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Identification of riboflavin: revealing different metabolic characteristics between Escherichia coli BL21(DE3) and MG1655

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One sentence summary: E. coli BL21 was found to be able to generate riboflavin in AM1 medium, which may attributable to the increased anabolism and reduced catabolism of riboflavin.

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ABSTRACT

There are many physiological differences between Escherichia coli B and K-12 strains, owing to their different origins. Deeper insight into the metabolic and regulative mechanisms of these strains will inform improved usage of these industrial workhorses. In the present study, we observed that BL21 fermentation broth gradually turned yellow during cultivation. By spectral analysis and liquid chromatography–mass spectrometry identification, we confirmed for the first time that the yellow substance accumulated in the fermentation broth is riboflavin. Comparing the enzyme sequences involved in riboflavin metabolism between BL21 and MG1655, we identified a site mutation on the 115 residue of bifunctional riboflavin kinase/FMN adenylyltransferase (RibF) in BL21. This His115Leu mutation was found to reduce enzyme activity to 55% of that of MG1655, which is probably one reason for riboflavin accumulation in BL21. Quantitative PCR analysis showed that genes of the entire branch of the riboflavin and FAD biosynthesis pathways in BL21 were up-regulated. Several physiological and metabolic characteristics of BL21 and MG1655 were found to be different, and may also be related to the riboflavin accumulation.

Keywords: riboflavin; Escherichia coli; BL21(DE3); MG1655; metabolic difference

INTRODUCTION

Escherichia coli has been a workhorse, not only in fundamental biological studies but also in various biotechnological applications. Almost all laboratory strains of E. coli are derivatives of non-pathogenic K-12 or B strains. Since two K-12 strains—MG1655 and W3110—are sequenced in 1997 (Blattner et al. 1997; Hayashi et al. 2006), increasing omics analyses (Choi, Lee and Lee 2003; Franchini and Egli 2006; Han and Lee 2006; Nandakumar, Cheung and Marten 2006; Ishii et al. 2007) and in silico metabolic modeling (Covert et al. 2004; Feist et al. 2007) have been performed in K-12. In contrast, there have been few studies for B strains, although they have been widely used for large-scale production of recombinant proteins, ethanol and other biomolecules (Choi, Keum and Lee 2006). Recently, the genome sequences of two B strains, REL606 and BL21(DE3), have been determined. Comparative omics analysis of B and K-12 shows that they are very similar (Schneider et al. 2002) and closely related (Yoon et al. 2012), although the strains show many important behavioral differences in cell metabolism and physiology (Swartz 1996). The B strains have a lower acetate production than K-12.
derivatives. A common explanation for that is the activated glycolate shunt, which is the main pathway for acetate utilization. Deletions of araC and icrR in E. coli MG1655 endowed the host with central metabolic fluxes similar to that of E. coli BL21(DE3) under glucose abundant conditions (Waegeman et al. 2011).

Riboflavin (vitamin B2), the precursor of coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), is an essential component of basic metabolism (Massey 2000). Riboflavin is required for a wide variety of cellular processes and has a key role in the metabolism of fats, ketone bodies, carbohydrates and proteins (Depeint et al. 2006). Many microorganisms including fungi, and also plants, synthesize riboflavin, while humans and animals need external riboflavin supply to avoid symptoms of deficiency (Stahmann, Revuelta and Seulberger 2000).

Riboflavin biosynthesis has been studied in both gram-positive and gram-negative bacteria, such as Bacillus subtilis and E. coli (Richter et al. 1992, 1997). Riboflavin is formed from one GTP molecule and two molecules of ribulose 5-phosphate (Ru-5-P), through a seven-step pathway (Bacher et al. 2000). In B. subtilis, the encoding genes of riboflavin biosynthesis reside within one rib operon (Vitreschak et al. 2002). In contrast, these genes in E. coli do not converge to a single operon but are scattered on the chromosome (Bacher et al. 2001). It has been reported that reducing the enzymatic activity of bifunctional riboflavin kinase/FMN adenylyltransferase (encoded by the ribB gene) could result in riboflavin overproduction in B. subtilis (Mack, van Loon and Hohmann 1998). In E. coli, this enzyme is encoded by ribF, which is an essential gene for survival. By modulating the expression of ribF, riboflavin production was improved in E. coli (Lin et al. 2014). However, the exact regulation mechanism of riboflavin in E. coli has not been revealed.

In the present study, we showed that E. coli BL21 was able to generate riboflavin under normal cultivation conditions. Comparative analysis of E. coli strains BL21 and MG1655 indicated that riboflavin accumulation may be a reason for their metabolic difference. By transcription and enzymatic analysis, we found that the riboflavin accumulation in BL21 is caused by not only a single mutation of ribF but also a certain regulation mechanism which up-regulates the whole FAD synthesis pathway.

**MATERIAL AND METHODS**

**Bacterial strains and plasmids**

The strains of E. coli and plasmids used in the study are summarized in Table 1. *Escherichia coli* DH5α was used as the host for molecular cloning. *Escherichia coli* BL21(DE3) was used as the host for protein expression. Plasmid pET28a was used as the expression vector for the enzyme activity assay of different RibFs. Genomic DNA was isolated from wild-type E. coli MG 1655 and BL21(DE3) with a TIANamp Bacterial DNA kit (Tiangen Biotech). The two ribF genes were respectively amplified from the genome DNA of E. coli MG 1655 and BL21(DE3) using the primer pair PriBF FP (CCTTTGCGGATGATGCTGCGGATGCATACA) and PriBF RP (ACTTCGAGAAGCCCGTTTCAAGC). To construct the expression plasmids, PCR fragments were digested with the restriction enzymes NcoI and Xhol and then ligated into the corresponding sites of pET28a, which was cut with the same restriction enzymes to generate plasmids pRibhmis (ribF from MG1655) and pRibhbis (ribF from BL21(DE3)).

**Table 1.** Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Relevant characteristics</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli K-12 MG1655</td>
<td>F−, λ−, F’ recB-50, rpsL−1</td>
<td>CGSC no: 6300</td>
</tr>
<tr>
<td>E. coli B BL21(DE3)</td>
<td>F− ompT gal dcm lon hisdR (Ru-5-P)−1 Δ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 min5])</td>
<td>TransGen</td>
</tr>
<tr>
<td>pET28a</td>
<td>pMB1 origin, protein expression vector</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>pRibhmis</td>
<td>pET28a containing ribF from BL21</td>
<td>This study</td>
</tr>
<tr>
<td>pRibhmis</td>
<td>pET28a containing ribF from MG1655</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Culture conditions**

LB medium (10 g l−1 tryptone, 5 g l−1 yeast extract and 10 g l−1 NaCl) was used for all DNA manipulations and protein expression. During cultivation and fermentation, AM1 mineral salts medium (Martinez et al. 2007) supplemented with 10 g l−1 glucose was used. Kanamycin (25 mg ml−1) was added to provide selective pressure during cultivation when necessary. To induce expression of plasmid-borne genes, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to cultures, with a final concentration of 0.2 mM.

**Analysis methods during fermentation**

Cell growth was detected by measuring optical density at 600 nm with a spectrophotometer (Thermo Scientific Instruments LLC, USA). To detect the yellow fluorescent substance in the fermentation broth, 2 μl of culture supernatant was spotted onto filter papers and exposed to ultraviolet light, to check for the fluorescence. The excitation–emission spectra were measured with a fluorescence spectrophotometer (RF-5301PC, Shimadzu Corp., Japan).

Glucose and organic acids were determined by high-performance liquid chromatography (HPLC) (Shimadzu) coupled with a refractive index detector (RID-10A), as reported previously (Chen et al. 2011). Samples were filtered through a 0.22 μm syringe filter and analyzed on an HPX-87H column (BioRad, USA) at 65°C with 5 mM H2SO4 as mobile phase and flow rate 0.6 ml min−1. Physiological parameters of specific growth rate, specific glucose uptake rate and specific product secretion rates were determined for the exponentially growing cells, according to methods described by Sauer et al. (1999). Intracellular FAD concentration was measured using an FAD Colorimetric/Fluorometric Assay Kit (Cat# K357-100; BioVision Inc., USA).

**HPLC and LC-MS analysis of riboflavin**

Supernatants were subsequently filtered through a 0.22 μm syringe filter, and a 20 μl aliquot of the filtered solution was injected into an HPLC system (Shimadzu) equipped with a UV-VIS detector (SPD-10A; excitation 470 nm; emission 530 nm). Riboflavin was analyzed on an Agilent ZORBAX SB-C18 column (4.6 mm ID × 250 mm) with 30% (v/v) methanol-0.1% (v/v) acetic acid as a mobile phase and flow rate 0.5 ml min−1.
Riboflavin was qualitatively analyzed by liquid chromatography–mass spectrometry (LC-MS) using a TurboMass Gold mass spectrometer (PerkinElmer Inc., USA). Riboflavin was identified by LC-MS based on retention time and fragmentation patterns of known standards.

**Protein expression of RibF**

*Escherichia coli* BL21(DE3) was transformed with plasmids pRibbhis and pRibRhis, and the resulting strains were aerobically cultivated overnight at 37 °C in LB medium. For shake flask culture, 2 ml of the overnight culture was added to a 300 ml Erlenmeyer flask containing 50 ml LB medium at 37 °C. Gene expression was stimulated by adding 0.2 mM IPTG after the culture had reached an optical density at 600 nm of 0.8–1.0. Flasks were transferred to 16 °C for soluble protein expression. After 20 h aerobic cultivation, cells were harvested by centrifugation at 14 000 × g at 4 °C for 10 min.

**SDS-PAGE analysis**

Harvested cells were washed three times with PBS buffer (pH 7.5) and resuspended in the same buffer, followed by ultrasonication at 4 °C (40% output, 10 min). After centrifugation at 14 000 × g at 4 °C for 10 min, the resulting supernatant was analyzed by 12% (w/v) SDS-PAGE. The gels were stained with Coomassie Brilliant Blue G-250. Protein was estimated by the method of Bradford (Bradford 1976) using a Bio-Rad protein assay and bovine serum albumin as a standard.

**Preparation of enzyme solutions**

All procedures were carried out at 0–4 °C. Cells were washed with cold PBS buffer (pH 7.5). After centrifugation, cells were resuspended in 10–15 ml of the aforementioned buffer solution. After ultrasonication, cells were centrifuged for 20 min to remove cell debris and unbroken cells. The resulting supernatants were collected and used to measure protein content and flavokinase activity. Total protein content was determined using an Easy Protein Quantitative Kit (TransGen Biotech Inc., Beijing, China).

**Enzyme activity assay of RibF**

Flavokinase activity is expressed as nanomoles of FMN formed from riboflavin and ATP. Flavokinase activity was measured in a final volume of 1 ml with 600 μl protein soluble supernatants and 400 μl PBS buffer (pH 7.5) containing 50 mM riboflavin, 3 mM ATP, 15 mM MgCl₂, and 10 mM Na₂SO₄. The mixture was pre-incubated at 37 °C for 5 min, and then the reaction was stopped by heating at 100 °C for 5 min (Nielsen, Rauschenbach and Bacher 1983). Proteins were removed from the substrates and product solution by centrifugation, and supernatant compositions were analyzed by HPLC.

**Quantitative real-time PCR (qRT-PCR)**

The gapA gene (glyceraldehyde-3-phosphate dehydrogenase A), whose expression level is relatively constant, was used as the control gene. After strains were cultivated in a shake flask for 3 h, total cellular RNA was extracted using an RNaseasy minikit (Tiangen). cDNA was generated by reverse transcription PCR using random 6-mers and oligo dT primers with a PrimeScript RT reagent kit (TaKaRa). qRT-PCR was carried out using the LightCycler 480 Real-Time PCR system (Roche) with SYBR Premix ExTaq II (TaKaRa). Measurement of each gene was repeated three times.

**RESULTS**

**Identification of riboflavin in fermentation broth of *E. coli* BL21(DE3)**

During the comparative experiments of strains BL21(DE3) and MG1655, we noticed that the color of the BL21 fermentation broth gradually turned yellow. This phenomenon occurred from the late exponential phase of BL21, whereas it did not arise in MG1655 (see Fig. S1, Supporting Information). The observed yellow substance emitted blue–violet fluorescence when exposed to UV light (Fig. S2, Supporting Information). Thus, we inferred that such obvious color change in the BL21 culture medium was attributable to the accumulation of a fluorescent substance. To identify the yellow substance in the fermentation broth, its excitation–emission spectra were measured with a fluorescence spectrophotometer. The results showed that the BL21 fermentation broth had a strong absorption peak at 350–450 nm and strong emission peak at 450–520 nm, which are proximate to the excitation and emission peaks of vitamin B₂ or riboflavin. MG1655 did not have such obvious peaks in this wavelength range (Fig. S3, Supporting Information). Thus, we preliminarily inferred that the yellow substance in the BL21 fermentation broth was riboflavin.

To verify this, the fermentation broths of both *E. coli* strains were collected and analyzed with HPLC and LC-MS. A peak at 21.37 min was identified in BL21 but not MG1655. This peak was subsequently proven to be riboflavin via LC-MS (Fig. 1). Upon diluting the riboflavin to the same concentration as in the fermentation supernatant, it displayed a similar light yellow color (Fig. S4, Supporting Information). Therefore, we concluded that the fluorescent yellow substance in the BL21 fermentation broth was riboflavin.

**Analysis of relationship between riboflavin accumulation and cell physiology changes**

Growth behavior was compared for BL21 and MG1655. When cultivated in AM1 medium in the presence of 10 g l⁻¹ glucose, both strains showed a similar glucose consumption rate, but the growth rate of MG1655 was 1.5 times faster than that of BL21 (Table 1). MG1655 produced 1.2 g l⁻¹ acetic acid after 14 h cultivation (Fig. 2a). Acetic acid is the main byproduct of MG1655, which is consistent with the literature. However, the acetic acid secretion rate of BL21 was 50% lower than that of MG1655 (Table 2). Additionally, lactic acid was detected in the BL21 culture, which reached its maximum yield of 1.7 g l⁻¹ at 14 h (Fig. 2b). In BL21, riboflavin accumulated together with cell growth (Fig. 2c). Even when cell growth entered the stationary phase, generation of riboflavin continued at a certain rate. In contrast, only a small amount of riboflavin was produced in the exponential phase of MG1655 (Fig. 2c).

In vivo, riboflavin is metabolized into FMN and FAD, which are known as the ligand of pyruvate dehydrogenase, succinate dehydrogenase and other important central metabolic enzymes. To find if riboflavin accumulation also caused the accumulation of FAD, the intracellular concentration of FAD in both MG1655 and BL21 was measured. The result showed that the intracellular FAD concentration in BL21 was about 1.5 times that in MG1655 (Table 3). FAD accumulation may enhance cell respiration, thereby affecting metabolic flux.
Figure 1. Identification of riboflavin by HPLC. (a) Riboflavin standard sample and (b) fermentation broth of BL21 (the arrows point at the peak of riboflavin); (c) mass spectra of the riboflavin standard sample; (d) mass spectra of the identified riboflavin peak in BL21 fermentation broth.

Table 2. Comparison of the physiological parameters between BL21 and MG1655.

<table>
<thead>
<tr>
<th></th>
<th>Growth rate (h⁻¹)</th>
<th>Glucose uptake (mmol g⁻¹ h⁻¹)</th>
<th>Lactate secretion (mmol g⁻¹ h⁻¹)</th>
<th>Acetate secretion (mmol g⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21</td>
<td>0.48 ± 0.01</td>
<td>9.14 ± 0.92</td>
<td>0.84 ± 0.03</td>
<td>2.85 ± 0.13</td>
</tr>
<tr>
<td>MG1655</td>
<td>0.67 ± 0.01</td>
<td>9.68 ± 0.79</td>
<td>0</td>
<td>6.11 ± 0.10</td>
</tr>
</tbody>
</table>

Exploring possible reasons for riboflavin accumulation

To understand the physiological causes for riboflavin accumulation in BL21, we analyzed the transcription of ribF and three genes involved in riboflavin biosynthesis of strains BL21 and MG1655. Of these three, ribB encodes 3,4-dihydroxy-2-butanone-4-phosphate synthase, ribE encodes riboflavin synthase β chain and ribC encodes riboflavin synthase α chain. These three gene products catalyze a three-step reaction of ribose-5-phosphate, thereby yielding riboflavin. Our results showed that the expression level of these genes in BL21 is approximately two-fold higher than that in MG1655 (Fig. 2d). The transcription of ribF in BL21 was also higher than that in MG1655, and comparable to that of genes involved in biosynthesis.

By comparing the protein sequences of the riboflavin synthesis pathways of BL21 and MG1655, we discovered that BL21
underwent a mutation in RibF (Fig. S5). Histidine 115 (polar) mutated to leucine (neutral). Leucine 116 is a conserved amino acid among all flavokinases in different bacteria and is important in catalysis. To compare the enzymatic differences between them, ribF genes from BL21 and MG1655 were cloned into expression vector pET28a. The expression of RibF was confirmed by SDS-PAGE (Fig. 3). The enzymatic analysis indicated that the RibF enzyme specific activity of BL21 was 534.03 nmol h$^{-1}$ mg$^{-1}$ of protein which is only 55% of that in MG1655 (954.19 nmol h$^{-1}$ mg$^{-1}$ of protein). Together with the result from transcription analysis, we suggest that riboflavin accumulation in BL21 occurred most probably because of an up-regulation of FAD synthesis pathway and the reduced RibF enzyme activity.

**DISCUSSION**

Compared with K-12, *E. coli* B strains show a number of phenotypes, such as faster growth in minimal media, lower acetate production and higher expression levels of recombinant proteins. BL21(DE3) is a specifically engineered B descendant harboring the T7 RNA polymerase gene (Studier and Moffatt 1986), and has become the most widely used strain for biotechnological applications. An increasing number of researchers prefer BL21 as the metabolic engineering host for production of chemicals and other biomolecules (Choi, Keum and Lee 2006).
Understanding the regulation mechanism and metabolic differences between B and K-12 strains will inform improved usage of these strains in metabolic engineering. Transcriptome analysis of B and K-12 strains has proven that genes involved in the glyoxylate shunt, TCA cycle, fatty acid, gluconeogenesis and anaerobic pathways are expressed differently (Phue et al. 2007). In the present study, we found that BL21 could accumulate more riboflavin than MG1655. The derivatives of riboflavin, FMN and FAD, which participate in the delivery of hydrogen to the electron transport chain, are important cofactors in cellular respiration. Therefore, they are vital in cell physiology, although they exist in small amounts within cells. To find the reason why BL21 accumulated excessive riboflavin, the gene and protein sequences of the riboflavin synthesis pathways in BL21 and MG1655 were compared, which showed that the bifunctional enzyme RibF in BL21 underwent a His115Leu mutation. We suggest that the inadequate enzyme activity of RibF contributed to the riboflavin accumulation. qPCR analysis showed that riboflavin biosynthetic genes are all up-regulated. Up-regulation of the riboflavin and FAD biosynthesis pathways increased riboflavin and FAD accumulation. Although there is a possibility that the His115Leu mutation is the reason for increased riboflavin, we are inclined to believe that there may be a mechanism up-regulating all genes of the riboflavin pathway and causing accumulation of the two downstream products.

Acid secretion is a severe problem in E. coli fermentation because cells can barely survive at pH lower than 5.5 (Eiteman and Altman 2006). Our study revealed that BL21 secretes less acetic acid, consistent with the literature. Acid overflow is caused by an imbalance in the metabolic flux distribution at a certain metabolic node. In MG1655, the accumulation of acetic acid is caused by an imbalance in an active glycolytic pathway and a less efficient TCA cycle. In BL21, the TCA cycle appears more efficient; therefore, less acetic acid was accumulated (Phue et al. 2005). One theory that can be inferred is that cells can increase flow through the TCA cycle by up-regulating the expression of cofactors matched with enzymes in the TCA cycle, and thereby strengthen cellular respiration. Therefore, the accumulation of riboflavin and FAD is likely to affect cell respiration and be one of the reasons for the metabolic differences between these two strains.

Escherichia coli B strains and derivatives have salient features, such as low acetate secretion, faster growth in minimal media, protease deficiency (saiSree, Reddy and Gowrishankar 2001) and simple cell surfaces (Herrera et al. 2002) that enhance permeability. Therefore, these strains have been used as industrial hosts for protein and biomolecule production. The additional up-regulated riboflavin and FAD biosynthesis pathway in BL21 suggests that this strain may also be used for riboflavin production.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSLE online.

FUNDING

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Conflict of interest. None declared.

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