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MINI-REVIEW

Engineering cell physiology to enhance recombinant protein production in *Escherichia coli*

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Abstract The advent of recombinant DNA technology has revolutionized the strategies for protein production. Due to the well-characterized genome and a variety of mature tools available for genetic manipulation, *Escherichia coli* is still the most common workhorse for recombinant protein production. However, the culture for industrial applications often presents *E. coli* cells with a growth condition that is significantly different from their natural inhabiting environment in the gastrointestinal tract, resulting in deterioration in cell physiology and limitation in cell's productivity. It has been recognized that innovative design of genetically engineered strains can highly increase the bioprocess yield with minimum investment on the capital and operating costs. Nevertheless, most of these genetic manipulations, by which traits are implanted into the workhorse through recombinant DNA technology, for enhancing recombinant protein productivity often translate into the challenges that deteriorate cell physiology or even jeopardize cell survival. An in-depth understanding of these challenges and their corresponding cellular response at the molecular level becomes crucial for developing superior strains that are more physiologically adaptive to the production environment to improve culture productivity. With the accumulated knowledge in cell physiology, whose importance to gene overexpression was to some extent undervalued previously, this review is intended to focus on the recent biotechnological advancement in engineering cell physiology to enhance recombinant protein production in *E. coli*.

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Introduction

With many attractive features of the *Escherichia coli* gene expression system (Terpe 2006), the existing bioprocesses for recombinant protein production are still dominated by the use of this workhorse. Its importance is also recognized for biopharmaceutical production, which typically has several options in selecting the expression system (Andersen and Krummen 2002; Schmidt 2004), given the fact that 9 out of the 31 therapeutic proteins approved during 2003 and 2006 are produced in *E. coli*, secondary to the mammalian cell systems (Walsh 2006). In *E. coli*, the expressed foreign gene products can be located in various intracellular compartments, including the cytoplasm, inner membrane, periplasm, and outer member (Cornelis 2000; Lee et al. 2003), or can be secreted extracellularly (Choi and Lee 2004; Georgiou and Segatori 2005). Several excellent reviews have been published to address various technical issues in recombinant protein production. These proposed biochemical, genetic, and metabolic engineering strategies are primarily developed for high-level gene expression (Baneyx 1999; Baneyx and Mujacic 2004; Hartley 2006; Makrides 1996; Sorensen and Mortensen 2005; Swartz 2001; Weickert et al. 1996) and high-cell-density cultivation (Choi et al. 2006; Lee 1996; Riesenberg and Guthke 1999; Shiloach and Fass 2005). To optimize the culture performance, the two goals are supposed to be achieved simultaneously. However, high-level gene expression can make cells physiologically ill and stress-sensitive. The overexpressed foreign gene products can challenge cells

with different levels of toxicity and metabolic burden. On the other hand, due to the limitation in nutrient and/or oxygen availability, cells in dense cultures often have lower cellular activity and metabolic energy that are required for effective biosynthesis. A compromise in balancing the levels of gene expression and cell growth needs to be reached at a certain point to maximize the volumetric recombinant protein productivity. With the inherent adaptability to survive in an adverse environment, *E. coli* can overcome the above challenges by triggering its natural defense systems in response to various physiological stresses (Boor 2006). This review is primarily focused on technical issues relevant to engineering cell physiology for enhancing recombinant protein production. We first review all the potential factors that induce physiological stresses and result in physiological deterioration during high-level recombinant protein production in dense *E. coli* cultures. Then, we review several *E. coli* defense systems that are stress-responsive, i.e., stress regulons, and their potential applicability in stress monitoring and suppression. Finally, we review various strategies that have been successfully applied for the construction of genetically engineered strains with improved cell physiology and enhanced recombinant protein productivity.

Table 1 Summary of various factors negatively affecting cell physiology during high-level recombinant protein production in dense *E. coli* cultures

Factors	Physiological effects
Intracellular	
Presence of multicopy expression vectors	Cause metabolic burden and inhibit cell growth
Toxicity of gene products	Inhibit cell growth or even cause cell death
High-level gene expression	Cause metabolic burden, heat-shock stress, starvation, and disintegrated membrane structure
Protein misfolding	Cytoplasmic or extracytoplasmic stress can be induced by misfolding of the target gene products and/or housekeeping proteins
Extracellular	
Accumulation of toxic wastes or metabolites	Secretion of metabolites, in particular, acetic acid, that are caused by ineffective metabolism and/or gene overexpression can potentially inhibit cell growth and gene expression
Nutrient-limiting	Reduce cellular activity and cause starvation
Oxygen-limiting	Cause anaerobiosis and inhibit cell growth
Presence of inhabitants	Inhibit cell growth

Potential factors to induce physiological stress

Potential factors that negatively affect cell physiology during high-level recombinant protein production in dense *E. coli* cultures are listed in Table 1. These intracellular and extracellular factors are respectively associated with high-level gene expression and high-cell-density cultivation though some of them, such as acetate overaccumulation, could result from both.

Gene overexpression

Both the accumulated foreign gene products and the environmental impact associated with high-level gene expression can present cells with significant physiological challenges. The stress often occurs in the local compartment where recombinant proteins reside. The cytoplasm is the compartment where all recombinant proteins are first synthesized and are possibly located as the final expression destination. Some recombinant proteins are exported into the extracytoplasmic compartment for various applications (Cornelis 2000). Periplasm has several advantages for expressing eukaryotic proteins because of its oxidative environment suitable for disulfide bond formation and the feasibility of obtaining proteins with authentic N termini. This compartment also contains a less amount of total proteins that are isolated from the cytoplasm so that downstream purification would be facilitated. To purify recombinant proteins in the periplasm, the outer membrane is selectively disintegrated without damaging the inner membrane and, therefore, a stable inner membrane is often required during the cultivation for recombinant protein production. It has been reported that several factors, such as growth rate, medium composition, cultivation mode (i.e., batch, continuous, and fedbatch), and growth phase (i.e., exponential and stationary), will determine the integrity, stability, components, structure, and function of the inner membrane, which in turn influence the translocational efficacy, cell physiology, and recombinant protein productivity (Arneborg et al. 1993; Shokri and Larsson 2004; Shokri et al. 2002). High-level gene expression along with effective translocation can possibly result in periplasmic accumulation of misfolded recombinant and/or housekeeping proteins and deteriorate cell physiology (Pan et al. 2003; Sletta et al. 2004). In the case of cell-surface display, which has been extensively explored for various biotechnological and industrial applications (Benhar 2001; Lee et al. 2003), the host cells often experience a stressful condition because the outer membrane becomes fragile or even disintegrated upon frequent insertion of passenger-carrier fusions (Narayanan and Chou 2007).

Cells experiencing the physiological stress and metabolic burden associated with gene overexpression are subject to growth arrest or even lysis (Bentley et al. 1990; Glick 1995;

Neubauer et al. 2003). In addition to the above membrane-related issues, other factors can contribute to such growth retardation. First, the two primary biological activities in recombinant cells, i.e., cell propagation and expression of foreign gene products, compete the use of various intracellular resources for biosynthesis, such as amino acids, nucleotides, and metabolic energy. These resources are heavily consumed toward heterologous gene expression, particularly for the case of using strong promoter systems to regulate gene overexpression. Cell growth can be limited by the availability of these resources upon induction, a situation known as starvation. Due to the lack of internal reprogramming for adjusting a new balanced growth, cells can respond to the starvation via suicidal behaviors, such as destroying their ribosomes (Dong et al. 1995; Kurland and Dong 1996). It has been reported that growth-arrested cells can experience stasis-induced deterioration of proteins, ineffective peptide chain elongation, protein misfolding, and difficult carbon and nitrogen assimilation (Dukan and Nystrom 1998; Tamarit et al. 1998). Second, cell growth can be inhibited by the toxicity of the foreign gene products (in the form of precursor, intermediate, or mature protein), which accumulate as soluble or insoluble aggregates in various cellular compartments. Physiological stress can arise locally in either the cytoplasm or the periplasm, depending on the location of the accumulated polypeptide species. Third, the presence of plasmids can induce the metabolic burden by altering the cellular regulatory status through a complex interaction between hosts and vectors (Ricci and Hernandez 2000; Rozkov et al. 2004; Wang et al. 2006a). Excessive replication of plasmids after the induction for gene overexpression is observed as another contributing factor to metabolic burden (Grabherr et al. 2002; Hoffmann and Rinas 2001b).

Adverse culture condition

Batch and fedbatch cultivations are the most common industrial operation for recombinant protein production. Successful adaptation of *E. coli* cells to the bioreactor culture environment for both cell survival and protein overproduction relies on precise physiological sensing and cellular response. To enhance recombinant protein production, gene overexpression is often conducted in couple with active cell growth and, therefore, an induction under a slow growth rate should be avoided (Shokri and Larsson 2004). In batch cultures, cells experience in series the exponential phase, in which oxygen is fast consumed and acetate might be excreted due to the active growth, and the stationary phase, in which cell growth is ceased, total oxygen requirement is reduced, and nutrients become limiting. Recombinant proteins are preferably synthesized in the exponential phase because the expression of foreign gene products requires a threshold level of cellular activity and metabolic energy, of which cells are often lacking in the stationary phase. Upon the transition into the stationary phase, cell growth becomes arrested or even ceased and protein synthesis is inhibited as a result. Also, the stationary-phase cultures present physiological challenges to cells due to nutrient and possibly oxygen limitations. The reduced gene expression level in the absence of active cell growth has been well documented (Khmel 2005) and this becomes a serious issue limiting recombinant protein production because a decent cell growth rate appears to be critical for effective expression of the foreign gene products.

Fedbatch operation is a common industrial approach for achieving high-cell-density cultivation. Similar to the stationary phase in batch cultures, fedbatch cultures are often operated under a nutrient-limiting environment with a relatively slow growth. Such a slow growth rate can induce general stress responses normally observed in the stationary-phase cultures (Ihssen and Egli 2004). It has been reported that starvation in a variety of nutrients has its physiological implications for *E. coli* cells. For example, the level of several defense proteins responding to oxidative stress increases and these oxidative stress proteins prevent stasis-related accumulation of oxidative damage in growth arrested cells (Nystrom 1999). Also, the outer membrane permeability is adapted to overcome the challenge from starvation (Ferenci 1999). The stressful condition can result in an increase of the outer membrane permeability and, therefore, several periplasmic proteins can be secreted extracellularly in high-cell-density cultures (Rinas and Hoffmann 2004). Note that the stress response and the membrane structure/integrity of cells in stationary-phase or fedbatch cultures with a relatively slow grow rate can be to some extent different from those for chemostats in which the grow rate can be regulated in a wider range (Shokri and Larsson 2004; Shokri et al. 2002). Here, we primarily focus on the starvation response for batch (i.e., the stationary phase) or fedbatch cultures that are commonly adopted for industrial applications. Both the limited cell growth and nutrient availability lead to reduced intracellular levels of biosynthetic resources that are heavily required for gene overexpression, including various building blocks of biomolecules, cellular activity, metabolic energy, and protein synthesis machinery, etc. It has been reported that the expression of many genes for amino acid biosynthesis is downregulated with an increased cell density and this starvation-like effect seems to be the major reason for the reduced expression of the foreign gene products in high-cell-density cultures (Yoon et al. 2003). As a result, high-cell-density cultivation and high-level gene expression can hardly be obtained simultaneously. In addition, deterioration of cell physiology in dense cultures can be mediated by

unbalanced carbon (usually glucose) metabolism (i.e., either glucose overfeeding or underfeeding), the presence of inhibitory materials, and the lack of key factors in cultures for biosynthesis. Glucose underfeeding can make cells be exposed to starvation. On the other hand, glucose overfeeding can result in incomplete carbon metabolism and partial diversion of the main carbon flux into acetate formation. Acetate is an organic acid known to be toxic to *E. coli* due to both the pH-based and anion-specific effects for inhibiting biosynthesis (Warnecke and Gill 2005). Acetate overexcretion limiting recombinant protein production occurs frequently in anaerobic high-cell-density or aerobic fast-growing cultures. Because glucose overfeeding and fast growth rate are known to be the primary causes for acetate excretion, cell growth needs to be strictly regulated in dense cultures. In addition, oxygen can easily become limiting because the oxygen demand for a large population of actively growing cells in fedbatch cultures is often high. The oxygen limitation results in anaerobiosis of the culture and can affect cell physiology via the accumulation of acetate (Phue and Shiloach 2005). Though an aerobic environment seems to be preferable for *E. coli* cultivation, acetate excretion can be induced by a fast growth rate in aerobic cultures. Also, the presence of oxygen can oxidize electron carriers to form hydrogen peroxide or superoxide anion (O_2^-), resulting in oxidative stress (Storz and Imlay 1999).

Cellular response to physiological stress

Heat-shock and general stress response

Cells growing in high-cell-density cultures for high-level recombinant protein production are subject to major physiological stresses, including heat shock, starvation, and stationary-phase stress, while other challenges, such as anaerobiosis, oxidative stress, osmotic pressure, phage shock, and pH stress can also exist. The response to physiological stress is induced to alleviate the impact and readjust the metabolism so that cells with physiological improvement can be more adaptive to or defensive from various stressful conditions (Boor 2006; Hoffmann et al. 2002; Hoffmann and Rinas 2004b; Weber et al. 2002). Proteins that are induced in response to physiological stress, known as stress-responsive proteins, are often involved in protecting various macromolecules, in particular, proteins, DNA, and membrane, from damage or in repairing them if they get damaged. Through the binding of the sigma factors recognizing the promoters of target stress-responsive genes to the core RNA polymerase to form functional holoenzymes for selective transcription of these genes, the synthesis of a subgroup of stress-responsive proteins is regulated at the transcriptional level. In *E. coli*, there are six sigma factors (Gruber and Gross 2003), among which only σ^S (RpoS), σ^{32} (σ^H or RpoH), and σ^E (σ^{24} or RpoE) are discussed here for their potential effects in suppression of the physiological stresses associated with recombinant protein production. These regulons have been identified to regulate the expression of subgroups of stress-responsive proteins. The σ^S regulon, interplaying with the σ^{70} (RpoD), is the major one to induce general stress response as cells enter into the stationary phase or encounter the situation that can cause growth arrest, such as starvation, osmotic challenge, and low pH (Hengge-Aronis 1999; Lacour and Landini 2004). To overcome the environmental challenge of the stationary-phase culture, cells become more resistant to stress through various changes in morphology, intracellular contents, gene expression, and metabolism (Hengge-Aronis 1999; Huisman et al. 1996; Makinoshima et al. 2002, 2003). The physiological adaptation process is mediated by triggering the expression of a variety of stationary-phase genes (Ishihama 1997, 1999; Tani et al. 2002). The σ^{32} regulon is responsible for triggering the cytoplasmic heat-shock response (Arsene et al. 2000). The partially overlapping regulons of σ^E and Cpx are the major regulons to trigger the extracytoplasmic heat-shock response (Alba and Gross 2004; Dartigalongue et al. 2001; Rizzitello et al. 2001; Ruiz and Silhavy 2005). Many of the heat-shock proteins carry the protease activity for degrading misfolded proteins and/or the chaperone activity for preventing protein misfolding or renaturing misfolded proteins (Hoffmann and Rinas 2004a). DnaK/J-GrpE and GroEL/ES are the major cytoplasmic chaperone systems, whereas Lon, ClpP, and FtsH are the major cytoplasmic proteases. Both of the two groups of proteins are induced via the σ^{32} pathway in response to the heat shock (Arsene et al. 2000). DegP and FkpA are the major periplasmic chaperones induced via the σ^E regulon, although DegP, with both protease and chaperone activities (Krojer et al. 2002), can be also induced via the Cpx pathway in response to the extracytoplasmic stress (Connolly et al. 1997). The stress regulon systems can be partially overlapped, namely, the same stress-responsive proteins can be induced by different stress sources or via different regulons (Wade et al. 2006). Also, one type of stress can possibly activate multiple regulons simultaneously. Recent biotechnological development in transcriptomic (Dharmadi and Gonzalez 2004) and proteomic analyses (Han and Lee 2006) offers an effective tool for systematic identification of the stress-responsive proteins and characterization of the response mechanism (Durrschmid et al. 2003; Rhodius et al. 2006; Weber et al. 2005; Zheng et al. 2001). Also, precise prediction of the stress-responsive promoters recognized by the sigma factor has been demonstrated (Rhodius et al. 2006).

Stress response via the above three regulons can be induced as a result of recombinant protein production.

High-level gene expression can induce the heat-shock response via the σ^{32} (for cytoplasmic stress) or σ^{E} (for extracytoplasmic stress) pathway (Hoffmann and Rinas 2001a). On the other hand, the stressful condition in high-cell-density (e.g., fedbatch) cultures, such as nutrient-limiting and slow growth, is similar to that in stationary-phase cultures and can induce the general stress response via the σ^{S} or σ^{E} pathway (Yoon et al. 2003). It has been reported that the slow growth rate, instead of high cell density or the nature of growth-limiting nutrients, controls the σ^{S}-related general stress response (Ihssen and Egli 2004). While the heat-shock response via the σ^{32} or σ^{E} pathway is often induced by high-level gene expression, the general stress response via the σ^{S} pathway can be suppressed under the same stressful condition (Schweder et al. 2002). Also, the stationary-phase culture conditions can induce the heat-shock-like response that is σ^{32}-independent (Diaz-Acosta et al. 2006). In addition to the role in response to the extracytoplasmic stress in *E. coli*, the σ^{E} regulon has been shown to have a physiological function associated with dead-cell lysis in the early stationary phase (Nitta et al. 2000). Other pathways leading to the stress response associated with recombinant protein production, such as phage-shock response (Aldor et al. 2005; Haddadin and Harcum 2005), oxidative stress response (Ritz et al. 2000), pH stress response (Tucker et al. 2002), and the response in which small heat-shock proteins are involved (LeThanh et al. 2005), have also been reported.

Stress monitoring

A thorough understanding of physiological stress and its corresponding cellular response will offer more insight to improve cell physiology for industrial applications. Nevertheless, the physiological improvement cannot be demonstrated without cell physiology being properly monitored. In the research area of recombinant protein production, cell physiology is recognized as a vague term generally in correlation with cell's health, viability, metabolic activity, and protein-producing ability, etc., although quantitative definition of cell physiology has been explored (Konstantinov 1996). As stated above, various stress-responsive regulons have been identified as the cell's natural defense system in response to physiological stress through the synthesis of a subset of stress-responsive proteins expressing the protease and/or the chaperone activities. As a practical application, these stress-responsive proteins can be used not only as "sensors" for monitoring cell physiology but also proper candidates to suppress physiological stress. To facilitate physiological monitoring, various strains containing a fusion allele of a stress-responsive promoter and a reporter gene have been constructed (Vollmer and Van Dyk 2004). These stress-responsive reporter systems have acted as in situ "biosensors" in various biotechnological applications, such as process monitoring in bioremediation (Hazen and Stahl 2006), toxicity measurement (Sorensen et al. 2006), investigation of the genotoxicity mechanism (Aubrecht and Caba 2005), in vivo protein aggregation (Schultz et al. 2006), and oxidative stress measurement (Lee and Gu 2003). The applicability of these approaches primarily relies on a successful search of the stress-responsive genes whose promoter activity can properly represent the cell physiology. Other techniques based upon instrumental measurement of the key culture variables are also adopted for physiological monitoring during recombinant protein production though their practical applicability remains to be justified (Bachinger et al. 2001; Borth et al. 1998; Bunin et al. 1996; Cserjan-Puschmann et al. 1999; Hoffmann and Rinas 2001a; Hoffmann et al. 2000; Ignatova 2005; Lewis et al. 2004; Lin et al. 2004; Looser et al. 2005).

Physiological improvement to enhance culture performance

Because the overproduction of heterologous gene products can result in metabolic burden and physiological stress, which in turn limit recombinant protein productivity, strategies have been developed for alleviation of these negative factors to restore cell physiology. Genetic and metabolic strategies to develop superior host/vector systems to enhance recombinant protein production have been widely explored by coexpression or knockout of certain key gene(s). Proper identification of the key gene(s) affecting cell physiology under various stressful conditions becomes critical for physiological improvement. While individual genes have been constantly searched in many physiological studies, recent progress in transcriptomic and proteomic analyses offer a systematic approach for fast identification of tens or hundreds of genes/proteins that are stress-responsive, either upregulated or downregulated (Aldor et al. 2005; Durrschmid et al. 2003; Franchini and Egli 2006; Haddadin and Harcum 2005; Raman et al. 2005; Weber et al. 2005; Yoon et al. 2003). However, rational selection of the proper gene(s) among these candidates for engineering cell physiology is still challenging. Cell physiology is usually engineered by tuning the expression dosage of this key gene(s) through gene knockout for downregulation or heterologous expression for upregulation. Expression vectors for flexible coexpression of multiple genes have been developed (Tolia and Joshua-Tor 2006). Downregulation can also be fine-tuned with antisense for gene silencing (Kemmer and Neubauer 2006; Kim and Cha 2003). These strategies are developed for mimicking or even boosting the natural response for suppression of the physiological stresses arising from

recombinant protein production. Potential factors that could be manipulated to improve cell physiology during high-level recombinant protein production in dense *E. coli* cultures are listed in Table 2.

Manipulation of stress-responsive genes

Because the heat-shock proteins are natural defense proteins frequently with protease and/or chaperone activities that are upregulated for repairing damaged proteins under stressful conditions, they are generally considered as the candidate for upregulation to improve recombinant protein production. Coexpression of chaperones, either cytoplasmic or periplasmic, has been applied to improve the folding of many foreign gene products, which are otherwise difficult to be expressed solubly (see below). Coexpression of proteases that do not selectively attack the desirable gene products can be effective in improving cell physiology, presumably by degrading the misfolded proteins with a certain level of toxicity (Kadokura et al. 2001; Pan et al. 2003). Coexpression of the heat-shock sigma factors, i.e., σ^{32} or σ^{E}, can stimulate the production of the heat-shock proteins to improve cell physiology (Thomas and Baneyx 1996). Coexpression of the small heat-shock proteins of IbpA and IbpB can reduce the physiological stress associated with protein misfolding via attenuating the accumulation of stress proteins (LeThanh et al. 2005). Several chemicals, e.g., benzyl alcohol or ethanol, are known to be able to induce the heat-shock response and supplementation of these chemicals in cultivation media has a folding-improving effect similar to chaperone coexpression (de Marco et al. 2005; Thomas and Baneyx 1996). Heat-shock response can be indirectly induced to suppress the toxicity of recombinant proteins by coexpression of a key gene product that activates the stress regulon (Snyder et al. 1995). The above biochemical and genetic strategies can be synergistically combined to optimize these improving effects. Suppression or knockout of the expression of downregulated heat-shock responsive proteins has not been explored yet. On the other hand, because the upregulated proteins in response to the stationary-phase stress are usually the factors leading to reduced cellular and metabolic activities, which negatively affect recombinant protein production, they are considered as the target for suppression to improve the recombinant protein yield although cell physiology might not necessarily be improved (Chou et al. 1996; Jeong et al. 2004). In contrast, the downregulated proteins that are involved in response to the stationary stress or the key proteins that potentially eliminate the stationary response can be the possible target for upregulation to improve the culture performance (Jeong and Lee 2003). Similarly, increasing the expression dosage of downregulated proteins that are involved in the limiting step within a biosynthetic pathway upon gene overexpression appears to be effective to improve recombinant protein productivity (Choi et al. 2003; Han et al. 2003).

Table 2 Summary of various factors that could be manipulated to improve cell physiology during high-level recombinant protein production in dense *E. coli* cultures

Factors	Physiological effects
Stress-responsive genes	Enhance the cellular response for suppressing physiological stresses
Folding effectors	Rescue misfolded proteins
Metabolic factors	Reduce metabolic burden or redirect carbon flux for more effective metabolism
Stationary-phase genes	Reduce stationary-phase responses and increase cellular activity
Others	Heuristic approaches for physiological improvement

Manipulation of folding effectors

The physiological impact arising from the misfolding and aggregation of recombinant proteins and the induced cellular response have been well documented at the molecular level (Villaverde and Carrio 2003). Several folding modulators and heat-shock proteins, including trigger factor, GroEL/ES, and DnaK/J-GrpE in the cytoplasm (Baneyx and Mujacic 2004) and Skp, SurA, DegP, DsbC, and FkpA in the periplasm (Mogensen and Otzen 2005), with foldase, chaperone, and/or *cis–trans* peptidyl-prolyl isomerase activities are involved in protein folding and targeting at various protein formation stages. Strategies based upon the coexpression of these folding accessory proteins have been adopted for effective expression of recombinant proteins via enhancing the target proteins' solubility, structural stability, translocation/secretion efficacy, or even disulfide bond formation (Hayhurst and Harris 1999; Heo et al. 2006; Kurokawa et al. 2000; Narayanan and Chou 2007; Nishihara et al. 2000; Sandee et al. 2005; Xu et al. 2005), and cell physiology is frequently improved as a result. Coexpression of chaperones can be also used to suppress the growth inhibition caused by the expression of toxic gene products (de Marco and De Marco 2004). Synergistic integration of multiple biological functions from these folding helpers to enhance recombinant protein production has been demonstrated (de Marco and De Marco 2004; Nishihara et al. 2000; Schlapschy et al. 2006).

Manipulation of metabolic factors

A technical issue limiting recombinant protein production is the inhibition of cell growth and protein synthesis associated with the extracellular accumulation of acetate, which is

a major fermentation byproduct frequently produced by *E. coli* cells in anaerobic high-cell-density and aerobic fast-growing cultures. Because carbon overfeeding, in particular, glucose, appears to be a major factor resulting in the acetate buildup, genetic strategies, primarily including mutant isolation (Contiero et al. 2000; De Anda et al. 2006; Peng and Shimizu 2006) and coexpression of key exogenous factors (Cho et al. 2005) to reduce the carbon assimilation rate (hence, the cell growth rate) without affecting recombinant protein synthesis ability (Picon et al. 2005), are shown to be effective in improving cell physiology and culture performance. On the other hand, metabolic engineering of cells to redirect or reduce carbon assimilation flux has been extensively explored to reduce acetate accumulation (Farmer and Liao 1997; Gosset 2005; Kim and Cha 2003; Vemuri et al. 2006; Yang et al. 2000; Zhu et al. 2001). Another viewpoint has been proposed that acetate does not inhibit cell growth and protein synthesis and its formation simply represents an inefficient consumption of glucose (March et al. 2002). As such, acetate excretion can be reduced simultaneously with enhanced cell growth and protein synthesis by boosting glucose assimilation through some other metabolic pathways (March et al. 2002). Cell physiology and recombinant protein production can be improved by increasing the availability of NADH, a common energy carrier and cofactor involved in various biosynthesis pathways (San et al. 2002).

Manipulation of stationary-phase genes

Because the expression of the stationary-phase genes is induced for cell's adaptation to the adverse condition in stationary-phase cultures, they are the logical target for tuning cell physiology. The σ^S-dependent stationary-phase genes have recently been characterized in detail (Lacour and Landini 2004). The *rpoS* mutation has been shown to have a positive effect on recombinant protein production in fedbatch cultures with a stationary-like environment (Jeong et al. 2004). The knockout mutant of the *rmf* gene, a stationary-phase gene encoding ribosome modulation factor that is involved in ribosome multimerization, shows a phosphate-starvation-like response in gene expression and is effective as a host for the production of recombinant proteins and metabolites (Imaizumi et al. 2005, 2006). In light of various physiological consequences for cells surviving in stationary-phase cultures that are similar to the natural environment in terms of nutrient limitation/starvation, it is generally recognized that the wild-type strains are not well adapted for many industrial applications. Mutant strains with physiological advantages for recombinant protein production, such as reduced acetate secretion, resistance to various physiological stresses (heat shock, osmotic challenges, and starvation), shorter lag phases, and sustained metabolic activities, can be screened in a lab environment for improving their potential industrial applicability (Adams 2004; Madan et al. 2005; Sonderegger et al. 2005; Weikert et al. 1997, 1998). A typical example with a known linkage is the phenotype of growth advantage in stationary phase, which is associated with the *rpoS819* mutation allele (Zambrano et al. 1993) and several other genetic loci (Zinser and Kolter 1999). However, very limited information regarding the linkage between the above desired phenotypes and their corresponding genotypes is available, making it difficult to develop rational genetic strategies for improving cell physiology (Zinser and Kolter 2004).

Manipulation of other factors

Various other approaches have been applied to alleviate the physiological impact from the toxicity, stress, and biosynthesis limitation associated with recombinant protein production. Promoters with extremely tight regulation to avoid tiny expression leakage in the noninduced condition are used to minimize the toxicity of the foreign gene products (Guzman et al. 1995). Because insoluble protein aggregates are generally recognized to be less toxic in vivo and are easier to harvest compared to their otherwise soluble partners, the foreign gene products can be overexpressed as insoluble inclusion bodies. However, the feasibility of this approach is dependent upon the performance of protein refolding. Another strategy to alleviate the toxicity is extracellular secretion of the foreign gene products through biochemical or genetic permeation of the outer membrane. Because there are two membrane barriers in gram-negative bacteria such as *E. coli*, the approach is limited only to periplasmic proteins. The gene overexpression system with a fine tuning in the expression and translocation efficacies to reduce the recombinant protein toxicity and stress has been applied for practical applications (Sletta et al. 2004; Soriano et al. 2002). Coexpression of the Sec export machinery or the *kil* gene encoding killing protein can increase the intracellular synthesis flux for recombinant protein production via releasing proteins extracellularly (Ignatova et al. 2003). Cell physiology can be affected by the blocking of the twin-arginine translocation (Tat) pathway for protein translocation into the periplasm and coexpression of phage shock protein (PspA), a stress protein in response to the deficiency of the Tat pathway, can relieve this secretion block (DeLisa et al. 2004). Manipulation of the nonstress-responsive genes has also been applied to suppress physiological stress. Coexpression of the bacterial hemoglobin and flavohemoglobin genes can improve cell physiology by alleviating the stress from oxygen limitation (Khosla and Bailey 1988; Khosla et al. 1990) and nitrosative and oxidative challenges (Frey et al.

2002) or reducing the toxicity of recombinant proteins (Chien et al. 2004). Both the glutamate and arginine-decarboxylase acid survival pathways and the concomitant availability of glutamate and arginine during acid challenge are identified to be associated with cell-density-dependent acid survival (Cui et al. 2001). The pH stress can be reduced by heterologous expression of functional arginine kinase from *Limulus polyphemus* (Canonaco et al. 2003). The oxidative stress can be suppressed by coexpression of a eukaryotic glutathione reductase from *Brassica campestris* (Yoon et al. 2005). Coexpression of a metal ion efflux protein from a eubacterial thermophile of *Thermus thermophilus* can improve cell physiology for recombinant protein production by protecting cells from toxic heavy metal ions (Spada et al. 2002). Finally, cell physiology can be improved by eliminating certain biosynthesis limitations associated with gene overexpression. The synthesis of rRNA can be activated by a small DNA bending protein, Fis, to enhance recombinant protein production in fedbatch cultures whose productivity is often limited by the number of available protein synthesis machinery (Richins et al. 1997). Genetic strategies to assist intracellular carbon assimilation, by which the limited building blocks for biosynthesis can be replenished, appear to be effective in alleviating metabolic burden, restoring cell growth, and improving recombinant protein synthesis (Flores et al. 2004; March et al. 2002; Wang et al. 2006b).

Conclusions

Due to the multiplicity of physiological impact arising from high-level gene expression and high-cell-density cultivation, the two production goals can hardly be achieved simultaneously and, therefore, the culture yield becomes limited. Most of the physiological stresses and their corresponding cellular response associated with recombinant protein production have been well characterized. However, the mechanistic link between the stress response and deteriorated culture performance is still not completely understood. Most of the genetic strategies for engineering cell physiology developed so far are still limited to single-gene manipulation and their feasibility appears to be rather target-protein-dependent. Another bioprocess obstacle is the lack of suitable techniques for monitoring cell physiology during the course of cultivation. It is hoped that, with many novel research tools and more integrative information based upon the studies in genomics, proteomics, metabolomics, and systems biology (Gill 2003; Ibarra et al. 2002; Lee et al. 2005; Park et al. 2005), these technical gaps will soon be filled for better understanding, monitoring, or even regulating cell physiology to enhance recombinant protein production in *E. coli*.

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