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Nitrate- and Nitrite-Sensing Protein NarX of *Escherichia coli* K-12: Mutational Analysis of the Amino-Terminal Tail and First Transmembrane Segment

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Nitrate and nitrite control of anaerobic respiratory gene expression is mediated by dual two-component regulatory systems. The sensors NarX and NarQ each communicate nitrate and nitrite availability to the response regulators NarL and NarP. In the presence of nitrate, the NarX protein acts as a positive regulator ("kinase") of both NarL and NarP activity. In the presence of nitrite, the NarX protein acts primarily as a negative regulator ("phosphatase") of NarL activity but remains a positive regulator of NarP activity. In other topologically similar sensory proteins, such as the methyl-accepting chemotaxis proteins, the transmembrane regions are important for signal transduction. We therefore used localized mutagenesis of the amino-terminal coding region to isolate mutations in *narX* that confer an altered signaling phenotype. Five of the mutations studied alter residues in the amino-terminal cytoplasmic tail, and five alter residues in the first transmembrane segment. Based on patterns of target operon expression in various regulatory mutant strain backgrounds, most of the mutant NarX proteins appear to have alterations in negative control function. One mutant, with a change of residue Leu-11 to Pro in the cytoplasmic tail, exhibits strikingly altered patterns of NarL- and NarP-dependent gene expression. We conclude that the amino terminus of the NarX protein is important for the differential response to nitrate and nitrite.

Escherichia coli is a facultative aerobe capable of using a variety of respiratory pathways to generate energy. In the absence of oxygen, nitrate (NO_3^-) is the most energetically favorable and thus preferred respiratory oxidant. Regulatory mechanisms that sustain the most efficient energy-producing pathways are directed by respiratory substrate availability. Synthesis of enzymes required for anaerobiosis is primarily regulated by the oxygen-responsive transcription activator Fnr (reviewed in references 19, 22, and 52). Modulation of anaerobic respiratory gene expression then depends upon the availability of alternative electron acceptors. A dual interacting two-component regulatory system consisting of homologous sensors, NarX and NarQ, and homologous DNA-binding regulators, NarL and NarP, couples gene expression to the availability of both nitrate and nitrite (NO_2^-) (reviewed in reference 56). The *narXL* complex operon is located at centisome 27.4 on the *E. coli* map, and the *narP* and *narQ* genes are located at centisomes 48.7 and 54.8, respectively (25). Salient features of the Nar system include response by each sensor to both nitrate and nitrite, and communication, as phosphoryl transfer, between each sensor and both the NarL and NarP proteins (reviewed in reference 56).

The expression of 12 operons is known to be controlled by the Nar regulatory system. Two general categories of regulation have emerged, namely, that which is dependent upon the NarL protein only and that which is dependent upon both the NarL and the NarP proteins (reviewed in reference 56). Expression of the *narG* and the *fdnG* operons (encoding nitrate reductase and formate dehydrogenase-N, respectively) is induced approximately 100-fold by nitrate and 10-fold by nitrite (46). This regulatory pattern presumably reflects different levels of phospho-NarL accumulation in nitrate- and nitrite-induced cells. Competition between the phosphorylated forms of the NarL and NarP proteins for common binding sites controls *napF* (*aeg-46.5*, encoding periplasmic nitrate reductase) operon expression (9, 46). Phospho-NarL evidently antagonizes the phospho-NarP-dependent nitrate and nitrite induction of *napF* operon expression (13).

The concentration of phosphorylated NarP and NarL proteins in vivo is determined by their interaction with the nitrate- and nitrite-sensing protein-histidyl "kinases" NarX and NarQ. Sequence comparison among these *E. coli* sensory proteins and the NarQ homolog ($NarQ_{Hi}$) of *Haemophilus influenzae* (18) reveals several regions of sequence conservation in the carboxyl-terminal (cytoplasmic) portion that presumably demarcate shared two-component-type functions, including nucleotide binding, autophosphorylation, and phosphoryl transfer (reviewed in references 42 and 57). In vitro evidence for the latter two activities has been presented for the *E. coli* proteins (4, 51, 60). The amino-terminal (periplasmic) portion includes two potential transmembrane helices and a conspicuous region, designated the P-box (4), in which 15 of 18 consecutive residues are identical. Apart from the P-box, little sequence conservation exists between the approximately 120-amino-acid-long amino-terminal domains of the NarX, NarQ, and $NarQ_{Hi}$ proteins.

Genetic analysis of the effects of *narX* and *narQ* null alleles on nitrate and nitrite gene regulation revealed dissimilarity between NarX and NarQ protein function. Either the NarX protein or the NarQ protein is sufficient for normal nitrate regulation of *narG*, *fdnG*, or *frdA* (encoding fumarate reductase) operon expression (reviewed in reference 56). However, normal nitrite regulation of these operons requires the NarX protein (46). The basis for this distinction appears to be the differential response of the NarX protein to nitrate and nitrite. In response to nitrate, the NarX and NarQ protein kinase

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activities, as inferred from target gene expression, are virtually indistinguishable. In response to nitrite, the NarX protein apparently functions primarily as a phospho-NarL "phosphatase" and, by opposing nitrite-induced NarQ kinase activity, keeps the level of phospho-NarL protein relatively low (reviewed in reference 56).

Five missense alleles of the *narX* gene were isolated by screening for aberrant regulation of *frdA*, *fdnG*, and *narG* operon expression (12, 24). These mutations define a region of the NarX protein with presumed functional similarity to the linker region of the methyl-accepting chemotaxis proteins. Three other NarX residues, all highly conserved among two-component-type sensors, have been substituted for by oligonucleotide-directed mutagenesis (4, 45). In vivo phenotypes resulting from these substitutions at Asn-509 and His-513 fit with the prevailing two-component sensor paradigm (reviewed in references 39 and 57), whereas those resulting from substitutions at His-399, the presumed site of autophosphorylation, are dependent upon the substitute (4, 45; see also reference 56).

Analysis of NarX protein response to nitrate and nitrite has not been achieved in vitro. Moreover, to date, there has been poor correlation between the phenotypes conferred by several mutant NarX proteins in vivo and the corresponding activities of their amino-truncated versions in vitro (4). Thus, while awaiting more meaningful in vitro assays, we have pursued an in vivo approach to studying these questions by devising a genetic screen to isolate NarX mutants with decreased capacity to distinguish between nitrate and nitrite.

We constructed an allele of *narX*, containing several unique silent restriction sites, that allowed us to focus our mutagenesis on defined segments of the gene. In the course of establishing and testing our genetic approach, we isolated several candidate mutations after mutagenizing the DNA encoding the amino-terminal end of NarX. Since mutations near the amino termini of other sensory proteins have demonstrated an effect on signal transduction (40, 43, 58), we continued our mutagenesis of this portion of the *narX* gene. Ten distinct mutants were subsequently isolated and characterized.

MATERIALS AND METHODS

Strains, plasmids, and general methodology. The *E. coli* strains and plasmids used in this study are described in Table 1. Strains were constructed by P1 *kc*-mediated transduction of mutant alleles into verified single-copy *lacZ* fusion reference strains. The *nar* alleles used have been described previously (16, 17, 46, 54). DNA manipulations were standard (31). Restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs, Inc. (Beverly, Mass.). Modified T7 DNA polymerase (Sequenase) was from U.S. Biochemical Corp. (Cleveland, Ohio), and both T4 DNA polymerase and *Taq* DNA polymerase (in storage buffer A) were from Promega Corp. (Madison, Wis.). [α-^{35}S]dATP was from DuPont (Boston, Mass.). Synthetic oligonucleotides were purchased from both Integrated DNA Technologies, Inc. (Coralville, Iowa) and Gibco BRL (Gaithersburg, Md.).

Media, culture conditions, and enzyme assays. Defined, complex, and indicator media for routine genetic manipulations were used as described previously (31). Ampicillin, tetracycline, kanamycin, and chloramphenicol were generally used at 100, 20, 75 and 25 μg/ml, respectively. Agar and dehydrated media were from Difco Laboratories (Detroit, Mich.). Other components were from Sigma Chemical Co. (St. Louis, Mo.). For determination of mutant phenotypes, β-galactosidase activity was measured from cultures grown anaerobically in 3-[*N*-morpholino]propane-sulfonic acid (MOPS)-buffered minimal medium with glucose as the sole carbon source (55). $NaNO_3$ (40 mM), $NaNO_2$ (5 mM), and ampicillin (50 μg/ml) were added as indicated. Cultures for the NarX topology studies were grown anaerobically in rich medium with added $NaNO_3$ (40 mM). Standard assays were used for measuring β-galactosidase (35) and alkaline phosphatase (3, 14) activities. In all cases, cultures were assayed in duplicate, and reported activity values are averaged from at least two independent experiments.

DNA manipulations. The Altered Sites in vitro mutagenesis system (Promega) was used to construct unique, silent restriction sites to form plasmid pVJS1241 (Fig. 1). Mutagenesis was performed with plasmid pVJS1220. The silent restriction sites were then monotonously cloned into precursors of plasmid pVJS1241. Introduction of a *Bgl*II site downstream of the *narL* gene allowed deletion of all but 174 bp of the *narL* gene and, by destroying an *Nsi*I site, made the remaining *Nsi*I site unique. The entire DNA sequence of plasmid pVJS1241 from the *Kpn*I site to the end of the *narX* gene was determined on both strands by the dideoxy chain termination method (50). The resulting allele, encoding the NarX$^+$ protein but containing the new silent restriction sites, is designated *narX*†. PCRs were typically run in 1× thermophilic buffer (Promega) with 5 U of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 0.15 mM each deoxynucleoside triphosphate, 50 pmol of each primer, and approximately 20 fmol of plasmid template added (10). Amplified DNA was washed with 50:50 phenol-chloroform followed by chloroform and, after ethanol precipitation, used directly in the mutagenesis procedure described below. PCR-generated mutations were confirmed by DNA sequencing on both strands as described below.

Construction of gene fusions used for topology analysis. A 1.4-kb *Eco*RI-*Bgl*II restriction fragment from plasmid pVJS1257 (encodes an unpublished mutant *narX* allele with the appropriate *Bgl*II restriction site) was cloned into plasmid pNM482 (36) to create plasmid pVJS1269 harboring an in-frame translational fusion, Φ(*narX-lacZ*)*254*, between the first 51 codons of the *narX* gene and the *lacZ* gene. A similar fusion, Φ(*narX-lacZ*)*255*, at codon 220 of the *narX* gene was fabricated by cloning the 1.8-kb *Pst*I restriction fragment from plasmid pVJS1241 into plasmid pNM481 (36), creating plasmid pVJS1268. The Φ(*narX-phoA*)*261* gene fusion was made by cloning the *Sma*I fragment from plasmid pPHOK2 (48) into the *Nru*I site of plasmid pVJS1213, creating an in-frame fusion at codon 195 of the *narX* gene. Plasmid pPHOK2 encodes both the *'phoA* gene and a kanamycin resistance gene between two polylinkers. Five *narX*::Tn*phoA* gene fusions were constructed in strain VJS3818, containing plasmid pVJS1213, by transposition of Tn*phoA* (33). Nutrient agar plates containing chloramphenicol (25 μg/ml), kanamycin (25 μg/ml), and 5-bromo-4-chloro-3-indolyl phosphate (XP; 40 μg/ml) were used to identify Tn*phoA* fusions of interest. This combination of antibiotics selected for transposition of Tn*phoA* onto the medium-copy-number plasmid pVJS1213, while the XP helped identify fusions with alkaline phosphatase activity. Plasmids from blue kanamycin- and chloramphenicol-resistant clones were restriction mapped. All fusion join points were determined by DNA sequencing.

Mutant isolation. Preliminary experiments showed that strain VJS3041 [*narX narQ* Φ(*narG-lacZ*)] containing plasmid pVJS1241 (*narX*†) has a Lac$^+$ phenotype on MacConkey-lactose-ampicillin medium containing 40 mM $NaNO_3$ and a Lac$^-$ phenotype on both MacConkey-lactose-ampicillin medium containing 5 mM $NaNO_2$ and MacConkey-lactose-ampicillin medium alone. To identify alleles of *narX* unable to distinguish between nitrate and nitrite, we screened for mutants with a Lac$^+$ phenotype on MacConkey-lactose-ampicillin medium containing 5 mM $NaNO_2$. Mutant NarX proteins that respond to nitrite as if it were nitrate are predicted to confer increased levels of Φ(*narG-lacZ*) expression, resulting in a Lac$^+$ phenotype.

More specifically, we made random mutations in a restricted region of the *narX*† gene via standard PCR (10). Oligonucleotide primers were chosen such that they directed amplification of an 800-bp region of plasmid pVJS1241 containing the *narX*† gene sequence and two unique restriction sites, *Kpn*I and *Xho*I (Fig. 1). Amplified DNA was then cloned as a 360-bp *Kpn*I-*Xho*I restriction fragment into plasmid pVJS1241, and the resultant plasmids were transformed into strain VJS3041 [*narX narQ* Φ(*narG-lacZ*)]. Ampicillin-resistant colonies were then replica plated to MacConkey-lactose-ampicillin medium containing 5 mM $NaNO_2$ and incubated aerobically overnight at 37°C. We chose Lac$^+$ colonies and purified them on MacConkey-lactose-ampicillin medium. Plasmids from these potential mutants were restriction mapped, and those with a wild-type *Pst*I restriction map were retained. Linkage of the Lac$^+$ phenotype to the plasmid was then demonstrated by moving plasmids from strain VJS3041 to strain VJS5054 (as VJS3041 but *pcnB*) and again scoring the Lac$^+$ phenotype on MacConkey-lactose-$NaNO_2$ medium. β-Galactosidase activity from liquid cultures of these potential mutants was then measured after anaerobic growth in the presence of $NaNO_3$ (40 mM), $NaNO_2$ (5 mM), or no added electron acceptor. Those plasmids conferring a phenotype distinct from that of plasmid pVJS1241 in strain VJS5054 were retained, and the 360 bp of DNA between restriction sites *Kpn*I and *Xho*I was sequenced.

RESULTS

Membrane topology of the NarX protein. Sequence analysis of the NarX protein reveals two potential transmembrane regions. Residues 15 through 37 and 158 through 174 define long hydrophobic stretches flanked by charged residues. With acceptance of these as transmembrane regions (59), the NarX protein seems to adopt a conventional sensor topology (reviewed in references 42 and 57), with the two transmembrane regions employing the cytoplasmic membrane to divide the NarX protein into a large cytoplasmic region comprising the carboxyl-terminal 424 residues and a smaller periplasmic region comprising 121 residues near the amino terminus. We constructed a set of *phoA* and *lacZ* gene (translational) fusions to experimentally test this topological conjecture (see Materi-

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype	Source
E. coli K-12 strains[a]		
CC118		32
CC321		32
BW11397		62
RP7947	*pcnB1 zad-981*::Tn*10*d(Km) *leuB6 fhuA*	29
VJS632	Prototroph	54
VJS676	Same as that for VJS632 but Δ(*argF-lac*)*U169*	Lab collection
VJS2197	Same as that for VJS676 but λΦ(*narG-lacZ*)*250*	46
VJS4734	Same as that for VJS676 but λΦ(*napF-lacZ*)	13
Derivatives of *E. coli* VJS2197		
VJS2430	Δ*narX242 zch-2084*::Ω-Cm	46
VJS3040	*narQ251*::Tn*10*d(Tc)	46
VJS3041	Δ*narX242 narQ251*::Tn*10*d(Tc)	46
VJS4033	Δ*narX242 narL505*(V88A) *zch-2084*::Ω-Cm *narQ251*::Tn*10*d(Tc)	Lab collection
VJS5054	Δ*narX242 narQ251*::Tn*10*d(Tc) *pcnB1 zad-981*::Tn*10*d(Km)	This work
VJS5710	*narQ251*::Tn*10*d(Tc) *pcnB1 zad-981*::Tn*10*d(Km)	This work
VJS5719	Δ*narX242 zch-2084*::Ω-Cm *pcnB1 zad-981*::Tn*10*d(Km)	This work
VJS5720	Δ*narX242 narL505*(V88A) *zch-2084*::Ω-Cm *narQ251*::Tn*10*d(Tc) *pcnB1 zad-981*::Tn*10*d(Km)	This work
Derivatives of *E. coli* VJS4734		
VJS4799	*narL215*::Tn*10*d(Tc)	13
VJS5721	Δ(*narXL*)*235 narQ251*::Tn*10*d(Tc) *pcnB1 zad-981*::Tn*10*d(Km)	This work
VJS5723	Δ(*narXL*)*235*	This work
VJS5724	Δ(*narXL*)*235 narQ251*::Tn*10*d(Tc)	This work
VJS5742	Δ(*narXL*)*235 pcnB1 zad-981*::Tn*10*d(Km)	This work
VJS5743	*narQ251*::Tn*10*d(Tc) *narL249*::Ω	This work
VJS5744	*narQ251*::Tn*10*d(Tc) *narL249*::Ω *pcnB1 zad-981*::Tn*10*d(Km)	This work
Plasmids		
pACYC184	Tc^r Cm^r	5
pHG165	Ap^r; pUC8 polylinker	53
pNM481	Ap^r; *'lacZ lacY*$^+$ (*'lacZ* gene fusion vector)	36
pNM482	Ap^r; *'lacZ lacY*$^+$ (*'lacZ* gene fusion vector)	36
pPHOK2	Ap^r Km^r; *'phoA* (*'phoA* gene fusion vector)	48
pSELECT	Tc^r Ap^s; pUC19 polylinker	Promega
pVJS1	Ap^r; *narX*$^+$ *narL*$^+$	55
pVJS2	Ap^r; *narX*$^+$	55
pVJS104	Cm^r; T7 ϕ10 promoter in *Eco*RV-*Pst*I sites of pACYC184	1
pVJS1210	Cm^r; 1.6-kb *Bst*YI deletion from pVJS104	This work
pVJS1213	Same as that for pVJS1210 but *narX*$^+$ on a 3.4-kb *Hin*dIII-*Bgl*II pVJS2 fragment	This work
pVJS1220	Same as that for pSELECT but *narX*$^+$ *narL*$^+$ on a 5.0-kb *Hin*dIII fragment from pVJS1	This work
pVJS1241[a]	Same as that for pHG165 but *narX*† on a 4.5-kb *Pst*I-*Hin*dIII fragment	This work

[a] Strain and plasmid constructions are described in Materials and Methods; see also Fig. 1.

als and Methods). These fusion types were chosen because of their wide acceptance as topological indicators and their complementary activities (32). Hybrid proteins from *lacZ* gene fusions have significant β-galactosidase activity only when cytoplasmically localized, and those from *phoA* gene fusions have significant alkaline phosphatase activity only when periplasmically localized (32). Fusion locations are indicated in Fig. 1.

The results are consistent with the topology outlined above. Measurement of β-galactosidase activities from upstream (periplasmic) and downstream (cytoplasmic) *lacZ* fusions showed that only the latter hybrid gene product was highly active (5 versus 150 U; *'lacZ* vector activity was <1 U). Complementary information was provided by the five upstream (periplasmic) and one downstream (cytoplasmic) *phoA* fusions; activities for the upstream fusions ranged from 35 to 73 U, whereas the activity of the downstream fusion was equivalent to that of the *'phoA* vector itself with no cloned insert (<0.1 U).

Reporter strains for phenotypic analysis. Gene fusions of *lacZ* to both the respiratory (*narG* operon) and periplasmic (*napF* operon) nitrate reductase structural genes provide in vivo measures of Nar regulatory protein function. Null alleles (deletions or transposon insertions) of regulatory genes were introduced into fusion strains by transduction. The *pcnB1* allele (29) was used to mimic single-copy status for plasmid-borne *narX* alleles (see below). All strains are described fully in Table 1.

Strain VJS5054 [*narX narQ* Φ(*narG-lacZ*) *pcnB*] was used to analyze interactions between the NarX and NarL proteins. Regulation of Φ(*narG-lacZ*) expression in this strain requires the NarX protein's ability to control phospho-NarL concentration in response to nitrate and nitrite availability (46). Derivatives carrying the *narL505* (strain VJS5720; with a V-to-A mutation at position 88 [V88A]) or the *narQ*$^+$ (strain VJS5719) allele were used for some experiments (see below).

Strain VJS5721 [Δ(*narXL*) *narQ* Φ(*napF-lacZ*) *pcnB*] was used to analyze interactions between the NarX and the NarP proteins. Nitrate and nitrite regulation of Φ(*napF-lacZ*) expression in this background requires normal NarX protein

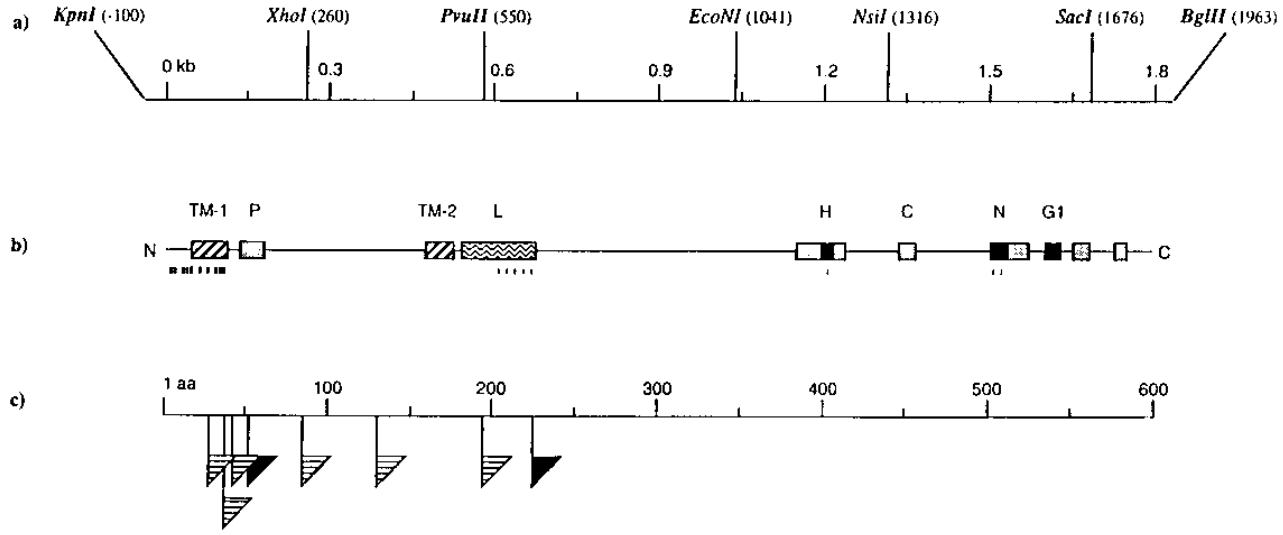

FIG. 1. (a) Partial restriction map of the *narX*† gene cloned into plasmid pVJS1241. Sites in boldface type were introduced via oligonucleotide-directed mutagenesis (see Materials and Methods). Numbers in parentheses indicate distances in base pairs from the translational start. The numbered scale indicates the distance in kilobases from the translational start. (b) Linear diagram of the NarX protein. Regions of sequence similarity among two component sensors are indicated by solid bars and labeled H, N, and G1 (42, 57). Some of the similar sequences among the *nar* sensors are shaded and labeled P or C (4). The linker box (L) has functional similarity with the methyl-accepting chemotaxis proteins (MCPs) (12). The presumed transmembrane regions are labeled TM-1 and TM-2. Positions of previously published mutations are indicated by lightface tick marks (see text). Boldface tick marks represent sites of mutations discussed in this work. (c) Flags indicate positions of *'lacZ* (solid) and *'phoA* (striped) gene (translational) fusions in *narX*. See Results for enzyme activities from the resultant hybrid proteins. The numbered scale indicates the distance in amino acid residues from the translational start.

function, including phosphoryl transfer to the NarP protein (46). The deletion of *narL* in this strain allows for full-level Φ(*napF-lacZ*) expression (9). A derivative carrying *narQ*$^+$ (strain VJS5742) was used for some experiments (see below).

Strain VJS5710 [*narX*$^+$ *narQ* Φ(*narG-lacZ*) *pcnB*] was used to determine the dominance relationships between *narX*$^+$ and the *narX* mutant alleles.

Evaluation of plasmid-based analyses. Nar regulatory phenotypes are sensitive to regulatory gene dosage (23). We previously used bacteriophage λ specialized transducing phage for single-copy analysis of *narX* and *narL* mutations (12, 16, 17). However, construction and verification of this phage are cumbersome when many mutant alleles are being analyzed. Thus, we hoped to use plasmids for ease of strain construction and therefore employed *pcnB* strains, with greatly reduced plasmid copy numbers, for phenotypic and complementation analysis as suggested previously (30). The *pcnB* gene encodes poly(A) polymerase, involved in ColE1-type plasmid copy number control (reviewed in reference 11). The cloning vector we employed, pHG165 (53), has the moderate copy number of its parent plasmid pBR322 in *pcnB*$^+$ strains. Our initial step was therefore to directly compare plasmid-*pcnB*-based analysis with single-copy-based analysis in our reporter strains. In these comparisons, strains carrying *narX*$^+$ at the normal chromosomal locus were indistinguishable from strains carrying *narX*† (with added silent restriction sites; see Materials and Methods) on the plasmid vector (Table 2). We conclude that these *pcnB* strains provide appropriate backgrounds for characterizing *narX* missense alleles.

Rationale and mutant isolation. An intriguing aspect of NarX protein function is its differential response to two structurally similar ligands, nitrate and nitrite (46). When either the NarX or NarQ protein signals availability of nitrate, or the NarQ protein signals availability of nitrite, the result is an approximately 100-fold, NarL-dependent induction in *narG* operon expression. When the NarX protein signals availability of nitrite, only an approximately fivefold, NarL-dependent induction ensues (46). By contrast, NarP-mediated *napF* operon induction (in *narL* null strains) is equally affected by nitrate or nitrite acting through either the NarX or the NarQ sensor. One long-range goal is to identify the basis by which the NarX protein responds differentially to nitrate and nitrite availability. We therefore designed a phenotypic screen to aid in the isolation of NarX mutants that do not differentiate between nitrite and nitrate for NarL-mediated transcriptional regulation. This screen relied upon the observation that strain VJS3041 [*narX narQ* Φ(*narG-lacZ*)] harboring plasmid pVJS1241 (*narX*†; see Materials and Methods) has a Lac$^+$ phenotype on MacConkey-lactose-ampicillin medium containing 40 mM $NaNO_3$ and a Lac$^-$ phenotype on both MacConkey-lactose-ampicillin medium containing 5 mM $NaNO_2$ and MacConkey-lactose-ampicillin medium alone. Thus, screening for mutants with Lac$^+$ phenotype on MacConkey-lactose-ampicillin me-

TABLE 2. Comparison of single-copy versus plasmid-*pcnB* analyses

narX allele[c]		β-Galactosidase sp act[a] in strain background[b]					
Chromosome	Plasmid	*narQ* Φ(*narG-lacZ*)			*narQ narL* Φ(*napF-lacZ*)		
		None[d]	NO_3^-[d]	NO_2^-[d]	None	NO_3^-	NO_2^-
Δ*narX*	No plasmid	33	43	36	120	160	130
	pHG165 (vector)	27	43	40	160	180	170
	narX†	39	3,540	95	1,570	5,870	4,820
narX$^+$	No plasmid	34	3,720	97	1,540	4,650	4,120
	narX†	43	3,660	95	—[e]	—	—

[a] β-Galactosidase activity was measured as described in Materials and Methods and is expressed in Miller units.
[b] See Table 1 for full genotypes. All strains carry *pcnB1*.
[c] *narX* allele located at the normal chromosomal locus or on plasmid pVJS1241.
[d] Anaerobic growth in the presence of no oxidant (none) or the indicated respiratory oxidant (NO_3^- or NO_2^-).
[e] —, not determined.

TABLE 3. Nucleotide and amino acid changes of *narX* alleles

Allele	Substitution[a]	
	Nucleotide	Amino acid
narX513	T17A	L6H
narX514	T19C	S7P
narX515[b]	T27C	L9P
narX516	T32C	L11P
narX517	A41G	Q14R
narX518[c]	T44C	V15A
narX519[b]	A52G	I18V
narX520[d]	T80C	L27P
narX521	A88T	M30L
narX522	C92T	A31V

[a] T17A, substitution of A for T at position 17, for example.
[b] Representative of two independent isolates.
[c] Representative of three independent isolates.
[d] This allele also contains a silent T-to-C change at position 99.

dium containing 5 mM $NaNO_2$ was predicted to identify NarX mutants that respond to nitrite as if it were nitrate. Interestingly, this screen should also identify NarX mutants that bestow constitutive Φ(*narG-lacZ*) expression and are invariably Lac^+. However, the majority of mutant alleles we recovered from this screen are specifically defective in nitrate and nitrite differentiation and remain Lac^- on MacConkey-lactose-ampicillin medium alone (see below) (61).

Imagining various scenarios for this differential response, we chose to construct a version of the *narX* gene containing several unique, silent restriction sites (*narX*† allele; see Materials and Methods) that would allow us to focus our mutagenesis on particular regions of the multifunctional *narX* gene product. Here, we report our mutational analysis of the extreme amino-terminal end of the protein. This region is important in signal transduction by other two-component sensors (40, 43, 58). Ten unique mutant alleles are listed in Table 3. The effects of these mutations on the phenotype of strain VJS5054 [*narX narQ* Φ(*narG-lacZ*) *pcnB*] were initially quantified by assaying β-galactosidase activity from liquid cultures grown anaerobically with added nitrate or nitrite or no added electron acceptor. Potentially interesting alleles were subjected to sequence analysis, followed by further phenotypic characterization to examine interaction with the *narL*, *narP*, and *narX* alleles. Representative mutant alleles were then further examined with respect to the *narL505* and *narQ* alleles. Each of these tests is described below.

Interaction with the NarL protein. Communication between mutant NarX proteins and the NarL protein was analyzed in strain VJS5054 [*narX narQ* Φ(*narG-lacZ*) *pcnB*]. The level of nitrate- or nitrite-induced Φ(*narG-lacZ*) expression presumably reflects phospho-NarL protein concentration (46). Thus, an alteration in the NarX protein's ligand sensing, signal transduction, or phosphoryl chemistry that influences phospho-NarL protein concentration can be inferred from Φ(*narG-lacZ*) expression levels.

Eight of the ten alleles were defined by their two- to sixfold increased sensitivity to nitrite (Table 4). Uninduced expression was essentially that of the wild type. Some of these mutant strains exhibited a partially defective response to nitrate, but in general, nitrate induction of Φ(*narG-lacZ*) expression was near normal. We denote these alleles as conferring a nitrite-hypersensitive phenotype. The *narX517* (Q14R) strain exhibited a strongly nitrite-hypersensitive phenotype but also displayed a 10-fold elevation in basal Φ(*narG-lacZ*) expression. The *narX516* (L11P) allele conferred high-level constitutive Φ(*narG-lacZ*) expression (Table 4).

Interaction with the NarP protein. Communication between mutant NarX proteins and the NarP protein was analyzed in strain VJS5721 [Δ(*narXL*) *narQ* Φ(*napF-lacZ*) *pcnB*]. The level of nitrate- or nitrite-induced Φ(*napF-lacZ*) expression in this strain presumably reflects the phospho-NarP protein concentration (13, 46). Thus, an alteration in the NarX protein's ligand sensing, signal transduction, or phosphoryl chemistry that influences the phospho-NarP protein concentration can be inferred from Φ(*napF-lacZ*) expression levels. We did not anticipate dramatic effects on Φ(*napF-lacZ*) expression from the nitrite-hypersensitive *narX* alleles because even the wild-type NarX protein does not distinguish between nitrate and nitrite with respect to NarP-mediated transcription. We were, however, interested in how the nitrite-hypersensitive mutant proteins would interact with the NarP protein because a nitrite-sensing NarX protein acts differentially with respect to the two *nar* response regulators.

The *narX*† allele conferred high-level basal Φ(*napF-lacZ*)

TABLE 4. Effects of *narX* mutations on Φ(*narG-lacZ*) and Φ(*napF-lacZ*) expression

narX allele[c]	β-Galactosidase sp act[a] in[b]:								
	VJS5054 [Φ(*narG-lacZ*) Δ*narX narQ*]			VJS5710 [Φ(*narG-lacZ*) *narX*$^+$ *narQ*]			VJS5721 [Φ(*napF-lacZ*) Δ(*narXL*) *narQ*]		
	None[d]	NO_3^-[d]	NO_2^-[d]	None	NO_3^-	NO_2^-	None	NO_3^-	NO_2^-
pHG165 (vector)	24	38	26	23	1,880	58	120	160	130
narX†	21	2,080	59	26	2,410	60	1,560	5,800	5,120
narX514 (S7P)	29	1,340	97	19	1,540	130	2,810	5,400	5,660
narX513 (L6H)	26	1,190	140	36	2,230	120	3,690	5,030	4,980
narX518 (V15A)	21	2,180	160	23	1,850	91	1,300	4,670	3,840
narX522 (A31V)	18	1,180	220	30	2,000	130	2,190	5,410	3,910
narX515 (L9P)	21	2,120	240	27	2,140	130	1,580	4,030	3,170
narX520 (L27P)	53	1,620	310	21	1,910	55	2,040	4,120	3,580
narX519 (I18V)	39	1,820	310	48	2,080	150	4,160	6,080	4,890
narX521 (M30L)	25	1,940	350	35	2,040	160	4,530	5,280	5,940
narX516 (L11P)	1,360	1,090	1,750	43	2,000	190	36	1,810	120
narX517 (Q14R)	240	1,550	1,240	20	2,160	420	3,360	5,240	3,900

[a] β-Galactosidase activity was measured as described in Materials and Methods and is expressed in Miller units.
[b] See Table 1 for full strain descriptions. All strains carry *pcnB1*.
[c] *narX* allele located on plasmid pVJS1241 derivative.
[d] Anaerobic growth in the presence of no oxidant (none) or the indicated respiratory oxidant (NO_3^- or NO_2^-).

TABLE 5. Influence of the *narL505* (V88A) allele on NarX phenotypes

narX allele[c]	β-Galactosidase sp act[a] in VJS5720[b]		
	None[d]	NO_3^-[d]	NO_2^-[d]
pHG165 (vector)	3,770	2,100	2,100
narX†	900	2,670	1,970
narX515 (L9P)	2,600	2,340	2,190
narX516 (L11P)	4,120	1,830	2,560
narX517 (Q14R)	4,200	2,190	2,240
narX521 (M30L)	2,270	2,500	2,430

[a] β-Galactosidase activity was measured as described in Materials and Methods and is expressed in Miller units.
[b] Φ(*narG-lacZ*) Δ*narX narL505 narQ pcnB1* (Table 1).
[c] *narX* allele located on plasmid pVJS1241 derivative.
[d] Anaerobic growth in the presence of no oxidant (none) or the indicated respiratory oxidant (NO_3^- or NO_2^-).

expression in strain VJS5721, with the consequence that nitrate and nitrite induction was only three- to fourfold. This high basal expression is due in large measure to the *narQ* null allele in this strain (61). Most of the nitrite-hypersensitive alleles provided slightly increased basal levels of Φ(*napF-lacZ*) expression with modest two- to threefold nitrate and nitrite induction (Table 4). The notably nitrite-hypersensitive *narX517* (Q14R), *narX519* (I18V), and *narX521* (M30L) alleles (see above) bestowed nearly constitutive Φ(*napF-lacZ*) expression. In the *narX516* (L11P) mutant, Φ(*napF-lacZ*) expression resembled Φ(*narG-lacZ*) expression in the wild type, i.e., both had a low basal level, a substantial nitrate induction, and a weaker nitrite induction. The induced level of Φ(*napF-lacZ*) expression in the *narX516* (L11P) strain did not significantly exceed the basal level expression of the wild type.

Complementation analysis. Dominance relationships were assessed in strain VJS5710 [*narX*$^+$ *narQ* Φ(*narG-lacZ*) *pcnB*] harboring *narX* mutant alleles on plasmid pVJS1241 derivatives. Φ(*narG-lacZ*) expression in these *narX*$^+$/*narX* heterozygous strains was compared with that in the homozygous strain. The subtle nature of most of the mutant phenotypes complicates interpretation of dominance relationships. We denote most of the nitrite-hypersensitive alleles as codominant (49), because for these mutations the phenotype of the *narX*$^+$/*narX* heterozygote was intermediate between those of the homozygous mutant and wild-type strains (Table 4). Exceptions are the *narX520* (L27P) allele, which was recessive to *narX*$^+$, and the *narX514* (S7P) allele, which conferred an even greater nitrite sensitivity in the presence of the *narX*$^+$ allele. By contrast, the strongly constitutive *narX516* (L11P) allele was recessive to *narX*$^+$, with the exception of a slightly elevated response to nitrite in the heterozygote (Table 4). The behavior of the *narX*$^+$/*narX*† homozygote is shown in Table 2.

Negative regulation (phosphatase) in *narX* mutants. The NarX protein is both a positive and negative regulator of NarL protein function, reflecting its kinase and phosphatase activities (reviewed in reference 56). The NarL505 (V88A) protein (encoded by the *narL505* allele) confers a constitutive phenotype with respect to Φ(*narG-lacZ*) expression (17, 46). In vitro, the NarL505 (V88A) protein requires phosphorylation for specific DNA-binding activity (28), so we infer that the constitutive, in vivo phenotype also requires phosphorylation. The NarX protein, with its phospho-NarL phosphatase activity, therefore causes decreased basal Φ(*narG-lacZ*) expression in a *narL505* strain. We reason that the mutant NarX proteins that retain negative function will likewise decrease this elevated basal expression.

Negative regulation was assessed in strain VJS5720 [*narX narL505 narQ* Φ(*narG-lacZ*) *pcnB*]. We chose a subset of four *narX* alleles for this analysis: the phenotypically unique *narX516* (L11P) and *narX517* (Q14R) alleles and the *narX515* (L9P) and *narX521* (M30L) alleles as representatives of the nitrite-hypersensitive class. The *narL505* strain expressed Φ(*narG-lacZ*) constitutively. Vector plasmid pHG165 had no effect, whereas introduction of the *narX*$^+$ allele reduced uninduced expression by threefold (Table 5). None of the four *narX* mutants decreased basal Φ(*narG-lacZ*) expression, suggesting that the mutant proteins have decreased phospho-NarL phosphatase activity.

Interaction with the NarQ protein. The amino acid sequences and the physiological functions of the NarX and NarQ proteins are highly similar, and active sensor proteins are generally thought to function as dimers (34, 41, 47). Furthermore, one report states that certain *narX* alleles confer dominant negative phenotypes with respect to *narQ*$^+$ (unpublished observations cited in reference 8). It is therefore plausible to imagine that the NarX and NarQ proteins may form heterodimers or otherwise influence each other's behavior. Clear evidence for dominant negative relationships would be informative in this regard (reviewed in reference 21).

The NarX protein is not a strong negative regulator of phospho-NarP accumulation in vivo (Table 4) (56). A dominant negative *narX* allele, in the classical sense, would affect NarQ function with respect to both the NarL and NarP proteins. Conversely, a NarX protein that has simply lost its negative control activity is predicted to have a much greater effect on NarL-mediated gene expression than on NarP-mediated gene expression in the presence of NarQ. We therefore examined the subset of four alleles in *narQ*$^+$ strain backgrounds to address potential sensor protein interactions with respect to the NarL and NarP proteins.

Neither *narX*$^+$ nor any of the four mutant *narX* alleles tested affected NarP-dependent Φ(*napF-lacZ*) expression in strain VJS5742 [Δ(*narXL*) Φ(*napF-lacZ*) *pcnB*] (Table 6). The *narX*$^+$ allele exhibited the expected behavior with respect to NarL-dependent Φ(*narG-lacZ*) expression in strain VJS5719 [*narX* Φ(*narG-lacZ*) *pcnB*], demonstrating a negative influence on NarQ-mediated positive control in the presence of nitrite. By contrast, the four *narX* mutant alleles tested all failed to influence NarQ-mediated nitrite induction of Φ(*narG-lacZ*) expression (Table 6). Thus, all four are denoted as functionally recessive to *narQ*$^+$.

TABLE 6. Influence of the *narQ*$^+$ allele on NarX phenotypes

narX allele[c]	β-Galactosidase sp act[a] in[b]:					
	VJS5719 [Φ(*narG-lacZ*) Δ*narX narQ*$^+$]			VJS5742 [Φ(*napF-lacZ*) Δ(*narXL*) *narQ*$^+$]		
	None[d]	NO_3^-[d]	NO_2^-[d]	None	NO_3^-	NO_2^-
No plasmid	42	2,190	2,640	180	5,500	5,040
pHG165 (vector)	42	2,300	2,200	180	6,760	6,020
narX†	24	2,110	400	210	6,860	5,710
narX515 (L9P)	41	2,290	2,080	170	6,870	6,360
narX516 (L11P)	1,800	2,220	2,770	200	7,220	6,860
narX517 (Q14R)	280	2,310	1,990	200	6,440	6,130
narX521 (M30L)	52	2,010	1,270	270	6,490	6,860

[a] β-Galactosidase activity was measured as described in Materials and Methods and is expressed in Miller units.
[b] See Table 1 for full strain descriptions. All strains carry *pcnB1*.
[c] *narX* allele located on plasmid pVJS1241 derivative.
[d] Anaerobic growth in the presence of no oxidant (none) or the indicated respiratory oxidant (NO_3^- or NO_2^-).

DISCUSSION

The Nar regulatory system provides a network from which to explore the basis for specificity in sensor-regulator interactions. This is evident from the response regulator-dependent, differential response to nitrite exhibited by the functionally similar and homologous NarX and NarQ sensor proteins. One goal of our ongoing mutational analysis of the NarX protein is to understand the basis for this differential function.

We focused our mutagenesis on the amino-terminal coding region and screened for *narX* mutants with a decreased ability to differentiate between nitrite and nitrate. This focused approach is an attempt to partition the NarX protein's complex signal transduction pathway into manageable elements. The set of *narX* mutations described here suggests an important role of the amino terminus in substrate differentiation, perhaps by maintaining competent signaling states in the NarX protein.

This analysis has highlighted our awareness that the NarX protein interacts rather differently with the two response regulators NarL and NarP. Previous work has shown that, in response to nitrite, the NarX protein acts primarily as a negative regulator (phosphatase) of NarL protein action but as a positive regulator (kinase) of NarP protein action (46). This difference in response regulator interaction was especially pronounced for several of the mutant alleles reported here.

The NarX protein has a conventional sensor membrane topology. Our topological analysis, employing *lacZ* and *phoA* gene fusions, supports the conclusion from sequence analysis that the NarX protein assumes a membrane topology reminiscent of that for the methyl-accepting chemotaxis proteins of *E. coli* and *Salmonella typhimurium* (2, 33, 37; reviewed in reference 20) and also suggested for many two-component sensors (42, 57).

Based on this postulated membrane topology, the 10 *narX* mutations discussed below affect two regions of the protein: five are located within the small amino-terminal cytoplasmic tail (residues 1 to 14) proximal to transmembrane region 1 (TM-1) and five are located within TM-1 itself (residues 15 to 37) (Fig. 1).

Nitrite-hypersensitive *narX* alleles. The phenotypes resulting from three of the cytoplasmic-tail mutations and all five of the TM-1 mutations defined a coherent class of *narX* mutant alleles. Most of the alleles conferred subtle phenotypes, so we chose the *narX521* (M30L) and *narX515* (L9P) alleles, having the strongest phenotypes, to represent the TM-1 and cytoplasmic-tail mutant groups, respectively. These mutations were codominant with the *narX*$^+$ allele, indicating that they are not complete loss-of-function alterations (49). The respective mutants exhibited nitrite-hypersensitive, NarL-dependent *narG* operon induction (Table 4). This nitrite-hypersensitive phenotype suggests that the mutations may only affect NarX phosphatase activity, which is evidently the predominant activity in the interaction of nitrite-stimulated NarX protein and the NarL protein (46). Defective negative control is further indicated by the failure of these mutants to alter constitutive expression in the *narL505* (V88A) mutant (Table 5). However, the *narX521* (M30L) mutant exhibited only partial NarQ-dependent nitrite induction of *narG* operon expression (Table 6), indicating retention of at least some negative control activity by this mutant protein.

Low wild-type levels of Φ(*narG-lacZ*) basal expression in the nitrite-hypersensitive mutant strains (Table 4) argue that the corresponding amino acid substitutions primarily affect the signaling state of the NarX protein, particularly, the nitrite-induced signaling state. These alterations may shift the normal nitrite-induced equilibrium between positive (kinase) and negative (phosphatase) functions (reviewed in references 44 and 56) toward the positive function with respect to the NarL protein. What might be the basis for this shift? A current model for the mechanism of transmembrane signaling, which is derived from solution ^{19}F nuclear magnetic resonance and X-ray crystallographic studies of the Tar chemosensor and disulfide cross-linking studies of both the Tar and Trg chemosensors, suggests that one of the conformational changes accompanying signal transduction is an intramolecular, swinging-piston movement of TM-2 relative to TM-1 (26, 27; reviewed in reference 2). With this signaling process in mind, lower phosphatase activity from the mutant alleles represented by NarX (M30L) may be best explained as an improper TM-1 based, nitrite-signaling conformation. In this view, the altered conformation would lower energy barriers that normally preclude the NarX protein from shifting further into its kinase mode in response to nitrite.

We note that the *narX521* (M30L) mutant also exhibited altered NarP-dependent regulation (Table 4), whereas most of the other nitrite-hypersensitive mutants were essentially normal with respect to NarP. The significance of this observation awaits clearer understanding of NarX-response regulator interactions.

Changes in the amino-terminal cytoplasmic tail. Three changes in the NarX amino-terminal cytoplasmic tail resulted in a phenotype similar to that for the TM-1 mutants and thus may also disrupt normal signaling conformations (see above). This contrasts with results from both a mutational analysis of the Tar chemosensor, in which no role was established for that protein's 8-residue amino-terminal tail (6), and analysis of mutations in the VirA protein's 17-residue tail that inactivate the protein (15). The other two *narX* alleles conferred unique phenotypes. Only nitrate was able to stimulate NarP-dependent control in the *narX516* (L11P) mutant (Table 4), and even then, *napF* operon expression was increased only to the normal uninduced basal level. By contrast, NarL-dependent transcription was constitutively activated, and this mutant exhibited no apparent negative control of NarL activity (Tables 4 to 6). Finally, the *narX516* (L11P) allele was recessive to *narX*$^+$, at least with respect to NarL-dependent expression. It appears that this mutant has a signal-independent, locked-output phenotype with respect to the NarL protein but is defective with respect to NarP interactions. We cannot as yet provide a simple explanation for this observation of distinctly different interactions between NarX516 (L11P) and the two *nar* regulators. However, a mutation in the amino-terminal cytoplasmic tail of the PgtB sensor (40) and specific mutations in the TM-1 regions of the sensors PgtB, VirA, and EnvZ also lead to signal-independent proteins locked into a kinase mode with respect to their cognate response regulators (40, 43, 58).

The phenotype of the *narX517* (Q14R) mutant was strongly nitrite hypersensitive, although it also had a somewhat elevated basal level of *narG* operon expression (Table 4). This phenotype is similar to that of the *pgtB* (R19Q) mutant (40). The altered residue in each case is presumably the most proximal to the cytoplasmic end of the TM-1 sequence. This position is potentially important in the orientation of these sensor proteins in the cytoplasmic membrane (59). However, the *narX517* (Q14R) mutant still responded to the presence of nitrate and nitrite, suggesting a properly oriented and localized protein. The mutant exhibited no negative control of NarL function (Tables 5 and 6), but it was unique in displaying a comparable NarL-dependent response to either respiratory substrate (Table 4). Thus, the conformation of the cytoplasmic tail, acting perhaps via TM-1, may be important in the ability of the NarX protein to differentially signal nitrate and nitrite availability.

Which NarX protein function responds to nitrate or nitrite? The NarX protein has both kinase and phosphatase activities with respect to the NarL protein (46, 51, 60). Nitrate or nitrite stimulation, and subsequent increases in phospho-NarL levels, could thus result from either an increase in net kinase activity, a decrease in net phosphatase activity, or both. These alternatives have been extensively considered in the context of other two-component regulatory systems (reviewed in references 38 and 44).

Our tests indicate that none of the NarX mutants examined retain significant phospho-NarL phosphatase activity (Tables 5 and 6), yet basal levels of NarL-mediated gene expression remained low in most of the mutant strains and the mutants still responded to nitrate and nitrite availability. These considerations imply that in the wild type, the NarX protein kinase activity is stimulated by nitrate or nitrite availability. An alternative hypothesis is that kinase activity is essentially constitutive and that nitrate or nitrite binding results in inhibition of phosphatase activity. If this were the case, then mutations that decrease phosphatase activity should result in consistently high levels of phospho-NarL. Only the NarX (L11P) mutant, which is apparently locked into a "kinase-on" conformation (Table 4), exhibited such a constitutive phenotype.

The elevated basal levels of NarP-mediated gene expression suggest that some level of NarX kinase activity is present even in the absence of inducer (Table 4). The NarX protein does not have a strong negative function with respect to the NarP protein, and in a *nar* wild-type strain, this basal activity is opposed by the NarQ protein's phospho-NarP phosphatase activity (61).

Together, these observations suggest a NarX protein whose unstimulated mode is a tight balance between phosphatase and limited kinase activities. The balance is then shifted toward the kinase by nitrate or nitrite activation of that function. Regulated kinase activity has been found in the chemotaxis system of *S. typhimurium* and is suggested by the mathematical model of porin gene regulation by the EnvZ protein (39, 49).

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