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The Molybdate-Responsive *Escherichia coli* ModE Transcriptional Regulator Coordinates Periplasmic Nitrate Reductase *(napFDAGHBC)* Operon Expression with Nitrate and Molybdate Availability

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Expression of the *Escherichia coli napFDAGHBC* operon (also known as *aeg46.5*), which encodes the periplasmic molybdoenzyme for nitrate reduction, is increased in response to anaerobiosis and further stimulated by the addition of nitrate or to a lesser extent by nitrite to the cell culture medium. These changes are mediated by the transcription factors Fnr and NarP, respectively. Utilizing a *napF-lacZ* operon fusion, we demonstrate that *napF* gene expression is impaired in strain defective for the molybdate-responsive ModE transcription factor. This control abrogates nitrate- or nitrite-dependent induction during anaerobiosis. Gel shift and DNase I footprinting analyses establish that ModE binds to the *napF* promoter with an apparent K_d of about 35 nM at a position centered at −133.5 relative to the start of *napF* transcription. Although the ModE binding site sequence is similar to other *E. coli* ModE binding sites, the location is atypical, because it is not centered near the start of transcription. Introduction of point mutations in the ModE recognition site severely reduced or abolished ModE binding in vitro and conferred a *modE* phenotype (i.e., loss of molybdate-responsive gene expression) in vivo. In contrast, deletion of the upstream ModE region site rendered *napF* expression independent of *modE*. These findings indicate the involvement of an additional transcription factor to help coordinate nitrate- and molybdate-dependent *napF* expression by the Fnr, NarP, NarL, and ModE proteins. The upstream ModE regulatory site functions to override nitrate control of *napF* gene expression when the essential enzyme component, molybdate, is limiting in the cell environment.

Molybdenum is an essential component of the molybdopterin cofactor in nearly all species, including bacteria, plants, and animals, where it is located at the active center of a certain oxidoreductases including nitrate reductase, dimethyl sulfoxide (DMSO) reductase, trimethylamine-*N*-oxide (TMAO) reductase, and biotin sulfoxide reductase. *Escherichia coli* has evolved a regulatory scheme to coordinate molybdenum uptake, in the form of molybdate, which is utilized for cofactor synthesis and assembly into the mature molybdoenzymes. The key regulatory element in this scheme is the ModE protein, a molybdate-responsive transcription factor the structure of which was recently determined (14). ModE was first identified in *E. coli* as the negative regulator of the high-affinity molybdate uptake system, encoded by the *modABCD* operon (11, 23, 33). Utilizing a combination of in vivo and in vitro approaches, ModE was shown to bind the *modA* promoter in a molybdate-dependent fashion (2, 11, 23). ModE also binds and regulates expression of the *moaADCDE* and *dmsABC* operons, which encode enzymes involved in the first steps of molybdate assimilation into molybdopterin and the DMSO reductase, respectively (1, 20, 21, 23). Recently ModE was shown to play a minor role in regulating the *hyc* and *nar* operons in *E. coli* (29). Finally, ModE orthologues have been identified in a wide number of bacteria, including *Azotobacter vinelandii*, *Rhodobacter capsulatus*, *Ralstonia eutropha* (*Alcaligenes eutrophus*), and *Thiosphaera pantotropha* (*Paracoccus denitrificans*) (reviewed in reference 13). ModE has been shown to regulate various molybdate-associated operons in several of these cases (18, 24, 32).

In this study, we examine the role of ModE in regulating the expression of the *napFDAGHBC* operon of *E. coli*. Sequence and biochemical analysis indicates this operon encodes a molybdenum-containing periplasmic nitrate reductase (10). Expression of the *E. coli napFDAGHBC* operon is positively regulated in response to anaerobiosis by Fnr and by the presence of nitrate and/or nitrite by NarP (5, 7, 26, 34). Here we show that in the absence of ModE, expression of the *napF* operon in response to both nitrate and nitrite is dramatically reduced. A ModE site was identified well upstream of the Fnr and NarP binding sites at the *napF* promoter, and we demonstrate that ModE binds this site in a molybdate-responsive manner to thereby coordinate enzyme synthesis with molybdate availability. We further demonstrate that by deleting the region containing the ModE binding site or by replacing $modE^+$ with a molybdate-independent *modE* allele (*modE**), induction of *napF-lacZ* expression in response to nitrate is rendered molybdate independent.

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MATERIALS AND METHODS

Bacterial strains, phages, plasmids, and culture conditions . The strains, phages, and plasmids used are listed in Table 1. P1 transductions were performed as described previously (17). For β-galactosidase assays, cells were grown either aerobically or anaerobically at 37°C with glucose (20 mM) in sodium phosphate-

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

Strain, plasmid, or phage	Origin	Relevant genotype or phenotype	Source or reference
Strains			
MC4100		F Δ*(argF-lac)U169*	Laboratory stock
PM6	MC4100	*modC modE*::Kan^r	20
PM8	MC4100	*modE*::kan^r	20
Plasmids			
pR415	pBR322	*lacZ*$^+$ *lacY*$^+$ *lacA*$^+$ Amp^r	30
pPM6	pACYC184	*modE*$^+$ Cm^r	20
pPM9	pACYC184	*modE**; encodes a molybdate independent *modE* allele, Cm^r	20
pHW2	pRS415	*napF-lacZ* operon fusion, Amp^r	Laboratory stock
pPM58 and -59	pRS415	*napF-lacZ*; operon fusions with upstream deletions in *napF* promoter region, Amp^r	This study
pPM69	pPM58	*napF-lacZ*; pPM58 with upstream region cloned back in correct orientation, Amp^r	This study
pPM70	pPM58	*napF-lacZ*; pPM58 with upstream region cloned back in reverse orientation, Amp^r	This study
pPM71	pPM69	*napF-lacZ*; pPM69 105-bp insert from the *cat* gene cloned in the *Eco*RI site, Amp^r	This study
pPM54 and -55	pRS415	*napF-lacZ*; operon fusions with mutations in the ModE binding motif, Amp^r	This study
Phages			
λRS45		*lacZ′ lacY*$^+$ *lacA*$^+$	30
λHW2	λRS45	Φ(*napF-lacZ*) (operon fusion)	Laboratory stock
λPM58 and -59	λRS45	Φ (*napF-lacZ*) (operon fusions with upstream deletions in *napF* promoter region)	This study
λPM69	λPM58	Φ(*napF-lacZ*) (λPM58 with upstream region cloned back in correct orientation)	This study
λPM70	λPM58	Φ(*napF-lacZ*) (λPM58 with upstream region cloned back in reverse orientation)	This study
λPM71	λPM69	Φ(*napF-lacZ*) (λPM69 with 105-bp insert from the *cat* gene cloned in the *Eco*RI site)	This study
λPM54 and 55	λRS45	Φ(*napF-lacZ*) (operon fusions with mutations in the ModE binding site)	This study

buffered minimal medium (pH 7) (6). Where indicated, sodium molybdate, sodium nitrate, and sodium nitrite were added at 100 μM, 40 mM, and 2.5 mM, respectively (6).

Recombinant DNA techniques. Transformation of *E. coli*, plasmid isolation, and DNA manipulations were performed as described previously (19). DNA sequencing with the Sequitherm Excel kit (Epicentre Technologies) and PCR amplifications were performed according to the manufacturer's instructions. One strand of all PCR products was sequenced entirely to verify accurate amplification (data not shown).

Plasmid constructions and site-directed mutagenesis. Segments of the *napF* promoter were PCR amplified from *E. coli* MC4100 to introduce flanking *Eco*RI and *Bam*HI restriction sites. The resulting fragments were cloned into the corresponding sites in plasmid pRS415 to generate the following operon fusions: pHW2, pPM58, and pPM59. Mutations in the ModE binding site were introduced into the promoter fragment cloned in pHW2 by splicing by overlap extension (15). All *napF-lacZ* fusions were transferred to λRS45 to generate the corresponding prophages, which were then integrated into the chromosome of the indicated strains in single copy as previously described (23, 31).

Gel shift assays and DNase I footprint analysis. ModE was purified as described previously (23). Gel shift assays and DNase I footprint analysis was performed as described previously (23). DNA fragments were PCR amplified and labeled by end filling with Klenow fragment. Maxam-Gilbert G reactions were run as size markers for the DNase I gel analysis (19).

β-Galactosidase assays. β-Galactosidase levels were determined by hydrolysis of 2-nitrophenyl-β-D-galactopyranoside (ONPG), and units of activity are expressed as nanomoles of ONPG hydrolyzed per minute per milligram of protein (6). The values presented are the average of three independent experiments that deviated less than 10% from the mean.

RESULTS

ModE is required for normal *napF-lacZ* expression under all growth conditions. To determine if ModE plays a role in the regulated expression from the *napF* promoter, we measured expression from a *napF-lacZ* operon fusion, λHW2 (Materials and Methods) (Fig. 1) in both wild-type (MC4100) and *modE*(PM8) backgrounds. Consistent with previous studies (5, 7, 26), *napF-lacZ* expression in the wild-type strain was increased fivefold in response to anaerobiosis and by an additional fivefold or sevenfold through the addition of either nitrate or nitrite, respectively (Table 2). In contrast, *napF-lacZ* expression was impaired in a *modE* strain under all conditions examined. Aerobic and anaerobic expression levels were lowered approximately two- and threefold, respectively. The anaerobic induction of *napF-lacZ e*xpression when either nitrate or nitrite was added was lowered by 9- and by 11-fold, respectively. Provision of *modE*$^+$ in *trans* by introduction of plasmid pPM6 restored *napF-lacZ* regulation to levels seen in MC4100 (Table 2).

To establish if the ModE control was molybdate responsive, we repeated the assays described above with the isogenic strain PM6, which is both *modE* and *modC*. The *modC* mutation blocks molybdate transport via the high-affinity *modABC* uptake system and can be phenotypically suppressed by supplementing the medium with large amounts (ca. 100 μM) of molybdate (27). The introduction of the *modC* mutation into the *modE* background had no further effect on *napF-lacZ* expression. However, when *modE*$^+$ was provided in *trans* (i.e., on plasmid pPM6), a wild-type pattern of *napF* regulation was seen, but only if the medium was supplemented with molybdate (Table 2). Interestingly, under anaerobic growth conditions, *napF-lacZ* expression in strain PM6 containing *modE*$^+$on plasmid pPM6 was slightly higher than in the wild-type strain grown under the same conditions. The addition of nitrate caused a modest reduction in *napF* gene expression (Table 2). When these assays were repeated with a strain containing a molybdate-independent *modE* allele, *modE** (expressed from plasmid pPM9), we observed an even greater increase in anaerobic expression. Again, addition of molybdate, and to a lesser degree nitrate, reduced this effect; this hyper-induction phenomenon is addressed below in the Discussion.

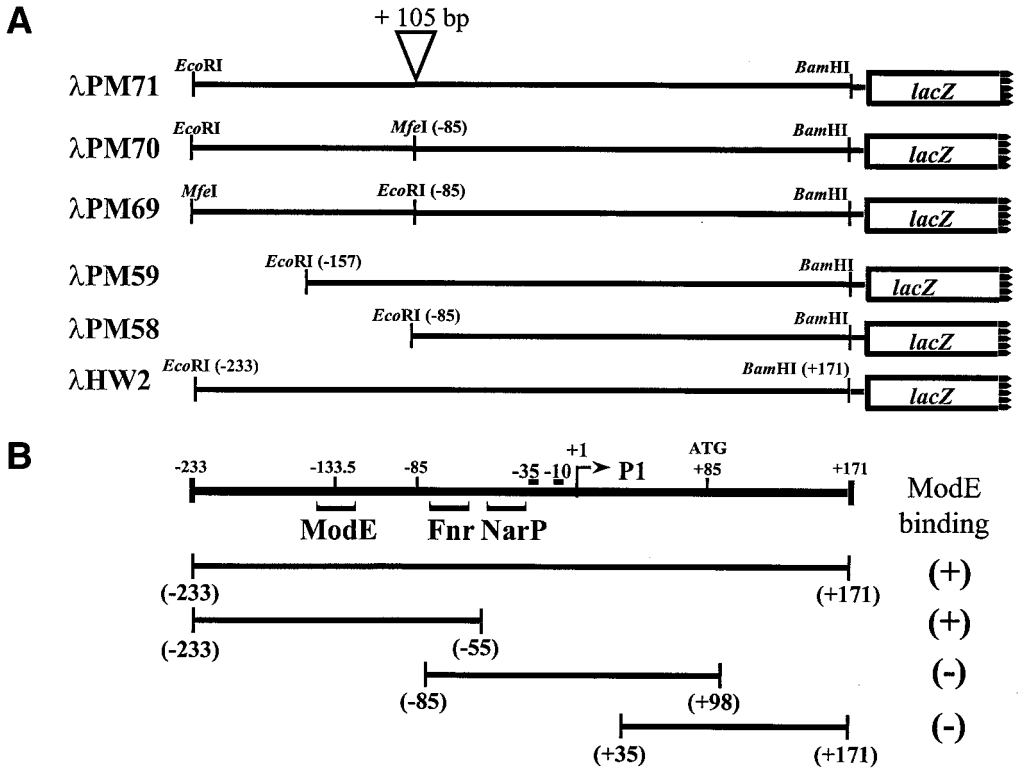

FIG. 1. Deletion analysis and mapping of the ModE binding site at the *E. coli napF* promoter. Shown in panel A are the various DNA fragments, with relevant restriction sites, used in the construction of the *napF-lacZ* operon fusions detailed in the text. Restriction site locations relative to the start site of transcription are indicated in parentheses. Shown below is a schematic representation of the *napF* promoter region. The transcription start site is indicated (5), and coordinates relative to this start site are given in base pairs. The locations of Fnr, NarP (7), and ModE binding sites are indicated with brackets. (B) The DNA fragments used to map the ModE binding site are shown. The ability (+) or inability (−) of ModE to bind a particular fragment in a gel shift assay with 128 nM ModE is indicated.

ModE binds the *napF* promoter well upstream of the start site of transcription. Inspection of the *napF* promoter region revealed a close match to the proposed *E. coli* ModE consensus recognition sequence reported by Anderson, McNicholas, and McNicholas (2, 20, 23). The putative binding site is centered at position −133.5 with respect to the *napF* transcript start site (Fig. 1). To establish that ModE binds the *napF* promoter fragment contained in λHW2 (Fig. 1), we performed gel shift assays with purified protein. ModE bound this fragment with high affinity and displayed an apparent dissociation constant (K_d) of 35 nM (Fig. 2A). When the gel shift was repeated in the presence of molybdate (100 μM) in the reaction buffer (Fig. 2B), a twofold decrease in the apparent K_d was observed (16 μM), consistent with molybdate binding (12). To rule out the possibility that other ModE binding sites exist elsewhere within the *napF* promoter region, we repeated the

TABLE 2. Effect of a *modE* allele on *napF-lacZ* expression in response to anaerobiosis and addition of nitrate

Strain	Relevant genotype[a]	β-Galactosidase activity[b]					
		+ O_2, NA	−O_2				
			NA	+NO_2^-	+NO_3^-	+Mo	+NO_3^-, +Mo
MC4100(pACYC184)	λHW2	50	280	1,900	1,250	265	1,300
PM8(pACYC184)	λHW2 *modE*	25	85	170	130	85	125
PM8(pPM6)	λHW2 *modE* (*modE*$^+$)	55	320	2,000	1,320	300	1,350
PM6(pACYC184)	λHW2 *modE modC*	25	90	ND[c]	130	90	135
PM6(pPM6)	λHW2 *modE modC* (*modE*$^+$)	30	425	ND	140	305	1,350
PM6(pPM9)	λHW2 *modE modC* (*modE*∗)	55	2,340	ND	1,440	330	1,400

[a] λHW2 is a prophage, inserted in the chromosome of the indicated strains in single copy, carrying a *napF-lacZ* operon fusion. Genes, present on multicopy plasmids are shown in parentheses.

[b] Units are given in nanomoles of ONPG hydrolyzed per minute per milligram of protein. Cells were grown in minimal glucose medium under aerobic and anaerobic conditions as described in the text. Sodium nitrate (NO_3^-), sodium nitrite (NO_2^-), and sodium molybdate (Mo) were added where indicated at 40 mM, 2.5 mM, and 100 μM, respectively; NA, no addition of nitrate, nitrite, or molybdate.

[c] ND, not determined.

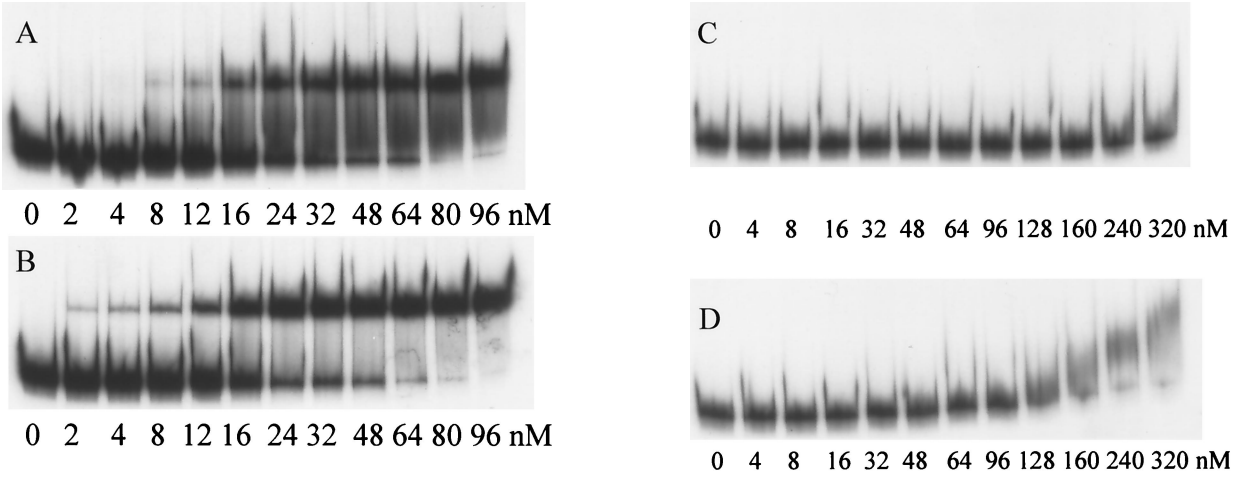

FIG. 2. Interaction of ModE with *napF* promoter DNA. Increasing amounts of purified ModE protein were incubated with a labeled *napF* promoter fragment from λHW2. (A) Wild-type *napF* promoter DNA and ModE without molybdate added. (B) Wild-type *napF* promoter DNA and ModE with 100 μM molybdate added. (C) Mutated *napF* promoter DNA from λPM54. (D) Mutated *napF* promoter DNA from λPM55.

gel shift assays with three truncated promoter fragments (Fig. 1B). These studies localized the ModE binding site between positions −233 and −55 and ruled out the presence of additional ModE sites located near the start of *napF* transcription (data not shown). To precisely identify where ModE binds, DNase I footprinting was performed (Fig. 3). ModE protected a 30-bp region (nucleotides −147 to −118) centered at position −133.5. Thus, this region contains a ModE binding site typical of others on the chromosome (22). Since several nucleotides were not completely protected (i.e., at positions −123, −125, −136, and −138), ModE may reside on one face of the DNA.

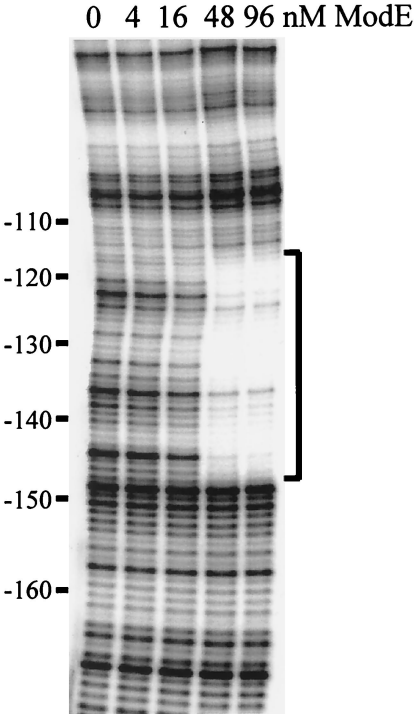

FIG. 3. DNase I footprint analysis of ModE interaction at *napF*. The pattern of protection when ModE is bound at *napF* in the presence of 100 μM molybdate is shown. The vertical bracket indicates the region of protection. Coordinates relative to the start site of transcription are given in base pairs.

Deletion of the ModE binding site relieves the need for *modE*. It was previously shown that the deletion of *napF* sequences upstream of position −85, which contains the ModE binding site centered at position −133.5, had no effect on wild-type *napF-lacZ* expression in response to either anaerobiosis or nitrate addition (7). To confirm this observation, we constructed a similar *napF-lacZ* fusion; a fragment of DNA spanning nucleotides −233 to −85 (with respect to the transcript start site) was deleted from λHW2 to give λPM58 (Fig. 1A). In the wild-type strain (MC4100) the pattern of *napF-lacZ* expression from λPM58 was similar to the full-length fusion contained on λHW2 (Table 3). However, in direct contrast to the ModE-dependent expression seen from λHW2, *napF-lacZ* expression from λPM58 was unaffected in a *modE* deletion strain. When we reintroduced the upstream DNA segment back into λPM58 to give λPM69, *napF-lacZ* expression was restored to *modE* dependency. (Note that in constructing these plasmids, we mutated 3 bp to introduce a unique *Eco*RI site at the downstream cloning junction [Fig. 1A].) Finally, to establish that DNA sequences 5′ of the ModE binding site were not required for the molybdate response, λPM59 was constructed where the nucleotides from −233 to −157 were deleted. (Nucleotide −147 marks the upstream boundary of the ModE binding site.) The pattern of *napF-lacZ* expression from λPM59 was identical to that of λHW2 in both wild-type and in *modE* backgrounds under all conditions tested (data not shown).

A correctly positioned ModE *cis*-acting site is essential for regulating *napF-lacZ* expression. The analysis presented above demonstrated that deletion of upstream DNA sequences containing the ModE binding site alleviated the in vivo requirement for molybdate and ModE for optimal *napF-lacZ* expression. To confirm that *modE* has an essential role in regulating *napF-lacZ* expression, we performed site-directed mutagenesis

TABLE 3. Effects of deletions and insertions in upstream DNA on *napF-lacZ* expression in response to anaerobiosis and nitrate addition

Strain	Relevant genotype[a]	β-Galactosidase activity[b]		
		+ O_2, $-NO_3^-$	$-O_2$	
			$-NO_3^-$	$+NO_3^-$
MC4100	λHW2	50	280	1,250
PM8	λHW2 *modE*	25	85	130
MC4100	λPM58	55	300	1,505
PM8	λPM58 *modE*	60	305	1,420
MC4100	λPM69	45	260	1,150
PM8	λPM69 *modE*	20	80	115
MC4100	λPM70	40	220	1,050
PM8	λPM70 *modE*	40	210	950
MC4100	λPM71	35	205	870
PM8	λPM71 *modE*	35	195	845

[a] Prophages λPM58, -59, -70, and -71, carrying various alterations in the upstream region of *napF* and their wild-type progenitor, λHW2, were inserted in the chromosome of the indicated strains in single copy.

[b] Units are given in nanomoles of ONPG hydrolyzed per minute per milligram of protein. Cells were grown in minimal glucose medium under aerobic and anaerobic conditions as described in the text. Sodium nitrate (NO_3) was added (at 40 mM) where indicated.

of conserved residues in the ModE recognition sequence centered at −133.5 relative to the *napF* promoter (Fig. 1). Two sets of 2-bp substitutions were introduced (Fig. 4), and the resultant promoter fragments were fused to *lacZ*, generating λPM54 and λPM55 (i.e., the λPM54 and λPM55 fusions each differ from λHW2 by only 2 bp). The effects of the mutations on ModE binding were assayed by in vitro gel shift assays (Fig. 2C and D). The promoter fragment from λPM54 no longer bound ModE (i.e., even when a 10-fold-higher level of ModE was used relative to the amount needed to shift the wild-type fragment). The altered promoter fragment from λPM55 displayed a sevenfold reduction in ModE binding.

The ModE binding site mutations were also evaluated in vivo by measuring β-galactosidase expression in a wild-type strain lysogenized with either λPM54 or λPM55 (Table 4).

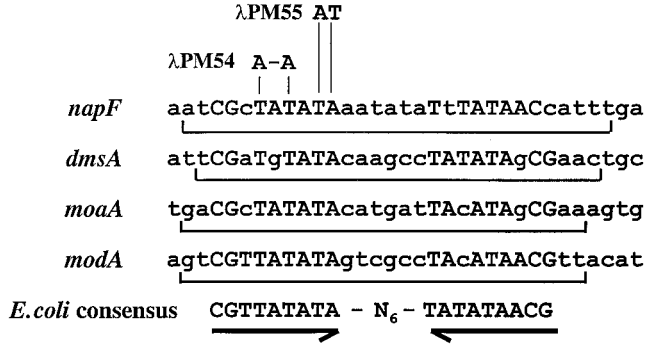

FIG. 4. Alignment of the ModE binding site at the *napF*, *dmsA*, *modA*, and *moaA* promoters with the proposed ModE consensus sequence. Nucleotides protected from DNaseI digestion are bracketed, and nucleotide matches to the ModE consensus sequence are shown in uppercase (2, 20, 23).

When cells were grown aerobically, anaerobically, or anaerobically with nitrate present, the ModE recognition site mutations markedly reduced *napF-lacZ* expression under each condition. Furthermore, introduction of a *modE* chromosomal deletion into strains carrying either λPM54 or λPM55 had no effect on gene expression (Table 4). Thus, the *cis*-acting mutations confer a $ModE^-$ phenotype.

Finally, to establish if the relative position of the ModE site was important, a 105-bp insertion was made at position −85 relative to the start of *napF* transcription to give λPM71 (Fig. 1). Although expression from the λPM71 fusion was slightly reduced when compared to that of the wild-type fusion (λHW2, Table 3), it was independent of ModE (i.e., repositioning the ModE site upstream by 10 helix turns was equivalent to deleting the ModE binding site region). In a similar manner, the orientation of the *Mfe*I-*Eco*RI fragment in λPM69 was reversed to invert the ModE site and move it from position −135.5 to a new position centered at −78.5 relative to the *napF* transcript start site (λPM70; Fig. 1). This rearrangement also abolished ModE control (Table 3).

Nitrate induction of *napF-lacZ* (λPM58) expression is molybdate independent. As noted above, replacement of the wild-type *modE* gene with a molybdate-independent allele, *modE**, abolished the requirement for molybdate for optimal *napF-lacZ* expression in response to nitrate addition when the upstream ModE binding site was present (i.e., λHW2; Table 2). To confirm that the molybdate requirement for *napF-lacZ* expression operates solely through *modE,* we introduced λPM58 (this fusion has a complete deletion of the ModE binding site) into a *modC* strain and measured gene expression in response to anaerobiosis and addition of nitrate. The *modC* mutation had no effect on *napF-lacZ* expression from λPM58 under any growth condition (data not shown), thus demonstrating that it is *modE* independent.

IHF plays a minor role in regulating *napF-lacZ* expression. A putative integration host factor (IHF) binding site was previously identified in the upstream region of the *napF* promoter (5). The ModE DNaseI footprinting experiments indicate that this proposed IHF site would overlap the left half of the ModE binding site by 5 bp. (The upstream boundary of the putative IHF site is at position −126, and the downstream boundary of the ModE binding site is at −122.) To determine if IHF plays a role in regulating *napF-lacZ* expression, we transduced a *himA* allele into wild-type (MC4100) and *modE* (PM8) strains that harbor λHW2. Compared to the wild-type strain, *napF-lacZ* expression in a *himA* strain was elevated twofold under all growth conditions (data not shown). Increased *napF-lacZ* gene expression was also seen in a *himA modE* strain, although the overall expression levels were lower due to the *modE* mutation (data not shown). Therefore, IHF serves a nonessential role in modulating *napF* gene expression.

DISCUSSION

The *E. coli napFDAGHBC* operon encodes a periplasmic nitrate reductase enzyme (10) similar to those encoded by the *nap* operons of other bacteria, including *Rhodobacter capsulatus*, *Ralstonia eutropha* (*Alcaligenes eutrophus*), and *Thiosphaera pantotropha* (3, 30). The *E. coli* enzyme is predicted to contain a molybdopterin moiety that raised the possibility that

TABLE 4. Effect of introducing mutations in the *modE* operator site on *napF-lacZ* expression in response to anaerobiosis and nitrate addition

Strain	Relevant genotype	Mutations in ModE binding site[a]	β-Galactosidase activity[b]			In vitro ModE binding[c]
			+ O_2, $-NO_3^-$	− O_2		
				$-NO_3^-$	$+NO_3^-$	
MC4100	λHW2	CGCTATATA-N_6-TTTATAACC	50	280	1,250	Wild type
PM8	λHW2 *modE*		25	85	130	
MC4100	λPM54	CGC<u>a</u>A<u>a</u>ATA-N_6-TTTATAACC	30	90	140	Absent
PM8	λPM54 *modE*		25	90	135	
MC4100	λPM55	CGCTATA<u>at</u>-N_6-TTTATAACC	30	100	150	Seven-fold reduction
PM8	λPM55 *modE*		30	85	125	

[a] Prophage λHW2 carries a copy of the wild-type ModE binding site. The mutations in the ModE binding site carried on the prophages λPM54 and -55 are underlined and shown in lowercase. All prophages are inserted in the chromosome of strains MC4100 and PM8 in single copy.

[b] Units are given in nanomoles of ONPG hydrolyzed per minute per milligram of protein. Cells were grown in minimal glucose medium under aerobic and anaerobic conditions as described in the text. Sodium nitrate (NO_3^-) was added (at 40 mM) where indicated.

[c] In vitro binding was measured via gel shift assays.

napFDAGHBC operon expression may be ModE dependent. Utilizing a *napF-lacZ* operon fusion, we demonstrate that a *modE* deletion impairs *napF-lacZ* expression by 10-fold. Sequence analysis of the *napF* promoter region identified a typical ModE binding site, centered at −133.5 bp with respect to the transcript start site. By utilizing a combination of gel shift and DNase I footprinting assays, we confirmed that ModE binds the *napF* promoter at this location with high affinity. Whereby the addition of molybdate modulated DNA binding by twofold in vitro (Fig. 2), molybdate addition caused a fivefold change in *napF* gene expression in vivo (Table 2). It is yet unclear if ModE interactions with molybdate act primarily to modulate DNA binding or, alternatively, to affect ModE interactions with other proteins involved in *napF* gene expression, including Fnr, NarP, NarL, and RNAP. The molybdate-dependent conformational changes within ModE are consistent with either model (9).

Interestingly, deletion of the ModE binding site at the *napF* promoter alleviates the requirement for *modE* without affecting *napF-lacZ* expression (Table 3). Given the distal 3′ location of the ModE binding site relative to ModE sites at other molybdate-regulated promoters, ModE-dependent *napF* regulation may somehow involve some type of DNA looping event to bring the bound ModE into contact with the other transcription activators. Since the introduction of a *himA* allele had only a twofold effect, it is unlikely that IHF plays a major role in this process. One possibility is that another general DNA binding protein occupies part of the ModE site and somehow suppresses *napF* gene expression when molybdate is limiting. Binding of ModE to the DNA under molybdate-sufficient conditions relives this control. It remains to be determined at the molecular level how ModE exerts its effects at the *napF* promoter.

Introduction of the molybdate-independent *modE** allele, which encodes a molybdate-independent variant of ModE, into a *modE modC* strain resulted in an unusually large (when compared to the wild-type strain) increase in *napF-lacZ* expression in response to anaerobiosis. The addition of molybdate and, to a lesser degree, nitrate resulted in a drop in gene expression. One explanation for these findings is that the inhibition of molybdate uptake, caused by the *modC* mutation, results in inactivation of the cell's complement of functional NarG and NapF nitrate reductase enzymes (i.e., inability to synthesize mature molybdoenzymes). Consequently, the cell is unable to metabolize any trace amounts of nitrate that may be present in the cell growth medium. As noted recently (34), trace amounts of nitrate would result in a large increase in *napF-lacZ* expression. The requirement for low levels of nitrate would also explain why *napF-lacZ* expression was lowered when nitrate was added to the medium (34). Thus, the provision of trace molybdate in the medium signals for the synthesis of the periplasmic molybdoenzyme for nitrate reduction under these conditions.

Nitrate induction of *napF-lacZ* expression in a *modE modC* double mutant was found to be independent of molybdate when *modE*$^+$ was replaced by a *modE** allele. Similarly, expression from a *napF-lacZ* fusion, which lacked the ModE binding site (λPM58), was unaffected by the introduction of a *modC* mutation. This finding is in direct contrast to those of previous studies that reported the cellular response to nitrate addition to be largely abolished in a *modC* background (6, 16, 25). These data were taken to imply that the Nar regulon senses molybdate as well as nitrate. Our studies strongly suggest that for expression originating from the *napF* promoter, the only molybdate-requiring component involved in mediating the response to nitrate addition is ModE.

The *napF* promoter is the fourth promoter at which we have characterized a ModE binding site. Based upon the ModE consensus sequence (Fig. 4), we searched for other putative ModE binding sites by using the PatScan program (8). Matches were checked to see if the site was located within the promoter regions of a gene or operon that encoded either molybdoenzymes or proteins involved in molybdate uptake and/or utilization. In *E. coli*, we found two additional candidates. One lies immediately upstream of an uncharacterized operon (accession no. g1787870) that encodes proteins that are highly homologous to the products of the *dmsABC* operon (4). The second match lies within 15 bp upstream of the translational start site of open reading frame 95 (ORF95; accession no. U28377), the product of which is unknown. However, ORF95 is directly upstream of *hybO*, the first gene of the *hybOABCDEFG* operon, which encodes the hydrogenase 2

complex (28). In *Haemophilus influenzae*, we also identified putative ModE binding sites upstream of the *moaACDE* and *modABC* operons and upstream of the *modA* and *torC* homologues (data not shown). Finally, as already noted (18), we also found matches upstream of the *anfA* homologue and *modABC* operons in both *Rhodobacter capsulatus* and *Azotobacter vinelandii*. Thus, ModE appears to play a global role in regulating molybdenum homeostasis in a number of bacterial species.

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