JOURNAL OF BACTERIOLOGY, Sept. 1993, p. 5867–5876
0021-9193/93/185867-10$02.00/0
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Vol. 175, No. 18

Structure and Function of a Periplasmic Nitrate Reductase in *Alcaligenes eutrophus* H16

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Received 29 April 1993/Accepted 7 July 1993

***Alcaligenes eutrophus* H16 shows three distinct nitrate reductase activities (U. Warnecke-Eberz and B. Friedrich, Arch. Microbiol. 159:405–409, 1993). The periplasmic enzyme, designated NAP (nitrate reductase, periplasmic), has been isolated. The 80-fold-purified heterodimeric enzyme catalyzed nitrate reduction with reduced viologen dyes as electron donors. The *nap* genes were identified in a library of *A. eutrophus* H16 megaplasmid DNA by using oligonucleotide probes based on the amino-terminal polypeptide sequences of the two NAP subunits. The two structural genes, designated *napA* and *napB*, code for polypeptides of 93 and 18.9 kDa, respectively. Sequence comparisons indicate that the putative gene products are translated with signal peptides of 28 and 35 amino acids, respectively. This is compatible with the fact that NAP activity was found in the soluble fraction of cell extracts and suggests that the mature enzyme is located in the periplasm. The deduced sequence of the large subunit, NAPA, contained two conserved amino-terminal stretches of amino acids found in molybdenum-dependent proteins such as nitrate reductases and formate dehydrogenases, suggesting that NAPA contains the catalytic site. The predicted sequence of the small subunit, NAPB, revealed two potential heme *c*-binding sites, indicating its involvement in the transfer of electrons. An insertion in the *napA* gene led to a complete loss of NAP activity but did not abolish the ability of *A. eutrophus* to use nitrate as a nitrogen source or as an electron acceptor in anaerobic respiration. Nevertheless, the NAP-deficient mutant showed delayed growth after transition from aerobic to anaerobic respiration, suggesting a role for NAP in the adaptation to anaerobic metabolism.**

Nitrate is a major nitrogen source for many bacteria. In the general assimilatory pathway, nitrate is converted via nitrite to ammonia, which is then assimilated into nitrogen metabolism. This metabolic route functions aerobically and anaerobically and involves assimilatory nitrate reductases which are repressed by ammonia. Nitrate can also serve as an electron acceptor for anaerobic respiration in the absence of oxygen. In this case nitrate is reduced by respiratory nitrate reductases to nitrite (reviewed in references 39 and 46). In a variety of bacteria, including *Alcaligenes eutrophus* H16 (24), the end product of nitrate respiration is dinitrogen. This denitrification pathway involves, in addition to the respiratory nitrate reductase, further respiratory reductases for nitrite, nitric oxide, and nitrous oxide (reviewed in reference 46).

A. eutrophus H16 is a gram-negative obligate respiratory bacterium which is able to grow heterotrophically on a broad range of substrates. Alternatively, it can grow autotrophically with hydrogen as an energy source, and in the absence of oxygen it can use nitrate or nitrite for anaerobic respiration (8). These alternative facultative metabolic pathways of *A. eutrophus* H16 are genetically linked to the megaplasmid pHG1 (13, 31). Physiological and genetic studies revealed the presence of three distinct nitrate reductase activities in this strain. One activity is subject to repression by ammonia and is insensitive to oxygen, indicating a nitrate-assimilatory function. A second, membrane-bound nitrate reductase activity is formed only in the absence of oxygen and is insensitive to repression by ammonia, pointing to a strictly respiratory function. In addition to these common enzyme types, a third nitrate reductase activity was detected in the soluble fraction of stationary-phase cells grown aerobically with ammonia. In contrast to the other two nitrate reductases, which are encoded on the chromosome of *A. eutrophus* H16, this third enzyme, designated NAP (nitrate reductase, periplasmic), appeared to be encoded by the megaplasmid pHG1 and was expressed independently of nitrate induction (43).

To elucidate the structure and function of NAP in *A. eutrophus*, the protein was purified to near homogeneity, the structural genes, designated *napA* and *napB*, were cloned, and their nucleotide sequences were determined. The physiological role of NAP in nitrate metabolism was investigated in studies with a mutant bearing a transposon insertion in one of the structural genes.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The bacterial strains, phages, and plasmids used are listed in Table 1. A series of plasmids carrying various segments of a 27.7-kb pHG1 DNA region was derived from cosmid pGE28 (44). Subclones pGE49 and pGE179 are deletion derivatives obtained by digestion with *Hin*dIII and *Cla*I. A 6.2-kb *Eco*RV-*Cla*I fragment of pGE49 was inserted into vector pVK102 to give pGE144. For this purpose, pVK102 was digested with *Hin*dIII, sticky ends were filled in by treatment with Klenow enzyme, and one blunt end was removed by digestion with *Cla*I. pGE182, which carries the 3-kb *Eco*RV-*Eco*RI fragment, is a cointegrate of pVK102 and pCH275 (see below). *Hin*dIII-linearized pCH275 was inserted into the *Hin*dIII site of pVK102, and pGE182 was constructed via a deletion of an *Eco*RI fragment.

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TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Relevant characteristics[a]	Reference or source
A. eutrophus		
H16	Nar$^+$ Nas$^+$ Nap$^+$ pHG1$^+$	DSM 428, ATCC 17699
HF210	Nar$^+$ Nas$^+$ Nap$^-$ pHG1$^-$; derivative of H16	17
HF326	Nar$^+$ Nas$^+$ Nap$^-$ *napA*::Tn*5*-B21	This study
E. coli		
S17-1	Tra$^+$ *recA pro thi hsdR chr*::RP4-2	37
DH1	*recA1 gyrA96 hsdR17 thi-1* λ$^-$	14
K38	F$^-$ *lacY1 leuB6 supE44 thi-1 thr-1 tonA21*	1
XL1-Blue	*recA1 endA1 gyrA96 thi hsdR17 supE44 recA1 lac* (F′ *proAB lacI*^{q} ZΔM15 Tn*10*)	Stratagene Cloning Systems, Inc.
Plasmids		
pVK102	Km^r Tc^r Mob$^+$ RP4*ori*	16
pSUP202	Ap^r Cm^r Tc^r Mob$^+$	37
pT7-5	Ap^r ColE1; T7 promoter	41
pGP1-2	Km^r; T7 RNA polymerase	41
pBluescript KS$^+$	Ap^r *lacZ′* f1*ori*; T7 promoter	Stratagene
pBluescript SK$^+$	Ap^r *lacZ′* f1*ori*; T7 promoter	Stratagene
pRME1	Ap^r Km^r; contains 1.2-kb Km^r cartridge	W. Messer
pGE28	27.7-kb pHG1 fragment in pVK102	44
pGE49	16-kb *Hin*dIII fragment of pGE28 in pVK102	This study
pGE144	6.2-kb *Eco*RV-*Cla*I fragment of pGE49 in pVK102	This study
pGE170	Tn*5* insertion derivative of pGE49	This study
pGE171	Tn*5* insertion derivative of pGE49	This study
pGE175	8-kb *Hin*dIII-*Cla*I fragment of pGE49 in pVK102	This study
pGE182	3-kb *Eco*RV-*Eco*RI fragment of pGE49 in cointegrate of pT7-5::pGE144	This study
pCH265	6.2-kb *Eco*RV-*Cla*I fragment of pGE49 in pSUP202	This study
pCH266	1.2-kb *Bam*HI Km^r cartridge from pRME1 in pCH265	This study
pCH270	*napA*::Tn*5*-B21 derivative of pCH266	This study
pCH275	6.2-kb *Eco*RV-*Cla*I (Klenow-treated) fragment in *Sma*I site of pT7-5	This study
pCH276	6.2-kb *Cla*I-*Eco*RV fragment in *Sma*I site of pT7-5	This study
pCH331	6.2-kb *Eco*RV-*Cla*I fragment of pGE49 in pBluescript KS$^+$	This study
pCH332	6.2-kb *Eco*RV-*Cla*I fragment of pGE49 in pBluescript SK$^+$	This study
pCH333	4.6-kb *Bgl*II-*Cla*I fragment of pGE49 in pBluescript KS$^+$	This study
pCH334	4.6-kb *Bgl*II-*Cla*I fragment of pGE49 in pBluescript SK$^+$	This study
Phages		
λ::Tn*5*-B21	Tc^r *b*221 *c*I857 *P*am80 Tn*5*-B21	38
λ::Tn*5*	Km^r *rex*::Tn*5*	4

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Mob, mobilizability; Tra, transfer of mobilizable plasmids; *ori*, origin of transfer.

Media and growth conditions. *Escherichia coli* strains were grown at 37°C in Luria broth (32). *A. eutrophus* strains were cultured aerobically and anaerobically in mineral medium. The following carbon sources were used: 0.2% (wt/vol) formate, 0.4% (wt/vol) pyruvate, 0.4% (wt/vol) gluconate, and 0.4% (wt/vol) fructose (43). Formate mineral medium contained 98 mM sodium potassium phosphate, pH 7.5. Incubation under anaerobic conditions was performed in 10-ml screw cap tubes filled to the brim with mineral medium containing 0.2% (wt/vol) potassium nitrate as the electron acceptor and 0.2% (wt/vol) ammonium chloride as the nitrogen source. The tubes were inoculated to an optical density of 0.1 at 436 nm with aerobically grown stationary-phase cells washed with 50 mM sodium potassium phosphate buffer, pH 7.5. Growth was monitored by measuring the optical density at 436 nm in a spectrophotometer (Spectronic 21; Milton Roy Company).

Purification of NAP. *A. eutrophus* H16 was grown aerobically to late stationary phase (50 h) in 5-liter Erlenmeyer flasks containing 3 liters of gluconate mineral medium at 30°C. The cells were harvested, and soluble extracts were prepared as described elsewhere (43). To prevent aggregation of NAP, a high salt concentration of 250 mM sodium potassium phosphate buffer, pH 6.5 (referred to below as phosphate buffer) was used throughout the purification, unless otherwise stated. All purification steps were carried out at 4°C. Proteins of the soluble fraction were precipitated by ammonium sulfate at 50% saturation. After centrifugation at 20,000 × *g* for 20 min, NAP was retained in the supernatant. The subsequent purification steps were carried out with a fast protein liquid chromatography system (Pharmacia LKB, Freiburg, Germany). The supernatant of the ammonium sulfate precipitation was applied to an alkyl Superose HR 5/5 column equilibrated with 1.7 M ammonium sulfate-containing phosphate buffer. Protein was eluted with a linear gradient of 1.7 to 0.2 M ammonium sulfate. Fractions containing NAP activity were desalted by Sephadex G-25 chromatography (column PD 10; exclusion capacity, >5,000) in the presence of 50 mM phosphate buffer (pH 6.5). Fractions were collected, pooled and applied to a MonoS HR 5/5 column equilibrated with 50 mM phosphate buffer (pH 6.5). Protein was eluted with a linear gradient of 0 to 250 mM phosphate buffer. The pooled fractions were concentrated by centrifugation (2,600 × *g*, 15 min) in Centriprep-10 concentrators (Amicon, Witten, Germany). Aliquots of 0.2 ml were subjected to gel filtration on a Superose 6 HR 10/30

column equilibrated with phosphate buffer. Fractions of 0.25 ml were eluted with the same buffer.

Molecular weight determination. The native molecular weight was determined by gel filtration with a Superose 6 HR 10/30 column. Molecular weight determinations of protein subunits were carried out by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19). Proteins were stained with Coomassie blue R250. A low-molecular-weight calibration kit (Pharmacia LKB) was used as a standard.

Protein determination. The protein contents in cell suspensions were determined by the method of Lowry et al. (22). The protein concentrations in cell extracts were determined by the bicinchoninic acid method, following the instructions of the manufacturer (Pierce, Rockford, Ill.). During chromatography, protein was monitored at 280 nm with a UV monitor (Pharmacia LKB).

Assay of NAP activity. NAP activity was determined by measuring the nitrite formed during reduction of nitrate, using the standard colorimetric method (21). The assay was performed under oxygen exclusion in a reaction mixture containing 1 ml of 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 5.5), 1 ml of 30 mM potassium nitrate, and 1 ml of 6 mM benzylviologen equilibrated with nitrogen gas and reduced with sodium dithionite at 50°C, as described previously (43). For qualitative detection of NAP activity during purification, a fast assay protocol was used under aerobic conditions with formate as the electron donor. The reaction was performed at 37°C in microtiter plates. Each well contained 50 μl of 50 mM MES buffer (pH 5.5), 50 μl of 30 mM potassium nitrate, and 50 μl of 1 M sodium formate. The reaction was started by the addition of 50 μl of extract and terminated after 10 to 20 min by the addition of 50 μl of nitrite reagent (21).

Analysis of the amino-terminal amino acid sequences. About 400 μg of the NAP preparation that had been obtained by gel filtration was desalted and separated by reversed-phase high-pressure liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan; two LC-6A pumps, SPD-7AV detector, Vydac C4 column [pore size, 300 Å; particle size, 5 μm, 75 by 4.6 mm]). For elution, 0.06% trifluoroacetic acid and 0.05% trifluoroacetic acid in acetonitrile (flow rate, 1 ml/min) were used. The chromatogram showed two peaks at 19.5 and 23.2 min. These fractions were collected and lyophilized. For amino-terminal sequence analysis, 75% of the first peak and 66% of the second peak were dissolved in trifluoroacetic acid and spotted onto a Polybrene-coated precycled glass fiber filter in a protein sequencer system (model 477A/120A; Applied Biosystems, Foster City, Calif.) with on-line HPLC analysis of the phenylthiohydantoin amino acids formed.

Cloning and sequencing. Standard DNA techniques were employed for cloning and sequencing (32). *nap* genes were identified in a cosmid library of megaplasmid pHG1 DNA (44) by DNA-DNA hybridization. Oligonucleotides based on the amino-terminal protein sequence were 5′ end labelled with [γ-^{32}P]dATP (Amersham Buchler, Braunschweig, Germany) and used as probes. For DNA sequence analysis, restriction fragments were inserted into the vector plasmids pBluescript KS/SK (Stratagene, Heidelberg, Germany). Serial deletions were generated by exonuclease III-S1 nuclease treatment with an Erase-a-Base kit (Promega Corp., Madison, Wis.). Double-stranded templates were isolated from *E. coli* XL1-Blue and sequenced by the dideoxynucleotide chain termination method (33). Sequencing reactions were done with [^{35}S]dATPαS, T7 DNA polymerase (Pharmacia LKB), or *Taq* polymerase (United States Biochemical Corp.) as recommended by the manufacturers. Compressions were resolved by using dITP or 7-deaza-GTP (Pharmacia LKB). Both strands of the *nap* region were sequenced. Gaps in the resulting sequence were filled by sequencing from oligonucleotide primers. DNA and protein sequences were analyzed with the PC/GENE software package (IntelliGenetics). Single and multiple alignments of derived amino acid sequences were done with the programs PCOMPARE, PALIGN, and CLUSTAL of PC/GENE, version 6.3.

Protein expression. The T7 RNA polymerase-promoter system (41) was used to detect expression of polypeptides from the *napA* and *napB* genes in *E. coli*. For these experiments a Klenow-treated 6.2-kb *Eco*RV-*Cla*I fragment was inserted in both orientations into the *Sma*I site of pT7-5, giving plasmids pCH275 and pCH276 (Table 1). The pT7-5 derivatives were transformed into *E. coli* K38 harboring plasmid pGP1-2. Transformants were induced at 42°C in the presence of rifampin (200 mg/ml) and L-[^{35}S]methionine (Amersham Buchler). Proteins were separated by SDS-PAGE in 12% (wt/vol) polyacrylamide gels (19).

Transposon mutagenesis. To introduce insertions into cloned *nap* genes, plasmid pGE49-harboring cells of *E. coli* S17-1 were infected with λ::Tn*5* (10). Tn*5* insertions were selected on kanamycin (50 μg/ml)- and tetracycline (10 μg/ml)-containing Luria broth plates. Doubly resistant transductants were mated with the megaplasmid-free derivative HF210 of *A. eutrophus*. Kanamycin- and tetracycline-resistant transconjugants were examined for NAP activity. Tn*5* insertions were mapped by restriction analysis.

Transposon Tn*5*-B21 (38) was used to introduce insertions into the megaplasmid pHG1 of *A. eutrophus*. This involved the cloning of an 8.6-kb *Cla*I fragment of plasmid pGE49 (Table 1) into the *Cla*I site of the tetracycline resistance gene of the suicide vector pSUP202 (37). Transformants of *E. coli* S17-1 were selected for tetracycline sensitivity and chloramphenicol and ampicillin resistance, yielding plasmid pCH265. To provide an appropriate selective marker for *A. eutrophus* H16, a kanamycin resistance cartridge was introduced into the *Bam*HI site of plasmid pCH265 as described previously (11), yielding plasmid pCH266. Transformants of *E. coli* S17-1 harboring pCH266 were infected with λ::Tn*5*-B21 (38). Transductants were selected as described previously (17). Tn*5*-B21 insertions on plasmid pCH266 were selected by mating tetracycline- and kanamycin-resistant transductants of *E. coli* S17-1 with *E. coli* DH1 as the recipient. Insertions were mapped by restriction analysis. Finally, an isogenic mutant of *A. eutrophus* H16 was obtained via a gene replacement protocol (17): plasmid pCH270 was transferred to *A. eutrophus* H16 by mating, and homogenote recombinants were selected as tetracycline-resistant, kanamycin-sensitive transconjugants.

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the EMBL, GenBank, and DDBJ data bases under accession no. X71385.

RESULTS

Purification of NAP. In order to provide a basis for the genetic analysis of the role of NAP in the physiology of *A. eutrophus*, we purified the NAP enzyme. The protein was isolated from cells that had been grown aerobically to the stationary phase in gluconate-ammonium mineral medium. Under these conditions, both the assimilatory nitrate reductase and the respiratory membrane-bound nitrate reductase

TABLE 2. Purification of NAP from *A. eutrophus*

Fraction	Vol (ml)	Total activity (U)	Sp act (U/mg of protein)	Yield (%)
Cell extract	218	468	0.4	100
Ammonium sulfate				
Precipitate	218	93	0.1	20
Supernatant	218	231	0.8	50
Alkyl Superose	34	120	2.4	26
Mono S	6.2	40	32.9	9
Gel filtration	3.5	14	29	3

are repressed (43). Eighty-fold purification of NAP was achieved in four steps: ammonium sulfate precipitation, separation by hydrophobic interaction, cation exchange, and gel filtration. Details of the purification protocol are given above, and a summary of the results is presented in Table 2 and Fig. 1. The enzyme preparation obtained by gel filtration showed three major and a few minor polypeptide bands after separation on SDS-PAGE (Fig. 1, lane 6).

Biochemical properties of NAP. Purification of NAP from the supernatant of the crude extract was possible without alkaline heat treatment or the aid of detergents. Thus, NAP is a soluble protein. The enzyme exhibited classical Michaelis-Menten kinetics with reduced benzyl viologen as the electron donor and nitrate as the substrate (see Materials and Methods). The K_m for nitrate was 0.12 mM, which is in the range reported for other bacterial nitrate reductases (40). The purified enzyme was active with viologen dyes such as methyl and benzyl viologen. No activity was observed with reduced pyridine and flavine nucleotides. Formate could serve as the electron donor for crude enzyme preparations but failed to mediate nitrate reduction by the purified NAP protein. The physiological electron donor for NAP is unknown.

The native molecular weight of purified NAP as determined by gel filtration was 92,000 ± 4,000 (data not shown). This value is in good agreement with the sum of the molecular masses of the two subunits (87 and 17 kDa) as estimated by SDS-PAGE of purified NAP (Fig. 1). The 87- and 17-kDa polypeptides were further purified by HPLC and used for determination of the amino-terminal protein sequence (Table 3). A slowly migrating 100-kDa polypeptide which copurified with NAP (Fig. 1) may be a contaminant or may even be a constituent of the NAP system which, however, is not essential for benzyl viologen-dependent nitrate reduction.

Cloning and localization of the *nap* structural genes. Previous studies on nitrate reductase-defective mutants indicated that the genetic determinants for NAP are located on the megaplasmid pHG1 of *A. eutrophus* (43). The experimentally determined amino-terminal sequences of the purified 87- and 17-kDa subunits provided the basis for the construction of two pairs of oligonucleotides (Table 3). These oligonucleotides were used to screen a cosmid library of megaplasmid pHG1 (44). A positive signal was obtained with cosmid pGE28 (data not shown). pGE28 contained a 27.7-kb insert and proved to be identical to a plasmid isolated from the same library in the course of cloning a gene for a flavohemoprotein (44). Plasmid pGE28 was introduced into the megaplasmid-free, NAP$^-$ derivative HF210 of *A. eutrophus* H16. Transconjugants exhibited wild-type levels of NAP activity (0.42 U/mg of protein).

Subcloning of pGE28 revealed that the smallest fragment

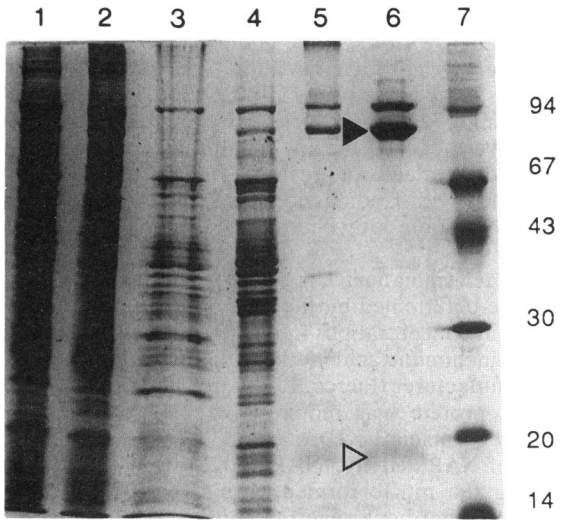


FIG. 1. Purification of the *A. eutrophus* H16 NAP protein. Protein samples collected at various stages of the purification procedure were separated on SDS-PAGE (12%) gels and stained with Coomassie blue. Lanes: 1, soluble extract; 2, pellet of the 50% ammonium sulfate precipitation; 3, supernatant of the ammonium sulfate precipitation; 4, eluate after alkyl Superose chromatography; 5, eluate after cation-exchange chromatography; 6, eluate after gel filtration; 7, molecular weight standards (sizes are given in thousands). NAPA is marked by a solid triangle, and NAPB is marked by an open triangle.

which restored NAP activity in *trans* was the 6.2-kb *Eco*RV-*Cla*I insert of pGE144 (Fig. 2). To localize the NAP genes more precisely, Tn*5* insertion mutants were isolated and analyzed for NAP activity. Insertions within a 1.5-kb *Sal*I-*Cla*I fragment of pGE144 (plasmids pGE170 and pGE171) did not affect NAP activity in the complementation test (Fig. 2), indicating that this region is not essential for NAP expression. On the other hand, a Tn*5*-B21 insertion in the 1-kb *Pst*I fragment of megaplasmid pHG1 (Fig. 2) led to a complete loss of NAP activity. This result clearly indicated that the genes for NAP reside on the internal segment of the 6.2-kb *Eco*RV-*Cla*I region. This was confirmed by Southern analysis of various restriction fragments of the 6.2-kb region with the oligonucleotide probes derived from the amino-terminal sequences of the purified subunits. The hybridizing fragments are marked by bars in Fig. 2. The genetic locus defined by the mutation was designated *nap*.

An in vivo T7 protein expression system (41) was used to detect proteins encoded by the 6.2-kb *Eco*RV-*Cla*I fragment. The 6.2-kb segment was inserted in both orientations downstream of the T7 promoter of vector pT7-5, yielding plasmids pCH275 and pCH276 (Table 1). Plasmid pCH275 directed the expression of two proteins (apparent M_rs, 70,000 and 17,000). The isogenic plasmid pCH276 carrying the same fragment in the opposite orientation did not produce any insert-linked proteins upon induction of the T7 promoter (data not shown). These results were again compatible with the mapping of the genes for NAP within the 6.2-kb *Eco*RV-*Cla*I fragment; in addition, they suggested that the genes are oriented from left to right on this fragment.

Nucleotide sequence analysis. A DNA region of 3,463 nucleotides located on the 6.2-kb *Eco*RV-*Cla*I fragment of pGE144 (Fig. 2) was sequenced. The nucleotide and derived amino acid sequences are presented in Fig. 3. Two open

TABLE 3. Amino-terminal protein sequences of mature NAPA and NAPB and oligonucleotides derived therefrom

NAPA[a]:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
A	N	F	V	T	D	S	E	V	T	K	L	K	W	S	K	A	P	C	R	F

5′-ACCAAGCTGAAGTGGTCGAAG-3′

5′-GCGAACTTCGTGACCGA-3′

NAPB[a]:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
G	L	V	D	A	M	R	G	P	T	A	I	A	N	E

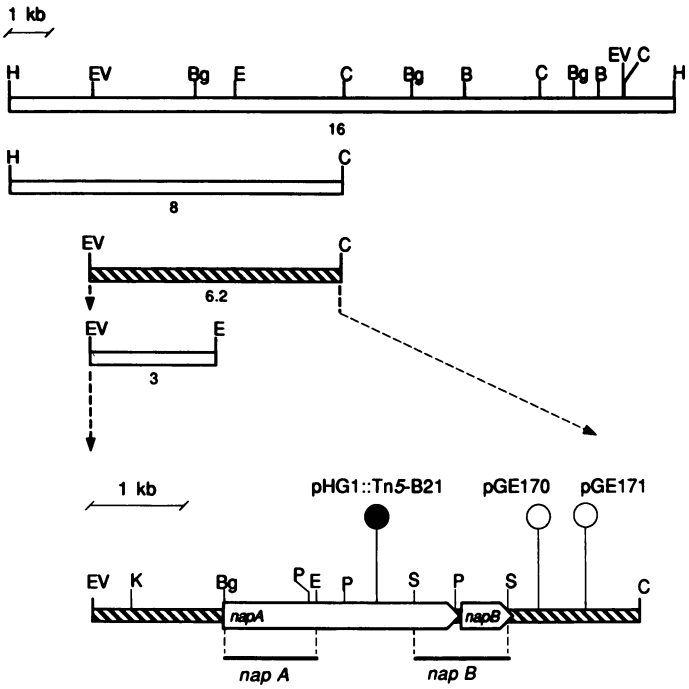
5′-GGCCCGACCGCGATCGCG-3′

5′-GGCCTGGTGGACGCGAT-3′

[a] The synthetic oligonucleotides were biased for the codon usage of *A. eutrophus* and used in Southern analysis as specific probes for *napA* and *napB*.

reading frames that are consistent with the molecular masses of the subunits of the purified NAP protein were identified. The open reading frames were designated *napA* and *napB*. The putative initiation codons of *napA* and *napB* and Shine-Dalgarno sequences are underlined in Fig. 3. *napA* starts at nucleotide position 296 and terminates with a TAG codon at position 2789. *napB* starts at position 2823 and terminates with a TAA codon at position 3330. The open reading frames are separated by an intergenic region of 32 nucleotides. The codon usages of *napA* and *napB* are typical of *A. eutrophus* genes (12, 42). Sequences resembling the *E. coli* consensus σ^{70} or σ^{54} promoter were not identified upstream of the *napA* initiation codon.

The *napA* gene of *A. eutrophus* encodes a polypeptide of 831 amino acids (M_r, 93,309), and the *napB* gene encodes a polypeptide of 169 amino acids (M_r, 18,924). These calculated molecular weights agree well with the sizes of the purified NAPA (87 kDa) and NAPB (17 kDa) subunits as determined by SDS-PAGE. The 21 amino-terminal amino acids of the mature NAPA subunit match amino acids 30



Plasmid	NAP activity U/mg
pGE49	0.63
pGE175	0.48
pGE144	0.49
pGE182	0
pGE170	0.47
pGE171	0.62
pHG1::Tn5-B21	0

FIG. 2. Physical and genetic maps of cosmid pGE49. The smallest fragment expressing NAP activity is indicated as a hatched bar. Open circles indicate Tn*5* insertions; the closed circle indicates a Tn*5*-B21 insertion. The open arrows indicate the actual boundaries of the *napAB* genes (drawn to scale), based on the sequence data (see text). Bars below the *Eco*RV-*Cla*I segment indicate the fragments that hybridize with the oligonucleotide probes shown in Table 3. Subcloned plasmids and plasmids with Tn*5* insertions are listed at the right, together with the NAP activity assayed in the corresponding transconjugants of *A. eutrophus* HF210. The values were determined with whole cells. The wild type contained 0.48 U/mg, and the pHG1-free derivative HF210 was devoid of NAP activity. B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I.

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TGCGCCCATACAGCCACTCCCATGCGGCGAGGAGTGGCATATCGCCGGCATCGTGGTGCACACCCTTCCCGACAGCCTAGATGCCCCAGGTGCGAGCGGC  100

CGCATTGAGGCATAACCGGCGCCGAGATCCACGCGGTCAGCGACGACGGCAAGCTGGTCGTCACCGTCGAGGCGCCCACGTCGCGCGCCATTGCCGCACA  200
                                                                                                  napA
CCTCACCTGCCTCCACCAGTTGGAGGGCGTGCTCTCCGCCGCGCTGGTCTACCAGCACAACGAAGACGCCGCAGCGATGAACGAGGAGATGGCCGATGAA  300
                                                                                                  M  K    2
GATCTCTCGTCGTGATTTCATCAAGCAGACCGCGATCACGGCTACCGCATCCGTGGCAGGCGTGACCCTGCCAGCGGGGGCCGCCAACTTTGTCACAGAC  400
 I  S  R  R  D  F  I  K  Q  T  A  I  T  A  T  A  S  V  A  G  V  T  L  P  A  G  A  A  N  F  V  T  D   35

AGCGAGGTGACCAAGCTGAAGTGGTCAAAGGCGCCATGCCGGTTCTGCGGCACAGGCTGCGGCGTCACCGTCGCCGTCAAGGACAACAAGGTGGTGGCCA  500
 S  E  V  T  K  L  K  W  S  K  A  P  C  R  F  C  G  T  G  C  G  V  T  V  A  V  K  D  N  K  V  V  A   68

CGCAAGGCGATCCGCAGGCCGAGGTCAACAAGGGCCTGAACTGCGTCAAGGGCTACTTCCTGTCCAAGATCATGTACGGGCAGGACCGGCTCACCAGGCC  600
T  Q  G  D  P  Q  A  E  V  N  K  G  L  N  C  V  K  G  Y  F  L  S  K  I  M  Y  G  Q  D  R  L  T  R  P  102

GCTCATGCGCATGAAGAACGGCAAGTACGACAAGAACGGCGATTTCGCGCCGGTCACCTGGGACCAGGCGTTCGACGAGATGGAGCGCCAGTTCAAGCGC  700
 L  M  R  M  K  N  G  K  Y  D  K  N  G  D  F  A  P  V  T  W  D  Q  A  F  D  E  M  E  R  Q  F  K  R  135

GTGCTGAAGGAAAAGGGTCCGACCGCGGTCGCATGTTCGGCTCCGGCCCAGTGGACGGTATGGGAAGGCTACGCGGCCGCCAAGCTCTACAAGGCAGGCT  800
 V  L  K  E  K  G  P .T  A  V  A  C  S  A  P  A  Q  W  T  V  W  E  G  Y  A  A  A  K  L  Y  K  A  G  168

TCCGCTCCAACAACATCGACCCCAATGCGCGGCATTGCATGGCATCGGCCGCGGCCGGCTTCATGCGCACCTTCGGCATGGACGAGCCGATGGGCTGCTA  900
F  R  S  N  N  I  D  P  N  A  R  H  C  M  A  S  A  A  A  G  F  M  R  T  F  G  M  D  E  P  M  G  C  Y  202

TGACGACTTCGAGGCCGCCGATGCCTTCGTGCTGTGGGGCTCGAACATGGCAGAGATGCACCCGATCCTGTGGACCCGCGTCACCGACCGCCGGCTGAGC 1000
 D  D  F  E  A  A  D  A  F  V  L  W  G  S  N  M  A  E  M  H  P  I  L  W  T  R  V  T  D  R  R  L  S  235

CACCCCAAGACCCGCGTGGTGGTGCTCTCCACCTTCACGCACCGGTGCTTCGACCTGGCCGACATTGGCATCATCTTCAAGCCGCAGACTGACCTGGCGA 1100
 H  P  K  T  R  V  V  V  L  S  T  F  T  H  R  C  F  D  L  A  D  I  G  I  I  F  K  P  Q  T  D  L  A  268

TGCTCAACTACATCGCCAACTACATCATCCGCAACAACAAGGTCAACAAGGACTTCGTCAACAAGCACACCGTCTTCAAAGAGGGCGTGACCGACATCGG 1200
M  L  N  Y  I  A  N  Y  I  I  R  N  N  K  V  N  K  D  F  V  N  K  H  T  V  F  K  E  G  V  T  D  I  G  302

CTACGGCCTGCGCCCGGACCATCCGCTGCAGAAGGCGGCCAAGAATGCCTCCGACCCCGGCGCGGCCAAAGTCATCACCTTCGACGAATTCGCGAAATTC 1300
 Y  G  L  R  P  D  H  P  L  Q  K  A  A  K  N  A  S  D  P  G  A  A  K  V  I  T  F  D  E  F  A  K  F  335

GTCTCGAAGTATGACGCCGACTACGTCAGCAAGCTGTCGGCCGTGCCCAAGGCCAAGCTCGACCAGCTCGCCGAACTGTACGCCGACCCCAACATCAAGG 1400
 V  S  K  Y  D  A  D  Y  V  S  K  L  S  A  V  P  K  A  K  L  D  Q  L  A  E  L  Y  A  D  P  N  I  K  368

TCATGTCGCTGTGGACCATGGGCTTCAACCAGCACACGCGCGGAACGTGGGCCAACAACATGGTCTACAACCTGCACTTGCTGACCGGCAAGATCGCCAC 1500
V  M  S  L  W  T  M  G  F  N  Q  H  T  R  G  T  W  A  N  N  M  V  Y  N  L  H  L  L  T  G  K  I  A  T  402

GCCGGGCAACAGCCCGTTCTCACTGACGGGGCAACCCTCCGCCTGCGGCACCGCGCGCGAGGTCGGCACCTTCTCGCACCGGCTGCCGGCGGACATGGTG 1600
 P  G  N  S  P  F  S  L  T  G  Q  P  S  A  C  G  T  A  R  E  V  G  T  F  S  H  R  L  P  A  D  M  V  435

GTCACCAACCCAAAGCATCGCGAAGAGGCCGAGCGCATCTGGAAGCTGCCGCCTGGCACCATCCCCGACAAGCCCGGCTACGACGCCGTGCTGCAGAACC 1700
 V  T  N  P  K  H  R  E  E  A  E  R  I  W  K  L  P  P  G  T  I  P  D  K  P  G  Y  D  A  V  L  Q  N  468

GCATGCTCAAGGACGGCAAGCTCAACGCCTACTGGGTGCAGGTCAATAACAACATGCAGGCCGCCGCCAACCTGATGGAGGAAGGGCTGCCGGGCTACCG 1800
R  M  L  K  D  G  K  L  N  A  Y  W  V  Q  V  N  N  N  M  Q  A  A  A  N  L  M  E  E  G  L  P  G  Y  R  502
                                                                                ▼
GAATCCCGCCAACTTCATCGTAGTGTCGGACGCCTACCCCACCGTGACCGCGCTGGCCGCCGACCTGGTCCTGCCCAGCGCGATGTGGGTCGAGAAGGAA 1900
 N  P  A  N  F  I  V  V  S  D  A  Y  P  T  V  T  A  L  A  A  D  L  V  L  P  S  A  M  W  V  E  K  E  535

GGCGCCTACGGCAACGCCGAACGCCGCACGCAGTTCTGGCACCAGCTGGTCGATGCTCCGGGCGAGGCGCGCTCGGACCTGTGGCAGCTGGTGGAGTTCG 2000
 G  A  Y  G  N  A  E  R  R  T  Q  F  W  H  Q  L  V  D  A  P  G  E  A  R  S  D  L  W  Q  L  V  E  F  568

CCAAGCGCTTCAAGGTGGAAGAGGTCTGGCCGCCCGAGCTGATCGCCAAAAAGCCGGAGTACAAGGGCAAGACCTTGTATGACGTGCTCTACCGCAACGG 2100
A  K  R  F  K  V  E  E  V  W  P  P  E  L  I  A  K  K  P  E  Y  K  G  K  T  L  Y  D  V  L  Y  R  N  G  602

CCAGGTCGACAAGTTCCCGCTCAAGGACGTCAACGCCGAATACCACAATGCCGAGGCCAAGGCCTTCGGCTTCTATCTCCAGAAGGGCCTGTTCGAGGAA 2200
 Q  V  D  K  F  P  L  K  D  V  N  A  E  Y  H  N  A  E  A  K  A  F  G  F  Y  L  Q  K  G  L  F  E  E  635
```

FIG. 3. Nucleotide sequence of the *A. eutrophus* H16 *nap* region. The amino acid sequence deduced from the *napA* and *napB* open reading frames is given in single-letter code below the nucleotide sequence. The signal peptides of NAPA and NAPB are italicized. Stop codons are indicated by asterisks, and Shine-Dalgarno sequences and initiation codons are underlined. The position of the Tn*5*-B21 insertion in *napA* is indicated by a solid triangle.

through 50 of the derived amino acid sequence. The 15 amino-terminal amino acids of the mature NAPB subunit match amino acids 36 through 50 of the derived amino acid sequence (Fig. 3). This indicates that the 5′ regions of *napA* and *napB* code for signal peptides of 28 and 35 amino acid residues, respectively (given in italics in Fig. 3). Hydropathy analysis (18) predicted that NAPA and NAPB are soluble proteins (data not shown). Taken together, these observations suggest that NAP is a periplasmic enzyme.

Sequence comparisons revealed local similarities between the NAP protein of *A. eutrophus* and the following molybdenum cofactor-containing enzymes: the catalytic subunits of the formate dehydrogenases of *Methanobacterium formicicum* (36), *E. coli* (3, 45), and *Wolinella succinogenes* (7) and the assimilatory nitrate reductase of *Klebsiella pneumoniae* M5al (20). Similarities were also found with the respiratory nitrate reductase (6), the dimethyl sulfoxide reductase (5), and the biotin sulfoxide reductase (25) of *E. coli*. The

```
TACGCCACCTTCGGCCGCGGTCACGGCCATGACCTGGCGCCGTTCGACGCCTACCACGAGGCGCGCGGCCTGCGCTGGCCCGTGGTCAACGGCAAGGAGA 2300
 Y  A  T  F  G  R  G  H  G  H  D  L  A  P  F  D  A  Y  H  E  A  R  G  L  R  W  P  V  V  N  G  K  E   668

CCCGCTGGCGCTACCGCGAAGGCAGCGACCCATACGTCAAGGCCGGCACCGGCTTCCAGTTCTACGGCAACCCCGACGGCAAGGCGGTGATCTTCGCCCT 2400
T  R  W  R  Y  R  E  G  S  D  P  Y  V  K  A  G  T  G  F  Q  F  Y  G  N  P  D  G  K  A  V  I  F  A  L  702

GCCCTACGAGCCGCCGGCCGAATCGCCCGACAAGGAATACCCGTACTGGCTAGTCACCGGCCGCGTGCTGGAGCACTGGCACTCCGGATCGATGACGCGG 2500
  P  Y  E  P  P  A  E  S  P  D  K  E  Y  P  Y  W  L  V  T  G  R  V  L  E  H  W  H  S  G  S  M  T  R   735

CGCGTGCCGGAGCTTTACCGTTCCTTCCCCAATGCGGTGGTGTTCATGCACCCGGAAGACGCGAAAGCGTTGGGGCTGCGCCGTGGCGTGGAGGTCGAGG 2600
 R  V  P  E  L  Y  R  S  F  P  N  A  V  V  F  M  H  P  E  D  A  K  A  L  G  L  R  R  G  V  E  V  E   768

TGGTGTCGCGGCGCGGCCGCATGCGCTCCCGCATCGAGACACGCGGGCGCGACGCGCCGCCGCGCGGGCTGGTGTTCGTGCCGTGGTTCGACGCCAGCCA 2700
V  V  S  R  R  G  R  M  R  S  R  I  E  T  R  G  R  D  A  P  P  R  G  L  V  F  V  P  W  F  D  A  S  Q  802

GCTGATCAACAAGGTAACGCTGGACGCTACCTGCCCGATCTCGCTGCAGACCGACTTCAAGAAGTGCGCGGTCAAGATCGTGAAGGTATAGCGTCGGTCG 2800
  L  I  N  K  V  T  L  D  A  T  C  P  I  S  L  Q  T  D  F  K  K  C  A  V  K  I  V  K  V  *            831
                                napB
AGACCAAGAACAGGGAGACGCCATGAAGCCAAGCCGATCCTGGGCAAGCCTGCTGGCCGTCTGCGCGGTGCTGCTGGCCGCGCTGGCCATGCAGGCGATC 2900
                                M  K  P  S  R  S  W  A  S  L  L  A  V  C  A  V  L  L  A  A  L  A  M  Q  A  I   26

TTTTTCCCCGCGCCCGCGCGCGCGCAGGGCCTGGTGGATGCCATGCGCGGCCCCACCGCCATCGCCAACGAGCCGCGCGCCCCGCTGCTCTACCCCACAG 3000
 F  F  P  A  P  A  R  A  Q  G  L  V  D  A  M  R  G  P  T  A  I  A  N  E  P  R  A  P  L  L  Y  P  T    59

AGAACAAGGACATCCGCCGCACGCGCAACTACACCATGCAGCCGCCGACGATCCCGCACAAGATCGACGGCTACCAGCTGGACAAGGACTTCAACCGCTG 3100
E  N  K  D  I  R  R  T  R  N  Y  T  M  Q  P  P  T  I  P  H  K  I  D  G  Y  Q  L  D  K  D  F  N  R  C   93

CATGTTCTGCCACGCGCGCACCCGCACCGAAGAGACCCAGGCGATCCCGGTCAGCATCACGCACTACATGGATCGCGACAACAACGTGCTGGCCGATGTT 3200
  M  F  C  H  A  R  T  R  T  E  E  T  Q  A  I  P  V  S  I  T  H  Y  M  D  R  D  N  N  V  L  A  D  V   126

TCGCCGCGGCGCTATTTCTGCACCCAATGCCACGTACCGCAGGCCGATACTAAGCCGCTGATCGGCAACAACTTCGTCGACGTGGACACCATCCTCAAGC 3300
 S  P  R  R  Y  F  C  T  Q  C  H  V  P  Q  A  D  T  K  P  L  I  G  N  N  F  V  D  V  D  T  I  L  K   159

GCAGGCCCGGCGCCAAGGGCGCCGCCAAATAAAACGAACAAAGTGGGTACGCCATGCTCGACCTGATCAAGCGCTACTGGCGGACGATCAACCGGCCCAG 3400
R  R  P  G  A  K  G  A  A  K  *                                                                        169

GGCCTATTTCAGCCTGGGTTTCTGACCCTGGGCGGGCTTCATTGCCGGCGTGATGTTCTGGGG                                        3463
```

FIG. 3—*Continued.*

amino-terminal part of NAPA shares two sequence elements with the catalytic subunits of the enzymes listed above (Fig. 4). The first cysteine-rich motif (Fig. 4A) may be involved in the coordination of iron-sulfur centers (6). The second element (Fig. 4B), covering a stretch of approximately 23 amino acid residues, may mark the active sites of the various molybdenum-containing enzymes (3).

Data base searches did not reveal obvious homologies between NAPB and other molybdoproteins. Nevertheless, an interesting feature of the *napB* translation product is that two putative heme *c*-binding sites (C-X-X-C-H-X) were found at nucleotide positions 93 to 97 and 133 to 137. These signatures are also present in the cytochrome *c* family of electron carrier proteins such as the periplasmic high-molecular-weight cytochrome *c* from *Desulfovibrio vulgaris* Hildenborough (26).

Phenotypic properties of a NAP$^-$ mutant. Comparison of the nucleotide sequence data and the results of restriction mapping showed that the Tn*5*-B21 insertion in plasmid pCH270 (Table 1) was located within the *napA* gene. Sequencing of the insertion borders pinpointed the insertion site at nucleotide position 1877 (Fig. 3). This allele was used to construct an isogenic *nap* mutant via gene replacement. The resulting mutant, HF326, was subjected to physiological studies. HF326 was devoid of NAP activity; assimilatory nitrate reductase and membrane-bound respiratory nitrate reductase activities were not affected in the mutant strain (data not shown).

To further explore the physiological function of NAP, the growth characteristics of mutant HF326 were examined. Under aerobic conditions, neither heterotrophic growth on a variety of carbon sources nor autotrophic growth with hydrogen as the energy source were affected. The ability to grow on nitrate as the nitrogen source was also unchanged in the mutant (data not shown). However, when nitrate was the terminal electron acceptor, the mutant strain showed a significant delay in growth after a shift from aerobic to anaerobic conditions (Fig. 5). This observation was reproducible, and one representative experiment is shown in Fig. 5. Growth retardation was dependent on the carbon source and was most pronounced with formate as the substrate (Fig. 5). In contrast to the megaplasmid-free strain, which lacks, in addition to NAP, essential functions for denitrification (31), the NAP$^-$ mutant retained the ability to denitrify.

DISCUSSION

Multiple nitrate reductase enzymes are found in various microorganisms, including certain nonsulfur photosynthetic bacteria (reviewed in reference 15), the aerobic denitrifyer *Thiosphaera pantotropha* (29, 30), and enterobacteria (20). In a previous report we presented evidence for the presence of three distinct nitrate reductase activities in *A. eutrophus* H16. One was assigned an assimilatory function, another showed features typical of the widespread membrane-bound respiratory nitrate reductases, and the third, designated NAP, remained to be classified. Unlike that of the more common nitrate reductases, the formation of NAP was not dependent on nitrate induction and anaerobiosis and was not sensitive to repression by ammonia (43).

The experiments reported here were designed to provide molecular data on NAP and to explore its physiological role.

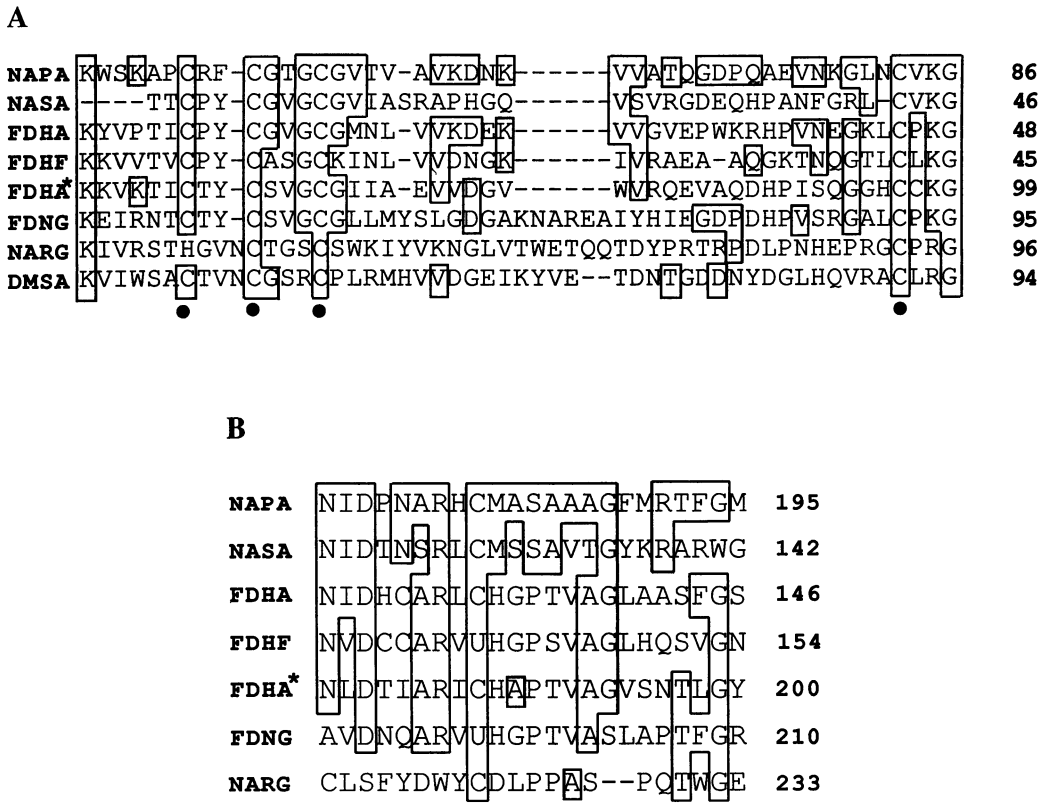

FIG. 4. Similar sequence elements in prokaryotic molybdenum cofactor-binding polypeptides. NAPA, nitrate reductase of *A. eutrophus* H16; NASA, assimilatory nitrate reductase of *K. pneumoniae* M5al (20); FDHA, formate dehydrogenase of *M. formicicum* (36); FDHF, formate dehydrogenase-H of *E. coli* (45); FDHA*, formate dehydrogenase of *W. succinogenes* (7); FDNG, formate dehydrogenase-N of *E. coli* (3); NARG, respiratory nitrate reductase of *E. coli* (6); DMSA, dimethyl sulfoxide reductase of *E. coli* (5). Amino acids identical to those in NAPA are boxed. (A) Cysteine cluster (marked by dots); (B) putative catalytic site. Amino acid residues are indicated in the standard single-letter code (U stands for selenocysteine).

NAP was isolated and partially characterized biochemically. Purification of the enzyme was carried out without alkaline heat treatment or the use of detergents, suggesting that NAP is a soluble protein. Comparison of the sequences of amino-terminal polypeptide chains of the mature NAP subunits with the amino acid sequences predicted from the nucleotide sequence pointed out the presence of signal peptides of 28 and 35 amino acids, respectively. These features are consistent with the assignment of NAP to the periplasm. Soluble periplasmic nitrate reductases have been found in *Rhodobacter capsulatus* (23), *Rhodobacter sphaeroides* f. sp. *denitrificans* (35), and *T. pantotropha* (2). Like *A. eutrophus*, some of these strains contain an additional membrane-bound nitrate reductase activity. Membrane-bound nitrate reductases usually consist of three subunits, α, β, and γ, in a ratio of 1:1:2. The α subunit has a molecular mass of approximately 150 kDa and contains the active site for nitrate reduction. The precise function of the β subunit, with a molecular mass of approximately 60 kDa, is unknown, but it has been suggested that it mediates subunit interaction and membrane association. The γ subunit has a molecular mass of approximately 20 kDa and is the nitrate reductase-specific cytochrome b_{556} (reviewed in reference 39).

In this report we present genetic evidence that NAP of *A. eutrophus* H16 is a dimer consisting of one 93-kDa polypeptide and one 19-kDa polypeptide. Mutant studies and complementation analysis gave no indication of additional protein components. This is consistent with the occurrence of one large- and one small-subunit species of NAP enzyme purified from *A. eutrophus*. Sequence analysis revealed two putative heme-binding sites typical of *c*-type cytochromes in the small-subunit polypeptide, indicating a role in the transport of electrons. The minimal catalytically active form of the periplasmic nitrate reductase of *R. sphaeroides* f. sp *denitrificans* consisted of a single catalytic subunit and *c*-type cytochrome (35). The periplasmic nitrate reductase of *R. capsulatus* was isolated as a complex of an 83-kDa molybdenum-containing subunit and a 13-kDa cytochrome c_{552} subunit (28). Thus, in contrast to the membrane-bound nitrate reductases, the periplasmic nitrate reductases appear to be composed of only two subunit species and contain *c*-type instead of *b*-type cytochromes.

Although the molybdenum and iron contents of NAP have yet to be experimentally determined, two lines of evidence argue that NAP is a molybdenum-containing protein. (i) Molybdenum-deficient mutants of *A. eutrophus* are impaired in several molybdenum cofactor-containing enzymes, including NAP (43). (ii) Sequence comparisons between NAPA and molybdenum cofactor-containing proteins such as formate dehydrogenase and nitrate reductase revealed two conserved segments, as follows. Four of the 11 cysteine residues are located at the amino-terminal part of the polypeptide chain and form a cluster of C-X_2-C-X_3-C-X_{27}-C resembling the 4Fe-4S centers found in bacterial ferredoxins

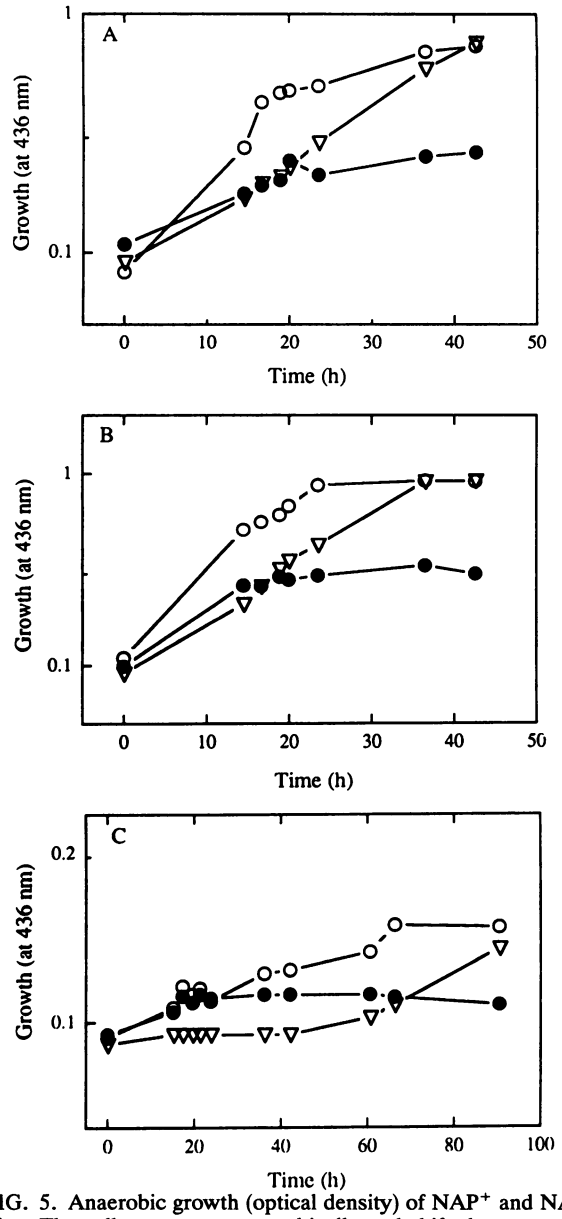

FIG. 5. Anaerobic growth (optical density) of NAP$^+$ and NAP$^-$ strains. The cells were grown aerobically and shifted to anaerobic conditions as described in Materials and Methods. The carbon sources used were fructose (A), pyruvate (B), and formate (C). Symbols: ○, wild-type H16; ▽, Nap$^-$ mutant HF326; ●, megaplasmid-free (Nap$^-$) derivative HF210.

(9). The NAPA cysteine cluster is unusual with respect to spacing and the lack of a proline residue normally found immediately after the fourth cysteinyl residue. Nevertheless, it suggests a structural model in which this part of the protein coordinates an iron-sulfur center, as proposed for similar cysteine clusters found in the formate dehydrogenases of *E. coli* (3) and the NAD-reducing hydrogenase of *A. eutrophus* (42). The second conserved segment of 23 amino acid residues may form the active site of molybdenum-dependent enzymes (3). This part of NAPA shows local homology to NASA of *K. pneumoniae* M5al (20) and formate dehydrogenases from various sources (3, 7, 36, 45).

Inactivation of NAPA by insertion mutagenesis led to a complete loss of NAP activity in *A. eutrophus*. Comparative growth studies with the NAP$^-$ mutant and the wild type, however, failed to show clear-cut effects. The mutant retained the ability to use nitrate as the nitrogen source and as the sole electron acceptor for anaerobic respiration. Although denitrification by the NAP$^-$ mutant was delayed, we conclude that NAP is not an essential function for nitrate assimilation and respiration. This is in accordance with previous observations on the physiological level indicating that the *nap* locus is unable to complement mutations in either the assimilatory or the membrane-bound nitrate reductase (43). In *T. pantotropha* the periplasmic nitrate reductase is expressed under both anaerobic and aerobic conditions. This enzyme is supposed to catalyze the first step in aerobic denitrification (2, 29). In contrast to *T. pantotropha*, *A. eutrophus* is incapable of denitrification under aerobiosis (34), and NAP activity is extremely low under complete oxygen exclusion (43). Thus, the role of NAP in the physiology of *A. eutrophus* is not clear.

We entertain two hypotheses on the physiological function of NAP. (i) NAP may be an oxidoreductase specific for certain reductants. In this context it is interesting that formate was the only physiological electron donor that mediated nitrate reduction with partially purified NAP preparations. The formate dehydrogenase-nitrate oxidoreductase complex of enterobacteria is one of the best characterized respiratory systems (39). (ii) NAP may be of benefit to *A. eutrophus* when the redox balance of the cells is disturbed. Such a situation could, in principle, occur during the transition from aerobic to anaerobic growth. NAP might permit reducing equivalents from reduced substrates to be transferred to nitrate as a preferred electron sink. This hypothesis explains the delayed growth of the NAP-deficient mutant under denitrifying conditions. The possibility that a major function of periplasmic nitrate reductase is the maintenance of redox balance for *R. capsulatus* and *T. pantotropha* has also been discussed (27).

Much more work is required to define precisely the structure and physiological function of periplasmic nitrate reductases. The genetic approach presented in this communication is one step toward this goal.

ACKNOWLEDGMENTS

We thank Edward Schwartz for critical reading of the manuscript. We are grateful to Valley Stewart for providing sequence data in press.

This work was supported by grants from the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

REFERENCES

1. **Appleyard, R. K.** 1954. Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K-12. Genetics **39**:440–446.
2. **Bell, L. C., D. J. Richardson, and S. J. Ferguson.** 1990. Periplasmic and membrane-bound respiratory nitrate reductases in *Thiosphaera pantotropha*; the periplasmic enzyme catalyzes the first step in aerobic denitrification. FEBS Lett. **265**:85–87.
3. **Berg, B. L., J. Li, J. Heider, and V. Stewart.** 1991. Nitrate-inducible formate dehydrogenase in *Escherichia coli* K-12. I. Nucleotide sequence of the *fdnGHI* operon and evidence that opal (UGA) encodes selenocysteine. J. Biol. Chem. **1266**:22380–22385.
4. **Berg, D. E., J. Dewis, B. Allet, and J. D. Rochaix.** 1975. Transposition of R factor genes to bacteriophage lambda. Proc. Natl. Acad. Sci. USA **72**:3628–3632.
5. **Bilous, P. T., S. T. Cole, W. F. Anderson, and J. H. Weiner.** 1988. Nucleotide sequence of the *dmsABC* operon encoding the anaerobic dimethyl-sulfoxide reductase of *Escherichia coli*.

Mol. Microbiol. 2:785–795.

6. **Blasco, F., C. Iobbi, G. Giordano, M. Chippaux, and V. Bonnefoy.** 1989. Nitrate reductase of *Escherichia coli*: completion of the nucleotide sequence of the *nar* operon and reassessment of the role of the α and β subunits in iron binding and electron transfer. Mol. Gen. Genet. **218:**249–256.
7. **Bokranz, M., M. Gutmann, C. Körtner, E. Kojro, F. Fahrenholz, F. Lauterbach, and A. Kröger.** 1991. Cloning and nucleotide sequence of the structural genes encoding the formate dehydrogenase of *Wolinella succinogenes*. Arch. Microbiol. **156:**119–128.
8. **Bowien, B., and H. G. Schlegel.** 1981. Physiology and biochemistry of aerobic hydrogen-oxidizing bacteria. Annu. Rev. Microbiol. **35:**405–452.
9. **Bruschi, M., and F. Guerlesquin.** 1988. Structure, function and evolution of bacterial ferredoxins. FEMS Microbiol. Rev. **54:** 155–176.
10. **de Bruijn, F., and J. R. Lupski.** 1984. The use of transposon Tn*5* mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids—a review. Gene **27:**131–149.
11. **Eberz, G., and B. Friedrich.** 1991. Three *trans*-acting regulatory functions control hydrogenase in *Alcaligenes eutrophus*. J. Bacteriol. **173:**1845–1854.
12. **Eitinger, T., and B. Friedrich.** 1991. Cloning, nucleotide sequence, and heterologous expression of a high-affinity nickel transport gene from *Alcaligenes eutrophus*. J. Biol. Chem. **266:**3222–3227.
13. **Friedrich, B., and E. Schwartz.** Molecular biology of hydrogen utilization in aerobic chemolithotrophs. Annu. Rev. Microbiol., in press.
14. **Hanahan, D.** 1983. Studies of transformation of *Escherichia coli* with plasmid. J. Mol. Biol. **166:**557–580.
15. **Hochstein, L. I., and G. A. Tomlinson.** 1988. The enzymes associated with denitrification. Annu. Rev. Microbiol. **42:**231–261.
16. **Knauf, V. C., and E. W. Nester.** 1982. Wide host range cloning vectors: a cosmid clone bank of an *Agrobacterium* Ti plasmid. Plasmid **8:**45–54.
17. **Kortlüke, C., K. Horstmann, E. Schwartz, M. Rohde, R. Binsack, and B. Friedrich.** 1992. A gene complex coding for the membrane-bound hydrogenase of *Alcaligenes eutrophus* H16. J. Bacteriol. **174:**6277–6289.
18. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
19. **Laemmli, U.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
20. **Lin, J. T., B. S. Goldman, and V. Stewart.** 1993. Structure of genes *nasA* and *nasB* encoding assimilatory nitrate and nitrite reductase in *Klebsiella pneumoniae* M5al. J. Bacteriol. **175:** 2370–2378.
21. **Lowe, R. H., and H. J. Evans.** 1964. Preparation and some properties of soluble nitrate reductase from *Rhizobium japonicum*. Biochim. Biophys. Acta **85:**377–389.
22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
23. **McEwan, A. G., H. G. Wetzstein, O. Meyer, J. B. Jackson, and S. J. Ferguson.** 1987. The periplasmic nitrate reductase of *Rhodobacter capsulatus*—purification, characterization and distinction from a single reductase for trimethyl-N-oxid, dimethylsulphoxide and chlorate. Arch. Microbiol. **147:**340–345.
24. **Pfitzner, J., and H. G. Schlegel.** 1973. Denitrifikation bei *Hydrogenomonas eutropha* Stamm H16. Arch. Microbiol. **90:**199–211.
25. **Pierson, D., and A. Campbell.** 1990. Cloning and nucleotide sequence of *bisC*, the structural gene for biotinsulfoxide reductase in *Escherichia coli*. J. Bacteriol. **172:**2194–2198.
26. **Pollock, W., R. Brent, M. Loutfi, M. Bruschi, B. J. Rapp-Giles, J. D. Wall, and G. Voourdouw.** 1991. Cloning, sequencing, and expression of the gene encoding the high-molecular-weight cytochrome *c* from *Desulfovibrio vulgaris* Hildenborough. J. Bacteriol. **173:**220–228.
27. **Richardson, D. J., and S. J. Ferguson.** 1992. The influence of carbon substrate on the activity of the periplasmic nitrate reductase in aerobically grown *Thiosphaera pantotropha*. Arch. Microbiol. **157:**535–537.
28. **Richardson, D. J., G. McEwan, M. D. Page, J. B. Jackson, and S. J. Ferguson.** 1990. The identification of cytochromes involved in the transfer of electrons to the periplasmic NO_3-reductase of *Rhodobacter capsulatus* and resolution of a soluble NO_3-reductase-cytochrome-c_{552} redox complex. Eur. J. Biochem. **194:**263–270.
29. **Robertson, L. A., and J. G. Kuenen.** 1984. Aerobic denitrification: a controversy revisited. Arch. Microbiol. **139:**351–354.
30. **Robertson, L. A., and J. G. Kuenen.** 1989. Combined heterotrophic nitrification and aerobic denitrification in *Thiosphaera pantotropha* and other bacteria. Antonie van Leeuwenhoek **57:**139–152.
31. **Römermann, D., and B. Friedrich.** 1985. Denitrification by *Alcaligenes eutrophus* is plasmid dependent. J. Bacteriol. **162:**852–854.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
34. **Sann, R.** 1990. Dissimilatorische Nitrit-Reduktase von *Alcaligenes eutrophus*: Enzymatik und Regulation. Ph.D. thesis, Free University of Berlin, Berlin, Germany.
35. **Satoh, T.** 1981. Soluble dissimilatory nitrate reductase containing cytochrome c from a photodenitrifier, *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*. Plant Cell Physiol. **22:**443–452.
36. **Shuber, A. P., E. C. Orr, M. A. Recny, P. F. Schendel, H. D. May, N. L. Schauer, and J. G. Ferry.** 1986. Cloning, expression, and nucleotide sequence of the formate dehydrogenase genes from *Methanobacterium formicicum*. J. Biol. Chem. **261:**12942–12947.
37. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Biotechnology **1:**784–791.
38. **Simon, R., J. Quandt, and W. Klipp.** 1989. New derivatives of transposon Tn*5* suitable for mobilization of replicons, generation of operon fusions, and induction of genes in Gram-negative bacteria. Gene **80:**161–169.
39. **Stewart, V.** 1988. Nitrate respiration in relation to facultative metabolism in enterobacteria. Microbiol. Rev. **52:**190–232.
40. **Stouthamer, A. H.** 1976. Biochemistry and genetics of nitrate reductase in bacteria. Adv. Microb. Physiol. **14:**315–375.
41. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
42. **Tran-Betcke, A., U. Warnecke, C. Böcker, C. Zaborosch, and B. Friedrich.** 1990. Cloning and nucleotide sequences of the genes for the subunits of NAD-reducing hydrogenase of *Alcaligenes eutrophus* H16. J. Bacteriol. **172:**2920–2929.
43. **Warnecke-Eberz, U., and B. Friedrich.** 1993. Three nitrate reductase activities in *Alcaligenes eutrophus*. Arch. Microbiol. **159:**405–409.
44. **Weihs, V., K. Schmidt, B. Schneider, and B. Friedrich.** 1989. The formation of an oxygen-binding flavohemoprotein in *Alcaligenes eutrophus* is plasmid-determined. Arch. Microbiol. **151:** 546–550.
45. **Zinoni, F., A. Birkmann, T. C. Stadtman, and A. Böck.** 1986. Nucleotide sequence and expression of the selenocysteine-containing polypeptide of formate dehydrogenase (formate-hydrogen-lyase-linked) from *Escherichia coli*. Proc. Natl. Acad. Sci. USA **83:**4650–4654.
46. **Zumft, W. G.** 1992. The denitrifying prokaryotes, p. 554–580. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes: a handbook on the biology of bacteria, 2nd ed., vol. 1. Ecophysiology, isolation, identification, applications. Springer-Verlag, Berlin.