Chapter 18
Glucose and Acetate Metabolism in *E. coli* – System Level Analysis and Biotechnological Applications in Protein Production Processes

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Contents

Abstract *Escherichia coli* is the main bacterial producer of heterologous proteins. The current production strategies aim at growing the bacteria to high density in order to achieve high levels of desired proteins. The major obstacle for reaching high cell densities with high product titers is the tendency of the bacteria to accumulate acetate during the unrestricted growth on glucose. Moreover, the high demand for precursors and energy required for the biosynthesis of the heterologous protein causes the cells to readjust their anabolic and catabolic reactions which, most often, aggravate the acetate problem. Implementing fed-batch protocols and employing more robust strains, such as *E. coli* B instead of K, can reduce acetate formation. Another approach is to implement metabolic engineering to minimize acetate formation by: (a) turning off genes which directly lead to the formation of acetate, (b) introducing

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genes that channel the carbon flow away from acetate towards other pathways, and (c) by reducing the glucose uptake through deleting or replacing genes of the sugar uptake system. Results show that a more general approach that focuses on global regulators and/or gene sets, encoding multiple pathways will be required to construct a robust strain capable of efficiently executing the production of recombinant proteins at high growth rates without the formation of toxic byproducts such as acetic acid.

18.1 Introduction

Escherichia coli is the major bacterial platform for producing heterologous proteins, which is usually done by growing the recombinant microorganism to high density on glucose as the carbon source. This topic has been the subject of numerous studies since the early 1970s, exploring the limits of bacterial culture density in order to achieve maximum productivity. Research strategies have focused on improving cultivation conditions, process related approaches and manipulation of the bacteria's physiology. The developed growth strategies, together with optimization of media composition, application of fed-batch and dialysis culture techniques have made it possible to grow *E. coli* to cell densities of up to 190 g/L dry cell mass (Shiloach and Fass 2005). High-cell density culture techniques have been successfully employed for large-scale production of recombinant proteins with high yield and high productivities (Choi et al. 2006).

The biosynthesis process exposes the bacteria to metabolic stress which is being reflected in the operation of their Central Carbon Metabolism and is associated with higher acetate production (Dittrich et al. 2005a, Tao et al. 1999). Acetate accumulation is considered an obstacle to enhanced recombinant protein production; it is also considered as one of the factors responsible for the reduced biomass yield in large scale fed-batch cultivation (Enfors et al. 2001, Phue and Shiloach 2005). Research is currently being directed to understand this behavior and the response of various *E. coli* strains to the growth conditions during the cultivation and production process. Few factors, among them, the overloading of the TCA cycle and limitations around the pyruvate node as well as local pockets of oxygen limitations in large-scale cultures, are considered to be the main reason for this phenomenon; therefore, concentrated effort is being directed to prevent the overloading and the related processes and to allow the uninterrupted oxidation of the carbon source. This chapter describes the operation of the Central Carbon Metabolism, possible explanations for the acetate production and ways for its reduction, in particular during the recombinant protein production process. It includes the following sections: a general description of the Central Carbon Metabolism of *E. coli*, a review of acetate production and consumption, a Systems Biology approach to the Central Carbon Metabolism in *E. coli K* (JM109) and *B* (BL21), the effect of recombinant protein production on glucose catabolism, and metabolic engineering approaches to overcome bottlenecks in primary metabolism.

18.2 The Central Carbon Metabolism in *E. coli* – General Description

The Central Carbon Metabolism of *E. coli* in general and specifically the glucose metabolism are well-known, well-studied and well-characterized topics (EcoCyc 2008, EcoSal ASM 2008, KEGG 2008, Nelson and Cox 2003). This metabolism can be described by several interconnected metabolic pathways as seen in Fig. 18.1.

The major pathways are glycolysis (Embden-Meyerhof-Parnas EMP), TCA cycle, glyoxylate shunt, pentose-phosphate pathway, anaplerotic reactions, acetate production, and acetate assimilation.

Glucose assimilation starts with its uptake into the cell via the phosphotransferase system (PTS). D-Glucose is transported by PTS and ultimately enters the cell as glucose-6-phosphate with the concomitant consumption of phosphoenolpyruvate (PEP) and the release of pyruvate. Although the PTS is the dominant transport system it is important to mention that there are alternative high-affinity glucose transport systems (e.g. *mgl*) which are activated at low glucose concentrations (Death

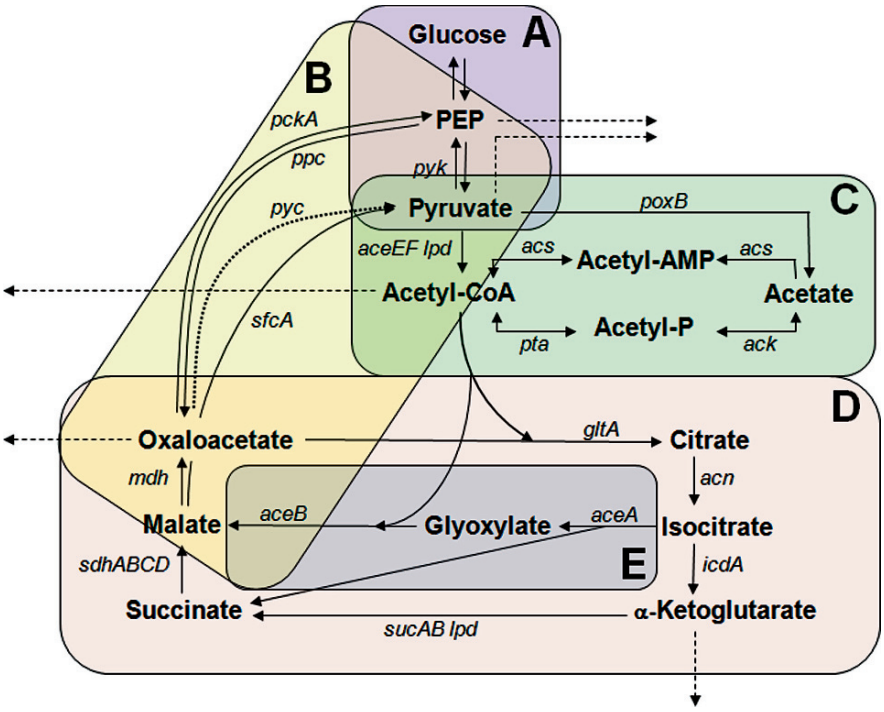

Fig. 18.1 Simplified view of the Central Carbon Metabolism of *E. coli* comprising (**A**) glycolysis and gluconeogenesis, (**B**) anaplerotic reactions, (**C**) acetate formation and assimilation, (**D**) TCA cycle, and **E**. glyoxylate shunt. Arrows with broken lines indicate removel of metabolites for biosynthesis. The arrow with the dotted line indicates an anaplerotic reaction catalysed by pyruvate carboxylase (an enzyme not present in wildtype *E. coli*)

and Ferenci 1994, Franchini and Egli 2006, Wick et al. 2001). Glucose transport can also occur via the galactose-proton-symport system (*galP*)(Chen et al. 1997). In these cases, phosphorylation of glucose is carried out by the cytoplasmic enzyme glucokinase (*glk*) (Meyer et al. 1997)

The glucose-6-phosphate can then be directed into three different routes: it can enter the glycolytic pathway through conversion to fructose-6-phosphate via the phosphoglucose isomerase (*pgi*) reaction, it can enter the oxidative branch of the pentose-phosphate pathway (*zwf*), or it can be converted by phosphoglucomutase (*pgm*) to glucose-1-phosphate for sugar nucleotide synthesis. When entering the glycolytic pathway, the fructose-6-phosphate is converted to fructose-1,6-bisphosphate by 6-phosphofructokinase (*pfkA* and *pfkB*), and then undergoes reversible aldol condensation by fructose bisphosphate aldolase (*fba*) to glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate. The continuation of this pathway is the interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate by triosephosphate isomerase (*tpiA*), followed by oxidative phosphorylation of glyceraldhyde-3-phosphate to 1,3-bisphosphoglycerate by glyceraldehyde-3-phosphate dehydrogenase (*gapA*) and the synthesis of ATP by phosphogylcerate kinase (*pgk*) producing 3-phophogylcerate. The two evolutionarily unrelated phosphoglycerate mutases (*gpmA*, *gpmM*) convert the 3-phosphoglycerate to 2-phosphoglycerate. Enolase (*eno*) catalyzes the dehydration of 2-phosphoglycerate to phosphoenolpyruvate (PEP), which contains a high-energy phosphate group that is used both for ATP synthesis and glucose transport by PTS. PEP is converted to pyruvate by two distinct pyruvate kinases (*pykF* and *pykA*). The reverse reaction, the conversion of pyruvate to PEP during gluconeogenesis is catalyzed by phosphoenolpyruvate synthase (*pps*).

Pyruvate is the end-product of glycolysis; it is oxidized to acetyl-CoA and CO_2 by the pyruvate dehydrogenase complex (composed of pyruvate dehydrogenase E1, dihydrolipoamide transacetylase E2, and dihydrolipoamide dehydrogenase E3; *aceEF*, *lpd*).

Acetyl-CoA is a pivotal molecule which can participate is several reactions: it can enter the TCA cycle, it can be used for fatty acids and triglycerides biosynthesis and it can be diverted towards acetate production. Accumulation of acetyl-CoA can affect the glucose utilization by causing accumulation of pyruvate and enhancing acetate production.

The TCA cycle plays two essential roles in the carbon metabolism: it is responsible for the total oxidation of acetyl-CoA, and serves as a source of intermediates for the biosynthesis of several amino acids. The general flow of the TCA cycle is as follows: Acetyl-CoA, formed by the oxidation of pyruvate condenses with oxaloacetate to form citrate by citrate synthase (*gltA*). The citrate is transformed to isocitrate by two genetically distinct aconitases (*acnA* and *acnB*). Next, isocitrate dehydrogenase (*icdA*) performs the oxidative decarboxylation of isocitrate to α-ketoglutarate with generation of NADPH. Following is another oxidative decarboxylation step in which α-ketoglutarate is converted to succinyl-CoA and CO_2 by the α-ketoglutarate dehydrogenase complex (*sucAB*, *lpd*). The succinyl-CoA is converted to succinate by succinyl-CoA synthetase (or succinate thiokinase; *sucCD*). Succinate is oxidized

to fumarate by succinate dehydrogenase (*sdhABCD*); the fumarate is reversibly hydrated to malate by three distinct fumarases (*fumA*, *fumB*, *fumC*). In the last reaction of the citric acid cycle, NAD-linked L-malate dehydrogenase (*mdh*) catalyzes the oxidation of malate to oxaloacetate.

The TCA cycle is interconnected to the glyoxylate shunt which is essential for growth on carbon sources such as acetate or fatty acids. This pathway allows the net conversion of acetyl-CoA to metabolic intermediates. In the glyoxylate shunt, isocitrate is cleaved by isocitrate lyase (*aceA*), forming succinate and glyoxylate. Isocitrate lyase competes with the TCA cycle enzyme isocitrate dehydrogenase (*icdA*) for isocitrate. The bifunctional enzyme isocitrate dehydrogenase kinase/phosphatase (*aceK*) regulates the activity of isocitrate dehydrogenase to allow isocitrate lyase to effectively compete for isocitrate. The formed glyoxylate condenses with a second molecule of acetyl-CoA to yield malate in a reaction catalyzed by malate synthase (*aceB*). The malate is subsequently oxidized to oxaloacetate, which can condense with another molecule of acetyl-CoA to start another turn of the TCA cycle. The operation of the TCA cycle can be affected by the removal of the cycle intermediates for biosynthesis of various cell compounds; this can cause accumulation of acetyl-CoA that potentially can affect also the activity of the glycolytic pathway. On the other hand, an active glyoxylate shunt can reduce the accumulation of acetyl-CoA and eliminate interference with both glycolysis and TCA cycle activities.

The role of the anaplerotic reactions is to replace intermediates. These reactions are considered part of the Central Carbon Metabolism and they include: the conversion of PEP to oxaloacetate by PEP carboxylase (PPC shunt, *ppc*), the conversion of oxaloacetate to PEP by PEP carboxykinase (*pck*), the conversion of pyruvate to oxaloacetate by pyruvate carboxylase (not present in wildtype *E. coli*; *pyc*), and the conversion of malate to pyruvate (and *vice versa*) by the malic enzyme (*sfcA*). The glucoenogensis, the conversion of pyruvate to glucose, can also be considered as anaplerotic reaction in which the organism converts excess glucose to glycogen.

Acetate production and assimilation are also part of the Central Carbon Metabolism. Acetate is produced from pyruvate and acetyl-CoA and consumed by conversion back to acetyl CoA. This component of the Central Carbon Metabolism includes the following reactions: acetate production from pyruvate by pyruvate oxidase B (*poxB*), acetate production from acetyl-CoA via acetyl phosphate by phosphotransacetylase (*pta*) and acetate kinase (*ack*), acetate consumption through acetyl-AMP by acetyl CoA synthetase (*acs*), and by the reverse action of acetate kinase (*ack*) and phosphotransacetylase (*pta*).

Lastly, the Central Carbon Metabolism also includes the pentose-phosphate (PP) pathway. The PP pathway serves several metabolic functions which include catabolism of pentoses, glucose, and gluconate, synthesis of pentoses, and providing precursors used in the biosynthesis of lipopolysaccharide, nucleotides, several amino acids and vitamins. This pathway includes two branches: oxidative and nonoxidative. In the oxidative branch, glucose-6-phosphate (G6P) is first oxidized by glucose-6-phosphate dehydrogenase (*zwf*) and then further converted by a series of enzymes to ribulose-5-phosphate (Ru5P) and CO_2. Two molecules of NADP are reduced in the dehydrogenase reactions of this process and can be used for reductive

biosynthesis, maintenance of redox balance, and regeneration of oxidative damage. The nonoxidative branch of the pathway comprises reversible reactions that perform the interconversion of the pentose phosphates ribulose-5-phosphate (Ru5P), ribose-5-phosphate (R5P), and xylulose-5-phosphate (Xu5P), and the transfer of either a glycoaldehyde group (transketolase) or a dihydroxyacetone group (transaldolase) among sugar phosphates.

18.3 Acetate Production and Consumption

As was mentioned in the previous section, acetate occupies an important place in the Central Carbon Metabolism of *E. coli*. Acetate accumulation can affect both the bacterial growth and the production of recombinant protein, and serves as an indicator that something went "wrong" in the glucose assimilation process. Acetate accumulation phenomenon has been reviewed comprehensively in the last few years (De Mey et al. 2007, Eiteman and Altman 2006, Shiloach and Fass 2005, Wolfe 2005) and, therefore, it will be described here with an emphasis on how several pathways affect acetate concentration and the possible role of few global controllers.

A genome-scale analysis of the integrated metabolic and transcriptional regulatory networks of *E. coli* shows that the genetic regulatory network responds primarily to the available electron acceptor and to the presence of glucose as the carbon source (Barrett et al. 2005). When carbon flux into the cells exceeds the amphibolic capacity of the central pathways, the flux is diverted to acetate excretion which diminishes the efficiency of carbon conversion to biomass (El-Mansi and Holms 1989). Inverse flux analysis has been used to predict the flux distribution based on the stoichiometries of the reactions in the metabolic network. This approach has been also applied to analyze acetate excretion in aerobic *E. coli* cultures (Delgado and Liao 1997, Farmer and Liao 1997). The results suggest that the anaplerotic pathways, including the reactions catalyzed by PEP carboxylase (*ppc*) and the glyoxylate shunt, are the most likely factors affecting acetate excretion in *E. coli*.

Similar to other metabolites, acetate concentration is the result of production and consumption. The main route for acetate production is from acetyl-CoA through acetyl-phosphate by the two enzymes: phosphotransacetylase (*pta*) and acetatekinase (*ack*). Another minor route for acetate production is directly from pyruvate by pyruvate oxidase B (*poxB*). Although the function of pyruvate oxidase B is not fully understood it is clear that it contributes significantly to aerobic growth efficiency (Abdel-Hamid et al. 2001, Flores et al. 2004a). Any reaction that affects the concentration of acetyl-CoA and pyruvate will, in turn, affect acetate production and hence concentration. Acetyl-CoA concentration is the result of production from glucose through the glycolytic pathway by the conversion of PEP to pyruvate by the reversible enzyme pyruvate kinase (*pyk*), and the conversion of pyruvate to acetyl-CoA by the irreversible reaction catalyzed by the pyruvate dehydrogenase complex (*aceEF*, *lpd*). Acetyl-CoA concentration is also affected by its consumption through the TCA cycle and its consumption for fatty acid biosynthesis. The anaplerotic enzyme PEP carboxylase (*ppc*) converts PEP to oxaloacetate, reducing acetyl-CoA

accumulation as a result of higher turnover of the cycle. Anaplerotic reactions can also reduce the acetyl-CoA concentration by lowering the pyruvate concentration through the conversion of PEP back to glucose and glycogen accumulation.

Acetate formation is also affected by the NADH/NAD ratio. Vemuri et al. (2006a) showed that several genes involved in the TCA cycle and respiration are repressed as the glucose consumption rate increases. Deletion of the gene coding for the regulatory protein ArcA (*arcA*) resulted in acetate reduction and increased the biomass yield due to the increased capacities of the TCA cycle and respiratory chain. Acetate formation was completely eliminated by reducing the redox ratio through expression of NADH oxidase (from *Streptococcus pneumonia*) in an *arcA* mutant, even at a very high glucose consumption rate (Vemuri et al. 2006b). NADH and NADPH can be converted into each other through reversible transfer of reducing equivalents between NAD and NADP. The pentose-phosphate pathway and isocitrate dehydrogenase (*icdA*) catalyzed reaction are generally considered as the major sources of the anabolic reductant NADPH which can be converted by the two native *E. coli* transhydrogenases (*pntAB* and *udhA*) into NADH and *vice versa* (Boonstra et al. 1999, Hoffmann et al. 2002, Sauer et al. 2004). Both transhydrogenases have divergent physiological functions: energy-dependent reduction of NADP with NADH by PntAB (Rydstrom 1977, Sauer et al. 2004), and reoxidation of NADPH by UdhA (Boonstra et al. 1999, Hoffmann et al. 2002, Sauer et al. 2004) thus providing *E. coli* primary metabolism with a high flexibility to cope with changing catabolic and anabolic demands.

Acetate assimilation is done by the enzyme acetyl-CoA-synthetase (*acs*) that converts acetate to acetyl-CoA through the intermediate acetyl-AMP. This route is being utilized when acetate is the carbon source and when there is a need to reabsorb the acetate formed when the bacteria grow at high rate, the latter is also known as the "acetate switch"(Wolfe 2005). The components of this switch are phosphotransacetylase (*pta*), acetate kinase (*ack*), and acetyl CoA synthetase (*acs*). This switching behavior is essential for alternating between periods of rapid growth in the presence of abundant nutrients and growth periods where these nutrients are in short supply. Kumari and co-workers (2000) showed that this switch occurs primarily through the induction of *acs*, and that the timing and magnitude of this induction depend, in part, on the direct action of the carbon regulator cyclic AMP receptor protein, synonym: cAMP-catabolite activator protein (*crp* or *cap*) and the aerobic/anaerobic transcriptional regulator (*fnr*). It also depends, probably indirectly, upon the glyoxylate shunt repressor (*iclR*), and its activator the transcriptional regulator of fatty acid metabolism (*fadR*). During aerobic growth of *E. coli* on acetate, phosphotransacetylase (*pta*) and the α-ketoglutarate dehydrogenase complex (*sucAB*, *lpd*) are in direct competition for their common co-factor, HS-CoA. This competition can create a bottleneck at the level of α-ketoglutarate dehydrogenase in the TCA cycle. Addition of pyruvate, glucose or any glycolytic intermediate to acetate-grown cells relieves the bottleneck by reversing the carbon flow through phosphotransacetylase to supply acetyl-phosphate and much-needed HS-CoA (El-Mansi 2005). Growth of *E. coli* on acetate as the sole source of carbon and energy requires operation of the glyoxylate shunt in connection with the

expression of the polycistronic *ace* operon (Cortay et al. 1989). Expression of the *aceK* gene is essential for growth on acetate (El-Mansi et al. 1987). The competition at the junction of isocitrate between isocitrate lyase (*aceA*) and isocitrate dehydrogenase (*icdA*) is resolved by the reversible phosphorylation/inactivation of isocitrate dehydrogenase and the operation of the glyoxylate bypass, the expression of which is subject to regulation at the transcriptional and translational levels as well as being dependent on growth rate (El-Mansi et al. 2006). The adaptation to acetate is connected to complex metabolic changes and alterations in gene expression in *E. coli* (Kirkpatrick et al. 2001, Oh et al. 2002, Rosenthal et al. 2008). For example, growth on acetate also induces expression of genes encoding malic enzymes (*maeA*, *sfcA*) and phosphoenolpyruvate synthase (*pps*) while causing repression of glycolytic and glucose phosphotransferase genes (Oh and Liao 2000).

Another mechanism that can affect acetate concentration is the carbon catabolite repression. It allows *E. coli* to alter its metabolism in response to the availability of specific sugar sources. The cAMP-catabolite activator protein (*cap*) complex regulates a number of *E. coli* genes involved in carbon metabolism (Krin et al. 2003). Kao et al. (2005) demonstrated that the gluconeogenic genes in *E. coli* provide a feedback loop to this global regulator in carbon source transition. PTS also plays a role in the carbon catabolite repression; inactivation of PTS components has been applied successfully as a strategy to abolish carbon catabolite repression, resulting in *E. coli* strains that use sugar mixtures more efficiently, such as those obtained from lignocellulosic hydrolysates (Gosset 2005).

A cAMP-independent catabolite repression mechanism found in *E. coli* involves the catabolite repressor/activator (*cra*), which formerly was designated as the fructose repressor (*FruR*), a pleiotropic transcriptional regulatory protein that controls the direction of carbon flux through metabolic pathways (Ramseier et al. 1993). When catabolites bind to Cra, it dissociates from the DNA, causing both catabolite activation and catabolite repression (Saier 1996). Cra controls the expression of genes encoding key enzymes of major pathways of carbon metabolism (Ramseier et al. 1995). Cra exerts a negative effect on the expression of genes encoding glycolytic and Entner-Doudoroff enzymes, while exerting a positive effect on genes encoding the TCA cycle, the glyoxylate shunt and gluconeogenic enzymes (Bledig et al. 1996, Kaga et al. 2002, Negre et al. 1996, Ow et al. 2007).

Based on the above information, several genetic modifications were implemented with an effort to reduce acetate accumulation. These methods are described in more details in the final section of this chapter.

18.4 Systems Biology Approach to the Central Carbon Metabolism in *E. coli* K (JM109) and B (BL21)

E. coli B (BL21) and *E. coli* K (JM109) respond differently to glucose concentration in their growth media, especially when the glucose concentration is 10 grams per

liter or more. *E. coli* B is not sensitive to the high glucose concentration, its growth is not affected, and there is very low acetate accumulation. In contrast, *E. coli* K is sensitive to the high glucose concentrations, produces elevated levels of acetate and grows at a slower rate (Shiloach et al. 1996). Investigation of the difference between these two strains can serve as an excellent tool for understanding the regulation and control of the Central Carbon Metabolism when utilizing glucose as sole carbon substrate.

The traditional approach for evaluating and understanding the regulation and the operation of the Central Carbon Metabolism was to concentrate on specific enzymes and genes and sometimes on a specific pathway. More information on the interrelationship between the various pathways will allow a better understanding of the processes controlling glucose utilization, reducing acetate production and improving growth and recombinant protein production.

With the development of new methodologies - especially the high-throughput measurements of DNA, RNA and proteins, and the new mathematical modeling and algorithms - it is possible to examine simultaneously various pathways and to have a sense of the regulation and the operation from a broader perspective. The term for this global approach is Systems Biology (Barrett et al. 2005, Kitano 2002). One of the popular definitions of Systems Biology is the investigation of complex biological processes in a way that aims to understand how individual molecular components combine on a global scale to yield particular structure function relationships and behave in response to specific perturbations. Attempts to utilize global understanding, although in a rather limited way, were implemented long before the term Systems Biology was coined in 2002. The continuing research of the difference between the Central Carbon Metabolism of *E. coli* K and B can serve as an example for implementing the System Biology approach. During the past years several methodologies were implemented to evaluate the relationship between the various pathways of the Central Carbon Metabolism, and the overall response of the system to glucose and acetate concentrations. With the introduction of each new method additional information was obtained and more details became available. Although no powerful mathematical approaches, which currently are part of the Systems Biology, were implemented, better understanding of the metabolism was achieved.

Several factors could be responsible for the different behavior of *E. coli* B compared to *E. coli* K: reduced glucose transport into the cell, increased respiration/O_2 transfer rate, decreased flux from pyruvate to acetate, increased anaplerotic flux from PEP to oxaloacetate, increased flux through the glyoxylate shunt and increased TCA cycle flux. The initial assumption was that perhaps the glyoxylate shunt is fully operational in *E. coli* B and may not be operational or operates at a low rate in *E. coli* K. This assumption had to be proven.

The first attempt to look at the glyoxylate shunt question from a "Systems Biology perspective" was done by metabolic flux analysis together with measuring the concentration and activity of several key metabolites and enzymes, respectively (van de Walle and Shiloach 1998). Metabolic flux analysis (Savinell and Palsson 1992) compares fluxes through specific pathways by using the following assumptions: use known reaction stoichiometries, ignore nonlinearities in kinetics, ignore

regulations and assume that the network has been correctly drawn. In this particular case of evaluating the flux through the glyxoylate shunt, the calculations were based on measuring specific glucose uptake rate, specific acetate production rate, growth rate, CO_2 production rate, O_2 uptake rate, cell monomers content, and assuming an ATP/oxygen (P/O) ratio of 1.33 and pseudo steady state concentrations for the intracellular metabolites (van de Walle and Shiloach 1998). The purpose was to determine the flux through the TCA cycle and the glyoxylate shunt; however, because of the singularity of this methodology (Vallino and Stephanopoulos 1990), it was impossible to calculate simultaneously the flux of the two pathways and to receive a direct answer. For *E. coli* B we were able to determine independently the flux through the TCA cycle and the flux through the glyoxylate shunt. But for *E. coli* K only the flux through the TCA cycle could be calculated as this bacterium has only negligible amounts of isocitrate lyase (*aceA*). The results of this initial phase can be summarized as follows: the flux through isocitrate dehydrogenase (*icdA*) was higher in *E. coli* B than in *E. coli* K, isocitrate dehydrogenase was highly active in B and the flux to acetate through the acetate kinase-phosphotransacetylase system (*pta-ack*) was higher in K (van de Walle and Shiloach 1998). In addition, *E. coli* B had a higher internal isocitrate concentration and a lower pyruvate concentration (van de Walle and Shiloach 1998).

The second attempt was to measure simultaneously the flux through the glyoxylate shunt and the TCA cycle using ^{13}C labeled glucose (Noronha et al. 2000). This was done by measuring the distribution of the ^{13}C isotopomers of oxaloacetate and acetyl-CoA. It was concluded that in *E. coli* B, the glyoxylate shunt is active at 22% of the flux through the TCA cycle and is inactive in K. Additionally, in *E. coli* B the flux through the TCA cycle equals the flux through the PPC shunt, while in *E. coli* K the flux of the TCA cycle is only third of the flux through the PPC (Noronha et al. 2000).

The third attempt in utilizing the "Systems Biology perspective" to gain better understanding of the Central Carbon Metabolism was made possible due to the deciphering of the *E. coli* genome and the availability of DNA microarray technology (Blattner et al. 1997, Richmond et al. 1999). By using Northern blots and DNA microarrys, it was possible to simultaneously follow the transcription of genes which are part of several metabolic pathways, and to identify the activated pathways at different growth conditions (Phue et al. 2005, Phue and Shiloach 2004). Although this method allowed the identification of up-regulated and down-regulated genes, it did not provide information on the flux through the various pathways. The results of this study are shown in Fig. 18.2a and b.

In *E. coli* B, the various pathways of the Central Carbon Metabolism are activated whether the glucose concentration is low or high, at both concentrations the tested pathways operate similarly. In contrast, *E. coli* K was responding differently to the various glucose concentrations; its gene activity profile was similar to *E. coli* B only at a low glucose concentration.

The latest step in this effort was done by comparing the transcription level of a group of genes that compose specific metabolic pathways by the semiparametric algorithm using oligo-microarrays (Phue et al. 2007). It was found that as a group,

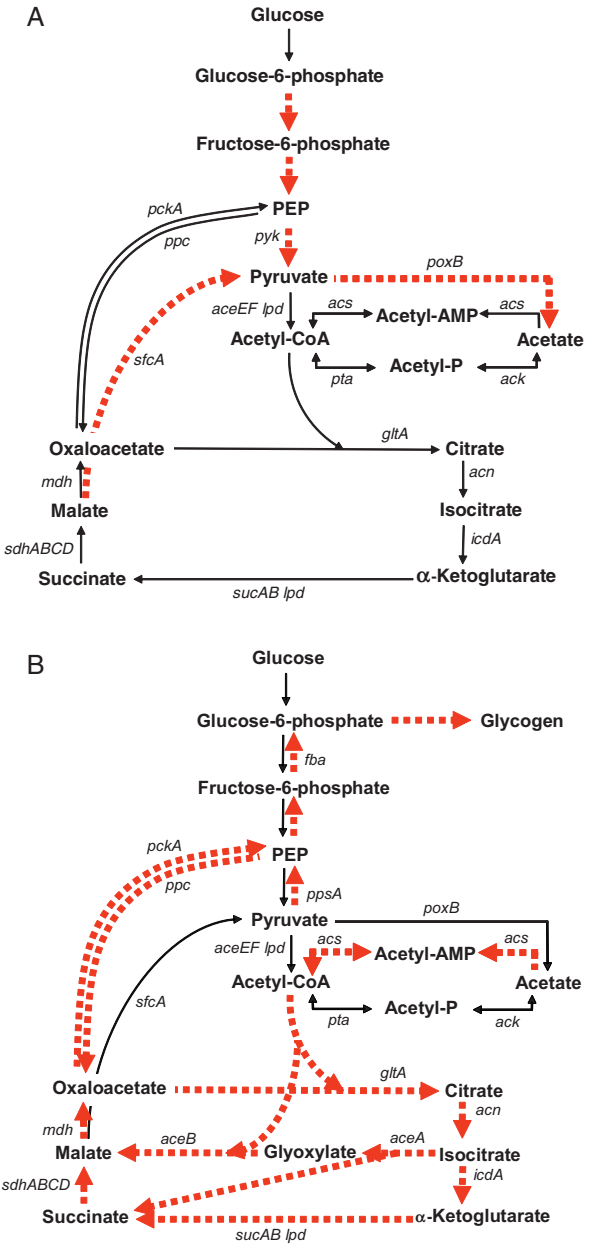

Fig. 18.2 Proposed glucose metabolism during growth of (**a**) *E. coli* K12 (JM101) and (**b**) *E. coli* B (BL21) under glucose excess conditions. The red arrows with broken lines indicate the activated pathways utilized by the different strains

the following pathways were transcribed differently in the two strains: glyoxylate shunt, TCA cycle, fatty acids biosynthesis, gluconeogensis, and anaplerotic pathways. There was no difference between the groups comprising transcription of either glycolysis or the pentose-phosphate pathway genes. This finding confirmed the

previous observation that the difference is not the result of a single gene but most likely the effect of one or more global controllers that influence the transcription of complete pathways.

With the information available so far, it is possible to have some explanation why *E. coli* B is producing less acetate when being exposed to high glucose concentration, and why it is utilizing glucose more efficiently than *E. coli* K. But it is impossible to point out why this is happening, why the TCA cycle flux in *E. coli* B is higher than in *E. coli* K, why the glyoxylate shunt is inactive at high glucose concentration in *E. coli* K and why the gluconeogenesis is active in *E. coli* B and not in *E. coli* K. The expectation is that high glucose concentration should activate the glyoxylate shunt; increase the TCA cycle activity, increase the acetate uptake and reduce the acetate concentration. All these actions are observed in *E. coli* B regardless of glucose or acetate concentration; it is puzzling that there is no activation in *E. coli* K, and there is constant activation in *E. coli* B, especially puzzling is the fact that *poxB* is less active in *E. coli* B. Perhaps, additional global analysis of the Central Carbon Metabolism will provide a better explanation. In the meantime, there are numerous efforts to improve *E. coli* K behavior by modifying the Central Carbon Metabolism. These approaches are described in the last section of this chapter.

18.5 Effect of Recombinant Protein Production on Glucose Catabolism

Escherichia coli is still the most prominent bacterial host for recombinant protein production with glucose as the common carbon substrate in recombinant protein production processes. This process can induce a variety of stress reactions in the bacterial host including flux alterations in primary metabolic pathways (Hoffmann and Rinas 2004). Calculations by Stouthamer revealed that protein synthesis is the most energy consuming process of all anabolic activities (Stouthamer 1977, Stouthamer 1980, Stouthamer 1986). According to these estimations, more than 50% of the ATP required for the formation of microbial cells during growth on defined medium with glucose as sole carbon and energy source is used for the polymerization of amino acids into proteins while only 4% is required for the synthesis of amino acids (Stouthamer 1986). Experiments by Anderson and von Meyenburg (1980) suggested that growth of *E. coli* in aerobic cultures under glucose excess conditions is limited by the rates of both respiration and ATP generation through oxidative phosphorylation. Thus, recombinant protein production might be potentially limited by bottlenecks in the energy-generating pathways. Under conditions of glucose excess, part of the glucose is not used for biomass and energy generation through the respiratory chain and proton motive force, rather is diverted towards the formation of overflow metabolites, mainly acetate, causing a reduction in the efficiency of glucose utilization.

Early experiments with genetically modified *E. coli* strains indicated that extracellular accumulation of acetate (Brown 1985, Jensen and Carlsen 1990, Meyer 1984, Shimizu 1988) or other overflow metabolites like glutamate (Rinas 1989) are associated with reduced yields of the recombinant protein produced. The recent

developments in the recombinant DNA technology and the widespread utilization of *E. coli* as a microbial protein production factory stimulated research associated with the understanding and solving of the "acetate problem". Although we still do not completely understand the complexity of acetate formation and are far from a non-acetate producing, metabolically balanced and robust *E. coli* designer strain, there has been progress in overcoming this difficulty and improving the recombinant protein production process (see following section). Early hypothesis suggested that metabolic bottlenecks leading to the formation of acetate are localized at the level of TCA cycle activity and in the respiratory chain (Anderson and von Meyenburg 1980, Majewski and Domach 1990). Therefore, acetate formation would be an alternative way for generating ATP, although at reduced efficiency. In fact, acetate formation is observed under conditions of energetic stress/deficiency when the carbon flux into the cells is bigger than the amphibolic capacity of the central pathways, for example, caused by artificially induced futile cycling (Chao and Liao 1994, Patnaik et al. 1992) or at rapid growth in glucose-limited chemostat cultures (Kayser et al. 2005).

It has not only been shown that recombinant protein synthesis is reduced during acetate accumulation, but also that induction of recombinant protein synthesis can lead to enhanced acetate excretion (Akesson et al. 1999, Wittmann et al. 2007). Also reported has been the enhanced pyruvate excretion as a result of recombinant protein synthesis, suggesting an alteration in the pyruvate oxidation pattern (George et al. 1992). An elevated intracellular pyruvate pool, together with enhanced pyruvate excretion was observed during recombinant protein production under glucose excess conditions using a temperature-inducible expression system (Wittmann et al. 2007). These observations suggest that enhanced acetate formation during recombinant protein production results from limitations around the pyruvate node.

Proteomic analyses of inclusion bodies, composed mainly of the recombinant protein product, revealed that dihydrolipoamide dehydrogenase (*lpd*), the common component of the pyruvate and the α-ketoglutarate dehydrogenase complexes, coaggregates during recombinant protein production. This probably leads to additional aggravation of the limitation around the pyruvate node (Rinas et al. 2007) and at the level of TCA cycle activity where α-ketoglutarate dehydrogenase activity is considered as a major bottleneck (El-Mansi 2004, Rinas 1989). Dihydrolipoamide dehydrogenase might be a critical protein since *lpd* knockout mutants of *E. coli* produced significantly more pyruvate and glutamate under aerobiosis (Li et al. 2006). Moreover, *E. coli* strains with deletion of both acetate producing pathways (*ack-pta* and *poxB*) accumulate pyruvate (Dittrich et al. 2005b). Pyruvate excretion in these strains can be prevented by overexpression of genes encoding the pyruvate dehydrogenase complex (Dittrich et al. 2005b) suggesting this complex enzyme as a potential metabolic engineering target for the generation of low acetate producing strains.

As indicated, recombinant protein synthesis driven by strong promoters is a high-energy consuming process potentially limited by bottlenecks in the energy-generating pathways. An example is recombinant protein production using temperature-inducible expression systems. This process caused an immediate drop of the adenylate energy charge, which serves as an indicator of the energetic status of the cells. This occurs at glucose limiting (Hoffmann et al. 2002) as well as at

glucose excess growth conditions (Wittmann et al. 2007). Under glucose excess conditions, protein synthesis, driven by the temperature-inducible lambda promoters, caused enhanced excretion of acetate and other byproducts (Wittmann et al. 2007) while protein synthesis under balanced carbon-limited conditions caused redirection of substantially more glucose into the energy-generating respiratory pathway (Hoffmann and Rinas 2001, Schmidt et al. 1999a). Thus, when recombinant protein synthesis is induced under carbon-limiting balanced growth conditions, which do not lead to the formation of acetate, a greater portion of glucose is diverted to carbon dioxide production compared to non-producing conditions (Hoffmann and Rinas 2001, Schmidt et al. 1999b). In balanced fed-batch conditions, about 40–45% of the glucose carbon is converted to carbon dioxide, which increases to 70% after temperature-induced recombinant protein production (Hoffmann and Rinas 2001). During IPTG-induced protein production in balanced carbon limited fed-batch cultures, the flux towards carbon dioxide formation increased from 44–46% of glucose carbon before induction to 50–52% after the onset of recombinant protein production (Schmidt et al. 1999b). An increased respiratory activity upon induction of recombinant protein synthesis has also been noted for other expression systems (Bhattacharya 1997, Lin and Neubauer 2000). The increase in protein synthesis rates upon induction in balanced carbon-limited fed-batch cultures correlated directly with an increase in respiratory activity (Hoffmann and Rinas 2001) together with enhanced glycolytic and TCA cycle activity and reduced pentose-phosphate pathway flux (Luo et al. 2008, Weber et al. 2002). In contrast to the catabolic response in balanced carbon-limited fed-batch cultures, cells reduce TCA cycle activity upon recombinant protein production under glucose excess in batch culture conditions (Wittmann et al. 2007).

Changes in the respiratory activity in response to recombinant protein production are primarily caused by changes on the level of catabolic enzyme activity and not on the amount of catabolic enzymes as the respiratory response is instantaneous (Hoffmann et al. 2002, Schmidt et al. 1999a,b). The cellular response towards recombinant protein production on the level of transcription and translation of genes encoding catabolic enzymes appears to be complex and very specific with respect to the recombinant protein produced and the conditions of induction. General conclusions are difficult to obtain; the most common observations include down-regulation of transcription of genes involved in energy generation, such as TCA cycle, respiration and AcrA-dependent genes (Durrschmid et al. 2008, Haddadin and Harcum 2005, Harcum and Haddadin 2006, Oh and Liao 2000). Proteome analysis indicated both decrease (Wagner et al. 2007) and increase in synthesis rate, or level of proteins (Durrschmid et al. 2008, Hoffmann et al. 2002, Jurgen et al. 2000) encoded by these genes. Contrasting findings, such as decreased transcript levels of TCA cycle and glyoxylate shunt enzymes associated with increased protein levels, have also been reported (Durrschmid et al. 2008).

Global transcriptome analysis of the cellular response towards recombinant protein production indicated that many genes of the glycolytic pathway (e.g. *fba*, *eno*) and PTS (e.g. *ptsG* and *crr*) were downregulated while the gene encoding glucokinase (*glk*) was strongly upregulated (Haddadin and Harcum 2005, Oh and Liao

2000). A strong upregulation of glucokinase in response to recombinant protein production has been noted not only through increased transcription but also through elevated enzyme levels (Arora and Pedersen 1995), indicating a shift in the utilization of the glucose uptake pathway in response to recombinant protein production. The impairment of glucose uptake during recombinant protein production (Lin et al. 2001, Neubauer et al. 2003) might be reflected by the transition from the utilization of the more common PTS towards alternative pathways for supplying overproducing cells with glucose-6-phosphate. On the other hand, reduced synthesis and leakage of periplasmic binding proteins involved in high-affinity glucose uptake (*mglB*) might also contribute to an impairment of glucose uptake under protein production conditions in high-cell density cultures (Rinas and Hoffmann 2004).

18.6 Metabolic Engineering Approaches to Overcome Bottlenecks in Primary Metabolism

The formation of acetic acid is a disturbing side reaction during rapid growth of *E. coli* on glucose (Luli and Strohl 1990). As a result, efforts have been undertaken to reduce the formation of acetic acid either by process control strategies or by metabolic engineering approaches. When implementing process control approaches, the aim is to reduce glucose uptake rate, generally done by limiting the glucose supply through fed-batch culture techniques (Korz et al. 1995, Lee 1996, Shiloach and Fass 2005). This approach has been successfully applied for recombinant protein production in high-cell density fed-batch cultures leading to recombinant protein levels in the range of 5–10 g L^{-1} with *E. coli* strains having tendency towards acetate formation under glucose excess conditions (Hoffmann and Rinas 2004, Schmidt et al. 1999a, Vallejo et al. 2002).

Metabolic engineering efforts have been implemented to generate strains which produce less acetate in protein production processes (for recent review refer to (Eiteman and Altman 2006)). Three major metabolic routes or combinations thereof have been applied to reduce acetate accumulation; (i) knocking out genes that directly lead to the formation of acetate, (ii) introducing genes that lead to redirection of the carbon flow away from glycolysis and acetate formation towards other pathways and metabolites, and (iii) reducing glucose uptake by deleting or replacing genes of the PTS.

Initial approaches focused on mutation or deletion of enzymes that lead to the formation of acetate, in particular blocking the acetate kinase-phosphotransacetylase (*ack-pta*) pathway (Bauer et al. 1990, Hahm et al. 1994). For example, a phosphotransacetylase mutant selected by classical mutagenesis techniques showed improved protein production properties in bioreactor cultures (Bauer et al. 1990). The downregulation of the acetate-generating pathway that includes the enzymes phosphotransacetylase (*pta*) and acetate kinase (*ack*) by using an antisense RNA strategy also improved recombinant protein production (Kim and Cha 2003). Most of these strains have been tested in laboratory-scale shake flask experiments and

did not show the robustness required for industrial application. *E. coli* strains that carry single mutations (e.g. *ack*, *pta*, *acs*, *poxB*) do not exhibit the robustness in high-cell density fed-batch cultures compared to the corresponding control strain (Contiero et al. 2000). Inactivation of the *poxB* gene results in slower growth rates and also leads to a reduced carbon conversion efficiency (percentage carbon flux to biomass)(Abdel-Hamid et al. 2001, Li et al. 2007), probably as a result of the activation of energetically less favorable metabolic pathways such as activation of *glk* and repression of PTS genes *ptsG* and *crr* (Li et al. 2007, Vemuri et al. 2005). The deletion of genes that lead to acetate formation (e.g. *ack*, *pta, poxB*) results in strains that secrete pyruvate (Chang et al. 1999, Diaz-Ricci et al. 1991, Dittrich et al. 2005b, Tomar et al. 2003) and other unusual by-products such as glutamate (Chang et al. 1999) into the culture medium. Taking advantage of this phenomenon, an *E. coli* strain that was engineered for optimal acetate production (Causey et al. 2003), was transformed into an efficient pyruvate producing strain by simply disrupting two genes that lead to acetate formation (*ack*, *poxB*)(Causey et al. 2004). The reduction of pyruvate formation through inactivation of the pyruvate kinase encoding genes (*pykA* and *pykF*) was also considered as a way to reduce acetate formation. The resulting strains metabolized glucose mainly via the PP pathway (Ponce et al. 1998, Siddiquee et al. 2004) and produced less acetate (Ponce 1999, Zhu et al. 2001), but also exhibited reduced growth rates when grown under glucose excess conditions (Ponce 1999, Ponce et al. 1995, 1998, Zhu et al. 2001).

Results obtained by inverse flux analysis suggested that increased flux through anaplerotic pathways (PPC shunt and glyoxylate bypass) should reduce acetate formation (Delgado and Liao 1997, Farmer and Liao 1997). In fact, deregulation of the glyoxylate bypass by disrupting *fadR*, reduced acetate formation without negatively effecting the growth rate (Farmer and Liao 1997, Peng and Shimizu 2006). Increasing the flux through the PPC shunt by overexpressing PEP carboxylase (*ppc*) further decreased acetate formation without impairment of the growth rate (Farmer and Liao 1997). On the other hand, deletion of *ppc* also reduced acetate formation but at the expense of a slower growth rate and a reduced glucose uptake rate (Peng et al. 2004).

Another approach for reducing acetete formation includes the generation of strains overexpressing heterologous genes that encode anaplerotic enzymes that replenish the TCA cycle, for example pyruvate carboxylase (*pyc*). These strains showed better performance in glucose excess batch culture (March et al. 2002) and also revealed reduced acetate production and higher cell yields in controlled chemostat cultures (Vemuri et al. 2005). Directing excess pyruvate away from acetate towards less toxic products through coexpression of other heterologous enzymes (such as acetolactate synthase (*alsS*) from *Bacillus subtilis* which finally leads to the formation of acetoin instread of acetate) also resulted in strains which produced less acetate and performed better as protein producers in batch and fed-batch cultures (Aristidou et al. 1994, 1995). Overexpression of the glucose-6-phosphate dehydrogenase encoding gene (*zwf*), which leads to an increased flux towards the pentose-phosphate pathway (by decreasing the glycolytic flux), resulted in a better performing production strain under carbon excess conditions (Flores et al. 2004b).

In this line, deletion of *zwf* resulted in elevated glycolytic flux and enhanced excretion of acetate and pyruvate (Hua et al. 2003).

Another approach to reduce formation of acetate and to improve protein production under carbon excess conditions involves the reduction of glucose uptake via the PTS (Backlund et al. 2008, Chou et al. 1994, Gosset 2005, Picon et al. 2005, Ponce 1999, Wong et al. 2008). As glucose uptake via the PTS is connected to the generation of pyruvate from PEP, the reduced pyruvate formation might lead to reduced acetate production. The majority of strains with modifications of the PTS produce less acetate, however, they do it at the cost of reduced growth rates (Backlund et al. 2008, Chou et al. 1994, Flores et al. 2002, Picon et al. 2005, Ponce 1999, Wong et al. 2008). Another approach to reduce acetate formation involves the inactivation of the PTS while forcing glucose transport through the galactose-proton symport system composed of the membrane localized galactopermease (*galP*) with subsequent glucose phosphorylation through cytoplasmic glucokinase (*glk*)(De Anda et al. 2006, Flores et al. 2007, Hernandez-Montalvo et al. 2003, Lara et al. 2008). These strains, when carrying multiple copies of *galP* and *glk* genes, exhibit growth rates similar to the PTS wildtype strains, in particular those with an *arcA* background (Flores et al. 2007, Hernandez-Montalvo et al. 2003), but at the same time exhibit increased acetate production rates compared to the PTS wildtype strains (Hernandez-Montalvo et al. 2003). Reducing the glucose uptake rate by fine-tuned expression of *galP* in a PTS mutant strain, reduced acetate formation, but this was associated with a slower growth rate compared with the PTS wildtype strain (Lara et al. 2008). Comparative studies on acetate formation by single global regulator gene knockout mutants (e.g. *arcA*, *arcB*, *cra*, *crp*, *cya*, *fnr*, and *mlc*) also revealed that reduced acetate formation is always connected to reduced glucose uptake and growth rates and *vice versa* (Perrenoud and Sauer 2005). In this way, avoiding utilization of the PTS by replacing glucose with fructose as carbon source also reduced acetate formation and improved protein production, but again at the cost of reduced growth rate (Aristidou et al. 1999).

In summary reducing acetate formation by metabolic engineering or any other means such as reduced glucose feeding or alternative carbon substrates without shifting the carbon flow to other unwanted by-products (e.g. pyruvate) is mainly achieved by reducing the glucose uptake rate concomitant to growth rate reduction.

To successfully generate robust strains producing significantly less acetate in carbon excess conditions it might be necessary to further consider the impact of global regulators, e.g. ArcA (Flores et al. 2007, Vemuri et al. 2006a,b) or FadR (Farmer and Liao 1997, Peng and Shimizu 2006) and the deletion or the introduction of whole gene sets that encode multiple pathways instead of focusing on single genes or pathways. For example, the reduction of the redox ratio in an $arcA^-$ background through expression of an heterologous NADH oxidase eliminated acetate formation even at high glucose consumption rates (Vemuri et al. 2006a). Moreover, eliminating native transcriptional control of a set of TCA cycle enzymes by chromosomal promoter mutation (*sdhCDAB-B0725-sucABCD*), resulted in a strain which produced less acetate and instead directed more glucose to carbon dioxide while maintaining high growth and glucose consumption rates (Veit et al. 2007).

18.7 Concluding Remarks

Enabling recombinant *E. coli* to grow to high density and to produce proteins without severely affecting its metabolism, its growth characteristics, and its protein biosynthesis capabilities is currently an important research and development topic. Comprehensive analysis of the Central Carbon Metabolism, among other metabolic pathways, is on-going in an effort to identify bottlenecks in the metabolism that might affect the growth and production process. So far, this analytical approach has yielded several targets that alleviate some of the growth constraints, especially improving the glucose oxidation process. It is likely that a System Biology approach that takes into account the relationships not only between the metabolic pathways of the Central Carbon Metabolism but also between others metabolic pathways and their relationship with global regulators and other effectors, may improve our understanding of the bacterial behavior under stress, and will result in improving the growth and production process. Although our knowledge of the *E. coli* metabolism and its regulation covers many aspects, at this point, there is not enough information to predict the possible response to different changes and different conditions. The goal is to have a global physiological overview of the *E. coli* metabolism and, accordingly, to construct a robust strain capable of efficiently executing the production of recombinant proteins.

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