Analysis of genes involved in nitrate reduction in *Clostridium perfringens*

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We have conducted the genetic analysis of fermentative nitrate reduction in *Clostridium perfringens*, a strict anaerobic bacterium. Nitrate reductase (NarA) was purified from the cytoplasmic fraction of the organism. Using a degenerate primer designed from its N-terminal amino acid sequence, a 9.5 kb fragment containing seven ORFs was cloned. The molecular mass and N-terminal amino acid sequence predicted from the nucleotide sequence of ORF 4 coincided with those determined for the purified NarA, indicating that ORF 4 corresponds to a *narA* gene. ORFs 5 and 6 encode a 15.4 kDa ferredoxin-like protein containing four iron-sulfur clusters and a 45 kDa protein homologous to NADH oxidase, respectively. Analyses involving primer extension and Northern blotting revealed that these three ORFs are transcribed as a polycistronic message. The ORF 5- and ORF 6-encoded proteins were shown by immunoblotting to be synthesized by cells grown in the presence of nitrate. Thus, these two proteins are likely to function as electron-transfer components in nitrate reduction in *C. perfringens*. The 9.5 kb fragment and a downstream region of 6.1 kb do not contain any genes involved in nitrate uptake or nitrite reduction. Instead, all 5 ORFs downstream of ORF 6 are homologous to genes reported for molybdopterin biosynthesis, unlike the genomic organization already determined for the respiratory and assimilatory nitrate-reduction systems. The evolutionary relationships between these two nitrate-reduction systems and the fermentative one based on the results of comparative genetic analysis are discussed.

Keywords: *Clostridium perfringens*, nitrate reductase, molybdopterin guanine dinucleotide, ferredoxin-like protein, NADH oxidase

INTRODUCTION

Nitrate is produced from molecular nitrogen mainly by bacteria through nitrogen fixation and nitrification, and is universally present as the principal storage form of nitrogen in soil. It is utilized by many bacteria, fungi, algae and plants as a nitrogen source via nitrate assimilation. The physiology and genetics of nitrate assimilation have been well studied in plants and fungi (Campbell & Kinghorn, 1990; Crawford & Arst, 1993; Crawford, 1995; Campbell, 1996), and more

recently in bacteria, such as *Synechococcus*, *Bacillus*, *Klebsiella* and *Azotobacter* (Lin & Stewart, 1998). Nitrate assimilation involves three pathway-specific steps: uptake of nitrate, reduction of nitrate to nitrite catalysed by nitrate reductase (Nar) and the six-electron reduction of nitrite to ammonium by nitrite reductase (Nir). Nitrate respiration is another metabolic process involving nitrate reduction; nitrate can be used as an electron acceptor under anaerobic conditions. Respiratory Nar couples substrate oxidation to nitrate reduction to generate the proton-motive force, and the resultant nitrite is finally converted to ammonium ions via ammonification or to gaseous nitrogen via denitrification. Respiratory Nar is widespread in both the Eubacteria (Berks *et al.*, 1995) and Archaea (Zumft, 1992; Völkl *et al.*, 1993) kingdoms. Its bioenergetics and genetics have been studied most extensively in *Para-*

Abbreviations: Nar, nitrate reductase; NarA, nitrate reductase of *Clostridium perfringens*; Nir, nitrite reductase; Mo-MGD, a molybdenum-molybdopterin guanine dinucleotide complex.

The GenBank accession number for the sequence reported in this paper is AB017192.

coccus denitrificans, *Escherichia coli* and *Rodobacter capsulatus* (Berks *et al.*, 1995; Gennis & Stewart, 1996).

Both assimilatory and respiratory NaRs are molybdo-enzymes containing a molybdopterin cofactor and an iron-sulfur cluster. They differ in that the former is soluble in the cytoplasm, while the latter is membrane bound. They also differ in the regulation of enzyme synthesis: the synthesis of the former is controlled by the availability of fixed nitrogen, e.g. glutamine and ammonium ions (Lin & Stewart, 1998), while the synthesis of the latter is independent of their availability. Another type of NaR, which is more similar to assimilatory NaR than to respiratory NaR (Berks *et al.*, 1995), has been identified in the periplasm, although its physiological function has not yet been established.

Nitrate reduction in strict anaerobes has attracted much less interest and is not well understood mainly because of the lack of suitable bacteria for physiological and genetic studies. *Clostridium perfringens*, a strict anaerobic pathogen, lives in soil, water, and the intestines of humans and other animals. Some strains of the organism can reduce nitrate to ammonium ions (Hasan & Hall, 1975). NaR (Seki-Chiba & Ishimoto, 1977) and NiR (Sekiguchi *et al.*, 1983) of *C. perfringens*, hereinafter designated NarA and NirA, respectively, were purified and their biochemical properties were characterized. NarA is a soluble molybdoenzyme whose molecular mass was estimated to be approximately 80 kDa (Seki-Chiba & Ishimoto, 1977; Seki *et al.*, 1987). The enzyme can catalyse NAD(P)H-dependent nitrate reduction in the presence of rubredoxin (Seki *et al.*, 1989) and a partially purified NAD(P)H-rubredoxin reductase (Seki *et al.*, 1988). Its location and molecular size are more similar to those of assimilatory NaRs than to those of respiratory NaRs. However, induction of the enzyme activity by nitrate is independent of the presence of ammonium ions in the culture medium, as in the case of respiratory NaRs (Hasan & Hall, 1975). NirA does not contain siroheme, unlike assimilatory NiRs, or haem or copper, unlike respiratory NiRs. Its molecular mass, 54 kDa, is significantly less than those reported for assimilatory and respiratory NiRs (63 kDa for higher plant NiRs, and 88–140 kDa for fungal and bacterial NiRs) (Sekiguchi *et al.*, 1983). The nitrite reduction by NirA generates hydroxylamine as the main product and possibly nitric oxide, but not ammonium ions (Sekiguchi *et al.*, 1983). Thus, nitrate reduction in *C. perfringens* differs in many respects from assimilatory and respiratory nitrate reduction. This unique nitrate-reduction system has been considered not to be linked to nitrate assimilation but to energy production: this is called nitrate fermentation (Takahashi *et al.*, 1963; Ishimoto *et al.*, 1974; Hasan & Hall, 1975).

NarA and NirA were well studied until the late 1980s. Since then, however, no further work has been conducted and much of this system remains to be elucidated. In an attempt to determine the molecular characteristics of NarA and NirA, we have undertaken the cloning and sequencing of their genes (*narA* and *nirA*). Nitrate is

reduced by bacteria to toxic compounds when it is ingested along with meals (Calmels *et al.*, 1996; Vermeer *et al.*, 1998). Considering that *C. perfringens* inhabits the intestines of humans and other animals, and also that it may generate potentially harmful byproducts of nitrate reduction such as hydroxylamine and nitrous oxides (Sekiguchi *et al.*, 1983), genetic analysis of nitrate reduction in *C. perfringens* is also of medical importance. Moreover, it would provide insights into the evolutionary relationships between the fermentative and other nitrate-reduction enzymes. In this study we have characterized the *nar* operon of *C. perfringens* and the downstream genes involved in molybdopterin synthesis, and compare them with already characterized homologous genes.

METHODS

Bacterial strains and plasmids. The *C. perfringens* strain used in this study was type A PB6K (Soda *et al.*, 1968). The plasmid vector, pT7Blue T-vector, and the host strain, *E. coli* NovaBlue (Novagen), were used for cloning DNA fragments obtained by PCR. To express the *narB* gene, we used pET-11a, a T7 RNA polymerase based expression vector (Novagen), and *E. coli* BL21(DE3) as the plasmid vector and host strain, respectively. To express the *narC* gene, we used pGEX-4T-2 and *E. coli* DH5 α as the plasmid vector and host strain, respectively. The plasmid vector pUC19 (Messing, 1983) and the host strain *E. coli* DH5 α (BRL) were used for all other recombinant DNA experiments.

Media and culture conditions. *C. perfringens* PB6K was precultured in cooked-meat medium (Nissui Pharmaceuticals) at 37 °C for 12 h. The preculture was diluted 200-fold with fresh medium, GAM (Nissui Pharmaceuticals) or PYG broth (7.5 g polypeptone, 5 g yeast extract, 2.5 g glucose and 50 μ l thioglycolic acid l⁻¹; Seki-Chiba & Ishimoto, 1977) and incubated at 37 °C. KNO₃ was added to the medium to a final concentration of 0.2% (w/v), when necessary. Transformed *E. coli* cells were selected on LB plates containing 20 g LB broth base (Gibco) and 15 g agar per litre, supplemented with 100 μ g ampicillin ml⁻¹, 0.2 mM IPTG (Wako Pure Chemicals) and 0.004% (w/v) X-Gal (Wako Pure Chemicals).

Assaying of NarA activity. Cultures were collected by centrifugation at 12000 g for 10 min at 4 °C. The cells were washed three times with 10 mM Tris/HCl (pH 8.0) by centrifugation. The cell suspension was passed twice through a French pressure cell at 10000 p.s.i. (6.9 MPa), followed by centrifugation at 18000 g for 20 min. The supernatant was used as the crude extract. NarA activity was determined using methyl viologen as a substrate, as described by Seki-Chiba & Ishimoto (1977). Enzyme activity was expressed as nmol nitrite formed min⁻¹. Protein concentrations were determined using the BCA protein assay reagent (Pierce) with BSA as a standard. All assays were carried out in triplicate.

SDS-PAGE and zymography. SDS-PAGE on a 7.5% polyacrylamide gel and staining with Coomassie brilliant blue R were performed as described by Laemmli (1970). To detect NarA activity by zymography, proteins were first separated on a gel under non-denaturing conditions and visualized by means of substrate-dependent oxidation of reduced methyl viologen, as described by Seki-Chiba & Ishimoto (1977).

Purification of NarA. *C. perfringens* PB6K was grown in 1 l PYG medium containing 0.2% (w/v) KNO₃ at 37 °C for 9 h.

A crude extract was prepared as described above. Purification was carried out by the method of Seki-Chiba & Ishimoto (1977) with the following modification. Briefly, ammonium sulfate was added to the crude extract to give 40% saturation, followed by centrifugation at 15000 *g* at 4 °C for 20 min. Ammonium sulfate was added to the supernatant to give 80% saturation and the precipitate was collected by centrifugation. The precipitate was dissolved in 15 ml 10 mM Tris/HCl (pH 8.0) and dialysed against the same buffer. The dialysed sample was applied to a DEAE Sephadex A-25 column (2.2 × 15 cm; Amersham Pharmacia Biotech). Proteins were eluted with a 400 ml linear gradient of 0–0.3 M NaCl in Tris/HCl (pH 8.0). Enzyme activity and the absorbance at 280 nm were monitored. The fractions eluted in the active peak were collected and the pooled fraction was dialysed against 10 mM Tris/HCl (pH 8.0). The dialysed sample was applied to an ion-exchange FPLC column (MonoQ, 1 ml bed volume; Amersham Pharmacia Biotech), that had been pre-equilibrated with 20 mM Tris/HCl (pH 8.0). Proteins were eluted with a 20 ml linear gradient of 0–0.3 M NaCl in the same buffer. The enzyme was eluted as a single peak at 170 mM NaCl. This step was repeated three times and the active fractions were combined.

Determination of the N-terminal amino acid sequence. The purified NarA was subjected to SDS-PAGE, followed by transfer to a PVDF membrane (Trans-Blot transfer medium; Bio-Rad). Proteins were visualized by staining with Coomassie brilliant blue R and the corresponding protein band was cut out. The excised membrane was wetted with acetonitrile and incubated in 100 mM Tris/HCl (pH 8.3) containing 6 M guanidine hydrochloride and 2 mg DTT ml⁻¹ at 37 °C for 2 h. Alkylation of cysteine residues was performed by incubation at 37 °C for 2 h after the addition of 4-vinylpyridine to a final concentration of 1% (w/v). After washing five times with 50% (v/v) methanol, the membrane was applied to a protein sequencer (model 492; Perkin Elmer) to determine the N-terminal amino acid sequence.

Cloning of a portion of the *narA* gene. A pair of degenerate oligonucleotide primers was designed to amplify a portion of the *narA* gene by PCR. Their sequences, 5'-ACITGYAA-YTAYTGYGCIYT-3' and 5'-CKYTTIATYTTICRCYTCYT-C-3', correspond to the amino acid sequences of Tyr⁷-Leu¹³ and Glu²¹-Arg²⁷, respectively, of NarA. *C. perfringens* chromosomal DNA was prepared as described previously (Matsushita *et al.*, 1994) and used as the template. PCR was performed with a thermal cycler (model TC1, Perkin Elmer), and the PCR product was purified from an acrylamide gel and cloned into the pT7Blue T-vector (Novagen), as described elsewhere (Matsushita *et al.*, 1999). The resulting plasmid, which was designated pNR1, was transformed into *E. coli* NovaBlue.

Cloning of the *nar* operon. The insert DNA in pNR1 was amplified, labelled with digoxigenin-11-dUTP (Roche Diagnostics) by PCR as described previously (Matsushita *et al.*, 1999) and used as a DNA probe. The chromosomal DNA was digested with *Pst*I and then subjected to Southern hybridization at 55 °C. DNA fragments around the positive signal were recovered from an agarose gel and ligated into pUC19. *E. coli* DH5 α was transformed with the ligation mixture. The colonies were screened by PCR as described previously (Matsushita *et al.*, 1999). Of the plasmids recovered from positive clones, a plasmid named pNR4 produced a PCR amplification product of the expected size. This plasmid was confirmed by nucleotide sequencing to contain the insert DNA, of which the deduced amino acid sequence coincided

with that determined for the purified NarA. This plasmid contained a 9.5 kb *Pst*I fragment. A further downstream region was detected on Southern hybridization using chromosomal DNA digested with *Hinc*II and a DNA probe prepared by PCR amplification of the region corresponding to nt 9286–9416 of the *Pst*I fragment. The 6.1 kb hybridizing fragment was cloned into pUC19, as described above. The resulting plasmid was designated pNR7.

DNA manipulations and nucleotide sequencing. Restriction endonucleases were purchased from Takara Shuzo, Toyobo and New England Biolabs. The DNA ligation kit was a product of Takara Shuzo. All recombinant DNA procedures were carried out as described by Sambrook *et al.* (1989). The nucleotide sequence was determined by the dideoxy chain-termination method (Sanger *et al.*, 1977), using an automated nucleotide sequencer (model ABI PRISM 377, Perkin Elmer). Plasmid template DNA was prepared using the Wizard plus miniprep DNA purification system (Promega). A Thermo Sequase fluorescent-labelled primer cycle-sequencing kit with 7-deaza dGTP (Amersham Pharmacia Biotech) and M13 dye primers (Perkin Elmer) were used for sequencing. An ABI PRISM dye-terminator cycle-sequencing ready reaction kit with AmpliTaq DNA polymerase FS (Perkin Elmer) and various synthetic primers were also used to determine ambiguous nucleotides and to fill in sequence gaps.

Southern hybridization. *C. perfringens* DNA (0.2 μ g) was digested with appropriate restriction enzymes, applied to a 0.8% agarose mini-gel and electrophoresed at 100 V for 1 h. Treatment of the gel and transfer of DNA to a nylon membrane (Hybond-N; Amersham Pharmacia Biotech) were performed as described previously (Matsushita *et al.*, 1994). After hybridization at 50 °C, the hybridized probes were detected with anti-digoxigenin-alkaline phosphatase Fab fragments (Roche Diagnostics) and a chemiluminescent dye (Lumi-Phos 530; Lumigen).

Northern hybridization and primer-extension analysis. *C. perfringens* cells were grown in GAM broth with or without 0.2% KNO₃ at 37 °C for 2 h until the culture reached an OD₆₀₀ of 0.8. Total RNA was prepared by the SDS-phenol method (Aiba *et al.*, 1981) and a sample (1 μ g) was separated on a denaturing agarose gel (1%). Hybridization was carried out at 50 °C using DNA fragments within the *narA* (nt 4629–5605), *narB* (nt 6115–6528), *narC* (nt 6845–7119), *mobB* (nt 7830–8261) and *moeA* (nt 9286–9416) genes as DNA probes. RNA molecular weight marker II (Roche Diagnostics) was used as size markers. Primer-extension experiments were performed using the same RNA sample as described above. A 30 nt primer, 5'-GAGACAATAGTTACAAGTAGATTG-TATTC-3', which is complementary to nt +8 to +37 of the *narA* gene coding region, was 5' end labelled with [γ -³²P]dATP [4.5 kCi/mmol⁻¹ (166.5 TBq mmol⁻¹); ICN Biochemicals] and hybridized with total RNA from *C. perfringens* cells. The hybrids were extended with reverse transcriptase (Superscript RT; Life Technologies) and the extension products were electrophoresed on a sequencing gel as described by Ba-Thein *et al.* (1996).

Amino acid sequence similarity search. A search for similar protein sequences was carried out using the BLAST algorithm (Altschul *et al.*, 1997) at the Center for Information Biology (National Institute of Genetics, Mishima, Japan). The default settings in the BLAST program were used without prefiltering low compositional complexity regions.

Expression of the *narB* and *narC* genes in *E. coli*. The *narB* gene was expressed in *E. coli* as follows. The DNA fragment

corresponding to NarB aa 1–137 was amplified using the two primers 5'-CATATGAAAAGAATAAAAAATCAATAGAGAT-3' and 5'-GGATCCTTATTCTTCTACCTCCTCTAAAGT-3', which contained *Nde*I and *Bam*HI sites, respectively (underlined). A 423 bp *Nde*I–*Bam*HI fragment containing the coding region of the *narB* gene was inserted into expression vector pET-11a. The resulting plasmid, named pET-NarB, was transformed into *E. coli* BL21(DL3), after the nucleotide sequence of the *narB* gene had been verified. The transformant was grown in LB broth containing 100 µg ampicillin ml⁻¹ at 37 °C. When the cultures reached an OD₆₀₀ of 0.6, IPTG was added to a final concentration of 1 mM. After incubation for 4 h, cells were harvested by centrifugation at 5000 g for 5 min at 4 °C. The cells were washed once with 10 mM phosphate buffer (pH 7.0) containing 1 mM EDTA, disrupted by two passages through a French pressure cell at 10000 p.s.i. (6.9 MPa) and centrifuged at 13000 g for 20 min. The precipitates containing large amounts of NarB inclusion bodies were washed three times with 10 mM phosphate buffer (pH 7.0) containing 4% Triton X-100 and 1 mM EDTA by centrifugation at 25000 g for 20 min to remove membrane proteins. To remove residual Triton X-100, the precipitates were washed twice with distilled water. The NarB protein was solubilized with 50 mM Tris/HCl (pH 8.5) containing 8 M urea and 10 mM DTT. The NarB protein recovered in the supernatant fraction was dialysed against the same buffer containing 4 M urea and 10 mM DTT. The dialysed sample was applied to a Sephadex G75 column (Amersham Pharmacia Biotech) and the protein recovered in the void fraction was used as the purified recombinant NarB protein, since this was shown to be homogeneous on SDS-PAGE.

The *narC* gene was expressed in *E. coli* as follows. The DNA fragment corresponding to the coding region of the *narC* gene was amplified using two PCR primers 3'-GGATCCATGAGATATATTGTTGTGGGGGCA-3' and 5'-CTCGAGTTAATATGTGTACTCTAAATTTTC-3', which contained *Bam*HI and *Xho*I sites, respectively (underlined). A 1.2 kb *Bam*HI–*Xho*I fragment was inserted into the pGEX-4T-2 vector (Amersham Pharmacia Biotech) to produce the NarC protein as a glutathione S-transferase fusion protein. After the nucleotide sequence of the fusion gene had been verified, the resulting plasmid, named pGST-NarC, was transformed into *E. coli* DH5α. The production and purification of the fusion protein were performed as described by the manufacturer.

Immunological methods. Polyclonal antibodies were raised in ddY mice by subcutaneous injection of 40 µg recombinant NarB or NarC protein in Freund's complete adjuvant (Amersham Pharmacia Biotech). Booster injections were given after 3 weeks and bleeding was performed after 4 weeks. Prior to use for Western blots, the antisera were preadsorbed with a large excess of a cell extract prepared from *E. coli* BL21(DE3) cells harbouring pET-11a overnight at 4 °C. The preadsorbed antisera were used at 1:100 dilution. For Western-blot analysis, 1 ml cultures of *C. perfringens* were centrifuged in a microfuge. The cell pellets were resuspended in 100 µl SDS-containing sample buffer and boiled for 3 min. Twenty microlitres of a sample was subjected to SDS-PAGE on either a 12.5 or 15% polyacrylamide gel and the proteins were blotted onto a nitrocellulose membrane. The replicates were incubated with mouse antiserum against the NarB or NarC protein and then subjected to immunostaining with goat anti-mouse IgG–alkaline phosphatase conjugate (Bio-Rad), and nitro blue tetrazolium (Sigma) and BCIP (5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt; Wako Pure Chemicals) as chromogenic substrates.

RESULTS

Cloning and sequencing of *narA* and neighbouring genes

To clone the *narA* gene, we purified NarA from *C. perfringens* PB6K to homogeneity, as shown by SDS-PAGE (Fig. 1a) and zymography (Fig. 1b, c). The molecular mass of NarA was determined to be approximately 74 kDa. The N-terminal amino acid sequence of NarA was determined to be MKRIQSTCNICALACNLDFYTEDGKIKRVVPT. PCR with the degenerate primers matching this sequence and the *C. perfringens* chromosome DNA amplified a DNA fragment of the expected size (62 bp). After this fragment had been cloned into the pT7Blue T-vector and its nucleotide sequence had been confirmed to coincide with the amino acid sequence, we performed Southern hybridization using it as a DNA probe. The 9.5 kb *Pst*I fragment detected was cloned into pUC19. The entire sequence of the insert fragment was determined (data not shown; accession number AB017192). Seven ORFs are present in this 9.5 kb fragment, the two reading frames at the 5' and 3' extremities being truncated (Fig. 2). The N-terminal amino acid sequence determined for the purified NarA coincided with that deduced from the sequence of ORF 4. The molecular mass of the protein encoded by ORF 4 was calculated to be 78436 Da, this being in agreement with the value determined for the

Fig. 1. Analysis of purified NarA by SDS-PAGE and zymography. Purified NarA [1 µg (a) or 2.5 µg (b, c)] was electrophoresed on an SDS-containing 7.5% acrylamide gel (a) and a 10% native acrylamide gel (b, c). The gels were subjected to staining with Coomassie brilliant blue R (a, b) or zymography (c), as described in Methods. Lane 1, markers; lane 2, purified NarA.

Fig. 2. The physical/genetic map of the *C. perfringens nar* gene cluster and its upstream and downstream regions cloned into pNR4 and pNR7. The restriction sites are: P, *Pst*I; E, *Eco*RI; X, *Xba*I; S, *Spe*I; H, *Hinc*II.

Table 1. Genes and gene products of the *nar* operon and neighbouring genes

Gene	Probable function	Calculated molecular mass (Da)	Product of the homologous gene	Source	Accession no.	BLAST <i>P</i> value
<i>dacA</i>	D-Alanyl-D-alanine carboxypeptidase	—	DacB	<i>B. subtilis</i>	P35150	8e-37
ORF 1	Electron-transfer subunit protein	36522	PyrZ	<i>B. subtilis</i>	P25983	2e-7
ORF 2	Unknown	46678	—	—	—	—
ORF 3	Unknown	16143	—	—	—	—
<i>narA</i>	NaR: catalytic subunit	78436	NasC	<i>B. subtilis</i>	P42434	3e-77
			FdhF	<i>E. coli</i>	P07658	6e-77
<i>narB</i>	NaR: electron-transfer subunit	15403	DmsB	<i>E. coli</i>	P18776	6e-77
			[4Fe-4S] iron-sulfur protein	<i>E. coli</i>	E65051	1e-15
<i>narC</i>	NaR: NADH oxidase subunit	45187	NasB	<i>B. subtilis</i>	P42433	4e-29
			NirB	<i>E. coli</i>	P08201	1e-26
<i>mobB</i>	Synthesis of MGD from molybdopterin	18097	MobB	<i>E. coli</i>	P32125	2e-20
<i>moeA</i>	Formation of thiomolybdenum complex	44871	MoeA	<i>E. coli</i>	P12281	3e-51
<i>mobA</i>	Synthesis of MGD from molybdopterin	23217	MobA	<i>B. subtilis</i>	AF012285	6e-9
<i>moeC</i>	Formation of thiomolybdenum complex	37407	MoeA-3	<i>Archaeoglobus fulgidus</i>	AE001095	1e-9
<i>moaA</i>	Synthesis of precursor Z from a guanosine derivative	37390	MoaA	<i>E. coli</i>	P30745	5e-45
ORF 12	Unknown	15593	—	—	—	—
ORF 13	Unknown	34404	—	—	—	—

purified NarA. Thus, ORF 4 was identified as the *narA* gene.

A similarity search of the protein database revealed that the proteins encoded by ORFs 4, 5 and 6 exhibit significant similarity to NasC (assimilatory NaR) of *B. subtilis*, DmsB (a ferredoxin-like electron-transfer subunit of dimethyl sulfoxide reductase) of *E. coli* and NasB (a NADH-oxidase subunit of assimilatory nitrate reductase) of *B. subtilis*, respectively (Table 1). This suggests that these three gene products form NaR and its electron-transfer carriers. Thus, ORFs 5 and 6 were tentatively designated the *narB* and *narC* genes, respectively. The protein encoded by ORF 7 shows homology to the *mobB* gene product of *E. coli*, which has been shown to be involved in the synthesis of the molybdenum-molybdopterin guanine dinucleotide complex (Mo-MGD), a cofactor of NaR of *E. coli*. There was no ORF with significant similarity to already known NiRs or proteins involved in nitrate transport.

That the NiR and NaR/NiR transporter genes are not in the vicinity of the *narA* gene differs from the genetic organization in *Synechococcus* sp., *B. subtilis* and *Klebsiella pneumoniae*, in which assimilatory NaR and NiR genes are clustered (Lin & Stewart, 1998).

In an attempt to find a *nirA* gene in a further downstream region, we cloned and sequenced the 6.1 kb *Hinc*II fragment that overlapped the 3' region of the 9.6 kb *Pst*I fragment (Fig. 2). A total of 14393 bp were sequenced by combining the nucleotide sequences of the two fragments (data not shown; accession number AB017192). A partial physical map, and the locations of *narA*, *narB*, *narC* and other ORFs are presented in Fig. 2. The protein sequences predicted from ORFs 7 to 11 showed significant homology to the products of the *mob*, *moa* and *moe* genes (Table 1), which are considered to be involved in the synthesis of Mo-MGD. Database searches did not reveal any sequences homologous to the other ORFs except for *dacA*, which exhibited signifi-

Fig. 3. Induction of *C. perfringens* NarA activity, and analyses of the *nar* operon by primer extension and Northern blotting. (a) Changes in NarA activity during the growth of *C. perfringens* in the presence and absence of KNO₃. Five millilitres of an overnight preculture in GAM broth without KNO₃ was inoculated into 1 l fresh GAM broth with (black symbols) or without (white symbols) 0.2% (w/v) KNO₃. Samples (100 ml) were taken at the indicated times. After the cells had been washed, cell lysates were prepared as described in Methods. Growth was monitored by measuring the OD₆₀₀ (□, ■). NarA activity (○, ●) was determined as described under Methods. Error bars represent standard deviations. (b) Primer-extension mapping of the 5' end of the *narA* mRNA. RNA was extracted from *C. perfringens* PB6K grown in GAM broth with or without 0.2% KNO₃ when cultures reached an OD₆₀₀ of 0.8. Sequencing ladders generated with the oligonucleotide used for the primer extension were loaded between the two reaction mixtures. Lane 1, RNA from cultures grown in the presence of KNO₃; lane 2, RNA from cultures grown in the absence of KNO₃. The DNA sequence of the sense strand around the first nucleotide in the transcript (designated +1) is shown and the -10 sequence is boxed. (c) Northern-blot analysis. Panels (i), (ii), (iii) and (iv) are blots with DNA probes specific to the *narA*, *narB*, *narC* and *mobB* genes, respectively. Lanes 1, markers; lanes 2, RNA from cultures grown in the presence of KNO₃; lanes 3, RNA from cultures grown in the absence of KNO₃.

cant similarity to DacB of *B. subtilis* (D-alanyl-D-alanine-carboxypeptidase, a penicillin-binding protein).

Identification of the *nar* operon

There are two putative rho-independent transcriptional terminators immediately upstream of the *narA* gene and downstream of the *mobB* gene, respectively, suggesting that the *narA*, *narB*, *narC* and *mobB* genes are transcribed as a polycistronic operon. Although a -10 consensus sequence, TATAAT, is present upstream of the *narA* gene, there is no sequence indicative of a -35 region. To determine the transcriptional-start site of this

operon, primer-extension analysis was performed on cells grown in GAM broth with and without KNO₃. As shown in Fig. 3a, NarA activity increased only slightly in the medium without KNO₃. On the contrary, the enzyme activity increased markedly in the presence of KNO₃, indicating that the enzyme synthesis is induced by KNO₃. RNA was prepared from the two cultures at an OD₆₀₀ of 0.8. No positive band was detected for RNA from cells grown in the absence of KNO₃. On the other hand, RNA from cells grown in the presence of KNO₃ gave a positive signal, which clearly indicated the transcriptional-start site was A at position +1 (Fig. 3b). Northern-blot analysis was also performed on the same

RNA samples using the DNA fragments from the *narA*, *narB*, *narC* and *mobB* genes as DNA probes. All blots had a band corresponding to approximately 5300 nt for the RNA from the cells grown in the presence of KNO_3 but not for that in the absence of KNO_3 (Fig. 3c). When Northern-blot analysis was performed using the DNA fragment within the *moaA* gene, a band was detected at the location corresponding to approximately 4800 nt, being distinct from that of *narABCmobB* transcripts (data not shown). These results indicate that the four genes are transcribed to give a polycistronic mRNA from the *narA* promoter.

Characterization of the *narA*, *narB* and *narC* gene products

NarA shows higher similarity to NarC (*B. subtilis* assimilatory Nar) (Poisson *P* value 3e-77) than NarG (*B. subtilis* respiratory Nar) (Poisson *P* value 3e-4). It also shows high similarity to FdhH, *E. coli* formate dehydrogenase H (Poisson *P* value 6e-77). A study on the three-dimensional structure of FdhH has well defined the amino acid residues ligating a [4Fe-4S] iron-sulfur cluster and Mo-MGD, a prosthetic group transferring an electron, as well as the catalytic site. The alignment of *C. perfringens* NarA with *E. coli* FdhH revealed that the following amino acid residues are conserved in the former: 15 of the 24 amino acid residues participating in the ligation of two Mo-MGD molecules; Lys⁴⁴, a residue transferring an electron from Mo-MGD to the [4Fe-4S] iron-sulfur cluster; and a 4-cysteine-residue motif for binding the [4Fe-4S] iron-sulfur cluster, as shown in Fig. 4a.

Alignment of the deduced amino acid sequences of NarB and homologous proteins showed a characteristic of NarB. Its deduced amino acid sequence includes four cysteine motifs, each of which belongs to a different [4Fe-4S] iron-sulfur cluster (Fig. 4b). This ferredoxin-like protein differs from ferredoxin in that it possesses four [4Fe-4S] iron-sulfur clusters, and also in that it consists of 178 aa residues, this being considerably longer than ferredoxin. It exhibits similarity to the electron-transfer subunits of many respiratory enzymes, which are referred to as 'four-cluster ferredoxins', since they possess motifs 1-4 differing in cysteine spacing (Gennis & Stewart, 1996), as shown in Fig. 4b.

The amino acid sequence deduced from the *narC* gene exhibits homology to those of NADH peroxidase (Poisson *P* value 1.2e-15) and sulfide dehydrogenase (Poisson *P* value 0.018). Their alignment revealed that NarC contains FAD- and NADH-binding domains, which were detected in the two enzymes on crystallographic analysis (Fig. 4c). These structural characteristics of NarB and NarC, and the linkage of the three genes suggest that they function to reduce nitrate in a cooperative manner such that an electron from NADH is first accepted by NarC, then transferred to NarB and finally to NarA.

Expression of the *narB* and *narC* genes

C. perfringens contains ferredoxin (Seki *et al.*, 1979) and rubredoxin (Seki *et al.*, 1989), both of which may function as an electron carrier for NarA (Seki *et al.*, 1988, 1989). Although it is clear that the *narB* gene is transcribed from the *narA* promoter upon induction by KNO_3 , the possibility that NarB is not synthesized cannot be ruled out. To determine whether or not NarB is produced upon induction, cells were grown in the presence and absence of KNO_3 , collected at an OD_{600} of 2.0 and subjected to Western-blot analysis (Fig. 5a). A band with the same mobility as that of the NarB protein was detected for the cells grown in the presence of KNO_3 but not for cells grown in its absence. However, other band(s) were also detected for both the induced and uninduced cells. They may be unknown protein(s) which are immunologically related to NarB or associated with the NarB protein. Synthesis of the NarC protein in induced and uninduced cells was also examined by Western-blot analysis (Fig. 5b). Antiserum was prepared from mice immunized with a GST-NarC fusion protein, because the fusion protein was insensitive to cleavage by thrombin. A band corresponding to a 45 kDa polypeptide, which coincided with the molecular mass predicted from the *narC* gene, was detectable for the induced cells. A band with the same mobility is weakly present in the uninduced cells. Since low but significant levels of nitrate reductase activity were detectable in the uninduced cultures grown at an OD_{600} of 2.0 (Fig. 3a), small amounts of NarB and NarC seem to be produced in the uninduced cells. The discrepancy that the NarC protein but not the NarB protein was detectable for the uninduced cells could be due to the difference in the immunoreactivity of the two antisera used. Thus, it seems very likely that the expression of the *narB* and *narC* genes at the protein level is coregulated with that of the *narA* gene.

DISCUSSION

In this study we have cloned and characterized the *nar* operon of *C. perfringens*. The results presented here not only support the earlier finding that NarA has a molybdenum cofactor, four non-haem irons and acid-labile sulfides (Seki-Chiba & Ishimoto, 1977; Seki *et al.*, 1987), but also provide additional information on NarA. The following are the characteristics of NarA suggested by the results of analysis of the *nar* operon: i) Mo-MGD is a likely cofactor of NarA, because it possesses the Mo-MGD-binding motif of *E. coli* FdhH and also because the *mobB* gene, which is involved in Mo-MGD synthesis, is present within the *nar* operon; ii) the iron-sulfur cluster of NarA seems to be the [4Fe-4S] type, as described above; iii) induction of NarA by KNO_3 occurs at the transcriptional level; and iv) NarA is coregulated along with NarB (a ferredoxin-like protein) and NarC (an NADH oxidase). The location of the *mobB* gene within the *nar* operon may reflect a functional linkage

Fig. 4. (a) Comparison of the deduced amino acid sequences of *C. perfringens* NarA and *E. coli* formate dehydrogenase H. The upper and lower sequences represent NarA and formate dehydrogenase H, respectively. Residue identities are indicated by asterisks and similarities by dots. The residues participating in the ligation of two Mo-MGD molecules are indicated by an open box. The lysine residue transferring an electron from the [4Fe-4S] iron-sulfur cluster to Mo-MGD, and the cysteine residues binding the [4Fe-4S] iron-sulfur cluster are indicated by an arrowhead and black backgrounds, respectively. (b) Alignment of the 4-cysteine motifs involved in ligation of the [4Fe-4S] cluster. The predicted amino acid sequences of NarB from *C. perfringens* (NarB, this work), DMSO reductase iron-sulfur subunit from *E. coli* (DmsB,

Fig. 5. Immunoblot analysis of NarB and NarC from *C. perfringens* cells. Cultures were grown in GAM broth with or without 0.2% KNO_3 and collected when the cultures reached an OD_{600} of 2. Cell lysates were prepared by boiling cell suspensions in SDS-sample buffer and electrophoresed through a 15% (a) or 12.5% (b) acrylamide gel. The proteins were blotted onto a nitrocellulose membrane and probed with anti-NarB (a) or anti-NarC (b) antisera. Lanes: 1, prestained size markers; 2, lysate prepared from KNO_3 uninduced cells; 3, lysate prepared from KNO_3 induced cells; 4 (a), purified NarB protein (b), purified GST-NarC fusion protein.

between MobB and NarA. The *mobA* and *mobB* gene products of *E. coli* are involved in the final step of Mo-MGD biosynthesis, conversion of molybdopterin to Mo-MGD. In the case of NarG, a membrane-bound respiratory NaR, NarJ is also required for insertion of Mo-MGD to its apoenzyme. Recently, MobB was demonstrated to be a GTP-binding protein (Eaves *et al.*, 1997) and NarJ was shown to be a specific chaperone

allowing the apoenzyme to incorporate Mo-MGD (Blasco *et al.*, 1998). In *E. coli*, the *mobA* and *mobB* genes are arranged as a single transcriptional unit, while a *narJ* gene is located within a *narGHIJ* operon. *C. perfringens* MobB seems to be involved in the final step of Mo-MGD biosynthesis like *E. coli* MobB. It may also play a role in the activation of NarA via incorporation of Mo-MGD like *E. coli* NarJ. This supposition requires further study on the activation of NarA in *C. perfringens*.

The organization of the *nar* operon suggests a functional relationship between NarA, NarB and NarC, i.e. the latter two proteins are linked to NarA. On the other hand, the *in vitro* reconstitution experiment performed by Seki *et al.* (1988) strongly suggested that rubredoxin, a 6 kDa iron-sulfur protein (Seki *et al.*, 1989), serves as an intermediary electron carrier for NAD(P)H-linked nitrate reduction, and that ferredoxin reduced by pyruvate dehydrogenase is linked to NarA instead of hydrogenase in the presence of nitrate. Their results, however, do not rule out a possible functional relationship between NarA, NarB and NarC. Considering the results of both our genetic analysis and their *in vitro* study, we assume that an electron-transfer pathway from NADH to NarA via NarC and NarB could be the main route of nitrate reduction, at least upon induction by KNO_3 , although ferredoxin and rubredoxin would also serve as intermediary electron carriers to NarA. To prove this, the NarA activity in the presence of NarB and NarC must be compared with that in their system.

Comparison of NaRs and their electron carriers between the three different nitrate-reduction systems would be informative for understanding their evolutionary relationships. *B. subtilis* possesses both respiratory and assimilatory NaRs, which are encoded by *narG* (Cruz Ramos *et al.*, 1995; Hoffmann *et al.*, 1995) and *nasC* (Ogawa *et al.*, 1995), respectively. The deduced amino acid sequence of *C. perfringens* NarA exhibits higher similarity to NasC than to NarG, indicating that *C. perfringens* NarA is more closely related to assimilatory NaRs. NasB, NADH dehydrogenase of *B. subtilis*, possesses three iron-sulfur clusters, while *Klebsiella oxytoca* NADH dehydrogenase, which is homologous to *B. subtilis* NasB, lacks such a cluster (Fig. 6). Instead, a region homologous to the clusters resides at the N-terminus of NaR of the latter organism (Fig. 6). Based on the homology between these iron-sulfur clusters and TodB, an electron-transfer subunit in the NADH-dependent toluene degradation system of *Pseudomonas putida*, the current *K. oxytoca* NaR and *B. subtilis* NADH-dependent dehydrogenase are postulated to

P18776), nitrate reductase 1 beta chain from *E. coli* (NarH, P11349), fumarate reductase iron-sulfur protein from *E. coli* (FrdB, P00364) and succinate dehydrogenase iron-sulfur protein from *E. coli* (SdhB, P07014) are shown, with amino acid residues being numbered on the left. The conserved cysteine residues are indicated by black backgrounds. (c) Alignment of nucleotide-binding motifs. The predicted amino acid sequence of NarC from *C. perfringens* (NarC, this work) and the known amino acid sequences of NADH peroxidase from *Streptococcus faecalis* (Npr, P37062) and sulfide dehydrogenase from *Chromatium vinosum* (Sdh, Q06530) are shown, with amino acid residues being numbered on the left. The residues forming FAD- and NAD-binding domains are indicated by open boxes.

Fig. 6. Comparison between homologous components involved in assimilatory and fermentative nitrate reduction. Abbreviations: Cp, *C. perfringens*; Bs, *B. subtilis*; Ko, *K. oxytoca*; NasC and NasB of *B. subtilis*, NaR and NAD(P) dehydrogenase, respectively; NasA and NasC of *K. oxytoca*, NaR and NAD(P) dehydrogenase, respectively. A domain or subunit predicted to function as an electron-transfer component is indicated by an arrowhead.

have resulted from fusion with an intermediate electron-carrier protein containing an iron–sulfur cluster (Lin & Stewart, 1998). Our finding that NarB is the most likely candidate for an intermediate electron carrier for NarA strengthens the fusion hypothesis, which might be extended to the origin of eukaryotic assimilatory NaRs containing all three components corresponding to NarA, NarB and NarC of *C. perfringens* (Campbell & Kinghorn, 1990).

The similarity between NarA and assimilatory NaR does not necessarily exclude the notion of Hasan & Hall (1975) that fermentative nitrate reduction might have evolved as the most primitive form of nitrate respiration. Instead, the fact that the ferredoxin-like protein is homologous to the electron-transfer subunits of many respiratory molybdoenzymes, including respiratory NaR, supports their hypothesis. The location of the *nirA* gene is also of interest in terms of the evolutionary relationship between NaRs. Genes for nitrite reductase are linked to those for NaR in the assimilatory system, but not in the respiratory system. The *nirA* gene is not present in the 14.4 kb region sequenced in this study. Possibly the *nirA* gene is not located in the vicinity of the *nar* genes in *C. perfringens*, although it cannot be ruled out that the *nirA* gene may be further downstream, distal to the genes for molybdenum cofactor biosynthesis. Thus, fermentative nitrate reduction may have evolved into both assimilatory and respiratory nitrate-reduction systems. Further detailed study on the *nar* operon and analysis of the *nir* gene(s) will facilitate elucidation of the evolutionary path of the fermentative nitrate-reduction system.

ACKNOWLEDGEMENTS

We are indebted to Shigehiko Taniguchi (Fujita Health University School Medicine, Toyoake-shi, Aichi, Japan) for encouraging us throughout this study. We also thank Nicholas J. Halewood for the invaluable assistance in preparation of the manuscript. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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Received 8 April 1999; revised 13 August 1999; accepted 27 August 1999.