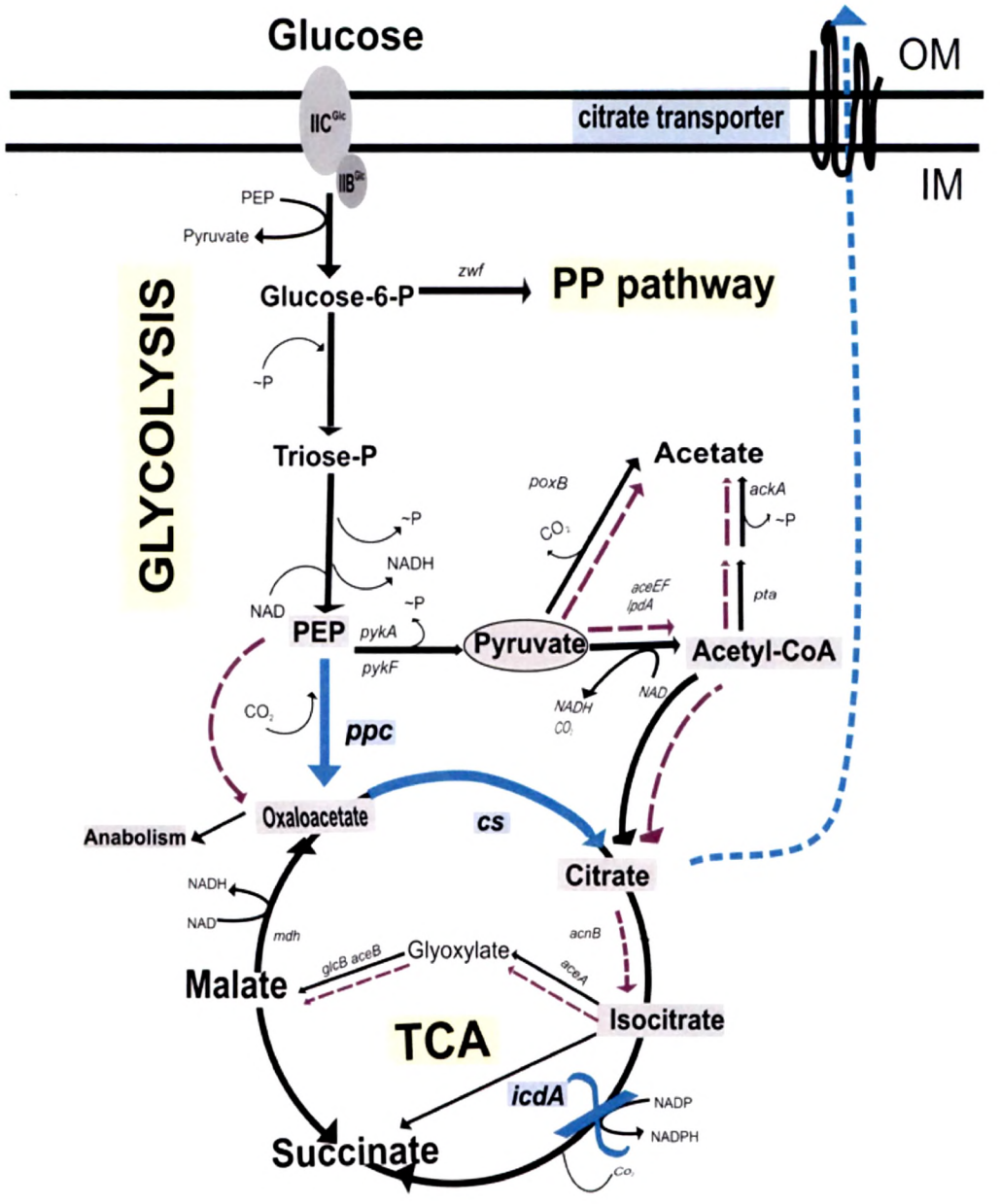

Fig.1.18: Metabolic basis for designing the genetic modifications in *E. coli* – Rationale. Blue boxes indicate the genes (over)expressed or down regulated. Blue lined (bold) indicate metabolic alterations imposed by the engineered genetic manipulations. X indicates block mediated by antisense or mutation. Magenta 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*, phosphoenol pyruvate carboxylase; *cs*, citrate synthase; *pyk*, pyruvate kinase; *ace*A, isocitrate lyase; *ace*B, malate synthase; *icd*A, isocitrate dehydogenase; *pox*B, pyruvate oxidase; *zwf*, glucose-6-phosphate dehydrogenase; *pta*, phosphotranacetylase; *ack*A, acetate kinase; *ace*EF and *lpd*, pyruvate dehydrogenase.