Involvement of Fnr and ArcA in Anaerobic Expression of the tdc Operon of Escherichia coli

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Anaerobic expression of the tdcABC operon in Escherichia coli, as measured by LacZ activity from single-copy tdc-lacZ transcriptional and translational fusions, is greatly reduced in strains lacking two global transcriptional regulators, Fnr and ArcA. The nucleotide sequence of the tdc promoter around −145 shows significant similarity with the consensus Fnr-binding site; however, extensive base substitutions within this region had no effect on Fnr regulation of the tdc genes. A genetic analysis revealed that the effect of Fnr on tdc is not mediated via ArcA. Furthermore, addition of cyclic AMP to the anaerobic incubation medium completely restored tdc expression in fnr and arcA mutants as well as in strains harboring mutations in the Fnr- and ArcA-dependent pfl gene and the Fnr-regulated glpA and frd genes. These results, taken together with the earlier finding that tdc expression is subject to catabolite repression by intermediary metabolites, strongly suggest that the negative regulatory effects of mutations in the fnr and arcA genes are mediated physiologically due to accumulation of a metabolite(s) which prevents tdc transcription in vivo.

When incubated anaerobically, Escherichia coli, a typical facultative anaerobe, represses a wide array of aerobic genes and concomitantly expresses certain others which encode key enzymes and proteins needed for anaerobic metabolism. Two global transcriptional regulators, Fnr (fumarate and nitrate reductase activator) and ArcA (a member of the two-component ArcBA sensor-regulator system), have generally been implicated in activating a large number of anaerobic genes and repressing many aerobic genes in oxygen-limiting growth conditions, respectively (reviewed in references 9, 15, 36, and 40). These regulatory proteins appear to have their individual specificities in regulating groups of genes and/or regulons. However, several operons in E. coli are under complex dual control of Fnr and ArcA. Both proteins serve as transcriptional activators of the pfl operon (30), whereas Fnr represses the cydAB operon while ArcA activates its transcription (9). A recent study on the expression of several aerobic and anaerobic respiratory pathway genes as a function of oxygen concentration revealed that Fnr functions optimally at low oxygen saturation, whereas ArcA controls gene expression at higher levels of oxygen (39). It is interesting that the anaerobic activation of arcA transcription is increased three- to fourfold in the presence of Fnr (2). These data clearly suggest that these two regulatory proteins are involved in overall coordination of expression of various anaerobic and aerobic genes in response to the varying oxygen status of the cell.

In molecular terms, Fnr, a sequence-specific DNA binding protein, recognizes a consensus palindromic nucleotide sequence, TTGATNNANATCAA (where N is any base), in the target promoters to activate transcription (36). The active form of Fnr is believed to be a homodimer with an iron-sulfur center which is redox sensitive (8, 16, 19, 27), although a recent model (24) proposed that Fnr exists as monomers, and two monomers occupy the two half-sites of the Fnr recognition sequence in DNA.

Regulation of gene expression by the ArcBA system involves a transphosphorylation event (12, 14). ArcB, the sensor component, undergoes autophosphorylation in response to some metabolic signal and then transfers the phosphate moiety to ArcA. The active phosphorylated form of ArcA (ArcA-P) serves as a regulator to control transcription. Recently, it has been reported that ArcA-P binds specifically to the target promoters of several oxygen-regulated genes, pfl, sodA, gltA, and lctPRD, in vitro (4, 22, 38). Although a primary nucleotide sequence, (A/T)GTTAATTA(A/T), required for ArcA-P binding has been derived from computer alignment of various promoters regulated by ArcA, further studies are needed to establish the consensus ArcA-P box (22). How the ArcBA system senses the availability of oxygen is not yet fully understood.

The anaerobically induced tdc operon of E. coli, which consists of three genes encoding a transcriptional regulator (TdcA), the biodegradative threonine dehydratase (TdcB), and a membrane-associated L-threonine-L-serine permease (TdcC) (Fig. 1A), is implicated in transport and metabolism of the amino acids during anaerobic growth to provide a source of metabolic energy (3, 6, 7, 37). Upstream of tdcABC and transcribed in opposite orientation is tdcR, which specifies a small protein that is essential for tdc transcription (10, 33). In addition to an anaerobic (microaerobic) environment and the two operon-specific transcription factors TdcA and TdcR, the efficient expression of the operon requires two global regulatory proteins, integration host factor (IHF) and the cyclic AMP (cAMP)-catabolite gene activator protein (CAP) complex (42, 44). Genetic and biochemical experiments revealed that TdcA, IHF, and CAP occupy their unique binding sites on tdcP at −175, −104, and −41, respectively, with respect to the tdcP transcription start site at +1 (Fig. 1B), and act in concert most likely by bending and looping the promoter DNA to form an active transcription complex (10, 42). We continue to examine the tdc operon as a well-defined model system to investigate the basic mechanisms underlying anaerobic gene expression. In this report, we present evidence that transcription of the tdc operon in vivo is greatly reduced in strains lacking the fnr and

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likely affect tdc gene products. Furthermore, both Fnr and ArcA most likely affect tdc expression via indirect means by influencing carbon metabolism during anaerobic growth.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth media.** The bacterial strains and plasmids used in this study are listed in Table 1. Recombinant tdc-lacZ transcriptional and translational fusions of λ phages were constructed by recombining λRZS with the corresponding plasmids, and single-copy λphages in appropriate hosts were obtained as described previously (31). Generalized transduction, using phage P1, was performed by the method of Miller (25). Strains were routinely grown in Luria-Bertani (LB) medium (25). TYE medium used for TYE medium (25) and host were obtained as described previously (31). Generalized transduction, using phage P1, was performed by the method of Miller (25). Strains were routinely grown in Luria-Bertani (LB) medium (25). TYE medium used for enzyme induction contained 2% tryptone and 1% yeast extract supplemented with 1% glucose, 10% d-glucose, 0.67% yeast extract, 0.5% L-tryptophan, 0.1% L-lysine, and 0.5% MgSO4.

Plasmid constructions. To isolate promoter mutations containing various base substitutions between −156 and −139, an 1.180-bp EcoRI-HindIII fragment (nucleotides 1197 to 2377) (32) from plasmid pSH241 was cloned into pSC1925. This study

Plasmid pSC1925 contains the promoterless coding region of tdcR and tdcA (B). The numbers with minus signs above the sequence represent upstream distance in nucleotides from the transcription start site of tdcA, designated +1. The boxed regions designated IHF (−104) and CAP (−41) represent sequences protected by IHF and CAP, respectively, during footprinting. The region TdcA (−135) with inverted repeat signifies the TdcA-binding site. The boldface letters between −152 and −139 in panel B show sequence similarity with the consensus Fnr-binding site.

![Diagram](https://via.placeholder.com/150)

**FIG. 1.** Organization of the tdc operon (A) and nucleotide sequence of the region between tdcR and tdcA (B). The numbers with minus signs above the sequence represent upstream distance in nucleotides from the transcription start site of tdcA, designated +1. The boxed regions designated IHF (−104) and CAP (−41) represent sequences protected by IHF and CAP, respectively, during footprinting. The region TdcA (−135) with inverted repeat signifies the TdcA-binding site. The boldface letters between −152 and −139 in panel B show sequence similarity with the consensus Fnr-binding site.

![Table](https://via.placeholder.com/150)

**TABLE 1.** Bacterial strains and plasmids used

<table>
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<th>Strain or plasmid</th>
<th>Genotype or derivation</th>
<th>Reference or source</th>
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<td>RK4353</td>
<td>araD139 Δ(argF-lac)U169 rpsL150 deoC1 relAI ptsF25 hbdD5301 gyrA219 non-9</td>
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<td>VJS7141</td>
<td>RK4353 ftn-271::Tn10</td>
<td>25</td>
</tr>
<tr>
<td>CAG18552</td>
<td>MG1655 zej-3144::Tn10 kan</td>
<td>35</td>
</tr>
<tr>
<td>QC2003</td>
<td>F' ΔlacU169 rpsL (sdh-lac) Δftr zdc-235::Tn9</td>
<td>2</td>
</tr>
<tr>
<td>QC2085</td>
<td>F' ΔlacU169 rps Δarc::Tn10</td>
<td>2</td>
</tr>
<tr>
<td>TL45</td>
<td>MC4100 glpR2 Δ(gfp-TglopA)4593 gvrA</td>
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<tr>
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<tr>
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<td>This study</td>
</tr>
<tr>
<td>SCM4200</td>
<td>MC4100 Δarc::Tn10 [P1 (QC2085) × MC4100]</td>
<td>This study</td>
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<tr>
<td>SCM4220</td>
<td>MC4100 Δftr zdc-235::Tn9 [Tn10 [P1 (QC2003) × SCM4200]</td>
<td>This study</td>
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<tr>
<td>SCM4250</td>
<td>MC4100 Δgpf-25 Δ (gfp::trpC184)</td>
<td>This study</td>
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<td>Plasmids</td>
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<tr>
<td>pY2W45</td>
<td>tdcR+ tdcP+ tdcA+ lacZ</td>
<td>43</td>
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<td>pY7W17</td>
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<td>pYW83</td>
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<td>pYW84</td>
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<td>This study</td>
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<td>pYW85</td>
<td>Base substitutions in tdcP (Fig. 2); otherwise identical to pSH241</td>
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<td>pYW86</td>
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<td>pCL1921</td>
<td>Sp+ Str+ lacPO-lacZ</td>
<td>20</td>
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<tr>
<td>pSC1925</td>
<td>Promoterless arcA+ cloned into the Smal site of pCL1921</td>
<td>This study</td>
</tr>
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</table>
AGT 3' (reverse), were used to amplify the arc^+ coding region by PCR, using the protocol supplied by Perkin-Elmer Cetus. The PCR product was purified by agarose gel electrophoresis, cut with SmaI, and cloned into SmaI-cleaved pCL1921. The plasmid was sequenced to verify the construct.

**Enzyme induction and assay.** Cells were grown aerobically at 37°C for approximately 16 h in LB medium, washed, and resuspended in fresh TYE. Small aliquots of cells were incubated anaerobically at 32°C in TYE without shaking for 6 to 8 h. The threonine dehydrogenase activity of toluene-treated cells was determined as described previously (11). Specific activity was expressed as nanomoles of malate oxidized per minute per milligram of protein. Malate dehydrogenase activity in cell extracts was measured spectrophotometrically as described by Kito (17), and specific activity was expressed as nanomoles of malate oxidized per minute per milligram of protein. β-Galactosidase activity in sodium dodecyl sulfate-chloroform-permeabilized cells was measured as described by Miller, and specific activity was expressed in Miller units [25].

**Other methods.** All DNA manipulations were carried out as suggested by Sambrook et al. (28). Restriction enzyme digests of DNA were performed according to the manufacturers' specifications. The Genetics Computer Group software package (University of Wisconsin, Madison) was used for DNA sequence analysis.

**RESULTS AND DISCUSSION**

**Fnr and ArcA requirements for tdc expression.** To examine the effects of fnr and arcA gene products on the anaerobic expression of the tdc operon, we constructed individual single-copy $tdc_{AB}$-

![Fig. 2. Nucleotide substitutions between -156 and -135 encompassing the putative Fnr-like sequence in $tdcP$ and relative lacZ expression (with respect to the wild type) from $tdc_{AB}$-lacZ fusions harboring the promoter mutations in fnr^+ and fnr strains. Boldface letters show base substitutions made in the wild-type $tdcP$ in pSH241. The boxed regions represent Fnr left half-site and Fnr right half-site. Also shown are the consensus Fnr and CAP recognition sequences.](http://jb.asm.org/)

![Table 2. Fnr and ArcA requirements for anaerobic tdc expression](http://jb.asm.org/)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>$\beta$-Galactosidase activity from lysogen*</th>
</tr>
</thead>
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<td></td>
<td></td>
<td>$\lambda$SH241b</td>
</tr>
<tr>
<td>RK4353</td>
<td>Wild type</td>
<td>560</td>
</tr>
<tr>
<td>VJS1741 fnr</td>
<td>65 (0.12)</td>
<td>205 (0.26)</td>
</tr>
<tr>
<td>MC4100</td>
<td>Wild type</td>
<td>920</td>
</tr>
<tr>
<td>SC4M200</td>
<td>$tdc_{AB}$-lacZ</td>
<td>47 (0.05)</td>
</tr>
</tbody>
</table>

*Expressed in Miller units. Numbers in parentheses are relative to the wild-type value of 1.0.

**Independent action of Fnr and ArcA.** Recently, Compan and Touati (2) reported that anaerobic activation of arcA transcription in E. coli requires Fnr. This observation raised the important question of whether Fnr affects tdc expression directly by influencing the tdc promoter ($tdcP$), similar to that seen with many Fnr-regulated genes, or, alternatively, acts indirectly via transcriptional activation of arcA. To distinguish between these two possibilities, we cloned a promoterless $arc^+$ coding region into expression vector pCL1921, yielding pSC1925 (see Materials and Methods), to drive arcA transcription from an Fnr-independent promoter. When this plasmid was introduced into an fnr mutant containing a $tdc_{AB}$-lacZ fusion, no stimulation of LacZ activity was detected from the lysogen (Table 3, experiment I). If the only role of Fnr in tdc expression were to activate arcA transcription, we would have seen LacZ activity in the fnr mutant harboring pSC1925. Thus, lack of tdc expression in fnr arcA^+ cells implies an independent function of Fnr. Control experiments included in Table 3 show that ArcA protein was indeed expressed from the multicopy pSC1925 plasmid in the presence (experiment II) and absence (experiment III) of Fnr: ArcA from pSC1925 restored tdc expression in the fnr- arcA strain and reduced malate dehydrogenase activity (encoded by mldh) in the fnr arcA double mutant. Iuchi and Lin (13) have shown that Mdh expression is elevated in arcA mutant during anaerobic incubation of E. coli. We conclude from these experiments that the effect of Fnr on tdc is independent of ArcA.
The Fnr-like site in tdcP is not involved in tdc expression. The evidence for Fnr regulation of gene expression is usually deduced from a combination of several types of experiments: a comparison of gene activity in fnr" and fnr strains, identification of a sequence in the target promoter having significant similarity with the consensus Fnr recognition site, and mutational substitutions of bases within the Fnr-like sequence in the promoter to assess the effect of Fnr protein. In a few cases, additional evidence has been obtained from footprinting experiments using wild-type and mutant Fnr proteins, which have been difficult to purify until recently because of their extreme oxygen lability (24).

The nucleotide sequence in tdcP around −145 shows significant similarity with the consensus Fnr-binding site with a two-base mismatch (shown in boldface letters in Fig. 1B). Because several Fnr-regulated promoters show some flexibilities in having different nucleotides in these mismatched positions (36), we inferred that Fnr might bind to tdcP DNA at this site to activate transcription and that mutations introduced within this 14-bp sequence would abolish tdc expression in an fnr" host. Accordingly, we made several nucleotide substitutions between −156 and −138 (shown in boldface letters in Fig. 2) by site-directed mutagenesis according to the Kunkel procedure (18) as detailed elsewhere (42). pYW77 had substitutions in three highly conserved bases within the Fnr consensus which are crucial for Fnr action, whereas pYW83 contained substitutions that created a perfectly matched Fnr consensus sequence. pYW85 and pYW86 each had five base substitutions to yield, respectively, Fnr left half-site and Fnr right half-site mutants. Some additional base substitutions were made to introduce new restriction sites for mutant screening.

Single-copy lysogens of the mutant plasmids harboring tdcaB-lacZ fusion were constructed in strains RK4353 (fnr") and JVS1741 (fnr). Assay of β-galactosidase activities of these Fnr site mutants in fnr" cells incubated anaerobically in TYE showed no significant difference in LacZ levels from that seen with the ASH241 lysogen harboring the wild-type tdcP sequence or that of the λYW83 lysogen containing a perfectly matched Fnr consensus site (Fig. 2). Furthermore, the mutant lysogens in fnr" cells had drastically reduced LacZ levels, similar to that found in ASH241, indicating that a functional Fnr protein is still necessary for tdc expression in the Fnr site mutants.

To rule out the unlikely possibility that the Fnr half-site mutants bind Fnr, we replaced the 22-bp Fnr-like sequence with a perfectly matched CAP site (1, 36) to yield pYW84. A single-copy lysogen of λYW84 showed high LacZ levels in fnr" strains (Fig. 2), similar to the Fnr site mutants. Thus, complete replacement of the Fnr-like site with a CAP site did not abolish the Fnr requirement for tdc expression. These results clearly show that the Fnr-like sequence around −145 in tdcP is not involved in the Fnr-dependent expression of the tdc operon. This finding was not totally surprising because the Fnr-binding sites are usually found closer (between −35 and −45) to the transcription start sites of mRNA (36). A computer search of the region between the tdcR and tdcA reading frames (Fig. 1) failed to find additional sequences with similarity to the 14-bp consensus Fnr-binding site.

Fnr and ArcA affect tdc expression by influencing anaerobic carbon metabolism. The minimal requirement for tdc expression is incubation of E. coli cells in a mixture of four amino acids, threonine, serine, valine, and isoleucine, supplemented with fumarate and cAMP (H4 medium) in an anaerobic environment (11). Because each component in this defined mixture is absolutely essential, it was proposed that not the amino acids themselves but some metabolite(s) derived anaerobically in reactions involving an electron acceptor may function as a putative inducer for tdc expression. In view of this finding, it is possible to envisage potential involvement of Fnr and/or ArcA in generating an anaerobic inducer for tdcP, because these regulatory proteins are known to regulate a number of E. coli genes whose products are needed for substrate fermentation or for anaerobic respiration. For example, Fnr is required for the expression of glpA, pfl, and frd, encoding, respectively, the anaerobic glyceraldehyde-3-phosphate dehydrogenase, pyruvate formate-lyase, and fumarate reductase, and ArcA enhances pfl operon expression (4, 15, 36); in arcA and fnr strains, lack of enzymes encoded by these genes would block carbon metabolism and thus alter metabolite levels in the cell (Fig. 3).

To test whether failure to express tdc in fnr and arcA mutants might be attributable to the absence of one or more of these enzymes, we constructed individual single-copy lysogens of tdcaB-lacZ in glpA, pfl, and frd mutants (all fnr" and arcA"") and their isogenic parent MC4100. Assay of β-galactosidase activities in these strains incubated anaerobically in TYE revealed greatly reduced LacZ levels in all three mutants relative to the wild-type strain (Table 4). These results indicate that interruption of carbon flow through the anaerobic pathway reactions leads to reduced tdc expression; they also suggest that mutations in the fnr and arcA genes appear to mimic the phenotypes of some mutants of carbon metabolism.

How does a block in anaerobic carbon metabolism decrease tdc transcription in vivo? We reported earlier that chromosomal TdcB expression was subject to catabolite repression by several intermediary metabolites such as glucose, gluconate,
The data presented in Table 4 indeed show the restoration of LacZ level from tdcAB-lacZ (11). These experiments have now been confirmed by using a lysogen in MC4100 (wild type) incubated anaerobically in TYE: at 50 mM, glucose, pyruvate, oxaloacetate, and succinate, individually, reduced LacZ activity three- to fourfold as compared to wild-type cells, a metabolite(s) derived during anaerobic reactions has a crucial role in activating tdc promoter. The results displayed in Table 4 suggest that the same putative inducer molecule appears to be present in cells lacking Fnr, ArcA, and the enzymes encoded by glpA, pfl, and frd, because all five mutants are able to express tdc in the presence of cAMP. The chemical identity of the inducing metabolite and how it activates tdc transcription remain unknown. Further investigation with mutants of anaerobic carbon metabolism and addition of fermentation end products to the growth medium to restore tdc expression would help in identifying the inducing metabolite.

A possible to argue in this context that abnormally elevated levels of cAMP-CAP may have an epistatic effect on the activation of tdc transcription. However, we found no significant effect of overexpressed wild-type CAP protein (with or without added cAMP) or a CAP* mutant protein (which acts independent of cAMP) in rescuing tdcR and tdcA mutants or enhancing tdc expression in the wild-type strain (data not shown).

Biochemical and genetic experiments performed to date clearly established that anaerobic metabolism is necessary for tdc operon expression in vivo; no tdc gene products are seen during aerobic incubation of cells in various media. In wild-type cells, a metabolite(s) derived during anaerobic reactions has a crucial role in activating tdc promoter. The results displayed in Table 4 suggest that the same putative inducer molecule appears to be present in cells lacking Fnr, ArcA, and the enzymes encoded by glpA, pfl, and frd, because all five mutants are able to express tdc in the presence of cAMP. The chemical identity of the inducing metabolite and how it activates tdc transcription remain unknown. Further investigation with mutants of anaerobic carbon metabolism and addition of fermentation end products to the growth medium to restore tdc expression would help in identifying the inducing metabolite.

An unusual aspect of tdc regulation revealed in this study was that the effects of frd and arcA mutations on tdc expression are mediated strictly through physiological means: accumulation of a metabolite(s) which represses tdc transcription. Interestingly, an opposite physiological situation was uncovered in earlier studies on the Fnr-dependent expression of the formate-hydrogen lyase (FHL) enzyme complex. Formate, a product of pyruvate metabolism catalyzed by the pfl gene product, functions as an inducer of the transcriptional activator, FhlA, which then activates the genes encoding the FHL complex (23, 26). In an frr mutant, reduced expression of Pfl would decrease the formate level in the cell, thus preventing Fhl expression. Thus, lack of a single regulatory gene product such as Fnr negatively affects the expression of two operons by two entirely different mechanisms. In view of these examples, it is reasonable to suggest that the roles of global regulatory proteins in specific gene transcription must be examined in the larger context of cellular metabolism.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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**TABLE 4. Decreased tdc expression in various mutants and its restoration by cAMP**

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<tr>
<th>Strain</th>
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† Expressed in Miller units. Numbers in parentheses are relative to the wild-type value of 1.0.