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The ResD Response Regulator, through Functional Interaction with NsrR and Fur, Plays Three Distinct Roles in *Bacillus subtilis* Transcriptional Control

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The ResD response regulator activates transcription of diverse genes in *Bacillus subtilis* in response to oxygen limitation. ResD regulon genes that are the most highly induced during nitrate respiration include the nitrite reductase operon (*nasDEF*) and the flavohemoglobin gene (*hmp*), whose products function in nitric oxide (NO) metabolism. Transcription of these genes is also under the negative control of the NO-sensitive NsrR repressor. Recent studies showed that the NsrR regulon contains genes with no apparent relevance to NO metabolism and that the ResD response regulator and NsrR coordinately regulate transcription. To determine whether these genes are direct targets of NsrR and ResD, we used chromatin affinity precipitation coupled with tiling chip (ChAP-chip) and ChAP followed by quantitative PCR (ChAP-qPCR) analyses. The study showed that ResD and NsrR directly control transcription of the *ykuNOP* operon in the Fur regulon. ResD functions as an activator at the *nasD* and *hmp* promoters, whereas it functions at the *ykuN* promoter as an antirepressor of Fur and a corepressor for NsrR. This mechanism likely participates in fine-tuning of transcript levels in response to different sources of stress, such as oxygen limitation, iron limitation, and exposure to NO.

Bacillus subtilis undergoes either nitrate respiration or fermentation to generate ATP when oxygen becomes limited (reviewed in reference 1). Growth under oxygen-limited conditions, particularly via nitrate respiration, requires the ResD-ResE two-component regulatory system (2, 3). During nitrate respiration in *B. subtilis*, unlike the case with denitrifiers, nitrite is reduced to ammonium instead of nitric oxide (NO). However, NO is generated at low concentrations from nitrite as a by-product of nitrate respiration in *B. subtilis* (4), as it is in *Escherichia coli* (5). Since accumulation of NO is cytotoxic, *B. subtilis* uses flavohemoglobin (Hmp) (6) and nitrite reductase (NasDEF) (7) to reduce NO levels by conversion of NO to nitrate (or N_2O under anaerobic conditions) (8–10) and by metabolism of nitrite to ammonium (7), respectively. NsrR, a member of the Rrf2 family, is known to control transcription of genes involved in NO detoxification in both Gram-positive and Gram-negative bacteria (reviewed in references 11 and 12). *B. subtilis* NsrR represses transcription of the *nasD* operon and *hmp* under anaerobic fermentative conditions (4). Transcription of these genes is dependent on the ResD response regulator and the ResE sensor kinase (6, 7). NsrR binds to the −35 region of the *nasD* promoter, resulting in disruption of the RNA polymerase (RNAP)-ResD-DNA complex (13). When NO is present endogenously via nitrate respiration or exogenously, NsrR-dependent repression of *nasD* and *hmp* is relieved. This derepression is attributed to the release of NsrR from the *nasD* promoter by direct interaction of NO with iron in the [4Fe-4S] cluster of NsrR (13, 14). More genes controlled by NsrR were identified by transcriptome analysis, which was validated by transcriptional *lacZ* fusions to promoters of the identified genes (15). Some of these NsrR-controlled genes belong to the Fur regulon, which functions in iron homeostasis (16), whereas others are known to participate in extracellular function and are corepressed by the AbrB and Rok repressors (17).

The NsrR-binding site at the *nasD* and *hmp* promoters contains a sequence of imperfect dyad symmetry; however, a similar sequence is not detected in promoter regions of NsrR-controlled genes that belong to the Fur or AbrB/Rok regulon. *In vitro* binding studies showed that the affinity of NsrR to the latter promoters is not enhanced by the presence of the [4Fe-4S] cluster, and hence, the binding is NO insensitive, unlike the binding of NsrR to the −35 region of the *nasD* promoter (15). On the other hand, *in vivo* transcription assays using a promoterless *lacZ* construct fused to the *sdpA* promoter (AbrB/Rok regulon) and the *ykuN* promoter (Fur regulon) strongly suggested that NO adversely affects NsrR repression. In order to determine whether these NsrR regulon genes are under the direct control of NsrR and if so whether the binding of NsrR is affected by NO *in vivo*, we carried out ChAP-chip (chromatin affinity precipitation [ChAP] coupled with tiling chip) and ChAP followed by quantitative PCR (ChAP-qPCR). We also used the *in vivo* approach to address how binding of one transcriptional regulator to a promoter DNA affects association of another regulator that targets the same DNA regulatory region. The study also revealed that ResD performs three distinct roles in transcriptional control in *B. subtilis*.

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MATERIALS AND METHODS

Construction of strains and culture conditions. *B. subtilis* strains used in this study are 168 derivatives (see Table S1 in the supplemental material) and were routinely cultured on Difco sporulation medium (DSM) agar (18). *ykuN-lacZ* expression was measured in the wild-type strain (ORB8458) and various mutant strains carrying a *ykuN*::pMutin insertion (19). Single and double mutants were constructed by transformation of ORB8458 with chromosomal DNA isolated from HB2501 (*fur*::*kan*) (20), LAB2511 (*resD*::*spc*) (15), and TF274 (*nsrR*::*cat*) (4). A triple mutant strain, ORB8512, harboring *ykuN-lacZ*, was constructed by transformation of ORB8510 (*ykuN*::pMutin *fur*::*kan resD*::*spc*) with TF274 chromosomal DNA. *nasD* expression was examined using SPβ-borne *nasD-lacZ* as previously constructed (7) except that 168 and its derivatives were used for SPβ phage transduction to generate ORB8620 (wild type), ORB8621 (*nsrR*::*cat*), ORB8622 (*resD*::*spc*), and ORB8626 (*resD*::*spc nsrR*::*cat*), which carry *nasD-lacZ*.

To construct a DNA cassette containing *nsrR-his*$_{12}$, the *nsrR* gene without the termination codon was amplified from *B. subtilis* genomic DNA using the primers nsrR-FF and nsrR-FR. The coding sequence of the His$_{12}$ tag and a tetracycline-resistance gene were amplified from plasmid pXT-cGFP-His12 (unpublished plasmid) using primers 12xhis-F and rPCR-tetR. The downstream region of the *nsrR* gene was amplified from *B. subtilis* genomic DNA using the primers nsrR-BF and nsrR-BR. Next, the three fragments were joined by recombinant PCR using the primers nsrR-FF and nsrR-BR and employed to transform wild-type *B. subtilis* 168 cells, followed by double crossover recombination, with selection for tetracycline resistance, to create the OC0010 strain. Markerless *B. subtilis* strains producing ResD and Fur with His$_{12}$ at their C-terminal ends (ORB8238 and ORB8440) were constructed by successive transformation with recombinant PCR products using the *E. coli mazF* gene as a counter-selection tool (21). In short, the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *mazF* gene was fused with the flanking sequences of the target gene with *his*$_{12}$ fused to the last codon of the *resD* or *fur* gene. The PCR product was used to transform *B. subtilis* 168, and the recombinant was selected for chloramphenicol resistance in the absence of IPTG. The recombinant was plated on DSM agar plates supplemented with IPTG in the absence of chloramphenicol, which resulted in an excision of the *mazF* cassette and introduction of *his*$_{12}$ at the 3′ end of the target gene. Expected introduction of *his*$_{12}$ was confirmed by PCR with the chromosomal DNA from the IPTG-resistant recombinant and sequencing of the PCR product. Sequences of oligonucleotides used in this study are listed in Table S2 in the supplemental material.

Concentrations of antibiotics used were as follows: chloramphenicol, 5 μg/ml; erythromycin plus lincomycin, 1 μg/ml and 25 μg/ml, respectively; spectinomycin, 75 μg/ml; kanamycin, 5 μg/ml; tetracycline, 12.5 μg/ml; ampicillin, 50 μg/ml.

ChAP-chip experiments. Chromatin affinity precipitation (ChAP) of DNA bound to NsrR-His$_{12}$, and ResD-His$_{12}$ was performed as previously described (22, 23) with some modifications. This method was originally developed to analyze *in vivo* protein complexes using the His$_{12}$ tag under denaturing conditions with a high concentration of imidazole (24) and applied to chromatin immunoprecipitation (ChIP)-chip analysis (22). Importantly, no protein is purified at detectable levels by silver staining if the wild-type 168 strain is used.

As for anaerobic NsrR-His$_{12}$ ChAP-chip and ChAP-qPCR experiments, an overnight aerobic culture of OC0010 in 2× YT liquid medium (18) was transferred (at a starting optical density at 600 nm (OD$_{600}$) of 0.06) to 250-ml glass bottles filled with 2× YT supplemented with 0.5% glucose and 0.5% pyruvate (anaerobic fermentation). Cultures anaerobically grown in 2× YT with 1% glucose and 0.2% nitrate (nitrate respiration) were used only for ChAP-qPCR. For NO treatment, 100 mM stock solution of spermine NONOate (sperNONOate) Cayman Chemical) was prepared by dissolving in 10 mM NaOH. SperNONOate was added to the fermentation cultures at a final concentration of 50 μM when the OD$_{600}$ reached around 0.6. For control cultures, the same volume of 10 mM NaOH was added. After 1 h of incubation, cells were treated with 1% formaldehyde, final concentration. ResD-His$_{12}$ ChAP, and Fur-His$_{12}$ ChAP were performed similarly using strains ORB8238 and ORB8440, respectively.

For aerobic NsrR-His$_{12}$ ChAP, strain OC0010 (*nsrR-his*$_{12}$) was precultured in S7$_{50}$ medium (25) with tetracycline and grown overnight under aerobic conditions. Cells were harvested from the culture tube at an OD$_{600}$ around 0.4 to 0.5, centrifuged, and washed with and resuspended in S7$_{50}$ medium. The cell suspension was used to inoculate S7$_{50}$ medium without tetracycline with a starting OD$_{600}$ of 0.04. Cells were cultured at 37°C with shaking at 200 rpm. Cells were harvested at T1 (1 h after the end of exponential growth) for the preparation of ChAP samples as described previously (22, 23). In short, cells were treated with formaldehyde before harvesting to cross-link target protein and DNA under native conditions. The material cross-linked to NsrR-His$_{12}$ or ResD-His$_{12}$ was purified using Dynabeads with a cobalt-based surface (Life Technologies, Carlsbad, CA), and DNA associated with the target protein was purified with phenol-chloroform followed by ethanol precipitation.

ChAP-chip data analyses were performed as described previously (22, 23). NsrR- and ResD-binding signals were analyzed and visualized *in silico* using a software package, Molecular Cloning Array Edition (imc_ae; in silico biology, inc.), as the values that divided signal intensities of DNA in the affinity-purified fraction (ChAP DNA) by those of DNA isolated from the whole-cell-extract fraction before the purification (control DNA). Protein-binding peaks were automatically detected as previously described with the following modification (26). The signals with values higher than threshold, which were determined as ≥2.0 for NsrR and ≥2.5 for ResD depending on their background levels, were concatenated when the distance of neighboring signals was less than 150 bp, and the regions containing ≥7 for NsrR and ≥10 for ResD probes were defined as protein-binding regions. Signals on rRNA, Spo0J binding regions, and highly transcribed regions, which make signals higher than the background level, were removed from the result as previously discussed (23). Binding intensity was shown as an average signal intensity per probe.

ChAP-qPCR experiment. qPCR was carried out on an Applied Biosystems StepOne Plus real-time PCR system with SYBR green PCR master mix (Applied Biosystems). Ten-fold-diluted DNA from the affinity-purified fraction (ChAP-DNA) and 100-fold-diluted DNA in the cell extract prior to affinity purification (input DNA) were used for the template. Amounts of PCR products were calculated against a standard curve obtained from a dilution series of 168 chromosomal DNA. Primers used for qPCR are listed in Table S2 in the supplemental material. Technical triplicates (at least) were used for qPCR on three independent biological replicates. The fold enrichment was calculated as follows. First, the average qPCR DNA amount of a tested gene in ChAP-DNA was divided by that of an *rpsD* control within the same ChAP biological sample (ChAP DNA ratio). Similarly, the average of the tested gene in input DNA in the same biological sample used for ChAP DNA was divided by that of *rpsD* in the same input biological sample (input DNA ratio). The fold enrichment of the tested DNA was obtained by dividing the ChAP DNA ratio by the input DNA ratio. The final value was the average of the fold enrichment value from three biological samples with the standard deviation.

Measurement of *lacZ* expression. To monitor transcription of *ykuN* and *nasD*, transcriptional *lacZ* fusions were introduced into the parental 168 strain and various mutant strains listed in Table S1 in the supplemental material. Cells cultured aerobically in 2× YT liquid medium overnight at 37°C were transferred to 2× YT supplemented with 0.5% glucose and 0.5% pyruvate or with 1% glucose and 0.2% nitrate (starting OD$_{600}$ = 0.02) and grown under anaerobic conditions. β-Galactosidase activity was measured at hourly intervals (27), and the activities at T−1 and T1 (1 h before and after the end of exponential growth, respectively) were shown as the average from at least three independent cultures.

Microarray data accession number. Raw data (CEL format) from the ChAP-chip experiments described here have been deposited in the ArrayExpress database (http://www.ebi.ac.uk/microarray-as/ae/) under accession code E-MEXP-3882.

TABLE 1 Target genes of NsrR and ResD identified by ChAP-chip analysis[a]

Category and gene	Relative signal intensity (fold)			Function/gene product
	NsrR, Aer	NsrR, An	ResD, An	
Binding to promoter region				
hmp	25.9	6.0	11.0	Flavohemoglobin
nasD	6.3	3.0	3.7	Nitrite reductase
ykuN	4.2	2.9	2.7	Flavodoxin
ydhB-ydhC	11.9	2.5		GntR-family Tc factor (*ydhC*), membrane protein (*ydhB*)
feuA	4.2	2.6		ABC transporter, iron uptake
ypqP	3.0		3.0	Capsular polysaccharide synthesis
yopS-yopR	5.7			SPß prophage
fpbC (*ypbR*)	3.9	2.3		Small protein, RNA chaperone
trpE	2.9			Anthranilate synthase
ydhK	2.4			General stress protein, ethanol survival
ctaO			6.1	Heme O synthesis, *ctaB* paralog
glpF			4.9	Glycerol uptake (*glpF*), glycerol kinase (*glyK*)
ctaA-ctaB			4.5	Heme A (*ctaA*)/heme O (*ctaB*) biosynthesis
ndk			3.7	Nucleotide diphosphate kinase
yjlC			3.6	*yjlC-ndh* operon, NADH dehydrogenase (*ndh*)
ytcP			3.5	ABC transporter
rsaE			3.2	nc-RNA
yppF-yppG			3.2	NudF subfamily (*yppG*)
lytF			3.1	Major autolysin
yrhG			3.0	Transporter
nsrR			3.0	NO-sensitive Tc factor
yvyD			2.7	General stress protein required for ribosome dimerization
Binding outside of promoter				
ynfE-ynfF	3.8			Phosphoprotein (*ynfE*), xylan utilization (*ynfF*)
yccF-natK (*yccG*)	3.2			Sensor kinase (*natK*)
spoIVCB	3.0		2.8	SigK
cotG	3.4			Spore coat protein
trpP (*yhaG*)	2.6			Tryptophan transporter
copZ (*yvgY*)	2.5			Copper transporter
ypqP (*yodU*)	2.3			Similar to capsular polysaccharide synthesis
fhuB		2.5		ABC transporter, iron uptake
cwlO (*yvcE*)			3.4	Endopeptidase-type autolysin
yozB			3.3	Unknown
yxiE			3.1	Phosphate starvation-induced stress protein
glmS			2.8	Glutamine-fructose-6-phosphate transaminase
ydbL			2.8	Unknown membrane protein

[a] Gene designations in parentheses are those used in the data deposited in the ArrayExpress database (http://www.ebi.ac.uk/microarray-as/ae/) (see Materials and Methods). Where NsrR and/or ResD interacts with an operon promoter, only the first gene in the operon is shown. Aer, aerobic conditions; An, anaerobic fermentative conditions.

RESULTS

NsrR ChAP-chip identified direct targets of NsrR. Our previous work showed that NsrR not only regulates transcription of genes involved in NO detoxification, such as *nasD* and *hmp*, but also controls genes that show no direct relevance to NO metabolism (15). To identify which genes are direct targets of NsrR, we uncovered the genome-wide binding profile of NsrR using formaldehyde-mediated cross-linking, followed by ChAP-chip analysis. In this experiment, codons specifying a 12× histidine tag (his_{12}) were fused to the 3′ end of *nsrR*, and *nsrR-*his_{12} was introduced at the *nsrR* locus of the 168 parental strain as the only *nsrR* allele. NsrR-His_{12}, like NsrR, repressed *nasD* transcription under anaerobic fermentative conditions but not during nitrate respiration (data not shown), confirming that NsrR-His_{12} is functional. The strain carrying *nsrR-*his_{12} was grown anaerobically in 2× YT supplemented with 0.5% glucose and 0.5% pyruvate (culture conditions for fermentation) (18), and cells around T1, where the highest *nasD* expression is detected in the *nsrR* mutant strain, were used to purify DNA associated with NsrR as described in Materials and Methods.

As expected from our previous studies (4), the ChAP-chip experiment indicated that NsrR associates with the *hmp-ykjA* and *nasDEF* operon promoters. Table 1 summarizes relative signal intensities normalized using DNA isolated from the whole-cell extract fraction before purification (control DNA) as described in Materials and Methods. In addition, NsrR bound to the promoter regions of certain Fur-repressed genes, such as *ykuNOP*, *feuABC-ybbA*, and *fbpC*, as well as the coding region of *fhuB*, which is consistent with our previous result that NsrR represses transcription of Fur regulon genes (15). The results indicate that NsrR controls transcription of these genes by direct interaction with the promoter regions. On the other hand, NsrR binding was not detected at the promoter regions of *sdpA* and *yukE*, expression of which was upregulated by the

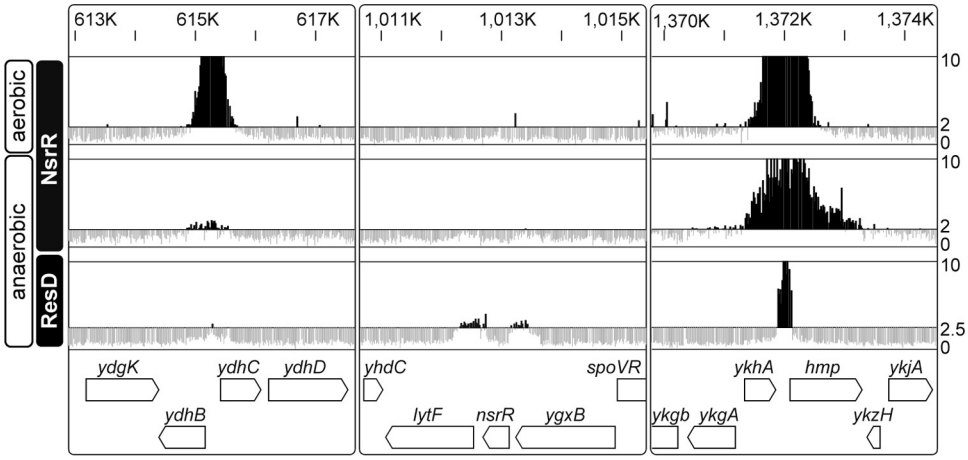

FIG 1 NsrR and ResD binding profiles, determined by ChAP-chip. Typical examples of NsrR and ResD binding signals in cells grown under aerobic and anaerobic fermentative conditions are shown. Protein-binding signals for each probe in the region are indicated alongside the genomic coordinates. The top line and bottom line in each column indicate signal intensities of 10 and 0, respectively. The middle lines for NsrR and ResD show threshold, 2.0 and 2.5, respectively. The gene organization is shown schematically at the bottom.

nsrR mutation in previous transcriptome and transcriptional *lacZ* fusion analyses (15). This result suggested that NsrR likely plays an indirect role in transcription of these genes. The study also identified potential targets of NsrR, namely, divergently transcribed *ydhB* and *ydhC*, which encode a membrane protein with unknown function and a GntR family transcription factor, respectively (Fig. 1 and Table 1).

To determine the extent of NsrR association with the genomic DNA under growth conditions other than anaerobic fermentative growth, we also carried out ChAP-chip analysis in aerobic cultures. Cells were grown in morpholinepropanesulfonic acid (MOPS)-buffered $S7_{50}$ defined medium (25) and harvested at T1. The result showed that NsrR binds to most of the sites identified in anaerobic cultures (summarized in Table 1). In addition, NsrR interaction was detected with 11 other regions including the promoter of tryptophan biosynthesis operon (*trpEDCFBA-hisC-tyrA-aroE*) and the coding region of *trpP* encoding a tryptophan transporter (28).

ResD associates with a few NsrR regulon genes. Our previous study indicated that ResD is involved in transcriptional regulation of all NsrR-controlled genes tested thus far (13). We wondered whether ResD controls NsrR-repressed genes by directly binding to promoter regions. To answer this question, a ChAP-chip experiment was performed using the *resD-his*$_{12}$ construct that was integrated into its native locus as a single copy. The *resD-his*$_{12}$ strain activated *nasD* similarly to activation by the wild-type strain (data not shown), indicating that the His_{12}-tagged protein is functional. The ChAP-chip results using anaerobic fermentative cultures showed that ResD binds to promoter regions of *hmp-ykjA*, the *nasDEF* operon, and an intergenic region between *ctaA* and the *ctaBCDEFG* operon, indicating that these genes are directly controlled by ResD (Table 1). *ctaA* and *ctaB* function in heme A and heme O biosynthesis, respectively, and the *ctaCDEF* genes code for subunits of cytochrome *c* oxidase. ResD-dependent activation of these genes was previously shown (29, 30). ResD bound other genes involved in respiration, such as *ctaO*, whose product participates in heme O synthesis (31), and the *yjlC-ndh* (NADH dehydrogenase) operon (32). ResD also associated with promoters of the adjacent genes *nsrR* and *lytF*, encoding a major autolysin (33) (Fig. 1). In addition to promoters of coding regions, ResD bound between the 3′ ends of *yjbG* and *yjbH*, where a sequence encoding a small noncoding RNA (nc-RNA), *ncr22* (alternatively called *ncr629* [34]) was previously identified (35). This nc-RNA is an ortholog of *rsaE* from *Staphylococcus aureus* (36). Transcriptomic and proteomic analyses showed that RsaE controls the synthesis of proteins involved in various metabolic pathways, suggesting that it facilitates the transition from the exponential to the stationary phase of growth (36). Based on the ChAP-chip results, genes with which both NsrR and ResD interact under anaerobic fermentative conditions are *hmp*, *nasD*, and *ykuN*. The *ykuN* operon contains two genes, *ykuN* and *ykuP*, which encode two short-chain flavodoxins (37). Since only a few genes were shown to interact with both NsrR-His_{12} and ResD-His_{12}, the binding profile detected in ChAP-chip is specific for the protein and not the His_{12} tag itself.

NsrR and ResD associate with DNA other than that in promoter regions. Although most of the NsrR- and ResD-binding sites reside in promoter regions as expected, some binding sites were found outside of promoter DNA. First, NsrR bound between 3′-intergenic regions of the *ynfE-ynfF* and *yccF-yccG* genes under aerobic conditions (Table 1). Currently no evidence is reported that nc-RNA is encoded within these intergenic regions and that the binding of NsrR to the 3′ ends of genes affects gene expression at these loci.

Second, NsrR and ResD appeared to associate with the coding regions of monocistronic genes (Table 1), although it is difficult to discern whether they bind to promoters or cover the entire transcription units in the case of these small genes. For example, NsrR bound to the coding sequence of *cotG* (a spore coat protein) under aerobic conditions. The entire region of *spoIVCB* encoding the N-terminal half of a sporulation-specific sigma factor, SigK, was bound by NsrR under aerobic conditions and by ResD under anaerobic conditions. Coding regions that are likely in contact with the ResD protein also include *yvcE* (*cwlO*), encoding endo-

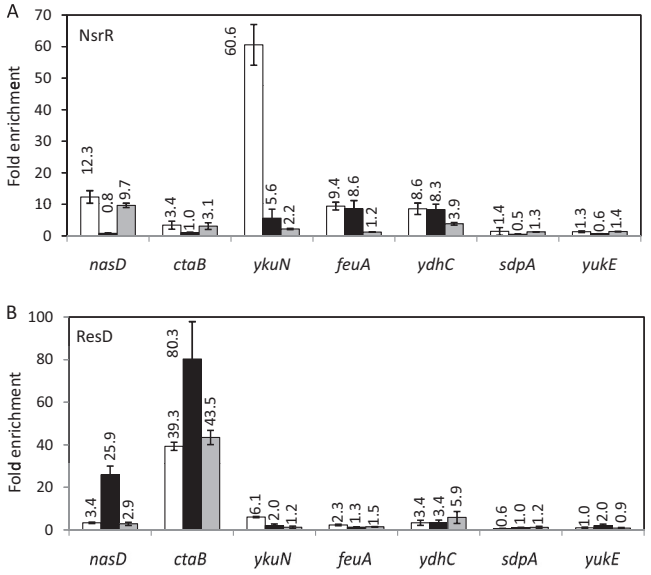

FIG 2 Interaction of NsrR and ResD *in vivo* with selected transcription units during anaerobic fermentative growth. (A) NsrR ChAP-qPCR. *B. subtilis* strains OC0010 (wild type; white bars), ORB8278 (*resD*::*cat*; black bars), and ORB8277 (*fur*::*kan*; gray bars) carrying *nsrR-his*$_{12}$ were anaerobically grown until T1 in 2× YT supplemented with 0.5% glucose and 0.5% pyruvate. NsrR ChAP was performed and DNA associated with NsrR was purified as described in Materials and Methods. (B) ResD ChAP-qPCR. *B. subtilis* strains ORB8238 (wild type; white bars), ORB8264 (*nsrR*::*cat*; black bars), and ORB8266 (*fur*::*kan*; gray bars) carrying *resD-his*$_{12}$ were grown for ResD ChAP. ChAP-qPCR was carried out as described in Materials and Methods. Fold enrichment was calculated with triplicates of each biological sample normalized with *rpsD* as described in Materials and Methods, and the average for three biological samples is shown above each bar with the standard deviation.

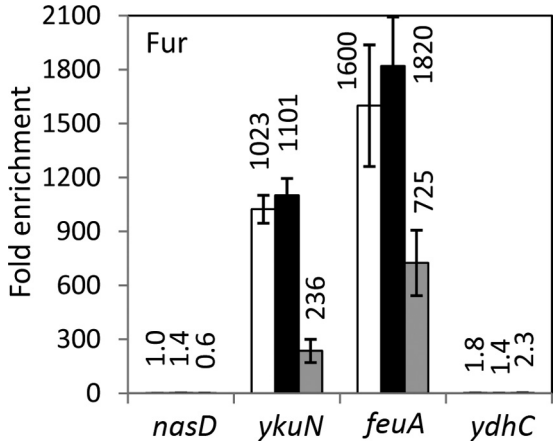

FIG 3 Interaction of Fur *in vivo* with selected transcription units during anaerobic fermentative growth. *B. subtilis* strains ORB8440 (wild type; white bars), ORB8501 (*nsrR*::*cat*; black bars), and ORB8502 (*resD*::*cat*; gray bars), carrying *fur-his*$_{12}$, were used for Fur ChAP-qPCR as described in the legend for Fig. 2.

peptidase-type autolysin, and *glmS*, which codes for glutamine-fructose-6-phosphate transaminase.

NsrR, ResD, and Fur affect each other's binding affinities to coregulated promoters. The results described above showed that ResD and NsrR control *nasD*, *hmp*, and *ykuN* transcription by directly interacting with promoter DNA. *In vitro* binding of Fur to *ykuN* was previously demonstrated (38). Fur is a sequence-specific transcriptional repressor that recognizes a 7-1-7 inverted repeat (39). While *ykuN* bears two Fur-binding elements, it is also the site of NsrR and ResD interaction, suggesting a complex interplay among multiple regulators to control transcription. To explore this possibility, we investigated how ResD, NsrR, and Fur (in the case of *ykuN*) might affect each other's interaction with *nasD* and *ykuN*. To this end, we used ChAP-qPCR in the wild-type and various mutant backgrounds.

NsrR binding to the *nasD* promoter during anaerobic fermentation was enriched around 12-fold compared to that to the *rpsD* promoter, and the absence of Fur did not have any significant effect on NsrR binding to *nasD* (Fig. 2A). On the other hand, the association of NsrR with *nasD* was abrogated to the level of *rpsD* in the *resD* mutant. This result showed that ResD but not Fur is required for NsrR to efficiently bind to the *nasD* regulatory region. We also examined NsrR binding to another ResD regulon gene, *ctaB*. A low level of enrichment of NsrR at the *ctaB* promoter was observed in the ChAP-qPCR experiment. Since NsrR association with *ctaB* was not detected in ChAP-chip analysis, whether NsrR interacts with *ctaB* remains inconclusive. Binding of NsrR to *ykuN* was reduced around 10-fold when ResD was absent, whereas the association of NsrR with *feuA*, another Fur regulon gene, was not affected by the *resD* mutation, which is in good agreement with ResD interaction with *ykuN* but not with *feuA*. Finally, the *fur* mutation substantially weakened NsrR association with the *ykuN* and *feuA* promoters. ChAP-chip showed that NsrR interacts with the *ydhB-ydhC* intergenic region (Fig. 1). ChAP-qPCR validated the result and further demonstrated that the binding of NsrR to *ydhC* was not affected by ResD and was reduced only 2-fold in the absence of Fur. This result showed that the effect of ResD on NsrR binding is promoter specific and that among the NsrR-interacting genes, the *resD* mutation most severely affects NsrR binding to *nasD* and *ykuN*.

ResD interaction with *nasD* was much lower than that with *ctaB*; however, binding to *nasD* increased 10-fold in the absence of NsrR, whereas only a 2-fold increase was observed in ResD binding to *ctaB* in the *nsrR* mutant background (Fig. 2B). The absence of Fur did not affect ResD binding to *ctaB* and *nasD*. In contrast, ResD binding to *ykuN* was reduced 3-fold in the *nsrR* mutant and completely abolished in the *fur* mutant. ResD weakly associated with *ydhC*, but the association was not changed in the *nsrR* mutant and was only slightly (if at all) higher in the *fur* mutant. Consistent with the results of ChAP-chip analysis, there was no association of either NsrR or ResD with the *sdpA* and *yukE* promoters (Fig. 2A and B).

To determine the effect of ResD and NsrR on binding of Fur to *ykuN*, we carried out Fur ChAP-qPCR. To this end, we constructed the strain producing Fur-His$_{12}$ at the native locus, and the functionality of the construct was examined using *ykuN-lacZ*. Fur-His$_{12}$, like native Fur, repressed *ykuN* transcription only in the presence of excess iron (data not shown). ChAP-qPCR experiments confirmed that Fur was highly enriched at Fur regulon promoters, *ykuN*, *feuA*, and *hmoA* (*yetG*), under anaerobic fermentative conditions (Fig. 3 and data not shown for *hmoA*). Unlike NsrR, which did not show any effect on Fur binding, ResD was shown to moderately affect binding to *feuA* (a 2-fold decrease for the *resD* mutant) and more strongly that to *ykuN* (a more than 4-fold decrease). Fur association with *nasD* or *ydhC* was not de-

TABLE 2 Genes that interact with NsrR, ResD, and Fur

Gene	Protein bound	Protein(s) that affects binding[a]: Positively	Negatively	Effect of NO on binding[b]
nasD	NsrR	ResD		Decrease
	ResD		NsrR	Increase
ykuN	NsrR	ResD, Fur		Decrease
	ResD	NsrR, Fur		Decrease
	Fur	ResD		No effect
feuA	NsrR	Fur		Decrease
	Fur	(ResD)		ND
ydhC	NsrR	(Fur)		(Decrease)
	ResD			ND
ctaB	NsrR	ResD		ND
	ResD		(NsrR)	ND

[a] Parentheses indicate a modest effect (around 2-fold) on binding.
[b] ND, not determined.

tected in the wild-type and mutant backgrounds. Table 2 summarizes the result described above, together with the effect of NO on binding of regulator proteins to certain genes, which is determined in the following section.

The ChAP-qPCR experiments mostly validated the ChAP-chip results and showed that multiple transcription regulators, by directly binding to the *feuA* (NsrR and Fur), *nasD* and *hmp* (NsrR and ResD), and *ykuN* (NsrR, ResD, and Fur) promoters, affect each other's binding affinity for the promoter DNAs.

NO affects NsrR binding *in vivo*. We have previously shown that NO does not affect binding activity of NsrR to the *ykuN* promoter *in vitro*, whereas transcription of *ykuN in vivo* is moderately upregulated in response to NO (15). In hopes of resolving the contradictory effects of NO, we examined whether NO affects *in vivo* DNA binding of NsrR. NsrR bound to the *nasD* promoter in cells grown in the absence of NO (fermentative conditions), whereas endogenous NO (through nitrate respiration) and exogenous NO (by the addition of 50 μM sperNONOate) almost completely eliminated NsrR binding to *nasD* (Fig. 4). This result is consistent with the previous *in vitro* binding data for *nasD* (13). Association of NsrR with *ykuN* was reduced 4-fold either during nitrate respiration or by exposure to sperNONOate, indicating that the binding is NO sensitive *in vivo*, albeit to a lesser extent than that to *nasD*. To further confirm the result, we also examined the effect of NO on NsrR binding to other promoters *in vivo* (Fig. 5). NsrR was enriched 130-fold at the *hmp* promoter compared to findings for *rpsD* in the absence of NO, and the enrichment was reduced to 4.8-fold during nitrate respiration and 0.9-fold after the addition of sperNONOate. This drastic effect of NO is similar to that observed with *nasD*. NO reduced NsrR binding to *ydhB-ydhC* and *feuA*, although a weak association of NsrR was detected as seen with *ykuN*. The ChAP-chip experiment showed that *yopS-yopR* and *ypqP* are the sites where NsrR bound under aerobic but not anaerobic conditions (Table 1). We detected by ChAP-qPCR a weak enrichment of NsrR at these promoters during anaerobic fermentative growth, but it suffered a 2.5- to 3.5-fold reduction in enrichment after NO treatment (Fig. 5). These results strongly suggested that NsrR binds to DNA in an NO-sensitive manner *in vivo*. In addition, the results showed that binding of NsrR to the NsrR-controlled *sdpA* and *yukE* promoters is at a level that is not significant under all conditions, including those in the presence of nitrate and NO (Fig. 5), indicating that nitrosylated NsrR, like holo-NsrR, does not bind to these promoters.

We next determined if NO has any effect on ResD binding and found that NO oppositely affects ResD binding to *nasD* and that to *ykuN* (Fig. 4). ResD binding to *nasD* increased, whereas its binding to *ykuN* was reduced, when NO was present. The results in Fig. 2B show that ResD associates with *nasD* in the *nsrR* mutant more than it does in the wild type, but its interaction with *ykuN* decreased in the *nsrR* mutant. Taken together, these results indicate that the opposite effects of NO on ResD binding between *nasD* and *ykuN* can be attributed to the NO-sensitive DNA-binding activity of NsrR. In other words, ResD *per se* does not likely sense NO.

Previous transcriptome analysis showed that transcription of both *nasD* and *ykuN* is upregulated by NO (40). Our previous and current studies demonstrated that NO reaction with NsrR is responsible for increased transcription of *nasD* (4, 13, 14). The Fur repressor contains a mononuclear iron, and nitrosylation of the

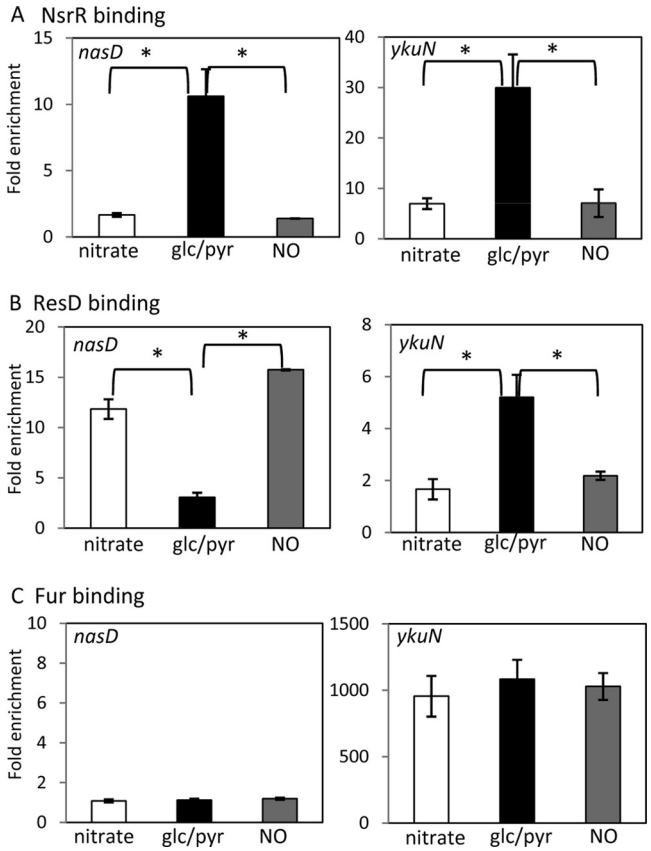

FIG 4 Effect of NO on association of NsrR, ResD, and Fur with *nasD* and *ykuN*. *B. subtilis* wild-type strains OC0010 (*nsrR-his*$_{12}$), ORB8238 (*resD-his*$_{12}$), and ORB8440 (*fur-his*$_{12}$) were grown in 2× YT supplemented with 1% glucose and 0.2% nitrate (nitrate respiration; white bars) or 0.5% glucose and 0.5% pyruvate (fermentation; black bars). The fermentative cultures were treated with 50 μM sperNONOate at an OD_{600} of 0.6 to 0.8 and further incubated for 1 h before harvesting cells (NO; gray bars). ChAP-qPCR was performed as described in the legend for Fig. 2 and Materials and Methods. Horizontal lines show statistical significance at $P < 0.05$.

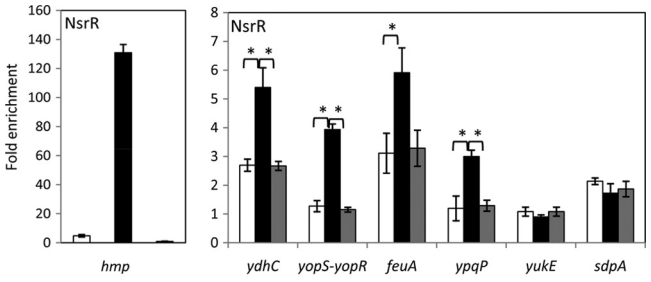

FIG 5 Effect of NO on association of NsrR with promoter DNAs. *B. subtilis* strain OC0010 (*nsrR-his*$_{12}$) was grown in 2× YT supplemented with 1% glucose and 0.2% nitrate (nitrate respiration; white bars) or 0.5% glucose and 0.5% pyruvate (fermentation; black bars). The fermentative cultures were treated with 50 μM sperNONOate at an OD_{600} of 0.6 to 0.8 and further incubated for 1 h before harvesting cells (NO; gray bars). ChAP-qPCR was performed as described in the legend for Fig. 2 and Materials and Methods. Horizontal lines show statistical significance at $P < 0.05$.

iron by NO was shown to inactivate Fur repressor activity in *E. coli* (41), which might also be the case with the *B. subtilis* Fur protein (40). The results described above indicated that NsrR directly participates in transcriptional control of *ykuN* and that NsrR binding to *ykuN* is NO sensitive. To determine whether Fur is also involved in NO-sensitive transcriptional repression of *ykuN*, we examined the effect of NO on Fur binding (Fig. 4). NO had no effect on Fur binding to *ykuN*, suggesting that NsrR plays a major role in NO-responsive transcriptional control of *ykuN*, at least under the current growth conditions.

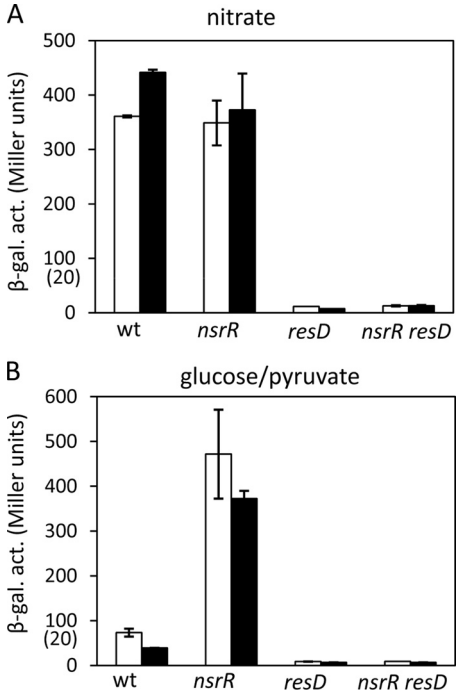

FIG 6 Effect of the *resD* and *nsrR* mutations on *nasD* transcription. The wild-type (wt) and mutant strains carrying *nasD-lacZ* were anaerobically grown in 2× YT supplemented with 1% glucose and 0.2% nitrate (A) (nitrate respiration) or 0.5% glucose and 0.5% pyruvate (B) (fermentation). Cells were harvested at hourly intervals, and β-galactosidase activities (β-gal. act.) at T−1 (white bars) and T1 (black bars) are shown. Error bars are standard deviations for triplicates. Numbers in parentheses on the *y* axes show values for the *resD* and *nsrR resD* mutants.

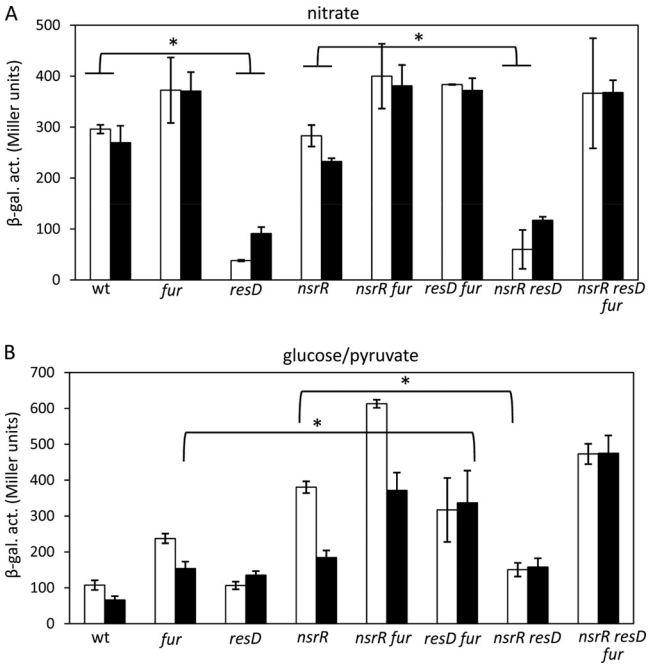

FIG 7 Effects of the *resD*, *nsrR*, and *resD* mutations on *ykuN* transcription. The wild-type and mutant strains carrying *ykuN-lacZ* were anaerobically grown in 2× YT supplemented with 1% glucose and 0.2% nitrate (A) (nitrate respiration) or 0.5% glucose and 0.5% pyruvate (B) (fermentation). Cells were harvested at hourly intervals, and β-galactosidase activities at T−1 (white bars) and T1 (gray bars) are shown. Error bars are standard deviations of data from triplicates.

Association of multiple transcription factors with the *ykuN* promoter controls transcription. We have shown that multiple transcriptional regulators establish contact with *nasD* and *ykuN* promoter DNA. To assess how these interactions affect transcriptional control, we examined *nasD-lacZ* and *ykuN-lacZ* expression in wild-type and mutant strains that grow under anaerobic conditions. The activities were measured in cells taken at hourly intervals, and the values at T−1 and T1 are presented in Fig. 6 and 7. *nasD* expression was repressed in the wild-type strain under fermentation conditions (with glucose and pyruvate), and the *nsrR* mutation relieved the repression (Fig. 6B). During nitrate respiration, *nasD* expression was fully derepressed in the presence of NsrR, indicating that NsrR lacks repressor activity when cells are exposed to NO (Fig. 6A). This result in the 168 background is similar to that in the JH642 background previously reported (4). Almost complete loss of *nasD* expression in the *nsrR resD* mutant demonstrated that the *nsrR* mutation does not bypass the requirement of ResD. The result confirms that ResD is an activator for *nasD* transcription and the release of NsrR from the −35 region is required for ResD-RNAP occupation at the promoter to form the transcription initiation complex.

In contrast to *nasD* transcription, ResD does not play a role as an activator in *ykuN* transcription, as evidenced by the result that full *ykuN* expression does not require ResD in the absence of Fur (Fig. 7). The result suggests that the role of ResD in *ykuN* expression is likely to modulate Fur repressor activity. During nitrate respiration, the NsrR repressor is inactive, and hence the *nsrR* mutation has no significant effect on *ykuN* transcription (comparing the wild type versus the *nsrR* mutant, the *fur* mutant versus the

nsrR fur mutant, and the *resD* mutant versus the *nsrR resD* mutant in Fig. 7A). β-Galactosidase activities were the lowest and similar in the *resD* and the *nsrR resD* mutants, indicating that Fur mainly repressed transcription. The major role of the Fur repressor was further confirmed by comparison of *ykuN* expression between the *resD* and *resD fur* mutants, since the repression of *ykuN* in the *resD* mutant was completely eliminated in the absence of Fur. However, Fur repressor activity becomes obvious only when cells lack ResD (compare the wild type versus the *resD* mutant and the *nsrR* mutant versus the *nsrR resD* mutant). Based on this result, we conclude that ResD functions as an antirepressor for Fur at the *ykuN* promoter. Under fermentative conditions, both Fur and NsrR repression was observed. Fur repression was antagonized by ResD at T−1 (compare the *nsrR* mutant versus the *nsrR resD* mutant in Fig. 7B), while NsrR repression was strengthened in the presence of ResD, particularly at T1 (compare the *fur* mutant versus the *resD fur* mutant). This result suggests a third role performed by ResD as a corepressor in *ykuN* transcriptional regulation.

DISCUSSION

Our previous transcriptome study identified possible NsrR-regulated genes (15), in addition to the originally identified *nasD* and *hmp* genes (4). However, whether these genes are direct targets of NsrR was inconclusive for three reasons. First, most of these candidate NsrR-controlled genes lack the NsrR-binding sequence previously identified in *nasD* and *hmp* interaction (11, 15). Second, NsrR binds *in vitro* to these genes with an affinity that is much lower than that observed for *nasD* and *hmp*. Third, NO does not affect *in vitro* binding of NsrR to newly identified genes, which is in sharp contrast with the important role of the [4Fe-4S] cluster in NsrR binding to *nasD* and *hmp* (13, 14).

Although NsrR was shown to bind *in vitro* to *sdpA* and *ykuN* with similar affinities (15), this study using ChAP-chip and ChAP-qPCR clearly distinguished direct targets of NsrR (such as *ykuN*) from indirect ones (*sdpA*). Identification of direct targets of NsrR prompted us to revisit the previously reported consensus NsrR-binding sequence using computational analysis of the target promoters. The results (see Fig. S1 in the supplemental material) identified CAKGDATYT (where K = G or T; D = A, G, or T; and Y = C or T) as a sequence commonly present in target genes. The identified sequence corresponds to the 5′ half of the imperfect 8-1-8 dyad symmetry sequence (ATR**TA**TYTtAAAt**A**tat, where R = G or A, and Y = C or T; bases in lowercase letters are not well conserved, and those in bold are critical bases as determined by mutational analysis) previously assigned as the consensus sequence of the NsrR binding site (15). Mutational analysis of the 8-1-8 sequence revealed that a deletion of the center T (the 9th nucleotide) leads to loss of NsrR binding and complete derepression of *nasD* expression (15), suggesting the important role served by the positioning of the two half-sites that constitute the sequence of partial dyad symmetry. The study also showed that the 4th and 5th nucleotides, T and A, at the left half of the 8-1-8 motif are important for NsrR binding, and the 14th nucleotide, A, at the right half is the site of a base substitution causing the most severe defect in both NsrR binding and repression among the base changes generated in the analysis. The most critical A, at the 14th nucleotide found in *nasD* and *hmp*, is not conserved among other promoters (see Fig. S1 in the supplemental material), which also lack the clear 8-1-8 motif. It is worth mentioning that previous ChIP-chip analysis of *E. coli* NsrR showed that an 11-1-11 motif, as well as a single 11-bp motif, functions as the NsrR-binding site *in vivo* (42). The study also showed that a base substitution at the half-site leads to a decrease in NsrR binding *in vivo*, although the effect was not confirmed *in vitro*. Taken all together, it is tempting to propose that the half-site serves as a low-affinity binding site and the full site as a high-affinity site. However, deletion and base substitutions of the proposed half-motif in the *ykuN* promoter did not show any effect on NsrR-dependent control of *ykuN in vivo*, nor did they affect *in vitro* binding by NsrR (unpublished results). Future work is required to unveil the nature of low-affinity NsrR-binding sequences in *B. subtilis*.

Previous transcriptome analysis showed that the Fur regulon, including *ykuN*, is induced by oxygen limitation, and the induction is higher in the presence of nitrate or nitrite, which contributes to NO generation (43). This notion was further confirmed by a later study demonstrating that NO upregulates iron homeostasis genes repressed by Fur (40). In this study, we demonstrated that NO reduces NsrR binding to the Fur-controlled *ykuN* and *feuA* promoters *in vivo*, thus upregulating transcription. The result does not fully solve the contradictory results of electrophoretic mobility shift assay (EMSA) showing that NsrR binds to *ykuN* in an NO-sensitive manner. Our current hypothesis is that binding of Fur to *ykuN* is NO sensitive only when another regulator(s), such as Fur and/or ResD, binds to the DNA. An alternative, although not mutually exclusive, possibility is that NO sensitivity of the NsrR-*ykuN* interaction is dependent on DNA topology, which is lacking when investigated *in vitro*, such as with EMSA. The hypothesis remains to be tested in future studies.

The current study further revealed that NsrR and ResD directly control *ykuN*, as well as *nasD* and *hmp*. ResD positively affects binding of NsrR to both *nasD* and *ykuN*, whereas NsrR plays opposite roles by inhibiting ResD binding to *nasD* and stimulating ResD interaction with *ykuN*. Based on this result and previous studies of *in vitro* NsrR binding to *nasD* (13), we propose how ResD and NsrR participate in transcriptional regulation of *nasD*. NsrR occupies the −35 region in the absence of NO, resulting in repression of *nasD* transcription. When NO is present, DNA binding affinity of NsrR is reduced and RNAP outcompetes NsrR for binding to the site. As shown previously, the presence of RNAP enhances ResD binding to *nasD*, thus stabilizing the *nasD*-ResD-RNAP transcriptional initiation complex (13). The topology of the *nasD* promoter DNA in the ternary complex might be favorable for NsrR to compete for the −35 region with RNAP once NO is consumed and the [4Fe-4S] cluster is repaired. This might explain the ChAP-qPCR result that indicates the positive role of ResD in NsrR binding, which ensures rapid silencing of ResD-activated transcription.

In *nasD* transcription, ResD functions as a transcriptional activator; however, the role of ResD is different in the case of *ykuN* transcription. ResD does not activate transcription, as evident from *ykuN* expression that is fully derepressed in the *resD fur nsrR* triple mutant (Fig. 7). This study showed that transcription of *ykuN* is controlled by two independent pathways involving the iron-sensing Fur repressor and the NO-sensitive NsrR repressor. Fur-dependent repression is antagonized by ResD, and ResD antirepressor activity does not involve a release of Fur from the *ykuN*

promoter. In fact, Fur binds better when ResD is present during anaerobic fermentation (Fig. 3) and nitrate respiration (data not shown). A similar antirepression mechanism was found in *comK* transcriptional control (44). In this case, ComK functions as an antirepressor of Rok and CodY without preventing binding of the two repressors to the *comK* promoter. ComK also acts as a transcriptional activator of late competence genes, such as *comG* (45), a role similar to that of ResD at the *nasD* promoter. However, our current study showed that ResD has a third role in transcription. The repression mediated by NsrR is exerted only in the presence of ResD (Fig. 7), where the two appear to act cooperatively in establishing promoter interaction (Fig. 2). Therefore, ResD either functions as a corepressor or in some way accentuates the repressor activity of NsrR, probably by enhancing NsrR binding. This mechanism likely functions as a safeguard to maintain the required levels of transcript under different stress conditions.

The *ykuN* operon is composed of genes encoding short-chain flavodoxins (*ykuN* and *ykuP*) and *ykuO*, the product of which is of unknown function. YkuN and YkuP are able to pass electrons to BioI (cytochrome P450), involved in biotin synthesis (37). They also participate in fatty acid desaturation as electron donors for acyl lipid desaturase (Δ5-Des) (46). *B. subtilis*, like some other bacteria, carries the oxygenase domain of NO synthase (bNOS) but lacks a reductase domain. YkuN and YkuP were shown to function as electron donors for bNOS and support NO production *in vitro* (47), although bNOS likely utilizes cellular reductases not specifically dedicated to bNOS *in vivo* (48). The role of YkuN/P in NO synthesis prompted us to examine whether NO produced by bNOS modulates NsrR activity. A null mutation in *nos* did not show any effect on NsrR-dependent repression (unpublished results) under either aerobic or anaerobic conditions, supporting the previously reported evidence that an intracellular source of NO affecting NsrR activity is generated by nitrate respiration (4). The lack of the effect by the *nos* mutation on NsrR activity under anaerobic conditions could be explained by the requirement of oxygen for NO production via bNOS. Even under aerobic conditions, the level of NO produced by bNOS could be lower than that generated during nitrate respiration, possibly due to lower production and/or higher instability of NO. The complex transcriptional regulation of *ykuNOP* by the multiple transcription factors might reflect the physiological roles of flavodoxins under different stress conditions. Flavodoxins and ferredoxins have analogous functions in shuttling electrons in redox-based reactions. Flavodoxins are more stress resistant than Fe-S-carrying ferredoxins, since the former proteins use flavin mononucleotide as a cofactor for redox activity. *B. subtilis* has a single ferredoxin gene (*fer*), and *fer* transcription is repressed by oxidative stress (diamide and hydrogen peroxide) and NO stress (during nitrate respiration) (49). Conversely, expression of the flavodoxin genes is induced under iron-limited conditions and during nitrate respiration to fulfill the function of replacing ferredoxin. Therefore, it makes physiological sense that the *ykuN* operon is under the negative control of Fur and NsrR. ResD could contribute to fine-tuning of *ykuNOP* transcription. In the presence of ResD and nitrate, cells undergo nitrate respiration, and Fur but not NsrR represses *ykuNOP*. Under these conditions, NO adversely affects both expression and activity of ferredoxin, and thus a flavodoxin(s) is needed as a substitute electron donor. This might explain why ResD antagonizes Fur repressor activity. Under fermentative conditions, there is less need for *ykuN* due to the presence of active ferredoxin; hence, *ykuN* expression is repressed by both Fur and NsrR.

Under aerobic conditions, NsrR binds to *ypqP* and *yodU*, which are located adjacent to *attL* and *attR* of the SPβ prophage, respectively (Table 1). Under anaerobic conditions, ResD also interacts with *ypqP*. The *ypqP* and *yodU* genes encode, respectively, the C-terminal and N-terminal portions of a putative capsular polysaccharide biosynthesis enzyme. The excision of SPβ prophage DNA from the *B. subtilis* genome results in generation of an intact *ypqP* coding sequence (50). The Rok transcriptional factor, which was originally isolated as the repressor of genetic competence in *B. subtilis* (51), was shown to bind to the left and right ends of the mobile element ICE*Bs1*, and the *rok* mutation led to a higher excision frequency of the element (52). Whether NsrR and/or ResD plays a similar role in stability of the SPβ prophage remains to be determined.

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