The *narX* and *narL* genes encoding the nitrate-sensing regulators of *Escherichia coli* are homologous to a family of prokaryotic two-component regulatory genes

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ABSTRACT

The nucleotide sequence of a 4.4-kilobase *Sac*II−*Ssp*I fragment encoding the *narXL* operon and a part of the *narK* gene of *Escherichia coli* has been determined. The *narX* and *narL* genes encode proteins of molecular weight 67,275 and 23,927, respectively, and are transcribed from a common promoter, *narXp*, locating within 429 bases upstream of *narX*. Transcription from *narXp* is not significantly induced by nitrate under anaerobiosis, whereas transcription from *narK* promoter, which overlaps *narXp* region and is transcribed divergently, is fully induced by nitrate. The N-terminal two-thirds of the NarL protein has extensive homology with those of a diverse set of prokaryotic regulatory proteins, including OmpR, PhoB, SfrA, UhpA, CheY, CheB, NtrC, DctD, FixJ, VirG, Spo0F, and Spo0A. A segment locating in the C-terminal half of the NarL protein seems to have potential most likely to form the helix-turn-helix structure characteristic of a class of DNA-binding protein. The protein is considered to play a role as a transcriptional activator of the nitrate reductase operon, *narCHJI*, and the *narK* gene. The C-terminal region of the NarX protein also has homology with other regulatory proteins known as counterparts of two-component regulatory systems, such as EnvZ, PhoR, PhoM, CpxA, NtrB, DctB, FixL, and VirA. Presence of two copies of hydrophobic segments in the N-terminal half of the NarX protein suggests the role as a transmembrane receptor sensing nitrate.

INTRODUCTION

Escherichia coli can exploit a variety of exogenous compounds as a terminal electron acceptor for respiratory electron transfer, including, in order of decreasing redox potential, oxygen, nitrate, trimethylamine N-oxide (Me_3NO), and fumarate. Use of these compounds is hierarchical, thereby maximizing buildup of proton motive force across the plasma membrane. This is effected by a complex regulatory network of gene expression that enables *E. coli* cells to take advantage of the available electron acceptor with the highest redox potential (reviewed in refs. 1 and 2).

The enzyme nitrate reductase consists of three membrane-bound subunits and contains molybdenum cofactor and heme (2). All three of the subunit polypeptides are encoded by the *narCHJI* operon located at 27 min on the *E. coli* chromosome (3, 4). The operon is positively controlled by an activator protein encoded by the closely linked *narL* gene in response to nitrate as the inducer (5), as well as by a pleiotropic transcriptional activator, the *fnr* gene product, which mediates anaerobic induction of several respiratory enzymes (6). In addition, the *narL* gene product has been shown to act as a repressor in the presence of nitrate on the operons *tor* and *frd* that encode Me_3NO and fumarate reductases, respectively (7, 8). Thus the NarL protein plays an important role as a genetic switch in the differential expression of these operons.

By cloning and operon fusion studies, regulatory region of the *nar* operon including

narL has been characterized (9). The *narL* gene is transcribed divergently with respect to the *narCHJI* operon accompanying the proximal *narX* gene. The *narX* and *narL* genes encoding proteins with estimated molecular weights of 66,000 and 28,000, respectively, may constitute an operon, and their expression is relatively insensitive to Fnr and nitrate. Therefore, one hypothesis to explain the hierarchical control of the reductase operons by nitrate is that the NarX and/or NarL proteins are transcriptional regulators of *nar*, *tor*, and *frd* operons eventually sensing the nitrate availability.

In the studies reported here, we have sequenced entire region of the *narX* and *narL* genes, which were subcloned from the insert of lambda 13H6 bearing upstream region of the *narCHJI* operon (10). The sequence revealed that NarX and NarL have homology in the deduced amino acid sequence with other prokaryotic regulatory proteins which belong to a two-component regulatory system and transduce environmental signals to transcriptional apparatus (11).

MATERIALS AND METHODS

Bacterial strains and media

E. coli K-12 strains used in this study were XL1-Blue (*recA1* Δ*lac endA1 gyrA96 thi hsdR17 supE44 relA1* { F′ *proAB lacI*^{q} *lacZ*Δ*M15* Tn*10*}, obtained from Stratagene), NM522 (*supE thi* Δ*(lac-proAB) hsdR5* { F′ *proAB lacI*^{q} *lacZ*Δ*M15*}) (12), and TNK50 (NM522 with Δ*narXL*::*kan*). The *narXL* deletion strains were constructed by the gene replacement method (13) utilizing homologous recombination of plasmid-encoded ΔnarXL::*kan* genes in pNR66 (see below) with the chromosomal *narXL* alleles in *polA12* strain MM383 (14). Subsequent P1 *vir* transductions were performed by the method of Miller (15) to obtain TNK50. Defined and complex media used for routine culture were as described previously (16). MacConkey nitrate agar was used to score Nar phenotype as described previously (17). When necessary, ampicillin (50 μg/ml), tetracycline (12.5 μg/ml), kanamycin (25 μg/ml), chloramphenicol (10 μg/ml), isopropyl-β-D-thio-galactopyranoside (IPTG, 24 μg/ml), or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, 40 μg/ml) was added. Induction by nitrate was accomplished in the medium containing 1% KNO_3.

Plasmids and DNA sequencing

The *narX* and *narL* genes sequenced in this study was from pNR17, which was constructed by subcloning an 8.4-kilobase (kb) *Sal*I−*Eco*RI fragment from lambda 13H6 into pBR322 (10). A 6.4-kb *Bal*I fragment of pNR17 bearing the complete coding region of the *narK*, *narX*, and *narL* genes was further subcloned in two orientation into *Sma*I site of pTN1058 to obtain pNR24 and pNR30 (Fig. 1). Derivatives of pNR24 and pNR30 were constructed by deleting various restriction fragments as shown in Fig. 1. The same 6.4-kb *Bal*I fragment of pNR17 was subcloned into *Eco*RI−*Dra*I sites of pBR322 after filling an end with T4 DNA polymerase. From the resultant plasmid, pNR57, most of the *narXL* coding sequence from *Xho*I site at 3.8 kb to *Bgl*II site at 1.9 kb (Fig. 1) was replaced with *kan* gene from pUC4K (18) to obtain pNR66.

DNA sequencing was done by the dideoxy-chain termination method (19) with [α-^{35}S]thio-dATP and modified T7 DNA polymerase (Sequenase, United States Biochemical Corp.). Single-stranded templates were prepared from derivatives of M13 phage (20) carrying various restriction endonuclease fragments from pNR24. Both strands were sequenced with minimum overlapping of 30 bases (strategies not shown). The DNA sequencing information was processed using the GENETYX software package (Software Development Co., Ltd., Tokyo).

Promoter analysis

Plasmid pTN1058 used for assaying promoter activity was constructed by replacing unique *Eco*RI site of pTN1051 (21) with the polylinker sequence of pUC18 from *Eco*RI site to *Sal*I site. The plasmid pTN1058 contains a terminator sequence within 120 bases upstream of the cloning sites which effectively prevents transcriptional reading through from the P4 and P5 promoters present in pBR322 (22, 23). There is an in-frame stop codon with the downstream *lacZ'* gene between the cloning sites and the initiation codon which prevents translational fusion from an inserted gene. Promoter activity in the *narK-narX-narL* region was determined by subcloning various restriction fragments into the multiple cloning sites of pTN1058. The activity was estimated by the α-complementation of the LacZ activity in XL1-Blue host on X-gal plate containing IPTG with or without nitrate under aerobic or anaerobic condition.

Gene product analysis

The NarX and NarL proteins were overexpressed in XL1-Blue harboring plasmid pNR71, pNR73, or pNR74 (Fig. 1) constructed in pHSG398 (24). The plasmids pNR71, pNR73, and pNR74 contained 2.9-kb *Dra*I−*Ava*I fragment, 2.0-kb *Dra*I−*Bgl*II fragment, and 1.2-kb *Nsi*I fragment, respectively, from pNR24 inserted into the multiple cloning sites of pHSG398 such that the *narX* and *narL* genes would be transcribed from the *lac* promoter.

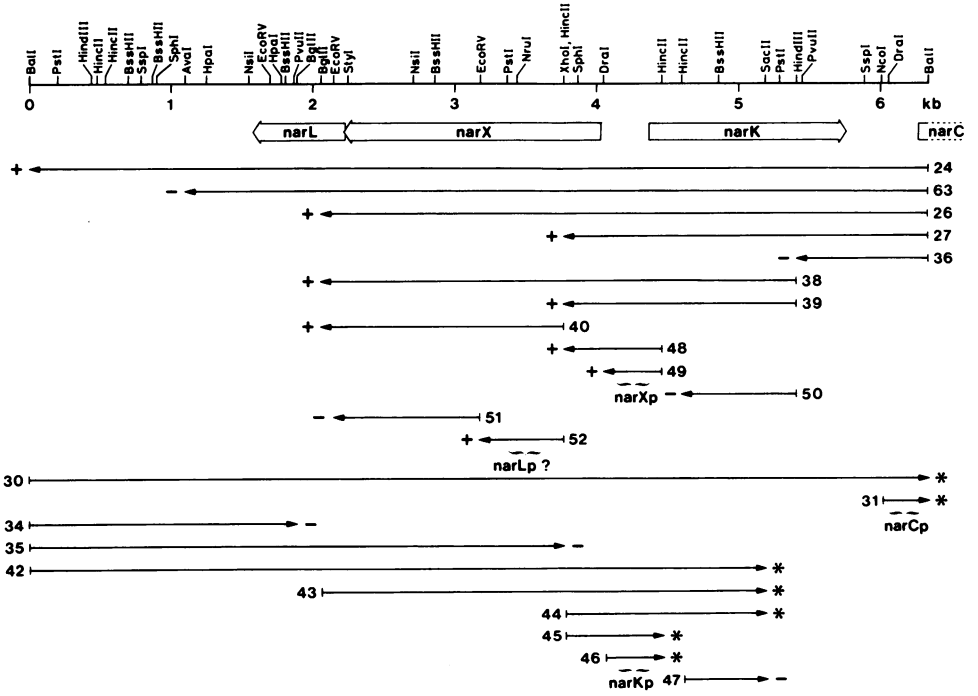

Figure 1. Genetic and physical map of the *narK-narX-narL* region. The top line represents restriction sites determined by the enzymatic digestion. In the middle portion show the locations of *narK*, *narX*, and *narL* coding sequences. Arrows in the bottom section give the length and orientation of the inserts shared by the plasmids pNR24 to pNR52 and pNR63 (24 to 52 and 63 in the figure) constructed in pTN1058 to detect promoter activity: −, no; +, constitutive; *, nitrate-inducible promoter activities determined by the LacZ assay. The promoter region of each *nar* operon was indicated as *narCp*, *narKp*, *narXp*, and *narLp*.

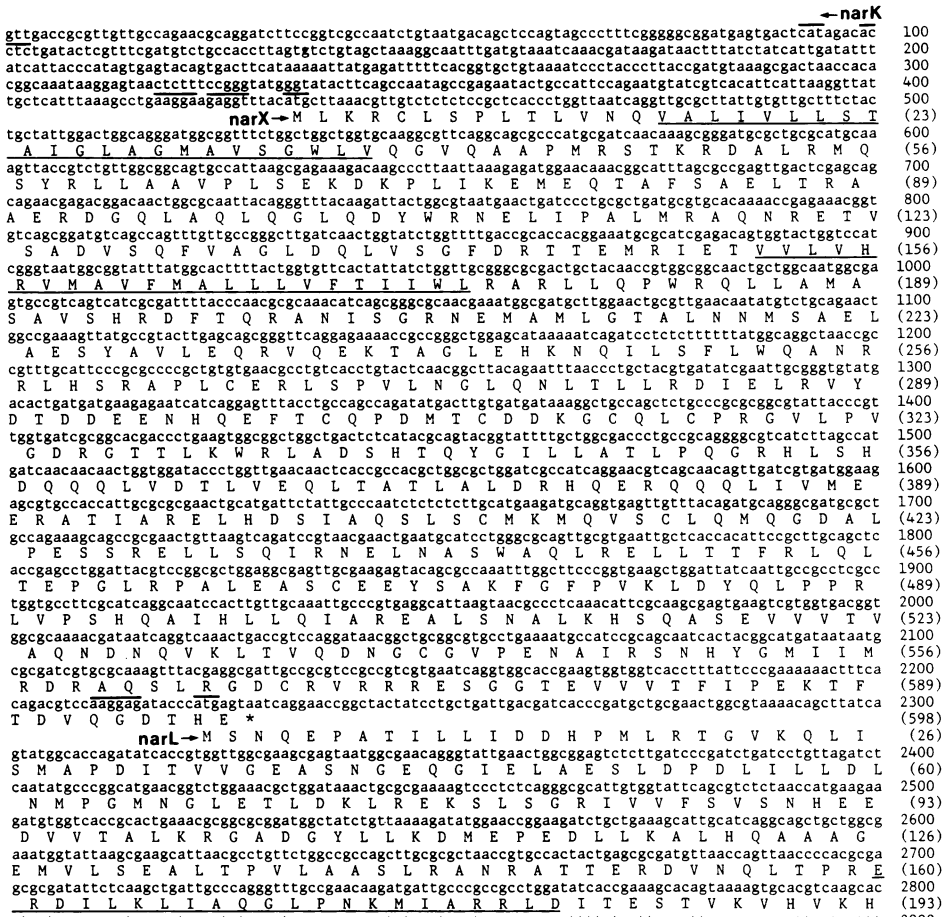

Figure 2. Nucleotide sequence of the *narXL* operon and deduced amino acid sequence of their products. Possible ribosome binding sites and ATG initiation codons are overlined. Two regions of sufficient hydrophobicity to span the membrane in NarX and one region of helix-turn-helix structure to be concerned with DNA binding in NarL are underlined.

Total cellular proteins accumulated after IPTG induction were dissolved in sodium dodecyl sulfate (SDS) sample buffer, and analyzed by SDS−polyacrylamide gel electrophoresis as described previously (25).

Enzyme assay

Aerobic culture were grown on a vigorously shaking platform at 37°C in midexponential phase. Anaerobic culture were grown in filled 20-ml syringes standing undisturbed at 37°C. β-Galactosidase activity was measured in toluene-permeabilized cells by monitoring the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside. Nitrate reductase activity was determined as previously described (26).

RESULTS

Subcloning of narX and narL genes

The recombinant plasmids pNR24 and pNR30 contain a 6.4-kb *Bal*I restriction fragment which, in addition to the *narX* and *narL* genes encoding the nitrate regulators, bears the *narK* gene and a part of the *narC* operon (9, 27). A detailed restriction map of this region was determined using the enzymes shown in Fig. 1, and is in good agreement with that reported previously (9, 28).

Previous studies have located the *narX* and *narL* genes in the region from 1.5 kb to 4.1 kb on the scale in Fig. 1 (9). Functionality of this region was verified using the *narXL* deletion strain TNK50 by analyzing nitrate induction of the reductase activity under anaerobic condition. Defect in the nitrate induction in TNK50 was restored by transforming with pNR71 as well as pNR24. The *narK* gene with unknown function is also mapped in the 4.3−6.0-kb region. Transcriptional control region of the *narC* operon was already sequenced (27), and shown to correspond to the 5.7−6.4-kb region. However, it was not clear whether the *narX* and *narL* genes constitute an operon. We determined the promoter activity in this region using pTN1058.

Promoter for the narX and narL genes

Anaerobically inducible and nitrate-inducible promoters could be detected in XL1-Blue host with plasmids pNR31, pNR42, pNR43, pNR44, pNR45, and pNR46 but not with pNR34, pNR35, and pNR47 (Fig. 1). Plasmid pNR31 contains a promoter of the *narC* operon (27, 29) and confers on the aerobically growing host cells characteristic blue color on X-gal medium as the center of the colony turned deeper blue with white periphery due to the development of anaerobic central area (4). The similar color reaction was also observed with plasmids pNR44, pNR45, and pNR46 bearing promoter region of *narK*.

The promoters in the *narX-narL* region were complicated. Plasmids pNR26, pNR27, pNR38, pNR39, pNR40, pNR48, pNR49, and pNR52 showed constitutive promoter activity that was essentially independent of anaerobiosis and the presence of nitrate. No promoter activity was detected with plasmids pNR36, pNR50, pNR51, and pNR63. The results suggest that there are at least two promoters operated separately at *Xho*I site in the *narX* coding region. The absence of promoter in the immediate upstream region of *narL* covered by pNR51 implicates that the *narX* and *narL* genes form an operon, which is transcribed from a common promoter located in the upstream region of *narX*. This is confirmed by the analysis of the nucleotide sequence of this region (see Fig. 2). The promoter activity detected with pNR52 seems to imply an additional promoter within the *narX* coding region, which may in part be responsible for the increased expression of *narL* relative to *narX* in the absence of added nitrate (9).

Nucleotide sequence of the narXL operon

The physical map of the *narXL* region (Fig. 1) was used as a basis for nucleotide sequencing of a 4.4-kb *Sac*II−*Ssp*I fragment from pNR24. The sequence data (Fig. 2) revealed two major open reading frames (ORFs) with a high coding probability and a leftward polarity, as supported from the findings on the promoter activity. No significant ORF with a high coding probability could be identified with the opposite orientation in the region shown in Fig. 2. We designated the first ORF of 1,794 nucleotides as *narX* and the second ORF of 648 nucleotides as *narL* from the following evidence. Both ORFs were preceded by a strong ribosome binding site sequence at appropriate distance from the first initiation codon ATG. The *narX* and *narL* genes, as mapped in this region with the correct orientation (9), encode proteins with apparent molecular weights of 62,000−66,000 and

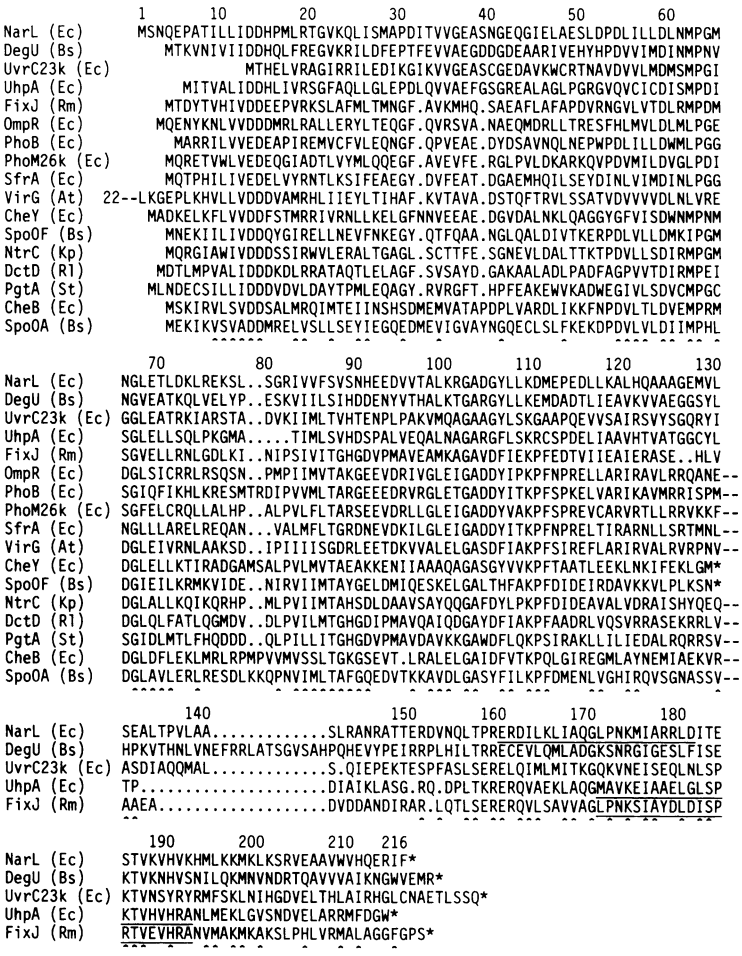

Figure 3. Sequence alignment of NarL with *E. coli* UvrC ORF2, UhpA, OmpR, PhoB, PhoM ORF2, SfrA, CheY, and CheB, *Rhizobium meliloti* FixJ, *Agrobacterium tumefaciens* VirG, *Bacillus subtilis* DegU, Spo0F, and Spo0A, *K. pneumoniae* NtrC, *R. leguminosarum* DctD, and *Salmonella typhimurium* PgtA. Conserved residues are marked below the sequence, defined as more than 70% of amino acids belonging to one of the groups P A G S T; H K R; Q N E D; I L V M F Y W; C. Possible helix-turn-helix DNA-binding motifs of NarL, UhpA, and FixJ are underlined.

25,000−28,000, respectively, in reasonable agreement with the calculated values of 67,275 and 23,927. Gene product analysis using pHSG398 expression vector located the *narX* and *narL* coding sequences between *Dra*I site at 407 and *Bgl*II site at 2395 and between *Nsi*I site at 1748 and *Nsi*I site at 2905, respectively (data not shown).

Structure of the NarX and NarL proteins

The deduced amino acid sequences of the NarX and NarL proteins were compared with sequences from National Biomedical Research Foundation data base. NarL was found to have extensive similarity over the first 120 amino acids to the N termini of the OmpR protein from *E. coli* (30, 31) and the NtrC protein from *Klebsiella pneumoniae* (32, 33).

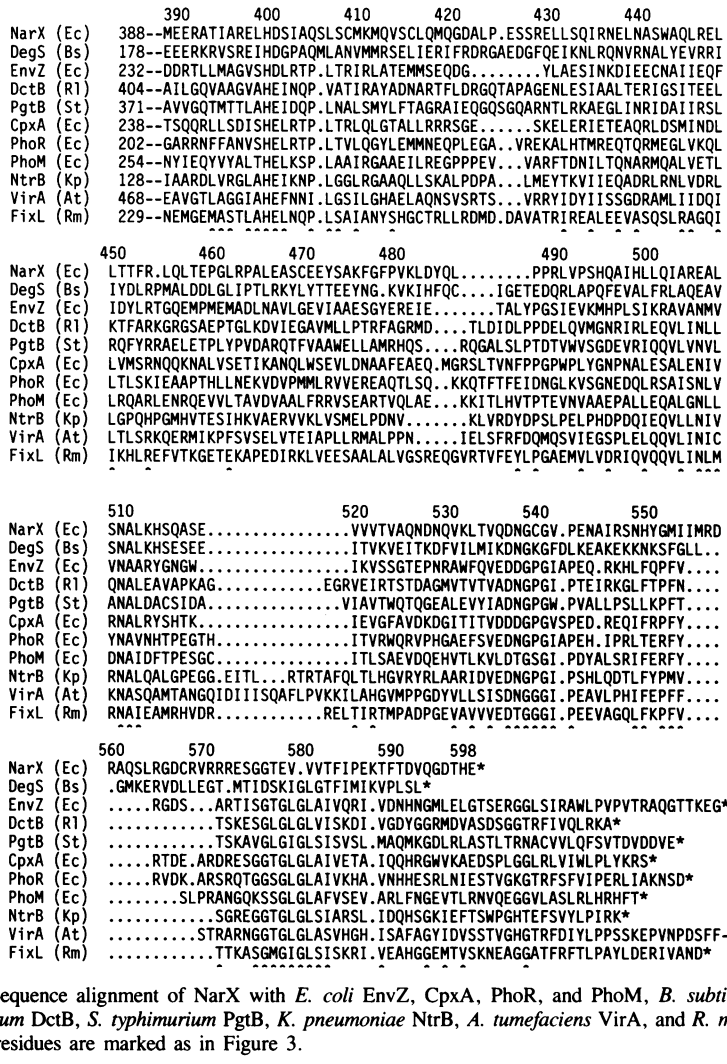

Figure 4. Sequence alignment of NarX with *E. coli* EnvZ, CpxA, PhoR, and PhoM, *B. subtilis* DegS, *R. leguminosarum* DctB, *S. typhimurium* PgtB, *K. pneumoniae* NtrB, *A. tumefaciens* VirA, and *R. meliloti* FixL. Conserved residues are marked as in Figure 3.

OmpR is involved in osmotic regulation of the synthesis of outer membrane proteins, and NtrC is involved in nitrogen control of several nitrogen assimilatory genes. It has been reported that OmpR and NtrC share homologous N-terminal domain with other bacterial regulatory proteins such as PhoB (34), PhoM ORF2 (35), SfrA (36), VirG (37, 38), DctD (39), PgtA (40), CheY (41, 42), Spo0F (43), CheB (41, 42), and Spo0A (44). This family also includes UhpA (45), FixJ (46), DegU (47, 48), and UvrC ORF2 (49), which, in addition to the homologous N-terminal domain, shared extensive similarity to NarL over their entire lengths (Fig. 3). Therefore, the most proteins of this family are subdivided into four different groups according to their C-terminal domain: (i) NarL, DegU, UvrC ORF2, UhpA, and FixJ; (ii) OmpR, PhoB, PhoM ORF2, SfrA, and VirG; (iii) NtrC, DctD, and PgtA; and (iv) CheY and Spo0F. The UhpA and FixJ proteins of the first group

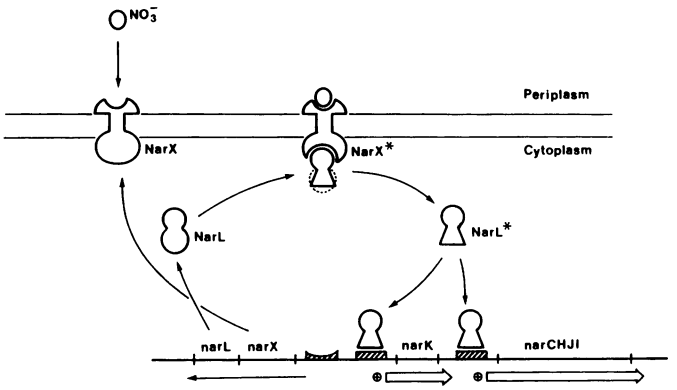

Figure 5. Model for *nar* gene activation in *E. coli*. See text for details.

were shown to have helix-turn-helix DNA-binding motif in the C-terminal domain (45, 46). By predicting the secondary structure of the NarL protein with a GENETYX program, we found the motif in the conserved C-terminal domain as a potential candidate (Fig. 2). It is also possible to assign the motif in the highly homologous region with FixJ and UhpA between Leu 171 and Lys 192, although this region is most likely to form the helix-beta-helix structure (Fig. 3). In any case, the bihelical region seems to confer NarL to interact with the control region of the nitrate-responsive *narC* and *narK* genes, consistent with genetically characterized phenotypes of *narL* (2, 5).

The regulatory proteins shown in Fig. 3 are known to regulate gene expression in combination with a second class of regulatory protein (11). Members of this second family, including EnvZ (30), DctB (39), PgtB (50), CpxA (51), PhoR (52), PhoM (35), NtrB (53), VirA (54), and FixL (46), share similarity to each other over their C-terminal 250 amino acids. NarX and DegS (47, 48) also shared this similarity to some extent (Fig. 4). Four or more conserved regions were recognized, some of which had extensively conserved residues with NarX. However, these two proteins are truncated and have poor homology in the very C terminus to a region which is highly conserved in the rest of the family. Most members of the EnvZ family except NtrB and DegS are transmembrane proteins and contain two regions of sufficient hydrophobicity outside of the conserved domain. We therefore screened the NarX sequence for potential transmembrane helices and found two such sequences, one at residues 15 to 37 and the other at residues 152 to 174 (Fig. 2). Thus, NarX is predicted to be a transmembrane protein, which acts as a sensor for nitrate and transduces signal of nitrate availability to the *narK* and *narC* operons via NarL.

DISCUSSION

The nucleotide sequence of the nitrate regulator operon, which is adjacent to the *narK* and *narC* operons at 27 min on the *E. coli* chromosome (3, 10), was determined. The *narXL* operon is transcribed essentially independent of anaerobiosis and nitrate availability. Two ORFs of the operon were designated *narX* and *narL* to indicate their order relative to the promoter on the basis of coding polarity and the size of the gene product. There is five-nucleotide overlap between the coding region of *narX* and *narL* which implies translational coupling of the genes. Under anaerobic condition, however, the *narL* gene

was expressed differently from the *narX* gene (9). An additional promoter present within the *narX* coding region may be responsible for the elevated expression of *narL* relative to *narX* in the absence of nitrate.

The *narL* gene encodes the pleiotropic transcriptional regulator which acts as an activator on the *nar* operon and a repressor on the *frd* and *tor* operons (5, 7, 8). Analysis of the deduced amino acid sequence of NarL revealed characteristic helix-turn-helix motif in the C-terminal region which is responsible for binding to the regulatory region of a target gene (Fig. 2). Extensive similarity of N-terminal amino acid sequence of NarL to other prokaryotic regulatory proteins such as OmpR and PhoB (Fig. 3) strongly suggests that NarL acts directly on the regulatory region of the *narK* and *narC* operons after some modification to an active form in the presence of nitrate. This modification is likely to be effected by the product of the proximal *narX* gene, since NarX has a homologous C-terminal domain with other family of regulatory proteins such as EnvZ and PhoR (Fig. 4), which are known as a counterpart of two-component regulatory systems (11). Thus, the NarX/NarL system belongs to sensor/regulator systems widely observed in prokaryotic gene regulation responding to a variety of environmental stimuli.

Most members of the EnvZ family are anchored in the inner membrane, and recognize physical or chemical signals through a periplasmic domain. The signal recognition domain has been assigned in non-conserved N terminus of each sensor class protein. According to the model (11), this domain transduces the stimulus to the conserved C-terminal cytoplasmic domain, and the activated C-terminal domain of the sensor protein then interacts with and modifies the conserved N-terminal domain of the regulator protein. The modification plays as a switch between inactive and active forms through a conformational change in the C terminus of the regulator. Structural similarity of the NarX/NarL system allows us to suppose the following mechanism operative in the positive control of the *nar* operon with nitrate (Fig. 5): NarX and NarL are constitutively produced independent of anaerobiosis and nitrate availability. Binding of nitrate to the periplasmic face of NarX triggers an allosteric change in the cytoplasmic portion of NarX, causing activation of NarX. Activated NarX then interacts with the N-terminal region of NarL, resulting in an allosteric or covalent modification of NarL to an active form. Activated NarL in conjunction with Fnr then activates transcription of the *narK* and *narC* operons, but represses transcription of other operons such as *frd* and *tor*.

It has been demonstrated that NtrB is a kinase/phosphatase capable of converting NtrC to a phosphorylated active form (55, 56) and that CheA is autophosphorylated at a His residue and able to transfer the phosphoryl group to CheB and CheY (57, 58). Similar observation was also made in the EnvZ/OmpR system (Mizuno, T. and Mizushima, S., 11th Annual Meeting of the Molecular Biology Society of Japan, 1988) and in the PhoR/PhoB system (Makino, K. *et al.*, *op. cit.*). These findings suggest the phosphorylation/dephosphorylation as a common mechanism for all members of sensor class to modulate their partners. In the NtrB/NtrC system, a His residue in NtrB and an Asp residue in NtrC were identified as the phosphorylated amino acids (59). It was also shown that PhoR was autophosphorylated at His 213, and transferred phosphoryl group to PhoB at Asp 53 to activate it (Makino, K. *et al.*, *op. cit.*). These His and Asp residues were conserved in all members of the sensor/regulator systems including NarX/NarL (Figs. 3 and 4). It is possible that the NarX protein activates the NarL protein through the similar mechanism.

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