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Involvement of NarK1 and NarK2 Proteins in Transport of Nitrate and Nitrite in the Denitrifying Bacterium *Pseudomonas aeruginosa* PAO1

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Two transmembrane proteins were tentatively classified as NarK1 and NarK2 in the *Pseudomonas* genome project and hypothesized to play an important physiological role in nitrate/nitrite transport in *Pseudomonas aeruginosa*. The *narK1* and *narK2* genes are located in a cluster along with the structural genes for the nitrate reductase complex. Our studies indicate that the transcription of all these genes is initiated from a single promoter and that the gene complex *narK1K2GHJI* constitutes an operon. Utilizing an isogenic *narK1* mutant, a *narK2* mutant, and a *narK1K2* double mutant, we explored their effect on growth under denitrifying conditions. While the Δ*narK1*::Gm mutant was only slightly affected in its ability to grow under denitrification conditions, both the Δ*narK2*::Gm and Δ*narK1K2*::Gm mutants were found to be severely restricted in nitrate-dependent, anaerobic growth. All three strains demonstrated wild-type levels of nitrate reductase activity. Nitrate uptake by whole-cell suspensions demonstrated both the Δ*narK2*::Gm and Δ*narK1K2*::Gm mutants to have very low yet different nitrate uptake rates, while the Δ*narK1*::Gm mutant exhibited wild-type levels of nitrate uptake. Finally, *Escherichia coli narK* rescued both the Δ*narK2*::Gm and Δ*narK1K2*::Gm mutants with respect to anaerobic respiratory growth. Our results indicate that only the NarK2 protein is required as a nitrate/nitrite transporter by *Pseudomonas aeruginosa* under denitrifying conditions.

Denitrification involves four separate nitrogen oxide reductases and ultimately reduces nitrate to dinitrogen (37). Respiratory nitrate reductase, which is the first enzyme in this denitrification pathway, has its active site on the cytoplasmic side of the membrane (23). The enzyme substrate, nitrate, is an ion and cannot be taken up by the simple process of passive diffusion (18). Both of these factors require the bacterium to synthesize a transport protein(s) to carry nitrate into the cytoplasm, where the reduction of nitrate to nitrite takes place. It has been demonstrated for *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, and *Escherichia coli* (7, 11, 24) that the product of nitrate respiration, i.e., nitrite, is immediately excreted to the external environment, presumably protecting the organism from potential toxic effects. These toxic effects are due to the ability of this anion to bind to the heme groups in electron carriers, thereby inhibiting the flow of electrons (25). Genetic and physiological data suggest that nitrate transport in some bacteria occurs through two different uptake systems. Thus, for the process of nitrate assimilation, ABC transporters as well as secondary transporters are postulated to be used. On the other hand, anaerobically, for the purpose of nitrate respiration, it is postulated that bacteria rely solely on secondary transporters (18).

Originally, John (14) demonstrated that membrane permeabilization of the cells significantly enhanced nitrate uptake, suggesting the need for a transport protein specific for nitrate. This was corroborated by several other studies which also demonstrated that external nitrate uptake in whole cells was restricted by a permeability barrier (10, 20). It was also observed that nitrate reduction and nitrate uptake were closely coupled, as *narG*-deficient mutants did not take up nitrate (24). Others demonstrated that nitrate uptake and reduction resulted in the immediate excretion of nitrite (7).

The first genetic locus identified as playing a role in nitrate uptake or nitrite excretion was *narK* of *E. coli* K-12 (4, 6, 20, 24, 33). Subsequently, other NarK-like proteins were identified by homology and by phenotype. NarK families of proteins belong to the major facilitator superfamily (MFS) of transmembrane transporters and are categorized as secondary transporters requiring the generation of a proton motive force (17). Homologues of NarK seem to be present in a multitude of organisms, where they may serve as either nitrate/proton symporters or as nitrate/nitrite antiporters.

E. coli is the paradigm for respiratory nitrate metabolism in bacteria. The current state of knowledge is based primarily on studies of this organism, which possesses two nitrate/nitrite transport proteins, NarK and NarU (2, 4, 13). These porters are separate from the *narG* operon, which contains the genetic information for the nitrate reductase enzyme complex. Since the first studies of *E. coli*, NarK homologues have been identified in a number of different organisms, such as *Bacillus subtilis* (5), *Staphylococcus carnosus* (8), *Thermus thermophilus* (22), *Paracoccus pantotrophus* (36), and *Mycobacterium tuberculosis* (32). Although these studies have enhanced the knowledge about nitrate/nitrite transport in bacteria, the actual mechanism(s) for nitrate transport remains controversial. The studies described here have identified the presence of a unique operon within an organism capable of denitrification. The system is novel among the *Proteobacteria*, as two genes, *narK1* and *narK2*, cluster with the *narGHJI* genes in a single operon.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The PAO1 *narG* mutant was

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant genotype or description	Source or reference
Strains		
E. coli		
DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ*lacU169* (ϕ80*lacZ*Δ*M15*)	Gibco
SM10	Km^r, Mobilizer strain	31
P. aeruginosa		
PAO1	Wild type	Al Darzins
Δ*narK1*::Gm strain	Gm^r, Δ*narK1*::Gm	This study
Δ*narK2*::Gm strain	Gm^r, Δ*narK2*::Gm	This study
Δ*narK1K2*::Gm strain	Gm^r, Δ*narK1K2*::Gm	This study
narG::*lacZ*Gm strain	Gm^r, ϕ(PA3875-*lacZ*Gm)	21
Δ*narK2*::Gm/p*narK1* strain	Gm^r Cb^r, Δ*narK1*::Gm with PA3877 in pUCP18	This study
Δ*narK2*::Gm/p*narK2* strain	Gm^r Cb^r, Δ*narK2*::Gm with PA3876 in pUCP18	This study
Δ*narK1K2*::Gm/p*narK1K2* strain	Gm^r Cb^r, Δ*narK1K2*::Gm with PA3877 and PA3876 in pUCP18	This study
Δ*narK2*::Gm/p*narK* strain	Gm^r Cb^r, Δ*narK2*::Gm with *E. coli narK* in pUCP18	This study
Δ*narK1K2*::Gm/p*narK* strain	Gm^r Cb^r, Δ*narK1K2*::Gm with *E. coli narK* in pUCP18	This study
PAO1/p*narK1K2* strain	Cb^r, wild-type PAO1 with PA3877 and PA3876 in pUCP18	This study
Plasmids		
pGem::*narK1*	Ap^r, 1.296-kb fragment containing PA3877 in the pGEM-T Easy vector (Promega)	This study
pGem::*narK2*	Ap^r, 1.407-kb fragment containing PA3876 in the pGEM-T Easy vector (Promega)	This study
pGem::*narK1K2*	Ap^r, 2.703-kb fragment containing PA3876 and PA3877 in the pGEM-T Easy vector (Promega)	This study
pCR::*narK E. coli*	Ap^r, 1.392-kb fragment containing *E. coli narK* in the pCR2.1 vector (Invitrogen)	This study
pEX18Ap	Ap^r *oriT mob sacB* gene replacement vector with multiple-cloning site from pUC18	12
pEX18Ap::*narK1*	Ap^r, ligation of a 1.296-kb EcoRI fragment of PA3877 of pGem::*narK1* into an EcoRI digest of pEX18Ap	This study
pEX18Ap::*narK2*	Ap^r, ligation of a 1.407-kb EcoRI fragment of PA3876 of pGem::*narK2* into an EcoRI digest of pEX18Ap	This study
pEX18Ap::*narK1K2*	Ap^r, ligation of a 2.703-kb EcoRI fragment containing PA3876 and PA3877 of pGem::*narK1K2* into an EcoRI digest of pEX18Ap	This study
pUCGM	Ap^r Gm^r *aacCl*	29
pEX18Ap:Δ*narK1*::Gm	Ap^r Gm^r, ligation of a 1-kb SmaI fragment of pUCGM containing *aacC1* into a blunt-ended NcoI-SalI deletion of pEX18Ap::*narK1*	This study
pEX18Ap:Δ*narK2*::Gm	Ap^r Gm^r, ligation of a 1-kb SmaI fragment of pUCGM containing *aacC1* into a blunt-ended XhoI-ApaI deletion of pEX18Ap::*narK2*	This study
pEX18Ap:Δ*narK1K2*::Gm	Ap^r Gm^r, ligation of a 1-kb SmaI fragment of pUCGM containing *aacC1* into a blunt-ended NotI-ApaI deletion of pEX18Ap::*narK1K2*	
pUCP18	Ap^r, broad-host-range cloning vector	28
p*narK1*	Ap^r, ligation of a 1.296-kb EcoRI fragment of pGem::*narK1* into the EcoRI site of pUCP18, complementation studies	This study
p*narK2*	Ap^r, ligation of a 1.407-kb EcoRI fragment of pGem::*narK2* into the EcoRI site of pUCP18, complementation studies	This study
p*narK1K2*	Ap^r, ligation of a 2.703-kb EcoRI fragment of pGem::*narK1K2* into the EcoRI site of pUCP18, complementation studies	This study
p*narK*	Ap^r, ligation of a 1.392-kb EcoRI fragment of pCR::*narK* into the EcoRI site of pUCP18, complementation studies	This study

previously isolated (21). All bacteria were grown at 37°C from single-colony isolates or overnight cultures in Luria-Bertani (LB) broth (Fisher Scientific). The medium was supplemented with nitrate at a final concentration of 1%. The cultures were also plated on LB medium, 1.5% agar (Difco, Detroit, Mich.). Plasmid integration during mutant construction was checked using *Pseudomonas* isolation agar (Difco, Detroit, Mich.)

Aerobic overnight cultures were incubated with shaking at 250 rpm unless otherwise noted. For anaerobic-growth cultures, conditions included magnetic stirring in 125-ml Erlenmeyer flasks with rubber stoppers equipped with ports for sample withdrawal and one-way gas release valves. To ensure complete anaerobiosis of the system, the medium was supplemented with 2% (wt/vol) Oxyrase (Oxyrase, Inc., Mansfield, Ohio) and flushed with argon. For the nitrate reductase assay and the whole-cell uptake study, the cultures were grown aerobically to an optical density at 660 nm (OD_{660}) of 0.5 to 0.6, after which time they were shifted to complete anaerobic conditions.

Antibiotics were used for *E. coli* at the following concentrations (μg/ml): ampicillin, 100; and gentamicin, 15. For *P. aeruginosa*, gentamicin and carbenicillin were used at 300 and 500 μg/ml, respectively.

Bioinformatics analyses. Gene, protein, and primer sequences for *P. aeruginosa* PAO1 and *E. coli* K-12 were obtained using the *Pseudomonas* genome database site (http://www.pseudomonas.com/) and *E. coli* K-12 genome database site (http://www.ecocyc.com/), respectively. Prediction of the molecular weights of the proteins, based on amino acid data, was made with individual proteomics tools available at the ExPASy mirror site (http://au.expasy.org/) of the Swiss Institute of Bioinformatics. A promoter search was carried out using the promoter prediction software site (http://www.fruitfly.org/). Sequence similarity

TABLE 2. Oligonucleotide primers used in this study

Primer	Location	Strand[a]	Sequence (5′ → 3′)
NarK1	*narK1*	+	CCTGTCACTACCTCCAAAG
NarK1	*narK1*	−	AGAAGCTGATATTGGACATG
NarK2	*narK2*	+	GTGCCTGTTCTTCCTCTC
NarK2	*narK2*	−	TTGGCGCTGTAGATGTAC
NarK1K2	*narK1*	+	CCTGTCACTACCTCCAAAG
NarK1K2	*narK2*	−	TTGGCGCTGTAGATGTAC
E. coli NarK	*narK*	+	CTGCTGCTCGAGTCAACTC
E. coli NarK	*narK*	−	TATAATTCGGTTTACAGGAAGG

[a] Forward and reverse primers are indicated by + and −, respectively.

comparisons between PAO1 NarK2 and *E. coli* K-12 NarK were carried out using the Multalin software (http://www.renabi.fr). Hydropathy profiles were generated as described previously (15) with a window size of 23 (http://www.bio.davidson.edu/courses/compbio/flc/home.html).

Manipulation of recombinant DNA and genetic techniques. All plasmid and chromosomal nucleic acid manipulations were by standard techniques (26). Plasmid DNA was transformed into *E. coli* DH5α-MCR (Gibco-BRL), SM10 (31), or *P. aeruginosa* PAO1. Restriction endonucleases, the Klenow fragment, and T4 DNA ligase were used as specified by the supplier (New England Biolabs). Plasmid DNA was isolated using the QIA prep spin kit (QIAGEN). DNA fragments were isolated from agarose gels using the Gene Clean kit (QBiogene). PCRs were performed using *Taq* DNA polymerase, PCR buffer, and deoxynucleoside triphosphates (Sigma Chemical Co.) in a Peltier thermal cycler. All the oligonucleotide primers used in this study are listed in Table 2 (Sigma-Genosys).

Construction of isogenic mutants. The open reading frames (ORFs) putatively responsible for the formation of NarK1 and NarK2 were identified by homology as PA3877 and PA3876, respectively, through the *Pseudomonas* Genome Project (34). The genes were amplified from PAO1 using primers based on sequence data from the *Pseudomonas* Genome Database (Table 2). All the strain constructions and manipulations are described in detail in Table 1. PCR fragments were initially cloned into the pGEM-T Easy (Promega) or pCR2.1 (Invitrogen) vector. The genes were inserted into pEX18Ap (12). Isogenic *narK1*, *narK2*, and *narK1 narK2* mutants were created by deletion of most of the ORFs, followed by insertion of *aacC1*, a gentamicin resistance marker from pUCGM (29). Single-copy chromosomal gene disruptions were created using a gene replacement technique previously described (27). Mutants were confirmed by PCR using primers specified in Table 2 (data not shown). All mutants were complemented by the use of pUCP18 plasmid vector (28) with the gene(s) of interest (Table 1).

Preparation of cell extracts to analyze nitrate reductase activity. To analyze cell extracts for enzyme activity, cultures were centrifuged and the cells washed five times with an equal volume of 0.1 M potassium phosphate buffer (pH 7.2). The cell suspensions were then sonicated five times at 4°C, with 15-second bursts and a rest interval of 1 min, in an ice bath using the Branson 150 sonicator, followed by centrifugation at 10,000 × *g* for 10 min to remove cell debris.

Determination of nitrate reductase activity. For the assay, 100 μl of cell extract was added to a 1.5-ml Eppendorf tube containing 700 μl 0.1 M potassium phosphate buffer (pH 7.2) followed by 50 μl of 1 M KNO_3. To start the reaction, 50 μl of freshly made 0.08% sodium hydrosulfite (dithionite) was added to 100 μl of methyl viologen, gently mixed, and added to cell extracts. The reaction proceeded for 2 min, after which time all contents were vigorously vortexed and the nitrite concentration was determined by the Griess reaction (16). Enzyme activity is defined as that amount of nitrate reductase required to produce 1 nmol nitrite min^{-1} mg^{-1} protein. All the assays were performed in triplicate and repeated at least twice with independent cultures.

Uptake of nitrate monitored by a nitrate ion-selective electrode. Whole cells were analyzed for rates of nitrate uptake using the Orion 9707 Ionplus nitrate electrode (Thermo Electron Co.) by a method previously described (11). Glucose (1 M) was used as an energy source, and the cells were spiked with 200 to 600 μM KNO_3 in an argon-generated anaerobic environment. All the assays were performed in triplicate and repeated at least twice with independent cultures.

Determination of the concentrations of extracellular nitrite. Extracellular nitrite was determined in whole-cell suspensions using the Griess reaction as previously described (4). All assays were performed in triplicate and repeated at least twice with independent cultures.

Determination of protein concentrations in whole-cell suspensions and cell extracts. The Bradford reagent (Sigma-Aldrich, St. Louis, Mo.) was utilized to determine the protein concentrations for both sonicated and whole-cell suspensions (3).

RESULTS

NarK1 and NarK2 as candidates for nitrate import or nitrite export. In the organism *P. aeruginosa* PAO1, the *narK1* and *narK2* genes are found in a cluster of genes which includes structural genes for the nitrate reductase enzyme complex (*narGHJI*) (Fig. 1), and these together appeared to comprise the *narK1K2GHJI* operon (http://www.pseudomonas.com). By extrapolation, the *narK1* gene encodes a protein of 431 amino acids with a molecular weight of 47.3, while the *narK2* gene encodes a protein of 468 amino acids with a molecular weight of 50.6 (http://au.expasy.org).

A comparison of peptide sequences between the NarK1 and NarK2 proteins yielded a similarity of 28% (http://www.renabi.fr). In contrast, a similarity of 74% was observed between NarK2 and NarK of *Escherichia coli* K-12. The NarK1 was found to be 59% similar to the NarK1 protein of *Thermus thermophilus*. Further, a hydrophobicity profile (http://www.bio.davidson.edu/courses/compbio/flc/home.html) indicates that both proteins contain 11 to 12 transmembrane helices. Such a helix profile is in complete agreement with the proposed roles for transporters.

The *narK1K2GHJI* operon. A promoter predictor program (http://www.fruitfly.org) indicated the presence of only one promoter for the *narK1*, *narK2*, and *narGHJI* genes, further suggesting that these genes might form one operon. This was verified by growing the Δ*narK1K2*::Gm mutant aerobically in LB broth supplemented with nitrate and gentamicin. In *P. aeruginosa* PAO1, respiratory nitrate reductase is normally induced only anaerobically in the presence of nitrate (19, 30). Because the Δ*narK1K2*::Gm mutant contains a gentamicin cassette insertion (Fig. 1) and consequently contains the gentamicin promoter, the respiratory nitrate reductase genes could be induced even aerobically in LB broth-nitrate through this promoter. Thus, under aerobic conditions, the Δ*narK1K2*::Gm mutant yielded normal amounts (330 ± 5 nmol nitrite min^{-1} mg^{-1} protein) of respiratory nitrate reductase activity, while no nitrate reductase activity was detected in the wild-type strain, further supporting the idea that all of these genes are contained in a single operon.

Effect of *narK1* and *narK2* mutations on anaerobic respiratory growth. All mutants and the respective complemented strains were grown anaerobically in LB broth supplemented with nitrate (Fig. 2). The Δ*narK1*::Gm mutant grew almost as rapidly as the wild type, yielding generation times of 2.6 ± 0.08 and 2 ± 0.4 h, respectively (Fig. 2A). In contrast, the Δ*narK2*::Gm mutant (Fig. 2B) was found to be severely impaired in nitrate-dependent anaerobic growth and yielded a generation time of 8.5 ± 0.6 h. Finally, the Δ*narK1K2*::Gm double mutant demonstrated almost no growth (Fig. 2C). A complementation of the Δ*narK2*::Gm mutant with p*narK2* completely rescued the mutant. However, the Δ*narK1K2*::Gm mutant was not fully complemented with p*narK1K2*, demonstrating only a slightly higher growth rate than the mutant (Fig. 2C). This was attributed to the overproduction of two mem-

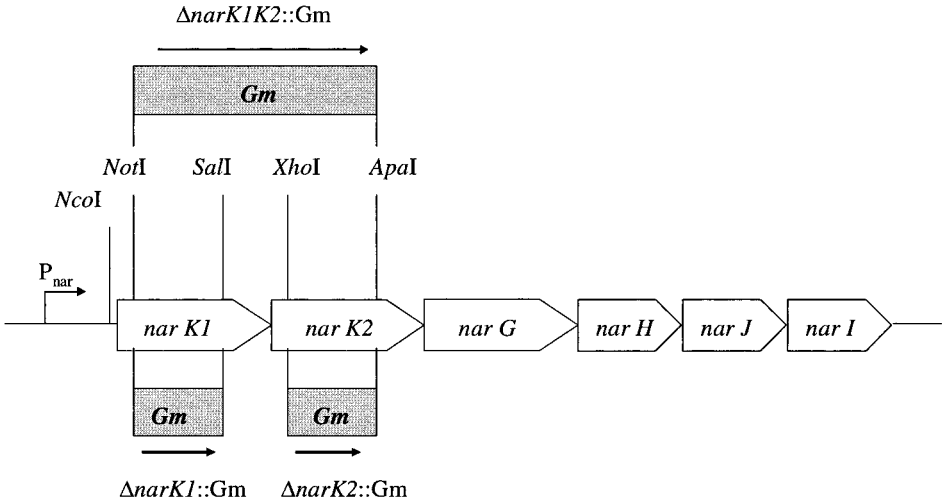

FIG. 1. Map of the *narK1K2GHJI* operon of *Pseudomonas aeruginosa.* The map shows the *narK1* and *narK2* genes to be upstream of the structural genes of nitrate reductase (*narGHJI*). Relevant restriction sites used to create deletions are shown. The endogenous promoter for the operon is shown as P_{nar}. The direction of transcription of both the operon and the gentamicin cassette (*Gm*) is shown with the help of arrows. The orientation of the Gm cassette in the gene disruptions was always positive with respect to the gene, as shown in the figure. The figure is not drawn to scale. The Δ*narK1*::Gm mutant was created by blunt-ending the Gm cassette into the NcoI-SalI deletion site. The Δ*narK2*::Gm mutant was created by blunt-ending the Gm cassette into the XhoI-ApaI deletion site. The Δ*narK1K2*::Gm mutant was created by blunt-ending the Gm cassette into the NotI-ApaI deletion site.

brane proteins due to the use of a high-copy-number plasmid (8). We have confirmed this inhibitory effect by transforming wild-type *P. aeruginosa* with p*narK1K2*. This strain grew slower than the wild type, yielding a generation time of 3.2 ± 0.6 h. As expected, the Δ*narK1K2*::Gm/p*narK1* complemented strain was also unable to grow (data not shown), implying a requirement for a functional NarK2 protein for respiratory nitrate reduction by *P. aeruginosa*.

Nitrate reductase activities in the *narK1*, *narK2*, and *narK1K2* mutants. The nitrate reductase activity was analyzed in cell extracts of all the strains using a nonphysiological electron donor, i.e., methyl viologen. For this purpose, strains were grown to an OD_{660} of 0.5 to 0.6 aerobically in LB broth supplemented with nitrate and gentamicin and then switched to anaerobic conditions for 3 h. The cell extracts were subsequently analyzed for methyl viologen-linked nitrate reductase activity (Table 3). Similar nitrate reductase activities were observed in all the strains, as was expected. This confirmed that the deletion-insertion mutagenesis of the genes did not affect the expression of the nitrate reductase genes and that the phenotypes observed were due to a defect in nitrate and/or nitrite transport.

Nitrate uptake. To investigate the role of *P. aeruginosa narK1* and *narK2* gene products in nitrate transport, anaerobically grown whole-cell suspensions were monitored for external nitrate using a nitrate electrode (Table 4). The Δ*narK1*::Gm mutant exhibited uptake rates similar to that of the wild type, consistent with the anaerobic growth rates observed. On the other hand, both the Δ*narK2*::Gm and the Δ*narK1K2*::Gm mutants were found to be severely impaired in their nitrate uptake ability, exhibiting uptake rates of 8.4 ± 0.3 nmol nitrate min^{-1} mg^{-1} protein and <1.5 nmol nitrate min^{-1} mg^{-1} protein, respectively. Complementation with p*narK2* and p*narK1K2* was found to rescue this phenotype. Furthermore, similar to the observation made for an *E. coli* nitrate reductase mutant, no nitrate uptake was observed in a *P. aeruginosa* PAO1 *narG* mutant (Table 4).

Nitrite accumulation by the *narK1*, *narK2*, and *narK1K2* mutants. Next, we wanted to see if there were any differences in nitrite extrusion between the wild type and the mutant strains. Samples were withdrawn during anaerobic nitrate-dependent growth, cells were removed, and the amount of nitrite was analyzed (data not shown). Given that the Δ*narK2*::Gm and Δ*narK1K2*::Gm mutants are unable to grow under these conditions and since cytoplasmic nitrite is a result of nitrate reduction, both of the mutants were expected to demonstrate limited nitrite excretion, which indeed was the case (data not shown). In contrast, although the Δ*narK1*::Gm mutant excreted visibly reduced amounts of nitrite compared to that excreted by the wild type, normalization of the data in terms of protein amounts abolished this difference, giving values of 60.5 ± 0.68 and 60.5 ± 0.7 μmol extracellular nitrite mg^{-1} protein for the wild type and Δ*narK1*::Gm mutant, respectively.

The isogenic *narK2* mutant was complemented by the *narK* gene of *Escherichia coli* K-12. Previous studies of nitrate/nitrite transport have been most extensively carried out on the NarK protein of *Escherichia coli* (4, 6, 13, 20, 24, 33). Thus, to establish the role of the *narK2* gene in PAO1, we cloned the *narK* gene of *E. coli* into a pUCP18 plasmid vector. The resulting strain was used to complement both the Δ*narK2*::Gm strain and the Δ*narK1K2*::Gm strain (Fig. 3). The results demonstrate that the *narK* gene of *E. coli* is capable of restoring anaerobic growth in PAO1 deficient in *narK2* and *narK1K2*. The growth rates of these complemented strains were not completely restored to wild-type levels, but that can be attributed to (i) high copy numbers of the membrane proteins being produced (8) and (ii) a nonidentical protein used for complementation.

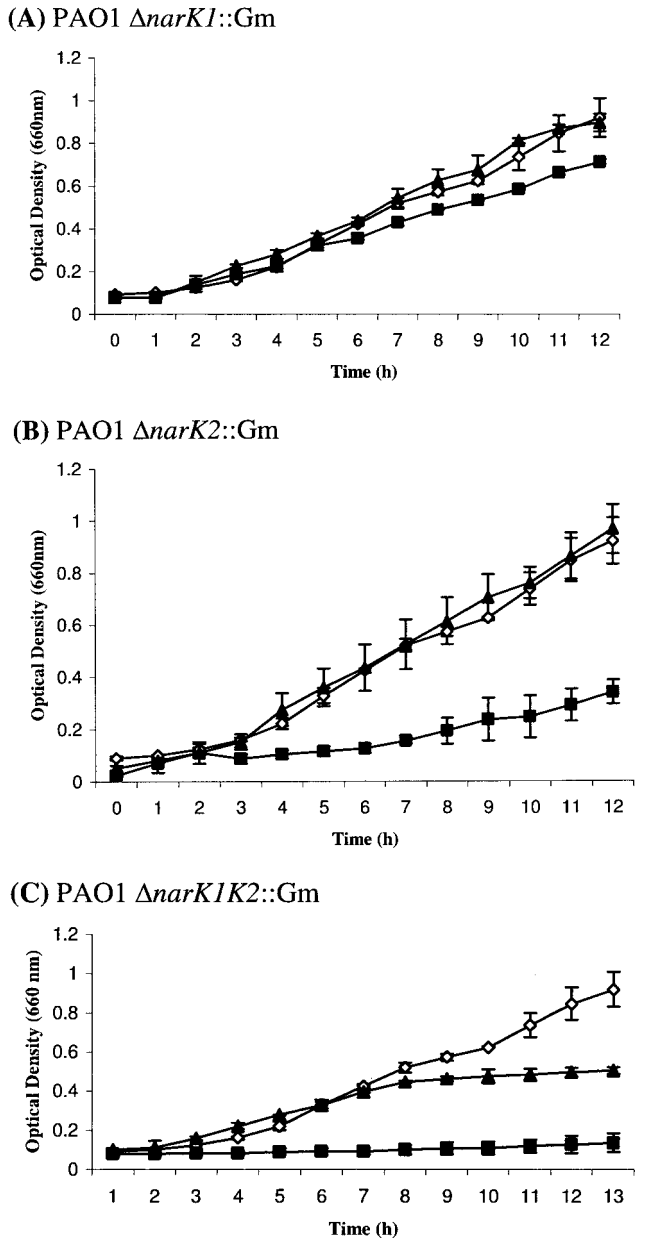

FIG. 2. Anaerobic growth of *Pseudomonas aeruginosa* PAO1 in LB medium supplemented with nitrate. All the inocula were prepared by growing the strains overnight in shaker-grown starter cultures in LB medium, which were then transferred to LB medium supplemented with 1% nitrate and the appropriate concentrations of gentamicin and/or carbenicillin and switched to anaerobic conditions using oxyrase and argon gas. (A) Anaerobic growth of PAO1 (◇), Δ*narK1*::Gm strain (■), and Δ*narK1*::Gm/p*narK1* complemented strain (*narK1* complement) (▲). (B) Anaerobic growth of PAO1 (◇), Δ*narK2*::Gm strain (■), and Δ*narK2*::Gm/p*narK2* complemented strain (*narK2* complement) (▲). (C) Anaerobic growth of PAO1 (◇), Δ*narK1K2*::Gm strain (■), and Δ*narK1K2*::Gm/p*narK1K2* complemented strain (*narK1K2* complement) (▲).

DISCUSSION

The goal of the present study was to elucidate the involvement of the *narK1* and *narK2* genes in *P. aeruginosa* denitrification. In this regard, isogenic *narK1*, *narK2*, and *narK1K2* mutants were created and verified. These studies confirmed that the *narK1* and *narK2* genes are in an operon with the *narGHJI* genes. The literature suggests that this is unusual since the *narG* operon of *E. coli* is distinctly separate from *narK* and *narU* (2, 33) as is *narK1* and *narK2* of *Thermus thermophilus* (22) and *narK* of *P. stutzeri* (9). Only *Paracoccus pantotrophus* (36) has a nitrate/nitrite transporter in the same operon as the genes for the nitrate reductase complex.

Studies of anaerobic, nitrate-dependent growth showed the Δ*narK1*::Gm mutant to be only slightly affected in growth, while both the Δ*narK2*::Gm and Δ*narK1K2*::Gm mutants were severely compromised compared to the wild type (Fig. 2). This suggests that these proteins serve different roles in nitrate-dependent, anaerobic growth. To make sure that these growth phenotypes were not due to an inactive nitrate reductase, all mutants were checked and confirmed for the presence of nitrate reductase activity (Table 3). These results are in contrast to the results of a study of *narK1* and *narK2* of *Thermus thermophilus* (22). In that study, a single mutation of *narK1* or *narK2* did not severely restrict anaerobic growth. Only when both of these genes were mutated was the organism severely restricted in anaerobic growth at the expense of nitrate. Fur-

TABLE 3. Reduced methyl viologen-linked nitrate reductase activities of the *P. aeruginosa* wild type and mutants grown anaerobically

Strain[a]	Nitrate reductase activity (nmol nitrite min^{-1} mg^{-1} protein)[b]
PAO1	334 ± 9
Δ*narK1*::Gm strain	310 ± 10
Δ*narK1*::Gm/p*narK1* strain	330 ± 12
Δ*narK2*::Gm strain	278 ± 12
Δ*narK2*::Gm/p*narK2* strain	328 ± 13
Δ*narK1K2*::Gm strain	229 ± 8
Δ*narK1K2*::Gm/p*narK1K2* strain	283 ± 4

[a] All strains were grown aerobically in LB medium supplemented with 1% nitrate to an OD_{660} of 0.5 to 0.6 and then shifted to anaerobiosis for 3 h.
[b] Enzyme activities were determined in cell extracts using reduced methyl viologen as the electron donor. Means and standard errors were calculated from three independent cell suspensions.

TABLE 4. Effects of mutations in *narK1*, *narK2*, *narK1K2*, and *narG* strains on rates of nitrate uptake

Strain[a]	Rate of nitrate uptake (nmol nitrate min^{-1} mg^{-1} protein)[b]
PAO1	175 ± 37
Δ*narK1*::Gm strain	192 ± 7
Δ*narK2*::Gm strain	8 ± 0.3
Δ*narK2*::Gm/p*narK2* strain	<1.5
Δ*narK1K2*::Gm strain	254 ± 31
Δ*narK1K2*::Gm/p*narK1K2* strain	186 ± 47
narG::*lacZ*Gm strain	0[c]

[a] All strains were grown aerobically in LB medium supplemented with 1% nitrate to an OD_{660} of 0.5 to 0.6 and then shifted to anaerobiosis for 3 h. Washed whole cells were suspended in 20 mM Tris-HCl buffer (pH 7.4) and monitored under argon-generated anaerobic conditions.
[b] Glucose (1 M) was used as the energy source, and the nitrate concentration was 200 to 600 mM. Means and standard errors were calculated from three independent cell suspensions.
[c] Not detected.

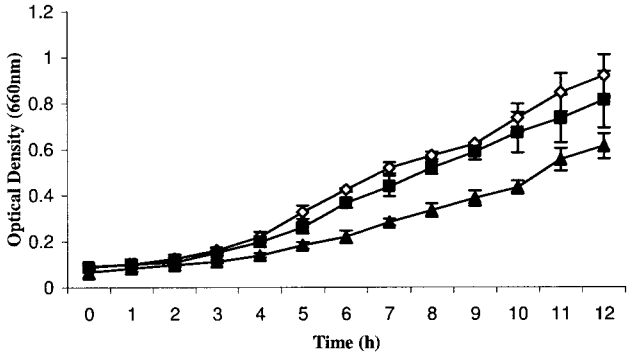

FIG. 3. Complementation of the Δ*narK2*::Gm mutant and the Δ*narK1K2*::Gm mutant with p*narK*. The *narK* gene cloned into the pUCP18 plasmid vector was obtained from *E. coli* K-12. All strains were grown overnight in LB medium and were then transferred to LB medium supplemented with 1% nitrate and an appropriate concentration of gentamicin and carbenicillin and switched to anaerobic conditions. The anaerobic growth of PAO1 (◇), Δ*narK2*::Gm strain complemented with p*narK* (■), and Δ*narK1K2*::Gm strain complemented with p*narK* (▲) is shown.

thermore, in *T. thermophilus*, complementation of the double mutant with either *narK1* or *narK2* restored the ability of the organism to grow anaerobically.

Nitrate uptake studies utilizing a nitrate-specific electrode yielded some interesting insights into the NarK1 and NarK2 protein function (Table 4). The Δ*narK1*::Gm mutant demonstrated nitrate uptake rates similar to that of the wild type. In contrast, both the Δ*narK2*::Gm and the Δ*narK1K2*::Gm mutants had very low, yet different, rates of nitrate uptake. This difference in nitrate uptake rate between the Δ*narK2*::Gm mutant and the double mutant was more than fivefold, indicating that both of the proteins may be involved with nitrate uptake. In addition, we observed no uptake of nitrate in a PAO1 *narG* mutant, thus connecting intracellular nitrite generation with nitrate uptake. This is consistent with the observation made for an *E. coli narG* mutant (24) and that of Ramirez et al. with *T. thermophilus* (22).

It is well established that during the denitrifying growth of *P. aeruginosa* in batch culture, there is a sequential reduction of nitrogen oxides (35). Similar results have been observed for other denitrifiers (1). The first product of denitrification, nitrite, is very toxic to the cells and thus excreted immediately upon reduction. This extracellular accumulation of nitrite continues to occur until the nitrate supply is exhausted. Moreover, previous studies have shown that nitrite reductase is located in the periplasm but does not participate in nitrite reduction until nitrate disappears from the external medium (30). Thus, we wanted to see if any of our mutants differentially accumulated nitrite in comparison to the wild type. The results indicate that both the Δ*narK2*::Gm and Δ*narK1K2*::Gm mutants demonstrated limited nitrite excretion but that the Δ*narK1*::Gm mutant excreted amounts of nitrite equivalent to that of the wild type. It is to be expected that a restriction in nitrate uptake would also limit nitrite production, and thus the results obtained for the Δ*narK2*::Gm and Δ*narK1K2*::Gm mutants may be explained in this manner. The *narK1* mutation did not seem to affect external nitrite accumulation when the wild type and mutant were normalized in protein content.

In a separate experiment, we used the *narK* gene of *E. coli* to complement our *narK2* mutant. This experiment was conducted because previous studies of nitrate/nitrite transport had been most extensively carried out with *E. coli* (2, 6, 20, 33), and recent studies concluded that the protein may operate as a nitrate/nitrite antiporter (4, 13). These conclusions were in contrast to the results reported for vesicle and proteoliposomes using ^{13}N nitrate (24), which did not support the antiporter mechanism. In the current study, the NarK protein of *E. coli* complemented both the Δ*narK2*::Gm mutant and the Δ*narK1K2*::Gm mutant of *P. aeruginosa* with respect to anaerobic, nitrate-dependent growth. This suggests that, functionally, the NarK2 protein of *P. aeruginosa* is similar to the NarK protein of *E. coli*. However, the issue of antiport versus uniport remains to be conclusively experimentally proven.

To summarize, in contrast to studies of other denitrifiers, such as *T. thermophilus* and *P. pantotrophus* (22, 36), the NarK1 protein is not as important for the anaerobic nitrate-dependent growth and survival of *P. aeruginosa*. However, both the anaerobic growth studies and nitrate uptake studies indicate some involvement of the NarK1 protein in *Pseudomonas* denitrification. For now, its role still remains enigmatic. One possibility is that the NarK1 protein is capable of taking up very small amounts of nitrate. Given that in a Δ*narK1*::Gm mutant the *narK2* functions at normal levels, a slight deficiency created by the lack of NarK1 is "masked" by the presence of NarK2. Therefore, no differences in nitrate uptake are observed between the wild type and the Δ*narK1*::Gm mutant. However, these differences become apparent on comparison of the Δ*narK2*::Gm and the Δ*narK1K2*::Gm strains. The fivefold difference observed between the two strains may be indicative of small amounts of nitrate uptake mediated by the NarK1 protein. Thus, the NarK1 protein may function in *P. aeruginosa* secondarily to NarK2. In the absence of NarK2, NarK1 would not be able to promote wild-type levels of nitrate-dependent, anaerobic growth but may provide just enough energy for the organism to sustain itself while it seeks other energy sources. Future studies would be needed to confirm the exact role of this protein.

Finally, in literature, the NarK-like proteins have been divided into two distinct subgroups: type I and type II (18). Both *E. coli* NarK and *P. aeruginosa* NarK2 have been classified as members of the type II group (18). On the other hand, *P. aeruginosa* NarK1 has been classified as a member of the type I group (18). Our results agree with the classification scheme for NarK2. However, it is difficult at the present time to corroborate the classification of NarK1, since its function is still unknown.

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