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The *narK* gene product participates in nitrate transport induced in *Escherichia coli* nitrate-respiring cells

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The nucleotide sequence of the *Escherichia coli narK* gene, which is located in the upstream region of the *narCHJI* operon, was determined. The *narK* gene encodes a very hydrophobic protein with 463 amino acid residues (M_r 49 693). A *narK* deletion mutant, under conditions for the induction of nitrate respiration, was unable to perform nitrate transport. Loss of transport activity was recovered by transforming the mutant with a *narK*$^+$ plasmid. Thus, we conclude that the *narK* gene encodes a transmembrane protein participating in nitrate transport. In the *narK* promoter region, we defined a unique sequence that we designate as a 'nitrate box', functioning as a putative NarL-binding site, in addition to the consensus sequence of the 'anaero-box'.

Nitrate transport; Gene, *narK*; Nitrate respiration; (*E. coli*)

1. INTRODUCTION

In *Escherichia coli*, nitrate respiration is fully induced by nitrate under anaerobic conditions (review [1]). The respiratory system is composed of a nitrate reductase complex (α- and β-subunits of nitrate reductase and cytochrome b_{NR}) [1–6] and a nitrate transport system [1,7]. So far three *nar* operons have been designated, i.e., *narLX, narK* and *narCHJI*. Four component proteins associated with the electron-transfer system are known to be encoded in the *narCHJI* operon [8–11]: The α- and β-subunits and cytochrome b_{NR} are the respective products of the *narC, narH* and *narI* genes [11,12]; the *narJ* gene product is required for the assembly of the nitrate reductase-cytochrome b_{NR} complex to be fully active in the membrane, however the details remain to be clarified [12]. The *narLX* genes have been recently demonstrated to encode two-component regulatory proteins for the *nar* operons [13]. The *narK* gene was previously supposed to encode a regulatory protein for nitrate repression of other variant anaerobic respiratory systems [12], but the product of the *narK* gene has never been qualified as such a regulatory protein [14–16].

Here, we have determined the nucleotide sequence of the *narK* gene, in succession to that of the *narLX* genes [13]. From our sequence analysis, the deduced product of the *narK* gene was shown to have the characteristics of a transmembrane protein. Using a *narK* deletion mutant, we demonstrated that the *narK* gene product participates in nitrate transport induced in nitrate-respirating cells.

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2. EXPERIMENTAL

2.1. *Bacterial strains and growth conditions*

The bacterial strains used were *E. coli* K-12 XL1-Blue (*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1* [F′ *proAb lacI*^{q} *lacZ M15*]) [17], TNK33 (F$^-$ *trpA9825, glnA*$^+$ revertant of M5004) [18], and TNK34 (F$^-$ *ΔnarK::kan*). The *narK* deletion mutants were constructed via a gene replacement method [19]; homologous recombination of the plasmid-encoded *ΔnarK::kan* gene in pNR64 (see below) was carried out with the chromosomal *narK* allele in *polA12* strain MM383 [20]. Then,

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P1 *vir* transductions were performed to obtain TNK34 by the method of Miller [21]. The *narK* deletion mutant was screened on MacConkey-nitrate agar plates as described [12] and the deletion confirmed with the map distance calculated from the cotransduction frequency with a *trpA* marker [21].

Cells were grown in L broth supplemented with appropriate antibiotics [22], unless otherwise noted. Ampicillin was used at 50 μg/ml, tetracycline at 12.5 μg/ml, and kanamycin at 25 μg/ml. Anaerobic cultures were grown in stationary, filled bottles at 37°C with or without 1% nitrate and 1% formate.

2.2. *Subcloning and DNA sequencing*

The *narK* gene was subcloned from pNR26 [13] originating from λ13H6, which is one of the clones in the *E. coli* chromosome library of Kohara et al. [23]. The plasmid pNR53 bearing the $narK^+$ gene was constructed by subcloning a 2.2 kb *Xho*I-*Nco*I fragment of pNR26 into the *Eco*RI-*Nco*I sites of pACYC184DP (obtained by deleting from pACYC184 a 0.41 kb *Pvu*II fragment bearing the promoter and N-terminal coding region of the *cat* gene) after filling an end with T_4 DNA polymerase. To obtain *ΔnarK::kan*, an *Eco*47III-*Hin*dIII fragment which contains most of the *narK* coding sequence was replaced with *kan* gene from pUC4K [24]. The plasmid pNR64 was constructed by subcloning the resultant *ΔnarK::kan* gene together with the flanking regions into the *Eco*RI-*Dra*I sites of pBR322.

DNA sequence analysis was performed by the dideoxy chain-termination method [25] with [α-^{35}S]thio-dATP and modified T_7 DNA polymerase (Sequenase, US Biochemical). Single-stranded templates were prepared from derivatives of M13 phage [26] bearing various fragments of the *narK* gene. Computer analyses of the DNA sequence and hydrophobicity plots were performed by using the GENETYX software package (Software Development, Tokyo).

2.3. *Transport assay*

Changes in nitrate concentration in the transport assay medium were monitored by a nitrate electrode (Orion, model 93-07) as in [7].

3. RESULTS AND DISCUSSION

3.1. *Analysis of the coding nucleotide sequence*

The nucleotide sequence of *narK* is shown in fig.1. The sequence contains one significant open reading frame (from nucleotide 505 to 1893) encoding a protein with 463 amino acids (M_r 49693). A typical sequence (GAGGT) for a ribosomal binding site was found 4 bases upstream of the first initiation codon ATG. The hydropathy index of the amino acid residues for the *narK* gene product was estimated as shown in fig.2. The protein was revealed to comprise 12 highly hydrophobic regions, each composed of about 20 residues (fig.2). Therefore, the NarK protein thus characterized as a strongly hydrophobic transmembrane protein appears to be qualified enough to form a channel-like structure. We assumed that the gene product is a nitrate transporter on the basis of: (i) the presence of a nitrate transporter has been demonstrated [1,7]; (ii) except for the *narK* gene, no coding capacity for the transport protein is apparently sustained in either the *narCHJI* operon or the downstream [9,10] and upstream regions [13,16]; (iii) the *narK* gene product is neither involved directly in the electron-transfer system [12] nor qualified as a regulatory protein [14–16]; and (iv) the *narK* gene product shows remarkable characteristics as a typical transmembrane protein, as mentioned above.

3.2. *Nitrate transport activity*

To investigate the assumption, we measured activity of nitrate transport for the *narK* deletion mutant TNK34 after induction of nitrate respiration. As shown in fig.3, the nitrate-respiring wild-type strain TNK33 clearly exhibits nitrate transport activity. In contrast, no significant nitrate transport activity was observed in the *narK* deletion mutant TNK34. Upon addition of octylglucoside to the mutant cell suspension in order to increase membrane permeability for NO_3^- [7], NO_3^- is immediately transported and reduced to NO_2^- (fig.3). Transport ceased on addition of N_3^-, confirming that the observed decrease in NO_3^- is linked to the nitrate reductase activity (fig.3). We then transformed TNK34 with $narK^+$ plasmid pNR53 and measured the transport activity. Fig.3 shows that the activity was satisfactorily recovered by the presence of the $narK^+$ plasmid in the mutant TNK34. We therefore conclude that the *narK* gene product participates in nitrate transport. Probably, the product is a nitrate transport protein.

NO_3^- appears to be antiported as the accumulated NO_2^-, which is toxic, is expelled from the cells [1,7]. In fact, it was confirmed that nitrite is expelled from the cells almost simultaneously and stoichiometrically after reduction of nitrate [7]. Furthermore, the antiport of nitrite is indispensable for cells to cancel out the electrogenic potential formed by nitrate transport. Nitrate transport appears to be a type of facilitated diffusion rather than active transport by the proton-motive force, since the nitrate gradient is per-

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gcatgcgcagcgcatcccgctttgttgatcgcatgggcgctgcctgaacgccttgcaccagccagccagaaaccgccatccctgccagtccaatagcagt 100
                                                                                    ←narX
agaaagcaacacaataagcgcaacctgattaaccagggtgagcggagagagacaacgtttaagcatgtaaacctcttccttcaggctttaaatgagcaat 200
aaccttaatgaatgtgacgatacattctggaatggcagtattctcggctattggctgaagtatacccatacccggaaagagttactccttatttgccgtg 300
tggttagtcgctttacatcggtaagggtagggattttacagcaccgtgaaaaatctcataatttttatgaagtcactgtactcactatgggtaatgataa 400
atatcaatgatagataaagttatcttatcgtttgatttacatcaaattgcctttagctacagacactaaggtggcagacatcgaaacgagtatcagaggt 500
gtctatgagtcactcatccgccccccgaaagggctactggagctgtcattacagattggcgaccggaagatcctgcgttctggcaacaacgcggtcaacgt 600
narK→M S H S S A P E R A T G A V I T D W R P E D P A F W Q Q R G Q R (32)
attgccagccgcaacctgtggatttccgttccctgtctgctgctggcgttttgcgtatggatgttgttcagcgctgttgcggtgaacctaccgaaagtcg 700
 I A S R N L W I S V P C L L L A F C V W M L F S A V A V N L P K V  (65)
gctttaattttacgaccgatcagctatttatgttgactgcgctgccttcggtttctggcgcgttattacgtgttccatactccttttatggttcctatctt 800
G F N F T T D Q L F M L T A L P S V S G A L L R V P Y S F M V P I F (99)
cggtggtcgtcgctggacggcgttcagcaccggtattctgattattccttgcgtctggctgggttttgccgtgcaggatacctccacgccttatagcgtc 900
  G G R R W T A F S T G I L I I P C V W L G F A V Q D T S T P Y S V (132)
ttcatcatcatctctctgctatgcggctttgctggcgcgaacttcgcatccagtatggcaaacatcagcttcttctttccgaaacagaagcagggtggcg 1000
 F I I I S L L C G F A G A N F A S S M A N I S F F F P K Q K Q G G  (165)
cgctgggtctgaatggtggtctgggaaacatgggcgtcagcgtcatgcagttggttgctccgctggtggtatcactgtcgattttcgcagtatttggtag 1100
A L G L N G G L G N M G V S V M Q L V A P L V V S L S I F A V F G S (199)
ccagggcgtcaaacagccggatgggactgagctgtatctggcgaatgcgtcctggatatgggtgccgttccttgccatcttcaccattgcggcgtggttt 1200
  Q G V K Q P D G T E L Y L A N A S W I W V P F L A I F T I A A W F (232)
ggcatgaacgatcttgctacctcgaaagcctccatcaaggagcagttgccggtactcaaacggggtcatctgtggattatgagcctgctgtatctggcaa 1300
 G M N D L A T S K A S I K E Q L P V L K R G H L W I M S L L Y L A  (265)
ccttcggctccttcatcggcttctccgcgggctttgcaatgctgtcaaaaacgcagttcccggatgttcagatttctgcaatacgctttcttcgggccgtt 1400
T F G S F I G F S A G F A M L S K T Q F P D V Q I L Q Y A F F G P F (299)
tattggtgcgctggcgcgttctgcaggtggtgcattatctgaccgtctgggcggaactcgtgtcacgctggtgaactttattctgatggcgattttcagc 1500
  I G A L A R S A G G A L S D R L G G T R V T L V N F I L M A I F S  (332)
ggcctgctgttcctgaccttaccgactgacgggcagggcggaagcttcatggcgttcttcgcggtcttcctggcgctgttcctgacagctgggctgggta 1600
 G L L F L T L P T D G Q G G S F M A F F A V F L A L F L T A G L G  (365)
gtggttccactttccagatgatttcagtgatcttccgtaaactgacaatggatcgcgtgaaagcagaaggggttctgacgaacgtgcgatgcgtgaagc 1700
S G S T F Q M I S V I F R K L T M D R V K A E G G S D E R A M R E A (399)
ggcaaccgacacggcggcggcgctgggtttcatctctgcgattggcgcgattggtggcttctttatcccgaaagcgtttggtagctcgctggcattaacg 1800
  A T D T A A A L G F I S A I G A I G G F F I P K A F G S S L A L T  (432)
ggttcgccagtcggcgcaatgaaggtatttttgatttttctatatcgcctgcgtagtgattacctgggcggtatatggtcggcattctaaaaaataaaccg 1900
 G S P V G A M K V F L I F Y I A C V V I T W A V Y G R H S K K *    (463)
ttactcgtcatacttcgggttacatgtgctgcggctgcgttcattcaccccagtcacttactttagtaagctcctgggattcattcacttgccgccttcc 2000
tgtaaaccgaattatatagagtaaaatatttgattatcctttgcgcggcatgatgtcgcgcttttttatgcgtcatttagttacaacatactaatgtta 2100
tatggtttatttcgccggatttcattaagagccattaatatgttacccatggggaatactccttaatacccatctgcataaaaatcttaatagtttaaat 2200
aactacaggtataaaacgtcttaatttacagtctgttatgtggtggctgttaattatcctaaaggggtatcttaggaatttacttttatttttcatcccca 2300
tcactcttgatcgttatcaattcccacgctgtttcagagcgttaccttgcccttaaacattagcaatgtcgatttatcagagggccgacaggctcccacg 2400
ggagaaaaccgatgagtaaattcctggaccggtttcgctacttcaaacagaagggtgaaacctttgccgatgggcatggcca 2482
narC→M S K F L D R F R Y F K Q K G E T F A D G H G (23)
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Fig.1. DNA nucleotide sequence of the *narK* operon and deduced amino acid sequence of the product. Possible ribosome-binding site and initiation codon are overlined; the anaero-box (432–445, 2307–2320), nitrate box (283–291, 2157–2165), and ϱ-independent terminator (2043–2068) are underlined.

manently supported by the immediate conversion of imported nitrate to nitrite which is eventually expelled. Since the transport is controlled by oxygen, five Cys residues in NarK may play an im-

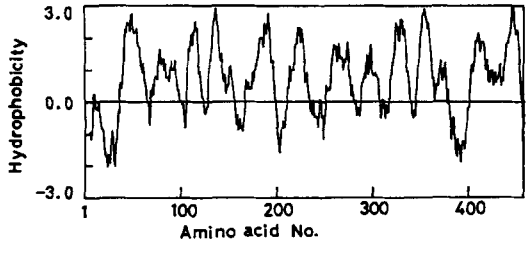

Fig.2. Hydropathy profile of NarK. Hydrophobicity was calculated at a span setting of 11 residues according to Kyte and Doolittle [32].

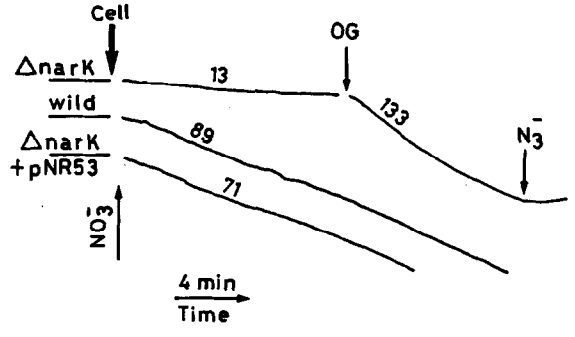

Fig.3. Uptake of NO_3^- as monitored using nitrate electrodes. ΔnarK, *narK*-deletion mutant TNK34; wild, TNK33; ΔnarK + pNR53, TNK34 with plasmid pNR53. OG, octylglucoside (added to 0.45% final concentration); N_3, sodium azide (final concentration 0.1 mM). The rates of uptake given beside traces are expressed in nmol nitrate/min per mg protein.

portant role in sensing redox potential to regulate the facilitator, as postulated previously [7].

Stewart and MacGregor [12] identified *narK* in Tn10 insertion mutants producing white colonies on MacConkey nitrate-trimethylamine *N*-oxide medium. The mutants exhibit a normal level of nitrate reductase activity and regulate the expression of the *nar* operon [12]. However, Rondeau et al. [9] reported that the *narK* mutant did not accumulate nitrite in the overlay assay, assuming that either the nitrate reductase produced by this mutant is not functional or the ability for the cells to remove nitrite is derepressed. Based upon our observations, however, their result is consistently interpreted as a lack of normal transport of nitrate.

3.3. *Analysis of nucleotide sequence of the regulatory regions*

The *narK* gene product was reported to be fully induced by nitrate under anaerobic conditions [16], as in the case of the *narCHJI* gene products. The common character in these two operons was confirmed in our promoter analysis of the *nar* gene [13]. Two elements of the sequence in the *narK* promoter region were found to be very homologous to the corresponding ones in the *narCHJI* promoter region, as shown in fig.1. In both promoter regions, an anaero-box (TTGATNNNNATCAA) [27,28] was identified as a putative Fnr-binding site, as expected from anaerobic induction of both products. Furthermore, we found a common sequence of TACTCCTTA in both promoter regions. Since the sequence is presumed to be an NarL-binding site in the *narCHJI* promoter region [29,30], the sequence TACTCCTTA should be a *cis*-element for nitrate induction and thus qualify to be termed a nitrate box.

Between the *narK* and *narC* genes, a typical sequence exists to serve as a ρ-independent terminator, indicating that the *narCHJI* operon is transcribed independently of the *narK* operon. It is interesting to note the fact that the overexpression of *narK* product by using a pUC vector, unlike the case of the *narCHJI* products, apparently disturbs normal membrane organization by excessive incorporation of the products, resulting in retarded growth and morphological change from normal rod to string chaining longer rods. Thus, it seems reasonable that expression of the *narK* gene is stringently regulated in a specific mode independent of the *narCHJI* operon.

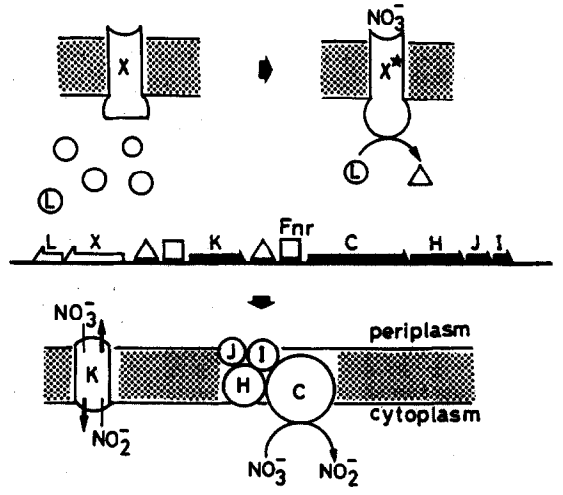

Fig.4. A schematic model for the induction mechanism of the nitrate respiratory system.

3.4. *A model for regulation and functions of the* nar *gene products*

Our model summarizing the induction mechanism of *narK* and *narCHJI* operons is illustrated in fig.4. Recently, we interpreted the *narX* gene product as being a nitrate-sensing protein activated specifically by nitrate, and the *narL* gene product, when activated by the active NarX, is supposed to be a *trans*-acting positive regulator that will bind to the nitrate box in the promoter region of *narCHJI* [13]. Similarly, the NarX activated by nitrate converts NarL into the active form, followed by this activated NarL and Fnr binding to the nitrate box and anaero-box, respectively, in the *narK* promoter region under anaerobic conditions (fig.4). The *narK*-gene product is probably a nitrate-transporting channel-like protein (nitrate/nitrite facilitator) with 463 amino acids. Nitrate is transported through the channel in a facilitative manner. Transported nitrate is reduced to nitrite by the nitrate reductase to generate the proton-motive force, and then the nitrite is expelled through the *narK* gene products. Similar mechanisms for nitrate transport and induction of proteins are expected to be operative in *Pseudomonas aeruginosa, Bacillus subtilis, Paracoccus denitrificans*, etc. [31].

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