

## Dual Overlapping Promoters Control *napF* (Periplasmic Nitrate Reductase) Operon Expression in *Escherichia coli* K-12

Valley Stewart,<sup>1,2\*</sup> Peggy J. Bledsoe,<sup>1</sup> and Stanly B. Williams<sup>2†</sup>

Section of Microbiology, University of California, Davis, California 95616,<sup>1</sup> and Section of Microbiology, Cornell University, Ithaca, New York 14853<sup>2</sup>

Received 2 June 2003/Accepted 21 July 2003

*Escherichia coli* elaborates a flexible respiratory metabolism, involving differential synthesis of isoenzymes for many oxidation and reduction reactions. Periplasmic nitrate reductase, encoded by the *napFDAGHBC* operon, functions with concentrations of nitrate that are too low to support respiration by membrane-bound nitrate reductase. The *napF* operon control region exhibits unusual organization of DNA binding sites for the transcription regulators Fnr and NarP, which activate transcription in response to anaerobiosis and nitrate, respectively. Previous studies have shown that the *napF* operon control region directs synthesis of two transcripts whose 5' ends differ by about 3 nucleotides. We constructed mutant control regions in which either of the two promoter –10 regions is inactivated. Results indicate that the downstream promoter (P1) was responsible for Fnr- and NarP-regulated *napF* operon expression, whereas transcription from the upstream promoter (P2) was activated only weakly by the Fnr protein and was inhibited by phospho-NarP and -NarL proteins. The physiological function of promoter P2 is unknown. These results establish the unconventional *napF* operon control region architecture, in which the major promoter P1 is activated by the Fnr protein bound to a site centered at –64.5 with respect to the transcription initiation site, working in conjunction with the phospho-NarP protein bound to a site centered at –44.5.

*Escherichia coli*, a facultative aerobe, synthesizes a diverse repertoire of anaerobic respiratory enzymes in response to electron acceptor availability (12, 34). Enzyme synthesis is controlled at the level of transcription initiation by the transcription activators Fnr, an iron-sulfur protein responsive to anaerobiosis (15, 16), and the NarL and NarP proteins, response regulators whose phosphorylation is controlled by the sensors NarX and NarQ in response to nitrate and nitrite (9, 33). Together, these regulatory proteins act to ensure a hierarchical response to electron acceptors in accordance with their standard redox potentials.

Periplasmic nitrate reductase (NapABC enzyme) is encoded by the *napFDAGHBC* operon, in which *napA* encodes the catalytic subunit, *napB* and *napC* encode cytochromes *c*, *napD* encodes a protein likely required for enzyme assembly, and *napF*, *napG*, and *napH* encode proteins that couple ubiquinol oxidation to nitrate reduction (2, 3, 13, 24). This operon was initially identified by Choe and Reznikoff as an anonymous anaerobically expressed gene at map coordinate 46.5 (*aeg-46.5* locus), whose expression is activated by the Fnr protein and inhibited by the NarL protein (6). In *E. coli*, the NapABC enzyme permits nitrate respiration during growth with low concentrations of nitrate (24, 31, 35).

Transcriptional control regions for several *E. coli* operons encoding anaerobic respiratory enzymes have been characterized. The *napF* operon transcriptional control region is exceptional. First, in the other operons, the Fnr protein binding site

is centered at or near position –41.5 with respect to the transcription initiation site (15) and the NarL-NarP binding sites are upstream of the Fnr binding site (9, 33). By contrast, the *napF* operon Fnr site is centered at –64.5 and the NarP-NarL site is located downstream, centered at position –44.5 (Fig. 1) (7, 9, 10). Second, nitrate and nitrite effectively activate transcription in *napP*<sup>+</sup> *narL* null strains, but not in *narL*<sup>+</sup> strains (6, 8, 10, 26). Evidence suggests that the phospho-NarL protein competes with the phospho-NarP protein for binding to the site centered at –44.5 but that only the phospho-NarP protein is able to activate transcription from this location (8, 10). Third, a ModE protein binding site centered at –134.5 confers molybdate-responsive *napF* operon expression, but deletion of this site renders expression independent of the *modE*<sup>+</sup> gene (21). The mechanism by which the molybdate-responsive ModE protein controls *napF* operon expression has not been determined. Finally, in continuous culture, expression of the *napF* operon is maximal during growth with a relatively low concentration of nitrate, 1 mM (35).

Both in vivo and in vitro analyses revealed two initiation sites for *napF* operon transcription (7, 10). The upstream site (denoted T2 in Fig. 1) was observed in aerated cultures, whereas the downstream site (denoted T1 in Fig. 1) was observed in anaerobic cultures supplemented with nitrate (7). In a defined in vitro transcription system, the T2 transcript is weakly synthesized irrespective of added Fnr protein, but its synthesis is inhibited in reactions containing phospho-NarP or phospho-NarL proteins. By contrast, the T1 transcript is synthesized only in reactions containing both Fnr and phospho-NarP proteins (10). Overlapping potential –10 elements are positioned appropriately with respect to these two transcription initiation sites (denoted P2 and P1 in Fig. 1). Here we report experiments designed to determine the roles of these two promoters

\* Corresponding author. Mailing address: Section of Microbiology, University of California, One Shields Ave., Davis, CA. 95616-8665. Phone: (530) 754-7994. Fax: (530) 752-9014. E-mail: vjstewart@ucdavis.edu.

† Present address: Department of Biology, University of Utah, Salt Lake City, UT 84112.

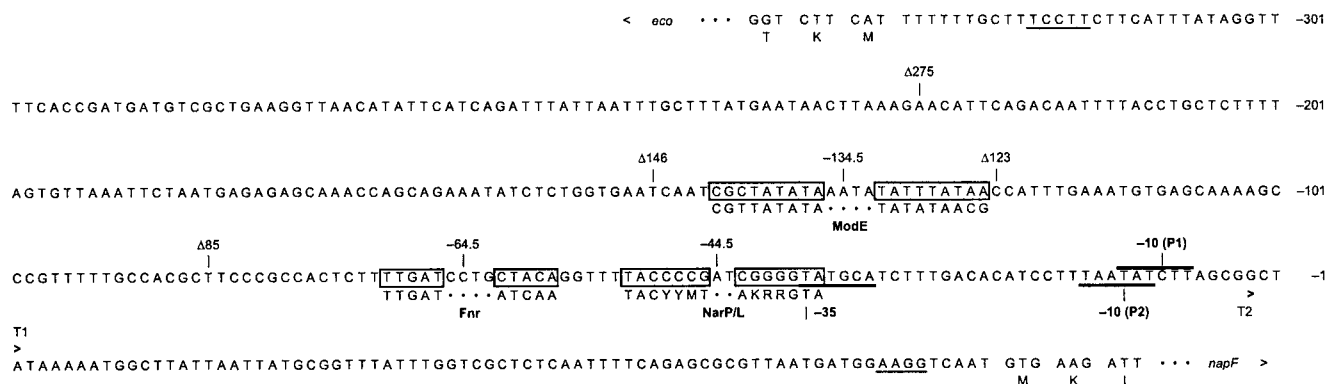


FIG. 1. The *napF* operon control region. Numbering is with respect to the transcription initiation site T1. Thick over- and underlines, -10 and -35 elements. Sequences for binding the NarP and NarL, Fnr, and ModE proteins are boxed; consensus sequences are shown below. Deletion end points of control region constructs used in this study are indicated. Thin underlines, Shine-Dalgarno regions for the *napF* gene and the divergently transcribed *eco* gene.

in controlling *napF* operon transcription. Results indicate that promoter P1 is largely responsible for Fnr- and NarP-regulated expression of the *napF* operon.

#### MATERIALS AND METHODS

**Strains and plasmids.** Strains and plasmids are listed in Table 1. Control region nucleotide sequences are depicted in Fig. 1. Genetic crosses were performed by Plk-mediated generalized transduction (22). Null alleles of *nar* regulatory genes (26) and the *napF1::Km* allele (31) have been described previously. Standard methods were used for restriction endonuclease digestion, ligation, transformation, and PCR amplification of DNA (20).

**Culture media and conditions.** Defined, complex, and indicator media for genetic manipulations were used as described previously (20). Defined medium to grow cultures for enzyme assays was buffered with 3-[*N*-morpholino]propane-sulfonic acid (MOPS) as previously described (32). The initial pH of this medium was set at 8.0 to ameliorate nitrite toxicity. Because the pK<sub>a</sub> of MOPS is 7.2, the buffering capacity of this medium continually increases as acidic fermentation products accumulate; at harvest, cultures typically had a pH of about 7.5.

Medium for batch cultures grown to the mid-exponential phase contained 80 mM glucose as the carbon source, and the respiratory oxidants NaNO<sub>3</sub> and NaNO<sub>2</sub> were added to 40 and 5 mM, respectively. Medium for overnight cultures arrested in the mid-exponential phase (30) contained glucose (6 mM), glucose plus NaNO<sub>3</sub> (4 and 10 mM, respectively), or glucose plus NaNO<sub>2</sub> (6 and 8 mM, respectively) as indicated in Tables 2 to 6. These concentrations were determined empirically to support growth to the mid-exponential phase (about 35 to 40 Klett units).

Cultures were grown at 37°C. Culture densities were monitored with a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., New York, N.Y.) equipped with a number 66 (red) filter. Anaerobic cultures for enzyme assays were grown in screw-cap tubes as described previously (32).

**Enzyme assay.** β-Galactosidase activities were determined at room temperature (approximately 21°C) by monitoring the hydrolysis of *o*-nitrophenyl-β-D-galactoside in CHCl<sub>3</sub>-sodium dodecyl sulfate-permeabilized cells. Specific activities are expressed in arbitrary units (22). All cultures were assayed in duplicate, and reported values are averages from at least two independent experiments.

**Construction of *napF* operon control region alterations.** Oligonucleotide-directed site-specific mutagenesis was used to introduce substitutions into the *napF* operon control region. Mutagenesis followed either the ampicillin selection protocol (17) or the QuikChange protocol (Stratagene Cloning Systems, La Jolla, Calif.) as described previously (1). PCRs were performed with a high-fidelity thermostable DNA polymerase (Accuzyme; Bionline USA, Reno, Nev.).

Plasmid pVJS1523 contains the *napF* operon control region on a 465-bp DNA fragment from an engineered *EcoRI* site at position -146 to an engineered *Bam*HI site downstream of *napF* codon 76 (8). Following each round of mutagenesis, the DNA sequence for the entire fragment was determined to eliminate isolates with spurious nucleotide substitutions. The control region cassettes were then recloned into the vector pRS414. The resulting Φ(*napF-lacZ*) operon

fusions were crossed into bacteriophage λRS45 (29), and monocopy lysogens were identified by a whole-colony PCR test (25).

**Transcript analysis.** Primer extension experiments were performed essentially as described previously (39). The primer 5'-CTTTGCGCCAGCGACCAG TGAG corresponds to *napF* codons 11 to 17 in the template strand, and therefore its proximal (3') end is 108 nucleotides (nt) downstream of the transcription initiation site T1. The primer was radiolabeled at the 5' end and used for extension reactions on RNA samples isolated from wild-type and mutant strains cultured to the mid-exponential phase anaerobically in the absence or presence of nitrate. Extension products were resolved on a thin polyacrylamide-urea slab gel and visualized by phosphorimager analysis. The same primer was used for parallel DNA sequencing reactions to provide size markers for the extension products.

5' rapid amplification of cDNA ends (RACE) analysis (11), also termed anchored PCR (18), used reagents purchased from Invitrogen Life Technologies (Carlsbad, Calif.) and was performed essentially as described by the manufacturer's instructions. Gene-specific primers were 5'-GGGTAACGCCAGGGTT TTCC for the first round (cDNA synthesis) and 5'-CTTAGTGAATCCGTAA TCATGGTCATAG for the second round (PCR). Anchor primers were 5'-GG CCAGGCGTCGACTAGTACGGGIHGGGIHGGGIH (where I stands for inosine) for dCTP-tailed cDNA products and 5'-GGCCACGCGTCGACTAGT ACCCCCCCCCCCCCCC for dGTP-tailed cDNA products.

#### RESULTS

**Transcription initiation sites.** Choe and Reznikoff reported results from primer extension experiments to define the 5' ends of *napF* operon mRNA species (7). The 5' ends of mRNA extracted from cultures grown anaerobically with nitrate mapped to the A residue indicated as T1 in Fig. 1, whereas those from aerated cultures mapped to the pair of G residues 3 to 4 nt upstream (indicated as T2). To confirm these results, we employed 5' RACE analysis, as described in Materials and Methods, on RNA extracted from anaerobic cultures grown in the absence or presence of nitrate. We sequenced approximately 12 of the resulting cDNA clones obtained from each of the two culture conditions over several independent experiments. Results (not shown) revealed 5' mRNA ends from anaerobic cultures at both G residues (T2) and 5' mRNA ends from anaerobic cultures with nitrate at the A residue (T1) and also at the T residue immediately upstream (Fig. 1). These results are virtually identical to those obtained with primer extension by Choe and Reznikoff (7).

To simplify discussion and retain consistency with previous

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype	Reference
<i>E. coli</i>		
SE1910	$\Delta modE2::Km$	14
VJS632	F <sup>-</sup> $\lambda^-$ prototroph	32
VJS676	As VJS632 but $\Delta(argF-lacIZYA)U169$	32
Derivatives of strain VJS676		
VJS4734	$\lambda\Phi(napF-lacZ)$ [ $\Delta 275$ ]	8
VJS4789	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146$ ]	8
VJS4796	$\lambda\Phi(napF-lacZ)$ [ $\Delta 123$ ]	8
VJS4799	$\lambda\Phi(napF-lacZ)$ [ $\Delta 275$ ] <i>narL215::Tn10</i>	8
VJS5123	$\lambda\Phi(napF-lacZ)$ [ $\Delta 275$ ] <i>narL215::Tn10 napF2::Km</i>	This study
VJS5723	$\lambda\Phi(napF-lacZ)$ [ $\Delta 275$ ] $\Delta(narXL)235$	This study
VJS5724	$\lambda\Phi(napF-lacZ)$ [ $\Delta 275$ ] $\Delta(narXL)235 narQ251::Tn10d(Tc)$	This study
VJS5743	$\lambda\Phi(napF-lacZ)$ [ $\Delta 275$ ] <i>narL249::<math>\Omega</math>-Sp narQ251::Tn10d(Tc)</i>	This study
VJS6931	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146; -35^-$ ]	This study
VJS6988	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146; P1^-$ ]	This study
VJS6990	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146; P2^-$ ]	This study
VJS7402	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146; P1^- P2^-$ ]	This study
VJS7431	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146; -35^- P1^-$ ]	This study
VJS7432	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146; -35^- P2^-$ ]	This study
VJS7439	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146$ ] <i>fmr-271::Tn10</i>	This study
VJS7440	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146; P1^-$ ] <i>fmr-271::Tn10</i>	This study
VJS7441	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146; P2^-$ ] <i>fmr-271::Tn10</i>	This study
VJS7442	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146$ ] <i>narL215::Tn10</i>	This study
VJS7443	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146; P1^-$ ] <i>narL215::Tn10</i>	This study
VJS7444	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146; P2^-$ ] <i>narL215::Tn10</i>	This study
VJS7452	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146$ ] <i>narP253::Tn10d(Cm)</i>	This study
VJS7453	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146; P1^-$ ] <i>narP253::Tn10d(Cm)</i>	This study
VJS7454	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146; P2^-$ ] <i>narP253::Tn10d(Cm)</i>	This study
VJS7455	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146$ ] <i>narL215::Tn10 narP253::Tn10d(Cm)</i>	This study
VJS7456	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146; P1^-$ ] <i>narL215::Tn10 narP253::Tn10d(Cm)</i>	This study
VJS7457	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146; P2^-$ ] <i>narL215::Tn10 narP253::Tn10d(Cm)</i>	This study
VJS8548	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146$ ] $\Delta modE2::Km$	This study
VJS8549	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146; P1^-$ ] $\Delta modE2::Km$	This study
VJS8550	$\lambda\Phi(napF-lacZ)$ [ $\Delta 146; P2^-$ ] $\Delta modE2::Km$	This study
VJS8551	$\lambda\Phi(napF-lacZ)$ [ $\Delta 123$ ] $\Delta modE2::Km$	This study
Plasmids		
pRS415	Ap <sup>r</sup> ; <i>lacZ</i> operon fusion vector	29
pVJS1523	Ap <sup>r</sup> ; <i>napF</i> control region; <i>EcoRI</i> (-146) to <i>BamHI</i> (+305) in pGEM7Zf(+)	8

publications, we assign the A residue (T1) +1 for numbering the sequence. For specific reference to the T2 transcript, we designate the G residue at position -3 the transcription initiation site (Fig. 1 and 2).

**Two mutationally separable *napF* operon promoters.** As noted previously (7, 10), the two transcription initiation sites are associated with overlapping potential -10 promoter ele-

ments (TAATATCTT; Fig. 1). The upstream element, which we denote P2, has two mismatches (underlined) from the -10 element consensus sequence (TAATAT versus TATAAT). The downstream element, which we denote P1, also has two mismatches (TATCTT). A potential -35 element has three mismatches from the consensus sequence (TATGCA versus TTGACA); it is located 16 bp upstream of the P2 -10 element

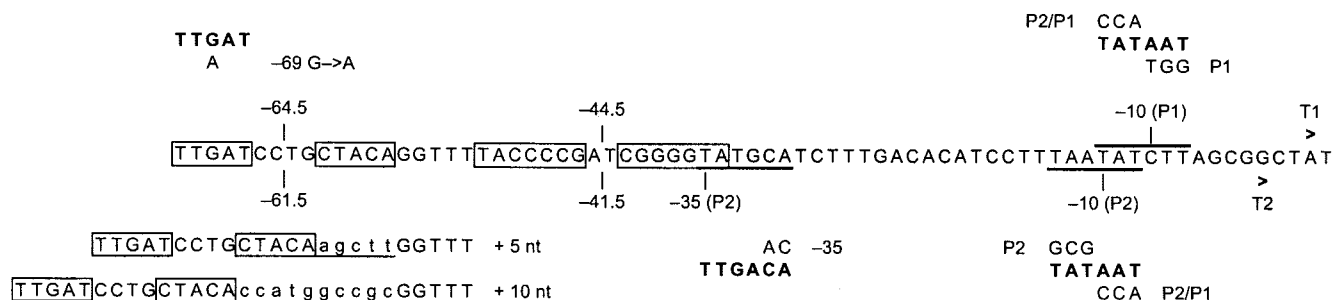


FIG. 2. Promoter region nucleotide substitutions. The binding sites for the Fnr protein and the NarP and NarL proteins are indicated by boxes. The consensus sequences for the Fnr, -35, and -10 elements are in boldface. Substitutions are indicated above or below the corresponding consensus sequences. Spacers of 5 and 10 bp between the Fnr binding site and the NarP and NarL binding sites are in lowercase letters and underlined.

TABLE 2. Alterations in the promoter region influence  $\Phi(\text{napF-lacZ})$  expression

Strain	Version of promoter <sup>b</sup> :			LacZ sp act <sup>c</sup>				Levels of activation by:		
	-35	P2	P1	-O <sub>2</sub>			-O <sub>2</sub>	NO <sub>3</sub> <sup>-</sup>	NO <sub>2</sub> <sup>-</sup>	
				+O <sub>2</sub> , -NO <sub>x</sub> <sup>-</sup>	-NO <sub>x</sub> <sup>-</sup>	+NO <sub>3</sub> <sup>-</sup>				+NO <sub>2</sub> <sup>-</sup>
VJS4789	+	+	+	35	280	2,790	3,750	8.0	10	13
VJS6990	+	-	+	27	180	4,130	5,380	6.7	23	30
VJS6988	+	+	-	95	280	55	130	2.9	0.2	0.5
VJS7402	+	-	-	<5	<5	<5	<5	— <sup>c</sup>	—	—
VJS6931	-	+	+	30	140	1,830	2,040	4.7	13	15
VJS7432	-	-	+	10	82	2,800	4,120	8.2	34	50
VJS7431	-	+	-	30	150	47	80	5.0	0.3	0.5

<sup>a</sup> Strains were cultured overnight in MOPS defined medium with limiting glucose. NO<sub>x</sub><sup>-</sup>, nitrate or nitrite.  
<sup>b</sup> Wild-type (+) or mutant (-) for indicated promoter elements (see Fig. 2). The P2<sup>-</sup> P1<sup>-</sup> alteration affects the common 3-bp core sequence.  
<sup>c</sup> —, no ratio to calculate.

and 19 bp upstream of the P1 -10 element (7). This -35 element overlaps the distal end of the NarP-NarL binding site centered at position -44.5 (Fig. 1).

We constructed mutant control regions carrying multiple contiguous substitutions designed to ablate these specific promoter elements individually. For simplicity, we omit description of several substitutions that were made and analyzed in exploratory experiments, the results from which are congruent with those presented here. Changes in the -10 region were designed to eliminate either the P2 or the P1 element without affecting the other (Fig. 2). Changes in the -35 region were designed to eliminate this element without affecting the NarP-NarL protein binding site (Fig. 2). Finally, a -10 region change was designed to eliminate both elements P2 and P1, by altering their common 3-bp core sequences.

Each set of nucleotide substitutions was introduced into a  $\lambda\Phi(\text{napF-lacZ})$  specialized transducing phage and integrated into the *E. coli* chromosome in monocopy as described in Materials and Methods. Strains were cultured in defined medium under four different growth conditions: aerated, anaerobic, anaerobic with nitrate, and anaerobic with nitrite.  $\beta$ -Galactosidase activity for each culture was measured.

The P2 element alterations had modest effects: relative to wild-type expression,  $\Phi(\text{napF-lacZ})$  expression was slightly reduced during anaerobic growth but slightly elevated during growth with nitrate or nitrite (Table 2). By contrast, the alterations to the P1 element had a strong effect on  $\Phi(\text{napF-lacZ})$  expression.  $\beta$ -Galactosidase activity in the aerated culture was elevated about threefold relative to that of the wild type, whereas expression in the anaerobic culture was exactly the same as that of the wild type. However, addition of nitrate or nitrite caused a modest decrease in  $\Phi(\text{napF-lacZ})$  expression relative to the anaerobic level. We conclude that the P1 element is essential for nitrate and nitrite induction of *napF* operon transcription.

The alterations to the common 3-bp core sequences of the P2 and P1 elements (Fig. 2) eliminated  $\Phi(\text{napF-lacZ})$  expression under all conditions (Table 2). This demonstrates that these two promoters are solely responsible for *napF* operon transcription. The alterations to the -35 element had relatively modest effects on  $\Phi(\text{napF-lacZ})$  expression both singly and in combination with alterations to the P2 and P1 elements (Table 2). Considering also the relatively weak match of this element to the -35 consensus and its proximity to the NarP-

NarL binding site, we therefore draw no conclusions regarding its role in *napF* operon expression.

We used primer extension analysis, as described in Materials and Methods, to examine mRNA 5' ends. During anaerobic growth in the absence of nitrate, the wild-type and P2<sup>+</sup> P1<sup>-</sup> constructs directed synthesis of modest levels of mRNA with 5' ends corresponding to transcript T2 (Fig. 3, lanes 1 and 2), whereas during anaerobic growth in the presence of nitrate the wild-type and P2<sup>-</sup> P1<sup>+</sup> constructs directed the synthesis of substantial levels of mRNA with 5' ends corresponding to transcript T1 (Fig. 3, lanes 5 and 6). We were unable to detect transcription from the P2<sup>-</sup> P1<sup>+</sup> and P2<sup>+</sup> P1<sup>-</sup> constructs during anaerobic growth in the absence and presence of nitrate, respectively (Fig. 3, lanes 3 and 4). Overall these patterns support the results from measuring  $\Phi(\text{napF-lacZ})$  expression. Fur-

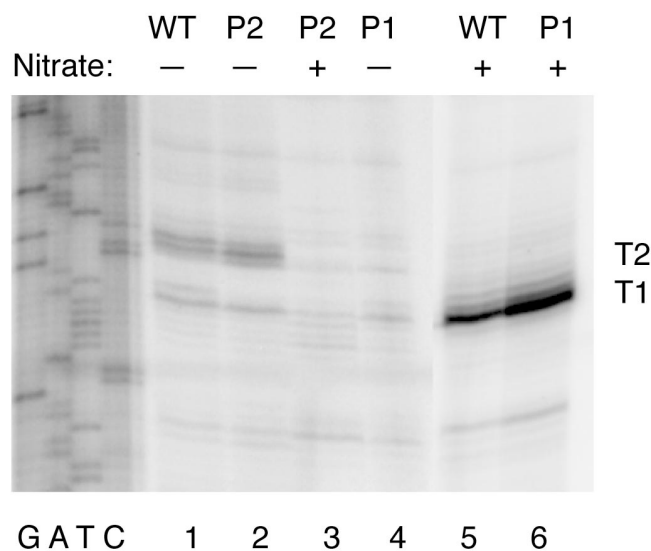


FIG. 3. Primer extension analysis of promoter mutations. RNA was extracted from wild-type (WT), P2<sup>-</sup> P1<sup>-</sup> (P2), and P2<sup>-</sup> P1<sup>+</sup> (P1) strains grown anaerobically in the absence (-) or presence (+) of nitrate as indicated. T1 and T2, transcription initiation sites (Fig. 1); G, A, T, and C, lanes for DNA sequencing reactions. These data are from a single experiment, with samples electrophoresed on the same gel; the gel lane images have been rearranged from the original order.



TABLE 3. Alterations in the promoter region and an *fnr* null allele influence  $\Phi(\textit{napF-lacZ})$  expression

Strain	Version of <sup>b</sup> :			LacZ sp act <sup>a</sup>			Level of activation by:	
	<i>fnr</i>	Promoter:		+O <sub>2</sub> , -NO <sub>x</sub> <sup>-</sup>	-O <sub>2</sub>		-O <sub>2</sub>	NO <sub>3</sub> <sup>-</sup>
		P2	P1		-NO <sub>x</sub> <sup>-</sup>	+NO <sub>3</sub> <sup>-</sup>		
VJS4789	+	+	+	38	230	2,220	6.1	10
VJS7439	-	+	+	41	50	150	1.2	3.0
VJS6990	+	-	+	10	130	3,200	13	25
VJS7441	-	-	+	7	41	370	5.9	9.0
VJS6988	+	+	-	97	240	73	2.5	0.3
VJS7440	-	+	-	120	100	43	0.8	0.4

<sup>a</sup> Strains were cultured overnight in MOPS defined medium with limiting glucose. NO<sub>x</sub><sup>-</sup>, nitrate or nitrite.

<sup>b</sup> +, wild type; -, mutant (see Fig. 2).

ther, this demonstrates that these control region substitutions did not result in the formation of alternate promoters.

**Effects of *fnr*, *narP*, and *narL* null alleles on expression from the two *napF* operon promoters.** The *fnr* null allele sharply reduced  $\Phi(\textit{napF-lacZ})$  expression from both the wild-type and P2<sup>-</sup> P1<sup>+</sup> control regions but by contrast had only a mild effect on expression from the P2<sup>+</sup> P1<sup>-</sup> control region (Table 3). These results indicate that the Fnr protein is a strong transcription activator for the P1 promoter but only a modest activator for the P2 promoter.

Previous experiments determined that, during anaerobic growth,  $\Phi(\textit{napF-lacZ})$  expression is induced about 10-fold by nitrate and about 20-fold by nitrite. In a *narL* null strain, nitrate induction is induced about 20-fold, whereas in a *narP* null strain induction by either nitrate or nitrite is virtually nil. This reflects the roles for the NarP and NarL proteins in activating and antagonizing, respectively, transcription initiation (8). These observations are recapitulated in Table 4.

Overall, expression patterns from the P2<sup>+</sup> P1<sup>+</sup> and the P2<sup>-</sup> P1<sup>+</sup> control regions were very similar. In both *narP*<sup>+</sup> *narL*<sup>+</sup> and *narP*<sup>+</sup> *narL* null strains,  $\Phi(\textit{napF-lacZ})$  expression was induced during growth with nitrate (Table 4). This induction was more pronounced in the *narL* null strains, reflecting the an-

tagonistic role for the phospho-NarL protein in controlling *napF* operon expression (8). Additionally, the *narP narL* double-null strains both exhibited about a 10-fold reduction in basal-level  $\Phi(\textit{napF-lacZ})$  expression. Previously, an analogous observation was made with a *narQ narX* double-null strain lacking both nitrate sensors (26). This suggests that low levels of phosphorylated NarP and NarL proteins may be present during growth in the absence of an inducer and are able to stimulate significant levels of transcription.

There was one striking exception to the similar expression patterns from these two control regions: the *narP* null *narL*<sup>+</sup> strain, carrying the P2<sup>-</sup> P1<sup>+</sup> control region, exhibited about a 25-fold induction in response to nitrate (Table 4; strain VJS7454). This was due in significant measure to the nearly 10-fold decrease in basal-level anaerobic expression in the absence of nitrate (28 Miller units in *narP* null *narL*<sup>+</sup> strains versus 200 Miller units in *narP*<sup>+</sup> *narL*<sup>+</sup> strains). Thus, the mutant control region differed from the wild type in its response to the NarL protein.

Anaerobic  $\Phi(\textit{napF-lacZ})$  expression from the P2<sup>+</sup> P1<sup>-</sup> control region was inhibited two- to threefold by nitrate when either the *narP*<sup>+</sup> or *narL*<sup>+</sup> gene was present, suggesting that

TABLE 4. Alterations in the promoter region and *narL* and *narP* null alleles influence  $\Phi(\textit{napF-lacZ})$  expression

Strain	Version of <sup>b</sup> :				LacZ sp act <sup>a</sup> without O <sub>2</sub>		Level of activation by NO <sub>3</sub> <sup>-</sup>
	<i>narL</i>	<i>narP</i>	Promoter:		-NO <sub>x</sub> <sup>-</sup>	+NO <sub>3</sub> <sup>-</sup>	
			P2	P1			
VJS4789	+	+	+	+	220	2,000	9.1
VJS7442	-	+	+	+	280	7,430	27
VJS7452	+	-	+	+	160	320	2.0
VJS7455	-	-	+	+	20	30	1.5
VJS6990	+	+	-	+	200	2,970	15
VJS7444	-	+	-	+	260	9,070	35
VJS7454	+	-	-	+	28	690	25
VJS7457	-	-	-	+	23	23	1.0
VJS6988	+	+	+	-	240	73	0.3
VJS7443	-	+	+	-	250	120	0.5
VJS7453	+	-	+	-	270	80	0.3
VJS7456	-	-	+	-	260	410	1.6

<sup>a</sup> Strains were cultured overnight in MOPS defined medium with limiting glucose. NO<sub>x</sub><sup>-</sup>, nitrate or nitrite.

<sup>b</sup> +, wild type; -, mutant (see Fig. 2).

TABLE 5. Alterations in the promoter region influence nitrate-responsive  $\Phi(\textit{napF-lacZ})$  expression independently of a *modE* null allele

Strain <sup>c</sup>	Deletion <sup>d</sup>	Version of <sup>b</sup> :			LacZ sp act <sup>a</sup> without O <sub>2</sub>		Level of activation by NO <sub>3</sub> <sup>-</sup>
		<i>modE</i>	Promoter:		-NO <sub>3</sub> <sup>-</sup>	+NO <sub>3</sub> <sup>-</sup>	
			P2	P1			
VJS4789	Δ146	+	+	+	380	2,440	6.4
VJS8548	Δ146	-	+	+	140	920	6.6
VJS6990	Δ146	+	-	+	290	3,890	13
VJS8550	Δ146	-	-	+	130	1,790	14
VJS6998	Δ146	+	+	-	270	54	0.2
VJS8549	Δ146	-	+	-	120	30	0.3
VJS4796	Δ123	+	+	+	410	2,590	6.3
VJS8551	Δ123	-	+	+	460	2,630	5.7

<sup>a</sup> Strains were cultured overnight in MOPS defined medium with limiting glucose.

<sup>b</sup> +, wild type; -, mutant (see Fig. 2).

<sup>c</sup> All strains carry the *modB*<sup>+</sup> allele.

<sup>d</sup> Control region deletion in  $\Phi(\textit{napF-lacZ})$  construct (see Fig. 1).

the NarL and NarP proteins antagonize expression from the P2 promoter.

Together, these results indicate that transcription initiation from the P1 promoter is strongly regulated by the Fnr, NarP, and NarL proteins and accounts for most of the observed  $\Phi(\textit{napF-lacZ})$  expression in wild-type strains. By contrast, transcription initiation from the P2 promoter seems to be nearly constitutive, exhibiting only weak activation by the Fnr protein and weak inhibition by the NarL and NarP proteins (Tables 3 and 4).

**Spacing between the Fnr and NarP protein binding sites.** To explore constraints on the spacing between the binding sites for the Fnr and NarP proteins, we constructed two *napF* operon control region variants with insertions of 5 and 10 nt (Fig. 2). For comparison, we also measured  $\Phi(\textit{napF-lacZ})$  expression from a mutant control region carrying a single-nucleotide substitution (-69G→A) in the Fnr site (8).

Wild-type  $\Phi(\textit{napF-lacZ})$  expression is induced approximately 10-fold by anaerobiosis and an additional 10-fold by nitrate. The previously described -69G→A change mimics an *fir* null allele: anaerobic induction and nitrate induction both are sharply decreased (8). The +5 and +10 spacing changes both reduced  $\Phi(\textit{napF-lacZ})$  expression to the same baseline level as the Fnr site substitution (data not shown). We concluded that the Fnr protein binding site cannot readily be moved relative to the NarP protein binding site and so did not pursue more-extensive analysis of this point.

**Effects of a *modE* null allele on expression from the two *napF* operon promoters.** Molybdate (MoO<sub>4</sub><sup>2-</sup>) availability controls anaerobic respiratory gene expression (23) through the molybdate-responsive transcriptional regulator ModE (28). Recently, McNicholas and Gunsalus (21) identified a ModE binding site centered at position -134.5 in the *napF* operon control region (Fig. 1) and demonstrated that the ModE protein controls  $\Phi(\textit{napF-lacZ})$  expression.

Previous 5' deletion analysis (8) resulted in two deletions (Δ146 and Δ123) that produce *napF* operon control regions that differ only in the presence or absence of the ModE protein binding site (Fig. 1). We therefore examined the effect of a *modE* null allele on  $\Phi(\textit{napF-lacZ})$  expression from these two

control region variants. The *modABC* operon encodes the high-affinity molybdate uptake system, which efficiently scavenges trace molybdate (28). Therefore, these experiments employed *modB* null strains in order to achieve internal molybdate limitation (23).

In the Δ146 strain, which retains the ModE protein binding site,  $\Phi(\textit{napF-lacZ})$  expression was induced during growth with added molybdate, and this induction required the *modE*<sup>+</sup> allele (data not shown). By contrast, in the Δ123 strain, in which the ModE protein binding site is deleted,  $\Phi(\textit{napF-lacZ})$  expression was independent of both molybdate limitation and the *modE*<sup>+</sup> allele (data not shown; Table 5). These results are fully congruent with those of McNicholas and Gunsalus (21).

We next examined the effect of a *modE* null allele on nitrate regulation of  $\Phi(\textit{napF-lacZ})$  expression from P2<sup>-</sup> P1<sup>+</sup> and P2<sup>+</sup> P1<sup>-</sup> control region variants in cultures of *modB*<sup>+</sup> strains. (These promoter alterations are in the Δ146 version of the *napF* operon control region, which contains the ModE protein binding site.) Results (Table 5) indicate that the *modE* null allele similarly affected expression from all three promoter combinations: overall expression was decreased about two- to threefold, but the patterns and magnitude of nitrate regulation were not affected. We conclude that promoters P1 and P2 are subject to equivalent regulation by the ModE protein.

**Effects of *narX*, *narQ*, and *napA* null alleles on *napF* operon expression.** A previous study of *napF* operon regulation (26) used a Nap<sup>-</sup> *nap::λplacMu53* insertion (6). The resulting strain exhibited elevated basal-level *napF* operon expression, as revealed through β-galactosidase activity, in comparison to the Nap<sup>+</sup>  $\lambda\Phi(\textit{napF-lacZ})$  specialized transductants employed in subsequent work (8). To determine the basis for this difference, we examined the effects on  $\Phi(\textit{napF-lacZ})$  expression of null alleles of the *narX* and *narQ* genes encoding the Nar sensors, as well as the *napA* structural gene. These experiments employed a *narL* null strain background to eliminate the antagonistic effects of the NarL protein on the induction of *napF* operon transcription.

A *narX* null allele had no effect, whereas a *narQ* null allele resulted in an approximately threefold elevation of basal expression and a twofold reduction in induced expression (Table

TABLE 6. Null alleles of the *narX*, *narQ*, and *napA* genes influence  $\Phi(\text{napF-lacZ})$  expression

Strain	Genotype <sup>b</sup>			LacZ sp act <sup>a</sup> without O <sub>2</sub>			Level of activation by:	
	<i>narX</i>	<i>narQ</i>	<i>napA</i>	-NO <sub>x</sub> <sup>-</sup>	+NO <sub>3</sub> <sup>-</sup>	+NO <sub>2</sub> <sup>-</sup>	NO <sub>3</sub> <sup>-</sup>	NO <sub>2</sub> <sup>-</sup>
VJS4799	+	+	+	290	6,270	5,280	22	18
VJS5723	-	+	+	280	6,140	4,950	22	18
VJS5743	+	-	+	930	3,320	3,630	3.6	3.9
VJS5123	+	+	-	940	7,000	6,980	7.4	7.4
VJS5724	-	-	+	170	140	130	0.8	0.8

<sup>a</sup> Strains were cultured to the mid-exponential phase in MOPS medium (defined medium with glucose). NO<sub>x</sub><sup>-</sup>, nitrate or nitrite.

<sup>b</sup> All strains carry a *narL* null allele (see Table 1 for full genotypes).

6). A *napA* null allele likewise produced an approximately threefold elevation of basal expression (Table 6). Thus, expression of the *napF* operon is sensitive to both *narQ* regulatory gene and *nap* structural gene integrity.

## DISCUSSION

The *napF* operon exhibits a unique pattern of expression and a unique control region architecture in comparison to other characterized *E. coli* Fnr- and Nar-regulated operons. Studies cited in this paper focus on determining the basis for the two distinct transcription initiation sites. Evidence suggests that overlapping -10 elements direct the synthesis of these transcripts. Interpretation of these results rests on the assumption that substitutions designed to inactivate one promoter have little influence on the activity or regulation of the other. This seems a reasonable supposition; neither of the -10 elements has an upstream TG (extended -10) motif, and therefore the changes designed to inactivate the promoter P2 -10 element are predicted not to influence the promoter P1 -10 element (4).

Analysis of synthetic promoters indicates that the Fnr protein, like the paralogous cyclic AMP receptor protein (Crp), can activate transcription when bound to a site centered at position -61.5 (5, 38). The *napF* operon P2 promoter appears to represent a natural example of this type of Fnr-dependent promoter. However, introduction of an *fnr* null allele had relatively little effect on  $\Phi(\text{napF-lacZ})$  expression in the P2<sup>+</sup> P1<sup>-</sup> strain (Table 3). Instead, it was the P2<sup>-</sup> P1<sup>+</sup> promoter, for which the Fnr binding site is centered at -64.5, that exhibited a strong requirement for the *fnr*<sup>+</sup> gene for  $\Phi(\text{napF-lacZ})$  expression. This conclusion is supported by results from in vitro transcription experiments in which the Fnr protein stimulated synthesis of the T1 transcript but not the T2 transcript (10). Interestingly, position -64.5 is not a permissive location for the Fnr protein to activate transcription from the synthetic promoter (38). One obvious difference between the synthetic promoter and the *napF* operon promoter is that transcription from the latter is also activated by the NarP protein bound to a site centered at position -44.5.

Based on results obtained with the P2<sup>-</sup> P1<sup>+</sup> and P2<sup>+</sup> P1<sup>-</sup> mutants, we conclude that the P1 promoter is the primary target for both Fnr and NarP activation of transcription initiation (Table 4). However, transcription from the P2 promoter was modestly inhibited (about threefold) by the NarP and NarL proteins in response to nitrate. These conclusions are

congruent with results from in vitro transcription experiments (10).

**Why two promoters for *napF* operon expression?** Examples of overlapping promoters abound in the literature. One familiar example is from the *E. coli gal* operon (36), in which the two promoters are reciprocally regulated by the Crp protein. Overlapping promoters of unknown physiological function are present in the *E. coli lac* operon control region (27).

In P2<sup>+</sup> P1<sup>+</sup> and P2<sup>-</sup> P1<sup>+</sup> strains cultured without nitrate,  $\Phi(\text{napF-lacZ})$  basal-level expression was reduced by approximately 10-fold in *narP narL* double-null mutants (Table 4). This suggests that the low levels of phospho-NarP (and phospho-NarL) are present even with no added inducer. In continuous cultures,  $\Phi(\text{napF-lacZ})$  expression is maximal during growth with only about 1 mM added nitrate (35). Thus, the *napF* control region seems to be tuned for efficient response to low nitrate levels, even the very low levels contaminating deionized water and commercial-grade culture medium components.

By contrast,  $\Phi(\text{napF-lacZ})$  basal-level expression from the P2<sup>+</sup> P1<sup>-</sup> control region remained high even in the *narP narL* double-null mutant (Table 4). We do not understand the mechanistic basis for this. Nevertheless, the results overall are consistent with the notion that the P2 promoter serves as the default promoter for *napF* operon expression under growth conditions when the P1 promoter is not activated (i.e., aerobiosis or anaerobiosis with no added nitrate or nitrite). This idea is supported by the different patterns of transcript synthesis in response to inducing versus noninducing conditions (Fig. 3) (7, 10). Furthermore, the P2 promoter might be activated under growth conditions that we have not explored.

**Activation of *napF* operon transcription by the phospho-NarL protein.** For strains with the wild-type control region,  $\Phi(\text{napF-lacZ})$  expression is activated in *narP*<sup>+</sup> *narL* null strains, but not in the reciprocal *narP* null *narL*<sup>+</sup> strains (8). Likewise, the phospho-NarP protein, but not the phospho-NarL protein, in conjunction with the Fnr protein, activates in vitro transcription from the *napF* P1 promoter (10). These observations led to the notion that the phospho-NarL protein cannot activate transcription when bound close to the promoter (position -44.5) (8). However,  $\Phi(\text{napF-lacZ})$  expression from the P2<sup>-</sup> P1<sup>+</sup> construct was efficiently induced by nitrate in a *narL*<sup>+</sup> *narP* null strain (Table 4). This induction was due in large measure to the roughly 10-fold decrease in basal expression described above. This indicates that, at least

in the P2<sup>-</sup> P1<sup>+</sup> mutant, phospho-NarL can activate transcription when bound adjacent to the promoter.

**Negative regulation of the NarP response regulator by the NarQ sensor.** Considerable evidence suggests that the NarX sensor serves as a negative regulator of NarL function but not NarP function (26, 31, 37). An equivalent role for the NarQ sensor as a negative regulator of NarP function was suggested by the patterns of *nrfA* (respiratory nitrite reductase) operon expression (26), but this conclusion was tempered by parallel studies on *napF* operon expression in which the operon exhibited high basal-level expression in the Nap<sup>-</sup> *nap::λlacMu53* insertion employed.

Nap<sup>+</sup> *λΦ(napF-lacZ)* specialized transductants exhibit relatively low basal-level *napF* operon expression (8). Indeed, introduction of a *napA* null allele resulted in elevated basal-level expression (Table 6). This apparent autoregulation of *napF* operon expression can be explained by low levels of nitrate as a contaminant in the culture medium (19). The likely physiological function of periplasmic nitrate reductase in *E. coli* is to scavenge nitrate in low concentration (24, 35), so it seems plausible that low-concentration nitrate might persist in cultures of Nap<sup>-</sup> strains.

Strikingly, the *narQ* null *narX*<sup>+</sup> strain also exhibited elevated basal-level *Φ(napF-lacZ)* expression, whereas the *narQ*<sup>+</sup> strains did not (Table 6). This implies a negative role for the NarQ protein in countering inappropriate, NarX-dependent phosphorylation in the absence of inducer. Presumably, this negative influence reflects phospho-NarP phosphatase activity. Thus, the Nar regulatory system has an element of symmetry, in which only the cognate sensor-regulator pairs (NarQ-NarP and NarX-NarL) exhibit negative regulatory interactions.

#### ACKNOWLEDGMENTS

We thank Li-Ling Chen for performing experiments on molybdate regulation of *napF* operon expression, K. T. Shanmugam for providing a culture of the *modE::Km* strain, R. K. Taylor for advice on the 5' RACE procedure, and Andrew Darwin for helpful critique of the manuscript.

This study was supported by Public Health Service grant GM36877 from the National Institute of General Medical Sciences.

#### REFERENCES

- Appleman, J. A., and V. Stewart. 2003. Mutational analysis of a conserved signal-transducing element: the HAMP linker of the *Escherichia coli* nitrate sensor NarX. *J. Bacteriol.* **185**:89–97.
- Berks, B. C., D. J. Richardson, A. Reilly, A. C. Willis, and S. J. Ferguson. 1995. The *napEDABC* gene cluster encoding the periplasmic nitrate reductase system of *Thiosphaera pantotropha*. *Biochem. J.* **309**:983–992.
- Brondijk, T. H., D. Fiegen, D. J. Richardson, and J. A. Cole. 2002. Roles of NapF, NapG and NapH, subunits of the *Escherichia coli* periplasmic nitrate reductase, in ubiquinol oxidation. *Mol. Microbiol.* **44**:245–255.
- Burr, T., J. Mitchell, A. Kolb, S. Minchin, and S. Busby. 2000. DNA sequence elements located immediately upstream of the -10 hexamer in *Escherichia coli* promoters: a systematic study. *Nucleic Acids Res.* **28**:1864–1870.
- Busby, S., and R. H. Ebright. 1999. Transcription activation by catabolite activator protein (CAP). *J. Mol. Biol.* **293**:199–213.
- Choe, M., and W. S. Reznikoff. 1991. Anaerobically expressed *Escherichia coli* genes identified by operon fusion techniques. *J. Bacteriol.* **173**:6139–6146.
- Choe, M., and W. S. Reznikoff. 1993. Identification of the regulatory sequence of anaerobically expressed locus *aeg-46.5*. *J. Bacteriol.* **175**:1165–1172.
- Darwin, A. J., and V. Stewart. 1995. Nitrate and nitrite regulation of the Fnr-dependent *aeg-46.5* promoter of *Escherichia coli* K-12 is mediated by competition between homologous response regulators (NarL and NarP) for a common DNA-binding site. *J. Mol. Biol.* **251**:15–29.
- Darwin, A. J., and V. Stewart. 1996. The Nar modulon systems: nitrate and nitrite regulation of anaerobic gene expression, p. 343–359. In E. C. C. Lin and A. S. Lynch (ed.), Regulation of gene expression in *Escherichia coli*. R. G. Landes Co., Georgetown, Tex.
- Darwin, A. J., E. C. Ziegelhoffer, P. J. Kiley, and V. Stewart. 1998. Fnr, NarP, and NarL regulation of *Escherichia coli* K-12 *napF* (periplasmic nitrate reductase) operon transcription in vitro. *J. Bacteriol.* **180**:4192–4198.
- Frohman, M. A., M. K. Dush, and G. R. Martin. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **85**:8998–9002.
- Gennis, R. B., and V. Stewart. 1996. Respiration, p. 217–261. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
- Grove, J., S. Tanapongpipat, G. Thomas, L. Griffiths, H. Crooke, and J. Cole. 1996. *Escherichia coli* K-12 genes essential for the synthesis of *c*-type cytochromes and a third nitrate reductase located in the periplasm. *Mol. Microbiol.* **19**:467–481.
- Grunden, A. M., R. M. Ray, J. K. Rosentel, F. G. Healy, and K. T. Shanmugam. 1996. Repression of the *Escherichia coli modABCD* (molybdate transport) operon by ModE. *J. Bacteriol.* **178**:735–744.
- Guest, J. R., J. Green, A. S. Irvine, and S. Spiro. 1996. The FNR modulon and FNR-regulated gene expression, p. 317–342. In E. C. C. Lin and A. S. Lynch (ed.), Regulation of gene expression in *Escherichia coli*. R. G. Landes Co., Georgetown, Tex.
- Kiley, P. J., and H. Beinert. 1998. Oxygen sensing by the global regulator, FNR: the role of the iron-sulfur cluster. *FEMS Microbiol. Rev.* **22**:341–352.
- Lewis, M. K., and D. V. Thompson. 1990. Efficient site directed in vitro mutagenesis using ampicillin selection. *Nucleic Acids Res.* **18**:3439–3443.
- Loh, E. Y., J. F. Elliott, S. Cwirla, L. L. Lanier, and M. M. Davis. 1989. Polymerase chain reaction with single-sided specificity: analysis of T cell receptor delta chain. *Science* **243**:217–220.
- Maloy, S., and V. Stewart. 1993. Autogenous regulation of gene expression. *J. Bacteriol.* **175**:307–316.
- Maloy, S. R., V. J. Stewart, and R. K. Taylor. 1996. Genetic analysis of pathogenic bacteria: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- McNicholas, P. M., and R. P. Gunsalus. 2002. The molybdate-responsive *Escherichia coli* ModE transcriptional regulator coordinates periplasmic nitrate reductase (*napFDAGHBC*) operon expression with nitrate and molybdate availability. *J. Bacteriol.* **184**:3253–3259.
- Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Pascal, M. C., J. F. Burini, J. Ratouchniak, and M. Chippaux. 1982. Regulation of the nitrate reductase operon: effect of mutations in *chlA*, B, D and E genes. *Mol. Gen. Genet.* **188**:103–106.
- Potter, L., H. Angove, D. J. Richardson, and J. Cole. 2001. Nitrate reduction in the periplasm of gram-negative bacteria. *Adv. Microb. Physiol.* **45**:51–112.
- Powell, B. S., M. P. Rivas, D. L. Court, Y. Nakamura, and C. L. Turnbough. 1994. Rapid confirmation of single copy lambda prophage integration by PCR. *Nucleic Acids Res.* **22**:5765. (Erratum, **23**:1278, 1995.)
- Rabin, R. S., and V. Stewart. 1993. Dual response regulators (NarL and NarP) interact with dual sensors (NarX and NarQ) to control nitrate- and nitrite-regulated gene expression in *Escherichia coli* K-12. *J. Bacteriol.* **175**:3259–3268.
- Reznikoff, W. S. 1992. The lactose operon-controlling elements: a complex paradigm. *Mol. Microbiol.* **6**:2419–2422.
- Self, W. T., A. M. Grunden, A. Hasona, and K. T. Shanmugam. 2001. Molybdate transport. *Res. Microbiol.* **152**:311–321.
- Simons, R. W., F. Houtman, and N. Kleckner. 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene*. **53**:85–96.
- Stewart, V., and P. J. Bledsoe. 2003. Synthetic *lac* operator substitutions for studying the nitrate- and nitrite-responsive NarX-NarL and NarQ-NarP two-component regulatory systems of *Escherichia coli* K-12. *J. Bacteriol.* **185**:2104–2111.
- Stewart, V., Y. Lu, and A. J. Darwin. 2002. Periplasmic nitrate reductase (NapABC enzyme) supports anaerobic respiration by *Escherichia coli* K-12. *J. Bacteriol.* **184**:1314–1323.
- Stewart, V., and J. Parales, Jr. 1988. Identification and expression of genes *narL* and *narX* of the *nar* (nitrate reductase) locus in *Escherichia coli* K-12. *J. Bacteriol.* **170**:1589–1597.
- Stewart, V., and R. S. Rabin. 1995. Dual sensors and dual response regulators interact to control nitrate- and nitrite-responsive gene expression in *Escherichia coli*, p. 233–252. In J. A. Hoch and T. J. Silhavy (ed.), Two-component signal transduction. ASM Press, Washington, D.C.
- Uden, G., and J. Bongaerts. 1997. Alternative respiratory pathways of *Escherichia coli*: energetics and transcriptional regulation in response to electron acceptors. *Biochim. Biophys. Acta* **1320**:217–234.



35. Wang, H., C. P. Tseng, and R. P. Gunsalus. 1999. The *napF* and *narG* nitrate reductase operons in *Escherichia coli* are differentially expressed in response to submicromolar concentrations of nitrate but not nitrite. *J. Bacteriol.* **181**:5303–5308.
36. Weickert, M. J., and S. Adhya. 1993. The galactose regulon of *Escherichia coli*. *Mol. Microbiol.* **10**:245–251.
37. Williams, S. B., and V. Stewart. 1997. Discrimination between structurally related ligands nitrate and nitrite controls autokinase activity of the NarX transmembrane signal transducer of *Escherichia coli* K-12. *Mol. Microbiol.* **26**:911–925.
38. Wing, H. J., S. M. Williams, and S. J. W. Busby. 1995. Spacing requirements for transcription activation by *Escherichia coli* FNR protein. *J. Bacteriol.* **177**:6704–6710.
39. Wu, S. Q., W. Chai, J. T. Lin, and V. Stewart. 1999. General nitrogen regulation of nitrate assimilation regulatory gene *nasR* expression in *Klebsiella oxytoca* M5a1. *J. Bacteriol.* **181**:7274–7284.