Nitrate- and Molybdenum-Independent Signal Transduction
Mutations in narX That Alter Regulation of Anaerobic
Respiratory Genes in Escherichia coli

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Escherichia coli can respire anaerobically by reducing nitrate, trimethylamine-N-oxide, dimethyl sulfoxide,
or fumarate. When nitrate is present, expression of the genes for fumarate (frdABCD), trimethylamine-N-
oxide, and dimethyl sulfoxide (dmsABC) is repressed while expression of the nitrate reductase (narGHJI) gene
is induced. This regulation requires molybdate and is mediated by the narX and narL gene products, which
together form a two-component regulatory system. We provide evidence that NarX is a nitrate and
molybdenum sensor which activates NarL when nitrate is available to cells. Mutants generated by hydroxy-
lamine mutagenesis were repressed for frdA-lacZ expression even when cells were grown in the absence
of nitrate. The mutations responsible for three of these nitrate independence (NarX*+) phenotypes were localized
to narX and further characterized in vivo for their ability to repress frdA-lacZ expression. Two of the mutants
(the narX64 and narX71 mutants) had a greatly reduced requirement for molybdenum to function but still
responded to nitrate. In contrast, a third mutant (the narX32 mutant) required molybdenum but did not exhibit
full repression of frdA-lacZ expression even when nitrate was present. These narX* alleles also caused the
induction of nitrate reductase gene expression and the repression of a dmsA-lacZ fusion in the absence
of nitrate. Each narX* mutation was determined to lie in an 11-amino-acid region of the NarX polypeptide
that follows a proposed transmembrane domain. We suggest that the conformation of the narX* gene products
is altered such that even in the absence of nitrate each of these gene products more closely resembles the wild-type
NarX protein when nitrate is present. These data establish a clear role for the narX gene product in gene
regulation and strongly suggest its role in sensing nitrate and molybdenum.

Escherichia coli can obtain energy via oxidative phosphorylation by reducing a variety of terminal electron acceptors.
These include, in order of decreasing potential energy, oxygen, nitrate, trimethylamine-N-oxide (TMAO), dimethyl
sulfoxide (DMSO), and fumarate (13, 34). Production of the enzymes (cytochrome o and d oxidase, nitrate reductase,
DMSO and TMAO reductase, and fumarate reductase) for the reduction of each of these respiratory substrates is
regulated by anaerobiosis and/or nitrate (2, 3, 17, 26, 29). During aerobic growth, the nitrate reductase (narGHJI),
DMSO and TMAO reductase (dmsABC), and fumarate reductase (frdABCD) genes are expressed at low levels while
cytochrome o (cyoABCDE) and d (cydAB) oxidase gene expression is high. Upon oxygen depletion and when nitrate
is present, narGHJI gene expression is elevated while the synthesis of the enzymes for the utilization of the other
alternative aerobonic electron acceptors is repressed (2, 3, 17–19, 26, 29). The fumarate reductase and DMSO and
TMAO reductase genes are expressed at high levels only when nitrate is either depleted or absent from the anaerobic
culture medium.

The mechanism for oxygen regulation of the respiratory pathway genes appears to function independently of nitrate
regulation. The aerobic or anaerobic response is mediated in part by the FNR protein (2, 3, 17, 21, 29), whereas the nitrate
response is mediated by at least two other regulatory proteins encoded by the narX (narR) and narL (frdR) genes (3,
14, 18, 19, 30, 31). Nitrate regulation also appears to require the presence of molybdate ions, as chlD strains, which are unable
to transport molybdate, are abnormal for nitrate control of narGHJI, dmsABC, and frdABCD gene regulation
(3, 15, 23, 30; L. V. Kalman, Ph.D. thesis, University of California, Los Angeles, 1990). The narX and narL genes are
adjacent to each other and map at 27 min on the E. coli chromosome (18, 19, 29, 31). They have been cloned, and
their DNA sequences have been determined (9, 24, 32). The genes encode proteins of 66 and 23 kDa, respectively
(9, 24, 32). Comparison of the predicted amino acid sequences of NarL and NarX with those of other bacterial proteins
reveals similarities to the two-component family of regulatory proteins (e.g., the ompR-envZ, virA-virG, and phoR-
phoB gene products; 9, 24, 32). In such systems, one protein component functions as an environmental sensor to detect
availability or change in an environmental signal. It then relates this information to a second protein, known as the
receiver protein, which responds to either activate or repress gene expression upon binding at a DNA regulatory site (25,
33). NarX is homologous in its N-terminal domain to the sensor proteins, and NarL is homologous at its C-terminal
domain to the receiver proteins (9, 24, 32). The NarX protein may thus be involved in sensing the availability of nitrate
and, possibly, molybdate. We propose that, in the presence of these two anions, NarX modifies NarL, which is then able
to activate nitrate reductase (narGHJI) gene expression and repress DMSO and TMAO reductase (dmsABC) and
fumarate reductase (frdABCD) gene expression.

In this study, we present further evidence for a model of NarX and NarL interaction. We isolated mutations of narX
(narX*) which caused the repression of fumarate reductase

* Corresponding author.
TABLE 1. E. coli strains, phages, and plasmids

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<tr>
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<td>TGI</td>
<td>MC4100</td>
<td>Δ(lac-pro) supE thi hsdD5(F' traD36 proA+B+ lacI tet) lacZD15</td>
<td>Amersham</td>
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<td>D. Westenberg</td>
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<table>
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and DMSO and TMAO reductase and the induction of nitrate reductase expression even when cells were grown in the absence of nitrate. The mutants also varied in their requirement for molybdenum ions. Two of the mutants no longer required molybdenum to repress frd gene expression, while a third narX+ mutant did. The locations of the mutations responsible for the NarX+ phenotypes were identified by DNA sequence analysis.

**MATERIALS AND METHODS**

**Bacterial strains, bacteriophages, and plasmids.** The *E. coli* K-12 strains, phages, and plasmids used in this study are listed in Table 1. The lysogenized JL100 in strain LK1351 was removed by coinfection as previously described (19), and λLK1 was introduced into the chromosome. This strain was subsequently transduced to *recA srl::Tn10* with a P1 lysate prepared on strain DW4 to yield strain LK3012 (narX* recA*). By a similar method, λLK1 was removed from LK3012(λLK1) (narX) and λPC25 (dmsA'·lacZ) was introduced to yield LK3012(λPC25) (narX*). Transductions and lysogenies were performed as previously described (27).

**Media.** For strain and plasmid constructions, cells were grown in Luria broth or on solid medium with chloramphenicol, tetracycline, or ampicillin present at a concentration of 30, 20, or 100 µg/ml, respectively, as needed. MacConkey agar (Difco)-lactose (1%)-fumarate (40 mM) plates containing the appropriate antibiotics were used for mutant isolation and screening. Sodium nitrate (40 mM) and sodium molybdate (100 µM) were added as needed. Cells were grown for enzyme assays in minimal medium supplemented with 40 mM glucose and 40 mM fumarate as previously described (18, 28). Nitrate, ampicillin, or molybdate was present as needed. The medium was made anaerobic by boiling and by cooling under a stream of oxygen-free nitrogen gas (19). The medium was dispensed by the Hungate
FIG. 1. Physical map and phenotype of wild-type and mutant narXL plasmids. A partial restriction map is shown above the locations of the narX and narL genes represented by the open boxes. The direction of transcription is from left to right as drawn. The extents of the wild-type and mutant regions of the various plasmids are indicated in the lower portion of the figure. Thick lines represent DNA exposed to hydroxylamine, while thin lines represent wild-type DNA. Dotted lines represent regions that were deleted. The mutant phenotype (i.e., white colony color on MLF medium) for a narX strain containing the various plasmids is indicated at the right.

procedure into 10-ml anaerobic culture tubes and sealed with butyl rubber stoppers and aluminum crimps. Tubes of medium were inoculated from overnight cultures grown under identical conditions, and the cells were allowed to double or four times in the mid-exponential phase prior to being harvested (optical density at 600 nm, 0.40 to 0.45; Kontron Uvikon 810 spectrophotometer).

Hydroxylamine mutagenesis. Approximately 5 µg of plasmid pLK63 (narX′L−) was treated with the chemical mutagen hydroxylamine for 3.5 h at 55°C as previously described (4). The DNA was diluted to 5 ml in 10 mM Tris–1 mM EDTA buffer (pH 7.5) and ethanol precipitated (22).

Enzyme assays. β-Galactosidase and nitrate reductase were assayed as previously described (18).

Plasmid construction. All transformation, plasmid isolation, and cloning procedures were performed as described previously (22). DNA fragments used for cloning experiments were isolated from 0.8% agarose gels and purified with GeneClean (Bio 101, La Jolla, Calif.). The 4.9-kb narXL-containing fragment from plasmid pLK63 or the corresponding hydroxylamine-treated regions from plasmids pLK63X4, pLK63X5, pLK63X6, pLK63X7, pLK63X8, and pLK63L3 were cloned into plasmid vector pZ152 to yield plasmids pLK5000, pLK5400, pLK5500, pLK5640, pLK5710, and pLK5320, respectively. The orientation of the inserted fragment in these plasmids was the same as that in pLK9 (18).

Derivatives of plasmids pLK5640, pLK5710, and pLK5320 were constructed to determine the locations of the mutations responsible for the nitrate dependence phenotype (Fig. 1). For example, the PstI fragment or the BgIII–BamHI fragment was deleted to yield plasmids lacking either the intact narX gene or the intact narL gene, respectively. The −3.5-kb BamHI–XhoI fragment, the −2-kb PstI fragment, or the 0.4-kb XhoI–PstI fragment was exchanged with the corresponding wild-type sequence in plasmid pLK5000 to further localize the region responsible for the mutant phenotype (Fig. 1). Plasmids pLK5644, pLK5714, and pLK5324 contain the 400-bp mutagenized region between the XhoI and PstI sites (Fig. 1) cloned into pLK5000 in place of the corresponding wild-type fragment. For DNA sequence analysis, the 400-bp XhoI–PstI fragment from plasmids pLK5644, pLK5714, pLK5324, and pLK5000 was isolated and cloned into the large SalI–PstI fragment of plasmid vector pUC18 or pUC19.

DNA sequence analysis. A double-stranded DNA template from plasmids pUC18 and pUC19 and containing the XhoI–PstI fragment derived from plLK5324, pLK5644, or pLK5714 was prepared by alkaline lysis. The DNA template was primed and sequenced by the dideoxy chain termination method.

RESULTS

Isolation of narX* mutants. To obtain mutations in the narX and/or narL genes that caused repression of a frdA′–lacZ protein fusion even when cells were grown in the absence of nitrate, we mutagenized plasmid pLK63 (narX′ narL′) with the chemical mutagen hydroxylamine. Treated DNA was transformed into strains LK3015 (narX narL), and LK3018 (narX′ narL), and the resulting transformants were plated onto MLF medium containing chloramphenicol. Either strain containing plasmid pLK63 (narX′ narL′) formed red colonies on this medium, because expression of the frdA′–lacZ fusion located on the lysogenic XLK1 phage is derepressed in the absence of nitrate (18). Mutants that contained either a narX* or a narL* allele, which would cause repression of frdA′–lacZ expression in the absence of nitrate, were expected to form white colonies on this medium. Of 405 transformants examined, 6 had a white colony phenotype after 24 h of aerobic incubation. These were restreaked for purity, and plasmid DNA was isolated and stored for further study. The plasmids were designated pLK63X4 to pLK63X8 and pLK63L3 (Table 1).

Localization of the mutations to narX. To determine whether the mutation contained on each mutant plasmid was
located in narX, narL, or the plasmid vector, we isolated the 4.9-kb HindIII fragment from each plasmid (pLK63X4, pLK63X5, pLK63X6, pLK63X7, pLK63X8, and pLK63L3) and recloned it into a nonmutagenized plasmid vector (pZ152) to create plasmids pLK5400, pLK5500, pLK5640, pLK5710, pLK5810, and pLK5320, respectively. When plated on MLF-ampicillin plates, strain LK3015 containing plasmid pLK5640, pLK5710, or pLK5320 formed white colonies, indicating that the mutation(s) resided in the 4.9-kb HindIII insert and not in the vector. Strain LK3015 containing plasmid pLK5400, pLK5500, or pLK5810 formed red colonies, indicating that the parent plasmid (pLK63X4, pLK63X5, or pLK63X8, respectively) contained a mutation in the vector. Further examination of these plasmids revealed that they were present in high copy numbers that apparently resulted in the mutant phenotype by overproduction of the NarL protein (data not shown). These three mutants were not examined further.

To determine whether the mutation(s) responsible for the mutant phenotype was in the narX gene or the narL gene, we either deleted or replaced regions of the 4.9-kb HindIII fragment in plasmids pLK5320, pLK5640, and pLK5710 (Fig. 1). The resulting plasmids were transformed into LK3015 (narXL+) and plated on MLF-ampicillin indicator medium to test for the mutant phenotype. Formation of white colonies indicated that the subcloned DNA fragment contained the mutation(s) responsible for the nitrate independence (mutant) phenotype. The lesions were found to be located within a 400-bp region of the narX gene between the XhoI and PstI sites. One of the narX* plasmids that contained a BglII BamHI deletion (represented in Fig. 1, line 3) failed to exhibit the mutant phenotype unless a wild-type narL* gene was provided in trans. This result was also seen previously and indicates that the narX gene copy number must be similar to that of narL (see Table 3 in reference 19).

DNA sequence analysis of narX* mutants. To identify the location of each mutation, we cloned the 400-bp XhoI-PstI fragment from plasmids pLK5644, pLK5714, and pLK5324 into plasmids pUC18 and pUC19 and sequenced it. A single C-to-T transition was identified in each mutant plasmid (Fig. 2). The mutations in clones pLK5714, pLK5324, and pLK5644 resulted in an amino acid replacement of a glycine residue at amino acid position 205 with arginine, a glutamate at position 208 with lysine, and an alanine at position 215 with threonine, respectively. These alleles were called narX32, narX64, and narX71, respectively.

**Phenotype of mutant narX* clones in narL, narX, chiD, and wild-type strains.** To determine the phenotype caused by the narX* alleles in various chromosomal backgrounds, we transformed plasmids pZ152, pLK5000, pLK5320, pLK5640, and pLK5710 into the following strains: LK3018 (narL recA), LK3015 (narX recA), LK3014 (chiD recA), and LK3017 (recA). Transformants were plated on MLF-ampicillin indicator medium with or without 100 μM molybdate (Table 2). Strains containing plasmid pZ152 or pLK5000 formed red colonies on the indicator medium because of their inability to repress expression of the fdrA- lacZ fusion. In contrast, the narX, narL, or wild-type strain containing a narX* plasmid formed white colonies on this indicator medium (Table 2). These results indicate that the mutant narX* alleles contained on each plasmid were dominant (in multicity) over the wild-type copy of narX on the chromosome.

**Molybdenum ions are required in addition to nitrate for normal repression of fumarate reductase and TMAO and**

![FIG. 2. Location and sequence of nitrate- and molybdate-independent narX* mutations. The NarX sensor protein is represented by the box, with the solid areas indicating the proposed locations of the hydrophobic transmembrane regions. The amino acid (aa) residues are indicated above the line. The DNA and predicted amino acid sequences of the region following the second transmembrane domain of NarX are indicated in the lower portion of the figure. The base and amino acid changes for each narX* mutation are indicated.](image-url)
TABLE 3. Effect of wild-type and narX* mutant plasmids on nitrate repression of frdA'-lacZ expression in narX (LK3012) and narX* (LK3013) strains

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a Cells of strain LK3012 (narX) or LK3013 (narX*) containing the indicated plasmids were grown in minimal glucose-fumarate-ampicillin medium anaerobically as described in Materials and Methods. When needed, nitrate was present in the medium at an initial concentration of 40 mM. b Expressed as nanomoles of p-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein. ND, Not determined.

d Mutant allele.

DMSO reductase gene expression (3, 15; Kalman, Ph.D. thesis). Transport of molybdenum by E. coli is dependent on the chiD locus, which is thought to encode a molybdate ion transport system (11). The addition of high levels of molybdenum to the growth medium can correct the chiD defect (7). Interestingly, strain LK3014 (chiD) containing either plasmid pLK5640 or plasmid pLK5710 displayed a white colony phenotype when plated on MLF-ampicillin indicator medium whether molybdate was added or not. These results suggest that the mutant narX64 or narX71 gene product in this strain did not require molybdenum or nitrate for repression of frdA'-lacZ expression. Strain LK3014 containing plasmid pLK5320, however, formed red colonies when plated in the absence of added molybdenum and white colonies in its presence. These results suggest that the narX32 mutant required molybdenum but not nitrate for repression of frdA'-lacZ expression.

Effect of narX* clones on frdA'-lacZ gene expression. To further examine the effect of the narX* mutations on frdA'-lacZ expression in narX or narX* backgrounds, we grew strains containing each of the various mutant plasmids, vector pZ152, or parent plasmid pLK5000 under anaerobic conditions in the presence or absence of nitrate and determined β-galactosidase levels (Table 3). The wild-type strain containing plasmid vector pZ152 exhibited a 35-fold repression of frdA'-lacZ expression by nitrate, while a narX strain containing the same plasmid was only repressed approximately 1.4-fold when grown in the presence versus the absence of nitrate. The addition of plasmid pLK5000, which encodes wild-type narX* and narL* into strain LK3012 caused a 193-fold repression upon the addition of nitrate. The narX and narX* strains containing the narX32* plasmids pLK5320 and pLK5324 showed a three- to fourfold repression of frdA'-lacZ expression relative to that in a strain containing a narX* plasmid when grown without nitrate. This result indicated that the plasmids encoded a narX32* gene product that did not require the presence of nitrate to function. Rather, it appeared to be locked in a mildly activated state even when nitrate was present.

This mutant protein was also relatively insensitive to the addition of nitrate to the medium. The other narX* mutants, the narX64 and narX71 mutants, showed a somewhat different response to nitrate. When either a narX or a narX* strain containing plasmid pLK5640, pLK5644, pLK5710, or pLK5714 was grown in medium lacking nitrate, the expression of frdA'-lacZ was repressed about two- to threefold relative to that in a strain containing a control plasmid (pZ152). When nitrate was present in the medium, frdA'-lacZ expression in these strains was further repressed to levels observed in a strain containing plasmid pLK5000. Thus, these two narX* alleles (narX64 and narX71) still responded to the presence of nitrate. The similar β-galactosidase activities in strains containing related plasmids (pLK5320 or pLK5324, pLK5640 or pLK5644, and pLK5710 or pLK5714) indicated that the mutation responsible for the mutant phenotype was located within the 400-bp XhoI-PstI fragment. Plasmids pLK5324, pLK5644, and pLK5714 were used for all subsequent experiments.

To determine whether multiple copies of the narL* gene or the narX* gene alone could account for the above-described findings, we transformed a narX strain and a wild-type strain with plasmid pLK634 (narL*) or plasmid pLK633 (narX*) and assayed for frdA'-lacZ expression (Table 3). When grown in the absence of nitrate, all strains exhibited similar levels of β-galactosidase activity relative to either strain with plasmid pZ152. These results indicated that the presence of a multicopy narL* or narX* plasmid did not account for the narX* effect on frdA'-lacZ expression when cells were grown without nitrate. These findings were reported previously (19).

Effect of the chiD allele and molybdenum on nitrate regulation of frdA'-lacZ expression. The effect of molybdenum availability on the function of the narX* alleles was determined by assaying a chiD strain, LK3014, containing the mutant narX* plasmids (Table 4). Plasmid pLK5324 containing the narX32 allele encoded a molybdate-sensitive NarX protein, as evidenced by the two- to threefold repression of frdA'-lacZ expression when this ion was present in the culture medium, which lacked nitrate. However, the narX32 allele was also relatively insensitive to nitrate, as the addition of this compound caused only a further threefold repression of frdA'-lacZ expression. In contrast, the other two narX* mutants (the narX64 and narX71 mutants) were less sensitive to molybdenum, as shown by a relatively small increase in frdA'-lacZ repression when it was added to the medium. However, the chiD strain containing either of these
two narX\* mutant alleles exhibited a marked repression of frdA'-lacZ expression in response to nitrate. It is interesting to note that the absence of NarX protein in pLK5000, in the chiD strain allowed nitrate-dependent repression of frdA'-lacZ expression even when molybdate was not added to the medium. The addition of molybdate, however, caused an additional 10-fold repression of frdA'-lacz expression. This result may indicate that the molybdate requirement was partially overcome when wild-type NarX and NarL were present in multiple copies or that molybdate was not effectively limited by the chiD allele.

**Effect of narX\* mutations on expression of a dmsA'-lacZ fusion.** A 12-fold nitrate repression of dmsABC gene expression was previously shown to be mediated by narL and narX (3). To determine the effect of the various narX\* alleles on this process, we monitored the expression of a dmsA'-lacZ fusion in strain LK3012(pACP25) bearing each of the narX\* plasmid derivatives. Cells were grown as described for the frdA'-lacz expression studies reported in Table 3. The results of these studies indicate that the mutant narX\* alleles had a similar but less dramatic effect on dmsA'-lacz expression than seen on frdA'-lacz expression (data not shown).

**Effect of narX\* mutations on nitrate reductase activity.** The induction of nitrate reductase gene expression in cells grown in the presence of nitrate has been shown to be mediated by narL and narX (14, 18, 19, 29–31) and narX (19). To establish the effect of the various narX\* alleles on nitrate reductase synthesis, we measured the levels of this enzyme in a narX strain (LK3012) containing each narX\* plasmid (Table 5). When strains containing the narX32, narX64, and narX71 alleles were grown in the absence of nitrate, each contained elevated levels of nitrate reductase (e.g., 71-, 29-, and 31-fold, respectively); there was a 2-fold increase in strain LK3012 containing plasmid pLK5000 (narX\*L\*') relative to that in a strain containing plasmid pZ125. When nitrate was added to the culture medium, levels of the enzyme were further elevated in the narX64 and narX71 plasmid-bearing strains (Table 5). As shown previously for the regulation of frdA'-lacz and dmsA'-lacz expression, these two NarX\* proteins still exhibited partial nitrate-sensing ability, whereas the narX32 gene product did not. As an additional control, a narL\* plasmid that lacked narX\* (pLK634) was introduced into a narX\* strain to test whether excess NarL, produced trans over NarX could activate nitrate reductase production in a similar fashion. It could not. The addition of narL\* in cis to narX\* (e.g., pLK5000) also did not cause a significant activation of nitrate reductase activity when cells were grown in the absence of nitrate. The NarX\* phenotype is apparently not due to the overproduction of NarL or NarX.

**DISCUSSION**

In previous studies, we demonstrated that the repression of fumarate reductase and DMSO and TMAO reductase gene expression can result in the derepression of frdA'-lacZ expression in response to nitrate is controlled by the narX (frdR1, narR) and narL (frdR2) gene products (3, 18, 19). Mutations in either gene result in the derepression of frdABCD gene expression or the inability to induce nitrate reductase synthesis. The sequence similarity of the narX and narL gene products to other known regulatory proteins suggests that they function as a two-component regulatory system (9, 24, 32). NarX shares a conserved C-terminal domain with the sensor group of proteins, while NarL shares a conserved N-terminal domain with the receiver group of proteins. On the basis of hydrophobicity studies and the sequence similarity to the sensor proteins, the NarX protein is predicted to contain two transmembrane regions, whereas the NarL protein is predicted to contain one transmembrane region, which is necessary for insertion into the cytoplasmic membrane, while the 400-amino-acid C-terminal portion resides in the cytoplasm. We propose that this regulatory protein must sense the presence of nitrate and possibly molybdenum to convert NarL to an active form. NarL is then able to bind DNA to either repress or activate gene expression. To provide genetic evidence for this model, we mutagenized a plasmid containing the narX\* and narL\* gene and screened for mutants which were defective in nitrate sensing and/or the NarX-NarL interaction.

Three alleles of narX (narX32, narX64, and narX71) that caused enhanced repression of fumarate reductase and DMSO and TMAO reductase gene expression and induction of nitrate reductase activity when cells were grown in the absence of nitrate were obtained. The narX32, narX64, and narX71 mutations were localized to a small region of narX by subcloning and DNA sequence experiments. We found that each allele contained a single-amino-acid change within an 11-amino-acid segment of the NarX protein (Fig. 2). This region immediately follows the second proposed transmembrane domain and is predicted to be at or near the inner surface of the cytoplasmic membrane. When cells were grown in the absence of nitrate, each narX\* mutation resulted in the repression of frdA'-lacz and dmsA'-lacz expression and in the induction of nitrate reductase synthesis, compared with a wild-type strain (Table 3 and 5). Upon the addition of nitrate, two of the narX\* alleles, narX64 and narX71, caused complete repression and induction of the respiratory enzymes while the remaining allele (narX32) did not. Strains bearing plasmids with the narX64 and narX71 alleles were also relatively independent of molybdenum for activity, unlike strains with the narX32 allele.

The above-described findings support a working model for the action of the narX\* mutants (Fig. 3). Upon interaction with the environmental signal, nitrate, a conformational change that occurs in NarX allows NarX to activate NarL. The activated NarL can then bind at its DNA recognition sites located upstream of the various nitrate-controlled genes to either repress frdABCD and dmsABC expression or activate narGHJI gene expression. The NarX\* proteins reported in this study also suggest an additional mechanism which is more like that of wild-type NarX after it has interacted with nitrate. The protein in the intermediate or partly activated conformation can then activate NarL even when cells are grown without nitrate. It appears that the
narX64 and narX71 gene products are still able to interact with nitrate and cause the further activation of NarL. In contrast, the narX32 gene product does not respond in the same way to the presence of nitrate, suggesting either that it is somehow impaired in its ability to detect nitrate or that it is impaired in its ability to further activate NarL.

Since molybdenum must be transported into the cell for the proper function of NarX and NarL (as shown by the inability of a chiD molybdate transport mutant to cause the repression of fumarate reductase expression in the presence of nitrate; 15, 19; Kalman, Ph.D. thesis), this ion must somehow interact with the cytoplasmic region of one of these two regulatory proteins. Nitrate conceivably interacts with the NarX protein region exposed to the periplasm, whereas the site(s) for molybdenum interaction may lie elsewhere in NarX, although this idea has yet to be tested. It is also by no means clear whether the locations for molybdenum and/or nitrate interaction are limited to the NarX protein. Additional proteins could conceivably aid in this process. However, it is probable that the conformation of each mutant NarX* protein is more like that of the wild-type NarX protein when nitrate is present, so as to preempt the effect of molybdenum and/or nitrate binding.

The NarX and NarL regulatory proteins clearly participate in detecting molybdate availability in the cell environment as well as nitrate availability. This type of regulation allows the activation of nitrate reductase gene expression only under conditions in which a functional molybdenum enzyme can be formed. It prevents the unnecessary synthesis of the nitrate reductase holoenzyme when molybdenum is unavailable to the cell in sufficient amounts even when nitrate is present. Fumarate reductase, which does not require molybdenum as an essential cofactor for catalytic activity, may then be synthesized to enable the cell to respire with fumarate.

The NarX* mutants isolated in this study clearly implicate NarX in nitrate and molybdenum signaling and show it to be required for the repression and activation of genes regulated in response to nitrate. These data also establish that NarL can act both as an activator and as a repressor under the same cell growth conditions. Previous reports indicated that narX strains could still induce the expression of nitrate reductase (30, 31) and repress frdA-'lacZ expression and thus concluded that NarX may not be essential for nitrate regulation. The NarX* mutants isolated in this work, however, indicate that NarX is clearly involved in the regulation of nitrate-controlled respiratory genes.

It has been previously suggested that there may be an internal promoter for the expression of narL that is located within the narX gene (5, 24). Thus, it may be argued that the properties of the NarX* mutants may simply be the result of a mutation in this promoter that results in the overexpression of NarL (5). We ruled out this possibility by measuring frdA-'lacZ activity in narX strains containing a plasmid encoding narX32L, narX64L, or narX71L as well as a plasmid (pLK634) encoding narL* alone (e.g., to determine the effect of having narX* on one plasmid and narL* on another). In this combination, readthrough from narX* into narL is not possible, since the genes are contained on separate plasmids. We found that the repression of frdA-'lacZ expression occurred in the absence of nitrate in strains containing plasmids bearing narX32, narX64, or narX71 but not in strains containing narX* (data not shown). These results indicate that the ability to regulate frd gene expression in the absence of nitrate is not simply due to the overproduction of NarL by readthrough of narL transcription from the narX gene but rather is due to an altered activity of the NarX protein.

As with other two-component systems, such as NtrB-NtrC, OmpR-EnvZ, VirA-VirG, and CheA-CheY (6, 10, 12, 16, 20, 35), it is possible that the interaction of NarX and NarL involves phosphorylation. If this is true, the NarX* mutants may be altered in the rate of autophosphorylation and/or in the rate of phosphorylation and dephosphorylation of the NarL protein. NarX* mutants presumably activate NarL even when cells are grown in the absence of nitrate. By this scheme, NarL would be phosphorylated and active even in the absence of nitrate. Analysis of narL mutants that relieve the NarX* phenotype will be interesting in this regard.
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