Supplemental Experimental Procedures

Construction of an E. coli *strain overproducing Strep-tag NsrR*

In order to generate NsrR fused to a Strep-tag at the C-terminus, the plasmid pMMN771 was constructed as follows. 100 pmole of complementary oligonucleotides oMN08-459 (5´-TAA*GGATCC*TGGAGCCACCCGCAGTTCGAAAAATAG*CTCGAG*AATAA-3´) and oMN09-460 (5´-TTATT*CTCGAG*CTATTTTTCGAACTGCGGGTGGCTCCA*GGATCC*TTA-3´) (BamHI and XhoI sites are italicized) were mixed in 30 μl of BamHI buffer (New England Biolabs). The mixture was heated at 95°C for 3 min, 65°C for 2 min, and 37°C for 2 min. The annealed oligonucleotide was digested with BamHI and XhoI and cloned into pET23a (Novagen) digested with the same enzymes to generate pMMN771. The insertion of the Strep-tag oligonucleotides in pMMN771 was confirmed by DNA sequencing. The *nsrR* gene was amplified by PCR from chromosomal DNA of *B. subtilis* JH642 strain using oligonucleotides oMN05-296 (5´-GGCGCGGG*CATATG*AAGTTAACCAATTATAC-3´: NdeI site is italicized) and oMN08-458 (5´-CAA*GGATCC*TTCCTTCATTTTTAAAAGC-3´: *Bam*HI site is italicized). The PCR product was digested with NdeI and BamHI and ligated with the NdeI-BamHI sites of pMMN771 to generate pMMN772. The *nsrR* sequence was verified by DNA sequencing. NsrR produced by *E. coli* carrying plasmid pMMN772 has the Strep-tag (WSHPQFEK) fused to the C-terminus of NsrR with a short linker (GS). The Strep-tag is followed by the stop codon (TAG).

Construction of a B. subtilis *strain overproducing the C-terminal His_6-tagged NsrR*

In order to overproduce the C-terminal His_6-tagged NsrR in *B. subtilis*, pMMN810 was constructed using pHT01 plasmid (MoBiTec). The *nsrR* gene with the His_6-coding region was amplified from pMMN740 using oligonucleotides oMN06-311 (5´-GGAG*GGATCC*ATGAA GTTAACCAATTATAC-3´: BamHI site is italicized) and oT7term (5´-GCTAGTTATTGCT CAGCG-3´). The PCR product was digested with BamHI and ligated with pHT01 digested with SmaI and BamHI to generate pMMN810. *B. subtilis* JH642 was transformed with pMMN810 and a transformant (ORB7838) was selected for chloramphenicol resistance.

Anaerobic purification of Strep-tag NsrR

E. coli BL21(DE3)/pLysS carrying pMMN772 was cultured in 1-L Luria-Bertani (LB) medium supplemented with ampicillin and chloramphenicol. At an OD_{600} of 0.4, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After incubating at 37°C for 3 h, cells were collected by centrifugation at 5,000 *g*, resuspended in the culture medium, and transferred into a 1-L sealed bottle. Cells were suspended in 20 ml of buffer B (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0 mM EDTA, 5 mM DTT) supplemented with an EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics) and were broken by passing through a French press placed in a plastic anaerobic glove bag (Glas-Col, LLC) that was continuously flushed with argon. The cleared lysate was recovered by centrifugation in a sealed tube at 15,000 *g* for 20 min. Subsequent purification steps were performed in an anaerobic chamber containing less than 1 ppm O_2 (Omni-Lab System; Vacuum Atmospheres Co.). All buffers and solutions were purged with argon and kept in the anaerobic chamber before use.

Cleared lysate was mixed with 5 ml Strep-Tactin® Superflow™ agarose resin (Novagen) in buffer B. After 1 h incubation, the column was washed with 10 volumes of buffer A. Strep-tag NsrR was eluted with buffer B containing 2.5 mM desthiobiotin. Fractions containing Strep-tag NsrR were pooled and buffer B was exchanged for buffer C (20 mM Tris-HCl pH 8.5, 5 mM DTT, and 10% glycerol) using a HiTrap™ Desalting column (GE Healthcare). The protein was then applied to a HiTrap™ Q XL (GE Healthcare) ion exchange column. The column was washed with 5 column volumes of buffer C followed by buffer C containing 100 mM NaCl and eluted with buffer C containing 400 mM NaCl.

Anaerobic purification of NsrR-His$_6$ from B. subtilis

ORB7838 was cultured aerobically at 37°C in 3-L LB supplemented with chloramphenicol and *nsrR* expression was induced with 0.5 mM IPTG when OD_{600} of the cultures reached around 0.4. After incubation for 3 h, cells were harvested and suspended in 15 ml buffer A (Yukl *et al*., 2008) containing 25 mg lysozyme and incubated in the anaerobic chamber at room temperature for 30 to 40 min. After passing the lysozyme-treated cells through a French press twice, we purified NsrR-His$_6$ as described in the purification from *E. coli* except the washing step of the Ni-NTA column with 100 mM imidazole was omitted.

Spectroscopic characterization of NsrR

NsrR protein was transferred to a cuvette fitted with a rubber septum in an anaerobic chamber. UV-vis spectra were obtained using a Cary 50 spectrophotometer (Varian). Room temperature

RR spectra were obtained using a 90° scattering geometry. All spectra were collected on a custom McPherson 2061/207 spectrograph (set at 0.67 m with variable gratings) equipped with a Princeton Instruments liquid-N_2-cooled CCD detector (LN-1100PB). Excitation at 488 nm was provided by an Innova I90C-3 argon ion laser and Rayleigh scattering was attenuated using a long-pass filter (RazorEdge®, Semrock). Frequencies were calibrated relative to indene and CCl_4 and are accurate within ± 1 cm^{-1}.

Complementation of nsrR *with Strep-tag NsrR*

To examine whether Strep-tag NsrR complements the *nsrR* null mutation in *B. subtilis*, the plasmid pMMN777 was constructed by cloning the erythromycin-resistance gene into pMMN772 in a similar way to construct pMMN749 (Yukl *et al*., 2008). pMMN777 was used to transform *B. subtilis* strain JH642 to generate ORB7554. ORB7554, which was obtained by a single crossover recombination at the *nsrR* locus, resulted in transcription of Strep-tag *nsrR* from the native *nsrR* promoter and concomitant inactivation of the native *nsrR*. ORB7554 was transduced with SPβ phage carrying a transcriptional *nasD-lacZ* fusion (Nakano *et al*., 1998) to generate ORB7555. Complementation experiments were carried out by determining expression of *nasD-lacZ* in ORB7555 together with LAB2854 (wild type carrying *nasD-lacZ*) (Nakano *et al*., 1998) and ORB6188 (*nsrR* mutant carrying *nasD-lacZ*) (Nakano *et al*., 2006). Cells were grown under anaerobic conditions in 2xYT supplemented with 1% glucose and 0.2% KNO_3 or 2xYT supplemented with 0.5% glucose and 0.5% pyruvate with appropriate antibiotics. Cells were collected at 1-h intervals and β-galactosidase activity was measured as previously described (Yukl *et al*., 2008).

Table 1: *B. subtilis* Strains and Plasmids Used in This Study

Strain or plasmid	Relevant feature	Source/reference
***B. subtilis* strains**		
JH642	parental strain	James Hoch
LAB2854	SPβ*c2del2*::Tn*917*::pMMN392 (*nasD-lacZ*)	(Nakano *et al*., 1998)
ORB6188	*nsrR*::*cat* SPβ*c2del2*::Tn*917*::pMMN392 (*nasD-lacZ*)	(Nakano *et al*., 2006)
ORB6559	*nsrR*::*spc*	This study
ORB6629	*nsrR*::*spc thrC*::*nsrR* (C92A)	This study
ORB6630	*nsrR*::*spc thrC*::*nsrR* (C106A)	This study
ORB6631	*nsrR*::*spc thrC*::*nsrR* (wt)	This study
ORB6632	*nsrR*::*spc thrC*::*nsrR* (C100A)	This study
ORB6640	*nsrR*::*spc thrC*::*nsrR* (C92A) SPβ*c2del2*::Tn*917*::pMMN392	This study
ORB6641	*nsrR*::*spc thrC*::*nsrR* (C106A) SPβ*c2del2*::Tn*917*::pMMN392	This study
ORB6642	*nsrR*::*spc thrC*::*nsrR* (wt) SPβ*c2del2*::Tn*917*::pMMN392	This study
ORB6643	*nsrR*::*spc thrC*::*nsrR* (C100A) SPβ*c2del2*::Tn*917*::pMMN392	This study
ORB7838	pMMN810	This study
Plasmids		
pCm::Sp	replacement of chloramphenicol resistance with spectinomycin resistance	(Steinmetz & Richter, 1994)
pDG646	erythromycin-resistance cassette	(Guérout-Fleury *et al*., 1995)
pDG795	*thrC* integration vector	(Guérout-Fleury *et al*., 1996)
pDG1515	tetracycline-resistance cassette	(Guérout-Fleury et al., 1995)
pET23a(+)	expression vector for C-terminal 6xHis-tagged protein	Novagen
pHT01	expression vector in *B. subtilis*	MoBiTec
pHG56	pUC18 with *nsrR* (wt)	This study
pHG64	pUC18 with *nsrR* (C92A)	This study
pHG66	pUC18 with *nsrR* (C106A)	This study
pMMN666	pDG795 with *nsrR* (C92A)	This study
pMMN667	pDG795 with *nsrR* (C106A)	This study
pMMN668	pDG795 with *nsrR* (wt)	This study
pMMN669	pDG795 with *nsrR* (C100A)	This study
pMMN732	pProEX-1 with *nsrR* (C100A)	This study
pMMN740	pET23a(+) with *nsrR* (wt)	(Yukl *et al*., 2008)
pMMN771	Strep-tag fusion vector	This study
pMMN772	pMMN771 with *nsrR* (wt)	This study
pMMN805	pET23a(+) with *nsrR* (C100A)	This study
pMMN810	pHT01 with *nsrR* (wt)	This study

[a] All *B. subtilis* strains are *trpC2 pheA1*.

Supporting References

Guérout-Fleury, A., K. Shazand, N. Frandsen & P. Stragier, (1995) Antibiotic-resistance cassettes for *Bacillus subtilis*. *Gene* **167**: 335-336.

Guérout-Fleury, A. M., N. Frandsen & P. Stragier, (1996) Plasmids for ectopic integration in *Bacillus subtilis*. *Gene* **180**: 57-61.

Nakano, M. M., H. Geng, S. Nakano & K. Kobayashi, (2006) The nitric oxide-responsive regulator NsrR controls ResDE-dependent gene expression. *J. Bacteriol.* **188**: 5878-5887.

Nakano, M. M., T. Hoffmann, Y. Zhu & D. Jahn, (1998) Nitrogen and oxygen regulation of *Bacillus subtilis nasDEF* encoding NADH-dependent nitrite reductase by TnrA and ResDE. *J. Bacteriol.* **180**: 5344-5350.

Steinmetz, M. & R. Richter, (1994) Plasmids designated to alter the antibiotic resistance expressed by insertion mutations *in Bacillus subtilis*, through in vivo recombination. *Gene* **142**: 79-83.

Yukl, E. T., M. A. Elbaz, M. M. Nakano & P. Moënne-Loccoz, (2008) Transcription factor NsrR from *Bacillus subtilis* senses nitric oxide with a 4Fe-4S cluster. *Biochemistry* **47**: 13084-13092.

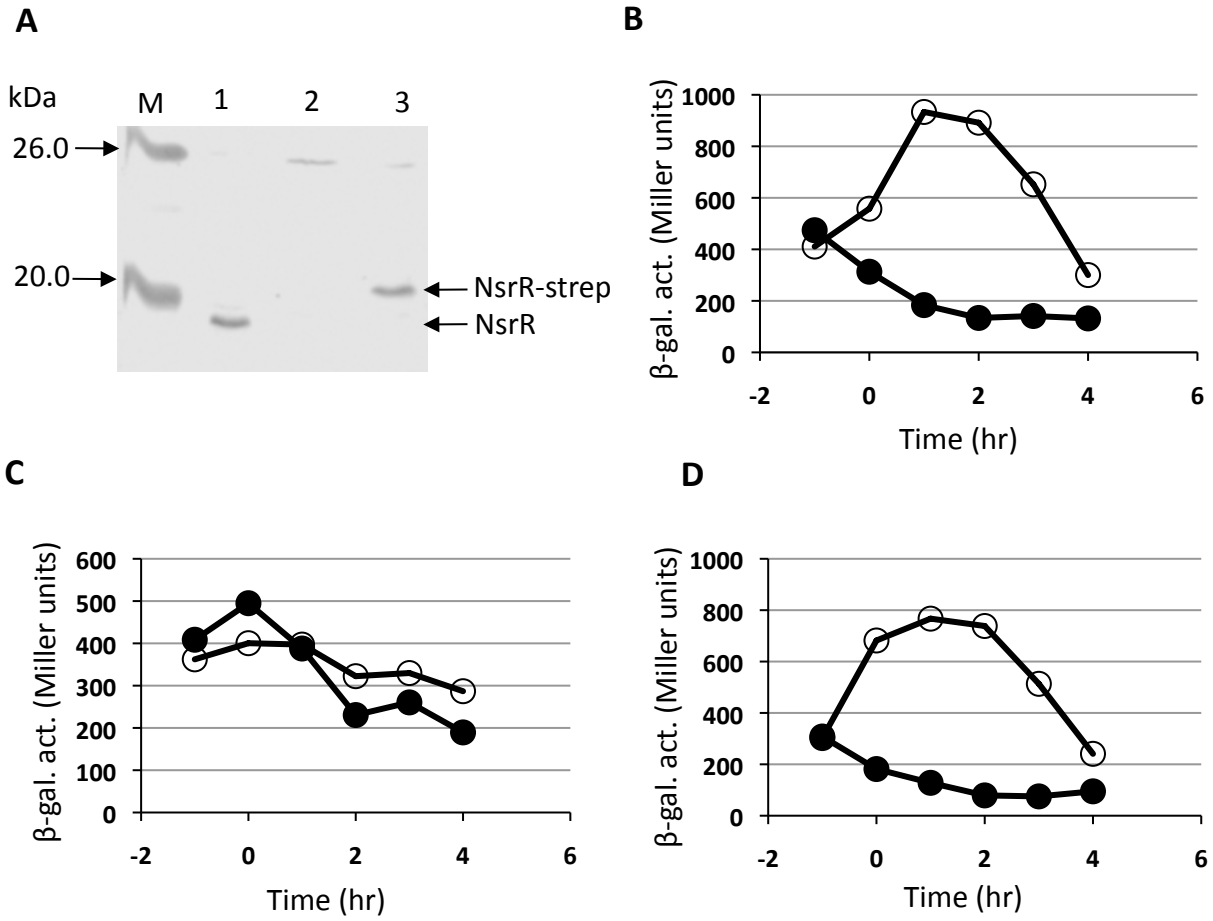

Figure S1. Complementation analysis of Strep-tag NsrR in *B. subtilis*.
A. Western blot analysis of NsrR. Lane 1, LAB2854 (wild type); lane 2, ORB6188 (*nsrR*); lane 3, ORB7555 (*nsrR* carrying *nsrR*-strep).
B to D. Expression of *nasD-lacZ* in LAB2854 (B), ORB6188 (C), and ORB7555 (D) cultured anaerobically in 2xYT supplemented with 1% glucose and 0.2% nitrate (open circles) or 0.5% glucose and 0.5% pyruvate (closed circles). T_0 represents the end of exponential growth.

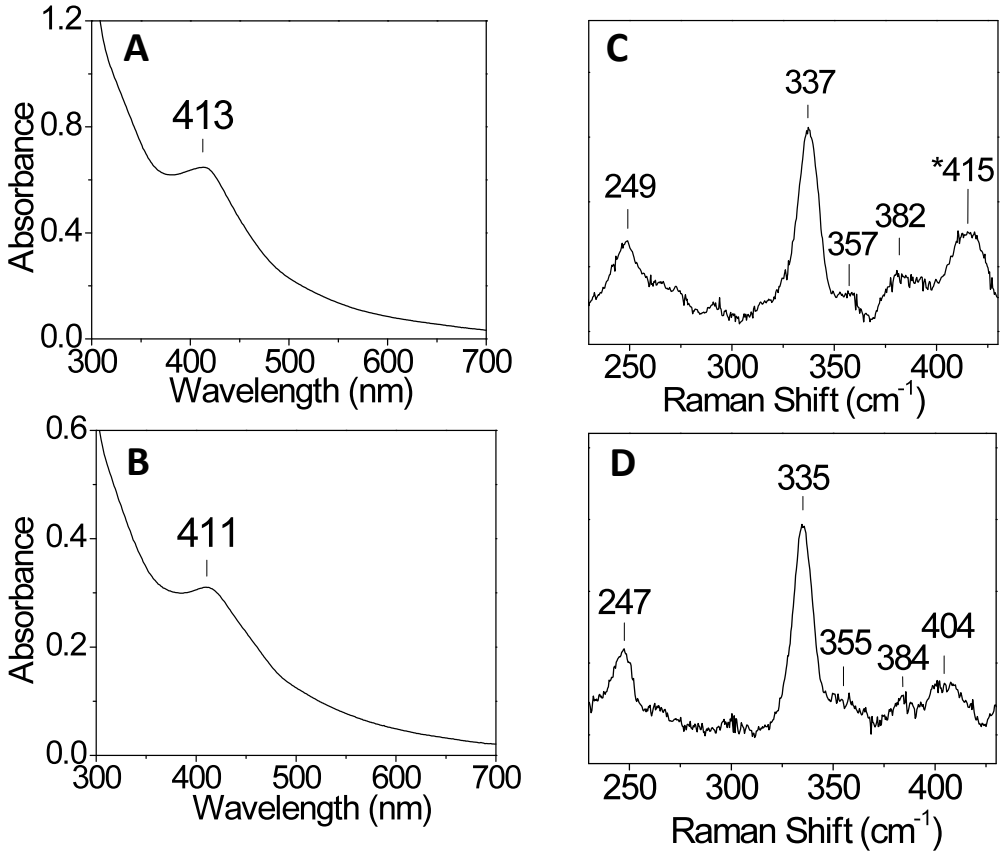

Figure S2. Spectroscopic analysis of Strep-tag NsrR isolated from *E. coli* in comparison with His_6-tagged NsrR previously characterized (Yukl *et al*., 2008). UV-vis absorption spectrum of Strep-tag NsrR (A) and NsrR-His_6 (B). Room temperature resonance Raman spectrum of Strep-tag NsrR (C) and NsrR-His_6 (D). λ_{exc} = 488 nm, 70 mW. The peak arising from the presence of glycerol is marked with * in C. All spectra were taken in the presence of 5 mM DTT.

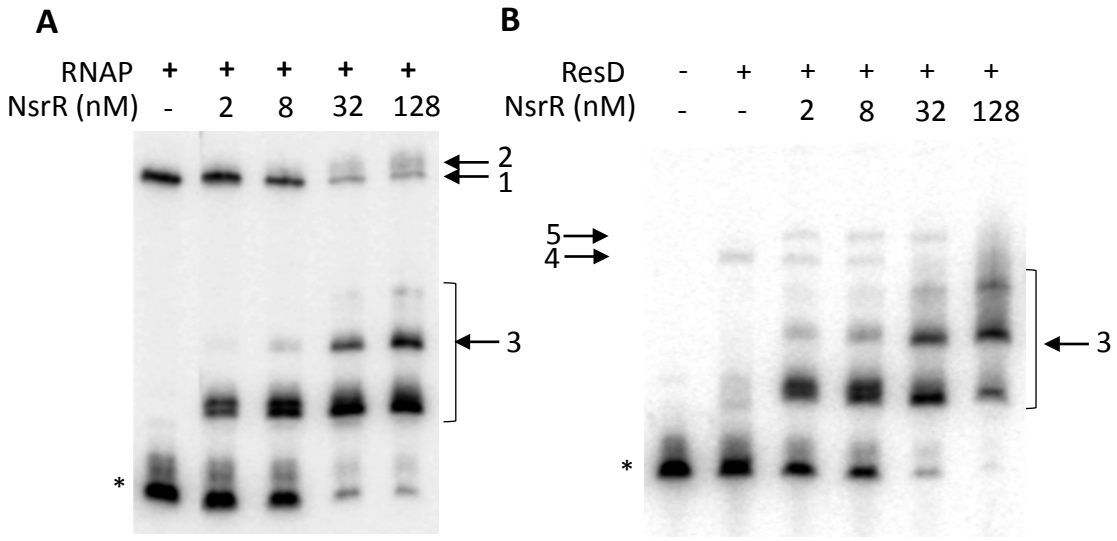

Figure S3. Analysis of RNAP, ResD~P, and NsrR binding to the *nasD* promoter. The *nasD* (-114 to -4) was used as a probe. All EMSA reactions and electrophoresis were carried out in an anaerobic chamber. A single asterisk shows the free probe. Arrows: 1, *nasD*-RNAP; 2, *nasD*-RNAP-NsrR; 3, *nasD*-NsrR; 4, *nasD*-ResD~P; 5, *nasD*-ResD~P-NsrR.

A. The probe (0.2 nM) was incubated with RNAP and increasing concentrations of NsrR-His_6 purified under anaerobic conditions.

B. The probe was incubated with 1 μM ResD phosphorylated with 1 μM ResE, 15 nM RNAP, and increasing concentrations of NsrR-His_6 purified under anaerobic conditions.

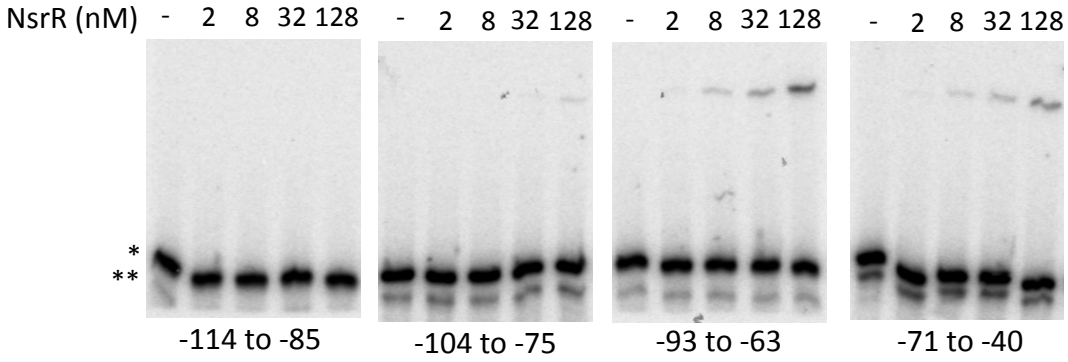

Figure S4. Binding assay of NsrR to the *nasD* promoter. The sequence of the *nasD* fragments used as probes is shown in Figure 1. The radiolabelled probe (0.1 nM) was incubated with increasing concentrations of wild-type NsrR-His_6 purified under aerobic conditions. A single asterisk shows the double-stranded DNA and a double asterisk shows the single stranded DNA, unannealed radiolabelled DNA oligonucleotide.

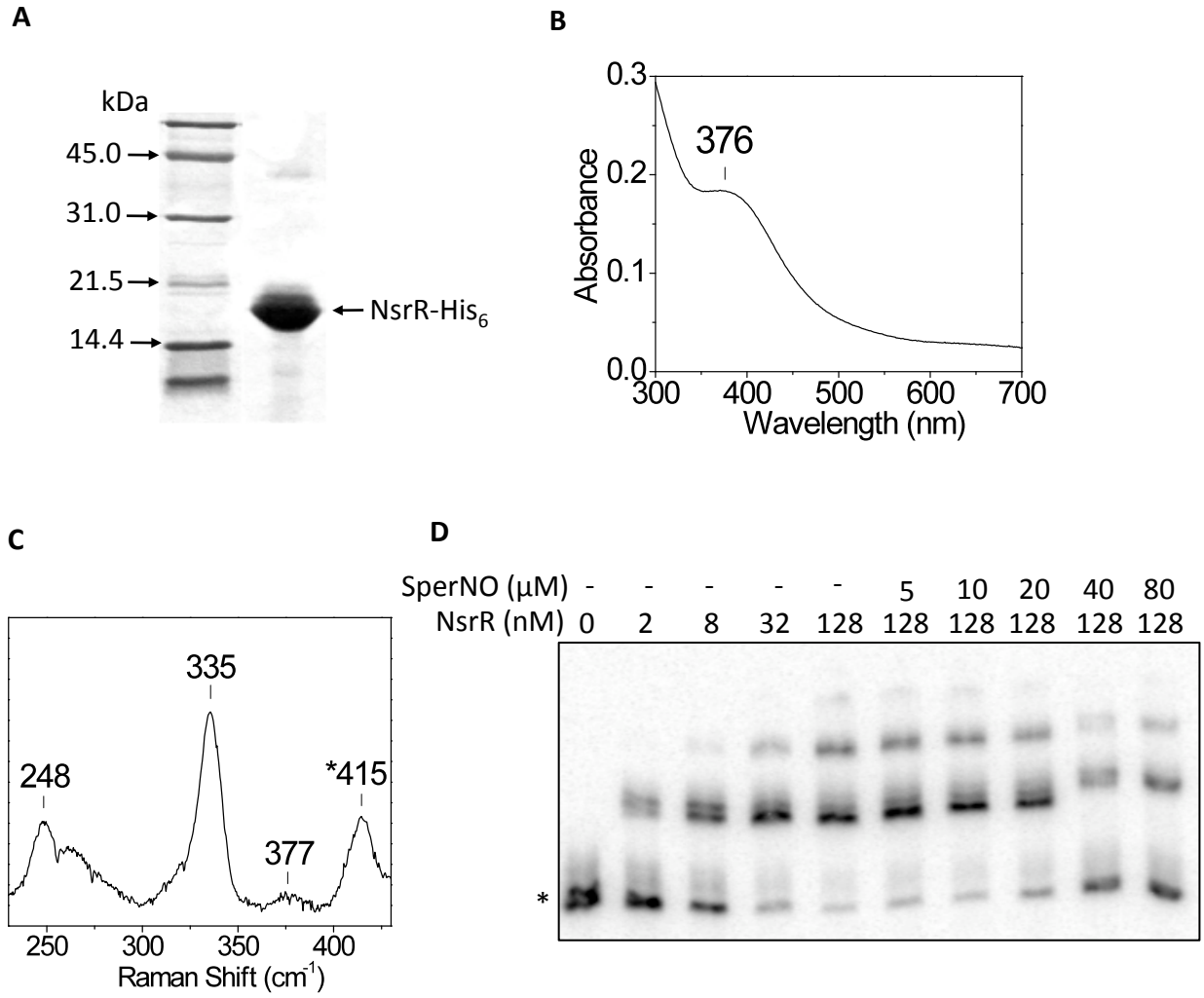

Figure S5. Characterization of NsrR-His_6 isolated from *B. subtilis*.
A. Anaerobically purified NsrR-His_6 from aerobic *B. subtilis* cultures.
B. UV-vis absorption spectrum in the presence of 5 mM DTT.
C. Room temperature resonance Raman spectrum in the presence of 5 mM DTT. λ_{exc} = 488 nm, 70 mW. The peak arising from the presence of glycerol is marked with * .
D. Binding of NsrR-His_6 to *nasD* and effect of sperNO on the binding. The *nasD*(-114 to -4) probe (0.1 nM) was incubated with increasing concentrations of NsrR. The reaction using 128 nM NsrR was also treated with increasing concentrations of sperNO. The asterisk shows the free probe. EMSA reaction and electrophoresis were carried out in the anaerobic chamber.