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Identification and Isolation of a Gene Required for Nitrate Assimilation and Anaerobic Growth of *Bacillus subtilis*

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The *Bacillus subtilis narA* locus was shown to include *narQ* and *narA*. The putative product of *narQ* is similar to FdhD, which is required for formate dehydrogenase activity in *Escherichia coli*. NarA showed homology to MoaA, a protein involved in biosynthesis of the molybdenum cofactor for nitrate reductase and formate dehydrogenase. Analysis of mutants showed that *narA* but not *narQ* is required for both nitrate assimilation and respiration.

Two of the important roles that nitrate reduction plays in bacterial physiology are nitrogen assimilation (4) and anaerobic respiration (25, 26). In nitrogen assimilation, nitrate can serve as a sole source of nitrogen by reduction through nitrite to ammonium, which is incorporated into macromolecules through the activities of glutamine synthetase and glutamate synthase. In respiration, nitrate is used as an alternative electron acceptor when oxygen is unavailable. Certain bacteria, such as strict aerobes, can carry out assimilatory nitrate reduction but cannot use nitrate as an electron acceptor for anaerobic growth. In contrast, bacteria such as *Escherichia coli* and *Salmonella typhimurium* grow anaerobically, using nitrate as a terminal electron acceptor. These organisms, however, do not assimilate nitrogen through nitrate reduction during aerobic growth. Other bacteria, including *Klebsiella pneumoniae*, *Klebsiella aerogenes*, and *Pseudomonas aeruginosa*, carry out both assimilatory and respiratory nitrate reduction.

Nitrate and nitrite reductases are two key enzymes involved in nitrate assimilation as well as in anaerobic respiration. Two *Bacillus subtilis* mutations, *narA* (11) and *narB* (33), leading to defective nitrate utilization as a sole nitrogen source, have been described and mapped to unlinked positions on the chromosome. In a separate report, the isolation of the *narB* locus (now referred to as *nasABCDEF*) was described (16). The *nasA* gene seems to encode a nitrate transporter. The *nasB* operon consists of five genes. NasB and NasC are likely to be subunits of nitrate reductase, NasD and NasE are probably subunits of nitrite reductase, and NasF is involved in the formation of siroheme, a cofactor of nitrite reductase. Nitrate reductase is also known to contain a cofactor containing molybdenum. Molybdenum cofactor is also required for the activities of several other enzymes, including formate dehydrogenase, sulfite oxidase, and xanthine dehydrogenase (19, 22).

In this report, we describe the isolation of the *narA* locus in *B. subtilis* by complementation of the *B. subtilis narA1* strain QB692 (11) (Table 1). Cells of this strain were transformed with a chromosomal DNA library (7) constructed in pMK4 (29) (Table 2), a shuttle vector for *B. subtilis* and *E. coli*, and plated on minimal glucose medium (MMG) supplemented with nitrate (2, 16). Plasmids were recovered from Nar^+ transformants and used to retransform QB692 cells to confirm that the Nar^+ phenotype was due to the plasmid. One plasmid (pDIA5338) was shown to complement with respect to growth on a medium containing nitrate as the sole nitrogen source.

To demonstrate that utilization of nitrate was not due to functional bypass of *narA*, we showed that linearized pDIA5338 could restore the Nar^+ phenotype by homologous recombination. Plasmid pDIA5338 was used to probe a *B. subtilis* chromosomal library constructed in λFixII (Stratagene, La Jolla, Calif.) (9) to isolate neighboring DNA. Of six positive lambda clones, λ*narA4* contained the largest insert; its 18-kb insert was sequenced on both strands. The sequence will be reported elsewhere. Southern hybridization analysis showed the absence of cocloning or rearrangements in the insert DNA (data not shown). DNA sequences were compiled by the Xbap program of Dear and Staden (6) and analyzed by using the DNA Strider 1.1 software (13). Partial sequence of the *B. subtilis* DNA insert in pDIA5338 was used to localize the *narA* locus in λ*narA4*, and this region was then subcloned. Plasmid pλ*narA4* is a pUC19 derivative containing a 5.2-kb *Sal*I fragment, and pλ*narA18* is a derivative of pUC18 with a 2.5-kb *Sst*I fragment (Fig. 1). Within the region encompassed by these two plasmids were two potential open reading frames with similarity to known genes, as determined by using the FASTA (18) (in Swissprot release 27) and BLAST (1) programs. The *narQ* gene would encode a protein of 262 amino acids that is similar to FdhD of *E. coli* (20) (28% identity) and *Wolinella succinogenes* (3) (30% identity). FdhD is required for formate dehydrogenase activity, but its specific role is unknown (12, 24, 27). *narA* would encode a protein of 341 amino acids with high similarity to *E. coli* MoaA (32% identity), a protein suggested to be responsible for the formation of a precursor of molybdopterin (8, 23). The *narA* gene starts 15 bp downstream of the *narQ* stop codon and is followed by a potential factor-independent transcription termination signal.

Plasmid pMMN235, containing the intact *narQ* and *narA* genes, was used to transform a *narA1* strain (CU495), and erythromycin-resistant transformants generated by Campbell-type integration were tested for ability to grow on MMG-nitrate medium. Integration of pMMN235 restored a Nar^+

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TABLE 1. *B. subtilis* strains used

Strain	Genotype	Source or reference
JH642	*trpC2 pheA1*	J. Hoch
CU495	*trpC2 narA1*	S. Zahler (11)
QB692	*sacA321 narA1*	11
LAB1727	*trpC2 pheA1* Δ*nasB*::pMMN191	16
LAB1826	*trpC2 pheA1 narA*::pMMN212 (P*spac-narA*)	This report
LAB1846	*trpC2 pheA1* Δ*narQ*::pMMN219	This report
LAB1851	*trpC2 pheA1* Δ*narQ*::pMMN219 *narA*::pMMN212 (P*spac-narA*)	This report
LAB2037	*trpC2 pheA1* Δ*narA*::pMMN242	This report

phenotype to the *narA1* mutant. Since the *B. subtilis* DNA in pDIA5338, which was shown to restore a Nar^+ phenotype in *trans*, contains a 5.5-kb fragment extending from the 3′ end of *narQ* (containing 480 bp DNA of *narQ*) through the entire *narA* gene, it is very likely that the *narA1* mutation lies in *narA*.

Deletion mutant alleles of *narQ* and *narA* were constructed and tested for nitrate assimilation. A 127-bp *Eco*RV fragment internal to the *narQ* gene was replaced by a phleomycin resistance ($Phleo^r$) gene from pMMN108. The resultant plasmid, pMMN207, was digested with *Sma*I in vector DNA and ligated with a filled-in *Eco*RI fragment containing a chloramphenicol resistance (Cm^r) gene from pMMN7 (a pGEM4 derivative with a Cm^r gene) to create pMMN219. Cells of *B. subtilis* JH642 were transformed with pMMN219 with selection for $Phleo^r$ followed by a screen for Cm^s to find transformants (e.g., LAB1846) that had arisen by double-crossover recombination. A 360-bp *Nru*I-*Bcl*I fragment (360 bp) internal to *narA* was also replaced by the $Phleo^r$ gene from pMMN108. The resultant plasmid, pMMN241, was digested with *Bam*HI and ligated with a Cm^r fragment isolated from *Bam*HI-digested pMMN7 to create pMMN242. JH642 was transformed with pMMN242, and a $Phleo^r$ Cm^s transformant generated by double-crossover recombination (LAB2037) was isolated. Another *narA* mutant was constructed in which expression of *narA* was under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P*spac* promoter. A 0.58-kb *Ase*I-*Hin*dIII fragment containing the 3′ end of *narQ* and the 5′ end of *narA* was inserted into pDH88 (32), a plasmid with an IPTG-inducible P*spac* promoter, which had been digested with *Hin*dIII and *Sma*I. The resultant plasmid, pMMN212, was used to transform JH642 by Campbell integration with selection for Cm^r to generate LAB1826. A strain containing Δ*narQ* and P*spac-narA* (LAB1851) was created by transformation through Campbell integration of Δ*narQ* strain LAB1846 with pMMN212. Table 3 summarizes the growth rates of various mutant strains. The *narA1* (CU495) and Δ*nasB* (LAB1727) mutants did not grow well with nitrate as the sole nitrogen source, as shown previously (11, 16). Observed initial growth might be due to residual ammonium from the precultures. Strain LAB1826, containing *narA* under the control of an IPTG-inducible promoter, did not grow with nitrate as a sole source of nitrogen but grew as

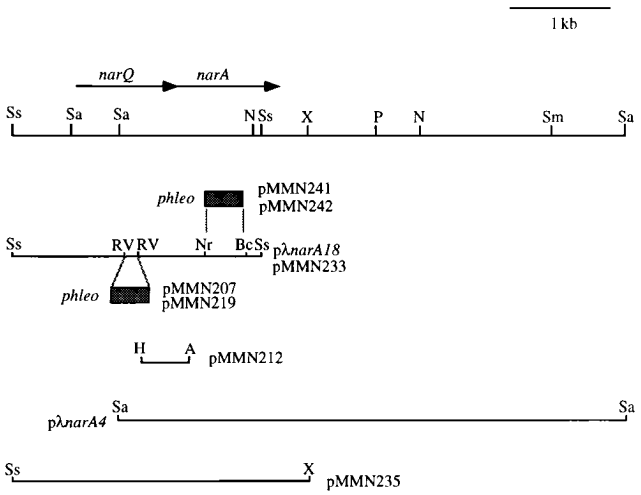

FIG. 1. Physical map of the *narQA* region. A restriction enzyme map of the *narQA* region is shown. Restriction site abbreviations: A, *Ase*I; B, *Bcl*I; H, *Hin*dIII; N, *Nsi*I; Nr, *Nru*I; P, *Pst*I; RV, *Eco*RV; Sa, *Sal*I; Sm, *Sma*I; Ss, *Sst*I; X, *Xba*I. Only relevant *Ase*I, *Bcl*I, *Eco*RV, *Hin*dIII, and *Nru*I recognition sites are included. The arrows represent two open reading frames, *narQ* and *narA*. The lines below the restriction enzyme map indicate cloned segments in the corresponding plasmids shown next to each line. The boxes represents a $Phleo^r$ gene (*phleo*) which was used to replace *narQA* DNA.

TABLE 2. Plasmids used

Plasmid	Relevant characteristics[a]	Reference
pDH88	P*spac* promoter, *lacI* Amp^r Cm^r	32
pLEW424	Derivative of pJDC9, Erm^r	5, 30
pMK4	Shuttle vector derived from pUC9 and pC194, Amp^r Cm^r	29
pDIA5338	pMK4 with 5.5-kb *narQA* region, Amp^r Cm^r	This report
pλ*narA4*	pUC19 with 5.2-kb *Sal*I fragment of *narQA*, Amp^r	This report
pλ*narA18*	pUC18 with 2.5-kb *Sst*I fragment of *narQA*, Amp^r	This report
pMMN7	Derivative of pGEM4, Amp^r Cm^r	
pMMN108	Derivative of pGEM4, Amp^r $Phleo^r$	
pMMN207	pλ*narA18* replaced 0.13-kb *Eco*RV fragment by $Phleo^r$, Amp^r $Phleo^r$	This report
pMMN212	pDH88 with 0.58-kb *narQA*, Amp^r Cm^r	This report
pMMN219	pMMN207 with Cm^r fragment, Amp^r $Phleo^r$ Cm^r	This report
pMMN233	pLEW424 with 2.5-kb *Sst*I fragment of *narQA*, Erm^r	This report
pMMN235	pLEW424 with 3.0-kb *narQA*, Erm^r	This report
pMMN241	pλ*narA18* replaced 0.36-kb *Nru*I-*Bcl*I fragment by $Phleo^r$, Amp^r, $Phleo^r$	This report
pMMN242	pMMN241 with Cm^r fragment, Amp^r $Phleo^r$ Cm^r	This report

[a] Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Erm^r, erythromycin resistance; $Phleo^r$, phleomycin resistance. The location of the *B. subtilis* DNA insert in each plasmid is shown in Fig. 1.

TABLE 3. Aerobic growth of mutants in MMG–0.2% KNO_3 medium[a]

Time (h)	A_{600}						
	JH642 (wild type)	CU495 (*narA1*)	LAB1727 (Δ*nasB*)	LAB1851 (Δ*narQ* P*spac-narA*)		LAB2037 (Δ*narA*)	LAB2037[b] (Δ*narA*)
				−IPTG	+1 mM IPTG		
13	0.3435	0.1077	0.1146	0.1816	0.2883	0.1730	
19	0.7446	0.1267	0.1294	0.2133	0.7521	0.2047	0.0784
23	1.5755	0.1345	0.1372	0.2299	1.5740	0.2131	0.1038
38	2.1720	0.1646	0.1620	0.3104	2.4720	0.2551	
45							1.6116

[a] Absorbances of cultures at 0 h were 0.02.
[b] Cells were cultured in MMG–0.2% KNO_2.

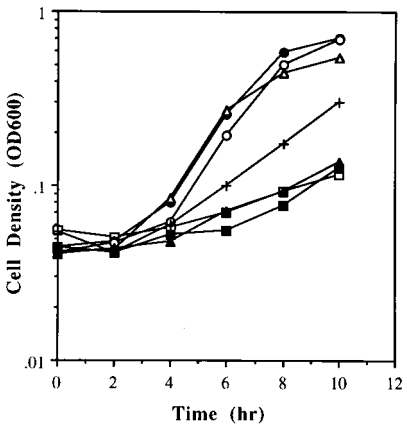

FIG. 2. Growth of *B. subtilis* strains under anaerobic conditions. Cells were incubated overnight in rich medium (2XYT) (14) supplemented with 0.2% KNO_3, transferred at 0 h to the same medium supplemented with 2% Oxyrase to produce an anaerobic condition, and further incubated without shaking. JH642 was also grown in 2XYT without nitrate. When indicated, 1 mM IPTG was added at 0 h. Cell density was measured by optical density at 600 nm (OD_{600}). ●, JH642 (wild type); □, JH642 without nitrate; ■, CU495 (*narA1*); ○, LAB1727 (Δ*nasB*); +, LAB1851 (Δ*narQ* P*spac-narA*) without IPTG; △, LAB1851 with IPTG; ▲, LAB2037 (Δ*narA*).

TABLE 4. Enzyme activities of mutant strains

Strain (genotype)	Nitrate reductase (nmol of nitrite/mg of protein/min)			Formate dehydrogenase[a] (nmol of DCPIP reduced/mg of protein/min)
	Assimilatory[b]	Respiratory[c]		
		0 h	7 h	
JH642 (wild type)	3.2	<0.01	76	48
CU495 (*narA1*)	<0.01	<0.01	<0.01	NT[d]
LAB1727 (Δ*nasB*)	<0.01	<0.01	60	NT
LAB1851 (Δ*narQ* P*spac-narA*)				
−IPTG	0.07	<0.01	9.2	1.9
+1 mM IPTG	2.8	<0.01	70	2.6
LAB2037 (Δ*narA*)	<0.01	<0.01	<0.01	<0.01

[a] Phenazine methosulfate-linked formate dehydrogenase activity was measured by the reduction of 2,6-dichlorophenol indolphenol (DCPIP) (31).

[b] Measured from cells grown in MMG supplemented with 0.2% glutamate (15) by the standard method with dithionite-reduced methyl viologen as the reductant (28).

[c] Cells cultured in 2XYT–0.2% KNO_3 were shifted at 0 h from an aerobic (shaking vigorously) to an anaerobic (incubating without shaking after addition of Oxyrase) condition.

[d] NT, Not tested.

well as the wild type (JH642) in the presence of IPTG, showing that *narA* is required for nitrate assimilation (data not shown). This finding was confirmed by the growth phenotype of a *narA* deletion mutant (LAB2037; Table 3). The *narQ* deletion mutant LAB1846 grew in MMG-ammonium but not in MMG-nitrate (data not shown). This result indicates either that *narQ* is required for nitrate assimilation or that the *narQ* mutation is polar on *narA*. To differentiate between these possibilities, the growth phenotype of LAB1851, which bears the *narQ* deletion and P*spac-narA*, was examined. In the absence of IPTG, the strain did not grow well in MMG-nitrate medium. However, in the presence of IPTG, it could utilize nitrate as the sole nitrogen source (Table 3). This result indicates that *narA* but not *narQ* is required for nitrate assimilation and that *narA* and *narQ* are likely to constitute an operon. Table 3 also shows that the *narA* mutant can grow in MMG-KNO_2, indicating that the *narA* mutation impairs nitrate but not nitrite assimilation.

B. subtilis is generally regarded as an aerobe and was reported not to grow anaerobically, even in the presence of nitrate (10). However, there is a report that *B. subtilis* can grow anaerobically when nitrate is added as a terminal electron acceptor (21). The isolation of genes similar to those of the nitrate/nitrite reductase system of *E. coli* (25) prompted us to reexamine anaerobic growth of *B. subtilis*. Wild-type JH642 cells did not grow anaerobically on Luria-Bertani (LB) or LB-KNO_2 agar but did grow well on LB agar supplemented with 0.2% KNO_3 when incubated in an anaerobic jar by using a gas-generating kit (Becton Dickinson, Cockeysville, Md.). To test the effects of *narQ* and *narA* mutations on anaerobic growth, cells were grown in rich medium containing KNO_3 and Oxyrase [Oxyrase, Inc., Mansfield, Ohio) (Fig. 2). A mutant with a deletion mutation in *nasB* (LAB1727) grew as well anaerobically as did JH642, indicating that nitrate reductase encoded by *nasB* is not required for nitrate respiration. In contrast, the *narA1* (CU495) and Δ*narA* (LAB2037) mutants did not grow anaerobically. Since LAB1851 carrying Δ*narQ* and P*spac-narA* grew well anaerobically in the presence of IPTG and less well in the absence of IPTG, we conclude that *narA* is required for anaerobic respiration and that *narQ* is dispensable for anaerobic respiration.

The results presented above show that NarA is essential for both nitrate assimilation and anaerobic respiration, while nitrate reductase encoded by *nasBC* is required only for the assimilatory pathway. Since these results imply the existence of two distinct nitrate reductases, we measured nitrate reductase activities from cells cultured under different conditions. Assimilatory nitrate reductase activity was detected from the *narQ* mutant (LAB1851 in the presence of IPTG) and wild-type cells, but no activity was observed from the *narA1*, *nasB*, and *narA* deletion mutants (Table 4). When cells were grown in 2XYT-nitrate under aerobic conditions, no nitrate reductase activity was detected (0 h after a shift; Table 4), indicating that neither assimilatory nor respiratory nitrate reductase is produced under this condition. JH642 (wild type), LAB1727 (Δ*nasB*), and LAB1851 (Δ*narQ* P*spac-narA*) grown in the presence of IPTG induced nitrate reductase activity 7 h after a shift to anaerobic conditions, but in CU495 (*narA1*) and LAB2037 (Δ*narA*), no induction was seen. These results argue that a *nasB*-independent nitrate reductase is used for anaerobic respiration.

To test the role of the *narQ* gene product in formate dehydrogenase activity, enzyme activity was examined in the wild-type, *narQ*, and *narA* strains grown aerobically in 2XYT containing 2 μM sodium molybdate and sodium selenate. Wild-type cells had high phenazine methosulfate-linked formate dehydrogenase activity, but LAB1851 (Δ*narQ* P*spac-narA*) had very low activity when cells were cultured either with or without IPTG. LAB2037 (Δ*narA*) has no detectable activity, indicating that formate dehydrogenase from *B. subtilis* also contains molybdenum cofactor. This result indicates that both *narA* and *narQ* are required for formate dehydrogenase activity. Instability of formate dehydrogenase activity from cells cultured anaerobically prevented comparative assays.

B. subtilis has been widely believed to be a strict aerobe. But as reported elsewhere (21) and shown here, *B. subtilis* can grow anaerobically in the presence of nitrate as a terminal electron acceptor. Soil, the natural habitat of *B. subtilis*, includes an abundance of anaerobic environments, and anaerobic bacteria such as *Clostridium* spp. commonly exist in the upper layers of soil (17). To our knowledge, *narA* is the first gene reported to be required for anaerobic growth in *B. subtilis*. Because of the

importance of nitrate as a source of nitrogen for many organisms inhabiting soil and aquatic environments, its utilization by *B. subtilis* may present a fruitful and informative area of study.

Nucleotide sequence accession number. The *narQ* and *narA* sequences have been assigned GenBank/EMBL/DDJB accession number Z35277.

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