

Nitrogen and Oxygen Regulation of *Bacillus subtilis* *nasDEF* Encoding NADH-Dependent Nitrite Reductase by TnrA and ResDE

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The nitrate and nitrite reductases of *Bacillus subtilis* have two different physiological functions. Under conditions of nitrogen limitation, these enzymes catalyze the reduction of nitrate via nitrite to ammonia for the anabolic incorporation of nitrogen into biomolecules. They also function catabolically in anaerobic respiration, which involves the use of nitrate and nitrite as terminal electron acceptors. Two distinct nitrate reductases, encoded by *narGHI* and *nasBC*, function in anabolic and catabolic nitrogen metabolism, respectively. However, as reported herein, a single NADH-dependent, soluble nitrite reductase encoded by the *nasDE* genes is required for both catabolic and anabolic processes. The *nasDE* genes, together with *nasBC* (encoding assimilatory nitrate reductase) and *nasF* (required for nitrite reductase siroheme cofactor formation), constitute the *nas* operon. Data presented show that transcription of *nasDEF* is driven not only by the previously characterized *nas* operon promoter but also from an internal promoter residing between the *nasC* and *nasD* genes. Transcription from both promoters is activated by nitrogen limitation during aerobic growth by the nitrogen regulator, TnrA. However, under conditions of oxygen limitation, *nasDEF* expression and nitrite reductase activity were significantly induced. Anaerobic induction of *nasDEF* required the ResDE two-component regulatory system and the presence of nitrite, indicating partial coregulation of NasDEF with the respiratory nitrate reductase NarGHI during nitrate respiration.

Changes in oxygen tension and reduced nitrogen supply are common to the habitat of the soil bacterium *Bacillus subtilis*. When external oxygen is limited, *B. subtilis* is able to use an alternative electron acceptor, namely, nitrate or nitrite, and grows by anaerobic respiration (for reviews, see references 11 and 15). Nitrite reduction catalyzed by *B. subtilis* nitrite reductase does not result in a proton gradient and coupled ATP generation. Instead, nitrite enhances anaerobic growth by serving as an electron sink. However, this process can still be designated as respiration in a broad sense according to the simple definition of a process utilizing an inorganic electron acceptor. A respiratory nitrate reductase responsible for nitrate respiration is encoded by the *narGHJI* operon, the expression of which is highly induced by oxygen limitation (1, 5, 8). The induction of *narGHJI* depends on an anaerobic regulatory protein, FNR (1, 16). Oxygen-sensitive nitrite reductase activity was also detected in *B. subtilis* cells grown anaerobically in the presence of nitrate and nitrite (4). The nitrite reductase activity is dependent on the ResD-ResE two-component signal transduction system (16, 20) but not on FNR (1), as shown by examining nitrite-dependent anaerobic growth of *resDE* and *fnr* mutant cultures (4).

The complete *Bacillus* genome sequence was recently reported (7), and *B. subtilis* appears to have only one nitrite reductase

that is encoded by *nasD* and *nasE*. The NasDE nitrite reductase was previously identified as an assimilatory enzyme (17). *nasD* and *nasE* are part of an operon also containing two upstream genes, *nasB* and *nasC*, that encode subunits of assimilatory nitrate reductase and a downstream *nasF* gene required for biosynthesis of siroheme, a cofactor of nitrite reductase. The *nasA* gene, which is divergently transcribed from the *nas* operon, is thought to encode a nitrate transporter (Fig. 1) (17). Both *nasB* and *nasC* are transcribed from the *nasB* promoter, and transcription possibly extends through *nasD* to *nasF* (17). Both *nasA* and *nasB* promoter activities are high during nitrogen-limited growth and repressed in the presence of a good nitrogen source (14). The transcriptional activation of *nas* as well as that of other genes during nitrogen-limited growth was shown to be mediated by the TnrA regulatory protein, which binds to an upstream *cis*-acting site of nitrogen-regulated genes (22, 23).

NasBC nitrate reductase is not detected at all in cells grown anaerobically in rich medium (3), suggesting that transcription from the *nasB* promoter is repressed under these nitrogen-excess conditions. If nitrite reductase encoded by *nasDE* is indeed the enzyme required for nitrite respiration, the expression of *nasDEF* must be directed from another promoter during anaerobiosis. As reported herein, NasDE nitrite reductase functions as both an assimilatory and a dissimilatory enzyme, and an intergenic promoter specific for *nasDEF* transcription is activated both by oxygen limitation and by nitrogen limitation.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. subtilis* strains used in this study are derivatives of JH642 (*trpC2 pheA1*) (Table 1). A *nasF-lacZ* transcriptional fusion was constructed as follows. A 730-bp fragment containing the 3' end of *nasE* and the 5' end of *nasF* was isolated by digesting plasmid pMMN217 (17) with

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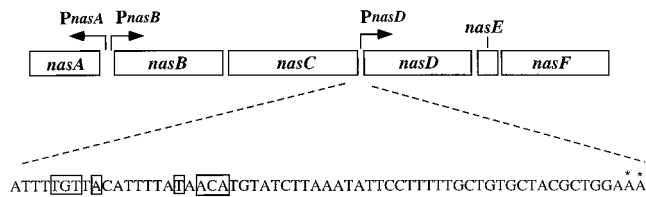


FIG. 1. Map of the *nas* region. The open boxes represent open reading frames that encode *NasA*, *-B*, *-C*, *-D*, *-E*, and *-F*. The locations of the promoters are shown by P, and arrows represent the directions of transcription. The nucleotide sequences of the *nasD* promoter region are shown beneath the operon diagram. Asterisks indicate transcription start sites determined by the primer extension analysis in Fig. 4. Nucleotides in boxes are the putative TnA binding site.

restriction endonucleases. The fragment thus obtained was inserted into promoter-probe vector pTKlac (6) to create pMMN224. Plasmid pMMN224 was introduced by transformation into JH642 with selection for chloramphenicol resistance (Cm^r). In the transformant (LAB1848) where pMMN224 was integrated into the *nasF* gene by Campbell-type recombination, the *lacZ* gene is transcribed from the *nasB* and *nasD* promoters as described in Results. pMMN224 was also introduced into *nasB* (LAB1727) and *nasD* (LAB1972) mutants to generate strains LAB2012 and LAB2008, respectively. The *nasB* and the *nasD* deletion mutants were constructed by replacing internal regions of the *nasB* and the *nasD* genes with antibiotic resistance markers as previously described (17). In order to construct a *nasC-lacZ* fusion, plasmid pMMN391 was first constructed. pMMN391 is a derivative of pTKlac (6) that carries a 970-bp internal fragment of *nasC* in front of the promoterless *lacZ* gene. LAB2844 cells carrying *nasC-lacZ* were generated by transformation of JH642 with pMMN391. A 350-bp fragment containing the *nasC* and *nasD* intergenic region along with the *nasD* promoter was obtained and inserted into pTKlacZ upstream of the promoterless *lacZ* gene. The resultant plasmid, pMMN392, was introduced by transformation into an SPβ lysogen, where it integrated into the resident SPβ prophage. A phage lysate carrying the *nasD-lacZ* fusion was introduced into wild-type and various mutant strains as described previously (24). The construction of *DresDE* (16, 20), *fnr* (1, 16), and *AnarGH* (5, 10) mutants was described elsewhere. A *tnrA* mutant (SF62) was obtained from Susan Fisher (22).

Examination of growth phenotype of *nasD* and *nasE* mutants. *B. subtilis* cells were grown anaerobically at 37°C in supplemented Luria-Bertani medium containing 10 mM (NH₄)₂SO₄ and NaNO₂ as described elsewhere (4, 5). Cells were also grown aerobically at 37°C in minimal medium containing 3.4 mM Na₃ citrate, 0.8 mM MgSO₄, 20 mM K₃PO₄ (pH 7.0), 1 mM tryptophan, 0.8 mM phenylalanine, 1 mM glucose, and 10 mM (NH₄)₂SO₄ or NaNO₂ as nitrogen source. Cells grown overnight in the minimal medium containing 10 mM (NH₄)₂SO₄ and 10 mM NaNO₂ were washed with the same medium without nitrogen source and were diluted 1:100 in fresh medium supplemented with 10 mM (NH₄)₂SO₄ or NaNO₂. LAB1982 and LAB1943 cells were grown in the presence of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Growth was monitored by measuring the optical density at 578 nm.

Measurement of nitrite reductase activity. *B. subtilis* cells used for measurement of nitrite reductase activity were grown aerobically or anaerobically as described above, except that aerobically grown cells were not washed before inoculating the fresh medium to support residual growth of the *tnrA* and the *nasD/E* mutant strains. Cells were harvested at late exponential growth phase, washed with 10 mM Tris-HCl (pH 7.4) and 10 mM MgCl₂, and disrupted with a French press. The crude lysate was centrifuged at 5,000 × g for 5 min at 4°C to remove intact cells and cell debris. The cell extract was used for enzyme assays. Subcellular fractionation was performed by centrifugation of the cell extract at 100,000 × g for 45 min at 4°C to separate the membrane (pellet) and cytosolic (supernatant) fractions. The membrane fraction was washed twice with distilled water to avoid cytosolic contamination. Contamination of both fractions was checked by measuring 5-aminolevulinic acid dehydratase as a marker enzyme for the cytosolic fraction and respiratory nitrate reductase as a marker for the membrane fraction, and no significant contamination of either fraction was observed. All procedures were done under strict anaerobic conditions for extracts prepared from both anaerobic and aerobic cultures because nitrite reductase is sensitive to oxygen, as previously described (4). Nitrite reductase activity of the cell extracts was measured under nitrogen by the nitrite-dependent oxidation of NADH in a cuvette containing 50 mM Tris-HCl (pH 7.5), 1 mM NADH, 1 mM KNO₂, and 50 to 200 μl of cell extract (containing 20 mg of protein per ml) at room temperature. Reactions were started by the addition of nitrite, and oxidation of NADH was monitored spectrophotometrically at 366 nm. Control reaction mixtures were incubated in the absence of external electron acceptors to determine the endogenous rate of NADH oxidation; this small background activity was subtracted from activities with nitrite to obtain net activity. One unit of enzyme activity corresponds to the reduction of 1 mmol of nitrite per min.

Measurement of β-galactosidase activity. *B. subtilis* cells grown overnight on DS (Difco sporulation) agar medium were used to inoculate 2× YT (yeast

extract-tryptone) liquid medium (12) supplemented with 1% glucose and 20 mM phosphate buffer (pH 7.0), which contained either 0.2% KNO₃ or 10 mM KNO₂, to an optical density at 600 nm of 0.02. Alternatively, cells grown aerobically overnight in TSS liquid medium (18) with 0.2% NH₄Cl were used to inoculate TSS medium containing 0.2% KNO₃ with or without 0.2% NH₄Cl. Anaerobic growth of cultures was carried out as described previously (16). To obtain cells grown aerobically with various nitrogen sources, cells grown overnight in TSS liquid medium with 0.2% NH₄Cl were used to inoculate TSS liquid medium with 0.2% glutamate, 0.2% NH₄Cl, 0.2% KNO₃, or 10 mM KNO₂. Samples were withdrawn at 1-h intervals, and maximum activities attained at late exponential to early stationary growth are shown in the tables. Assays for β-galactosidase were carried out as previously described (9).

Primer extension analysis. *B. subtilis* LAB2854 was grown aerobically or anaerobically in 2× YT medium supplemented with 1% glucose, 20 mM phosphate buffer (pH 7.0), and 0.2% KNO₃ to obtain cells for RNA purification. LAB2854 was also cultured under aerobic conditions in TSS medium with 0.2% NH₄Cl or 0.2% KNO₃. Total RNA was extracted from cells harvested at mid- to late exponential growth as previously described (13). The 5' end of *nasD* mRNA was localized by primer extension analysis with the Promega system. Forty micrograms of total RNA and 2 pmol of an end-labeled primer (5' CCCGGC CATTCCATTACCA 3') were combined in a hybridization mix containing primer extension buffer (50 mM Tris-HCl [pH 8.3], 50 mM KCl, 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM deoxynucleoside triphosphates, and 0.5 mM spermidine, supplied by the manufacturer). Primer-RNA hybridization was facilitated by heating for 1 min at 90°C, followed by a 2-min incubation at 60°C and a slow cooling of the hybridization mix to room temperature. Primer extension was performed at 42°C for 30 min with avian myeloblastosis virus reverse transcriptase. After treatment with RNase, the extension products were separated by electrophoresis in 6% polyacrylamide-8 M urea gels. The precise size of the primer extension product was determined by comparison with DNA sequencing reactions by the dideoxy chain-termination method (19) and the same oligonucleotide used for primer extension.

RESULTS

***B. subtilis* cells possess a *nasDE*-dependent nitrite reductase activity during aerobic and anaerobic growth.** Previous results strongly suggest that *nasD* and *nasE* encode subunits of nitrite reductase (17). We examined whether aerobic nitrite reductase activity is indeed detected in wild-type cells but not present in *nasD* or *nasE* mutants. NADH-dependent nitrite reductase activity was detected in a cell extract prepared from the wild-type strain grown with nitrite, but activity with NADPH was much lower; no activity was detected when ammonium was

TABLE 1. *B. subtilis* strains used in this study

Strain	Relevant characteristics	Source or reference
JH642	<i>trpC2 pheA1</i>	J. A. Hoch
LAB1727	<i>trpC2 pheA1 ΔnasB::erm</i>	17
LAB1848	<i>trpC2 pheA1 nasF::pMMN224</i>	This study
LAB1943	<i>trpC2 pheA1 ΔnasE::phleo Pspac-nasF</i>	17
LAB1972	<i>trpC2 pheA1 ΔnasD::phleo</i>	17
LAB1982	<i>trpC2 pheA1 ΔnasD::phleo Pspac-nasEF</i>	17
LAB2008	<i>trpC2 pheA1 ΔnasD::phleo nasF::pMMN224</i>	This study
LAB2012	<i>trpC2 pheA1 ΔnasB::erm nasF::pMMN224</i>	This study
LAB2135	<i>trpC2 pheA1 ΔresDE::tet</i>	16
LAB2136	<i>trpC2 pheA1 fnr::spc</i>	16
LAB2844	<i>trpC2 pheA1 nasC::pMMN391</i>	This study
LAB2854	<i>trpC2 pheA1 SPβc2del2::Tn917::pMMN392</i>	This study
LAB2862	<i>trpC2 pheA1 ΔresDE::tet SPβc2del2::Tn917::pMMN392</i>	This study
LAB2863	<i>trpC2 pheA1 fnr::spc SPβc2del2::Tn917::pMMN392</i>	This study
LAB2908	<i>trpC2 pheA1 tnrA::Tn917</i>	This study
LAB2911	<i>trpC2 pheA1 tnrA::Tn917 SPβc2del2::Tn917::pMMN392</i>	This study
LAB2408	<i>trpC2 pheA1 ΔnarGH::phleo</i>	10
LAB2966	<i>trpC2 pheA1 ΔnarGH::phleo SPβc2del2::Tn917::pMMN392</i>	This study
MH5081	<i>trpC2 pheA1 ΔresDE::tet</i>	20
THB1	<i>trpC2 pheA1 ΔnarGH::tet</i>	5
THB2	<i>trpC2 pheA1 fnr::spc</i>	4

TABLE 2. NADH-dependent nitrite reductase activities of various *B. subtilis* strains grown under aerobic and anaerobic conditions

Strain	Nitrite reductase activity \pm SD (10^{-4} U/mg of protein) ^a		
	Anaerobic growth	Aerobic growth	
		Nitrite	Ammonium
JH642 (wild type)	334 \pm 12 ^b	146 \pm 14 ^b	ND
THB1 ($\Delta narGH$)	351 \pm 21	164 \pm 18	ND
LAB1943 ($\Delta nasE Pspac-nasF$) ^c	ND	ND	ND
LAB1972 ($\Delta nasD$)	ND	ND	ND
LAB1982 ($\Delta nasD Pspac-nasEF$) ^c	ND	ND	ND
MH5081 ($\Delta resDE$)	ND	ND	ND
THB2 (<i>fnr</i>)	527 \pm 21	348 \pm 24	ND

^a NADH-dependent nitrite reductase activities were measured with cell extracts prepared from *B. subtilis* strains grown aerobically in minimal medium with nitrite and anaerobically in supplemented Luria-Bertani medium with nitrate and nitrite as described in Materials and Methods. Values reported are the averages of at least three experiments performed in triplicate. ND, not detectable (less than 40×10^{-4} U/mg of protein).

^b Activity with NADPH was less than 10% of that with NADH.

^c Cells were grown in the presence of 1 mM IPTG.

used as sole nitrogen source (Table 2). However, a cell extract prepared from a *nasD* mutant (LAB1972) grown with nitrite had no detectable nitrite reductase activity. To eliminate the possibility of polar effects caused by the *nasD* mutation on the expression of the downstream *nasE* and *nasF* genes, we used two strains that were constructed previously (17). In these strains, a *Pspac* promoter was integrated downstream of the mutated genes, allowing induction of the genes residing 3' to the mutation (LAB1943, $\Delta nasE Pspac-nasF$; and LAB1982, $\Delta nasD Pspac-nasEF$) by IPTG. Previous results showed that expression of *nasE* and *nasF* from the *Pspac* promoter is sufficient to support growth with nitrite as sole nitrogen source (17). However, extracts prepared from LAB1943 and LAB1982 grown with nitrite or ammonium in the presence of IPTG had no significant nitrite reductase activity, indicating that both *nasD* and *nasE* genes are required for enzyme activity. Since it was previously shown that NasD and NasE have high homology to subunits of nitrite reductases (17), together these results confirm that the *nasD* and the *nasE* genes encode subunits of assimilatory nitrite reductase.

The possibility that NasDE nitrite reductase also functions in anaerobic respiration was examined by measuring nitrite reductase activity in cell extracts prepared from anaerobic cultures of the wild-type and *nas* mutant strains (Table 2). NADH-dependent activity was detected in wild-type, but not in LAB1943 and LAB1982, strains. This result clearly indicates that NasDE nitrite reductase is the dissimilatory nitrite reductase involved in anaerobic respiration. No nitrite reductase activity was detected in the membrane fraction, whereas nearly all activity was present in the cytosolic fraction, indicating that the NasDE enzyme is a cytoplasmic protein.

In order to examine effects of *nasD* and *nasE* mutations on aerobic nitrite assimilation, LAB1982 (*nasD*) and LAB1943 (*nasE*) strains were grown aerobically in minimal medium containing nitrite or ammonium as sole nitrogen source. No significant difference in growth was observed between the wild type and the mutants grown with ammonium as sole nitrogen source (Fig. 2A); however, the mutants were unable to grow when nitrite was used as sole nitrogen source (Fig. 2B). This result is consistent with the previously observed growth defects of the mutants (17). *B. subtilis* grows anaerobically by fermentation in rich medium in the absence of an electron acceptor (10). When nitrite was present, the anaerobic growth was en-

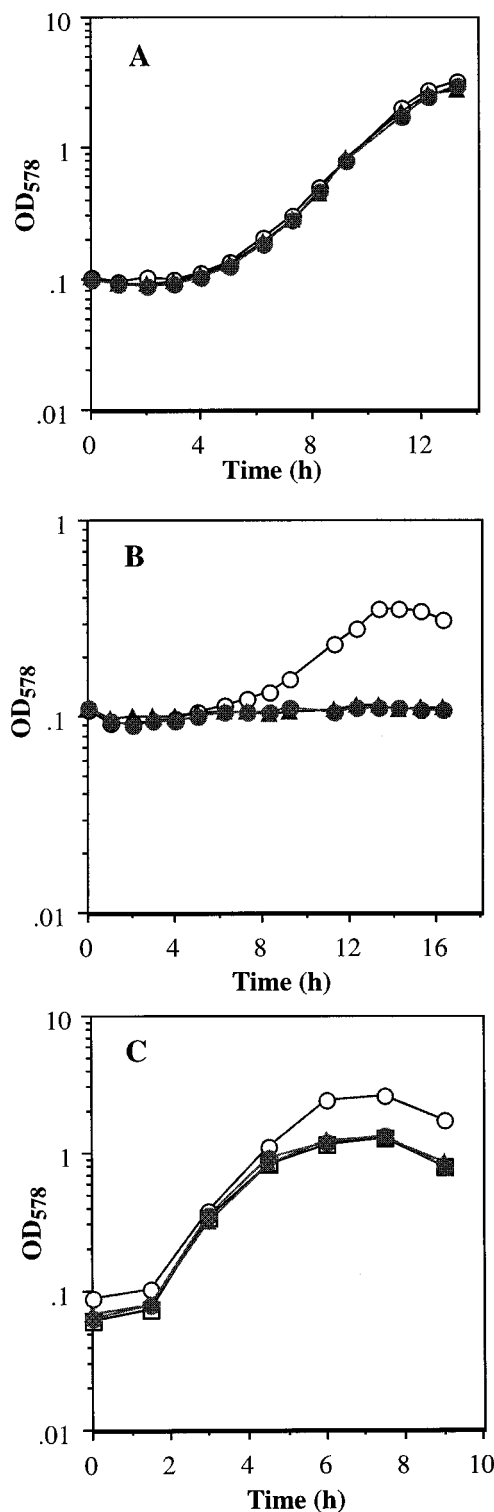


FIG. 2. Growth of *B. subtilis* wild-type, *nasD*, and *nasE* mutant strains: JH642 (wild type) (open circles), LAB1982 ($\Delta nasD Pspac-nasEF$) (closed circles), and LAB1943 ($\Delta nasE Pspac-nasF$) (closed triangles). The mutant strains were cultured in the presence of 1 mM IPTG. (A and B) Aerobic growth in minimal medium supplemented with 10 mM (NH₄)₂SO₄ (A) or with 10 mM NaNO₂ (B). (C) Anaerobic growth in rich medium containing 10 mM NaNO₂ and 10 mM (NH₄)₂SO₄. Open squares indicate growth of JH642 with ammonium only. OD₅₇₈, optical density at 578 nm.

TABLE 3. β -Galactosidase activities of *nasC/nasF-lacZ* fusions grown aerobically with various nitrogen sources

Nitrogen source ^a	β -Galactosidase activity (Miller units) ^b	
	LAB2844 (<i>nasC-lacZ</i>)	LAB1848 (<i>nasF-lacZ</i>)
Glutamate	53	22
Nitrate	19	20
Nitrite	19	22
Ammonium	0.4	0.9

^a Cells were grown aerobically in TSS minimal medium with 0.5% glucose containing the indicated nitrogen sources

^b Maximum activities during growth are shown (averages of three or more experiments). The standard error was less than 25%.

hanced as reported previously (4) and as shown in Fig. 2C. The growth behavior of the *nasD* and the *nasE* mutants in the presence of nitrite was identical to the fermentative growth of the wild-type cells. These results demonstrate that the NasDE enzyme functions both aerobically and anaerobically.

Effect of mutations in *resDE* and *fnr* on aerobic and anaerobic NasDE activity. ResD/E and FNR are known to be required for the transcription of genes that are expressed in response to oxygen limitation in *B. subtilis* (for reviews, see references 11 and 15). The two-component signal transduction system comprising the response regulator ResD and its cognate sensor kinase ResE (20) is required for induction of *fnr* transcription upon oxygen limitation and, thus, for the activation of other anaerobically induced genes requiring FNR for transcription (16). To determine if a *resDE* or an *fnr* mutation affects anaerobic nitrite reductase levels, nitrite reductase activity was examined in *resDE* and *fnr* mutant cells. A *resDE* mutant did not possess anaerobic NADH-dependent nitrite reductase activity, which confirmed the previous results with benzyl viologen as the electron donor (4). Furthermore, no nitrite reductase activity was detected in cell extracts of the *resDE* mutant prepared from cultures grown aerobically in minimal medium supplemented with ammonia or nitrite as sole nitrogen source. This result indicates that ResDE is required for both assimilatory and respiratory nitrite reductase activities.

Increased nitrite reductase activity was observed in cell extracts prepared from anaerobically grown *fnr* mutant (THB2) cultures compared to that of the wild type (4). Table 2 shows that the *fnr* mutant also possessed higher nitrite reductase activity during aerobic growth with nitrite than that of the wild-type strain, suggesting a slight negative effect of FNR on nitrite reductase activity during aerobic and anaerobic growth.

Regulation of nitrate and nitrite reductase genes. The results described above indicated that nitrite reductase encoded by *nasD* and *nasE* is essential for nitrite respiration during anaerobic growth. Our previous results showed that nitrate reductase encoded by *nasB* and *nasC* located upstream of the *nasD* gene functions only in nitrate assimilation during aerobic growth (3, 17). *B. subtilis* has another nitrate reductase (NarGHJI) that functions as a respiratory enzyme (1, 5, 8). Since NasBC is necessary only during aerobic growth under nitrogen-limited conditions and NasDE nitrite reductase must be produced in response to either nitrogen or oxygen limitation, it is very likely that expression of *nasDEF* is regulated differently from that of *nasBC*. In an attempt to analyze the regulation of nitrate reductase (*nasBC*) and nitrite reductase (*nasDEF*) genes, a promoterless *lacZ* gene was inserted into the *nasC* or *nasF* gene, and expression of *nasC-lacZ* and *nasF-lacZ* was examined under different culture conditions. Expression of *nasC* and *nasF* during growth in TSS minimal medium

with various nitrogen sources was examined (Table 3). It was previously shown that *nasB* operon transcription was derepressed during growth in the presence of poor nitrogen sources such as glutamate, proline, and nitrate (nitrogen-limited conditions) and repressed when growth media were supplemented with a good nitrogen source such as ammonium or glutamine (nitrogen-excess conditions). Table 3 shows that expression of *nasC* from the *nasB* promoter is regulated by the nitrogen source in the same manner as *nasB* expression (14). *nasF* expression is also observed to be derepressed by nitrogen limitation and repressed under conditions of nitrogen excess, which corresponds well to the observed nitrite reductase activities (Table 2). *nasC* expression is twofold higher in glutamate-containing medium than in nitrate- or nitrite-containing medium. However, *nasF* expression is equally derepressed in the presence of glutamate, nitrate, or nitrite. The significance of the difference is unknown; however, it may reflect *nasF* expression from the *nasD* promoter, which is higher in the presence of nitrate or nitrite than of glutamate as described below (Table 4). This result demonstrates that *nasC* and *nasF* are both under nitrogen regulation. Indeed, neither *nasC* nor *nasF* was expressed when cells were grown aerobically in a rich medium such as 2 \times YT (data not shown). When cells were cultured in 2 \times YT medium under anaerobic conditions, however, *nasF* expression was highly induced. In contrast, *nasC* was still severely repressed (Fig. 3). These results indicate that the nitrate reductase (*nasBC*) genes are activated only by nitrogen limitation but that the nitrite reductase (*nasDEF*) genes are induced by oxygen limitation as well as by nitrogen limitation. The pattern of *nasC* and *nasF* expression corresponds well to the function of their products.

An intergenic *nasD* promoter is essential for anaerobic induction of nitrite reductase genes. The results described above suggest that *nasDEF* is transcribed from an internal promoter under anaerobic conditions. Introduction of a *nasB* mutation had no significant effect on anaerobic *nasF* expression; however, a polar mutation in *nasD* abolished expression (Fig. 3). This suggests that there is likely an intergenic promoter upstream of *nasD* that is responsible for anaerobic *nasDEF* transcription. In order to test this possibility, the *nasC* and the *nasD* intergenic region was inserted into promoter-probe vector pTKlac (6), and the resulting *lacZ* fusion (called *nasD-lacZ*) introduced into the SP β prophage was observed to be activated both during nitrogen-limited growth (Table 4) and by anaerobiosis (Table 5) as was the case with *nasF-lacZ*. These findings indicate that *nasD*, *nasE*, and *nasF* are all transcribed from the *nasD* promoter. Anaerobic *nasD* expression was

TABLE 4. β -Galactosidase activities of *nasD-lacZ* fusions in wild-type and mutant strains grown under aerobic conditions

Nitrogen source ^a	β -Galactosidase activity (Miller units) ^b			
	LAB2854 (wild type)	LAB2862 (Δ <i>resDE</i>)	LAB2863 (<i>fnr</i>)	LAB2911 (<i>tnrA</i>)
Glutamate	14	13	20	0.4
Nitrate	40	21	43	0.6
Nitrite	88	29	84	<0.1
Ammonium	0.7	0.6	0.6	0.4
Nitrate plus ammonium	1.2	NE ^c	NE	NE
Nitrite plus ammonium	2.3	NE	NE	NE

^a Cells were grown aerobically in TSS minimal medium with 0.5% glucose containing the indicated nitrogen sources.

^b Maximum activities during growth are shown (averages of three or more experiments). The standard error was less than 25%.

^c NE, not examined.

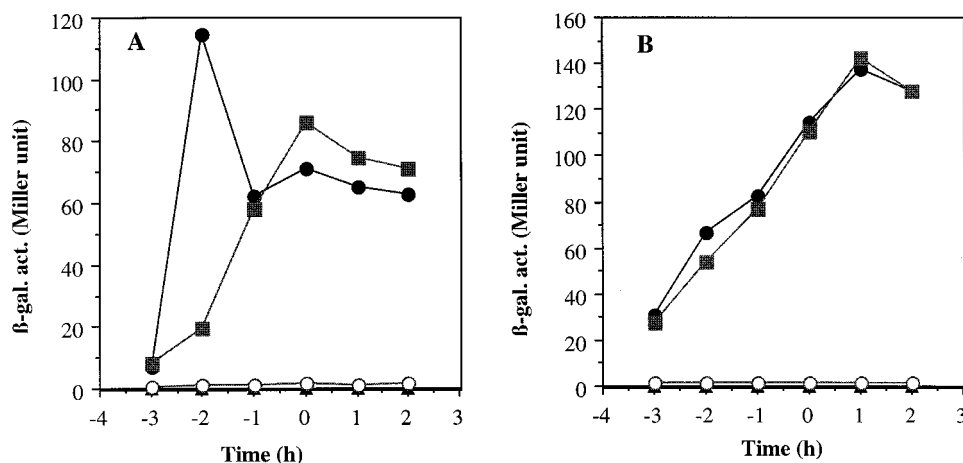


FIG. 3. Expression of *nasC-lacZ* and *nasF-lacZ* fusions. Cells were grown in $2\times$ YT medium containing 1% glucose and 20 mM phosphate buffer (pH 7.0) with 0.2% KNO_3 (A) or 10 mM KNO_2 (B) under anaerobic conditions. Time zero indicates the end of exponential growth. Closed circles, LAB1848 (wild type, *nasF-lacZ*); closed triangles, LAB2844 (wild type, *nasC-lacZ*); closed squares, LAB2012 (Δ *nasB nasF-lacZ*); open circles, LAB2008 (Δ *nasD nasF-lacZ*).

slightly but reproducibly higher when cells were grown in the presence of nitrite than when nitrate was present (Table 5). The expression of *nasD-lacZ* during aerobic growth in TSS medium with nitrite was two- and sixfold higher than that observed in cells grown with nitrate or glutamate, respectively (Table 4). However, previous work showed that *nasB* expression is slightly higher when glutamate is the sole nitrogen source than when nitrate is present (14). To determine if nitrite reductase gene expression is induced by its substrate nitrite under conditions of nitrogen excess, β -galactosidase activity was examined in LAB2854 cells grown in the presence of ammonium and nitrite (Table 4). β -Galactosidase activity was threefold higher in cells grown in TSS medium containing ammonium and nitrite than in cells grown without nitrite; however, the level was much lower than that observed in cells grown with nitrite alone, and addition of nitrate in the TSS-ammonium medium hardly stimulated *nasD* expression. These results suggest that aerobic *nasDEF* expression is not significantly induced by nitrate or nitrite under nitrogen-excess conditions.

Effect of mutations in *fnr*, *resDE*, and respiratory nitrate reductase genes on *nasD* expression. The results described above demonstrate that ResDE is indispensable for nitrite reductase activity and that FNR has a small negative effect on enzyme activity. To determine if the effect of *resDE* and *fnr* on nitrite reductase activity is caused by an effect on expression of *nasD*, *nasD-lacZ* expression was examined in *resDE* and *fnr* mutant cells. The maximal levels of β -galactosidase activity in *resDE* and *fnr* mutants grown anaerobically in $2\times$ YT medium with nitrate were 1 and 10%, respectively, of that detected in wild-type cells (Table 5). When cells were cultured in the presence of nitrite, however, the level of *nasD-lacZ* expression in the *fnr* mutant was restored to that of the wild-type strain. In contrast, nitrite had no stimulatory effect on *nasD* expression in the *resDE* mutant. FNR is required for transcription of anaerobically induced genes including the respiratory nitrate reductase (*narGHJI*) genes. To determine if the role of FNR in *nasD* expression is to transcribe *narGHJI* and thus to produce nitrite, an inducer for *nasD* transcription, *nasD-lacZ* expression was examined in a *narGH* mutant grown anaerobically in the presence of nitrate or nitrite. Table 5 shows that β -galactosidase activity in the *narGH* mutant was as low as that of the *fnr* mutant when cells were grown in the presence of nitrate,

and the activity was restored by the addition of nitrite. This result argues that FNR and the respiratory nitrate reductase are required for anaerobic *nasD* expression solely to produce nitrite while ResD and ResE have a more direct role in *nasDEF* regulation.

In contrast to the expression observed during anaerobic growth, aerobic *nasD* expression was not significantly affected by *resDE* or *fnr* mutations except that *nasD* expression in the *resDE* mutant was two- to threefold lower than that in the wild-type cells when grown in the presence of nitrate or nitrite (Table 4). These results show that ResDE and nitrite are indispensable for anaerobic induction of *nasD* expression but that ResDE and FNR play no major role in *nasD* expression under nitrogen-limited aerobic growth.

Effect of the *tnrA* mutation on *nasD* expression. Transcription of many nitrogen-regulated genes in *B. subtilis* including *nasB* is activated by TnrA, which responds to nitrogen-limited conditions (22). Table 4 shows that TnrA is also responsible for activation of *nasD* expression when cells are grown aerobically under poor nitrogen conditions (with glutamate, nitrate, or nitrite as nitrogen source). The *tnrA* mutant is unable to grow with nitrate or nitrite as sole nitrogen source; however, the

TABLE 5. β -Galactosidase activities of *nasD-lacZ* fusions in wild-type and mutant strains grown under anaerobic conditions

Strain	β -Galactosidase activity (Miller units) in medium ^a :			
	$2\times$ YT		TSS	
	Nitrate	Nitrite	Nitrate	Nitrate plus ammonium
LAB2854 (wild type)	339	566	394	296
LAB2862 (Δ <i>resDE</i>)	3.5	4.2	3.3	9.6
LAB2863 (<i>fnr</i>)	32	493	NE ^b	NE
LAB2911 (<i>tnrA</i>)	225	371	377	317
LAB2966 (Δ <i>narGH</i>)	30	384	NE	NE

^a Maximum activities during growth are shown (averages of three or more experiments). The standard error was less than 25%. Cells were grown anaerobically in TSS minimal medium with 0.5% glucose or in $2\times$ YT medium containing the indicated nitrogen sources.

^b NE, not examined.

growth defect per se is not responsible for the reduced *nasD* expression. In fact, LAB2844 is also unable to grow with nitrate as sole nitrogen source because the *nasC* gene is inactivated by the integration of the *lacZ* fusion; however, high *lacZ* expression was observed during incubation with nitrate (Table 3). In contrast to the aerobic expression, the *tnrA* mutation resulted in only slightly lower β -galactosidase levels in cells grown anaerobically in 2 \times YT medium containing either nitrate or nitrite (Table 5). These results demonstrate that *nasDEF* transcription from the *nasD* promoter is activated by TnrA during nitrogen-limited growth and by ResDE and nitrite under anaerobiosis.

ResDE, not TnrA, is required for *nasD* transcription during nitrogen-limited anaerobic growth. The results noted above raised a question whether TnrA or ResDE (or both) is involved in activation of *nasDEF* during nitrogen-limited anaerobic growth. In an attempt to answer this question, β -galactosidase activity from *nasD-lacZ* was examined in LAB2854 (wild type), LAB2862 (Δ *resDE*), and LAB2911 (*tnrA*) strains grown anaerobically in TSS medium containing nitrate or nitrate plus ammonium (Table 5). β -Galactosidase activity in the wild-type cells grown in the presence of nitrate and ammonium was comparable to the activity in the absence of ammonium, confirming that *nasD* expression is not repressed by conditions of nitrogen excess during anaerobic growth. However, mutation in *resDE* but not in *tnrA* abolished β -galactosidase activity under both nitrogen-excess and nitrogen-limited growth conditions. This result clearly shows that ResDE is mainly (if not exclusively) responsible for *nasDEF* expression during anaerobic growth regardless of nitrogen sources.

Identification of *nasD* transcriptional start sites. Primer extension analysis was carried out to identify the transcriptional start site for the *nasD* gene and to determine whether *nasD* is transcribed from the same start site when activated by nitrogen limitation as when activated by oxygen limitation. No primer extension product was observed with RNA isolated from cells grown aerobically in 2 \times YT medium or in TSS medium supplemented with a good nitrogen source (ammonium) (Fig. 4). When RNA was isolated from cells cultured anaerobically in 2 \times YT medium or aerobically in TSS medium with a poor nitrogen source (nitrate), primer extension products which corresponded to RNA with 5' ends located 30 and 31 bp upstream of the *nasD* start codon were detected. There is a consensus TnrA box (TGTNAN₇TNACA) centered 47 to 48 bases upstream of the transcriptional start site (Fig. 1). This result shows that *nasDEF* is most likely transcribed from the same transcriptional start sites during aerobic and anaerobic growth.

DISCUSSION

B. subtilis can utilize nitrate or nitrite as sole nitrogen source, and nitrate and nitrite are also utilized as electron acceptors for anaerobic respiration. While nitrate reduction is catalyzed by an aerobic assimilatory (NasBC) or an anaerobic respiratory (NarGHJI) enzyme, this paper shows that nitrite reduction is performed by a single soluble NADH-dependent reductase (NasDE). We also identified a *nasD* promoter utilized for *nasDEF* transcription that is induced by oxygen limitation via ResDE. Moreover, transcription of *nasDEF* was shown to be activated by TnrA during nitrogen-limited aerobic growth.

The TnrA-regulated promoters (*nrgAB*, *gabP* P2, *nasA*, and *nasB*) were shown to have a common dyad symmetry sequence (TGTNAN₇TNACA) upstream of the transcriptional start site (2, 14, 21). Recent studies have further demonstrated that the consensus dyad symmetry sequences function as TnrA binding sites and that high-level activation of the *nrgAB* promoter oc-

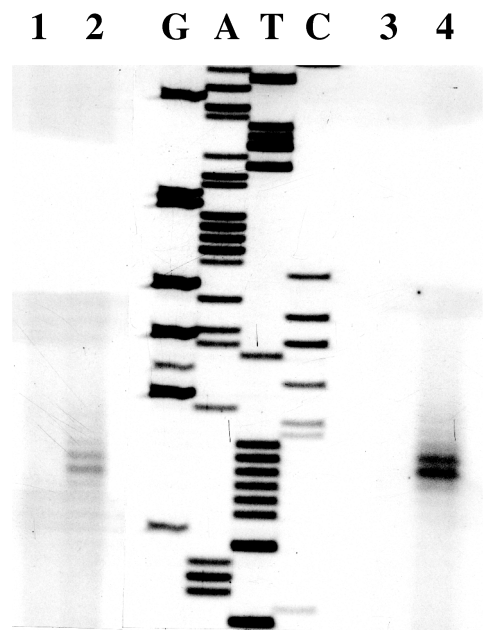


FIG. 4. Determination of the transcription start site of *nasD* by primer extension analysis. Total RNA was isolated from LAB2854 cells grown aerobically in TSS medium with 0.2% NH₄Cl (lane 1) or 0.2% KNO₃ (lane 2). RNA was also prepared from LAB2854 cells grown in 2 \times YT with 1% glucose, 20 mM phosphate buffer (pH 7.0), and 20 mM KNO₃ under aerobic (lane 3) or anaerobic (lane 4) conditions. The same oligonucleotide primer used for the primer extension analysis was used for sequencing by the dideoxy chain termination method (lanes G, A, T, and C).

curs only when the TnrA site is centered 49 to 51 bp upstream of the transcriptional start site (23). The TnrA site in the *nasD* promoter is located 47 to 48 bp, instead of 49 to 51 bp, upstream of the transcriptional start site, but it is not known at present if the TnrA-dependent control of *nasD* is significantly different from that of *nrgAB*. For the poor nitrogen sources tested, *nasD-lacZ* expression was highest when nitrite was present, with nitrate next, followed by glutamate. The stimulatory effect of nitrite (and nitrate probably after reduction to nitrite) is likely to be mediated mainly by ResDE since the *resDE* mutant exhibited much-reduced nitrite-dependent activation of *nasD* expression (Table 4). Furthermore, the positive effect of nitrite on the *nasD* expression is completely dependent on TnrA. These results suggest a possible interaction of TnrA and ResD in the presence of nitrite that is required for full induction of *nasD* transcription during nitrogen-limited aerobic growth. Since *nasC* expression is not increased during aerobic growth with nitrite (Table 3), the interaction between TnrA and ResD, if it exists, could be specific for *nasD* transcription among the TnrA-dependent promoters. As discussed below, under anaerobic conditions, ResDE and the presence of nitrite are sufficient to activate *nasD* transcription. Although significant levels of aerobic *nasD* expression were observed in the *resDE* mutant, aerobic nitrite reductase activity was not detected, suggesting that ResDE may affect nitrite reductase activity during aerobic growth. Alternatively, this difference may be attributed to the sensitivity limits of the nitrite reductase assay. As noted in Table 2, this spectroscopic assay cannot detect activity less than 40×10^{-4} U/mg of protein, indicating that the *resDE* mutant could have 30% of the wild-type activity, which corresponds to the result that *nasD-lacZ* activity in the *resDE* mutant is 30% of that in the wild-type strain during aerobic growth with nitrite (Table 4). In fact, the

resDE mutant is able to grow at a reduced rate with nitrite as sole nitrogen source, indicating that the low nitrite reductase activity in the *resDE* mutant is sufficient to allow cells to assimilate nitrite.

Anaerobically induced transcription of *nasDEF* during nitrogen-limited growth as well as under conditions of nitrogen excess is dependent on the ResDE two-component signal transduction system. The mutation in *resDE* nearly abolished *nasD* expression, indicating that TnrA fails to activate *nasDEF* expression under anaerobic conditions even when nitrogen is limited. There are some possible explanations for this latter result that can be tested experimentally. For example, expression of *tnrA* may be repressed during anaerobic growth; conversely, TnrA protein may not respond to nitrogen limitation or the activity of TnrA may be inhibited under anaerobic conditions. A previous study suggested that TnrA receives a signal for nitrogen availability that is generated by glutamine synthetase (22). Possibly, glutamine synthetase activity is affected by anaerobic respiration, resulting in loss of TnrA activity under these conditions. The *tnrA* mutant is unable to grow aerobically with nitrate or nitrite as sole nitrogen source, which is at least partly due to the inability to activate the *nas* genes (22). In contrast, the *tnrA* mutant can grow well anaerobically with nitrate or nitrite as sole nitrogen source (data not shown). This indicates that the respiratory nitrate reductase (NarGHJI) functions together with NasDE nitrite reductase during nitrogen-limited anaerobic growth. In fact, the *narGH* mutant was unable to grow anaerobically with nitrate as sole nitrogen source, but the *nasBC* mutant grew well (data not shown).

How is ResDE involved in transcriptional activation of *nasDEF* by oxygen limitation? ResD (response regulator) and ResE (kinase sensor) are known to be required for aerobic and anaerobic respiration in *B. subtilis* (20). Three anaerobically induced genes are known to be positively controlled by ResDE: namely, *fnr* (16), *hmp* (8), and *nasDEF*. Whether ResD phosphorylated by the ResE kinase binds directly to these promoters remains to be determined. Analysis of ResDE-controlled promoters is in progress, and the possibility that ResD directly binds to these promoters is under examination in order to elucidate ResDE-dependent anaerobic regulation in *B. subtilis*.

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