Curr Microbiol (2012) 64:412–417
DOI 10.1007/s00284-012-0087-6

High Growth Rate Downregulates *fumA* mRNA Transcription but Is Dramatically Compensated by Its mRNA Stability in *Escherichia coli*

Hsiao-Hsien Lin · Ching-Hsueh Lin · Shiaw-Min Hwang · Ching-Ping Tseng

Received: 13 November 2011 / Accepted: 9 January 2012 / Published online: 3 February 2012

Abstract Little is known about the association among the transcription, post-transcription, and protein production of the *fumA* gene. This study demonstrates that increasing growth rate (k) from 0.24/h to 0.96/h causes a marked eightfold reduction in *fumA* transcription as assessed using the β-galactosidase activity from *fumA* promoter fused with a *lacZ* reporter. It was further confirmed using Northern blot analysis. Most interestingly, the FumA protein levels remained unchanged over the growth rate, as indicated by Western blot analysis. Therefore, whether the reduced *fumA* mRNA expression under the high growth rate can be overcome by increasing the stability of the *fumA* mRNA was tested. The half-life of *fumA* mRNA was established to significantly increase by fivefold when the growth rate was increased to 0.96/h. This finding suggests that the cells could turn down the expression of *fumA* mRNA because of increased stability of its mRNA under the high growth rate. This notion indicates that mRNA stability plays an essential role in maintaining a critical cellular level of a given protein when the mRNA transcript is downregulated by a metabolic event.

H.-H. Lin · C.-H. Lin · C.-P. Tseng (✉)
Department of Biological Science and Technology, College of Biological Science and Technology,
National Chiao Tung University, 75 Po-Ai Street,
Hsinchu 30050, Taiwan, ROC
e-mail: cpts@cc.nctu.edu.tw

S.-M. Hwang
Bioresource Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC

Introduction

A recent transcriptomic and proteomic study showed that only 47% protein abundance is determined from the mRNA levels in *Escherichia coli* [10]. In other words, not all the protein abundance is mRNA directed and the mechanism involved in such a relationship is not readily known.

Fumarase (EC 4.2.1.2) is an enzyme that catalyzes the interconversion between fumarate and malate. Although three fumarase genes, *fumA*, *fumB*, and *fumC*, have been reported in *E. coli* [4], FumA is a major form that participates in the tricarboxylic acid (TCA) cycle [17, 20] during both aerobic and anaerobic growth. The *fumA* gene encodes the identical dimers of fumarase with a M_r 60,000 of each [18]. The *fumA* gene expression levels varied over a 20-fold range in response to the oxygen content of the culture medium [14]. Interestingly, the expression is higher in carbon-poor media than that in rich media [14].

Growth rate plays an important role in the transcription of a given mRNA of *E. coli* under continuous culture. High growth rate could either upregulate or downregulate the gene expression by lowering the cAMP, which serves as a transcriptional factor (when bound to cAMP-binding protein) to regulate the promoter activity of a given gene [1, 8, 13]. Whether the growth rate affects *fumA* gene expression, transcription, and protein levels, however, has not been well elucidated. In this study, the transcriptional expression of *fumA* was shown to be conversely by its growth rate ($k = 0.24$/h to 0.96/h), but the final protein abundance can be compensated for by the mRNA stability. The significance and potential application of this finding in pharmaceutical and industrial usages are discussed.

Materials and Methods

Bacterial Strains

Escherichia coli W3110 was used in continuous culture for the study of *fumA* expression. The *fumA–lacZ* fusion strain [14] was kindly provided by Dr. P. Gunsalus of UCLA, Los Angeles, California. The activity of β-galactosidase was measured according to the method described previously [11], with the data represented as the average of at least two or three independent experiments with a variation of no more than 5% from the mean.

Cell Growth

For continuous culture, the growth rate was controlled and carried out in a New Brunswick Bioflo III fermentor (New Brunswick Scientific, Edison, NJ) using a standard procedure previously described [6, 17]. The fermentor was fitted with a 1.5-L vessel and operated at a working volume of 1 L. A modified Vogel–Bonner medium (3.3-mM $MgSO_4$, 10-mM citric acid, 28-mM $NaNH_4HPO_4$, 37-mM K_2HPO_4, casamino acids 0.25 mg/L; pH 6.5) supplemented with glucose (2.25 mM) was used to limit cell growth (i.e., carbon-limited medium). The ventilation was maintained by saturating the culture medium with sterile air at a rate of 2 L/min. To vary the cell growth rate, the medium addition rate was adjusted from 4 to 16 mL/min ($k = 0.24$/h to 0.96/h). When the cells were shifted to a new growth rate, steady state was generally achieved in sixfold of the reactor residence. This was monitored through cell density at OD_{600} nm, which serves as an indicator for cells reaching equilibrium.

For batch culture, the cells were grown in modified Vogel–Bonner medium containing 10-mM glucose or 10-mM sodium acetate at 37°C. For RNA and protein analysis, growing cultures were harvested under the exponential phase at OD_{600} of 0.45–0.50.

Northern Blot Analyses and mRNA Half-life

DNA probe specific to *E. coli fumA* was amplified with fumA-f (5′-GTTACTGACGCCGGGGAAACTG-3′) and fumA-r (5′-CCAAGATTTTGCGCTTCGATCA-3′) primers. Northern blot analysis was performed with 10 μg of total RNA according to the standard protocol as previously described [9]. Briefly, RNA isolation was performed according to the procedures of the TRI reagent-RNA kit (Molecular Research Center, Cincinnati, OH). Total RNA was loaded and separated on 1% agarose–formaldehyde gel for the analysis of *fumA*. The integrity and quality of the RNA samples were confirmed by the presence of 23S and 16S rRNA. Following electrophoresis, the gels containing RNA were transferred to a positively charged nylon membrane (Millipore, Bedford, MA) and fixed by cross-linking the membrane with UV light. The membrane was then hybridized with digoxigenin (DIG)-labeled DNA probes for at least 12 h at 42°C. DNA probes specific to *E. coli fumA* were amplified with dNTPs and DIG-11-dUTP mixture (Roche Applied Science, Mannheim, Germany) similar to that previously described [9]. After hybridization, the membranes were washed and were detected using a chemiluminescent substrate (Roche Applied Science, Indianapolis, IN). The blots were then developed by an exposure to Kodak film. The levels and half-lives of the mRNAs are presented as the mean $\pm$ standard deviation (SD) of the band intensity analyzed by Northern blotting in triplicate, whereas the band intensity was determined via ZERO-Dscan Image Analysis System (Scanalytics, Billerica, MA). The half-life of the mRNA was determined by the initiation of mRNA transcription that was inhibited by the addition of rifampin to a final concentration of 500 μg/mL before harvesting the total cellular RNA. After adding rifampin, the cultured cells were harvested at different time points and chilled in dry ice/ethanol bath for 1 min before RNA extraction [9, 19].

Preparation and Purification of Recombinant FumA Protein and Its Antibodies

Recombinant FumA protein was expressed using a plasmid construct prepared using a similar previously described method [15]. The pQE30-*fumA* plasmid was transformed into *E. coli* BL21 (DE3) and cultured at 37°C until the OD_{600} reached between 0.3 and 0.5. To express the FumA protein, the cells were then induced with the addition of 1-mM isopropyl β-D-thiogalactoside and incubated for another 3 h. The protein expressed was monitored through 10% SDS-PAGE and stained with Coomassie blue. The recombinant FumA was isolated using a nickel column (Invitrogen Ni–NTA Purification System) according to the manufacturer's instructions. Anti-FumA antibody was prepared by immunizing rabbits with the purified recombinant FumA protein. The specificity of the antibody was tested through Western blot analysis using purified FumA and the cell lysate of a *ΔfumA* mutant.

Western Blot Analysis

Briefly, cells harvested at different growth rates were resuspended in 100-mM potassium phosphate buffer (pH 7.6) followed by a sonication and centrifugation at 4°C. A total of 15 μg of the extract protein from the lysate was electrophoresed on 10% SDS-PAGE for 2 h at 100 volt. After transferring to nitrocellulose paper, FumA was monitored using an antibody prepared against FumA

and developed with an ECL detection system (Amersham, Arlington Heights, IL).

Results and Discussion

Effect of Growth Rate on the Expression of *fumA* mRNA and FumA Protein Level

First, a standard procedure to control the *E. coli* growth rate was employed by maintaining cells in a continuous culture with a glucose-limit chemostat [6, 17]. Figure 1a demonstrates that the increase in cell growth rate (k) from 0.24/h to 0.96/h resulted in a substantial reduction in *fumA* transcription by eightfold based on the β-galactosidase activity from *fumA* promoter fused with a *lacZ* reporter gene. Northern blot analysis was further used to confirm that the growth rate negatively affected the overall *fumA* mRNA levels (Fig. 1b). To verify that the changes in mRNA levels were not because of the sample loading or dilution effect, intrinsic 23S and 16S rRNA were routinely assessed as a control. These levels were not affected by the growth rate under the same experimental condition (Fig. 1b). The identical control was also conducted for the other studies described in the following.

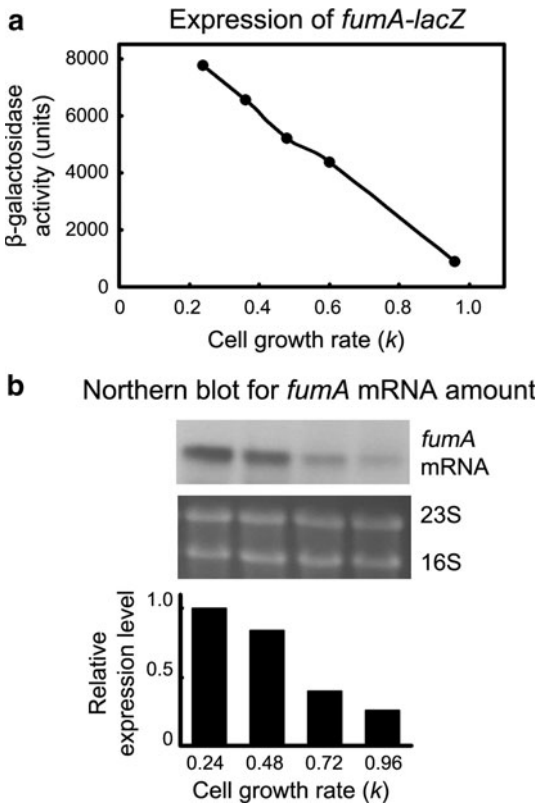

Fig. 1 Effect of growth rate on *E. coli fumA* gene expression. **a** Expression was determined using a *lacZ*-fused reporter. **b** Overall *fumA* mRNA levels were determined using Northern blot analysis. Each point represents the mean of duplicates. The 23S and 16S rRNA presented in total isolated RNA pool were used as an intrinsic control and stained with ethidium bromide

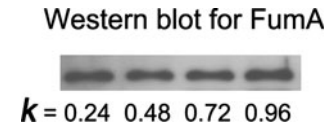

Fig. 2 Effect of growth rate on overall *E. coli* FumA levels assessed using Western blot analysis. Cell lysates from different growth rates were collected and adjusted to equal protein concentrations in which 15 μg was used for SDS-PAGE followed by a Western blot analysis

Given that FumA is one of the major enzymes in *E. coli* for using carbon sources to generate ATP through the Krebs cycles, an attempt was made to address why *E. coli* cells with limited *fumA* expression (under the high growth rate) remain metabolically active. Based on Western blot analysis, the FumA protein levels from the cells grown under the different growth rates were determined. Interestingly, the FumA protein levels were not significantly altered (Fig. 2) even though their overall mRNA levels were dramatically different. Furthermore, the enzymatic activity of FumA remained almost unchanged using the cell lysates obtained using different growth rates (data not shown). The latter result is consistent with a previous observation [17], revealing that growth rate has no effect on the cellular enzymatic activity.

Stability of *fumA* mRNA Under High Growth Rate

Reduced *fumA* mRNA expression under a high growth rate might therefore be compensated for by increased stability of *fumA* mRNA. To test this hypothesis, the half-lives of the *fumA* mRNA of cells grown at different growth rates were determined. Rifampin was added at zero time to block newly synthesized mRNA transcripts; the aliquots were then withdrawn at time intervals (from 0 to 8 min) before the cellular RNA isolation and Northern blot analysis [9]. Thus, the data obtained are essentially related to the effect of growth rate on the stability rather than the expression of *fumA* mRNA [19]. Figure 3a depicts that the half-life of *fumA* mRNA was significantly increased using Northern blot analysis. For example, at the third minute, *fumA* mRNA was almost completely diminished at 0.48/h, but remained abundant at 0.96/h. The calculation of the half-life in Fig. 3b shows an approximately fivefold increase as growth rate (k) increased from 0.24/h to 0.96/h.

Figure 3c reveals that the half-life of *fumA* is inversely correlated with the overall mRNA produced over growth rate (Fig. 1). The finding can be rationalized that the cells are not multiplying fast enough to produce newly synthesized mRNA of *fumA* at high growth rate, but the

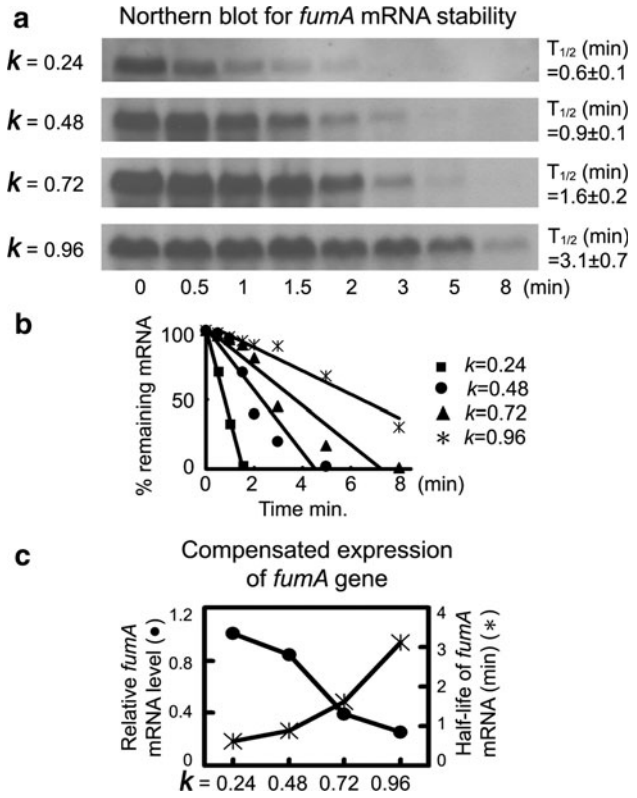

Fig. 3 Effect of growth rate on the *E. coli fumA* mRNA stability. **a** The stability of *fumA* mRNA was measured via Northern blot analysis. Rifampin was added to the cultured cells to inhibit new mRNA synthesis and harvested at the designed timing from 0 to 8 min. Analysis of the *fumA* mRNA levels was performed by fractionation of the total RNAs on 1% agarose–formaldehyde gel followed by transfer of the RNA onto a nylon membrane. The membrane was then hybridized with DIG-labeled *fumA* DNA. **b** The half-life of *fumA* mRNA was determined using the density images from the Northern blot analysis. Each point represents the mean of triplicates. **c** Inverse relationship between *fumA* gene expression and *fumA* mRNA half-life over growth rate

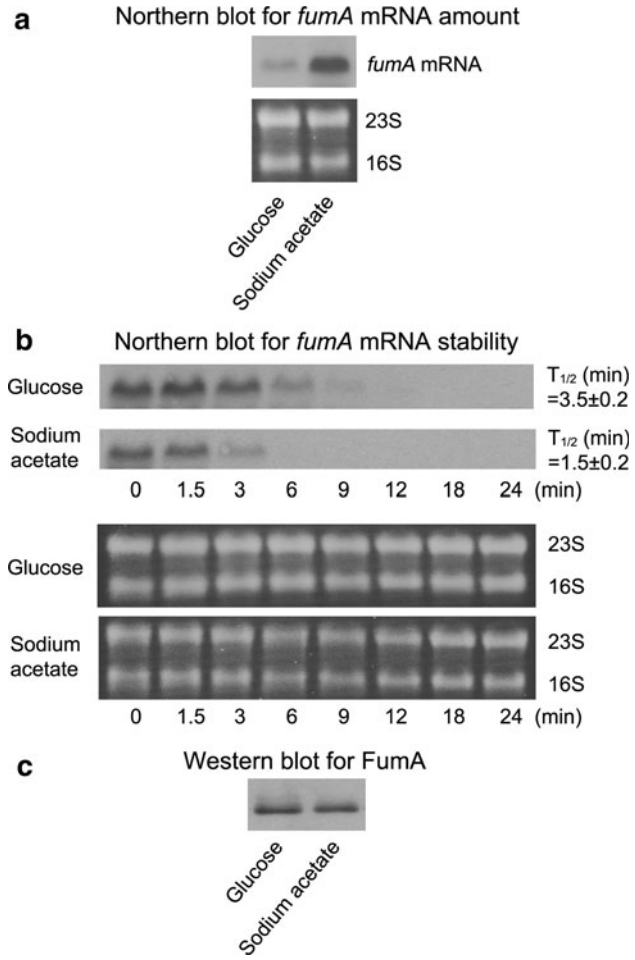

Fig. 4 Effect of sodium acetate on the stability of *E. coli fumA* mRNA. **a** *FumA* gene expression in culture medium containing 10-μM sodium acetate or glucose determined using Northern blot analysis. **b** Stability profiles of *fumA* mRNA determined using Northern blot analysis. **c** FumA protein levels assessed through Western blot analysis. At the same time, 23S and 16S rRNA served as an intrinsic control

availability of the FumA enzyme can be overcome by increasing the half-life of its mRNA.

Effect of Sodium Acetate on the Stability of *fumA* mRNA

To verify that the overall mRNA produced is inversely correlated with its half-life (Fig. 3c), glucose (10 mM) was substituted with sodium acetate (10 mM), a known substrate that increases *fumA* gene expression, in the medium [3, 14]. Figure 4a confirms such "increasing" effect produced by the sodium acetate. The stability of the *fumA* mRNA in the presence of glucose and sodium acetate was then examined. Figure 4b reveals that high *fumA* expression is related to the short mRNA half-life and vice versa when compared with Fig. 4a. Furthermore, sodium acetate did not affect the overall levels of the cellular FumA protein relative to that of glucose (Fig. 4c), which is consistent with the result mentioned earlier (Figs. 3, 4).

Remarks of the Significance of This Study

At present, the mechanism by which high growth rate lowers the *fumA* levels in cells remains elusive. Increasing the growth rate decreases the concentrations of intracellular cAMP [1, 8]. The cAMP levels were monitored over the continuous culture batches, which indicated that their levels are inversely correlated with the growth rate (data not shown). Furthermore, using a cAMP-deficient *E. coli* strain, the lack of cAMP in cells was confirmed to yield extremely low *fumA* expression (data not shown). We, therefore, speculate that high growth rate might initially

lower the cAMP level to cause the downregulation of *fumA*.

Another possibility beyond the scope of this study is the translation efficiency. Previous studies have shown a positive correlation between the protein translation and mRNA stability in *E. coli*. The initiation of translation leads the complex formation between mRNA and the ribosome, and the binding of ribosomes to the 5′-ends of mRNAs is a rate-limiting step in mRNA decay. A deficiency of ribosomes will prevent or reduce translation, usually shortening the mRNA half-life and causes the decreased translation of mRNA [5, 7]. Recent analyses of transcriptomic and proteomic data in *Saccharomyces cerevisiae* have also indicated that post-transcriptional regulation modulates the efficiency of mRNA translation into protein [2, 12]. Similarly, a fivefold increase in the *fumA* mRNA half-life was found to compensate for the protein translation at high growth rates, with an eightfold reduction in transcription (Figs. 1a, 2, 3a).

Large-scale absolute protein expression measurements (APEX) were used and applied to estimate the relative contributions of transcriptional- and translational-level gene regulation in the yeast and *E. coli* proteomes. Liu et al. [10] found that 73% of the variance in yeast protein abundance is positively correlated with mRNA abundance and concluded that yeast gene expression regulation occurs mostly through mRNA-directed mechanisms. Notably, only 47% of the variance in *E. coli* protein abundance is positively correlated with its mRNA abundance. Although the mechanism by which a given protein level does not depend on mRNA abundance is unclear, we provided a typical example to show that FumA protein level is not directly related to its mRNA abundance in *E. coli*. We also point out that the final protein level can be compensated for by increasing mRNA stability. The stability plays an essential role in maintaining critical levels of a given protein when mRNA transcription is downregulated by a metabolic event. Figure 4 reiterates that *fumA* mRNA is underexpressed in the medium with glucose, but its abundance is then overcome by increase in the mRNA half-life.

In recent years, the number of recombinant proteins used for therapeutic applications has dramatically increased. The production of these proteins has a remarkable demand in the market. *E. coli* cells offer a means for the rapid and economical production of recombinant proteins. These advantages, coupled with a wealth of biochemical and genetic knowledge, have enabled the production of some therapeutic proteins such as insulin and growth hormones. These demands have driven the development of a variety of strategies for achieving high-level protein expression, particularly those involving several aspects, such as transcriptional regulation, mRNA stability, translation initiation and termination (translational regulation), host design considerations, and fermentation factors that are available for manipulating expression conditions, including the growth rate [16]. These are the major challenges in obtaining a high yield of some targeted proteins at low cost. Sorting out the protein abundances that are regulated by their mRNA stability and growth rate from those directed by mRNA levels would help achieve the above challenges economically.

In summary, the interrelationship among the transcription, post-transcription, and translation of *fumA* has not been previously elucidated. This study indicates that the transcriptional expression of *E. coli fumA* is drastically and inversely regulated by its growth rate, but the final protein abundance can be compensated for by mRNA stability. This finding may provide a reference value for future industrial applications of the preparation of recombinant proteins, as well as malate and fumarate as building block chemicals in metabolically engineered *E. coli*.

Acknowledgments This work was supported by the National Council of Science (Grant no. NSC 98-2311-B-009-003 -MY2). We would like to thank Chair Prof. Simon J.T. Mao of National Chiao Tung University for his critical review of this manuscript.

References

1. Bettenbrock K, Sauter T, Jahreis K, Kremling A, Lengeler JW, Gilles ED (2007) Correlation between growth rates, EIIACrr phosphorylation, and intracellular cyclic AMP levels in *Escherichia coli* K-12. J Bacteriol 189:6891–6900
2. Brockmann R, Beyer A, Heinisch JJ, Wilhelm T (2007) Post-transcriptional expression regulation: what determines translation rates? PLoS Comput Biol 3:e57
3. Gosset G, Zhang Z, Nayyar S, Cuevas WA, Saier MH Jr (2004) Transcriptome analysis of Crp-dependent catabolite control of gene expression in *Escherichia coli*. J Bacteriol 186:3516–3524
4. Guest JR, Miles JS, Roberts RE, Woods SA (1985) The fumarase genes of *Escherichia coli*: location of the *fumB* gene and discovery of a new gene (*fumC*). J Gen Microbiol 131:2971–2984
5. Kaberdin VR, Blasi U (2006) Translation initiation and the fate of bacterial mRNAs. FEMS Microbiol Rev 30:967–979
6. Kayser A, Weber J, Hecht V, Rinas U (2005) Metabolic flux analysis of *Escherichia coli* in glucose-limited continuous culture. I. Growth-rate-dependent metabolic efficiency at steady state. Microbiology 151:693–706
7. Komarova AV, Tchufistova LS, Dreyfus M, Boni IV (2005) AU-rich sequences within 5′ untranslated leaders enhance translation and stabilize mRNA in *Escherichia coli*. J Bacteriol 187:1344–1349
8. Kuo JT, Chang YJ, Tseng CP (2003) Growth rate regulation of *lac* operon expression in *Escherichia coli* is cyclic AMP dependent. FEBS Lett 553:397–402
9. Lin HH, Hsu CC, Yang CD, Ju YW, Chen YP, Tseng CP (2011) Negative effect of glucose on *ompA* mRNA stability: a potential role of cyclic AMP in the repression of *hfq* in *Escherichia coli*. J Bacteriol 193:5833–5840
10. Lu P, Vogel C, Wang R, Yao X, Marcotte EM (2007) Absolute protein expression profiling estimates the relative contributions of transcriptional and translational regulation. Nat Biotechnol 25:117–124

11. Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 352–355
12. Mittal N, Roy N, Babu MM, Janga SC (2009) Dissecting the expression dynamics of RNA-binding proteins in posttranscriptional regulatory networks. Proc Natl Acad Sci USA 106: 20300–20305
13. Notley-McRobb L, Death A, Ferenci T (1997) The relationship between external glucose concentration and cAMP levels inside *Escherichia coli*: implications for models of phosphotransferase-mediated regulation of adenylate cyclase. Microbiology 143: 1909–1918
14. Park SJ, Gunsalus RP (1995) Oxygen, iron, carbon, and superoxide control of the fumarase *fumA* and *fumC* genes of *Escherichia coli*: role of the *arcA*, *fnr*, and *soxR* gene products. J Bacteriol 177:6255–6262
15. Peekhaus N, Conway T (1998) Positive and negative transcriptional regulation of the *Escherichia coli* gluconate regulon gene *gntT* by GntR and the cyclic AMP (cAMP)–cAMP receptor protein complex. J Bacteriol 180:1777–1785
16. Sorensen H, Mortensen K (2005) Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. J Biotechnol 115:113–128
17. Tseng CP, Yu CC, Lin HH, Chang CY, Kuo JT (2001) Oxygen- and growth rate-dependent regulation of *Escherichia coli* fumarase (FumA, FumB, and FumC) activity. J Bacteriol 183:461–467
18. Ueda Y, Yumoto N, Tokushige M, Fukui K, Ohya-Nishiguchi H (1991) Purification and characterization of two types of fumarase from *Escherichia coli*. J Biochem 109:728–733
19. Vytvytska O, Jakobsen JS, Balcunaite G, Andersen JS, Baccarini M, von Gabain A (1998) Host factor I, Hfq, binds to *Escherichia coli ompA* mRNA in a growth rate-dependent fashion and regulates its stability. Proc Natl Acad Sci USA 95:14118–14123
20. Woods SA, Schwartzbach SD, Guest JR (1988) Two biochemically distinct classes of fumarase in *Escherichia coli*. Biochim Biophys Acta 954:14–26