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Mechanisms of Adaptation to Nitrosative Stress in *Bacillus subtilis*▽†

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Bacteria use a number of mechanisms for coping with the toxic effects exerted by nitric oxide (NO) and its derivatives. Here we show that the flavohemoglobin encoded by the *hmp* gene has a vital role in an adaptive response to protect the soil bacterium *Bacillus subtilis* from nitrosative stress. We further show that nitrosative stress induced by the nitrosonium cation donor sodium nitroprusside (SNP) leads to deactivation of the transcriptional repressor NsrR, resulting in derepression of *hmp*. Nitrosative stress induces the sigma B-controlled general stress regulon. However, a *sigB* null mutant did not show increased sensitivity to SNP, suggesting that the sigma B-dependent stress proteins are involved in a nonspecific protection against stress whereas the Hmp flavohemoglobin plays a central role in detoxification. Mutations in the *yjbIH* operon, which encodes a truncated hemoglobin (YjbI) and a predicted 34-kDa cytosolic protein of unknown function (YjbH), rendered *B. subtilis* hypersensitive to SNP, suggesting roles in nitrosative stress management.

Nitric oxide (NO) is a free radical of major importance as a defense and signaling molecule (26). However, at high concentrations, NO is toxic to all cells. NO exerts toxic effects by binding to heme-, iron-, and copper-containing proteins. NO can be reduced or oxidized and thereby give rise to a series of compounds that are collectively referred to as reactive nitrogen species (RNS) (6, 34). Together with superoxide, NO rapidly forms the highly reactive intermediate peroxynitrite ($ONOO^-$), which in turn can form peroxynitrous acid (ONOOH), an unstable and reactive oxidizing species. Another important RNS, the nitrosonium ion (NO^+), can react with a variety of organic side groups, especially with thiols to form *S*-nitroso compounds (19). RNS react with many cellular components, including metals, lipids, thiols, and DNA, leading to membrane and DNA damage and to inhibition of respiration and other cellular activities. Biological and chemical processes contribute to the presence of RNS in nature. For example, through the action of NO synthases, phagocytic cells generate NO to inhibit infectious bacteria (6), and in soil NO is present as a result of microbial denitrification (3). In addition, bacteria that use nitrite as an electron acceptor can generate NO endogenously (4).

Bacteria have evolved a number of strategies for coping with the toxic effects exerted by RNS. They adapt by activating genes that encode proteins involved in detoxification, repair, and maintenance of homeostasis (reviewed in reference 30). In *Escherichia coli* an NO reductase, NorVW, and a flavohemoglobin play prominent roles in RNS detoxification. The latter enzyme is present in a wide variety of bacterial and eukaryotic microorganisms (30). Flavohemoglobins are oxygen-binding proteins composed of a heme-containing globin domain fused with a ferredoxin reductase-like flavin-binding domain (41). Under oxic conditions they efficiently oxidize NO to NO_3^-, and in the absence of oxygen they reduce NO to N_2O (9, 41). Not all bacteria encode flavohemoglobins but some, for example, *Mycobacterium tuberculosis*, possess a compact hemoglobin, called truncated hemoglobin (trHb), that could fulfill the same function (27). In the gram-positive soil bacterium *Bacillus subtilis*, both a flavohemoglobin (Hmp) and a trHb (YjbI) are present. The structure of YjbI is known (10), but its physiological role remains unclear. Expression of the *B. subtilis hmp* gene is induced upon exposure to NO or sodium nitroprusside (SNP) ($Na_2[Fe(CN)_5(NO^+)]$) (23), a nitrosating agent that can release NO^+ spontaneously at physiological pH (14). However, the physiological role of Hmp in nitrosative stress management has not been established. The promoter of the *hmp* gene is induced during nitrate respiration by a shift to anaerobic conditions but not during fermentative growth in *B. subtilis* (17). Expression of *hmp* has been shown to be dependent on the ResD-ResE two-component system (23). A recent study has employed DNA microarrays to analyze the response of the *B. subtilis* transcriptome to NO (21). It was found that nitrosative stress induces expression of *hmp* and members of the PerR, Fur, and sigma B general stress regulons. Studies on *E. coli* have shown differences in transcriptome responses to NO and NO^+ (33). In this study we present a genome-wide transcriptome analysis of the *B. subtilis* response to NO^+ (SNP) and compare our data to the transcriptome analysis of the response to NO. A major difference revealed is that none of the genes that are up-regulated by SNP are members of the Fur regulon. We show that Hmp but not gene products that depend on sigma B for expression is required for an adaptive response to SNP in *B. subtilis*. Cells lacking trHb and YjbH, a protein of unknown function, were found to be hypersensitive to SNP indicating a role in nitrosative stress management. Moreover, we have elucidated the mechanism by which *hmp* transcription is regulated in response to nitrosonium stress.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *B. subtilis* strains and plasmids used in this study are listed in Table 1. Oligonucleotides used as primers are listed in Table 2. *E. coli* strain TOP10 [*mcrA* Δ(*mrr-hsdRMS-mcrBC*)

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

Strain or plasmid	Relevant characteristics[a]	Source or reference[b]
Strains		
LAB2026	*trpC2 pheA1* Δ*hmp*::*cat* Cm^r	17
MH5081	*trpC2 pheA1 resDE*::*tet* Tc^r	36
1A1	*trpC2*	BGSC[c]
LUW219	Δ*yjbIH*::*spc* Sp^r	18
LUW222	*yjbI*ΩpMUTIN2mcs-*yjbI′* Em^r	This study
LUW229	*sigB*Δ*2*::*spc* Sp^r	18
LUW260	Δ*hmp*::*cat* Cm^r	LAB2026→1A1
LUW261	Δ*hmp*::*cat yjbIH*::*spc* Cm^r Sp^r	LUW219→LUW260
LUW264	Δ*resDE*::*tet* Tc^r	18
LUW272	Δ*yjbH*::*spc* Sp^r	This study
LUW340	Δ*nsrR*::*spc* Sp^r	This study
LUW365	Δ*resDE*::*tet* Δ*nsrR*::*spc* Tc^r Sp^r	MH5081 → LUW340
Plasmids		
pCR-Blunt II-TOPO	Cloning vector; Km^r	Invitrogen
pCW7	Expression vector capable of replication in *E. coli* and *B. subtilis*; Cm^r	This study
pCW7-*yjbI*	*yjbI* under the P*spac* promoter in pCW7	This study
pCW7-*yjbH*	*yjbH* under the P*spac* promoter in pCW7	This study
pCW7-*yjbIH*	*yjbIH* under the P*spac* promoter in pCW7	This study
pDG1726	Integration vector for *B. subtilis*; Sp^r Am^r	11
pDG1727	Integration vector for *B. subtilis*; Sp^r Am^r	11
pDG148	Expression vector capable of replication in *E. coli* and *B. subtilis*; Am^r Km^r	35
pDG148-*hmp*	*hmp* under the P*spac* promoter in pDG148	This study
pHP13	Expression vector capable of replication in *E. coli* and *B. subtilis*; Em^r Cm^r	12
pMUTIN2mcs	Integration vector for *B. subtilis*; Em^r Am^r	38
pΔ*nsrR*1	Km^r Sp^r	This study
pΔ*yjbIH*1	Ap^r Sp^r	18
pΔ*yjbH*1	Km^r Sp^r	This study
pYjbI2	Insertional Em^r plasmid carrying an internal part of *yjbI*	This study
pTOPOYjbIH	Km^r	This study

[a] All LUW strains are derivatives of 1A1 and thus carry the *trpC2* auxotrophic marker. Am^r, Cm^r, Em^r, Km^r, Tc^r, and Sp^r, resistance to ampicillin, chloramphenicol, erythromycin, kanamycin, tetracycline, and spectinomycin, respectively.
[b] Arrows indicate transformation and point from donor DNA to recipient strain.
[c] BGSC, Bacillus Genetic Stock Center, Department of Biochemistry, Ohio State University, Columbus.

ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nupG*] was used for the propagation of plasmids. *B. subtilis* strains were grown at 37°C in nutrient sporulation medium with phosphate supplemented with 0.5% glucose (NSMPG) (39). Tryptose blood agar base medium (TBAB) was used for growth of bacteria on plates. Growth for RNA extraction was performed in 250-ml baffled E-flasks containing 25 ml of NSMPG at 37°C and 100 rpm. For microarray analysis and real-time PCR, *B. subtilis* strains were grown to exponential phase (optical density at 600 nm [OD_{600}] = 0.6), at which point SNP, $NaNO_2$, or $NaNO_3$ was added to different final concentrations. Control cultures were left untreated. At 5 minutes after the addition, samples were taken out for RNA preparation. For reverse transcriptase PCR (RT-PCR),

TABLE 2. Oligonucleotide sequences

Oligonucleotide	Sequence (5′→3′)
yjbI1	GGCAGATGGGACAATCGTTTAACGCACC
yjbI4	CCAAGCTTCTATCGCAACTTGTTGATAC
yjbI5	CCGAATTCCAGCCCTACATGGTCCATTGCG
yjbI9	GGGCATGCTGCTGATGAAAGTCAAGCAGTCTTA
yjb14	CCGAATTCCTCCTTTCCAAGAAACGC
yjb16	TTATCGCTTCCGCAAGCCTTACCGC
yjb17	ATAGGCTCTCCTGCATATTTCTGAG
yjbH2	TTTTTCACATGATTGATATTCATCAG
yjbH4	TGATCTAGATGACAAACTATCAGCATGAGC
hmp1	GGTCTAGATGTTAGATAACAAAACAATCG
hmp2	GGGTCGACTCAAACGGACTGCGCCAAACTTA
yxgB1	CAATTTACTTAAAAGACACAAGCC
lytF1	CGAAATCATTGTGTTAATCTTCGC
hmpRTfw	CCACAAGCACCGCAGTATC
hmpRTrev	GCTTGCTCTTCAGCCTGTTC
nasDRTfw	TGACTTTGTGAGGCGTGTTC
nasDRTrev	TTGATATGCCATCGGGCTAC

B. subtilis 1A1 cells were grown to stationary phase. L-broth or L-agar was used for growth of *E. coli* strains (32). The following antibiotics were used when required: chloramphenicol (5 μg ml^{-1}), kanamycin (5 μg ml^{-1}), tetracycline (15 μg ml^{-1}), and a combination of erythromycin (0.5 μg ml^{-1}) and lincomycin (12.5 μg ml^{-1}) for *B. subtilis* strains and ampicillin (100 μg ml^{-1}) and chloramphenicol (12.5 μg ml^{-1}) for *E. coli* strains.

DNA techniques. DNA manipulations and *E. coli* transformations were carried out as described by Sambrook et al. (32). Isolation of *B. subtilis* chromosomal DNA and transformation of *B. subtilis* strains with chromosomal or plasmid DNA were performed as described by Hoch (13). DNA sequencing was carried out on isolated plasmid DNA or PCR products using the BigDye terminator v3.1 cycle sequencing reaction kit (Applied Biosystems) and an ABI Prism 3100 DNA sequencer (PE Biosystems).

Construction of mutants. The template used for the PCRs described below was chromosomal DNA isolated from *B. subtilis* strain 1A1. The *yjbH* mutant was constructed as follows. A fragment containing *yjbIH* and the region downstream of *yjbH* was amplified by PCR using primer pair yjbI1/yjbI9 (Table 2) and inserted into pCR-Blunt II-TOPO. The recombinant plasmid was transformed into *E. coli*. The resulting plasmid, pTOPOYjbIH, was isolated, cut with NruI and BglII and ligated to a 1,484 bp NruI/BamHI fragment from pDG1727 containing a spectinomycin resistance gene to give pΔ*yjbH1*. This plasmid was used to transform *B. subtilis* 1A1 to spectinomycin resistance. Transformants were screened by PCR amplification to identify recombinants, which carried a deletion of the *yjbH* gene arising from a double-crossover event (data not shown). One obtained recombinant (LUW272) was chosen for further analysis. The *yjbI* and *yjbH* genes were inactivated as follows. To create an insertion in *yjbI* and a conditional *yjbH* mutation, a 270-bp HindIII-EcoRI fragment containing an internal part of *yjbI* was produced by PCR using the primer pair yjbI4/yjbI5 (Table 2) and ligated with the HindIII-EcoRI-digested integration vector pMUTIN2mcs, generating plasmid pYjbI2. Upon transformation into *B. subtilis* 1A1 and integration into the chromosome by recombination, pYjbI2 disrupts

the *yjbI* gene and places *yjbH* under the control of the isopropyl-D-thiogalactopyranoside (IPTG)-inducible promoter P*spac*. At the same time, a transcriptional fusion between the natural *yjbI* promoter and the *lacZ* gene is created. The resulting strain was designated LUW222. An *nsrR* (*yhdE*) mutant was constructed as follows. A fragment containing *nsrR* and part of the upstream (*ygxB*) and the downstream (*lytF*) genes was amplified by PCR using primer pair yxgB1/lytF1 (Table 2) and inserted into pCR-Blunt II-TOPO. The recombinant plasmid was transformed into *E. coli*. The resulting plasmid was isolated, cut with HpaI and SspI, and ligated to a 1,498-bp PvuII fragment from pDG1726 containing a spectinomycin resistance gene to give p*ΔnsrR1*. This plasmid was used to transform *B. subtilis* 1A1 to spectinomycin resistance. Transformants were screened by PCR amplification to identify recombinants, which carried a deletion of the *nsrR* gene arising from a double-crossover event (data not shown). One obtained recombinant (LUW340) was chosen for further analysis.

Construction of plasmids. Plasmid pCW7 is a low-copy-number vector for overproduction of proteins in *B. subtilis.* It was derived from pHP13 and pDG148. It carries the pTA1060 origin of replication (37), the pTA1060 *rep*60 gene, the *cat* gene from pHP13, and the *spac*-I promoter and the *lacI* gene from pDG148. Details of the construction will be described elsewhere. Plasmids for autologous expression of *yjbI* and *yjbH* were derived from pCW7. Plasmid pCW7-*yjbIH* was constructed by amplifying *yjbIH* by using primers yjbI1 and yjbI9 (Table 2). The PCR product was cloned into pCW7 at XbaI and SalI restriction sites. Plasmid pCW7-*yjbH* was constructed by amplifying *yjbH* by using primers yjbH2 and yjbH4 (Table 2). In the primary annotation of the *yjbH* region, no apparent ribosome-binding site precedes the ATG start codon (16). By examination of the sequence upstream of the suggested translational start site of *yjbH*, the choice of start codon was refined. An alternative start codon (TTG) present upstream of the ATG codon is preceded by a putative ribosome-binding site (GGAGG). This alternative start codon would specify YjbH containing an additional 24 amino acid residues compared to that previously predicted. The *yjbI* translation termination codon overlaps the putative *yjbH* translation initiation codon by two bases. Such an overlap can lead to translational coupling. The alternative start codon was used in the *yjbH* expression plasmid. The *yjbH* PCR product was cut with XbaI and EcoRI and cloned into pCW7-*yjbIH* that had been cut with the same restriction enzymes. Plasmid pCW7-*yjbI* was constructed by deletion of a large part (a 753-bp SphI fragment) of *yjbH* in plasmid pCW7-*yjbIH.* Plasmid pDG148-*hmp* was constructed using primers hmp1 and hmp2 (Table 2). The amplified DNA fragment was cut with XbaI and SalI and cloned into pDG148 that had been cut with the same restriction enzymes. The template used for all the PCRs described above was chromosomal DNA isolated from *B. subtilis* strain 1A1.

Microarray analysis. The procedures used for microarray analysis, cDNA labeling, slide hybridization, data collection, and normalization have been described previously (18). Growth was performed as described above, and in exponential phase (OD_{600} = 0.6), SNP was added to a final concentration of 0.5 mM. RNA isolated from control cells and SNP-treated cells was used to prepare cDNA labeled with Cy3-dCTP and Cy5-dCTP, respectively.

RNA isolation and cDNA synthesis for real-time PCR and RT-PCR. Growth was performed as described above, and total RNA was isolated as described previously (18) and was purified further using the QIAGEN RNeasy Mini Protocol for RNA Cleanup with on-column DNase digestion with the QIAGEN RNase-free DNase set. RNA was quantified spectrophotometrically. Two micrograms of total RNA was used for cDNA synthesis with random hexamer primers and Superscript III RT from Invitrogen according to the manufacturer's protocol. A control without RT was also prepared to make sure that no chromosomal DNA contamination gave false-positive results in the PCR analyses.

RT-PCR. Growth, RNA isolation, and cDNA synthesis were performed as described above. PCR was performed using three different primer pairs: yjbI4/yjbI5, yjb16/yjb17, and yjbI4/yjb17 (Table 2). Each primer pair was used with three different templates: *B. subtilis* 1A1 chromosomal DNA, 1A1 cDNA prepared as described above, and control without RT. As an additional negative control, a fourth reaction was performed without template.

Real-time PCR. RNA isolation and cDNA synthesis were performed as described above. cDNA and no-RT controls were diluted fivefold in 10 mM Tris-HCl (pH 8.0) before use in real-time PCR analysis. Primers used for real-time PCR (hmpRTfw, hmpRTrev, nasDRTfw, and nasDRTrev) are listed in Table 2. Real-time PCR was performed in a Rotor-Gene 2072 real-time cycler (Corbett Research). Each 20-μl reaction mixture contained 2.75 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 1:20,000 (vol/vol) SYBR green (Sigma), 1 unit Platinum *Taq* polymerase (Invitrogen), 1× Platinum *Taq* PCR buffer, 250 nM of each primer, and 2 μl of the fivefold-diluted cDNA or no-RT control. To controls without template, 2 μl 10 mM Tris-HCl (pH 8.0) was added. A minimum of two reactions were performed for each biological replicate. Three biological replicates per strain were run with each primer pair. The relative transcript abundance was determined using the comparative quantitation feature of the software Rotor-Gene version 4.6, where the take-off point of each individual reaction is calculated from the second derivative of the raw data. This take-off point is then used, together with the reaction efficiency, to calculate the relative concentration of the samples compared to a selected control sample, which is set to 1. For each primer pair, one cDNA preparation from 1A1 cells not treated with SNP was chosen as the default control sample. This sample was included in all runs. The control sample was, just like the other samples, always run in duplicate, and the lowest value of the two was set to 1. For graphs, all data were normalized so that the average of all 1A1 cDNA preparations from cells not subjected to SNP treatment was set to 1. A two-way Student *t* test assuming equal variances was used to compare data.

Determination of cell survival after exposure to SNP. *B. subtilis* strains were grown to exponential phase (OD_{600} = 0.6), at which point a sublethal amount of SNP (0.5 mM) was added. Control cultures were left untreated. Growth was continued for 30 min, after which two 1 ml-samples of the culture were withdrawn and treated with a toxic amount of SNP (10 mM) or a corresponding volume of water for 30 min at room temperature (20 to 25°C). Viable counts were performed, and survival rates were calculated as the CFU after treatment with a toxic dose of SNP divided by the CFU in the same culture treated with water. Each assay was repeated at least three times.

Miscellaneous methods. Protein concentrations were estimated using the bicinchoninic acid method (Pierce) with bovine serum albumin as the standard. Polyclonal antisera against YjbI and YjbH were obtained by immunizing rabbits with purified YjbI and YjbH produced in *E. coli*. For immunoblot analysis, cell extracts were incubated for 10 min at 95°C in the presence of 0.4% (wt/vol) sodium dodecyl sulfate, 2.9 mM 2-mercaptoethanol, 5% (vol/vol) glycerol, and 12 mM Tris buffer (pH 6.8), and the proteins were then fractionated by Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to Hybond P membranes (GE Healthcare). Immunodetection was carried out by chemiluminescence using the ECL system (Pierce; Super Signal). Primary antisera were used at a 1,000-fold dilution, and secondary antibodies (donkey anti-rabbit immunoglobulin G conjugated to horseradish peroxidase [Amersham Biosciences]) were used at a 5,000-fold dilution.

RESULTS AND DISCUSSION

***Bacillus subtilis* exhibits an adaptive response to SNP.** To investigate how *B. subtilis* responds to nitrosative stress we exposed cells to the nitrosating agent SNP ($Na_2[Fe(CN)_5(NO^+)]$). When wild-type *B. subtilis* cells were exposed to a high dose of SNP (10 mM), only 1% were found to survive. However, if the cells were first induced with a sublethal dose of SNP (0.5 mM), their ability to survive the subsequent challenge with a high dose of SNP increased 10-fold (Fig. 1), showing that there is an adaptive response to SNP in *B. subtilis*. Fluorescence microscopic visualization by live-dead staining (LIVE/DEAD BacLight bacterial viability kit; Molecular Probes) gave essentially the same result (data not shown).

To exclude the possibility that cyanide liberated from SNP is responsible for the growth inhibition, *B. subtilis* cells were exposed to 10 mM $K_3[Fe(CN)_6]$. The survival of cells treated with $K_3[Fe(CN)_6]$ was not affected relative to that of control cultures (data not shown).

Transcriptome analysis of the response to SNP. To investigate the cellular response to SNP at the transcriptional level, *B. subtilis* cells were grown aerobically to exponential phase and were then left untreated or exposed to 0.5 mM SNP. This amount of SNP did not affect growth. Cells were harvested 5 min after SNP addition to capture the earliest transcriptional responses elicited by the nitrosative stress. The results obtained from five pairs of cultures (control and SNP treated) showed that 181 genes were significantly up-regulated and that 89 were repressed in cells exposed to SNP (Fig. 2). Primarily, SNP affected the transcription of genes assigned to the follow-

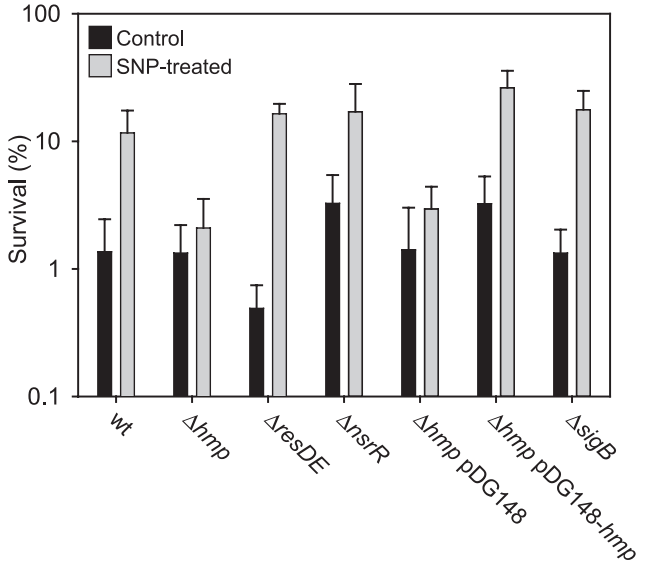

FIG. 1. Analysis of the adaptive response to SNP in *B. subtilis*. Cultures were grown aerobically and treated with a sublethal concentration (0.5 mM) of SNP or left untreated (for details, see Materials and Methods). The cultures were then challenged with 10 mM SNP for 30 min, and dilutions were plated on TBAB medium. Viable counts were performed, and survival rates were calculated as the CFU after treatment with a toxic dose of SNP divided by the CFU in the same culture treated with water. Strains 1A1 (wild type [wt]), LUW260 (Δ*hmp*), LUW264 (Δ*resDE*), LUW340 (Δ*nsrR*), LUW260/pDG148, LUW260/pDG148-*hmp*, and LUW229 (Δ*sigB*) were used. The results shown are the means from at least three independent experiments. Error bars indicate the standard deviation.

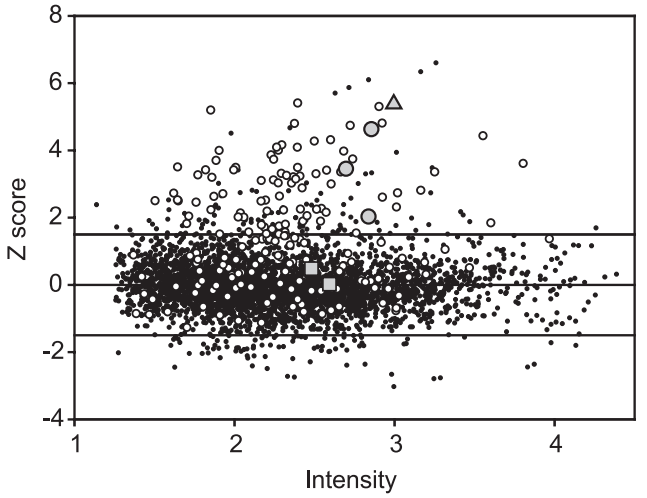

FIG. 2. Nitrosative stress-regulated transcription in *B. subtilis*. Changes in gene expression levels as determined by microarray analysis of mRNA levels in exponentially aerobically growing 1A1 (wild-type) cells treated with 0.5 mM SNP for 5 min are shown. The signal intensity represents the base 10 logarithm of the square root of the product of Cy3 and Cy5. The intensity-dependent Z score measures the number of standard deviations by which a particular data point differs from the local mean. The horizontal lines mark the Z-score threshold value (1.5 or −1.5) that is considered significant. Data points above or below these lines represent genes that are significantly up- or down-regulated, respectively, in SNP-treated cells. Open circles, transcripts reported to depend on σ^B; gray circles outlined in black, *nasDEF*; triangle, *hmp*; squares, *yjbIH*. For the transcriptome data set, see supplemental data 1 in the supplemental material.

ing categories, based on annotations in the Comprehensive Microbial Resource database (29): transport and binding proteins, energy metabolism, amino acid biosynthesis, and protein synthesis. This reflects the wide variety of targets for stress elicited by SNP. Of the SNP-induced genes, more than half could be assigned to the σ^B-controlled general stress regulon (Fig. 2; see Fig. S1A in the supplemental material). Genes belonging to the ResDE (42) and Rex (18) regulons were also up-regulated in response to SNP (see Fig. S1B in the supplemental material). The ResDE and the Rex regulons are induced by decreases in oxygen availability or changes in the redox status of the cells. NO is known to rapidly inhibit respiration by binding to heme-copper terminal oxidases, leading to changes in redox balance (20).

The adaptive response to SNP requires the flavohemoglobin encoded by *hmp*. In our microarray data set, *hmp*, encoding a flavohemoglobin, is among the most highly up-regulated (~100-fold) genes in response to SNP (Fig. 2). The up-regulation of *hmp* expression in response to SNP treatment was confirmed using quantitative real-time PCR (qRT-PCR), showing an approximately 50-fold increase in the amount of *hmp* transcript after SNP induction (Fig. 3A). Flavohemoglobins have been implicated in NO detoxification and protection against nitrosative stress in several bacteria and unicellular fungi (8, 41). To investigate the possible role of Hmp in the adaptive response to SNP, an *hmp* null mutant was analyzed. In the mutant, the adaptive response to SNP was completely abolished: no difference in survival between induced and uninduced cells was observed (Fig. 1).

NsrR (YhdE) regulates *hmp* and *nasD* in response to nitrite and nitrosative stress. Induction of *hmp* has been suggested to require the two-component system ResDE (23), and several members of the ResDE regulon were induced by SNP (see Fig. S1B in the supplemental material). However, in a *resDE* null mutant, no decrease in induced survival was observed (Fig. 1). A likely explanation for this was provided by microarray analysis (see supplemental data 3 in the supplemental material) and qRT-PCR experiments, which showed that *hmp* is up-regulated in the *resDE* mutant after SNP induction, although to a lesser extent than in the wild type (20-fold in the *resDE* mutant, compared to 50-fold in the wild type as determined using qRT-PCR) (Fig. 3A). Apparently, the induced *hmp* expression observed in the *resDE* mutant was sufficient to develop an adaptive resistance to SNP under the conditions tested.

Recent studies show that NsrR, a member of the Rrf2 family of transcription factors, acts as a regulator in response to nitrosative stress in *E. coli* and *Nitrosomonas europaea* (1, 2). Putative NsrR-binding sites are present in front of two genes in the *B. subtilis* genome: *hmp* and *nasD*. The *nasDEF* operon encodes an NADH-dependent nitrite reductase (NasDE) that catalyzes the reduction of nitrite to ammonium and NasF, an enzyme involved in the synthesis of siroheme, a cofactor of NasDE (25). The *nasDEF* operon is a member of the ResDE regulon (42). In our microarray data set, *nasD*, *nasE*, and *nasF* are highly up-regulated in response to SNP (50-, 20-, and

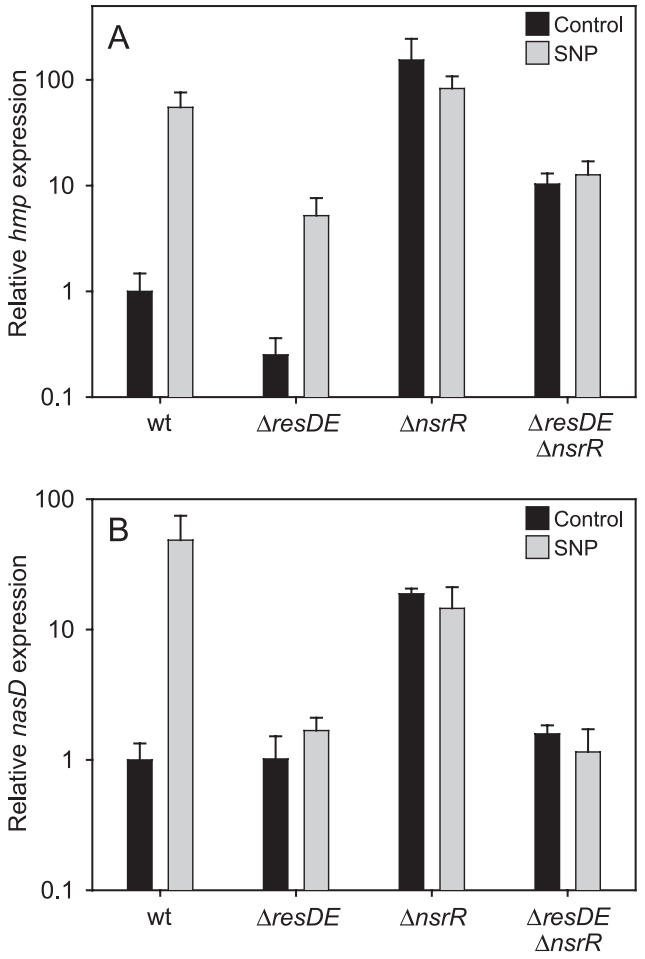

FIG. 3. Changes in *hmp* (A) and *nasD* (B) expression in response to SNP. cDNA was prepared from cultures grown aerobically and treated with 0.5 mM SNP or grown without SNP treatment, and the relative *hmp* and *nasD* expression was determined using qRT-PCR. Strains 1A1 (wild type [wt]), LUW264 (Δ*resDE*), LUW340 (Δ*nsrR*), and LUW365 (Δ*nsrR* Δ*resDE*) were used.

6-fold, respectively) (Fig. 2; see supplemental data 1 in the supplemental material). This up-regulation was confirmed for the first gene in the operon, *nasD*, using qRT-PCR, showing an approximately 50-fold increase in the amount of *nasD* transcript after SNP induction (Fig. 3B). No significant up-regulation of *nasD* in response to SNP was seen in a *resDE* mutant when analyzed by microarray analysis (see supplemental data 3 in the supplemental material) and qRT-PCR (Fig. 3B). In an *nsrR* (previously *yhdE*) null mutant strain, *hmp* and *nasD* transcript levels similar to those present after SNP induction in wild-type cells were observed both before and after SNP induction (Fig. 3A and B), indicating that NsrR works as a repressor of these genes. In an *nsrR resDE* double mutant, *hmp* transcription levels before and after SNP induction were similar to those of the *resDE* mutant after SNP induction (Fig. 3A), while *nasD* expression stayed at the same level as before SNP induction in the wild type and as both before and after SNP induction in the *resDE* mutant (Fig. 3B). These results suggest that ResDE is essential for the up-regulation of *nasD* but not *hmp* expression, while NsrR acts as a repressor of both *hmp* and *nasD* in the absence of nitrosative stress. Thus, even though *hmp* and *nasDEF* are controlled by the same regulators, *hmp*, unlike *nasDEF*, can be expressed under conditions that do not activate ResD.

During the course of this work, Nakano et al. identified *B. subtilis* NsrR as a negative regulator of *hmp* and *nasDEF* by using *lacZ* fusion experiments (24). However, in contradiction to our data, it was suggested that an *nsrR* mutation does not cause derepression of *nasD* under aerobic conditions.

Expression of *hmp* is not enough to yield an increased resistance to SNP. The deregulated expression of *hmp* in the *nsrR* mutant resulted in a small increase in SNP tolerance under noninducing conditions (Fig. 1). When the *nsrR* mutant was induced with a sublethal concentration of SNP, the cells exhibited a more than 10-fold-higher survival (Fig. 1). These results were confirmed when the *hmp* null mutant was complemented with a plasmid bearing the *hmp* gene under the control of the IPTG-inducible P*spac* promoter. Expression of *hmp* from the plasmid resulted in a marginal increase in cell survival under noninducing conditions compared with that of the isogenic strain carrying only the vector without the *hmp* insert. When the complemented *hmp* mutant was first induced with a sublethal level of SNP, a more than 10-fold-higher survival was observed (Fig. 1). This indicates that Hmp alone is not sufficient to provide a large increase in resistance to SNP, but an additional factor, induced by SNP, needs to be present. Alternatively, in the absence of nitrosative stress, Hmp is inactive due to superoxide-generated self-destruction. It has been shown that *E. coli* Hmp is irreversibly inactivated in the presence of O_2 and NADH due to superoxide formed during turnover. However, in the presence of NO, superoxide production is abolished (40). Thus, we speculate that a small amount of NO induces *hmp* expression and protects the flavohemoglobin from self-destruction.

Nitrite induces *hmp* and *nasD* expression. Nakano et al. (24) suggested that it is NO and not nitrite that is sensed by NsrR, and this has also been implied for NsrR in *Neisseria gonorrhoeae* (28). However, in *N. europaea* (1, 2), it has been suggested that nitrite, not NO, controls the activity of NsrR. In our experiments, induction with nitrite yielded *hmp* and *nasD* expression at approximately the same level as after SNP induction. However, the concentration of nitrite required to reach this expression level was 40-fold higher than the SNP concentration used (data not shown). Control experiments with 20 mM $NaNO_3$ gave no induction of *hmp* or *nasD* (data not shown). Nitrite is a source of NO in vivo, and it is possible that the small amount of NO formed from nitrite interacts with NsrR to cause derepression of *hmp* and *nasD*. However, treatment with 20 mM nitrite did not give an increased resistance to SNP (data not shown). We speculate that the amount of NO needed to activate NsrR is smaller than that required to protect Hmp from superoxide-generated self-destruction.

The *yjbIH* operon confers an apparent constitutive protection against SNP. trHbs have been reported to protect bacterial cells from nitrosative stress. For example, in *M. tuberculosis* a trHb (trHbN) catalyzes the oxidation of NO into nitrate (27). *B. subtilis* possesses a trHb encoded by the *yjbI* gene. The chromosomal context suggests that the *yjbI* gene is part of a *yjbIH* operon. To verify cotranscription of *yjbI* and *yjbH*, total RNA was isolated and used to perform RT-PCR with a pair of

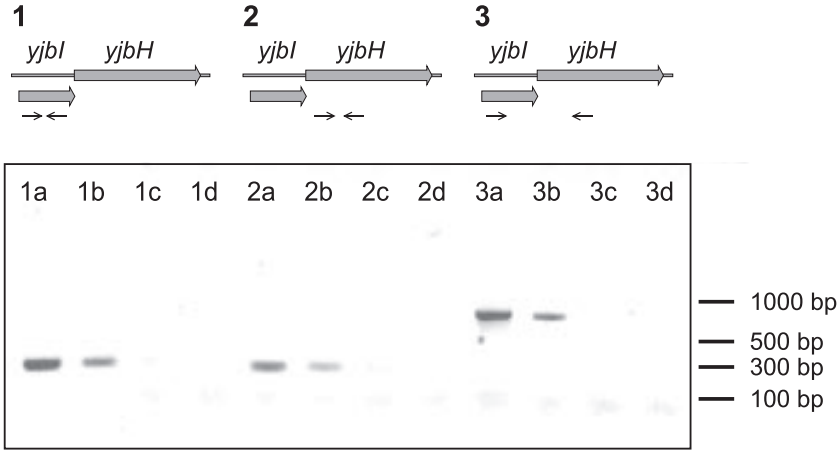

FIG. 4. Organization of the *yjbIH* locus in *Bacillus subtilis*. RT-PCR analysis of *yjbI* and *yjbH* is shown Amplification reactions were carried out using pairs of oligonucleotide primers as schematically illustrated by arrows below the map of the *yjbIH* locus. The DNA products were analyzed by agarose gel electrophoresis. For the samples loaded in lanes 1a, 2a, and 3a, chromosomal DNA was used as the template; in lanes 1b, 2b, and 3b, cDNA was used as the template; in lanes 1c, 2c, and 3c, RNA was used as the template (RT was absent in the cDNA synthesis reaction); and in lanes 1d, 2d, and 3d, no template was present.

primers that allows coamplification of both genes. A single product of the expected size was detected, indicating that *yjbI* and *yjbH* are cotranscribed in vivo (Fig. 4). To investigate the roles of *yjbI* and *yjbH* in nitrosative stress, we compared SNP resistance in the wild type and in *yjbI*, *yjbH*, and *yjbIH* mutant strains. When the *B. subtilis* cells lacking *yjbI*, *yjbH*, or both *yjbI* and *yjbH* were exposed to a toxic dose of SNP, an approximately 100-fold decrease in cell survival compared to that of the wild-type strain was observed (Fig. 5). Expression of *yjbIH* from a low-copy-number plasmid in cells lacking *yjbIH* resulted in wild-type survival levels (Fig. 5). In contrast, expression of only *yjbI* in cells lacking *yjbIH* did not restore the survival levels (Fig. 5).

As expected, expression of *yjbH* from a low-copy-number plasmid in cells lacking *yjbH* resulted in wild-type survival levels (Fig. 5). However, expression of only *yjbH* from a plasmid in cells lacking *yjbIH* also resulted in wild-type survival levels (Fig. 5). This finding is contradicted by data obtained with the *yjbI* insertion mutant LUW222, where the *yjbI* gene was inactivated by insertion of the suicide plasmid pYjbI2. pYjbI2 disrupts the *yjbI* gene and places *yjbH* under the control of the IPTG-inducible P*spac* promoter. When the *yjbI* mutant LUW222 was grown in the presence of 1 mM IPTG, survival levels were very similar to those observed in the absence of IPTG, suggesting that the tolerance to SNP could not be restored by expression of *yjbH* (Fig. 5).

Immunoblot analysis confirmed that YjbH antigen is present in cell extracts of LUW222 grown in the presence of IPTG (data not shown). However, the level of YjbH antigen in cells complemented with *yjbH* on a plasmid was at least 20-fold higher. Thus, complementation in LUW219/pCW7-*yjbH* could be due to the nonphysiological concentration of YjbH.

The steady-state levels of YjbI and YjbH in strains containing *yjbIH* or *yjbI* on a plasmid as determined using immunoblotting show that the levels of YjbI and YjbH increased more than 10-fold compared to those in the wild type (Fig. 6). The increased levels of YjbI and YjbH do not appear to promote tolerance to SNP above the levels observed for the wild-type cells, indicating that the amount of these proteins is not limiting in wild-type cells or that they are not directly involved in the SNP detoxification mechanism.

Deletion of *yjbI* and/or *yjbH* did not affect the adaptive response to SNP (Fig. 7), and the expression levels of *yjbI*, measured with microarray analysis (Fig. 2) and qRT-PCR (data not shown), remained constant after SNP induction. The expression of *yjbI* was not affected by mutations in *nsrR* or *resDE* (data not shown).

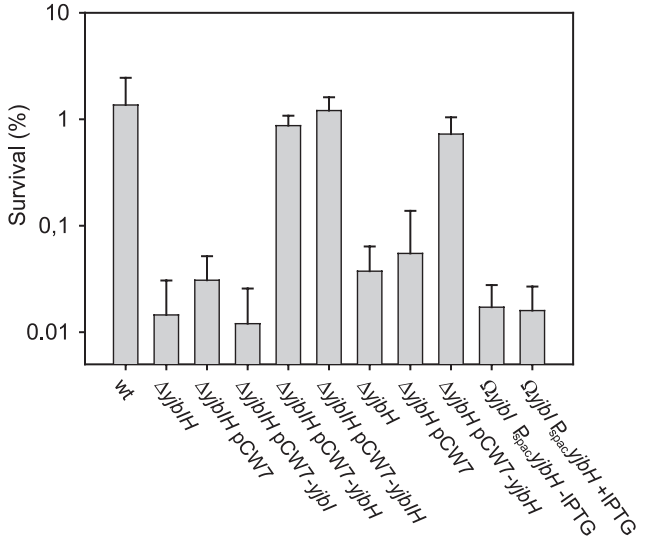

FIG. 5. Effects of nitrosative stress on the viability of different *B. subtilis* strains. Aerobically growing cultures were challenged with 10 mM SNP for 30 min and then plated on TBAB medium. CFU are plotted as a percentage of the values for untreated cultures. The results are the means from at least three independent experiments. Error bars indicate the standard deviation. Strains 1A1 (wild type [wt]), LUW219 (Δ*yjbIH*), LUW219/pCW7, LUW219/pCW7-*yjbI*, LUW219/pCW7-*yjbH*, LUW219/pCW7-*yjbIH*, LUW272 (Δ*yjbH*), LUW272/pCW7, LUW272/pCW7-*yjbH*, LUW222 (*yjbI* insertion) without IPTG, and LUW222 with IPTG were used.

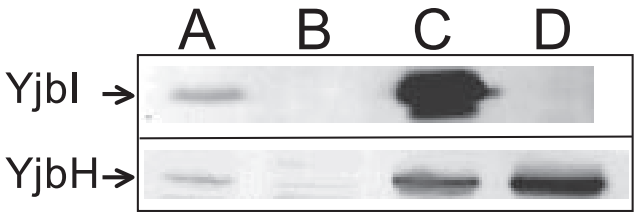

FIG. 6. Overproduction of YjbI and YjbH in *B. subtilis* cells. Immunoblot analysis of cell extracts (10 μg total protein loaded in each lane) from different *B. subtilis* strains, using YjbI or YjbH antiserum, is shown. The blots were overexposed to demonstrate the presence of small amounts of YjbI and YjbH antigen. Lanes: A, 1A1/pCW7; B, LUW219 (Δ*yjbIH*)/pCW7; C, LUW219/pCW7-*yjbIH*; D, LUW219/pCW7-*yjbH*.

Deletion of *hmp* in the *yjbIH* mutant significantly reduced the induced tolerance to SNP (Fig. 7). Taken together, the results indicate that deletion of either *yjbI* or *yjbH* affects the uninduced tolerance to SNP. We have previously shown that a *yjbIH* mutation affects growth and oxygen consumption (18). It is possible that the deletion of *yjbI* or *yjbH* affects the cells in a more global way that makes them hypersensitive to nitrosative stress.

The *sigB* general stress response is not required for the adaptive response to SNP. To further examine the induction of the general stress response by SNP, a transcriptome analysis of the response to SNP in a *sigB* null mutant was performed using four pairs of cultures. As expected, genes belonging to the *sigB* regulon that were induced by SNP in the wild type were not induced in the *sigB* mutant. However, interestingly, a group of genes previously reported to be *sigB* dependent (*yvyD*, *yxjG*, *ywfH*, *ywhH*, *ynfC*, and *gabP*) (31) were up-regulated also in the *sigB* mutant (see Fig. S1C in the supplemental material). These genes may have been incorrectly annotated to the *sigB* regulon, or they may have additional mechanisms for induction as indicated by the presence of sequences that are similar to those found at the −10 and −35 regions of sigma A promoters upstream of *yxjG*, *ywfH*, and *ynfC*. Another group of genes (*yonA*, *yjlB*, *yisZ*, *yufS*, *yddC*, and *ytkC*) that were previously not assigned to the *sigB* regulon were affected by the *sigB* mutation (see Fig. S1A in the supplemental material). These genes are, at least under the conditions tested, either directly or indirectly dependent on *sigB* for induction by SNP.

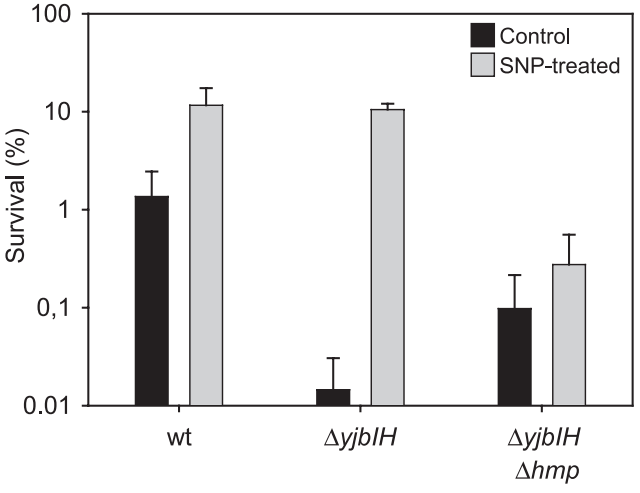

FIG. 7. The induced resistance to SNP is not affected by YjbI or YjbH. Cultures were grown aerobically and treated with a sublethal concentration (0.5 mM) of SNP or left untreated. The cultures were then challenged with 10 mM SNP for 30 min and plated on TBAB medium. Viable counts were performed, and survival rates were calculated as the CFU after treatment with a toxic dose of SNP divided by the CFU in the same culture treated with water. The results shown are the means from at least three independent experiments. Error bars indicate the standard deviation. Strains 1A1 (wild type [wt]), LUW219 (Δ*yjbIH*), and LUW261 (Δ*yjbIH* Δ*hmp*) were used.

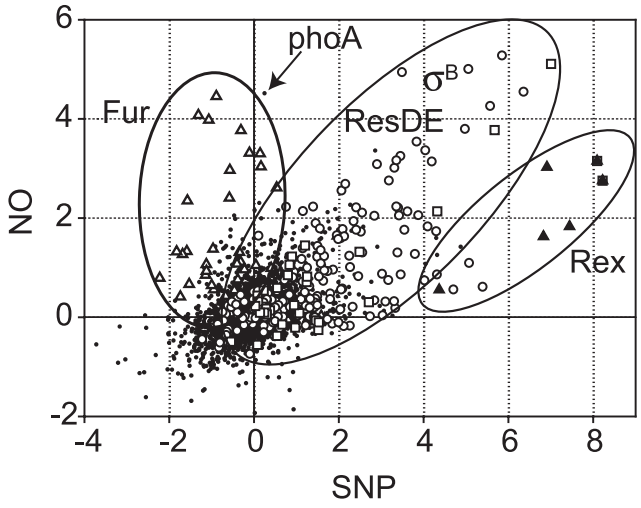

FIG. 8. Graphical comparison of the SNP and NO stimulons of *B. subtilis*. The fold changes ($\log_2$) of gene expression levels of the two data sets are plotted. The data for the NO stimulon are those reported for *B. subtilis* cells treated with 50 μM NO for 5 min (21). The data for the SNP stimulon were obtained in this work (Fig. 2). Members of the Fur (open triangles), σ^B (open circles), ResDE (open squares), and Rex (closed triangles) regulons are highlighted. The signal corresponding to the *phoA* transcript is indicated with an arrow.

To test whether the σ^B regulon affects the sensitivity of *B. subtilis* to nitrosative stress, the *sigB* null mutant was exposed to SNP. The *sigB* mutant showed no increased sensitivity to SNP, nor did the mutation affect the induced resistance (Fig. 1), suggesting that the proteins encoded by the σ^B-dependent genes are involved in a nonspecific protection against stress. To exclude the possibility that Hmp might have masked any effect of the *sigB* mutation, a *sigB hmp* double mutant was tested for SNP tolerance. No difference in survival between the *sigB hmp* mutant and the *hmp* mutant was observed (data not shown).

Comparison of the SNP and NO stimulons in *B. subtilis*. Recently, the genome-wide response of *B. subtilis* to NO gas under aerobic growth conditions was reported (21). NO activates the *sigB* stress response via the energy-signaling pathway, in contrast to SNP, which activates it via the environmental pathway, underscoring the different actions of NO and SNP (21). To analyze the spectrum of responses of *B. subtilis* to different types of nitrosative stress under aerobic conditions, we compared the SNP and NO stimulons in a scatter plot (Fig. 8). Most genes showed similar expression patterns in response to SNP and NO. However, a notable difference is the apparent lack of derepression by SNP of genes controlled by the ferric uptake regulator (Fur). This could reflect the chemical differ-

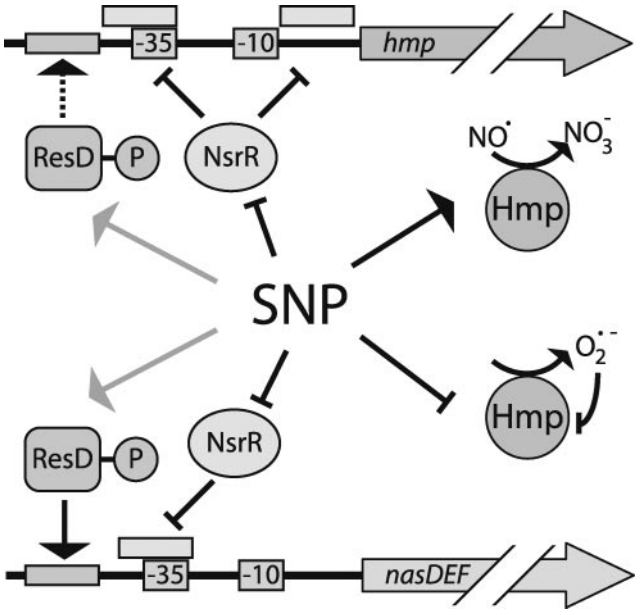

FIG. 9. Model of *hmp* and *nasDEF* regulation in response to nitrosative stress. During nitrosative stress inflicted by SNP, nitrosylation of NsrR results in derepression of *hmp* and *nasDEF* (consistent with the model previously suggested by Nakano et al. [24]). Under oxic conditions Hmp detoxifies NO to NO_3^-. However, when Hmp is present in the absence of NO, superoxide that inactivates the enzyme is produced. ResD, presumably in its phosphorylated form, is required for expression of *nasDEF* and enhances transcription from the *hmp* promoter (indicated by the dashed arrow). The mechanism of activation of ResD by SNP is not known (indicated by the gray arrows).

ences between SNP and NO. The former is a nitrosonium donor that efficiently could form *S*-nitrosothiol by covalent addition to cysteine residues. The S-N bonds could subsequently be cleaved, resulting in release of NO and the formation of S-S bonds. Inactivation of *E. coli* Fur by NO has been shown to involve the formation of a dinitrosyl-iron complex (5). The apparent lack of inactivation of *B. subtilis* Fur by SNP could reflect kinetic differences, could be due to differences in the intracellular concentration of NO, or could depend on the difference in medium composition between the two experiments. For the SNP analysis, growth was performed in an iron- and phosphate-rich medium. This is also the likely reason why *phoA* is highly induced by NO but not by SNP, since phosphate causes repression of *phoA* expression (Fig. 8). Similar differences have been seen in the response of the *E. coli* transcriptome to various sources of NO or NO^+ (7, 15, 22).

Conclusions. In this work we demonstrated that the flavohemoglobin encoded by the *hmp* gene has a vital role in an adaptive response to protect *B. subtilis* from nitrosative stress. Expression of *hmp* is regulated by the transcriptional repressor NsrR (Fig. 9). Upon exposure to SNP, it is likely that nitrosylation of NsrR results in derepression of *hmp*. NsrR also regulates expression of the nitrite reductase encoded by the *nasDE* genes. The fact that the protonated form of nitrite, nitrous acid, can spontaneously yield N_2O_3 (19), a powerful nitrosating agent, provides a rationale for the coregulation of *hmp* and *nasDEF* by the transcriptional repressor NsrR and the activator ResD. We also investigated the role of the trHb YjbI in RNS detoxification. Several studies have indicated a role for trHbs in the detoxification of RNS. We show that the *yjbI* gene is cotranscribed with *yjbH*, a gene that encodes a 34-kDa cytosolic protein belonging to COG2761 in the database of clusters of orthologous groups of proteins (COGs). This COG contains DsbA-like proteins that may be involved in disulfide bond formation or other redox-based processes. Expression of *yjbIH* is independent of NsrR and SNP. Cells lacking *yjbIH* are hypersensitive to nitrosative stress, possibly due to secondary effects caused by the lack of YjbIH. As in previous studies, we found that nitrosative stress induces the sigma B-controlled general stress regulon. However, a *sigB* null mutant did not show increased sensitivity to SNP, suggesting that the sigma B-dependent stress proteins are involved in a nonspecific protection against stress whereas Hmp is crucial for SNP detoxification.

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