JOURNAL OF BACTERIOLOGY, June 1992, p. 3667–3675
0021-9193/92/113667-09$02.00/0
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Vol. 174, No. 11

Mutational Analysis Reveals Functional Similarity between NARX, a Nitrate Sensor in *Escherichia coli* K-12, and the Methyl-Accepting Chemotaxis Proteins

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Received 9 January 1992/Accepted 24 March 1992

During anaerobic growth, nitrate induces synthesis of the anaerobic respiratory enzymes formate dehydrogenase-N and nitrate reductase. This induction is mediated by a transcription activator, the *narL* gene product. The *narX* gene product may be involved in sensing nitrate and phosphorylating NARL. We isolated *narX* mutants, designated *narX, that caused nitrate-independent expression of the formate dehydrogenase-N and nitrate reductase structural genes. We used λ *narX* specialized transducing phage to genetically analyze these lesions in single copy. Two previously isolated *narX** mutations, *narX32* and *narX71*, were also constructed by site-specific mutagenesis. We found that each of these alleles caused nitrate-independent synthesis of formate dehydrogenase-N and nitrate reductase, and each was recessive to *narX*$^+$. The *narX** mutations lie in a region of similarity with the methyl-accepting chemotaxis protein Tsr. We suggest that the *narX** proteins have lost a transmembrane signalling function such that phosphoprotein phosphatase activity is reduced relative to protein kinase activity.**

Enterobacteria can use a variety of compounds, including nitrate (NO_3^-) and fumarate, as terminal electron acceptors for anaerobic respiration. Formate-nitrate oxidoreductase is a major anaerobic respiratory chain in *Escherichia coli*. Formate dehydrogenase-N, encoded by the *fdnGHI* operon (3), transfers electrons derived from formate oxidation to nitrate reductase, encoded by the *narGHJI* operon. Both formate dehydrogenase-N and nitrate reductase are cytoplasmic membrane-bound enzyme complexes that contain molybdenum cofactor, iron-sulfur centers, and heme (for a review, see reference 39). Fumarate reductase, a cytoplasmic membrane-bound enzyme complex encoded by the *frdABCD* operon, allows cells to use fumarate as a terminal electron acceptor (for a review, see reference 24).

A dual regulatory mechanism ensures that electron acceptors with the greatest redox potential are used preferentially for respiration. During aerobic growth, anaerobic respiratory enzymes are not synthesized. In the absence of oxygen, synthesis of formate dehydrogenase-N and nitrate reductase is induced by nitrate, while synthesis of other respiratory enzymes, such as fumarate reductase, is repressed by nitrate. Anaerobic expression of these operons requires the product of the *fnr* gene, FNR (for a review, see references 17 and 39). Nitrate induction of the *fdnGHI* and *narGHJI* operons requires NARL, the *narL* gene product (3, 38, 41). NARL also mediates nitrate repression of genes encoding alternate respiratory enzymes, including *frdABCD* (15, 18, 40). Our current model for the regulation of the *fdnGHI*, *narGHJI*, and *frdABCD* operons in response to anaerobiosis and nitrate is shown in Fig. 1.

The *narL* gene is located at 27 min on the *E. coli* genetic map, just upstream of the *narGHJI* operon (38, 41). Null alleles of *narL* eliminate nitrate induction of *fdnGHI* and *narGHJI* expression and nitrate repression of *frdABCD* expression (3, 15, 38, 41). Molecular genetic analysis of the *narL* region led to the discovery of a closely linked gene, *narX* (19, 41). The sequences of NARX (30, 42) and NARL (13, 30, 42) share substantial similarity with the histidine protein kinase (sensor) and response regulator components, respectively, of two-component regulatory systems (for a review, see reference 44). Signal transduction between members of two-component regulatory pairs involves protein phosphorylation; the histidine protein kinase is autophosphorylated on a histidyl residue, and this phosphate is subsequently transferred to an aspartyl residue on the response regulator (for a review, see reference 44).

The simple model is that NARX and NARL form a nitrate-responsive regulatory pair; NARX senses the presence of nitrate and phosphorylates NARL, which thereupon regulates transcription. Indeed, genetic evidence suggests that NARL is a phosphorylated transcriptional regulator (10). Analysis of *narX*, however, suggests a more complicated model (9, 40, 41). In particular, null alleles of *narX* have no effect on nitrate induction of *fdnGHI* or repression of *frdABCD* (9). This observation suggests that *narX*$^+$ is not essential for normal nitrate regulation. We therefore postulated the existence of a hypothetical second, possibly redundant, nitrate-responsive sensor, designated NARQ (9) (Fig. 1). *narQ* null mutations have been isolated, and the *narQ* gene has been cloned and sequenced (31).

The NARX protein probably shares a similar overall topology with the methyl-accepting chemotaxis proteins (MCPs), with periplasmic amino-terminal and cytoplasmic carboxyl-terminal domains. MCPs respond to external chemoattractants and chemorepellants by transmitting a signal to the cytoplasm, thereby causing appropriate changes in bacterial swimming behavior (for a review, see reference 43). Previous genetic analysis of the serine chemoreceptor, Tsr, identified a cytoplasmic region, the linker, as functionally important for proper signal transduction (2).

We report here our genetic characterization of *narX* mutants, designated *narX**, that caused aberrant regulation

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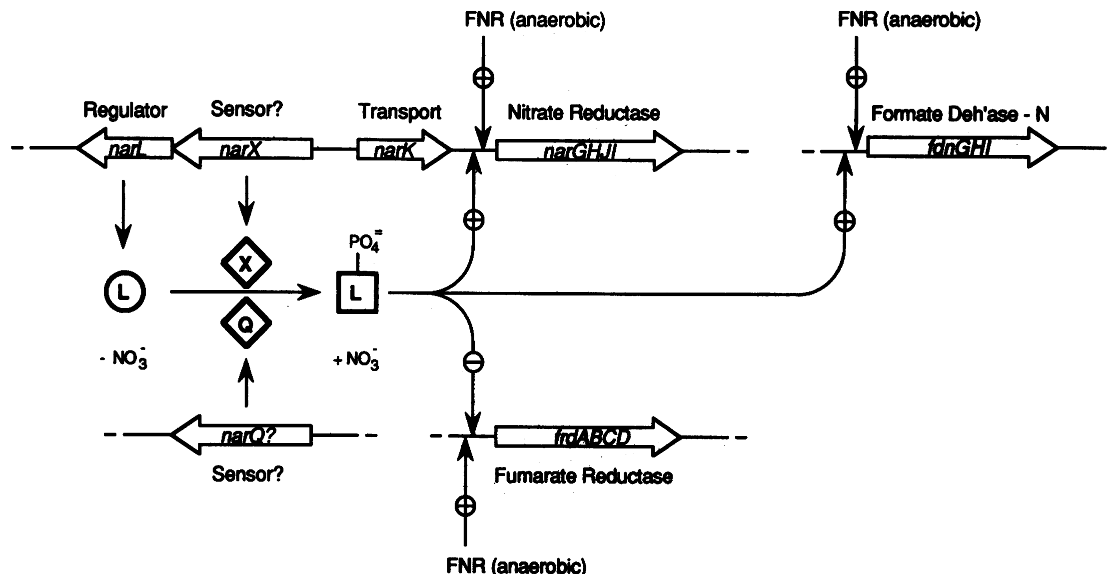

FIG. 1. Model for regulation of *narGHJI*, *fdnGHI*, and *frdABCD* expression. The *nar*, *fdn*, and *frd* regions are not to scale. Open arrows indicate protein-coding regions and their direction of transcription. +, positive regulation (activation); −, negative regulation (repression). NARL is hypothesized to be a nitrate-responsive DNA-binding protein which, when activated by protein phosphorylation, activates *narGHJI* and *fdnGHI* and represses *frdABCD* transcription. NARX and the hypothetical NARQ may be involved in modulating NARL activity via phosphorylation. FNR is required for anaerobic induction of *narGHJI*, *fdnGHI*, and *frdABCD*. NARK is involved in nitrate uptake (6). Adapted from reference 10.

of gene expression. Each of the four *narX** alleles studied carried single-residue substitutions in a region sharing sequence and positional similarity with the linker region of Tsr and other MCPs. This indicates that the NARX linker is important for transmembrane signal transduction. Each *narX** allele was recessive to *narX*$^+$. We suggest that these mutations result in loss of a transmembrane signalling function, such that phosphoprotein phosphatase activity is reduced relative to that of protein kinase.

MATERIALS AND METHODS

Strains, plasmids, and genetic methods. *E. coli* strains, λ specialized transducing phage, and plasmids used in this study are described in Table 1. Genetic crosses were performed by bacteriophage P1 *kc*-mediated transduction (26).

Standard methods were used for restriction endonuclease digestion, ligation, and transformation (5). Restriction enzymes and T4 DNA ligase were from New England BioLabs, Inc. T4 DNA polymerase and Sequenase were from United States Biochemical Corp.

Media. Defined, complex, and indicator media for routine genetic manipulations were used as previously described (5, 26). Kanamycin (Km) and ampicillin (Ap) were used at 75 and 200 μg/ml, respectively. Chloramphenicol (Cm) was used at 25 μg/ml, except where indicated. Agar and dehydrated media were from Difco Laboratories (Detroit, Mich.). Other components were from Sigma Chemical Co. (St. Louis, Mo.).

Cultures for β-galactosidase and nitrate reductase assays were grown in 3-[*N*-morpholino]propanesulfonic acid (MOPS)-buffered minimal medium with glucose as the sole carbon source (29, 41). The initial pH of this medium was set at 7.8. For experiments involving growth of plasmid-carrying strains, one-half-strength MOPS-glucose medium was supplemented with tryptone (0.4%), yeast extract (0.125%), and NaCl (0.125%). L-Tryptophan (0.2 mM) was added to defined media, and $NaNO_3$ (40 mM) was added as indicated. Cultures of *chlD* strains were supplemented with K_2SO_4 (2 mM [23]) and Na_2MoO_4 (100 μM) as indicated.

Culture conditions. Culture densities were monitored with a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., New York, N.Y.) equipped with a number 66 (red) filter. All cultures were grown at 37°C. Anaerobic cultures for β-galactosidase and nitrate reductase assays were grown in screw-cap tubes as previously described (41).

Enzyme assays. β-Galactosidase and reduced methyl viologen-nitrate reductase assays were measured in $CHCl_3$–sodium dodecyl sulfate-permeabilized cells as previously described (10). β-Galactosidase and nitrate reductase activities are expressed in terms of cell density (A_{600}) determined by the formulae of Miller (26) and Stewart and Parales (41), respectively. Each culture was assayed in duplicate. Reported values are averaged from at least two independent experiments.

Mutagenesis. Oligonucleotide-mediated site-specific mutagenesis followed the procedure of Kunkel et al. (22). Primers used to generate the desired changes were as follows: 5′-GGGCGCAAC<u>A</u>AAATGGCGA-3′ to convert Glu-208 of NARX to Lys, and 5′-GCAAACATCAGC<u>A</u>GGCGCAACG-3′ to convert Gly-205 of NARX to Arg. The mutagenesis was performed on pVJS836, which was constructed by subcloning the *Hin*dIII-to-*Nsi*I fragment, containing the upstream region of *narX*, into pGEM-7Zf(+) (Fig. 2). Base changes were identified by DNA sequence analysis (pVJS830 contained the E208K change and pVJS832 contained the G205R change). The *narX* mutations were placed in *cis* to either *narL*$^+$ or Δ*narL241* by ligating the 0.7-kb *Bcl*I fragments from either pVJS830 or pVJS832 into the *Bcl*I deletion in pVJS828 or pVJS829 (Fig. 2). Δ*narL241* is an internal *Bgl*II deletion that removes approximately one-third of the *narL* coding region (9). In each case, the entire DNA

TABLE 1. Strains, plasmids, and phage

Strain	Genotype	Source or reference
E. coli strains[a]		
ES1578	*mutD5 ara-14 argE3*(Oc) *galK2 hisG4*(Oc) *kdgK51 lacY1 leuB6 mgl-51 mtl-1 rac rfbD1 rpsL31 supE44 thi-1 thr-1 tsx-33 xyl-5*	CGSC[b]
JM83	*ara* Δ(*lac-proAB*)*X111 rpsL* φ80d Δ(*lacZ*)*M15*	48
RK4353	*araD139* Δ(*argF-lac*)*U169 flhD5301 gyrA219 non-9 rpsL150 ptsF25 relA1 deoC1*	38
RZ1032	*dut-1 ung-1 thi-1 relA1 sbd-279*::Tn*10 supE44*	22
VJS285	As RK4353 but *recA1* λ Φ(*narG-lacZ*)*218*	38
VJS533	As JM83 but *recA56*	41
VJS691	Δ(*argF-lac*)*U619* Δ(*trpEA*)*2*	41
VJS882	As VJS691 but *narG234*::MudJ	3
VJS1250	As VJS691 but *fdnG108*::MudJ	3
VJS1510	As VJS691 but *frdA401*::MudJ Δ(*narXL*)*235 recA1*	9
VJS1576	As VJS691 but *frdA401*::MudJ	9
VJS2007	As VJS691 but *fdnG108*::MudJ Δ(*narXL*)*235 recA1*	9
VJS2536	As VJS691 but *frdA401*::MudJ *recA1*	10
VJS2641	As VJS691 but *fdnG108*::MudJ Δ(*narXL*)*235 chlD247*::Tn*10*	10
VJS2642	As VJS691 but *frdA401*::MudJ Δ(*narXL*)*235 chlD247*::Tn*10*	This work
VJS2736	As VJS691 but *fdnG108*::MudJ *recA1*	10
Plasmids		
pGEM-7Zf+	Ap^r, T7 φ10 promoter	Promega
pHG165	Ap^r, pUC8 polylinker	37
pVJS1	Ap^r *narX*$^+$ *narL*$^+$ in *Hin*dIII site of pHG165	41
pVJS206	Ap^r Cm^r, as pVJS1 but *zch-2084*::Ω-Cm	9
pVJS211	Ap^r Km^r, as pVJS1 but Δ*narXL240*::Km	9
pVJS804	Ap^r Cm^r, as pVJS206 but *narX511* Δ*narL241*	This work
pVJS805	Ap^r Cm^r, as pVJS206 but *narX510* Δ*narL241*	This work
pVJS808	Ap^r Cm^r, as pVJS206 but *narX510 narL*$^+$	This work
pVJS809	Ap^r Cm^r, as pVJS206 but *narX511 narL*$^+$	This work
pVJS828	Ap^r Cm^r, as pVJS206 but Δ*narX238 narL*$^+$	This work
pVJS829	Ap^r Cm^r, as pVJS206 but Δ*narX238* Δ*narL241*	This work
pVJS830	Ap^r, as pVJS836 but *narX32 narL*$^+$	This work
pVJS832	Ap^r, as pVJS836 but *narX71 narL*$^+$	This work
pVJS834	Ap^r Cm^r, as pVJS206 but *narX32 narL*$^+$	This work
pVJS836	Ap^r, *Hin*dIII to *Nsi*I of *narXL* in pGEM-7Zf(+)	This work
pVJS844	Ap^r Cm^r, as pVJS206 but *narX71 narL*$^+$	This work
Phage		
λ p*chlC3*	*narX*$^+$ *narL*$^+$ *att*$^+$ *int*$^+$ *imm*^{21}	38
λ VJS107	As λ p*chlC3* but Δ(*narXL*)*237*::Km	9
λ VJS108	As λ p*chlC3* but *narX*$^+$ *narL*$^+$ *zch-2084*::Ω-Cm	9
λ VJS111	As λ p*chlC3* but *narX*$^+$ Δ*narL241 zch-2084*::Ω-Cm	9
λ VJS135	As λ p*chlC3* but *narX510* Δ*narL241 zch-2084*::Ω-Cm	This work
λ VJS136	As λ p*chlC3* but *narX511* Δ*narL241 zch-2084*::Ω-Cm	This work
λ VJS137	As λ p*chlC3* but *narX510 narL*$^+$ *zch-2084*::Ω-Cm	This work
λ VJS138	As λ p*chlC3* but *narX511 narL*$^+$ *zch-2084*::Ω-Cm	This work
λ VJS139	As λ p*chlC3* but *narX32 narL*$^+$ *zch-2084*::Ω-Cm	This work
λ VJS140	As λ p*chlC3* but *narX71 narL*$^+$ *zch-2084*::Ω-Cm	This work
λ VJS141	As λ p*chlC3* but *narX32* Δ*narL241 zch-2084*::Ω-Cm	This work
λ VJS142	As λ p*chlC3* but *narX71* Δ*narL241 zch-2084*::Ω-Cm	This work

[a] All strains are F$^-$ and are λ$^-$ except as indicated.
[b] CGSC, Coli Genetic Stock Center, courtesy of B. J. Bachmann.

sequence of the *Bcl*I fragment was determined to ensure that additional mutations were not introduced during the mutagenesis.

The λ *narX*$^+$ Δ*narL241* specialized transducing phage (λ VJS111) was mutagenized by growth on a *mutD5* strain, ES1578 (34, 47).

Isolation of *narX mutants.** Independent mutagenized lysates of λ VJS111 were used to transduce indicator strains VJS882 [Φ(*narG-lacZ*)] or VJS1250 [Φ(*fdnG-lacZ*)]. Samples of saturated cultures (0.1 ml each) were mixed with approximately 10^4 PFU of mutagenized lysate and incubated at room temperature for 15 min. Mixtures were plated on MacConkey-lactose-chloramphenicol medium and incubated aerobically overnight at 37°C. Lac$^+$ (nitrate-independent) colonies were purified on MacConkey-lactose medium.

Linkage of the Lac phenotype to the λ phage was tested by making lysates from putative nitrate-independent strains and transducing an indicator strain. The resulting Cm^r lysogens were then examined for the Lac phenotype.

Genetic manipulation of *narX* mutations. To allow their study in single copy, *narX* mutations located on plasmid vectors were recombined onto λ phage. We have previously developed and described methods to exchange *narX* (and *narL*) alleles between λ specialized transducing phage and plasmid vectors (9). To transfer alleles from plasmids to phage, lysates of λ VJS107 (Δ*narXL*::Km) were made on

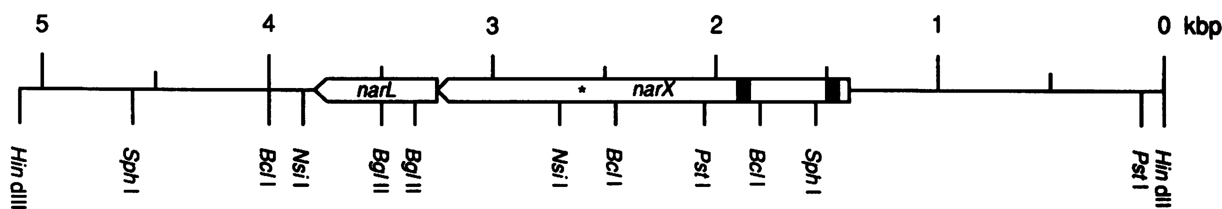

FIG. 2. Partial restriction map of the *narXL* region. The asterisk marks the position of the conserved His residue in the *narX* coding region, and the black boxes represent the presumed membrane spanning regions. Adapted from reference 9.

strains carrying *narX** derivatives of the Ap^r plasmid pVJS206 (*narX* narL*$^+$ *zch*::Cm). Recombinants were isolated by selecting for Cm^r transductants, with subsequent screening for Km^s and Ap^s. To transfer alleles from phage to plasmids, lysogens (λ *narX* zch*::Cm) carrying the Ap^r plasmid pVJS211 (Δ*narXL*::Km) were grown for several generations and then spread onto plates containing 200 μg of chloramphenicol per ml to enrich for recombinants carrying the *zch*::Cm marker on the plasmid. Plasmid DNA was isolated from a pool of Cm^r colonies, and recombinants were isolated by selecting for Cm^r transformants, with subsequent screening for Ap^r and Km^s. The structures of the recombinant plasmids were confirmed by restriction analysis. *narL*$^+$ was reconstructed from Δ*narL241* by ligating in the 0.2-kb *Bgl*II fragment to make the *narX* narL*$^+$ derivatives pVJS808 and pVJS809.

All *narX** alleles were studied in single copy in a variety of strain backgrounds. Single lysogens were verified by their level of resistance to chloramphenicol as previously described (9).

DNA sequence analysis. Mutations were first mapped to one of three 600-bp regions of *narX* by a series of subcloning experiments. The *Pst*I or *Nsi*I fragments from pVJS804 and pVJS805 were ligated into *narXL* containing plasmids deleted for the same fragments, to reconstruct an intact *narXL* region. The resulting subclones were then reduced with *Bgl*II to make Δ*narL241*. These latter plasmids were used to transform strain VJS285 [φ(*narG-lacZ*)], selecting for Cm^r. Transformants were screened for the nitrate-independent Lac^+ phenotype on MacConkey-lactose-chloramphenicol plates.

DNA sequencing of double-stranded DNA was carried out as previously described (10) by using the dideoxynucleotide chain termination method with modified T7 DNA polymerase (45) and [α-^{35}S]dATP labeling (4). Oligonucleotides were synthesized at the Oligonucleotide Synthesis Facility of the Cornell University Biotechnology Program.

Computer-assisted sequence similarity analysis. Computer analyses used the Genetics Computer Group program suite (7) running on the BIOVAX computer of the Cornell University Biotechnology Program. The program BLAST (basic local alignment search tool [1]) was used for similarity searches of the sequence data bases NBRF/PIR (version 28) and SWISS-PROT (version 19). Parameters were $S = 48$ (cutoff score), $T = 17$ (word pair score), and $w = 4$ (word pair length).

Statistical analysis to evaluate the similarity between the linker regions of Tsr and NARX employed the jumbling test of Doolittle (8). The program SHUFFLE (7) was used to create eight randomized versions each for both of the 80-residue sequences shown in Fig. 3. Each of the randomized Tsr sequences was then aligned with each of the randomized NARX sequences in pairwise fashion by using the algorithm of Needleman and Wunsch (28).

RESULTS

Isolation of nitrate-independent *narX* alleles. We used localized mutagenesis to isolate *narX* mutations, designated *narX**, that caused expression of the *narGHJI* and *fdnGHI* operons in the absence of nitrate. λ VJS111, which carries *narX*$^+$ Δ*narL241*, was grown on a *mutD5* strain, which causes increased levels of mutagenesis because of inefficient misincorporation repair. The resulting phage were used to transduce *narX*$^+$ *narL*$^+$ Φ(*narG-lacZ*) and Φ(*fdnG-lacZ*) indicator strains, and Cm^r Lac^+ transductants were selected on MacConkey-lactose-chloramphenicol medium. The efficiency of lysogenization, as measured by Cm^r transductants, was approximately 10^{-1}/PFU, and that of transduction to Lac^+ was approximately 10^{-1}/PFU. Seven independent *narX** alleles were isolated, three of which are described in this report.

The *narX** alleles studied were recessive to *narX*$^+$ (see below). This was unexpected, as they were isolated in a *narX*$^+$ background. The slightly elevated noninduced level of Φ(*narG-lacZ*) and Φ(*fdnG-lacZ*) expression in a *narX**/*narX*$^+$ strain resulted in red colony color on MacConkey-lactose medium, which led to initial detection of *narX** mutants. Previous studies have revealed that MacConkey-lactose medium often yields apparently constitutive mutants

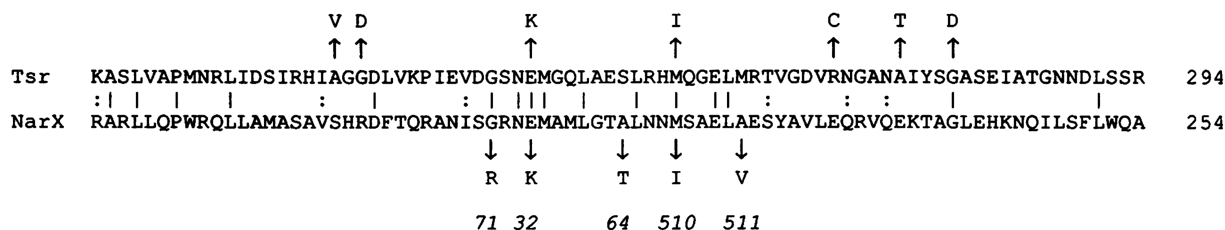

FIG. 3. Sequence alignment of the linker regions of Tsr and NARX in the standard single-letter code. The first residue in each sequence is that which immediately follows the last hydrophobic residue in the second transmembrane spanning region. Arrows indicate residues changed in mutants. Symbols: |, identical residues; :, analogous residues (Arg-Lys, Asn-Gln, Ile-Val, and Ser-Thr).

TABLE 2. Nucleotide and amino acid changes of *narX* alleles

Allele	Nucleotide		Amino acid	
	Site[a]	Change	Site[a]	Change
narX32[b]	648	G → A	208	Glu → Lys
narX71[b]	639	G → A	205	Gly → Arg
narX510[c]	683	G → A	219	Met → Ile
narX511[c]	697	C → T	224	Ala → Val

[a] Positions are numbered from the *narX* translation initiation site (30).
[b] Allele isolated by Kalman and Gunsalus (20).
[c] Allele isolated in this study.

whose phenotypes, when subsequently determined by enzyme assays, are nearly wild type (10, 36).

We also attempted, by screening for white colonies on MacConkey-lactose-nitrate-chloramphenicol medium, to isolate *narX* mutants that interfered with nitrate induction of Φ(*narG-lacZ*) and Φ(*fdnG-lacZ*). No *narX* mutants were recovered in this screen.

DNA sequence analysis. The *narX** alleles chosen for further characterization were recombined from λ phage onto plasmids (9). Subcloning localized each of the *narX** mutations to a 600-bp region of *narX* (see Materials and Methods). One mutation mapped upstream of the *Pst*I site in *narX*, while the other two mapped between the *Pst*I and *Nsi*I sites in *narX* (Fig. 2). The corresponding 600-bp region of each *narX** allele was sequenced. Two independent mutants contained one change, designated *narX511*, and the third isolate contained another change, designated *narX510*. Predicted amino acid changes for each of the *narX** alleles are shown in Table 2.

Construction of *narX mutants.** While this work was in progress, Kalman and Gunsalus (20) reported the isolation and characterization of three *narX** mutants. None of the seven mutations that we isolated corresponded to those of Kalman and Gunsalus. Thus, to directly compare their results with our own, we used site-specific mutagenesis to construct two of these alleles, *narX32* and *narX71*.

Phenotypic analysis. Nitrate regulation involves both induction and repression of specific operons. In order to help understand the mechanisms involved, we determined the effect of *narX** alleles on this regulation. Thus, we examined *narGHJI*, Φ(*fdnG-lacZ*), or Φ(*frdA-lacZ*) expression in Δ(*narXL*) strains carrying different λ*narX** *narL*$^+$ phage (Table 3). *narX32* conferred the strongest nitrate-independent expression of *narGHJI* and Φ(*fdnG-lacZ*), while *narX511*, *narX71*, and *narX510* had progressively weaker effects. Overall, each of the alleles conferred stronger nitrate-independent expression on *narGHJI* than on Φ(*fdnG-lacZ*). None of the *narX** alleles caused nitrate-independent repression of Φ(*frdA-lacZ*), while *narX32* strains seemed to be partially defective in Φ(*fdnG-lacZ*) induction and Φ(*frdA-lacZ*) repression (Table 3).

Complementation analysis. To assess dominance relationships, we compared *narGHJI*, Φ(*fdnG-lacZ*), and Φ(*frdA-lacZ*) expression in haploid strains with that in merodiploid strains (*narX*$^+$/λ*narX**). Each of the *narX** alleles was recessive to *narX*$^+$ (Table 3).

Kalman and Gunsalus (20) reported that multiple copies of *narX32* and *narX71* are dominant to a single copy of *narX*$^+$. These experiments employed plasmids carrying both *narX** and *narL*$^+$. Our own experiments revealed that these alleles, as well as *narX510* and *narX511*, were recessive to *narX*$^+$ in merodiploid strains (Table 3). To examine this difference in

TABLE 3. Complementation analysis of *narX** alleles in merodiploid strains

narX allele[a]	Enzyme sp act[b]			
	Δ(*narXL*)235[c]		*narX*$^+$ *narL*$^{+d}$	
	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$
narGHJI[e]				
narX$^+$	12	780	11	720
narX510	56	780	20	880
narX71	200	820	32	760
narX511	240	800	37	810
narX32	660	760	25	610
Φ(*fdnG-lacZ*)[f]				
narX$^+$	8	1,040	8	790
narX510	19	1,010	9	870
narX71	35	1,010	11	810
narX511	46	1,030	13	790
narX32	85	670	11	710
Φ(*frdA-lacZ*)[g]				
narX$^+$	68	6	60	7
narX510	68	6	56	8
narX71	73	6	59	7
narX511	73	6	61	8
narX32	77	27	60	15

[a] *narX* allele on the λ phage.
[b] Determined as described in Materials and Methods and expressed in arbitrary units. Cultures were grown anaerobically with or without nitrate as indicated.
[c] Δ(*narXL*) at the *nar* locus; λ *narX*$^+$ *narL*$^+$ or λ *narX** *narL*$^+$ at the λ attachment site. Parental strains were VJS1510 [Φ(*frdA-lacZ*) Δ(*narXL*) *recA*] and VJS2007 [Φ(*fdnG-lacZ*) Δ(*narXL*) *recA*].
[d] *narX*$^+$ *narL*$^+$ at the *nar* locus; λ *narX*$^+$ Δ*narL* or λ *narX** Δ*narL* at the λ attachment site. Parental strains were VJS2536 [Φ(*frdA-lacZ*) *narX*$^+$ *narL*$^+$ *recA*] and VJS2736 [Φ(*fdnG-lacZ*) *narX*$^+$ *narL*$^+$ *recA*].
[e] Nitrate reductase enzyme activity from *narGHJI*.
[f] β-Galactosidase activity from a Φ(*fdnG-lacZ*) operon fusion.
[g] β-Galactosidase activity from a Φ(*frdA-lacZ*) operon fusion.

results, we measured nitrate reductase and β-galactosidase activities in *narGHJI*$^+$ Φ(*frdA-lacZ*) strains carrying the *narX32* or *narX71* alleles on λ phage and on multicopy plasmids (Table 4). The plasmid-carrying strains grew very poorly in defined medium, so we used an enriched medium to allow exponential growth of these strains. Enriched medium altered the patterns of gene expression; nitrate reductase induction was reduced about twofold, while Φ(*frdA-lacZ*) expression was elevated about fivefold (Table 4). Despite these quantitative differences, the qualitative patterns of regulation in merodiploid strains in enriched medium compared with that in defined medium were similar; in both cases, *narX*$^+$ was dominant to *narX** (compare Tables 3 and 4). In contrast, multiple copies of the *narX** alleles (along with multiple copies of *narL*$^+$) were dominant to a single chromosomal copy of *narX*$^+$ (Table 4), in agreement with the results of Kalman and Gunaslus (20).

Effect of *narX mutations on molybdate regulation.** Induction of *narGHJI* and Φ(*fdnG-lacZ*) expression and repression of Φ(*frdA-lacZ*) require molybdate in addition to nitrate (11, 16). Molybdate limitation is achieved by using a *chlD* strain, defective in molybdate uptake (33), and is relieved by adding 100 μM (excess) molybdate to the growth medium (12). Kalman and Gunsalus (20) concluded that *narX71* and *narX64* confer relatively molybdate-independent phenotypes. We examined the phenotypes conferred by *narX** mutations under conditions of limiting molybdate by mea-

TABLE 4. Complementation of *narX** alleles in merodiploids and in plasmid-carrying strains

narX allele[a]	Enzyme sp act[b]			
	Δ(*narXL*)*235*[c]		*narX*$^+$ *narL*$^{+d}$	
	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$
narGHJI[e]				
λ *narX*$^+$	12	400	9	340
λ *narX71*	80	260	30	340
λ *narX32*	230	160	29	210
p *narX*$^+$	12	340	16	360
p *narX71*	160	410	130	380
p *narX32*	480	440	410	400
Φ(*frdA-lacZ*)[f]				
λ *narX*$^+$	230	16	170	22
λ *narX71*	210	8	260	17
λ *narX32*	140	64	250	30
p *narX*$^+$	190	2	260	2
p *narX71*	130	2	120	1
p *narX32*	50	30	60	14

[a] *narX* allele on the λ phage or plasmid.
[b] Determined as described in Materials and Methods and expressed in arbitrary units. Cultures were grown anaerobically in enriched medium, with or without nitrate as indicated.
[c] Δ(*narXL*) at the *nar* locus; λ *narX*$^+$ *narL*$^+$ or λ *narX** *narL*$^+$ at the λ attachment site, or plasmids p*narX*$^+$ *narL*$^+$ or p*narX** *narL*$^+$. Parental strain was VJS1510 [Φ(*frdA-lacZ*) Δ(*narXL*) *recA*].
[d] *narX*$^+$ *narL*$^+$ at the *nar* locus; λ *narX*$^+$ Δ*narL* or λ *narX** Δ*narL* at the λ attachment site, or plasmids p*narX*$^+$ *narL*$^+$ or p*narX** *narL*$^+$. Parental strain was VJS2536 [Φ(*frdA-lacZ*) *narX*$^+$ *narL*$^+$ *recA*].
[e] Nitrate reductase enzyme activity from *narGHJI*.
[f] β-Galactosidase activity from a Φ(*frdA-lacZ*) operon fusion.

suring the expression of Φ(*fdnG-lacZ*) and Φ(*frdA-lacZ*) in haploid *narX** *chlD* strains (Table 5). In the absence of nitrate, Φ(*fdnG-lacZ*) expression in *narX71*, *narX510*, and *narX511* strains was essentially unchanged by the addition of molybdate (Table 5). In this sense, the constitutive pheno-

TABLE 5. Effects of molybdate on Φ(*fdnG-lacZ*) and Φ(*frdA-lacZ*) regulation by *narX**

narX allele[a]	β-Galactosidase sp act[b]			
	$-NO_3^-$		$+NO_3^-$	
	$-MoO_4^{2-}$	$+MoO_4^{2-}$	$-MoO_4^{2-}$	$+MoO_4^{2-}$
Φ(*fdnG-lacZ*)[c]				
narX$^+$	9	6	45	750
narX510	12	12	56	860
narX71	19	24	62	860
narX511	24	29	56	860
narX32	22	110	25	380
Φ(*frdA-lacZ*)[d]				
narX$^+$	60	53	86	5
narX510	60	58	68	3
narX71	64	56	82	4
narX511	62	61	70	4
narX32	64	52	76	27

[a] Δ(*narXL*) at the *nar* locus; λ *narX*$^+$ *narL*$^+$ or λ Δ*narX* *narL*$^+$ at the λ attachment site. Parental strains were VJS2641 [Φ(*fdnG-lacZ*) Δ(*narXL*) *chlD*::Tn*10*] and VJS2642 [Φ(*frdA-lacZ*) Δ(*narXL*) *chlD*::Tn*10*].
[b] Determined as described in Materials and Methods and expressed in arbitrary (Miller) units. Cultures were grown anaerobically with or without nitrate and molybdate, as indicated.
[c] β-Galactosidase activity from a Φ(*fdnG-lacZ*) operon fusion.
[d] β-Galactosidase activity from a Φ(*frdA-lacZ*) operon fusion.

TABLE 6. Effects of Δ*narX* on molybdate regulation of Φ(*fdnG-lacZ*) and Φ(*frdA-lacZ*)

narX allele[a]	β-Galactosidase sp act[b]			
	$-NO_3^-$		$+NO_3^-$	
	$-MoO_4^{2-}$	$+MoO_4^{2-}$	$-MoO_4^{2-}$	$+MoO_4^{2-}$
Φ(*fdnG-lacZ*)[c]				
narX$^+$	10	8	96	1,080
Δ*narX238*	9	20	42	1,020
Φ(*frdA-lacZ*)[d]				
narX$^+$	44	36	68	4
Δ*narX238*	44	35	53	2

[a] Δ(*narXL*) at the *nar* locus; λ *narX*$^+$ *narL*$^+$ or λ Δ*narX* *narL*$^+$ at the λ attachment site. Parental strains were VJS2641 [Φ(*fdnG-lacZ*) Δ(*narXL*) *chlD*::Tn*10*] and VJS2642 [Φ(*frdA-lacZ*) Δ(*narXL*) *chlD*::Tn*10*].
[b] Determined as described in Materials and Methods and expressed in arbitrary (Miller) units. Cultures were grown anaerobically with or without nitrate and molybdate, as indicated.
[c] β-Galactosidase activity from a Φ(*fdnG-lacZ*) operon fusion.
[d] β-Galactosidase activity from a Φ(*frdA-lacZ*) operon fusion.

types conferred by these alleles might be described as molybdate independent. In the presence of nitrate, however, each of these strains required excess molybdate for full induction of Φ(*fdnG-lacZ*) and full repression of Φ(*frdA-lacZ*) expression (Table 5). Therefore, in this context, these alleles were fully dependent upon molybdate. By contrast, the *narX32* strains responded to molybdate even in the absence of nitrate, as found by Kalman and Gunsalus (20). We also compared the molybdate dependence of Φ(*fdnG-lacZ*) and Φ(*frdA-lacZ*) expression in *narX*$^+$ and Δ*narX* strains (Table 6). Little difference in molybdate response was observed, suggesting that *narX*$^+$ is not essential for normal molybdate regulation.

Computer-assisted sequence similarity analysis. We observed that the region of NARX defined by the *narX** mutations shares similarity with the linker region of Tsr, an MCP. Two methods (see Materials and Methods) helped to evaluate the possible significance of this similarity. First, we used BLAST to search two data bases for sequences similar to the 80-residue region of NARX shown in Fig. 3. In both cases, the best match (after NARX itself) was with the linker region of Tsr; the homologous regions of other MCPs were also detected. The NARX-Tsr similarity score with BLAST was 59. The designers of BLAST suggest that "only . . . score[s] over 55 are likely to be distinguishable from chance similarities" by using the parameters employed (1).

Second, we employed the jumbling test of Doolittle (8). The 64 pairwise comparisons of the randomized NARX and Tsr interdomain linkers (Fig. 3) gave an alignment score of 26.1 ± 2.3 (mean ± standard deviation; range, 21.0 to 33.1). Comparison of the authentic NARX and Tsr interdomain linkers gave an alignment score of 37.6, which is 5.0 standard deviation units above the mean.

DISCUSSION

The sequence of *narX* suggests that its product is a sensor that signals the presence of nitrate to the *narL* gene product. This paper describes the isolation and genetic characterization of *narX** mutations that conferred nitrate-independent expression of *narGHJI* and Φ(*fdnG-lacZ*). *narX** mutations have also been described by Kalman and Gunsalus (20).

Functional sequence similarity between NARX and Tsr.

NARX, like most two-component sensors, is predicted to contain an amino-terminal periplasmic domain bounded by two hydrophobic transmembrane regions and a carboxyl-terminal cytoplasmic domain (30). This overall topology is shared by the MCPs involved in signal transduction for chemotaxis (43). NARX is different from many sensors in that it contains a long stretch of amino acid residues between the second transmembrane spanning region and the beginning of the histidine protein kinase domain (44). We observed that this region is similar to the analogous region, designated the linker, in MCPs (Fig. 3). Mutational analysis of the gene for one MCP, *tsr*, led to the proposal that the linker is important for transmembrane signal transduction (2). Two changes of Tsr that each confer an altered transducer phenotype (E248K and M259I [2]) are identical to two of the *narX** changes, *narX32* and *narX510* (Fig. 3). All other *narX** mutations reported to date lie within this region of shared sequence similarity. The E248K and M259I mutations in *tsr* were isolated and studied on multicopy plasmids (2), so it is not known whether they are dominant or recessive to tsr^+ in merodiploids.

This similarity between Tsr and NARX was originally detected by visual inspection. Subsequently, we employed computer-assisted analyses to evaluate the statistical significance of this similarity. Two different methods, described in Results, both give the same conclusion; the observed similarity, while marginal, is probably significant. The biological significance seems clear. The region of similarity between Tsr and NARX occurs in precisely congruent positions in the two proteins, immediately following the second transmembrane domain. Neither protein shares sequence similarity in other domains, although their overall topologies are likely to be similar. Most importantly, identical mutations within this region in the two proteins (Fig. 3) affect transmembrane signalling.

This linker region similarity between Tsr and NARX is not shared by any of the other sensor components we have inspected, including those tabulated by Stock et al. (44). Data base searches also failed to detect this region in other proteins (except for other MCPs). This suggests that the mechanism of transmembrane signal transduction by NARX may differ from that of other two-component sensor proteins and is more similar to that of the MCPs.

Phenotypes conferred by *narX alleles.** We examined the effect of different *narX** alleles on the expression of *narGHJI*, Φ(*fdnG-lacZ*), and Φ(*frdA-lacZ*) in haploid *narX** strains. *narX32* conferred the strongest nitrate-independent phenotype, while *narX511*, *narX71*, and *narX510* were progressively weaker (Table 3). Collectively, the *narX** alleles affected the three nitrate-regulated operons in a differential manner. Each allele conferred higher levels of nitrate-independent *narGHJI* expression than Φ(*fdnG-lacZ*) expression. None of the alleles, when present in single copy, caused nitrate-independent repression of Φ(*frdA-lacZ*); we do not understand the reason for this. However, analogous patterns of *narGHJI*, Φ(*fdnG-lacZ*), and Φ(*frdA-lacZ*) expression have been observed previously (9, 10, 41). Differences in NARL binding sites upstream of these operons and the sensitivity of NARL for these sites may explain these observations (10). Additionally, the phenotypic effect of each *narX** allele required $narL^+$ (data not shown).

Complementation analysis. We examined dominance relationships by comparing the expression of *narGHJI*, Φ(*fdnG-lacZ*), and Φ(*frdA-lacZ*) in haploid *narX** and merodiploid $narX^+$/*narX** strains. Each of the *narX** alleles was recessive to $narX^+$ (Table 3). By contrast, *narX** mutations in multicopy (in conjunction with multicopy $narL^+$) were dominant to a single copy of $narX^+$ (20) (Table 4). We believe that merodiploid analysis provides the more credible view of $narX^+$/*narX** dominance relationships (35).

Effect of *narX alleles on molybdate regulation.** In addition to nitrate and anaerobiosis, molybdate is necessary for normal regulation of nitrate reductase, formate dehydrogenase-N, and fumarate reductase synthesis. It is not known whether NARX directly responds to the presence of molybdate in controlling gene expression (20) or whether an independent regulatory system monitors molybdate availability (9). Our analysis of *narX** mutants indicated that these strains still required molybdate for proper regulation of *narGHJI*, *fdnGHI*, and *frdABCD* expression (Table 5).

The role of NARX. Single-base substitution *narX** mutations differed phenotypically from Δ*narX* deletions, which are presumed to be null alleles (9). By definition, the phenotype of a null allele represents the complete loss of function. In the case of *narX*, this loss of function still allows normal nitrate regulation, which led to the hypothesis that a redundant sensor, NARQ, must substitute for NARX at least for nitrate induction in Δ*narX* strains (9). Indeed, the *narQ* gene has recently been identified and is currently being studied (31).

The nitrate-independent phenotypes conferred by the *narX** alleles were recessive to $narX^+$. Generally, recessive lesions result from loss of function. In addition, the *narX** mutations arose at a relatively high frequency, further consistent with the idea that they represent loss of a function. For comparison, a dominant allele of *narL* that causes nitrate-independent gene expression was found at an approximately 100-fold-lower frequency than the *narX** alleles (10).

Analogous EnvZ mutants are somewhat dominant in diploid analysis (32). However, the *envZ* mutations studied in this case are in different regions than the *narX** mutations, as EnvZ does not have a linker region.

Thus, we have two different types of loss-of-function alleles, Δ*narX* and *narX**, that have different phenotypes. This suggests that NARX has (at least) two activities, both of which are lost in Δ*narX* strains, but only one of which is defective in *narX** strains. The *narX** strains, in addition, must be defective in a negative regulatory function, since these alleles confer a constitutive phenotype. Similar logic was previously employed in analyzing mutants of *ntrB* and *ntrC*, encoding a two-component regulatory pair involved in nitrogen regulation (25).

We do not know the relative stabilities of the wild-type and mutant NARX proteins. Significantly lower stability of the NARX* proteins would provide an alternate explanation for the observed recessive behavior of the *narX** alleles. However, it is interesting to note a recent in vitro analysis of Tar, the aspartate chemoreceptor, in which heterodimers between normal and severely truncated Tar monomers were isolated and tested for transmembrane signal transduction (27). Heterodimers in which one monomer consisted solely of the periplasmic domain were still capable of propagating their transmembrane signal in response to aspartate. This suggests that for MCPs, at least, a heterodimer consisting of one wild-type subunit and one loss-of-function mutant subunit would behave essentially as a wild type in transmembrane signalling.

What are the two functions of NARX? The paradigm of two-component regulatory systems suggests that *narX* encodes a bifunctional protein, with both protein kinase and phosphoprotein phosphatase activities (14, 21, 46). Δ*narX* strains must lack both activities. Kinase activity, at least,

would be supplied by the hypothetical NARQ protein, a redundant nitrate sensor. By contrast, if *narX** strains were deficient in phosphoprotein phosphatase activity in relation to protein kinase activity, the net effect would be an accumulation of phosphorylated NARL even in the absence of the inducer, nitrate. Further experiments will test these ideas.

ACKNOWLEDGMENTS

We are grateful to Tom Silhavy for helpful discussions, Ted Thannhauser for synthesizing oligonucleotides, and Douglas Berg for constructive editorial suggestions on early versions of the manuscript. We thank the members of our laboratory for their helpful advice and interest.

This study was supported by Public Health Service grants RO1 GM36877 (awarded to V.S.) and T32 GM07617 (predoctoral traineeship for L.A.C.) from the National Institute of General Medical Sciences.

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