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MINI-REVIEW

Metabolic engineering of *Escherichia coli* to improve recombinant protein production

Min Liu[1,2] · **Xinjun Feng**[1] · **Yamei Ding**[3] · **Guang Zhao**[1] · **Huizhou Liu**[1] · **Mo Xian**[1]

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Abstract *Escherichia coli* is one of the most widely used strains for recombinant protein production. However, obstacles also exist in both academic researches and industrial applications, such as the metabolic burden, the carbon source waste, and the cells' physiological deterioration. This article reviews recent approaches for improving recombinant protein production in metabolic engineering, including workhorse selection, stress factor application, and carbon flux regulation. Selecting a suitable host is the first key point for recombinant protein production. In general, it all depends on characteristics of the strains and the target proteins. It will be triggered cells physiological deterioration when the medium is significantly different from the cell's natural environment. Coexpression of stress factors can help proteins to fold into their native conformation. Carbon flux regulation is a direct approach for redirecting more carbon flux toward the desirable pathways and products. However, some undesirable consequences are usually found in metabolic engineering, such as glucose transport inhibition, cell growth retardation, and useless metabolite accumulation. More efficient regulators and platform cell factories should be explored to meet a variety of production demands.

✉ Huizhou Liu
liuhuizhou@qibebt.ac.cn

✉ Mo Xian
xianmo@qibebt.ac.cn

[1] CAS Key Laboratory of Biobased Materials, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao 266101, China

[2] University of Chinese Academy of Sciences, Beijing 100049, China

[3] Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China

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Introduction

The recombinant protein production is a complex and enormous project. The commercial production of recombinant proteins has gained great interest and development in the past few decades (De Anda et al. 2006; Pavlou and Reichert 2004). The impressive progress is that many therapeutic and industrial proteins have been brought into practical applications (Chen 2012), such as rennin, interferons, proteases, and insulin. *Escherichia coli*, a common inhabitant of the mammalian intestines, undoubtedly has been widely used for recombinant protein production because of its fast growth, facility in genetic manipulation and low cost (Baneyx 1999; Terpe 2006). However, obstacles also exist in both academic researches and industrial applications. Firstly, the expression of plasmid-encoded proteins consumes various energy and resources, which leads to the growth inhibition. This so-called metabolic burden provides stress selection for plasmid-free cells and affects plasmid stability (Chou 2007;Glick 1995; Hoffmann and Rinas 2001). Previous researches have put forward to some significant strategies to overcome it, like reducing the plasmid copy number (Jones et al. 2000; Summers and Sherratt 1984), changing the plasmid-coded antibiotic resistance genes (Hong et al. 1995), integrating the desire genes into chromosome and applying the plasmid addiction system (PAS) based on selective killing to prevent the plasmid-free cells survival (Gao et al. 2014; Vidal et al. 2008). The chromosomal integration and PAS have the features of higher plasmid stability, lower production cost, less environmental pollution, and so on (Gao et al. 2014). The two strategies have

been widely used for improving the industrial production of recombinant proteins. Secondly, the accumulation of insoluble proteins may lead to the deterioration in cell physiology. The native defense systems of *E. coli* are triggered to adapt the adverse condition (Chou 2007). Coexpression of some molecular chaperones can help proteins to fold into their correct conformation and degrade the misfolded proteins. Lastly, the undesired metabolites formation is also a serious problem in recombinant protein production, especially the acetate excretion (Eiteman and Altman 2006).

Acetate excretion is a serious problem because it affects cell density, biomass accumulation, and recombinant protein production even at concentrations as low as 0.5 g/L (Farmer and Liao 1997; Koh et al. 1992; Nakano et al. 1997). Acetate has adverse effects on the macromolecules synthesis of DNAs, RNAs, proteins, and lipids (Cherrington et al. 1990). These macromolecules usually play essential roles in cellular activities, such as the genes transcription-translation, signal transduction, stress responses, and cell regulation. It is commonly revealed that acetate is distributed about 10–30 % carbon flux from glucose as the sole carbon in aerobic fermentation (Farmer and Liao 1997). The rate of acetate accumulation is directly related to the rate of cell growth and the rate of glucose consumption. In the fed-batch fermentation, *E. coli* begins to generate acetate only when the cells grow above a threshold specific growth rate. This threshold is usually related to the cells capacity for consuming oxygen (Eiteman and Altman 2006). In general explanation, acetate overflow is a manifestation of imbalance between glucose uptake and limited activity of the tricarboxylic acid (TCA) cycle and respiratory chain (El-Mansi and Holms 1989). The insufficient replenishment of coenzyme A may be another problem for acetate overflow (El-Mansi 2004). Strategies for overcoming acetate accumulation are mainly through reducing the speed of glucose uptake or controlling cell growth below the threshold specific growth rate (Eiteman and Altman 2006). However, Kaspar Valgepea and his colleagues proposed a new theory for acetate overflow by systems biology approach. They elucidated that acetate overflow was triggered by acetyl-CoA synthetase (Acs) downregulation resulting in reduction of acetate assimilation and disruption of the PTA-ACS node (Valgepea et al. 2010). Overexpression of Acs may be an effective way to improve the acetate assimilation. Since acetate is produced mainly through two pathways. One pathway is catalyzed by pyruvate oxidase (encoded by *pox*) and the other pathway is catalyzed by phosphotransacetylase (encoded by *pta*) and acetate kinase (encoded by *ack*) (Fig. 1). Acetate accumulation will be eliminated or reduced by deleting one or more acetate-formation-related genes.

This article reviews the recent strategies for improving recombinant protein production in three aspects, selecting a suitable workhorse for proteins production, coexpressing stress factors to help proteins to fold into their native conformation and redirecting more carbon flux to the desirable pathways and products.

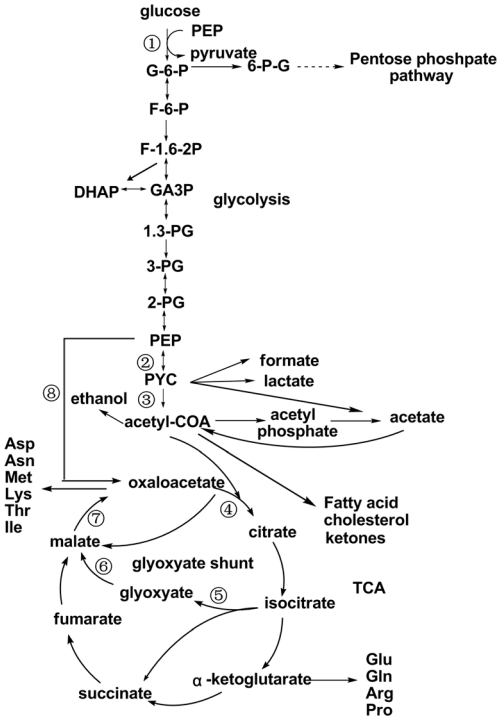

Fig 1 Key metabolic pathways of *E. coli* involved in glycolysis, TCA cycle, and glyoxylate shunt. Glucose is transported principally by the phosphotransferase system (PTS) and accepted a phosphate group from PEP. PEP carboxylase and glyoxylate shunt are the most important anaplerotic reactions to replenish the intermediate metabolites of TCA cycle. The TCA cycle intermediates oxaloacetate and α-ketoglutarate are metabolized to produce ten amino acids. Not all pathways are indicated. ① Glucose-phosphoenolpyruvate phosphotransferase system (PTS); ② pyruvate kinases; ③ pyruvate dehydrogenase complex; ④ citrate synthase; ⑤ isocitrate lyase; ⑥ malate synthase; ⑦ malate dehydrogenase; ⑧ PEP carboxylase

Workhorse selection

E. coli, a common inhabitant of the mammalian intestines, undoubtedly has become one of the most widely used strains for heterologous gene expression. Its products have extended to the fields of medicines, industrial materials, food additives, and scientific researches. The products, such as antibodies and enzymes are commonly used in scientific researches. In *E. coli*, K-12 strains (W3110, MG1655, JM109, and BW25113) and B strains (BL21, BL21(DE3), BL21(DE3) pLysS, and BL21(DE3) Rosetta) are the most common strains for laboratorial and biotechnological applications (Terpe 2006). It is really difficult to decide which strain is the best one for heterologous gene expression. It often depends on the characteristics of the strains and the target proteins. So, workhorse selection is the first key point for recombinant protein production in metabolic engineering. This part aims to compare the

similarities and differences between the two strains and provide some help for selecting an appropriate host.

B strains are derived from K-12 strains and locate the higher position in the evolutionary tree of *E. coli*. They are highly similar in genome sequence based on the comparison of insertion sequence (IS) elements (Schneider et al. 2002). However, B strains experienced the treatments of P_1 transduction and UV irradiation during the process of evolution (Daegelen et al. 2009). These evolutions resulted in some differences between K-12 and B strains in phenotype, genome, cellular metabolism, and physiology:

1. The phenotype comparison. Low expression of motility-related genes and lack of flagellar biosynthetic genes make B strains nonmotile (Yoon et al. 2012). It is an important property for B strains because flagellar biosynthesis consumes a lot of energy and is not necessary for industrial applications. For that reason, B strains have a higher growth rate in minimal medium than K-12 strains (Pósfai et al. 2006). Differences in cells envelope composition influence on its integrity and permeability, which further result in alterations of materials transport (Yoon et al. 2009). The existence of the second type II secretion (T2S) system for protein extracellular secretion qualify B strains as the first choice for recombinant proteins extracellular production (Herrera et al. 2002; Yoon et al. 2009). However, B strains are not as good as K-12 strains in emergency system sensitivity and cell stability because of the nonmotile feature (Yoon et al. 2009).
2. The genome comparison. K-12 strains have received more attention than B strains in the past decades. It is mainly attributed to the fact that K-12 strains are much easier for constructing a variety of mutants by recombinant DNA techniques (Swartz 1996). However, during the process of B derivatives evolution, BL21(DE3) was inserted the DE3 part from λ phage and this part carried T7 RNA polymerase gene under the control of *lacUV5* promoter (Studier and Moffatt 1986). The plasmid vectors harboring T7 promoter system have the capacity for high-level expression of recombinant proteins in BL21(DE3). Thanks to this evolution, BL21(DE3) has become the most widely used strain for recombinant protein production (Terpe 2006). Even so, there are also some undesirable problems for the industrial applications, like proteins misfolding, high basal expression of toxic proteins, codon bias, and rare codons (Terpe 2006). More BL21 derivatives have been provided to meet the developing need of industrial productions. BL21(DE3) Rosetta is a specifically engineered B descendant that contains some rare codon-related tRNAs for eukaryotic proteins expression. It also has the functions of promoting the formation of disulfide bonds and helping proteins to fold into their native conformation. Host strains harboring the vector of pLysS or pLysE can effectively reduce the basal level of proteins, because the two vectors express T7 lysozyme, a natural inhibitor of T7 RNA polymerase. B strains are naturally lack of the OmpT outer membrane protease and the major protease Lon (Jiang et al. 2002; Srivastava et al. 1999). Additionally, B strains exhibit upregulations of some amino acid biosynthetic genes (Yoon et al. 2009). All these features are desirable for improving the recombinant protein production.
3. The cellular metabolism and physiology comparison. B strains show less acetate accumulation than K-12 during the process of high cell density cultivation with glucose as the sole carbon. No differences are found in genes involved in acetate production and the central metabolic pathways between K-12 and B strains (Jeong et al. 2009). The common explanation is that the metabolic genes are under different regulations. B strains have higher activity in TCA cycle and glyoxylate shunt. Glyoxylate shunt is the main pathway for acetate assimilation and promotes more conversion of acetate to acetyl-CoA (Phue et al. 2005; Yoon et al. 2009). B strains have many desirable features for recombinant protein production, like faster growth in minimal medium, higher proteins expression level, higher ability for proteins extracellular secretion, less proteins degradation during the purification, wider choice for plasmid vectors and lower acetate accumulation (Yoon et al. 2009).

In spite of this, B strains are not the perfect host for recombinant protein production (Xu et al. 2006). So, a host will be better for use if it harbors more advantages from B and K-12 strains. Several groups have done this job in *E. coli* K-12 strains through metabolic engineering to get the similar physiological and metabolic features to BL21(DE3).

A previous research revealed that inactivation of the local regulator IclR and the global regulator ArcA simultaneously in the host cell *E. coli* K-12 MG1655 increased the biomass yield from 0.43±0.02 to 0.63±0.01 cmol/cmol glucose and decreased the acetate yield from 0.16±0.01 to 0.04±0.02 cmol/cmol glucose under batch conditions (Waegeman et al. 2011). The results approximate the values observed in BL21(DE3) because BL21(DE3) naturally possesses low expression of *arcA* and *iclR* genes (Waegeman et al. 2011). The Arc system regulates numerous operons expression under both aerobic, microaerobic, and anaerobic conditions. It is composed of the cytosolic response regulator ArcA and the membrane bound sensor kinase ArcB. ArcB is autophosphorylated at the expense of ATP and then sequentially transfers phosphoryl group to ArcA, the phosphorylated ArcA binds to the promoter region of the TCA cycle genes and regulates these genes expression (Nizam et al. 2009; Perrenoud and Sauer 2005). Isocitrate lysate regulator (IclR) represses the expression of *aceBAK* operon which codes for

the glyoxylate shunt enzymes of isocitrate lyase (AceA), malate synthase (AceB), and isocitrate dehydrogenase kinase/phosphatase (AceK) (Yamamoto and Ishihama 2003). Glyoxylate shunt is the main pathway for acetate utilizing. A combined deletion of *arcA* and *iclR* in *E.coli* K-12 MG1655 shows the similar biomass yield and acetate accumulation to *E. coli* BL21(DE3). This phenomenon is attributed to the improved activity of TCA cycle and glyoxylate shunt by analysis of metabolic flux. The follow-up studies had investigated that the increased biomass and decreased acetate accumulation led to a higher green fluorescent protein (GFP) yield. However, the GFP yield decreased again when the cell density was greater than 2 g/L CDW. It is probably due to the proteins degradation. Further knockout *lon* and *ompT* genes in *E. coli* K-12 MG1655△*arcA*△*iclR*, the quadruple mutant strain showed a higher GFP yield of 27±5 mg g^{-1} CDW and was similar to the yield 30±5 mg g^{-1} CDW of *E. coli* BL21(DE3) (Waegeman et al. 2013).

Compared to K-12, B strains have many desirable features for recombinant protein production and make them to be the most widely used strains. However, the cell stability and the emergency system sensitivity are weak because of the nonmotile phenotype. Given this, the developing trend of engineering bacteria should combine more advantages form K-12 and B strains. It is also important to develop some new derivatives to meet a variety of production demands. Establishing a platform cell factory that produces more than one product in a batch fermentation will be more competitive for a company.

Stress factor application

The culture medium for recombinant protein production is significantly different from the cells natural inhabiting environment, resulting in cell physiological deterioration and growth inhibition. A review has focused on the recent biotechnological advancements in cells physiological engineering to improve recombinant protein production. It reviews all the potential factors that induce the cell physiological stresses and elaborates several *E. coli* defense systems for these adverse conditions (Chou 2007). This paper provides another strategy to enhance the recombinant protein production by coexpressing the stress factors.

Dps is a nonspecific DNA-binding protein which belongs to the family of the stress proteins. It is an important component of the nucleoid when cells grow in the stationary phase (Azam et al. 1999). Dps protects DNA from the damages of oxygen radical and acetic stress by forming extremely stable complexes with DNA. The combination has no need of specific DNA sequences (Azam et al. 1999; Zhao et al. 2002). It was demonstrated that coexpression of Dps increased the yield of target protein Polh/GFP about 2.5-fold (Kim et al. 2003). Findings from this study provided strong evidence that stress proteins had be successfully used for recombinant protein production.

It is a common problem that many proteins with multiple disulfide bonds fail to fold into their native conformation and are easy to accumulate as inclusion bodies. Chaperones and foldases are the major classes of the folding accessory factors in *E. coli*. Some of them have been proved to promote the correct folding of proteins (Hartl and Hayer-Hartl 2002; Walter and Buchner 2002). A report has elucidated the effects of coexpressing some folding accessory factors, including DnaK, DnaJ, GrpE, TF, GroEL/ES, DsbABCD, and Trx. The productivity of horseradish peroxidase (HRP) is significantly enhanced, and the cell growth inhibition is relieved by coexpressing Dsb (Kondo et al. 2000). In the production of proteins with several disulfide bonds, Dsb can effectively facilitate the formation of correct disulfide bonds and the formation of active proteins (Kurokawa et al. 2000).

However, the limitation of suitable technologies for monitoring cell physiology during the process of cultivation is the major obstacle. It is hoped that the stress factors will soon be applied to improve recombinant protein production with more integrative information based upon the development of systems biology, metabolomics, genetics, and proteomics.

Carbon flux regulation

Carbon flux regulation is an effective approach for improving the carbon source utilization and recombinant protein production by redirecting the carbon flux to the desirable pathways. Four methods that can regulate the carbon flux are discussed in this part, such as the modifications of global regulators and noncoding small RNAs, the inhibition of acetate accumulation, and the applications of anaplerotic reactions.

Genetic modifications of global regulators

The transcription and translation of genetic information are regulated by multiple mechanisms at different levels, such as transcription level, posttranscription level, and translation level. It is generally considered that transcriptional regulation is the dominant mode in *E. coli* genes expression regulation. Researchers have found many important regulators and elucidated their effects in the regulatory network, such as Mlc, ArcA, ArcB, IclR, Fnr, and Cra. A global regulator usually controls several operons that belong to different groups and exhibit pleiotropic phenotypes (Gottesman 1984; Perrenoud and Sauer 2005). Modifications of a global regulator show different variations in metabolic pathways and the carbon flux. Based on this, genetic modification of a global regulator is a simple and effective way to improve recombinant protein production in *E. coli*.

Mlc (encoded by *mlc*) is a global regulator of carbohydrate metabolism (Decker et al. 1998; Kimata et al. 1998). Mlc represses the expression of phosphoenolpyruvate:sugar phosphotransferase system (PTS) operon related to several sugars uptake and utilization (Kim 1999; Kimata et al. 1998). However, the intracellular concentration of Mlc is limited due to its autoregulation and a low efficiency of *mlc* translation (Hosono et al. 1995; Nam et al. 2001). Mlc-overproducing mutants were constructed by site-directed mutagenesis and one of the mutants SR754 exhibited lower glucose consumption rate and acetate accumulation rate than its wild strain MC4100. In addition, the mutant SR754 also improved the GFP production about ten times and showed four times more β-galactosidase activity compared with MC4110 (Cho et al. 2005). The overexpression of *mlc* strengthens the inhibition of sugar uptake and utilization. It is a useful way to reduce acetate accumulation and improve recombinant protein production in metabolic engineering.

The members of Arc system, ArcA and ArcB are also the global regulators that have been widely used in metabolic engineering. Knockout of *arcA* gene reduced acetate yield by 44 % and improved β-galactosidase yield by 30 % compared to the control strain (Vemuri et al. 2006b). Other researchers have explained these results from the changes of genes expression and enzymes activity. They investigated the effects of deleting *arcA* and/or *arcB* on genes expression and enzymes activity of TCA cycle and glyoxylate shunt (Nizam et al. 2009). In the *arcB* mutant, TCA cycle genes such as *gitA*, *icdA*, *fumA*, *mdh*, and glyoxylate shunt gene *aceA* were upregulated. The activity of ICDH and MDH were 1.5- and 5.6-fold higher than the parent strain *E. coli* BW25113. The effects were more obvious by further deleting *arcA* (Nizam et al. 2009). Since TCA cycle is an important provider of energy and precursors, while glyoxylate shunt is the main pathway of acetate-utilizing, the upregulation of many genes expression in these two pathways leads to the higher recombinant protein production and lower acetate accumulation. It is worth mentioned that the redox ratio of NADH/NAD has a strong link with acetate overflow and recombinant protein production (Vemuri et al. 2006a). The acetate overflow will occur when the crucial redox ratio surpass 0.06. Overexpression of the heterologous NADH oxidase (NOX) from *Streptococcus pneumonia* reduced the redox ratio and eliminated acetate overflow. β-Galactosidase production was improved by 120 % when NOX was overexpressed in *arcA* mutant (Vemuri et al. 2006b). Adding nicotinic acid was another way to reduce the ratio of NADH/NAD, and it also increased the enzyme activity of TCA cycle, such as CS, ICDH, and MDH (Nizam et al. 2009). Compared to the two-component Arc system, Fnr is a one-component global regulator and belongs to the family of CRP-related regulators. Fnr also controls some TCA cycle gene expression under anaerobic condition with the similar functions to the Arc system (Körner et al. 2003; Perrenoud and Sauer 2005). It will be a potential global regulator in recombinant protein production under anaerobic condition.

The global regulator Cra (also called FruR) influences on distribution of the carbon flux (Ramseier 1996). The regulatory effects of Cra are mostly opposite to that of ArcA/B. Inactivation of Cra, Entner Doudoroff (ED) pathway and Pentose Phosphate pathway are upregulated. Glyoxylate shunt and TCA cycle are downregulated (Sarkar and Shimizu 2008; Sarkar et al. 2008). These changes lead to increases in the specific glucose uptake rate and the acetate production rate for the Cra mutant as compared to the parent strain. 6-Phosphogluconate dehydratase (Edd) is the key enzyme in ED pathway which catalyzes a dehydration of 6-phosphogluconate to form KDPG. Further deleting *edd* gene may avoid large carbon flux rerouting through inefficient ED pathway. Unfortunately, the acetate of the double mutant is overflowed as well as the case of the single *cra* mutant. However, the *cra*, *edd*, *iclR* triple mutant BW25113 shows the highest cell yield and the least acetate production (Sarkar et al. 2008). Knockout of *iclR* can the glyoxylate shunt and reduce acetate production. It is also demonstrated that inactivation of *c* activates *ra* significantly alleviates growth inhibition from the burden of maintaining a plasmid (Ow et al. 2007). The plasmid-bearing *cra* knockout mutant (P+*cra*) has a higher specific growth rate of 0.92 h^{-1} compared to the plasmid-bearing wild strain (P+WT) of 0.75 h^{-1} (Ow et al. 2007).

The global transcriptional regulators have been widely used for improving the recombinant protein production or alleviating the growth retardation from the metabolic burden. However, the effects of these regulators usually involve in many metabolic pathways and exhibit pleiotropic phenotypes. It is very difficult to aim at a single pathway or a certain gene by modifying the global regulators. The results will be more complex and unpredictable when the several global regulators are combined modified. In order to have the better industrial applications, we also need deeper researches on their regulatory mechanisms and metabolic effects.

Applications of noncoding small RNAs

The noncoding small RNAs have been attracted increasing attentions in recent years. More than 70 noncoding small RNAs have been identified with different length of nucleotides. The noncoding small RNAs regulate their target genes expression at posttranscription level (Vanderpool and Gottesman 2004). They can affect the stability and translation of mRNAs through forming base pairing with the help of Hfq, an RNA chaperone (Aiba 2007; Waters and Storz 2009). The small RNA SgrS is a member of this group. It is observed that SgrS inhibits the translation of *ptsG* and consequently reduces the concentration of the glucose transporter $EIICB^{glu}$.

EIICB^{glu} is encoded by *ptsG* gene and it is an important component of PTS (Aiba 2007). The concentration of EIICB^{glu} is also regulated by the global regulator Mlc at *ptsG* transcription level (Kimata et al. 1998). However, when cells grow in high glucose concentration, only *E. coli* BL21 shows the change of SgrS gene expression (Negrete et al. 2010). The SgrS expression and glucose transport of *E. coli* K-12 strains are not affected under this condition (Negrete et al. 2010). The expression of SgrS may be an additional mechanism that contributes to the lower acetate accumulation in *E. coli* BL21. Based on the difference of SgrS expression between *E. coli* K-12 and B strains, it will be possible to reduce acetate accumulation in *E. coli* K-12 by overexpressing the small RNA SgrS. Negrete et al. have researched the effects of overexpressing SgrS in *E. coli* K-12 (MG1655 and JM109) strains on cell growth, glucose consumption, and acetate excretion. The cell growth pattern of parental *E. coli* K-12 and SgrS overexpressing *E. coli* K-12 was very similar. The glucose consumption of the SgrS overexpressing strains was slightly slower. However, the acetate excretion was significantly different, there was almost no acetate accumulation in SgrS overexpressing strains (Negrete et al. 2013). These observations opened a new way to reduce the acetate excretion by external controlling the noncoding small RNAs.

Csr (the carbon storage regulator) system has been discovered to regulate over 700 genes expression of *E. coli* at posttranscription level (Timmermans and Van Melderen 2010). CsrA and CsrB are the major components of Csr system. CsrA is a RNA-binding protein that binds to the Shine-Dalgarno (SD) site of its target mRNAs and blocks ribosome binding (Liu and Romeo 1997). CsrB is a 350-nucleotide noncoding small RNA molecule. It antagonizes the effects of CsrA-induced translation inhibition by forming a large globular ribonucleoprotein complex (Liu et al. 1997; Romeo 1998). In fact, the intracellular level of CsrB is the key determinant of CsrA activity in the cell. It is demonstrated that overexpression of CsrB can improve the desirable compounds production and decrease acetate formation (McKee et al. 2012). Since CsrB extensively exists in most species of prokaryotes, this approach may also be widely used in metabolic engineering.

The noncoding small RNAs regulate genes expression at posttranscription level. Compared to the transcriptional regulators, their regulations may be more purposeful and pertinent. However, researches on genetic modifications of the small RNAs are just emerging. Many attempts are also needed for applying them in metabolic engineering.

Overcoming acetate accumulation

For the carbon flux regulation, another genetic approach is to direct carbon flow to a desirable product or to cut off it to the by-products, especially acetate. An excellent review has described many approaches to retard the acetate excretion in both fermentation process and genetic modifications (Eiteman and Altman 2006). The cells growing on glucose usually undergo a metabolic switch associated with the excretion and assimilation of acetate. Acetate is produced in exponential growth phase by Pta-AckA pathway. It is converted to acetyl-CoA by the catalysis of acetyl-CoA synthetase (Acs) when the cells begin the transition to stationary phase (Kumari et al. 2000). Acs plays a critical role in acetate assimilation and utilizes it to generate ATP and metabolic intermediates via the TCA cycle and the glyoxylate shunt. Acs activates acetate to acetyl-CoA by two enzymatic steps with producing the intermediates of acetyladenylate and pyrophosphate. Compared to Pta-AckA pathway, this reaction is irreversible because the intracellular enzyme pyrophosphatase removes the intermediate pyrophosphate (Fig. 2). For that reason, overexpression of acetyl-CoA synthetase offers a significant strategy to increase acetate assimilation and reduce acetate excretion. It is also a potential tool to redirect more carbon flux toward the desirable pathways and products in metabolic engineering (Lin et al. 2006). The expression of *acs* is closely related to the carbon sources in culture (Kumari et al. 2000; Shin et al. 1997). Cells grow slowly on acetate as the sole carbon and show high *acs* transcription level, whereas cells quickly shut off *acs* transcription when they grow initially on acetate and then expose to glucose. So, *acs* transcription may be induced by acetate. Meanwhile, an article has revealed that the timing and/or magnitude of this induction depend on many factors (Kumari et al. 2000). On the one hand, it is directly related to the carbon regulator cyclic AMP receptor protein (CRP) and the oxygen regulator FNR. On the other hand, it probably indirectly depends on the IclR, its activator FadR, and many genes involved in acetate metabolism, such as *aceA*, *poxB*, *ackA*, and

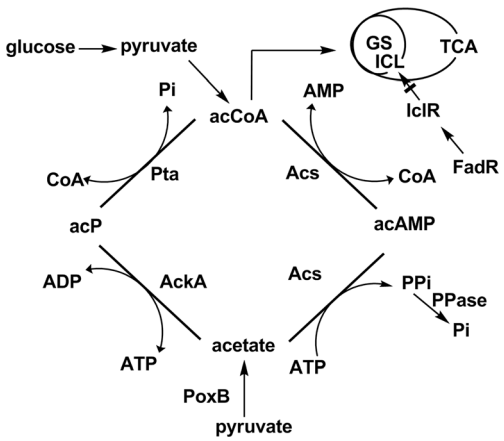

Fig 2 Pathways of acetate synthesis and utilization in *E. coli* (Kumari et al. 2000). *acCoA* acetyl-CoA, *acAMP* acetyl-AMP, *acP* acetyl P, *CoA* coenzyme A, *PPi* pyrophosphate, *Pta* phosphotransacetylase, *AckA* acetate kinase, *Acs* acetyl-CoA synthetase, *PPase* pyrophosphatase, *PoxB* pyruvate oxidase, *GS* glyoxylate shunt, *TCA* tricarboxylic acid cycle, *ICL* isocitrate lyase, *IclR* repressor of the glyoxylate shunt operon aceBAK, *FadR* regulator of fatty acid metabolism that also activates *iclR*

pta. It was found that cells lacking only *ackA* or both *ackA* and *pta* exhibited similar reduction of *acs* transcription. The exogenous addition of acetate to these mutants had no effects on *acs* transcription level (Kumari et al. 2000). IclR probably operates upon *acs* transcription indirectly through controlling the synthesis of glyoxylate shunt enzymes. Thus, it was easy to explain the fact that *acs* transcription pattern of *aceA* mutant was indistinguishable from that of *iclR* and *fadR* mutants. Because isocitrate lyase (encoded by *aceA*) is the first enzyme of glyoxylate shunt and the feedback mechanism to *acs* senses both high and low glyoxylate shunt acitivity (Kumari et al. 2000). Overall, the mechanisms are varied and complex to regulate *acs* expression in *E. coli*.

Based on the above results, there are two important methods to overcome acetate production. Firstly, inactivation of acetate formation genes may be the most simple and effective way to eliminate or reduce acetate accumulation, such as the *poxB*, *ackA*, and *pta* gene. More carbon flux is redirected to the desirable pathways and products. The utilization and conversion of carbon source are significantly improved by overcoming acetate formation. However, the modifications of acetate metabolic pathways usually have negative impacts on cell growth (De Mey et al. 2007). A possible explanation is that ATP is released in the process of acetate production by Pta-AckA pathway. Besides, these perturbations may lead to accumulations of other by-products, such as lactate and pyruvate. Secondly, enhancement of acetate assimilation is an indirect way to overcome acetate accumulation. Acetyl-CoA synthetase (Acs) is the main pathway to absorb acetate and generate ATP and metabolic intermediates via the TCA cycle and the glyoxylate shunt. The *acs* expression is regulated by many factors in *E. coli*. It seemingly includes direct regulation by CRP and FNR to activate transcription initiation and indirect regulation by transcription factors and acetate metabolic genes to control carbon flux. However, these mechanisms are very complex and varied. Compared to these complex regulations in *E. coli*, external overexpression of *acs* may be a simple approach for improving acetate assimilation and reducing acetate accumulation.

Anaplerotic reactions

The expression of foreign genes overconsumes the energy and materials from TCA cycle which leads to surplus of acetyl-CoA. The additional acetyl-CoA usually converts to acetate by the activation of Pta-AckA pathway. In fact, the anaplerotic reaction is an important way to replenish the intermediate metabolites in TCA cycle. Two possible anaplerotic reactions that can be used are the phosphoenolpyruvate (PEP) carboxylase (PPC) and glyoxylate shunt when *E. coli* grows on glucose (Fig. 1). Improving the proportion of carbon flux through these anaplerotic reactions can reduce acetate accumulation and improve protein production. PPC is regulated by many metabolites. It is activated by fructose-1,6-diphosphate (FDP), GTP, acetyl-CoA and guanosine tetraphosphate (ppGpp) and inhibited by malate and aspartate. As mentioned above, the glyoxylate shunt is regulated by many transcriptional regulators, including *iclR*, *arcA/B*, *fruR*, and *fadR*. Previous results showed that acetate yield was below the concentration of 0.2 g/L in the *fadR* mutant with PPC overexpression. Deletion of *fadR* alleviates the inhibition of the glyoxylate shunt and overexpression of PPC replenishes the intermediate metabolite oxaloacetate. However, there was no significant difference in biomass between the control and the mutant strain (Farmer and Liao 1997). Unfortunately, PEP is the main phosphate group donor for the PEP phosphotransferase system in glucose transport. Overexpressing PPC diminishes the supply of PEP needed for the PTS-mediated glucose transport and decreases the growth rate both in aerobic and anaerobic conditions (Chao and Liao 1993; Gokarn et al. 2000). Besides PPC and the glyoxylate shunt, pyruvate carboxylase (PYC) is another anaplerotic pathway which catalyzes pyruvate to oxaloacetate. PYC usually presents in some prokaryotes but is not found in *E. coli* (Attwood 1995). In batch fermentations, cells containing pyruvate carboxylase from *Rhizobium etli* (*R. etli*) significantly increased biomass by 41 %, the production of β-galactosidase was increased by 68 %, and the concentration of acetate was decreased by 57 %. The presence of pyruvate carboxylase allows the cells to use glucose more efficiently and redirects more carbon flux toward biomass and protein formation and away from acetate secretion (March et al. 2002). In addition, L-aspartate ammonialyase (aspartase) is an extra supply of intermediate metabolites in TCA cycle. The physiological effect of aspartase catalyzes the deamination of L-aspartic acid to produce fumarate. When cells grew in glucose minimal medium containing aspartate, the aspartase-producing strain improved the production of green fluorescent protein and β-galactosidase by 5-fold (Wang et al. 2006). It is a potential way to improve the recombinant protein production with coexpressing an enzyme that converts its substrate to the intermediate metabolite in the TCA cycle.

Comparing all these anaplerotic reactions, PPC and glyoxylate shunt are the most important anaplerotic reactions to replenish intermediate metabolites in TCA cycle. The two reactions are regulated by many metabolites and transcriptional factors, respectively. PYC is not a native enzyme of *E. coli* and the gene *pyc* is usually introduced from other prokaryotic organisms. The activity of PYC is to convert pyruvate to oxaloacetate at the expense of ATP. Overexpression of *pyc* allows *E. coli* to use glucose more effectively, an effect same as that by *ppc*. However, PEP involves in PTS-mediated glucose transport, overexpression of *ppc* has negative effects on glucose transport and cell growth. The aspartase-mediated anaplerotic reaction can greatly improve the recombinant protein production without impairing cell growth. The reasonable explanation is that this reaction can provide an additional

carbon source except glucose for use in *E. coli*. The augment of the carbon flux in TCA cycle ensures the requirement for cell growth. In industrial applications, the relationship between the cost of the substrate and the resulting profit must be taken into consideration.

Conclusion

Escherichia coli is a good host for recombinant protein production because of its fast growth, low cost, and easy manipulation. However, obstacles also exist in both academic researches and industrial applications. Firstly, *E. coli* contains different types of strains. It is the first key point to choose a suitable one for recombinant protein production. In general, it all depends on the characteristics of the strains and the target proteins. Secondly, the cells physiological deterioration and enzymes inactivation may be triggered when the medium is significantly different from the cell's natural environment. Coexpression of the stress factors can help proteins to fold into their native conformation. Lastly, carbon waste and by-products formation are the two problems that cannot be ignored. Previous researchers have put forward to some approaches to redirect more carbon flux toward the desirable

Table 1 Metabolic engineering methods applied to improve recombinant proteins production and reduce acetate accumulation in *Escherichia coli* bioprocesses

Methods	Descriptions	References
Workhorse selection	Depend on characteristics of the strains and the proteins	
Knockout of *iclR*, *arcA*, *lon*, and *ompT* genes in *E.coli* K-12 strains	Prevented protein degradation, showed the similar GFP yield and acetate accumulation to that of *E. coli* BL21(DE3)	(Waegeman et al. 2011)
Stress factors application		
Coexpression of the stress factors	Helped proteins fold into their native conformation, improved target proteins production	(Kim et al. 2003; Kondo et al. 2000)
Carbon flux regulation	Redirect more carbon flux towards the desirable pathways and products	
Global regulators	Exhibit pleiotropic phenotype, regulate genes expression at transcription level	
Overexpression of *mlc*	The GFP and β-galactosidase yield increased about ten times and four times, respectively.	(Cho et al. 2005)
Knockout of *arcA* and/or *arcB*	Improved the activity of TCA cycle and glyoxylate shunt, reduced acetate yield, and increased proteins production	(Nizam et al. 2009; Vemuri et al. 2006b)
Knockout of *fnr*	Controlled some TCA cycle gene expression under anaerobic condition, had the similar regulatory function to *arcA*/*arcB*	(Körner et al. 2003)
Knockout of *Cra*	Alleviated growth retardation from the introduction of plasmids	(Ow et al. 2007)
Noncoding small RNAs	Regulate genes expression at posttranscription level	
Overexpression of SgrS	The cell growth and glucose consumption was not affected and had no acetate accumulation.	(Negrete et al. 2010)
Overexpression of CsrB	Improved the production of fatty acid, 1-butanol, and amorphadiene and decreased acetate formation.	(McKee et al. 2012)
Overcome acetate accumulation	Improve the carbon utilization	
Overexpression of *acs*	Increased acetate assimilation	(Lin et al. 2006)
Knockout of acetate-formation-related genes	Eliminated or reduced acetate formation	(De Mey et al. 2007)
Anaplerotic reactions	Replenish intermediate metabolites of TCA cycle	
Overexpression of *ppc* in *fadR* strain	Acetate yield was reduced more than fourfold, the cell growth and glucose transport were diminished.	(Chao and Liao 1993; Gokarn et al. 2000)
Heterogeneous expression of *pyc*	The cell mass was increased by 41 %; the production of model protein was increased by 68 %; the acetate concentration was decreased by 57 %.	(March et al. 2002)
Coexpression of aspartase with adding its substrate Asp into the medium	The yield of GFP and β-galactosidase was increased 5-fold	(Wang et al. 2006)

pathways and products. Global regulators often involve in many metabolic pathways and exhibit pleiotropic phenotypes. They regulate genes expression at the transcription level. Modification of a global regulator results in alternations of many genes and pathways related to the carbon metabolism. The noncoding small RNAs are another kind of regulators that affect the stability of their target mRNA at posttranscription level. It is not common to improve the recombinant protein production by modifying the noncoding small RNAs. The observations of SgrS and CsrB open a new way to reduce the acetate excretion and improve recombinant protein production through the noncoding small RNAs. Beyond that, inactivation of acetate formation genes is a direct way to reduce acetate accumulation. Anaplerotic reactions can replenish intermediate metabolites in TCA cycle and improve the carbon source utilization. In summary, this article mainly reviews recent approaches for improving recombinant protein production in metabolic engineering (Table 1). However, some undesirable consequences are usually found in genetic modifications, such as glucose transport inhibition, cell growth retardation, and useless metabolite accumulation. More efficient regulators and platform cell factories should be explored to meet a variety of production demands.

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