The Nitric Oxide-Responsive Regulator NsrR Controls ResDE-Dependent Gene Expression

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The ResD-ResE signal transduction system is essential for aerobic and anaerobic respiration in *Bacillus subtilis*. ResDE-dependent gene expression is induced by oxygen limitation, but full induction under anaerobic conditions requires nitrite or nitric oxide (NO). Here we report that NsrR (formerly YhdE) is responsible for the NO-dependent up-regulation of the ResDE regulon. The null mutation of *nsrR* led to aerobic derepression of *hmp* (flavo-hemoglobin gene) partly in a ResDE-independent manner. In addition to its negative role in aerobic *hmp* expression, NsrR plays an important role under anaerobic conditions for regulation of ResDE-controlled genes, including *hmp*. ResDE-dependent gene expression was increased by the *nsrR* mutation in the absence of NO, but the expression was decreased by the mutation when NO was present. Consequently, *B. subtilis* cells lacking NsrR no longer sense and respond to NO (and nitrite) to up-regulate the ResDE regulon. Exposure to NO did not significantly change the cellular concentration of NsrR, suggesting that NO likely modulates the activity of NsrR. NsrR is similar to the recently described nitrite- or NO-sensitive transcription repressors present in various bacteria. NsrR likely has an Fe-S cluster, and interaction of NO with the Fe-S center is proposed to modulate NsrR activity.

Nitric oxide (NO) is a most versatile signaling molecule whose effects are observed throughout all biological kingdoms. Because NO is highly reactive, it can interact with transition metals, cause S-nitrosylation, and react with superoxide to generate peroxynitrite, which is a strong oxidant. Nitrosylation of metals and cysteine is particularly suitable for controlling gene expression because it is a reversible modification (reviewed in references 7 and 36). Accumulated evidence indicates that NO controls bacterial gene expression by regulating the activities of transcription factors. Such factors include NnrR, NNR, DnrD, and DNR that activate denitrification genes in *Rhodobacter sphaeroides* (21), *Paracoccus denitrificans* (39), *Pseudomonas stutzeri* (40), and *Pseudomonas aeruginosa* (3), respectively. These transcription factors all belong to the cyclic AMP receptor protein/fumarate nitrate reductase regulator (FNR) family. In addition to DNR, *P. aeruginosa* has an NO-responsive, σ^{54} -dependent transcriptional regulator, FhpR, which activates the flavo-hemoglobin gene (2). FhpR is homologous to NorR, a member of the NtrC family of response regulators, although phosphorylation does not play a role in activation of NorR, unlike many response regulators (32). NorR, by responding to NO, controls the NO reductase gene in *Ralstonia eutropha* (32). Because deletion of the GAF (cyclic GMP-stimulated phosphodiesterases, adenylate cyclases, and FhlA) domain in the N-terminal region of NorR results in a constitutive (NO-independent) phenotype, it was proposed

that the GAF domain inhibits the ATPase activity of the central AAA⁺ domain, and the inhibition is released by receiving the NO-dependent signal (32). This assumption was experimentally proven by a recent study of *Escherichia coli* NorR (11), which showed that the NorR GAF domain contains a nonheme iron center that binds NO to form mononitrosyl iron. The nitrosylation stimulates the ATPase activity of NorR, thereby activating transcription of the *norW* genes encoding NADH-dependent NO reductase.

A similar nitrosylation of iron modulates activities of other transcription factors. For example, NO interacts with the [2Fe-2S] center in SoxR that controls gene expression in response to superoxide stress (12). Interaction of SoxR with NO leads to a dinitrosyl-iron formation in the [2Fe-2S] center. In vitro transcription experiments showed that the NO-modified SoxR is able to activate *soxS* at a level similar to that of oxidized SoxR. Similarly, NO reacts with the [4Fe-4S] cluster of FNR to generate a dinitrosyl-iron-cysteine complex (9). FNR represses expression of *hmp* in *E. coli* grown under anaerobic conditions. In contrast to the effect of nitrosylation on SoxR, nitrosylation of FNR reduces binding to the *hmp* promoter, leading to induction of *hmp*. Another example of an NO-sensitive regulator is the ferric uptake regulation protein (Fur), which contains nonheme ferrous iron. When cellular iron levels become low, Fur loses iron, and the apo repressor protein is unable to bind to its target DNA; hence, genes involved in iron uptake are expressed. The exposure to NO results in iron nitrosylation of Fur, leading to loss of DNA-binding activity through an uncharacterized conformational change (10).

We have previously shown that ResDE-dependent gene expression is stimulated by NO (25). The ResD-ResE signal transduction system is involved in the control of aerobic and anaerobic respiration in *Bacillus subtilis*. NO-dependent stim-

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TABLE 1. *B. subtilis* strains and plasmids

Strain or plasmid	Characteristic or description	Reference or source
Strains		
JH642	Parental strain (<i>trpC2 pheA1</i>)	J. A. Hoch
LAB2135	<i>resDE::tet</i>	30
LAB2252	SP β c2 Δ 2::Tn917::pMMN288 (<i>fnr-lacZ</i>)	30
LAB2408	<i>narG::phleo</i>	27
LAB2518	SP β c2 Δ 2::Tn917::pML107 (<i>hmp-lacZ</i>)	29
LAB2854	SP β c2 Δ 2::Tn917::pMMN392 (<i>nasD-lacZ</i>)	27
LAB2966	<i>narG::phleo</i> SP β c2 Δ 2::Tn917::pMMN392 (<i>nasD-lacZ</i>)	27
MH5636	His ₁₀ - <i>rpoC</i>	33
ORB3782	<i>narG::phleo</i> SP β c2 Δ 2::Tn917::pML107 (<i>hmp-lacZ</i>)	This study
ORB3927	<i>resDE::tet</i> SP β c2 Δ 2::Tn917::pML107 (<i>hmp-lacZ</i>)	This study
ORB4007	<i>hmp::pML67</i> (<i>hmp-lacZ</i>) Cm ^r	This study
ORB6120	<i>hmp::pML67</i> (<i>hmp-lacZ</i>) Spc ^r	This study
ORB6126	<i>resDE::tet hmp::pML67</i> Spc ^r	This study
ORB6179	<i>nsrR::cat</i>	This study
ORB6187	<i>nsrR::cat</i> SP β c2 Δ 2::Tn917::pMMN288 (<i>fnr-lacZ</i>)	This study
ORB6188	<i>nsrR::cat</i> SP β c2 Δ 2::Tn917::pMMN392 (<i>nasD-lacZ</i>)	This study
ORB6189	<i>nsrR::cat</i> SP β c2 Δ 2::Tn917::pML107 (<i>hmp-lacZ</i>)	This study
ORB6199	<i>resDE::tet nsrR::cat</i>	This study
ORB6220	<i>resDE::tet nsrR::cat</i> SP β c2 Δ 2::Tn917::pML107 (<i>hmp-lacZ</i>)	This study
ORB6436	<i>narG::phleo nsrR::cat</i>	This study
ORB6437	<i>narG::phleo</i> SP β c2 Δ 2::Tn917::pMMN288 (<i>fnr-lacZ</i>)	This study
ORB6439	<i>narG::phleo nsrR::cat</i> SP β c2 Δ 2::Tn917::pMMN288 (<i>fnr-lacZ</i>)	This study
ORB6440	<i>narG::phleo nsrR::cat</i> SP β c2 Δ 2::Tn917::pMMN392 (<i>nasD-lacZ</i>)	This study
ORB6441	<i>narG::phleo nsrR::cat</i> SP β c2 Δ 2::Tn917::pML107 (<i>hmp-lacZ</i>)	This study
TF274	<i>nsrR::cat</i>	This study
Plasmids		
pC194	<i>Staphylococcus aureus</i> origin; Cm ^r	18
pCBB31	pUC19 with the <i>cat</i> gene from pC194	This study
pGBKT7	Gal4 DNA binding domain	Clontech
pMMN448	pUC18 with <i>hmp</i> promoter (−191 to +79)	29
pMMN638	pGBKT7 with <i>nsrR</i>	This study
pMMN648	pPROEX1 with <i>nsrR</i>	This study
PPROEX-1	Expression vector with His ₆ tag	BRL
pSN64	pTYB4 with <i>sigA</i>	This study
pTKlac	Promoter-probe vector	19

ulation of the ResDE regulon is more profound under anaerobic conditions than aerobic conditions. The effect of NO on gene expression requires both ResD and ResE, but expression of *hmp* also exhibits ResDE-independent transcriptional control. We proposed that the ResE sensor kinase likely perceives a signal, either NO itself or a signal derived from NO, leading to phosphorylation of ResD (25). As previously described, ResE does not contain cysteine, which eliminates the possibility of S-nitrosylation by NO, and there is no evidence that ResE binds a metal. Therefore, it has remained mysterious how NO up-regulates ResDE-dependent gene expression. In the manuscript, we will present evidence that the activity of NsrR, a recently identified nitrite (NO)-responsive transcriptional regulator (34), is responsible for the up-regulation of the ResDE regulon in response to NO.

MATERIALS AND METHODS

Strains and plasmids. All *B. subtilis* strains used in this study are derivatives of JH642 except TF274, which is a derivative of the strain 168 (Table 1). Plasmids

TABLE 2. Oligonucleotides

Oligonucleotide	Sequence (5' to 3')	Purpose
oHG-1	GGCATCATCAGAGCAAGC AAAAAT	<i>nasD</i> template
oHG-7	AAATGCCCGGTTTTAAGG	<i>nasD</i> template
oMN98-24	GGAATTCAGAGGTGGC GTTA	<i>fnr</i> template
oMN98-25	CGGGATCCAGCAATTC ATAC	<i>fnr</i> template
oMN99-89	GGAATTCCTAAAAACAT AAGT	<i>hmp</i> template
oMN99-90	CGGGATCCAGTGCTTT TAAT	<i>hmp</i> template and primer extension
oMN05-296	GGCGCGGGCATATGAAG TTAACCAATTATAC	pMMN638
oMN05-297	GTCTCCCGGGCTATTCC TTCATTTTTAAAA	pMMN638
oSN02-39	CATGCCATGGCTGATAAA CAAACCAC	PSN64
oSN02-40	TTCAAGGAAATCTTTCAA ACGTTTAC	PSN64
oSN03-86	CATGTTTTTATCACCTAAA AGTTTACCAC	<i>rpsD</i> template
oSN03-87	CGATACACCTTATTGATA AGGAACAAAATG	<i>rpsD</i> template
PUC-F	GTITTTCCAGTCACGACG	<i>nsrR</i> mutation
PUC-R	GAATTGTGAGCGGATAAC	<i>nsrR</i> mutation
yhdE-F1	TGTCCTTGCTCAAGT TGTC	<i>nsrR</i> mutation
yhdE-R1	GTATCCGCTCACAATTC ACTTCATAATAAGACC	<i>nsrR</i> mutation
yhdE-F2	CGTCGTGACTGGGAAAAC CAAAGAAGATATCATG	<i>nsrR</i> mutation
yhdE-R2	AAGC ACTGGAAGTATAGCTT GAAG	<i>nsrR</i> mutation

and oligonucleotides are listed in Table 1 and Table 2, respectively. *E. coli* DH5 α [λ^- ϕ 80d λ cZ Δ M15 Δ (*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(r_K[−] m_K[−]) *supE44 thi-1 gyrA relA1*] was used for plasmid construction. The *nsrR* mutant (TF274) was constructed as follows. Upstream and downstream regions of *nsrR* were amplified by PCR with the primer sets yhdE-F1/yhdE-R1 and yhdE-F2/yhdE-R2, respectively. The *cat* (chloramphenicol acetyltransferase) gene was amplified by PCR from plasmid pCBB31 using primers PUC-F and PUC-R. The 5' ends of yhdE-F2 and yhdE-R1 are complementary to the sequence of PUC-F and PUC-R, respectively. Three PCR products were mixed and used as templates for the second PCR with primers yhdE-F1 and yhdE-R2. The resultant PCR fragment amplified via overlap extension was used for transformation of *B. subtilis* 168. The region amplified by PCR was sequenced, and one conserved mutation (GCA to GCG for Ala at residue 479) in the *yggB* gene was identified. The *nsrR* mutation was moved by transformation to the JH642 background to construct isogenic strains.

Antibiotics were used at the following concentrations: chloramphenicol, 5 μ g/ml; spectinomycin, 75 μ g/ml; tetracycline, 10 μ g/ml; erythromycin and lincomycin, 1 μ g/ml and 25 μ g/ml; ampicillin, 25 μ g/ml.

TF array analysis. Transcription factor (TF) array analysis was carried out similarly to a previously described method (17). In short, 285 genes encoding known and putative transcription factors were mutated by insertion of the *cat* gene. Chromosomal DNA isolated from *B. subtilis* carrying each mutation was spotted into 96-microwell plates. Competent cells of ORB 6120 (wild type) and ORB6126 (*resDE*) carrying *hmp-lacZ* (spectinomycin-resistance) were added into each well, and after transformation each culture was spotted onto Luria-Bertani (LB) agar plates supplemented with chloramphenicol. After overnight incubation at 37°C, colonies of the transformants were replica plated with a 96-pick replicator to LB agar containing chloramphenicol, spectinomycin, and 40 μ g/ml of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal).

Primer extension analysis. The wild-type strain (JH642) was grown anaerobically in 2 $\times$ yeast extract-tryptone (YT) medium (30) supplemented with 1% glucose and 0.2% KNO₃. Cells were harvested at an optical density at 600 nm (OD₆₀₀) of around 0.3. The *resDE nsrR* mutant (ORB6199) was grown aerobically in the same medium supplemented with tetracycline and chloramphenicol until an OD₆₀₀ of 0.5 was reached. RNA was purified using the RNeasy kit (QIAGEN) according to the manufacturer's instructions. Primer extension anal-

ysis was carried out as previously described (31) except that 4 µg of RNA was used with the primer oMMN99-90 that hybridizes with the *hmp* RNA between nucleotides 60 and 79 from the 5' end of the transcript. Sequencing reactions were performed using a Thermo Sequenase Cycle Sequencing Kit (United States Biochemical) with the primer oMMN99-90 and pMMN448 as template.

Measurement of β -galactosidase activities. *B. subtilis* cells were cultured either in 2× YT supplemented with 1% glucose and 0.2% KNO₃ or in 2× YT supplemented with 0.5% glucose and 0.5% pyruvate. An NO donor, spermine NONOate (Cayman Chemical), was dissolved in 10 mM NaOH at a concentration of 100 mM. An NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt (carboxy-PTIO) was purchased from Molecular Probes and dissolved in water to a concentration of 100 mM. Cells were harvested at 0.5- to 1-h intervals and β -galactosidase activity was measured as previously described (23).

Purification of proteins. In order to overproduce NsrR in *E. coli*, plasmid pMMN648 was constructed as follows. The *nsrR* coding region was amplified by PCR from JH642 chromosomal DNA using the primers oMN05-296 and oMN05-297. The resultant PCR product was digested with NdeI and SmaI, and the released *nsrR* fragment was inserted into PGBKT7 (Clontech) that was digested with the same enzymes to generate pMMN638. The *nsrR* gene recovered from pMMN638 by digesting with NdeI and BamHI was cloned into the NdeI-BamHI sites of a His₆-tagged expression vector pPROEX-1 (BRL) to construct pMMN648. *E. coli* BL21(DE3)/pLysS [F⁺-*ompT* *hds5*_{B(rB-mB)}-*gal dcm* (DE3) pLysS (Cm^r)] was transformed with pMMN648 plasmid DNA. The resultant transformant was cultured at 37°C in 1 liter of LB medium supplemented with ampicillin and chloramphenicol. At an OD₆₀₀ of 0.5, 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was added to the culture, which was further incubated for 6 h at 30°C. Purification of NsrR was carried out under aerobic conditions. The harvested cells were broken by multiple passages through a French press, and a cleared lysate was recovered by centrifugation at 15,000 × g for 20 min. The lysate was mixed with 3 ml of Ni²⁺-nitrilotriacetic acid (Ni-NTA) resin (QIAGEN) in buffer A (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole). The slurry was rotated slowly at 4°C for 1 h and applied to a column. The column was washed with 30 volumes of buffer A and then 10 volumes of buffer A containing 50 mM imidazole. His₆-NsrR was eluted with buffer A containing 300 mM imidazole. Fractions containing His₆-NsrR were pooled and dialyzed against buffer B (50 mM Tris-HCl, pH 7.6, 100 mM NaCl) and used for production of anti-NsrR antibody in rabbits (Josman, LLC).

RNA polymerase (RNAP) was purified from *B. subtilis* MH5636 (33), which produces an RNAP β' subunit fused to a His₁₀ tag. Purification of RNAP using Ni-NTA and HiQ chromatography was described elsewhere (31).

In order to purify σ^A protein, plasmid pSN64 was constructed as follows. The *sigA* gene was amplified by PCR using primers oSN02-39 and oSN02-40 and JH642 chromosomal DNA as a template. The PCR product was digested with NcoI and cloned into pTYB4 digested with NcoI and SmaI. *E. coli* ER2566 (New England Biolabs) carrying pSN64 was grown at 37°C in LB medium supplemented with ampicillin. ER2566 genotype is F⁺ λ^- *fluA2* [*lon*] *ompT* *lacZ*::*T7 gene1 gal sulA1* Δ (*mcrC-mnr*)114::IS10 *R*(*mcr-73::miniTn10-Tet*)² *R*(*zgb-210::Tn10*)(*Tet*)⁺ *endA1* [*dcm*]. When the OD₆₀₀ reached approximately 0.5, IPTG was added to a final concentration of 0.5 mM, and the cultures were further incubated at 25°C for 3.5 h. Cells were harvested by centrifugation and resuspended with buffer C (25 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1 mM EDTA, 1 mM MgCl₂, 10% glycerol) containing 0.05% Triton X-100. Cells were broken by passages through a French press, and the lysate was centrifuged at 20,000 × g for 20 min. The supernatant was applied onto a chitin column equilibrated with buffer C. After being washed with buffer C, the column was washed with buffer D (25 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM MgCl₂, 10% glycerol). The intein tag was cleaved by incubating the column at 4°C overnight with buffer D containing 50 mM dithiothreitol. Proteins were eluted with buffer D, and fractions containing SigA protein were combined and applied onto a HiQ column equilibrated with buffer D and eluted with a 100 mM to 500 mM KCl linear gradient. Fractions containing SigA were pooled and dialyzed against buffer D before being frozen at -70°C.

In vitro runoff transcription. The linear templates used for in vitro transcription assays were amplified by PCR using the oligonucleotides listed in Table 2 as previously described (14, 31). In vitro transcription was carried out as described in a previous paper (14) with minor modification. ResD and ResE (each, 1 µM) were incubated at room temperature for 15 min in 20 µl of transcription buffer (25 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 5 mM MgCl₂, 0.25 mM ATP, 50 µg of bovine serum albumin per ml, 10% glycerol, and 0.4 U of RNase inhibitor [RNasin; Promega]). RNAP, σ^A (both at 25 nM), various amounts of NsrR, and 5 nM template were added, and the reaction mixtures were incubated at room temperature for 10 min. ATP, GTP,

CTP (each, 100 µM), UTP (25 µM), and 5 µCi of [α -³²P]UTP (800 Ci/mmol) were added to start transcription. After incubation at 37°C for 25 min, the reaction was stopped, and transcripts were analyzed on a 6% polyacrylamide-urea gel. The gel was analyzed with a PhosphorImager (Molecular Dynamics), and the intensity of bands was quantified using ImageQuant.

Western blot analysis. To determine the NsrR amount, JH642 was aerobically and anaerobically cultured at 37°C in 2× YT supplemented with 1% glucose and 0.2% KNO₃. JH642 was also cultured in 2× YT with 0.5% glucose and 0.5% pyruvate under anaerobic conditions. At an OD₆₀₀ of 0.2, 100 µM spermine NONOate was added to the cultures grown in the presence of glucose and pyruvate, and the culture was further incubated for 1 h before harvest. As a negative control, ORB6179 (*nsrR::cat*) was used, which was cultured anaerobically in 2× YT supplemented with glucose and pyruvate. Cultures were harvested at an OD₆₀₀ of around 0.3 (for anaerobic cultures) or 0.5 (for aerobic cultures). Proteins were prepared from the harvested cells as described previously (4), and 15 µg of total protein was applied to a sodium dodecyl sulfate-polyacrylamide (15%) gel for electrophoresis. Western blot analysis was carried out using anti-NsrR antibody as previously described (29).

To determine whether the *nsrR* mutation affects the ResD level, JH642 and ORB6179 were cultured anaerobically in 2× YT supplemented with 1% glucose and 0.2% KNO₃ or 2× YT supplemented with 0.5% glucose and 0.5% pyruvate. Cells were harvested at an OD₆₀₀ of around 0.6, when expression of ResDE-controlled genes is the highest. Ten micrograms of total protein was applied to a sodium dodecyl sulfate-polyacrylamide (12%) gel for electrophoresis, and Western blot analysis was carried out using anti-ResD antibody (29).

RESULTS

A null mutation in *yhdE* (*nsrR*) results in aerobic derepression of *hmp*. Our previous results showed that NO up-regulates *hmp* expression under aerobic and anaerobic conditions, and it does so, albeit to a lesser extent, in the absence of ResDE (25). In the hope of identifying a regulator involved in ResDE-independent activation of *hmp* by NO, a TF array experiment was carried out. In this assay we first used ORB6126, a *resDE* mutant carrying *hmp-lacZ*. Two transformants exhibited a Lac⁺ phenotype. One such transformant was shown to carry a *cat* insertion in the *lacR* gene. Since *lacR* encodes a repressor of the *B. subtilis* β -galactosidase gene, the observed Lac⁺ phenotype was caused by activation of the endogenous β -galactosidase and not by activation of *hmp-lacZ*. The other Lac⁺ clone was identified as the *yhdE* mutant (Fig. 1A and B).

The result suggested that YhdE is a transcriptional repressor of *hmp* and that *hmp* is expressed in the absence of ResDE if *yhdE* is mutated. In this TF array experiment around 10% of transformants were not recovered, probably because some mutations, when combined with the *resDE* mutation, caused adverse effects on growth. Therefore, we repeated the TF array using a wild-type strain (ORB6120) carrying *hmp-lacZ*. All transformants were obtained in this experiment, and again the *lacR* and *yhdE* mutants showed a Lac⁺ phenotype (Fig. 1C). In addition, a *spo0A* mutant appeared pale blue on the X-Gal plate (data not shown), suggesting that Spo0A negatively regulates ResDE-dependent *hmp* expression, which is consistent with the previous observation that Spo0A exerts a negative role in *resDE* expression (37).

In order to confirm the TF array results, we used a transcriptional *hmp-lacZ* fusion to examine the effect of the *yhdE* mutation on aerobic *hmp* expression in the wild-type and *resDE* mutant strains. Figure 1D shows that *hmp* was expressed poorly in aerobic cultures of the wild-type strain as expected, whereas the expression was substantially increased in the *yhdE* mutant. It also shows that the aerobic derepression of *hmp* caused by the *yhdE* mutation was partially independent of the

FIG. 1. TF array experiment to identify a transcriptional regulator of *hmp*. The figure shows only 9 of 285 spots. (A) Assignment of a mutation in each array is indicated. *yhdE* is now named *nsrR*. (B) ORB6126 (*hmp-lacZ resDE*) was used as the recipient of transformation. (C) ORB6120 (*hmp-lacZ*) was used for transformation. The *resDE nsrR* and *nsrR* mutants expressed *hmp-lacZ* under aerobic conditions, as indicated by the blue area, on LB agar supplemented with X-Gal. Details are described in Materials and Methods. (D) Effect of the *nsrR* mutation on *hmp-lacZ* expression is shown in the wild-type (left) and *resDE* mutant (right) strains grown aerobically in 2 $\times$ YT supplemented with 1% glucose and 0.2% KNO₃.

ResDE two-component regulatory system. These results confirmed the TF array results indicating that YhdE is likely a transcriptional repressor of *hmp*. Similar *lacZ* assay experiments showed that the *yhdE* mutation does not bring about aerobic derepression of *fnr* and *nasD*, unlike *hmp* (data not shown).

Since anaerobic *hmp* expression is highly dependent on ResDE (22), we wondered if ResDE-independent transcription of *hmp* in the *yhdE* mutant under aerobic conditions utilizes a transcription start site different from that activated by ResD in anaerobic cultures. Primer extension analysis of RNA isolated from wild type cultured under anaerobic conditions confirmed the previously identified transcription start site (22) (Fig. 2). The experiment also showed that *hmp* is transcribed from the same start site in the aerobically grown *resDE yhdE* mutant cells.

A protein-protein BLAST search showed that YhdE belongs to a newly identified NsrR subfamily of the Rrf2 family (34) (see details in Discussion). NsrR from *Nitrosomonas europaea* is a nitrite-responsive repressor of *nirK* encoding nitrite reductase (5). In addition, a recent work showed that *E. coli nsrR* (*yjeB*) encodes a negative regulator of genes that are up-regulated by nitrosative stress (6). In light of these studies, we hypothesized that the previously observed ResDE-indepen-

FIG. 2. Identification of the transcription start site of *hmp*. Primer extension analysis was carried out using RNA isolated from ORB6199 (*resDE nsrR*) grown under aerobic conditions (lane 1) and JH642 (wild type) grown under anaerobic conditions (lane 2). Nucleotide sequence ladders with the same primer as that used for the primer extension experiment are shown on the left. The transcription start site is marked with an asterisk.

FIG. 3. Effect of the *nsrR* mutation on anaerobic expression of *hmp-lacZ* (A), *nasD-lacZ* (B), and *fnr-lacZ* (C). *B. subtilis* strains were grown anaerobically in 2× YT supplemented with 1% glucose and 0.2% KNO₃ (closed symbols) or with 0.5% glucose and 0.5% pyruvate (open symbols). (A) Circle, LAB2518 (wild type); square, ORB6189 (*nsrR*). (B) Circle, LAB2854 (wild type); square, ORB6188 (*nsrR*). (C) Circle, LAB2252 (wild type); square, ORB6187 (*nsrR*). Time zero is the end of the exponential growth phase.

dent induction of *hmp* upon nitrosative stress during aerobic growth is likely the result of inactivation of YhdE repressor activity. Hence, YhdE was renamed NsrR.

NsrR plays an important role in ResDE-dependent transcription during anaerobic growth. The result described above indicated that NsrR represses *hmp* expression under aerobic conditions. We have previously shown that oxygen limitation is required but not sufficient for anaerobic induction of *hmp* as well as other genes of the ResDE regulon. To attain full induction of ResDE-controlled genes, nitrate, nitrite, or NO is also needed. During nitrate respiration the membrane-bound respiratory nitrate reductase (NarGHI enzyme) catalyzes reduction of nitrate to nitrite. Nitrate reductase is required for nitrate-dependent up-regulation but not for nitrite- and NO-dependent activation (22, 25, 27, 28). Since the final product of nitrate respiration in *B. subtilis* is ammonium and NO is not an obligatory product of nitrate respiration, if and how NO is produced during nitrate respiration remains unknown. The previous observation of nitrite- and NO-dependent activation of ResDE-controlled genes is particularly intriguing, given that NsrR from *N. europaea* and *E. coli* was proposed to be a nitrite- and NO-sensitive repressor, respectively (5, 6, 34), although it has not been shown whether nitrite and NO directly regulates the repressor activity of NsrR. Therefore, we next examined whether NsrR has any role in *hmp* expression under anaerobic conditions.

As shown previously, *hmp* expression was low when wild-type cells were grown anaerobically under fermentative conditions (in the presence of glucose and pyruvate), and the expression was highly induced during growth by anaerobic respiration (in the presence of glucose and nitrate) (Fig. 3A). The *nsrR* mutation caused a dramatic increase in *hmp* expression in the absence of nitrate but resulted in decreased expression in the presence of nitrate. Importantly, the *nsrR* mutant becomes insensitive to nitrate (Fig. 3A). A similar effect by the *nsrR* mutation was observed during examination of anaerobic *nasD* and *fnr* expression (Fig. 3B and C). These results indicated that NsrR serves as a negative regulator for ResDE-dependent transcription in anaerobic cultures in the absence of nitrate. The results also suggested that full induction of ResDE-dependent gene expression observed in the presence of nitrate requires NsrR. Anaerobic expression of *hmp*, *nasD*,

and *fnr* in the *nsrR* mutant still required ResDE, although ResDE-independent *hmp* transcription at the level similar to aerobic expression was detected in the *nsrR* mutant (data not shown).

NO modulates NsrR activity. Unlike the wild-type strain, ResDE-dependent gene expression is not stimulated by nitrate in the *narG* mutant, but stimulation can be restored in the *narG* mutant by the addition of nitrite (22, 27, 28). Since NO had a stimulatory effect similar to nitrite, we hypothesized that NO is an inducer of the ResDE regulon, and nitrate is required for full induction because nitrate reductase reduces it to nitrite, which is then converted to NO (25). One could envisage that NsrR senses NO and regulates expression of the ResDE-controlled genes. In fact, *E. coli* NsrR, a repressor of genes that are up-regulated by nitrosative stress, was shown to be sensitive to NO (6). On the other hand, NsrR of *N. europaea* was reported to be sensitive to nitrite and not to NO (5). Therefore, we next examined whether NsrR-dependent gene regulation in *B. subtilis* is sensitive to NO. The *narG* and *narG nsrR* strains carrying *hmp-lacZ* were cultured in 2× YT supplemented with 0.5% glucose and 0.5% pyruvate. The *narG* mutant is unable to grow anaerobically by nitrate respiration, but it grows well in the presence of glucose and pyruvate by generating ATP through fermentation (26). Although the *narG* strain grew anaerobically in the glucose-pyruvate medium, *hmp* expression was very low (Fig. 4A). Consistent with the result shown in Fig. 3A, introduction of the *nsrR* mutation into the *narG* mutant caused a dramatic increase in *hmp* expression before the addition of spermine NONOate (Fig. 4A, time zero). We then investigated the effect of NO by using spermine NONOate as a source of NO. Spermine NONOate dissociates at neutral pH with a half-life of 39 min at 37°C, releasing 2 mol of NO per mole. Addition of 100 μ M spermine NONOate to the *narG* strain at an OD₆₀₀ of 0.2 resulted in a large increase in *hmp* expression. The *hmp* expression in the *narG nsrR* mutant was increased over time, which is likely due to accumulation of phosphorylated ResD. Unlike the *narG* strain, the expression in the *narG nsrR* mutant was hardly affected by exposure to spermine NONOate, although the expression began to increase 1 h after the addition (Fig. 4A). A similar stimulatory effect of NO on *nasD* and *fnr* expression was ob-

FIG. 4. Effect of spermine NONOate on anaerobic expression of *hmp-lacZ* (A), *nasD-lacZ* (B), and *fnr-lacZ* (C). *B. subtilis* strains were grown anaerobically in 2 $\times$ YT supplemented with 0.5% glucose and 0.5% pyruvate. At time zero, samples were withdrawn to measure β -galactosidase activity, and spermine NONOate was added to the rest of the tubes at a final concentration of 100 μ M. β -Galactosidase activities were measured in cells harvested at indicated times. Open symbols and closed symbols indicate cultures without and with spermine NONOate, respectively. (A) Circle, ORB3782 (*narG*); square, ORB6441 (*narG nsrR*). (B) Circle, LAB2966 (*narG*); square, ORB6440 (*narG nsrR*). (C) Circle, ORB6437 (*narG*); square, ORB6439 (*narG nsrR*).

served in the *narG* mutant but not in the *narG nsrR* mutant (Fig. 4B and C).

The observed effect of spermine NONOate strongly suggested that NO eliminates NsrR negative control, but we could not completely exclude the possibility that NO is oxidized to nitrite using a small amount of oxygen likely present in our oxygen-limited conditions. In attempts to test this possibility, we used an NO scavenger, carboxy-PTIO. Carboxy-PTIO interacts with NO and generates nitrite (1, 16); thus, it is useful for distinguishing whether nitrite or NO causes observed effects on gene control. Induction of *nasD* expression in the *narG* mutant at 0.5 and 1 h after the addition of 100 μ M spermine NONOate was eliminated by simultaneously adding 1 mM

carboxy-PTIO to the cultures (Fig. 5). Carboxy-PTIO is known to interact with NO by a 1:1 stoichiometry (1); however, we found under our culture conditions that carboxy-PTIO was consumed, probably by radicals produced by *B. subtilis*, and that equimolar amounts were not sufficient to scavenge NO. Thus, we used an excess amount of carboxy-PTIO over NO. Expression of *nasD* was not significantly affected by 1 mM carboxy-PTIO alone in the *narG* and *narG nsrR* strains, indicating that the effect of carboxy-PTIO in the NO-treated cultures was not caused by the scavenger itself. As shown before (27), nitrite (5 mM) also induced *nasD* expression in the *narG* mutant (Fig. 5). The increased *nasD* expression by nitrite was also abolished in the presence of carboxy-PTIO,

FIG. 5. Effect of carboxy-PTIO on NO- or nitrite-dependent induction of *nasD-lacZ* expression. Spermine NONOate (sper/NO; 100 μ M) or nitrite (5 mM) was added at time zero to the *narG* (LAB2966) or *narG nsrR* (ORB6440) strain grown anaerobically in 2 $\times$ YT supplemented with 0.5% glucose and 0.5% pyruvate. β -Galactosidase activity was measured at 0 h (before the addition of spermine NONOate or nitrite; white column) and at 0.5 h (gray column) and 1 h (black column) after the addition of spermine NONOate or nitrite. Carboxy-PTIO (1 mM) was added at time zero where indicated. The experiments were repeated two to three times, and average results are shown with standard deviations.

FIG. 6. Western blot analysis of NsrR (A and B) and ResD (C and D) in cells grown under different culture conditions. (A) Coomassie-stained gel. (B) Immunoblot using anti-NsrR antibody. Cleared lysates were prepared from the following: lane 1, ORB6179 (*nsrR*) cultured anaerobically in 2× YT supplemented with 0.5% glucose and 0.5% pyruvate; lane 2, JH642 (wild type) cultured anaerobically in 2×YT supplemented with 0.5% glucose and 0.5% pyruvate; lane 3, JH642 cultured anaerobically in 2×YT supplemented with 1% glucose and 0.2% KNO₃; lane 4, JH642 cultured anaerobically in 2×YT supplemented with 0.5% glucose and 0.5% pyruvate, followed by exposure to 100 μM spermine NONOate for 1 h; lane 5, JH642 cultured aerobically in 2×YT supplemented with 1% glucose and 0.2% KNO₃. Molecular mass markers are shown on the lane marked with M. (C and D) Western blot analysis of ResD protein in cells grown under anaerobic conditions. (C) Coomassie-stained gel. (D) Immunoblot using anti-ResD antibody. Cleared lysates were prepared from JH642 (lane 1) and ORB6179 (lane 2) cultured in 2×YT supplemented with 1% glucose and 0.2% KNO₃, and JH642 (lane 3) and ORB6179 (lane 4) cultured in 2×YT supplemented with 0.5% glucose and 0.5% pyruvate.

demonstrating that NO, generated from nitrite, is the direct effector of NsrR.

How does NO affect regulation through NsrR? One possibility is that the intracellular concentration of NsrR is regulated by NO, while the other possibility, which is more likely, is that NO affects NsrR activity. In order to examine the former possibility, NsrR levels were examined in cells cultured under different conditions (Fig. 6A and B). Western blot analysis using anti-NsrR antibody indicated that NsrR was slightly more abundant in anaerobic cultures than aerobic cultures. It also showed that the addition of nitrate or NO to anaerobic cultures, which induces ResDE-dependent gene expression, did not decrease NsrR levels. These results argue that the effect of NO on expression of the ResDE-controlled genes is not caused by regulation of the NsrR concentration by NO; instead, NsrR activity is likely regulated.

Next, we examined whether NsrR affects *resD* expression; ResD amounts were compared between wild type and the *nsrR* mutant (Fig. 6C and D). Western blot analysis using anti-ResD antibody showed that the ResD level was similar between wild type and the *nsrR* mutant cultured anaerobically in the presence or absence of nitrate. Therefore, it is unlikely that the effect of NsrR on ResDE-dependent gene expression is due to altered ResD expression.

NsrR directly represses *hmp* and *nasD* transcription. During aerobic purification of NsrR, we found that NsrR was brownish immediately after elution from an Ni-NTA column, and UV/visible light absorption spectra of the NsrR protein showed an absorbance at 440 nm and a shoulder at around 550 nm (unpublished data), indicating that *B. subtilis* NsrR is an Fe-S protein. However, the absorbance of the purified protein quickly disappeared under aerobic conditions, which suggests that the Fe-S center is sensitive to oxygen, as previously observed with many Fe-S proteins. Although it is very likely that, thus purified, NsrR protein lost the Fe-S center and, therefore, we were unable to assess the effect of NO on NsrR activity, we used *in vitro* transcription assays to examine whether the apo-NsrR protein retained repressor activity (Fig. 7). We used *rpsD* encoding ribosomal protein S4 as a control because the *nsrR* mutation does not affect *rpsD* expression *in vivo* (data not shown). At a concentration of 0.5 μM, NsrR repressed ResDE-dependent *hmp* and *nasD* transcription to 30 to 40%, indicating that NsrR directly represses transcription probably by binding to *hmp* and *nasD*. In contrast, *fmr* transcription was repressed only at a higher amount of NsrR. Since NsrR hardly affects *rpsD* transcription at the concentration we used, *fmr* expression could also be directly repressed by NsrR but at the higher concentration.

FIG. 7. Effect of NsrR on ResDE-dependent transcription in vitro. (A) The indicated amount of NsrR protein was incubated with ResD (1 μM), ResE (1 μM), and RNAP (50 nM). A 50 nM concentration of σ^A was also included. *rpsD* was used as a negative control. The asterisks indicate the positions of each transcript, *hmp* (86 bases), *nasD* (96 bases), *fnr* (101 bases), and *rpsD* (71 bases). (B) The intensity of each band was quantified with ImageQuant and is shown as percent transcript detected in the absence of NsrR. The data shown are the average of two independent experiments, and the standard deviations are indicated.

DISCUSSION

Our previous study showed that expression of ResDE-controlled genes, *nasD* and *hmp*, in particular, is activated by NO (24, 25). This NO-dependent stimulation is much stronger under anaerobic conditions than aerobic conditions. Since *B. subtilis* is not a denitrifier, reduction of nitrite generates ammonium, not NO. Therefore, it is not obvious if and how NO is endogenously generated during nitrate respiration. However, recent studies of nondenitrifying *E. coli* indicated that NO is produced from nitrite during nitrate respiration, and production requires periplasmic cytochrome *c* nitrite reductase (Nrf) and cytoplasmic siroheme-dependent nitrite reductase (Nir) (8). *B. subtilis* has only the siroheme-dependent nitrite reductase encoded by *nasDE*. Although it remains to be elucidated how *B. subtilis* generates NO, this work showed that NO, which is produced from nitrite under anaerobic condi-

tions, plays a pivotal role in ResDE-dependent gene activation through NsrR.

B. subtilis NsrR is a protein of 16.5 kDa that belongs to the NsrR subfamily of the Rrf2 family of putative transcriptional regulators found in widely divergent bacteria (34). Rrf2 family proteins have a predicted helix-turn-helix motif in the N terminus, which is likely involved in DNA binding. The NsrR subfamily is distinguished from other Rrf2 proteins because of conserved cysteines at the C terminus. *B. subtilis* has three Rrf2 family proteins, NsrR, CymR (YrzC), a master regulator of sulfur metabolism (13), and YwgB. Among the three *B. subtilis* proteins only NsrR has the conserved cysteines and is therefore classified into the NsrR subfamily. Several studies reported on gene regulation by the NsrR subfamily proteins. Rrf2 of *Desulfovibrio vulgaris* negatively regulates the *hmc* operon that encodes the high-molecular-mass cytochrome redox protein complex (20). *N. europaea* NsrR represses *nirK*, which encodes nitrite reductase, and the repres-

FIG. 8. Putative NsrR-binding sites in the *hmp* and *nasD* promoters. (A) Sequences similar to the proposed NsrR-binding site for gram-positive bacteria (34) are shown by numbered lines. The 3A and 3B sites are half-sites, and the 3B site is on the noncoding strand. The ResD-binding sites proximal to the promoter identified in a previous work (14) are marked by double lines labeled ResD. The most proximal ResD-binding site (ResD-B) is present on the opposite strand from the other ResD boxes. An arrow with a circle shows the start site and direction of transcription (22, 27). (B) Comparison of the putative NsrR-binding sites. Highly conserved and weakly conserved nucleotides in the putative consensus sequence are shown in uppercase bold and lowercase letters, respectively. An imperfect dyad symmetry sequence is shown by arrows. Abbreviations: R, A or G; Y, C or T.

sion is reversed by nitrite (5). RirA of *Rhizobium leguminosarum* is an iron-responsive regulator. Mutations in the *rirA* gene lead to constitutive expression of genes that are normally repressed by iron (38).

Although some circumstantial evidence was presented that NsrR homologs are transcriptional regulators (5, 41), direct evidence came from the *in vitro* transcription study that showed that IscR of *E. coli* directly represses the *iscSUA* operon encoding Fe-S cluster assembly proteins (35). Electron paramagnetic resonance analysis indicated that IscR contains a [2Fe-2S] cluster and that its full repressor activity requires the Fe-S cluster assembly proteins. This suggested that the [2Fe-2S] cluster functions in regulation of IscR repressor activity (35). A recent study of *E. coli* NsrR indicated that NsrR is a negative regulator of genes that are up-regulated by NO, and the *nsrR* mutation resulted in constitutive expression (6). Because NsrR-dependent repression requires iron, it was proposed that NsrR is either directly or indirectly inactivated by iron limitation. Taken together, these studies suggest that NsrR carries an Fe-S cluster, and apo-NsrR, caused by iron limitation or lack of the Fe-S cluster assembly protein, is unable to bind to DNA. Interaction of NO with the Fe-S cluster, which could bring about iron-nitrosylation or removal of iron, likely results in weakened DNA-binding activity. We have shown that aerobically purified NsrR directly represses *hmp* and *nasD* expression. The NsrR protein used in this experiment lacks an Fe-S cluster, as judged by the UV/visible light absorption spectra. Although we cannot exclude the possibility that the protein sample contains a small fraction of holo-NsrR, it is likely that the apo form of NsrR could bind to *hmp* and *nasD*.

All of the NsrR homologs so far identified were shown to be repressors except that IscR directly activates and represses a

different set of genes (15). Consistently, putative NsrR-binding sites, identified through comparative genomic analysis, are mostly located near the transcription start site (6, 34). We have shown that the NsrR plays a negative role as a direct repressor for *hmp* and *nasD* and possibly for *fur*.

In the hope of uncovering a clue to understanding the transcriptional control by NsrR, we examined whether there might be a putative NsrR-binding sequence in the regulatory region of *hmp*, *nasD*, and *fur*. Rodionov and coworkers applied comparative genomic approaches to predict DNA binding motifs for various NsrR orthologs (34). The predicted NsrR-binding motif is well conserved in gram-positive bacteria including most *Bacillus* and *Streptomyces* species, and candidate sites were observed only upstream of *hmp* (34). Sequences similar to the proposed NsrR-binding site for the gram-positive bacteria (gATGyAT-N3-ArATryat, where y is C or T and r is G or A, and highly conserved nucleotides are shown in uppercase letters) (34) were found in the *hmp* and *nasD* promoter regions (Fig. 8A). Two putative NsrR-binding sites were detected in the region overlapping the *hmp* transcription start site (sites 1 and 2), suggesting that these sites are targets for NsrR-dependent negative regulation. We detected two half-sites upstream of the *hmp* promoter, one of which is located on the noncoding strand. Our previous hydroxyl radical footprinting experiments showed that phosphorylated ResD binds in tandem to the same face of the DNA helix in the *hmp* promoter except for the most promoter-proximal ResD-binding site (Fig. 8, ResD-B) (14). Interestingly, this ResD-binding site is likely on the same helical face as one of the putative NsrR-binding half-sites (Fig. 8, shown as 3B). The other half-site (3A) and the ResD-binding site (Fig. 8, ResD-A) adjacent to the most promoter-

proximal site appear to be situated on the same face of the helix. It is tempting to speculate that one monomer of ResD interacts with NsrR bound to 3A and that the other ResD monomer interacts with NsrR occupying 3B. In the *nasD* promoter, a putative NsrR-binding site was detected immediately downstream of the most promoter-proximal ResD-binding site. In contrast to the *hmp* and *nasD* promoters, we could not detect a putative NsrR-binding site in the *fur* promoter. In this context it is worth mentioning that a recent study with IscR indicated that there are at least two different classes of IscR binding sites (15). Our study also suggested that NsrR may play a positive role in ResDE-dependent transcription. Although future studies are needed to elucidate in more detail the mechanism by which NsrR regulates ResDE-dependent gene regulation, this study has uncovered the link between oxygen limitation and NO, both of which are required for transcriptional activation of the ResDE regulon.

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