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Regulation of fumarase (*fumB*) gene expression in *Escherichia coli* in response to oxygen, iron and heme availability: role of the *arcA, fur*, and *hemA* gene products

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Abstract

Three distinct fumarases, FumA, FumB and FumC, have been reported in *Escherichia coli.* While the *fumA* and *fumC* gene products are expressed under aerobic cell growth conditions, the FumB fumarase appears to be more abundant during anaerobic growth. To study the transcriptional regulation of the *fumB* gene, a *fumB-lacZ* operon fusion was constructed and analyzed in a single copy under a variety of cell culture conditions. Expression of *fumB-lacZ* was fourfold higher under anaerobic than aerobic growth conditions. This anaerobic response is modulated by the ArcA and Fnr proteins, which function independently as anaerobic activators of *fumB* gene expression. Cellular iron limitation in a *fur* mutant caused *fumB-lacZ* expression to decrease sevenfold while cellular heme limitation decreased *fumB* gene expression twofold. In addition, *fumB-lacZ* expression was shown to vary depending on the DNA superhelicity. This study further delineates the regulation of the *fumB* gene in cell growth.

Keywords: Fumarase gene expression; *fumB*; Aerobic vs. anaerobic growth; *Escherichia coli*

1. Introduction

Escherichia coli contains three fumarase genes, *fumA, fumB,* and *fumC*, that catalyze the interconversion of fumarate and L-malate [1,2]. The *fumA* and *fumC* genes are located at 35.5 min of the *E. coli* linkage map [3] while the *fumB* gene is located at 93.5 min [1]. The physiological function of each *E. coli* fumarase has been studied by using a triple mutant transformed with a plasmid containing one of the three fumarase genes. The FumA enzyme appeared to be a component of the tricarboxylic acid (TCA) cycle since it was synthesized predominantly under aerobic conditions [4]. Expression of the *fumA* gene was lowest during anaerobic cell growth suggesting a role for FumA as an aerobic fumarase. Anaerobic expression of *fumA-lacZ* from the *fumA* promoter was derepressed in both an *arcA* and an *fnr* mutant indicating that both ArcA and Fnr function as anaerobic repressors [5]. The FumB enzyme was shown to be more abundant under anaerobic conditions, especially during anaerobic respiratory growth with glycerol plus fumarate [4]. Furthermore, the anaerobic expression of the *fumB* gene is reduced in an *fnr* mutant suggesting that Fnr is a transcrip-

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tional activator [4]. Transcription of the *fumC* gene was shown to be complex: it was dependent on both the *fumA* and *fumC* promoters [5]. It has been reported that synthesis of FumC was increased by the addition of oxidizing agents, and this increase was assumed to be dependent on the *soxRS* gene products [6]. Recently, it has been proved that both the superoxide control and the iron control of *fumC* expression require the SoxR regulatory protein. FumC was thus proposed to substitute for FumA when environmental iron is limiting or when superoxide radicals accumulate [5]. In this study, we further examined the regulation of *fumB* gene using a *fumB-lacZ* operon fusion.

2. Materials and methods

2.1. Bacterial strains, bacteriophages, and plasmids

The genotypes of the *E. coli* K-12 strains, plasmids and the bacteriophage are listed in Table 1. The *arcA*, *fur*, *topA* and *fis* strains were constructed by introducing the indicated mutation into strain MC4100 λCPT7 (*fumB-lacZ*) by P1 transduction followed by selection for resistance to the appropriate drug [7]. The PC2 (*fnr*) λCPT7 lysogen and PC10 (*hemA*) λCPT7 lysogen were constructed by infecting PC2 and PC10 with a high titer λCPT7 lysate as previously described [8].

2.2. Construction of fumB-lacZ operon fusion

To construct the *fumB-lacZ* fusion, the 3.16-kb *Hin*dIII-*Hpa*I fragment of *fumB* gene was cloned from the Kohara library into pTZ19 to give plasmid pCPT4. The 1.49-kb *Beg*II-*Pst*I fragment was then inserted into M13 mp19 to give M13CTP5 [9]. By using oligonucleotide-directed mutagenesis, a new *Eco*RI site was introduced into the *fumB* gene at position +86 relative to the start of *fumB* translation to give M13CPT6. A 896-bp *Bgl*II-*Eco*RI fragment containing the 5′ end of the *fumB* gene and the upstream 810 bp was isolated from M13CPT6 and inserted into the *Bam*HI-*Eco*RI sites of pRS1247 to generate the *fumB-lacZ* operon fusion designated pCPT7. The junction between the *fumB* promoter regions and the *lacZ* gene was confirmed by double-strand DNA sequencing analysis [10]. The fusion was transferred to RZ5 to generate the correspond-

Table 1
Escherichia coli strains, phages and plasmids

Strain, phage, plasmid	Derivation	Genotype or phenotype	Reference or source
Strains			
MC4100		F^- *ara*D139 Δ(*argF*−*lac*)*U169rpsL 150 relA1 flb-5301 deoC1 ptsF25 rbsR*	[11]
PC2	MC4100	*fnr*	[13]
C35	MC4100	*arcA* Kan^r	[13]
PC40	MC4100	*hemA41* Kan^r	[5]
SJP 2	MC4100	*fur*::Tn*5*	[5]
SJP 4	MC4100	*fis*-767Kan^r	[5]
SJP 6	PC2	*fnr arcA* Kan^r	[5]
RS 2	MC4100	*pyrF287topAgal125rps* λ^-	[16]
Phages			
λRZ5			[11]
λCPT7	pCPT7	Φ(*fumB-lacZ*)*lac*Y^+*lac*A^+	This study
M13mp19			[9]
M13CPT5	M13mp but with 1.48-kb *Bgl*II-*Pst*I fragment		This study
M13CPT6	M13 CPT5 but with an *Eco*RI site		This study
Plasmids			
pRS1247		*lacZ lac*Y^+ *lac*A^+	[11]
pCPT4	pTZ19	*fumB*$^+$	This study
pCPT7	pRS1247	Φ(*fumB-lacZ*)*lac*Y^+*lac*A^+	This study

ing λCPT7, which was then introduced into the MC4100 chromosome as previously described [11]. Single lysogen was identified and purified for subsequent study. The wild-type locus of *fumB* was stably integrated at the lambda attachment site on the chromosome.

2.3. Cell growth

For strain manipulation and maintenance, cells were grown in Luria broth or on solid medium. When required, ampicillin and kanamycin were added to the medium at concentrations of 100 and 50 μg ml^{-1}, respectively. For β-galactosidase assay, cells were grown in glucose (40 mM) minimal medium (pH 7.0) [12], unless otherwise indicated [13]. Aerobic and anaerobic growth were performed as previously described [13]. Flasks or tubes containing medium were inoculated from the overnight cultures grown under the same conditions, and the cells were allowed to double four of five times while in mid-exponential phase prior to harvest for analysis (optical density at 600 nm of 0.4–0.5; Kontron Uvikon 810 spectrophotometer). Trimethylamine-*N*-oxide (TMAO), sodium nitrate, or fumarate was added at an initial concentration of 40 mM [12]. Where indicated, δ-aminolevulinic acid (δ-ALA), 2,2-dipyridyl or ferrous sulfate was added at final concentrations of 24 μg ml^{-1} or 150 or 80 μM, respectively.

2.4. β-Galactosidase assay

β-Galactosidase assays were performed as previously described [12]. One unit of β-galactosidase is defined as the hydrolysis of 1 nmol *o*-nitrophenyl-β-D-galactopyranoside (ONPG) per min per mg protein. All values shown represent the average of at least three determinations and did not vary more than 10% from the mean.

3. Results

3.1. Effect of oxygen and other electron acceptors on fumB-lacZ expression

To examine the effect of anaerobic respiratory conditions on *fumB* expression, an *E. coli* wild-type strain containing a *fumB-lacZ* fusion was grown in the presence and absence of the alternative electron acceptors oxygen, nitrate, TMAO, or fumarate (Table 2). Consistent with the earlier observations of Woods and Guest [4], *fumB* expression was lowest during aerobic cell growth and elevated fourfold when cells were grown anaerobically under fermentative conditions (minimal glucose medium). When cells were grown anaerobically with nitrate present, *fumB-lacZ* expression was twofold lower than when no electron acceptors were added. Interestingly, *fumB* expression was highest when fumarate and TMAO were present as electron acceptors during anaerobic growth in a glucose minimal medium. When glycerol was substituted for glucose as the carbon source, conditions in which the cells derive energy from electron transport-linked phosphorylation reactions, *fumB-lacZ* expression was lowest in the presence of oxygen. It was elevated four- to seven-fold when nitrate, TMAO or fumarate was added in glycerol minimal medium (Table 2). The results of these *fumB-lacZ* studies suggest that FumB is oper-

Table 2
Effect of alternative electron acceptors on *fumB-lacZ* expression

Electron acceptor added[a]	β-Galactosidase activity (U)	
	Minimal glucose	Minimal glycerol
None	120	NG[b]
Oxygen	30	32
Nitrate	74	140
TMAO	150	190
Fumarate	140	230

[a]Cells were grown in 40 mM glucose or glycerol minimal medium either aerobically or anaerobically as described in the text. Sodium nitrate, TMAO, or fumarate was added at an initial concentration of 40 mM.
[b]NG, no growth.

ative during conditions of anaerobic respiration since *fumB* expression is elevated during these conditions.

3.2. *Effect of the arcA, fnr, topA and fis gene products on fumB-lacZ expression*

The aerobic/anaerobic control of the *fumA*, *fumB* and *fumC* genes is mediated by the *fnr* gene product [4,5]. Expression of the *fumA* and *fumC* genes is also negatively regulated by the ArcA protein [5]. To examine the individual and combined effects of mutations in these two genes, the λCPT7 fusion was introduced into isogenic *fnr, arcA*, and *fnr arcA* strains. Single lysogens were identified and purified for subsequent study. A fourfold difference in *fumB-lacZ* expression occurred in response to oxygen availability in the wild-type parent strain (Table 3). Fnr acts as a positive regulator of *fumB-lacZ* expression during anaerobic conditions as previously proposed [4]. Expression of *fumB-lacZ* was decreased by 50% in an *fnr* mutant. The results showed that ArcA was also a positive regulator of *fumB* gene expression. The level of *fumB-lacZ* expression in the *arcA* deletion strain decreased 30% under anaerobic growth conditions relative to the wild-type strain, whereas aerobic expression was unaffected. In addition, *fumB-lacZ* expression decreased fourfold in the *fnr* and *arcA* double mutant strain anaerobically. Thus, the Fnr and ArcA proteins appear to function independently of each other to regulate *fumB* gene expression.

There is increasing evidence that DNA supercoiling varies in response to environmental signals such as osmolarity or anaerobic growth [14,15]. In a *topA* mutant strain, expression of *fumB-lacZ* decreased threefold under aerobic conditions and lowered 30% under anaerobic conditions (Table 3). When the *topA* allele was introduced into *arcA* and *fnr arcA* double mutant strains [16], *fumB-lacZ* expression was lowered about fourfold and fivefold, respectively. These results indicate that a change of DNA superhelicity negatively regulates *fumB* gene expression. The Fis protein is known to be involved in regulation of a variety of genes in *E. coli* [17]. Under the conditions tested, a *fis* mutation activated *fumB* gene expression about 30% during anaerobic conditions relative to the parent strain (Table 3).

Table 3
Effect of *arcA, fnr, topA, himA*, and *fis* mutations on *fumB-lacZ* gene expression[a]

Genotype	β-Galactosidase activity (U)	
	Aerobic	Anaerobic
Wild-type	30	120
fnr	30	56
arcA	28	85
fnr arcA	18	35
topA	12	94
fnr topA	12	46
arcA topA	12	31
fnr arcA topA	14	26
fis	45	160

[a]Cells were grown in glucose minimal medium under aerobic or anaerobic conditions as described in the text.

Table 4
Effect of iron availability on *fumB-lacZ* expression

Addition[a]			β-Galactosidase activity (U)	
O_2	Dip	Fe^{2+}	Wild-type	*fur*
+	−	−	30	20
+	+	−	15	14
+	−	+	57	22
+	+	+	50	15
−	−	−	120	28
−	+	−	16	15
−	−	+	134	30
−	+	+	125	32

[a]Cells were grown in a minimal glucose medium aerobically or anaerobically as described in the text. Dipyridyl (Dip) and ferrous sulfate (Fe^{2+}) were added at initial concentrations of 150 μM and 80 μM, respectively, as indicated.

3.3. *Effect of iron and heme availability on fumB-lacZ expression*

Iron is an essential component of the FumA and FumB fumarase activities [18]. Because cellular iron limitation stimulates *fumC* gene expression [5], the effect of if iron limitation on *fumB* gene expression was tested. When cells were grown in the presence of the iron chelator 2,2-dipyridyl to limit iron, *fumB-lacZ* expression was decreased sevenfold during anaerobic growth (Table 4). When iron was added in excess, *fumB-lacZ* returned to the level seen when no 2,2-dipyridyl was present. Under aerobic conditions, expression of *fumB-lacZ* was elevated twofold when iron was added in excess to the medium. To test whether a *fur* mutant which is defective for iron

Table 5
Effect of heme availability on *fumB-lacZ* gene expression

Addition[a]		β-Galactosidase activity (U)	
Oxygen	δ-ALA	Wild-type	*hemA*
+	−	30	22
+	+	28	25
−	−	120	63
−	+	110	92

[a]Cells were grown in a minimal glucose medium aerobically or anaerobically as described in the text. δ-ALA was added at an initial concentration of 24 μg ml^{-1}.

regulation and uptake affects *fumB-lacZ* expression, a *fur* allele was introduced into the *fumB-lacZ* fusion strain [19]. Anaerobic levels of β-galactosidase were reduced to the level seen under aerobic conditions (Table 4). Addition of excess iron to the medium did not restore *fumB-lacZ* expression. These results indicate that Fur is involved in the observed iron regulation.

Heme is a cofactor of several enzymes needed for energy generation during anaerobic growth [20]. We examined the effects of heme availability on *fumB-lacZ* expression since it affects *fumA* and *fumC* gene expression [5]. In the *hemA* mutant strain which is defective for heme biosynthesis, *fumB-lacZ* expression was reduced twofold during anaerobic growth (Table 5). Expression was partially restored to wild-type levels by providing the cells exogenously with δ-ALA, the end product of the reaction catalyzed by the *hemA* gene product. Whether this regulatory effect is direct or indirect is unclear.

4. Discussion

Fumarase participates in the TCA cycle during aerobic growth, and in the reductive pathway from oxaloacetate to succinate during anaerobic growth. It was previously shown that the *fumA* and *fumC* genes are most highly expressed during aerobic conditions [5], whereas the *fumB* gene was expressed at a higher level under anaerobic cell growth conditions [4]. As the other TCA cycle genes are regulated by ArcA or both ArcA and Fnr repressors [5,19,21], ArcA and Fnr also function as negative regulators of the *fumA* and *fumC* genes while Fnr is an anaerobic activator of the *fumB* gene [4]. In this study, we further demonstrate that this anaerobic regulation of *fumB* gene expression is mediated by both ArcA and Fnr proteins that function independently as anaerobic activators of *fumB-lacZ* expression. It is similar to their effects on the respiratory genes *cydAB, frdABCD, dmsABC* and *narGHJK* [22]. The fact that anaerobic cell growth in a glycerol medium with the electron acceptor fumarate present results in a relatively high level of *fumB* gene expression suggested that FumB contributes to the fermentative and non-cyclic TCA pathway [4].

Iron is an essential component of both the FumA and FumB fumarases, while FumC does not require iron for its activity [23]. However, iron limitation only affects the *fumC* gene but has no effects on *fumA* gene expression [5]. In contrast to the observation that *fumC* gene expression was activated by 2,2-dipyridyl, *fumB-lacZ* expression decreased when 2,2-dipyridyl was added. This repression can partially be restored by adding excess iron (Table 4). Since iron is required for Fnr activity [24], the decrease of *fumB-lacZ* expression by the chelator may be due to lowered Fnr activity. FumA and FumB are members of the iron-dependent hydrolases [3,23]. Earlier studies suggested that the *fumA* gene is activated by Fur whereas the *fumB* and *fumC* genes were not significantly affected by Fur under aerobic conditions [25]. In this study, we found that the expression of the *fumB* gene was abolished in the *fur* deletion strain under anaerobic conditions where *fumB* is more highly expressed. Thus, iron control of *fumB* expression was *fur*-dependent. Although the *fumA* and *fumB* genes are homologous and their encoded products are structurally similar, FumA has a higher affinity for fumarate than for L-malate whereas FumB exhibits the reverse pattern of affinities for these two compounds [23]. Therefore, a hierarchical control of *fumA* and *fumB* genes by ArcA and Fnr during anaerobic growth suggested that the synthesis of the FumB fumarase under anaerobic conditions gives *E. coli* the flexibility to meet its requirement for diverse environmental conditions.

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