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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

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Two sensor proteins, NarX and NarQ, mediate nitrate regulation of anaerobic respiratory gene expression. Either of these sensors is sufficient to signal the presence of nitrate to the response regulator protein, NarL, a transcriptional activator and repressor. Two observations suggested the existence of a second response regulator that is also involved in nitrate regulation. First, *narL* null mutants retain residual nitrate induction of *fdnG* operon expression; this residual induction is absent in *narX narQ* double-null strains. Second, nitrate induction of *aeg-46.5* operon expression is substantially enhanced in *narL* null strains (M. H. Choe and W. S. Reznikoff, J. Bacteriol. 173:6139–6146, 1991). We found that this nitrate induction requires either the NarX or the NarQ protein, consistent with the existence of a second response regulator. We designate this second regulator NarP. We isolated insertion mutants that are defective in *aeg-46.5* operon expression. These insertions are in the *narP* gene, which encodes a response regulator that is 44% identical to the NarL protein. Null alleles of *narP* abolished *aeg-46.5* induction and also eliminated the residual NarL-independent nitrate induction of *fdnG* operon expression. Both the NarX and NarQ proteins communicate with both the NarP and NarL proteins. We found that the primary signal for NarP-dependent *aeg-46.5* operon induction is nitrite rather than nitrate. By contrast, nitrite is a relatively weak signal for NarL-dependent induction. In *narX* null strains, nitrite was an efficient signal for NarL-dependent induction, and this induction required the NarQ protein. We conclude that, in wild-type strains, the NarQ protein communicates the presence of nitrite to both the NarP and NarL proteins and that the NarX protein inhibits this communication with the NarL protein.

Escherichia coli is a facultative aerobe and can synthesize a variety of respiratory chains during anaerobic growth. Anaerobic induction of respiratory enzyme synthesis is mediated by the activator protein Fnr (reviewed in reference 35). The energetically most efficient anaerobic respiratory chain is formate-nitrate oxidoreductase, which consists of formate dehydrogenase-N (encoded by the *fdnGHI* operon), quinone, and nitrate reductase (encoded by the *narGHJI* operon) (reviewed in reference 38). During anaerobic growth, nitrate (NO_3^-) induces the synthesis of formate dehydrogenase-N and nitrate reductase through the action of the activator protein NarL (2, 37; reviewed in reference 39). The NarL protein also represses the synthesis of alternate respiratory enzymes such as fumarate reductase (encoded by the *frdABCD* operon) during anaerobic growth with nitrate (17, 19, 40). Thus, anaerobic respiratory enzyme synthesis is subject to hierarchical regulation, with dual control by anaerobiosis and nitrate ensuring that the organism makes the most efficient use of terminal electron acceptors for respiratory energy production (reviewed in reference 39).

Recently, Choe and Reznikoff (6) used operon fusions to identify a number of anaerobically expressed genes. Two of these genes, termed *aeg-46.5* and *aeg-93*, show unconventional regulation in response to nitrate. Expression of the *aeg-46.5* operon is weakly induced by nitrate during anaerobic growth. In a *narL* null mutant, *aeg-46.5* basal expression is elevated and transcription remains nitrate inducible. This shows that the NarL protein represses *aeg-46.5* expression even in the absence of nitrate. Regulation of the *aeg-93* operon resembles that of the *nrf* operon, encoding formate-dependent respiratory nitrite reductase (26). Additionally, the *nrf* genes map at 93 min (9). Thus, it is likely that the *aeg-93* operon fusion resides in one of the *nrf* genes. Expression of the *aeg-93* (*nrf*) operon is strongly induced by nitrate only in a *narL* null mutant. Thus, the regulation of the *aeg-46.5* and *aeg-93* (*nrf*) operons suggests that a NarL-independent pathway for nitrate regulation exists in *E. coli* (6, 26). Furthermore, these results indicate that the NarL protein acts differently at different target operons.

The NarL protein is homologous to response regulators of two-component regulatory systems (reviewed in reference 27). Molecular genetic analysis identified the NarX protein (41), a cognate sensor (histidine protein kinase) (reviewed in reference 27). The *narX* and *narL* genes form a complex operon tightly linked to the *narGHJI* operon (14, 41). By analogy to other two-component regulatory systems, autophosphorylated NarX protein acts as a substrate for NarL phosphorylation ("NarL kinase activity") in the presence of nitrate. Genetic analysis reveals that the NarX protein also plays a negative role in controlling NarL activity, probably through stimulating dephosphorylation of NarL-phosphate ("NarL-phosphate phosphatase activity") in the absence of nitrate (8, 15). Recently, Walker and DeMoss purified the NarX and NarL proteins and demonstrated in vitro autophosphorylation of NarX, transfer of phosphate from NarX-phosphate to NarL, and accelerated dephosphorylation of NarL-phosphate in the presence of NarX protein (44).

Nonpolar *narX* null deletions confer no observable phenotype with respect to nitrate regulation (14). This observa-

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TABLE 1. Strains, plasmids, and phages

Strain, plasmid, or phage	Genotype	Source
E. coli strains		
CAG12098	*zeg-722*::Tn*10*	32
CAG12177	*zeh-298*::Tn*10*	32
RZ4546.5	*aeg-46.5*::λ*plac*Mu53	6
RZ4593	*aeg-93*::λ*plac*Mu53	6
VJS632	Prototroph	41
VJS676	VJS632 Δ(*argF-lac*)*U619*	41
VJS691	VJS632 Δ(*argF-lac*)*U619* Δ(*trpEA*)*2*	41
VJS1357	VJS691 Δ(*narXL*)*235*	14
VJS1945	VJS676 λΦ(*fdnG-lacZ*)121_{UCA}	1
Strain VJS1945 derivatives		
VJS3038	Δ(*narXL*)*240*::Km	29
VJS3042	*narQ251*::Tn*10*d(Tc)	29
VJS3043	Δ*narX242 zch-2084*::Ω-Cm *narQ251*::Tn*10*d(Tc)	29
VJS3081	*narL215*::Tn*10*	29
VJS4325	*narP253*::Tn*10*d(Cm)	This work
VJS4326	*narL215*::Tn*10 narP253*::Tn*10*d(Cm)	This work
VJS4327	*narL215*::Tn*10 narQ252*::Tn*10*d(Cm)	This work
VJS4335	Δ*narX242*	This work
VJS4336	Δ(*narXL*)*240*::Km *narQ251*::Tn*10*d(Tc)	This work
VJS4337	Δ(*narXL*)*240*::Km *narP253*::Tn*10*d(Cm)	This work
VJS4338	*narL249*::Ω *narQ251*::Tn*10*d(Tc) *narP253*::Tn*10*d(Cm)	This work
VJS4339	Δ(*narXL*)*240*::Km *narQ251*::Tn*10*d(Tc) *narP253*::Tn*10*d(Cm)	This work
VJS4340	Δ*narX242 narP253*::Tn*10*d(Cm)	This work
VJS4341	*narQ251*::Tn*10*d(Tc) *narP253*::Tn*10*d(Cm)	This work
VJS4342	Δ*narX242 narQ251*::Tn*10*d(Tc) *narP253*::Tn*10*d(Cm)	This work
Plasmids		
pHG165	Ap^r	36
pVJS332	$narP^+$ in 8-kb *Bam*HI fragment in pHG165	This work
pVJS334	$narP^+$ in 1.8-kb *Eco*RI-*Bam*HI subclone of pVJS332	This work
Phages		
λNK1324	ATS-transposase Tn*10*d(Cm) delivery vehicle	20
λVJS206	Φ(*narG-lacZ*)*250* (gene fusion)	28
λVJS208	Φ(*frdA-lacZ*)*402* (gene fusion)	29
λVJS252	Φ(*fdnG-lacZ*)121_{UCA} (gene fusion)	1

tion led to the identification of a second nitrate sensor, the NarQ protein (5, 14, 29). Genetic analysis shows that either the NarX or the NarQ protein is sufficient for essentially wild-type nitrate regulation of target operon expression (29; reviewed in reference 39). This identification of the *narQ* gene, which is unlinked to any other *nar* regulatory gene, raised at least two questions: do physiological signals other than nitrate act to control target gene expression, and does a second response regulator also interact with the NarQ and NarX proteins? The work reported in this article addresses these questions. We have identified the structural gene for a second nitrate-responsive response regulator, the NarP protein. We provide genetic evidence that either the NarX or the NarQ protein can interact with the NarL and NarP proteins to regulate target operon expression in response to nitrate. Finally, we found that nitrite (NO_2^-), the reduction product of nitrate, also acts to control target operon expression through the NarX, NarQ, NarL, and NarP proteins.

MATERIALS AND METHODS

Strains, plasmids, and genetic methods. *E. coli* strains, plasmids, and λ specialized transducing phages used in this study are described in Table 1. For each gene and operon fusion studied, we employed P1 *kc*-mediated transduction (23) to construct a set of isogenic strains carrying the appropriate null alleles of the *nar* regulatory genes. The alleles *narL215*::Tn*10* (37), *narL249*::Ω (15), Δ(*narXL*)*240*::Km, Δ*narX242* and *zch-2084*::Ω-Cm (14), *narQ251*::Tn*10*d (Tc) and *narQ252*::Tn*10*d(Cm) (29), and *narP253*::Tn*10*d (Cm) (this work) were used. Only the strain set carrying λΦ(*fdnG-lacZ*) is listed in Table 1; strain sets bearing the other fusions were also derived from VJS676 and carried the same combinations of alleles. The Δ(*narXL*)*235* allele was used instead of the Δ(*narXL*)*240*::Km allele for the *aeg*::λ*plac*Mu53 fusion strains, which are kanamycin resistant (Km^r); the λ*plac*Mu53 fusions were transduced into strain VJS1357, and other markers were subsequently introduced as appropriate.

Media, culture conditions, and enzyme assays. Defined, complex, and indicator media for routine genetic manipulations were used as described previously (10, 23). Cultures for β-galactosidase assays were grown in 3-[*N*-morpholino]propanesulfonic acid (MOPS)-buffered minimal medium with glucose as the sole carbon source (25) as described previously (41). $NaNO_3$ (40 mM) or $NaNO_2$ (5 mM) was added as indicated. β-Galactosidase assays in permeabilized cells were described previously (23). All cultures were assayed in duplicate, and reported values are averaged from at least two independent experiments.

Mutant isolation. The indicator strain for mutagenesis carried *aeg-46.5*::λ*plac*Mu53 (6) and Δ(*narXL*)*235*. Mutagen-

esis was performed by insertion of Tn*10*d(Cm) by using λNK1324 as described previously (20). Mutagenized cultures were plated on nutrient agar-tetrazolium-lactose medium (23) supplemented with 40 mM $NaNO_3$ plus chloramphenicol (25 μg/ml). After aerobic incubation, lactose-nonfermenting (red) colonies were chosen for further analysis.

Cloning the *narP* gene. We used complementation analysis to determine which of the Kohara phages (21) contain the *narP* gene. Twenty phages carrying DNA around the 46.5-min region were tested because the *narP* gene is linked to *aeg-46.5* (see below). The indicator strain for complementation carried *aeg-46.5*::λ*plac*Mu53, Δ(*narXL*)*235*, and *narP253*::Tn*10*d(Cm). The Kohara phages lack *c*I repressor, so we used λ *c*I$^+$ as a helper phage. We spotted the Kohara phages plus helper phage on lawns of the indicator strain spread on tryptone plates. These plates were replica printed to MacConkey-lactose-nitrate plates. Lac$^+$ (red) lysogens were observed at spots containing either phage 20F6 or 19D1. We cloned the *narP*$^+$ gene on an 8-kb *Bam*HI fragment from phage 20F6 into the plasmid pHG165. To identify the desired clone, the indicator strain was again used to screen for the NarP$^+$ phenotype. Deletion subcloning yielded a 1.8-kb *Eco*RI-*Bam*HI fragment which carried the *narP*$^+$ gene (plasmid pVJS334).

Insertions of Mu dII1734 (MudK) into pVJS334 were isolated as described elsewhere (4). One *narP*::MudK insertion in pVJS334 was crossed to the *E. coli* chromosome as previously described (41). The *narP*::MudK insertion was then found to be greater than 98% linked to the *narP*::Tn*10*d(Cm) lesions; this confirmed that the cloned gene was allelic with the chromosomal gene.

DNA sequencing. The entire *narP* gene sequence was determined on both strands by the dideoxy chain-termination method, by using primers complementary to the ends of bacteriophage MudK as described previously (1). We used seven MudK insertions in pVJS334, located at approximately 150-bp intervals along the *narP* gene, to obtain the sequence. The *narP* flanking region was sequenced by using primers complementary to the *lacZ* gene on pHG165. Computer-assisted DNA sequence analysis employed the package of programs assembled by the Genetics Computer Group (12).

Nucleotide sequence accession number. The DNA sequence reported in this article has been deposited in the GenBank nucleotide sequence data base under accession number L11273.

RESULTS AND DISCUSSION

Rationale. Several observations led us to suspect that a second response regulator is involved in nitrate control of anaerobic respiratory gene expression. First, we and others recently discovered a second nitrate-responsive sensor protein, NarQ (5, 29), raising the question of whether there is a second response regulator as well. Second, we had previously observed slight but reproducible nitrate induction of *fdnG* operon expression in *narL* null strains (2). This residual induction was absent in *narX narQ* double-null strains (29), indicating that NarX and NarQ communicate the presence of nitrate to a response regulator other than the NarL protein. Finally, Choe and Reznikoff (6) reported NarL-independent nitrate induction of an anaerobically expressed operon fusion termed Φ(*aeg-46.5-lacZ*). We subsequently found that NarL-independent nitrate induction of Φ(*aeg-46.5-lacZ*) expression was eliminated in the *narX narQ* double-null strain (this work; see below). We therefore elected to isolate mutations in the gene (*narP*) encoding this second nitrate regulatory protein.

With dual sensors, NarX and NarQ, we wondered whether signals other than nitrate could influence regulation. We had previously noted relatively weak nitrite induction of *fdnG* and *narG* operon expression (2). Also, Tyson et al. (43), in studying *nirB* (NADH-nitrite reductase) operon expression, concluded that a regulatory protein in addition to the NarL protein is involved in mediating nitrite regulation. Thus, we elected to examine the effects of nitrite as well as nitrate on target operon expression.

Our strategy was first to identify and characterize the *narP* gene and then to examine the effects of *narP*, *narL*, *narX*, and *narQ* null alleles singly and in combination on the expression of various target operons.

Identification and mapping of *narP*. We built a strain carrying a deletion of the *narL* and *narX* genes as well as the Φ(*aeg-46.5-lacZ*) operon fusion. We mutagenized this strain with Tn*10*d(Cm) and screened for mutants with decreased β-galactosidase expression on indicator plates, as described in Materials and Methods. We recovered several candidates, which were characterized by genetic and phenotypic tests. Several of the mutants carried Tn*10*d(Cm) in the *narQ* gene, validating the mutant screen [recall that *narX narQ* double-null strains have very low Φ(*aeg-46.5-lacZ*) expression]. Other candidates had phenotypes similar to the *narQ* mutants but did not carry insertions in the *narQ* gene. Six such strains were chosen for further analysis; each proved to carry a *narP*::Tn*10*d(Cm) insertion.

In the course of using generalized transduction to backcross the candidate *narP*::Tn*10*d(Cm) insertions, we observed approximately 70% linkage to the Φ(*aeg-46.5-lacZ*) operon fusion, which is marked with Km^r. To further localize the position of *narP*::Tn*10*d(Cm) insertions, we performed reciprocal crosses with the *zeg-722*::Tn*10* and *zeh-298*::Tn*10* insertions, which are located in the 46- to 47-min region of the genetic map (32). Results from a variety of crosses revealed a gene order of *narP*–(*aeg-46.5*, *zeg-722*)–*zeh-298* (data not shown). This places *narP* to the counterclockwise side of *aeg-46.5*.

The close linkage of *narP* and *aeg-46.5* raises the question of whether these genes form an operon. However, the chromosomal Lac phenotype of *narP* strains was fully complemented by a small (1.8-kb) clone (see Materials and Methods), indicating that the two genes are separately transcribed.

Our sequence analysis (see below) also identified the end of an IS*5* insertion at the *Eco*RI end of the *narP* subclone. This observation helps to confirm the physical map location of the *narP* gene (Fig. 1; see legend for details).

Molecular cloning and sequence of *narP*. The *narP*$^+$ gene was cloned by complementation from the Kohara phage library as described in Materials and Methods. Further subcloning delimited the *narP*$^+$ gene to a 1.8-kb *Eco*RI-*Bam*HI fragment. A *narP*::MudK insertion constructed in the plasmid was recombined into the chromosome and mapped to confirm the identity of the *narP* sequence.

The presumed translation initiation region for the *narP* gene is 5′-CTCAGGAGACTACTATG-3′, where the Shine-Dalgarno region and initiation codon are underlined. The sequence (Fig. 2) reveals an open reading frame whose predicted 24-kDa product is clearly homologous to the *narL* gene product; the two proteins share 44% identical residues with only one small gap to maintain alignment (Fig. 3). In fact, data base searches show that the NarL and NarP

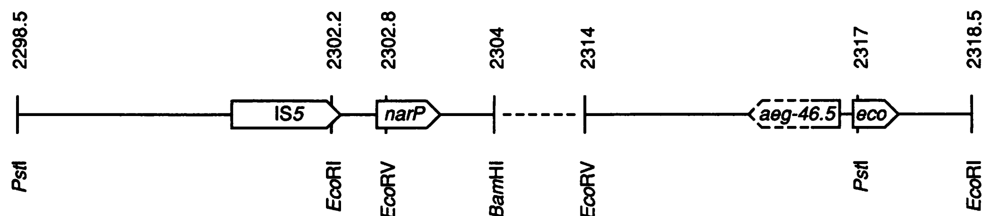

FIG. 1. Physical map of the *narP* region. Coordinates (in kilobases) are taken from the revised Kohara map (30). The relative positions of the *Eco*RI, *Eco*RV, and *Bam*HI sites in the vicinity of the *narP* gene were determined by DNA sequencing (this work) and match those of the revised map (30). The IS*5* insertion has been described previously (3, 24). The sequence of the 5′ untranslated region of the *aeg-46.5* gene was found to overlap with that of the *eco* gene (7). Since the map location of the *eco* gene is known (16), we have inferred the relative position of the *aeg-46.5* gene and that the two genes are transcribed divergently. The extent of the *aeg-46.5* gene is unknown (7). No known genes are located between *narP* and *aeg-46.5*. The physical map locations and the convergent transcriptional directions of the *narP* and *aeg-46.5* genes demonstrate that the two genes are not in the same operon.

proteins are more similar to each other than either is to any other identified protein. The identities tend to cluster around highly conserved regions involved in phosphorylation of response regulators and around the helix-turn-helix motifs (Fig. 3).

The deduced NarL protein is in a small subset of response regulators that includes UhpA and DegU (reviewed in reference 27). The predicted NarP sequence shares similar features with members of this family. In particular, the carboxyl termini of the NarP and NarL proteins contain predicted helix-turn-helix motifs of the type that is found in other DNA-binding proteins such as MalT, FixJ, and RcsA (Fig. 3) (18, 42).

The NarX and NarQ proteins are also members of a minor subfamily of sensor proteins, including UhpB and DegS, and likewise are more similar to each other than either is to any other identified protein. It is therefore appealing that the response regulators, NarL and NarP, are members of the cognate subfamily.

Regulation of *aeg-46.5* operon expression. We identified the *narP* gene in screens for altered *aeg-46.5* operon expression, so we initially focused on *aeg-46.5* regulation in various *nar* mutant backgrounds. Strains carrying each possible combination of the four *nar* regulatory gene null alleles were constructed and assayed for β-galactosidase synthesis as described in Materials and Methods. The results are shown in Table 2.

In wild-type strains, Φ(*aeg-46.5-lacZ*) expression was weakly induced by nitrate and substantially induced by nitrite. As found by Choe and Reznikoff (6), introduction of a *narL* null allele caused increased Φ(*aeg-46.5-lacZ*) expression in both the absence and presence of nitrate. Likewise, nitrite induction was elevated in the *narL* null strain. By contrast, the *narP* null allele reduced Φ(*aeg-46.5-lacZ*) expression to basal levels, irrespective of growth conditions; the *narP narL* double-null strain had the same phenotype (Table 2). These results show that the NarP protein activates Φ(*aeg-46.5-lacZ*) expression in response to nitrate and nitrite and that the NarL protein antagonizes this activation.

In *narL* null strains, either *narQ*$^+$ or *narX*$^+$ was sufficient for maximal NarP-mediated nitrate and nitrite induction, although this induction was somewhat more pronounced in *narQ*$^+$ strains (Table 2). This shows that either the NarX or NarQ protein is sufficient to phosphorylate the NarP protein in response to either nitrate or nitrite. By contrast, *narX narQ* double-null strains were devoid of Φ(*aeg-46.5-lacZ*) expression in response to either nitrate or nitrite, showing that at least one of the sensor proteins is required for this expression. Indeed, it was this latter observation that led us to search for the *narP* gene. Finally, the *narX narQ narL* triple-null mutant reveals that the high uninduced basal level of Φ(*aeg-46.5-lacZ*) expression required either the *narX*$^+$ or *narQ*$^+$ gene, indicating that this expression is due to relatively low-level, nitrate- and nitrite-independent phosphorylation of the NarP protein by the NarX and NarQ proteins.

We do not understand the relative action of the NarL and NarP proteins in controlling Φ(*aeg-46.5-lacZ*) expression, and we do not understand the role of phosphorylation in this control. Therefore, the complex phenotypes conferred by *narX* and *narQ* null alleles in *narL*$^+$ *narP*$^+$ strains are difficult to interpret. In the presence of nitrate, the *narX* strain behaved essentially as the wild type did, while the

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ATGCCTGAAGCAACACCTTTTCAGGTGATGATTGTGGATGATCATCCACTTATGCGACGCGGTGTTCGTCAGTTACTGGAGCTTGATCCT   90
M  P  E  A  T  P  F  Q  V  M  I  V  D  D  H  P  L  M  R  R  G  V  R  Q  L  L  E  L  D  P    30
                                                                   EcoRV
GGCTCTGAAGTGGTCGCCGAAGCGGGCGACGGCGCGAGCGCTATCGATCTGGCGAATAGACTGGATATCGACGTGATCTTGCTGGATCTC  180
G  S  E  V  V  A  E  A  G  D  G  A  S  A  I  D  L  A  N  R  L  D  I  D  V  I  L  L  D  L    60

AATATGAAAGGTATGAGTGGCCTGGATACTCTCAATGCCTTGCGCAGGGATGGCGTTACCGCGCAAATTATTATCCTGACCGTATCCGAT  270
N  M  K  G  M  S  G  L  D  T  L  N  A  L  R  R  D  G  V  T  A  Q  I  I  I  L  T  V  S  D    90

GCCTCCAGCGATGTCTTTGCGCTGATAGACGCAGGCGCAGACGGTTATCTGTTGAAAGACAGCGACCCGGAAGTATTGCTGGAAGCGATT  360
A  S  S  D  V  F  A  L  I  D  A  G  A  D  G  Y  L  L  K  D  S  D  P  E  V  L  L  E  A  I   120

CGTGCCGGAGCGAAAGGCAGCAAAGTCTTTAGCGAACGCGTCAATCAGTACTTACGTGAACGTGAAATGTTTGGCGCGGAAGAAGATCCC  450
R  A  G  A  K  G  S  K  V  F  S  E  R  V  N  Q  Y  L  R  E  R  E  M  F  G  A  E  E  D  P   150

TTCAGCGTGCTGACGGAGCGCGAGCTGGATGTTCTGCACGAGCTGGCACAGGGGCTGTCAAATAAACAGATTGCCTCGGTGTTGAATATT  540
F  S  V  L  T  E  R  E  L  D  V  L  H  E  L  A  Q  G  L  S  N  K  Q  I  A  S  V  L  N  I   180

TCCGAGCAGACAGTAAAAGTACATATTCGCAATCTGCTGCGTAAACTCAATGTCCGCTCACGCGTGGCGGCCACCATTCTGTTCCTGCAA  630
S  E  Q  T  V  K  V  H  I  R  N  L  L  R  K  L  N  V  R  S  R  V  A  A  T  I  L  F  L  Q   210

CAACGCGGGGCACAATAA                                                                          648
Q  R  G  A  Q                                                                               215
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FIG. 2. Nucleotide sequence of the *narP* gene and predicted amino acid sequence in the standard single-letter code. The predicted translational start and stop sites are underlined. An *Eco*RV site that aids alignment to the *E. coli* physical map (see Fig. 1) is indicated.

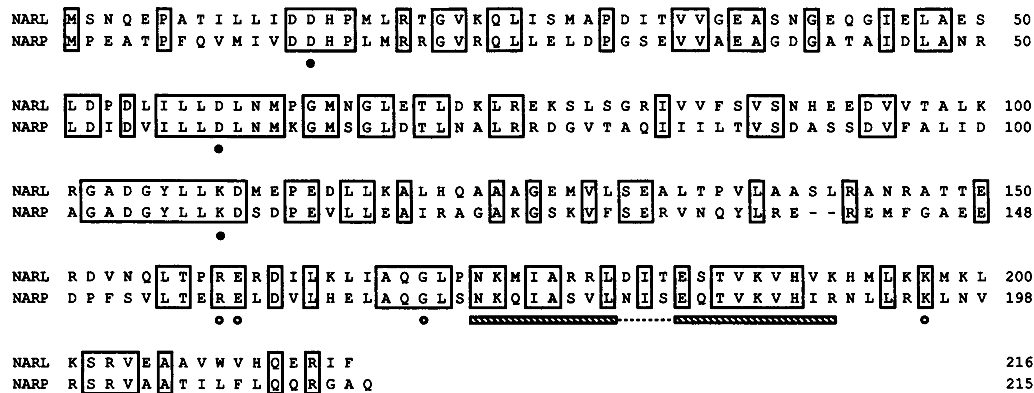

FIG. 3. Sequence alignment of NarP and NarL in the standard single-letter code. Identical residues are boxed. Symbols: –, gaps introduced to maintain alignment; ●, conserved residues (Asp and Lys) found in response regulator proteins; ○, conserved residues in the DNA-binding domains of the FixJ-RcsA subfamily. The presumed helix-turn-helix motif is underlined.

narQ strain exhibited uninducible Φ(*aeg-46.5-lacZ*) expression. By contrast, in the presence of nitrite, the *narX* and *narQ* strains were equally uninducible (Table 2). We have considered a variety of explanations for these observations but prefer to reserve interpretation until we better understand the roles of the NarP and NarL proteins in this control.

Finally, we examined the effects of an *fnr* null allele on Φ(*aeg-46.5-lacZ*) expression in *narL* and *narP* strains. Expression was very low irrespective of *nar* genotype (data not shown), showing that the *fnr* mutation is epistatic to *narL* and *narP* mutations, as expected.

The Φ(*aeg-46.5-lacZ*) operon fusion results from an insertion in the *aeg-46.5* operon, thus disrupting the function of its encoded product(s). This could potentially affect the observed pattern of regulation, if, for example, the *aeg-46.5* operon product(s) plays an essential metabolic role in anaerobiosis. However, we monitored nitrate reductase synthesis and nitrite production or consumption in *aeg-46.5* cultures, and both aspects were indistinguishable from those of the wild-type in both nitrate- and nitrite-grown cultures (data not shown).

Regulation of *fdnG* operon expression. We next examined the effects of *nar* regulatory genes on Φ(*fdnG-lacZ*) expression. Previous work from our laboratory has shown that the *fdnG* operon is induced approximately 100-fold by growth with nitrate and that either *narX*$^+$ or *narQ*$^+$ is sufficient for normal induction. In addition, approximately fourfold induction by nitrate remains in *narL* null strains, but this low-level induction is abolished in *narX narQ* double-null strains. Finally, the *fdnG* operon is weakly induced during growth with nitrite (2, 29).

Wild-type and *narP* null strains exhibited similar patterns of Φ(*fdnG-lacZ*) expression, namely, strong induction by nitrate and weak induction by nitrite (Table 3). The *narL* null strain exhibited weak nitrate and nitrite induction. However, the *narL narP* double-null strain, like the *narX narQ* strain, was devoid of Φ(*fdnG-lacZ*) induction by either nitrate or nitrite (Table 3). This shows that the NarP protein is responsible for the residual induction observed in *narL* null strains. We do not know how or where the NarP protein acts in controlling *fdnG* operon expression.

In *narL*$^+$ strains, irrespective of *narP* genotype, introduction of a *narQ* null allele had only a small effect on nitrate and nitrite induction of Φ(*fdnG-lacZ*) expression (Table 3), as expected from previous observations. However, the situation was quite different in the *narX* null derivatives, where nitrite induction was elevated to nearly the same levels as nitrate induction in the wild type (Table 3). This is a very striking result, for it provides a clear-cut regulatory phenotype for a *narX* null *narQ*$^+$ strain (14, 29). We have argued previously that the NarX protein plays a negative role in NarL-mediated gene expression (8, 15). This observation provides clear evidence for a negative function of the NarX protein: eliminating NarX (but not NarQ) by mutation caused significant NarL-dependent induction of gene expression in the presence of nitrite.

An interpretation of this result, that nitrite is an efficient Φ(*fdnG-lacZ*) inducer in a *narX* null strain, is that the

TABLE 2. Φ(*aeg-46.5-lacZ*) expression in *nar* regulatory mutants

Genotype[a]	β-Galactosidase sp act[b]											
	narL$^+$ *narP*$^+$			*narL narP*$^+$			*narL*$^+$ *narP*			*narL narP*		
	None[c]	$+NO_3^-$[c]	$+NO_2^-$[c]	None	$+NO_3^-$	$+NO_2^-$	None	$+NO_3^-$	$+NO_2^-$	None	$+NO_3^-$	$+NO_2^-$
narX$^+$ *narQ*$^+$	51	130	520	300	1,540	1,360	25	19	24	25	27	18
narX narQ$^+$	26	140	47	400	1,630	1,420	27	20	18	23	25	18
narX$^+$ *narQ*	35	28	43	360	1,100	920	25	20	25	24	28	19
narX narQ	25	31	19	26	31	21	22	24	17	26	27	20

[a] Combination of *narL*, *narP*, *narX*, and *narQ* wild-type or null alleles as indicated.
[b] β-Galactosidase specific activity was measured as described in Materials and Methods and is expressed in Miller units.
[c] Anaerobic growth with no acceptor (None), with nitrate ($+NO_3^-$), or with nitrite ($+NO_2^-$) as indicated.

TABLE 3. Φ(*fdnG-lacZ*) expression in *nar* regulatory mutants

Genotype[a]	β-Galactosidase sp act[b]											
	narL⁺ *narP*⁺			*narL narP*⁺			*narL*⁺ *narP*			*narL narP*		
	None[c]	$+NO_3^-$[c]	$+NO_2^-$[c]	None	$+NO_3^-$	$+NO_2^-$	None	$+NO_3^-$	$+NO_2^-$	None	$+NO_3^-$	$+NO_2^-$
narX⁺ *narQ*⁺	6	1,000	24	7	34	23	7	910	18	7	6	5
narX narQ⁺	18	920	800	6	35	21	14	890	870	7	5	4
narX⁺ *narQ*	6	910	10	9	16	15	6	890	12	6	6	5
narX narQ	6	7	5	9	6	5	6	5	7	6	7	5

[a] Combination of *narL*, *narP*, *narX*, and *narQ* wild-type or null alleles as indicated.
[b] β-Galactosidase specific activity was measured as described in Materials and Methods and is expressed in Miller units.
[c] Anaerobic growth with no acceptor (None), with nitrate ($+NO_3^-$), or with nitrite ($+NO_2^-$) as indicated.

equilibrium of the NarX protein, in response to nitrite, is shifted more toward NarL-phosphate phosphatase than NarL kinase activity. Thus, in the wild type, much of the NarL-phosphate formed by the NarQ protein in response to nitrite would be dephosphorylated by the NarX protein. In the mutant lacking the NarX protein, NarL-phosphate would accumulate, leading to substantial Φ(*fdnG-lacZ*) induction.

In the *narL* null strain, nitrate and nitrite induction were consistently higher in the *narX* than in the *narQ* derivative. This mirrors our observations with Φ(*aeg-46.5-lacZ*) expression, where the *narQ* strain was slightly defective in nitrate induction in the *narL* null background (Table 2). Taken together, these observations suggest that the NarQ protein phosphorylates the NarP protein to a somewhat higher level than does the NarX protein, although the differences are rather subtle.

Regulation of *narG* operon expression. Expression of the *narG* operon is induced approximately 100-fold by nitrate, and this induction is abolished by *narL* null alleles (37). Either the NarX or NarQ protein is sufficient for normal regulation (29). *narG* operon expression is also induced by nitrite (2).

A *narP* null allele had no effect on nitrate or nitrite induction of Φ(*narG-lacZ*) expression (Table 4). Conversely, a *narL* null allele abolished both nitrate and nitrite induction, irrespective of the presence of *narP*⁺ (Table 4). Thus, the *narP* gene has no effect on Φ(*narG-lacZ*) expression.

In a *narX* null mutant, nitrite was as effective as nitrate in inducing Φ(*narG-lacZ*) expression, as observed also with Φ(*fdnG-lacZ*) expression (compare Tables 3 and 4). Conversely, nitrite induction of Φ(*narG-lacZ*) expression in the *narQ* null strain was less efficient than in the wild type (compare Tables 3 and 4). As described above, we believe that these observations reflect a negative regulatory role for the *narX*⁺ gene in nitrite regulation.

Regulation of *frdA* operon expression. Expression of the *frdA* operon is repressed approximately 10-fold by nitrate, and this repression is abolished by *narL* null alleles (17, 40). Either the NarX or NarQ protein is sufficient for full repression (5, 29), while repression is abolished in a *narX narQ* double-null strain. Φ(*frdA-lacZ*) expression was slightly stimulated by nitrite (Table 5).

Again, a *narP* null allele had no effect on Φ(*frdA-lacZ*) expression, while a *narL* null allele abolished nitrate repression irrespective of the presence of *narP*⁺ (Table 5). Thus, the *narP* gene had no effect on Φ(*frdA-lacZ*) expression.

Nitrite was an effective repressor of Φ(*frdA-lacZ*) expression in a *narX* null mutant, while it was without effect in a *narQ* null strain and in the *narX narQ* double-null strain. Again, we believe that nitrite repression in the *narX* null strain reflects a negative regulatory role for the NarX protein.

Hierarchy of NarL-mediated regulation. Several observations have led us to believe that expression of the *narG*, *fdnG*, and *frdA* operons is differentially sensitive to NarL-mediated regulation. First, *narX* insertion alleles, which reduce (but do not eliminate) *narL* gene expression through polarity, cause only a slight decrease in nitrate induction of *narG* operon induction while virtually eliminating nitrate repression of *frdA* operon expression (14, 41). Nitrate induction of *fdnG* operon expression in such strains is decreased by a greater extent than is *narG* expression, but it is not eliminated (2). These observations suggest a hierarchy of *narG* > *fdnG* > *frdA* with respect to the threshold of NarL-phosphate necessary for regulation. Other observations with missense constitutive alleles of *narL* (15) and *narX* (8) support this hierarchy: in each case, the constitutive allele causes relatively high-level expression of Φ(*narG-lacZ*), intermediate expression of Φ(*fdnG-lacZ*), and little or no repression of Φ(*frdA-lacZ*).

TABLE 4. Φ(*narG-lacZ*) expression in *nar* regulatory mutants

Genotype[a]	β-Galactosidase sp act[b]											
	narL⁺ *narP*⁺			*narL narP*⁺			*narL*⁺ *narP*			*narL narP*		
	None[c]	$+NO_3^-$[c]	$+NO_2^-$[c]	None	$+NO_3^-$	$+NO_2^-$	None	$+NO_3^-$	$+NO_2^-$	None	$+NO_3^-$	$+NO_2^-$
narX⁺ *narQ*⁺	33	3,100	380	30	23	25	32	2,860	280	25	28	31
narX narQ⁺	57	2,880	3,320	29	21	23	—[d]	—	—	—	—	—
narX⁺ *narQ*	30	2,950	140	—	—	—	—	—	—	—	—	—
narX narQ	29	29	34	—	—	—	—	—	—	—	—	—

[a] Combination of *narL*, *narP*, *narX*, and *narQ* wild-type or null alleles as indicated.
[b] β-Galactosidase specific activity was measured as described in Materials and Methods and is expressed in Miller units.
[c] Anaerobic growth with no acceptor (None), with nitrate ($+NO_3^-$), or with nitrite ($+NO_2^-$) as indicated.
[d] —, not determined.

TABLE 5. Φ(*frdA-lacZ*) expression in *nar* regulatory mutants

Genotype[a]	β-Galactosidase sp act[b]											
	narL⁺ *narP*⁺			*narL narP*⁺			*narL*⁺ *narP*			*narL narP*		
	None[c]	$+NO_3^-$ [c]	$+NO_2^-$ [c]	None	$+NO_3^-$	$+NO_2^-$	None	$+NO_3^-$	$+NO_2^-$	None	$+NO_3^-$	$+NO_2^-$
narX⁺ *narQ*⁺	110	10	160	110	120	140	110	12	130	110	120	130
narX narQ⁺	110	9	11	110	120	150	—[d]	—	—	—	—	—
narX⁺ *narQ*	110	17	140	—	—	—	—	—	—	—	—	—
narX narQ	110	130	130	—	—	—	—	—	—	—	—	—

[a] Combination of *narL*, *narP*, *narX*, and *narQ* wild-type or null alleles as indicated.
[b] β-Galactosidase specific activity was measured as described in Materials and Methods and is expressed in Miller units.
[c] Anaerobic growth with no acceptor (None), with nitrate ($+NO_3^-$), or with nitrite ($+NO_2^-$) as indicated.
[d] —, not determined.

The patterns of nitrite induction in wild-type strains provide further support for this hierarchy (compare Tables 3, 4, and 5). Nitrite induction of Φ(*narG-lacZ*) expression was greater than that of Φ(*fdnG-lacZ*) expression, while Φ(*frdA-lacZ*) expression was essentially indifferent to nitrite. Taken together with the above-described results, this reflects, in our opinion, differential sensitivities of the various control regions to the amount of NarL-phosphate. Relatively low levels of NarL-phosphate seem to be sufficient for substantial *narG* operon induction, while relatively high levels of NarL-phosphate seem to be required for *frdA* operon repression. If the NarX protein is predominantly (but not exclusively) a NarL-phosphate phosphatase in the presence of nitrite, then relatively little NarL-phosphate would accumulate in wild-type cells grown with nitrite. This low level of NarL-phosphate would be sufficient for relatively strong Φ(*narG-lacZ*) expression but insufficient for observable Φ(*frdA-lacZ*) repression.

The arrangement of NarL protein-binding sites in the *narG* and *fdnG* operon control regions is quite different (13, 22), and the control regions also differ in their requirement for an accessory factor, integration host factor (28). Thus, the differential sensitivities of these two control regions for NarL-phosphate could reflect different binding-site affinities or different mechanisms of transcription activation. Likewise, the levels of NarL-phosphate necessary to repress *frdA* operon expression depend on binding affinity and mode of action. Therefore, the mechanistic bases for this hierarchy of sensitivity to NarL-mediated regulation are currently unknown.

Regulation of *aeg-93* (*nrf*) operon expression. Expression of Φ(*aeg-93-lacZ*) is induced by nitrate in a *narL* null strain (6). This regulation, which is analogous to but distinct from that of the *aeg-46.5* operon, also suggested the existence of a nitrate-responsive transcription factor in addition to the NarL protein. Thus, we examined Φ(*aeg-93-lacZ*) expression in response to nitrate and nitrite in the various *nar* regulatory gene mutants (Table 6).

In *narL*⁺ strains, Φ(*aeg-93-lacZ*) expression was efficiently induced by nitrite but not nitrate irrespective of the *narP* gene. By contrast, Φ(*aeg-93-lacZ*) expression in the *narP*⁺ *narL* null strain was induced equally well by either nitrate or nitrite. This shows that the NarL protein represses Φ(*aeg-93-lacZ*) expression in response to nitrate but activates expression in response to nitrite. The NarP protein, by contrast, mediates activation by both nitrate and nitrite.

In the *narL* null *narP*⁺ strain, either *narX*⁺ or *narQ*⁺ was sufficient for full induction by either nitrate or nitrite (Table 6), demonstrating again that either the NarX or NarQ protein can activate the NarP protein in response to either nitrate or nitrite. In the absence of nitrate and nitrite, Φ(*aeg-93-lacZ*) expression was elevated in the *narQ narL* null strain. This implies that the NarQ protein negatively regulates the NarP protein, much like the NarX protein negatively regulates the NarL protein. However, basal expression of Φ(*aeg-46.5-lacZ*) was not elevated in the *narQ narL* strain (Table 2).

Regulation by nitrate and nitrite followed essentially identical patterns in *narL*⁺ strains, irrespective of the presence of the *narP* gene. This shows that NarL-mediated repression is phenotypically dominant to NarP-mediated activation. That the same regulatory pattern was observed in both *narL*⁺ *narP*⁺ and *narL*⁺ *narP* null strains implies that induction of Φ(*aeg-93-lacZ*) expression in *narL*⁺ strains reflects NarL-mediated activation. Although more complicated hypotheses are not excluded, this is the simplest one that explains currently available data.

In *narL*⁺ strains, nitrate prevented Φ(*aeg-93-lacZ*) expression equally well in *narX* and in *narQ* null strains,

TABLE 6. Φ(*aeg-93-lacZ*)[a] expression in *nar* regulatory mutants

Genotype[b]	β-Galactosidase sp act[c]											
	narL⁺ *narP*⁺			*narL narP*⁺			*narL*⁺ *narP*			*narL narP*		
	None[d]	$+NO_3^-$	$+NO_2^-$	None	$+NO_3^-$	$+NO_2^-$	None	$+NO_3^-$	$+NO_2^-$	None	$+NO_3^-$	$+NO_2^-$
narX⁺ *narQ*⁺	27	46	630	23	540	580	29	37	670	17	67	110
narX narQ⁺	25	62	58	27	520	590	25	43	46	18	68	100
narX⁺ *narQ*	33	64	630	100	490	570	40	53	660	22	80	120
narX narQ	23	200	210	25	92	150	68	160	460	19	55	99

[a] The *aeg-93* locus probably corresponds to *nrf*, which encodes formate-dependent respiratory nitrite reductase (9).
[b] Combination of *narL*, *narP*, *narX*, and *narQ* wild-type or null alleles as indicated.
[c] β-Galactosidase specific activity was measured as described in Materials and Methods and is expressed in Miller units.
[d] Anaerobic growth with no acceptor (None), with nitrate ($+NO_3^-$), or with nitrite ($+NO_2^-$) as indicated.

showing that either the NarX or NarQ protein is able to activate NarL-mediated repression (Table 6). This suggests that high levels of NarL-phosphate act to repress Φ(*aeg-93-lacZ*) expression, irrespective of the presence of NarP-phosphate.

The pattern of regulation by nitrite was quite different. In *narX*$^+$ strains, nitrite was an effective inducer of NarL-mediated Φ(*aeg-93-lacZ*) expression. By contrast, in the *narX* null *narQ*$^+$ strain, nitrite had little effect on NarL-mediated Φ(*aeg-93-lacZ*) expression. We have argued above that *narX*$^+$ strains have relatively low levels of NarL-phosphate when cultured in the presence of nitrite, while *narX* null *narQ*$^+$ strains accumulate relatively high levels of NarL-phosphate. Thus, the data for the single-sensor mutants suggest that NarL-mediated induction of Φ(*aeg-93-lacZ*) expression occurs only when NarL-phosphate levels are relatively low (nitrite-grown *narX*$^+$ cultures). When NarL-phosphate levels are relatively high (nitrate-grown cultures and nitrite-grown *narX* null *narQ*$^+$ cultures), Φ(*aeg-93-lacZ*) is not expressed.

This hypothesis seemingly presents a paradox. We concluded above that higher levels of NarL-phosphate result in more efficient induction of *fdnG* operon expression, for example, but here we argue that lower levels of NarL-phosphate result in more efficient induction of *aeg-93* (*nrf*) operon expression. Can these ideas be reconciled? A difference in these models is that high levels of NarL-phosphate are postulated to repress *aeg-93* (*nrf*) operon expression, while the NarL protein does not repress *fdnG* operon expression under any conditions. Thus, NarL-phosphate plays a dual role at the *aeg-93* (*nrf*) control region.

An explanation for this dichotomy follows the model for OmpR-mediated regulation of the *ompF* gene (31, 33, 34). Silhavy and students have interpreted their genetic analyses to reveal *ompF* gene activation when OmpR-phosphate levels are relatively low and *ompF* gene repression when OmpR-phosphate levels are relatively high. The key to this model is that repression (by high levels of OmpR-phosphate) is phenotypically dominant to activation (by low levels of OmpR-phosphate). Our own genetic analysis of *aeg-93* (*nrf*) regulation is much less advanced than that for the *ompF* gene, but this type of model provides an attractive working hypothesis to guide future experimentation.

Residual nitrate and nitrite induction of Φ(*aeg-93-lacZ*) expression remained in *narL narP* double-null, *narX narQ* double-null, and all combinations of triple-null strains (Table 6). In fact, some induction remained even in the quadruple-null mutant. This implies that yet a third nitrate- and nitrite-responsive regulatory system is involved in regulating *aeg-93* (*nrf*) operon expression. Elucidation of this system remains a challenge for future work.

Finally, we examined the effects of an *fnr* null allele on Φ(*aeg-93-lacZ*) expression in *narL* and *narP* strains. Expression was very low irrespective of *nar* genotype (data not shown), showing that the *fnr* mutation is epistatic to *narL* and *narP* mutations, as expected.

The growth physiology of the *aeg-93* (*nrf*) insertion strain was tested in parallel with that of the *aeg-46.5* strain (see above), and again no difference from that of the wild type was observed (data not shown).

Hypothesis. The interplay between two response regulators, two sensors, two regulatory metabolites, and five target operons inevitably means that complex and diverse regulatory interactions are involved in different contexts. We have attempted to organize and summarize our findings and interpretations in a comprehensive hypothesis. This is a

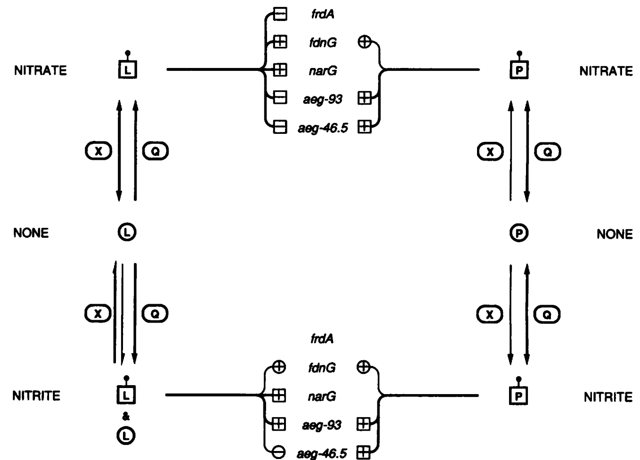

FIG. 4. Hypothesis for nitrate- and nitrite-regulated gene expression. See text for details. Activation and repression of target operon expression are indicated by + and −, respectively. Strong interactions are boxed, while relatively weak interactions are circled. NarL and NarP are represented by circled L and circled P, respectively, while the phosphorylated forms are represented by the same letters boxed. The sensors NarX and NarQ are represented by X and Q, respectively. In the presence of nitrite, a relatively small proportion of NarL molecules is phosphorylated. Target operons are as follows: *frdA*, fumarate reductase; *fdnG*, formate dehydrogenase-N; *narG*, nitrate reductase; *aeg-46.5*, anaerobically expressed gene of unknown function at 46.5 map units; *aeg-93*, probably formate-nitrite reductase (*nrf*).

working hypothesis which we believe adequately explains most of the currently available data, although alternate explanations exist for some points. This hypothesis, depicted schematically in Fig. 4, will help to guide future work aimed at understanding nitrate and nitrite control of anaerobic respiratory gene expression. For reference, Table 7 summarizes the observed regulatory patterns.

First, consider NarL-mediated regulation. In the presence of nitrate, either the NarX or NarQ protein is sufficient to form high levels of NarL-phosphate. This amount of NarL-phosphate fully activates *narG* and *fdnG* operon expression and fully represses *frdA*, *aeg-46.5*, and *aeg-93* (*nrf*) operon expression. The NarX protein also negatively regulates the NarL protein by acting as a NarL-phosphate phosphatase in the absence of nitrate. The NarQ protein apparently does not have a similar negative role (29; and this work).

In the presence of nitrite, the NarQ protein acts to phosphorylate NarL with an efficiency approaching that seen in nitrate-grown cultures. However, the NarX protein acts predominantly as a NarL-phosphate phosphatase, although some NarL kinase activity is also present. The net effect is that only intermediate levels of NarL-phosphate accumulate in wild-type nitrite-grown cells. This intermediate level of NarL-phosphate activates the *narG* operon relatively efficiently, activates the *fdnG* operon and represses the *aeg-46.5* operon relatively weakly, and has no effect on expression of the *frdA* operon. This intermediate level of NarL-phosphate is insufficient to repress expression of the *aeg-93* (*nrf*) operon and instead actually activates its expression.

Second, consider NarP-mediated regulation. The NarP protein apparently has no effect on *narG* or *frdA* operon expression. In the presence of nitrate or nitrite, the NarP protein is phosphorylated by either NarQ or NarX to ap-

TABLE 7. Summary of *nar* regulatory gene product action[a]

Operon	Function	Action in the presence of:	
		Nitrate[b]	Nitrite[b]
narGHJI	Nitrate reductase	NarL (X, Q) activates; NarP, no effect	NarL (X) inhibits; (Q) activates; NarP, no effect
fdnGHI	Formate dehydrogenase-N	NarL (X, Q) activates; NarP (X, Q) weakly activates	NarL (X) inhibits; (Q) activates; NarP (X, Q) weakly activates
frdABCD	Fumarate reductase	NarL (X, Q) inhibits; NarP, no effect	NarL, no effect; NarP, no effect
aeg-46.5	Unknown	NarL (X, Q) inhibits; NarP (X, Q) activates	NarL (X, Q) inhibits; NarP (X, Q) activates
aeg-93	Formate-nitrite reductase[c]	NarL (X, Q) inhibits; NarP (X, Q) activates	NarL (X) activates; (Q) inhibits; NarP (X, Q) activates

[a] Interactions in the wild type were deduced from the results presented in Tables 2 to 6 (see also Fig. 4).
[b] Sensor proteins (X and Q, NarX and NarQ, respectively) involved in a given regulation are shown in parentheses.
[c] The *aeg-93* locus probably corresponds to *nrf*, which encodes formate-dependent respiratory nitrite reductase (9).

proximately the same extent, although phosphorylation by NarQ may be slightly more efficient. NarP-phosphate weakly activates *fdnG* operon expression and efficiently activates *aeg-46.5* and *aeg-93* (*nrf*) operon expression. However, the final level of *aeg* operon expression is set by competition between NarP-mediated activation and the efficiency of NarL-mediated repression.

A potential complication for any comprehensive hypothesis is the fact that one regulatory metabolite, nitrite, is formed by metabolism of the other regulatory metabolite, nitrate. Our cultures are grown with excess nitrate, which thus persists in the culture medium well past the time of culture harvest. Nonetheless, nitrate-grown cultures steadily accumulate nitrite during the course of growth (11). This nitrite accumulation could influence some of the regulatory pathways depicted in Fig. 4. Further analysis will attempt to clearly separate the effects of nitrate, nitrite, and nitrate plus nitrate. However, enterobacteria growing in the wild probably encounter nitrate and nitrite in various concentrations and proportions, so the results with nitrate-grown batch cultures probably reflect a reasonable approximation of normal regulation.

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