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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

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The tricarboxylic acid cycle enzyme fumarase catalyzes the interconversion of fumarate to L-malate. *Escherichia coli* contains three biochemically distinct fumarases. While the *fumA* and *fumB* genes encode heat-labile, iron-containing fumarases, the *fumC* gene product is a heat-stable fumarase which does not require iron for activity. To study how the *fumA* and *fumC* genes are regulated, we constructed *lacZ* operon fusions to the *fumA* and/or *fumC* upstream regions. Expression of the *fumA* and *fumC* genes was lowest during anaerobic cell growth, in support of the proposed roles of FumA and FumC as aerobic fumarases. Transcription of the *fumC* gene was shown to be complex: it was dependent on both the *fumA* and *fumC* promoters. Anaerobic expression from the *fumA* promoter was derepressed in both an *arcA* and a *fnr* mutant, while expression from the *fumC* promoter was derepressed in only the *arcA* strain. The *fumA* promoter was also shown to be catabolite controlled, whereas the *fumC* promoter was relatively unaffected by the type of carbon used for cell growth. Cellular iron limitation stimulated *fumC* but not *fumA* expression. Superoxide radicals also caused increased *fumC* gene expression; *fumA* expression was unaffected. Both the superoxide control and the iron control of *fumC* expression required the SoxR regulatory protein. These studies suggest different physiological roles for the FumA and FumC fumarases. The iron-containing FumA fumarase is the more abundant enzyme under most conditions of aerobic cell growth except when iron is limiting; FumC, which lacks iron, appears to be a backup enzyme that is synthesized optimally only when iron is low or when superoxide radicals accumulate.

Fumarase, or fumarate hydratase (EC 4.2.1.2), catalyzes the interconversion of fumarate and L-malate. It is widely distributed in a variety of organisms because of its central role in the tricarboxylic acid (TCA) cycle during aerobic cell metabolism (17). In facultative anaerobes such as *Escherichia coli*, fumarase also participates in the reductive pathway from oxaloacetate to succinate during anaerobic growth. The existence of three fumarase genes in *E. coli*, *fumA*, *fumB*, and *fumC*, has been reported (12, 13). The *fumA* and *fumC* genes are adjacent and are located at 35.5 min on the *E. coli* linkage map near the *manA* gene, which encodes mannose 6-phosphate isomerase (25). The *fumB* gene is located at 93.5 min on the *E. coli* linkage map near the *mel* operon (12).

Comparison of the *fumA*, *fumB*, and *fumC* gene sequences reveals that they comprise two biochemically distinct types of enzymes with respect to their size and cofactor requirements (43). The *fumA* and *fumB* genes are homologous and encode products of identical size (ca. 60,000 Da) which form thermolabile dimers of M_r 120,000. They are classified as class I enzymes (13) and are members of the iron-dependent hydrolases, which include aconitase and malate hydratase (25, 34, 43, 47). The purified FumA contains a 4Fe-4S center when active; it can be inactivated upon oxidation to give a 3Fe-4S center (8, 47). FumA activity can be restored by anaerobic incubation with iron and thiol. The *fumC* gene does not show any homology with either the *fumA* or *fumB* gene, but it exhibits extensive homology with the fumarase genes of *Bacillus subtilis*, *Saccharomyces cerevisiae*, and mammals (19, 20, 26, 32, 37, 45). It also has similarity to the aspartase gene, *aspA*, of *E. coli* (38, 42). FumC is classified as a class II enzyme that forms thermostable tetramers of M_r 80,000 (43). Class II enzymes do not require iron for their activity (47).

The physiological role of each *E. coli* fumarase is not yet entirely clear, although this issue has been addressed both genetically and biochemically. By using a *fumA fumB fumC* triple mutant transformed with a plasmid containing one of the three *fum* genes, the activity of each fumarase was assayed under different cell growth conditions (41). Since FumA was synthesized predominantly under aerobic conditions, it is proposed to be a component of the TCA cycle. The decrease in FumA synthesis seen when cells were grown in the presence of glucose suggested that the Crp-cyclic AMP complex was involved in this process. Crp binding sites were proposed to exist within the *fumA* promoter region (41). The FumB enzyme was shown to be more abundant under anaerobic conditions (41). However, little could be concluded about the physiological significance of FumC, as its synthesis was reported to occur at a low constitutive level (41). It was recently reported that synthesis of the heat-stable fumarase was increased by the addition of oxidizing agents and that this increase was abolished in a *soxRS* strain (23). The *soxRS* genes encode positive regulators for controlling synthesis of proteins in response to oxidative stress (10, 39). FumC was thus proposed to be a substitute enzyme for FumA when cells are exposed to oxidizing conditions.

In this study, the expression of the *fumA* and *fumC* genes was investigated by constructing and analyzing *fumA-lacZ*, *fumC-lacZ*, and *fumAC-lacZ* operon fusions. The transcriptional control of the *fumAC* genes was shown to be complex: *fumC* was shown to be transcribed from the *fumC* promoter as

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TABLE 1. *E. coli* K-12 strains, phages, and plasmids used

Strain, phage, or plasmid	Derivation	Genotype or phenotype	Reference or source
Strains			
MC4100		F^- *araD139* Δ(*argF-lac*)*U169 rpsL 150 relA1 flb-5301 deoC1 ptsF25 rbsR*	35
PC2	MC4100	*fnr*	5
PC35	MC4100	*arcA* Kan^r	5
PC40	MC4100	*hemA41* Kan^r	P. Cotter
SJP3	MC4100	*himA*Δ*82* Tet^r	This study
SJP4	MC4100	*fis-767* Kan^r	This study
SJP6	PC2	*fnr* Δ*arcA* Kan^r	This study
GC4468		F^- Δ(*lac*)*4169 rpsL*	2
DJ901	GC4468	Δ(*soxR-zjc-2205*) *zjc-2204* Kan^r	9
JTG1078	GC4468	*soxR105 zjc-2204* Kan^r	9
IS4	MC4100	*narXL* Kan^r	3
Phages			
λRZ5			36
λSJP24	pSJP24	φ(*fumA-lacZ*) $lacY^+$ $lacA^+$	This study
λSJP25	pSJP25	φ(*fumC-lacZ*) $lacY^+$ $lacA^+$	This study
λSJP27	pSJP27	φ(*fumAC-lacZ*) $lacY^+$ $lacA^+$	This study
M13 mp19			24
M13 SJP186	M13 mp18 but with 2.35-kb *Eco*RI fragment		This study
M13 SJP187	M13 SJP186 but with a *Bam*HI site		This study
M13 SJP191	M13 mp19 but with 2.12-kb *Hin*dIII-*Pst*I fragment		This study
M13 SJP192	M13 SJP191 but with a *Bam*HI and *Eco*RI site		This study
Plasmids			
pRS1247		*lacZ* $lacY^+$ $lacA^+$	36
pRS415		*lacZ* $lacY^+$ $lacA^+$	36
pFumA1			John McCoy
pSJP8	pTZ19	$manA^+$ $fumA^+$ $fumC^+$	This study
pSJP22	pSJP8 but with *Bam*HI and *Eco*RI sites		This study
pSJP24	pRS415	φ(*fumA-lacZ*) $lacY^+$ $lacA^+$	This study
pSJP25	pRS1247	φ(*fumC-lacZ*) $lacY^+$ $lacA^+$	This study
pSJP27	pRS415	φ(*fumAC-lacZ*) $lacY^+$ $lacA^+$	This study

well as from the *fumA* promoter. While both the *fumA* and *fumC* genes were expressed optimally under aerobic conditions, their levels of expression differed with respect to cell growth with different carbon compounds, with iron limitation or heme availability, and with superoxide-generating agents. These studies imply distinctly different roles for the two aerobic fumarase enzymes depending on the environmental conditions that the cell encounters.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. The genotypes of the *E. coli* K-12 strains, plasmids, and bacteriophages used are listed in Table 1. The *arcA*, *himA*, *fis*, *hemA*, and *narXL* mutations were introduced into strain MC4100 carrying the indicated *fum-lacZ* fusion by P1 transduction (27). A high-titer λ lysate of λSJP24, λSJP25, or λSJP27 was used to introduce the *fum-lacZ* fusions into strain PC2 (*fnr*) (5).

Construction of *fum-lacZ* operon fusions. The 3.45-kb *Hin*dIII-*Eco*RI fragment of pFumA1 containing the 35.5-min region of the *E. coli* chromosome was cloned into pTZ19U to give plasmid pSJP8. The 2.12-kb *Hin*dIII-*Pst*I fragment of pSJP8 was inserted into the corresponding sites of M13 mp19 to give M13 SJP191. By using oligonucleotide-directed mutagenesis, a new *Eco*RI site was introduced into the *manA* gene, which encodes mannose 6-phosphate isomerase. A new *Bam*HI site was then added into the *fumA* gene, which is transcribed divergently from the *manA* gene (21). This newly generated mutant M13 was designated M13 SJP192. The 2.12-kb *Hin*dIII-*Pst*I fragment of M13 SJP192 was used to replace the corresponding fragment of pSJP8 to give plasmid pSJP22.

To construct the *fumA-lacZ* fusion, the 0.45-kb *Eco*RI-*Bam*HI fragment of pSJP22 was inserted into pRS415 (36) to generate the operon fusion plasmid pSJP24 (Fig. 1). To construct the *fumC-lacZ* fusion, the 1.88-kb *Bam*HI-*Eco*RI fragment of pSJP22 was inserted into pRS1247, a promoterless *lacZ* operon fusion vector, to give pSJP25, which carries a *fumC-lacZ* operon fusion. The 2.35-kb *Eco*RI fragment from pSJP22 was cloned into pRS415 to form a *fumAC-lacZ* fusion and designated pSJP27. This *fumAC-lacZ* fusion includes the *fumA-manA* promoter region present in the *fumA-lacZ* fusion as well as the intact *fumA* gene and the *fumC* promoter region present in the fragment used for the *fumC-lacZ* fusion (Fig. 1). The junction between the *fumAC* promoter regions and the *lacZ* gene was confirmed by double-stranded DNA sequencing analysis (33). The fusions were transferred to λRZ5 to generate the corresponding λSJP phages (Table 1), which were then introduced into the MC4100 chromosome as previously described (36).

Cell growth. For strain manipulations and maintenance, cells were grown in Luria broth (LB) or on solid media. When required, ampicillin was added to the medium at a concentration of 100 mg/liter. For β-galactosidase assay, cells were grown in glucose (40 mM) minimal medium (pH 7.0) (4) unless otherwise indicated (5). For assay of cells grown on other carbon sources, each compound was added at a final concentration of 40 mM. Buffered LB (50 mM KPO_4 [pH 7.0]) was prepared either with or without glucose (40 mM) supplementation.

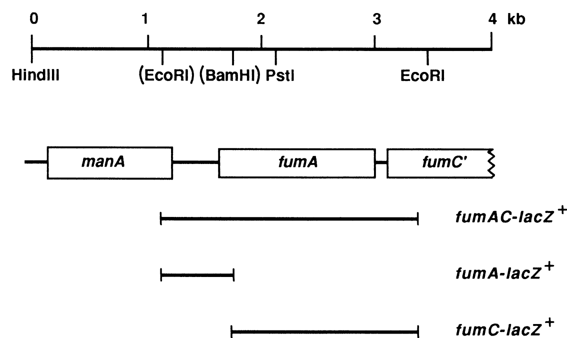

FIG. 1. Physical map of the *fumAC* gene region. The DNA fragments used to construct the *fumA-lacZ* (λSJP24), *fumAC-lacZ* (λSJP27), and *fumC-lacZ* (λSJP25) fusions are indicated below. Restriction enzyme sites used for cloning are indicated at the top. The restriction sites shown in parentheses were introduced by site-directed mutagenesis.

Aerobic growth and anaerobic growth were carried out as previously described (5). High aeration of cultures during aerobic growth was accomplished by shaking 10-ml culture volumes in 150-ml flasks. Flasks or tubes containing the indicated medium were inoculated from the overnight cultures grown under the same conditions, and the cells were allowed to double four or five times under mid-log exponential phase prior to harvesting for analysis (optical density at 600 nm [OD_{600}] of 0.4 to 0.5; Kontron Uvikon 810 spectrophotometer). Anaerobic cultures were harvested at an OD_{600} of 0.25 (Spectrophotometer 21; Milton Roy Co.). When desired, 2,2′-dipyridyl or ferrous sulfate was added at a final concentration of 0.15 or 0.08 mM, respectively. To examine the effect of heme availability on *fum-lacZ* expression, cells were grown in buffered LB supplemented with 30 mM pyruvate and with or without exogenous δ-aminolevulinic acid (δ-ALA) at 40 μg/ml (6).

To test the effect of paraquat (superoxide radical formation) on gene expression, the cells were grown in buffered LB supplemented with 40 mM glucose. When the cells reached an OD_{600} of 0.1 (Kontron Uvikon 810 spectrophotometer), paraquat was added to a final concentration of 0.1 mM. Cell samples were taken every 15 min and assayed for β-galactosidase activity.

β-Galactosidase assay. β-Galactosidase levels were determined by hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as previously described (4). Protein concentration was estimated by assuming that a culture absorbance of 1.4 at OD_{600} (Kontron Uvikon 810 spectrophotometer) corresponds to 150 μg of protein per ml as previously described (27). Units of β-galactosidase are expressed as nanomoles of ONPG hydrolyzed per minute per milligram of protein (4). β-Galactosidase values represent the averages of at least four experiments with a variation of no more than 10% from the mean.

Primer extension analysis. mRNA was isolated from a wild-type strain grown aerobically in LB as previously described (28). Primer extension reactions were performed with synthetic oligonucleotides complementary to positions +47 to +65 of the *fumC* gene relative to the predicted start of translation. The radiolabeled DNA was prepared by incorporation of [α-^{32}P]CTP, using reverse transcriptase (35). DNA sequencing reactions were performed with an appropriate template to provide a nucleotide sequence ladder for comparison.

RESULTS

Construction of *fumA*, *fumC*, and *fumAC* operon fusions to *lacZ*$^+$. As the *fumC* gene is located immediately downstream of the *fumA* gene, it is possible that the *fumC* and *fumA* genes are cotranscribed from the *fumA* promoter. To test this and to further examine how the *fumA* and *fumC* promoters are regulated in response to different cell growth conditions, three operon fusions were constructed (Fig. 1). Two fusions, λSJP24 and λSJP25, have the *fumA* and *fumC* upstream regions, respectively. The third fusion, λSJP27, has the combined *fumA* and *fumC* upstream regions and thus contains an intact *fumA* gene. The expression of each *lacZ* fusion was examined with respect to anaerobic regulation, catabolite repression, and exposure to cell stress conditions. The latter included limiting the cell for iron, since the FumA fumarase contains an iron-sulfur center whereas FumC does not, and for heme, since heme-containing proteins are involved in ATP generation from NADH generated by the TCA cycle reactions. Lastly, we tested the effect of exposing the cell to paraquat, which generates superoxide radicals, as this has been shown to elevate levels of heat-stable fumarase activity (23).

The *fumC* gene is expressed from the *fumA* and *fumC* promoters. When levels of expression of the *fumA-lacZ*, *fumC-lacZ*, and *fumAC-lacZ* fusions were compared in cells grown aerobically in a glucose minimal medium, it was evident that the *fumC* gene is expressed from its own promoter and from the upstream *fumA* promoter (Table 2). The level of *fumAC-lacZ* expression was 1.5-fold higher than the level of *fumC-lacZ* expression. When cells were grown in other types of media, this ratio was increased. In an acetate minimal medium, the level of *fumAC-lacZ* expression was sixfold greater than the level of *fumC-lacZ* expression. Similar results were seen under anaerobic cell growth conditions in most media tested (Table 2). These findings suggested that *fumC* gene expression is more complex than indicated by prior studies (41); a reassessment was thus warranted to address the role of the *fumA* promoter in *fumC* gene expression.

TABLE 2. Expression of *fumA-lacZ*, *fumAC-lacZ*, and *fumC-lacZ* expression in different media

Medium[a]	β-Galactoside activity (U)					
	fumA-lacZ		*fumC-lacZ*		*fumAC-lacZ*	
	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$
Glucose	1,150	290	210	40	320	80
Xylose	2,640	790	320	80	670	260
Galactose	2,860	1,180	250	120	650	400
Glycerol	4,340	NG[b]	330	NG	870	NG
Acetate	11,600	NG	310	NG	1,980	NG
Succinate	6,260	NG	410	NG	1,160	NG
Fumarate	7,790	NG	410	NG	1,210	NG
LB	2,420	1,490	250	40	540	210
LB-glucose	580	180	110	20	220	40
LB-pyruvate	3,030	320	200	20	460	60

[a] Cells were grown in a minimal basal medium (pH 7.0) with the indicated additions. Aerobic and anaerobic cultures were grown as described in the text.
[b] NG, no growth.

Effect of carbon substrates on *fumA* and *fumC* gene expression. Strains containing the *fumA-lacZ*, *fumC-lacZ*, and *fumAC-lacZ* fusions were grown in media of different compositions to evaluate how the type of carbon substrate affects *fum* gene expression (Table 2). During aerobic cell growth, *fumA-lacZ* expression varied over a 20-fold range. In contrast, *fumC-lacZ* expression remained relatively constant: it did not vary by more than about twofold for most media examined. Expression of the *fumAC-lacZ* fusion was intermediate and varied over a ninefold range. These findings indicate that the carbon control of *fumC* expression occurs primarily from the upstream *fumA* promoter rather than from the *fumC* promoter. In minimal media, *fumA-lacZ* expression increased in the order glucose, xylose, galactose, glycerol, and the TCA intermediates succinate and fumarate. The highest level of *fumA-lacZ* expression was observed when cells were grown in an acetate minimal medium. The lowest expression was in a buffered LB-glucose medium or in a minimal medium containing glucose. During anaerobic cell growth, *fumA-lacZ* expression was also controlled by the type of carbon used, although to a somewhat lesser extent than during aerobic cell growth (i.e., 8-fold versus 20-fold).

Effects of oxygen and other electron acceptors on *fumA-lacZ* and *fumC-lacZ* expression. To examine how cell respiration conditions and anaerobic fermentation conditions affect *fumA-lacZ* and *fumC-lacZ* expression, cells were grown in the presence and absence of the alternative electron acceptors oxygen, nitrate, TMAO, and fumarate (Table 3). Expression of *fumA-lacZ* and *fumC-lacZ* was highest during aerobic cell growth. When cells were grown anaerobically in a glucose minimal medium, conditions in which the cells must carry out fermentative metabolism, expression of *fumA-lacZ* and *fumC-lacZ* was four- to fivefold less than under aerobic conditions. These results indicate that both FumA and FumC are aerobic fumarases. When cells were grown anaerobically with nitrate, TMAO, or fumarate present as the electron acceptor, *fumA-lacZ* and *fumC-lacZ* expression was less than twofold higher than when no electron acceptors were added. However, if glycerol was substituted for glucose, conditions in which the cells derive energy from electron transport-linked phosphorylation reactions, *fumA-lacZ* expression was elevated three- to sixfold. Interestingly, the level of *fumA-lacZ* expression in cells grown anaerobically in the glycerol medium was higher than when cells were grown aerobically in glucose minimal medium.

TABLE 3. Effects of alternative electron acceptors on *fumA-lacZ* and *fumC-lacZ* expression[a]

Electron acceptor added[a]	β-Galactosidase activity (U)			
	fumA-lacZ		*fumC-lacZ*	
	Glucose	Glycerol	Glucose	Glycerol
None	290	NG[b]	40	NG
Oxygen	1,150	3,880	210	330
Nitrate	370	1,710	50	70
TMAO	490	1,490	70	160
Fumarate	350	2,040	40	120

[a] Cells were grown in 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.

These results suggest that the TCA cycle is operative during conditions of anaerobic respiratory growth. A similar pattern of gene expression was seen with the *fumC-lacZ* fusion (Table 3). Lastly, the level of *fumA-lacZ* expression was generally 5- to 10-fold higher than the level of *fumC-lacZ* expression in any given medium.

Effects of the *arcA* and *fnr* gene products on *fumA-lacZ* and *fumC-lacZ* expression. Since a four- to fivefold difference in both *fumA-lacZ* and *fumC-lacZ* expression was observed in response to oxygen availability, we examined whether the ArcA or Fnr regulatory protein was involved in this control as shown for other genes in *E. coli* (15). Strains containing a deletion of the *arcA* gene, the *fnr* gene, or both genes were constructed and analyzed following aerobic or anaerobic cell growth (Table 4). The level of *fumA-lacZ* expression in the *arcA* deletion strain was increased under both anaerobic (10-fold) and aerobic (2-fold) conditions compared with the wild-type parent strain. Expression of the *fumC-lacZ* fusion in the *arcA* deletion strain was elevated sixfold during anaerobic growth relative to the wild-type strain, whereas aerobic expression was increased only modestly (Table 4). Finally, the pattern of *fumAC-lacZ* expression was intermediate between those seen for the *fumA-lacZ* and *fumC-lacZ* fusions. These findings demonstrate that ArcA is a negative regulator of *fumA* and *fumC* gene expression. The ArcA control of *fumC* gene expression is also complex: it requires the combined interactions of ArcA at both the *fumA* and *fumC* promoters.

The Fnr regulatory protein also appears to control *fumA* and *fumC* gene expression (Table 4). The level of *fumA-lacZ* expression in the *fnr* deletion strain was threefold higher under anaerobic conditions than in the isogenic parent strain. In contrast, the *fnr* mutation resulted in a minor effect on *fumC-lacZ* expression (i.e., less than twofold increase under anaerobic conditions). Thus, the aerobic/anaerobic control of *fumA* and *fumC* gene expression involves ArcA repression at both the *fumA* and *fumC* promoters, while Fnr regulates *fumC* at a distance from the *fumA* promoter. Interestingly, *fumA-lacZ* expression in the *fnr arcA* double-mutant strain was derepressed 17-fold during anaerobic growth, compared with 10-fold in the *arcA* strain and threefold in the *fnr* strain, relative to the wild-type parent strain. Thus, the Fnr and ArcA proteins appear to function independently of each other to control *fumA* expression.

Comparison of the *fumA-lacZ*, *fumAC-lacZ*, and *fumC-lacZ* gene expression data reveals several other significant differences with respect to the aerobic/anaerobic control of the *fumA* and *fumC* genes (Table 4). First, *fumA* expression is always 5- to 10-fold higher than *fumC* expression. This finding suggests that the FumA fumarase is the major fumarase during aerobic cell growth. Second, the observation that the level of *fumA-lacZ* expression is also higher than the level of *fumAC-lacZ* expression during either aerobic or anaerobic growth (Tables 2 and 4) suggests that a portion of the *fumA* transcripts originating from the *fumA* promoter terminate before reading through to the *fumC* gene (e.g., compare *fumA-lacZ* expression with *fumAC-lacZ* expression). It is not yet known if this posttranslational process is somehow regulated. Third, the role of the *fumA* promoter in *fumC* gene expression varies depending on the type of carbon compound used for cell growth (Table 2), which suggests that the FumC fumarase serves in much the same capacity for cell metabolism as does the FumA fumarase.

To determine if expression of either the *fumA* or *fumC* gene is controlled by the nitrate-responsive NarX-NarQ-NarL two-component regulatory system (14), a *narXL* deletion strain was also analyzed following anaerobic growth in the presence and absence of nitrate (data not shown). Introduction of the *narXL* deletion did not alter expression of either *fumA-lacZ* or *fumC-lacZ* relative to the wild-type parent.

Effects of *arcA* on carbon control of *fumA-lacZ* and *fumC-lacZ* expression. We examined whether the *arcA* gene contributes to the carbon control of *fumA* and *fumC* gene expression. In an *arcA* strain, *fumA-lacZ* expression did not vary by more than about twofold under aerobic conditions compared with the wild-type strain (Fig. 2A and B). Anaerobically, the effect of an *arcA* gene deletion on *fumA-lacZ* expression was more dramatic: expression was derepressed 5- to 20-fold compared with the wild-type cells grown in corresponding media.

A similar pattern of ArcA control was also seen for *fumC-lacZ* expression during aerobic and anaerobic cell growth (Fig. 2C and D). However, the absolute level of expression was always considerably higher (ca. 15-fold) for *fumA-lacZ* than for *fumC-lacZ*. ArcA appears to aid in modulating the carbon control, but it is not sufficient to account for the observed catabolite control of either the *fumA* or *fumC* promoter.

Effects of *himA* and *fis* gene products on *fumA* and *fumC* expression. The integration host factor and Fis proteins are known to be involved in regulation of a variety of genes in *E. coli* (40, 46). We tested if these proteins affect *fumA* and *fumC* expression (Table 4). Under the conditions tested, *himA* and *fis* mutations had little effect on either *fumA-lacZ* or *fumC-lacZ* expression.

Effects of heme availability on *fumA-lacZ* and *fumC-lacZ* expression. Since heme is a cofactor of several enzymes needed for energy generation, including succinate dehydrogenase, cytochrome *o* and *d* oxidases, and nitrate reductase, we examined the effects of heme availability on *fumA* and *fumC* expression.

TABLE 4. Effects of *arcA*, *fnr*, *himA*, and *fis* mutations on *fum-lacZ* gene expression[a]

Genotype	β-Galactosidase activity (U)					
	fumA-lacZ		*fumC-lacZ*		*fumAC-lacZ*	
	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$
Wild type	1,150	290	210	40	320	80
fnr	1,160	870	220	70	350	170
arcA	2,310	3,070	250	230	450	600
fnr arcA	2,260	4,910	290	250	ND[b]	ND
himA	1,100	560	230	70	ND	ND
fis	970	260	220	60	290	80

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

[b] ND, not determined.

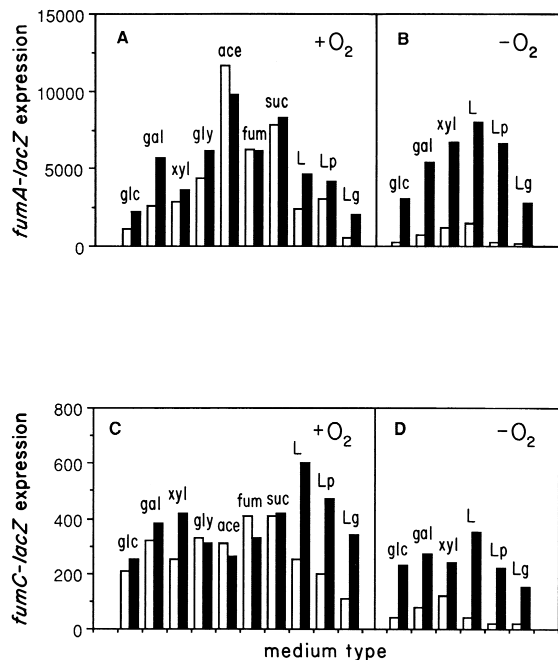

FIG. 2. Effects of a Δ*arcA* mutation on *fumA-lacZ* expression and *fumC-lacZ* expression in cells grown in minimal media with various types of carbon compounds and in rich media. (A and C) Aerobic cell growth; (B and D) anaerobic cell growth. The open bars represent the wild-type strain, and the solid bars represent the Δ*arcA* strain. Cells were grown in minimal medium supplemented with the indicated carbon compound (40 mM) or in buffered LB. Units are given in nanomoles of ONPG hydrolyzed per minute per milligram of protein. glc, glucose; gal, galactose; xyl, xylose; gly, glycerol; ace, acetate; fum, fumarate; suc, succinate; L, LB; Lp, LB-pyruvate; Lg, LB-glucose.

To block heme production, a *hemA* mutation was introduced into the *fumA-lacZ* and *fumC-lacZ* fusion strains. This effect can be suppressed by providing the cells exogenously with δ-ALA, which is the end product of the reaction catalyzed by the *hemA* gene product. In the *hemA* strain, *fumA-lacZ* expression was reduced more than twofold during aerobic growth (Table 5). Expression was partially restored to wild-type levels by the addition of δ-ALA. Under aerobic conditions, *fumC-lacZ* expression was also slightly decreased (less than twofold) in the *hemA* strain. Strikingly, an 11-fold increase in *fumC-lacZ* expression was observed under anaerobic conditions in the *hemA* strain compared with the wild-type strain. The anaerobic expression of *fumC-lacZ* was further increased 40-fold by addition of δ-ALA to the *hemA* strain. This effect was unanticipated.

TABLE 5. Effects of heme availability on *fumA-lacZ* and *fumC-lacZ* expression

Addition[a]		β-Galactosidase activity (U)			
		fumA-lacZ		*fumC-lacZ*	
Oxygen	δ-ALA	Wild type	*hemA*	Wild type	*hemA*
+	−	3,030	1,220	200	130
+	+	1,950	1,780	210	190
−	−	300	230	20	230
−	+	340	300	30	1,250

[a] Cells containing the *fumA-lacZ* or *fumC-lacZ* fusion were grown in buffered LB-pyruvate (30 mM) medium either aerobically or anaerobically as described in the text. δ-ALA was added to a final concentration of 40 μg/ml.

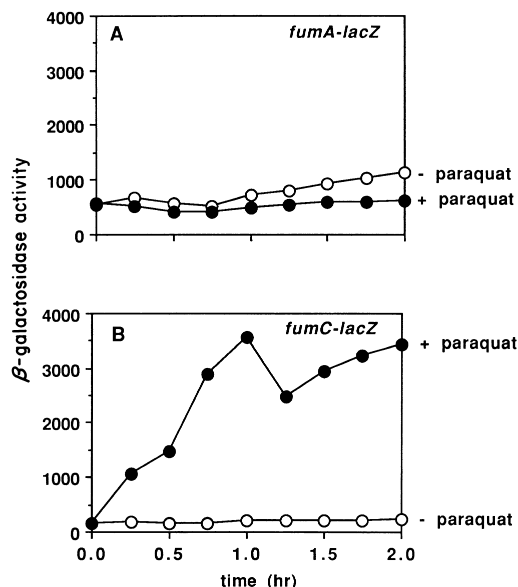

FIG. 3. Effects of paraquat on *fumA-lacZ* and *fumC-lacZ* expression. Cells were grown in buffered LB with 40 mM glucose to an OD_{600} of 0.1. For induction, paraquat was added to a final concentration of 0.1 mM. Cells were removed every 15 min after the addition of paraquat and assayed for β-galactosidase activity. The β-galactosidase activities were plotted against time after induction. Units are given in nanomoles of ONPG hydrolyzed per minute per milligram of protein.

Effect of the oxidizing agent paraquat on *fumA-lacZ* and *fumC-lacZ* expression. It was shown previously that the heat-stable fumarase (FumC) activity was increased by the presence of the superoxide-generating agent paraquat (23). To test whether this increase is due to altered transcription from the *fumA* or *fumC* promoter, we analyzed the expression of the *fumA-lacZ* and *fumC-lacZ* fusions in the presence of this agent (Fig. 3). Cells were grown to an OD_{600} of 0.1 and dispensed into two Erlenmeyer flasks. Paraquat (0.1 mM) was then added to one of the flasks, and at various times, cell samples were removed from each flask and β-galactosidase levels were determined. Enzyme activity was plotted versus the time following the addition of paraquat. Expression of the *fumA-lacZ* fusion was reduced about twofold by the addition of paraquat. In contrast, *fumC-lacZ* expression increased almost immediately after addition of paraquat, and it continued to increase during the course of the experiment. By 2 h, *fumC-lacZ* expression was 20-fold higher than in the nontreated cells. Hence, transcription of *fumC* but not *fumA* is induced by the superoxide-generating agent paraquat; this control occurs solely from the *fumC* promoter.

Superoxide-induced gene expression in *E. coli* has been shown to be mediated by the *soxR* gene product (1, 45). To further explore the role of SoxR in the induction of *fumC-lacZ* gene expression, two different *soxR* mutants were examined. One strain contained a deletion of the *soxR* locus, and the other had a *soxR** mutation that results in the synthesis of an altered SoxR protein in the absence of superoxide (9). The induction of *fumC-lacZ* expression by paraquat was completely abolished in the Δ*soxR* mutant (Fig. 4). In contrast, *fumC-lacZ* expression in the *soxR** mutant was 20-fold higher than in the wild-type strain. Addition of paraquat caused a further increase (ca. fourfold) after 2 h compared with when no paraquat was added. The *soxR** mutation appears to cause an elevated level of *fumC* expression in the absence of superoxide, while

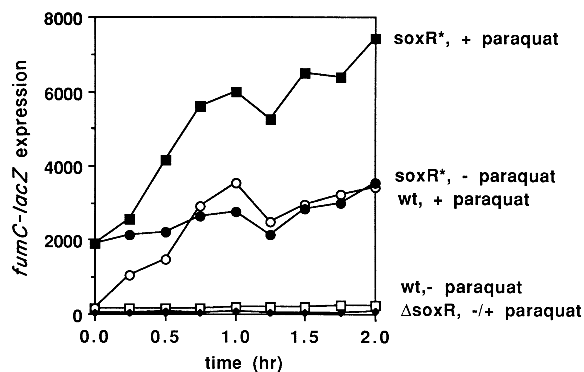

FIG. 4. Effect of the *soxR* locus on induction of *fumC-lacZ* expression by paraquat. Wild-type (wt) and Δ*soxR* and *soxR** mutant cells were grown in buffered LB with 40 mM glucose up to an OD_{600} of 0.1. For induction, paraquat was added to a final concentration of 0.1 mM. Cells were removed every 15 min after addition of paraquat and assayed for β-galactosidase activity. The β-galactosidase activities were plotted against time after induction. Units are given in nanomoles of ONPG hydrolyzed per minute per milligram of protein.

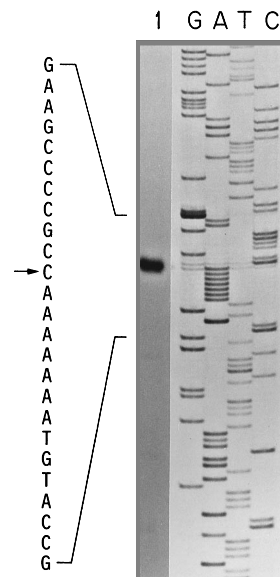

FIG. 6. Location of the in vivo mRNA 5′ terminus of the *fumC* transcript. Lane 1 represents primer extension reactions using mRNA prepared from cells grown aerobically; lanes T, G, C, and A show the DNA sequencing reaction products from the corresponding region upstream of *fumC*. The arrow indicates the position which corresponds to the observed end of the *fumC* mRNA. The primer corresponded to nucleotide positions +47 to +65 relative to the *fumC* initiation codon.

the addition of superoxide further stimulates its response. Neither the Δ*soxR* nor the *soxR** mutant had any effect on *fumA-lacZ* expression (data not shown).

Effect of iron availability on *fumA-lacZ* and *fumC-lacZ* expression. Iron is an essential component of the FumA fumarase, whereas FumC does not need iron for its activity (47). To test if iron limitation affects *fumA* or *fumC* expression, cells were grown in the presence of the iron chelator 2,2′-dipyridyl. Under these iron-limiting conditions, *fumA-lacZ* expression was not greatly affected during aerobic or anaerobic growth (data not shown). However, under the same conditions, *fumC-lacZ* expression was elevated eightfold aerobically and threefold anaerobically (Fig. 5). When iron was added in excess, *fumC-lacZ* expression returned to the level seen when 2,2′-dipyridyl was not added.

The iron control of *fumC-lacZ* expression was also examined in the Δ*soxR* and *soxR** strains grown under iron-limited or iron-excess conditions (Fig. 5) and compared with *fumC-lacZ* expression in a wild-type strain. During aerobic cell growth, the iron starvation induced *fumC-lacZ* expression sevenfold above the level seen in the nonstained wild-type strain. However, expression was abolished in the Δ*soxR* strain. Thus, iron control of *fumC* expression is SoxR dependent. In the *soxR** strain, *fumC-lacZ* expression was constitutive under all conditions examined. During anaerobic cell growth, *fumC-lacZ* expression was lowered considerably, but it exhibited the same pattern of gene expression as observed in each of the strains grown aerobically.

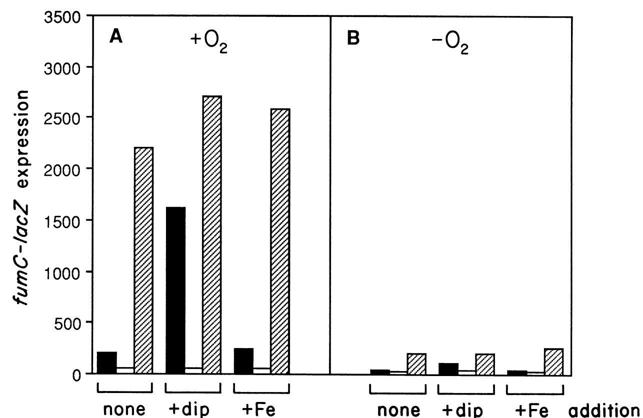

FIG. 5. Effects of iron limitation on induction of *fumC-lacZ* expression during aerobic (A) and anaerobic (B) cell growth. Solid bars represent the wild-type strain, open bars represent the Δ*soxR* strain, and hatched bars indicate the *soxR** mutant. Cells were grown in minimal medium supplemented with glucose (40 mM) without any addition (none), with the iron chelator 2,2′-dipyridyl (150 μM; +dip), or with excess iron (80 μM $FeSO_4$; +Fe). Units are given in nanomoles of ONPG hydrolyzed per minute per milligram of protein.

Location of the *fumC* mRNA 5′ end. Primer extension reactions were performed to locate the start site for *fumC* transcription. A single 5′ mRNA terminus corresponding to a position 120 nucleotides upstream of *fumC* translation start was identified (Fig. 6). This site is just 5′ of a proposed SoxS binding site for the *fumC* gene (7). It is not clear if this site represents the actual 5′ end for *fumC* transcription or if it is a termination/processing site for transcription from the *fumA* promoter. However, no additional mRNA termini were observed in these experiments.

DISCUSSION

Why does *E. coli* contain three distinct fumarases? One experimental approach to answer this question has been to examine the patterns of *fumA*, *fumB*, and *fumC* gene expression under different cell growth conditions. It was previously shown that the *fumA* gene was expressed at a twofold higher level during aerobic than during anaerobic conditions, while the *fumB* gene was expressed at a higher (ca. 1.5-fold) level under anaerobic conditions (41). In contrast, *fumC* expression was reported to be low and relatively constant (41). In this study, we performed a more detailed analysis of *fumA* and *fumC* gene expression. The *fumA* and *fumC* genes are each expressed at significantly higher levels (ca. fourfold) during aerobic than during anaerobic cell growth (Table 2). The expression is also catabolite controlled and responsive to iron limitation and oxidative stress. These data are consistent with

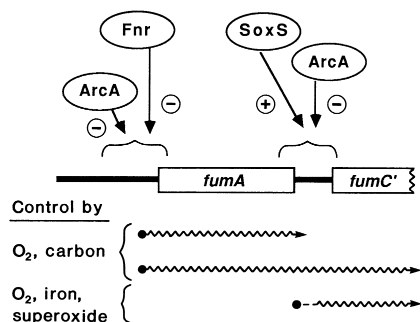

FIG. 7. Regulation of the *fumA* and *fumC* genes by the *fnr*, *arcA*, and *soxS* gene products. The *fumA* gene is transcribed from its own promoter, while *fumC* transcription occurs from both the *fumA* and *fumC* promoters. The transcripts are indicated by wavy lines. Whereas the *fumA* promoter is regulated by both Fnr and ArcA, transcription from the *fumC* promoter is regulated by ArcA and SoxR. Control by carbon, oxygen, iron, and superoxide is indicated.

the notion that the FumA and FumC enzymes both participate in the TCA cycle reactions in which FumA is the major enzyme used under normal growth conditions. FumC is most abundant under conditions of iron limitation and oxidative stress. The genes for each enzyme are also expressed at high levels in the cell under anaerobic conditions.

Aerobic/anaerobic control is mediated by ArcA and Fnr. We show that the aerobic/anaerobic control of the *fumA* and *fumC* genes is mediated by the ArcA and Fnr regulatory proteins (Table 4). This control is complex: it involves action by ArcA and Fnr as negative regulators of *fumA* and *fumC* expression from the *fumA* promoter. ArcA also serves to negatively regulate *fumC* expression from the *fumC* promoter (Fig. 7). A prior investigation of *fumA* and *fumC* expression led to somewhat different conclusions (41). However, those studies were performed with *fumA-lacZ* and *fumC-lacZ* gene fusions that contained relatively small regions of upstream DNA compared with the fusions used in this study. Second, the role of the *fumA* promoter in *fumC* gene expression was not examined. Of the TCA cycle genes examined thus far, only the promoters for the *fumA* and *sdhCDAB* genes are controlled by both ArcA and Fnr (this study and references 18 and 31). The *gltA*, *acnA*, and *mdh* genes appear to be controlled by ArcA only (11, 29, 30).

The *fumC* promoter provides the response to iron limitation and superoxide radicals. The *fumC* gene is expressed in part by readthrough from the *fumA* promoter. However, the *fumC* promoter alone is responsible for the induction of *fumC* expression during iron limitation or when the cell is exposed to oxidative stress (Fig. 3 to 5). Expression from the *fumC* promoter is increased eightfold upon iron limitation and 20-fold by the superoxide-generating agent paraquat. In contrast, no iron control of the *sdhCDAB*, *mdh*, or *gltA* genes was observed (29–31). As *fumC* gene expression is greatly increased as iron becomes limiting, the cell continues to express the *fumA* gene as it did with iron in excess. This finding suggests that the cell may be unable to produce a functional FumA enzyme which requires the assembly of a 4Fe-4S iron sulfur center for its activity. The FumC enzyme that lacks iron is ideally suited to meet the cell's need for fumarase activity as iron becomes limited. It is unknown whether the *fumB* gene is iron controlled.

It was previously shown that synthesis of the heat-stable FumC fumarase was increased by paraquat (23). We demonstrate that this is due to specific transcription from the *fumC* rather than the *fumA* promoter (Fig. 3). This induction is mediated by the SoxR protein, since it is abolished in a Δ*soxR* mutant and induced in a *soxR** strain even without the presence of paraquat. Interestingly, induction of *fumC* transcription in the *soxR** mutant still responded to superoxide radicals as seen when paraquat was added to the medium. The SoxR protein required iron for activation of the *soxS* promoter (16). The constitutive expression of *fumC-lacZ* in a *soxR** strain is consistent with the *soxR** phenotype (Fig. 5). This would allow constitutive expression of the SoxS protein, which serves as an activator for many paraquat-induced genes (1, 22, 44). Whether other SoxRS-controlled genes are also iron controlled like *fumC* is unknown.

Catabolite repression of *fumAC* expression is provided by the *fumA* promoter. The expression of the *fumA* and *fumC* genes is also regulated in response to the type of carbon compound used for cell growth (Table 2). This control occurs under both aerobic and anaerobic conditions: during aerobic conditions, a 20-fold range in *fumA* gene expression was seen, whereas a 10-fold range was seen for *fumAC*. It is evident that the carbon control of *fumC* occurs from the *fumA* promoter, as *fumC-lacZ* expression from λSJP27 did not vary more than about twofold. Thus, transcription readthrough from the *fumA* promoter is a significant factor in *fumC* expression under all cell growth conditions. The expression pattern of several other TCA cycle genes (i.e., *sdhCDAB*, *gltA*, and *mdh*) also varies with the type of carbon source used (29–31). This pattern generally shows increased gene expression in the order glucose, galactose, xylose, glycerol, and the TCA intermediates succinate and fumarate. It is not yet known whether Crp provides this control over the *fumA* and *fumC* genes. Crp control has been shown for the aconitase gene, *acnA* (11).

Proposed roles for the FumA, FumB, and FumC fumarases in *E. coli*. The pattern of oxygen control of *fumA* transcription indicates that the FumA fumarase is an enzyme of the TCA cycle (Fig. 8), in support of the prior proposal by Wood and Guest (41). Together, carbon and oxygen modulate *fumA* expression over a 65-fold range (Table 2). We also show transcription of the *fumA* gene in response to oxygen is regulated by two global regulatory proteins, ArcA and Fnr. FumB appears to be slightly more abundant (ca. 1.5-fold) under anaerobic conditions. On the basis of these studies, we propose the *fumC* gene product to be an alternative aerobic TCA cycle enzyme that can substitute for FumA under conditions of extreme iron limitation and during oxidative stress. The synthesis of low amounts of the FumC fumarase by transcription from the *fumA* and *fumC* promoters may provide *E. coli* with the flexibility to adapt to changing environments of iron and superoxide stress. As the *fumC* gene is regulated by oxygen, carbon supply, iron availability, and oxidative stress, the cell has evolved a rather complex strategy for meeting its requirement for fumarase activity during adaption to diverse environmental conditions. Like *fumA* expression, *fumC* expression can vary over a considerable range (ca. 45-fold; Table 3).

Why does the cell make an iron-containing FumA fumarase

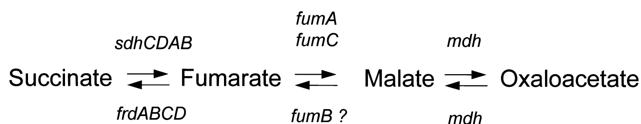

FIG. 8. Proposed roles of the *fumA*, *fumB*, and *fumC* genes in the TCA cycle and noncyclic pathway in *E. coli*. The reactions catalyzed by the indicated gene products are indicated: *gltA*, citrate synthase; *fumA*, FumA fumarase; *fumB*, FumB fumarase; *fumC*, FumC fumarase; *sdhCDAB*, succinate dehydrogenase; *frdABCD*, fumarate reductase.

if it can also synthesize the FumC fumarase? The differential expression of the *fumA* and *fumC* genes under iron-limiting conditions suggests that the FumA enzyme is somehow better suited to the cell's needs under normal conditions than the FumC fumarase. FumC is a backup enzyme and is by implication more costly either to synthesize or to retain by the cell.

ACKNOWLEDGMENTS

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